Anti-leukemia immunity is enabled by unmasking cross-reactive antigens with vaccination and checkpoint blockade

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Abstract

BCR-ABL$^+$ acute lymphoblastic leukemia patients have transient responses to current therapies. However, the fusion of BCR to ABL generates a potential leukemia-specific antigen that could be a target for immunotherapy. To address how BCR-ABL$^+$ leukemia escapes immune surveillance, we developed a peptide: MHC-II tetramer that labels endogenous BCR-ABL-specific CD4$^+$ T cells. Naïve mice harbored a small population of BCR-ABL-specific T cells that proliferated modestly upon immunization. We saw that BCR-ABL specific T cells were cross-reactive with an endogenous peptide derived from ABL. Despite this cross-reactivity, the remaining population of BCR-ABL reactive T cells proliferated upon immunization with the BCR-ABL fusion peptide and adjuvant. In response to BCR-ABL$^+$ leukemia, BCR-ABL specific T cells proliferated and converted into regulatory T cells (Treg cells), a process that was dependent on cross-reactivity with self-antigen, TGFβ1, and MHC-II antigen presentation by leukemic cells. Treg cells were critical for leukemia progression in C57Bl/6 mice, as transient Treg cell ablation led to extended survival of leukemic mice. In an effort to find immunotherapy approaches for BCR-ABL$^+$ B-ALL, we found that robust MHC-II expression, coupled with appropriate costimulation, correlated with lower leukemic burden. We next assessed whether checkpoint blockade or therapeutic

vaccination could improve survival in mice with pre-established leukemia. Consistent with the low mutation load in our leukemia model, we found that checkpoint blockade alone had only modest effects on survival. In contrast, robust heterologous vaccination with BAp peptide generated a small population of mice that survived long-term. Checkpoint blockade strongly synergized with heterologous vaccination to enhance overall survival in mice with leukemia. Enhanced survival did not correlate with numbers of BAp:I-A^b-specific T cells, but rather with increased expression of IL10, IL17, and GrzmB and decreased expression of PD1 on these cells. Thus, despite a paradigm in the field that numerous neo-antigen specific T cells are required for effective anti-cancer immunity, a rare cross-reactive $CD4^+$ T cell population mediates anti-leukemia immunity in our model.

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Chapter 1 An overview of interactions between cancer and T lymphocytes

Introduction

The focus of this thesis is to increase our understanding of the interactions between the immune system and cancer. While the early studies of the immune system clarified that the immune system resided, by-and-large in the primary (bone marrow and thymus) and secondary (lymphatic) lymphoid organs, sporadic reports over the past thirty years have made it clear that immune cells are a major part of every tissue in the body (1-5). Further, a new era of tools and ideas about immunology have fueled an examination of the immune system in diseases and tissues, which were not always thought to have an immune component (6-8). In parallel with these discoveries in immunology, a new concept has developed in cancer biology; that cancers are comprised of tissue-level organization (9-11). By that logic, I hypothesize that if cancers are tissues and tissues normally contain immune cells, there should be an immune cell component to all cancers-including leukemia. Further, just as the immune cells in skeletal muscle tissue play a critical role in muscular pathologies (6), I hypothesize that the immune cells in “leukemia tissue” play a critical role in leukemia pathology. I sought to test these fundamental hypotheses in my thesis.

Specifically, I sought to characterize if an immune “response” to leukemia existed, if this response was-at “baseline”-beneficial or detrimental to the host, and finally if this immune response could be modulated to improve therapeutic outcome of the host.

Cancer: A complex disease with a genetic basis.

Cancer has long been understood to initiate as a genetic disease, arising from mutations that lend a selective advantage to a cell (12, 13). These mutations lead to pathologies associated with bulk size, metastasis, and/or secretion of toxic compounds. Cancers have been recognized in humans since at least ancient Egyptian cultures, and therapeutic interventions for cancer have existed for just as long. However, cancer was not a leading cause of death until recently. This industrial revolution led to a medical renaissance, particularly with the discovery and mass-production of penicillin and other antibiotics. These treatment options led to an increased lifespan in humans, which allowed people to live long enough to die of cancer instead of other infections. Thus, as the world became industrialized, cancer became a leading health problem. By the 1970’s the Nixon administration made cancer therapy a national effort.

Since cancer is a genetic disease that develops because of mutations, it seems plausible that these mutations could become therapeutic targets. In fact, most cancers arise from multiple mutations. Early evidence for this came from the studies of patients with Familial Adenomatous Polyposis (FAP), which results from autosomal dominant loss of one allele of the tumor suppressor APC (14). While this is mildly detrimental to FAP patients, loss of the second allele of APC occurs, which is the 'second hit' necessary to initiate transformation of colonic epithelia. While most cancers clearly arise from multiple mutations, an argument exists that cancers may arise with one mutation. BCR-ABL^{+} leukemia may sometimes be one such cancer. While the chromosomal translocation BCR-ABL is a single mutation event, it leads to activation of multiple signaling cascades that together transform the normal cell into a malignant cell. BCR-ABL is present in nearly all chronic myeloid leukemia (CML) in humans. BCR-ABL is also present in a about 25% of B Acute Lymphoblastic leukemia (B-ALL) in humans, and this percentage positively correlates with the age of the patient population (BCR-ABL is more common in B-ALL of older adults than of younger adults).

Despite early understanding of the genetic basis for cancer, this knowledge has not let to huge advances in treatment until recently, for which there are at least two main reasons: 1) the study of computational and molecular biology has made

huge progress, allowing scientists to better understand complex living systems; and 2) a fundamental change in our understanding of cancer: cancers are not just made up of the mutated “cancer cells”, but also a heterogeneous network of other cell types.

The Cancer Stem Cell Theory Implies Immune Involvement in Malignancy

Solid cancers are heterogeneous masses. Histology makes it clear that a tumor is more than a single cell type. One hypothesis for this heterogeneity is that, just as the heart has many different cell types from different lineages working together, a tumor also has many different cell types that work together for the function of the tumor. The cancer stem cell theory suggests that a cancer stem cell can give rise to many different cancer cell types which help to form the tumor tissue (9). Thus, the cancer stem cell theory supports the idea that a tumor contains cells that are not derived from the cancer stem cell, but were recruited to the tumor (implicitly, to support tumor progression). Since immune cells are an important component of all other tissue types (1), the cancer stem cell theory implies that immune cells must be a component of tumors as well - and that the

immune cells that are recruited to a tumor should be beneficial for tumor progression.

If there is a tissue stroma associated with a solid tumor like a melanoma, then there should be a tissue stroma associated with a liquid tumor like leukemia. However, it is less-clear how the “tumor stroma” in a leukemia would be defined, since the leukemia is ostensibly liquid and moving throughout the body.

Leukemia: current treatments and knowledge gaps

Leukemia is a broad group of pathologies

The focus of my thesis research is modeling the immune response to leukemia. Leukemia is a broad name that means white cells in the blood. Thus, leukemia is white blood cell cancer. Most leukemias do surface in the blood, but they can arise in other site in the body such as the lymph nodes (15).

While there are many types of leukemia, one way to categorize this disease is by the cell of origin. By this classification, there are two main types of leukemia: leukemia arising from lymphocytes (lymphoid leukemia) and leukemia arising from myelocytes (myeloid leukemia). Within these lineages, leukemias can be classified based on their activity. There are chronic leukemias and acute leukemias, with chronic leukemias generally developing slower with a lower blast

percentage, while acute leukemias develop rapidly and have a high blast percentage (15). Acute leukemia of lymphoid origin is referred to as Acute Lymphoblastic Leukemia, or ALL.

Current therapies for ALL

Most ALL cases are treated first by cytotoxic chemotherapeutics to achieve remission. Remission is generally not durable, and patients often require a long-term treatment solution. For many patients, this long-term option is another round of chemotherapy followed by bone marrow transplant (16). Despite these therapeutic interventions, long-term survival for adult ALL remains below fifty percent.

Current ALL chemotherapies are derivatives of chemicals that were discovered in the past 150 years. The industrial era of the late 1800's and early 1900's led to a renaissance in chemistry, with creation of massive chemical companies like Bayer and Pfizer. While there were many industrial uses for the chemicals produced by these companies, the companies recognized the potential utility for their small molecules in exerting biological effects. The chemical renaissance of the late 1800's also led to the dawn of the era of chemical warfare around the time of World War I. Critical observation by medical workers during and after

World War I noted that mustard gas victims had a pronounced leukopenia. This early observation led to laboratory investigations into how these small molecules interacted with cancer, and it was found that these molecules could inhibit DNA fidelity and block replication in rapidly dividing cells like cancer cells. Chemotherapeutics based on mustard gas went on to become chlormethine, one of the first chemotherapeutics; and derivatives of these therapies are still used today (17, 18).

The era of chemotherapy focused on targeting the cancer cell by identifying exquisitely small differences between the cancer cells and 'normal' cells in the body. However, the therapeutic window for chemotherapies is very small, since there are minimal differences between a cancer cell and a normal cell. In leukemia, there are a variety of clinical trials being conducted that explore cytotoxic chemotherapeutics to treat ALL. Most of these therapies target cell division through various pathways, since cancer cells divide rapidly while most normal cells do not. One regimen called hyper-CVAD (a course of cyclophosphamide, vincristine, and doxorubicin alternated with a course of dexamethasone and Ara-C) is used in some aggressive ALL cases, sometimes in combination with the tyrosine kinase inhibitor Gleevec (19). Trials of hyper-CVAD report side effects including hospitalization of the majority of patients,

fungal infection, pneumonia, sepsis, neurotoxicity, and disseminated intravascular coagulothapies, all with a long-term response rate of about 35% (20). Thus, there has been a long-standing effort to identify other therapeutic modalities for cancer besides surgery and chemo/radiotherapy.

Recently, a different long-term solution was found for a small family of leukemias called Chronic Myeloid Leukemia. This long-term solution is maintenance therapy with one of a family of small molecules called tyrosine kinase inhibitors (TKI) (21-24). This class of drug targets an enzyme family called tyrosine kinases, which are a component of cell signaling cascades. Normal cells respond to growth signals from the extracellular matrix. These growth signals often use tyrosine kinases to communicate the extracellular signals into changes in the normal cell. In cancer cells, tyrosine kinases are often mutated so that they transmit signal even in the absence of extracellular growth signals. Thus, the TKIs are designed to block the aberrant signaling that occurs through tyrosine kinases and by doing so, inhibit cancer progression. Patients with CML often respond well to TKI treatment because the majority of CMLs express a chromosomal translocation that juxtaposes the Breakpoint Cluster Region gene (*BCR*) and the Src family kinase *ABL*. This chromosomal translocation, termed

BCR-ABL is also expressed in about 25% of adult B-lineage ALLs (B-ALL) (25). Since BCR contains a docking site for GRB2 and ABL contains a tyrosine kinase domain, BCR-ABL may be able to rapidly activate the Ras and Akt signaling cascades (26). Additionally, BCR-ABL can phosphorylate STAT5, which may lead to increased survival of transformed cells (27-30). These signaling events can lead to transformation of the BCR-ABL$^+$ cell. The first major TKI was designed to specifically target the kinase domain of ABL in BCR-ABL. This drug, Gleevec, is still used for induction in BCR-ABL$^+$ B-ALL and CML, as well as maintenance therapy for BCR-ABL$^+$ CML (26).

Much of the work in this thesis discusses immune responses to BCR-ABL. This may seem clinically paradoxical since BCR-ABL is already a well-established treatment target for TKIs as discussed above. However, there are multiple reasons for our investigation of BCR-ABL as an immunotherapy target. First, while BCR-ABL is present in nearly all CML, BCR-ABL is also present in a subset of Acute Lymphoblastic Leukemias (ALL) (23, 31), making BCR-ABL a relevant clinical target in ALL. Second, resistance develops to BCR-ABL-targeted TKIs, particularly in BCR-ABL$^+$ B-ALL. Multiple mechanisms have been identified that lead to Gleevec resistance. The most common (and that which is usually the case in Gleevec-resistant BCR-ABL$^+$ CML) is acquisition of mutations in BCR-

ABL that render Gleevec unable to bind the ATP binding pocket of BCR-ABL (32, 33).

The other mechanism of resistance is more specific to BCR-ABL$^+$ B-ALL. One of the targets of BCR-ABL kinase activity is STAT5 (28, 29). Phosphorylated STAT5 (pSTAT5) in-turn leads to transcription of cell cycle progression genes such as Cyclin D1, Cyclin D2, and BclXL that lead to G1/S transition and evasion of apoptosis (34) (35). B-ALL, but not CML, cells express large quantities of another transcription factor BCL6. Multiple lines of evidence in a variety of cell types suggest that BCL6 and STAT5 reciprocally regulate one-another (36-40). Indeed, in BCR-ABL$^+$ B-ALL cells, increased pSTAT5 inhibits BCL6, and increased BCL6 inhibits STAT5 transcription. The Melnick group showed that when BCR-ABL$^+$ B-ALL cells were treated with Gleevec, pSTAT5 levels decrease, allowing an over-accumulation of BCL6 (41). BCL6 also drives a pro-transformation transcriptional program (one mechanism of which may be to inhibit the tumor suppressor TP53 (42)) that then transforms the BCR-ABL$^+$ B-ALL cells through a STAT5-independent mechanism.

New small molecule therapies are being developed that target Gleevec-resistant point mutants. One such drug, Ponatinib, has had some clinical success, however the therapeutic window of this drug is relatively small (21).

Thus, there is a shortfall in treatment options for BCR-ABL^{+} B-ALL, and new therapeutic avenues should be explored.

Treatment options for ALL: Surgery

Surgery was the first treatment option used for cancer and is still a mainstay of cancer therapy today. One problem with surgical therapy for cancer is that a requisite is being able to access most of the cancer cells. In the case of leukemia, surgery is often not used, because there are not yet strategies to surgically debride leukemia from a patient. An interesting dichotomy arises with ALLs regarding surgical therapy. Specifically, B-ALL often arises as leukemia (blood cancer) while T-lineage ALL often presents as lymphoma (lymph node cancer) (15, 43). Thus, T-ALL is generally easier to surgically resect, given the discreet location of lymph nodes, while B-ALL remains challenging to access in the blood.

Treatment options for ALL: Targeting the Stroma

Initial efforts for cancer therapy focused on targeting the cancer cell themselves. However, by the mid-1990s, it was clear that the tumor was a complex tissue-like structure. Around this time, scientists started some of the first studies that

targeted a non-tumor cell type for improved therapeutic outcome in cancer (targeting vascular tissue in the tumor to prevent circulation and thus starve the tumor of nutrients) (44-46). It has since become clear that the approach of targeting VEGF is not always ideal, as resistance mechanisms may be developed instead of preventing cancer progression (47). Nonetheless, these types of therapies set a precedent for treating cancer by targeting cell types that were part of the tissue stroma of the tumor. Just as the vasculature is part of that stroma, so are immune cells. Thus, this precedent opened the door for cancer immunotherapy, by showing that, in some cases, there was a therapeutic benefit to targeting therapy to the tissue stroma of the tumor instead of to the cancerous cells themselves. While endothelial cells that are the canonical target of VEGF support the tumor and this supportive action can theoretically be limited, immune cells in the tumor may be able to play a truly detrimental role to the tumor by actually killing tumor cells. To that end, much work has gone into understanding the role of immune cells in solid tumors, particularly melanoma (48-50). These findings have progressed into clinical trials and even first-line therapies in a few malignancies (51-53).

Clearly, immune cells make up a component of tumors. Methods to modulate the immune system to protect the host from cancer fall into five main categories:

adjuvants, vaccinations, bone marrow transplant, adoptive cell therapy, and inhibitory checkpoint blockade. These methods will be detailed below.

Adjuvants

Immunological adjuvants were the first use of immunotherapy for cancer, and arose from straightforward clinical observations. One striking story is from a Canadian physician named Hunter who describes a patient with a facial tumor. Shortly after this tumor diagnosis, the patient develops erysipelas at the tumor site. Erysipelas is a cutaneous infection often with the bacteria *Streptococcus pyogenes* or *Serratia marcescens.* In this case the infection produced a fluid-filled abscess and the tumor was cleared shortly thereafter, allowing the patient to die of a heart attack some years later (54). Around the same time as Dr. Hunter, a New York physician named William Coley's was creating suspensions of heat-killed bacteria to inject into tumors (55). For a time, the "Coley's Toxins" were so popular that they were marketed by the pharmaceutical company Parke-Davis. However, the formulation proved challenging to correctly produce, and the treatment fell out of style with physicians and government regulators.

One hypothesis of how the Coley's Toxins elicit their effect is as an adjuvant. An immunological adjuvant helps to activate innate immune cells, which then allows for increased activation and programming of adaptive immune cells. Adjuvants can be chemicals and other non-biological substances (for example, alum), or they may also be cytokines that may directly activate innate and adaptive immune cells. One advantage of using cytokines instead of Coley's Toxins is that Coley's Toxins are not well defined, while cytokines are individual proteins that can be purified to a pharmaceutical grade. The downside is that a single cytokine may not provide the broad biological impact as Coley's Toxins-due to the complex nature of Coley's Toxins. In part because of the defined nature of cytokines, these became the next step of immunotherapy. Numerous clinical trials were conducted that treated cancer patients with cytokines like Type I Interferon (56) and IL-2. Clinical trials were set up testing Type I Interferon in ALL patients, but ideal regimens are still being established.

Adjuvants are still used in the clinic and in clinical trials, however their popularity is limited by the side effects associated with them. However, one example that has made some clinical headway is the therapeutic vaccination ProstVac (57) (58). ProstVac is a vaccinia virus-based vaccine that encodes antigens from prostate cancer as well as important immune costimulatory molecules (59).

Exactly how ProstVac modulates the immune response is not fully understood, but side effects have been relatively mild compared to standard chemotherapy (no patients died in the initial trials of ProstVac) (57).

Vaccination

Another approach for cancer immunotherapy is vaccination. Immunology, as applied to medicine, has a long-standing tradition of vaccination. Vaccination takes advantage of immunological memory-a component of the adaptive immune system. The idea developed that vaccination could be used to activate adaptive immunity in response to cancer-and potentially this would be one mechanism of cancer immunotherapy. The most famous “cancer vaccine”, Gardasil, does not actually target a cancer at all. Instead, Gardasil targets a family of viruses that often are associated with cancer. Thus, by preventing viral infection, Gardasil helps prevent cervical cancer. Few other prophylactic vaccinations for cancer have been developed into full therapies, however the prophylactic vaccination still provides a useful “proof-of-principle” for the utility of a memory immune response against cancer.

Bone Marrow Transplant

Cancers can adapt to the host immune system by inducing immune suppression (60-62). This in-turn protects the cancer from the host immune response (63, 64). Because of this base-level immune suppression, immune cells in the cancerous host often have minimal effect on the tumor. While some therapies aim to reprogram these host immune cells to increase functionality, other therapies look to place new, functional, immune cells into the host, such that these might be programmed to effectively control cancer (65). The basis of this is bone marrow transplant. Bone marrow transplant is a therapy for many diseases. In some cases, the idea with bone marrow transplant is to match the donor and host bone marrow as close as possible. This is ideal for minimizing the autoimmune side effects, which can occur from bone marrow transplants. However, in the case of cancer treatment, physicians aim to slightly mismatch donor and host bone marrow. This allows the donor-derived adaptive immune cells to recognize host cells as “non-self”. This can then lead to the donor-derived adaptive immune cells killing the host cancer cells. This effect is referred to as “Graft-vs. -Leukemia” or GVL. The benefit of GVL is weighed against the detriment of “Graft-vs. -Host” (GVH) which is the broad term for autoimmune complications arising from bone marrow transplant.

Adoptive Cell Therapy

Instead of transferring fresh immune cells into a cancer host by bone marrow transplant, another approach is to reprogram the host's immune cells *ex vivo*, followed by adoptive transfer of these cells back into the host (66). Such therapies are referred to as "Adoptive Cell Therapy" or ACT. The basic concept is to harvest immune cells from a patient with cancer and culture these cells in a dish *ex vivo* with the correct signals so that the cells become highly activated and programmed to control cancer progression. These cells are allowed to proliferate many times, and then the cells are adoptively transferred back into the host. This therapy option has proven effective in clinical trials (66, 67).

Immune Inhibitory Checkpoint Blockade

Instead of harvesting the immune cells from a patient and culturing them *ex vivo*, the main area of research and clinical application currently is using approaches to re-activate immune cells inside of the cancer host. This greatly increases the therapeutic window and decreases the side effects, since no cells are transferred (a major infection risk that also requires aggressive immunosuppression). Instead, this approach uses antibodies that specifically bind to, and block,

molecules that normally inhibit an immune response(49, 68-72). Such molecules (like PDL1 and CTLA4) are often expressed at high levels on tumor cells, and likely help the tumor to control and modulate the immune cells in the tumor stroma.

Cancer and the Immune System

The evidence showing that infections correlated with improved outcome to cancer was fortified by causative evidence in humans in the past few years that linked immune status to cancer progression (51). These findings came from clinical trials examining checkpoint blockade therapies that showed immune modulation could lead to improved outcome for some cancers.

T cells in Cancer

Of all the immune cells, T cells are probably the most studied in relation to cancer. T cells are hematopoietic cells that develop further in the thymus. During thymic development, T cells are engendered with a T cell receptor (TCR) which recognizes cognate antigen in the context of a Major Histocompatibility Complex (MHC). TCR development occurs in a sufficiently random process that T cells should be engendered with individual TCRs capable of recognizing nearly any possible amino acid sequence bound to MHC. Most T cells recognize

foreign antigens. Since cancer derives from cells that are normally in the body, most proteins expressed by cancers are not foreign antigens but instead are 'self-antigens'. This presents a problem for T cell mediated cancer immunotherapy. However, the rules of 'self' and 'foreign' are not clearly defined, and this provides some wiggle-room for T cell based cancer immunotherapy.

T cell development and central tolerance

Because T cells can have a highly destructive capability, thymic development has also evolved to remove most T cell clones that recognize self-peptides bound to MHC (termed central tolerance). The processes enabling central tolerance are still being understood, but part of this process involves signaling to T cells that cross-react with self, such that a pro-apoptotic protein called Bim is activated in these cross-reactive T cells (73). Bim leads to activation of intrinsic apoptosis mechanisms, which destroys the cross-reactive T cell. Indeed, *Bim*$^{-/-}$ mice have increased abundance of T cells, with enrichment of cross-reactive clones (63, 74, 75).

CD4^{+} *T cells in cancer*

Some T cells express the co-receptor CD4, allowing these T cells to utilize the TCR to recognize antigen presented in the context of MHC-II (p: MHC-II). In

addition to differences in the structure of MHC-I vs. MHC-II, the other major difference is where and when MHC-II is expressed as compared to MHC-I. While MHC-I is expressed on essentially every cell in the body, MHC-II is canonically expressed on “professional” Antigen Presenting Cells (APC), which are cells specifically differentiated to process and present antigen (mainly B cells, macrophages and dendritic cells). Therefore, $CD4^+$ T cells normally only recognize antigen presented by these cell types. This is with the caveat that MHC-II expression can be induced on other cell types, often by IFNγ.

CD4+ T cells are classically called “T-helper” cells, and the nomenclature commonly delineates subsets of CD4+ T cells as “Th_x”. The role of T helper cells often is to “Help” the immune response-that is modulate it in various ways. A few examples of what ‘help’ may manifest as include activating CD8+ T cells and improving antigen presentation (the job of Th_1 cells), inducing neutrophil and granulocyte activation (Th_{17} cells), or slowing down CD8+ T cell function and antigen presentation (Treg cells). These T-helper subsets are commonly classified based on the abundance of certain transcription factors (TBET for Th_1, RORGT for Th_{17}, FOXP3 for Treg, etc.) and cytokine production (IFNγ for Th_1, IL17 for Th_{17}, and TGFβ for Treg, etc.) Given the broad range of potential functions of CD4+ T cells, these cells can potentially be beneficial or detrimental

to a host organism as it's immune system responds to the pathogen insult of cancer.

Th_1 cells are the first lineage of T cell discovered, by Tim Mossman in the mid 1980's (76). Because of the cytokines produced by Th_1 cells, these cells are important for clearing intracellular pathogens by inducing macrophage activation (77-79), for inducing B cell class switching to IgG (80) and for helping CD8+ T cells respond to infections (81). In coordination with expression of the transcription factor Tbet (82), Th_1 cells are classically thought to produce IFN-gamma, interleukin-2 (IL2) and TNF-alpha, though they are described as producing a host of other cytokines in response to various stimuli (83).

Tregs are CD4+ T cells that express the transcription factor FOXP3. These cells canonically have an immune-suppressive function, which may manifest in a variety of mechanisms. Recent literature supports that Tregs can induce immune-suppression by secreting anti-inflammatory cytokines (63, 84), reprogramming antigen presenting cells (APC) to an immune-suppressive phenotype, internalizing cytokines required for T cell activation, contact-dependent inhibition of effector cells by a Granzyme B-mediated mechanism

(85), and secreting molecules that dampen metabolism of surrounding immune cells. In normal circumstances, Tregs are a necessary part of maintaining immune homeostasis in the body. Evidence for the necessity of Tregs comes from humans and mice with genetic ablations of FOXP3, which leads to a body-wide autoimmune syndrome called immunodysregulation polyendocrinopathy enteropathy X-linked syndrome (IPEX) (86-89). Tregs may also play a critical role in suppressing the immune response at the end of an infection, and also in maintaining equilibrium with commensal organisms. In the context of cancer, Tregs generally are thought to be detrimental to the host and advantageous for cancer progression. A positive correlation between Tregs and tumor progression has been suggested (90). Interestingly, the role of Tregs varies depending on the type of tumor model used. For example, Tregs appear to be required for cancer progression in one orthotopic breast cancer model and our own studies of BCR-ABL^{+} B-ALL (63, 64), but Tregs do not play as fundamental of a role in an acute myeloid leukemia model (62).

Th_{17} cells are a recently discovered lineage of cell that is critical for recruitment and activation of granulocytes (91, 92). The role of Th_{17} cells in tumors is somewhat controversial and may be highly dependent on the tumor type.

However, there is firm evidence in mice supporting either a benefit or a detriment for Th_{17} cells in tumors of various types (93-96).

Another recently described lineage of T helper cell is the cytolytic (T_{CL}) cell. These cells are purported to directly kill target cells through Granzyme/perforin or Fas-mediated mechanisms (83, 97). This lineage of $CD4^+$ T cell is thought to lyse target cells in a p: MHC-II dependent manner. This lineage is theoretically important since many B lineage leukemic cells express MHC-II, and thus could potentially be targets for lysing by T_{CL} cells. These cells are generally characterized by expression of Granzyme B, Perforin, and Fas signaling components(83).

Studying T cells in Cancer Immunotherapy

T cells are a major arm of adaptive immunity, and their function in response to many pathogen insults including cancer-has been well characterized. However, the vast majority of studies that examine T cell responses examine the bulk $CD4^+$ or $CD8^+$ T cell response. This is because technology was only available in the past 30 years to examine antigen-specific T cell responses to a peptide: MHC complex (98-100). The advent of molecular cloning of TCRs, and then TCR

transgenic mice allowed investigators to interrogate an antigen-specific immune response by adoptive transfer of TCR transgenic T cells into mice, followed by challenge with the pathogen of interest. This approach still had drawbacks, as these transferred T cells were a monoclonal population (all T cells had exactly the same TCR, and thus responded in a monoclonal response). To examine the polyclonal T cell response to a pathogen insult required another technological breakthrough. By the mid 1990's, a number of crystal structures of p: MHC-I combinations had been solved, and there was an understanding of certain pathogen peptides that bound to MHC-I. This information was used to generate a p: MHC-I labeled with biotin, which could then be attached to Streptavidin linked to a fluorochrome. These so-called p: MHC "tetramers" (a reference to the 4-sided tetrameric assembly of p: MHC's on the streptavidin) allowed labeling of endogenous T cells engendered with a TCR that bound to the labeled p: MHC-I (101). This breakthrough technology allowed investigators to use conventional flow cytometry to analyze $CD8^+$ T cells as they responded to cognate p: MHC-I *in vivo* and thus gain a highly detailed level of understanding of the immune response (102).

The p: MHC tetramer was a major breakthrough because it allowed investigators the resolution necessary to discern and directly enumerate distinct antigen-

specific T cells. Simply examining bulk activated T cells in response to a given pathogen was not sufficient, since even Specific Pathogen-Free mice could be responding to many pathogen insults at a given time. So understanding which *in vivo* responses were due to the pathogen of interest, and which were due to other peptide: MHC encountered by the T cells was impossible prior to the p: MHC tetramer.

In the late 1990's to early 2000's, similar technology was developed to interrogate $CD4^+$ T cells by using p: MHC-II tetramers. This technology potentially allowed for *in vivo* labeling of antigen-specific $CD4^+$ T cells. However, a biological hurdle associated with how peptides bind to MHC-II limits the feasibility of p: MHC-II tetramers for any given peptide. While MHC-I binds to peptides with a binding cleft that is "closed" on the ends (thus allowing only ~9-11 amino acids to bind the MHC-I), MHC-II has an "open" binding cleft, where potentially very long peptides might bind MHC-II. This means that computational algorithms based strictly on interaction of amino acids with the MHC-II binding cleft may not be useful since binding points outside of the binding cleft may be important for a given peptide: MHC-II interaction. Because of this drawback, there is a relative paucity in understanding the role of $CD4^+$ T cell responses to pathogen insults. For example, to the best of our knowledge, our study is the

first to characterize a leukemia-reactive $CD4^+$ T cell population responding to leukemia *in vivo*.

Conclusions

Cancer is now understood to comprise an entire tissue, with its own vasculature, stroma, and immune features. While many studies have examined the role of exogenous T cells to interact with the tumor (via adoptive transfer), there is scant understanding of how the endogenous T cells interact with the tumor. Cancer has an amazing capacity to undergo natural selection. Cancer cells have a highly unstable genome, and this allows many rounds of mutation to achieve a desired outcome. As such, it seems clear that a cancer should be able to acquire mutations that render it a favorable advantage over the immune system. A main focus of this thesis is to examine cancer and immunology with an appreciation for the capacity of the tumor to have selective advantages. As such, a common idea that I am exploring in this thesis is that, just as a tumor requires vascular stromal tissue to maintain the life of the tumor, maybe the tumor also requires immune cells for survival. Perhaps, at steady state, the immune response to a tumor is fully beneficial to the tumor.

Chapter 2 Tracking BCR-ABL specific CD4+ T cells during steady state and responding to leukemia

Introduction

[1]Cancer immunotherapy is an effective clinical approach in malignancies with high rates of non-synonymous mutations (52, 103-105). Most cancer immunotherapy approaches currently focus on neo-antigen specific T cells, which ideally respond to mutations in proteins that drive tumorigenesis (69, 106-108). However, identifying non-synonymous immunogenic mutations in driver

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[1] The majority of Chapter 2 is previously published.

63. Manlove, L. S., Berquam-Vrieze, K.E., Pauken, K.E., Williams, R.T., Jenkins, M.K., Farrar, M.A. 2015. Adaptive immunity to leukemia is inhibited by cross-reactive induced regulatory T cells. *The Journal of Immunology*.

genes is not always possible, thereby necessitating the use of either multiple antigens, or cross-reactive self-antigens, to prevent immune escape. This problem is illustrated in B cell acute lymphoblastic leukemia (B-ALL), which has few non-synonymous mutations (109). However, B-ALL is characterized by chromosomal translocations that give rise to fusion proteins encoding neo-antigens that drive transformation (31). We focused on BCR-ABL$^+$ B-ALL, which creates a neo-antigen at the fusion of BCR to ABL. Immunotherapy is an attractive goal in BCR-ABL$^+$ B-ALL because current therapies elicit only transient responses and long-term survival is poor. CD4$^+$ T cells from patients with BCR-ABL$^+$ B-ALL can secrete IFNγ upon *ex vivo* restimulation with peptides from the BCR-ABL fusion, but these responses are inadequate to eradicate leukemia in vivo (110, 111). To understand why BCR-ABL specific immunity fails to eliminate BCR-ABL$^+$ B-ALL in mice, we identified BCR-ABL specific CD4$^+$ T cells and tracked their responses to leukemia in vivo.

To examine anti-leukemia T cell responses we made use of a BCR-ABL$^+$ B-ALL mouse model that recapitulates the human disease (112). To track anti-leukemia T cell responses, we generated a BCR-ABL peptide (BAp): MHC Class II tetramer reagent. We demonstrate that an adaptive immune response is elicited

against BCR-ABL^{+} B-ALL and this response limits leukemia progression. BAp:I-A^{b}-specific T cells exist in mice and proliferate in response to immunization with BAp peptide plus an adjuvant. Inoculation with live BCR-ABL^{+} leukemic cells also resulted in proliferation of BAp:I-A^{b}-specific T cells. However, these cells were converted into Treg cells and thus unable to eliminate leukemia. Importantly, transient Treg ablation with *Foxp3$^{DTR/DTR}$* mice resulted in extended lifespan of leukemic mice, which correlated with increased number of CD44^{hi}, Ly6C^{+} BAp:I-A^{b}-specific T cells, suggesting that induction of Treg cells by the leukemia led to decreased priming and Th1-like CD4^{+} T cell differentiation.

Materials and Methods

Mice

C57BL/6 mice and *Cdkn2a$^{-/-}$* (strain 01XF6, B6, 129-Cdkn2a^{tm1Cjs}/Nci, (113)) mice came from the National Cancer Institute. *Foxp3-GFP* (stock# 006772) mice came from Jackson Laboratories (Bar Harbor, ME). *Bim$^{-/-}$*, *OT-IxRag2$^{-/-}$, Foxp3$^{DTR/DTR}$, OT-IIxRag2$^{-/-}$* and *SM1xRag2$^{-/-}$* mice were generated locally as previously described (75, 98, 114-116). Mice were housed at the University of Minnesota in specific pathogen free conditions and all experiments were

approved by IACUC. *Statb5CA* transgenic mice were previously described and have been maintained by our lab (117).

Immunizations

Mice were immunized with Complete Freund's Adjuvant (CFA)+BAp subcutaneously in the hind flank.

Anti-TGFβ in vivo treatment

Mice were treated with anti-TGFβ (clone 1D11, Bio X Cell) or isotype (clone MOPC21, Bio X Cell) with 1mg i.p. on the same day that the mice were inoculated with leukemia, followed by 200μg i.p. every-other-day for fourteen days.

Anti-IL10R in vivo treatment

Mice were treated with anti-IL10R (clone 1B1.3A, Bio X Cell) or isotype (clone HRPN, Bio X Cell) with 0.3mg i.p. every-third-day.

Diphtheria Toxin Treatment

Mice were treated with 0.2µg/mouse diphtheria toxin (List Biologicals) by i.p. injection every-other-day unless otherwise described in figure legends. Treg depletion was analyzed by monitoring GFP^+, $CD4^+$ cells.

Leukemia model

The BCR-ABL^+ B Acute Lymphoblastic Leukemia model has been previously described (118). Briefly, *Cdkn2a*$^{-/-}$ mouse bone marrow cells were transduced with viral supernatant containing a BCR-ABL (P190)-IRES-GFP retrovirus (119). Cells were cultured in RPMI1640+10%FBS+1%Penicillin-Streptomycin+1%L-Glutamine+0.001%beta-mercaptoethanol in a 37°C incubator. 2,500 live cells were adoptively transferred i.v., into host mice without prior irradiation. The SP1 cells were derived from leukemia in a *STAT5bCAxPax5*$^{+/-}$ mouse as previously described (120). MHC-$II^{-/-}$ leukemia was generated by crossing *Arf*$^{-/-}$ mice with I-$A^{b-/-}$ mice to generate *Arf*$^{-/-}$*, I-A*$^{b-/-}$ mice. Bone marrow from this genotype was transduced with the BCR-ABL IRES GFP retrovirus to generate MHC-$II^{-/-}$, BCR-ABL^+ GFP^+ leukemic cells.

Tetramer production

The BAp:I-A^b tetramer and others used herein were produced as described (121). Purified monomer was tetramerized with SA-PE or SA-APC.

Tetramerization was conducted as described below with an example:

1) Convert concentration of monomer from mg/mL to mM. Divide concentration by 0.066, for instance, 3.529 mg/mL/0.066=49.38 mM.
Sample calculation: **C_1V_1 monomer=C_1V_1 SA-PE**
(49.38 mM)(X)=(9.5 mM[SA])(50 ml) when 9.5 mM[SA] and 7 mM[PE] (but this varies from vial to vial)

2) **X=9.61ul, but because we want 4 monomers for every molecule of SA, we multiply 9.61 by 4.5 to ensure saturation.**

3) **9.61x4.5= 43.28**

4) **43.28+50ml SA-PE= 93.28 ml total.** Spec this mixture to determine the molarity of PE (choose fluorescence on the spec instead of protein)

5) Use this molarity for the next calculation (example: 3.8 mM)

6) **(3.8 mM [PE])=(9.5 mM[SA])/7mM [PE])=5.15 mM[SA]**

7) **(5.15 mM[SA])(93.3 ml)=(1mM)(X) X=480.495**

8) **480.495ml - 93.3 ml = 387.195 ml**

9) **Add 387.195 ml PBS-A** to the tube to obtain a 1 mM concentration of tetramer

Antigen-Specific CD4+ T cell Enrichment Strategy

I-A^b tetramer-binding cell enrichment was performed as described (121). Tetramers loaded with PE and APC were used together, and tetramer double-positive events were analyzed to increase sensitivity and specificity (122). Cells were enriched with either Miltenyi or StemCell anti-APC and anti-PE reagents following manufacturer's protocols. The enriched fractions were mixed with Accucheck counting beads (LifeTechnologies, Grand Island NY) for cell enumeration. The following procedure details the enrichment strategy for StemCell kits, which is what was used most of the time.

Buffers:

"Sorter Buffer"

0.5% Serum

1x phosphate-buffered saline (Dulbecco's)

*all flow analysis will also be run in this buffer. These enrichments tend to leave quite a few dead/dying/sticky cells. Running in very low concentrations

1) Sacrifice the mice with cervical dislocation/CO_2 in WMBB 2-240

2) Spray the outside of the mouse with 70% EtOH

3) Open the mouse with a midline dermal incision, leaving the peritoneum intact

4) Use manual force to extend the incision circumferentially about the midline of the mouse

5) gently pull the skin down and up, to deglove the mouse

6) Excise the inguinal, axillary, brachial, superficial cervical, submandibular, mesenteric, and para-aortic lymph nodes, as well as the spleen(SLO).

7) Place all Secondary Lymphoid Organs into 1 well of a 6 well plate filled with 1mL ice-cold sorter buffer. Cells should be on ice for the rest of procedure, unless otherwise stated.

8) Deglove any remaining skin on the 2 hind legs.

9) Use snips to sever the femur immediately distal to the iliac joint.

10) Use snips and Kim wipes to clear excess tissue from the long bones.

11) Load a 5mL syringe with a 26ga needle. Aspirate 2mL of ice-cold sorter

buffer through the long bone lumen using the syringe. Repeat for femurs, tibias, and fibulas.

12) Cut small squares (~1cm x 1cm) of 70um mesh netting.

13) Use forceps to place the square in the center of the bottom of the well of the 6-well plate. Use forceps to pile all Secondary Lymphoid Organs on top of the mesh. Use the plunger of a 5mL syringe to mash the Secondary Lymphoid Organs through the 70um mesh square.

14) Move all tissue/liquid 15mL polypropylene conical tube.

15) Rinse mesh in the 6 well plates w/ another 2mL sorter buffer. Then, do another mash + rinse. We want to make absolutely sure we recover all cells at this step. Top off the conical w/ sorter buffer and spin down 1600rpm 5min 4C in enclosed BSL-2 containers.

16) Aspirate liquid into bleach-prefilled vacuum flasks with secondary containment

17) add Fc block (clone 2.4G2 Serum Free Media + 2%mouse serum + 2% rat serum) to a final volume of 2x the pellet itself-using the gradations in the 15mL

conical to guide you. So if your pellet is about 200uL, then add another 200uL of sorter buffer.

18) resuspend the pellet by vigorously sliding the 15mL conical over a 15mL tube rack.

19) Add tetramer to the sample at a final concentration of ~10nM , depending on the dose response curve for the current lot of tetramer. The tetramer should say what its concentration is. For 2W1S:IA^b, it seems to be at 1uM (i.e. 1000nM) usually, so a 1:100dilution of tetramer into Fc block /cell pellet should suffice.

-so, if you have 200uL pellet, then add 200uL Fc Block + 4uL Tetramer.

20) Vortex and incubate for 1 hour at **room temperature** in the dark.

21) Wash with 15mL cold sorter buffer at 1600rpm 5min 4C in enclosed BSL-2 centrifuge.

22) Aspirate liquid into a bleach-prefilled vacuum flask with secondary containment and add 1mL sorter buffer to the pellet.

23) Vigorously slide the tubes over a 15mL tube rack to resuspend.

24) Add 15uL StemCell PE selection cocktail + 15uL StemCell APC selection cocktail

25) Vortex quickly and incubate **room temperature** for 15 minutes in the dark.

26) Set up StemCell magnets at **room temperature**

27) add 45uL StemCell enrichment beads to each pellet (they should still be at ~1mL volume)

28) incubate 10 min at **room temperature** in the dark.

29) Add 1mL ice cold sorter buffer to each tube (the total volume should now approximate 2.5mL, which is the optimal volume for loading onto the StemCell magnet)

30) Put a piece of 70um nylon mesh on top of a FACS tube

31) cut the last ~4mm off of a p1000 tip (the goal is to widen the bore just slightly) and set a p1000 to ~800uL.

32) Use the pipet to gently resuspend the cells/2.5mL liquid.

33) Aspirate the cells into the FACS tube through the 70um mesh. Be aware, it may take multiple pieces of 70um mesh to aspirate all the liquid into the FACS

tube, since these will get clogged quickly. If they get clogged, just throw out that piece of mesh and get a new one.

34) Put the FACS tubes on the magnet for 5min **room temperature**

35) Either tip the StemCell magnet rack so that the FACS tubes empty into 15mL conical tubes, or manually decant the FACS tubes into 15mL conical tubes.

36) Add 2.5mL sorter buffer to the FACS tube and return to the magnet (if it was ever removed from the magnet-it's fine to remove or not remove the FACS tube from the magnet after decanting, but make sure the FACS tube is in the magnet while decanting!)

37) incubate for 5min **room temperature** and decant as above. Repeat one more time after this for a total of 3x

38) After 3 washes (thus leaving you with a total of 7.5mL decanted liquid), evaluate the FACS tube and make an executive decision about if more washes may be needed. They will help increase your enrichment efficiency at the expense of time and cell viability.

39) equalize volumes in the 15mL conicals w/ sorter buffer and spin down in an enclosed BSL-2 centrifuge.

40) Add 50uL of antibody cocktail to the cell pellets (I normally take (40uL x # of samples *0.10) + (40uL x # of samples) = volume of sorter buffer; plus 1.1uL of each antibody/sample. A normal panel might be:

APC: Tetramer (already stained)

PE: Tetramer (already stained)

BV421: CD25 (PC61)

BV510: CD19 (1D3)/CD69

BV650: CD45.1/CD8a (55-6.7)

BV785: CD44 (IM7)

PECy7: Ly6C

PerCPCy5.5: CD4 (RM4/5)

APCef780:

NK1.1

CD11B

CD11C

CD45.1

B220

F4/80

FITC: PD1 (J43)

-I would be cautious running over 10 colors, particularly if fix-perming. [As will all F.C.] the analysis seems to run cleaner when less compensation is involved. The catch here is that the event number may bee exceedingly low, so I wouldn't want to mis-report because of a weird F.C. error.

41) Incubate on ice 30min dark.

42) Wash samples w/ 3mL sorter buffer in enclosed BSL-2 centrifuge

43) Aspirate the supernatant into a bleach-prefilled vacuum flask with secondary catchment and resuspend in 600uL sorter Buffer + 100uL Counting beads in a FACS tube (i.e. 12 x 75mm polystyrene tube).

44) Move the samples to the flow cytometry core facility to analyze. Make sure to run bleach through the cytometer upon finishing, followed by water. As well, the waste tank for the cytometer must be bleach-prefilled.

45) Clean up by scrubbing tools with hot water, Bac-Down soap, and 1:1000 bleach. Make sure to rinse the tools vigorously to remove bleach.

46) Mouse carcasses need to be closed into airtight bags and returned to the BSL-2 cooler in WMBB 1-514 if BSL-2 experiments.

47) All exposed consumable wastes should be double-bagged in autoclave bags and sterilized by autoclaving.

48) All liquid waste should soak for >20 minutes with bleach, and then can be washed down the sink drain with excess water.

Intravenous (i.v.) labeling

I.v. labeling was performed using 15μg anti-CD45.1 antibody conjugated to PerCP-Cy5.5 (eBioscience). Antibody was suspended in 200μL PBS and the mixture was injected by tailvein i.v. injection. Three minutes later, mice were euthanized and SLO was harvested.

Histology

Histology was performed as previously described and antibodies are listed below (123). A brief protocol is detail below for most staining conditions.

1. Fix slides in Acetone (-20 C) for 10 minutes. Allow to air dry ~5 minutes

2. Apply PAP Hydrophobic Pen around the edges of the slides. Allow drying for 3-5 minutes

3. Rehydrate slides in PBS. Sections will need up to 500μL of PBS for complete coverage. NOTE: After this step, DO NOT allow the slides to dry out. This will compromise ability to image the slide.
4. Blocking Solution: Apply 200 uL of 5% BSA PBS solution. Place in humidified chamber for 45 minutes to one hour.
5. Add primary antibody stain mixture to slides (in PBS - volume of 150 uL). Stain for 60 minutes.

FITC/AF488: Goat anti-GFP (probably 1:100 is good)

PE/AF555:

APC/AF647: rat anti-Foxp3 (1:100, clone FJK16S)

UV/DAPI:

6 Wash slides 3x in PBS. Apply 200 uL of PBS to wash. Let sit for 1-3 minutes after each application of PBS.

7. Add secondary antibody stain mixture to slides (in PBS - volume of 150 uL) for 60 mins

FITC/AF488: Bovine anti-goat AF488 (1:200)

PE/AF555:

APC/AF647: donkey anti-rat AF647

UV/DAPI:

8. Wash slides 2x in PBS. Apply 200 uL of PBS to wash. Let sit for 1-3 minutes after each application of PBS.

9. Incubate slides with 200 uL of 5% rat serum in PBS for 20 minutes.

10. Add other antibodies - in this case CD3 PE (5% rat serum in PBS - volume of 150 uL) for 60 mins (quick spin CD3 down to remove deconjugated PE.)

FITC/AF488:

PE/AF555: Hamster anti-mm CD3 (1:100, 145-2C11 clone)

APC/AF647:

UV/DAPI: Rat anti-mm/hs B220 (BV421) (1:100)

11. Wash slides 3x in PBS. Apply 200 uL of PBS to wash. Let sit for 1-3 minutes after each application of PBS.

12. Apply 40 uL of prolong gold per slide (20 uL per section). Apply coverslip.

Antibodies

Antibodies for flow cytometry and histology include CD3 PE, CD4 (RM4-5) PerCPCy5.5, CD8 (53-6.7) BV650, CD11c (N418) PE, FOXP3 (FJK16S) PE, were purchased from BD Biosciences (San Jose, CA); NK1.1 (PK136), CD11b (M1/70), CD11c (N418), B220 (RA3-6B2), and F4/80 in APC-eFluor780; PD1 (J43) FITC, CD73 eFluor450, FR4 PE-Cy7, PDL1 PerCP-eFluor710, MHC-II I-Ab eFluor450, IL7R PE, and all ELISpot antibodies were purchased from eBiosciences (San Diego, CA), and IgM (Fab') APC was purchased from Jackson Immunoresearch (West Grove, PA). Rat IgG1 (HRPN) PerCP-Cy5.5 Isotype and Rat IgG2a (2A3) violetFluor450 Isotype were purchased from Tonbo Biosciences (San Diego, CA). Cells from enriched fractions were analyzed on an LSR-II Fortessa cytometer (BD Biosciences, San Jose CA) and data was analyzed in FlowJo (Treestar, Ashland OR). Intracellular cytokine staining was done using BD Cytofix/Cytoperm reagents and protocols (BD Biosciences, San Jose CA). Anti-IL10 (JES5-16E3, PE, eBiosciences), TGFB1(LAP) (TW7-16B4, ef710, eBiosciences), IFNγ (XMG1.2, BV421, BD Horizon), and TNFα (MP6-XT22, AF488, eBiosciences) were stained by this procedure.

ELISPOT

C57BL/6 mice were immunized with e1a2 peptide (GEGAFHGDAEALQRPVASDF, 2 mg/ml) or 2W1S peptide (EAWGALANWAVDSA, 0.2 mg/ml) emulsified in Complete Freund's Adjuvant (CFA) (Sigma-Aldrich, St. Louis, MO) and harvested two weeks later. $CD4^+$ T cells were enriched using a mouse CD4 T cell Isolation Kit (Miltenyi, Bergisch Gladbach Germany). Naïve mouse splenocytes were harvested, irradiated and mixed at a 1:1 ratio with the immunized $CD4^+$ T cells in wells coated with IFN-gamma capture antibody (AN-18) at a concentration of 15mg/mL. Synthetic overlapping peptides that spanned the BCR-ABL p190 fusion (Genscript, Piscataway, NJ) were added to individual wells. 2W1S peptide and Concanavalin A were used as positive controls. Samples were developed using a biotinylated IFN-gamma detection antibody (R4-6A2) and streptavidin-Alkaline Phosphatase with 5-Bromo-4-chloro-3'-indolyphosphate (BCIP) and Nitro-blue tetrazolium (NBT) developing agents and photographed and analyzed with a ImmunoSpot S6 Microanalyzer using the ImmunoCapture 6.3 and ImmunoSpot 5.0 Pro DC software (Cellular Technology Ltd. (Shaker Heights, OH).

Statistics

Standard normality tests suggested departures from normality, so non-parametric tests (Mann-U Whitney test for two groups, Kruskal-Wallace & Dunns' Test for more than two groups) were used unless otherwise stated. Normality assessments and non-parametric tests were done in GraphPad Prism (LaJolla, CA). Linear regressions and correlation coefficients were estimated in GraphPad Prism.

Modeling BCR-ABL$^+$ B Acute Lymphoblastic Leukemia in mice allows study of the host anti-leukemia immune response

The adaptive immune system mounts a response to BCR-ABL$^+$ leukemia

To examine whether BCR-ABL$^+$ leukemia elicited an adaptive immune response in mice, we adoptively transferred *Cdkn2a*$^{-/-}$ BCR-ABL$^+$ pre-B cells into healthy, immune-competent recipient mice. This model incorporates two genetic alterations common in high-risk human B-ALL: BCR-ABL and loss of the tumor suppressor ARF (124). Further, these cells can be transferred into unconditioned host mice where they faithfully form B-ALL (118). The leukemic cells express

CD19, B220, IL7R, MHC-II, and low levels of immunoglobulin heavy chain (IgH) and therefore closely resemble human progenitor B-ALL (Figure 2-1).

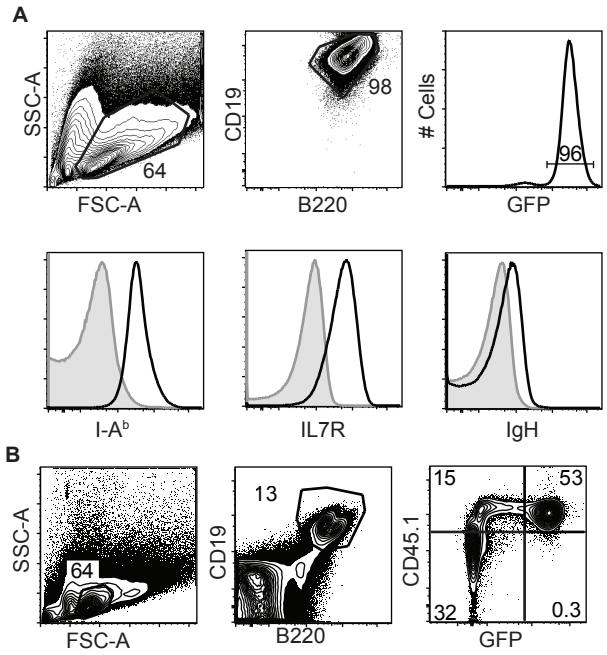

Figure 2-1: BCR-ABL$^+$ B-ALL forms in mice.

A. Phenotype of BCR-ABL$^+$ cells grown *in vitro*. Cells were analyzed after 3 weeks in culture, and express CD19, B220, GFP, I-A^b, IL7R, and Ig Heavy Chain. Isotype controls are in grey and experimental stains are in black; shown are representative stains. **B**. BCR-ABL$^+$ leukemic cell phenotype *in vivo*. Live lymphocytes are gated for CD19$^+$ B220$^+$, and CD45.1$^+$GFP$^+$ events are leukemic cells.

In syngeneic (C57BL/6) hosts, leukemia arose in the bone marrow and the splenic red pulp with small numbers of leukemic cells in the lymph nodes (Figure 2-2). Of note, the majority of leukemic cells were i.v.+ in the spleen and i.v.- in

the lymph nodes, again supporting that the leukemic cells in the spleen were associated with the red pulp.

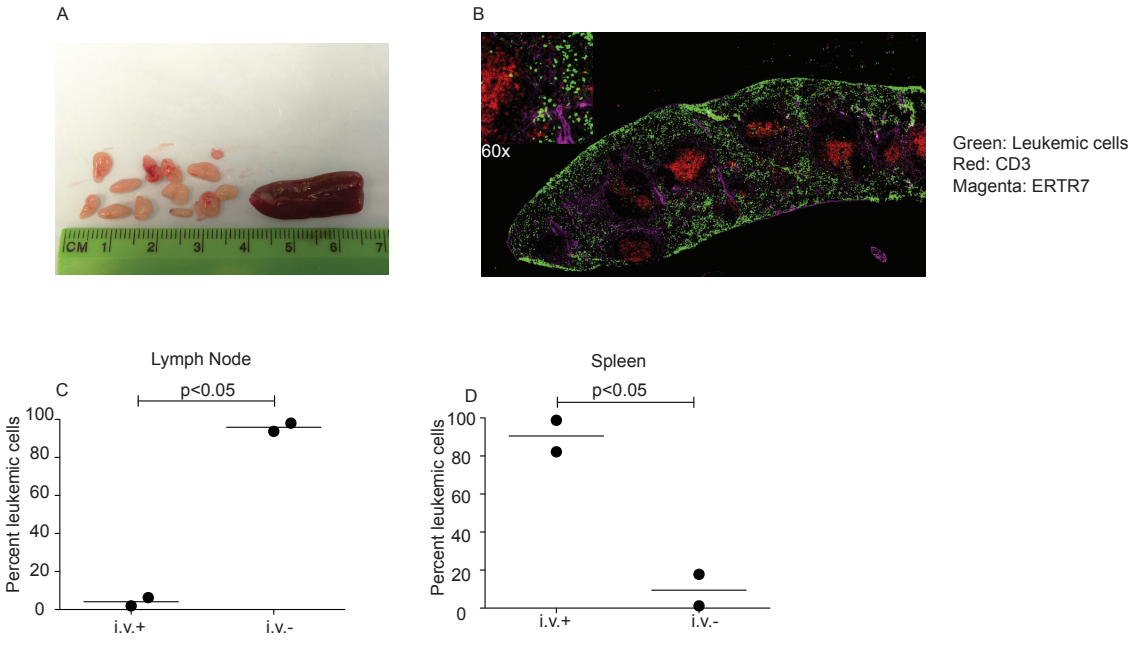

Figure 2-2: Representative leukemic tissue and histology

A. Representative lymph nodes and spleen from a mouse with leukemia. **B**. Representative histology of a leukemic mouse spleen at 14 days post-transfer. Leukemic cells are GFP^+(Green) and are localized in the red pulp ($ERTR7^+$, magenta). T cells are stained with CD3 in Red. **C, D.** Leukemic mice were injected with anti-CD45.1(PerCP-Cy5.5) i.v. and harvested three minutes later. Flow cytometry was used to analyze the fraction of leukemic cells which were i.v.+ or i.v.- in the Spleen or Lymph Nodes. Two independent repeats were conducted, statistics were analyzed by Mann-Whitney U test.

To determine if the adaptive immune system had any control over $BCR\text{-}ABL^+$ B-ALL progression we compared leukemic cell counts (referred to hereafter as leukemic burden) in C57BL/6 and OT-Ix*Rag2*$^{-/-}$ mice. OT-Ix*Rag2*$^{-/-}$ mice were

used because they have T cells but no leukemia-specific adaptive immunity. The percentage of leukemic (CD45.1$^+$, GFP$^+$) B cells in the bone marrow (BM) was significantly higher in OT-Ix*Rag2*$^{-/-}$ mice than in C57BL/6 mice (Figure 2-3).

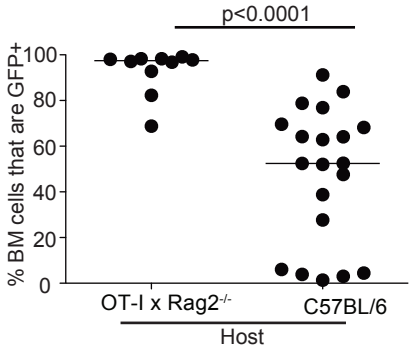

Figure 2-3: Leukemic burden varies with presence of adaptive immunity

GFP$^+$ cells in BM as a percentage of the CD19$^+$B220$^+$ cells in C57BL/6 and OT-Ix*Rag2*$^{-/-}$ hosts. Twenty days after leukemic cell transfer BM cells were harvested from WT or OT-Ix*Rag2*$^{-/-}$ hosts and CD19$^+$B220$^+$GFP$^+$ cells were gated as in A. GFP$^+$ cells in BM as a percentage of the CD19$^+$B220$^+$ cells are graphed. P-value from Mann-Whitney U Test. Data shown from three separate experiments.

Steady-state vs. peptide-CFA immunization of two leukemia-specific T cell populations

BAp:I-A^b-specific T cells in naïve mice.

We studied the interaction between BCR-ABL$^+$ B-ALL and CD4$^+$ T cells by tracking leukemia-specific T cells. Since BCR-ABL is a somatic mutation, the peptide spanning the fusion of BCR to ABL could potentially elicit a T cell

response. Using an ELISpot assay, we found a MHC-II binding peptide in the BCR-ABL fusion that elicited a $CD4^+$ T cell response (Figure 2-4), and constructed a peptide:I-A^b monomer wherein this peptide (BAp) was covalently linked to biotinylated I-A^b (referred to as BAp:I-A^b). Of note, it is surprising that the BAp peptide was peptide #8. BAp is made up of sequence that is ~85% from ABL and only ~15% from BCR. Our hypothesis was that a peptide such as peptide #5 (which is approximately 50:50 derived from BCR vs. ABL) would have been the most immunogenic. However, if major contact residues are the defining feature of TCR recognition of cognate peptide: MHC, then previously published work would support that peptide #8 should be the best binding peptide, since it most closely aligns with high-quality contact residues (125).

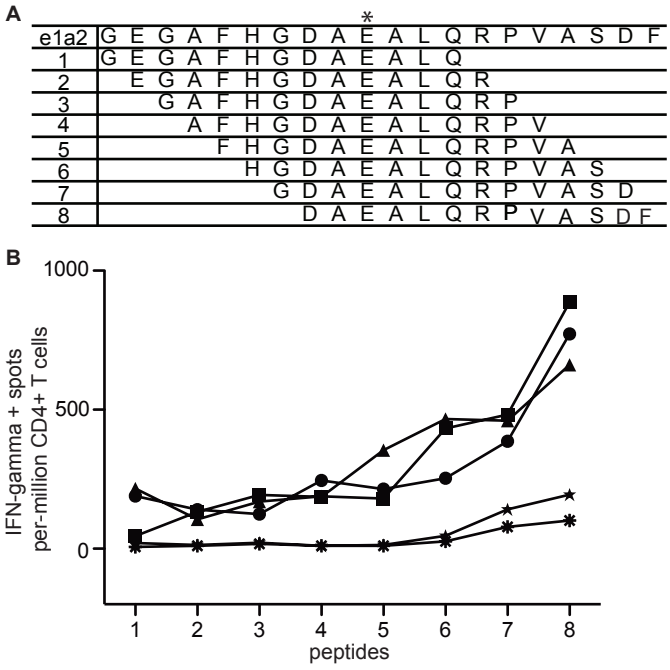

Figure 2-4: BCR-ABL elicits an immune response in C57BL/6 mice

A. Immunized mice expressed IFNγ when exposed to BCR-ABL. Peptides 1-8 on the X-axis are 13aa peptides that progressively span the BCR-ABL e1a2 fusion (top panel, asterisk represents the fusion junction). Mice were immunized with the full e1a2 20aa peptide. Two weeks later, cells were harvested from SLO and separate wells were pulsed with individual peptides 1 through 8 and then stained with anti-IFNγ. **B**. Five mouse repeats are shown.

We labeled BAp:I-A^b-specific T cells by tetramerizing BAp:I-A^b monomer with Streptavidin-PE or Streptavidin-APC. Using this reagent, we detected ~8 BAp:I-A^b-specific $CD4^+$ T cells per mouse (Figure 2-5). No BAp:I-A^b-specific T cells were detected in OT-IIx*Rag2*$^{-/-}$ and SM1x*Rag2*$^{-/-}$ mice, where all T cells are specific for an ovalbumin or a flagellin peptide bound to I-A^b, respectively,

demonstrating specificity of the BAp:I-A^b tetramer for the BAp:I-A^b-specific T cell TCR.

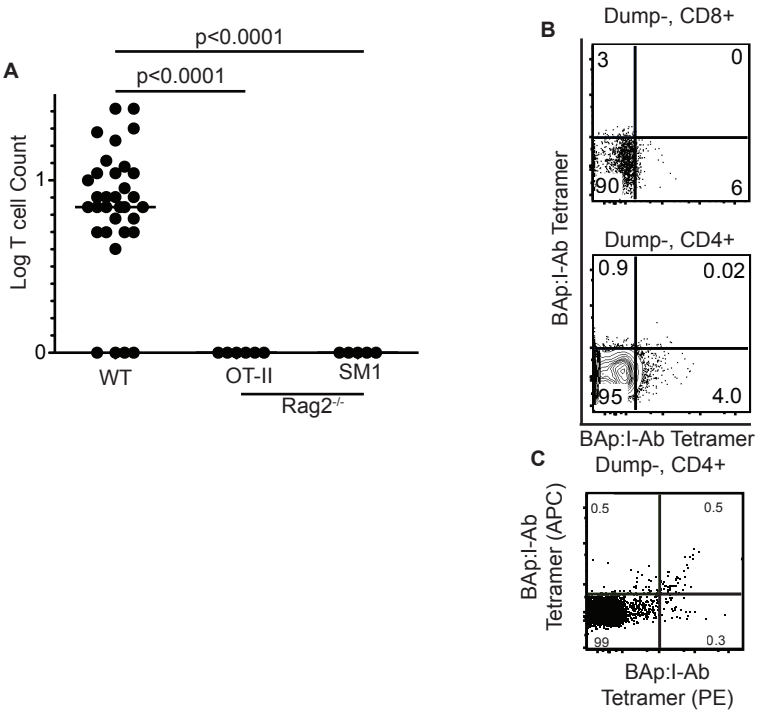

Figure 2-5: BAp:I-A^b tetramer detects T cells in C57BL/6 mice

A. Enumeration of BAp:I-A^b-specific T cells from C57BL/6, OT-IIx*Rag2$^{-/-}$, and SM1xRag2$^{-/-}$*. Shown are 44 mice from 10 experiments, y-axis is the Log(Y+1) of the BAp:I-A^b-specific T cell count. **B**. Naïve BAp:I-A^b-specific T cells are Dump$^-$, CD8$^-$, and CD4$^+$. Dump$^-$CD8$^+$ (top panel) or Dump$^-$CD4$^+$ (bottom panel) T cells were gated on and stained with BAp:I-A^b-APC (Y-axis) and BAp:I-A^b-PE (X-axis). **C**. Representative flow plot of T cells from a C57BL/6 mouse BAp:I-Ab tetramers labeled with APC and PE, 14 days after immunization with BAp peptide plus adjuvant.

The BAp:I-A^b-specific T cells typically expressed low levels of canonical activation markers including CD11a and PD1 in naïve mice, however expression of CD44 and FR4 were significantly, albeit modestly, increased on BAp:I-A^b-specific T cells in naïve mice (Figure 2-6).

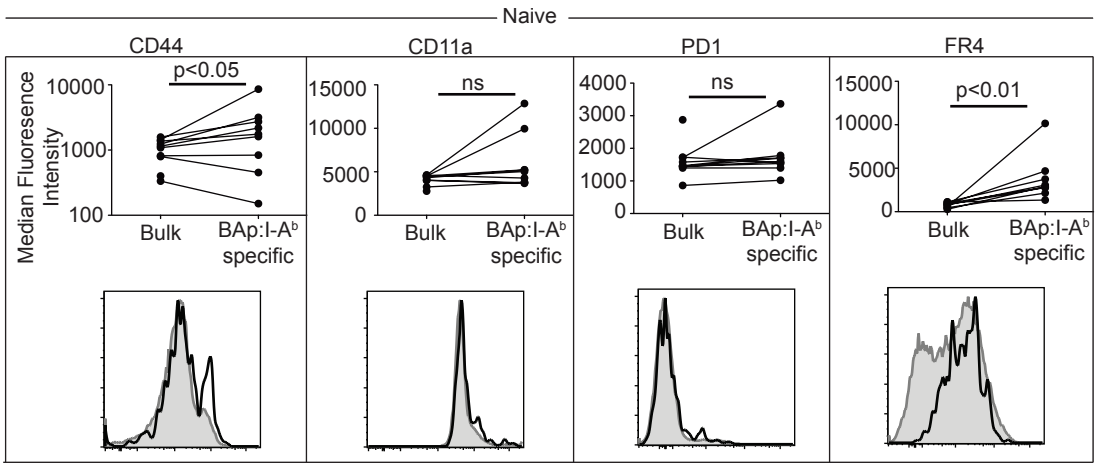

Figure 2-6: BAp:I-A^b tetramer-binding cell phenotype at steady-state

Phenotype of BAp:I-A^b-specific T cells from naïve mice (black lines) compared to the bulk CD4$^+$ population from the same mouse (grey lines). Shown are results from three independent experiments whose events were then concatenated for the histograms. All comparisons were made with Mann-Whitney U test (2 groups). Shown are 10 mice from 3 independent experiments, whose events were then concatenated for the histograms.

Nonetheless, all of these markers were uniformly expressed at high levels following BAp peptide + CFA immunization demonstrating that the BAp:I-A^b-specific T cells responded to cognate peptide immunization (Figure 2-5, Figure

2-7). Taken together, these findings show that BAp:I-A^b-specific T cells are capable of responding to BAp antigen.

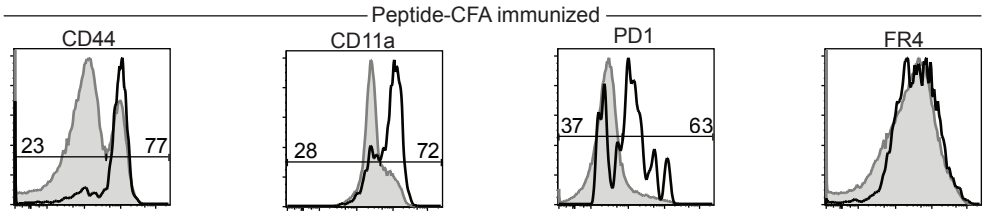

Figure 2-7:BAp:I-A^b tetramer-binding cell phenotype upon immunization

BAp:I-A^b-specific T cells from mice immunized with peptide plus adjuvant 14 days later (black lines). Grey histograms are bulk $CD4^+$ T cells. Shown are 10 mice from 3 independent experiments, whose events were then concatenated for the histograms. The numbers are percentage of cells falling into each given gate.

BAp:I-A^b-specific T cells respond to BCR-ABL^+ B-ALL

Despite their limited numbers, we hypothesized that BAp:I-A^b-specific T cells might still respond to BCR-ABL^+ leukemia. To test this hypothesis, we tracked BAp:I-A^b-specific T cells following adoptive transfer of leukemic cells into C57BL/6 mice and observed a significant accumulation of BAp:I-A^b specific T cells at 14 and 25 days post-transfer of BCR-ABL^+ leukemic cells (Figure 2-8). CD44 expression on BAp:I-A^b-specific T cells also increased in a stepwise fashion at 14 and 25 days post-transfer, suggesting that these cells were recognizing antigen in leukemic mice (Figure 2-8).

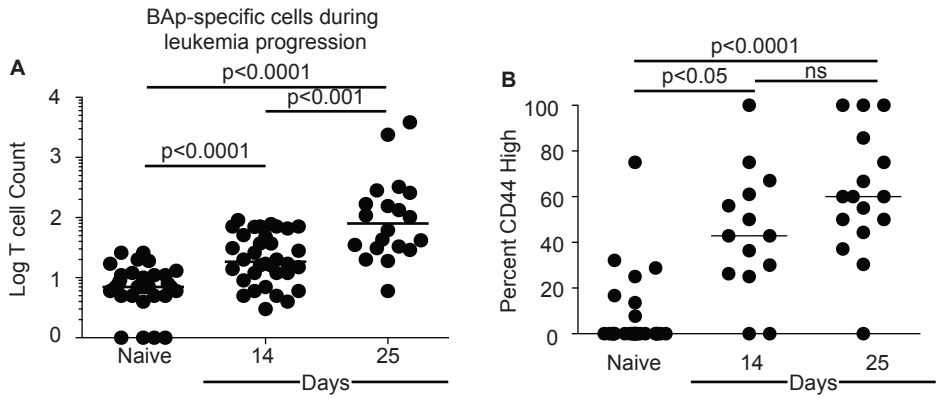

Figure 2-8: BAp:I-A^b-specific T cells experience antigen and respond to BCR-ABL$^+$ leukemia *in vivo*

A. 2,500 live BCR-ABL$^+$ leukemic cells were transferred into mice. BAp:I-A^b-specific T cells were counted at fourteen days or twenty-five days post-inoculation. Y-axis is the Log (Y+1) of the BAp:I-A^b-specific T cell count. Cumulative results from more than three experiments are shown; data is from greater than three independent experiments. **B.** Percent CD44-high expression on BAp:I-A^b-specific T cells. The data was collected as in A.

This response was antigen-specific as BAp:I-A^b-specific T cells did not proliferate or upregulate CD44 in response to a BCR-ABL negative leukemia. Thus, BAp:I-A^b-specific cells proliferate but do not eliminate leukemia. Additionally, we inoculated mice with a previously described BCR-ABL$^-$ adoptive transfer B-ALL "SP1" and found that BCR-ABL$^-$ B-ALL did not induce expansion or upregulation of CD44 on BAp:I-A^b-specific T cells (Figure 2-9). While the experiments using SP1 cells yielded very few BAp:I-A^b-specific T cells (which makes phenotypic analysis of these cells challenging) the result of having detected few BAp:I-A^b

tetramer-binding events in SP1 leukemic mice also supports the conclusion that BAp:I-A^b-specific T cells are likely not proliferating in response to BCR-ABL^- (SP1) leukemia.

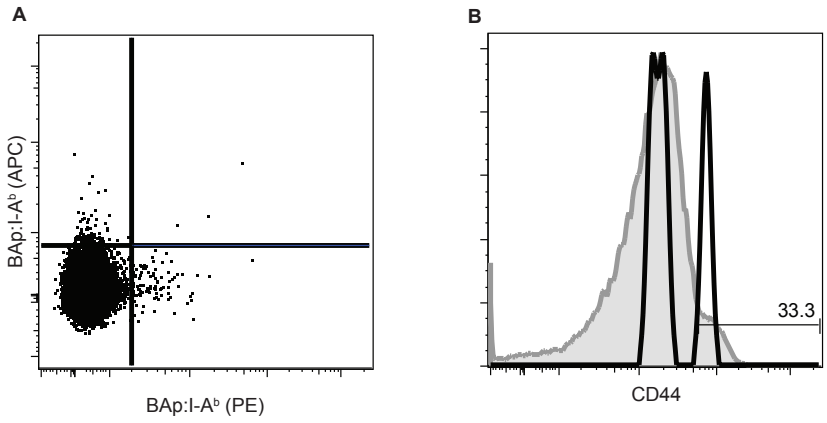

Figure 2-9: BAp:I-A^b-specific T cells do not proliferate or experience antigen in response to BCR-ABL^- B-ALL

A. C57BL/6 mice were adoptively transferred with SP1 leukemia (120). Fourteen days later, SLO were harvested from three independent mice and stained with BAp:I-A^b tetramer. Samples were analyzed by flow cytometry. Shown is $Dump^-CD4^+CD8^-$, Tetramer double positive. In SP1-leukemic mice, three events were uncovered. **B**. CD44 expression was analyzed on BAp:I-A^b-specific T cells from the mice harvested in A. 30% of the BAp:I-A^b-specific T cells were $CD44^+$ and the majority (66%) were $CD44^-$, suggesting these cells had not experienced cognate BAp:I-A^b.

$BCR\text{-}ABL^+$ leukemic cells also expressed a GFP reporter, making GFP a leukemia-specific neo-antigen in leukemic C57BL/6 mice. We used a GFP:I-A^b-specific tetramer (126) to examine if neo-antigen specific T cells behave similarly to BAp:I-A^b-specific T cells in response to leukemia. While BAp:I-A^b-specific T

cells proliferated as much in response to leukemia (53-fold) (Figure 2-10) as they did in response to BAp peptide plus adjuvant (47-fold) (Figure 2-11), GFP:I-A^b-specific T cells proliferated only 17% as much in response to leukemia (66-fold) (Figure 2-10) as GFP peptide plus adjuvant (366-fold) (Figure 2-11). Importantly, the ratio of GFP:I-A^b specific T cells to GFP^+ leukemic cells was not different than the BAp:I-A^b-specific T cell-to-leukemic cell ratio (Figure 2-10). Thus, the proliferation of neo-antigen specific T cells responding to $BCR\text{-}ABL^+$ B-ALL is inhibited.

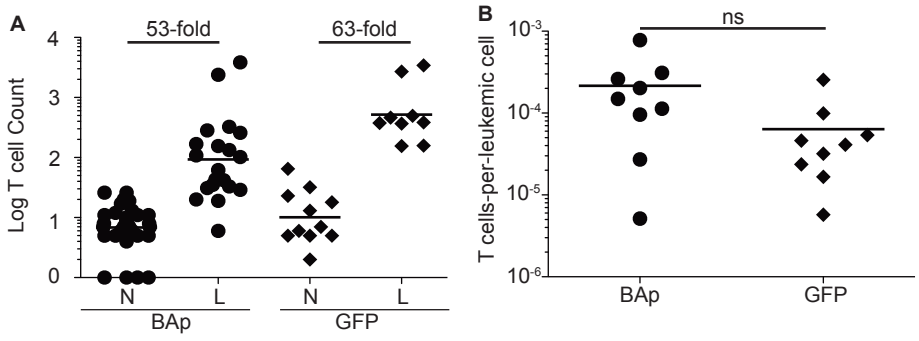

Figure 2-10:GFP:I-A^b-specific T cells respond to BCR-ABL-IRES-GFP^+ leukemia

A. Fold increase in antigen-specific T cell count between naïve (N) mice and leukemic (L) mice. **B**. Target-to-effector ratio of leukemic mice, either tracking BAp-specific T cells or GFP-specific T cells; three independent experiments shown. All comparisons were made with Mann-Whitney U test (2 groups) or Kruskal-Wallace test (>2 groups).

Evidence for cross reactivity vs. neo-antigen specificity of leukemia-specific T cell populations

BAp vs. GFP:I-A^b-specific T cell responses to CFA + peptide immunization

We next examined whether BAp:I-A^b specific T cells had comparable proliferative capacity to other antigen-specific naïve T cells. We found that in response to peptide plus adjuvant immunization, BAp:I-A^b-specific T cells proliferated modestly (47-fold, Figure 2-11). BAp:I-A^b-specific T cell proliferation resembled that observed with GFP:I-A^b-specific T cells in *Foxp3-GFP* mice (18-fold), where GFP is a self-antigen. In contrast, immunization with GFP in C57BL/6 mice (in which GFP is a foreign antigen) resulted in a 366-fold expansion of GFP:I-A^b specific T cells (Figure 2-11). Thus BAp:I-A^b-specific T cell expansion resembled that seen for T cells that recognized low abundance self-antigens (GFP in *Foxp3-GFP* mice).

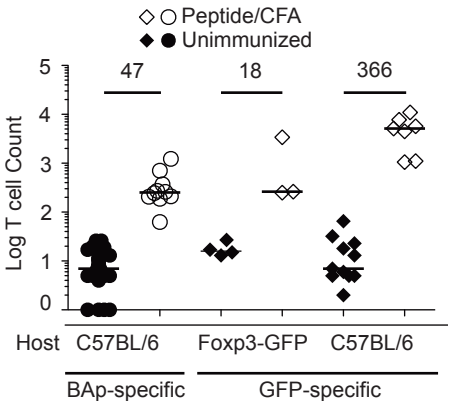

Figure 2-11: BAp:I-A^b-specific T cells proliferate similarly to known cross-reactive T cells

C57BL/6 or *Foxp3-GFP* mice were immunized with BAp or GFP peptide + CFA. Secondary lymphoid organs were harvested 14 days later and BAp:I-A^b-specific or GFP:I-A^b-specific T cells were enumerated. Y-axis is the Log (Y+1) of the BAp:I-A^b-specific T cell count or the GFP:I-A^b-specific T cell count. The number above the line is the fold change from median (indicated by black bars). All fold expansions shown resulted in significantly higher T cell counts than in unimmunized mice; >2 independent experiments were conducted for each immunization shown.

Cross-reactivity of BAp:I-A^b-specific T cells with the self peptide ABL

T cell-based cancer immunotherapy is thought to be more effective when T cells recognize cancer cell neo-antigens with minimal cross-reactivity for self-antigens (127). Thus, we addressed if BAp:I-A^b-specific T cells recognized neo-antigens or cross-reactive self-antigens. Recent studies have shown that small peptide:MHC-II specific T cell repertoires are predictive of cross-reactivity with

self-antigen (128). The BAp peptide (DAEALQRPVASDF) closely resembles the endogenous ABL peptide (LEEALQRPVASDF) (85% identical, see underlined portion), so we tested if BAp:I-A^b tetramer-binding T cells could recognize the overlapping ABL peptide. When mice were immunized with adjuvant plus ABL peptide, BAp:I-A^b tetramer-binding cells proliferated 46-fold, which is similar to the proliferation seen after immunization with BAp peptide plus adjuvant (Figure 2-12). This evidence suggests that BAp:I-A^b-binding T cells are cross-reactive with the self-peptide ABL. These results may explain the occasional expression of CD44 that we saw on BAp:I-A^b-specific T cells in naïve mice (Figure 2-6).

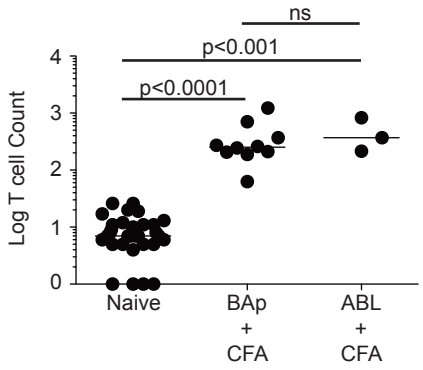

Figure 2-12: BAp:I-A^b-specific T cells cross-react with a peptide from ABL

C57BL/6 mice were immunized with BAp peptide or ABL peptide emulsified in CFA. 14 days later, secondary lymphoid organs were harvested as previously described. Y-axis is the Log (Y+1) of BAp:I-A^b tetramer-binding events; data is from three independent experiments. All comparisons were made with Kruskal-Wallace Test (>2 groups).

BAp:I-A^b specific T cell numbers in naïve mice are controlled by central tolerance

The precursor frequency of BAp:I-A^b-specific T cells was quite low (Figure 2-5), thus we examined whether immune central tolerance may be limiting the number of BAp:I-A^b-specific T cells in naïve mice. *Bim*$^{-/-}$ mice, which have defective thymic negative selection, had significantly (~5 fold) more BAp:I-A^b-specific T cells (Figure 2-13) (74, 75, 129). Thus, ~80% of the BAp:I-A^b-specific T cell progenitors were deleted during thymic negative selection.

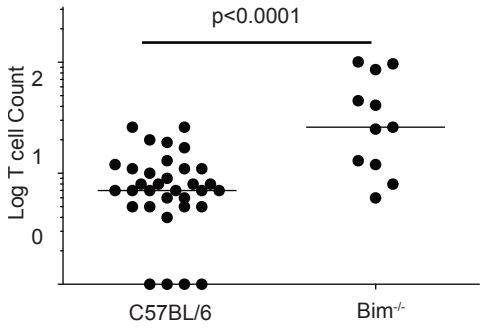

Figure 2-13: *Bim*$^{-/-}$ mice have more BAp:I-A^b-specific T cells at steady state than C57BL/6 mice

BAp:I-A^b-specific T cells were enumerated from *Bim*$^{-/-}$ mice and compared to C57BL/6. Y-axis is the Log (Y+1) of the BAp:I-A^b-specific T cell count and three independent experiments are shown. Significance was calculated with the Mann-Whitney U test.

Bim$^{-/-}$ BAp:I-A^{b}-specific T cells do not have increased tetramer Mean Fluorescence Intensity

We next reasoned that the BAp:I-A^{b}-specific T cell clones, which were rescued in *Bim*$^{-/-}$ mice, might have high affinity for cognate BAp:I-A^{b}. To examine this issue, we analyzed the tetramer mean fluorescence intensity of BAp:I-A^{b} tetramer binding cells and normalized this value to that found in the matching bulk CD4^{+} T cell compartment. Interestingly, we found that the tetramer mean fluorescence intensity was significantly higher in the C57BL/6 mice than in the *Bim*$^{-/-}$ mice (Figure 2-14).

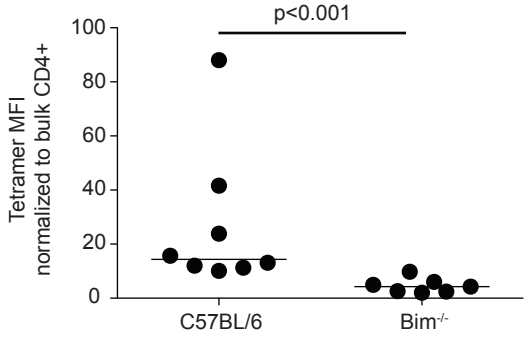

Figure 2-14: *Bim* deficiency does not allow BAp:I-A^{b}-specific T cells to bind tetramer with higher affinity or avidity.

BAp:I-A^{b}-specific T cells were harvested from C57BL/6 or *Bim*$^{-/-}$ mice and BAp:I-A^{b} tetramer mean fluorescence intensity (PE channel) was measured. Shown are 3 independent experiments, p-value is from Mann-Whitney U Test. Significance was calculated with the Mann-Whitney U test.

Bim$^{-/-}$ BAp-specific T cells do not respond better to peptide/CFA immunization

In addition, when we immunized *Bim*$^{-/-}$ mice with BAp peptide emulsified in CFA, we saw no increase in the number of BAp:I-A^{b}-specific T cells recovered from *Bim*$^{-/-}$ mice (Figure 2-15). Thus, *Bim*$^{-/-}$ mice have an increased number of BAp:I-A^{b}-specific T cells, but these cells do not appear to bind cognate BAp:I-A^{b} with higher affinity, nor do these cells accumulate to higher numbers upon immunization.

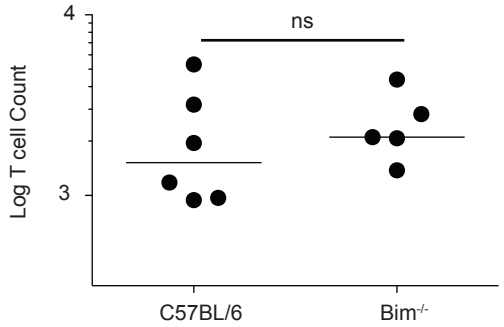

Figure 2-15: *Bim* deficiency does not allow BAp:I-A^{b}-specific T cells to proliferate more in response to peptide/CFA immunization

C57BL/6 or *Bim*$^{-/-}$ mice were immunized with BAp peptide emulsified in CFA, and BAp:I-A^{b}-specific T cells were harvested from the spleen and lymph nodes 14 days later and these cells were enumerated. Y-axis is the Log (Y+1) of the BAp:I-A^{b}-specific T cell count. Shown is data from 2 independent experiments, significance was calculated with the Mann-Whitney U test.

Bim$^{-/-}$ mice do not allow for more protection after prophylactic vaccination

Bim is also important in contraction of T cells following acute infection (130). Since Complete Freund's Adjuvant leaves an antigen depot and thus likely does not truly cause contraction or create immunological memory, we reasoned that *Bim*$^{-/-}$ BAp:I-A^{b}-specific T cells might respond better to an acute vaccination where antigen was formally cleared. Thus, we infected mice with LCMV-Armstrong and provided exogenous synthetic BAp peptide at three and five days post-infection (Figure 2-16). We have used this infection strategy as a method to boost strong antigen presentation of BAp:I-A^{b} and robust expansion of the BAp:I-A^{b}-specific T cells. We then re-challenged mice with BCR-ABL^{+} leukemia greater than 40 days later, with the hypothesis *Bim*$^{-/-}$ mice would be better able to reject leukemia upon rechallenge if contraction had not limited the BAp-specific T cells to the same degree that it would in *Bim*-replete mice. To the contrary, we found that *Bim*$^{-/-}$ hosts actually trended towards succumbing to leukemia faster than *Bim*-replete hosts. If *Bim* expression was inducing aggressive central tolerance and/or contraction after infection that inhibited BAp:I-A^{b}-specific T cell function the prediction would have been that *Bim*$^{-/-}$ mice would survive longer with

leukemia, since the leukemia-specific T cells were more abundant. Thus, this data provides evidence supporting the conclusion that *Bim* deficiency does not unleash BAp:I-A^b-specific T cells to optimally function-either during central tolerance or during contraction following acute infection.

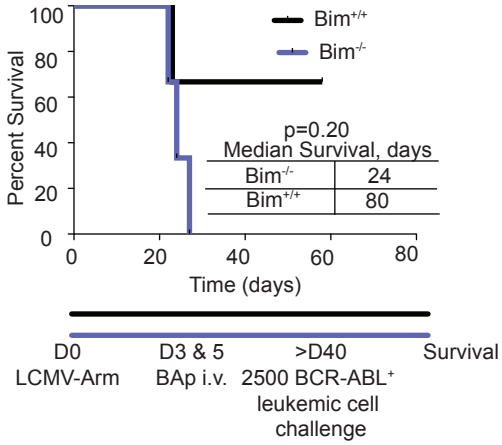

Figure 2-16: *Bim*$^{-/-}$ mice are not better protected from leukemia challenge after LCMV+BAp vaccination

Bim$^{-/-}$ or *Bim*-replete hosts were vaccinated with Lymphocytic Choriomeningitis Virus (LCMV)-Armstrong with the addition of 200µg synthetic BAp peptide provided i.v. at both day 3 and day 5 post infection (see Chapter 4 methods, "Infections and Immunizations"). Mice were re-challenged with 2500 BCR-ABL$^+$ Leukemic cells at greater than 40 days post-infection and survival was analyzed. Statistics were taken from the Log-Rank (Mantel-Cox) test. Median survivals are reported.

Conclusion

Recent studies demonstrate that augmenting immune responses can be an effective treatment for cancer (107, 131-134). While the immune system recognizes both tumor-associated self-antigens and tumor-specific neo-antigens,

cell-based immunotherapy approaches have focused on utilizing neo-antigen specific T cells, which should not be subject to immune tolerance. Many of these approaches utilize mutations arising in the malignancy that may not drive cancer progression, thus potentially allowing immune escape. Further, most of these treatments have been employed in cancers with many non-synonymous mutations. In this study, we examine the immune response to a cancer with few non-synonymous mutations by investigating the adaptive immune response to the oncoprotein BCR-ABL. Our approach was informed by using BAp:I-Ab tetramers, to track cross-reactive leukemia-specific T cells, and GFP:I-Ab tetramers to track neo-antigen specific T cells. A major finding from our studies is that BCR-ABL+ leukemia induces tolerance to both cross-reactive tumor associated self-antigens and tumor-specific neo-antigens. However, the mechanism of tolerance differed depending on whether the tumor-derived antigen was a cross-reactive tumor antigen or a neo-antigen. While leukemia induced conversion of BAp:I-Ab-specific T cells into Treg cells, it inhibited proliferation of GFP:I-Ab-specific T cells without driving Treg induction. We speculate that cross-reactive antigen-specific Treg cells are one mechanism by which leukemias induce tolerance to tumor-specific neo-antigens.

The murine host may also allow for immune tolerance to leukemia. In our model, the majority of BAp:I-A^b specific T cells are clonally deleted in the thymus, which may result in too few BAp:I-A^b specific T cells to effectively clear leukemia. For example, previous studies have shown that increasing the number of antigen-specific $CD4^+$ T cells enhanced anti-tumor immunity (106). Though we could recover more BAp:I-A^b-specific T cells in *Bim*$^{-/-}$ mice, our data do not support that the increased number of these cells correlates with increased functionality. In support of this, immunization of *Bim*$^{-/-}$ mice led to the same number of BAp:I-A^b-specific T cells as seen in C57BL/6 mice that were immunized in the same way. Thus, central tolerance may remove BAp:I-A^b-specific T cells but other mechanisms also exist that limit functional immune responses to cancer.

Chapter 3 Regulatory T cell dynamics during the immune response to leukemia

Introduction

[2]T-helper cells can play both beneficial and detrimental roles in the immune response to cancer. Specifically, while Th1-like cells are thought to benefit the host in clearing a cancer pathogen insult, regulatory T cells (Treg) often play a detrimental role to the host by inhibiting the host immune response to cancer. The Farrar lab has a long-standing interest in Treg development, and as such I was lucky to have numerous tools and knowledge sources at my disposal for exploring Tregs in response to leukemia. Therefore, I devoted one chapter of my

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[2] A portion of the Chapter 3 data and text is previously published.

Manlove, L.S., Berquam-Vrieze, K.E., Pauken, K.E., Williams, R.T., Jenkins, M.K.m Farrar, M.A. 2015. Adaptive Immunity to Leukemia Is Inhibited by Cross-Reactive Induced Regulatory T cells. Journal of Immunology, September 16, 2015.

thesis specifically to highlighting a number of findings regarding Tregs and leukemia.

Treg Development in the thymus and periphery

While many thymocytes develop with under selective “positive” and “negative” pressures, some Tregs probably develop through an alternative pathway termed “agonist selection”. Agonist-selected T cells are allowed to survive thymic development despite being engendered with a TCR that has high affinity for cognate self peptide:MHC (73). A portion of the peripheral Treg pool is thought to develop through the agonist-selected pathway, thus residing in the periphery with a high affinity for cognate self-peptide: MHC-II. A major feature of this developmental track is a dependence of these “thymic-derived” Tregs on Interleukin-2 (IL2), which has been well studied by our lab and others (135-138).

[3]STAT5 Activation Drives Thymic Treg Lineage Commitment

$CD4^+CD25^+FOXP3^+$ Tregs that develop in the thymus (also known as 'natural Tregs') constitute 2-4% of CD4 single positive (CD4SP) thymocytes, yet this relatively small population plays a critical role in maintaining peripheral tolerance and preventing autoimmunity. The TCR repertoire of these natural Tregs overlaps with that of non-regulatory T cell populations but is skewed to favor TCRs that interact with higher affinity to self-antigens in the thymus (140-145). The molecular mechanisms that drive Treg development have been tied to three primary signaling modules. First, TCR signaling plays a key role as TCRs with higher affinity TCRs for self-antigen are preferentially selected into the Treg

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[3] A portion of this introduction is previously published as a review article. The portions included in this introduction were co-authored, and full acknowledgement is given to Shawn Mahmud and Michael Farrar for their extensive contributions.

Mahmud, S. A., L. S. Manlove, and M. A. Farrar. 2013. Interleukin-2 and STAT5 in regulatory T cell development and function. *Jak-Stat* 2: e23154.

lineage (142, 146). Second, the costimulatory receptor CD28 also plays an important role as *Cd28*$^{-/-}$ and *B71/2*$^{-/-}$ mice both show clear defects in Treg development (147-150). Third, signals emanating from the interleukin-2 receptor are also required for Treg differentiation in the thymus (138, 151). These observations culminated in the development of a two-step model of thymic Treg development, in which a TCR- and CD28-dependent, but cytokine-independent first step generates an IL2-responsive intermediate "Treg progenitor" that lacks FOXP3 expression. Subsequently, a TCR-independent, IL2/STAT5-dependent second step results in the rapid conversion of Treg progenitors into mature FOXP3+ Tregs (137, 152). Additionally, recent work suggests that an intermediate step between TCR signaling and CD25 expression may be costimulation via Tumor Necrosis Factor Receptor Super Family (TNFRSF) (153).

Upon interacting with medullary APCs presenting self-peptide:MHC II complexes, strong TCR signals in a fraction of CD4SP thymocytes cause them to differentiate into Treg progenitors, marked by elevated expression of the high-affinity IL2Rα chain (CD25), the IL2Rβ chain (CD122), and the costimulatory TNF receptor superfamily member, glucocorticoid-induced TNF-related protein (GITR)

(137, 152). The emergence of this $CD4^{+}CD25^{+}CD122^{hi}GITR^{hi}FOXP3^{-}$ Treg progenitor population requires canonical activation of the NF-κB pathway downstream of TCR and CD28 ligation. Paired activation of LCK from these receptors(149) signals through the canonical NF-κB pathway to ultimately promote nuclear translocation of c-REL and REL-A(149, 154-157). The requirement for NF-κB activation in Treg differentiation is demonstrated by the absence of thymic Tregs—and importantly, Treg progenitors—in animals deficient in *Cd28, Prkcq, Carma1, Bcl10,* and *Rel* (148, 149, 158-160). Further studies revealed that c-REL binds the conserved non-coding sequence 3 (CNS3) located in the *Foxp3* promoter to promote epigenetic modification of *Foxp3* rendering it permissive for subsequent transcription initiation (161).

The conversion of $FOXP3^{-}$ Treg progenitors into mature $FOXP3^{+}$ Tregs in the thymus occurs via a TCR-independent but IL2/STAT5-dependent process(137, 152). Ligand binding by the high affinity IL2R complex leads to phosphorylation of three key tyrosine residues located in the cytoplasmic domain of IL2Rβ by the kinases JAK1 and JAK3. Phosphorylation of Tyr-338 recruits the SH2-containing adaptor molecule, SHC, facilitating activation of the RAS/MAPK/ERK and PI3K/AKT pathways via GRB2 and GAB2, respectively. Phosphorylation of IL2Rβ

at Tyr-510 (and to a lesser degree Tyr-392) is critical for recruiting and activating STAT5 (162). The importance of IL2R signaling in thymic Treg differentiation is clearly demonstrated by the fact that the lethal autoimmunity in mice lacking *Il2rb* is due to a failure to generate thymic Tregs, and this phenotype is completely restored by adoptive transfer of small numbers of wild type Tregs(163). Moreover, retroviral transduction of *Il2rb*$^{-/-}$ bone marrow with wild type *Il2rb*, or a mutant construct capable of activating only STAT5 via Tyr-510, restored thymic Treg generation in bone marrow chimeric mice. In contrast, restoration of Treg development did not occur when mutant constructs capable of activating RAS/PI3K, but not STAT5, were transduced into *Il2rb*$^{-/-}$ bone marrow cells and engrafted into recipient mice(138). Likewise, crossing *Il2rb*$^{-/-}$ mice to transgenic mice expressing a constitutively active form of STAT5b (*Stat5b-CA* mice) restored Treg development in the thymus(138). Additional support for the role of STAT5 in Treg development came from two studies that demonstrated that conditional deletion of STAT5 in DP thymocytes (i.e., *Cd4-Cre x Stat5a/b*$^{FL/FL}$ mice) had minimal effects on CD4SP thymocytes with the exception of CD4^{+}FOXP3^{+} thymic Tregs (138, 164). Together, these findings indicate that STAT5 activation downstream of IL2R is required for thymic Treg development.

Two groups have demonstrated that $CD4^+CD25^+FOXP3^-$ thymocytes are direct precursors of FOXP3+ Tregs, which require only an additional IL2R/STAT5-dependent signal to express FOXP3. First, Hsieh and colleagues showed that adoptive transfer of $CD4^+CD25^+FOXP3^-$, but not $CD4^+CD25^-FOXP3^-$ thymocytes, into the thymii of wild type hosts resulted in the development of $CD4^+FOXP3^+$ Tregs. Similar results were observed upon adoptive transfer into MHCII-deficient mice demonstrating that the conversion process did not require additional signals via the TCR(152). In addition, stimulation of sorted Treg progenitors with IL2 *in vitro* led to induction of *Foxp3* mRNA within a few hours followed by the development of $CD4^+FOXP3^+$ Tregs 24 hours later. These findings were subsequently confirmed by Burchill and colleagues(137).

IL2, STAT5 and induced Tregs

An important feature of peripheral tolerance is the conversion of naïve $CD4^+$ T cells into induced regulatory T cells (iTregs) in peripheral lymphoid organs. iTregs have important roles in protecting against chronic inflammatory conditions, and likely play a key role in regulating immune responses to commensal microorganisms(165, 166). The differentiation of iTregs, like nTregs in the thymus, requires both TCR and IL2-dependent signals. However, unlike nTregs,

iTregs require Transforming Growth Factor-B (TGFβ) for their differentiation (167). Moreover, while the CARMA1/NFkB pathway is required for the development of nTregs it actually antagonizes iTreg differentiation(168). Finally, the stability of iTregs is lower than that of nTregs (169, 170), a feature that correlates with the greater degree of DNA methylation of the CNS2 region of the *Foxp3* gene in iTregs versus nTregs (171). Thus, iTregs differ in several ways from nTregs.

A role for IL2 in iTreg development was established many years ago in studies documenting the role of both TGFβ and IL2 in iTreg differentiation (172, 173). Likewise, STAT5 also plays an important role in iTreg differentiation in vitro (164). More recent studies have demonstrated that IL2 and STAT5 also play critical roles in maintaining stability of the iTreg lineage (169). Specifically, these studies demonstrated that transfer of iTregs into lymphopenic hosts resulted in loss of FOXP3 expression in the transferred iTregs. This result could be blocked by co-administration of agonist IL2: anti-IL2 complexes indicating that IL2 was required to maintain FOXP3 expression in iTregs in vivo. These studies further documented that the loss of *Foxp3* expression correlated with re-methylation of the CNS2 region (also referred to as regulatory T cell Specific Demethylated

Region or TSDR) of the *Foxp3* gene, and that IL2 stimulation prevented this re-methylation process. The mechanism by which this occurs remains unclear. However, STAT5 binding sites are found in the CNS2 region, which may be important for maintaining *Foxp3* expression. It is also possible that STAT5 directly initiates demethylation of this region as naïve $CD4^+FOXP3^+$ T cells are converted into $CD4^+FOXP3^+$ iTregs. Arguing against this possibility is evidence that STAT5 binds poorly to its cognate DNA binding site when it is methylated (174, 175). However, only one of the three potential STAT5 binding sites found in the CNS2 region contains a CpG motif that could be methylated (138). Thus, whether IL2 and STAT5 promote demethylation of CNS2 requires additional study.

As mentioned above with regard to thymic Treg development, STAT5 interacts with a number of potential binding partners. The role of these binding partners in iTreg development remains to be defined. Interestingly, recent work from the O'Shea lab has shown that micro RNAs activated by TGFB and Retinoic Acid Receptor (RAR) suppress the expression of one of these binding partners, the co-repressor NCOR2 (176). Specifically, this study demonstrated that conversion of iTregs into T_{FH} is limited when *mir10a* is expressed at high levels,

such as is the case following TGFβ and RAR signaling in the periphery. It appears that *mir10a* may have a role in fixing the iTreg cell lineage by suppressing conversion of iTregs into either Th_{17} or T_{FH} cells. Additional studies are needed to more precisely define the role of NCOR2 and other STAT5 interacting partners on the development and maintenance of regulatory T cells.

What cells give rise to induced Tregs?

An area of current interest is understanding if all naïve $CD4^+$ T cells have an equal chance to become induced Tregs (i.e., is iTreg induction strictly due to extrinsic factors, or are some $CD4^+$ T cells intrinsically more capable of becoming iTregs?). Reviews have suggested that the molecular mechanisms are in place for Th17 and Treg cells to trans-differentiate (177), and it is well-established that the protein Foxp3 binds to a plethora of other transcription factors which may prepare chromatin for plasticity into the Th17 (or other) lineages (178, 179). However, our data may be the first supporting that a subset of the naïve $CD4^+$ T cell population is poised to differentiate into iTreg cells (63). Additionally, the Mueller lab at the University of Minnesota has recently uncovered evidence supporting the conclusion that “anergic” $CD4^+$ T cells in the periphery are poised

to differentiate into Tregs. Anergic (sometimes called “exhausted) T cells may be phenotypically quite similar to naïve cells, since anergic T cells may downregulate canonical markers of antigen experience like CD44 (180). Thus, it is possible that no naïve $CD4^+$ T cell is poised to differentiate into the Treg lineage, and instead these cells are all “anergic”.

Studying Treg functionality in mice

Studying Treg functionality is a delicate business because ablation of Tregs is rapidly lethal (88, 89). Various depletion strategies have been used in the literature, including treatment with cyclophosphamide (181), anti-CD25 (182, 183), and anti-FR4 (184) to deplete Tregs. Experimentally, a commonly-used model now is the *Foxp3*$^{DTR/DTR}$ mouse, which has the diphtheria toxin receptor knocked in to the *Foxp3* locus (115). These mice can then be treated with exceedingly low doses of Diphtheria Toxin (DT) leading to selective, transient ablation of the $Foxp3^+$ Treg cells.

The role of Tregs in cancer is variable. Many findings in human cancers show an infiltration of Tregs into the tumor stroma. However, these studies are correlative with disease outcome and not causative (185-188). Further, clinical Treg depletion has been used for treating cancer, but these therapies are non-specific

and it is not clear that the cancer therapy is caused by Treg depletion as opposed to some other effect of the treatment. However, what is clear from mouse models using the *Foxp3*$^{DTR/DTR}$ platform is that Tregs have different functionality in different models. For example, depletion of Tregs using *Foxp3*$^{DTR/DTR}$ was sufficient to prolong survival of mice with Renca cell kidney cancer (189). However, in a mouse model of breast cancer, Treg depletion with *Foxp3*$^{DTR/DTR}$ mice increased survival minimally on its own (where it also decreased metastasis formation), but synergized with ionizing radiation to prolong survival even further (64). Taken together, these results all suggest that *Foxp3*$^{DTR/DTR}$-mediated Treg depletion likely will have a positive impact on outcome in our studies, but it is unclear how this will synergize with other therapies.

Tregs clearly have complex roles in regulating immune homeostasis. The purpose of this chapter is to address how Tregs fit into the puzzle of anti-leukemia immunity in one mouse model of BCR-ABL^{+} B-ALL.

Leukemia-responding Tregs in steady state, immunization, and response to leukemia

T cells specific for cross-reactive leukemia antigens are converted into pTreg cells by leukemia.

Our studies provide evidence that BAp:I-A^b-specific T cells respond to BCR-ABL^+ B-ALL despite cross-reactivity with self-antigen. However, leukemia still progresses in C57BL/6 mice, which suggests that mechanisms of tolerance inhibit the immune responses by cross-reactive T cells. Indeed, BAp:I-A^b-specific T cells positively correlated with leukemic burden in naïve mice inoculated with leukemia (Figure 3-1), suggesting that the BAp:I-A^b-specific T cells might prevent immune responses to B-ALL.

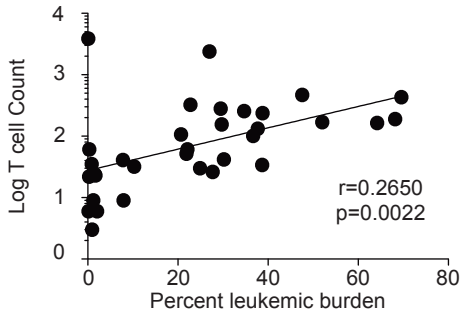


Figure 3-1: BAp:I-A^b-specific T cells positively correlate with leukemic burden

Log (Y+1) of the BAp:I-A^b-specific T cell counts (Y-axis) correlates with leukemic burden (depicted as the percent of bone marrow B cells, which are GFP+) on the X-Axis. More than three experiments are shown, Spearman correlation is shown.

A possible explanation was that BAp:I-A^b-specific T cells were regulatory T cells (Treg cells). Both naïve *Foxp3-GFP* mice and *Foxp3-GFP* mice immunized with BAp plus adjuvant typically had no or few BAp:I-A^b specific Treg cells whereas the majority of BAp:I-A^b specific T cells in leukemic mice were FOXP3-GFP$^+$ (Figure 3-2). This contrast supports the conclusion that B-ALL converts BAp:I-A^b-specific T cells into pTreg cells, and provides a mechanism for host tolerance of leukemia.

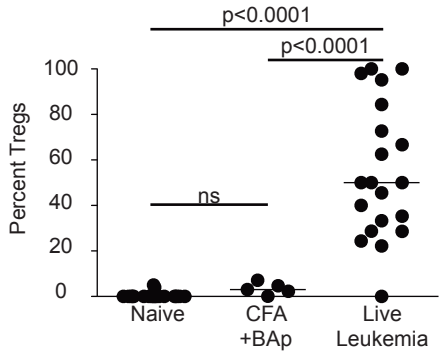


Figure 3-2: Leukemia selectively induces BAp:I-A^b-specific Tregs

Mice were unimmunized, immunized with CFA+BAp, or injected with 2500 BCR-ABL$^+$ cells; percentage of BAp:I-A^b-specific Tregs were analyzed 14 days post-inoculation. >2 independent experiments for each group are shown, Kruskal-Wallace test performed (>2 groups).

Peripheral cross-reactivity enhances conversion to Treg lineage in response to leukemia

We next determined if leukemia-specific Treg polarization was related to the cross-reactivity of BAp:I-A^b-specific T cells with self-antigen. To address this question, we examined both neo-antigen specific T cells and cross-reactive T cells responding to leukemia. The leukemic cells used in these experiments express both GFP and BCR-ABL, thus GFP was a leukemia-specific neo-antigen in C57BL/6 mice and BCR-ABL was a cross-reactive antigen in C57BL/6 mice. We enumerated GFP:I-A^b-specific Treg cells responding to leukemia in *Foxp3*-

RFP mice (where GFP is a non-self antigen) and *Foxp3-GFP* mice (where GFP is a self-antigen). Most GFP:I-A^b-specific T cells did not polarize into FOX3-RFP^+ Treg cells in response to leukemia (1.5% +/- 1.7%). In contrast, significantly more GFP:I-A^b-specific T cells were Treg cells in leukemic FOXP3-GFP mice (8.3% +/- 31.2%, $p<0.005$; Figure 3-3).

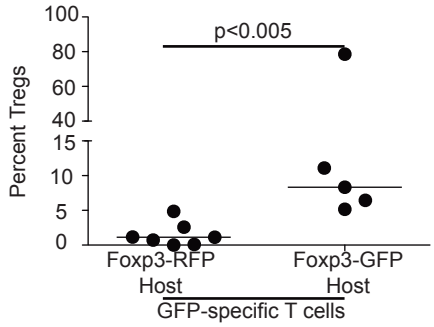


Figure 3-3: Cross-reactivity with self-antigen potentiates leukemia-specific Treg conversion

Percent of BAp:I-A^b-specific T cells that are $FOXP3^+$ Treg cells in *Foxp3-dsRed* or *Foxp3-GFP* mice. 2,500 BCR-ABL^+ B-ALL cells were transferred into *Foxp3-RFP* or *Foxp3-GFP* mice and BAp:I-A^b-specific T cells were enumerated 14 days later. Y-axis is percentage of BAp:I-A^b-specific T cells that are FOXP3+ Treg cells in their respective hosts. >2 independent experiments are shown for each group, Mann-Whitney U test performed (2 groups).

Importantly, 75% of naïve *Foxp3-GFP* mice contained no GFP:I-A^b-specific Treg cells (Figure 3-4), suggesting that GFP:I-A^b-specific Treg cells seen in leukemic *Foxp3-GFP* mice were induced Treg cells.

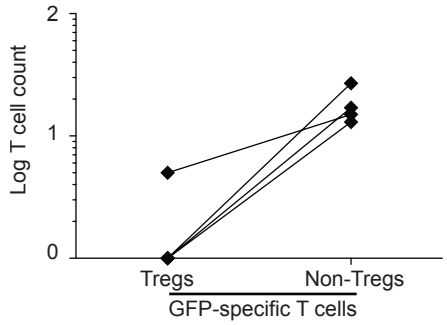


Figure 3-4: 75% of naïve GFP:I-A^b-specific T cells are not Tregs at steady-state

SLO were harvested from naïve Foxp3-GFP mice, and GFP:I-Ab-specific T cells were enumerated; FOXP3-GFP expression was evaluated on these cells. The Y-axis shows the log(Y+1) of GFP:I-Ab-specific T cell count from these mice.

Thus, peripheral CD4$^+$ T cells that cross-react with self have increased capacity to become pTreg cells in response to leukemia.

Histologically, Treg cells were localized in the splenic red pulp, adjacent to red pulp macrophages and leukemic cells (Figure 3-5).

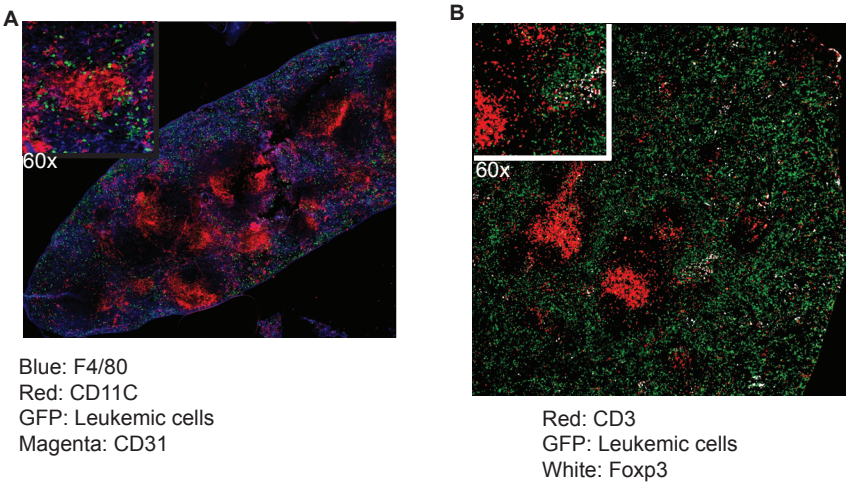


Figure 3-5: Localization of leukemic cells, Tregs, and red-pulp macrophages in leukemic mice

A. Representative histology of spleen from a C57BL/6 mouse 14 days after leukemic cell transfer. Blue is F4/80, Red is CD11C, Magenta is CD31, and Green is GFP^+(leukemic cells). b. Representative histology from spleen as in A, showing $FOXP3^+$ Treg cells in the splenic red pulp. Red is CD3, White is FOXP3, Green is GFP^+ (leukemic cells).

Red pulp macrophages have been shown to induce Treg cells in vitro (190). Thus, Tregs responding to leukemia antigens are preferentially cross-reactive, and localized in an immune-suppressive microenvironment near the leukemic cells.

The role of Tregs in the anti-leukemia immune response

Tregs allow leukemia progression in C57BL/6 mice.

To determine if Treg cells inhibited the immune response to BCR-ABL^{+} leukemia, we selectively ablated Treg cells using *Foxp3*$^{DTR/DTR}$ mice (115). Transient depletion of Treg cells led to a 98% decrease in leukemic burden in the BM at fourteen days post-inoculation with leukemia (Figure 3-6). Depletion of Tregs following leukemia inoculation also led to an increased survival of mice as compared to untreated C57BL/6 mice (Figure 3-7).

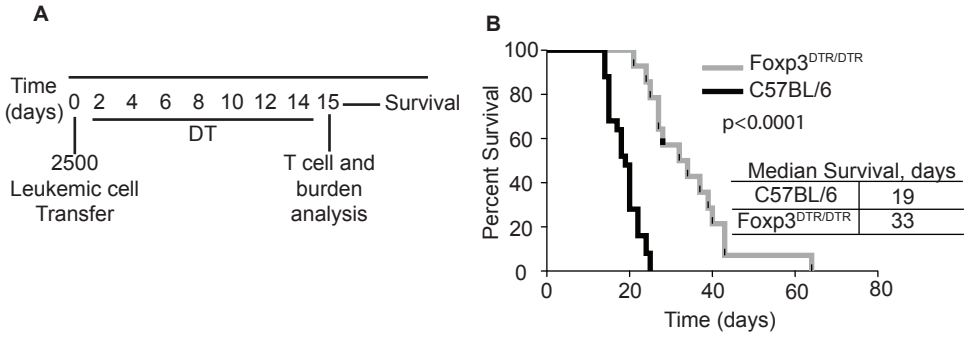


Figure 3-6: Diphtheria Toxin treatment strategy and survival in leukemic *Foxp3*$^{DTR/DTR}$ mice

A. *Foxp3*$^{DTR/DTR}$ or C57BL/6 mice were inoculated with 2500 leukemic cells and then treated with Diphtheria Toxin (DT) at 0.25µg/kg DT daily, and compared to C57BL/6 mice treated with DT. **B**. Foxp3$^{DTR/DTR}$ mice were treated as in C and survival was analyzed. P-values from Log-rank test; three independent experiments shown.

In parallel BAp:I-A^b-specific T cells proliferated ~30-fold more in DT-treated $Foxp3^{DTR/DTR}$ mice than in DT-treated C57BL/6 mice (Figure 3-7).

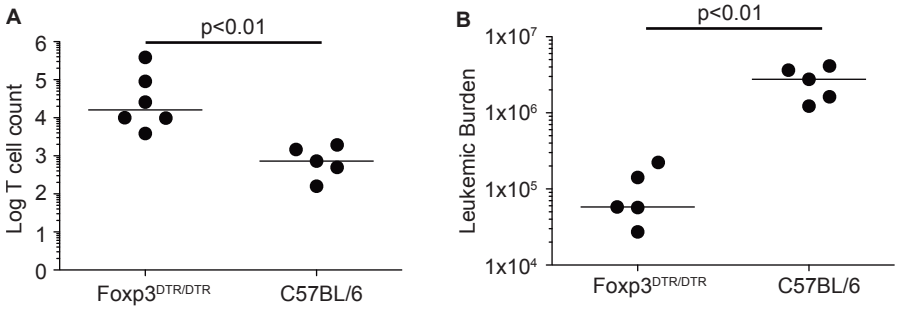


Figure 3-7: Treg depletion allows increased BAp:I-A^b-specific T cell numbers in correlation with decreased leukemic burden

A. Log(Y+1) of the BAp:I-A^b-specific T cell count in *$Foxp3^{DTR/DTR}$* mice and C57BL/6 mice inoculated with leukemia and treated with DT as in A. Three independent experiments are shown. **B**. Mice were harvested at 14 days post-inoculation and leukemic cells were enumerated from the spleen and lymph nodes.

Treg Depletion Timing and Leukemia Survival

We noticed that some Foxp3DTR mice survived longer when given leukemia and treated with DT compared to only being treated with DT (and not having leukemia) (Figure 3-8). This hinted that the leukemic cells had an

immunosuppressive capacity in-and-of themselves. Interestingly, there was no statistically significant difference in overall survival as analyzed by the Log-Rank (Mantel-Cox) test. However, the hazard ratio between the two groups was 0.46, and the shapes of the curves suggest that at least some leukemic mice outlive the DT-treated non-leukemic mice. As well, the mean survival for leukemic mice is slightly longer than for non-leukemic mice. Further, this analysis may be hampered because the endpoints are implicitly different between the DT+leukemia mice and the DT-leukemia mice. Most of the DT+leukemia mice were euthanized bilateral hind-limb paralysis, as per IACUC protocol. Meanwhile, the DT-leukemia mice never developed hind limb paralysis, and thus never reached the same endpoint. Instead, the DT-leukemia endpoint was when mice would not voluntarily eat. This timepoint coincided with many overt signs of IPEX. Thus, we conclude that the leukemic cells may be able to "rescue" the immunosuppression lost in some leukemic DT-treated Foxp3$^{DTR/DTR}$ mice, but that a survival analysis may not be the best depiction of this.

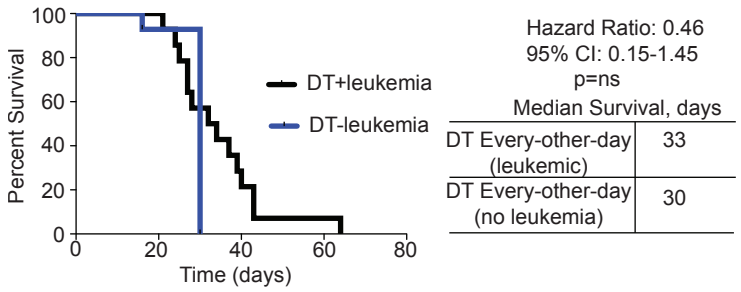
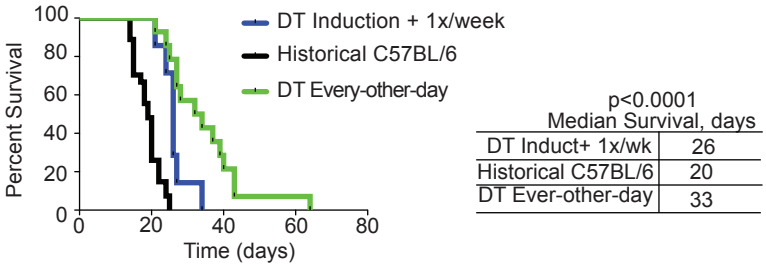


Figure 3-8: Survival of Treg-depleted non-leukemic mice compared to Treg-depleted leukemic mice hints that leukemia protects Treg-depleted mice from IPEX-like pathologies

Foxp3$^{DTR/DTR}$ mice were either adoptively transferred with 2500 BCR-ABL^{+} leukemic cells and then treated with 0.2μg Diphtheria Toxin (DT) i.p. every-other-day for the remainder of their survival (DT + leukemia, black line) or not transferred with leukemia, but treated with DT as above (DT – leukemia, blue line). Survival was compared by the Log-rank (Mantel-Cox) test, which resulted in an insignificant difference in survival. Also reported is the hazard ratio (0.46), the 95% confidence interval of the hazard ratio, and the median survival.

We then wanted to explore if perhaps some of the DT-treated leukemic Foxp3$^{DTR/DTR}$ mice were dying from IPEX and not from leukemia. To test this, we treated leukemic Foxp3$^{DTR/DTR}$ mice with an 'induction' dose of DT (0.2ug every-other-day for one week) followed by weekly DT treatment. This dose should cause far less Treg depletion than every-other-day DT treatment, and therefore should limit IPEX-related pathologies. Contrary to our hypothesis, we found that the induction + 1x/week DT treated mice had decreased overall survival compared to treating with DT every-other-day. Thus, this data suggests that constant "tamping-down" of the Treg population is required to allow survival in leukemic mice-even though this dosage may flirt with inducing IPEX. This data

could be supporting a model where *some sort* of immune-suppressive cell is required to prevent IPEX in mice; this cell could be a leukemic cell, a Treg, or a combination of the two. Depleting Tregs without providing leukemic cells induces IPEX-like symptoms (Figure 3-8). Additionally, our data shows that depleting Tregs to a high degree for a long time (DT every-other-day) allows mice to survive long from both leukemia and IPEX, while depleting Tregs to a lesser degree (induction + 1x/week) allows mice to survive for a significantly shorter amount of time (these mice uniformly developed bilateral hind-limb paralysis, suggesting that they had developed leukemia). Thus, equilibrium may occur between Tregs and leukemic cells that allows for sufficient immune suppression to prevent IPEX, but sufficient immune activation to limit leukemia.



p<0.0001
Median Survival, days

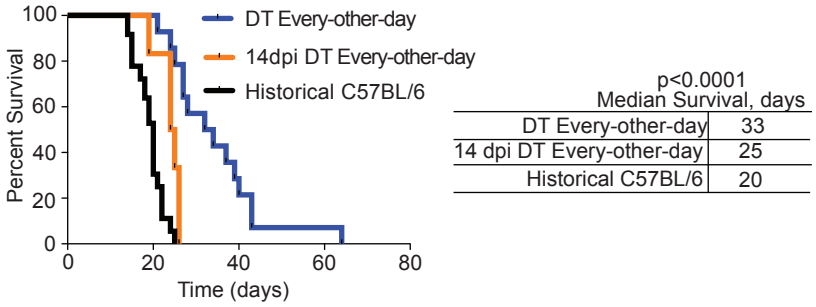
DT Induct+ 1x/wk	26
Historical C57BL/6	20
DT Ever-other-day	33

Figure 3-9: High-level Treg depletion over the course of leukemia progression is required for increased survival by Treg depletion

Foxp3$^{DTR/DTR}$ or C57BL/6 mice were adoptively transferred with 2500 BCR-ABL^{+} leukemic cells and then *Foxp3*$^{DTR/DTR}$ were treated with 0.25μg DT every-other-day for 1 week, followed by 0.25μg DT 1x-per-week after that (DT Induction + 1x/week, blue line), *Foxp3*$^{DTR/DTR}$ were treated with 0.2μg DT every-other-day for the duration of survival (DT Every-other-day, green line) or C57BL/6 mice were untreated and survival was analyzed (Historical C57BL/6, black line). Survival was compared by Log-Rank (Mantel-Cox) test. Median survival is also reported.

Another issue was when, during the course of leukemogenesis, Tregs were eliciting their immune suppressive function. While we showed that Treg depletion for the entire span of leukemia (Figure 3-5) was critical for survival of leukemic mice, this analysis did not clarify if Tregs were important for leukemia survival at the onset of leukemia transfer, or at later stages of leukemia progression. To address this question, Foxp3$^{DTR/DTR}$ mice were inoculated with leukemic cells and Tregs were depleted starting 14 days after leukemic cell transfer. At this timepoint, the leukemia is well established throughout the body (Figure 2-2). "Late" Treg depletion (starting at 14 days-post-inoculation) resulted in increased survival compared to historical C57BL/6 leukemic mice, but

decreased survival as compared to DT treatment every-other-day (Figure 3-10). Since late Treg depletion resulted in an intermediate increase in survival of leukemic mice, this data supports a role for Tregs both early in leukemia onset and late during the maintenance/progression of leukemia.



p<0.0001

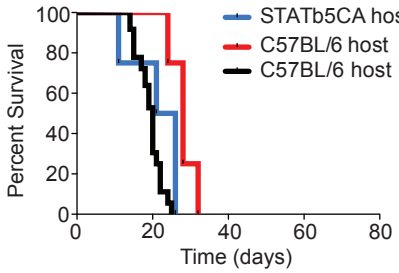
Median Survival, days	
DT Every-other-day	33
14 dpi DT Every-other-day	25
Historical C57BL/6	20

Figure 3-10: Treg depletion during advanced-stage leukemia has a partial benefit to the host

Foxp3$^{DTR/DTR}$ or C57BL/6 mice were adoptively transferred with 2500 BCR-ABL^{+} leukemic cells and then *Foxp3*$^{DTR/DTR}$ were treated with 0.2μg DT every-other-day for the duration of survival (DT Every-other-day, blue line), *Foxp3*$^{DTR/DTR}$ were treated with 0.2μg DT every-other-day for the duration of survival starting at 14 days post-leukemia inoculation (14dpi DT Every-other-day, orange line) or C57BL/6 mice were untreated and survival was analyzed (Historical C57BL/6, black line). Survival was compared by Log-Rank (Mantel-Cox) test, each group contained at least two independent experiments comprising >8 mice. Median survival is also reported.

Transgenic constitutively active STAT5 mice have similar leukemic burden to C57BL/6 hosts

Previous work has shown that certain mouse models such as *Statb5CA* transgenic mice, have increased numbers of Tregs. The *STAT5bCA* mouse has a number of phenotypes in immune cells, including a notable increase in the number of Tregs (137). Since Treg depletion allowed increased survival of leukemic mice, our hypothesis was that STAT5bCA mice, with their increased number of Tregs, would have a decreased survival when given leukemic cells. Indeed, leukemic STAT5bCA mice survived for significantly less time than leukemic C57BL/6 mice in the matched experiment, though the survival of STAT5bCA mice closely overlays that of historical C57BL/6 leukemia survival (Figure 3-11). Nonetheless, thus, these data suggest that STAT5bCA host mice (which have an increased number of Tregs) survive for less time than C57BL/6 host mice (which should have a 'normal' number of Tregs), which supports our conclusions that Tregs allow for more aggressive leukemia and thus decreased survival from leukemia.



p<0.05
Median Survival, days

STAT5bCA	24
Historical	20
C57BL/6	28

Figure 3-11: Leukemic *STATb5CA* mice have less survival than leukemic C57BL/6 mice

STATb5CA (blue line) or littermate-control C57BL/6 (orange line) or historical C57BL/6 mice (black line) were adoptively transferred with 2500 BCR-ABL^{+} leukemic cells. Survival was analyzed by Log-Rank (Mantel-Cox) test. Also shown is median survival.

Tregs inhibit priming and Th1-like skewing of BAp:I-A^{b}-specific T cells

We next sought mechanistic insight into how the presence of Tregs inhibited the leukemia-specific immune response. We found that the percent of BAp:I-A^{b}-specific T cells that were CD44^{hi} was significantly higher in DT-treated Foxp3$^{DTR/DTR}$ mice, suggesting that Tregs inhibit priming of BAp:I-A^{b}-specific T cells (Figure 3-12). We reasoned that Ly6C^{+} Th1-like cells might also be important in controlling leukemia progression (191-193). Indeed, the fraction of CD44^{+} and Ly6C^{+} BAp:I-A^{b}-specific T cells was increased in DT-treated *Foxp3$^{DTR/DTR}$* mice, suggesting that Tregs inhibit BAp:I-A^{b}-specific T cell priming

and effector differentiation during the immune response to leukemia (Figure 3-12).

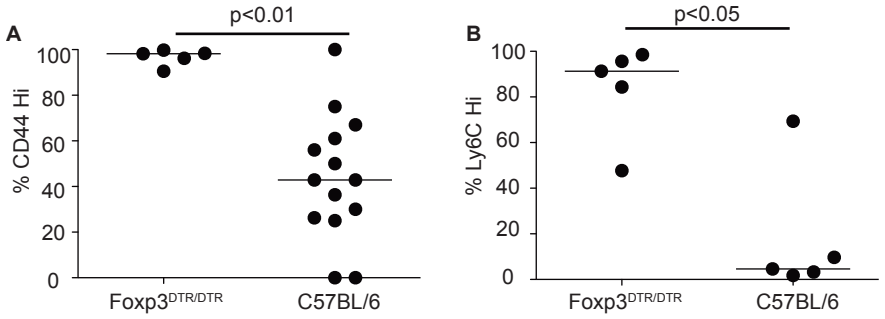


Figure 3-12: Treg depletion allows increased antigen experience and Th1-like phenotype in BAp:I-A^{b} specific T cells from leukemic mice

A. Experiments were conducted as in Figure 3-6 A; shown is the percent CD44^{hi} BAp:I-A^{b}-specific T cells. **B**. Experiments were conducted as in Figure 3-6 A; shown is the percent Ly6C^{hi} BAp:I-A^{b}-specific T cells. All comparisons were made with Mann-Whitney U test (2 groups).

Treg conversion in response to leukemia is dependent on TGFβ

Tregs were clearly important for leukemia progression (Figure 3-6). However the mechanism that drives BAp:I-A^{b}-specific T cells into the Treg lineage in mice with leukemia remains unclear. One possible explanation is that the leukemic cells might produces cytokines that drive Treg conversion. To address this point, we analyzed cytokine production by leukemic cells and by BAp:I-A^{b}-specific T cells

responding to leukemia. When responding to leukemia, both leukemic cells and BAp:I-A^b-specific T cells produced the immune-suppressive cytokines IL10 and TGFβ1 (Figure 3-13).

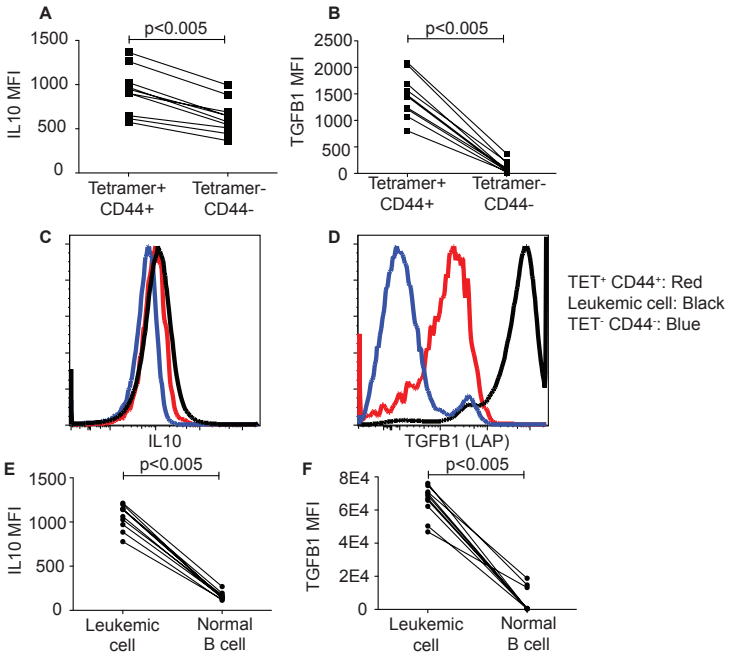


Figure 3-13: TGFβ1 and IL10 are produced during leukemia by leukemic cells and BAp:I-A^b-specific T cells

A, B. BAp:I-A^b-specific T cells were gated on BAp:I-A^b Tetramer $(APC)^+$, $CD44^+$ and were stained for cytokines. Shown is the median fluorescence intensity of IL10 (A) and TGFβ1 (B). Three independent experiments are shown, comparisons were made with Wilcoxan Matched-Pairs test (2 paired groups). **C, D.** Shown is the overlay of IL10 (C) and TGFβ1 (D) expression on BAp:I-A^b $Tetramer^-$, $CD44^-$ $CD4^+$ T cells (Blue), BAp:I-A^b $Tetramer^+$, $CD44^+$ $CD4^+$ T cells (Red), and Leukemic cells (Black). Shown are 10 mice from three independent experiments, whose results were concatenated. **E, F.** Leukemic cells were gated as $CD45.1^+$, GFP^+ and were stained for cytokines. Shown is the median fluorescence intensity of IL10 (E) and TGFβ1 (F). Three independent experiments are shown, comparisons were made with Wilcoxan Matched-Pairs test (2 paired groups).

Since TGFβ1 is important for conversion of naïve $CD4^+$ T cells into peripheral Treg cells (139, 167, 194), we reasoned that TGFβ1 might drive BAp:I-A^b-specific Treg conversion in response to leukemia. When leukemic mice were treated with anti-TGFβ antibody or isotype control, anti-TGFβ treatment led to significantly less BAp:I-A^b-specific Tregs, and significantly more proliferation of BAp:I-A^b-specific T cells than isotype treatment or historical analyses of leukemic mice at the same timepoint (Figure 2-8,Figure 3-14). These results suggested that TGFβ1 was critical for efficient induction of leukemia-specific Tregs in this model.

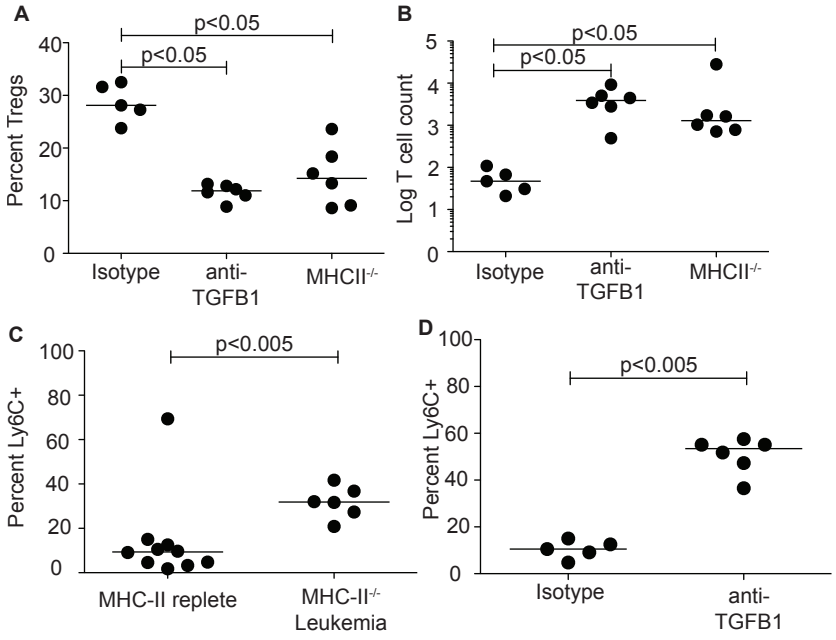


Figure 3-14: TGFβ1 and leukemic cell MHC-II potentiate Treg induction and limit BAp:I-A^b-specific T cell expansion in leukemic mice

A. Mice were inoculated with MHC-II replete leukemia or MHC-II$^{-/-}$ leukemia as labeled, or treated with Isotype or anti-TGFβ antibody. BAp:I-A^b-specific Tregs were gated as previously described and the percent Tregs was analyzed. Results are from three independent experiments, Kruskal-Wallace and Dunns' test used to establish significance. **B**. Mice were treated as in A, and BAp:I-A^b-specific T cell count was analyzed as previously described. Results are from three independent experiments, Kruskal-Wallace and Dunns' test used to establish significance. **C**. BAp:I-A^b-specific T cells from mice inoculated with MHC-II$^{-/-}$ leukemia or MHC-II replete leukemia and harvested 14 days later were analyzed for Ly6C expression. Shown are three independent experiments, significance was established with Mann-Whitney U test. **D**. BAp:I-A^b-specific T cells from mice inoculated with leukemia and treated with anti-TGFβ or isotype control were analyzed for Ly6C expression 14 days later. Shown are three independent experiments, significance was established with Mann-Whitney U test.

Treg induction is dependent on MHC-II presentation by leukemic cells

Leukemic cells and Tregs were also in relatively close contact in the splenic red pulp (Figure 3-5). Thus, we reasoned that the leukemic cells might present antigen in the context of MHC-II, and initiate Treg induction by simultaneously providing TGFβ1. To test this hypothesis, we generated MHC-II$^{-/-}$ leukemic cells and inoculated Foxp3-GFP mice with them. When we analyzed BAp:I-A^{b}-specific Treg induction 14 days later, we found that significantly fewer BAp:I-A^{b}-specific T cells were Tregs in response to MHC-II$^{-/-}$ leukemia than in response MHC-II replete leukemia (either from historical experiments or during experiments done at the same time with isotype treatment Figure 3-2, Figure 3-14). In parallel, MHC-II$^{-/-}$ leukemia or anti-TGFβ treatment led to significant increases in Ly6C expression on BAp:I-A^{b}-specific T cells, suggesting that these cells might be converted into a Th1-like lineage instead of the Treg lineage (Figure 3-14). Additionally, MHC-II$^{-/-}$ leukemia correlated with significantly more proliferation of BAp:I-A^{b}-specific T cells in leukemic mice, again suggesting that the decreased BAp:I-A^{b}-specific Treg conversion might allow for increased proliferation of the BAp:I-A^{b}-specific T cells (Figure 3-14). Consistent with these findings, we observed a significant increase in survival of mice inoculated with MHCII$^{-/-}$

leukemia compared to MHCII-replete leukemia (Figure 3-15). Together, these findings support a mechanism of host protection from leukemia where antigen presentation on MHC-II by the leukemic cells induces immune suppression (and thus leukemia progresses in the host) while antigen presentation on MHC-II by myeloid cells induces immune system activation (and thus leukemia regresses in the host).

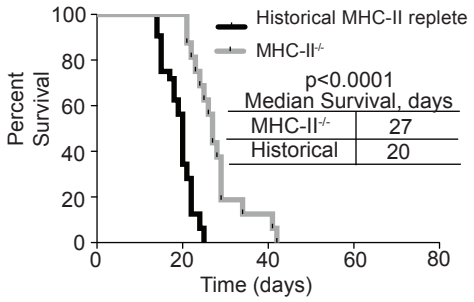


Figure 3-15: MHC-II expression on leukemic cells modulates survival of leukemic mice.

Mice inoculated with MHC-II$^{-/-}$ leukemia or MHC-II replete leukemia were aged until leukemia-induced morbidity and survival to morbidity was analyzed. P-values from Log-rank (Mantel-Cox) test; three independent experiments shown.
Thus, antigen presentation by the leukemic cells enhances induction of BAp:I-A^{b}-specific T cells into the Treg lineage, inhibits proliferation of Ly6C+ BAp:I-A^{b}-specific T cells and reduces survival of leukemic mice.

Previous reports suggest that IL10 signaling is a common feature of immune-suppressive APCs, which may indirectly lead to Treg induction (190, 195). Particularly given histology showing that Tregs, leukemic cells, and red pulp

macrophages were juxtaposed in close proximity in the red pulp (Figure 3-5), we reasoned that IL10 signaling might help induce leukemia-specific Tregs in BCR-ABL^{+} B-ALL. We treated leukemic mice every-third-day with IL10R blockade (clone 1B1.3A) or isotype (clone HRPN) and analyzed survival. We found that Il10R blockade treatment did not change overall survival of mice, though formally the median survival was slightly increased (Figure 3-16). However, we also found that IL10R blockade did increase BAp:I-A^{b}-specific T cell count and decreased the fraction of BAp:I-A^{b}-specific T cells that were Tregs (Figure 3-16). Importantly, these experiments were not compared to isotype control, but instead to historical data.

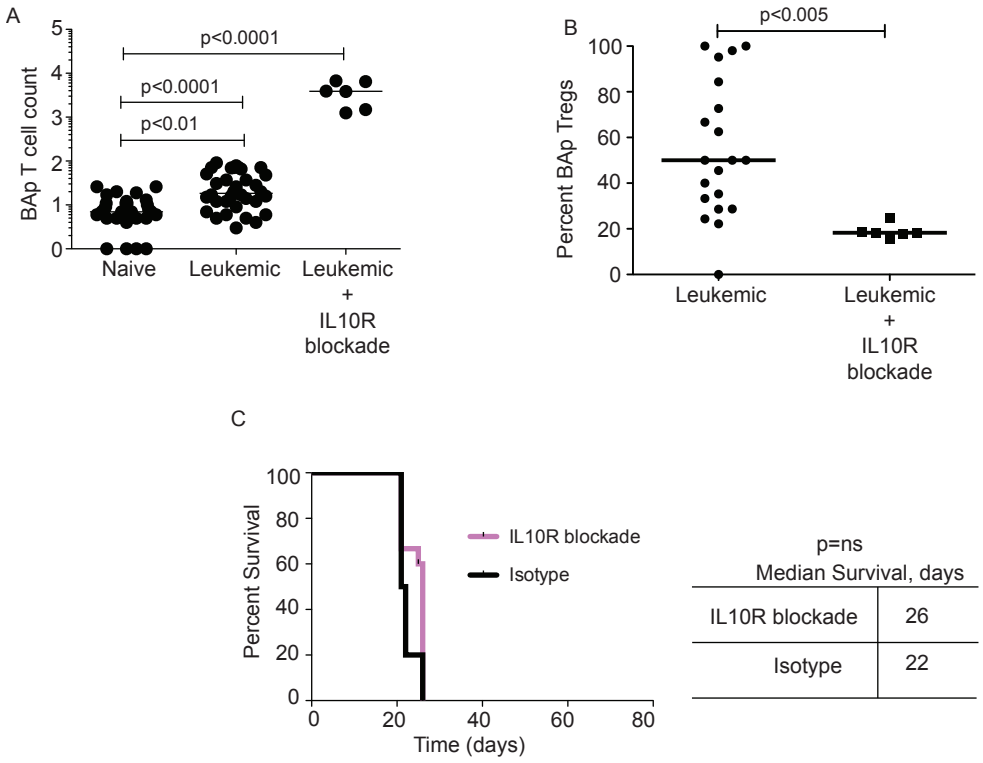


Figure 3-16: IL10 signaling modulates BAp:I-A^b-specific T cell count and phenotype, but does not change overall survival in steady-state leukemias.

A. Naïve, leukemic, or IL10R blockade-treated leukemic mice where harvested and BAp:I-A^b-specific T cells were enumerated. Shown is the Log(Y+1) of the BAp:I-A^b-specific T cell count from the SLO. Significance from Kruskal-Wallace and Dunn's test, all groups contain at least two independent experiments. **B**. BAp:I-A^b-specific Tregs were analyzed from historical or IL10R blockade-treated mice. Significance from Mann-Whitney U test, both groups contain at least two independent experiments. **C.** Leukemic mice were treated with IL10R blockade antibody or isotype control. Survival was analyzed by Log-rank (Mantel-Cox) test, at least two independent experiments were conducted per-group.

Conclusion

A key question in cancer immunotherapy is whether T cell tolerance can be prevented or reversed. Our findings demonstrate that BCR-ABL specific tolerance can be limited by Treg depletion, which correlates with increased expression of Ly6C on the BAp:I-A^b-specific T cells. Ly6C is expressed on Th1-like effector cells that produce high levels of IFNγ, IL2, and Granzyme B (192). Importantly, Th1 cells are classically thought to be critical for tumor control (196). Thus our data suggest that $LY6C^+FOXP3^-$ BAp:I-A^b-specific T cells are useful biomarkers of a robust immune response to leukemia (Figure 3-12). Our data also suggests that global excess of Tregs, like that found in *Stat5bCA* transgenic mice, has a negative impact on survival of leukemic mice (Figure 3-11). It should be noted that the *Stat5bCA* mice have a number of other abnormalities (for example, an excess of $CD8^+$ T cells, progenitor B cells, and granulocytes), which may impact this analysis (117). Nonetheless, there is clearly a correlation between the excess quantity of Tregs in *Stat5bCA* mice and decreased survival from leukemia seen in this model.

Depletion of Tregs is not compatible with life, yet leukemic mice with long-term Treg depletion survived. This observation begged the experiment to analyze if

the leukemic cells were “rescuing” mice from IPEX that would normally be induced in Treg-depleted mice (Figure 3-8). Indeed, 50% of leukemic, Treg-depleted mice lived longer than non-leukemic, Treg-depleted mice. This data hints that perhaps leukemic cells have sufficient immune-suppressive functions that they can replace Tregs, however further analysis needs to be done. For instance, the only experiments done here are using survival as readout. Survival is probably not the best readout for this experiment since the mice die of different pathologies depending on if leukemia is present or absent. Nonetheless, this experiment may be seen as a “jumping-off point” for future studies exploring this interesting question.

In this analysis, we embarked on a number of studies to ascertain the timing of leukemia progression relative to Treg induction, and when these Tregs are important. We found that Tregs were important for leukemia progression both at early timepoints as well as later during leukemia progression, since Treg depletion starting at a late stage of leukemia onset still had a partial impact on increasing survival of leukemic mice (Figure 3-10). Additionally, early depletion of Tregs followed by a light, once-weekly DT treatment also led to partial increase in survival of leukemic mice, suggesting that strong Treg depletion late in leukemia progression also contributed to the survival seen in leukemic

Foxp3$^{DTR/DTR}$ mice which received DT every-other-day from leukemia onset until morbidity (Figure 3-9). Taken together, these observations support the conclusion that Tregs are required for optimal leukemia progression both at early times during leukemia onset and at late times during leukemia progression.

Our studies clearly demonstrate that Treg induction is an important mechanism for suppressing anti-leukemia immune responses. We identified TGFβ1 production and MHC-II presentation by leukemic cells as key molecular mechanisms that allowed conversion of BAp:I-A^{b}-specific T cells into the Treg lineage. We uncovered leukemic cells and BAp:I-A^{b}-specific T cells as two prominent sources of TGFβ1. Genetic ablation of MHC-II or antibody-based depletion of TGFβ resulted in decreased conversion of BAp:I-A^{b}-specific T cells into Tregs and a corresponding increase in proliferation of BAp:I-A^{b}-specific T cells in response to leukemia. Thus, our data support the concept that TGFβ1 and antigen presentation by leukemic cells are critical components in the extensive conversion of BAp:I-A^{b}-specific T cells into the Treg lineage (Figure 3-14).

Temporal regulation of TGFβ1 production by leukemic cells and BAp:I-A^b-specific T cells remains to be ascertained in this model. One likely model is that initial TGFβ1 production by the leukemic cells induces BAp:I-A^b-specific Treg conversions, which then produce TGFβ1 as well. In this model, leukemic cell-derived antigen presentation and TGFβ1 initiate Treg conversion, and BAp:I-A^b-specific T cell-derived TGFβ1 maintains Treg polarization. Importantly, such a mechanism would allow BAp:I-A^b-specific Tregs to suppress proliferation of other anti-leukemia T cells via TGFβ1 (such as GFP:I-A^b-specific T cells in our model).

Regardless of the temporal expression patterns of TGFβ1, the data presented herein point to the importance of the leukemia microenvironment. Specifically, the leukemia microenvironment has copious amounts of TGFβ, as well as antigen presentation by the leukemic cells, which protects the leukemia from the host immune response. This model requires leukemic cells to present antigen on MHC-II as well as produce TGFβ1, thus suggesting that these two mechanisms may synergize to induce immune suppression.

IL10 signaling has both pro-tumor and anti-tumor roles in other settings (197-199). In our model, IL10R blockade (and ostensibly disruption of IL10 signaling)

led to appropriate BAp:I-A^b-specific T cell phenotypes (increased expansion of the cell population and a decreased fraction of which were Tregs). However, IL10R blockade did not extend survival of leukemic mice (Figure 3-16). It is therefore likely that IL10 signaling has many roles in the immune response to BCR-ABL^+ B-ALL, and perhaps it plays a detrimental role to the host despite its apparent ability to induce canonically better anti-leukemia BAp:I-A^b-specific T cells (i.e., more proliferation and a lower fraction that are $Foxp3^+$).

Our findings provide evidence that Treg depletion can modestly extend survival of leukemic mice and that this correlates with increased priming and Th1-skewing of BAp:I-A^b-specific T cells. Finally, even though current immunotherapy is largely focused on tumor neo-antigens, our studies support the notion that cross-reactive T cells can play a role in the immune response to leukemia. Therefore our work may inform future therapeutic options which are aimed at reducing the number or function of Treg cells present in cancer.

Chapter 4 Immunotherapy approaches to treat leukemia: an informed approach based on leukemia molecule expression

Introduction:

[4]Patients with B-cell acute lymphoblastic leukemia (B-ALL) harboring the BCR-ABL chromosomal translocation have very poor outcomes (200, 201). Current therapies for BCR-ABL$^+$ B-ALL include cytotoxic chemotherapeutics, tyrosine kinase inhibitors, and bone marrow transplantation. These treatments are both toxic and often transiently effective, indicating that new treatment options are urgently needed. One such option is immunotherapy. Recent work in cancers with high numbers of non-synonymous mutations has demonstrated that immunotherapy involving neutralization of PD1 and CTLA4 (checkpoint blockade) is an effective treatment option (107, 131-133). Whether immunotherapy involving checkpoint blockade strategies will also be effective in cancers with few non-synonymous mutations, such as ALL (109), remains unclear

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[4] The Majority of Chapter 4 has been submitted for publication (Luke Manlove, first author) November 2015.

To determine whether immunotherapy is an effective option for treating B-ALL we used a mouse model of BCR-ABL$^+$ B-ALL to characterize the host immune response to this leukemia (63, 118, 202). We found that the host adaptive immune system can respond to BCR-ABL$^+$ B-ALL and slow leukemia progression. Although B-ALL cells are believed to have low numbers of non-synonymous mutations (109), the fusion between BCR and ABL does generate an MHC class II restricted peptide antigen that can be recognized by a small population of endogenous BAp:I-A^b-specific T cells in mice. The BAp:I-A^b-specific T cells were predominately induced to become Treg cells in response to leukemia, which suppressed an effective immune response to BCR-ABL$^+$ B-ALL (111). Thus, T cells do respond to BCR-ABL$^+$ leukemia in this mouse model, but the response was immune suppressive in nature, and detrimental to host survival.

Herein, we address if the immune response to leukemia could be modulated thus making BCR-ABL$^+$ B-ALL malleable to checkpoint blockade-based T cell immunotherapy. Three signals are thought to be required to activate a T cell

response including (1) peptide:MHC interactions with the T Cell Receptor, (2), costimulation through CD28, and (3) cytokines and/or costimulatory molecules to polarize a response (203-205). We reasoned that all three signals would need to be appropriately aligned for an efficient anti-leukemia T cell response to occur. In addressing this issue, we found that mice with BCR-ABL$^+$ B-ALL cells expressing high levels of MHC class II, along with a high ratio of the costimulatory molecule CD40 relative to the co-inhibitory molecule PDL1, had significantly reduced leukemic burden. We then demonstrated that prophylactic vaccination with BAp peptide could induce BAp-specific memory T cells that protect mice from BCR-ABL$^+$ B-ALL re-challenge. Thus BAp peptide presentation is important for prophylactically generating a protective anti-leukemia immune response.

We also wanted to understand if vaccination could be used as a therapeutic treatment for leukemia, as opposed to a prophylactic treatment. Repeated immunization of mice with established leukemia with LCMV plus BAp peptide (i.e., homologous vaccination) had only a modest effect on overall survival. In contrast, a distinct therapeutic heterologous vaccination involving sequential infection with LCMV plus BAp, transgenic LM-BAp and VSV plus BAp led to the

generation of a small number of mice that survived long-term. Antibodies against the co-inhibitory molecules (anti-PDL1 and anti-CTLA4) and co-stimulatory molecules (anti-CD40) checkpoint blockade had modest effects in a therapeutic setting, but when combined with heterologous vaccination significantly enhanced long-term survival. Importantly, this approach did not alter the number of BAp:I-A^b specific T cells but did significantly alter their cytokine expression pattern. Thus, cancers with low numbers of non-synonymous mutations are susceptible to checkpoint blockade if combined with an appropriate vaccination strategy using leukemia specific antigens.

Materials and Methods

Mice

C57BL/6 mice and *Cdkn2a*$^{-/-}$ (strain 01XF6, B6, 129-Cdkn2a^{tm1Cjs}/Nci, (113)) mice came from the National Cancer Institute. *Foxp3-GFP* (stock# 006772) and *Ifng*$^{-/-}$ (stock# 002287) mice came from Jackson Laboratories (Bar Harbor, ME). *OT-IxRag2*$^{-/-}$ mice were generated locally as previously described(116). *Ifng*$^{-/-}$ and *Ifngr*$^{-/-}$ mice were obtained Mice were housed at the University of Minnesota in specific pathogen free conditions or BSL-2 facilities, and all experiments were approved by IACUC.

Listeria monocytogenes generation

Acta- Listeria monocytogenes strain 1942 (from Dr. Sing Sing Way) expressing BAp peptide from a plasmid was constructed as previously described (206-208). Briefly, the pAM401 vector was digested with PstI and SalI to linearize the vector. The digested product was electrophoresed on a 1% agarose gel and purified using a Qiagen QiaQuik gel extraction kit (Qiagen, Germantown MD). BAp peptide sequence was synthesized by the BioMedical Genomics Center at the University of Minnesota, with a 5' PstI restriction site and a 3' SalI restriction site (NEB, Ipswich, MA). The linear pAM401 vector was ligated to the synthesized BAp DNA sequence using T4 Ligase (NEB, Ipswich, MA) at room temperature for 5 minutes. Ligation reactions were transformed into DH10B *Escherichia coli* (NEB, Ipswich, MA) and plated on plates containing Chloramphenicol (Sigma-Aldrich, St. Louis, MO). Colonies were picked and screened by restriction digestion with PvuII, HindIII, and BamHI (NEB, Ipswich, MA). Clones that gave the expected restriction digest pattern were sequenced to confirm that they contained the appropriate insert. DNA sequence was translated into peptide sequence and aligned to confirm that the BAp peptide was in the same reading frame as SIINFEKL (a control peptide in the pAM401 construct). Correctly

assembled clones were electroporated into penicillin-pretreated *acta- L. monocytogenes* strain 1942 at 1500V, 2500 microfarads, and 400 Ohms in sucrose-containing media. Electroporated bacteria were then plated on Brain-Heart Infusion agar plates supplemented with 20μg/mL chloramphenicol. Correct clones were picked and tested by infection.

Infections and Immunizations

10^7 Colony-forming Units (CFU) of *L. monocytogenes* expressing BAp (LM+BAp) were injected intravenously through the tailvein (i.v.). *Listeria monocytogenes* expressing BAp (LM-BAp) or 2W1s peptide (LM-2W) were grown overnight in Brain Heart Infusion supplemented with 20μg/mL chloramphenicol. The next morning, bacteria were then subcultured into fresh Brain Heart Infusion with chloramphenicol and analyzed by optical spectrometry at O.D.600 after 4hrs of incubation at 37C. When bacterial cultures reached a density of O.D.600 of 0.5, 10^7 Colony-forming Units (CFU) were washed twice in Phosphate-buffered Saline (PBS) and injected into mice by tail vein injection in 400μL PBS. Injections were conducted in a BSL-2 facility. Mice were housed in a BSL-2 facility following

infection. Mice vaccinated with LCMV-Armstrong received 10^5 plaque forming units (pfu) intraperitoneally (i.p.) at day 0. Vesicular Stomatitis Virus-Indiana was used at $5x10^5$ pfu i.v. at day 0. Vaccinia (Western Reserve) was delivered i.v. At day 3 and day 5, mice were injected i.v. with 200µg BAp Mice were harvested at indicated timepoints.

Leukemia model

The BCR-ABL$^+$ B Acute Lymphoblastic Leukemia model has been previously described (118). Briefly, *Cdkn2a*$^{-/-}$ mouse bone marrow cells were transduced with viral supernatant containing a BCR-ABL (P190)-IRES-GFP retrovirus (119) and cultured for adoptive transfer as previously described (63).

Tetramer production

The BAp:I-A^b tetramer and others used herein were produced as described (121). Purified monomer was tetramerized with SA-PE or SA-APC and cells were enriched as previously described (63).

In-vivo antibody treatment

Mice were treated with anti-PDL1 10F.9G2 and anti-CTLA 9H10 (Bio X Cell, West Lebanon NH) with 100µg i.p. every-other-day (unvaccinated) and 200µg i.p. twice per week (vaccinated). Mice were treated with anti-CD40 (FGK4.5) (Bio X Cell, West Lebanon NH) 200µg i.p. every-other-day.

Antibodies

Antibodies for flow cytometry and histology include CD3 PE, CD4 (RM4-5) PerCPCy5.5, CD8 (53-6.7) BV650, CD11c (N418) PE, FOXP3 (FJK16S) PE, CD80 (16-10A1) APC, CD86 (GL1) PE-Cy7, CD19 BV605, B220 (RA3-6B2) Horizon V500, IFNγ (XMG1.2) BV650, LAP (TW7-16B4) PE, TNF alpha (MP6-XT22) BV421, IL17A (TC11-18H10) AlexaFluor488, and PSGL1 (2PH1) BV421 purchased from BD Biosciences (San Jose, CA); NK1.1 (PK136), CD11b (M1/70), CD11c (N418), B220 (RA3-6B2), and F4/80 in APC-eFluor780; PD1 (J43) FITC, CD73 eFluor450, FR4 PE-Cy7, PDL1 PerCP-eFluor710, MHC-II I-Ab eFluor450, IL10 (JESS-16E3) PE, Granzyme B (NGZB) PE-Cy7, GARP (YGIC86) eFluor450, and all ELISpot antibodies were purchased from eBiosciences (San Diego, CA), and IgM (Fab') APC was purchased from

Jackson Immunoresearch (West Grove, PA). Rat IgG1 (HRPN) PerCP-Cy5.5 Isotype and Rat IgG2a (2A3) violetFluor450 Isotype were purchased from Tonbo Biosciences (San Diego, CA). Cells from enriched fractions were analyzed on an LSR-II Fortessa cytometer (BD Biosciences, San Jose CA) and data was analyzed in FlowJo (Treestar, Ashland OR).

Statistics

Standard normality tests suggested departures from normality, so non-parametric tests (Mann-U Whitney test for two groups, Kruskal-Wallace & Dunns' Test for more than two groups) were used unless otherwise stated. Normality assessments and non-parametric tests were done in GraphPad Prism (LaJolla, CA). Principal component analysis was conducted in R (prcomp function) (209). Linear regressions and correlation coefficients were estimated in GraphPad Prism and R.

A detailed description of the PCA and corresponding linear regression are included herein. We performed a principal component analysis (PCA) on the following five phenotype metrics collected on each mouse: PDL1, MHC-II, CD40,

CD80, CD86. We added one to each metric, and then log-transformed the resulting value so that our data met the PCA assumption of joint normality. Components were estimated using the prcomp function in the stats package in R.

First, pairs plots of the raw manifest variables and log-transformed manifest variables were created. Principal component analysis is a method for reducing the dimension of a dataset by transforming an initial set of possibly-correlated manifest variables into a set of new, orthogonal variables, which are referred to as “components”. PCA describes the multivariate correlation in the dataset. While the method generates as many components as there are measured variables in the dataset, most of the variation in the dataset can usually be captured with only a few components. Each component consists of a value that describes the proportion of variation in the original dataset explained by the component, and a set of loadings that describe the extent to which each manifest variable correlates with that component. Let X be an $n \times k$ matrix containing measurements of k different manifest variables for n sampled individuals.

Estimation is obtained through an Eigen-decomposition of the square matrix $X'X$.

Eigenvalues correspond to proportions of variance in the original dataset captured by each component, and eigenvectors describing correlations between each manifest variable and each component. Although the method constructs as many principal components as there are manifest variables in the dataset, interpretation is limited to those components that explain the preponderance of variation in the dataset. The number of components to interpret is often determined using a screeplot, showing the proportion of variance explained by each component.

A screeplot for the components identified for the log-transformed immunogenicity phenotype metrics is shown in Figure 1D. Based on the screeplot, we interpreted the first two components. Each manifest variable's loading on each component is shown in Table 1.

We extracted PC scores for PC1 and PC2 for each mouse in our dataset. We used a linear regression model to relate these PC scores to percent leukemic burden measured on these same mice. The regression model consisted of four terms: an intercept (β_0), main effects for each PC, (β_1 and β_2), and an interaction term between the two PCs (β_3). Let y_i be percent leukemic burden in the i^{th}

studied mouse, let $X_{1,i}$ be the i^{th} mouse's PC1 score, and let $X_{2,i}$ be the i^{th} mouse's PC2 score. Then the regression model we fit can be written as:

$$y_i = \beta_0 + \beta_1 (X_{1,i}) + \beta_2 (X_{2,i}) + \beta_3 (X_{1,i}) (X_{2,i}) + \varepsilon_i$$

The model was fit using the lm function in R (1). An overall F-test clearly suggested that at least some of the coefficients in the model differed significantly from zero (F-statistic = 23.76 on 3 and 26 degrees of freedom; $p < 0.0001$).

Specifically, the model detected strong relationships between the first two PCs and leukemic burden, and a marginally significant interaction effect in our dataset.

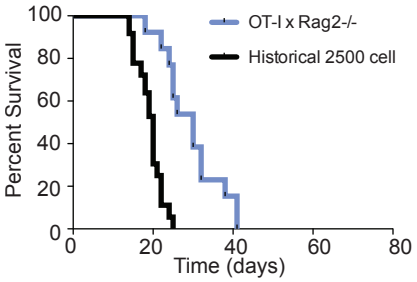
In general, the intercept term corresponds to expected leukemic burden for mice with average scores on both PC1 and PC2. Specifically, under this model we expect that mice with average PC1 and PC2 scores have an average leukemic burden of 62.64 percent. For mice with an average score of PC2, but who are

one unit above average on PC1, we expect an average leukemic burden of 52.64 percent (62.64 – 10.00). For mice with an average score on PC1, but who are one unit about average on PC2, we expect an average leukemic burden of 43.34 percent (62.64 – 19.30). For mice that are one unit above average on both PC1 and PC2, we expect an average leukemic burden of 27.38 percent (62.64 – 10.00 – 19.30 – 5.96).

Adaptive Immunity: Benefits and Detriments to leukemic mouse survival

We first sought to address the efficacy of the mouse adaptive immune response to leukemia in the steady-state (where the only pathogen insult in SPF mice is that from BCR-ABL^{+} leukemia). Our initial data suggested that leukemia progressed faster in OT-I x *Rag2*$^{-/-}$ mice (Figure 2-3), but we also analyzed the survival of these mice when adoptively transferred with leukemic cells (Figure 4-1). Contrary to our predictions, we found that OT-I x *Rag2*$^{-/-}$ mice survived longer with leukemia than C57BL/6 mice. During this analysis, we noticed that the way in which the mice became moribund was different between C57BL/6 hosts and OT-I x *Rag2*$^{-/-}$ hosts. Specifically, C57BL/6 hosts uniformly developed

bilateral hind-limb paralysis while the OT-I x *Rag2*$^{-/-}$ developed a broad range of leukemia-associated pathologies of which the most prominent was severe lymphadenopathy. Thus, we think that apparent dichotomy between leukemic burden and survival is in part due to the endpoints used in the survival analysis.



p<0.0001

Median Survival, days	
OT-I x Rag2$^{-/-}$	30
Historical 2500 cell	20

Figure 4-1: Adaptive immunity causes poor survival in steady-state leukemic mice

OT-I x Rag2$^{-/-}$ hosts (blue line) or C57BL/6 hosts (black line) were adoptively transferred with 2500 BCR-ABL^{+} leukemic cells and survival was analyzed. Log-rank (Mantel-Cox) test was used to analyze statistics. Median survival is also reported.

We also reasoned that adaptive anti-leukemia immunity probably was dependent on CD4^{+} T cells, CD8^{+} T cells, or both. To test this, we adoptively transferred mice with BCR-ABL^{+} leukemia and treated with CD4 and/or CD8a depleting antibodies at previously published doses (210). Again, to our surprise, we found that none of these depleting regimens had any obvious phenotype as far as changing survival of the leukemic mice (Figure 4-2). From these results, we conclude that at steady-state, the T cell compartment of the adaptive immune

system is having very little effect on leukemia progression. Indeed, since we know that during this time course a substantial fraction of one leukemia-specific T cell population (BAp:I-A^b-specific T cells) are Tregs (Figure 3-2), perhaps the only effect that the immune system has on leukemia in steady-state conditions is a deleterious one for the host.

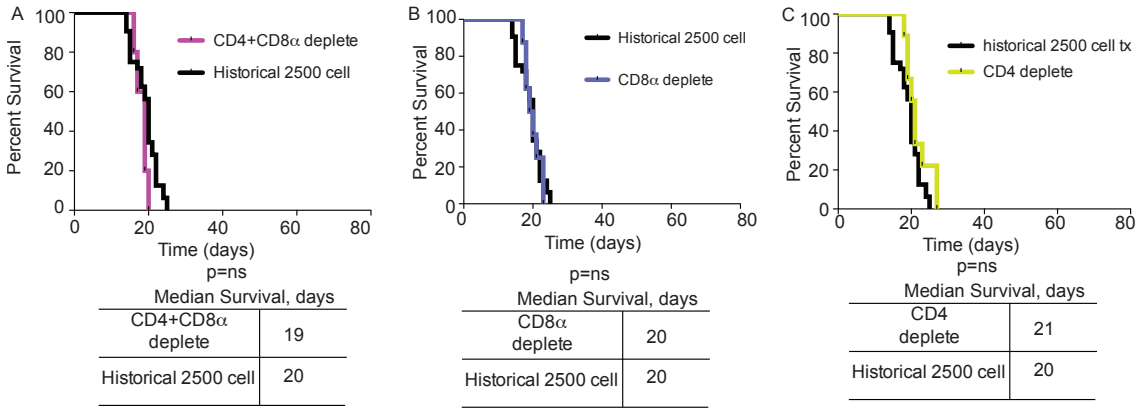


Figure 4-2: T cell depletion does not change survival of steady-state leukemic mice

C57BL/6 mice were adoptively transferred with 2500 BCR-ABL$^+$ leukemic cells and survival was analyzed. **A**. Mice were either untreated (black line) or treated with induction doses of 750μg anti-CD4 (GK1.5) and 750μg of anti-CD8 (YTS 169.4) and then followed by weekly doses of 250μg of the above antibodies for the duration of survival. **B**. Mice were treated as in A, except with only anti-CD8 being provided (though at the same doses as in A). **C**. Mice were treated as in A, except with only anti-CD4 being provided (though at the same doses as in A). All statistics come from the Log-rank (Mantel-Cox) test. Also shown is the median survival.

Antigen presentation and costimulatory markers are expressed in certain patterns on leukemic cells

We previously showed that there was a higher fraction of live leukemic cells in the BM and SLO of *OT-I x Rag2*$^{-/-}$ mice than in C57BL/6 mice. Further, the range of leukemic burdens was quite broad in the C57BL/6 hosts (IQR=11%-69%) but less-so in the OT-I x Rag2$^{-/-}$ hosts (IQR=90%-98%) (Figure 2-3:) (63). To understand why there was such a range in leukemic burden, we looked for characteristic differences in the leukemic cells from mice with low leukemic burden compared to leukemic cells from mice with high leukemic burden. Since B cells can function as antigen presenting cells, we examined the expression of MHC-II to stratify the leukemic burden based on expression of surface markers. MHC-II expression inversely correlated with leukemic burden (mice that had low fraction of leukemic cells in the bone marrow had high MHC-II expression on the leukemic cells, Figure 4-3).

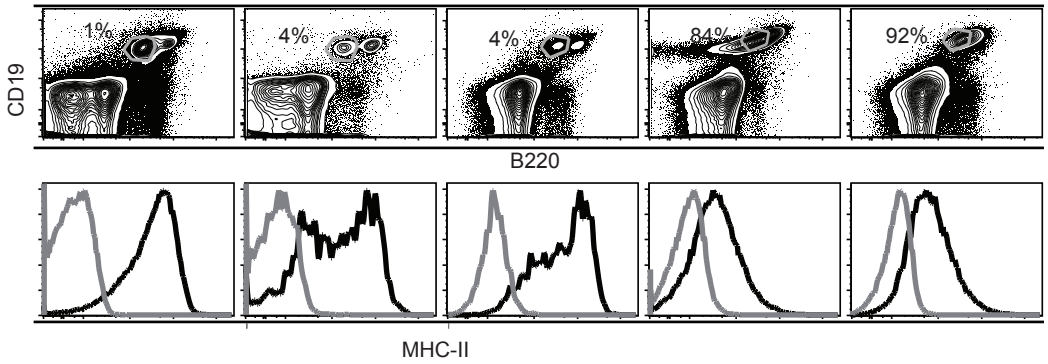


Figure 4-3: MHC-II expression on leukemic cells in relation to leukemic burden

Representative dot plots and histograms from five mice with varying leukemic burden (gated as live, singlet, $CD19^{+}$, $B220^{low}$ cells. Black curves are MHC-II, grey curves are isotype. Listed are the percentages of live singlet events that fall into the $CD19^{+}$, $B220^{low}$ gate.

Since antigen presentation without any costimulation is ineffective at inducing a T cell response, we examined the expression of the costimulatory molecules CD40, CD80, CD86, and PDL1. These costimulatory molecules individually correlated with leukemic burden to a minimal degree (Figure 4-4).

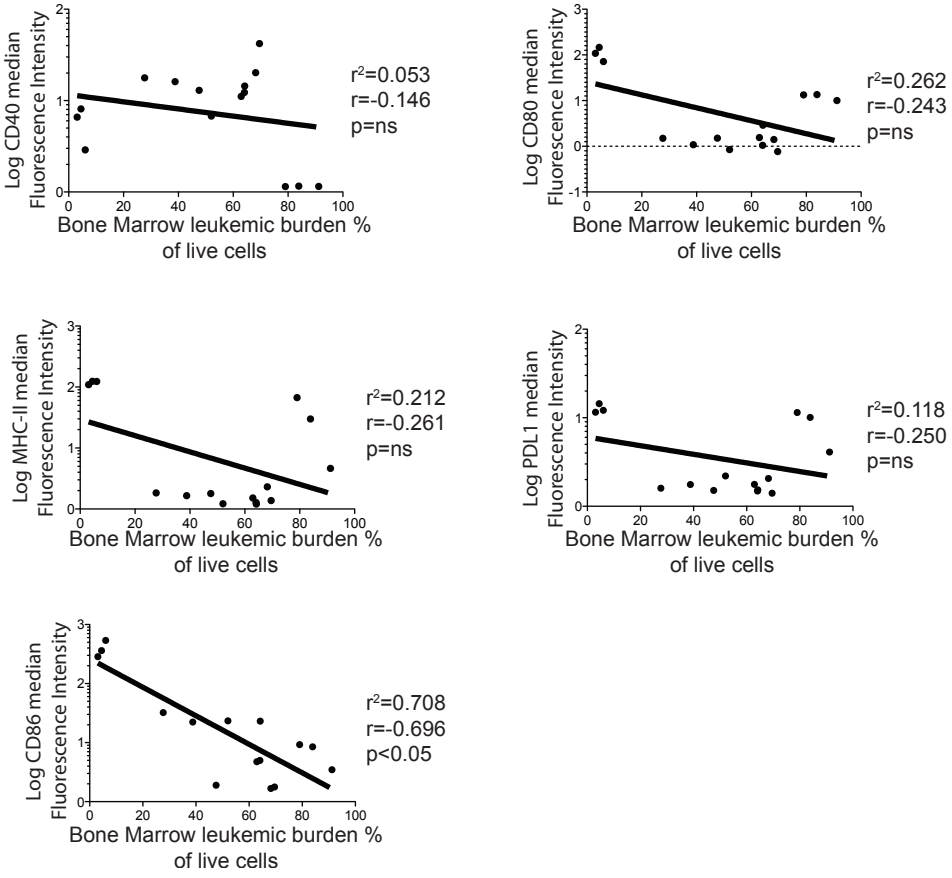


Figure 4-4: Individual costimulatory molecules have minimal correlative power with leukemia progression

Individual correlations between each contributor to the PCA (CD40, MHC-II, CD86, CD80, and PDL1) and leukemic burden-depicted as the percentage of leukemic cells that comprise live cells in the bone marrow. The black line on each graph is the best fit. Shown also is the r value, r^2 value, and the p-value (from Spearman correlation test). All correlations, except for that of CD86 with leukemic burden, are insignificant.

Thus, we next sought to identify if there was an ensemble of costimulatory molecules that correlated with leukemic burden, by using Principal Components Analysis (Figure 4-5).

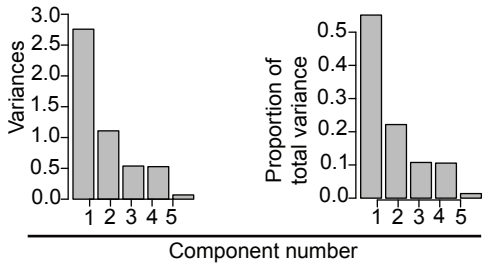


Figure 4-5: PC1 and PC2 account for 77% of variability in leukemia progression

Screeplots of Principal Components (PC). Y-axis shows proportion of variance accounted for by each PC. Principal component analysis was derived from 27 separate mice in 3 experiments.

The first component described a positive correlation between MHC-II, CD80, CD86, and PDL1. The second component was driven by a negative correlation between CD40 and PDL1 (Figure 4-6).

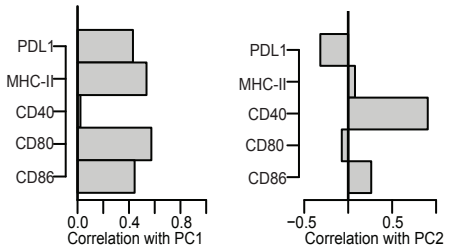


Figure 4-6: PC1 and PC2 are defined by distinct costimulation patterns

Correlations of measured variables with first two principal components. PDL1, MHC-II, CD80, and CD86 correlate positively with PC1; CD40 and (to a lesser extent) CD86 correlate positively, while PDL1 correlates negatively, with PC2.

These first two components described 77% of the variation in leukemic burden that we observed in mice (Figure 4-7).

	PC1	PC2	PC3	PC4	PC5
Standard Deviation	1.67	1.05	0.73	0.73	0.26
Proportion of Variance	0.55	0.22	0.11	0.11	0.014
Cumulative Proportion	0.55	0.77	0.88	0.99	1.00

Figure 4-7: Description of PCs and descriptive power of each PC

PCs are listed and shown is the standard deviation around each PC, the proportion of variance described by each individual PC, and the cumulative proportion of variance described.

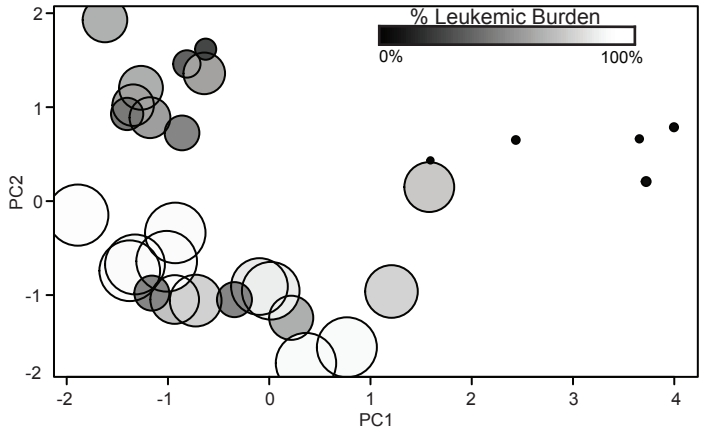


Figure 4-8: Distribution of individual mouse scores on PCs 1 and 2.

Mouse leukemic burden is indicated by dot size and dot shade, larger white dots indicate mice with higher leukemic burden, and smaller black dots indicate mice with lower leukemic burden.

Mice that had low leukemic burden tended to have high scores for both PC1 and PC2 (Figure 4-8). Therefore, we used linear regression to examine relationships between the first two PC scores and leukemic burden (Figure 4-9). Both high PC1 and high PC2 scores were associated with significantly decreased leukemic burden ($p<0.001$). A low PC1 score (score = -2) was predictive of high leukemic burden (left panel) regardless of PC2 score. In contrast, leukemias with higher PC1 scores (score = 0-2), showed dependence on PC2 in predicting leukemic burden. These results demonstrate that leukemic burden inversely correlates

with the capacity of leukemic cells to activate $CD4^+$ T cells as assessed by MHC-II, CD40, CD80, CD86, and PDL1 expression on the leukemic cells.

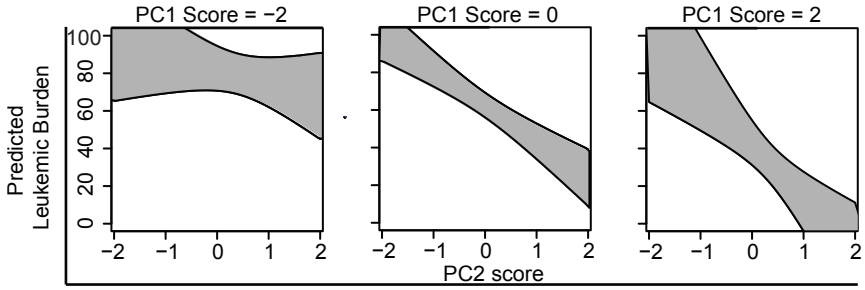


Figure 4-9: PC1 and PC2 synergize to predict leukemia progression

Predicted leukemic burden as a function of PC2 scores at three separate levels of PC1 (low, average, and high). Grey regions denote 95% confidence bounds.

Modulation of individual costimulatory molecules modestly improves survival of leukemic mice

Our PCA suggested that an ensemble of costimulatory molecules (CD80, CD86, PDL1, CD40) functioned as a cohesive unit to modulate anti-leukemia immunity. Nonetheless, it was possible that individual targeting of antigen presentation and costimulatory molecules might change the disease course. Further, recent findings suggest that co-inhibitory molecule blockade is advantageous for disease outcome in other cancers (132). We tested if antibody targeting of

PDL1, CTLA4, or CD40 would be sufficient to change leukemia progression. Antibody blockade of PDL1 and CTLA4 (either individually, or in combination) led to a modest, but significant increase in survival of leukemic mice (Figure 4-10). Additionally, treatment of leukemic mice with an anti-CD40 antibody that is characterized as an agonist also led to a modest, yet significant increase in survival (211) (Figure 4-10). Thus, the components defined by our PCA do not individually identify therapeutic targets in this model, again supporting that these molecules must be addressed as an ensemble.

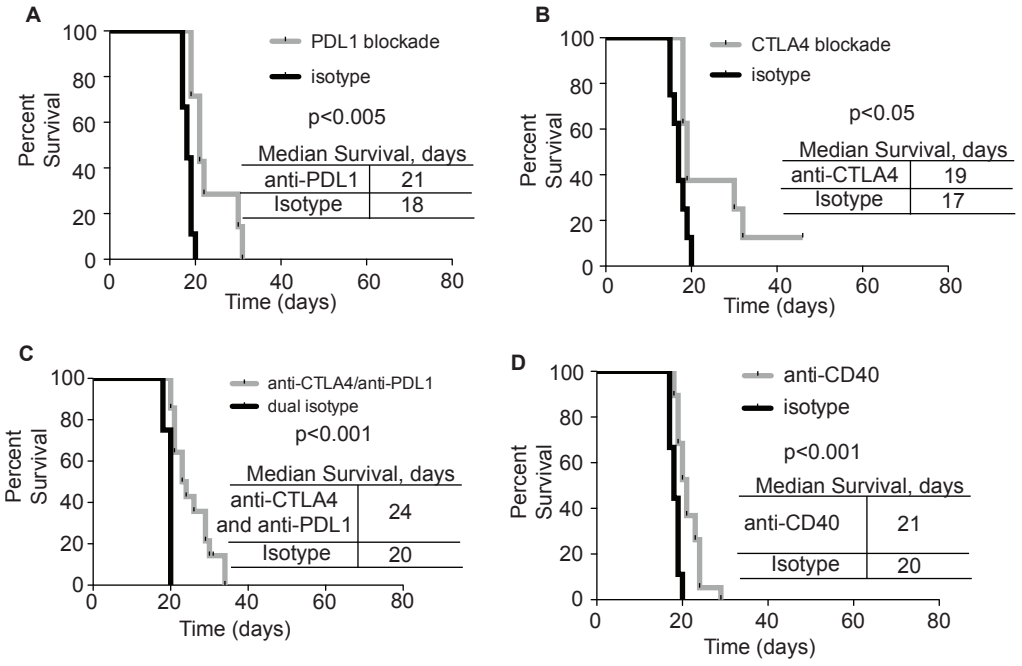


Figure 4-10: Individual costimulatory molecule-targeted therapy is only modestly effective in BCR-ABL$^+$ B-ALL

A. C57BL/6 mice were inoculated with 2500 leukemic cells and treated every-other-day with 100μg anti-PDL1 (clone 10F.9G2) until moribund. Hazard ratio of 0.10 with a 95% confidence interval from 0.025 to 0.42. **B**. Mice were treated as in A, except with 100μg anti-CTLA4 (clone 9H10). Hazard ratio of 0.21 with a 95% confidence interval from 0.057 to 0.76. **C**. Mice were treated as in A, except with 100μg anti-PDL1 (clone 10F.9G2) plus 100μg anti-CTLA4 (clone 9H10). Hazard ratio of 0.012 with a 95% confidence interval from 0.0018 to 0.18. **D**. Mice were treated as in A, except with 200μg anti-CD40 (clone FGK4.5). Hazard ratio of 0.084 with a 95% confidence interval from 0.022 to 0.31. Two or more independent experiments shown in each panel, Log-Rank (Mantel-Cox) test was used to establish significance in all panels.

BAp-specific T cells can be primed by acute infection plus exogenous peptide.

None of the immune checkpoint modulations we tested yielded substantial efficacy at extending survival of leukemic mice. However, we have previously shown that Ly6C$^+$ BAp:I-A^b-specific T cells correlate with anti-leukemia immunity upon Treg depletion (63). We attempted to re-create an inflammatory environment by using a variety of pathogen insults to generate many Ly6C$^+$ BAp:I-A^b-specific T cells. We infected mice with *Listeria monocytogenes* expressing the BAp peptide (termed LM+BAp), which caused a 65-fold increase in BAp:I-A^b-specific T cell numbers. In parallel, we infected mice with either LCMV-Armstrong, Vaccinia (Western Reserve) or VSV-Indiana and then delivered 200µg BAp peptide i.v. at 3 and 5 days post-infection. This allowed us to utilize the inflammation caused by acute viral infection to induce a strong BAp:I-A^b-specific CD4$^+$ T cell response (termed LCMV+BAp, Vacc+BAp, or VSV+BAp). At peak infection LCMV+BAp caused a 74-fold proliferation of BAp:I-A^b-specific T cells, while VSV+BAp caused a 114-fold proliferation of BAp:I-A^b-specific T cells (Figure 4-11). These results show that BAp:I-A^b-specific T cell proliferation can be initiated by immunization.

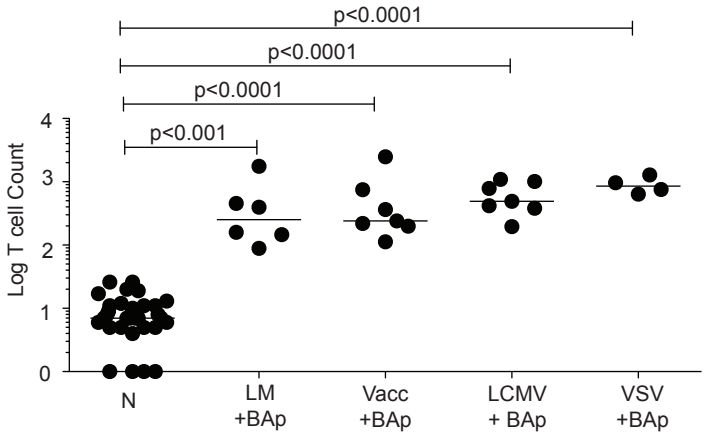


Figure 4-11: Infection in the presence of BAp yields proliferation of BAp:I-A^b-specific T cells

Naïve mice (N) were immunized with LM+BAp, LCMV+BAp, VSV+BAp or Vaccinia+BAp. Secondary lymphoid organs were harvested at peak and BAp:I-A^b-specific T cells were enumerated. More than two independent experiments are shown for each infection. Kruskal-Wallace and Dunn's test used to establish significance.

Additionally we found that LCMV+BAp induced a high frequency of Ly6C$^+$ memory BAp:I-A^b-specific T cells following leukemia re-challenge, while LM+BAp induced substantially fewer Ly6C$^+$ memory BAp:I-A^b-specific T cells following leukemia re-challenge (Figure 4-12). Since our previous work showed that Ly6C was expressed on the majority of BAp-specific T cells upon Treg depletion (which also resulted in significantly less leukemic burden and significantly longer survival of leukemic mice), we reasoned that acute viral infections were better adjuvants

for inducing protective BAp-specific immunity, since they too resulted in a higher fraction of BAp-specific T cells expressing Ly6C.

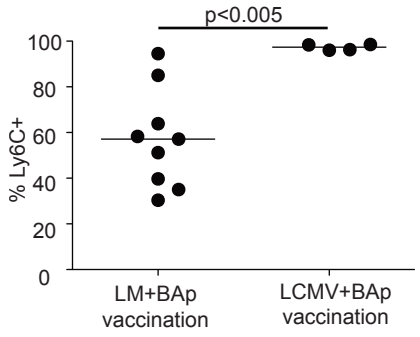


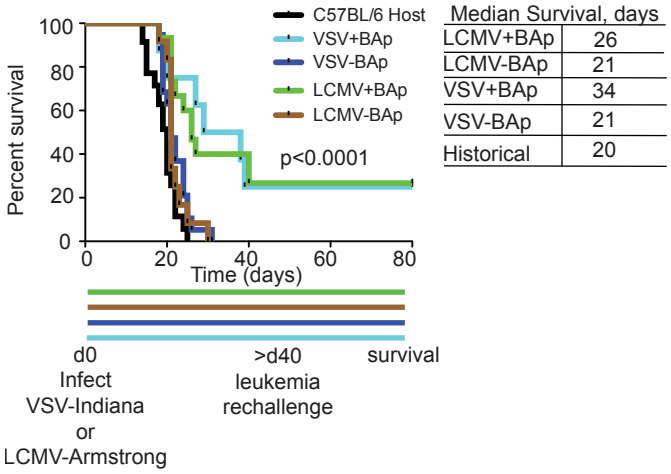
Figure 4-12: BAp:I-A^b-specific T cell Ly6C expression following leukemia rechallenge

Percent Ly6C+ BAp:I-A^b-specific T cells from mice vaccinated with either LM+BAp or LCMV+BAp at d0 and then rechallenged with leukemia at d30. Two or more independent experiments conducted for each infection. Mann-Whitney U Test used to establish significance.

BAp-specific adaptive immunity confers long-term survival for leukemic mice.

The PCA suggested that MHC-II expression, and thus antigen presentation, was important in describing the immune response to leukemia. We have identified one peptide from BCR-ABL (BAp) that is processed and presented on MHC-II *in vivo*. Thus, we hypothesized that immunization with BAp plus strong adjuvants cells might mediate protection from BCR-ABL$^+$ B-ALL in mice. To test this, we infected mice with either LCMV-Armstrong (+/- BAp peptide) or VSV-Indiana (+/-

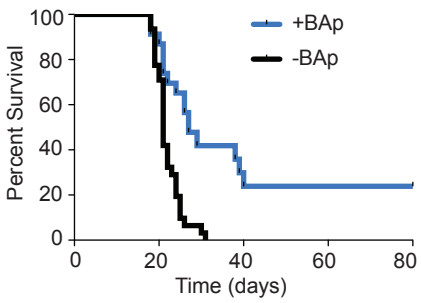
BAp peptide) and re-challenged mice with BCR-ABL^{+} leukemia >40 days later; a memory timepoint when no acute inflammation remained. (Figure 4-13). Only mice that were infected with an acute viral pathogen plus BAp peptide exhibited long-term survival; mice that were infected with just an acute viral pathogen in the absence of BAp succumbed to leukemia rapidly. Indeed, when comparing all vaccinated mice from Figure 4-13, the presence of BAp during the acute viral infection was sufficient to drive a hazard ratio of 0.24, suggesting that mice vaccinated "+BAp" had a five-fold higher probability of surviving at a given timepoint (Figure 4-14). Thus, BAp-specific adaptive immunity can confer long-term survival in this model. This evidence supports the conclusion that MHC-II antigen presentation of BAp is critical for effective prophylactic vaccination, though this analysis provided little functional data for how prophylactic vaccination controlled leukemia upon re-challenge.



Median Survival, days	
LCMV+BAp	26
LCMV-BAp	21
VSV+BAp	34
VSV-BAp	21
Historical	20

Figure 4-13: Viral prophylactic vaccination with BAp confers long-term survival in some leukemic mice

Prophylactic vaccination with BAp peptide allows long-term survival in leukemic mice. Mice were vaccinated with LCMV-Arm +/- BAp or VSV-I +/- BAp Greater than 40 days later, mice were rechallenged with 2500 BCR-ABL^{+} leukemic cells and rested until morbidity. Log-Rank (Mantel-Cox) test was used to establish significance, three or more independent experiments used for each group.



Hazard Ratio: 0.24
95% CI: 0.12 - 0.46

p<0.0001

Median Survival, days	
+BAp	27
-BAp	21

Figure 4-14: Presence of BAp during acute infection induces long-term survival of leukemic mice

Vaccinated mice +/- BAp (data taken from experiment depicted in Figure 4-13) were divided into two groups strictly based on if the vaccinations included exogenous BAp peptide (+BAp, blue line) or did not include exogenous BAp peptide (-BAp, black line). Significance from Log-rank (Mantel-Cox) test. Hazard ratio, and 95% Confidence Intervals flanking the hazard ratio, are shown.

Interferon-γ potentiates anti-leukemia immunity during prophylactic vaccination

Many reports have documented the importance of Interferon-γ (IFNγ) in anti-tumor immunity (196, 212). In $CD4^+$ T cells, IFNγ is normally produced by Th_1 cells. We have previously shown that most BAp:I-A^b-specific T cells responding to BCR-ABL^+ leukemia at steady-state are Treg cells and thus are likely making minimal IFNγ. Consistent with this idea, we found that the ability of host T cells to make IFNγ did not substantially affect survival following inoculation with

leukemic cells since *Ifng*$^{-/-}$ hosts succumbed to leukemia similarly to C57BL/6 hosts (Figure 4-15).

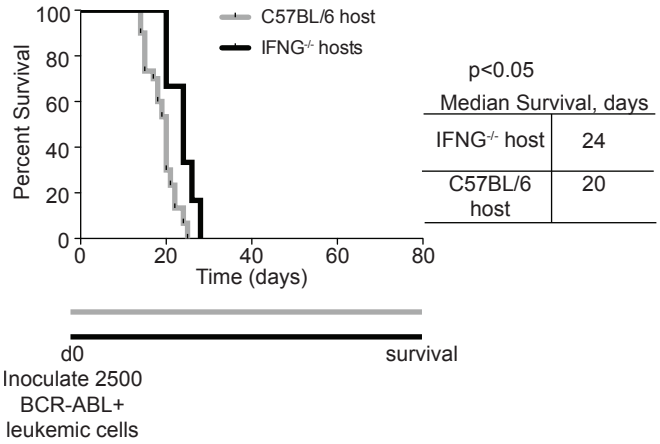


Figure 4-15: IFNγ is not critical for leukemic mouse survival at steady-state

Naïve C57BL/6 mice or Ifng$^{-/-}$ mice were adoptively transferred with 2500 BCR-ABL^{+} leukemic cells. Survival was analyzed with the Log-Rank (Mantel-Cox) test used to establish significance. Two or more experiments shown for each group.

Despite the lack of a clear role for IFNγ at steady-state, we hypothesized that IFNγ might play a role in the adaptive immune response to leukemia following prophylactic vaccination. To test this, we prophylactically vaccinated *Ifng*$^{-/-}$ mice with LCMV-Arm+BAp and re-challenged with leukemia at more than 40 days post-vaccination. Survival of vaccinated *Ifng*$^{-/-}$ host mice was statistically the same as that of unvaccinated mice (Figure 4-16). Comparatively, *Ifng*-replete

mice had long-term survival from LCMV-Arm+BAp vaccination. Thus, IFNγ production was one critical mechanism for effective anti-leukemia immunity following prophylactic vaccination.

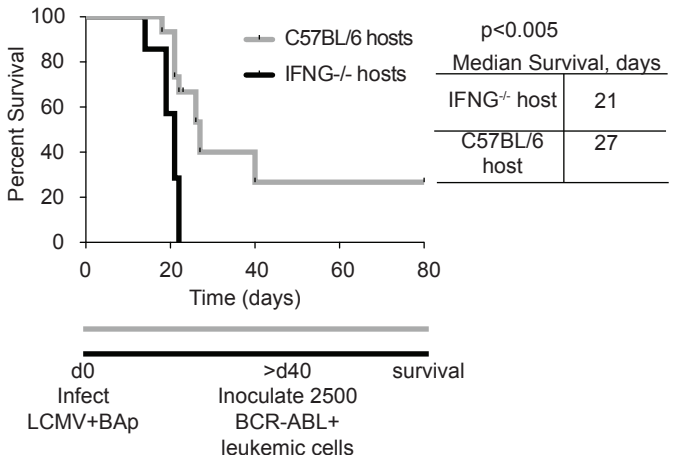


Figure 4-16: IFNγ is required for anti-leukemia immunity after prophylactic vaccination

Naïve C57BL/6 mice or *Ifng*$^{-/-}$ mice were vaccinated with LCMV-Armstrong + BAp peptide as in Figure 4. Log-Rank (Mantel-Cox) test used to establish significance. Two or more independent experiments used in each group.

Specific pathogens mediate effective prophylactic vaccination for BCR-ABL+ B-ALL.

Immune memory is a critical component of prophylactic vaccination. To determine if effective BAp:I-A^{b}-specific memory T cells were formed by

vaccination, we infected mice with LM+BAp or LCMV+BAp and waited 40 days to enumerate BAp:I-A^b-specific memory T cells. We recovered significantly more memory BAp:I-A^b-specific T cells from LCMV+BAp infected mice than from LM+BAp infected mice (Figure 4-17). We then vaccinated mice with either LCMV+BAp or LM+BAp and re-challenged by transferring 2500 leukemic cells into the mice 40 days later. BAp:I-A^b-specific T cell numbers were assessed 14 days after leukemia re-challenge (i.e., 54 days after initial infection). Vaccination with LCMV+BAp, but not LM+BAp, led to a significant increase in the number of BAp:I-A^b-specific T cells following leukemia challenge and decreased leukemic burden (4-fold, Figure 4-17, Figure 4-18). Thus, the increase in BAp:I-A^b memory T cell numbers following LCMV+BAp but not LM+BAp vaccination correlated with disease outcome.

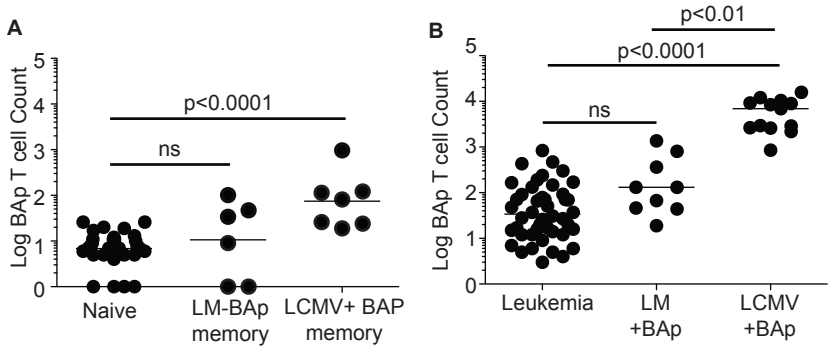


Figure 4-17: BAp:I-A^b-specific T cells form memory and respond to leukemia re-challenge

A. Mice were infected as in Figure 4-11 and rested 30 days, when BAp:I-A^b-specific T cells counts were compared to naïve counts of BAp:I-A^b-specific T cells. Shown are BAp:I-A^b-specific Log (Y+1) T cell counts of BAp:I-A^b-specific memory cells following vaccinations, gated on CD11a^{high}CD44^{high}. Two or more independent experiments shown for each infection. **B**. Mice were unvaccinated or vaccinated with LCMV+BAp or LM+BAp, and 2500 BCR-ABL$^+$ cells were transferred 40 days post-infection. Shown are BAp:I-A^b-specific Log (Y+1) T cell counts; two or more independent experiments shown for each infection.

Ly6C denotes effective anti-leukemia CD4$^+$ T cells

The quality and quantity of BAp:I-A^b-specific memory T cells was different comparing LM+BAp vaccination to LCMV+BAp vaccination. We observed that the inter-quartile range of leukemic burdens in the mice vaccinated with LCMV+BAp was broad (IQR=1.4 x10^6, Fig 6C), showing that protection mediated by LCMV+BAp vaccination was more effective in some mice than in others. We previously observed that Ly6C expression was increased on BAp:I-A^b-specific T cells when Tregs were depleted (63). Therefore, we examined whether Ly6C

expression on BAp:I-A^b-specific T cells correlated with leukemic burden. Mice with high leukemic burden despite prophylactic vaccination (and thus considered 'failed vaccinated mice'), had a significantly lower percent of $Ly6C^+$ BAp:I-A^b-specific T cells than the 'successfully vaccinated mice' (Figure 4-18). Additionally, significantly more BAp:I-A^b-specific T cells expressed Ly6C after LCMV+BAp vaccination (which lowered leukemic burden) than LM+BAp (which had no effect on leukemic burden) (Figure 4-12). Importantly, the number of $Ly6C^+$ BAp:I-A^b-specific T cells was inversely correlated with leukemic burden in LCMV+BAp vaccinated mice. In contrast, leukemic burden did not correlate with total $CD4^+Ly6C^+$ cells in these mice (Figure 4-18). Our data do not directly support a causative role for $Ly6C^+$ BAp:I-A^b-specific T cells responding to leukemia. However, our findings do suggest that interaction of leukemia and leukemia-specific $CD4^+$ T cells may impact the expression of Ly6C, or that $Ly6C^+$ BAp:I-A^b-specific T cells may induce less leukemia progression.

Prophylactic vaccination controls the fraction of BAp:I-A^b-specific T cells which are induced into the Treg lineage after leukemia rechallenge

We also observed that Treg cells made up a significantly smaller portion of the BAp:I-A^b-specific T cell population in mice that were prophylactically vaccinated with LCMV+BAp than unvaccinated mice (Figure 4-19). These results support a functional role for $Ly6C^+FOXP3^-$ BAp:I-A^b-specific T cells during the immune response to leukemia following prophylactic vaccination.

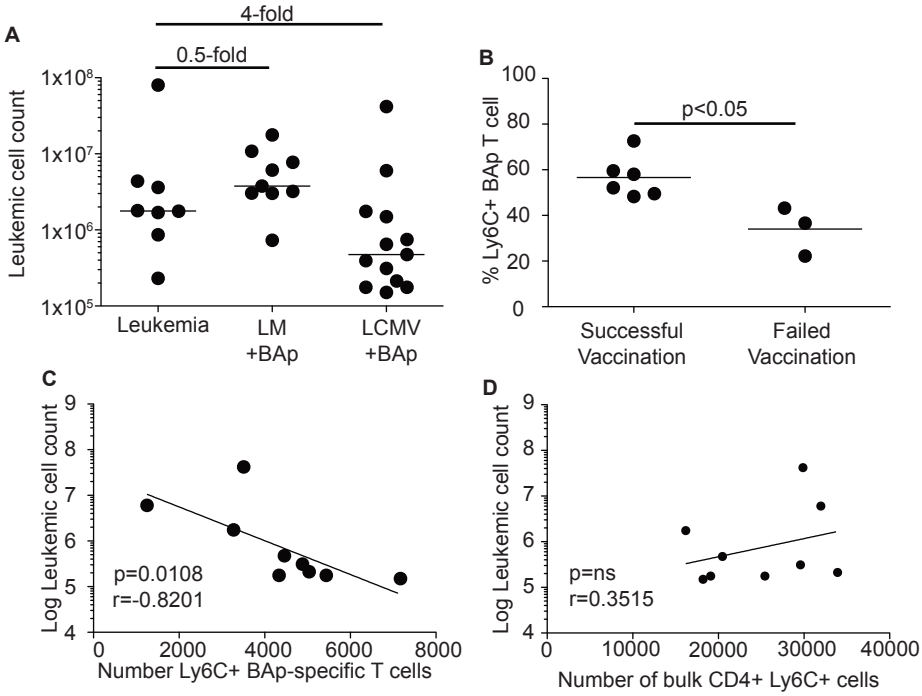


Figure 4-18: LCMV-Armstrong, but not *L. monocytogenes*, yields effective prophylactic vaccination against leukemia

A. Mice were treated as in Figure 4-11 and leukemic burden was analyzed. Lines are median values, numbers represent fold changes in median. **B**. Percent $Ly6C^+$ on BAp:I-A^b-specific T cells harvested from LCMV+BAp-vaccinated mice. Two or more independent experiments shown for each infection. **C**. $Ly6C^+$ BAp:I-A^b-specific T cell count negatively correlates with leukemic burden from secondary lymphoid organs. Spearman correlation used to calculate statistical significance. **D**. $Ly6C^+CD4^+$ T cell count does not correlate with leukemic burden from SLO. Spearman correlation used to calculate statistical significance.

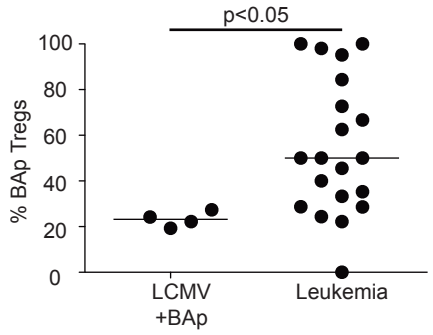


Figure 4-19: Prophylactic LCMV-Arm+BAp vaccination prevents mass-conversion to Treg lineage by BAp:I-A^b-specific T cells after leukemia rechallenge

Foxp3GFP mice were prophylactically vaccinated with LCMV-Arm+BAp and rechallenged with 2500 BCR-ABL$^+$ leukemic cells >40 days later. BAp:I-A^b-specific Tregs were enumerated. Shown is the fraction of total BAp:I-A^b-specific T cells that were Tregs in the SLO.

Antigen presentation and costimulation on leukemic cells are modulated by prophylactic vaccination.

Our principal component analysis (Figure 4-5) suggests that high ratios of CD40:PDL1 and MHC-II:PDL1 may be predictive of low leukemic burden. We examined the leukemic cells from LCMV+BAp vaccinated mice and LM+BAp-vaccinated mice. First, we found that leukemias in mice that were successfully vaccinated with LCMV+BAp had significantly higher expression of CD40 and MHC-II than their "failed vaccination" counterparts (Figure 4-20).

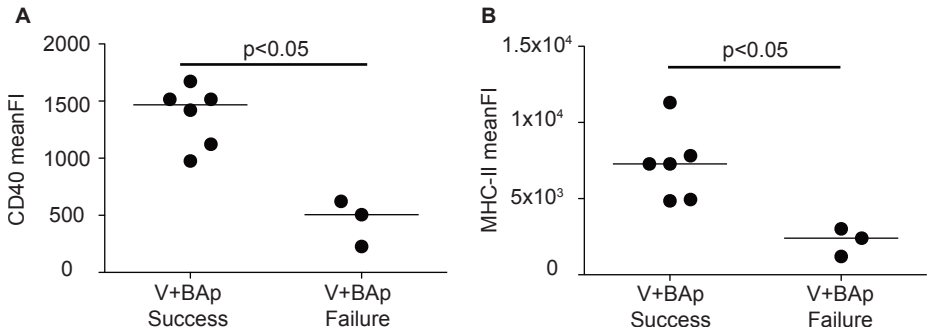


Figure 4-20: CD40 and MHC-II expression is higher on leukemic cells from successfully-vaccinated mice

A. Mice were vaccinated with LCMV+BAp and inoculated with leukemia 30 days later. CD40 Mean Fluorescence Intensity from leukemic cells harvested from Successful vaccinated mice or Failed vaccinated mice derived from Figure 6C. Mann-Whitney U Test used to establish significance. **B**. MHC-II Mean Fluorescence Intensity from leukemic cells harvested from Successful vaccinated mice or Failed Vaccinated mice. Mann-Whitney U test used to establish significance.

Second, in fitting with the predictions stemming from our PCA, we found that CD40:PDL1 and MHC-II:PDL1 increased on LCMV+BAp vaccinated mice (which was an effective vaccination regimen) but not significantly on LM-BAp vaccinated mice (an ineffective vaccination regimen, Figure 4-21). Thus, prophylactic vaccination with acute viral pathogens plus BAp peptide results in protection from leukemia in correlation with our established biomarkers for leukemia immune response. It is unclear why these same predictors do not apply to LM+BAp vaccinated mice.

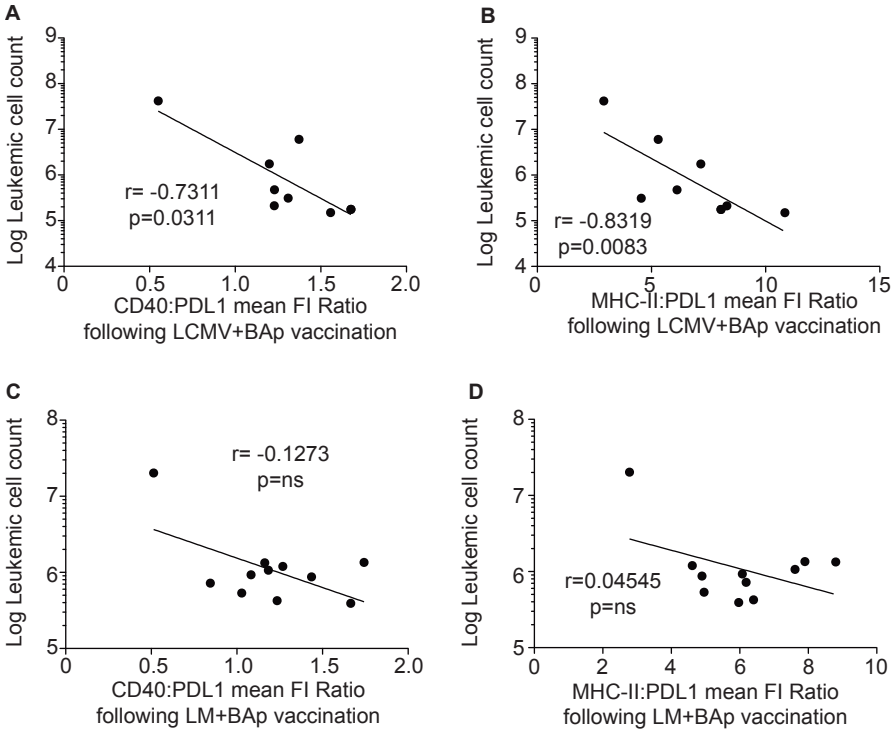


Figure 4-21: CD40, PDL1, and MHC-II follow PCA predictions in leukemias treated with viral, but not bacterial, vaccination

A. Ratio of Mean Fluorescence Intensity of CD40: PDL1 on leukemic cells was calculated from mice vaccinated with LCMV+BAp, and a correlation was calculated between this ratio (X-axis) and the log leukemic cell count (Y-axis). Values from Spearman Correlation. **B**. Ratio of Mean Fluorescence Intensity of MHC-II: PDL1 on leukemic cells was calculated from mice vaccinated with LCMV+BAp, and a correlation was calculated between this ratio (X-axis) and the log leukemic cell count (Y-axis). Values from Spearman Correlation. **C**. Ratio of Mean Fluorescence Intensity of CD40: PDL1 on leukemic cells was calculated from mice vaccinated with LM+BAp, and a correlation was calculated between this ratio (X-axis) and the log leukemic cell count (Y-axis). Values from Spearman Correlation. **D**. Ratio of Mean Fluorescence Intensity of MHC-II: PDL1 on leukemic cells was calculated from mice vaccinated with LM+BAp, and a correlation was calculated between this ratio (X-axis) and the log leukemic cell count (Y-axis). Values from Spearman Correlation.

Therapeutic heterologous vaccination drives long-term survival

We hypothesized that a pro-inflammatory environment might counter leukemia-derived immune suppression, while also inducing BAp-specific adaptive immunity, and thus inhibit leukemia progression. To test this hypothesis, we therapeutically vaccinated mice, which had established leukemia, using either homologous vaccinations with LCMV-Armstrong+BAp peptide or heterologous vaccinations with LCMV-Armstrong+BAp, LM+BAp, and VSV-Indiana+BAp (Figure 4-22). Homologous vaccination with LCMV+BAp significantly prolonged survival although all mice ultimately succumbed to leukemia. Heterologous vaccination should create a more robust pro-inflammatory response, since antibodies created during the primary infection will not neutralize the secondary and tertiary infections. Indeed, heterologous vaccination was significantly more effective and led to long-term survival (>twice the median untreated survival) in ~10% of mice. Thus, repeated vaccination with heterologous agents was an effective treatment strategy in mice with BCR-ABL^{+} B-ALL.

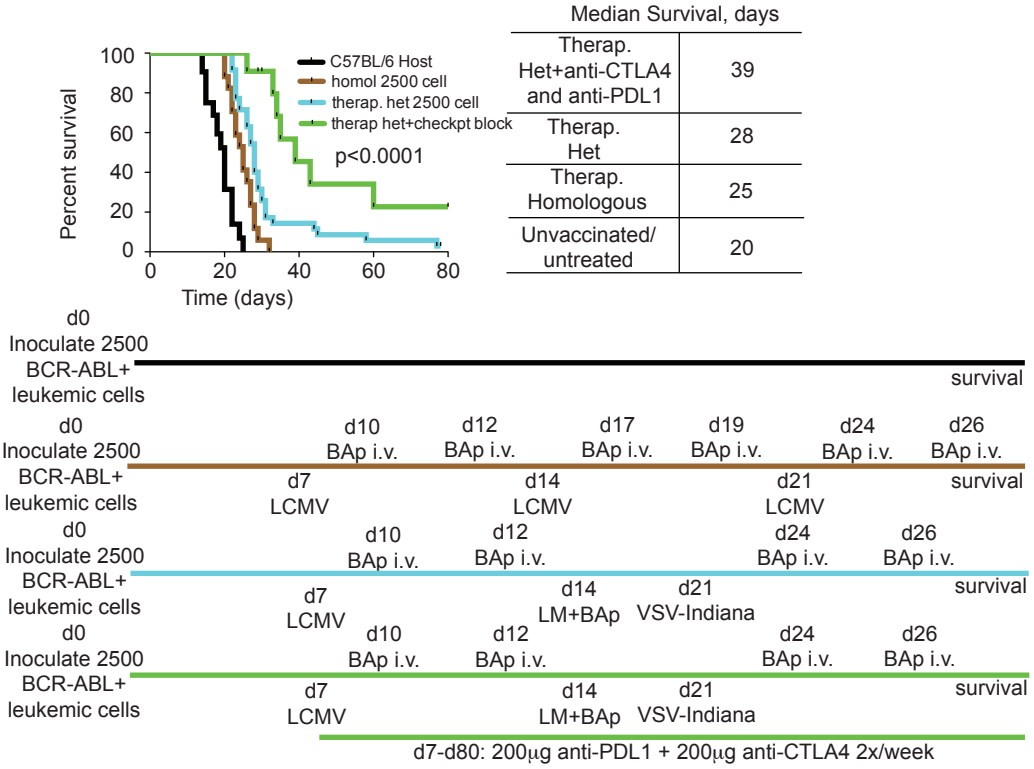


Figure 4-22: Therapeutic vaccination confers long-term survival to BCR-ABL$^+$ leukemic mice, and synergizes with checkpoint blockade

Mice were inoculated with 2500 BCR-ABL$^+$ leukemic cells at d0, and then rechallenged with one of four different treatments. 1) No treatment (Black); 2) Homologous vaccination with Lymphocytic Choriomeningitis Virus (LCMV) Armstrong at day 7, day 14, and day 21, with 200μg exogenous BAp peptide delivered i.v. at three and five days post-infection (days 10, 12, 17, 19, 24, 26) (Brown); 3) Heterologous vaccination with LCMV-Armstrong at day 7, LM-BAp at day 14, and Vesicular Stomatitis Virus (VSV) Indiana at day 21, with 200μg exogenous BAp peptide delivered i.v. at three and five days post-infection (days 10, 12, 17, 19, 24, 26), N=52 (Blue); 4) as in 3, except with 200μg of anti-PDL1 (10F.9G2) and 200μg of anti-CTLA4 (9H10) twice per week from day 7 to day 80, N=11 (Green). Surviving mice were euthanized at day 80 post-leukemia inoculation. Shown are survival curves, and the Log-Rank (Mantel-Cox) test was used to analyze statistics.

The immune response to acute viral and bacterial infection is canonically pro-inflammatory. However since the mice have active leukemia, high doses of leukemia antigens may still be available during this pro-inflammatory state, which may cause chronic antigen stimulation, a situation where PDL1 signaling is highly expressed (213). Additionally, our initial findings showing that CD44 was not highly expressed on all BAp:I-A^b-specific T cells responding to leukemia suggest that BAp-specific T cell priming is not optimal (63). CTLA4 blocks interaction of CD28 with B7-1 and B7-2 molecules, thereby reducing T cell priming (214). Thus, we hypothesized that therapeutically vaccinated mice that were treated with dual PDL1/CTLA4 checkpoint blockade might show improved survival. This treatment strategy led to a significant increase in survival beyond that seen for either PDL1+CTLA4 blockade (Figure 4-10) or therapeutic vaccination (Figure 4-22), with 31% of mice surviving long-term. Since this long-term survival is far past the timepoint when inflammation would remain from the therapeutic vaccination, it suggests that an adaptive immune response is mediating long-term survival.

To understand some of the mechanisms allowing effective therapeutic vaccination, we compared homologous, heterologous, and heterologous plus

checkpoint blockade treatments and assessed BAp:I-A^b-specific T cell expansion and effector function. To do this, we inoculated mice with BCR-ABL^+ leukemia and started therapeutic vaccination at the same timepoint. We then harvested the mice 21 days later and enumerated BAp:I-A^b-specific T cells. We found that all regimens induced robust proliferation (~1250-fold over naïve precursor numbers); however, there was no difference in the number of BAp:I-A^b-specific T cells recovered between any of the three treatment groups (Figure 4-23).

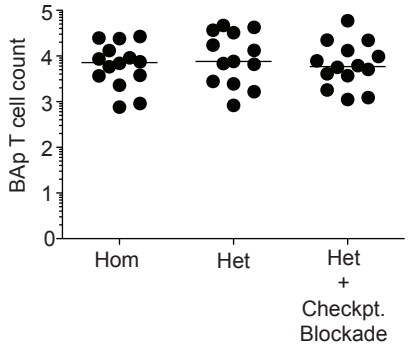


Figure 4-23: Number of BAp:I-A^b-specific T cells does not correlate with therapeutic vaccination outcome

Mice were treated as in Figure 4-22, but treatment was started on the same day as leukemia challenge. Mice were euthanized at day 21 and BAp:I-A^b-specific T cells were harvested and enumerated. Shown are BAp:I-A^b-specific Log (Y+1) T cell counts; two or more independent experiments shown for each infection. Groups were compared with Kruskal-Wallace and Dunn's test, with no significant differences found.

Since total numbers of BAp:I-A^b-specific T cells did not help give insight into the mechanisms that allowed effective therapeutic vaccination, we examined the

phenotype of the BAp:I-A^b-specific T cells recovered. We reasoned that BAp:I-A^b-specific T cells should take on a more Th1-like phenotype in response to therapeutic vaccination, and that this phenotype should correlate with improved disease outcome.

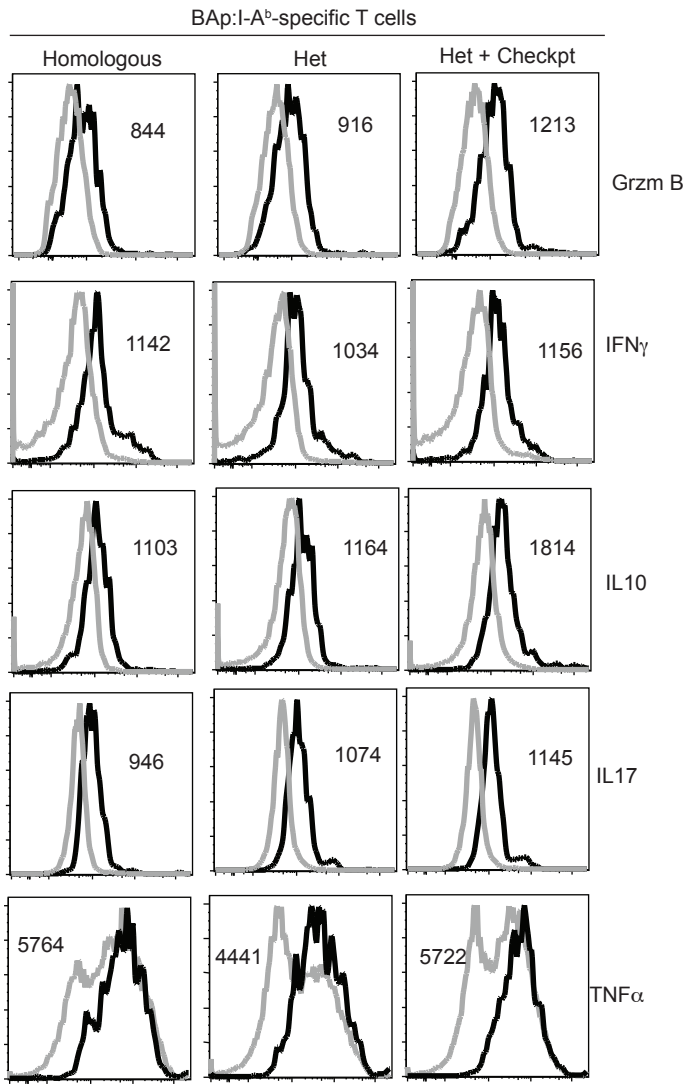


Figure 4-24: Cytokine production from BAp:I-A^b-specific T cells during therapeutic vaccination

BAp:I-A^b-specific T cells were harvested from mice as in Figure 8B, and stimulated *ex-vivo* with PMA and Ionomycin to analyze potential cytokine production. Shown is concatenated data from 10 individual mice in two or more independent experiments. The numbers on each histogram are the mean fluorescence intensity

However, we found no significant increase in IFNγ or TNFα (two canonical Th1 cytokines). Interestingly, we found that a larger fraction of BAp:I-A^b-specific T cells produced more IL10, Granzyme B, and both IL17 and Granzyme B together (Figure 4-24, Figure 4-25), all of which have previously been associated with pro-inflammatory tumor clearance (93, 197, 215, 216).

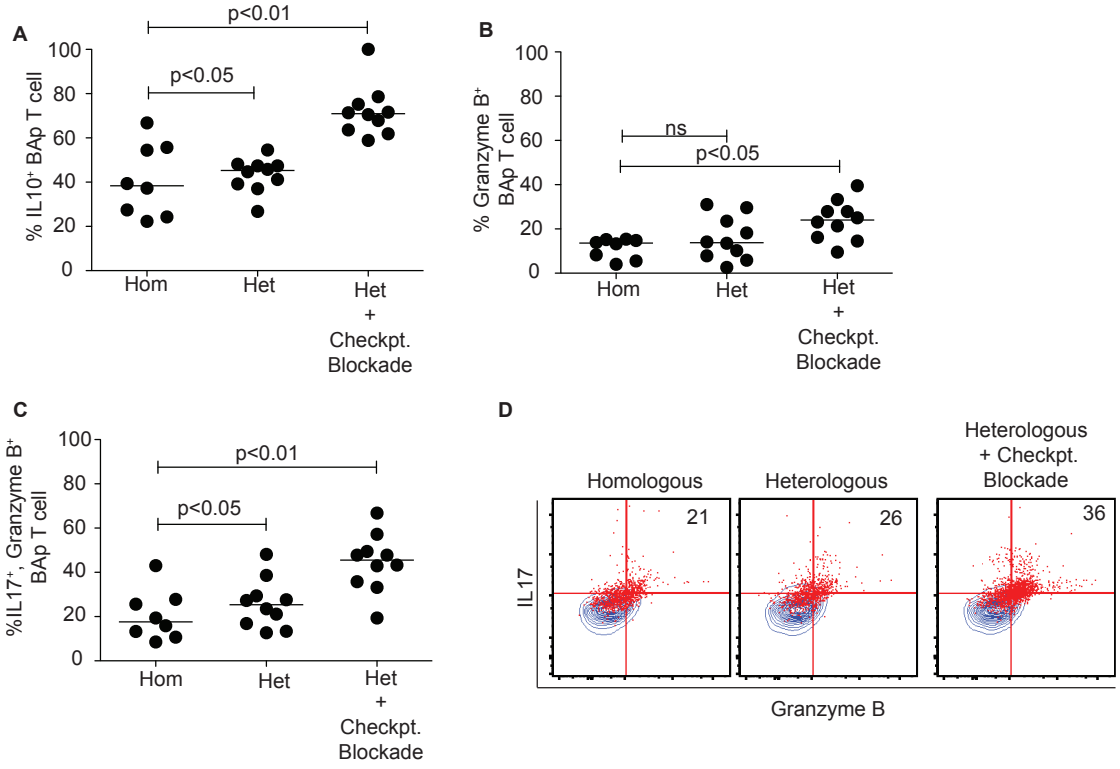


Figure 4-25: IL10, Granzyme B, and IL17 are produced more in correlation with effective therapeutic vaccination

A. Based on concatenated histograms from Figure 4-24, gates were drawn to delineate "positive" vs. "negative" fraction of cells and applied to individual mice. Percent positive is shown on the Y-axis for IL10. Kruskal-Wallace and Dunn's test used to establish significance. **B**. Granzyme B was analyzed. Kruskal-Wallace and Dunn's test used to establish significance. **C**. Percent of BAp:I-A^b-specific T cells which are double-positive for IL17 and Granzyme B is shown and analyzed. Statistics were analyzed by Kruskal-Wallace and Dunn's test. **D**. BAp:I-A^b-specific T cells were harvested as in Figure 9A and concatenated events from 10 mice were gated to show $IL17^+$, Granzyme B^+ percentages of BAp:I-A^b-specific T cells. Numbers on the graph are percentage of double-positive events in the gates as shown.

Additionally, we examined expression of PD1, a molecule that is canonically associated with anti-inflammatory stimuli. We found that PD1 expression on

BAp:I-A^b-specific T cells positively correlated with leukemic burden in all therapeutically vaccinated mice (Figure 4-26), and that PD1 expression was lowest on heterologous vaccination plus checkpoint blockade-treated mice. Together, these results provide evidence that poly-functional CD4$^+$ leukemia-specific T cells can produce a combination of IL10, IL17, and Granzyme B, correlated with effective anti-leukemia adaptive immunity.

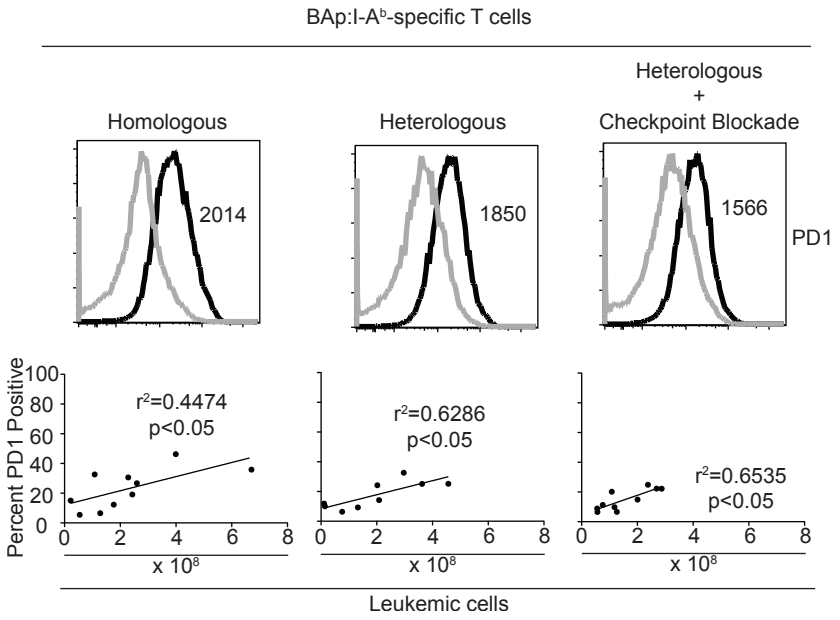


Figure 4-26: PD1 expression on BAp:I-A^b-specific T cells decreases as therapeutic vaccination efficacy increases

PD1 expression shown on histograms from concatenated samples receiving either Homologous, Heterologous, or Heterologous + Checkpoint Blockade treatment. PD1 expression on BAp:I-A^b-specific T cells was analyzed and linear regression was used to compare to leukemic burden in the same mouse. Data from Spearman Correlation shown.

Leukemia pathology changes between vaccinated and unvaccinated mice

We also noticed an interesting phenotype change when treating mice with vaccination. For example, in unvaccinated mice, the mice uniformly were euthanized when they became moribund from bilateral hind-limb paralysis (a phenotype which parallels that previously observed in this model, Richard Williams personal communication). Also, unvaccinated mice had relatively small lymph nodes and large spleens (Figure 2-2). However, in therapeutic heterologous vaccinated mice, about fifty percent of the mice developed severe lymphadenopathy (morphologically resembling a lymphoma) and displayed no signs of hind limb paralysis when they were euthanized. It is unclear why the phenotype changed, but this could suggest that either massive inflammation is occurring in the lymph nodes of vaccinated mice (thus resulting in a huge influx of immune cells) or that leukemic cells re-localize towards the lymph nodes in leukemic mice (thus resulting in a huge influx of leukemic cells).

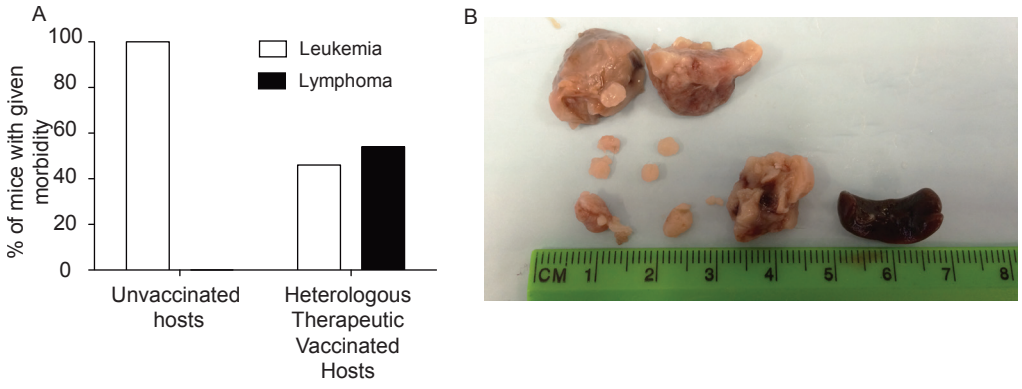


Figure 4-27: Cause of morbidity differs when comparing unvaccinated to vaccinated host mice

Unvaccinated or therapeutic heterologous vaccinated host mice were euthanized following IACUC protocols and the gross cause of morbidity was analyzed. "Leukemia" denotes mice with no clearly enlarge lymph nodes and relatively enlarged spleen (see Figure 2-2) and lymphoma denotes at least one enlarged lymph node and relatively small spleen. **A.** Twenty unvaccinated and twenty vaccinated mice were compared. 100% of unvaccinated mice had bilateral hind-limb paralysis and 46% of therapeutic heterologous vaccinated mice had bilateral hind-limb paralysis. **B.** Representative spleen and lymph node from one therapeutic heterologous vaccinated mouse at the time of morbidity.

Discussion

BCR-ABL$^+$ B-ALL is poorly responsive to current therapies. One approach that has not been well-explored in this disease is checkpoint blockade-based immunotherapy. Checkpoint blockade works well in malignancies with many non-synonymous mutations to achieve long-term survival (52, 127, 217-220). Presumably, this is because the increased number of non-synonymous mutations allows for a concurrent increase in the number of neo-antigen specific

T cells. Comparatively, in cancers with low numbers of non-synonymous mutations, like ALL (221), checkpoint blockade-based immunotherapy targeting the endogenous immune response is relatively unstudied. Cancers like ALL have fewer non-synonymous mutations, likely resulting in fewer neo-antigen specific T cells, and at least some cross-reactive T cells that respond to ALL (63). In fact, current paradigms suggest that cancers with low numbers of non-synonymous mutations are minimally affected by targeting the endogenous immune response with checkpoint blockade. Herein, we show that an endogenous cross-reactive T cell response can be effective in controlling BCR-ABL^{+} B-ALL, but this requires both checkpoint blockade and an aggressive heterologous vaccination strategy.

Our data suggests that, at steady-state, a very minimal adaptive immune response occurs against leukemia-and any adaptive immune response that exists may benefit the leukemia instead of the host. Supporting this, we saw that *OT-I xRag2*$^{-/-}$ mice actually survived longer with leukemia (albeit with a higher leukemic burden) than C57BL/6 mice (Figure 4-1). This analysis may be confounded because *OT-I xRag2*$^{-/-}$ mice did not develop bilateral hind-limb paralysis to the same extent as C57BL/6 mice, but nonetheless this data is worth

considering. Additionally, antibody depletion of $CD4^+$ cells, $CD8\alpha^+$ cells, or both in combination, resulted in essentially no change in survival from leukemia (though mice in these experiments uniformly succumbed to bilateral hind-limb paralysis) (Figure 4-2). Taken together, these data support the conclusion that the steady-state adaptive immune response to BCR-ABL^+ leukemia is essentially non-existent.

Despite our findings that the steady-state adaptive immune response to leukemia was minimal, we found that a strong immune response correlated with decreased leukemic burden. MHC-II expression on leukemic cells correlated with disease outcome, which hinted that $CD4^+$ T cells were important for anti-leukemia immunity. We verified a role for MHC-II presentation for one leukemia antigen (BAp) by prophylactically vaccinating mice in the presence or absence of BAp and analyzing survival. These experiments (Figure 4-3, Figure 4-13, Figure 4-14) provide evidence supporting a role for MHC-II mediated presentation of BAp in anti-leukemia immunity. This response requires IFNγ and correlates with increased induction of Ly6C on the BAp:I-A^b-specific T cells (Figure 4-16, Figure 4-18). Incidentally, prophylactic vaccinations with viral adjuvants that induce Type I Interferon (either LCMV-Armstrong or VSV) were more functional than

bacterial adjuvants that preferentially induce IL12 (81) (Figure 4-18). This fits with previous clinical findings where Type I Interferon treatment increased the survival of some patients with lymphoblastic leukemia (56).

MHC-II expression on leukemic cells (and ostensibly, antigen presentation to $CD4^+$ T cells) positively correlated with disease outcome, and this correlation was stronger when CD40 was also expressed on leukemic cells. Conversely, leukemic cell expression of PDL1 correlated with worse outcome. These results suggest that both the quantity of antigen presentation (i.e. expression of MHC-II) and the quality of antigen presentation (i.e., CD80, and CD86, CD40, and PDL1) by leukemic cells dictate anti-leukemia immunity. We used Principal Component Analysis and linear modeling to predict disease outcome of leukemic mice by looking at expression patterns of an ensemble of 5 surface molecules related to antigen presentation and costimulatory molecules on the leukemic cells (Figure 4-5). This analysis highlighted the importance of multiple costimulatory molecules on the leukemic cells in disease outcome. Additionally, this analysis strongly suggested a role for targeting the immune checkpoint molecules PDL1 and CTLA4 as a therapy for BCR-ABL^+ B-ALL.

Costimulatory and co-inhibitory molecules play a role in cancer progression (71, 218, 222-226). In our model we observed statistically significant, yet likely biologically irrelevant, increases in survival upon monotherapy with either anti-PDL1 or anti-CTLA4, or dual checkpoint blockade therapy with both anti-PDL1 and anti-CTLA4 (Figure 4-10). Since our leukemia model likely has few non-synonymous mutations (52, 127), these observations fit the paradigm that large numbers of non-synonymous mutations are critical for potent, long-term anti-tumor immunity.

Immune checkpoint blockade therapy alone was only minimally effective in treating leukemic mice in our model. Thus, we explored therapeutic vaccination immunotherapy. Two lines of evidence precipitated this strategy. First, previous reports show that therapeutic heterologous vaccination can be effective in other cancers, albeit in those with higher mutation rates (227). Second, it was clear that MHC-II mediated antigen presentation was important for leukemia outcome, and the pathogens used in our therapeutic vaccination scheme all induce MHC-II expression on APCs (228-230). Fittingly, when mice were therapeutically

vaccinated with these pro-inflammatory pathogens, we saw increased survival (Figure 4-22). To achieve the most ideal vaccination strategy, we used heterologous vaccination. In this approach, we used multiple infectious adjuvants to yield an optimal pro-inflammatory environment for adaptive immune activation. Similar approaches have been used prophylactically (231) and therapeutically for cancer (227, 232). However, our study examines therapeutic heterologous vaccination in combination with checkpoint blockade specifically to target $CD4^+$ T cells in cancer-an underexplored field.

We observed long-term survival from therapeutic vaccination using a heterologous regimen of different infections, but not when using a homologous vaccination regimen. There are multiple possibilities to explain this result. First, oncolytic viruses induce robust anti-tumor immunity, which is improved when used in combination with checkpoint blockade (58). Our therapeutic vaccination regimen did include one lytic virus (Vesicular Stomatitis Virus) (216, 233). Thus, the increase in long-term survival we saw with therapeutic heterologous vaccination could be due to lytic infection. However our prophylactic vaccination experiments demonstrate that the lytic effect is not critical as both LCMV (a lysogenic virus) or VSV (a lytic virus) induced a similar degree of immunity to

BCR-ABL+ B-ALL in mice, long after these acute infections would have been cleared, arguing against an oncolytic virus mechanism. Instead, we favor the idea that heterologous vaccination induces a cytokine milieu that potently activates T cells. Indeed, our data support this conclusion since BAp:I-A^b-specific T cells have increased functionality after heterologous vaccination (Figure 4-25).

We also showed that therapeutic vaccination synergized with anti-PDL1 and anti-CTLA4 therapies. When mice received therapeutic heterologous vaccination in combination with anti-PDL1 and anti-CTLA4, 31% had long-term survival, compared to 10% for therapeutic heterologous vaccination alone, and 0% for anti-PDL1, anti-CTLA4, and anti-PDL1 + anti-CTLA4. These results suggest that even malignancies with few non-synonymous mutations (like B-ALL) can be responsive to immunotherapies that classically work well only in malignancies with high levels of non-synonymous mutations (52). Importantly, such results are contingent upon aggressive therapeutic vaccination approaches. One possible explanation for this could be that leukemia-derived antigen is available for the entire duration of the therapeutic vaccination regimen. This chronic antigen stimulation may lead to further increases in PDL1 and CTLA4 expression on

leukemic cells, which may explain the synergy between therapeutic vaccination and dual checkpoint blockade.

Our data provide initial mechanistic insights into how therapeutic vaccination therapy can lead to leukemia rejection by the C57BL/6 host. During the therapeutic vaccination response, we saw that many BAp:I-A^b-specific T cells were poly-functional (producing Granzyme B and multiple cytokines such as IFNγ, TNFα, IL10, and IL17). Importantly, we were able to delineate some cytokines that were produced more in the most-effective vaccination regimen (therapeutic heterologous vaccination + checkpoint blockade). With this regimen, we saw a significantly increased fraction of BAp:I-A^b-specific T cells that produced Granzyme B, IL10 and a combination of Granzyme B + IL17. This observation suggests that effective therapeutic vaccination induces formation of poly-functional leukemia-specific $CD4^+$ T cells. Additionally, we saw a trend towards decreased PD1 expression on BAp:I-A^b-specific T cells that correlated significantly with decreased leukemic burden (Fig 9A, B). Previous literature supports a potential role of T cell-derived IL10 in anti-tumor immunotherapy (197-199). As well, IL17 and Granzyme B have both been implicated in T cell responses to cancer (93, 215, 234). We envision two possibilities for how and

when BAp:I-A^b-specific T cells might elicit anti-leukemia immunity after therapeutic vaccination. First, since the most effective therapeutic vaccination regimen we used (heterologous vaccination + checkpoint blockade) also yielded the greatest fraction of Granzyme B producing BAp:I-A^b-specific T cells, it is formally possible that these cells directly kill MHC-II^+ BCR-ABL^+ leukemic cells. Indeed, higher expression of MHC-II on the leukemic cells generally correlates with an improved outcome (Figure 4-3). Second, it is possible these poly-functional BAp:I-A^b-specific T cells also induce the most potent BAp:I-A^b-specific memory T cells, which may be required for long-term leukemia control. In support of this, Th17 cells responding to tumor in other models have a capacity for long lifespan, which may be associated with memory formation (93, 235). Therefore, our observations provide data supporting that poly-functional BAp:I-A^b-specific T cells are induced by effective therapeutic vaccination, and that these cells go on to help control leukemia.

The current paradigm is that neo-antigen specific T cells have more capacity to respond to tumors because the repertoires of these cells have not been impinged upon by thymic central tolerance (127). Implicit in this concept is that cross-reactive T cells will be less-able to respond to tumors since the repertoires of

these cells ostensibly have been limited by thymic central tolerance. We have previously shown multiple lines of evidence that BAp:I-A^b-specific T cells are cross-reactive with self antigen and that the BAp:I-A^b-specific T cell repertoire is limited by thymic central tolerance (63). Nonetheless, we observed here that BAp-specific adaptive immunity is crucial for anti-leukemia immunity following prophylactic vaccination, despite the cross-reactivity of this peptide with self antigen. Thus, our observations provide a counterpoint to the idea that neo-antigen specific T cells are a prerequisite for effective endogenous anti-cancer T cell responses (221). Taken broadly, our observations suggest that fusion proteins created by chromosomal translocations may be viable immunotherapy targets even when the fusions do not create neo-antigens. This is particularly relevant since chromosomal translocations often result in “driver” mutations, thus leaving minimal opportunity for cancer immunoediting to occur.

Checkpoint blockade is thought to work best in tumors with high numbers of non-synonymous mutations. Our results support this concept as checkpoint blockade was only minimally effective in B-ALL, a leukemia that generally has lower numbers of non-synonymous mutations. Importantly though, we demonstrate that aggressive heterologous vaccination synergizes with checkpoint blockade to

unmask a strong immune response focused on a well-characterized driver mutation (BCR-ABL) that is capable of controlling a highly aggressive and uniformly fatal form of leukemia. Therefore our work establishes immunotherapy approaches to induce long-term survival in leukemias like B-ALL that have classically been considered refractory to checkpoint blockade-based immunotherapy.

Chapter 5 Conclusions

We have learned much, but I am still left shaking my head in awe at the amazing complexity of living systems. When working with living systems, it seems that nothing can be taken for granted and there may be a surprise around every corner. Indeed, even an "unsurprising" result is hugely satisfying. The thesis project outlined in this document has many interesting results, which I will briefly outline below.

We found that a subtle adaptive immune response exists to BCR-ABL^{+} leukemia even in the steady-state (Figure 2-1, Figure 4-1, Figure 4-2). However, this response is complex and is largely mediated by Tregs (Figure 3-1, Figure 3-2) that lend a benefit to the leukemia. A part of this adaptive immune response

recognizes a peptide from the BCR-ABL fusion protein (Figure 2-4) and we were able to use this fusion peptide (BAp) to construct a peptide: MHC-II tetramer that specifically and sensitively labeled BAp:I-A^b-specific T cells (Figure 2-5). In non-leukemic, unimmunized mice, the BAp:I-A^b-specific T cells already showed hints of having an abnormal repertoire. Indeed, a small fraction of these cells were $CD44^+$, suggesting that some BAp:I-A^b was effectively presented to $CD4^+$ T cells in non-leukemic, unimmunized mice (Figure 2-6). Additionally, the population of BAp:I-A^b-specific T cells recovered in these mice was strikingly small (Figure 2-5), especially when compared to other $CD4^+$ tetramer-binding T cell populations enumerated with a similar technique (128). Additionally, the BAp:I-A^b-specific T cells proliferated poorly in response to peptide/CFA immunization, which is a characteristic that is associated with cross-reactive T cells (Figure 2-11). However, in these situations the BAp:I-A^b-specific T cells do express surface markers that suggest these cells have experienced cognate peptide: MHC-II via the TCR (Figure 2-7). These lines of evidence all suggest that BAp:I-A^b-specific T cells are likely cross-reactive with self antigen. We were able to find one probable cross-reactive self antigen in ABL (Figure 2-12) that elicited an expansion of BAp:I-A^b tetramer-binding cells upon immunization. Additionally, it seemed that the quantity, but not quality, of BAp:I-A^b-specific T cells was

controlled in part through thymic negative selection (Figure 2-13, Figure 2-14, Figure 2-15).

Despite a small precursor frequency and phenotypic cross-reactivity, BAp:I-A^b-specific T cells did respond to BCR-ABL^+ (but not BCR-ABL^-) leukemia (Figure 2-8, Figure 2-9). In this setting, BAp:I-A^b-specific T cells positively correlated with leukemic burden, and indeed most of the BAp:I-A^b-specific T cells were induced into the Treg lineage in response to leukemia (Figure 3-1, Figure 3-2). This robust induction to the Treg lineage is likely due in part to the cross-reactivity of BAp:I-A^b-specific T cells, since a model cross-reactive $CD4^+$ T cell population (GFP:I-A^b-specific T cells in a GFP^+ host mouse) was also induced into the Treg lineage in response to leukemia (Figure 3-3, Figure 3-4).

Tregs were important for allowing leukemia progression in C57BL/6 hosts, as Treg depletion in *Foxp3*$^{DTR/DTR}$ hosts let to significantly increased survival of leukemic mice (Figure 3-6). This correlated with increased numbers of BAp:I-A^b-specific T cells, increased Th1-like phenotype, and increased antigen experiences; suggesting that Tregs were suppression proliferation, polarization,

and priming of leukemia-specific T cells at steady-state (Figure 3-12). Interestingly, Tregs were likely required for leukemia progression both at initial leukemic cell transfer, as well as later during leukemia progression (Figure 3-9, Figure 3-10). This may suggest that Tregs have multiple immune-suppressive mechanisms in leukemic mice. As well, leukemic cells may have a functionally immune-suppressive role since leukemic Treg-depleted mice developed less IPEX-like pathologies than non-leukemic Treg-depleted mice (Figure 3-8).

In leukemic mice, Tregs, leukemic cells, and red pulp macrophages were all located in close proximity in the red pulp of the spleen (Figure 3-5). This environment is canonically immune-suppressive, with red pulp macrophages likely producing classic immune-suppressive cytokines TGFβ and IL10. Additionally, BAp:I-Ab-specific T cells and leukemic cells produced both of these cytokines during the steady-state immune response to leukemia (Figure 3-13). Indeed, blockade of either TGFβ or IL10R led to decreased Treg induction (Figure 3-14, Figure 3-16) and TGFβ blockade also resulted in increased BAp:I-A^b-specific T cell numbers during the immune response to leukemia (Figure 3-14). Intriguingly, MHC-II expression on leukemic cells was also required for efficient Treg induction in correlation with increased survival of MHC-$II^{-/-}$ BCR-

ABL^+ leukemia-bearing mice (Figure 3-14, Figure 3-15). This result highlights the importance of proper antigen presentation in directing an immune response.

In exploring antigen presentation by leukemic cells during the immune response to leukemia, we found that a unique expression pattern of MHC-II, CD40, CD80, CD86, and PDL1 existed on leukemic cells. While these variables did not individually correlate with leukemic burden (and thus outcome) we were able to use Principal Components Analysis to derive components from expression levels of these variables (Figure 4-3, Figure 4-4, Figure 4-5, Figure 4-6). Indeed, high levels of MHC-II antigen presentation, along with cells that expressed higher CD40 and lower PDL1, correlated with improved disease outcome (Figure 4-9). In fact, the first two components of this PCA described 77% of the variability that we saw in leukemic burden in our model (Figure 4-7).

This PCA suggested that an entire ensemble of immune-regulatory markers would need to be therapeutically targeted to improve disease outcome, and not just any one costimulatory or coinhibitory marker individually. In support of this, we found that individual targeting of all the components except MHC-II yielded no

biologically relevant change in survival of leukemic mice (Figure 3-15, Figure 4-10). However, we also were aware that appropriate antigen presentation and costimulation *could* yield an effective anti-leukemia immune response under certain situations. In support of this, BAp peptide + acute viral prophylactic vaccination was sufficient to induce long-term survival of leukemic mice in an IFNγ-dependent manner (Figure 4-11, Figure 4-13). Effective prophylactic vaccination was characterized by viral, and not bacterial, prophylactic vaccination that produced $Ly6C^{+}FOXP3^{-}$ BAp:I-A^{b}-specific T cells (Figure 4-12, Figure 4-17, Figure 4-18, Figure 4-19, Figure 4-20, Figure 4-21).

While the prophylactic vaccination results were intriguing, we sought to apply this to mice that might “come in to the clinic with leukemia”, i.e. in a therapeutic setting. To increase the likelihood of success, we tried a variety of vaccination regimens, some of which were based on the idea of heterologous prime-boost. In these schemes, we vaccinated mice multiple times with different pathogens at each challenge. Therapeutic heterologous vaccination was effective at inducing long-term survival of leukemic mice (Figure 4-22). Additionally, the checkpoint blockade therapies that were minimally effective on their own showed substantial synergy with therapeutic heterologous vaccination. Functionally, our data

supports the conclusion that therapeutic heterologous vaccination + checkpoint blockade elicits improved outcome by changing the phenotype (and not the number) of responding T cells (Figure 4-23, Figure 4-24, Figure 4-25, Figure 4-26). Indeed, the most effective vaccination regimens induced BAp:I-A^b-specific T cells that produced more variety of cytokines with decreased PD1 expression. Finally, we found that the pathology with which leukemic mice present changes if mice are vaccinated. The unvaccinated mice died rapidly and 100% of these mice had bilateral hind-limb paralysis associated with minute lymph nodes and splenomegaly. Comparatively, therapeutic heterologous vaccinated mice developed severe lymphadenopathy and maintained hind-limb mobility. It remains to be seen what is the cause or effect of this changing phenotype.

This thesis contains some results that, at face-value, appear to be conflicting. Namely, MHC-$II^{-/-}$ leukemia allows mice to survive longer than MHC-II replete leukemia. But, MHC-II on leukemic cells *positively* correlates with *improved* disease outcome. I think that the explanation for this apparent dichotomy lies in comparing “steady-state” leukemic mice versus vaccinated leukemic mice. My theory is that antigen presentation by the leukemic cell is bad for the host (in part because of Treg induction). However, when many more APCs are present (as is

the case during the therapeutic vaccinations), the balance is shifted: now relatively more of the antigen presentation occurs by non-leukemic cells, and this leads to an improved immune response to leukemia.

As I have progressed into writing my thesis, I have gone through many of my experimental notes. Anecdotally, I hypothesized incorrectly 75% of the time. And yet, these experiments were still informative, and maybe even more so than when I predicted the result! As I close this chapter of my life, I am struck with mixed feelings of melancholy and excitement as I realize that in biology, nothing is what you think. Melancholy, because this implies that nothing I did *truly* matters; every "certainty" I found is just evidence for-or-against a theory-and is not "certain" at all. Excitement, because this means that *anything* can be learned. Thus my thesis, like so many before mine, helps to advance the field by providing another perspective.

Chapter 6 Bibliography

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