Cyclin B1: Abnormal Self /Tumor Antigen

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We previously identified the aberrantly expressed cell cycle regulator cyclin B1 as a tumor antigen that can elicit both humoral and cellular immune responses in cancer patients. While cyclin B1 is only transiently expressed in normal cells, cancers of many tissue origins constitutively overexpress the cell cyclin in the cytoplasm, which correlates with poorer patient prognosis. We propose that this tumor-specific overabundance and cytoplasmic location of cyclin B1 leads to the presentation of high—and therefore immunogenic—concentrations of cyclin B1 peptides to the immune system. Our studies focused on the source of immune responses against cyclin B1 and the significance of these immune responses in the setting of cancer.

To study the significance of the anti-cyclin B1 immune response in human cancer, we tested plasma from patients with non-small cell lung cancer (NSCLC) for anti-cyclin B1 IgG and demonstrated that a longer overall survival in patients with stage IB NSCLC is correlated with high levels of anti-cyclin B1 IgG. We also demonstrated that cyclin B1-specific antibody and T cell responses exist in healthy individuals who have no history of cancer. Further, the cyclin B1-specific T cells in healthy individuals are antigen experienced and in both $CD4^+$ and $CD8^+$ T cell compartments. We then sought to determine the potential significance of a preexisting anti-cyclin B1 immune response in the setting of cyclin B1+ tumor development. Using both transplantable and spontaneous mouse models of cyclin B1+ tumors, we demonstrated that

vaccination against cyclin B1 prior to the administration or spontaneous development of cyclin B1+ tumors inhibits tumor growth. Finally, given that viral infection has been shown to lead to overexpression of cyclin B1, we proposed that the anti-abnormal self protein, anti-tumor antigen immune responses we observed in healthy people were a result of a virus infection. The extension of that hypothesis is that infections with viruses can train the immune system to recognize abnormal expression of self proteins and therefore protect from cancers that abnormally express those proteins as well. We demonstrated that infection with ectromelia, a mouse pox virus, protects from tumor challenge.

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1.0 INTRODUCTION

1.1 IMMUNOSURVEILLANCE

The concept that the immune system could prevent tumor development was first put forth in 1909 by a German physician/immunologist Paul Ehrlich, albeit without experimental evidence (1). Half century later, Burnet and Thomas elaborated on this concept of "immunosurveillance" and postulated that cells of the immune system continually recognize and destroy neoplastic cells, although the idea was still based on self and non-self observations in cancer and transplant biology and had not yet been shown experimentally (2-4). Many researchers attempted to provide experimental evidence in support of this hypothesis by trying to demonstrate that immunodeficiency in people and in some mouse models leads to increased cancer development, but with little success. In retrospect, this lack of success can be attributed to the limited knowledge at that time of the many different immune effector mechanisms, only some of which were non-functional in the experimental models. A better experimental proof was obtained more recently in new animal models deficient in molecules important in multiple immune functions. For example, it could be clearly demonstrated that more tumors develop in mice in the absence of immune effector molecules IFN-γ and perforin (5, 6). Furthermore direct effects of lymphocyte depletion on cancer surveillance could be conclusively shown following the development of RAG-$2^{-/-}$ knockout mice. Whereas in previous mouse models lymphocyte

deficiency was incomplete, the RAG-2$^{-/-}$ mouse lacks all T, B, and NKT cells (7). When challenged with a carcinogen, RAG-2$^{-/-}$ mice develop cancers more frequently and at a higher rate than the wild type controls. The simple conclusion of these experiments is that the immune system can recognize cancerous cells and eliminate them.

However, the relationship between the immune system and cancer is more complex, as evidenced by the development of cancer despite immunocompetence. The term 'cancer immunoediting' has been proposed to describe this complex relationship (7). Dunn, Old, and Schreiber described three stages of immunoediting. The first is elimination, where anti-tumor immune responses successfully survey and destroy cells that have undergone neoplastic transformation. If this process is complete, no other stage of immunoediting occurs. This stage represents the traditional concept of immunosurveillance, and it is the only stage that occurs in people who never develop clinically detectable cancer. However, if a developing tumor escapes elimination, it enters the equilibrium phase of immunoediting, where the immune system still destroys some cancer cells, but in so doing, selects for increasingly immunoresistant cancer. This equilibrium, if left to proceed long enough, eventually turns into the final phase of cancer immunoediting: escape. In this final phase, the immune system has edited the cancer so completely that it can now grow in a host that has an otherwise competent immune system.

1.1.1 Tumor Antigens

Throughout the studies of cancer immunosurveillance, researchers have worked to identify antigens on tumor cells that elicit specific, anti-tumor immune responses. The goal has been to harness their antigenic potential to stimulate immune responses for cancer therapies and/or

cancer prevention. Tumor antigens identified to date can be subdivided into two categories: tumor specific and tumor associated antigens.

1.1.1.1 Tumor-Specific Antigens

Tumor-specific antigens are molecules that are expressed by cancer cells or premalignant cells but not by normal cells. The appeal of these antigens is their tumor specificity; vaccination or adoptive immune therapy against tumor-specific antigens would result in anti-tumor responses while leaving the healthy tissues intact.

The most common class of tumor specific antigens includes molecules that are mutated and are therefore expressed with a different protein sequence than they would be in normal tissues. As a result of the amino acid changes, the new molecule appears foreign to the immune system and the known mechanisms of tolerance or regulation against self do not hinder the production of an immune response. These new molecules arise from genetic mutations induced either randomly—as a result of environmental carcinogens and/or unrepaired DNA point mutations—or nonrandomly, as is the case with proto-oncogenes such as p53 and ras (8). While targeting these mutated protein products has the therapeutic benefit of tumor specificity, they also have the disadvantage of being unique for each patient, each tumor, and even each cell, as would be the case for a clonally heterogeneous tumor. As a result, immunotherapies against these antigens, such as vaccines, would have to be custom-made for every patient at best, and they could not be used in the preventative setting, where the eventual mutations are not yet known.

A second class of tumor-specific antigens is the collection of viral proteins expressed in cells infected with oncogenic viruses such as human papillomavirus (HPV), epstein barr virus (EBV), and hepatitis C virus (HCV) (9). Like the products of genetic mutations, these antigens

are also recognized as foreign by the immune system, and they are therefore able to be targets of robust immune responses without concerns of tolerance or autoimmunity. However, unlike the randomly altered self proteins, viral tumor antigens are expressed in all individuals with cancers caused by the same virus (shared antigens). As such, tumor antigens derived from oncogenic viruses are extremely good candidates for preventative and therapeutic vaccines. Still, despite the broad variety of tumors that are a result of persistent viral infections, these tumors only account for less than 15% of the total human tumor burden worldwide (10, 11).

1.1.1.2 Tumor Associated Antigens

Tumor associated antigens (TAA) are molecules that are expressed differently in tumor cells differs than in normal cells. This difference in expression from the normal cell can be temporal (expressed at a different developmental stage of the organism or the cell), spatial (expressed in a different cellular compartment), quantitative (expressed at much higher levels), or qualitative (subject to different posttranslational modification).

The first identified human tumor associated antigen was the human melanoma associated antigen 1 (MAGE-1), a member of the cancer-testis (CT) family of tumor antigens (12). These proteins, which include MAGE, GAGE, and NY-ESO-1, are expressed in germ cells, trophoblasts, and cancers (13). While CT antigens are self molecules, their restricted expression makes them partly similar to the tumor specific antigens, because the immune system can only encounter these molecules on cancer cells and not on differentiated normal cells; cancer-testis antigens expressed in spermatagonia cannot be recognized by T cells, since these cells do not express MHC class I or II, rendering the CT antigens on normal germ cells immunologically invisible (14, 15).

The second tumor associated antigen identified was tyrosinase, a member of what is now known as melanocyte differentiation tumor antigens that also include Melan-A/Mart-1 and gp100. These proteins are expressed in approximately 85% of melanomas and in normal differentiation of melanocytes (16, 17).

While the melanocyte differentiation antigens are usually separated into a category of their own, they truly belong to the broadest category of tumor associated antigens, that of overexpressed self proteins. Overexpressed tumor antigens include Her2/neu, wild type p53, the oncofetal antigens carcinoembryonic antigen (CEA) and alpha fetoprotein (AFP) as well as survivin and cyclin B1. The mechanism by which overexpression converts a self molecule into an immunologically recognizable antigen has not been completely elucidated. Most likely, low densities of the protein in the normal cell result in very low density and/or very infrequent presentation on MHC class I. As a result, the overexpressed protein either becomes more consistently presented to the immune system or the density of presentation crosses threshold needed for T cell stimulation (18). The members of the overexpressed TAA group with the broadest normal expression, including wild type p53 and cyclin B1, are perhaps the most interesting: their breadth of expression in normal tissue translates to aberrant expression in many tumor types; in addition, the fact they are frequently expressed in the periphery means that peripheral tolerance mechanisms must be avoided or overcome in order to generate the immune responses that have been documented to target them. Studies of these tumor antigens will also inform the mechanisms that govern the ignorance of normally expressed but recognition of overexpressed self proteins.

Importantly, as more is discovered about each of these tumor antigens, their categories and classifications increasingly blend into one another, as is seen with the melanocyte

differentiation antigens that are also overexpressed TAA. CEA and AFP are often placed into a category of their own (as onco-fetal antigens), since their normal expression is found in the developing embryo and/or fetus; but given that CEA was later found to be expressed in normal colonic tissue (19) and AFP is expressed during liver inflammation (20-22), they also fit into the overexpressed, self molecule TAA group. MUC1 is an example of a tumor antigen that can straddle several groups, including the tumor specific and overexpressed/tumor associated antigen categories (23). MUC1 is transmembrane glycoprotein normally expressed on the apical membrane of ductal epithelial cells. The tumor form of MUC1 is specific in that cancerous, modified glycosylation of the peptide backbone of MUC1 generates glycopeptides that serve as neoantigens and are not seen on healthy cells (23). However, modified MUC1 glycosylation also occurs in areas of inflammation, which means that—while healthy cells are currently considered MUC1 neo-antigen free—cells other than cancer can also express these glycoepitopes (24). As such, MUC1 is truly a tumor associated antigen, although the neoglycosylation does not neatly fit into one of the existing TAA categories. Beyond the neoantigen glycosylation, MUC1 (and therefore its unmodified peptide backbone) is also overexpressed on tumor cells (23); therefore, an antigen derived from the MUC1 protein core also serve as an overexpressed TAA.

In summary, the majority of shared tumor antigens are self molecules that are abnormally expressed in space, time, quantity, or quality—and often all four—such that these changes are recognized by the immune system. Studies regarding the significance of immune responses against these antigens as well as the barriers to their success as tumor-rejection targets have begun to define a problem-set for today's tumor immunologists.

1.1.2 Significance of the Immune Response to Tumor Antigens

The overarching goal of tumor antigen discovery and cancer immunosurveillance research is to prevent human death from cancer. Thus far, the major focus of tumor antigen research has been on vaccination strategies, prophylactic and therapeutic in the mouse and—with the exception of the HPV Gardasil™ vaccine and hepatits B vaccines—only therapeutic in human trials.

1.1.2.1 Vaccination-Induced Immune Responses in Cancer Patients

Mouse models of cancer vaccination have shown great promise, but the successes have not been translated to human cancer. While mouse models of vaccination demonstrate cancer prevention, Merck's Gardasil™ vaccine for the prevention of cervical cancer caused by human papillomavirus (HPV) and hepatitis B vaccines for the prevention liver cancer are the only prophylactic cancer vaccine used in humans. Nonetheless, they are directed towards viral proteins, which are tumor specific antigens and not tumor associated antigens. Without the ability to test other preventative vaccines, the timing of vaccination is perhaps the biggest limitation to the current human trials; not only is cancer already established in these studies, but the patients most often have advanced stage, metastatic cancer. The minimal success in human therapeutic vaccination—estimated to be between 3.3 and 10 percent (25, 26)—is therefore not surprising; in mice, therapeutic vaccination against cancer has not been successful (26), and in humans, therapeutic vaccination is also ineffective in the setting of infectious disease, including HPV (27). Still, the study of therapeutic vaccination strategies is extremely important: these studies inform the field of tumor-immune system interactions and help identify barriers to success; they may be useful in combination with immunotherapies that combat those barriers, such as the antibody that blocks the T cell negative costimulatory molecule CTLA4 (28, 29).

Despite advances in cancer prevention, it is almost certain that there still will be hundreds of thousands of new cancer diagnoses per year— as such, finding immunologic cures for existing cancer is a paramount task.

Administration of various vaccination regimens has been shown to elicit TAA-specific immune responses in many different types of cancer, including breast, prostate, colorectal, hematologic, and renal cell cancers as well as the most studied cancer, melanoma (25, 26, 30, 31). While the response rates to these cancers have been low, complete responses have been documented in several melanoma trials. Rosenberg, Yang, and Restifo collected data from 440 cancer patients enrolled in an even larger number of active immunization trials, the majority of whom (422) had metastatic melanoma. Of those patients, 3 experienced complete responses, and 11 experienced partial responses, as determined by the more stringent criteria used to obtain the 3.3% response rate, noted above. These individuals did not receive concurrent treatments known to have clinical benefit in metastatic melanoma, such as IL-2 and IFN-α. Complete responses have also been reported in vaccination trials for non small cell lung cancer and renal call carcinoma (32, 33).

Importantly, vaccination trials in melanoma and other cancers have noted correlations between immune responses elicited by vaccination and clinical response, although the latter is rare. These immune responses can be measured in several ways, including tetramer- and ELISPOT-detected T cells, antibody responses, delayed type hypersensitivity responses, and determinant spreading. In vaccination of renal cell carcinoma, a patient with a complete response had a 40-fold increase in antigen-specific IFNγ production after the vaccination, compared to 3.5 and 4.5-fold changes noted in the other patients who developed an antigen-specific IFNγ response (33). In a trial of vaccination of colorectal cancer using DC loaded with

an altered CEA peptide, the percentage of tetramer positive $CD8^+$ T cells induced by vaccination (total percentage and fold increase from pre-vaccination) was significantly correlated with clinical response (34). Altered-peptide, tetramer positive cells were also shown to be specific for the wild type sequence of the same peptide, with a post-vaccination frequency of >1%. In melanoma vaccine trials, clinical responses have been correlated with Melan-A specific $CD8^+$ T cells (35), the direct and recall responses to multi-peptides used in vaccination (36), and with the occurrence of determinant spreading, where $CD8^+$ T cells specific for the vaccination antigen were not significantly correlated with improved clinical response but the vaccine-induced response to other melanoma antigens was (37).

However, since immune responses to vaccinations are common and objective clinical response are rare, there is a disconnect between measurable immunologic parameters and successful cancer elimination. From both human and mouse studies, it is clear that treatment of advanced stage cancer with therapeutic vaccination alone is an insufficient strategy to achieve a cancer cure and that vaccination at earlier stages of disease shows the most promise (26). As more work is done to advance the possibility of preventative vaccination or vaccination in early stage cancer, it will be important to understand what clinical significance anti-TAA immune responses have in the pre-cancer or early stage cancer setting. In addition, given the successes seen in the adjuvant setting—when vaccines are administered after surgical resection of tumors (25, 38)—it will be important to evaluate the significance of naturally occurring anti-TAA immune responses in the prognosis of surgically treated cancer patients.

1.1.2.2 Naturally Occurring Immune Responses in Cancer Patients

Naturally occurring TAA-specific immune responses—those that develop spontaneously, without vaccination—have also been found in cancer patients. These natural humoral and

cellular immune responses were the main tool used to identify tumor antigens (atyping, serex, mage).

Anti-TAA Antibodies in Cancer Patients

The first attempts to identify serologic tumor antigens were conducted in the late 1970s by Lloyd Old and his colleagues. Sera from patients with melanoma, brain tumors, and renal cell carcinoma were used to interrogate autologous tumors in a search for tumor specific cell surface antigens using a process termed 'autologous typing' (39-41). In this method, cell lines from primary cancers were established and plated along with normal tissue cell line controls for use as reproducible targets for diluted, autologous sera. However, the molecular identities of proteins identified through this method were very difficult to obtain because biochemical purification and cloning techniques available at the time required higher titers of anti-tumor antibodies than were available in the human sera, and by the mid 1980s, only two had been identified (42). The early 1980s also brought the first discovery of anti-p53 antibodies in the sera of breast cancer patients (43).

The next major push for the identification of antigens recognized by antibodies in cancer patients took place during the early 1990s, when several groups began to use previously identified tumor antigens as probes in immunoprecipitation, western blot, and ELISA assays of cancer patient sera (44-46). Her2/neu and MUC1 were among the antibody-recognized antigens detected at that time. Since then, the list of tumor antigens against which cancer patients form antibodies has grown tremendously. The discovery process has been facilitated by the advent of the serological analysis of tumor antigens by recombinant cDNA expression cloning (SEREX). This technique uses cDNA libraries from human tumors and packages them into lambda-phage

vectors for expression in *E. coli*. The expressed proteins are then transferred to nitrocellulose and screened with autologous sera. Clones that elicit antibody binding are then subcloned to obtain monoclonality, and the original cDNA is excised and sequenced (47, 48).

Most studies of anti-tumor antigen antibodies in cancer have been performed on the tumor antigen p53, against which antibodies have been detected in patients with breast, lung, colon, hematologic, head and neck, gynecologic, skin, thyroid, bladder, prostate, brain, and hollow and solid gastrointestinal organ cancers (49). This breadth of cancer types is a reflection of the widespread incidence of p53 overexpression in cancers of all tissue origins.

The significance of these humoral responses in cancer prognosis varies both between and within tumor antigens. As might be expected from the number of studies that have documented p53-specific antibodies in cancer patients, p53 is one of the most frequently studied humoral antigens for cancer prognosis. In most studies, the presence of anti-p53 antibodies is an indication of a worse prognosis, as is the case for breast, lung, and colon cancers (49). However, one study has demonstrated that the presence of anti-p53 antibodies predicts a better response to radiotherapy in lung cancer (50). Unlike p53, antibodies specific for MUC1 have been exclusively associated with improved prognosis, as has been demonstrated in breast, pancreatic, lung, and ovarian cancers (51-54). Association with better prognosis has also been found for anti-CEA antibodies in colon cancer. Antibody responses to intracellular molecules are important in prognosis as a biomarker of an immune response that is mounted by T cells, whereas antibody responses to surface antigens could serve as biomarkers and as direct mediators of an anti-tumor immune response. And while it is interesting to note that improved prognosis is seen with antibodies specific for extracellular tumor antigens, this area of research lacks enough data to warrant any conclusions.

TAA-specific T cells in Cancer Patients

The prognostic effect of spontaneously occurring tumor-specific T cell responses has also been studied in cancer patients. Many of these studies have been conducted based on the number of tumor infiltrating lymphocytes (TIL), the specificity of which was not determined. In these studies, TIL were associated with improved prognosis in prostate cancer, melanoma, rectal cancer, and colorectal cancer (55-58). More specifically, other studies found that $CD8^+$ T cells (and their Th1 helper correlates) are the crucial element for improved prognosis, especially if those T cells demonstrated proliferative capabilities (58-63). However, like the humoral anti-tumor immune responses, some studies have pointed towards a correlation between TIL and a worse prognosis, since TIL can include $CD4^+$ regulatory T cells, which suppress the activity of tumor-specific effector T cells (64). Even the presence of proliferating $CD8^+$ T cells in the tumor cell nest has been associated with a poorer outcome (65). Still, the majority of evidence suggests that tumor infiltrating CTL are anti-tumor effectors.

Despite the evidence for the prognostic power of TIL, the same studies did not determine the TIL specificity. Specificities of TIL have been documented for over a decade, with the first boom of antigen-specific TIL discovery occurring in melanoma in 1994 (66-68). Since then, TIL with known tumor antigen specificity have been found in many other cancers, including non small cell lung cancer, head and neck cancer, as well as seminoma (69-71). The prognostic impact of spontaneously occurring TIL with known antigen specificity has been shown to correlate with an improved prognosis in a study of melanoma (72). Haanen *et al.* evaluated $CD8^+$ TIL in melanoma patients for reactivity with MART-1, gp100, tyrosinase, and MAGE-A1. MART-1, gp100, and tyrosinase-specific $CD8^+$ T cells were detectable in 12 of 16 patients, and

their presence correlated with overall survival ($p = 0.0094$), while the percentage of tumor infiltrating $CD8^+$ T cells did not.

Spontaneously arising $CD4^+$ and $CD8^+$ T cells specific for TAA have also been detected in the blood of cancer patients (73-76), but the studies on related prognosis have been limited. Autoimmune vitiligo has been associated with increases in MART-1-specific peripheral T cells (77) as well as improved prognosis in melanoma patients (78, 79); however, these data do not directly show that the presence of spontaneously-arising TAA-specific T cells in the peripheral blood improves prognosis.

The detection of antigen-specific T cells is often restricted to patients with known HLA, mostly HLA-A2 in melanoma, which allows for the use of tetrameric HLA molecules bound to both tumor antigen peptides and a fluorescent tag. These tetramers can then be used to identify antigen specific T cells in flow cytometry. Other methods of TAA-specific T cell identification require the availability of billions of PBMC and a week of time in order to generate autologous dendritic cells for use in stimulation assays. For clinical studies on cancers for which tetramers are not available or for studies in which the number of patients makes DC generation too expensive or time consuming, *ex vivo* identification of TAA-specific T cells can also be performed with the use of a peptide library. The use of peptide libraries allows the APC present in PBMC to present the spectrum of a tumor antigen's epitopes, and it has also led to the *ex vivo* identification of TAA-specific T cells in premalignancy (80).

In summary, while humoral and cellular TAA-specific immune responses have been found to spontaneously arise in cancer patients, very little has been learned about their prognostic significance. It is not clear, however, whether this is because the area is understudied or because studies in which no significance was found remain unpublished. Another important

consideration is the time at which these responses are assessed. Given that tumors are immunosuppressive, as discussed in 1.2.3.1, the prognostic significance of immune responses detected in the face of established cancer might be difficult to detect. As a result, the most informative studies would be those studies in which anti-TAA immune responses could be evaluated before cancer diagnosis (during premalignancy or a cancer-free state) or after the bulk of the cancer has been resected.

1.1.2.3 Naturally Occurring Immune Responses in Healthy Individuals

Humoral and cellular immune responses against tumor associated antigens have also been documented in healthy individuals, although these observations have received very little attention.

TAA-specific Antibodies in Healthy Individuals

Antibodies specific for TAA were found in healthy individuals as early as 1987, with the discovery of CEA-specific antibodies in normal sera (81). In 1994, these results were confirmed in a study of anti-CEA antibodies in gastrointestinal malignancies where "fewer than 10%" of 28 controls had anti-CEA antibody (82) and again in 2000, when a similar 11% of controls were positive for anti-CEA IgG or IgM (83). Also during the late 1990's, MUC1 and HER2/neu-specific antibodies were found in the sera of healthy controls (52, 84-86). Still, the focus of these studies was the idea that cancer patients had higher levels of antibody than controls, and not on the fact that healthy individuals could have anti-TAA immune responses. In 1998, with the use of cDNA expression libraries from tumors and SEREX, Old and colleagues found that 14

of 48 antigens were also reactive with normal sera, and stated that this was "presumptive but not conclusive evidence that these antigens are unrelated to cancer" (87).

The clinical significance of these pre-existing anti-tumor antibodies has helped to solidify the concept that anti-TAA antibodies can exist in healthy people. MUC1 specific antibodies in healthy women have been shown to correlate with a decrease risk of ovarian cancer (88). In addition, antibodies specific for p53, while not reported to be found in a healthy population, have been shown to exist in people at high risk for development of cancer due to chronic lung diseases and environmental lung exposures. In these individuals, anti-p53 antibodies were able to predate and predict the development of cancer (89-91). Also, while not demonstrated in healthy individuals, antibodies specific for AFP have been found in patients with liver cirrhosis and chronic hepatitis (92), and antibodies specific for p53 have been found in Barret's esophagous (93). Still, the studies of TAA-specific antibodies in the sera of healthy individuals are in their infancy, perhaps in part because it is assumed that healthy people should not have antibodies specific for tumor antigens.

TAA-specific T cells in Healthy Individuals

T cell responses specific for tumor antigens have been found in healthy individuals as well, but these studies have been almost entirely performed after weeks of in vitro stimulation, implying that T cells could be generated from naïve precursors when sufficient, tolerance-breaking stimuli were applied. Examples of tumor antigens for which T cells have been generated include melanoma-associated chondroitin sulfate proteoglycan (94), cytochrome p450 1B1 (95), survivin (96, 97), and tyrosinase related protein 1 (98).

Other studies have been able to find TAA-specific T cell directly *ex* vivo, but these cells were not considered antigen-experienced. $CD8^+$ T cells specific for Melan-A/Mart-1 were found in 6 of 10 healthy HLA-A2 individuals, but the phenotype of the tetramer-positive cells was $CD45RA^{hi}/RO^-$, which was considered to be naive (99). However, CD45RA has more recently been found on effector $CD8^+$ T cells; therefore, the $CD8^+$ T cells specific for Melan-A in these healthy individuals could have been effector (but not memory) CTL. One particularly thorough study identified CEA-specific $CD4^+$ T cells in the blood of healthy individuals (100). The study noted that 46% of people (n = 50) had naïve CEA-specific $CD4^+$ T cells that could be stimulated to proliferate *in vitro*. Most interestingly, PBMC from individuals that did not show a proliferative response to CEA instead secreted IL-10, also with CEA-specificity (20 of 23 non-proliferators); neutralization of IL-10 then revealed antigen experienced ($CD45RO^+$) $CD4^+$ T cells that could proliferate specifically in response to CEA. The authors concluded that healthy individuals had CEA-specific $CD4^+$ precursors that were either ignorant of the peripheral existence of the self antigens or exposed to CEA but specifically suppressed by IL-10-secreting Tr1 cells. Still, while CEA is widely expressed in tumor tissues, as a self antigen it is only expressed at low levels in normal colon (19). Therefore, peripheral tolerance might be expected to be less complete than for ubiquitously expressed tumor antigens, such as cyclin B1 and p53. To date, no studies have shown $CD8^+$ and $CD4^+$ memory T cell responses specific for a self and tumor associated antigen. The significance of these pre-existing anti-tumor T cell responses for cancer risk also has yet to be reported.

1.1.3 Summary

In summary, tumor antigens are most often altered (abnormal) self antigens. Immune responses to these antigens can be generated or boosted in cancer patients through vaccines, but a clear correlation between anti-tumor immune responses and clinical outcome has not been established. Naturally occurring anti-TAA antibody responses in cancer patients have a variable prognostic value, while studies on the prognostic value of spontaneously arising TAA-specific T cells in cancer have been rare.

These unimpressive results are not surprising, since TAA-specific immune responses have a very low likelihood of success against an immunosuppressive, dense tumor mass. A much higher likelihood of success is predicted for immune responses induced before the onset of cancer or immune responses that exist after the bulk of the cancer has been surgically resected. Study of this in humans is impeded by the limited application of prophylactic vaccination. However, reports of pre-cancerous TAA-specific antibody and T cell responses in healthy individuals have begun to accumulate, even though they are rarely recognized as such. Studies of the significance of naturally occurring anti-TAA immune responses in healthy individuals—to question the source of the response and the significance for cancer risk—are rare.

1.2 CHALLENGES FOR IMMUNOSURVEILLANCE

To date, many studies have shown that it is possible to induce immune responses specific for altered self molecules that are also tumor antigens both *in vitro* and *in vivo*. However, while these "self-antigen" specific T and B cells can exist in the peripheral repertoire, their ability to

prevent the development of cancer or destroy existing cancer is clearly limited in some individuals. The mechanisms by which these cells are kept from completely preventing cancer when stimulated spontaneously or from completely destroying cancer when stimulated by vaccination are not yet fully elucidated. These mechanisms are likely a combination of central and peripheral culling of the reactive repertoire, normal immune regulation, and tumor-induced suppression.

1.2.1 Tolerance and the Tumor Associated/Self Antigen

Given that all tumor associated antigens are expressed in one form or another by normal cells and are therefore encoded in the genome, they are considered to be self proteins subject to the rules that govern immune tolerance to self. Because of that assumption, many studies on the TAA-specific humoral and cellular immune responses in cancer patients have focused on approaches that will break tolerance and in that way effect tumor destruction. Later in this thesis we will discuss the merits of this assumption.

1.2.1.1 B Cell Tolerance

When a given antigen is not recognized or responded to by the immune system, the immune system is considered to be nonresponsive to that antigen. The first tolerance mechanism affecting B cells are triggered in the bone marrow, where an immature B cell that expresses IgM is exposed to self antigen. If a B cell receptor is ligated by a surface-bound mutli-valent self antigen, as is the case for cell surface antigens present on bone marrow stromal cells, the B cell is either deleted (clonal deletion) (101, 102) or stimulated to undergo receptor editing, where a new light chain is rearranged to reveal a different antigen specificity (103). In addition, if an

immature B cell in the bone marrow encounters a sufficient amount of soluble self antigen to cross-link the surface IgM, the B cell becomes anergic, or unable to respond to its cognate antigen (104). The source of these self proteins—whether presented on stromal cells or released into the bone marrow milieu as soluble antigens—has not been elucidated. Self-reactive, anergic B cells can then migrate into the periphery, where they exhibit a shortened life-span when in competition with mature non-self reactive B cells (105, 106). Still, the state of B cell anergy is considered to be variable and plastic, with the potential for reversal (107). In addition, it should be noted that studies of anergic B cells have been conducted in mice and while both humans and mice are known to produce high numbers of self-reactive B cells (108), an anergic population of self-reactive B cells has yet to be identified in humans (109).

Also relevant to anti-tumor antigen immune responses are those self-reactive B cells that are not affected by negative selection in the bone marrow. These B cells can be of such low avidity and affinity that they never receive the negative stimuli associated with binding multi-valent surface-bound or soluble self antigen. More significantly, it is possible that B cells with high avidity and affinity for self protein simply never encounter that antigen in the bone marrow, and are thus considered to be clonally ignorant (110). As a result, these B cells are able to mature normally and enter the periphery, where they can produce auto-antibody when stimulated.

Recently, evidence has begun to show that some autoreactive antibody might be produced by B cells that were originally not self-reactive (111). This switch to autoreactivity can happen during the process of somatic hypermutation, when a B cell stimulated by antigen in a secondary lymphoid organ undergoes additional variable region editing in order to select for higher affinity antibody (affinity maturation). This editing can result in memory IgG B cells that

can later develop into plasma cells (112), which would enable the secretion of autoantibody without the restriction of central or peripheral tolerance mechanisms. A final mechanism for autoantibody production has been suggested by mouse models of allelic inclusion, where receptor editing in the bone marrow can lead to the production of naïve B cells with two productive receptors. While the non-self reactive receptor could enable the B cell to escape central tolerance, the self-reactive receptor could be engaged in the periphery to initiate the production of autoantibody (113-116).

1.2.1.2 T Cell Tolerance

The first and main mechanism of T cell tolerance occurs by deletion of self-reactive T cells. This deletion takes place in the cortico-medullary junction and medulla of the thymus, where double positive ($CD4^+$, $CD8^+$) T cells are screened for reactivity to self peptide/self MHC, which if present leads to deletion of self-reactive T cells by apoptosis (117, 118). Cells in the thymus are able to screen for self-reactive T cells by virtue of their ability to transcribe portions of the genome that are tissue restricted (expressed in fewer than 5 of 45 tissues (119)) (such as the melanocyte differentiation antigens and the cancer testis antignes) or restricted in the developmental stage of an organism (such as the oncofetal antigens). This presentation of a large repertoire of self antigens is also mediated by the antigens brought into the thymus by way of antigen-carrying cells from the periphery (120). However, not all screening for self-reactive T cells in the thymus leads to deletion. In both mouse and human models, some self-reactive T cells are also converted to antigen specific regulatory ($CD4^+$, $CD25^+$, $FoxP3^+$) T cells, which function in peripheral tolerance (121, 122).

Thymic deletion and Treg conversion (central tolerance) are incomplete, and non regulatory T cells with self-reactive receptors can move into the peripheral circulation. These

potentially auto-reactive cells are then subjected to another round of deletional and non-deletional tolerance mechanisms. Deletional mechanisms have been demonstrated in mouse models of diabetes (123) and melanoma tumor antigen expression. In the later experiments, peripheral deletion of self-reactive T cells was observed in the lymph nodes (including those that did not drain from sites that normally express the antigen) but not the spleen; the lymph nodes were then shown to produce auto-antigen transcript at high levels, a process that was radioresistent and therefore possibly conducted by lymph node stromal cells (124).

Nondeletional mechanisms of peripheral tolerance include the induction of anergy and specific suppression by centrally or peripherally induced regulatory populations of T cells. For a naïve T cell to become an effector T cell, it needs to be stimulated through both the T cell receptor (signal 1) and CD28 (signal 2). Signal 2 is delivered through ligation of CD28 by CD80 or CD86 on professional antigen presenting cells. Receipt of signal 1 without signal 2—as would be the case when a self-peptide reactive $CD8^+$ T cell encounters self-peptide presented on MHC class I of a healthy cell in the periphery—leads to anergy, the inability of a T cell to respond to its cognate antigen (125, 126).

Given that T cells specific for TAA can be induced through vaccination or arise spontaneously, peripheral deletion must be incomplete just as central deletion is. Induction of anergy must be incomplete (or reversible (127)) as well. Incomplete induction of peripheral anergy could be due immunologic ignorance, where self-reactive T cells never encounter their antigen in the periphery. In this concept, the peripheral expression of the antigen is non-existent or infrequent—as would be the case for CEA and the cancer-testis antigens. Peripheral anergy induction might also be incomplete due to antigen expression below the threshold of recognition, which may be the case for the melanocyte differentiation antigens (18) and cyclin B1.

The final arm of peripheral T cell tolerance is the active suppression of non-anergic, self-reactive T cells. This could take place while the T cell is still naïve—such as is the case for the peripheral induction of Tr1 and T_H3 cells—or after the T cell has become an effector—as would be seen for antigen-specific suppression by previously generated regulatory T cells (128).

1.2.2 Breaking Tolerance

All forms of nondeletional tolerance (anergy, ignorance, and active suppression) still result in the peripheral presence of T cells with self-specific receptors. As a result, they are available for recruitment in effector anti-self immune responses, as long as the tolerance mechanisms can be overcome, or broken. Currently, the field of cancer immunotherapy is largely directed towards vaccination strategies that can break tolerance to tumor antigens in both the $CD4^+$ and $CD8^+$ T cell compartments (129). These strategies have included the use of *ex vivo*-loaded dendritic cells, viral antigen vectors, and adoptive transfer of *ex vivo*-stimulated T cells. However, the attempts to break tolerance are based on the concept that tolerance is the default state for all TAA-specific T cells in cancer patients. Implicit in this concept is the idea that vaccination will introduce autoimmune responses that were otherwise nonexistent. As a result, the prospect of autoimmunity is then a barrier to the use of prophylactic or early-stage therapeutic vaccination, as it suggests that vaccination could induce disease. While autoimmune vitiligo has been noted in treatment of melanoma (130), and severe ocular autoimmune disease was noted in melanoma trials with anti-CTLA-4 therapy (131), this may not be the case for all tumor associated and self antigens. Even for tumor antigens where tolerance is broken either by vaccine or spontaneous mechanisms, the level and frequency of antigen presentation on the normal cell might result in a

much higher threshold for response; as a result, immune responses boosted by cancer vaccination could induce effective anti-tumor immune responses while leaving the healthy cells intact.

1.2.3 Cancer Immunoevasion

Even if tolerance for a TAA-specific T cells is broken (by vaccination or by a spontaneous mechanisms), there are still significant barriers to functional, anti-tumor immune responses. These include the mechanisms by which cancers can immunosuppress as well as the ways in which cancers can specifically escape immunosurveillance.

1.2.3.1 Cancer Immunosuppression

Malignancy has long been associated with immunosuppression, even in the absence of chemotherapy and radiotherapy. This has been most well documented for hematologic malignancies, for which pre-therapy immunosuppression is associated with clinical illness (132, 133); however, solid cancers have also been associated with decreased recall delayed type hypersensitivity and *in vitro* T cell response to mitogens (134). Beyond this systemic immunosuppression, tumors have been shown to specifically suppress tumor antigen-specific $CD4^+$ and $CD8^+$ T cells, in both mouse and human systems. In the mouse, naïve, transgenic $CD4^+$ T cells specific for a tumor antigen were rendered unresponsive after encounter with the tranplanted tumor *in vivo* (135). TAA-specific immunosuppression has also been observed for endogenous (self) antigen in the human system. A study by Lee, *et al.*, demonstrated that tyrosinase-specific $CD8^+$ T cells in a melanoma patient were significantly expanded (>2% of total peripheral $CD8^+$ T cells) but functionally deficient (136); *in vitro*, the T cells were unable to lyse targets or respond with IFNγ, even after stimulation with IL-2. This was in contrast with

intact immune responses against control antigens from CMV and EBV. In addition, a mouse model of tumor antigen-specific T cell responses demonstrated that adoptively transferred TAA-specific $CD4^+$ T cells not only become anergic, but that the majority either remain ignorant to antigen or are converted to regulatory T cells after injection into mice bearing tumor-antigen-expressing tumors (137).

One mechanism by which this tumor antigen-specific immunosuppression may be mediated is through the quality of the APC that encounters the tumor antigen-specific T cell. Dendritic cells can determine the fate of naive T cells, including induction of activation or tolerance; this decision, in turn, is partially dependent on the context in which the DC encounters the antigen (138-140). Therefore, given that DC can be made less immunostimualtory through exposure to cytokines such as IL-10, TGF-β, and VEGF (141, 142)—all of which have been shown to be secreted by tumors—tumors can influence DC to specifically suppress anti-tumor T cell responses (143).

1.2.3.2 Cancer Evasion of T cell Responses

Even if a TAA-specific $CD8^+$ T cell that was primed to be an effector encounters a tumor, there are still mechanisms by which the tumor can avoid recognition. One of these evasion mechanisms is a decrease in antigen processing and presentation machinery in the cancer cell. For example, tumors have been demonstrated to have β2-microglobulin mutations (144, 145), which would lead to a lack of MHC class I; they have been shown to decrease MHC class I through transcriptional regulation and selective loss of HLA alleles (144), which may have presented the targeted TAA epitope; they have been shown to undergo antigen loss (146-148); and cancer cells have shown defects in antigen processing at the level of the immunoproteosome (149) and the function of TAP (150, 151), which is necessary to transport processed peptides

from the cytosol into the endoplasmic reticulum, where they can be loaded onto MHC class I. In addition, cancer cells have also been demonstrated to express molecules that activate negative regulatory pathways on T cells. One example is programmed death receptor ligand-1 (PD-L1). PD-L1 has been shown to be expressed on tumor cells (152-154), which can enable to binding of PD-1 on T cells, leading to inhibition of activation and apoptosis of CTL (155-157).

1.2.4 Summary

Several mechanisms of tolerance and cancer immune evasion can hinder a successful anti-tumor immune response. However, tolerance is a very leaky process, and it includes the production of cells that are ignorant of antigen and therefore able to be recruited in an appropriately-primed immune response. This immune response would then have the greatest likelihood of success if it were generated in the absence of cancer-specific immunosuppression—either before the cancer develops or after it has been resected.

1.3 CYCLIN B1

Cyclin B1 is a self protein and a tumor antigen that is required for the transition from G2 to M phase of the cell cycle. In the normal cell, cyclin B1 is expressed very transiently as the cell moves into mitosis, after which it is immediately ubiquitinated for proteasomal degradation (158).

1.3.1 Biology in the Normal and Cancerous Cell

Cyclin B1 is expressed in the cytoplasm during interphase (159) where it accumulates until a threshold level is reached for the formation of the active maturation promoting factor (MPF), a complex of cyclin B1 and cdk1 (160-163). The kinase portion of the MPF is inactive until both cyclin B1 is bound and Wee1 family kinases dephosphorylate two tyrosine residues (164). After activation, the MPF accumulates in the nucleus—possibly by active transport (165)—where it initiates the breakdown of the nuclear envelope and the commencement of mitosis (166). In the normal cell, cyclin B1 expression is tightly regulated after initiation of mitosis, when it is ubiquitinated by the anaphase-promoting complex (APC) and degraded in the metaphase-to-anaphase transition (167).

Cyclin B1 overexpression and accumulation in the cytoplasm has been documented in many cancer types, including melanoma, cervical cancer, non-small cell lung cancer, acute myelogenous leukemia, large B cell lymphoma, non-Hodgkin's lymphoma, breast cancer, gastric carcinoma, colorectal carcinoma, and several head and neck cancers, including those of the esophagous, tongue, and larynx (168-184). In addition, while the normal cell requires destruction of cyclin B1 for entry into mitosis (167), tumor cells overexpress cyclin B1 throughout the cell cycle (185). Cyclin B1 overexpression has also been correlated with a poorer patient prognosis or a more malignant phenotype in lung, gastric, breast, tongue, esophageal and laryngeal cancers (171, 174, 177, 182-184, 186, 187).

One mechanism of cyclin B1 overexpression is through p53 loss of function (deletion or mutation), which has been shown to inhibit cyclin B1 expression in a pRb-dependent fashion. While p53 functions through several mediators to inhibit MPF activity (188-192), it also directly represses cyclin B1 transcription (193). Evidence of the causal connection between p53 loss of

function and cyclin B1 overexpression in cancer was first shown by Yu *et al* with the use of a colorectal carcinoma cell line that has both a wild type p53 clone and a clone in which p53 has been knocked out. In the $p53^{-/-}$ clone, there is a high level of cyclin B1 expression. When p53 expression was restored with a recombinant adenovirus carrying p53 DNA, cyclin B1 levels dropped as p53 levels increased. In addition, a survey of p53 expression in 14 tumor cell lines demonstrated an inverse relationship between cyclin B1 expression and wild type p53 expression in 8; only one cell line had simultaneous expression of both proteins.

1.3.2 Cyclin B1 as a Tumor Associated/Self Antigen

Given that cyclin B1 is transiently expressed between interphase and metaphase in the normal cell, it is possible that cyclin B1-specific T cells are able to avoid the induction of anergy or peripheral deletion associated with normal cell presentation of self peptides. Furthermore, cells undergoing stress would be less likely to spill cyclin B1 protein into the extracellular compartment for phagocytosis by immature DC, since responses to stress—such as p53 expression—lead to a repression of cyclin B1 synthesis. As the target of the resulting non-anergic T cells, cyclin B1 is different from all other identified tumor antigens since it is ubiquitously but transiently expressed by all dividing, healthy cells.

1.3.2.1 Identification of Cyclin B1 as a Tumor Antigen:

In the study of the relationship between cyclin B1 and cancer, Kao, *et al.* identified cyclin B1 as a tumor antigen (194). T cell responses specific for the self protein cyclin B1 were identified after a fraction of cyclin B1-homologous peptides eluted from immunoprecipitated MHC class I of a breast adenocarcinoma cell line (MS) were found to prime naïve T cells (194).

The identified peptides could prime T cells from healthy donors and elicit T cell responses in the PBMC of cancer patients. Importantly, while the fraction of peptides tested contained only peptides with at least one mutated residue, the gene encoding cyclin B1 in the original tumor cells was still normal. Therefore, any errors in sequence were likely accumulated during translation. Since the eluted peptides contained these patient-specific mutations, the wild-type homologue (CB9) was also tested in T cell stimulation assays in order to ensure that the antigenicity of cyclin B1 could be generalized to all cyclin B1-overexpressing tumors. Results of ELISPOT data gathered from four patients with squamous cell carcinoma of the head and neck (SCCHN) showed MHC class I dependent T cell stimulation by both the wild type and tumor-derived cyclin B1 peptides. The responses were different for each patient, but eluted peptide P4 and wild type peptide CB9 showed stimulation in more than one patient. The identified T cell responders were then shown to have functional anti-tumor activity as they were able to kill HLA-matched tumor cells from the original breast adenocarcinoma cell line. *In vitro* stimulated T cells from a SCCHN patient were able to lyse the MS cells better than controls, a function that was increased by transfection with costimulatory CD80.

These data were the first to identify a cell cyclin as a T cell tumor antigen. While others had shown the presence of cyclin overexpression (186, 195, 196) and cyclin-specific antibodies in cancer (197), none had shown a direct T cell response.

1.3.2.2 T Cell Dependent Antibody Responses Against Overexpressed Cyclin B1

Antibodies specific for cyclin B1 have been detected in hepatocellular, breast, gastric, prostate, and colorectal cancers (197, 198). In gastric and lung cancers, recursive partitioning analyses applied to seven tumor antigens, including p53, showed the presence of anti-cyclin B1 antibody to be the main discriminating factor between cancer cases and healthy controls.

Antibodies specific for cyclin B1 were also a crucial discriminator in four other cancers (199). However, these analyses used the same very low coating concentration for each of the 7 tumor antigens, and therefore, they were likely not optimized for detection of anti-cyclin B1 antibody. Finally, *in silico* analysis of cyclin B1 identified many potential cyclin B1 epitopes that could bind to HLA-B and HLA-DR (200).

After the identification of T cell responses in cancer patients, Suzuki *et al.* worked to determine the frequency of T cell-dependent anti-cyclin B1 (IgG) in patients with several different cancer types, with particular attention to lung cancer (198). Pancreatic, breast, colon, and lung cancer patients all had significantly higher anti-cyclin B1 antibody levels than did the 5 to 10 healthy controls tested in each assay. In the same publication, anti-cyclin B1 IgG was found in patients who presented with non-cancerous lung disease and were classified as heavy or light/non-smokers. The anti-cyclin B1 antibody level was significantly higher in the heavy smokers, although the numbers were small (9 heavy vs. 11 light smokers). This indicated that cyclin B1 overexpression could occur in tissues at risk of developing cancer and was supported by cyclin B1 overexpression observed with both dysplastic and metaplastic lung tissue. This data is complemented by the research of Geddert, *et al.*, which demonstrated CB1 overexpression in metaplasia, dysplasia, and carcinoma in the evolution of Barret's esophagus (201).

1.3.3 Summary

Cyclin B1 is a tumor antigen that is overexpressed in many different cancers and indicates a worse prognosis for the majority of cancers in which it is overexpressed. Cyclin B1 can be recognized by cyctoxic T cells and T cell-dependent antibody in patients with cancer, and it is a

promising candidate for vaccination, since naïve T cells in healthy individuals could be primed *in vitro* to respond to the wild type cyclin B1 peptide, CB9. Also, the fact that cyclin B1 is overexpressed when tumors lose function of p53 means that cyclin B1 offers an additional advantage as a tumor antigen and target for broad clinical use: while p53 mutations can vary in immunogenicity, cyclin B1 is overexpressed—and subsequently immunogenic—as a result p53 deregulation and deletion (185, 202). In addition, since cyclin B1 is required for entry into mitosis, it is unlikely that a growing tumor could select against cyclin B1, resulting in tumor escape through antigen loss. Finally, given that cyclin B1 is transiently expressed in the normal cell, normal cyclin B1 expression may lead to the presentation of cyclin B1 peptides in low density, low frequency, and low duration; this, in turn, indicates that cyclin B1 expression in the healthy cell is insufficient to elicit autoimmunity or induce peripheral tolerance.

1.4 STATEMENT OF THE PROBLEM

Our lab identified cyclin B1 as a tumor antigen that can be recognized by T cells and antibodies in cancer patients. However, there were no data regarding the significance of this immune response in cancer patients. Antibodies specific for other tumor antigens—such as p53 and MUC1—have been shown to have prognostic significance for cancer patients. Given that the overexpression of cyclin B1 most often correlates with a poor prognosis and a more malignant phenotype in cancer, we hypothesized that cyclin B1-specific IgG and T cells would serve as a biomarker for early diagnosis or prognosis. As described in chapter 2, to explore anti-cyclin B1 immune responses as a biomarker for prognosis, we collected plasma samples from 162 lung cancer patients and tested them for anti-cyclin B1 IgG. We also obtained tumor

sections from many of the patients and evaluated them for cyclin B1 overexpression. Our analyses included an evaluation of the effect of anti-cyclin B1 IgG on overall and progression-free survival in all patients and patients with identified cyclin B1-positive tumors. We also evaluated the effect of high or low levels of cyclin B1 IgG on the overall and progression-free survival of patients in each stage of disease.

In the study of anti-cyclin B1 IgG in lung cancer, we also collected healthy, non-smoking, age-approximated controls in order to have an anti-cyclin B1 IgG 'negative' population to use in a statistical definition of 'positive'. In the process, healthy individuals were also found to have a high level of anti-cyclin B1 IgG. We then hypothesized that cellular and humoral immune responses specific for cyclin B1 are present in individuals without cancer and their presence could have an effect on the development of cyclin B1-positive tumors. As described in chapter 3, we tested plasma from 65 healthy individuals for anti-cyclin B1 IgG, and we tested T cells from blood bank donors for the presence of memory, cyclin B1-specific $CD4^+$ and $CD8^+$ T cells. Our studies also worked to identify cyclin B1 T cell epitopes with the use of a peptide library. In addition, we evaluated whether cyclin B1-specific immune responses can protect mice from the growth transplantable or spontaneous cyclin B1-positive tumors.

Finally, we attempted to predict and identify events in healthy controls that would lead to an immune response against this self protein/tumor antigen in the absence of cancer. A literature search revealed several observations that viral infections can cause cells to aberrantly express self molecules, among them cyclin B1, that we hypothesized could become visible to the immune system, thereby training it to recognize these changes in subsequent viral infections and cancers that induce similar patterns of self-protein expression. Chapter 4 describes the results of mouse experiments where mice were infected with the mouse orthopox virus ectromelia or given

non-infectious (UV-irradiated) modified vaccinia virus. After the acute infection, mice were challenged with a transplantable tumor and monitored for the affects of infection on tumor growth and overall survival.

2.0 ANTI-CYCLIN B1 ANTIBODY AS A BIOMARKER OF PROGNOSIS IN NON-SMALL CELL LUNG CANCER

2.1 INTRODUCTION

Non-small cell lung cancer (NSCLC) is the leading cause of cancer death worldwide (203). Currently, treatments for lung cancer include surgery, chemotherapy, and radiotherapy, with recent advances in immunotherapy, such as the monoclonal anti-VEGF antibody (204). Still, the death rates in lung cancer soar. This is due in part to the frequent late presentation of the disease (205), a factor that is difficult to avoid given the lack of recommended screening modalities (national comprehensive cancer network). And while screening for lung cancer by computed tomography (CT) has begun to show some benefit in detecting early stage disease (206), there is still a striking need for more effective therapeutics. One understudied area of lung cancer therapeutics is vaccination against tumor antigens. The most notable success in lung cancer vaccination was seen in the adjuvant setting, where complete responses were achieved when autologous tumor cells engineered to express GM-CSF were administered to 2 stage IV NSCLC patients after they were rendered disease free by surgery (38). Vaccination in the adjuvant setting may be more successful than vaccination without surgical resection, since cancers are known to be highly immunosuppressive (129).

Given the successes achieved with vaccine-induced anti-tumor antigen immune responses administered after surgical resection, we wanted to determine the post-surgery prognostic significance of naturally induced (rather than vaccine-induced) anti-tumor antigen immune responses. To do this, we focused on cyclin B1, which we previously identified as a tumor antigen that can elicit both cytotoxic T cell and antibody responses in cancer patients. Overexpression of cyclin B1 has been shown to correlate with increased invasiveness and metstatic capabilities *in vitro* (183) as well as a worse prognosis in several human cancers, including non-small cell lung cancer. As a result, it is possible that immune responses against cyclin B1 could control the most dangerous, residual cancer cells—and potentially cancer stem cells—that exist after surgical resection. We hypothesized that cyclin B1-specific antibody in the plasma of NSCLC patients would correlate with an improved prognosis.

We assessed anti-cyclin B1 IgG in 123 patients with NSCLC in plasma samples collected between 2002 and 2005. Given that IgG is a T-cell dependent isotype, anti-cyclin B1 IgG serves as a biomarker of an anti-cyclin B1 T cell response. Patients were followed for 2-5 years at the time of this analysis. Overall survival and progression-free survival were studied among all patients and among the subset of patients for whom tumor sections were available for staining and identified as cyclin B1-positive tumors. As a continuous variable for all stages, anti-cyclin B1 IgG did not correlate with overall or progression-free survival. However, when stages were evaluated separately, patients with stage IB NSCLC who had anti-cyclin B1 antibodies above the median survived significantly longer than those with anti-cyclin B1 antibodies below the median.

2.2 MATERIALS AND METHODS

2.2.1 Blood Collection

Blood was collected from individuals diagnosed with lung cancer at the time of a surgical procedure, usually the resection of tumor. All blood collection was performed under protocols approved by the University of Pittsburgh institutional review board.

2.2.2 ELISA for Anti-Cyclin B1 Antibody

ELISA plates (Thermo, Milford, MA) were each coated with 0.65µg recombinant human cyclin B1 protein in 50µl PBS per well. Plates were sealed overnight at 4˚C and washed 5 times with PBS before use. Cyclin B1-coated wells and empty, background wells were then coated with blocking buffer (2.5% BSA in PBS) for 1 hour. Plasma samples were diluted 1:100 in blocking buffer in 96-well polypropylene plates (Nunc, ThermoFisher) along with five control samples that represented the range of the assay. A multichannel pipette was then used to transfer 50µl of each diluted sample to the ELISA plates. Samples were allowed to incubate for 1 hr and were subsequently washed 5 times with 1% PBS-Tween. Anti-human IgG (Sigma) was diluted in blocking buffer and incubated on the plates for 1 hour. Plates were then washed as before and incubated with alkaline phosphatase substrate (Sigma*Fast*™ Tabs, Sigma) for 1 hour in the dark. 3M NaOH was added to stop the reaction and plates were read immediately at 405nm. After subtraction of background, samples run on separate days were normalized using the 5 sample controls. Briefly, the controls on all days were averaged and the difference between the overall mean and the mean on a given day was applied to all samples on that day.

2.2.3 Immunohistochemistry

Slides of tumor sections were deparaffinized and hydrated by transferring through Xylem, 100%EtOH, 95%EtOH, 70%EtOH, and several changes of dionized (DI) water. Heat-induced epitome retrieval was performed using a 20 minutes of decloaking in Dako High PH Buffer. Slides were allowed to cool and rinsed in DI water. Slides were then rinsed in Tris Buffered saline (TBS) for 5 minutes before treatment with 5% hydrogen peroxide. After another TBS rinse, the anti-cyclin B1 antibody (clone GNS-1, BD Pharmingen, working dilution 1:125) was added and incubated for 30 minutes. The slides were rinsed in TBS again and the Envision Mouse polymer (Dako) was applied. Another TBS rinse was followed by development with DAB + chromagen for 10 minutes. DI was used to rinse, after which slides were counterstained with filtered DAKO Hematoxylin for 90 seconds. After a tap water wash, slides were dipped in ammonia and washed under running tap water.

2.2.4 Statistical Methods

The association of the survival time and progression free survival time with all available explanatory variables was examined via Cox proportional hazards regression. This analysis included only patients within NSCLC lung cancer, and without neoadjuvant chemo/radiation therapy before tissue collection. Pathological stage was classified as follows: IA, IB, IIA/B, III and IV. Clinical stage was used for those cases where pathological stage was not available. Histology was classified as follows: Group 1 including Squamous cell carcinoma (SCCA); Group 2 including Adenocarcinoma, Adenosquamous carcinoma, BAC, Carcinoid and Malignant Carcinoid; Group 3 including Large cell carcinoma, Non-small cell carcinoma

(NSCLC) and Undiff NSCLC. Small cell carcinoma was excluded from this analysis. Smoking status was classified as follows: active or smoker NOS with smoking exposure between 1 and 50 Pack-Year (PY); active or smoker NOS with smoking exposure between 51 and 75 PY; active or smoker NOS with smoking exposure greater or equal than 76 PY; ex-smoker with smoking exposure between 1 and 50 PY; ex-smoker with smoking exposure between 51 and 75 PY; ex-smoker with smoking exposure greater or equal than 76 PY; never smoker.

2.3 RESULTS

2.3.1 Distribution of patients

Of the 162 lung cancer patients for whom plasma was collected and tested for anti-cyclin B1 antibody response, 123 fit the criteria for statistical assessment of prognosis. Exclusions were based on an absence of follow-up data or the receipt of neoadjuvant therapy before blood collection. Of the 123 included patients, 117 had sufficient data for studies of progression free survival (25^{th} and 50^{th} percentiles were 10 and 21 months), and 121 had sufficient data for analyses of overall survival (median overall survival, 34 months; 25^{th} and 75^{th} percentiles were 16 months and 79 months) (Table 2-1). Tumor stages were represented with similar frequency, although nonresectable stage IV NSCLC represented only 14% of cases (Table 2-2). Of these 123 patients, 51 and 47 patients had sufficient outcome data for progression free and overall survival (respectively) in combination with tumor sections that could be identified as cyclin B1-positive.

Table 2-1: Patient survival, normalized cyclin B1 IgG, and age.

Variable	N	Mean	Median	Minimum	Maximum	Range	Std Dev
Overall Survival (month)	121	-	34	0.3	104.7	104.4	19.93
Progression Free Survival (month)	117	-	21	0.2	95.6	95.4	19.78
Normalized CB1 IgG	123	0.32	0.26	0.01	1.53	1.52	0.26
Age at tissue collection	123	70.02	72	47	86	39	8.72

Table 2-2: Patient and tumor characteristics

Variables	Level	Frequency	Percent
Stage	IA	24	20%
	IB	31	25%
	IIA/B	21	17%
	III	30	24%
	IV	17	14%
Histology Group	Squamous cell carcinoma(SCCA)	35	28%
	Adenocarcinoma Adenosquamous carcinoma BAC Carcinoid Malignant carcinoid	67	55%
	Large cell carcinoma Non-small cell carcinoma(NSCLC) Undiff NSCLC	21	17%
Sex	Female	67	54%
	Male	56	46%
Race	African American	8	7%
	White	115	93%
Smoking Status	Active, smoker NOS,1-50 PY	24	20%
	Active, smoker NOS,51-75 PY	10	8%
	Active, smoker NOS,>76 PY	15	13%
	Ex-smoker,1-50 PY	41	34%
	Ex-smoker,51-75 PY	11	9%
	Ex-smoker,>76 PY	8	7%
	Never smoker	11	9%

2.3.2 NSCLC Patients Have a High Frequency of Anti-Cyclin B1 IgG and Cyclin B1-Positivie Tumors.

Plasma from all 162 NSCLC patients was tested for anti-cyclin B1 IgG by ELISA. Figure 2-1 demonstrates that the majority of NSCLC patients have detectable levels of anti-cyclin B1 IgG. In addition, while only 99 patients had tumor available for cyclin B1 staining (only 77 of whom could be matched to the patient ID number connected to the outcome data and plasma samples and only 51 of whom had sufficient outcome data), 90% of tumors were positive for cyclin B1 (defined as >15% of cancer cells staining positively). 78% of those tumors expressed very high levels of cyclin B1, defined as >50% of the tumor cells staining positively. Anti-cyclin B1 IgG did not correlate with age, sex, histology group, race, stage, smoking status/pack-year history or cyclin B1 status of the tumor (data not shown).

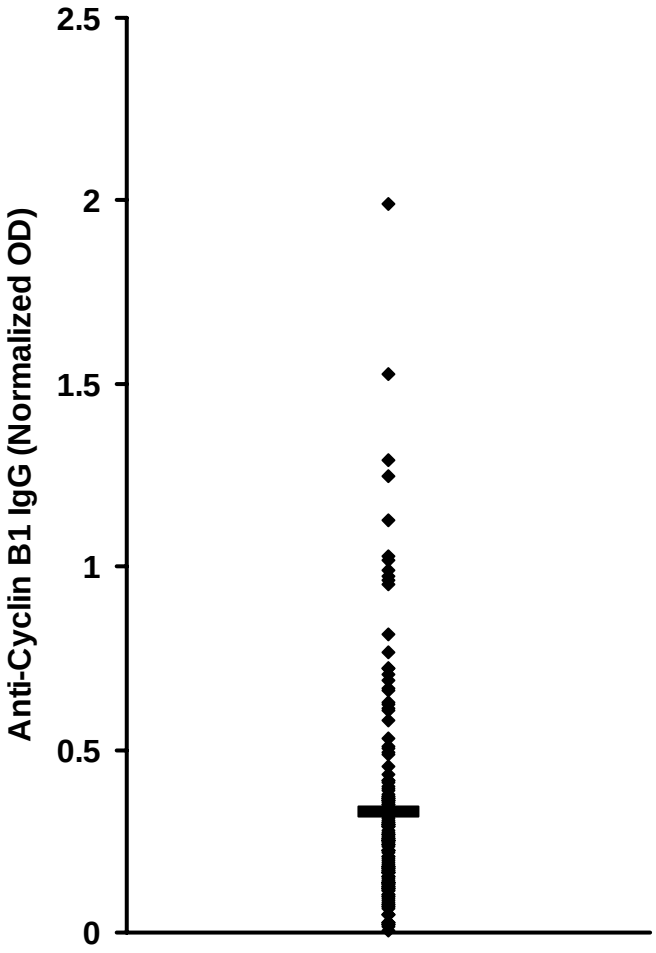

Figure 2-1: Anti-cyclin B1 IgG is detectable in the majority of NSCLC patients.

Plasma from 162 NSCLC patients was tested for the presence of anti-cyclin B1 IgG by ELISA. Mean normalized OD is indicated. The majority of patients have a clear anti-cyclin B1 IgG signal (median OD = 0.253).

2.3.3 Overall Survival Among All Patients is Not Significantly Correlated with Anti-Cyclin B1 IgG

Analysis of 121 NSCLC patients demonstrates that anti-cyclin B1 IgG does not correlate with overall survival among all stages when measured as a continuous variable (Table 2-3). This is in contrast to stage and age at tissue collection, both of which were significantly correlated with

overall survival, as would be expected. Given that anti-cyclin B1 immune responses would only be expected to be clinically significant for cyclin B1-poitive tumors, the same analysis was performed on 51 patients who had known cyclin B1-positive tumors (Table 2-4). While the effect of cyclin B1 IgG was still not significant, elimination of the estimated 10% of patients with cyclin B1 negative tumors reduced the p value from 0.84 to 0.43.

2.3.4 Progression-Free Survival Among All Patients is Not Significantly Correlated with Anti-Cyclin B1 IgG

Analysis of 117 NSCLC patients demonstrates that anti-cyclin B1 IgG does not correlate with progression-free survival among all stages when measured as a continuous variable (Table 2-5). This is in contrast to stage, which was significantly correlated with overall survival as would be expected. The same analysis was performed on 47 patients who had known cyclin B1-positive tumors (Table 2-6). Elimination of the estimated 10% of patients with cyclin B1 negative tumors reduced the p value from 0.68 to 0.213.

Table 2-3: Overall survival among all non-small cell lung cancer patients

Variables	Level	OS P-value	Level P-value	Hazard Ratio
Normalized CB1 IgG		0.84	0.84	1.125
Age at tissue collection		**0.048**	0.048	**1.035**
Stage	IA	**0.008**	0.0006	**0.134**
	IB		0.0281	**0.364**
	IIA/B		0.1075	**0.464**
	III		0.2095	**0.568**
	IV		(Reference)	-
Histology	Squamous cell carcinoma(SCCA)	0.18	0.1305	1.933
	Adenocarcinoma Adenosquamous carcinoma BAC Carcinoid Malignant carcinoid		0.7409	1.139
	Large cell carcinoma Non-small cell carcinoma(NSCLC) Undiff NSCLC		(Reference)	-
Sex	Female	0.75	0.75	0.911
	Male		(Reference)	-
Race	African American	0.67	0.67	1.224
	White		(Reference)	-
Smoking Status	Active, smoker NOS,1-50 PY	0.5	0.5252	0.7
	Active, smoker NOS,51-75 PY		0.7979	1.196
	Active, smoker NOS,>76 PY		0.3082	1.833
	Ex-smoker,1-50 PY		0.8932	0.932
	Ex-smoker,51-75 PY		0.777	1.195
	Ex-smoker,>76 PY		0.4225	0.575
	Never smoker		(Reference)	-

Table 2-4: Overall survival among patients with cyclin B1-positive tumors

Variables	Level	OS P-value	Level P-value	Hazard Ratio
Normalized CB1 IgG		0.43	0.43	2.287
Age at tissue collection		**0.003**	0.003	**1.106**
Stage	IA	**0.033**	0.0021	**0.018**
	IB		0.0782	**0.181**
	IIA/B		0.1196	**0.202**
	III		0.1185	**0.222**
	IV		(Reference)	-
Histology Group	Squamous cell carcinoma(SCCA)	0.43	0.9804	0.981
	Adenocarcinoma Adenosquamous carcinoma BAC Carcinoid Malignant carcinoid		0.3624	0.486
	Large cell carcinoma Non-small cell carcinoma(NSCLC) Undiff NSCLC		(Reference)	-
Sex	Female	0.23	0.23	0.477
	Male		(Reference)	-
Race	African American	0.72	0.72	0.715
	White		(Reference)	-
Smoking Status	Active, smoker NOS,1-50 PY	0.061	0.9292	0.896
	Active, smoker NOS,51-75 PY		0.5474	2.136
	Active, smoker NOS,>76 PY		0.7265	1.754
	Ex-smoker,1-50 PY		0.4136	0.342
	Ex-smoker,51-75 PY		0.3914	3.444
	Ex-smoker,>76 PY		0.0921	0.081
	Never smoker		(Reference)	-

Table 2-5: Progression-free survival among all non-small cell lung cancer patients

Variables	Level	PFS P-value	Level P-value	Hazard Ratio
Normalized CB1 IgG		0.68	0.68	1.275
Age at tissue collection		0.17	0.17	1.027
Stage	IA	**0.0019**	0.0003	**0.15**
	IB		0.0076	**0.286**
	IIA/B		0.0906	**0.451**
	III		0.3257	**0.648**
	IV		(Reference)	-
Histology Group	Squamous cell carcinoma(SCCA)	0.52	0.3382	0.637
	Adenocarcinoma Adenosquamous carcinoma BAC Carcinoid Malignant carcinoid		0.2651	0.652
	Large cell carcinoma Non-small cell carcinoma(NSCLC) Undiff NSCLC		(Reference)	-
Sex	Female	**0.03**	0.03	0.504
	Male		(Reference)	-
Race	African American	0.49	0.49	1.459
	White		(Reference)	-
Smoking Status	Active, smoker NOS,1-50 PY	0.492	0.5675	0.717
	Active, smoker NOS,51-75 PY		0.3485	1.828
	Active, smoker NOS,>76 PY		0.72	0.794
	Ex-smoker,1-50 PY		0.297	0.555
	Ex-smoker,51-75 PY		0.7575	0.816
	Ex-smoker,>76 PY		0.8657	0.883
	Never smoker		(Reference)	-

Table 2-6: Progression-free survival among patients with cyclin B1-positive tumors

Variables	Level	PFS P-value	Level P-value	Hazard Ratio
Normalized CB1 IgG		0.213	0.213	6.55
Age at tissue collection		0.106	0.106	1.061
Stage	IA	**0.04**	0.009	**0.04**
	IB		0.028	**0.057**
	IIA/B		0.1771	**0.243**
	III		0.1612	**0.272**
	IV		(Reference)	-
Histology Group	Squamous cell carcinoma(SCCA)	0.31	0.127	0.282
	Adenocarcinoma Adenosquamous carcinoma BAC Carcinoid Malignant carcinoid		0.3194	0.437
	Large cell carcinoma Non-small cell carcinoma(NSCLC) Undiff NSCLC		(Reference)	-
Sex	Female	**0.0378**	0.0378	**0.224**
	Male		(Reference)	-
Race	African American	0.85	0.85	1.231
	White		(Reference)	-
Smoking Status	Active, smoker NOS,1-50 PY	0.42	0.2447	0.25
	Active, smoker NOS,51-75 PY		0.347	0.287
	Active, smoker NOS,>76 PY		0.4162	0.273
	Ex-smoker,1-50 PY		0.1071	0.15
	Ex-smoker,51-75 PY		0.8838	0.821
	Ex-smoker,>76 PY		0.205	0.131
	Never smoker		(Reference)	-

2.3.5 High Levels of Anti-Cyclin B1 IgG Correlates with a Longer Overall Survival in Patients with Stage IB NSCLC.

Whereas the previous analyses were performed with cyclin B1 as a continuous variable for all stages at once, we next assessed overall and progression-free survival using cyclin B1 as a binary variable—with levels above and below the median for the group determined to be high and low anti-cyclin B1 IgG, respectively—and for each stage. Figure 2-2 shows that high levels of anti-cyclin B1 IgG correlate with a significantly increased overall survival in stage IB NSCLC (log rank test, p = 0.0026). The same was not true for progression-free survival (p = 0.11, Figure 2-3).

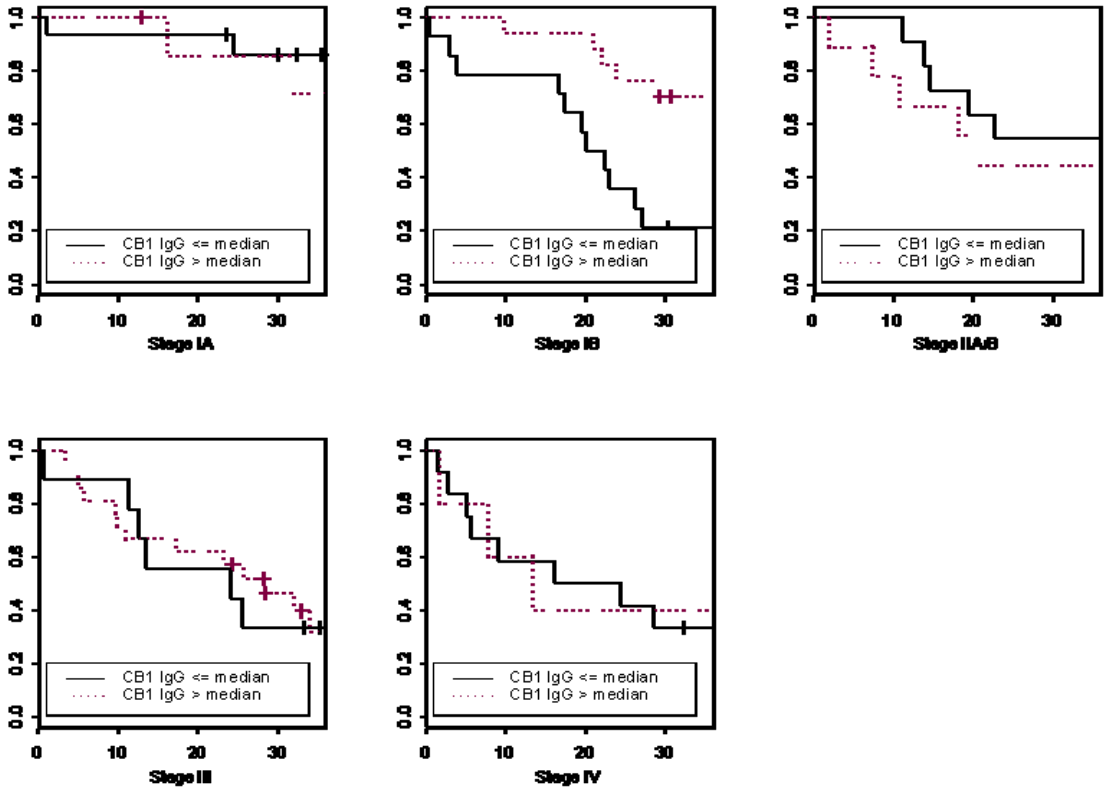

Figure 2-2: Overall survival in patients above and below the median anti-cyclin B1 IgG

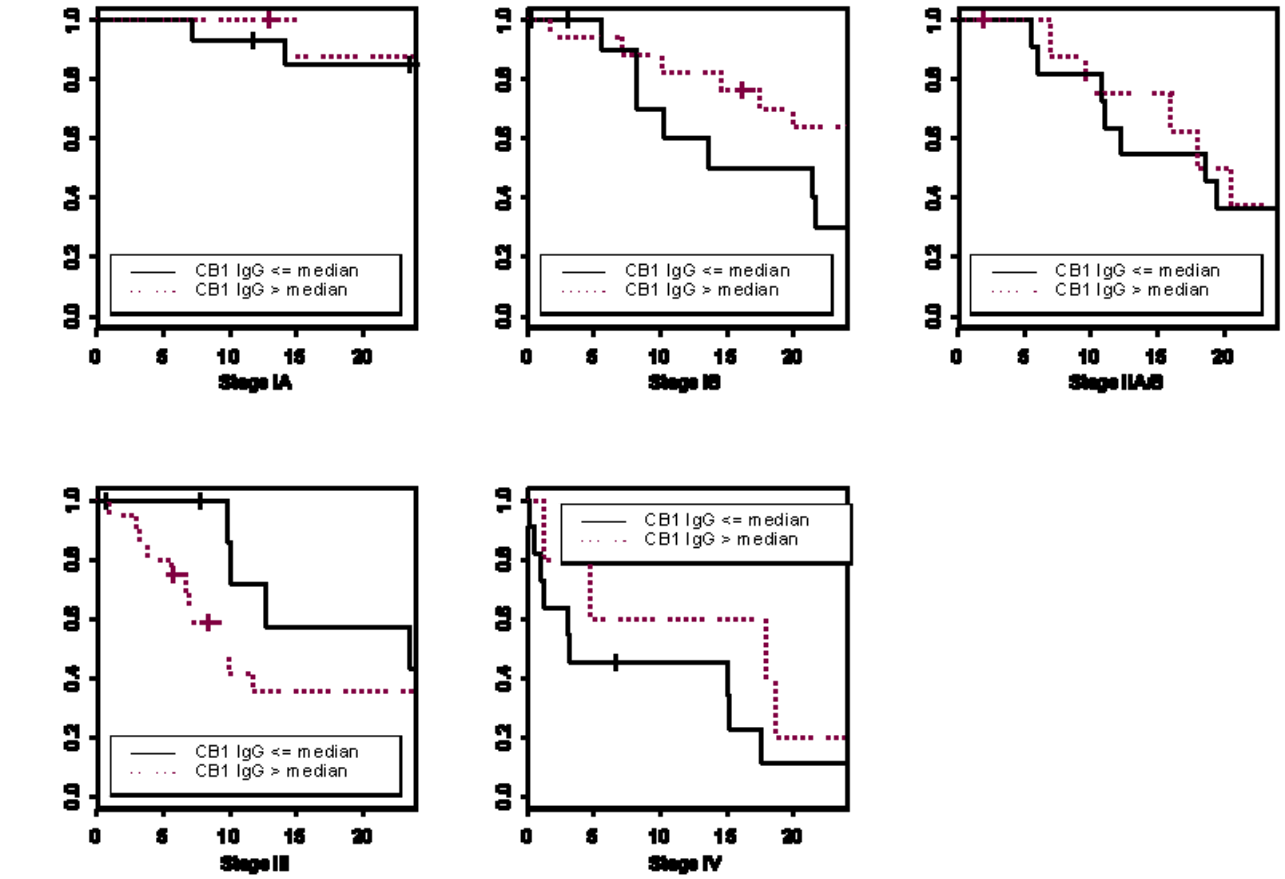

Figure 2-3: Progression-free survival above and below the median anti-cyclin B1 IgG.

2.4 DISCUSSION

In this study, we demonstrate that the presence of high levels of anti-cyclin B1 IgG at the time of surgical resection predicts a longer overall survival for patients with stage IB lung cancer. Stage IB is defined as a tumor mass greater than 3 cm with no lymph node or distant metastases. It is possible to completely resect the visible tumor in stage IB, and studies have reported 5-year

survival rates of 38%, 46%, 49.9%, 59%, 58%, and 68% (207-211). As a result, we were not surprised that stage IB presented our best opportunity to detect the prognostic significance of anti-cyclin B1 IgG; tumor resection allows for assessment of an anti-tumor immune response in the absence of the immunosuppressive cancer, and unlike other stages with very high and very low survival rates, the approximate 50% survival rate in stage IB means that a study of survival would have the best power in this stage. We also expected that our results would be best found in the overall survival group, since the analyses of progression-free survival would require consistent follow-up evaluation for every individual in order to be sure that all recurrences were documented as closely to the true recurrence date as possible. Also, given that many patients who are otherwise lost to follow up can still be included in analyses of overall survival based on government reports of death, the numbers of patients in the final analysis of overall survival are also better than for progression-free survival in our study.

Ours is one of a very limited number of studies that evaluate the prognostic significance of anti-tumor associated antigen (TAA) antibody responses in human cancer. To date, most studies have focused on the prognostic significance of anti-p53 antibodies and have found correlations with worse prognosis (49). In a NSCLC study by Lai *et al*., anti-p53 antibodies significantly correlated with a decrease in survival, with a median survival time of 141 days for anti-p53 positive patients vs. 239 for anti-p53 negative patients (212). However, 75 of the 111 NSCLC patients in this study had cancer that was stage IIIb or higher, for whom no resection (and only chemotherapy or radiotherapy) was recommended. In addition, 8 of the 9 anti-p53 antibody positive patients were in the non-resectable groups. As a result, the study does not address the significance of anti-TAA immune responses after surgical resection of cancer. The correlation between high antibody level and advanced NSCLC cancer was reproduced by another

study in which the majority of anti-p53 antibodies were found in advanced disease and correlated with a decreased survival in those stages as well (213). However, this correlation is not always the case, as a study by Bergqvist *et al*. demonstrated that patients without nodal or distant metastases had a higher level of anti-p53 antibodies than patients with advanced disease; the study did not observe a correlation between anti-p53 antibody and survival (214). Still, the studies of anti-p53 antibodies in NSCLC do not analyze the effect of the antibodies for individual stages, and they could therefore miss the significance of an anti-TAA antibody response after the removal of an immunosuppressive tumor. One study has evaluated the significance of anti-p53 antibodies in resectable (stage Ia-IIIa) NSCLC. The authors still found that the p53-specific antibodies correlated with a worse overall survival (215). Unlike p53, antibodies specific for MUC1 have been shown to predict an increased overall survival in NSCLC, although the study included only those patients with stages IIIB and IV NSCLC (53).

Only one other study has evaluated antibodies specific for self molecules in the prognosis of NSCLC. Blaes *et al.* examined sera of 61 patients with resectable NSLC (stages I-IIIa and resectable IIIb) for the presence of anti-neuronal and anti-nuclear antibodies (ANA) (216). Anti-neuronal antibodies were significantly correlated with increased survival in all stages, while ANA were significantly correlated with increased survival only in stage III. However, given the small numbers of stage I and II patients ($n = 32$) and p values approaching significance ($p = 0.11$), it is possible that an appropriately powered study would have also shown significance of ANA in these stages as well. Importantly, the ANA assays were performed on HEp-2 cells, which are derived from a human laryngeal carcinoma. As a result, the observed antibody binding could in fact be to self antigens that are also tumor antigens. The authors concluded that the autoantibodies represented a successful immune response against the tumor.

Taken together, the studies of the two anti-tumor antigen antibodies (anti-p53 and anti-MUC1) and autoantibodies in NSCLC have shown that whether prognosis is improved or worsened is based on the target molecule. While the anti-p53 studies indicated that antibodies are a sign of an unmanageable tumor burden, the anti-MUC1 and autoantibody studies concluded that antibodies indicated successful immunosurveillance. In addition, given that the majority of NSCLC patients present with late stage disease (205), it is not surprising that only one of these studies addressed the significance of anti-TAA immune responses in patients who have been rendered tumor-free by surgery (216).

We also demonstrated that anti-cyclin B1 antibodies do not correlate with age, stage, smoking history, sex, or cyclin B1 status of the tumor. While we are currently improving our data on the cyclin B1 staining of the tumor (increasing the number of analyzed tumors and converting the measurements from discreet scoring to a continuous quantification), the lack of a positive correlation between anti-cyclin B1 IgG and the tumor stage (burden) or cyclin B1 status indicates that the antibody responses may not have been mounted in direct response to the cancer. This is in contrast to anti-p53 antibodies, which have not only been correlated with a higher tumor stage (212, 213) in NSCLC but also have been suggested to indicate tumor relapse in the setting of adjuvant chemotherapy in NSCLC, and therefore be related to tumor burden after resection (217).

Importantly, our studies of significance in the analyses of anti-cyclin B1 antibody as a continuous variable in the overall survival of NSCLC are not yet complete. In 2 years, we will have 5 year survival data on all patients. In addition, we are currently working to obtain the remaining tumor samples that have not yet been assessed for cyclin B1 staining. Together, these

additional data will increase our ability to determine the effects of anti-cyclin B1 IgG on overall survival among patients with cyclin B1-positive tumors.

When all 5-year survival data have been obtained, we will also perform the assessment of anti-cyclin B1 IgG as a continuous variable in overall survival with the exclusion of patients who did not undergo surgery that resulted in complete resection (mostly stages IIIB and IV). This will be in greater concordance with our hypothesis model, in which the effect of anti-TAA immune responses are most successful in the absence of an immunosuppressive tumor mass, as would be the case for immune responses existing prior to the development of cancer or—in this case—after it has been surgically removed.

3.0 HEALTHY PEOPLE HAVE ANTI-CYCLIN B1 IMMUNE RESPONSES THAT WHEN ELICITED IN MICE BY VACCINATION CONTROL GROWTH OF CYCLIN B1+ TRANSPLANTABLE AND SPONTANEOUS TUMORS

3.1 INTRODUCTION

Overexpression of cyclin B1 has been documented in many human cancers, including colorectal, lung, cervical, and head and neck carcinomas (182, 186, 187, 195, 218). Additionally, this overexpression has been shown to correlate with a worse prognosis in lung, laryngeal, esophageal, and tongue cancers (177, 182-184, 186, 187). In normal cells, cyclin B1 synthesis and nuclear function are required for entry into mitosis, after which the cyclin is rapidly degraded. In cancer cells, cyclin B1 is expressed constitutively in all stages of the cell cycle (185). Additionally, while cyclin B1 is localized and exerts its transient function in the nucleus of a normal proliferating cell, cancer-associated overexpression is evident primarily in the cytoplasm where it can be subject to proteasomal processing and presentation in MHC class I. We previously reported isolation of cyclin B1 peptides from MHC class I molecules of tumor cells that were recognized by tumor-specific memory T cells present in patients with cyclin B1 overexpressing tumors (194). We later showed that cancer patients have cyclin B1-specific antibodies of IgM, IgG and IgA isotypes (198).

Cyclin B1 overexpression in many tumors is secondary to the loss of p53 function either through p53 mutations or a deletion (185). Considering that alterations in p53 function happen in many tumors early in the process of transformation, cyclin B1 is a good candidate antigen for immunotherapy and immunoprevention of a large number of human tumors. Additionally, since cyclin B1 is required for entry into mitosis, it is unlikely that a growing tumor could select against cyclin B1, resulting in tumor escape through antigen loss.

While studying anti-cyclin B1 immune responses in cancer patients, we discovered that many healthy individuals also had anti-cyclin B1 immunity. In order to determine how frequently this may occur, and eventually to determine the potential significance of these immune responses, we collected blood samples from individuals in different age groups and with no known history of cancer. Given that all published data focused on cancer patients and we were the first to interrogate anti-cyclin B1 immunity in healthy individuals, the goal of our studies was to examine both humoral and cellular immunity to cyclin B1. We found that, in addition to antibodies, healthy individuals have both $CD4^+$ and $CD8^+$ antigen-experienced T cells specific for cyclin B1. This begged the question of whether these responses could be in any way involved in immune surveillance of cyclin B1 overexpressing tumors. To directly test this, we used transplantable and spontaneous cyclin B1 overexpressing mouse tumor models. Cyclin B1 is highly conserved among species and the homology between mouse cyclin B1 and human cyclin B1 is 85%. We determined that just like in human cancer (185), mouse tumors lacking p53 overexpress cyclin B1. This allowed us to use a $p53^{-/-}$ mouse that spontaneously develops cyclin B1 overexpressing tumors and cell lines established from these tumors. We found that cyclin B1 based vaccines can elicit strong cyclin B1-specific humoral and cellular immune

responses that can reject a transplantable tumor challenge or inhibit spontaneous tumor growth in $p53^{-/-}$ mice and significantly increase overall survival.

3.2 MATERIALS AND METHODS

3.2.1 Synthesis of Mouse and Human Cyclin B1 Recombinant Proteins

Mouse cyclin B1 and human cyclin B1 were subcloned into pDEST-17, a T7 driven vector with an N terminal His-tag (Invitrogen, Carlsbad, CA). Bl21 codon+ BL21 RIPL bacteria cells (Invitrogen) were transformed and grown overnight in shaker flasks at 37°C, in a constitutive expression state. Recombinant cyclin B1 was found in inclusion bodies and extracted with guanidine-HCL under reducing conditions. Solubilized inclusion bodies lysate was passed over a Q-Sepharose FF (Amersham Biosciences, Piscataway, NJ) packed column monitored by an AKTA prime chromatography system, and the protein was collected in flow through fractions. Fractions were run over Nickel-HP 5 ml HighTrap columns (Amersham Biosciences) in a modified refolding protocol. The protein was loaded onto the column, washed with guanidine, exchanged into a 6M urea buffer, and then slowly exchanged into a 3M Urea buffer. The protein was eluted in an imidazole gradient, and fractions were collected and run on a gel to determine which ones contained the protein. Full-length cyclin B1, both mouse and human, runs on Tris/glycine gels under reducing conditions at the predicted weight of 54kD and only fractions containing full-length protein were collected. Protein was concentrated on 30 MWCO Amicon filters and

assayed for endotoxin. Protein purity was analyzed by coomassie blue, Western blot, HPLC, and Limulus amebocyte lysate assay (LAL, for endotoxin assessment).

3.2.2 Blood Donors

Blood was collected from individuals ages 25-79 after receipt of consent. Blood collection at the University of Pittsburgh and the Rockefeller University was approved by the institutional review board of each university. Buffy coats were obtained from the Allegheny County Health Department Blood Bank. Blood used for peptide library stimulations was collected from individuals enrolled in a University of Pittsburgh biomarker study for lung cancer risk. Individuals for the biomarker study were ages 55-79, heavy smokers, and negative for lung cancer by computed tomography

3.2.3 Measurement of Human Anti-Cyclin B1 Antibody Responses

Wells of 96-well ELISA plates (Thermo, Milford, MA) were each coated with 0.65µg recombinant human cyclin B1 protein in 50µl PBS. Plates were sealed overnight at 4˚C and washed with PBS before use. Cyclin B1-coated wells and empty, background control wells were then blocked with 2.5% BSA in PBS (blocking buffer) for 1 hour. Plasma samples were diluted 1:400 in blocking buffer in 96-well polypropylene plates (Nunc, Thermo Fisher, Rockford, IL) along with five control samples that represented the range of the assay. 50µl of each diluted sample was then transferred to the ELISA plates. Samples were incubated for 1 hr and were subsequently washed with 1% PBS-Tween. Anti-human IgG (Sigma, St. Louis, MO) was diluted in blocking buffer and incubated on the plates for 1 hour. Plates were then washed as

before and incubated with alkaline phosphatase substrate (Sigma*Fast*™ Tabs, Sigma) for 1 hour in the dark. 3M NaOH was added to stop the reaction and plates were read immediately at 405nm. After subtraction of background, samples run on separate days were normalized using the 5 sample controls. Briefly, the controls on all days were averaged and the difference between the overall mean and the mean on a given day was applied to all samples on that day.

3.2.4 Generation and Loading of Dendritic Cells

Peripheral Blood Mononuclear Cells (PBMC) from buffy coats were incubated in AIMV (Gibco, Invitrogen) at 37˚C for 1 hour to obtain adherent monocytes. Non-adherent cells were removed for future use. Monocytes were then cultured in complete, serum-free AIMV media for 6 days in the presence of 400U/ml GM-CSF (R&D, Minneapolis, MN) and 1000U/ml IL-4 (R&D). Additional media and GM-CSF/IL-4 were added again on day 4. On day 6, DC were harvested and loaded at 30μg/ml for each protein. Both ovalbumin (OVA, Sigma) and cyclin B1 proteins were treated for LPS removal with Detoxi-Gel (Pierce, Thermo Fisher). After 4 hours, a maturation cocktail of 10μg/ml TNFα (R&D), 10μg/ml IL-6 (R&D)**,** and 1μg/ml PGE_2 (Sigma) was added. DC were then allowed to mature for 2 more days before use in assays.

3.2.5 T cell Stimulation with Dendritic Cells

PBL that did not adhere during monocyte isolation were frozen in 10% DMSO in FBS for use in assays. Frozen cells were thawed and washed on the day of use and either used directly in assays as total PBL or subjected to $CD8^+$ T cell purification (Miltenyi, Auburn, CA). PBL or $CD8^+$ T cells were plated with autologous, protein-loaded or unloaded DC at a DC:T cell ratio of 1:10 in

complete RPMI supplemented with 10% human serum (Gemini Bio-Products, West Sacramento, CA), 10ng/ml IL-12 (R&D), and 5ng/ml IL-6 (R&D). PBL used in proliferation assays were labeled with 5μM CFSE, following the manufacturer's protocol (CellTrace™, Invitrogen) and as detailed below. Proliferation was assed by flow cytometry on day 7. For assessment of secreted cytokines, blocking antibodies for MHC class I (W6/32) and CD4 (RPA-T4, 2.5μg/ml; Biolegend, San Diego, CA) were added to the indicated T cell cultures before the addition of DC and refreshed every two days. IFNγ concentrations in the supernatant were assessed by ELISA and following the manufacturer's protocol (OptEIA; BD Biosciences, San Jose, CA). Cultures were set up in triplicates for supernatant and proliferation assays.

3.2.6 Intracellular Cytokine Staining

Cyclin B1-loaded, OVA-loaded, and unloaded DC were combined with autologous T cells in triplicate so that the assay could be performed at three time points after the start of the co-culture. Six hours after the co-culture was begun, brefeldin A (BD Biosciences) was added to the first set of the triplicate for 11 hours, after which cells were fixed, permeabilized, and stained for cell surface markers following the manufacturer's protocol (BD Cytofix/Cytoperm™). This is indicated as the 24 hour time period. Brefeldin A was then added to the remaining two sets of the triplicate at 30 and 54 hours after culture (indicated as 48 hour and 72 hour time periods, respectively).

3.2.7 Peptide Library Synthesis

Cyclin B1 peptides were designed to span the entire cyclin B1 protein sequence and to represent the available T cell epitopes. The peptides were approximately 12-15 amino acids in length with an 11 amino acid overlap, for a total of 118 cyclin B1 peptides. The peptides were synthesized in collaboration with the Proteomics Resource Center at the Rockefeller University using a previously published method (80). Peptides were optimized for synthesis with the epitope library fragment generation program PeptGen, (available at http://www.hiv.lanl.gov). Integrity of each peptide was verified by matrix-assisted laser desorption/ionization (MALDI) mass spectrometry using a delayed extraction spectrometer system (Voyager; PerSeptive/Applied Biosystems, Foster City, CA).

3.2.8 T cell Assays Employing the Peptide Library

Peripheral blood was collected into vaccutainers containing heparin sulfate and processed to obtain PBMC by density centrifugation. PBMC were washed in PBS and labeled with 5μM CFSE (CellTrace™, Invitrogen) in warmed 0.1% FBS in PBS for 10 minutes at 37˚C. The CFSE was quenched with 3 volumes of ice cold complete RPMI and incubated for 5 minutes before washes with complete RPMI. PBMC were then resuspended in media containing anti-CD28 and anti-CD49d antibodies for additional costimualtion. Wells coated with anti-CD3 were used as positive controls. Peptides were added for a final concentration of 2μg/ml. No peptide was added to negative control wells. After 6 days, cells were stained for cell surface markers and tested for proliferation by CFSE dilution using flow cytometry.

3.2.9 Animals and Cell Lines

C57Bl/6 female mice, 6-8 weeks old, were purchased from The Jackson Laboratory. C57Bl/6 p53 deficient female mice, TSG-p53N12-M, 5-6 weeks old, were purchased from Taconic Transgenics. All animals were housed in the University of Pittsburgh Animal Facility. The LO2 cell line, a lymphoma derived from a p53 deficient mouse, was a kind gift from Dr. Soren Buus in University of Copenhagen, Denmark. It was maintained in vitro in RPMI-1640 medium (Gibco, Invitrogen) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Cellgro; Media Tech Inc**.,** Manassas**,** VA.), penicillin (100U/ml), streptomycin (100μg/ml), 0.3% glutamine (Gibco, Invitrogen), 0.1mM non-essential amino acids, 1 mM sodium pyruvate, 10μM β-mercaptoethanol (Gibco, Invitrogen), at 37^0C in 95%O_2/5%CO_2. LO2 cells were subcutaneously inoculated into syngeneic C57Bl/6 mice to establish a transplantable tumor model. All animals were housed in the University of Pittsburgh Animal Facility.

3.2.10 Immunohistochemical Staining of Cyclin B1 in Tumor Sections

Sections from mice were fixed in formalin (Thermo Fisher) for 24 hours and then embedded and sectioned (3-5μm). After drying overnight at 37°C, samples were deparaffinized, dehydrated, and stained with anti-cyclin B1 antibody (BD Biosciences) in the Transplant Surgery Experimental Pathology Laboratory in University of Pittsburgh. Briefly, samples were blocked with 2.5% BSA (Sigma) for 30 min at room temperature and stained with anti-cyclin B1 antibody. The avidin-biotin peroxidase method was then applied according to manufacturer's protocol using the Vectastain ABC Elite staining kit (Vector laboratories, Inc., Burlingame, CA).

3.2.11 Construction of Mouse and Human Cyclin B1 pcDNA3.1 DNA vectors

Human cyclin B1 cDNA derived from a Hela cell line was a gift from Dr. Qimin Zhan at the University of Pittsburgh. Mouse cyclin B1 cDNA was an RT-PCR product derived from the mouse $p53^{-/-}$ LO2 cell line. Briefly, RT-PCR was performed using primers ATGGCGCTCAGGGTCACTAG (forward) and CAGTCTATTGGAGTTATGCCTTTG (reverse). A band at approximately 1.3kbp migrated on a 1.2% E-Gel Agarose gel (Invitrogen). The mouse cyclin B1 band was eluted using a MiniElute Kit (Qiagen, Valencia, CA) and subcloned into PCR2.1-TOPO vector (Invitrogen) and used to transform One-Shot TOP10 competent cells (Invitrogen) as described by the manufacturer. Colonies were picked for culture, and plasmids were isolated and identified positively by an EcoRI digest. Both cDNAs were then subcloned into the BamHI-XhoI site of the pcDNA3.1 expression vector (Invitrogen). All inserts were verified by DNA sequencing.

3.2.12 Recombinant Protein Vaccination

C57Bl/6 mice were immunized subcutaneously with 25µg/100µl/mouse recombinant human cyclin B1 (hCB1) protein, mouse cyclin B1 protein (mCB1), or 100µl PBS as a control. At the time of immunization or PBS treatment, an immunostimulatory (IS) patch containing 20µg heat-labile enterotoxin (LT) (provided by IOMAI Corporation) was applied to the immunization site. Repeat injections and LT/IS patch application were repeated twice in 3 week intervals. Sera were collected to measure antibody response. Seventeen days after the last immunization, 3 mice per group were sacrificed to study T cell responses. The remaining mice from the human

cyclin B1 and PBS groups, as well untreated, age-matched mice, were challenged with $1x10^6$ LO2 cells subcutaneously. Tumor growth and survival were monitored.

3.2.13 DNA Priming/Protein Boost Vaccination and Tumor Measurements

C57Bl/6 mice were shaved on the abdomen with a No. 40 clipper 24 hours prior to treatments. Experimental groups were immunized with pcDNA3.1 vector alone or the vector carrying either mouse or human cyclin B1 cDNA. The cDNA (4μg) was coated on 1-3μm gold particles (Bio-Rad, Hercules, CA) and fired into the shaved abdominal skin using a helium-powered gene gun. Three weeks later, mice were boosted with protein in the presence of heat-labile enterotoxin (LT) via an immunostimulatory (IS) patch (IOMAI Corporation). Briefly, 25μg/100μl/mouse of human or mouse cyclin B1 was injected subcutaneously, with PBS injections used as controls. In order to prevent grooming during the immunization procedure, mice were anesthetized intramuscularly with 100mg/kg ketamine (Phoenix Scientific Inc., St. Joseph, MO) mixed with 11mg/kg xylazine (Phoenix Scientific). The shaved skin was hydrated by rubbing with saline-drenched gauze. Hydrated skin was lightly blotted with dry gauze prior to immunization. The skin was abraded with sandpaper using gentle pressure 10 times in one direction. The area was then blotted with saline-saturated gauze and blotted dry. An immunostimulatory (IS) patch containing 20μg of heat-labile enterotoxin (LT) was applied to the treated skin. Seventeen days after protein boost immunizations in the transplantable tumor experiments, $1x10^6$ LO2 tumor cells were injected subcutaneously. Tumor growth and animal survival were monitored. In the $p53^{-/-}$ mice, the immunization protocol was begun at 6 weeks of age, with 15 mice in each group. Mice were monitored for survival, the development visible tumors more than 2cm in diameter, or distress that met IACUC standards for euthanasia.

3.2.14 Measurement of Mouse Anti-cyclin B1 T Cell Responses

Seventeen days after the last immunization, 3 mice in each group of the transplantable LO2 tumor experiments were sacrificed for T cell studies. IFNγ ELISPOT assay were performed as described previously (219). Briefly, nitrocellulose plates (Millipore, Billerica, MA) were coated with anti-IFNγ capture antibody (BD Biosciences) overnight at 4°C. DC were loaded with mouse cyclin B1 protein for 2-6 hours and mixed with autologous T cells at a DC/T cell ratio of 1:10 for 20 hours at 37°C. The cells were seeded at 10^5 cells/well. All assays were performed in serum-free AIMV medium (Gibco, Invitrogen). The plates were then washed with 0.1% Tween 20 in PBS and stained with anti-IFNγ mAb (BD Pharmingen) for 2 hours at 37°C. The plates were washed, and either an avidin-peroxidase complex or an alkaline phosphatase-labeled avidin D antibody (Vector Laboratories) was added to the plates for 1 hour. The plates were then developed using either AEC substrate (Sigma) or BCIP/NBT solution (KPL, Inc., Gaithersburg, MD), and spots were quantified microscopically with an inverted phase-contrast microscope (Carl Zeiss, Inc.) along with a computer-assisted image analysis system (Immumoassay). Anti-CD4 or anti-CD8 blocking antibodies (BD Biosciences) were added to the cultures for blocking experiments.

3.2.15 Measurement of Mouse Anti-cyclin B1 Antibody Responses

In the transplantable tumor model experiments, sera were drawn 2 weeks after the last immunization. Anti-cyclin B1 antibody titers were measured by ELISA. Briefly, cyclin B1 specific IgG levels were determined in sera with the use of 96-well ELISA plates (Immulon-2HB, Dynex Laboratories) coated overnight with 0.1μg/well cyclin B1. Plates were blocked with

0.5% casein/Tween–20 for 2 hours and washed. Samples were serially diluted (two fold) on ELISA plates and incubated overnight at 4°C. IgG was detected with horseradish peroxidase conjugated goat anti-mouse IgG (Bio-Rad) and 2'2-azino-bis (3-ethylbenzthiazoline sulfonic acid) substrate (ABTS, KPL, Inc.). The enzyme reaction was stopped using 1% sodium dodycyl sulfate. Antibody titers are reported as ELISA units, which correspond to the inverse dilution of the serum that yielded an OD_{405} of 1.0. For the $p53^{-/-}$ experiments, sera were drawn 3 weeks after final immunization and tested for anti-cyclin B1 IgG by ELISA. ELISA for the $p53^{-/-}$ experiments was conducted as in the human assays described above, with the following exceptions: wells were coated with 0.6μg mouse cyclin B1; the secondary was horseradish peroxidase-congugated anti-mouse IgG (Sigma); and the substrate (TMB, BD Biosciences) was incubated for 30 minutes before reading at 450nm.

3.3 RESULTS

3.3.1 Healthy Individuals Have Anti-Cyclin B1 Humoral Immune Responses.

We collected blood from individuals with no history of cancer in order to determine the prevalence and intensity range of anti-cyclin B1 antibodies. Considering that there is a wide range of antibody titers in cancer patients, from very low to very high, we wanted to find out if this range would be recapitulated in the healthy population as well, or if we would find instead individuals with antibody and those without antibody. Blood was drawn from individuals ages 20-75, and plasma was collected and stored at -80˚C. Plasma was diluted 1:400 and tested by ELISA for the presence of anti-cyclin B1 antibody. Results were normalized between assays

through the use of standard samples that were run on each plate, each day. As shown in Figure 1A, many healthy individuals had detectable levels of cyclin B1-specific IgG, and the levels ranged from very low to very high. Since cyclin B1 is overexpressed in cancer and the risk of neoplastic events is thought to increase with age, we sought to determine whether the prevalence or levels of anti-cyclin B1 IgG increased with age as well. We found no correlation between age and antibody levels in an adult population (Figure 1B). In addition, the anti-cyclin IgG was predominantly IgG1 and IgG3 (data not shown), which have been suggested to be indicators of Th1-mediated immune responses (47, 220).

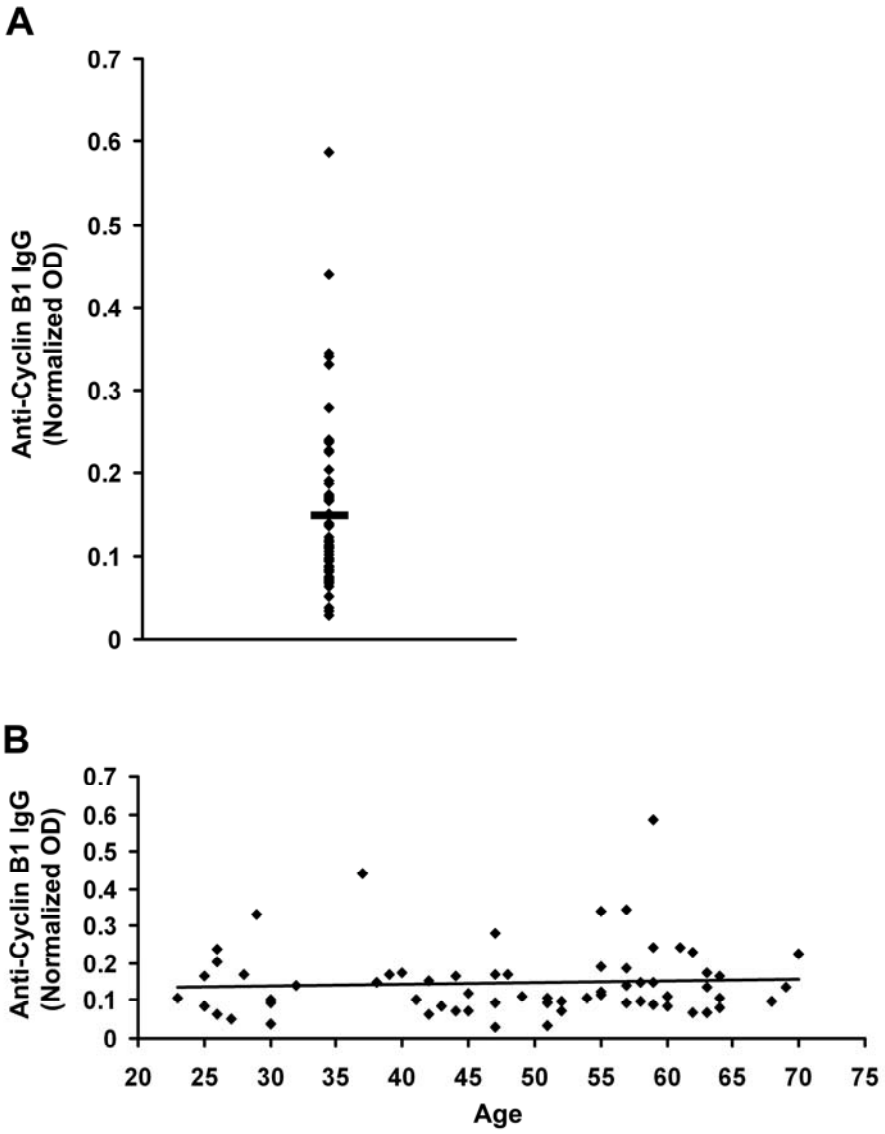

Figure 3-1 Healthy individuals have anti-cyclin B1 IgG.

Plasma samples from healthy donors (n = 65) with no history of cancer were tested for anti-cyclin B1 IgG by ELISA at a dilution of 1:400. (A) Anti-cyclin B1 IgG from all individuals was normalized to 5 standard controls. Bar indicates mean OD. (B) Levels of anti-cyclin B1 IgG are independent of age (Pearson correlation, $p = 0.63$). A.) Plasma from healthy donors without a known history of cancer was tested for anti-cyclin B1 IgG by ELISA at a dilution of 1:400. B.) Levels of IgG are independent of age (Pearson correlation, $p = 0.63$).

3.3.2 Healthy individuals Have Cyclin B1-Specific T Cells.

Because IgG is a T cell-dependent isotype, it can be expected, even though it is not always the case, that individuals positive for anti-cyclin B1 IgG would also have cyclin B1 specific T cells. We previously observed T cell responses in cancer patients with cyclin B1 overexpressing tumors (194). Therefore, we sought to determine whether cyclin B1-specific T cells exist in healthy individuals as well. The T cell studies were performed using PBMC obtained from blood bank donors. Plastic adherence was used to separate monocytes and grow dendritic cells (5 days in IL-4 and GM-CSF), and the remaining lymphocytes were frozen for use in subsequent studies. DC were loaded for 4 hours with cyclin B1, ovalbumin (OVA), or no protein, the later two serving as negative controls. Loaded DC were then cultured for 2 days in the presence of TNFα, IL-6, and PGE_2 to promote their maturation, before they were co-cultured with autologous PBL or purified $CD8^+$ T cells for subsequent assays. ELISA assessment of the culture supernatants showed that total PBL from a blood bank donor produced IFNγ in response to cyclin B1 but not to OVA (Figure 2A). This response was dependent on T cell receptor signaling, since receptor blockade with antibodies that prevent signaling through MHC class I and CD4 reduced the IFNγ to control levels. These data are representative of responses we saw in similar experiments performed on PBL from three other blood bank donors (data not shown). PBL from the same buffy coat were labeled with CFSE, and proliferation was assessed by flow cytometry on day 7 of culture. As is shown in Figure 2B, the $CD4^+$ T cells proliferated significantly more in response to cyclin B1-loaded DC than to DC that were unloaded or loaded with OVA. We repeated the same culture conditions with purified $CD8^+$ T cells. After 10 days, the $CD8^+$ T cells cultured with cyclin B1-loaded DC produced significantly more IFNγ than the control cultures (Figure 2C).

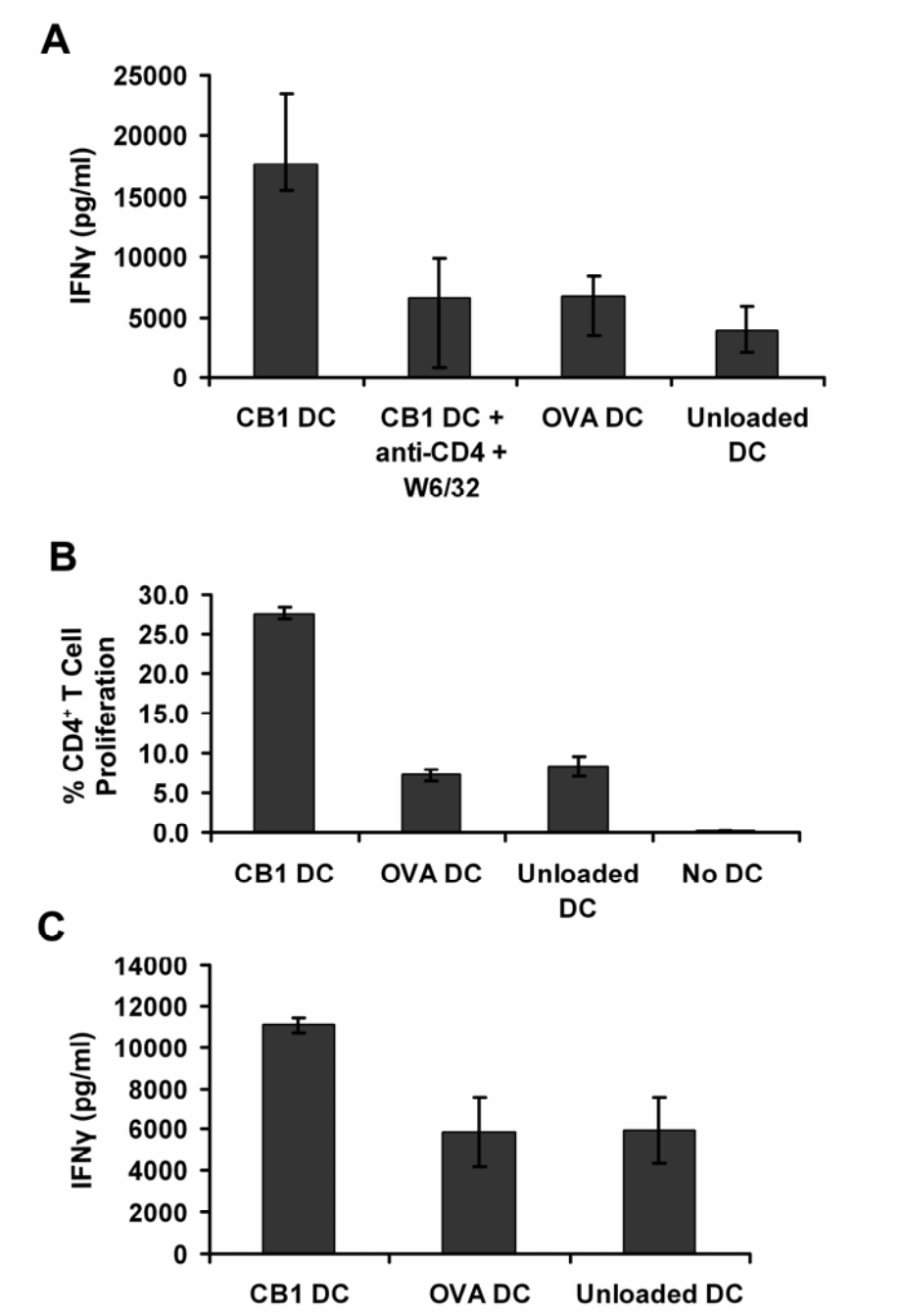

Figure 3-2 Healthy Individuals have T cells specific for cyclin B1.

(A) T cells: Monocyte-depleted PBMC were cultured with autologous DC that were loaded with ovalbumin (OVA), cyclin B1 (CB1) or unloaded. Supernatant from the 7th day of culture was tested by ELISA for IFNγ. W6/32: MHC class I blocking antibody. (B) CD4^{+} T cells: PBMC from the same donor as in (A) were labeled with CFSE and cultured with autologous DC in the presence or absence of indicated antigen or without DC. Percent of proliferating CD4^{+} cells was assessed by flow cytometry after 7 days of culture. C.) CD8^{+} T cells: CD8^{+} T cells were purified from PBMC (a second donor) and cultured with autologous DC with and without indicated antigens. Supernatants were tested after 10 days of culture for IFNγ by ELISA. Bars indicate standard deviation.

3.3.3 Cyclin B1-Specific T cells in Healthy Individuals Are Antigen Experienced.

Since the T cell responses we observed were after a week or more of culture, which could theoretically be sufficient to prime naïve cells, we performed kinetic studies for detection of cyclin B1-specific T cells that were previously primed *in vivo*. Naïve cells do not exhibit effector function within the first days after exposure to antigen, but antigen experienced (memory) cells do. PBL from the blood bank donor in Figures 2A and 2B were cultured with cyclin B1-loaded, OVA-loaded and control (unloaded) DC for assessment of IFNγ production during the first, second, and third day of culture. Brefeldin A (BFA) was added to one set of the triplicate co-culture for 11-hours during the first day. Intracellular IFNγ accumulated during this 11 hour incubation is indicated as the 24-hr time period. (Figure 3). The second and third set of the triplicates were then incubated with BFA for 11 hours during the second and third day, indicated as the 48 and 72 hour times periods, respectively. In the first 24 hours, both CD4^{+} and CD8^{+} T cells produced more IFNγ than controls (Figure 3). This effect was magnified over the 48 and 72 hour periods.

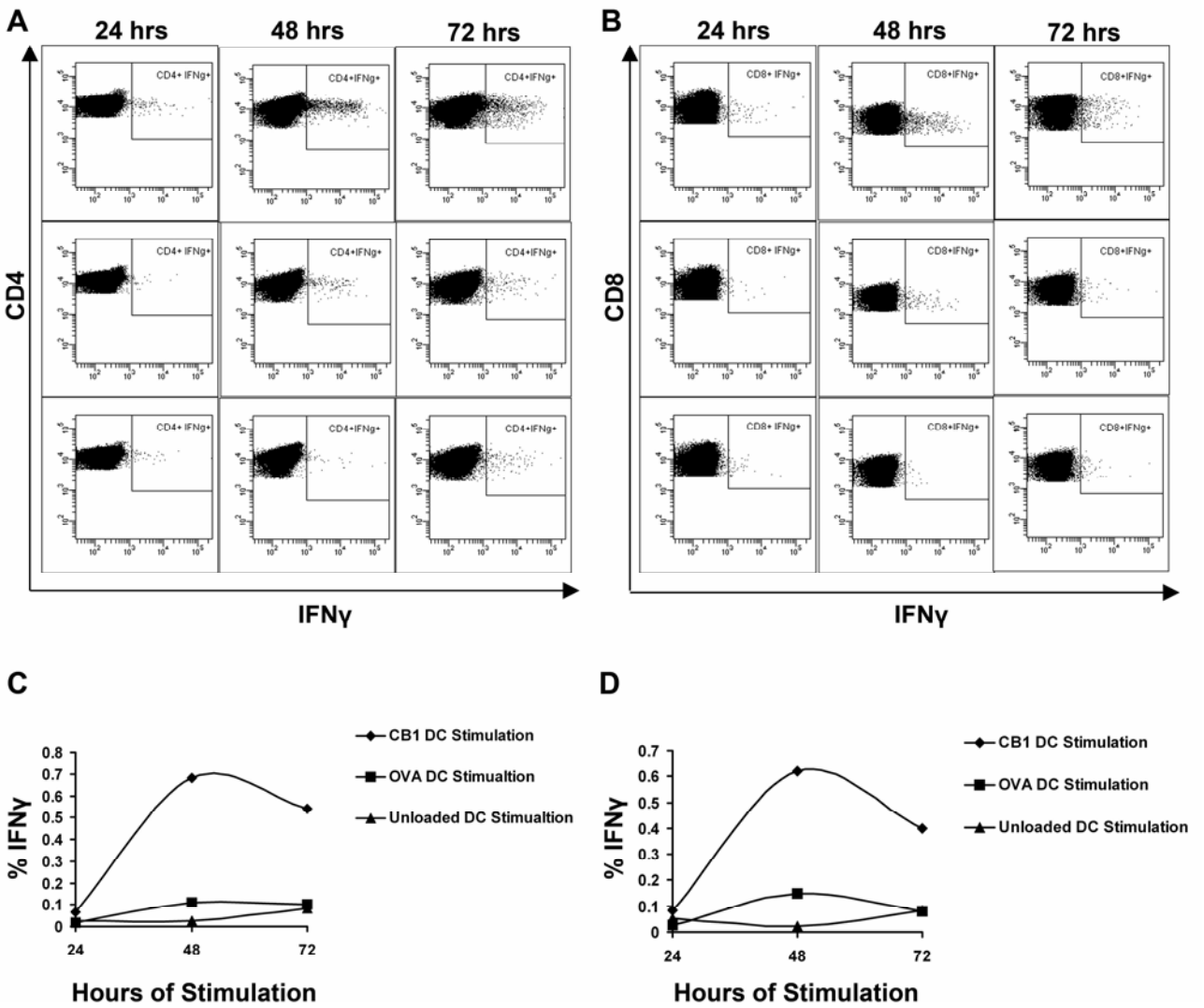

Figure 3-3 Cyclin B1-specific T cells from healthy individuals function as memory cells.

PBMC were processed to obtain DC and peripheral blood lymphocytes. Brefeldin A (BFA) was added for 11 hours to one set of a triplicate culture at 6, 30, and 54 hours after combination of DC with PBL. After incubation with BFA, $CD4^+$ T cells (A and C) and $CD8^+$ T cells (B and D) were assessed for intracellular IFNγ. Flow plots for each culture condition are shown in A and C. Top row, PBL stimulated with cyclin B1-loaded DC; middle row, PBL stimulated with OVA-loaded DC; bottom row, PBL stimulated with unloaded DC. B and D show a graphical representation of the percentage of IFNγ-positive cells for $CD4^+$ (B) and $CD8^+$ (D) T cells.

3.3.4 Cyclin B1 Amino Acids #215-233 Contain Frequently Recognized T Cell Epitopes.

Cyclin B1 is a relatively large protein of 54 kD and can be processed into numerous class I and class II restricted peptides that could serve as T cell epitopes. We were interested in knowing if there were certain domains in the protein that were more likely to be recognized by T cells than others. This could be important in selecting a repertoire of peptides to elicit or boost T cell responses in patients. We developed an assay that uses a cyclin B1 peptide library composed of peptides 12-15 amino acids in length, a size that has been previously shown to elicit both $CD4^+$ and $CD8^+$ T cell responses (221, 222), overlapped by approximately 11 amino acids and spanning the whole cyclin B1 protein sequence. We first tested pools of peptides and then individual peptides from pools that induced T cell proliferation. Blood donors in these studies were individuals enrolled in a lung cancer screening study who were confirmed to be free of lung cancer by CT scan. PBMC were isolated and labeled with CFSE. Cells were then plated in 96-well plates to which were added peptide pools or individual peptides. Negative control wells contained no peptides and positive control wells were coated with anti-CD3 antibody. Cells were cultured in media containing anti-CD28 and anti-CD49d antibodies to promote co-stimulation and better activation. Six days after stimulation, cells were tested by flow cytometry for CFSE dilution. Repeated experiments with different peptide pools and individual peptides from these pools identified 3 peptides that spanned amino acids #215-223 and repeatedly stimulated T cells from multiple blood donors. As shown in Figure 4, all individuals tested have either $CD4^+$ or $CD8^+$ T cells that are specific for one or more of these three peptides.

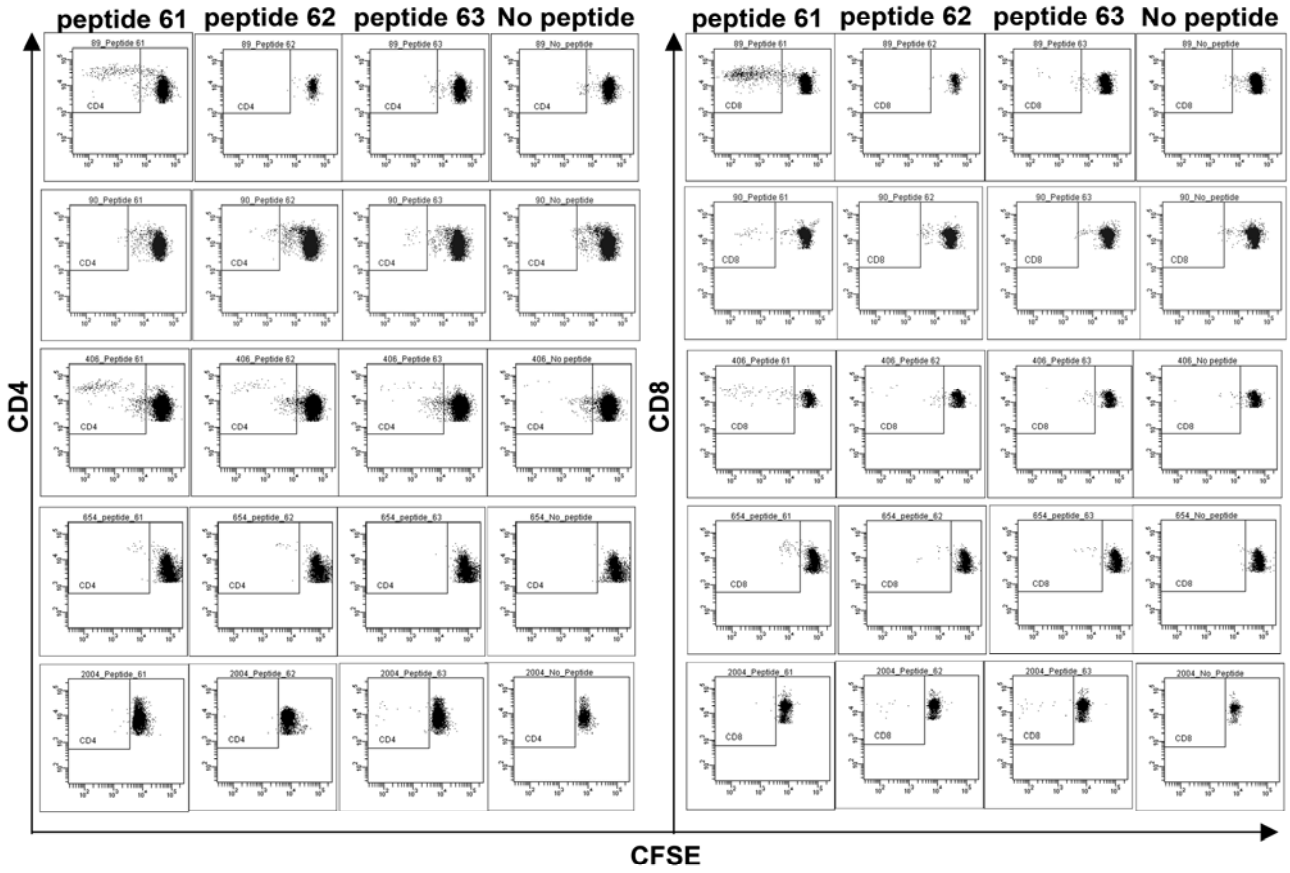

Figure 3-4 Identification of cyclin B1 peptides, targets of spontaneously arising immunity.

PBMC from 5 heavy smokers who are negative for lung cancer by computed tomography (CT) scan were labeled with CFSE and stimulated with 2μg/ml recombinant cyclin B1 peptides. Peptides 61, 62, and 63 overlap to span an 18 amino acid region from amino acids 215-233 of the cyclin B1 protein. After 6 days of culture, PBMC were stained with cell surface markers and proliferation was assessed by flow cytometry. peptide 61: KFRLLQETMYMTVSI; peptide 62: LQETMYMTVSIIDRF; peptide 63: MYMTVSIIDRFM.

3.3.5 Spontaneous Tumors in p53 Deficient Mice Overexpress Cyclin B1.

Given that healthy individuals have both humoral and cellular immune responses that are specific for the self and tumor antigen cyclin B1, we explored the potential significance of anti-cyclin B1 immune responses that exist prior to the onset of cancer. Our first approach was to use a transplantable tumor model with a lymphoma cell line (LO2) that was derived from a $p53^{-/-}$ mouse. We previously published that cyclin B1 overexpression characterizes tumors with non-functional p53 (185). This suggested that tumors arising in $p53^{-/-}$ mice would spontaneously overexpress cyclin B1. Figure 5 shows immunohistochemical staining of cyclin B1 overexpression in a spontaneous mouse lymphoma versus normal cells in a $p53^{-/-}$ mouse and tissues of WT mice for comparison. Cells in the primary tumor arising in the $p53^{-/-}$ thymus (Fig 5 A) show uniformly increased expression of cyclin B1 with some cells having very high expression. Tumor cells metastasizing to the spleen (Figure 5C) and other organs (not shown) are high expressers of cyclin B1, suggesting that the cells in the primary tumor with the highest level of cyclin B1 expression may be those that preferentially metastasize to the periphery. It is important to point out that normal spleen cells in the $p53^{-/-}$ thymus and spleen do not overexpress cyclin B1 (WT thymus (Figure 5B) and WT spleen (Figure 5D) are used for comparison) even though they all lack p53, and thus overexpressed cyclin B1 is a bona fide tumor antigen.

The LO2 cell line was established from one such tumor, and clones with highest cyclin B1 expression were selected and propagated for tumor rejection studies.

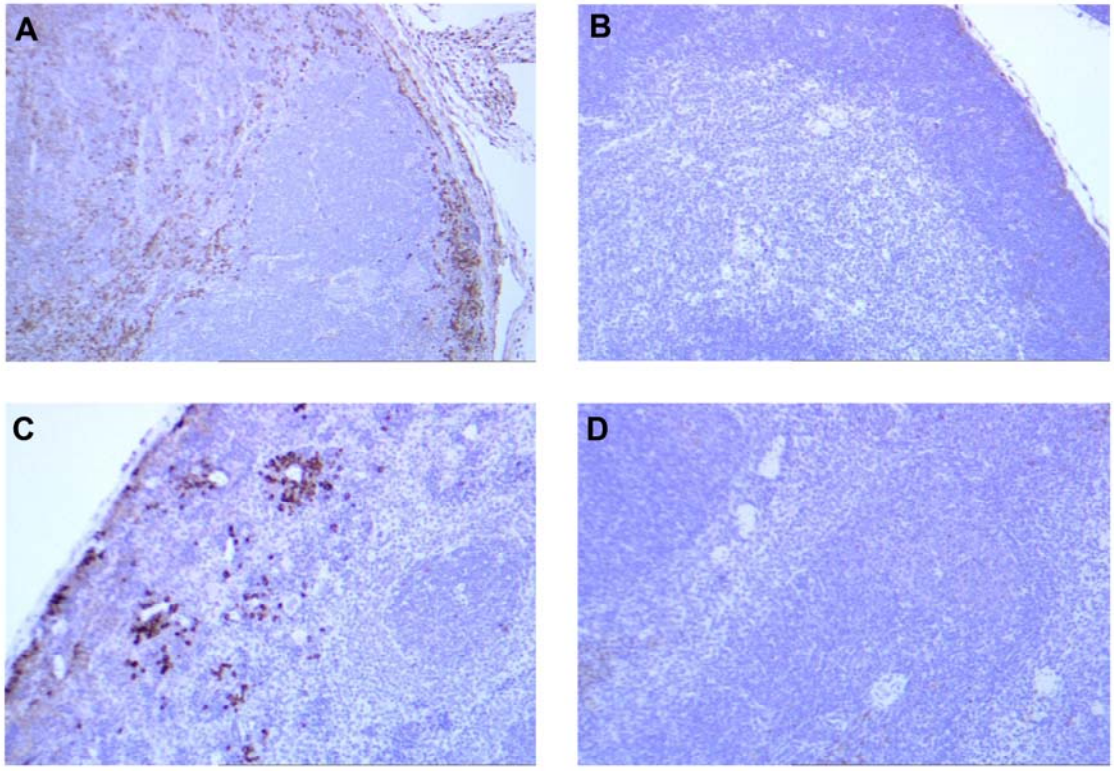

Figure 3-5 Spontaneous tumors that develop in p53$^{-/-}$ mice overexpress cyclin B1.

Thymus (B) and spleen (D) from a wild type mouse do not overexpress cyclin B1. In a p53$^{-/-}$ mouse, the primary tumor (thymoma (A)) and metastases to the spleen (C) exhibit cyclin B1 overexpression. Non-tumor cells in both the thymus and spleen of the p53$^{-/-}$ mouse are all negative for cyclin B1 overexpression.

3.3.6 Vaccines Based on Either Mouse or Human Cyclin B1 Induce Humoral and Cellular Immune Responses in Mice

Because overexpressed cyclin B1 in tumors is neither mutated nor altered postranslationally, we expected that immune responses in healthy mice would be subject to self-tolerance and therefore

difficult to elicit. For that reason we chose to test as immunogens both mouse (self) and human (highly homologous but could be considered non-self) cyclin B1 in two different forms, recombinant protein and DNA. As adjuvant, we chose transdermal delivery at the site of antigen injection of heat labile enterotoxin (LT) via an immunostimulatory (IS) patch (223, 224). Figure 6 shows that both mouse and human cyclin B1 are immunogenic in mice and generate cross-reactive immunity. In Figure 6A, we show that immunization with the mouse cyclin B1 protein elicits T cells specific for both mouse and human cyclin B1. In Figure 6B, we show that immunization with human cyclin B1 protein elicits high titer antibodies reacting against both human (right panel) and mouse (left panel) cyclin B1. Figure 6C shows that all the cyclin B1-specific T cell reactivity can be blocked with anti-CD4 antibody. When these mice were challenged with LO2 tumor subcutaneously, neither vaccine was able to inhibit tumor growth. Thus, antibody and $CD4^+$ T cell responses alone, which was the response induced with the recombinant protein immunization, were clearly not the desired tumor rejection response.

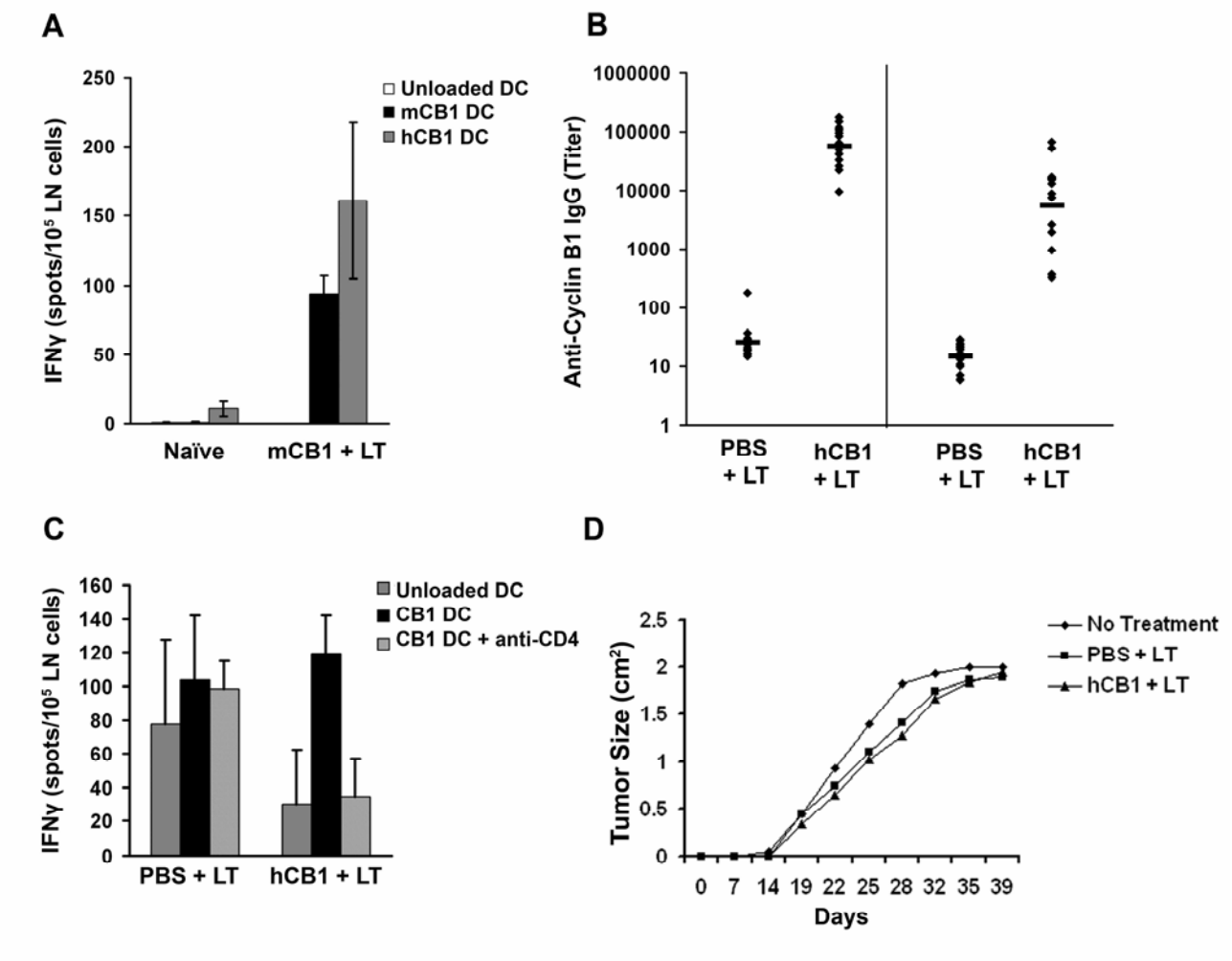

Figure 3-6 Cyclin B1 immunity elicited by immunization with recombinant cyclin B1 protein.

C57Bl/6 mice were immunized subcutaneously with either mouse (A, mCB1) or human (B,C,D, hCB1) recombinant cyclin B1 protein and boosted with the protein + LT/IS patch twice in 3 week intervals. Mice immunized with PBS (A, B, C, D) or not treated (D) were used as controls. Error bars indicate standard deviation. (A) Mice immunized with mouse cyclin B1 protein generate IFNγ producing T cells specific for both mouse and human cyclin B1. (B) Both anti-human (left) and anti-mouse (right) cyclin B1 antibody responses were elicited by human cyclin B1 protein immunization. Bars indicate geometric mean. (C) Cyclin B1-specific T cell responses were elicited by priming and boosting with human cyclin B1 protein and the LT/IS patch. The response can be blocked with anti-CD4 antibody. LT patch alone induced non-specific T cell activation. (D) In comparison to untreated mice, neither LT alone nor human cyclin B1 protein + LT was able to prevent or delay tumor growth.

In contrast, when we used the DNA prime, protein boost approach, we were able to induce effective anti-tumor immunity. Fifteen mice per group were immunized with 4μg of pcDNA3.1 control vector (group 1) and vectors encoding either mouse cyclin B1 (group 2) or human cyclin B1 (group 3). Three weeks later, mice were boosted with 25 μg of human cyclin B1 protein followed by the application of an immunostimulatory (IS) patch containing 20μg of heat-labile enterotoxin (LT). The boost was repeated in another three weeks. Mice without any vaccination (no treatment) were used as no treatment controls. Mice primed with pcDNA3.1 vector and boosted with LT/IS patch only were used as adjuvant controls (vector + adjuvant). Sera were collected for antibody detection. Seventeen days after the last immunization, three mice per group were sacrificed for assessment of *in vitro* T cell responses, and the rest of the mice were challenged with LO2 cells. Figure 7A shows that the DNA prime/ protein boost vaccination regimen induces cyclin B1-specific T cell responses, and the induced responses can only partially be blocked by anti-CD4 antibody (groups 2 and 3). The same results were obtained by boosting with mouse cyclin B1 protein (data not shown). These results implied successful priming of $CD8^+$ T cells. We confirmed that cyclin B1 specific T cell responses can also be blocked by anti-CD8 antibody in mouse cyclin B1 protein boosting experiment (data not shown).

Cyclin B1 DNA prime protein boost vaccination also successfully elicited both anti-human and anti-mouse cyclin B1 antibody responses (Figure 7B). While the DNA-only or protein-only vaccines could elicit immune responses but not protection against tumor challenge (data not shown and Figure 6), the combined DNA/protein boost vaccination approach significantly delayed tumor growth. By the 25^{th} day after tumor challenge, groups that received DNA vaccines with protein boosts had significantly lower mean tumor volume and significantly more tumor-free mice than the control and the protein-only group (Figure 7C). By the 42^{nd} day

after tumor challenge, all mice in the control groups were sacrificed due to excessive tumor burden, while 2 mice in group 1, 6 mice in group 2 and 5 mice in group 3 remained tumor free (data not shown). Figure 7D shows that at 70 days after tumor challenge, 58% of the mice survived in the mouse cyclin B1 DNA vaccine primed group (group 2), 42% in the human cyclin B1 DNA vaccine primed group (group 3), and only 16% in the control vector primed group (group 1). 100% mice that were not vaccinated with cyclin B1 vaccines died (group no treatment and vector + adjuvant). Importantly, it appears that self-tolerance was not a factor, as the mouse cyclin B1 DNA vaccine protected equally or even slightly better than the human cyclin B1 DNA vaccine. Similar results were obtained when the mouse cyclin B1 DNA vaccine was boosted with mouse cyclin B1 protein (data not shown).

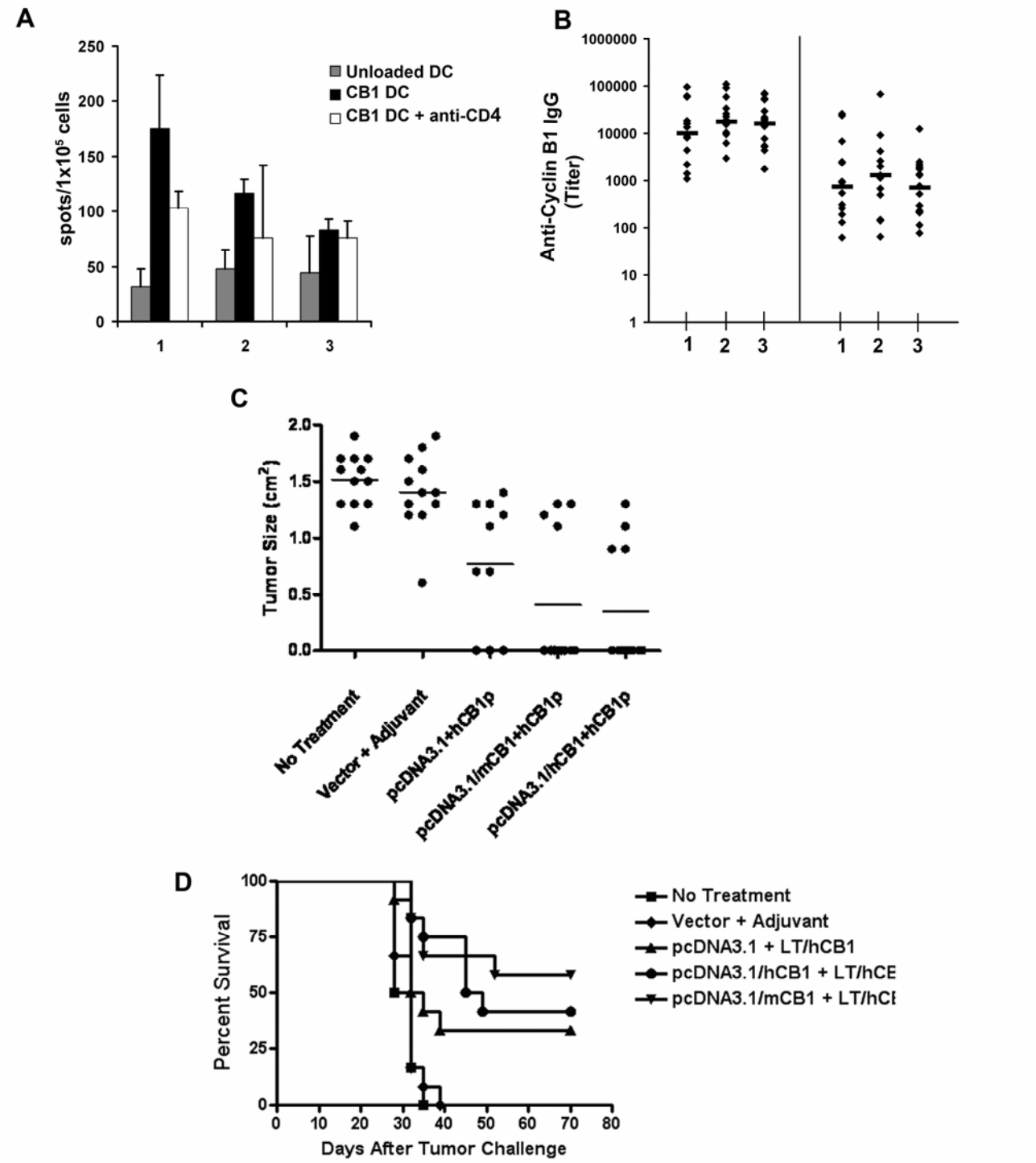

Figure 3-7 Cyclin B1 DNA prime/protein boost vaccination elicited cyclin B1-specific cellular and humoral responses and delayed tumor growth.

Mice were primed with DNA vaccine encoding either pcDNA 3.1 empty vector (group 1), mouse cyclin B1 (mCB1, group 2), or human cyclin B1 (hCB1, group 3) cDNA. All three groups were boosted with human cyclin B1 recombinant protein and LT/ IS patch two times in 3 week intervals. Mice without any vaccination were used as no treatment controls. Mice primed with pcDNA3.1 vector alone and treated with LT/IS patches were used as the adjuvant control. Spleens were isolated and stimulated with cyclin B1 loaded and unloaded DC for ELISPOT assessment of IFNγ release. Error bars indicate standard deviation. (A) DNA prime/protein boost vaccination elicited cyclin B1 (CB1) specific T cell responses (B) Vaccination successfully elicited both anti-human (left) and anti-mouse (right) cyclin B1 antibody responses. Bars indicate geometric mean. (C) On day 25 after tumor challenge, mice without cyclin B1 immunization all grew tumors (no treatment and adjuvant + vector groups), while 2 of 10 mice in group 1 were tumor free. In contrast, 8 of 12 mice in groups 2 and 3 were tumor free. Bars indicate mean tumor size. (D) At 70 days after tumor challenge, survival for each group was 58% (group 2), 42% (group 3), and 16% (group 1). In contrast, all mice in the no treatment and adjuvant + vector control groups were sacrificed due to tumor burden.

3.3.7 Cyclin B1 DNA Prime/Protein Boost Vaccine Delays Spontaneous Tumor Growth and Prolongs Overall Survival in $p53^{-/-}$ Mice.

In order to approximate better the development of human cancer and the effects of a pre-existing anti-cyclin B1 immune response, we moved from a transplantable to a spontaneous tumor model, the $p53^{-/-}$ mouse. We initiated vaccination of $p53^{-/-}$ mice at 6 weeks of age prior to any evidence of tumor growth. The mice were divided in three groups receiving the following treatments: mouse cyclin B1 DNA vaccine with a mouse cyclin B1 protein boost in the presence of an LT/IS patch; empty-vector DNA control vaccine followed by the LT/IS patch; and no treatment. Serum was collected 3 weeks after immunization to test for anti-cyclin B1 IgG (Figure 8A). Mice were monitored for survival and/or tumor development and eliminated from the survival curve after death, or after the mice met IACUC standards for euthanasia. Figure 8A shows that

vaccinated mice developed a strong IgG response, which indirectly also indicates that a specific helper T cell response was elicited. The results we presented earlier show that this vaccine protocol elicits CD8 responses as well. The immune response induced by this cyclin B1 vaccine is capable of controlling tumor development and prolonging overall survival in this highly susceptible mouse spontaneous tumor model (Figure 8B).

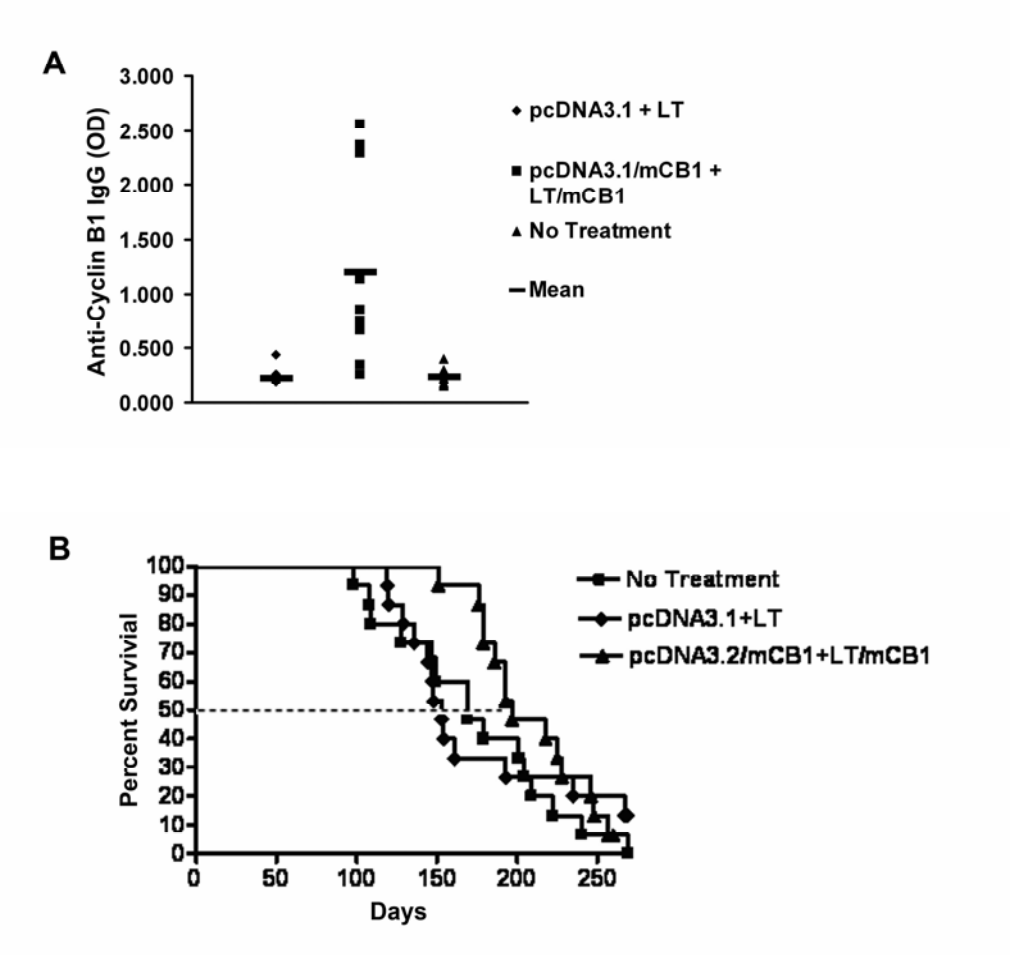

Figure 3-8 Cyclin B1 DNA vaccine at 4-6 weeks of age elicits an anti-cyclin B1 immune response and delays spontaneous tumor growth in p53$^{-/-}$ mice.

p53$^{-/-}$ mice were vaccinated with pcDNA3.1 with mouse cyclin B1 (mCB1) cDNA and boosted with the LT/IS patch + mCB1 protein twice at 3 week intervals. Control mice received empty DNA vector and the LT/IS patch or no treatment. (A) DNA prime/protein boost vaccination elicits anti-cyclin B1 IgG. Bars indicate mean

OD. (B) Cyclin B1 vaccination retarded spontaneous tumor growth. The dotted line indicates the time point at which 50% of the mice in each group died.

3.4 DISCUSSION

In this study, we demonstrate that healthy individuals have immune responses against the self-protein cyclin B1 that was previously found to be abnormally expressed in tumor cells and thus considered a tumor associated antigen. This immune response consists of both antibodies and T cells. Various levels of cyclin B1-specific IgG are present in most healthy individuals, independent of age. Furthermore, healthy individuals have cyclin B1 specific memory $CD4^+$ and $CD8^+$ T cells. Ours is the first study that directly addressed the possibility that healthy individuals can have strong T cell memory and antibody responses specific for ubiquitous self-proteins, many of which are known tumor associated antigens, with no apparent adverse autoimmune consequences.

There are several other tumor associated antigens that are also constitutively expressed by normal, adult tissues accessible to the immune system. A partial list includes the mucin glycoprotein MUC1, expressed on normal ductal epithelial cells and overexpressed in hypoglycosylated form on tumor cells (23); wild-type p53, expressed by normal cells undergoing genetic stress or apoptosis and also constitutively overexpressed by tumor cells (225); normal melanocyte differentiation antigens overexpressed in melanoma cells (75); carcinoembryonic antigen (CEA), found in low levels in healthy colon (19) but overexpressed in tumor cells; and cyclin B1, the subject of our studies. The fact that these molecules are seen by the immune

system as tumor antigens by virtue of their overexpression, constitutive expression, mislocalized expression, or all of the above—rather than by random mutations— makes them completely shared between individuals and therefore practical targets for immunotherapy. Among these tumor antigens, cyclin B1 is notable for the wide array of tissues in which it is both normally- and, in cancer, over-expressed. In this way, cyclin B1 is a tumor antigen that is not only shared between individuals but also shared between cancers of many different tissue origins.

Humoral and cellular immune responses to these self-molecules/tumor antigens have been studied in cancer patients for evidence of their role in cancer immunosurveillance and disease outcome. Antibodies specific for p53 have been shown to correlate with a worse prognosis in breast, lung, and colon cancers (49), while MUC1-specific antibodies are associated with a better prognosis in breast, pancreatic, lung, and ovarian cancer (51-54). In addition, studies of people at high risk for development of cancer due to chronic lung diseases and environmental lung exposures have demonstrated that anti-p53 antibodies can predate and predict the development of cancer (89-91). We are currently investigating the importance of anti-cyclin B1 antibodies in the prognosis of lung cancer (Vella, *et al*., in preparation) as well as in the prediction of lung cancer development in a high risk population (226) and Egloff *et al.* in preparation). Similarly, the presence of T cells in tumors has been associated with better prognosis in lung, ovarian, and colon cancer (58, 227, 228). The specificity of these T cells was not determined, nor is it known if they were generated in response to the growth of the tumor or preexisted as memory cells, such as we find for cyclin B1 in healthy people.

While ours was the first study to focus on preexisting (pre-tumor) responses to tumor associated antigens, careful analysis of published papers dealing with these responses in cancer patients reveals that similar observations were made by others but not given proper importance.

T cells specific for some tumor associated antigens could be expanded in vitro from healthy individuals such as for melanoma-associated chondroitin sulfate proteoglycan (94), cytochrome p450 1B1 (95), survivin (96, 97), and tyrosinase related protein 1 (98). Additionally, four studies have identified T cells in healthy individuals without the need for *in vitro* expansion. These T cells were specific for Melan-A/Mart-1 ($CD8^+$, naïve), Her2/neu ($CD8^+$, effector), CEA ($CD4^+$, naïve/ignorant or suppressed) and wild type p53 ($CD8^+$, mostly $CD45RA^+$) (99, 100, 229, 230). These and similar studies have focused primarily on the difference in responses to these molecules between healthy individuals and cancer patients or on the fact that self-specific T cells exist in the periphery for use in vaccination. They did not question how and why these responses existed in healthy people. If this is discussed at all, it is in the context of tumor immunosurveillance with the assumption that these responses might be the result of cleared neoplastic events. However, the fact that we do not see increases in anti-cyclin B1 antibody levels as an adult population ages (Figure 3-1B, (226), and data not shown), despite a higher likelihood of accumulated neoplastic events, suggests that these immune responses are unrelated to neoplastic events. Instead, we propose that abnormal expression (overexpression, constitutive expression, mislocalization, abnormal posttranslational modification) of self-molecules can be a result of non-neoplastic events as well, such as infections and acute and chronic inflammation. We have previously published that several non-neoplastic events known to affect MUC1-expressing tissues correlate with anti-MUC1 antibody in healthy individuals, including mumps virus infection of MUC1+ salivary glands, and mastitis affecting MUC1+ breast ducts (88).

Perhaps the most compelling evidence for a non-neoplastic stimulus for self/tumor antigen specific antibodies was demonstrated in a mouse model of viral infection (231). Mice infected with either vaccinia virus or lymphocytic choriomeningitis virus and then screened by

SEREX for changes in their antibody profiles, were found to have generated antibodies against several viral antigens but also against many host cell proteins involved in cell cycle progression and cell adhesion, some of which had previously been characterized as tumor-associated antigens. This study is of particular relevance to cyclin B1 data we report here as it suggests the possibility that a viral infection may lead to abnormal expression of cyclin B1 and generation of anti-cyclin B1 immunity. Two recent reports lend further support to this hypothesis by showing that infection of human fibroblasts with varicella zoster virus (VZV) (232) and human cytomegalovirus (HCMV) (233) induces overexpression of cyclin B1 in the cytoplasm that strongly resembles its abnormal expression in cancer cells. The extension of our hypothesis would propose that anti-cyclin B1 immune memory developed in the context of certain viral infections could serve as effective immunosurveillance against cyclin B1 overexpressing tumors.

Data we report from our mouse experiments confirm that preexisting immune responses against cyclin B1 can protect against the growth of both transplantable and spontaneous cyclin B1-overepxressing tumors. The mouse experiments also demonstrated that, while all vaccines elicited anti-cyclin B1 immune responses, only the DNA prime/protein boost vaccination strategy was able to provide significant protection. Since DNA vaccines can transfect antigen presenting cells as well as the myocytes and keratinocytes at the injection site, they are able to elicit immune responses via endogenous and exogenous antigen presentation pathways (234). Therefore, we attribute the success of the DNA prime, protein boost vaccine to the priming of cyclin B1-specific $CD8^+$ T cells as well as the humoral and $CD4^+$ T cell responses elicited with the protein-only vaccine. As an extension into our human studies, it is thus likely that immune responses against cyclin B1 vary among individuals in their anti-tumor protective potential and

thus in spite of high prevalence of antibodies and T cells that we observed, some people can nevertheless develop cyclin B1 overexpressing tumors.

Inasmuch as our studies show that anti-cyclin B1 immunity is safe and can be protective against tumors, cyclin B1 based vaccines may be a useful strategy to boost preexisting anti-cyclin B1 immunity in individuals who are at high risk for developing cyclin B1+ tumors. The presence of memory responses against self-antigens also known to be tumor associated antigens has been considered a case of broken self-tolerance. This has created an expectation that in order to elicit effective anti-tumor immunity, cancer vaccines must be able to break self-tolerance (235, 236). While there is some evidence from clinical trials of cancer vaccines or other forms of immunotherapy that effective anti-tumor immunity sometimes correlates with autoimmunity (237, 238), most of the time immune responses against tumor associated antigens do not recognize normal cells. We would like to propose that this is because the immune system likely maintains self-tolerance against the normal expression of these molecules and only responds to changes in their expression brought about by infections or malignant transformation.

Immunotherapy based on these antigens in the setting of early disease or for cancer prevention has been slow in developing because of the concern that it might elicit autoimmunity. However, if immunotherapy is directed against abnormal expression and abnormal forms of these antigens, which appear to be detected as foreign by the immune system, the chances of inducing autoimmunity are greatly diminished. The success and safety seen in mouse studies of preventative vaccinations based on these antigens might then be considered safe enough to be expanded into the clinical setting, where they would be expected to induce or boost already existing immunity against abnormal self molecules expressed on tumor cells.

4.0 A NEW PARADIGM IN CANCER IMMUNOSURVEILLANCE

4.1 INTRODUCTION

While studies of cancer immunosurveillance in humans focus on anti-tumor immune response in cancer patients, we and others have found that these immune responses can exist in healthy individuals as well (52, 81, 84-86, 88, 99, 100). Our studies on the tumor antigen cyclin B1 have demonstrated that healthy individuals can have memory $CD4^+$ and $CD8^+$ T cells specific for the self protein. We also demonstrated that T cell dependent antibody (IgG) specific for cyclin B1 exists in the sera of healthy individuals. We initially assumed that these immune responses represented immunologic memory of previous neoplastic events. However, the fact that anti-cyclin B1 IgG was not correlated with age in healthy individuals or the level of cyclin B1 overexpression in cancer patients led us to search for non-neoplastic events that could induce anti-tumor antigen immune responses. Two studies using human foreskin fibroblasts and infection with varicella zoster virus (232) and human cytomegalovirus (233) demonstrated that infection with these viruses induced overexpression of cyclin B1 indistinguishable from what is seen in cancer cells. We therefore hypothesized that viral infections could cause aberrant expression of self proteins, such as cyclin B1, that resemble the aberrant expression that occurs in a cancer cell. The extension of this hypothesis is that viral infection could therefore train the immune system to recognize aberrant expression of self molecules on tumor cells and result in

protection against the development of cancer. To test this hypothesis, we infected mice with ectromelia virus, a mouse orthopox virus that causes acute infection (239). We then waited 60 days and challenged ectromelia-exposed and unexposed mice with transplantable tumor and observed that ectromelia-exposed mice survived significantly longer than controls.

4.2 MATERIALS AND METHODS

4.2.1 Mice, MVA, and Ectromelia

C57BL/6J mice were purchased from Harlan Winkelmann. IFN-I-R–deficient (A129) mice were originally obtained from Michel Aguet (University of Zurich, Zurich, Switzerland) and backcrossed to C57BL/6 mice for 8 generations. The MVA used for this study was MVA-BN, developed by Bavarian Nordic (European Collection of Cell Cultures, V00083008). MVA was propagated and titered on primary chicken embryo fibroblasts (CEFs) that were prepared from 11-day-old embryonated pathogen-free hen eggs (Charles River Laboratories) and cultured in RPMI-1640 medium supplemented with 10% FCS. All viruses used in animal experiments were purified twice through a sucrose cushion. In order to UV-inactivate the MVA, concentrated MVA stocks were UV irradiated with a UV Chamber (Genelinker GS; Bio-Rad) for 15 minutes under sterilizing conditions.

4.2.2 Vaccinations and Ectromelia Infections

For C57Bl/6 mouse experiments, mice were separated into two groups and treated with either UV-inactivated MVA (UV-MVA) (n= 10) or MVA (n = 20), at a dose of $1x10^8$ $TCID_{50}$. Six days later, the UV-MVA control group received the $1x10^4$ $TCID_{50}$ of UV-MVA, while the experimental group received 10^4 pfu ectromelia virus. IFN-I-$R^{-/-}$ mice were separated into three treatment groups. The first group was left untreated (n = 6); the second received $1x10^8$ $TCID_{50}$ MVA followed by a same-day administration of $1x10^3$ pfu ectromelia (n = 5); and the third group was vaccinated with $1x10^8$ $TCID_{50}$ MVA and $1x10^4$ pfu ectromelia. Subcutaneous injections were performed in the inguinal region by applying a total of 1×10^8 $TCID_{50}$ of MVA by injecting 2 times a volume of 250 µl each. Mice were anesthetized with ketamine/xylamine, and viruses were applied by intranasal dropwise installation in a total volume of 50 µl..

4.2.3 Tumor Challenge

Sixty days after the last vaccination or virus infection, mice were challenged with $1x10^6$ LO2 cells (a lymphoma cell line derived from a $p53^{-/-}$ C57Bl/6 mouse that overexpresses cyclin B1). Cells were injected in 100µl volumes into the flank of the mice. Tumors were monitored for growth, and mice were sacrificed when tumors exceeded animal care restrictions.

4.2.4 Generation of Dendritic Cells

Bone marrow was removed from the femurs and tibia of female, C57Bl/6 mice into complete RPMI (cRPMI). After passage through a 70µm cell strainer, cells were spun down and treated

with red blood cell lysis buffer (Sigma) and washed 3 times in cRPMI. Cells were resuspended at a concentration of 10^4 cells/ml in cRPMI and cultured in T75 flaks with 50ng/ml GM-CSF (Technogene) and 10g/ml IL-4 (R&D). Cytokines were refreshed on days 2 and 5. On day 6, cell scrapers (Nunc, Rochester, NY) were used to remove adherent cells from the flasks. All cells were collected for DC isolation using a $CD11c^+$ magnetic bead isolation kit (Miltenyi), following the manufacturers protocol. Isolated cells were then split into 3, 50ml conicals (Falcon) for protein loading. Both cyclin B1 and OVA proteins were treated for LPS removal (Pierce Detoxi-Gel). Briefly, the detoxifying resin was washed in sodium deoxycholic acid, according to the manufacturers protocol and the agarose beads were then combined with the proteins in equal volume. After 1 hour of incubation, the beads were spun down by centrifugation and the protein supernatant was removed for use in loading. Proteins were added to DC at a concentration of 30μg/ml and incubated for 3.5 hours at 37°C. Without removing proteins, DC were then matured with 5μg/ml Poly (I:C) (Alexis) for 2 days.

4.2.5 Serum ELISA

Blood was collected 3 and 6 weeks after last challenge with either Ectromelia or control vaccination. Serum was collected after density centrifugation for use in ELISAs. Wells of 96-well ELISA plates (Thermo, Milford, MA) were each coated with 0.6μg recombinant human cyclin B1 protein (IOMAI, Gaithersburg, MD) in 50μl PBS. Plates were sealed overnight at 4˚C and washed 5 times with PBS before use. Cyclin B1-coated wells and empty, background wells were then coated with blocking buffer (2.5% BSA in PBS) for 1 hour. Plasma samples were diluted in blocking buffer in 96-well polypropylene plates (Nunc, ThermoFisher). A multichannel pipette was then used to transfer 50μl of each diluted sample to the ELISA plates.

Samples were allowed to incubate for 1 hr and were subsequently washed 5 times with 1% PBS-Tween. HRP-tagged anti-mouse IgG (Sigma) was diluted in blocking buffer and incubated on the plates for 1 hour. Plates were then washed as before and incubated with TMB substrate (BD Biosciences) for 30 minutes in the dark. 2N H_2SO_4 was added to stop the reaction and plates were read immediately at 450nm. Background was subtracted to obtain final OD.

4.2.6 T cell Assays

Subcutaneous lymph nodes were excised pressed through a cell strainer before counting and plating at a density of $4.25x10^5$ cells/200µl/well in a 96-well plate. For blocking experiments, lymph node cells were combined with antibodies specific for CD4 (GK1.5, BD biosciences) and CD8 (53-6.7, BD Biosciences) at a concentration of 5µg/ml. Cyclin B1-loaded, OVA-loaded, and Unloaded DC were washed and added to wells for a DC: lymph node cell ratio of 1:20. Splenic cells were also collected from mice and passed through a cell strainer. After treatment with red blood cell lysis buffer, cells were counted and $1x10^6$ cells were plated in cRPMI in a 48 well plate. Blocking antibodies and DC were added as for lymph nodes, with a DC:T cell ratio of 1:69. To compensate for the low numbers of DC available, 10µg/ml cyclin B1 and OVA were added to wells with cyclin B1- and OVA-loaded DC, respectively. Supernatants were collected on day 4, and media was replaced, at which point the antibodies were added again to maintain T cell blocking. Supernatants were drawn again on day 6. Supernatants were assessed for IFNγ production by ELISA, following the manufacturers protocol (BD OptEIA).

4.3 RESULTS

4.3.1 Ectromelia Infection Protects from Tumor Challenge

In order to determine whether a viral infection can train the immune system to recognize aberrant self molecules on cancer cells, we tested whether infection could protect against the growth of a transplantable tumor. We used ectromelia virus, a mouse orthopox virus that serves as a model for human variola virus (smallpox). Not only are the genomes of variola and ectromelia similar, but mice are the natural host to ectromelia as humans are to variola, and mice develop symptoms that are similar to the human disease (239). In order to test the effects of systemic viral infection without losing mice to the disease, prior to the administration of the virus we vaccinated with modified vaccinia ankara (MVA), an attenuated vaccinia strain that exhibits limited replication ability in mammals (240). High dose MVA vaccination has been shown to protect the more susceptible Balb/c mice from severe illness (241). We used MVA-BN, a highly attenuated strain that was generated from Modified Vaccinia Ankara strain 571.

To test whether infection protects against tumor growth, 20 C57Bl/6 mice were vaccinated with $1x10^8$ $TCID_{50}$ MVA and infected with $1x10^4$pfu ectromelia 6 days later. As controls, 10 mice were treated with UV-inactivated MVA (UV-MVA) at both time points. These control mice were therefore exposed to the immune stimulus of viral molecules but not exposed to infection by either ectromelia or the attenuated MVA. 60 days after the acute ectromelia infection or UV-MVA vaccination, mice were challenged with $1x10^6$ LO2 cells, a mouse lymphoma that was developed in a C57Bl/6 $p53^{-/-}$ mouse and demonstrated to overexpress cyclin B1.

Two weeks after the challenge, mice were monitored for tumor growth and survival. As can be seen in Figure 4-1, mice infected with ectromelia survived longer than those receiving noninfectious viral exposure ($p < 0.0001$).

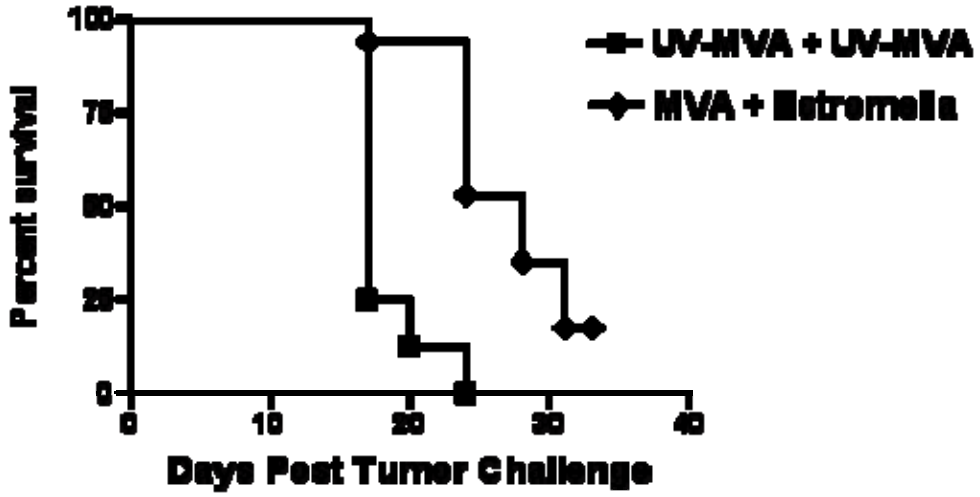

Figure 4-1 Ectromelia infection confers protection against tumor challenge

Mice were vaccinated with MVA and subsequently infected with ectromelia (n = 20) or treated with UV-inactivated MVA alone (n = 10). 60 days after the infection or UV-MVA treatment, mice were challenged with the $p53^{-/-}$ mouse lymphoma cell line LO2 and monitored for survival. Mice were removed from the survival curves once tumor volume reached 2 mm^3. Mice infected with MVA and ectromelia survived significantly longer than mice treated with UV-MVA ($p<0.0001$).

Similar studies were performed on interferon I receptor deficient (IFN-1-$R^{-/-}$) mice, which are more susceptible to viral infection since they cannot respond to IFNα. These mice have been used to test the ability of MVA to protect immunocompromised mice from ectromelia challenge (242). MVA treatment of IFN-1-$R^{-/-}$ mice can be performed on the same day of ectromelia infection and still provide protection from lethal disease (242). MVA-treated IFN-1-$R^{-/-}$ mice were infected with $1x10^3$ (n= 5) and $1x10^4$ (n = 4) pfu of ectromelia while one group

was left untreated (n = 6). Three months after ectromelia infections, mice were challenged with $1x10^6$ LO2 cells and monitored for tumor size. Figure 4-2 demonstrates a trend towards a dose response observed 9 days after tumor challenge. Higher virus exposure led to lower mean tumor volumes.

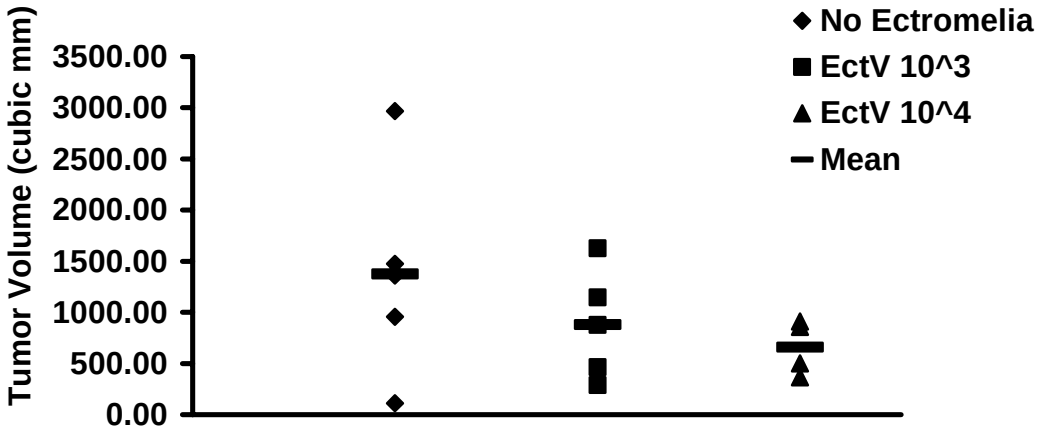

Figure 4-2 Infection of IFN-1-R$^{-/-}$ mice protects from tumor challenge in a dose-dependent fashion

Mice were either left untreated or vaccinated with $1x10^8$ $TCID_{50}$ MVA on the same day of ectromelia infection with $1x10^3$ or $1x10^4$ pfu of ectromelia. LO2 challenge was performed 90 days after infection, and tumor size was measured 9 days after the LO2 challenge.

4.3.2 Ectromelia Infection Did Not Induce a Significant Anti-cyclin B1 Antibody Response

Our preliminary studies demonstrated that mice vaccinated with MVA and infected with ectromelia but not mice vaccinated with MVA alone developed anti-cyclin B1 IgG that was

readily detectable 7 days after ectromelia infection. However, when the larger groups of mice were tested for antibody 3 and 6 weeks after infection, significant differences were not reproducibly detectable (data not shown). Three weeks post-infection was previously shown to be the peak time point of orthopox-virus induced antibody responses in mice (231).

4.3.3 Cyclin B1-Specific T Cells Are Found in the Lymph Nodes, but Not the Spleen, of All Mice, Regardless of Ectromelia Infection

Three mice from each of the UV-MVA and MVA/Ectromelia groups were sacrificed on the day of LO2 challenge in order to obtain spleens and lymph nodes for T cell assays. Spleens and lymph nodes were also obtained from non-age-matched, untreated mice. Cells from each organ were combined with $CD11c^+$ bone-marrow derived DC that were loaded with cyclin B1 or DC that were loaded with OVA or no protein, as controls. Blocking experiments were performed on cyclin B1-stimulated cultures in order to demonstrate that T cells were specifically recognizing presented cyclin B1 through the T cell receptor. Supernatants were removed 4 and 6 days after the start of the co-culture and tested for secreted IFNγ by ELISA. As can be seen in Figure 4-3, cyclin B1 specific T cells could be detected in lymph nodes from mice of all groups. In contrast, cyclin B1 specific T cells were not detectable in the spleens of any group (Figure 4-4); instead, the splenic T cells of the UV-MVA and MVA + Ectromelia groups had higher background levels of activation than the untreated mice.

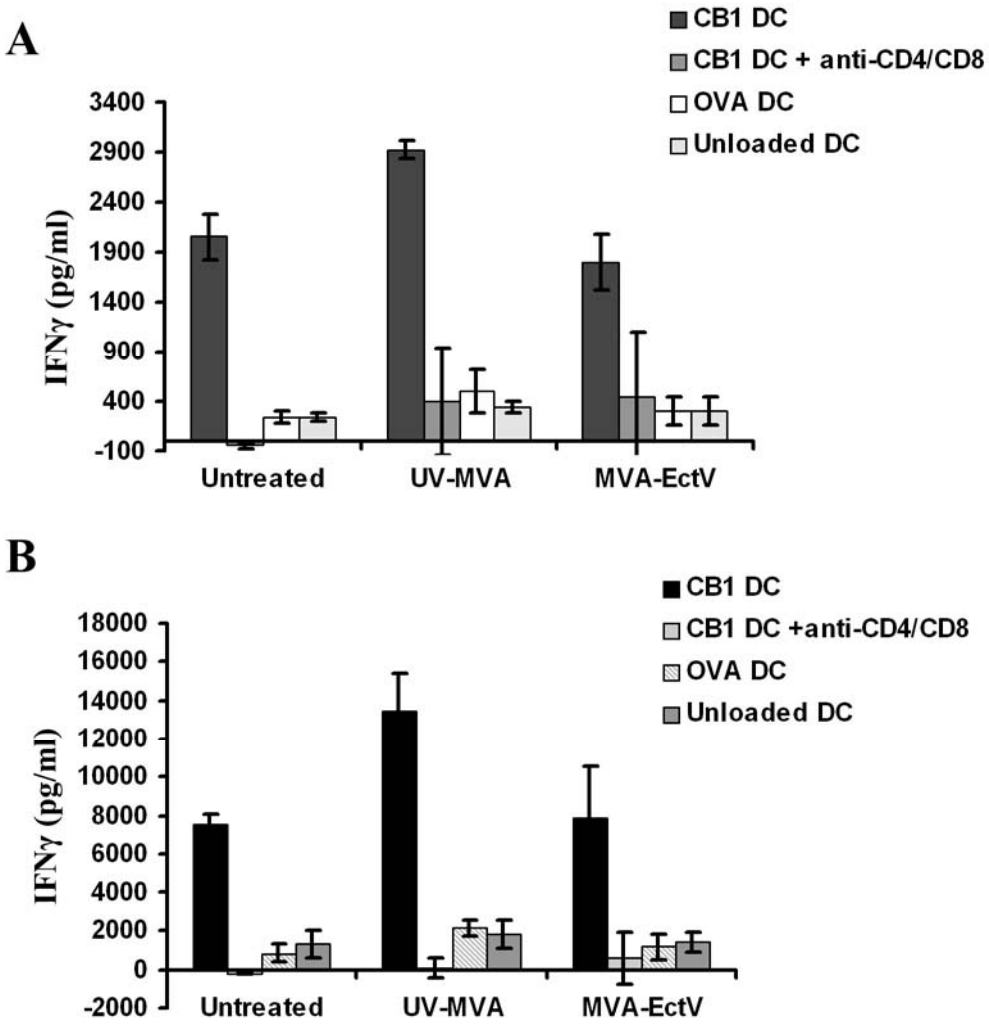

Figure 4-3 Cyclin B1-specific T cells were found in the lymph nodes of all mice

Lymph nodes from 2 untreated, UV-MVA treated, and MVA + Ectromelia treated mice were harvested 60 days after the final vaccination or virus treatment. Lymph nodes from the non age-matched untreated mice were harvested on the same day. Cells were combined with CB1-loaded, OVA-loaded, or Un-loaded DC. Culture supernatants were harvested on days 3 (A) and 6 (B) and tested for IFNγ. Error bars represent differences between the 2 mice for each group.

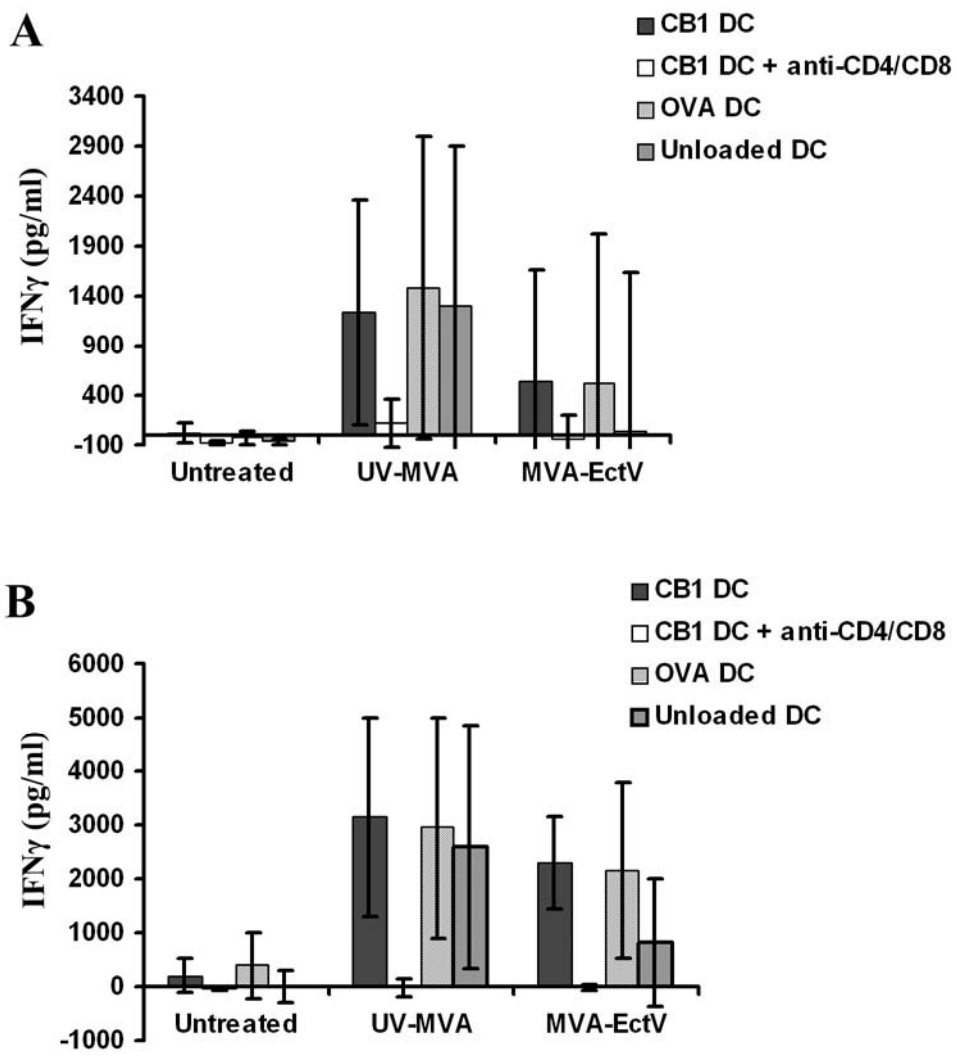

Figure 4-4 Cyclin B1-specific T cells were found in the lymph nodes of all mice

Spleens from untreated (n = 3), UV-MVA treated n = 2), and MVA + Ectromelia treated (n = 3) mice were harvested 60 days after the final vaccination or virus treatment. Spleens from the non age-matched untreated mice were harvested on the same day. Processed ells were combined with CB1-loaded, OVA-loaded, or Un-loaded DC. Culture supernatants were harvested on days 3 (A) and 6 (B) and tested for IFNγ.

4.4 DISCUSSION

In this preliminary study, we demonstrate that infection with modified vaccinia ankara (MVA) and ectromelia virus can increase overall survival in a model of transplantable tumor growth. The fact that the tumor challenge took place 60 days after the start of the acute infection is strong evidence that the anti-tumor effects were mediated through the induction of specific, adaptive effector T cells and generation of memory cells, and not through a general state of innate immune stimulation that results from triggering toll like receptors and other pattern recognition molecules. This is further supported by the fact that UV-MVA and MVA + Ectromelia infected mice demonstrated the same level of background T cell activation in the spleen (Figure 4-4) but displayed significant differences in their ability to control tumor growth. Interestingly, the control mice had immune responses to cyclin B1 in the absence of any manipulation. Specifically, Figure 4-3 demonstrates that even untreated mice have immune responses that are specific for cyclin B1, while Figure 3-5 demonstrates that this is not the case for all C57Bl/6 mice. This may be a feature of the facility in which these mice are kept (the mice in Figure 4-3 are housed in a facility where viral infections are routinely performed).While the survival study did not control for the possibility that the highly attenuated MVA-BN could be responsible for some of the protection, the importance of the ectromelia infection was reinforced by the dose response noted in the INF-I-$R^{-/-}$ experiment, where infection with $1x10^4$ pfu of ectromelia resulted in a smaller mean tumor volume than infection with $1x10^3$ pfu (with the same MVA dose).

The idea that immune responses to infection can help destroy cancer is not novel, and it has been observed with the use of Coley's toxins (a combination of endotoxins, exotoxins, and enzymes from *Serratia marcescens* and *Streptoccocus pyogenes*) in soft-tissue cancers (243) and

the attenuated *Mycobacterium bovis* (BCG) in bladder cancer (244). In these settings, the exposure to immune stimuli and subsequent release of inflammatory and pyrogenic cytokines and chemokines stimulate innate and/or pre-existing adaptive immune responses to destroy immunogenic tumors (244, 245). However, these immune stimuli do not specifically expand previously naïve anti-tumor T cells, and like IFNα and IL-2 in melanoma, they simply increase the activity of pre-existing anti-tumor innate and adaptive responses. Our study uncouples the non-specific immune stimulus from the cancer protection, since the acute ectromelia infection precedes the tumor challenge by 60 days and does not produce higher non-specific levels of T cell responsiveness or IFN-γ secretion than the control group that received non-infectious viral proteins (Figures 4-3 and 4-4).

To our knowledge we are the first to propose that pre-cancer viral infections induce aberrant expression of self proteins in the context of viral adjuvants and therefore lead to the generation of specific, anti-self/tumor antigen immune memory that can subsequently protect against the development of cancer. Evidence that viral infection can induce aberrant expression of self has been shown *in vitro* when infection of human forseskin fibroblasts with VZV (232) and HCMV (233) induced overexpression of tumor antigen cyclin B1. In addition, virus-induced overexpression of a tumor antigen has also been shown *in vivo*, as 23% of patients with chronic hepatitis C infection without hepatocellular carcinoma (HCC) were shown to have elevated expression (serum levels) of the HCC tumor antigen alpha fetoprotein (AFP) (246). However, the example of hepatitis C also highlights the fact that some viral infections cause cancer rather than protect from it.

Not only have viral infections been shown to induce aberrant expression of self molecules, but there is also evidence that infections can reduce the risk of melanoma. In a case-

control (retrospective) study from the European Organization for Research and Treatment of Cancer, analysis of 603 melanoma patients and 627 population controls demonstrated that severe infections (those inducing fever greater than 38.5˚C) correlated with a significantly lower melanoma risk later in life (247). The majority of identified infections were bacterial. In addition, a second study on the same populations demonstrated that the risk of melanoma was also reduced in individuals who received the live vaccines for tuberculosis (BCG) and smallpox (vaccinia virus) (248). Interestingly, the effects of these separate infections (BCG, vaccinia, and severe childhood infections) were not cumulative; individuals who received repeated vaccinations or vaccinations and childhood severe illnesses were not less likely to develop melanoma than those who were exposed to vaccination or severe infection alone. As such, the authors suggested that vaccination and severe infections worked to protect from melanoma through the same mechanism, which we propose to be the expression of aberrant self. The idea that live vaccines can provide protection (although not specifically noted as protection from cancer) was also demonstrated by a study that surveyed 1,893 adults from Guinea-Bissau for the presence of a vaccinia scar and followed them for 4 years, from 1998 to 2002 (249). Rates of all-cause mortality (excluding accidents and violence) demonstrated that those who received vaccinia vaccination had a decreased likelihood of death.

Perhaps the most notable support for the idea that this virus-induced protection from cancer is antigen specific was published by Ludewig and Sahin, *et al*. (231). The authors infected mice with vaccinia virus (VV, a cytopathic virus) and lymphocytic choriomeningitis virus (LCMV, non cytopathic). Mice were bled during the peak production of antibodies and tested for autoreactivity using the serological identification of antigens by recombinant expression cloning (SEREX) method. The target antigens for the SEREX screen were expressed

from cDNA libraries that were generated either from a mixture of uninfected mouse organ tissues (lung, spleen, and liver) or the lungs of VV-infected mice. The authors noted that virus infection, especially with the cytopathic VV, induced high titers of autoantibody. Interestingly, 22 of the 36 antigens recognized by antibodies in VV-infected mice and 7 of 14 antigens recognized in LCMV-infected mice were orthologues of human antigens that had been previously identified to be the targets of human anti-tumor antibodies. This supports our hypothesis that virus infection can induce immune responses that are not only anti-self but also anti-tumor.

Interestingly, the authors noted that the antigens recognized by LCMV-infected and VV-infected mice had a limited overlap (231); therefore, unlike the studies of human melanoma risk (248), these data indicate that infection with different organisms would widen the breadth of anti-tumor immune responses. Also of note was the difference in the number of non-viral antigens recognized between the SEREX libraries prepared from the uninfected organs and those prepared from infected organs. Mice infected with VV recognized 8 self antigens from the uninfected tissues and 14 self antigens from VV-infected lung. Although the authors did not explicitly state the conclusion, we interpret this data to mean that virus infection induced aberrant expression of self proteins that were not present in the uninfected tissues. The authors concluded that many human antigens identified by SEREX may actually be "afterglows of infection-associated immunopathology," rather than responses that were generated in response to cancer.

The concept that viruses can induce autoimmune T and B cells is also not novel. The connection between virus infection and autoimmunity has been heavily researched (250). Several models for viral induced autoimmunity have been proposed, including viral proteins that

are cross-reactive with self (molecular mimicry) and bystander activation—a situation in which autoreactive cells are nonspecifically activated either directly by viral TLR ligands or indirectly by viral maturation of antigen presenting cells that picked up self antigen from infected cells (251). Whereas the bystander hypothesis would suggest that different viruses would elicit the same autoimmune profile, the SEREX studies by Ludewig and Sahin, *et al*. provide evidence to the contrary, since LCMV and VV infections induced different autoantibody profiles.

Despite the attempts to identify viruses that cause autoimmunity, no smoking gun has been found (251). This may be because no single viral infection is responsible for this anti-self, anti-tumor immune response. Instead, immune responses to self molecules aberrantly expressed in the setting of viral infections may be similar for many different viruses; this, in turn, may be a mechanism of anti-viral protection evolved by the immune system. In order to protect from as-of-yet unseen viruses when the new antigens cannot be immediately recognized by adaptive, memory immune responses, the immune system instead learns to recognize the aberrant expression of self molecules. In this way, viral infections not only train the immune system to recognize cells that aberrantly express self molecules when infected with subsequent, unrelated viruses, but the viruses also train the immune system to recognize cells that aberrantly express self molecules when altered by carcinogenesis.

5.0 SUMMARY

The studies presented here demonstrate that the self protein cyclin B1, initially defined as a tumor antigen due to evidence of cyclin B1-specific immunity in patients with cyclin B1 overexpressing tumors, is also recognized by the immune system of healthy individuals, leading to cellular and humoral immune memory. These data demonstrate that cyclin-B1 specific $CD4^+$ and $CD8^+$ T cells are neither centrally nor peripherally deleted. In addition, the fact that the T cells responded rapidly with the production of IFNγ indicates that these T cells were not rendered anergic upon their initial encounter of cyclin B1, as would be expected to occur under normal, non-stimulatory circumstances in the absence of costimulation. This suggests that cyclin B1-specific T cells encountered cyclin B1 peptides *in vivo* in the context of co-stimulation and, judging by their cytokine profile, under Th1-polarizing conditions. This is also reflected in the predominance of IgG1 and IgG3 isotypes of anti-cyclin B1 antibodies that are driven by Th1 type helper T cells.

The fact that healthy individuals can have robust anti-cyclin B1 humoral and cellular immune responses without overt autoimmune disease has several implications. First, it demonstrates that tolerance to cyclin B1 does not need to be broken in order to boost immunity for the prevention and therapy of cyclin B1-positive cancer. Second, it indicates that T cells specific for cyclin B1 do not encounter sufficient presentation of their cognate peptides on healthy tissues to cause disease. As a result, it is possible that tumor antigens such as cyclin B1,

for which tolerance has already been 'broken,' may be the safest vaccine candidates. Third, it suggests that there may be many tumors that abnormally express self antigens to which an immune response has already been mounted due to other events occurring prior to the tumor.

The other studies presented here explore the significance of anti-cyclin B1 immune responses. Chapter 2 discusses the prognostic significance of anti-cyclin B1 IgG in NSCLC patients—as a biomarker of a T cell response—after surgical resection of the tumor. We found that anti-cyclin B1 IgG predicts a longer overall survival in stage IB lung cancer. These results become more interesting in light of the immune responses observed in healthy individuals. Specifically, we proposed that TAA-specific immune responses would have a better chance of destroying cancer cells, or keeping them in check, once the bulk of the immunosuppressive tumor is removed. Conversely, it could be argued that the immune responses generated against cancer were formed by that immunosuppressive environment and that they may therefore be permanently handicapped, regardless of tumor resection. However, our data in chapter 3 suggest that the immune responses were formed in the absence of cancer and in an immunostimualtory, Th1-polarizing environment. As a result, immune responses specific for cyclin B1 may be rescued by removal of the tumor and/or vaccination to boost the preexisting response.

While chapter 2 evaluated the significance of anti-cyclin B1 immune responses after cancer has already formed, chapter 3 addressed their significance in the prevention of cancer. Mice treated with cyclin B1 DNA vaccines followed by cyclin B1 protein boosts survived longer than control mice in both transplantable and spontaneous models of cyclin B1-overexpressing tumors. Furthermore, the elicited cyclin B1 immune responses were not associated with any autoimmune pathology, as has been observed in melanoma vaccination in both humans and mice. This indicates that boosting pre-existing cyclin B1 immunity may safely delay or prevent

cancer in humans. However, a question still remained: why are there pre-existing cyclin B1-specific immune responses in healthy people?

Chapter 4 addressed this question. We proposed that viral infection can induce the aberrant expression of cyclin B1 and other self proteins and therefore stimulate immune responses against abnormal self. The extension of this hypothesis was that viral infections can train the immune system to recognize aberrant self, including the abnormal expression of self proteins that occurs in cancer. While we were unable to demonstrate that ectromelia infection induces cyclin B1-specific immune responses—in part because they were already present in the control mice, and possibly because ectromelia might not induce cyclin B1 overexpression—we show that viral infection protects mice from tumor challenge. This was likely due to immune responses against several aberrantly expressed self antigens during the viral infection that are similarly aberrantly expressed on the tumor cells used in the challenge.

Chapter 4 supports a version of the hygiene hypothesis that can be applied to tumor immunity. The current hygiene hypothesis presents the idea that exposure to bacteria, viruses, and parasites helps set the immune system to predominantly Type 1 immunity thus prevents hypersensitivity-mediated diseases (such as allergy and asthma) that are mediated by Type 2 immunity. The tumor immunologist's hygiene hypothesis follows in suit but focuses on specific adaptive immunity and adaptive immune memory. Viral infections can induce aberrant expression of specific self proteins along with the pro-inflammatory viral molecules. This setting trains the immune system to recognize shared abnormal self-molecule expression patterns in cells that are under stress. As such, viral infections, vaccinations that cause controlled viral infection, or vaccinations that target the molecules that are abnormally expressed may in fact train the immune system to recognize both infection and cancer.

In summary, the immune system of healthy individuals and cancer patients can recognize cyclin B1. This immunologic event correlates with protection from cancer in mice and enhanced overall survival in patients with stage IB lung cancer. However, the pre-existing immune responses in healthy individuals are clearly not enough to prevent the development of cyclin B1-positive cancer in all people, and as such, vaccinations that boost the cyclin B1-specific immune response—either before cancer, as therapy, or in the adjuvant setting after tumor resection—are necessary to reveal the full anti-tumor potential of cyclin B1 immunity. Because cyclin B1 expression in cancer is abnormal, the cyclin B1-specific immune responses elicited by natural processes and vaccines do not see 'self' but rather 'abnormal self.' As such, vaccination against cyclin B1 and other tumor antigens for which tolerance has been naturally broken should not elicit autoimmune pathology.

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