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Deleterious changes to the T cell compartment following immunotherapy

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Cell and Molecular Biology

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Abstract

The combination of anti-CD40 and interleukin-2 is a potent immunotherapy regimen that results in synergistic anti-tumor responses. This has been demonstrated in multiple murine tumor models of metastatic disease with various tumor types. The primary anti-tumor responses elicited by this combination are capable of inducing tumor regression and prolonged survival. However, the generation of secondary T cell responses after irradiated tumor vaccine is abrogated after anti-CD40 and IL-2. This abrogation also occurs after other immunotherapeutic approaches that prompt the production of large amounts of interferon-gamma (IFNγ). These observations correlated with a significant skewing of the T cell compartment. First, we observed a selective decreased of conventional $CD4^+$ T cells following immunotherapy. Second, we observed a more than five fold expansion of memory phenotype cells which were incapable of generating responses to new antigens. The data presented here suggest that despite initial tumor regression, potent systemic immunotherapy may impair responses to new immunological challenges.

Selective $CD4^+$ T cell death after immunotherapy results in an alteration in the ratio of $CD4^+$ T cells to CD8+ T cells and impairs the generation of a secondary immune response. Our data suggest that this phenomenon after immunotherapy is the result of the selective upregulation of programmed death-1 (PD-1) and its IFNγ responsive ligand, B7-H1. We show that the expression of PD-1 is restricted to the surface of $Foxp3^{neg}$ $CD4^+$ T cells and that $CD8^+$ T cells and $CD4^+$

Foxp3+ regulatory T cells remain PD-1 low after immunotherapy. Furthermore, the expression of PD-1 correlates with $CD4^+$ T cell death after immunotherapy. In the absence of IFNγ either by the use of mice lacking IFNγ ($IFN\gamma^{-/-}$) or the receptor for IFNγ ($IFN\gamma R^{-/-}$), B7-H1 remains low after immunotherapy. Subsequently, $CD4^+$ T cells expand in response to immunotherapy in the absence of IFNγ responsive B7-H1.

We observed a significant expansion of memory phenotype T cells after cytokine based immunotherapy which correlated with impairment of proliferative responses to new antigens. Memory T cells are more sensitive to cytokine stimulation than naïve T cells. Therefore, we used a young thymectomized mouse model to determine if pre-existing memory T cells were preferentially expanded by immunotherapy. The thymectomized mouse model allowed us to evaluate long term T cell responses to immunotherapy in the absence of de novo T cell generation. Using this model, we observed expansion of memory T cells, within both the CD4+ and CD8+ T cell compartments without a major sacrifice of the size of the naïve T cell compartment.

Compared to memory T cell expansion, there was relatively small change in the naïve T cell compartment. Naïve CD8+ T cell numbers were unchanged by immunotherapy and naïve CD4+ T cells were decreased by less than half. Memory T cells were still significantly expanded after 30 days of rest. Furthermore, the persistent expansion of memory T cells correlated with a

maintained decrease in proliferative function to new antigens. Taken together, these data demonstrate a long term consequence of immunotherapy to the phenotypic makeup and, importantly, the function of the T cell compartment.

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List of abbreviations

Ag: Antigen

αCD40/anti-CD40: agonist CD40 mAb

APC: Antigen presenting cell

CD: Cluster designation

CTLA-4: Cytotoxic T lymphocyte antigen-4

CD: Dendritic cell

EAE: Experimental allergic encephalomyelitis

HPV: Human papilloma virus

IFN-γ: Interferon gamma

i.p.: intraperitoneal

i.v.: intravenouse

IL: Interleukin

KO: knockout

mAb: monoclonal antibody

MFI: Median fluorescence intensity

MHC: Major histocompatability complex

NK cell: Natural killer cell

NKG2D: Natural Killer Group 2, Member D

PD-1: Programmed death-1

RCC: Renal cell carcinoma

rhIL-2 or IL-2: recombinant human interleukin-2

TAA: Tumor associated antigens

TAM: Tumor associated macrophage

Tconv: $CD4^+$ $Foxp3^{neg}$ conventional T cell

Thmx: Thymectomized

TIL: Tumor infiltrating lymphocyte

TLR: Toll like receptor

Treg: $CD4^+$ $Foxp3^+$ regulatory T cell

Introduction

Augmentation of immune responses holds great promise for the treatment of cancer. A role for the immune system in combating malignant growth was first described in the late 1800's by William Coley who observed an abnormally high incidence of tumor regression in patients who had suffered from acute bacterial infections (1). Coley's report instigated a new field in pre-clinical research which focused on manipulating immune responses to target and destroy tumors. The use of immuntoherapeutics is potentially promising, but there are still tremendous hurdles to be overcome. Below is a discussion of the evidence in favor immunotherapy and the challenges that still remain.

1. The difficulties facing potent and sustained immune responses to cancer

Directing T cell responses to cancer antigens is problematic. It is generally accepted that the weak antigenic property of tumor associated antigens (TAAs) is due to their origination from self tissues or that are intracellular. The lack of a sufficient antigen for immune recognition is true for the majority of tumors, but this is not true for all malignancies. Tumors with strong viral antigen components to which immune responses can occur, i.e. cervical cancer, Burkitt's lymphoma and adult T cell leukemia, among others escape immune control. This escape from immune surveillance demonstrates the extent of immunosuppression that tumors employ to evade immune recognition.

Immune responses to cancer have been classically divided into innate and adaptive arms due to specificity of response. Natural killer (NK) cells are often considered as a rapid defense mechanism against virally infected and neoplastic cells. Unlike T cells, NK cells are not restricted by MHC recognition and are therefore effective against tumor cells which have downregulated MHC class I. The presence of NK cells is limited within solid tissues and their distribution limits their effectiveness against solid tumors. While NK cells are attractive candidates to help reduce metastases, historically it has been believed that their lack of antigen specificity translates to a lack of immunological memory and sustained responses (2).

Adding to the complexity of tumor-immune cell interactions are the immunological suppression mechanisms used by tumors both locally in their tumor microenvironment and/or systemically. Some tumors are capable of inducing the production of Th2 type cytokines that skew the local immune response away from a pro-inflammatory type toward a humoral type which is not harmful to tumor growth (3). Additionally, tumor microenvironments can support inhibitory cell types i.e. regulatory T cells and myeloid suppressor cells (4-6). Tumor associated macrophages (TAMs) are one such example and can inhibit effector cell responses within tumors (7). Tumor cells can also upregulate suppressive or death ligands to reduce the function of migrating, activated effector cells (8-10). Systemically, tumors can promote myeloid suppressor cells that upon interaction with T cells in the lymph node or other peripheral lymphoid organs inhibit the

generation of an anti-tumor response (11). Thus, even if T cell responses to the tumor occur, suppression may obviate protection.

2. Evidence for immune responses to cancer in man

Despite the many suppressive networks employed by tumors, there is an abundance of evidence, both experimental and anecdotal, to suggest the immune system is cognizant of the presence of a tumor. However, the stage at which the tumor is recognized by the immune system is questionable and may depend on the tumor type. Animal studies as well as isolation of human tumors have demonstrated the presence of tumor infiltrating lymphocytes (TILs). TILs are specific for tumor associated antigens and display an activated phenotype (12). Additionally, there is a favorable association between the number of TILs found within a tumor and the prognosis of patients. The relationship between TILs and prognosis has been demonstrated with many different neoplasms including, but not limited to, breast and colorectal cancer (13-17). However, *in vitro* studies have suggested that the effector capabilities of TILs are inexplicably dampened (18, 19). Therefore, the enrichment of TILs found within a tumor and enhancement of TIL would likely result in an effective therapeutic approach.

Dudley et al. have worked to augment the effector function of TILs by *ex vivo* expansion with interleukin-2 (IL-2) before adoptive transfer into melanoma patients (20). Although limited to patients with metastatic melanoma, the observations were initially promising with substantial reduction of tumor burden.

Despite initial responses, this therapy was unable to sustain long term (> 2years) tumor regression (20). The generation of antigen loss variants was not discussed but is one possible explanation for these observations. However, these data likely demonstrated that the presence of specific activated T cells may have been efficient at reducing the tumor burden to near undetectable limits, but concurrent memory was not attained and the tumor eventually escaped immune control.

Historically, there have been compelling examples of immune surveillance to human and mouse tumors. One such example was from two individual kidney transplant patients, each receiving a kidney from a donor after a fatal car accident. Both recipients later developed metastatic melanoma tissue typed to the kidney donor. This recurrence of tumor was despite the donor having been in remission for well over ten years at the time of the accident (21). This observation demonstrated a potential role for immune recognition and control of human tumors.

3. Immune responses to tumors in mouse models

Studies in immunodeficient and transgenic mouse models have clearly demonstrated a direct role of immune effector functions in shaping the immunogenictiy of tumor cells throughout tumor growth. The observation that tumors develop in immunodeficient mice occurred despite being housed in specific pathogen free environments led to a hypothesis of tumor-immune cell interaction called “immunoediting” (22). Tumor immunoediting occurs in three

stages; elimination, equilibrium and escape. During the elimination phase, cells of the innate and adaptive immune system are capable of recognizing neoplastic cells and destroying them. Interferon-gamma (IFNγ) is an important effector cytokine for this process and spontaneous tumors rendered resistant to, or sensitive to IFNγ signaling have been demonstrated to be more or less responsive to immune destruction, respectively. Despite more recent data suggesting a cytostatic role for IFNγ after potent immune stimulation (23), the role for IFNγ in tumor cell elimination was originally thought to be due to direct activation of effector T cells and direct induction of MHC I on tumor cells (22). IFNγ upregulation of MHC I may be a key factor in tumor recognition and elimination as it is instrumental in the recognition of normal cellular components which have become TAAs (22).

During the second phase of cancer immunoediting, tumor equilibrium, tumor cells that survived the elimination phase undergo consistent targeting and destruction by cells of the immune system. This constant targeting of tumor cells by immune effectors can be beneficial or detrimental to tumor development. The equilibrium stage has the potential to last for a long period of time, resulting in seemingly permanent regression, or to select for a less immunogenic variant. IF a less immunogenic variant is selected, the third stage of tumor immunoediting is attained, tumor escape (22). This process demonstrates the potential dichotomy in the actions of immune cell recognition and targeting of tumor cells. It also

demonstrates however, that continuous recognition of new TAAs may be needed not for eradication, but for control of neoplastic disease.

A compelling example of an acquired immune response to auto-antigens in the presence of tumor was recently demonstrated using a mouse model of prostate cancer. In spontaneous adenocarcinoma, Savage et al. demonstrated that TILs from tumor bearing mice, unlike T cells isolated from their non-tumor bearing counterparts, recognized a ubiquitously expressed self antigen, histone H4 (12). While the recognition of histone H4 did not result in complete tumor regression, it did result in a significant reduction in tumor size (12). This report demonstrated that TILs are capable of recognizing normal cell components when associated with a tumor. What was not addressed in this study however was whether there was an oncogenic role of histone H4 and why this intracellular protein had become immunogenic. This report instigates questions the developmental stage of the tumor that immune cells are capable of recognizing which may lead to answers as to why tumors are capable of escaping complete immune eradication.

Part of the aforementioned question was answered recently with the demonstration that carcinogen induced tumors are not only recognized by T cells, but that immunological recognition is capable of maintaining a state of tumor “equilibrium” (24). In a mouse model of spontaneous sarcoma, approximately 20% of the exposed mice never developed tumors. However, upon depletion of T cells (both $CD4^+$ and $CD8^+$) from the non-tumor bearing cohort, subcutaneous

tumors quickly developed. From these T cell depletion studies it was concluded that immune-controlled tumor equilibrium was responsible for the lack of tumor progression in the original “tumor free” cohort. Furthermore, the authors demonstrated a role for immunoediting of TAAs by tumor transfer studies from tumor bearing mice into naïve recipients. Tumors that developed spontaneously as opposed to those that developed as the result of T cell depletion had different levels if immunogenicity and therefore displayed different growth rates (24). These data suggest a direct role for immune effectors in the destruction and/or control of tumor growth.

Immune responses can be generated against either the tumor itself or the stroma which is a critical support network for tumor growth to maintain tumor equilibrium (25, 26). Using antigen specific $CD8^+$ T cell adoptive transfers, Zhang et al. demonstrated tumor regression when cells of the tumor stroma were pulsed with tumor antigen. Pulsing resulted in tumor antigen cross presentation to T cells infiltrating the site (25). Furthermore, the transferred $CD8^+$ T cells maintained a state of tumor equilibrium and consistently destroyed myeloid-derived stromal cells which are vital to tumor growth (26). This study demonstrated that directing antigen specific responses toward the tumor stroma and not the tumor cells directly may be another strategy for which the application of immunotherapeutics can be used. Taken together, these publications have demonstrated that immune recognition of TAAs can result in efficient tumor destruction or the control of tumor growth.

However, the question still remains; how do the tumors bypass immune control? How do they escape? Numerous studies have demonstrated that immune cell recognition can lead to tumor escape (22). One such mechanism is through the emergence of an antigen loss variant, during which tumor cells expressing a TAA are destroyed and thus less antigenic cells are selected. Thus is can be inferred that in the presence of a tumor, cells of the immune system can disregard the evolutionary pressures to avoid autoimmunity and respond to “self” determinants. This self recognition includes responses to ubiquitously expressed “self” proteins and can occur at some undetermined time after tumor initiation. However, these responses may be inefficient at complete tumor regression possibly as an evolutionary pressure to avoid autoimmunity since autoimmunity is rarely observed after such approaches.

While mouse studies are helpful in showing what can be done, they are limited in their mimicry of the human situation. Some of the primary flaws are that studies often utilize young, inbred mice and results are rarely demonstrated in more than one strain. Additionally, the mice are housed under specific pathogen free conditions. Furthermore, the majority of the immune repertoire of a young (8-16wk old) mouse is phenotypically naïve. However, cancer predominantly affects elderly. Many studies have demonstrated that the aged immune system is significantly altered in comparison to the young immune system both phenotypically and in its ability to generate an immune response (27-29). These

observations suggest that an aged immune system may have more difficulty in reaching tumor equilibrium.

4. Cell surface receptors capable of inhibiting T cell responses

Immunotherapuetic strategies for the treatment of malignant disease frequently aim to enhance immune responses through either the administration of agents designed to elicit a Th1 response or through the blockade of inhibitory receptor ligand interactions (30). Inhibitory receptors on T cells are powerful mediators of effector function. One such surface receptor is cytotoxic T cell antigen-4 (CTLA-4). Other important although less studied, are the receptors of the T cell immunoglobulin and mucin domain (TIM) family of proteins, most notably, TIM-3.

CTLA-4 is a cell surface receptor upregulated on T cells after activation through the T cell receptor (TCR) (31). Ligation of CTLA-4 by one of its two ligands, B7-1 and B7-2 can result in dampening the cell cycle (32). Additonally, CTLA-4 ligation has been implicated in the conversion of conventional CD4+ T cells to regulatory CD4+ T cells through the upregulation of the transcription factor Foxp3 (32). Due to the potency with which CTLA-4 acts to inhibit T cell function, blocking antibodies to this receptor have been of interest to immunotherapeutic regimens for malignant disease. Specifically, two blocking antibodies to CTLA-4 which are currently in clinical trial, ipilimumab (phase II) and tremelimumab (phase III) have shown promising results in melanoma patients when combined with either tumor vaccine or interleukin-2, respectively (33, 34). These reports suggest that the

blockade of inhibitory T cell receptors in combination with immunostimulation may be superior to either agent alone.

Tim-3 is another T cell inhibitory receptor which is upregulated upon T cell activation and is associated with the progression of multiple diseases. Ligation of TIM-3 dampens effector T cell proliferation and cytokine secretion (Reviewed in (35)). TIM-3 has been extensively studied in mouse models of experimental autoimmune encephalitis (EAE). Blockade of the TIM-3 receptor pathway in a mouse model of EAE accelerated disease progression suggesting an important role for this pathway in peripheral tolerance (35). TIM-3 has been associated with T cell dysfunction in other diseases as well. Upregulation of TIM-3 has recently been associated with T cell exhaustion of human CD8+ T cells in patients infected with Human Immunodeficiency Virus (HIV) (36). These results suggested that increased TIM-3 expression on CD8+ T cells from HIV patients correlated with disease progression. Furthermore, it was suggested that a compensatory relationship may exist between the T cell inhibitory receptor programmed death-1 (PD-1) and TIM-3 during chronic HIV infection (36). Therefore, these reports highlight the multiple T cell inhibitory pathways that may need to be overcome to establish maximal T cell effector function by immunotherapy.

Effector T cell responses can additionally be inhibited through the cell surface receptor programmed death-1 which is increased on cells after TCR mediated

activation (37, 38). Ligation of PD-1 on the T cell surface can elicit T cell senescence or cell death (38). Additionally, one of the ligands for PD-1, B7-H1 is highly IFNγ responsive (39). This IFNγ responsiveness makes the PD-1 pathway of particular interest to any immunotherapeutic regimens which promote a pro-inflammatory environment as a mechanism of action.

5. The Role of Programmed Death-1 and Programmed Death Ligand-1

Following anti-CD40 and IL-2 immunotherapy, $CD8^+$ T cells significantly expand whereas $CD4^+$ T cell numbers remain relatively static. This stasis of the CD4+ T cell compartment is due to elevated apoptosis and/or necrosis of the activated $CD4^+$ T cells and was dependent on interferon-gamma (IFNy) (23). One possible explanation would be the interactions between programmed death-1 (PD-1) and its IFNy dependent ligand, programmed death ligand-1 or B7-H1.

PD-1 is found on most cells of hematopoietic origin and its gene expression has been associated with programmed cell death of thymocytes after TCR ligation (37). The presence of surface PD-1 on activated T cells is important for peripheral tolerance of $CD8^+$ T cells to self expressed antigens in the tissues (40). Ligation of PD-1 by B7-H1 is also important for tolerance to self antigens early in T cell development (41). PD-1 expression on T cells may be used to indicate disease progression in a variety of disease settings with different pathologies. These include HIV, Hodgkin's lymphoma, schistosomiasis and rheumatoid arthritis (42-44). In the case of rheumatoid arthritis, $PD\text{-}1^+$ $CD4^+$ T cells can

accumulate in the synovial fluid of affected patients and represent a population of anergic T cells (45). The multiplicity of diseases with which PD-1 expression is correlated may indicate its importance in T cell effector responses.

Two ligands are known to recognize and bind PD-1; B7-H1 (PDL-1, CD274) and B7-DC (PDL-2, CD273) (46). B7-DC, which is primarily found on dendritic cells, is not highly responsive to IFNγ. However, B7-H1 is highly responsive to IFNγ and after ligation of PD-1, elicits either apoptosis or senescence. B7-H1 is found on many cell types, both hematopoietic and non-hematopoietic in origin (47). Surface B7-H1 on tumor cells can contribute to tumor evasion of immune response as it is upregulated both *in vivo* and *in vitro* on tumor cells in response to IFNγ (48). Because of the potential to modulate immune responses in a positive or negative manner, the PD-1/B7-H1 pathway is under investigation with respect to cancer therapy (49-51).

Limited reports have discussed a differential role for PD-1 on the surface of $CD4^+$ T cell subsets. In one report, PD-1 ligation by B7-H1 at the site of *H. Pyloir* infection resulted in T cell anergy. Furthermore, co-culture of $CD4^+$ T cells from *H. Pylori* infected donors with infected epithelial cells *in vitro* resulted in an expansion of Treg cells when B7-H1 was present. B7-H1 was critical for the expansion of regulatory T cells (Tregs) as the inclusion of B7-H1 blocking antibodies to the cultures abrogated Treg expansion (52). This report was among the first to demonstrate a direct role for B7-H1 in the promotion of Tregs.

Differential expression pattern of PD-1 between resting Treg cells and activated conventional $CD4^+$ T cells (Tconv) cells has been recently discussed after CD3 stimulation *in vitro*. First, they showed that Treg cells, which classically lack appreciable surface expression of PD-1, contain large amounts of PD-1 in intracellular vesicles. Importantly, TCR ligation of Treg cells resulted in significant upregulation of PD-1 onto surface of Treg cells (53).

Immunotherapy represents a potentially powerful means to treat cancer. However, strong stimuli administered as immunotherapeutic agents likely enhance both effector cells and regulatory cells. This unintentional expansion of regulatory cells may have important consequences with respect to the efficacy of immunotherapy. First, a reduction in the CD4:CD8 balance coinciding with an increase in Treg cells making up the $CD4^+$ compartment may be detrimental to the generation of sustained anti-tumor responses. Also, a shift in the balance of regulatory and effector cells, favoring cells of inhibitory phenotype may increase the amount of drug required to gain the desired pro-inflammatory result. This is especially important for drugs which have proven to be effective but are restricted due to toxicity, such as IL-2 (54). As most pro-inflammatory regimens elicit their responses directly through the production of IFNy, the PD-1 pathway may be one of great importance.

Expression patterns of PD-1 and B7-H1 are important indicators of disease outcome (42-44). However, they may also be important indicators of the efficacy of immunotherapy. B7-H1 can be used as a “molecular shield” by tumors in response to IFNγ (48). Effective immunotherapeutic applications to treat cancer are sometimes associated with the development of autoimmune disease (55). Therefore, blocking inhibitory receptors such as PD-1, B7-H1 and CTLA-4 may have direct impact on immunosuppressive mechanisms used by the tumor in addition to breaking tolerance (38, 41, 55).

In combination with immunotherapeutic stimuli, blockade of PD-1 and/or B7-H1 may be necessary to achieve a maximal response. Blocking antibodies against PD-1 and B7-H1 are best applied in combination. It was recently shown that combined blockade, but neither antibody alone, was capable of enhancing anti-tumor effects (38). Blockade of one side of this pathway is usually met with only partial effects. This report illustrates a problem that should be considered when designing immunotherapies that are dependent on the blockade of an inhibitotyr pathway.

6. Memory T cell responses and cancer

Immunological memory has classically been defined by immunological response time. Upon rechallenge with the appropriate antigen, memory cells react rapidly to destroy pathogens or infected host cells. Immunological memory has therefore been employed for the control of widespread disease through the application of

vaccination. Various infectious diseases can be controlled or even eradicated by vaccination. However, some diseases such as cancer have remained difficult to vaccinate effectively against. The difficulty in generating an effective anti-tumor response through vaccination has primarily been attributed to the presence of active disease, therefore in this context, vaccination is being used as a treatment regimen rather than for prevention.

The potential effectiveness of preventative vaccination are illustrated by the successes of vaccination to viruses, such as the virus responsible for smallpox. Vaccination it now is being used for the prevention of Human Papilloma Virus (HPV) for the prevention of cervical cancer. Unlike Smallpox, cancer vaccines must target a disease that has slowly developed out of self tissues. In some cases, tumor development has occurred for well over 20 years before the emergence of clinical symptoms (21). The observation that tumors can be present for such an extended period of time before detection is the result of two major factors which allow for tumors to grow unabated; first, non-viral TAAs are weakly immunogenic and are inefficient at promoting immune responses, second, tumors themselves employ immunosuppressive pathways to avoid immune detection.

Cancer is a general term for hundreds of different diseases, which illustrates a major problem associated with its management (21). Many different vaccination strategies have been explored for the treatment of various types of cancer, both

in mouse models and clinical trials. Some have shown potentially promising results although this has been limited (21, 56). Thus, more research is needed to continue the search for effective anti-tumor vaccines as a means to boost anti-tumor responses. Due to multiple mechanisms of immunological suppression, the generation of antigen specific immunity and memory to TAAs is difficult, especially considering that most tumor vaccines are administered after disease onset. Additionally, many immunotherapeutic regimens, even those that seemed promising initially, did not confer lasting immunity to TAAs (30). Therefore, it is possible that in the context of an existing tumor, two therapy regimens must be applied; one that targets the initial tumor and one that generates lasting antigen specific T cell immunity to TAAs.

Currently vaccination for cancer is limited to prevention and is inefficient at treating existing disease. One such example is the HPV vaccine which is a preventative vaccine that has potential to impact cancer rates (57, 58). Gaining FDA approval in 2006, this vaccine protects against the two most common strains of HPV (HPV16 and HPV18) which are responsible for 60-70% of all cases of cervical cancer in the United States annually (59). Recent reports have suggested that the HPV vaccine is ineffective against active HPV infections (www.cdc.gov/std/hpv) however the reason for its inefficiency during active infection has not yet been established. Although the evidence is not yet sufficient to make definitive claims, it is likely that vaccination of infected women will not result in similar protection against HPV induced cervical cancer.

7. Memory T cell Responses and Immunotherapy

While the use of immunotherapy to promote T cell responses for the treatment of certain cancers has made significant progress, it has been hampered by relatively low response rates with regard to overall survival and has been applied to a limited number of cancers. One area that is lacking thus far is an understanding of the importance (or not) of memory T cell generation after immunotherapy for cancer treatment. There are multiple models of memory T cell formation (60, 61). When properly primed, memory T cells are tenacious and capable effectors that can remove antigen-positive cells before any signs of re-encounter have occurred (**Fig. 1**). For this reason, the generation of memory T cells in an individual with cancer is desirable for durable and sustained anti-tumor responses (60, 61).

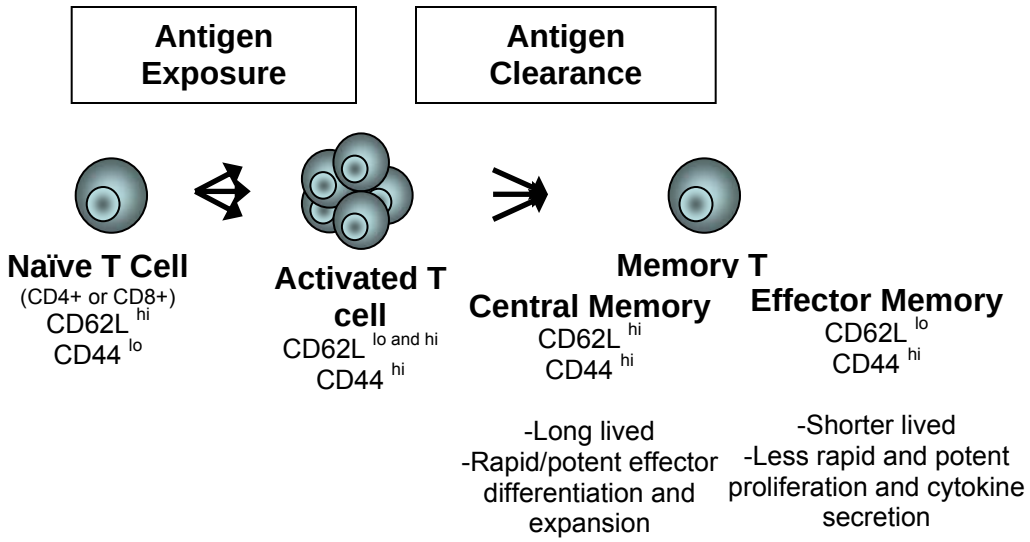

Figure 1. Generation of memory T cells. Two subtypes of memory T cells are generated following an immune response. Central memory ($CD62L^{hi}$ $CD44^{hi}$) T cells are longer lived and are activated more rapidly as determined by cytokine production and death ligand upregulation. Effector memory ($CD62L^{lo}$ $CD44^{hi}$) T cells are shorter lived and less rapid effector cells, but are maintained in the periphery for a extended period of time after infection.

The generation of antigen-specific T cell responses to cancer has been the focus of some groups attempting to increase the numbers of antigen-specific T cells in patients through *ex vivo* expansion followed by adoptive transfer (62). However the results are reported in complete response rates without discussion of the overall survival time of patients. This may be an indicator that immunological memory is not being attained or that down regulation pathways may be exerted after immunotherapy (20, 63, 64). For adoptive lymphocyte transfer to be efficacious, it is imperative to understand how T cells persist in an environment where extensive immune stimulation is being applied. One logical question that

has yet to be answered experimentally would be what effect does the use of potent immune stimuli have on immunological memory? Previous work from our lab has demonstrated that immunotherapy resulting in high production of IFNγ impaired secondary T-cell responses, despite initial anti-tumor responses capable of eliciting tumor regression (23, 65). These findings have created a paradox with respect to the use of strong immunotherapeutic agents to treat cancer. While strong immune stimulation may be necessary for the initial destruction of weakly immunogenic tumors, their use may also impair the development of immunological memory or sustained anti-tumor responses.

Strong cytokine stimuli can have deleterious effects on antigen-specific T cells both directly and indirectly (66-70). More recently, it was demonstrated that the use of potent immune stimulatory agents, such as an agonist CD40 mAb (anti-CD40) and interleukin-2 (IL-2) or CpG and IL-12, as a source of therapy can result in the deletion of cells that are vital to the antigen specific response and memory cell generation (23, 71). CD40 is an important immunological receptor vital to immune activation (72). Bartholdy *et al.* reported a loss of LCMV-virus specific $CD8^+$ T cells after treatment with an anti-CD40. Their observations highlighted that despite anti-CD40 delivering artificial “help” to $CD8^+$ T cells during a viral infection, instead of being primed, $CD8^+$ T-cells were in fact deleted. This resulted in a lack of sustained anti-viral responses (71). Our laboratory has previously shown that combined immunotherapy consisting of anti-CD40 and IL-2 could markedly increase the survival of tumor-bearing mice (65). However, if

mice were vaccinated with irradiated tumor prior to immunotherapeutic administration, they did not generate a significant memory response and were not protected against a later live tumor challenge (23). Furthermore, we found that while $CD8^+$ T cells expand following immunotherapy, $CD4^+$ T cell numbers did not increase. Importantly, this lack of $CD4^+$ T-cell expansion was dependent on IFNγ.

8. Is immunological memory important for tumor regression or tumor equilibrium?

Thus far, we have discussed two potential problems that complicate effective vaccination to cancer antigens. First, cancer antigens are often weak in eliciting an immune response. Second, cancer antigens are generated over long periods of time, often without “danger” signals. The amount of time that cancer antigens are present may affect the ability of immune cells to recognize and target them. One reason for this is peripheral tolerance. Peripheral tolerance is a process by which T cells are tolerized to self antigen exposure in the periphery. One proposed mechanism for tolerance in the periphery is through self peptide expression by antigen presenting cells (APCs) in the periphery without appropriate co-stimulation (73). Another proposed mechanism of peripheral tolerance is the co-expression of inhibitory molecules such as B7-H1 alongside MHC-peptide complexes on the surface of normal cells (74). B7-H1 upregulation by tumors in response to INFγ is an important example of one peripheral tolerance mechanisms which are used by tumors as a mechanism of immune

suppression (48). Danger signals that occur during infection with a foreign pathogen or as the result of a high level of cellular necrosis are required for appropriate immunological activation (75). Expression of inhibitory ligands which can elicit tolerance immune cell tolerance may support the tumor presence for long periods of time without any sign of danger to the host.

The tumor burden at this time of immunotherapy may affect the therapeutic efficacy. Many tumors can be associated with high levels of necrotic cell death which should elicit a dianger signal. However, this often occurs at much later stages in cancer progression. At this time, due to the tumor burden, generating immune responses to tumor antigens may be a futile exercise. In order to generate effective immunological memory against cancer antigens, vaccination strategies must overcome these mechanisms of T cell suppression to self peptides that are associated with the tumor. However, one first needs to determine whether immunological memory is effective for tumor regression and maintenance of tumor free survival.

The *ex vivo* induction and transfer of autologous IL-2 activated lymphocytes into tumor bearing individuals can have profound effects on primary tumor regression, but this therapy has not conferred long lasting survival of patients (20). However, it is possible that optimal anti-tumor responses would be expected by combining highly activated T cells for initial tumor destruction followed by a regimen designed to generate immunological memory to maintain a state of tumor

equilibrium. It has not been sufficiently addressed whether tumor regression and tumor equilibrium require the same type of immune effector cell.

Using systemic adjuvant therapy to enhance the immune response generated to a tumor antigen vaccine has been the goal of many mouse models and clinical trials (reviewed in (30)). Toll like receptor (TLR) agonists are one such method of enhancing cancer vaccine efficacy. A TLR 9 agonist was used in melanoma patients by combination with a cancer vaccine strategy and resulted in a markedly higher expansion of melanin-A specific $CD8^+$ T cells in the peripheral blood of treated patients (76). However, it was noted that the majority of antigen specific T cells generated with TLR-9 agonist plus vaccine were of effector memory phenotype. Effector memory T cells do not persist for an extended period of time after infection and long term disease free status was not reported (30). This report demonstrated one problem facing current immunotherapy regimens which is the development of lasting protection.

Systemic administration of IL-2 has been administered as part of many clinical cancer vaccine trials to support the antigen specific expansion of effector cells (30). Most clinical trials utilizing IL-2 have been limited in the amount of cytokine that can be administered as IL-2 is associated with severe toxicity (77, 78). However, with the exception of one report (79), clinical trials using low dose IL-2 have not demonstrated a beneficial role of this cytokines addition to cancer vaccine regimens (80-83). The administration of other systemic proinflammatory

cytokines as well as the blockade of inhibitory cells and surface markers have also been used in conjunction with cancer vaccines in clinical trials to maximize vaccine efficacy (30). Some combined treatments have had modest results, but problems associated with toxicity are usually associated with effective enhancement of the immune response as well as difficulties in sustaining T cell responses.

9. Absence of T cell memory after strong immune stimulation

There are two subtypes of memory T cells, effector memory ($CD44^{hi}$ $CD62L^{lo}$) and central memory ($CD44^{hi}$ $CD62L^{hi}$). In addition to the original, basic definition of T cell memory which was defined by the expression of CD44 and CD62L, CD127, the alpha receptor for IL-7 can separate memory T cell subsets (84). CD127 is highly expressed on memory cells that have an antigen specific origin, but is not highly expressed on memory cells that have upregulated a memory phenotype due to homeostatic proliferation or some of the memory T cells which have developed due to recognition of self antigens (84). The necessary signal(s) for a T cell to develop into these a specific subset is debated (85, 86). However, it is generally accepted that there is a different physiological role for each subset (87). Central memory T cells (T_{CM}) are those typically regarded as immunological memory cells. They are described as the longer lived memory cell and are, at their most basic definition characterized by the expression of adhesion molecule L-selectin (CD62L) in addition to the classic memory cell identifier CD44.

Both CD28 and CD127 are suggested to be important for the longevity of memory cells and central memory T cells that can persist in the lymph nodes long after an antigen is cleared (88, 89). While the long term presence of $CD28^{null}$ T cells, both $CD4^+$ and $CD8^+$, have been described in humans, they have been linked with immune incompetence associated with normal aging or with chronic inflammation (90). Upon secondary antigen exposure, central memory T cells rapidly produce cytokines and undergo a high level of cellular proliferation (85, 86). It is generally accepted that the central memory T cells have an advantage to prolonged survival because of heightened cytokine receptor expression (84). The difference in survival between different memory T cell subsets indicates that cancer therapies should be designed to elicit central memory T cell responses.

Conversely, effector memory T cells are maintained for less time than central memory T cells and remain in the periphery during for their life cycle. Mouse models have demonstrated that effector memory T cells persist for a relatively short period after antigen exposure, only lasting about 2-3 weeks (87). Effector memory T cells are classified by their expression of the memory marker CD44 and their appreciable lack of lymph node adhesion molecules, most notably L-selectin (CD62L). Effector memory cells also respond quickly to antigen re-exposure most likely with less rapidity and potency than central memory cells (86) **(Fig 1)**.

To understand T cell memory it is important to understand the critical collaboration between $CD4^+$ and $CD8^+$ T cells. $CD4^+$ T cells are known for their functional role as helpers of an immune response and are needed during the primary immune response to prime $CD8^+$ T cells properly for sustaining immunological memory (91, 92). The powerful effector capabilities of memory $CD8^+$ T cells make them an exceptionally attractive candidate for cancer therapy. While some tumor models seem to be directly sensitive to direct killing by $CD4^+$ T cells (93), these cells are not generally regarded as potent effectors in mouse tumor models. However, $CD4^+$ T cells are vital to the generation and possibly the maintenance of effective $CD8^+$ T cell-mediated immunity, which is especially important for most immunotherapy approaches (91, 92, 94-96).

In various tumor models, our laboratory demonstrated that immunotherapy-dependent $CD4^+$ T cell death resulted in the destruction of immunological memory when immunotherapy was administered immediately after irradiated tumor vaccine (23). The lack of sustained T cell memory occurred despite primary anti-tumor responses by the same systemic immunotherapy regimen (65). Cells expanded during immunotherapy were predominantly effector memory phenotype. In this model of potent systemic immunotherapy, the observed level of $CD8^+$ T cell expansion strongly argues against selective expansion of antigen specific cells, which would have resulted in long term immunological memory. This demonstrates a potential problem with combination immunotherapies as

cytokine support of vaccination may be beneficial to the primary or metastatic tumor burden but detrimental to the generation of long lasting immunity.

Why then, if potent immune stimulation is associated with the expansion of memory phenotype cells, does this not correspond with long lived immunity **(Fig 2)**. Verneris *et al.* reported one possible mechanism. They reported that $CD8^+$ T cells can acquire MHC-unrestricted killing mechanisms after T cell receptor (TCR) crosslinking and high dose IL-2 *in vitro* (97). Cytotoxicity of these cells was displayed toward many different target cells in an NKG2D-mediated fashion. NKG2D is an activating receptor found on the surface of activated NK cells, T cells and macrophages (98, 99). The ligands for NKG2D are stress ligand and their recognition elicits killing of the cell which is expressing the stress ligand(s) (98). In the Verneris *et al.* report, NKG2D mediated killing was dependent on the adaptor protein DAP-10 which was only upregulated in the presence of high dose and not low dose IL-2 (97). This study demonstrated a possible mechanism through which potent systemic immune stimulation could be eliciting a large population of effector T cells that are capable of primary tumor regression, but that are not antigen specific in nature. Therefore these cells would not persist as long lasting memory cells as demonstrated in (**Fig 2**).

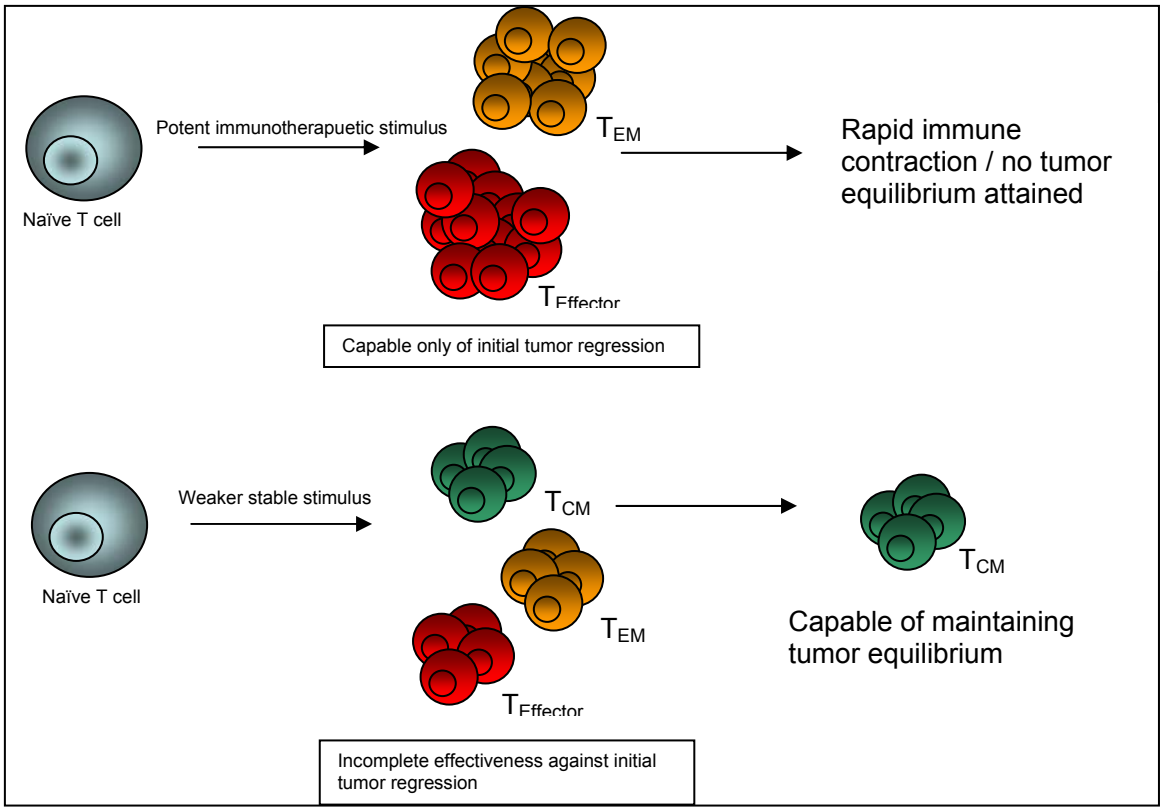

Figure 2. Divergence in memory retention depending on stimulus. This schematic demonstrates the hypothesis that potent immune stimulus initiates powerful effector memory (T_{EM}) and effector ($T_{Effector}$) T cells. This response is capable of inducing initial tumor regression, but long term memory is not attained to tumor antigens. Alternatively, weaker more stable stimulus elicits $T_{EM,}$ $T_{Effector,}$ and central memory (T_{CM}) T cells which are capable of long lived antigen recognition and therefore tumor equilibrium.

Long term immunological studies in a mouse model of sepsis have shown that immediately following the induction of sepsis, dendritic cells (DCs) were depleted from the lung and the spleen (100). Furthermore, it was demonstrated that once the DC population returns, their function, as determined by IL-12 secretion, is severely depressed for over 6 weeks after sepsis onset (100). IL-12 enhances

the survival time of activated CD8+ T cells and its availability may be a limiting factor in CD8+ T cell responses to solid tumors (101, 102). Therefore, this study may have demonstrated a currently unidentified problem associated with the administration of potent systemic immune stimulation for the treatment of cancer. A consequence of activation and expansion of the APC population may in the context of systemic inflammation, result in selective APC depletion and persistent APC desensitization to stimulation. This would potentially result in blunted responses, and in the context of cancer, may result in relapse.

For the two chapters of this dissertation, the consequences of potent immunotherapy in the form of anti-CD40 in combination with IL-2 were evaluated. Through these experiments it was first demonstrated that potent immunotherapy selectively promotes Treg cells in the CD4+ T cell compartment. Selective Treg promotion inversely correlates with the selective upregulation of inhibitory PD-1 on CD4+ Tconv cells. Treg expansion may work to reduce the efficacy and/ or the longevity of beneficial effector cells. Second, these results suggest that T cells of a memory phenotype are attained through the application of immunotherapy. Furthermore, the data demonstrate in a mouse model of complete thymic involution, that the administration of immunotherapy significantly expands memory phenotype cells while having minimal effect on the naïve T cell compartment. Further, this was correlated with a persistent reduction in T cell proliferative responses. These results suggest that immuntoehrapy which is

capable of inducing a successful initial anti-tumor response may be detrimental to the generation of new immune responses.

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Chapter 1: Regulatory and Conventional $CD4^+$ T cells Show Differential Effects Correlating with PD-1 and B7-H1 Expression After Immunotherapy

Abstract:

Recently, our laboratory reported that secondary $CD8^+$ T-cell mediated anti-tumor responses were impaired following successful initial anti-tumor responses using various immunotherapeutic approaches. While immunotherapy stimulated significant increases in $CD8^+$ T cell numbers, the number of $CD4^+$ T cells remained unchanged. The current investigation revealed a marked differential expansion of $CD4^+$ T-cell subsets. Successful immunotherapy surprisingly resulted in an expansion of $CD4^+$ $Foxp3^+$ T-regulatory (Treg) cells concurrent with a reduction of conventional $CD4^+$ T cells (Tconv), despite the marked anti-tumor responses. Following immunotherapy, we observed differential upregulation of PD-1 on the surface of $CD4^+$ $Foxp3^+$ regulatory T cells (Treg cells) and $CD4^+$ $Foxp3^-$ T cells (Tconv cells). Interestingly, it was the ligand for PD-1, B7-H1 (PDL-1) that correlated with Tconv loss after treatment. Furthermore, interferon gamma knockout (IFNγ-/-) and interferon gamma receptor knockout (IFNγR-/-) animals lost upregulation of surface B7-H1 even though PD-1 expression of Tconv cells was not changed and this correlated with $CD4^+$ Tconv increases. These results suggest that subset specific-expansion may contribute to marked shifts in the composition of the T-cell compartment, potentially influencing the effectiveness of some immunotherapeutic approaches that rely on interferon gamma (IFNγ).

Introduction:

Immunotherapeutic use of an agonist CD40 mAb in combination with IL-2 has been shown to have synergistic anti-tumor effects in mouse models of advanced renal cell carcinoma (RCC) and lung carcinoma (1). More recently, we reported that treatment of mice after immunization combined with this and other immunotherapeutic regimens can lead to an interferon-gamma (IFN-γ) dependent loss of $CD4^+$ T cells and subsequent inability to mount an effective memory response after a delayed live tumor challenge despite successful initial anti-tumor responses (2). Additionally, other investigators using a viral antigen challenge model have shown similar effects after administration of anti-CD40 alone. Administration of anti-CD40 to LCMV-infected mice was associated with the loss of virus specific $CD8^+$ T cells upon secondary challenge *in vitro*. While a loss of $CD4^+$ T cells was also observed, the dominant effector outcome was due to the loss of $CD8^+$ T cells and was mediated by the Fas-FasL pathway (3). The majority of tumor models focus on $CD8^+$ T cell effector pathways. However, in addition to helping generate tumor-antigen specific $CD8^+$ T-cell memory, recent studies suggest a more direct role for $CD4^+$ T cells in some anti-tumor responses (4). While we previously reported a loss in $CD4^+$ T cell numbers after anti-CD40 and IL-2 immunotherapy despite increases in $CD8^+$ T cells, the mechanism underlying this lack of $CD4^+$ T-cell expansion was not clear.

$CD4^+$ T cells are a very diverse lymphocyte population with respect to the cytokines they can produce, and understanding their polarization toward stimulatory or inhibitory activity is important for understanding how they can affect treatment in a disease setting (5). Regulatory $CD4^+$ T cells expressing the hallmark forkhead transcription factor 3 (Foxp3) (Treg cells) are of particular interest with respect to cancer immunotherapy due to their potent immunosuppressive effects. It has therefore been suggested that their presence should be evaluated with all immunotherapeutic regimens since increases in Treg cells can be counterproductive to the desired outcome (6). We therefore examined the effects of CD40-based immunotherapeutic regimens on $CD4^+$ T cell subsets and key markers correlating with their expansion or loss. Our current observations presented herein report a differential expression pattern of the cell surface marker Programmed Death-1 (PD-1, CD279) in response to anti-CD40 and IL-2 immunotherapy on the surface of conventional $CD4^+$ T cells (Tconv) and Treg cells. PD-1 is found on most cells of hematopoietic origin and its surface expression has been associated with programmed cell death of thymocytes after TCR ligation (7, 8). PD-1 upregulation after T-cell activation has been implicated as being important for the peripheral tolerance of $CD8^+$ T cells to tissue antigens, as well as self antigens early in their development (9-11).

We observed markedly increased expression of PD-1 on the surface of $CD4^+$ Tconv cells, but not Treg cells after treatment with anti-CD40 and IL-2. Additionally, B7-H1 was upregulated in an IFN-γ dependent fashion, consistent

with previous reports,(12) and we found this upregulation of B7-H1 to correlate with the observed loss of $CD4^+$ T cells. These findings caused us to look more closely at $CD4^+$ T cell subsets in the context of immunotherapy-induced alterations of $CD4^+$ T cell subsets and overall changes in the composition of the T-cell compartment. The results reported herein led us to the hypothesis that IFN-γ dependent upregulation of B7-H1 after immunotherapy is met with a differential expression of PD-1 on conventional $CD4^+$ T cell versus Treg cells. From these results, we suggest that differential expression patterns of the regulatory marker PD-1 following immunotherapy contribute to the loss of Tconv cells while simultaneously allowing Treg cells to expand. This may have ramifications in the length and extent of anti-tumor effects after immunotherapy.

Materials and Methods:

Mice. Female C57BL/6 and BALB/c mice were purchased from the Animal Production Area of the National Cancer Institute (Frederick, MD). B6.129S7-I*fng*^{tm1Ts} (IFNγ$^{-/-}$) and B6129S7*Ifng*R (IFNγR-/-) as well as some aged matched control WT C57BL/6 mice were purchased from Jackson Laboratories (Bar Harbor, ME). Mice were between 8 and 12 weeks at the start of experiments and housed in microisolator cages or in the case of genetically engineered and aged matched control mice, on a Hepa filtered vent rack. Under all settings, mice were housed under specific pathogen free conditions. All experiments were in accordance with IACUC guidelines.

Reagents and cell Lines. Agonist rat anti-mouse CD40 (FGK115B3) was purified by ammonium sulfate precipitation from ascites. The endotoxin level of the anti-mouse CD40 antibody was <1endotoxin unit/mg antibody as determined by quantitative chromogenic limulus amebocyte lysate kit (QCL-1000, Bio Whittaker, Cambrex, Walkersville, MD). Recombinant human Interleukin-2 (IL-2; TECIN. Teceleukin) was provided by the National Cancer Institute. Recombinant human IL-15 (IL-15) was purchased from Peprotech (Rocky Hill, NJ). Purified rat IgG was purchased from Jackson ImmunoResearch Laboratories (West Grove, PA).

Cell Preparations. Spleen and lymph node cells were prepared by gentle dissociation and filtered to remove excess debris followed by washing two times in DPBS containing 5% FBS (Hyclone, Logan UT) and 1% Penicillin/Streptomycin (Mediatech, Herndon, VA). Cell counts were determined by a lyse/no wash procedure with known concentration of fluorescent beads or on a Coulter Z1 particle counter (Coulter Electronics, Arlington, TX). Blood was collected in tubes containing EDTA, Red blood cells were lysed in blood samples with FACSLyse (BD Biosciences, San Jose, CA).

Antibodies and Flow Cytometry. Cell suspensions from lymph node or spleen or whole blood were incubated with antibodies labeled with fluorescein (FITC) R-Phycoerythrin (PE), PE-cyanine 5 (PC5) and/or PE-Cyanine 7 (PC7) and PE-Texas Red (PE-TXred) followed by wash and resuspension in PBS + 5%FBS (Hyclone, Logan UT) + 1% Penicillin/Streptomycin (Mediatech, Herndon, VA.). Intracellular Foxp3 labeling was completed using the Ready Set Go, Foxp3

labeling Kit (eBiosciences, San Diego, CA) and all samples were resuspended in 1% formaldehyde (Sigma) in 1X Dulbeccos Phosphate Buffered Saline (Mediatech, Herndon, VA). Antibodies were purchased from either eBiosciences or BD Biosciences. Listmode data files were collected on a three color FACScan flow cytometer using Cell Quest software (Becton Dickinson, San Jose, CA), on a four-color Beckman Coulter XL/MCL using system II software or on a 5-color FC 500/MPL (Beckman Coulter, Fullerton CA)**.** All data sets were analyzed using FlowJo software (Treestar, Ashland, OR).

Treatment Protocol. Agonist rat anti-mouse CD40 (FGK115B3) was administered i.p. at 65ug to mice in experiments utilizing transgenic mice (IFNγ-/- or IFNγR-/-) or 80ug per dose for 5 consecutive days. Recombinant human IL-2(IL-2), 0.5 – 1.0 $X10^6$ IU/dose was administered i.p. four times per week in two sets of two injections; the second injection in a set being 8-20hrs from the previous one. In experiments where IL-15 was used in combination with anti-CD40, 2.5ug of recombinant human IL-15 (IL-15) was administered i.p. twice daily in place of IL-2 injections.

Statistics. Statistical analysis was performed using Prism software (Graphpad Software Inc.) Flow cytometry data were analyzed using student's t test; a Welch's correction was applied to data sets with significant differences in variance. Survival data were analyzed using a Log Rank Test. A minimum of 3 mice per group was used and all experiments. Experiments using C57BL/6 mice were repeated at least 3 times. BALB/c experiments were performed once with 3 mice per group to support the observations made using C57BL/6 mice. Data

were tested for normality and variance, a p-value of <0.05 was considered significant.

Results:

Systemic Immunotherapy Results in a Selective Loss of Conventional $CD4^+$ T cells but not Regulatory $CD4^+$ T cells. Evaluation of splenic $CD4^+$ T cell percentages 11 days after the start of immunotherapy shows a marked lack of expansion of $CD4^+$ T cells compared with $CD8^+$ T cells (Fig. 1A). Despite reported initial anti-tumor effects (1) this was found to be due to cell death as $CD4^+$ T cells were shown to be entering into the cell cycle after immunotherapy even though no expansion was taking place. Similar to our previous observations in multiple immunotherapeutic models (2), evaluation of $CD4^+$ and $CD8^+$ T cells shortly after immunotherapy administration resulted in an alteration of the normal ratio between $CD4^+$ and $CD8^+$ T cells (Fig. 1B). We next examined the effect of immunotherapy on the different $CD4^+$ T cell subsets. Surprisingly, while $CD4^+$ T cells did not expand as a whole population, the regulatory subset of $CD4^+$ T cells defined by the expression of Foxp3 (Treg Cells) significantly ($P < 0.05$) expanded following administration of immunotherapy (Fig. 1C). In addition to total cell number, Treg cell expansion concurrent with the lack of Tconv cell expansion resulted in Treg cells making up a larger percentage of the $CD4^+$ T cell compartment (Fig. 1D). Since IL-2 and not IL-15 is reported to be a strong promoter of Treg cells *in vivo*, anti-CD40 was combined with IL-15 to determine if this combination would also result in a significant expansion of Treg cells. In

addition to anti-CD40 and IL-2, anti-CD40 and IL-15 combined immunotherapy resulted in similar preferential expansion of Treg cells and not Tconv (Fig. 1E and F). This appears to be due to a dominant effect of CD40 as IL-15 alone did not promote Treg expansion (data not shown). These results suggest that administration of immunotherapy results in an early loss of Tconv cells and simultaneous expansion of Treg cells, despite the occurrence of marked anti-tumor effects.

Systemic Immunotherapy Results in a Differential Expression of PD-1 on the Surface of Conventional and Regulatory T cells in Conjunction with B7-H1 Upregulation on all $CD45^+$ splenocytes. PD-1/B7-H1 ligation has been shown to have inhibitory and even pro-apoptotic effects on $CD8^+$ T cells (7, 13). However, the effect of immunotherapy on this pathway with regard to $CD4^+$ T cells has not previously been investigated. Therefore, we assessed surface PD-1 expression on $CD4^+$ Tconv cells and $CD8^+$ T cells as well as $CD4^+$ Treg cells by flow cytometry immediately following administration of an anti-CD40 and IL-2 regimen. Following immunotherapy, we observed a significant increase in the percentage of Tconv cells expressing PD-1 on the cell surface ($P < 0.001$) which was not observed in the Treg cell subset ($P > 0.05$). We also observed a significant increase in the percentage of $CD8^+$ T cells that expressed surface PD-1 after treatment with anti-CD40 and IL-2 (Fig. 2A and B), however the fold increase in the percentage of $CD8^+$ T cells expressing surface PD-1 was significantly ($P < 0.01$) lower than $CD4^+$ Tconv cells (Fig. 2C). The percentage of

$CD8^+$ T cells from control treated animals did not significantly differ from naïve animals (Fig. 2D). While we observed a higher baseline percentage of $CD8^+$ T cells that were also PD-1^+, $CD8^+$ T cells had a consistently lower ($P < 0.0001$) level of receptor expression than $CD4^+$ T cells as determined by median fluorescence intensity (data not shown). Further studies performed in both C57BL/6 mice and BALB/c mice showed that administration of anti-CD40 and IL-2 induced similar expression patterns of PD-1 on the surface of $CD4^+$ T cells in both strains (Fig. 2E). In addition to PD-1, we also examined the expression of the PD-1 ligand, B7-H1. B7-H1 is widely expressed on most hematopoietic cell types (12), therefore we evaluated its expression by flow cytometric analysis on all $CD45^+$ splenocytes. Following anti-CD40 and IL-2 administration, we observed a significant ($P < 0.0001$) increase in the median fluorescence intensity of surface B7-H1 on $CD45^+$ splenocytes (Fig. 3A and B). B7-H1 expression was also significantly ($P< 0.05$) higher on the surface of the $CD11c^+$ population of leukocytes (data not shown) however, the expression was not limited to myeloid or lymphoid cells therefore we evaluated surface B7-H1 expression on all hematopoietic ($CD45^+$) cells. We did observe some variation in the baseline level of B7-H1 in our control treated animals between experiments; however, a comparison between naïve and control treated animals did not show an effect of the rat Ig and PBS treatment in the relative levels of B7-H1 on $CD45^+$ cells (Fig. 3C). These data show that anti-CD40 and IL-2 results in the upregulation of B7-H1 on $CD45^+$ cells, while simultaneously increasing surface PD-1 expression on conventional $CD4^+$ T cells and not on Treg cells. These changes correlate

directly with the observed loss in $CD4^+$ T cell numbers suggesting that changes in the expression of B7-H1 and PD-1 may contribute to the decrease in conventional $CD4^+$ T cells in the absence of similar effects on $CD4^+$ Treg cells.

Surface PD-1 Expression on $CD4^+$ Tconv Cells is not Changed in the Absence of IFNγ After Immunotherapy. Previous data from our laboratory indicated that the selective loss of $CD4^+$ T cells following anti-CD40 and IL- 2 was dependent on IFNγ (2). Therefore, we evaluated the relative levels of PD-1 on the surface of $CD4^+$ T cells from wild type mice versus mice lacking either IFNγ (IFNγ-/-) or the IFNγ receptor (IFNγR-/-). After treatment with anti-CD40 and IL-2, we found surface expression of PD-1 on $CD4^+$ T cells was still significantly ($P < 0.001$) upregulated in IFNγR-/- mice (Fig. 4A and B) and IFNγ-/-mice (Fig. 4C and D). Surface upregulation of PD-1 on $CD4^+$ T cells occurred in the absence of IFNγ signaling despite increases in $CD4^+$ T cell numbers following treatment (2). These data suggest that IFNγ is not influencing the observed reduction in $CD4^+$ T cells through direct alteration of the surface expression of PD-1 on $CD4^+$ T cells.

IFN-γ Dependent B7-H1 Expression on Hematopoietic Cells Correlates with $CD4^+$ T cell Loss. Since surface expression of PD-1 on $CD4^+$ T cells in IFNγR-/- and IFNγ-/-mice did not directly correlate with IFNγ dependent loss of $CD4^+$ T cells despite restoration of $CD4^+$ T cell expansion, we evaluated the relative levels of B7-H1 expression following immunotherapy. Surface expression of B7-

H1 and not PD-1, is reported to be dependent on IFNγ (12). To examine a possible correlation between B7-H1 expression and immunotherapy induced $CD4^+$ T cell loss, flow cytometric analysis was used to determine the relative levels of B7-H1 on $CD45^+$ cells from IFNγR-/- mice and IFNγ-/-mice after administration of anti-CD40 and IL-2. As expected, $CD45^+$ splenocytes from wild type mice showed a significant ($P < 0.001$) upregulation of surface B7-H1 expression after treatment with anti-CD40 and IL-2. In contrast, $CD45^+$ splenocytes from both IFNγR-/- mice (Fig. 5A and B) and IFNγ-/-mice (Fig. 5C and D) did not show elevated cell surface B7-H1 expression after treatment with anti-CD40 and IL-2. These data suggest that direct effects of IFNγ on B7-H1 expression patterns correlated with the observed loss of $CD4^+$ T cells following anti-CD40 and IL-2. As selective immunotherapy induced expression of PD-1 on $CD4^+$ Tconv cells and not Treg cells this allowed for Treg cells to avoid the inhibitory effects mediated by anti-CD40 and IL-2 upregulation of B7-H1 on all $CD45^+$ cells. This selective upregulation results in a marked alteration of the $CD4^+$ Tconv: $CD4^+$ Treg: $CD8^+$ T cell ratio which may contribute to the loss of secondary responses at a later time, after immunotherapy.

Discussion:

In this manuscript, we show that tumor immunotherapy regimens that can lead to successful initial anti-tumor responses paradoxically result in a lack of $CD4^+$ Tconv cell expansion concurrent with $CD4^+$ Treg cell expansion and this correlates with PD-1/B7-H1 expression patterns following immunotherapy.

Specifically, we present evidence that upregulation of the inhibitory molecule PD-1 on Tconv cells following immunotherapy is a likely mechanism that contributes substantively to an imbalance between potentially beneficial Tconv cells and deleterious Treg cells. Treg cells are important mediators of the inflammatory immune response through their inhibitory actions on $CD4^+$ and $CD8^+$ T cells as well as NK cells (14, 15). Their presence has been shown to hinder the promotion of an effective immune-mediated anti-tumor response (6). The selective expansion of Treg cells after immunotherapy described here may present a mechanism by which the immune system attempts to down-regulate itself after being exposed to such a powerful stimulus such as anti-CD40 and IL-2. Therefore, these cells may be a critical determining factor in the outcome of at least some immunotherapeutic approaches to cancer treatment.

In a previous publication, we reported substantial effects of anti-CD40 and IL-2 on the ratio of $CD4^+$ to $CD8^+$ T cells. In tumor-bearing mice, we showed long term effects that changes in this ratio can have on memory $CD8^+$ T cell responses (2). Based on our current observations, we cannot rule out the possibility that PD-1 ligation is also having an effect on $CD8^+$ T cells. However we observed higher increases in PD-1 expression on $CD4^+$ T cells in terms of MFI and the percent of PD-1 positive cells. Therefore, we think that in our model PD-1 is having a more pronounced effect on $CD4^+$ Tconv cells than on $CD8^+$ T cells. The data presented in this publication extend our previous observations to suggest that when subsets of $CD4^+$ T cells are carefully evaluated, substantial

differences in important subsets can be detected. For example, our findings suggest that the ratio of $CD4^+$ Tconv cells to $CD8^+$ T cells may be lower than was originally thought. This is due to a preferential expansion of Treg cells in the $CD4^+$ T cell compartment after treatment with either anti-CD40 and IL-2 or anti-CD40 and IL-15. While the consequences of such a preferential expansion following immunotherapy have not been previously described, this may contribute to the loss of secondary responses, and also may shorten the duration of the initial anti-tumor response. It is therefore reasonable to suggest that combination immunotherapy in conjunction with Treg cell depletion may further enhance the effectiveness of this approach. One potential way to reduce the induction of Treg cells would be to use IL-15 instead of IL-2 (16), as IL-2, and not IL-15, is known to be a strong promoter of Treg cell expansion (17-19). However, we did not observe a difference in the expansion of Treg cells between the two immunotherapeutic regimens, and IL-15 alone did not induce Treg cell expansion (data not shown). This suggests Treg cell expansion may rely more on the administration of anti-CD40 than on the administration of IL-2.

Agonist CD40 mAb administration has been shown to suppress the immune response to LCMV infection resulting in an increase in viral titers after treatment. In this LCMV model, loss of antigen specific $CD8^+$ T cells was observed. Interestingly, in this model a significant decrease in $CD4^+$ T cells was also observed after treatment with anti-CD40 alone, however any potential correlation with PD-1 expression was not discussed (3).

The use of anti-CD40 and IL-2 provides a model for investigating the disadvantages and benefits of potent immune stimulation. This model magnifies differences that may occur in the effectiveness of initial versus long term immune-mediated tumor responses. In this regard, our recent studies indicate that strong immunotherapeutic regimens such as anti-CD40 and IL-2 combined therapy can hamper long term responses to antigen through deleterious changes in the $CD4^+$:$CD8^+$ T cell balance (2). Alterations in this balance have been of great interest for some time (20, 21). Most recently there has been a debate about potentially "helpless" $CD8^+$ T cells being incapable of responding to secondary antigenic challenge, which can occur even when primary response capabilities function with complete normalcy and strength (22, 23).

Surface expression patterns of PD-1 on human $CD4^+$ T cells can be used as an indication of disease outcome in various human disease settings such as rheumatoid arthritis, schistosomiasis and Hodgkin's lymphoma (24-26). Similarly, PD-1 expression occurs on the $CD8^+$ T-cells of patients infected with HIV who show long term progressor status (27). However, few reports have discussed the relative expression patterns on $CD4^+$ T cell subsets, and to our knowledge, no reports have discussed a differential response of $CD4^+$ T cell subsets to immunotherapy dependent on this pathway. While we observed a differential expression of PD-1 on the surface of Tconv cells and Treg cells after anti-CD40

and IL-2, we did not find a difference in the expression pattern of other immune markers such as Fas or DR5 after treatment (data not shown).

PD-1 has two known ligands, B7-H1 (PDL-1, CD274) and B7-DC (PDL-2, CD273) (12). B7-H1 is found on many cell types including lymphocytes and myeloid cells as well as cells that are not of hematopoietic origin (8). B7-DC is primarily found on dendritic cells and is not upregulated in response to IFN-γ; therefore, we have focused on effects of B7-H1. Ligation of PD-1 by B7-H1 is capable of eliciting either apoptosis or senescence (7, 12). B7-H1 is inducibly upregulated on tumor cells both *in vivo* and *in vitro*, and is therefore thought to be important in tumor evasion of immune responses (28). PD-1 engagement by B7-H1 has been shown to have potent inhibitory effects on immune stimulation (29, 30) resulting in the promotion of $CD8^+$ T cell tolerance to self antigens in the periphery (9). Therefore, the PD-1/B7-H1 pathway is currently under intense investigation since manipulating it has the potential to modulate immune responses in a positive or negative manner (31-33).

Given our data presented here, further studies in tumor bearing mice could address the question of whether selective upregulation of PD-1 on Tconv cells and not Treg cells following immunotherapy might allow the tumor to dampen the effectiveness of tumor infiltrating lymphocytes, while not affecting the immune inhibiting function of Treg cells. B7-H1 upregulation may additionally promote immune suppression by supporting cell conversion to a suppressive phenotype.

In addition to Tconv cells being negatively affected through ligation of PD-1 by B7-H1, Treg cells may benefit from this interaction (34). In *H. Pylori* infection, T-cell anergy at the site of infection has been shown to be dependent on PD-1 ligation by B7-H1. It was shown that the presence of B7-H1 promoted an increase in Treg cell frequency when $CD4^+$ T cells from *H. Pylori* infected donors were co-cultured with *H. Pylori* infected epithelial cells *in vitro*. This Treg cell expansion was abrogated when anti-B7-H1 antibody was included (34).

Blockade of PD-1 and/or B7-H1 as well as other inhibitory markers such as CTLA-4 have recently been of interest when attempting to break tolerance (11, 35), (36). Combined PD-1 and B7-H1 blockade, but not B7-DC is reported not only to enhance $CD8^+$ T cell mediated anti-tumor responses, but also to reverse anergy in $CD8^+$ T cells (11). It is important to note, however that blockade of the PD-1/B7-H1 pathway usually only results in a partial removal of its inhibitory effect. Studies in our model aimed at the blockade of PD-1 or B7-H1 singly with anti-CD40 and IL-2 had no effect *in vivo* possibly due to the massive expansion of $PD\text{-}1^+$ and $B7\text{-}H1^+$ cells which would require very high levels of blocking antibody to obtain results (data not shown). This demonstrates one of the potential problems when exerting very strong stimulatory signals to amplify immune responses. Studies using PD-1 or B7-H1 knockout mice may be the best way to determine if anti-tumor responses after immunotherapy are enhanced by the removal of PD-1 or B7-H1 signaling.

Relieving immune responses from the strict control that is mediated through PD-1/B7-H1 may be beneficial for the development of more effective anti-tumor immunotherapeutic approaches. Herein we report a previously undescribed preferential expansion of Treg cells which occurs in parallel to the loss of effector cells after administration of anti-CD40 and IL-2. It is of interest that this preferential Treg expansion still resulted in marked initial anti-tumor effects (1, 2). Because of its potent immunomodulating capabilities, the PD-1 and B7-H1 receptor/ligand interactions provide a potentially important component that should be further considered with regard to immune changes and overall responses that can be induced by different forms of immunotherapy. Our observations highlight the strong opposing force that the immune system has to potent stimuli. The different regulatory mechanisms utilized to protect from over stimulation may hinder efforts toward more effective anti-tumor immunotherapies.

Chapter 1 References:

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Figure Legends:

Figure 1. Anti-CD40 and IL-2 combination immunotherapy results in skewing of normal lymphocyte ratios. Spleens from C57BL/6 mice harvested on day 11 of treatment with anti-CD40 and IL-2 showed expansion of $CD4^+$ $Foxp3^+$ regulatory T cells. (A) Animals that had received immunotherapy showed a significant ($P < 0.05$) increase in the number of splenic $CD8^+$ T cells but not in $CD4^+$ T cells as determined by flow cytometry. (B) After treatment with anti-CD40 and IL-2, animals showed a significant ($P < 0.0001$) decrease in the ratio of $CD4^+$ to $CD8^+$ T cells in the spleen. (C and D) Despite the lack of expansion of splenic $CD4^+$ T cells after immunotherapy, we observed a significant ($P < 0.05$) increase in Treg cells in animals that had been treated with anti-CD40 and IL-2. This increase in Treg cells was determined to be in total $CD4^+$ $Foxp3^+$ cell numbers (C) and as a percentage (D) of the total $CD4^+$ T cell population. (E and F) IL-15 was used in combination with anti-CD40 in place of IL-2 and Treg cells were analyzed by flow cytometry for expansion (E) in cell numbers and (F) as a percentage of all $CD4^+$ T cells. Data in A-D was repeated at least three times with similar results, the data in E and F was repeated two times. Analysis for all parts of figure 3 were analyzed using an unpaired student t test, a Welch's correction was applied for any set of data with significantly different variances.

Figure 2. Regulatory T cells fail to upregulate PD-1 as a result of anti-CD40 and IL-2 immunotherapy. Relative levels of PD-1 on the surface of $Foxp3^+$ $CD4^+$ Treg cells were compared with conventional $Foxp3^-$ $CD4^+$ T cells 11 day

after initiation of immunotherapy by flow cytometry. (A) Representative histograms of PD-1 labeling show unlabeled control (gray line), Rat Ig/PBS treated animals (dashed black line) and anti-CD40 and IL-2 treated animals (solid black line). (B) The relative percentage of Treg cells expressing surface PD-1 was not significantly ($P > 0.05$) greater in animals which had received anti-CD40 and IL-2 immunotherapy. However, surface PD-1 expression was significantly ($P < 0.01$) higher on conventional $CD4^+$ T cells and $CD8^+$ T cells from animals that had received immunotherapy. (C) The increase in the percentage of cells expressing surface PD-1 as a fold increase in animals treated with anti-CD40 and IL-2 over control treated animals. (D) The percent of $CD8^+$ T cells expressing surface PD-1 is not significantly ($P > 0.05$) increased when animals are treated with the control therapy. (E) Representative histograms show similar upregulation of surface PD-1 on $CD4^+$ T cells from C57BL/6 mice as well as BALB/c mice; unlabeled control (gray line), Rat Ig/PBS treated animals (dashed black line) and anti-CD40 and IL-2 treated animals (solid black line). The data in A and B were repeated at least three times with similar results, the data in C was collected one time. A student t-test, with a Welch's correction for any data sets with significant differences in variance was used in B, a one way ANOVA was used in parts C and D.

Figure 3. Anti-CD40 and IL-2 immunotherapy results in an increased surface expression of B7-H1 on all hematopoietic cells. Mice were treated with anti-CD40 and IL-2 according to the standard regimen listed in the Materials

and Methods section. 11 days after treatment initiation, spleen were harvested and analyzed by flow cytometry for surface B7-H1 expression. (A) B7-H1 (PDL-1) expression was determined by median fluorescence intensity analysis and is significantly ($P < 0.0001$ by unpaired student t test) higher on splenocytes from animals which received anti-CD40 and IL-2. (B) Representative histograms show a similar pattern in both C57BL/6 mice as well as BALB/c mice; unlabeled control (gray line) and labeling with anti-B7-H1 antibody (clone MIH5) on $CD45^+$ splenocytes from control treated animals (dashed black line) and animals treated with anti-CD40 and IL-2 (solid black line). (C) A comparison of the MFI of B7-H1 on $CD45^+$ cells in control treated animals versus anti-CD40 and IL-2 treated animals, a one way ANOVA was used for statistical analysis. Each experiment was made up of three mice per group. The data shown in B and C were collected on two different flow cytometers (Beckman Coulter XL and FacScan, respectively) and the Y axes are representative of the MFI as reported by the two instruments. Experiments shown in A were completed at least three times with similar results, data in B and C was completed one time (n=3 for each).

Figure 4. Surface expression of PD-1 on $CD4^+$ T cells is not affected by IFN-γ. Surface PD-1 expression on $CD4^+$ T cells was evaluated by flow cytometry 11 days after immunotherapy initiation in animals lacking either IFN-γ (IFNγ-/-) or the IFN-γ Receptor (IFNγR-/-). (A) Representative histograms of PD-1 in IFNγR-/- mice show unlabeled control (gray line), Rat Ig/PBS treated animals (dashed black line) and anti-CD40 and IL-2 treated animals (solid black line). (B) Cells

isolated from the spleens of IFNγR-/- and WT C57BL/6 animals showed a significantly ($P < 0.001$) higher percentage of $CD4^+$ T cells in the spleen expressing surface PD-1 from animals which had been administered immunotherapy. (C) Histograms of PD-1 labeling in IFNγ-/- mice show unlabeled control (gray line), Rat Ig/PBS treated animals (dashed black line) and anti-CD40 and IL-2 treated animals (solid black line). (D) Evaluation of splenocytes from IFNγ-/- mice 11 days after the start of immunotherapy showed a higher ($P < 0.001$) percentage of $CD4^+$T cells expressing surface PD-1 in animals that had been administered anti-CD40 and IL-2. Experiments using IFNγR-/- animals were completed one time with three animals per group and were supported by IFNγ-/- data which was completed three times with three animals per group. An unpaired student t-test was used to determine significant differences between animals which had received immunotherapy and the control immunotherapy, a Welch's correction was used for data sets with significant differences in variance.

Figure 5. B7-H1 expression correlates with IFN-γ. B7-H1 expression on all $CD45^+$ leukocytes was evaluated in wild type animals and animals deficient in either interferon-gamma (IFNγ-/-) or the interferon-gamma receptor (IFNγR-/-). Unlike WT mice where a significantly ($P < 0.001$) higher level of B7-H1 was observed on cells from animals which had received immunotherapy, B7-H1 was not higher after immunotherapy in either IFNγR-/- (A and B) or IFNγ-/- (C and D) mice. An unpaired student t test was used to determine significant differences between animals which had received immunotherapy and the control

immunotherapy. (A and C) Representative histograms of IFNγR-/- mice and IFNγ-/- mice respectively show control labeling (gray line), labeling of splenocytes from animals which received Rat Ig and PBS (black dashed line) or anti-CD40 and IL-2 (black solid line).

Figure 1

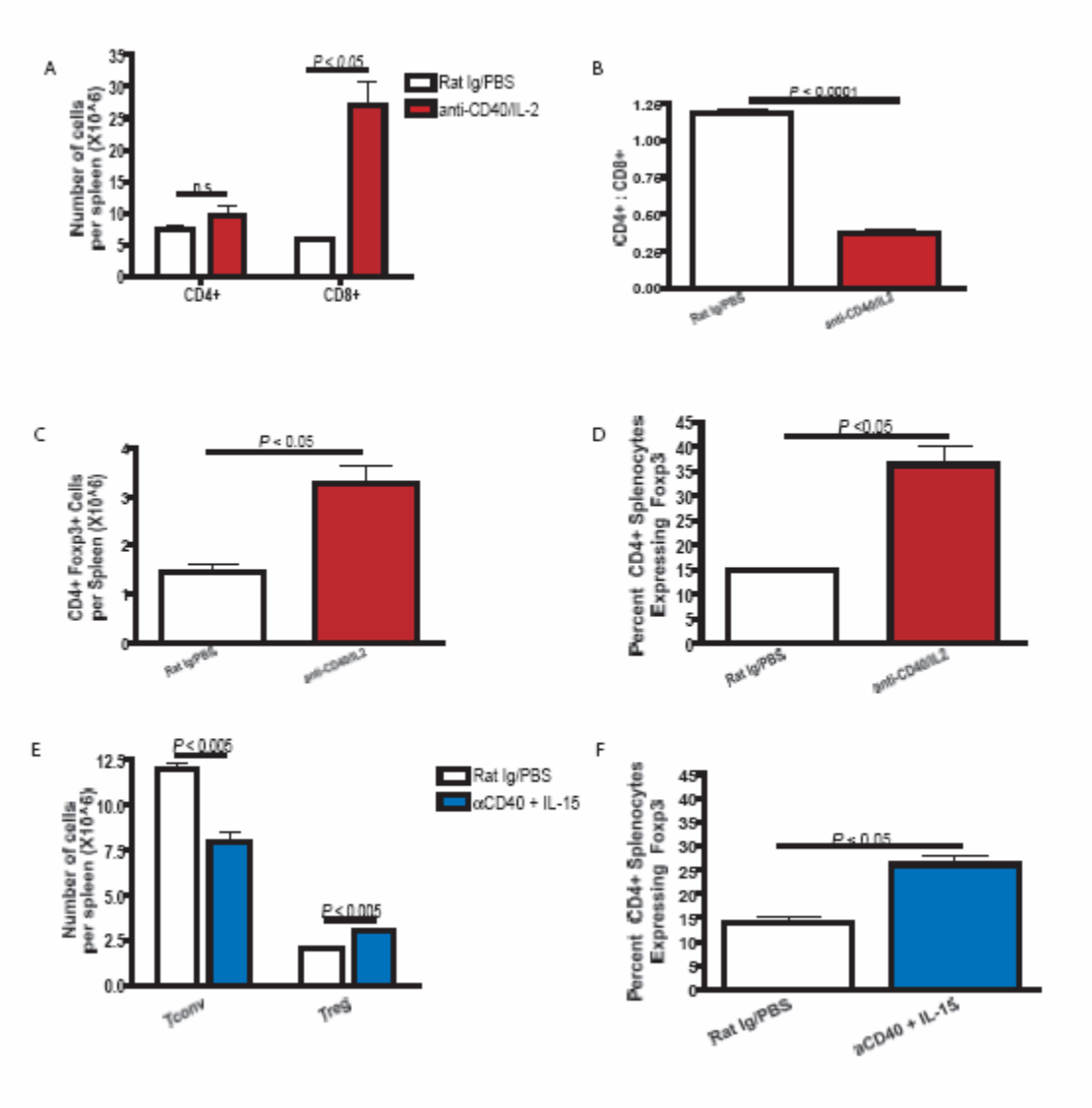

A
Number of cells per spleen (X10^6)
P≤0.05
Rat Ig/PBS
anti-CD40/IL-2
CD4+
CD8+
B
CD4+ : CD8+
P < 0.0001
C
CD4+ Foxp3+ Cells per Spleen (X10^6)
P < 0.05
D
Percent CD4+ Splenocytes Expressing Foxp3
P <0.05
E
Number of cells per spleen (X10^6)
P< 0.005
αCD40 + IL-15
Tconv
Treg
F
Percent CD4+ Splenocytes Expressing Foxp3
P < 0.05
αCD40 + IL-15

Figure 2

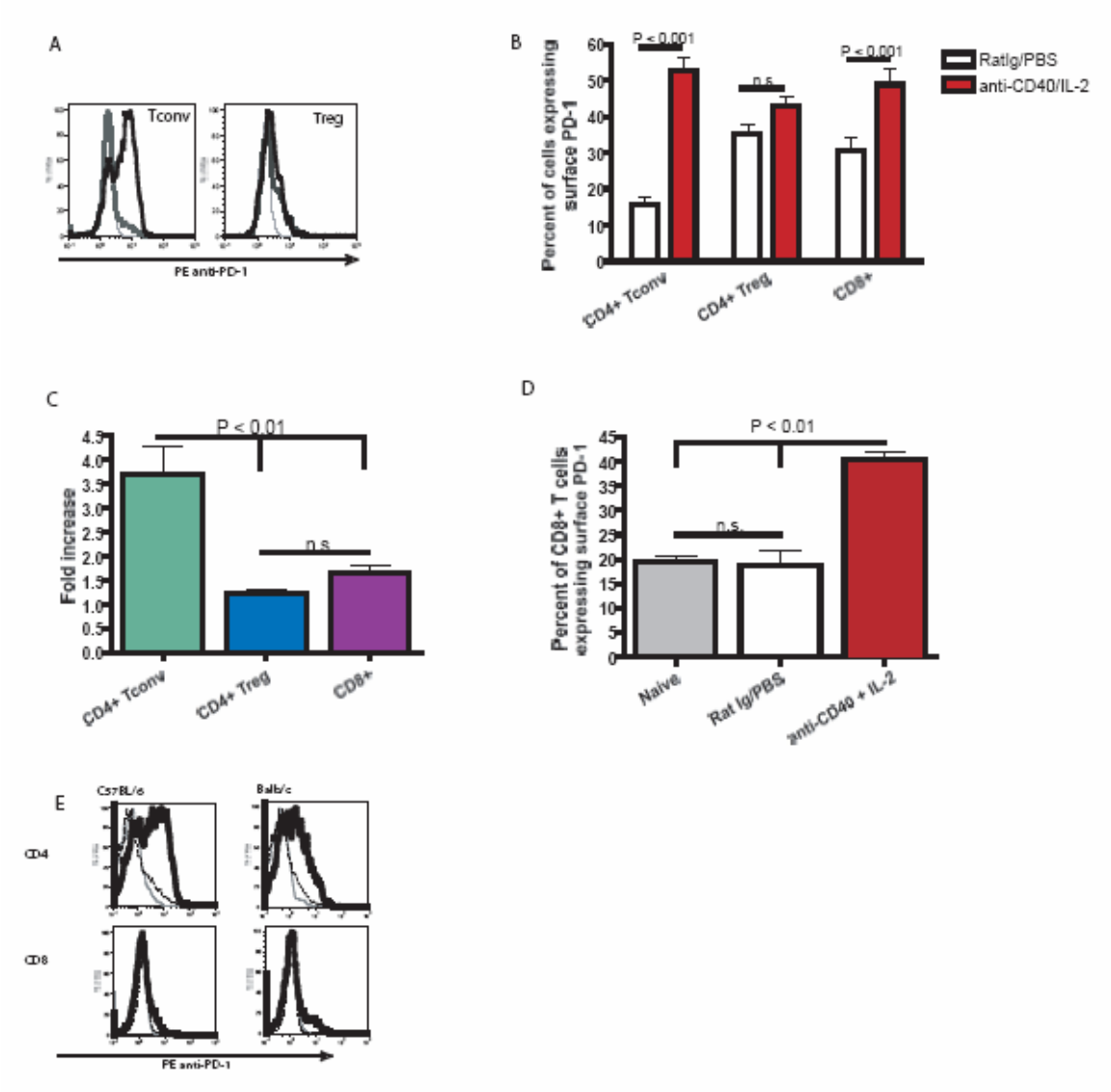

A
Tconv
Treg
PE anti-PD-1
B
P < 0.001
P < 0.001
n.s.
Percent of cells expressing surface PD-1
RatIg/PBS
anti-CD40/IL-2
CD4+ Tconv
CD4+ Treg
CD8+
C
P < 0.01
n.s.
Fold increase
CD4+ Tconv
CD4+ Treg
CD8+
D
P < 0.01
n.s.
Percent of CD8+ T cells expressing surface PD-1
Naive
Rat Ig/PBS
anti-CD40 + IL-2
E
C57BL/6
Balb/c
CD4
CD8
PE anti-PD-1

Figure 3

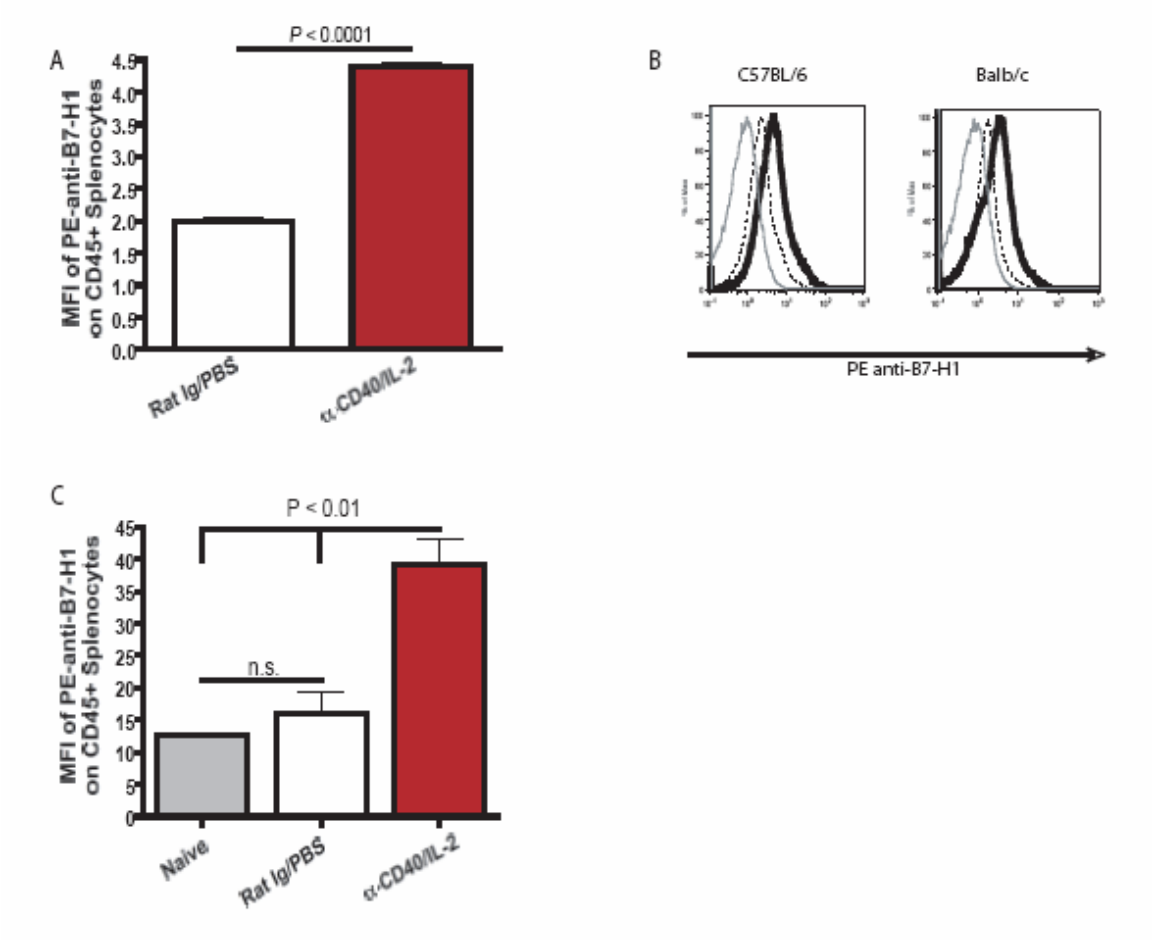

A
P < 0.0001
MFI of PE-anti-B7-H1 on CD45+ Splenocytes
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Rat Ig/PBS
α-CD40/IL-2
B
C57BL/6
Balb/c
PE anti-B7-H1
C
P < 0.01
n.s.
MFI of PE-anti-B7-H1 on CD45+ Splenocytes
45
40
35
30
25
20
15
10
5
0
Naive
Rat Ig/PBS
α-CD40/IL-2

Figure 4

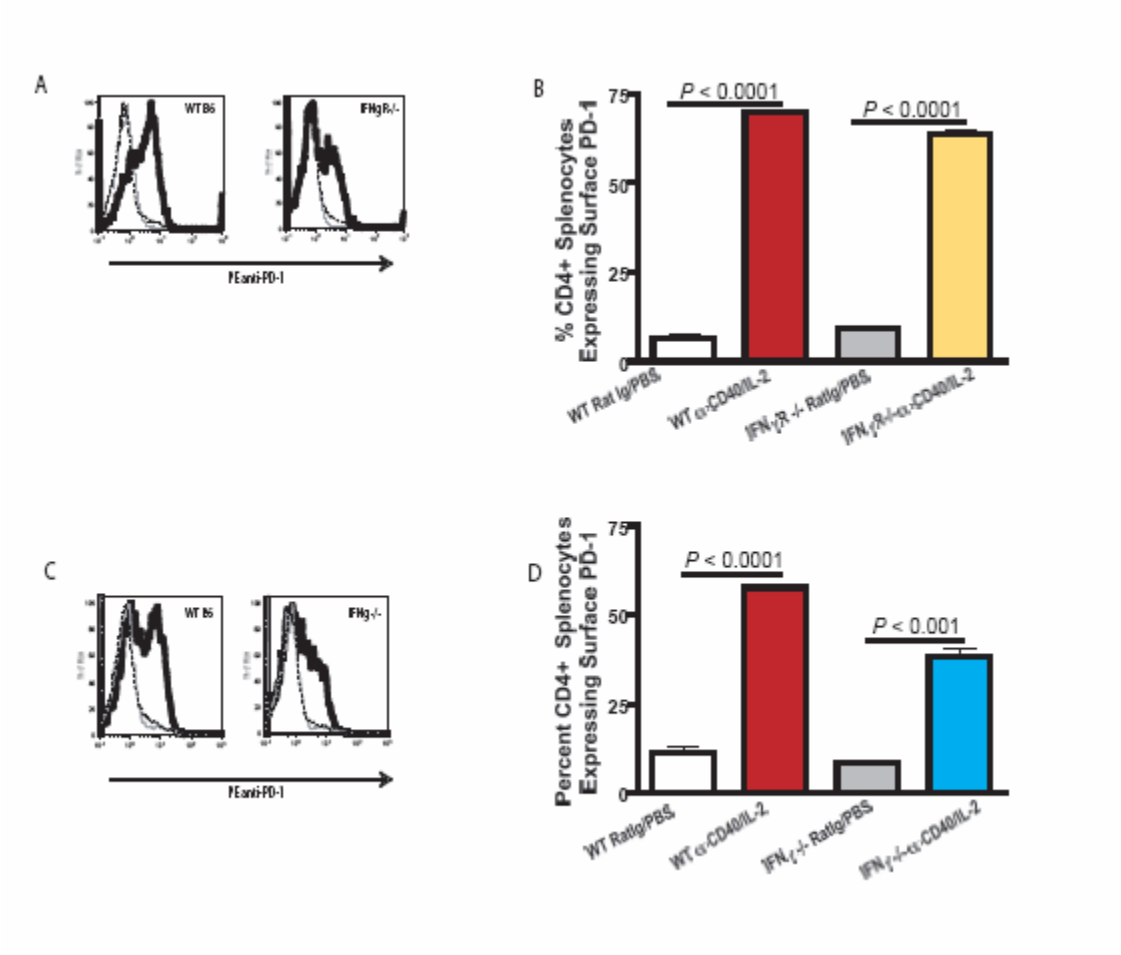
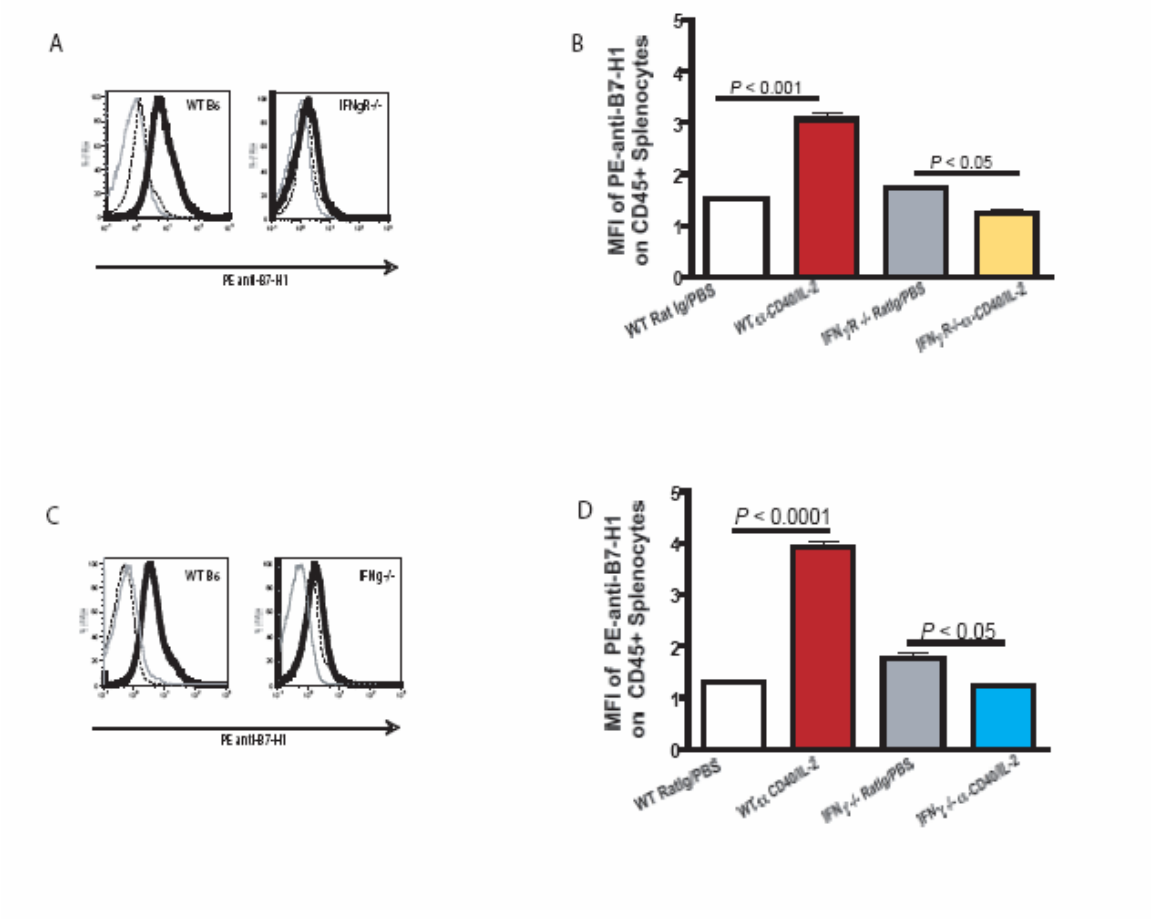

A
WT B6
IFNgR-/-
PEanti-PD-1
B
P < 0.0001
P < 0.0001
% CD4+ Splenocytes Expressing Surface PD-1
75
50
25
0
WT Rat Ig/PBS
WT α-CD40/IL-2
IFN-γR -/- Ratlg/PBS
IFN-γR-/-α-CD40/IL-2
C
WT B6
IFNg-/-
PEanti-PD-1
D
P < 0.0001
P < 0.001
Percent CD4+ Splenocytes Expressing Surface PD-1
75
50
25
0
WT Ratlg/PBS
WT α-CD40/IL-2
IFN-γ -/- Ratlg/PBS
IFN-γ-/-α-CD40/IL-2

Figure 5


A
WT B6
IFNgR-/-
PE anti-B7-H1
B
MFI of PE-anti-B7-H1 on CD45+ Splenocytes
P < 0.001
P < 0.05
WT Rat Ig/PBS
WT α-CD40/IL-2
IFN-γR -/- Rat Ig/PBS
IFN-γR-/- α-CD40/IL-2
C
WT B6
IFNg-/-
PE anti-B7-H1
D
MFI of PE-anti-B7-H1 on CD45+ Splenocytes
P < 0.0001
P < 0.05
WT Rat Ig/PBS
WT α-CD40/IL-2
IFN-γ -/- Rat Ig/PBS
IFN-γ -/- α-CD40/IL-2

Chapter 2: Cytokine based immunotherapy reduces the size of the memory, but not naïve, T cell compartment even in the absence of a thymus

Abstract

Systemic IL-2 administration is an efficient way to expand and activate T cells. This approach is used as an immunotherapy for malignant disease either alone or in combination with vaccination strategies. Here, we evaluated the effect of administration of a potent immunotherapeutic regimen on the expansion of T cells. Using a thymectomized mouse model, we found that pre-existing memory T cells were expanded more than five fold after the administration of anti-CD40 and high dose interleukin-2. This increase in memory T cells contrasted with little change in the CD4 naïve and less than a two-fold decrease in the CD8 naïve T cells. Importantly, the lack of expansion of naïve T cells correlated with reduced or even abrogated T cell proliferation to new antigens. Evaluation of the T cell compartment, 30 days after discontinuing therapy revealed that the decrease in the ratio of naïve to memory T cells persisted and correlated with impaired T cell proliferation. The lower frequency of naïve T cells occurred in treated young control mice as well as in treated adolescent thymectomized mice in which replacement of naïve T cells was curtailed. Our results demonstrate a negative and long lasting impact of immunotherapy.

Introduction

Systemic potent immunotherapy resulting in beneficial primary anti-tumor responses can also paradoxically impair immune responsiveness. The use of systemic cytokine administration as an immunotherapeutic agent has been used for the treatment of malignant diseases both in murine models and clinically (1). We have demonstrated that the combination of an agonist anti-CD40 mAb and interleukin-2 results in synergistic primary anti-tumor efficacy but abrogates secondary anti-tumor responses induced by irradiated tumor vaccination (2, 3). In this paper, we address how the secondary immunity could have been abrogated.

Our previous publications provided insights for addressing the loss of immune responsiveness after immunotherapy. Specifically, the anti-CD40/IL-2 immunotherapy reduced the ratio of CD4+ T cells to CD8+ T cells (2). The reduced ration required IFN-γ (2). This phenomenon was the result of selective CD4+ T cell death which correlated with the expression of programmed death-1 protein (PD-1) on CD4+ T cells and appearance of IFN-γ responsive PD-1 ligand, B7-H1 on all CD45+ cells (4). Furthermore, after immunotherapy PD-1 was present on conventional CD4+ T cells but not on regulatory CD4+ T cells, thus facilitating preferential Treg expansion after therapy (4). Taken together, these three reports from our laboratory clearly demonstrated different effects of the

anti-cD40/IL-2 immunotherapy on specific T cell subsets and that assessment of long term alterations in the CD4 and CD8, memory and naïve, T cell populations should be investigated further.

The accumulation of memory T cells has been correlated with decreased responsiveness to new antigens and may be a major factor in age-associated immune defects (1, 5, 6). We have demonstrated that anti-CD40/ IL-2 immunotherapy results in significant expansion of memory phenotype T cells corresponding with a decrease of naïve phenotype T cells (2). It has been hypothesized that memory T cells are capable of responding to new pathogens containing epitopes that closely resemble their original cognate antigen (5). However, recognition of new antigens by memory T cells is considered to be exceptionally rare (5). Furthermore, in a model of tolerization to the male antigen, it was demonstrated that tolerized T cells persisted as memory cells and were capable of secreting large amounts of the suppressive cytokine interleukin-10 upon re-challenge with the antigen to which they were tolerant (1, 7). Thus, the expanded memory populations are restricted to formerly encountered antigens and can potentially suppressive to T cell proliferation, which would affect naïve as well as memory cells. Thus there are increasing reports which suggest that the size and antigen specificity of the memory T cell compartment may have significant impact on the generation of an immune response to a new antigen.

Therefore, immunotherapy-induced enrichment of memory cells may be detrimental to the development of immune responses to new antigens and was a focus of this investigation.

Memory T cells can be expanded and activated in response to many cytokines directly. In contrast, cytokines render naïve T cells more responsive to TCR engagement but have limited ability to activate naïve T cells directly (8, 9). One potential mechanism for the differential sensitivity to cytokine is a difference in the level of cytokine receptors on the surface of naïve and memory T cells. For example, T cells with a memory phenotype have higher surface expression of CD122, which is part of the common gamma chain signaling complex (10). However, increased sensitivity to cytokine stimulation suggests that systemic cytokine administration alone as an immunotherapy may have more impact on the existing memory T cell population than on the naïve T cell population.

Naïve T cells are difficult to measure because they rapidly convert to a memory phenotype upon activation and/or by homeostatic proliferation (11). The response of naïve T cells to immunotherapy is especially important in models of the aged who lack thymic function as a result of progressive loss of thymic mass with aging. We used a model of thymic ablation to determine the effects of immunotherapy on the naïve and memory T cell compartments in the absence of *de novo* T cell generation in the thymus. We hypothesized that immunotherapy

alone would selectively expand pre-existing memory T cells and potentially impair T cell responses to new antigens.

Materials and Methods:

Mice. Female C57BL/6 mice were obtained from the National Cancer Institute (Frederick, MD). 'ThmX' mice were surgically thymectomized at 6-8 weeks of age by Charles River Laboratories. Mice were between 8 and 20 weeks at the start of experiments and housed in micro isolator cages or on a Hepa filtered vent rack, under specific pathogen free conditions. All experiments were in accordance with IACUC guidelines.

Reagents and cell Lines. Agonist rat monoclonal anti-mouse CD40 antibody (FGK115B3) was purified by ammonium sulfate precipitation from ascites. The endotoxin level of the anti-mouse CD40 antibody was <10 endotoxin unit/mg antibody as determined by the quantitative chromogenic limulus amebocyte lysate kit (QCL-1000, Bio Whittaker, Cambrex, Walkersville, MD). Recombinant human Interleukin-2 (IL-2; TECIN. Teceleukin) was provided by the National Cancer Institute. Purified rat IgG was purchased from Jackson ImmunoResearch Laboratories (West Grove, PA).

Cell Preparations. Spleen and lymph node cells were prepared by gentle dissociation and filtered to remove debris, followed by washing two times in PBS containing 5% FBS (Hyclone, Logan UT) and 1% Penicillin/Streptomycin (Mediatech, Herndon, VA).

***In vitro* mixed lymphocyte reaction (MLR) cultures.** Responder cells were prepared by the gentle dissociation of spleens from the experimental groups of C57BlL/6 (H2b) mice. Stimulator cells were prepared in the same manner from BALB/c (H2d) mice and irradiated (30Gy). Stimulator cells were plated at a starting $3.5X10^5$ cells/well and two-fold serial dilutions were made in 96 well plates. Seven$X10^5$ irradiated BALB/c splenocytes were added to each well resulting in responder to stimulator ratios starting at 0.5: 1 and decreasing twofold, ending at 0.06: 1. The cultures were incubated for 96 hours, pulsed with tritiated thymidine (1µci/well) and incubated for an additional 16-18hrs. The plates were harvested and counted in the absence of scintillation on a β-plate reader (Packard). Three individual wells were analyzed for each data point.

Antibodies and Flow Cytometry. Cell suspensions from lymph node or spleen or whole blood were incubated with monoclonal antibodies labeled with fluorescein (FITC), R-Phycoerythrin (PE), PE-cyanine 5 (PC5), PE-Cyanine 7 (PC7) and/or PE-Texas Red (PE-TXred) followed by washing and resuspension in PBS + 5%FBS (Hyclone, Logan UT) + 1% Penicillin/Streptomycin (Mediatech, Herndon, VA.). The clones used for these studies were the following; FITC-anti-mouse CD62L (MEL-14), PE anti-mouse-CD8a (53-6.7), PC5 anti-mouse-CD4 (GK1.5), and PC7 anti-mouse CD44 (IM7). Antibodies were purchased from either eBiosciences or BD Biosciences. Listmode data files were collected on a four-color Beckman Coulter XL/MCL using system II software or on a 5-color FC 500/MPL (Beckman Coulter, Fullerton CA)**.** All data sets were analyzed using

FlowJo software (Treestar, Ashland, OR). Cell counts were determined by a label-no wash flow cytometric procedure. Fluorescent beads at a known concentration were added to each sample. From the bead counts, the analysis volume was determined and converted to absolute cell counts.

Treatment Protocol. Agonist rat anti-mouse CD40 monoclonal antibody (FGK115B3) was administered i.p. at 80 ug per dose for 5 consecutive days. Recombinant human IL-2(IL-2), 1.0 $X10^6$ IU/dose was administered i.p. four times per week in two sets of two injections; the second injection in a set being 8-20 hrs from the previous one.

Statistics. Statistical analysis was performed using Prism software (Graphpad Software Inc.) Flow cytometry data were analyzed using Student's t test; a Welch's correction was applied to data sets with significant differences in variance or an ANOVA. A minimum of 3 mice per group were used in all experiments. Experiments were repeated at least 3 times. Data were tested for normality and variance and a p-value of <0.05 was considered significant.

Results

The frequency of naïve T cells drops immediately after immunotherapy but recovers a month later. One month of rest following immunotherapy results in a significant restoration of the percent of T cells expressing a naïve phenotype.

Immunotherapy results in the apparent reduction of naïve T cells with a concurrent expansion of memory phenotype cells. To explore further the long term ramifications of the immunotherapeutic regimen (illustrated in **Suppl. Fig1**) which we found elicited primary anti-tumor responses [ref], we evaluated the phenotypes of splenic T cells of mice following the administration of immunotherapy. These mice were young (8-10wk old) C57BL/6 mice that were still producing T cells in their thymuses. Similar to our previous observations, immediately after immunotherapy was stopped, we observed a significant decrease in the percentage of naïve phenotype ($CD44^{lo}$, $CD62L^{hi}$) T cells and a corresponding increase in the percentage of memory phenotype ($CD44^{hi}$) cells of both, CD4+ (**Fig 1A**) and CD8+ (**Fig 1B**) T cell subsets (2). For example, in Fig.1A the control mice had 68.7% CD4+ naïve T cells while the mice immediately after therapy had only 11.6% naïve CD4+ T cells. Furthermore, we observed that by 42 days after therapy initiation, the percentages of naïve T cells within the CD4+ and CD8+ T cell compartments had returned to levels which resembled those of the control treated mice ((2), **Fig 1**). In Figure 1A, the controls had 61.8% naïve T cells a month after therapy while the mice given therapy had recovered from 11.6% to 53.3%. These data suggest that naïveT

cells are being negatively impacted by immunotherapy, either by being outnumbered by proliferating memory cells or by direct loss of naïve cells (or both). Therefore, we questioned how the therapy would affect T cells from a mouse with limited naïve T cell production. We first tried aged mice, and then used thymectomized mice, to discriminate between selective expansion of memory cells with unaffected (surviving) naïve T cells *vs.* direct detrimental effects of immunotherapy on naïve T cells (that would remain unreplaced by new T cells in the aged or thymectomized mice).

Selection of thymectomized mice as a relevant model for the aged immune system.

Aged animals are more susceptible than young animals to toxicities associated with cancer therapeutics and immunotherapeutics (12, 13). Age is associated with increases in the size of the memory T cell compartment and a decrease in the size of the naïve T cell compartment. Furthermore, most of the demand for immunotherapy in humans is in individuals whose thymic function is severely decreased or even absent, namely adults or elderly. Therefore, to determine how the response to immunotherapy would be different in an aged mouse, we administered immunotherapy to aged (18-22mo) mice. The doses were the same as for young (8-10wk) mice. However, aged mice died of toxicity-related complications within three days of therapy initiation (**fig 2**).

Thymectomized mice have been used as a relevant model for the aged (1, 14). We observed significant decreases in the relative frequency of naïve phenotype T cells within the CD4 and the CD8 T cell compartments between young control *vs.* young thymectomized and young control *vs.* aged mice (**Suppl. Fig2**). Also, similar to previous reports (15), we found there to be a higher number of memory phenotype cells in the spleens of aged mice (data not shown). Thymectomized mice and aged mice also displayed similar impaired proliferative responses to new allo-antigens as monitored by the mixed leucocyte response (MLR) (**Suppl. Fig3**).

We were capable of administering immunotherapy to young thymectomized mice without lethal toxicity (data not shown). These data suggest that immunotherapy in young thymectomized mice would facilitate the investigation of long term alterations of immunotherapy on naïve cells exposed to the immunotherapy.

Naive T cell recovery after immunotherapy is less pronounced in mice lacking a thymus. To address whether the restoration of naïve T cells a month after immunotherapy described in Figure 1 was the product of *de novo* thymic output, we administered anti-CD40 and IL-2 to mice which had received a surgical thymectomy (thmX) approximately 30 days prior to the start of immunotherapy. We observed an overall skewing of the T cell compartment toward a predominantly memory phenotype immediately following anti-CD40 and IL-2 in the spleens of both thmX and unaltered B6 mice (**Fig 3**). For example,

the effector memory and central memory combined CD4 + T cells were 21% for the control mice and 70% for the thymus-intact mice immediately after therapy, and 36% *vs.* 84% for the thymectomized untreated *vs.* anti-CD40/IL-2 –treated We noted that mice lacking a thymus had an increased baseline in the percentage of memory T cells (21% *vs.* 36%). However, naïve T cell recovery in the spleens of thmX mice one month after therapy was less pronounced compared with the unaltered C57BL/6 mice (**Fig 3 C and D**), 14.2% *vs.* 45.9%. These observations were more dramatic in the CD4+ T cell compartment that appeared to have suffered more of a naïve T cell reduction as the result of immunotherapy (**Fig 3A and C**). These data suggest that *de novo* T cell generation by the thymus has at least some contribution to the apparent naïve T cell recovery observed 30 days after immunotherapy. From these calculations, the naïve cells were either reduced in number during therapy or outnumbered by memory T cells with greater proliferation rates. Total naïve T cell numbers in the animals on day 11 in the thymectomized control vs. anti-CD40/IL-2 treated animals are needed to determine if the naïve cells were directly affected.

Naïve T cell loss as a fraction of the population is greater than the loss of naïve T cell numbers.

We used thmX mice to determine if alterations could be observed in the naïve ($CD62L^{hi}$ $CD44^{lo}$) T cell and other cell compartment (Figures 4, 5, and 6 for naïve, central memory and effector memory phenotype T cells). We previously demonstrated that potent immunotherapy results in heightened CD4+ T cell

death (2), so we first focus on loss of naïve CD4 T cells. Indeed, there was a major drop in the both the % (fig 4A) and the number (fig 4C) of naïve CD4 T cells right after therapy. Compare the while and blue bars right after therapy: from 40x to 10?% naïve of all CD4 T cells (1A) and from 3M to 1M total naïve CD4 T cells in the spleen (1B). Consistent with the previously observed pronounced effects on CD4 T cells, while the CD8 naïve T cells decreased in percentage by day 11 of therapy (4C), their total numbers were being largely unaffected (4D) (probably due to proliferation of the memory T cells). A month later, we observed a persistent decrease in the fraction of T cells expressing a naïve phenotype in both the CD4+ (**Fig 4A**) and CD8+ (**Fig 4B**) T cell compartment. This observation was despite a more than five-fold decrease in the percentage of naïve phenotype cells within both T cell compartments. These data suggest that despite naïve T cells decreasing as a result of absent thymic output, immunotherapy had a minimal negative impact on the naïve T cell compartment.

Memory CD8 T cells remain increased in cell numbers after immunotherapy. Memory T cells are a vital part of immunity to pathogens; however an increased fraction of memory T cells is associated with immunological dysfunction in the aged. An evaluation of spleen cells from mice which had been treated with immunotherapy indicated both an immediate and a persistent increase in the total number of central memory CD8+ T cells (figure 5, see 5D for the total number of central CD8 T cells). Interestingly, we noted an inherent difference in central memory phenotype CD4+ versus CD8+ T cells both as a fraction of the T

cell compartment and in absolute numbers (**Fig 5**), with the CD8 cells increasing in numbers (5D) while the CD4 cell recoveries were similar (5C). Central memory T cells are defined by their longevity in addition to their powerful effector functions (16). However, the CD4+ T cell compartment lacked cells of a central memory phenotype both prior to and after the administration of immunotherapy, suggesting that immunotherapy may selectively expand an existing effector memory population.

Both CD4 and CD8 effector and effector memory (EM) phenotype T cells underwent marked expansion in response to immunotherapy. Eleven days after immunotherapy began, analysis by flow cytometry indicated that this memory subset was expanded more than three-fold by immunotherapy, regardless of the presence of the thymus (**Fig 6**). Subsequently, upon examination of spleen cells one month after therapy, we observed a persistent expansion of effector/EM phenotype cells after therapy except in CD4+ T cells from thmX mice (**Fig 6 vs. 6C**), despite significant losses of these cells in number during the month post therapy.

T cell impairment after immunotherapy has immediate and persistent consequences on immune function, even in mice with thymuses. Immediately following immunotherapy we compared the proliferative responsiveness of splenocytes from young (10-14wk) C57BL/6 mice (with

thymuses, Fig &a&B) . We noted immediately following immunotherapy, a complete abrogation in the *ex vivo* proliferative response to allogeneic stimulation of splenocytes from the treated mice (**Fig 7A**). Even at the highest responder responder numbers there was little uptake of radiolabeled thymidine. These data suggest that non-specific expansion of memory T cells correlated with an impairment of proliferative function to new antigens.

We observed a persistent decrease in the proliferative function of splenocytes from C57BL/6 mice with thymuses (**Fig 7B**). Furthermore, ex vivo proliferative responses of splenocytes isolated from thmX mice on day 42 were also reduced (**Fig 7C**). The reduction in proliferative responses after treatment was less severe in thmX mice than in young unaltered mice (**Fig 7 B vs. C**). The T cell proliferative responses were much lower in the control thmX mice, compared to the control mice with thymuses (note the 10 fold difference in the CPM scale). The very poor MLR response of the thymectomized mice given immunotherapy indicates that the MLR requires naïve T cells,since the effector/EM cells were present in the thymectomized mice receiving immunotherapy. These data suggest that immunotherapy results in a sustained reduction in T cell proliferative response to new antigens which may have more important consequences to the generation of new immune responses to infectious agents.

Discussion

The data presented here indicate that an immunotherapeutic regimen which resulted in a more than five-fold expansion of memory T cells did so with comparatively minimal long term impact on the size of the naïve T cell compartment. However, there was a profound initial reduction of CD4 naïve T cells after therapy. The restoration of the numbers of naïve T cells a month after immunotherapy was independent of thymic function. Furthermore, we demonstrated that memory T cells remained expanded one month later and that the ratio of naïve to memory T cells after immunotherapy correlated with proliferative function to new antigens. The reduced function was less pronounced in mice which lacked a thymus however, a small reduction in these mice resulted in a sustained near abrogation of a proliferative response to new antigens. These studies were important in demonstrating that what appeared to be small changes in the T cell repertoire may be very important changes in the absence of a thymus.

Many factors could contribute to the observed correlation between reduced ratios of naïve to memory T cells and reduced proliferative responses to new antigens. First, memory T cells have minimal response to new antigens. The increased percentage of memory T cells in the responder cell populations after immunotherapy may have reduced the number of capable responder cells in the assay. However, adjustment of the R: S ratios to reflect total CD4+ to stimulator

also demonstrated a reduced response curve after immunotherapy (data not shown). Second, intrinsic T cell defects associated with increased time out of the thymus may help to explain reduced function of thmX mice independent of immunotherapy and compounded impairment after immunotherapy. Increased time in the periphery is associated with reduced CD4+ T cell function as determined by proliferation and cytokine production (15). This may be the result of insensitivity to TCR signaling, but the factors leading up to this insensitivity have yet to be described. Additionally, reports which have evaluated how long a CD4+ T cell needs to be away from the thymus to become impaired are lacking. Finally reduced proliferative responses could be the result of regulatory T cells (Tregs) in the splenocytes of treated mice. We have previously demonstrated that Tregs are increased in response to this regiment of immunotherapy (4). While Tregs may be a contributing factor, they are unlikely to have caused the magnitude of impairment that was observed.

Memory and naïve T cells have different sensitivity to cytokines for proliferation (8, 17) and the selective expansion of memory T cells after immunotherapy may be a significant factor in the reduced function to new antigens. Our previous observations, when extrapolated, indicate that after anti-CD40 + IL-2, there is some level of TCR mediated signaling, but that it is limited to conventional CD4+ T cells. This was indicated by the selective upregulation of programmed death-1 (PD-1) on conventional CD4+ T cells (4). PD-1 was first described on T cells after in vitro stimulation through the TCR (18, 19). One subsequent publication has

indicated that PD-1 can be upregulated in response to super-physiological amount of common gamma chain cytokines (20). However, we have found difficulty in reproducing such results through the cultured of splenocytes with high doses of IL-2 or IL-15 (Unpublished observations). Furthermore, we observed that naïve CD4+ T cells alone are reduced in number following the immunotherapy that contained super-physiological IL-2. While anti-CD40 antibody treatment results in a remarkable upregulation of MHC II on B cells and other antigen presenting cells (3), the source of the peptide that would be capable of eliciting CD4+ T cell recognition in this context is unclear.

The results presented here may be especially important in the context of tumor bearing hosts. Tanchot et al. demonstrated in 1997 that transgenic TCR CD8+ T murine cells which have been tolerized to their cognate antigen persisted in the periphery as memory phenotype T cells (7). Additionally, the tolerized cells in this system were still capable of responding to TCR re-stimulation. Upon encounter with their cognate antigen, the tolerized cells responded rapidly and produced large amounts of interleukin-10 (7). It was suggested from these results that persistence and production of anti-inflammatory cytokines may be an important peripheral mechanism to avoid autoimmunity. In mouse models of many cancers, it has been well established that one mechanism of immune evasion for tumors is the induction of immunological tolerance (21). One pathway in which the phenomenon has been extensively studied is the PD-1 pathway (22). These data suggest that immunotherapy regimens may specifically enhance these tolerant

cells with a memory phenotype and this would, in the context of cancer, be strongly counter productive to the immunotherapeutic goal in which long term effector immunity would be needed to curtail remissions and metastases.

The basic observation presented here remains clear; immunotherapy results in a prolonged dampened proliferative response by T cells to new antigens. Furthermore, this correlates with a persistent reduction in the naïve/memory ratio. This effect may additionally have more dire consequences to the immune response in the absence of a young, optimally functional thymus. Deciphering which cell types are responding to stimulation by new antigens and how these cells are being affected by the other cells present would be exceptionally difficult. Naïve T cells acquire a memory phenotype upon activation or homeostatic proliferation (11), making adoptive transfer studies impossible to monitor. Therefore, we evaluated the size of the individual compartments in thymectomized mice. This evaluation helped to gain insight into whether significant conversion of naïve T cells to memory was occurring. We found that the size of the naïve T cell compartment was only slightly altered when compared with the expansion of the memory T cell compartment. Interestingly, the naïve T cell compartment appeared to have a functional defect. These data are especially applicable to cytokine therapy regimens designed for adults or elderly.

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Chapter 2 Figure Legends

Figure 1. Naïve T cells were initially depleted after immunotherapy but the subset recovered a month later. Eight-10wk old C57BL/6 mice were treated with anti-CD40 and IL-2 immunotherapy as described in the materials and methods. The cells illustrated were all CD3+ T cells. Expression of CD44 and CD62L was evaluated by flow cytometry on **A**) CD4+ and **B**) CD8+ T cells from spleens. CD44 and CD62L expression was used to determine naïve ($CD44^{lo}$,$CD62L^{hi}$), central memory ($CD44^{hi}$, $CD62L^{lo}$), or effector memory ($CD44^{hi}$, $CD62L^{lo}$) phenotypes of T cells. The data shown are representative of at least 3 independent experiments each consisting of 3 animals per group.

Figure 2. Aged mice succumbed to immunotherapy-related toxicity. Young (2-2.5 months) or aged (18-22mo) C57BL/6 mice were treated with anti-CD40 and IL-2 immunotherapy. Mice were monitored for toxicity and euthanized if they became moribund. The ata shown are representative of at least 3 independent experiments with 6 mice in the aged anti-CD40 and IL-2 groups and 3 mice in all other groups. Statistical analysis was completed by applying a log-rank test.

Figure 3. Naïve T cells were depleted during immunotherapy and remained depleted in thymectomized mice a month after immunotherapy. Young C57BL/6 mice which were surgically thymectomomized at 6-8 weeks of age or their littermates with intact thymi were treated with anti-CD40 and IL-2 as

described in the materials and methods. Flow cytometry was used to determine the memory subsets as described in figure 1. **A**) CD4+ and **B**) CD8+ splenic T cells from C57BL/6 mice and **C**) CD4+ and **D**) CD8+ splenic T cells from 10-14wk old B6 thymectomized mice. Data shown are representative of at least 3 independent experiments each consisting of 3 animals per group.

Figure 4. Naïve T cell numbers were only slightly decreased after immunotherapy. Young (10-14wk) surgically thymectomized C57BL/6 mice or their unaltered littermates were treated with anti-CD40 and IL-2 immunotherapy. 11 and 42 days after immunotherapy, the percentages and numbers of T cells with a naïve phenotype ($CD62L^{hi}$ $CD44^{lo}$) were determined by flow cytometry. (**A and B**), the fraction of naïve T cells within each subset was determined for **A**) CD4+ and **B**) CD8+ T cells after immunotherapy. (**C and D**), the number of T cells with naïve phenotypes for **C**) CD4+ and **D**) CD8+ T cells in the spleen was determined. Data were collected from of 3 mice per group and are representative of 3-5 independent experiments. Statistical analyses were made using a two-way ANOVA with *P<0.05, **P<0.01 and ***P<0.001.

Figure 5. CD8+ T cells but not CD4+ T cells with the central memory phenotype increased in number after anti-CD40 + Il-2 treatment. Young (10-14wk) surgically thymectomized C57BL/6 mice or their littermates with intact thymuses were treated with anti-CD40 and IL-2 immunotherapy. 11 and 42 days after immunotherapy, the percentages and total numbers of T cells with a central

memory phenotype ($CD62L^{hi}$ $CD44^{hi}$) were determined by flow cytometry. (**A and B**), the percentage of central memory T cells within each subset was determined for **A**) CD4+ and **B**) CD8+ T cells after immunotherapy. (**C and D**), the total number of central memory phenotype was determined for **C**) CD4+ and **D**) CD8+ T cells in the spleen. Data consist of 3 mice per group and are representative of 3-5 independent experiments. Statistical analyses were made using a two-way ANOVA test with *P<0.05, **P<0.01 and ***P<0.001.

Figure 6. Effector memory T cells increased in number during immunotherapy and remained significantly elevated one month after immunotherapy. Young (10-14wk) thymectomized C57BL/6 mice and their littermates with intact thymuses were treated with anti-CD40 and IL-2 immunotherapy. 11 and 42 days after immunotherapy, the percentages and numbers of T cells with an effector or effector memory phenotype ($CD62L^{lo}$ $CD44^{hi}$) were determined. (**A and B**), the percentages of effector/effector memory T cells within each subset were determined for **A**) CD4+ and **B**) CD8+ T cells after immunotherapy. (**C and D**), indicate the number of effector/effector memory phenotype of **C**) CD4+ and **D**) CD8+ T cells in the spleen. Data consist of 3 mice per group and are representative of 3-5 independent experiments. Statistical analyses wwere made using two-way ANOVA tests with *P<0.05, **P<0.01 and ***P<0.001.

Figure 7. The MLR responses of anti-CD40/IL-2 treated mice were compromised immediately after therapy and the deficit persisted one month later in mice with and without thymuses. Anti-CD40 and IL-2 was administered to young (10-14wk) C57BL/6 mice or young (10-14wk) thymectomized C57BL/6 mice. **A**) On day 11 after therapy, spleens were removed and proliferative responses to new antigens was determined in an *ex vivo* mixed lymphocyte culture as described in the materials and methods. (**B and C**) day 42 after immunotherapy, T cell proliferative responses were measured against allo-antigens using spleen cells from both **C**) control mice with intact thymuses and **D**) thymectomized C57BL/6 mice. Data are representative of at least three independent experiments. Statistical analyses in all parts of the figure were made by applying a two-way ANOVA.

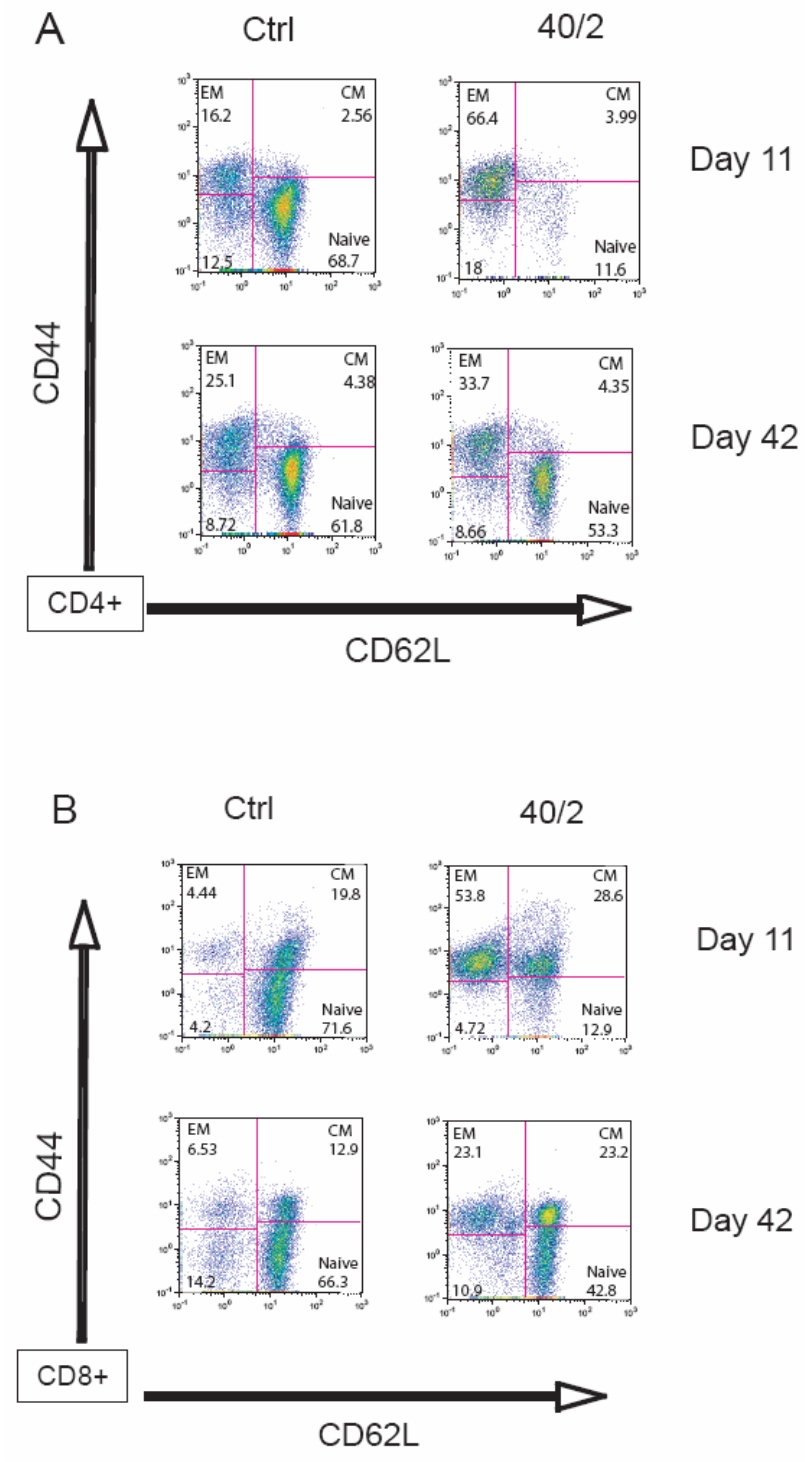
Supplementary Figure 1. The anti-CD40 and interleukin-2 immunotherapy regimen. Anti-CD40 (FGK115.B3) was administered intraperitoneally at 80ug/dose for five consecutive days. Recombinant human interleukin-2 (IL-2) was administered i.p. on days 1, 4, 8 and 11. On days 1 and 4, $1X10^6$ IU of IL-2 was injected twice, with anti-CD40 between the two injections and at least 4 hrs between each injection. On days 8 and 11, $1X10^6$ IU of IL-2 was injected twice with a minimum of 6 hrs between doses.

Supplementary Figure 2. Naïve T cells were reduced in aged and thymectomized mice. Spleen cells taken from young control or thymectomized

(12-14wk) or aged (18-22mo) mice. The splenocytes were analyzed by flow cytometry for the expression of CD3, CD4, CD8 and CD44 on T cells. Memory T cells were classified as having high surface expression of CD44 and naïve T cells were classified as having low surface expression of CD44. Data consisted of 2-3 mice per group and are representative of 2-3 independent experiments. Statistical analysis was completed using a two-way ANOVA with *P<0.05, **P<0.01 and ***P<0.001.

Supplementary Figure 3. Splenocytes from aged and thymectomized mice compared to splenocytes of young mice had lower proliferative MLR responses. C57Bl6 Spleen cells from aged (18-22mo) and young control and young thymectomized (14-18wks) were evaluated for proliferation after allo-stimulation in a mixed lymphocyte culture as described in the materials and methods. Briefly, splenocytes were co-incubated with irradiated allogeneic BALB/c spleen cells for four days and their proliferative responses were measured by the incorporation of 3H-thymidine. Data are representative of at least 2 independent experiments each consisting of 2-3 mice per group. A two-way ANOVA was applied for statistics with * P < 0.05, **P < 0.01 and ***P < 0.001.

Figure 1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CD62L

Figure 2

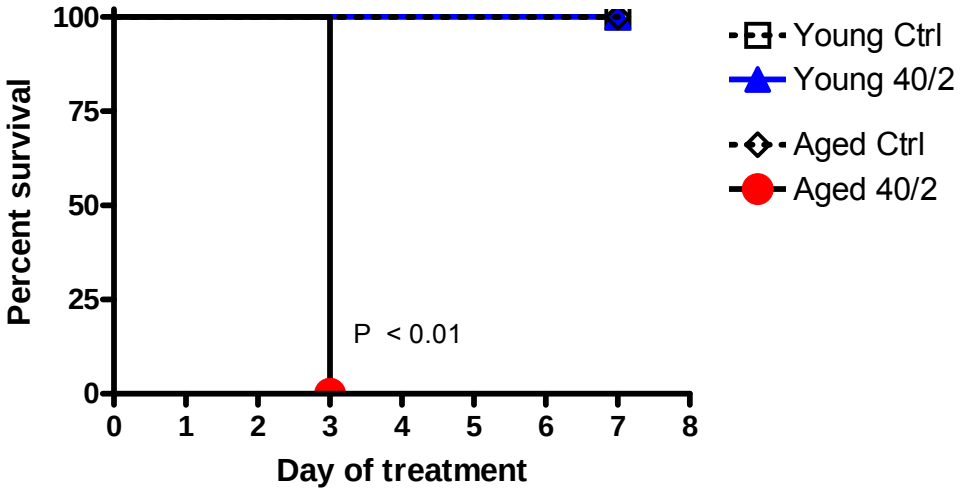

100
75
50
25
0
Percent survival
P < 0.01
0
1
2
3
4
5
6
7
8
Day of treatment
Young Ctrl
Young 40/2
Aged Ctrl
Aged 40/2

Figure 3

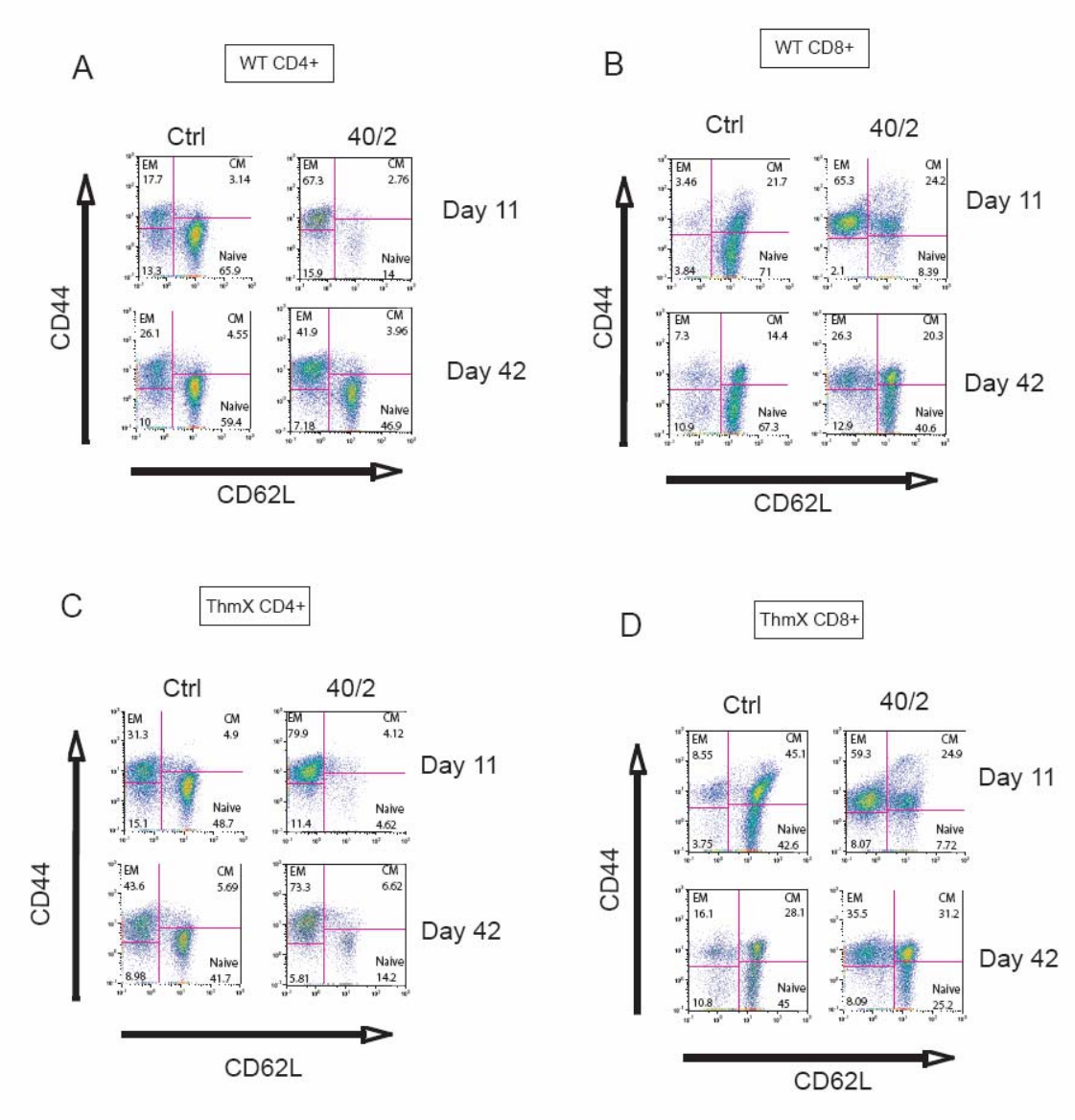

A
WT CD4+
Ctrl
40/2
Day 11
Day 42
CD44
CD62L
EM 17.7
CM 3.14
Naive 65.9
13.3
EM 67.3
CM 2.76
Naive 14
15.9
EM 26.1
CM 4.55
Naive 59.4
10
EM 41.9
CM 3.96
Naive 46.9
7.18
B
WT CD8+
Ctrl
40/2
Day 11
Day 42
CD44
CD62L
EM 3.46
CM 21.7
Naive 71
3.84
EM 65.3
CM 24.2
Naive 8.39
2.1
EM 7.3
CM 14.4
Naive 67.3
10.9
EM 26.3
CM 20.3
Naive 40.6
12.9
C
ThmX CD4+
Ctrl
40/2
Day 11
Day 42
CD44
CD62L
EM 31.3
CM 4.9
Naive 48.7
15.1
EM 79.9
CM 4.12
Naive 4.62
11.4
EM 43.6
CM 5.69
Naive 41.7
8.98
EM 73.3
CM 6.62
Naive 14.2
5.81
D
ThmX CD8+
Ctrl
40/2
Day 11
Day 42
CD44
CD62L
EM 8.55
CM 45.1
Naive 42.6
3.75
EM 59.3
CM 24.9
Naive 7.72
8.07
EM 16.1
CM 28.1
Naive 45
10.8
EM 35.5
CM 31.2
Naive 25.2
8.09

Figure 4

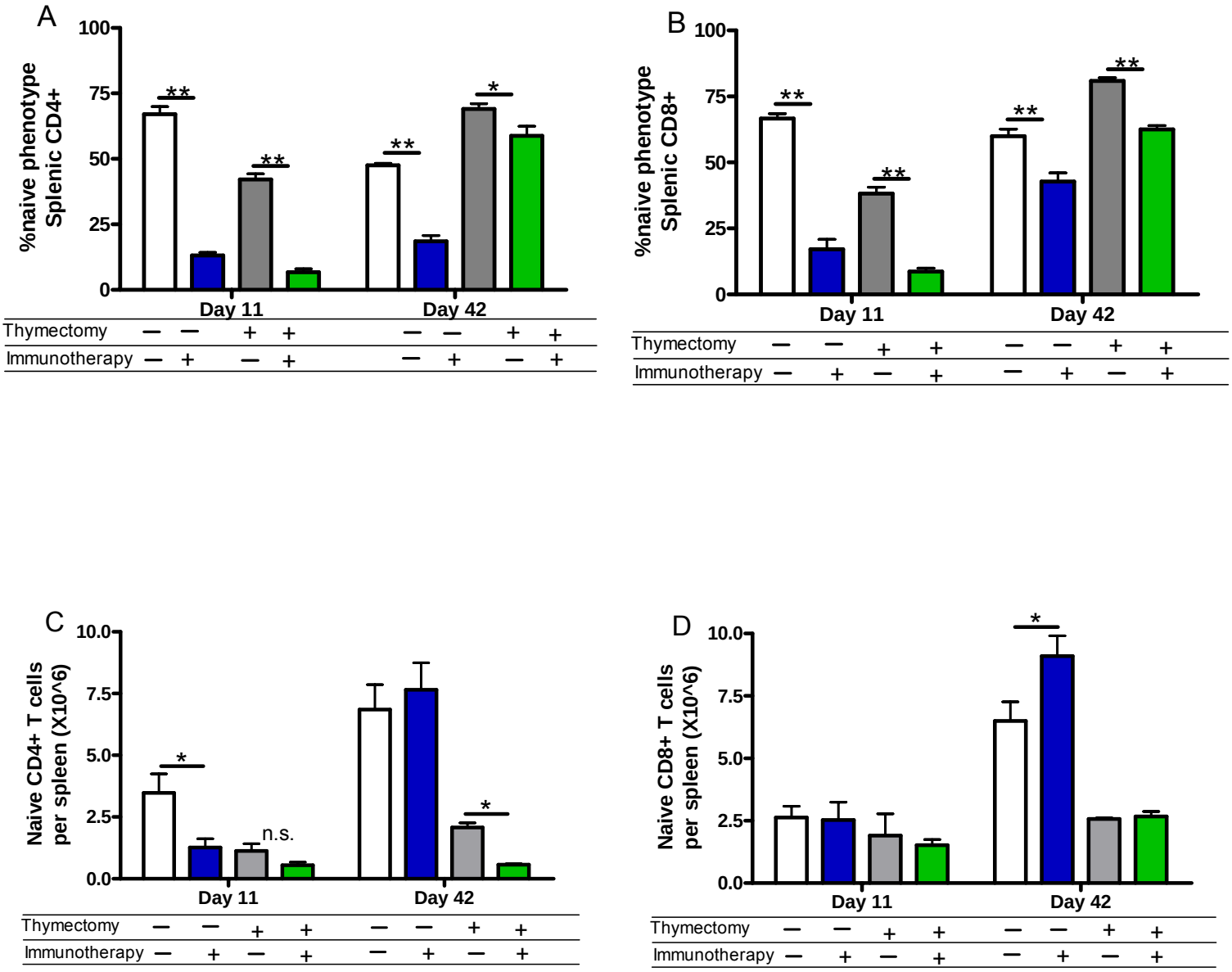

A
%naive phenotype Splenic CD4+
100
75
50
25
0
**
**
**
*
Day 11
Day 42
Thymectomy — — + + — — + +
Immunotherapy — + — + — + — +
B
%naive phenotype Splenic CD8+
100
75
50
25
0
**
**
**
**
Day 11
Day 42
Thymectomy — — + + — — + +
Immunotherapy — + — + — + — +
C
Naive CD4+ T cells per spleen (X10^6)
10.0
7.5
5.0
2.5
0.0
*
n.s.
*
Day 11
Day 42
Thymectomy — — + + — — + +
Immunotherapy — + — + — + — +
D
Naive CD8+ T cells per spleen (X10^6)
10.0
7.5
5.0
2.5
0.0
*
Day 11
Day 42
Thymectomy — — + + — — + +
Immunotherapy — + — + — + — +

Figure 5

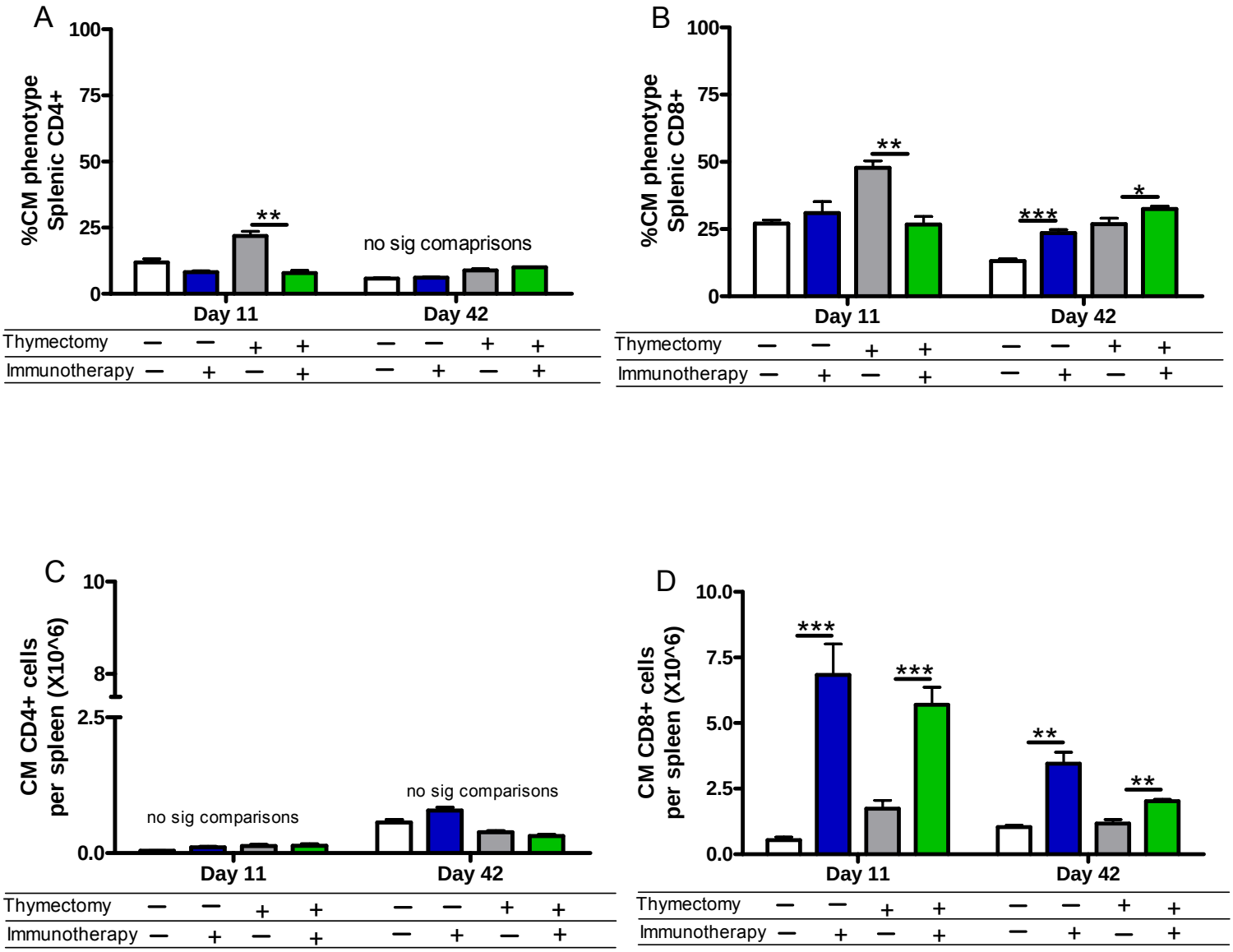

A
%CM phenotype Splenic CD4+
100
75
50
25
0
**
no sig comaprisons
Day 11
Day 42
Thymectomy
Immunotherapy
B
%CM phenotype Splenic CD8+
**
***
*
C
CM CD4+ cells per spleen (X10^6)
10
8
2.5
0.0
no sig comparisons
no sig comparisons
D
CM CD8+ cells per spleen (X10^6)
10.0
7.5
5.0
2.5
0.0
***
***
**
**

Figure 6

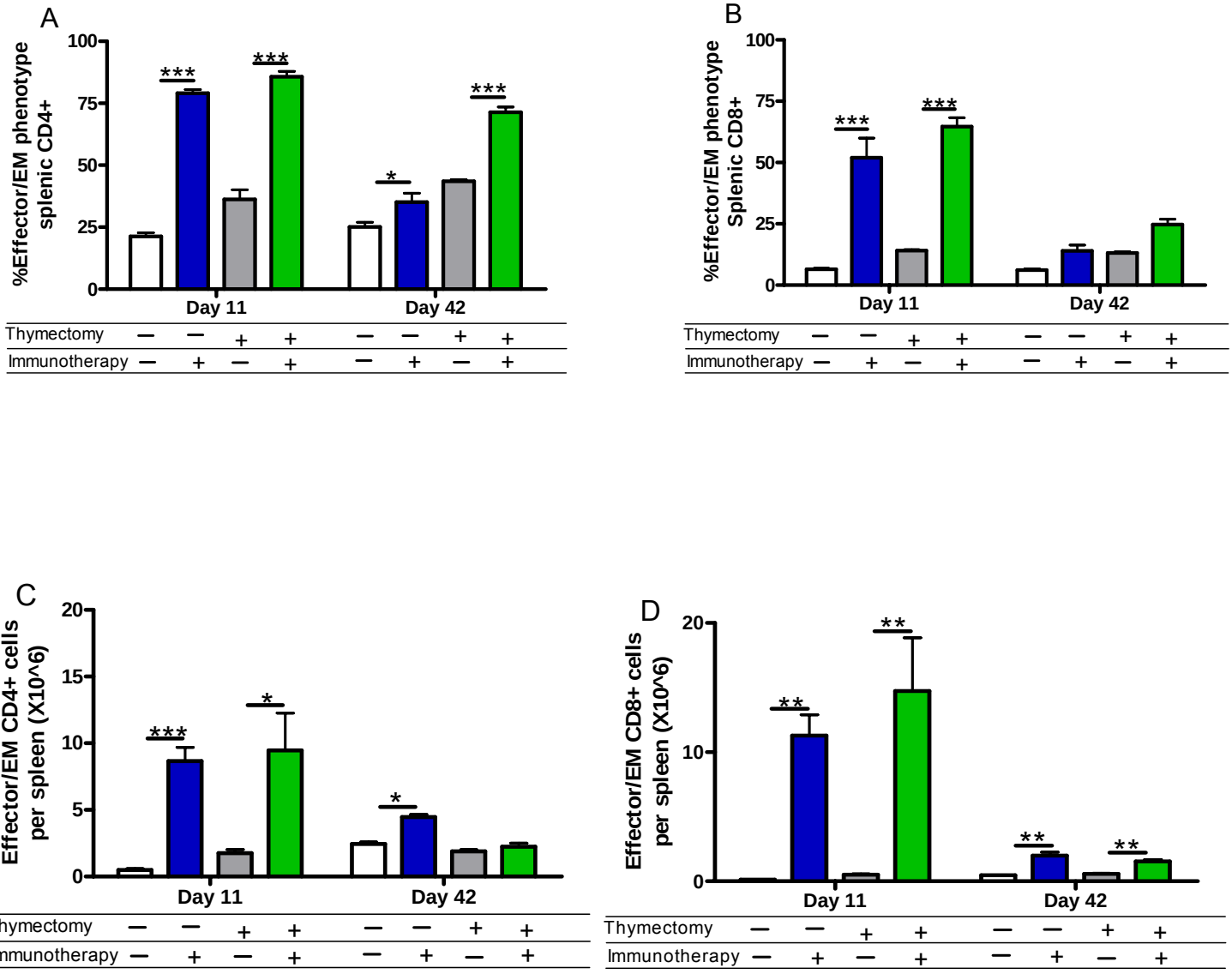
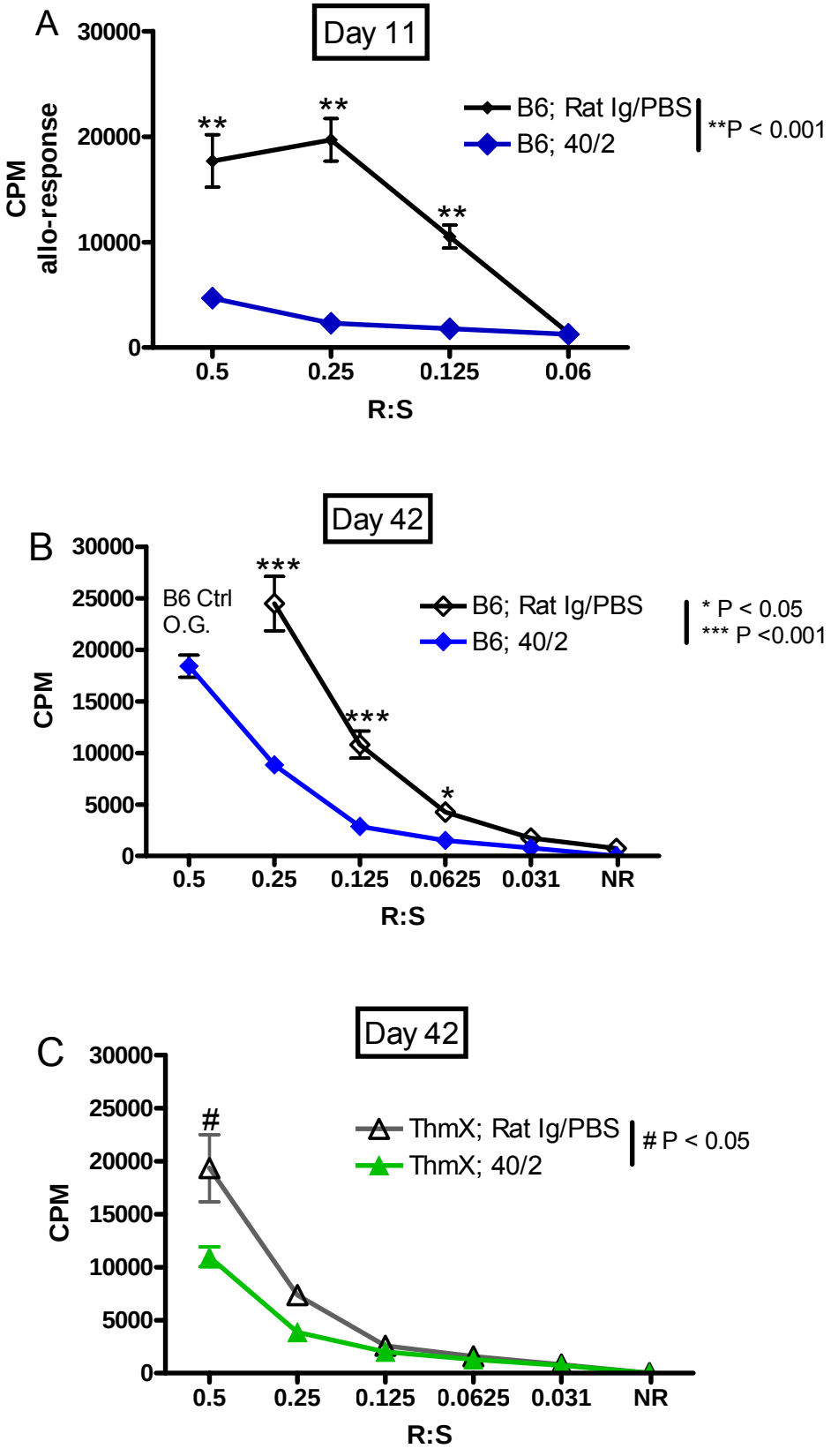
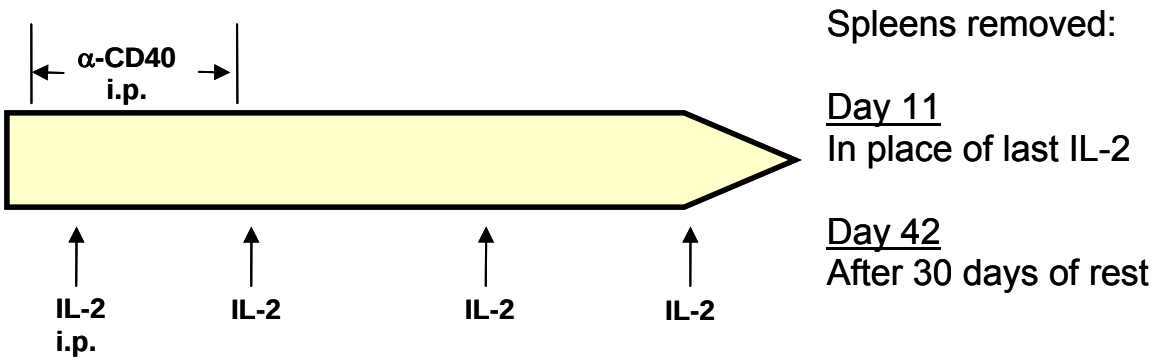

A
%Effector/EM phenotype splenic CD4+
100
75
50
25
0
***
***
*
***
Day 11
Day 42
Thymectomy — — + + — — + +
Immunotherapy — + — + — + — +
B
%Effector/EM phenotype Splenic CD8+
100
75
50
25
0
***
***
Day 11
Day 42
Thymectomy — — + + — — + +
Immunotherapy — + — + — + — +
C
Effector/EM CD4+ cells per spleen (X10^6)
20
15
10
5
0
***
*
*
Day 11
Day 42
Thymectomy — — + + — — + +
Immunotherapy — + — + — + — +
D
Effector/EM CD8+ cells per spleen (X10^6)
20
10
0
**
**
**
**
Day 11
Day 42
Thymectomy — — + + — — + +
Immunotherapy — + — + — + — +

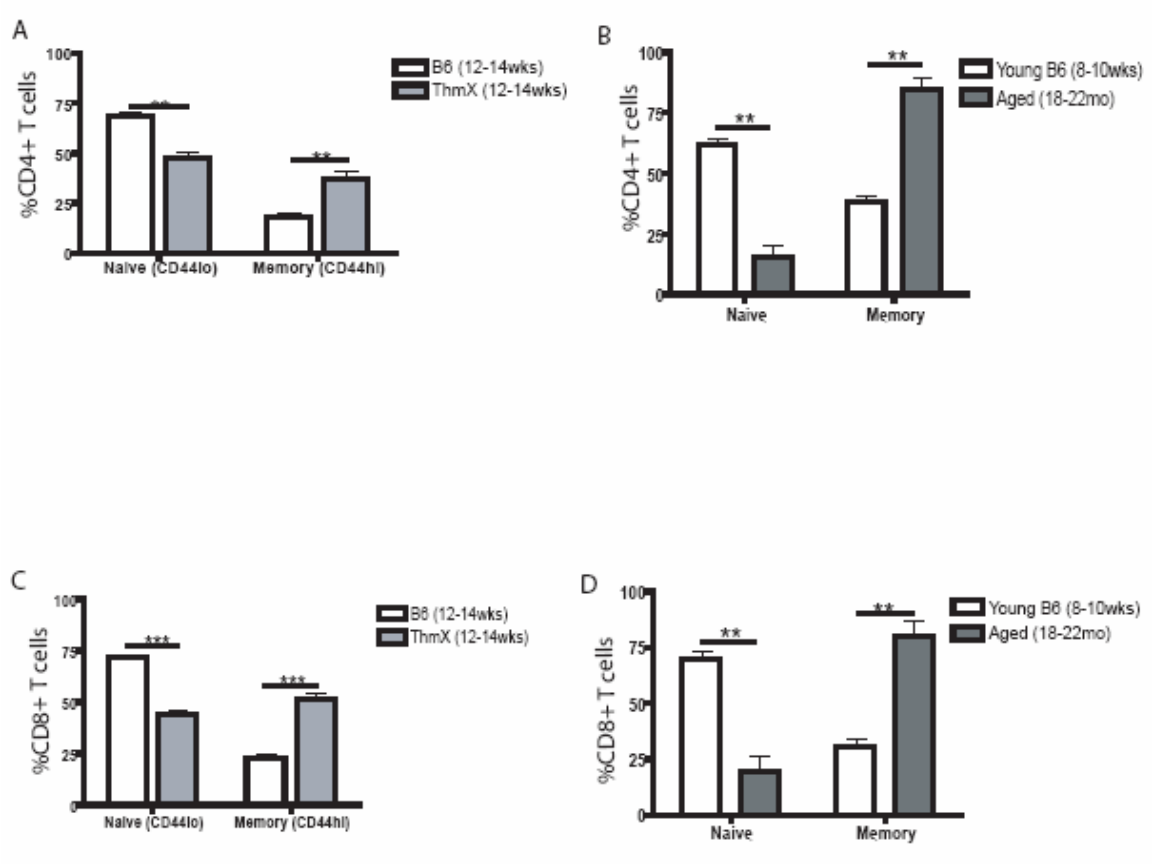
Figure 7


A
Day 11
CPM
allo-response
30000
20000
10000
0
**
**
**
B6; Rat Ig/PBS
B6; 40/2
**P < 0.001
0.5
0.25
0.125
0.06
R:S
B
Day 42
B6 Ctrl
O.G.
***
***
*
B6; Rat Ig/PBS
B6; 40/2
* P < 0.05
*** P <0.001
25000
15000
5000
0.0625
0.031
NR
C
Day 42
#
ThmX; Rat Ig/PBS
ThmX; 40/2
# P < 0.05

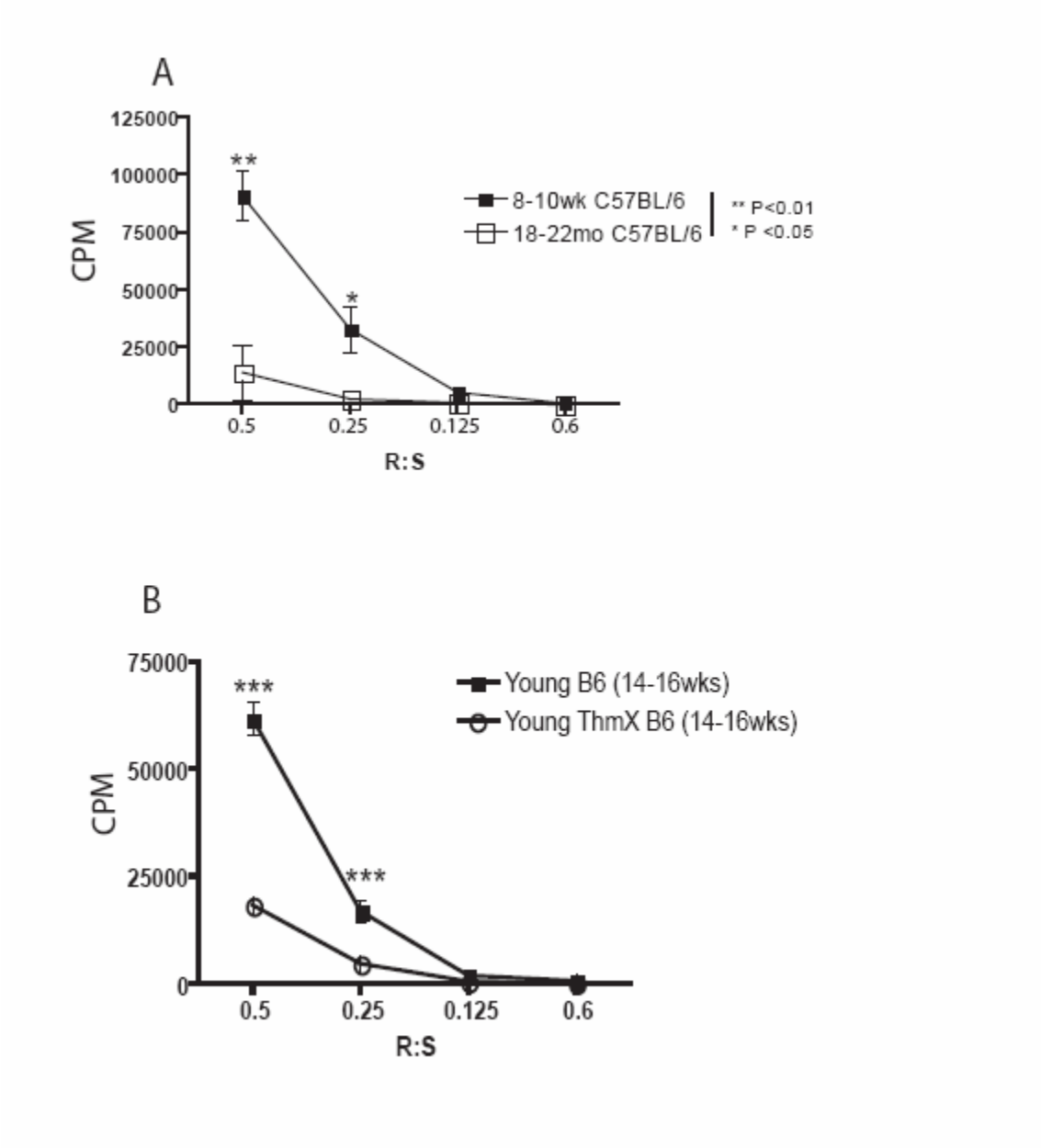
Supplemental Figure 1



Supplemental Figure 2



Supplemental Figure 3



Personal Perspectives

PD-1 is an important inhibitory receptor for the inhibition of T cell responses, but its role in immunotherapy may prove to be much more complex. Within the tumor microenvironment, the ligation of this receptor is most likely instrumental to the production of tolerant tumor-associated (TAA) specific T cells. However, due to increasing reports of similar and potentially compensatory cell surface markers, PD-1 may work in a compensatory manner with other cell surface receptors to inhibit T cell responses. As introduced above, other inhibitory markers such as CTLA-4 and TIM-3 also play key roles in T cell inhibition. This suggests that these receptors may be able to compensate for a blockade of PD-1 signaling. My personal interpretation of this is that cancer immunotherapeutics are fighting an uphill battle against evolutionary pressures against autoimmunity. The intricate systems that have been put into place by evolutionary trial and error are difficult at best to decipher. This is especially true in the context of each individual tumor microenvironment and each therapeutic regimen. However, reports describing the response of individual pathways to immunotherapeutic regimens are important beginnings to understanding how each pathway may be able to compensate for each other.

Blockade of numerous inhibitory pathways simultaneously was a logical next step, but not likely, in my opinion to result in a usable regimen. Autoimmune disease is likely to result, and tumor regression may be a beneficial outcome. However, the removal of multiple pathways may also elicit an unanticipated level of cytokine

secretion by activated T cells. A balance between toxicity and tumor growth would then need to be established. This may result in prolonged tumor survival, but quality of life would be a justifiable question, one that preclinical research is rarely designed to evaluate.

An important question that remains is the functionality of the immune response to new antigens after cancer therapy. This is especially true if the immunotherapeutic regimen is designed to elicit autoimmune-like responses as the mechanism for the desired outcome. In this setting, do self antigens act as consistent stimulation similar to those that would be present during chronic infection? If yes, then how would immunotherapy alter the ability to fight off common viruses which are inevitable pathogens?

Answering the above mentioned questions in a clean immunological setting is difficult. In my experience, alterations in the T cell phenotype, presence, function and response to stimulus are heavily influenced by the presence or absence of a thymus and the duration of said presence or absence. Furthermore, the dynamic properties of immune cells are beyond our current understanding. In a setting where ex-vivo T cell function appears to be almost completely abrogated, response to cytokine stimulus can still result. Responses to infections however may not be as complete, and this is the answer that truly matters.

In the model system described in chapter 2, we used a thymectomized mouse model to answer a question of the long term affect on T cells of potent immunotherapy. However, the observation that resulted was the expansion of memory T cells specifically by immunotherapy. This observation to me was very exciting and could be taken further as suggested in the discussion of that section to include the effect of immunotherapy on tumor tolerant memory T cells. Separating their immediate effector function from their sustained or memory effector function would be of great interest.

Finally is the question of what all of the results presented here mean to cancer therapy in humans. While this question appears easy at first, it is not. This is primarily because of the environment to which the immunotherapy is being applied. For example, as I see it, different tumor types interact differently with the immune environment surrounding them. Different tumors employ different strategies for immune evasion or even promotion. This environment is going to be enhanced through the application of immunotherapeutics. Depending on how the tumor has manipulated this environment, the therapeutic goal may be enhanced or repressed. However, one take away message I would like to leave is that systemic immunotherapy is not selective, and that it is important to know what environment you are going into to be able to best maximize your results.