

**ENHANCED ANTI-TUMOR IMMUNITY BY BREAKING IMMUNE
TOLERANCE**

By

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Dedicated to my family

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Table of Contents

Dedication	ii
Acknowledgements	iii
List of Figures	vi
List of Tables	viii
Abstract	ix

Chapters

1. Introduction	1
1.1 Immune Surveillance.....	1
1.2 T-cell development	5
1.3 T cell tolerance and anti-tumor immunity	7
1.4 APCs and the development of Tregs in the thymus	17
1.5 TRAMP.....	19
2. Targeting Lymphotoxin-mediated Negative Selection in the Thymus to Prevent Prostate Cancer in Mice with Genetic Predisposition.....	21
2.1 Abstract.....	21
2.2 Introduction	22
2.3 Materials and Methods.....	24
2.4 Results	26
2.5 Discussion	30
3. B7 Blockade Alters the Balance between Regulatory T cells and Tumor-reactive T Cells for Immunotherapy of Cancer	44

3.1 Statement of Clinical Relevance	44
3.2 Abstract.....	45
3.3 Introduction	45
3.4 Materials and Methods.....	48
3.5 Results	52
3.6 Discussion	60
4. The Role of Bone Marrow-derived APCs and Thymic Stroma-derived APCs in Regulatory T Cell (Treg) Development.....	78
4.1 Abstract.....	78
4.2 Introduction	79
4.3 Materials and Methods.....	82
4.4 Results	83
4.5 Discussion	90
5. Concluding Remarks	103
References	108

List of Figures

2.1	LT α deficiency prevents clonal deletion of tumor-reactive T cells in the TRAMP mice.....	34
2.2	LT α deficiency inhibits development of prostate cancer	36
2.3	Identification of a time window to avoid lymphocyte infiltration associated with LT β Rlg treatment	37
2.4	LT β Rlg treatment rescued tumor reactive T cells from clonal deletion in the thymus	38
2.5	LT β Rlg reduced apoptosis of transgenic T cells in the TRAMP/TGB transgenic mice	41
2.6	LT β Rlg treatment reduces size of prostate cancer and prevents metastasis	42
3.1	Anti B7-1/B7-2 antibody treatment reduced Tregs in both the thymus and periphery of normal mice and delayed the development of palpable tumors	65
3.2	Anti B7-1/B7-2 antibody treatment enhanced TAg-specific CTLs and partially rescued clonally deleted TAg-specific T cells in the TRAMP mice	67
3.3	Anti B7-1/B7-2 mAbs treatment in mice with established prostate cancer inhibited cancer progression.....	69
3.4	Anti-B7 treatment reduces the number and size of metastatic lesions in the TRAMP mice	71
3.5	Anti-B7 blockade in tumor-bearing mice increased infiltration of T cells into tumors but did not cause autoimmunity.....	72
3.6	Anti B7-1/B7-2 mAbs treatment in mice bearing MC38 colon carcinoma	74

3.7 Anti-CD25 treatments of mice with established prostate cancer inhibited cancer progression	75
3.S1 CD4⁺ cells from the lymphocyte gate express CD3	77
4.1 B7 signal is required for Treg generation.....	93
4.2 B7 expression on bone marrow-derived APCs and thymic stroma-derived APCs in the four groups of chimerical mice.....	94
4.3 B7 expression on bone marrow-derived APCs is sufficient for production of CD4⁺FoxP3⁺ cells.....	97
4.4 B7 signaling is critical for the development of Tregs.....	98
4.5 B7 expression on thymic stroma-derived APCs is critical to Treg function	99
4.6 Impaired function of Treg from group II chimeric mice.....	101

List of Tables

2.1	Inflammation induced by LTβRlg at 4 but not 6 or 11 weeks of age	33
4.1	B7 expression on different APCs in the four groups of chimeric mice ...	
	92

Abstract

The immune system plays a major role in the surveillance against tumors. To avoid attack from the immune system, tumor cells adapt different strategies to escape immune surveillance. Different approaches of immunotherapy have been developed based on the understanding of these strategies.

Due to the random process of T cell receptor (TCR) rearrangement, autoreactive T cells responding to self-antigens are generated during the development of T cells in the thymus. To avoid autoimmune disease, the immune system must be tolerant to self-antigens. Immune tolerance can be divided into central tolerance and peripheral tolerance. Central tolerance is imposed during the development of T cells in the thymus, while peripheral tolerance occurs after T cells are exported into the periphery. Recent studies showed that inducing immune tolerance is one of the major strategies used by tumor cells to escape from immune surveillance. Since tumor cells express self-antigens that can be expressed in the thymus, tolerance to these tumor antigens can be induced in the thymus by clonal deletion. Another important mechanism of immune tolerance involves the function of regulatory T cells (Tregs). Tregs can be produced in either the thymus or in the periphery, but act in the periphery, particularly in the target tissue. The increased number of Tregs found in cancer patients and the existence of tumor specific-Tregs indicates that these cells may suppress the

anti-tumor immune response in the periphery. Therefore, both central tolerance and peripheral tolerance allow tumor cells to avoid an anti-tumor response.

With the understanding of signal pathways involved in immune tolerance, it is possible to break immune tolerance to tumor cells by targeting molecules involved in these pathways and thus, enhance anti-tumor immunity. In chapter II, using the endogenous prostate cancer model TRAMP, we targeted the $LT\alpha$ gene to break central tolerance. Our data demonstrated that both the targeted mutation of the $LT\alpha$ gene and soluble receptor blocking of the $LT\alpha$ protein rescued T cells with high avidity to tumor antigen in the thymus. These treatments also substantially reduced the risk of prostate cancer, with almost complete ablation of metastasis. In chapter III, we targeted the co-stimulatory molecules B7-1 and B7-2 to break both central tolerance and peripheral tolerance. In these studies, both the rescue of tumor antigen-specific T cells and a reduction of Tregs were achieved by anti-B7-1 and anti-B7-2 mAbs. Correspondingly, delayed tumor development was observed in an endogenous prostate cancer model and in a transplanted MC38 tumor model treated with anti-B7 antibodies. In chapter IV, to further understand the development of Tregs in the thymus, we studied the roles of thymic APCs in Treg development. We found both the thymic stroma-derived APCs and bone marrow-derived APCs are required for Treg development in the thymus.

Chapter I

Introduction

1.1 Immune Surveillance

Accumulating evidence supports the notion that the immune system plays a major role in the surveillance against tumors (1-4). Evidence of immune surveillance comes from both animal models and clinical observations. Mice with a wide variety of immunodeficiencies have a high rate of tumor incidence and are more susceptible to transplanted or chemical carcinogen-induced tumors (1, 4). Immunosuppressed patients following stem cell- or organ-transplantation were shown to have a high incidence of tumors (5-7). However, many patients develop cancer even in the presence of an apparently normal immune system. This indicates that tumor cells are able to escape immune surveillance (8). Three distinct strategies adopted by tumor cells to escape from tumor immunity include: 1) lack of recognition, 2) lack of susceptibility and 3) active induction of immune tolerance.

1.1.1 Lack of Recognition

To avoid attack from the immune system, the most efficient way is to shut down the immune response at early steps. Antigen processing and presentation is

an early step needed to prime and activate T cells. It plays an essential role in the initiation of the immune response. Mutations and deletions of genes encoding components of the antigen processing and presentation pathway are commonly observed in tumor cells(9). Because antigens are presented to T cells through MHC/peptide complexes, impaired antigen presentation may be obtained by disruption of the expression of MHC molecules. Indeed, HLA molecules have been shown to be completely lost in several murine and human tumors including cervical-, lung-, prostate-, and renal-cell carcinoma. Molecules important for assembly of the MHC class I molecule, such as β 2m, LMP-2, LMP-7, TAP1 and TAP2 were observed to be mutated or depleted in these tumors(9-12). Alteration of tumor antigen expression is another mechanism for tumor cells to escape from the immune response. It has been shown that some tumor variants completely lose tumor antigen expression and are therefore no longer recognized by the immune system(13, 14). The balance in signaling through activating and inhibitory receptors is able to affect Immune recognition. NK and T cells both express a number of costimulatory and inhibitory receptors. Evidence suggests that tumors may evade T and NK cell recognition by shifting the signaling balance towards inhibition(15-18).

1.1.2 Lack of susceptibility

One of the most efficient ways for immune surveillance to eliminate tumor cells is to kill them. Correspondingly, tumor cells have adopted several strategies to lower their susceptibility to cell death, and thus escape attack by the host. First, tumor cells overexpress inhibitors to molecules critical for inducing apoptosis. For

example, in order to counter the granule exocytosis pathway induced by CTLs and NK cells, the granzyme B inhibitor PI-9/SPI-6 has been found to be overexpressed in several different types of human tumors including melanoma, breast, colon and cervical carcinoma (19, 20). The expression of cathepsin B, a cysteine proteinase that inactivates perforin, was found to be expressed by tumor cells to escape from perforin-mediated killing (21-23). Second, tumor cells can overexpress anti-apoptotic molecules. The FLICE-inhibitory protein (FLIP) acts against apoptosis by binding to the death-inducing signaling complex (DISC) (24). Overexpression of FLIP was observed in melanomas resistant to death receptor signaling(25). An in vivo B cell lymphoma model also proved that immune escape could result from overexpression of FLIP(26). Third, tumor cells have been shown to block death signaling via releasing soluble receptors. For instance, elevated levels of soluble Fas were found in sera from cancer patients and were associated with poor prognosis in melanoma (27, 28). Fourth, tumor cells can lose expression and/or function of death ligands or receptors. Missense mutations in the Fas gene leading to decreased expression of functional Fas have been found in several different tumor types including multiple myeloma, non-Hodgkins lymphoma and melanoma (29-31).

1.1.3 Induction of immune tolerance

One of the central roles of the immune system is to discriminate “self” from “non-self”. To avoid autoimmunity, the immune system has evolved mechanisms of immune tolerance to check and/or delete autoreactive T cells (32-34). Since tumors arise from transformed host cells, it is not surprising that tumor cells make

use of immune tolerance to avoid attack from the immune system. Studies showed that both central tolerance and peripheral tolerance are exploited by cancer cells in order to avoid attack from the immune system. For example, the elimination of high avidity tumor-specific T cells happened in central tolerance and the suppression of tumor response by regulatory T cells happened in peripheral tolerance.

A common mechanism of central tolerance is to deplete autoreactive T cells with high avidity TCR to self-antigens by negative selection in the thymus. During the past decades, a main finding in tumor immunology is that the majority of tumor antigens identified so far may be categorized as self-antigens (35-41). Moreover, tumor antigens are found to be expressed in the thymus (42). Therefore, tumor-specific T cells with high avidity to these tumor antigens might have been depleted during their development in the thymus. Indeed, studies have shown that tumor-specific T cells usually have lower affinity TCR for their tumor antigen than normal T cells specific for an exogenous antigen (43-45). This finding suggested a role of central tolerance in tumor escape from immune surveillance.

Peripheral tolerance is another mechanism reported to be used by tumor cells to escape immune surveillance. Treg is a subpopulation of T cells with immunosuppressive capacity. These cells were first identified as being involved in the prevention of autoimmune disease by maintenance of peripheral tolerance (46-48). Cumulating evidence indicates that Tregs play an important role in preventing anti-cancer immune responses. Elevated numbers of Treg cells have been observed in the blood of cancer patients with malignant effusions (49, 50).

Meanwhile, infiltrated Tregs have been found at tumor sites in animal models and in cancer patients (51). Moreover, direct suppression of tumor-specific T cell immunity has been proved in ovarian carcinomas, in which Tregs were specifically recruited to the tumor site (52, 53). In application, depletion of tumor infiltrating CD4 cells led to the eradication of an established tumor (54, 55). These data demonstrated that Tregs are able to suppress tumor antigen-specific T cells, allowing tumor growth in the presence of tumor antigen-specific immunity.

An increased understanding of the molecular mechanisms of tumor escape has provided a theoretical basis for immunotherapy. In this study, we developed different cancer immunotherapies via targeting signaling pathways involved in the induction of immune tolerance.

1.2 T-cell development

Numerous studies have demonstrated the important role of T cell responses in mediating tumor immunity against tumor cells. In particular, antigen-specific CD8⁺ T cells with cytotoxic activity have been shown to be important effector cells in mediating tumor regression and rejection of established tumors.

1.2.1 T-cell development

T cells are a subset of lymphocytes which play a central role in cell-mediated immunity. The abbreviation 'T' stands for thymus since the thymus is the principal organ in which the T cell develops. T cells can be distinguished from other lymphocyte types by the presence of a T cell receptor (TCR). According to the expression of $\alpha\beta$ TCR and $\gamma\delta$ TCR, T cells can be categorized into two major subsets, $\alpha\beta$ T cells and $\gamma\delta$ T cells. $\alpha\beta$ T cells are the most abundant (95% of T

cells) and the best studied. In this dissertation, unless mentioned, the T cells described only refer to $\alpha\beta$ T cells.

$\alpha\beta$ T cells can be divided into two major subsets, namely helper T cells and cytotoxic T cells. T helper cells express the co-receptor CD4 and can only be activated by peptides presented by MHC II molecules. Once activated, they divide rapidly and secrete various types of cytokines that regulate or help the immune response. Depending on the cytokine signals received, these cells can differentiate into TH1, TH2, and TH17 type cells. Tregs are another subset of CD4 T cells which suppress the proliferation of T cells. Cytotoxic T cells are also known as CD8⁺ T cells due to the expression of the co-receptor CD8 at their surface. These cells only recognize peptides presented by MHC I molecules. Their main function is to destroy tumor cells and cells infected by virus or intracellular bacteria.

The microenvironment in the thymus directs the differentiation as well as the maturation of T cells. The thymus is a multi-lobed organ composed of cortical and medullary areas surrounded by a capsule. Hematopoietic progenitors derived from hematopoietic stem cells in the bone marrow populate the thymus and expand by cell division to generate a large population of immature thymocytes. These progenitors enter into the thymus through post-capillary venules at cortico-medullary junction areas, where they encounter networks of cortical epithelial cells (the thymic stroma) and undergo a period of proliferation. As they differentiate, they move from the outer cortex towards the inner medulla of the thymus. According to the expression of CD4 and CD8 molecules, T cell

development in the thymus undergoes four stages. The early thymocytes express neither CD4 nor CD8, and are therefore classified as double-negative ($CD4^-CD8^-$) cells. As they progress through their development, the TCR β chain is rearranged. Thymocytes with successful rearrangement of TCR β start to express both CD4 and CD8, and become double-positive (DP) thymocytes ($CD4^+CD8^+$). At the DP stage, thymocytes rearrange the TCR α chain. Thymocytes with rearranged TCR β and TCR α chains are then subject to positive and negative selection. Finally, those surviving thymocytes mature to single-positive ($CD4^+CD8^-$ or $CD4^-CD8^+$) thymocytes that are then released from the thymus to peripheral tissues.

1.3 T cell tolerance and anti-tumor immunity

During the development of T cells in the thymus, the TCRs are generated by random rearrangement of their TCR precursors. Part of the TCRs produced during this random process are able to respond to self-antigens. Inevitably, autoreactive T cells with self-responding TCRs are produced during this random process. In order to avoid autoimmune disease, immunological tolerance is induced by the immune system to establish and maintain unresponsiveness to self-antigens. T cell tolerance can be categorized as central tolerance and peripheral tolerance. Central tolerance occurs during T cell development in the thymus, while peripheral tolerance occurs after lymphocytes leave the thymus.

1.3.1 Central Tolerance

The negative selection of T cells that leads to the deletion of thymocytes whose T cell receptors have a 'high affinity' for self antigen is a process known as central tolerance. During T cell maturation in the thymus, T cell precursors

randomly rearrange their TCRs to generate functional TCR. A virtually unlimited repertoire of TCR specificities are created via this random process. Autoreactive T cells with TCRs which respond to self antigen are also produced during this process. To avoid autoimmunity, a self-tolerant TCR repertoire must be obtained in the thymus. Mechanisms of positive selection and negative selection in the thymus are crucial for the shaping of a self-tolerant TCR repertoire. T cells with high affinity TCRs for self-antigens are depleted via negative selection in the thymic cortex. While those with low to medium affinity to self-antigens, but with potency to respond to non-self antigens, are positively selected the medullar and/or cortex and released into the periphery.

Central tolerance is a double-edged sword however. While it protects us from autoimmune disease, it also impedes anti-tumor immunity. Tumor cells take advantage of immune tolerance to avoid attack from the immune system (56-60). A major obstacle to an effective anti-tumor immune response is the development of immune tolerance to tumor antigens, which causes cancer development even in the presence of an apparently normal immune system (42). Recent studies showed that the majority of human and mouse tumor antigens expressed on tumors are also expressed in the normal tissues from which they derive (35-41). Therefore, they are also self-antigens. Although these tumor antigens are often recognized by T cells from cancer patients (61-66), most cancers appear to progress in spite of significantly expanded CD8 T cells specific for the tumor antigens (61, 62). Several recent studies have suggested that responding T cells have a significantly lower avidity for the tumor antigens in an antigen sufficient

host than in an antigen deficient host, indicating that normal expression of tumor antigens promotes the elimination of high avidity T cells (45). It is likely that the mechanisms which tolerize the T-cell repertoire to self-antigens in order to avoid autoimmunity may also negatively impact the ability of these same T-cell specificities to mediate tumor immunity. The high avidity tumor-reactive T cells might have been deleted during negative selection in thymus.

1.3.1.1 AIRE and Central Tolerance

Autoreactive T cells are depleted via apoptosis during negative selection in the thymus. During negative selection, two signals are critical to trigger the apoptotic process, the TCR signal and the costimulatory signal.

In order to elicit optimal TCR signaling, self antigens need to be expressed and presented effectively in the thymus. The expression of self-antigens in the thymus has long been a matter of controversy until recently. For the ubiquitously expressed self-antigens, the expression and presentation of these antigens in the thymus will be able to elicit a TCR signal (32, 34). Thus, T cells with TCRs responsive to these antigens are deleted via negative selection in the thymus. For other self-antigens that are not so abundant and often expressed in a tissue-specific manner, it was assumed that such tissue-specific antigens (TSAs) were unavailable for presentation in the thymus (34, 67, 68). Under this model, it was believed that the TCR signal was missed due to the unavailability of such TSAs in the thymus, and therefore tolerance to such TSAs was precluded from central tolerance and could only be achieved through peripheral tolerance(69). It was found recently, however, that a wide range of peripheral antigens are

expressed in the thymus, such as insulin, glucagons, glutamic acid decarboxylase (38), tyrosine-phosphatase-like protein IA-2, trypsin (70, 71), chymotrypsin, amylase, elastases (72, 73), thyroid hormones, and neuroendocrine molecules (74), as well as unmutated tumor antigens, such as tyrosinase and P1A (75). The expression of these TSAs suggests that central tolerance does cover these peripherally restricted antigens. However, the molecular basis of the expression of such TSAs in the thymus was unclear until recently. The breakthrough came from Mathis and colleagues' work. They demonstrated that these TSAs are expressed on medullary thymic epithelial cells under the control of the Aire gene, through the autonomous "ectopic" transcription/translation of the corresponding genes (76). Aire was initially defined as the underlying gene that mutated in patients with autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED), an autosomal recessive, monogenic disorder characterized by organ specific autoantibodies and multiorgan autoimmune destruction (70). Mice with targeted mutation of Aire develop multiorgan autoimmune disease, implying a lack of tolerance to TSAs (71). Lost function of AIRE results in abrogated expression of peripheral antigens in the thymus. Impaired antigen presentation of thymic medullar epithelial cells was also observed (77). These data clearly established an important role of Aire in negative selection.

Since most of the tumor antigens identified so far are self-antigens, the expression of these tumor antigens in the thymus may result in deletion of tumor-specific T cells with high-avidity TCR to these tumor antigens. Therefore, blockade of central tolerance via manipulating AIRE signaling might be able to

rescue these high-avidity tumor-specific T cells from negative selection in the thymus. To be an effective therapy, a target needs to be susceptible to regulation by drugs. Unfortunately, as a transcription factor that mainly exists in the nucleus, AIRE is difficult to be targeted by large molecular drugs. Moreover, how AIRE regulates the expression and presentation of TSAs in the thymus is still elusive, which makes it difficult to alter its expression pharmacologically.

1.3.1.2 $LT\alpha$ and Central Tolerance

Fortunately, studies from another group recently established a link between AIRE and Lymphotoxin alpha ($LT\alpha$). With the clue that $LT\alpha$ deficient mice have a similar autoimmune phenotype as Aire deficient mice, Fu and colleagues demonstrated that expression of Aire is under the control of $LT\alpha$ (78). Importantly, $LT\alpha$ can be targeted because $LT\alpha$ signaling is mediated through the cell surface receptor $LT\beta R$. The decoy receptor of $LT\beta R$, $LT\beta RIg$, is capable blocking $LT\alpha$ signaling. Therefore, using $LT\beta RIg$ to target $LT\alpha$ signaling is a means to regulate central tolerance, and possibly be able to rescue high-avidity tumor specific T cells from negative selection in the thymus.

$LT\alpha$ was initially identified as a member of TNF family and $LT\alpha$ was originally named $TNF\beta$ (79). Later studies unveiled its critical role in the organogenesis of secondary lymphoid tissues (80). Lymphotoxin exists as two forms, secreted $LT\alpha$ and membrane-associated $LT\beta$ (81). The exact role of secreted $LT\alpha$ remains unknown, while the membrane-associated $LT\beta$ seems to not function by itself. When $LT\alpha$ is co-expressed with $LT\beta$, it forms a membrane-bound heterotrimer consisting of $LT\alpha 1\beta 2$ which binds as a functional ligand specifically to the $LT\beta R$, a

member of the TNF receptor family (81). Interestingly, $LT\alpha$ is expressed by activated lymphocytes, whereas the $LT\beta R$ is mainly expressed by non-haematopoietic and myeloid lineage cells (82-84). This expression pattern indicates that $LT\alpha$ functions as a communication link between lymphocytes and stromal cells, thus suggesting a possible role of $LT\alpha$ in the selection of lymphocytes in the thymus. It has been shown that both $LT\alpha$ deficiency and $LT\beta R$ deficiency are associated with autoimmune phenotypes to peripheral organs (78, 85). Consistently, perturbed tolerance in the thymus was found in these mice (86). These data provide evidence that $LT\alpha$ is involved in negative selection, although the underlying mechanisms remain controversial.

Three possible mechanisms for how $LT\alpha$ regulates negative selection have been proposed so far. First, $LT\alpha$ controls the expression of TSAs in the thymus. Fu and colleagues showed that mice with a targeted mutation of $LT\alpha$ have depressed expression of Aire and peripheral antigens. They also have shown that $LT\alpha$ directs the expression of Aire (78). However, other studies found there is no change in Aire expression in $LT\alpha$ deficient mice (87), challenging this Aire-dependent mechanism. Apart from AIRE, $LT\alpha$ may also modulate expression of other self-antigens that are apparently AIRE-independent. Using Type II collagen (CII) as the antigen, Fu's group found that the central tolerance to antigen is under the control of $LT\alpha$ but not AIRE (88), thus demonstrating the existence of an AIRE-independent pathway. Second, $LT\alpha$ was revealed to control the migration of thymocytes to the thymic medulla via regulation of the expression of thymic medullary chemokines. Deficiency of $LT\alpha$ resulted in

reduced expression of secondary lymphoid tissue chemokine (SLC) and EBV-induced molecule 1 ligand chemokine (ELC) which are required for thymocyte migration to the medulla (89). Reduced migration of thymocytes to the medulla causes impaired negative selection. Third, $LT\alpha$ maintains the structure of the thymic medulla. Defected thymic medulla structure was reported to disturb thymic negative selection. In $LT\alpha$ deficient mice, the architecture of UEA positive thymic epithelial cells (mature thymic epithelial cells) is disorganized (85).

Overall, these data demonstrate that $LT\alpha$ has an important role in negative selection, which pinpoints $LT\alpha$ as a potential target for regulating central tolerance. Moreover, a very interesting finding connected $LT\alpha$ with anti-tumor immunity. Reduced expression of $LT\alpha$ was reported to be associated with a low rate of tumor incidence. An NcoI polymorphism in the first intron of the human $LT\alpha$ gene was found to be correlated with reduced levels of $LT\alpha$ production (90). This polymorphism has been shown to be associated with decreased risk of cancer including prostate cancer, bladder cancer, testicular germ cell cancer, lung cancer, breast cancer, gastric cancer, lymphoma, myeloma and colorectal cancer etc. (91-101). Given the important role of $LT\alpha$ in negative selection, reduced expression of $LT\alpha$ may result in impaired negative selection. Accordingly, high-avidity tumor specific T cells may be rescued from the impaired negative selection, thus enhancing anti-tumor immunity. Therefore, targeting the $LT\alpha$ signal transduction pathway may be an effective immunotherapy towards enhancing anti-tumor immunity via breaking central tolerance.

1.3.1.3 B7 and Central Tolerance

The costimulatory signal is the second signal critical for triggering the apoptotic process during negative selection. The B7-CD28 interaction has been proved to be one of the major costimulatory pathways. Studies from our lab and others support a significant role for the B7-CD28 costimulatory pathway in negative selection (102, 103). Data from our lab demonstrated that perinatal blockade of B7-1 and B7-2 inhibits negative selection of highly pathogenic autoreactive T cells. Impaired negative selection was reported in CD28-deficient mice. Thus, blockade of B7-CD28 signaling might be able to manipulate central tolerance. However, we shall be careful of this idea because the B7-CD28 interaction is also required for T cell activation. T cells may not be activated or revert to an anergy status due to absence of costimulatory signal. A short-window blockade may rescue tumor specific T cells from negative selection and allow for activation after the B7-CD28 interaction is recovered. Besides central tolerance, the B7-CD28 interaction is also involved in peripheral tolerance.

1.3.2 Peripheral Tolerance

Peripheral tolerance occurs after lymphocytes leave the primary organs. T cells with low to medium affinity are able to exit into the periphery whereas high-affinity T cells are deleted via central tolerance. However, these low-affinity T cells that leave the thymus are relatively but not completely safe. Some of them still have TCRs that can respond to self antigens. To check or delete these released autoreactive T cells, the immune system has evolved mechanisms of peripheral tolerance, such as ignorance, anergy, cell death and dominant suppression by Tregs etc.

Ignorance refers to T cells specific for self-antigens present in circulation that are quite capable of making a response but are unaware of the presence of their self-antigen. This arises for two reasons. The first is that the antigen may simply be present at a concentration which is under the threshold to trigger a response. The second reason is that some antigens are sequestered from the immune system in locations which are not freely exposed to surveillance. These are termed immunologically privileged sites, such as the eye, CNS and testis. Except for TCR signaling, naive T cells also need co-stimulatory signals to become activated. However, the expression of these co-stimulatory molecules is restricted so that most tissue cells lack either B7-1/B7-2 or CD40 or both. Such cells also normally lack class II MHC molecules. Thus tissue cells normally present a spectrum of peptides from their endogenously synthesized proteins on self MHC class I in the absence of co-stimulation. Interaction of such cells with autoreactive T cells leads to the T cell becoming refractive to a later encounter with the same antigen even when co-stimulation is present. This refractory state is termed anergy. Cell death triggered via the binding of Fas ligand (FasL) to Fas is another mechanism of peripheral tolerance. Most activated T cells express Fas. Meanwhile, some cells of the body express FasL. When these activated T cells encounter these Fas expressing cells, binding of Fas to FasL triggers their death by apoptosis. For example, cells within the eye always express FasL and are thus ready to kill off any rogue T cells that might gain entry.

Suppression by Tregs is the only dominantly active mechanism in peripheral tolerance. The critical role of Tregs in the maintenance of peripheral

tolerance is demonstrated by the genetic disease IPEX (Immunodysregulation Polyendocrinopathy Enteropathy X-linked Syndrome) in humans and the Scurfy phenotype in mice, in which a mutation in FoxP3 results in severely impaired immune regulation (104-106). Except for the auto-reactive T cells described in previous mechanisms, there are many auto-reactive T cells which are capable of encountering and reacting to their cognate antigen. Tregs actively suppress the activation and expansion of these auto-reactive T cells. Tregs are a subgroup of T cells produced in the thymus. They are characterized by the expression of CD25 and FOXP3 (47). Cumulating evidence indicates that FoxP3 is essential for the differentiation and possibly the function of Tregs. Impaired Treg function or reduced Treg number was found to be associated with diverse autoimmune diseases, including multiple sclerosis, lupus and type-1 diabetes etc. (46-48, 107-109).

Since the majority of tumor antigens identified so far are self-antigens, Tregs may also suppress the immune response specific to these tumor antigens. Cumulating evidence supports a role for Tregs in restrained cancer immunity. For example, cancer patients have elevated numbers of Treg cells in the blood of malignant effusions (49, 50, 110). Treg cells are also recruited and accumulated at tumor sites in animal models and in cancer patients (51, 52, 111). Correlation between the number of CD4⁺CD25⁺ Treg cells in clinical samples of some, although not all cancers led to the hypothesis that Tregs may suppress the effector function of tumor antigen-specific T cells, allowing tumor growth in the presence of tumor antigen-specific immunity. Therefore, reducing Tregs in cancer

patient may be an effective immunotherapy. Consistent with this concept, the removal of CD4⁺CD25⁺ Treg cells by an anti-CD25 antibody promoted rejection of transplanted tumor cells. However, this approach has showed little efficacy in animals with spontaneous tumors, which better reflect the challenge of cancer immunotherapy. In a recent study using a transgenic model of prostate dysplasia, anti-CD25 mAb treatment at 12 weeks of age caused only a 25% reduction in the prostate mass at 20 weeks, although extended observation has not been carried out to document a long term effect. Given that activated T cells also express CD25, administration of anti-CD25 antibody may also deplete activated tumor-specific cytotoxic T cells.

Alternatively, it is worth considering conditions that are selectively required for the generation and maintenance of Tregs. It is now well-established that the B7-CD28 pathway is involved both in the thymic development and peripheral maintenance of Tregs (112). CD28^{-/-} and B7-1/B7-2^{-/-} NOD mice have markedly decreased numbers of CD4⁺CD25⁺ Treg cells in the thymus as well as in the periphery(113). Further studies indicated that B7:CD28 interactions are needed for both development and maintenance of CD4⁺CD25⁺ Treg cells (114). Moreover, as costimulatory molecules, the B7:CD28 interaction plays a significant role in negative selection, although not necessarily for all self antigens. As such, transient blockade of B7-1/2 may reduce Tregs while increasing the frequency of cancer-reactive T cells, thus overcoming the two major barriers to effective cancer immunity.

1.4 APCs and the development of Tregs in the thymus

Treg development within thymus requires the unique interaction of TCR with self-peptide/MHC complex expressed on antigen presenting cells in the thymus (115, 116). Recent studies showed that Tregs are developed from T cells with 'high affinity' TCRs for self antigen during negative selection (47). Therefore, induction of Tregs during negative selection may also be categorized as a mechanism of central tolerance. Antigen presenting cells (APCs) play a critical role in negative selection. There are two major groups of APCs in thymus. One group is the thymic epithelial cells, which are thymic stroma-derived. The other group is the thymic dendritic cells, which are bone marrow-derived. The role of these two groups of APCs in negative selection has been well demonstrated. However, their roles in Treg development remain unclear. Many studies showed that expression of antigen by thymic epithelium is the most effective way of inducing Treg cells (115), which suggests that the thymic epithelium is essential for Treg development. However, antigen expression by hematopoietic cells can be sufficient to induce Treg cells (117). The mature dendritic cells and antigen-processing dendritic cell are also reported to be able to expand Treg cells in the periphery (118). In addition, it was observed that a subpopulation of B7-1 and B7-2 expressing dendritic cells in the thymic medulla is critical for positive selection of high-affinity autoreactive T cells to differentiate into Treg cells (119). Our recent study also supports an important role of dendritic cells (DCs) in the induction of Tregs. It is not surprising to have these controversial results given that these studies focused on one group of APCs while ignoring the contribution of the other group. In order to fully understand the roles of these two group APCs in Treg

development, it is necessary to compare their contribution in one system.

The B7-CD28 pathway has been proven to be involved in both generation and maintenance of Tregs. Meanwhile, B7-1 and B7-2 are mainly present at high levels on professional APCs such as dendritic cells, and thymic epithelial cells (120, 121). Therefore, we were able to compare the contribution of thymic stroma-derived APCs and bone marrow-derived APCs to Treg development by limiting the B7 expression on these APCs.

In chapter IV, we differentiate the roles of thymic stroma-derived APCs and bone marrow-derived APCs in the thymic development of Treg cells via reconstitution of bone marrow chimerical mice by using B7-deficient mice and wild-type B6 mice. Our data indicates that B7 expression on bone-marrow derived cells is sufficient to produce normal numbers of Tregs. However, impaired function of these Treg cells is also observed. Surprisingly, the thymic epithelium, which was thought to be the most important antigen-presenting cells in inducing Tregs, only can induce $\frac{1}{2}$ of the Treg numbers in the thymus. Our results demonstrate that both bone marrow-derived cells and thymic stroma-derived cells are critical for Treg development in the thymus. In order to produce a normal number of functional Tregs, both bone marrow-derived APCs and thymic stroma-derived APCs are required.

1.5 TRAMP

TRAMP is a well established mouse model for prostate cancer with clearly defined progression of prostate cancer that resembles the human disease (122, 123). Metastasis (hematogenous and lymphatic) can be detected as early as 12

weeks after birth (124). By the time the mice are 24-30 weeks old, the prostate cancer becomes palpable in the abdomen. Except for studying cancer development, TRAMP is also a good model for studying immune tolerance to tumor antigen. The TRAMP mouse was made by using the minimal rat probasin promoter regulatory element sequence to target the expression of SV40 large T Antigen (Tag) to the epithelium of the mouse prostate. Functional analysis of TRAMP mice revealed that T cells were tolerant to SV40 Tag. Previous studies from our lab showed that negative selection is the major mechanism for immune tolerance in TRAMP mice(125). Several available lines of TCR transgenic mice specific for SV40 large T Antigen make TRAMP mice a very useful model for studying the effect of immune tolerance on anti-tumor immunity.

Chapter 2

Targeting Lymphotoxin-mediated Negative Selection in the Thymus to Prevent Prostate Cancer in Mice with Genetic Predisposition

2.1 Abstract

Identification of individuals with a genetic predisposition to cancer paves the way for chemoprevention of cancer, where small molecular drugs are used primarily to inhibit pathways of molecular oncogenesis. Lack of enough high affinity of T cells against tumor-associated antigens makes immune prevention difficult. Here we used TRAMP mice that spontaneously develop prostate cancer due to transgenic expression of SV40 large T antigen to test the potential of rescuing high affinity tumor-reactive T cells in individuals with a genetic predisposition to cancer. We report that targeted mutation of the *Lt α* gene rescued T cells specific for SV40 large T antigen and drastically delayed development of prostate cancer, with reduced cancer incidence and ablation of metastasis. Remarkably, short-term treatments with LT β RIg interrupted clonal deletion, reduced the size of primary cancer and completely prevented metastasis later in life without provoking autoimmune diseases. This study established

non-antigen-based immune prevention as a novel avenue to reduce cancer risk for those with a genetic predisposition to cancer.

2.2 Introduction

One of the most important advances in cancer research is the identification of individuals with increased susceptibility (1). Broadly speaking, genetic susceptibility can be conferred by inactive alleles of tumor suppressor genes or by hypermorphic alleles of oncogenes (2, 3). In extreme cases, inactivating mutations of tumor suppressor genes such as p53 (4), APC (5, 6) and BRCA1/2 (7-9) resulted in a nearly 80% life-long cancer risk, although the majority of susceptibility loci have a much less profound effect. Identification of genetically susceptible individuals calls for preventive measures to minimize the life-long cancer risk of these high risk populations.

The notion of chemoprevention was first demonstrated more than 30 years ago (10). Its efficacy has been demonstrated in several large clinical trials (11-13). Generally speaking, the drug or nutritional supplement must be administered repeatedly over the life time of the individual. Therefore, chemoprevention has a high burden of compliance and drug safety. On the other hand, thanks to immunological memory, immunity can last a life time without the stringent requirement of frequent boosting. It is of great interest to determine whether immune prevention can be adopted to reduce both risk and mortality of cancer.

The power of immune prevention is best demonstrated by large scale of prevention of various infectious diseases, including eradication of smallpox. The

classic notion of immune prevention is based on immunization with antigens expressed by the pathogens. Its adoption to cancer is limited by several factors. First, compared with pathogens, cancer antigens are poorly defined, unpredictable and more heterogeneous (14-16), which makes it considerably more difficult to design antigen-based vaccines for the purpose of prevention. Second, since cancers are derived from normal tissues, most of the high affinity T cells reactive to such peripheral tissue antigens in the cancer cells have been deleted (17). Lack of high affinity tumor-reactive T cells would, in theory, make immune prevention difficult to attain.

Recent studies have demonstrated that clonal deletion of T-cells reactive to peripheral antigens depends on their expression in the thymic medullar epithelial cells (18, 19). Since tumors are comprised of malignantly transformed cells from normal tissues and therefore likely express tissue-specific antigens, it is of interest to determine whether these T cells can be rescued for the purpose of immune prevention. Since the lymphotoxin α ($LT\alpha$) gene plays a major role in the development and function of medullary epithelial cells, (20, 21), especially in the context of clonal deletion of peripheral antigen-reactive T cells, blocking this pathway may allow rescue of tumor-reactive T cells and therefore, prevent the development of cancer. Using mice with a targeted mutation of $LT\alpha$ (22), we reveal here a valuable target for rescuing prostate cancer reactive T cells and for cancer immune prevention. More importantly, transient blockade of $LT\alpha$ rescued tumor-specific T cells, significantly reduced the sizes of prostate cancer and eliminated cancer metastasis.

2.3 Materials and Methods

Experimental animals

WT, TRAMP mice expressing the SV40 Tag controlled by rat probasin regulatory elements and $Lt\alpha^{-/-}$ mice, all in the C57BL/6 background, were purchased from the Jackson Laboratory (Bar Harbor, ME). The mice were bred at the animal facilities of the Ohio State University (Columbus, OH) and the University of Michigan (Ann Arbor, MI). Transgenic TGB and TCR-1 mice expressing TCR specific for different epitopes of SV40 large T antigen presented by different MHC loci have been described (23) (24).

Generation of TRAMP mice expressing TGB TCR (TGB-TRAMP) was described (25). $Lt\alpha^{+/+}$ TRAMP, $Lt\alpha^{+/-}$ TRAMP and $Lt\alpha^{-/-}$ TRAMP mice were obtained by breeding $Lt\alpha^{+/-}$ mice with $Lt\alpha^{+/-}$ TRAMP mice. The TCR-1 mice were bred with $Lt\alpha^{-/-}$ mice to obtain $Lt\alpha^{+/-}$ TCR-1 mice, which were crossed with the $Lt\alpha^{+/-}$ TRAMP mice to produce $Lt\alpha^{+/+}$ TCR-1TRAMP, $Lt\alpha^{+/-}$ TCR-1TRAMP and $Lt\alpha^{-/-}$ TCR-1TRAMP mice.

LT β Rlg treatment

For cancer prevention, 6 week old TRAMP mice were treated with 3 weekly injections of 100 μ g LT β Rlg or control IgGFc, intraperitoneally. Treated mice were examined at least weekly for palpable tumor in the lower abdomen. The prostate volume was measured by MRI at 30 weeks. Mice were euthanized at 32 weeks, and internal organs were collected for histology analysis.

For rescue of clonal deletion, 6 week old TRAMP/TGB mice were treated with 3 weekly injections of 100 μ g LT β Rlg or control IgGFc, intraperitoneally. Two

weeks after the last treatment, the mice were sacrificed and the total thymocytes and splenocytes were harvested and stained with fluorochrome-conjugated anti-CD4 (RM4.5), anti-CD8 (53-6.7), and anti-V β 8.1+8.2 (MR5-2) antibodies and analyzed by the LS2 flow cytometer (Becton & Dickinson, Mountainview, CA).

To test potential autoimmune side effects, 4 week, 6 week and 11 week old TRAMP mice were treated with 100 μ g LT β Rlg or control IgGFc every week, for a total of 3 injections intraperitoneally. Two weeks after the last treatment, the mice were sacrificed and peripheral organs were collected. Tissue sections from peripheral organs were stained with hematoxylin and eosin (H&E).

Histology

Mouse organs were fixed with 10% buffered formalin and were paraffin embedded. Tissue sections were stained with hematoxylin and eosin (H&E), and examined under a microscope. All pathological examinations were performed without knowing the treatment and the genotypes of the mice. At least three sections, 25 micron apart, were examined for each organ to ensure comprehensive evaluation.

Magnetic resonance imaging (MRI) of prostate

The progression of prostate cancer in the TRAMP model was measured by MRI as described (26). Briefly, MRI experiments were performed on a Varian system equipped with a 7.0-Tesla, 18.3-cm horizontal bore magnet (300-MHz proton frequency). For MRI examination, the mice were anesthetized with sodium pentobarbital (70 mg/kg intraperitoneally) and maintained at 37°C inside the magnet using a heated circulation water blanket, with pelvis motion (due to

respiration) minimized by a small plastic support placed before insertion into a 3-cm diameter quadrature birdcage coil (USA Instruments). Multislice images were acquired using a T_1 -weighted spin echo sequence (TR/TE = 880/13, field of view = 30 × 30 mm using a 128 × 128 matrix, slice thickness = 1.5 mm, and slice separation = 1.0 to 1.6 mm.). Each set contained 9 to 25 slices and enough sets were obtained to provide contiguous image data of the prostate tumor. Prostate volume was measured using the formula $V=4/3[(D_1+D_2)/4]^3\pi$, where D_1 and D_2 correspond to the longest and shortest (transverse and sagittal) diameter measured from the MRI image. The accuracy of this measurement was confirmed by comparing preneoplasia MRI volumes to postneoplasia actual prostate volumes in select cases.

2.4 Results

2.4.1 Targeted mutation of $LT\alpha$ limits clonal deletion of SV40 T antigen-specific T cells

One of our groups recently demonstrated a critical role for $LT\alpha$ in clonal deletion of T cells specific for tissue-specific antigens (20). As a first test to determine the utility of this pathway in cancer immune prevention, we took a transgenic approach to determine whether this pathway could be exploited to rescue cancer-reactive T cells. We crossed transgenic mice expressing TCRs specific for SV40 large T antigen (TCR-1) (24) to TRAMP mice expressing SV40 large T antigen under the control of the probasin promoter (27), with the null mutation in none, one or two alleles of the $LT\alpha$ gene. The development of the transgenic T cells was evaluated by flow cytometry.

As shown in Fig. 1a, in the TCR-1/TRAMP double transgenic mice, targeted mutation of one or both alleles of the $LT\alpha$ gene resulted in a significant increase in total thymic cellularity. A dramatic increase in % of DP cells and a significant decrease in the % of DN cells was observed among the transgenic TCR⁺ cells. Targeted mutation of both alleles of $LT\alpha$ eliminated the DN population while the DP and CD8 SP subsets expanded (Fig. 1b&c). In addition, the numbers of transgenic T cells were greatly increased in the spleens of $LT\alpha$ -deficient mice (Fig. 1d, e). Remarkably, partial rescue was observed in the heterozygous mice (Fig. 1). Therefore, $LT\alpha$ plays a critical role in clonal deletion of SV40-large T antigen-reactive T cells.

2.4.2 Targeted mutation of $LT\alpha$ inhibits development of spontaneous prostate cancer

To test the role for $LT\alpha$ in the onset of prostate cancer, we measured the size of prostates at 30 weeks by magnet resonance imaging (MRI) (26). Representative images are shown in Fig. 2a, while the summary of the data are shown in Fig. 2b. These data demonstrated that the size of the prostate was reduced by more than 3-fold in the TRAMP mice with either heterozygous or homologous deletion of $LT\alpha$ (Fig. 2b, c). At 34 weeks, the three groups of mice were sacrificed for double blind histology analyses of cancer development and metastasis. As shown in Fig. 2c, 100% of the WT mice developed malignant prostate cancer, with metastasis in 7/12 cases. Among them, one mouse had metastasis in the kidney only, while six others had metastasis in the lung including two that also had metastasis in the liver. In mice with the homozygous mutation,

only 45% (5/11) mice developed malignant tumors. Remarkably, 4/11 mice had normal prostate morphology, while two others had prostate intraepithelial neoplasia (28). Only 1/11 mice had metastasis, in both the liver and lung. A reduction of cancer incidence 13/16 was also observed in the heterozygous mice. Two heterozygous mice had completely normal prostate and one mouse had PIN. Moreover, only 1 in 16 heterozygous mice showed lung metastasis. Since lymph node development is preserved in the heterozygous mice (data not shown), the major reduction of metastasis cannot be attributed to the lack of lymph nodes, which occurred in mice with homozygous $LT\alpha$ mutation (22). X^2 analysis indicated a gene-dose dependent reduction both in rate of malignancy ($P=0.0071$) and metastasis ($P=0.0023$). Taken together, our data presented in Figure 1 and 2 demonstrate that targeted mutations of $LT\alpha$ rescued tumor-reactive T cells and increased host resistance to prostate cancer.

2.4.3 The administration of $LT\beta RIg$ rescues tumor-reactive T cells without provoking autoimmune inflammation

The fact that genetic inactivation of $LT\alpha$ conveys host resistance to prostate cancer raised an interesting possibility that $LT\alpha$ may be targeted for the purpose of immune prevention. Since aged $LT\alpha^{-/-}$ mice developed chronic inflammation, one has to be concerned with potential autoimmune side effects of this treatment (20, 21). In order to achieve this goal, we compared the inflammatory response when mice were treated with 3 weekly administrations of soluble murine $LT\beta RIg$ or Human IgGFc, starting at 4, 6 or 11 weeks of age. The mice were sacrificed 4 weeks after completion of the treatments. As shown in Table 1 and Fig. 3, while

infiltrates in liver and lung were observed in mice after receiving their first dose at 4 weeks of age, no inflammation or tissue injury were observed when the treatment was initiated at 6 or 11 weeks of age.

We have recently reported strong clonal deletion in transgenic mice TRAMP/TGB that both express TCR specific for SV40 large T cells and SV40 large T antigen (25, 29). The clonal deletion was characterized by a massive reduction of CD8⁺Vβ8^{hi} transgenic T cells (25). These features were recapitulated in the double transgenic mice receiving IgG Fc control (Fig. 4a). Interestingly, treatment with LTβRIg resulted in a 6-fold increase in DP cells and a nearly 3-fold increase in the CD8 SP subset (Fig. 4b lower panel and Fig. 4c). Correspondingly, the number of transgenic CD8 T cells was more than doubled in the spleen. In mice lacking the large T antigen, no increase of transgenic T cells in the thymus was conferred by fusion protein (Fig. 4f-h). In contrast the fusion proteins actually reduced the number of transgenic T cells in the thymus. Therefore, the LTβRIg treatment expanded SV40 T antigen-specific T cells only if the antigen was present.

To determine whether LTβRIg prevented deletion of antigen-specific T cells, we compared the % of apoptotic cells by staining with Annexin V. As shown in Fig. 5, LTβRIg significantly reduced the % of apoptotic cells regardless of the subsets of the transgenic thymocytes. This treatment, however, had no effect on apoptosis of T cells in the spleen. Therefore, the increase of transgenic T cells in the TRAMP/TGB mice was likely due to the rescue of T cells from clonal deletion in the thymus.

2.4.4 Short-term treatment with LT β Rlg reduces the progression of primary prostate cancer and prevents metastasis

LT β Rlg binds LT α with high affinity. To test whether LT β Rlg treatment can significantly affect the progression of prostate cancer, we treated TRAMP mice with 3 weekly injections of either LT β Rlg or control IgG, starting at six weeks. At 30 weeks the volume of the prostate was measured by MRI. As shown in Fig. 6a, on average, the LT β Rlg treatment at 6 weeks caused a greater than 50% reduction in prostate volume ($P < 0.01$).

We carried out histological analysis to characterize the effect of LT β Rlg treatment on the development of metastasis. As shown in Fig. 6b, 4 of 7 control-Ig treated TRAMP mice developed metastasis in the lung and/or liver, consistent with previous reports by others (27). Importantly, none of the LT β Rlg treated mice developed metastasis. Moreover, the lack of autoimmune disease was further supported by lack of inflammation in any of the organs studied (Fig. 6b and data not shown). Therefore, transient treatment of LT β Rlg reduced the size of primary lesion and completely prevented metastasis without provoking lymphocyte infiltration into organs.

2.5 Discussion

It is difficult to use a cancer vaccine as preventive measures for those with a genetic predisposition towards cancer development because of the multitude of mechanisms of immune tolerance, including clonal deletion to tissue-specific antigens (17, 25, 29) clonal anergy (30) as well as the unpredictability of tumor antigens (14-16). Here we devised a non-antigen-based strategy of immune

prevention that in theory can be applicable to tumors from a variety of tissue origins. The foundation of the strategy is the critical role of $LT\alpha$ in the clonal deletion of T cells specific for peripheral antigens (20, 21). Using TCR transgenic mice as the basic readout, we have demonstrated that short-term treatments with soluble $LT\beta$ Rlg rescued cancer-reactive T cells that would be otherwise deleted in the thymus. Corresponding to this, we found that TRAMP mice that received short term treatment of soluble $LT\beta$ Rlg at six weeks had significantly reduced tumor sizes at 30 weeks. More importantly, this treatment completely prevented the development of metastasis. Since the targeted mutation of $LT\alpha$ limits clonal deletion of SV40 T antigen-specific T cells and inhibits development of spontaneous prostate cancer, prevention by $LT\beta$ Rlg is likely due to its binding to $LT\alpha$.

It has been demonstrated that transgenic mice expressing SV40 T antigens developed tumors concomitant with the development of T-antigen-specific T cells (31). Therefore, merely priming antigen-specific T cells is insufficient to prevent tumor development. The quality of T cells, such as the antigens recognized and affinity for cancer antigens, also likely matters. Our data presented in this study indicates that blockade of $LT\alpha$ can efficiently prevent deletion of two lines of high affinity transgenic T cells specific for an antigen expressed in a prostate specific fashion as a transgene.

A major advantage of the $LT\alpha$ -blockade based immune prevention is the potential applicability to a number of different cancer types regardless of tumor antigens involved. Although it remains to be tested whether this strategy is

applicable to humans, it is of interest to note the association between $LT\alpha$ polymorphisms and risk of prostate cancer in man (32-34).

Since the prevention is to be applied to high-risk healthy patients, a primary concern is its potential autoimmune side effects. It has been reported that germline mutation of $LT\alpha$ causes multiple organ infiltration (20). Our extensive analysis of the $LT\beta RIg$ -treated mice indicated no lymphocyte infiltration into organs if the treatment was initiated after 4 weeks of age. The side effect when treated at 4 weeks of age was probably due to the more active thymopoiesis that occurs at a younger age. Since treatment at six weeks had significant preventive effect, our data demonstrates that it is possible to identify appropriate windows in which cancer immune prevention can be achieved without overt risk of autoimmune diseases. Taken together, this study has opened a new avenue to develop an immune intervention that prevents cancer development. This approach represents a major departure from the principle of cancer vaccine as it alleviates the need to identify tumor antigens. It is envisaged that subjects that carry high risk alleles may be treated with reagents to block $LT\alpha$ or other critical pathways for tolerance to peripheral antigens in order to reduce their future cancer risk and improve clinical outcomes if they do develop cancer.

Table 2.1 Inflammation induced by LT β Rlg at 4 but not 6 or 11 weeks of age

Organs	Liver		Lung		Kidney		Prostate	
	hlgGFc	LT β Rlg	hlgGFc	LT β Rlg	hlgGFc	LT β Rlg	hlgGFc	LT β Rlg
4W	0/7	6/7	0/7	3/7	0/7	0/7	0/7	0/7
6W	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3
11W	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3

Figure Legend

Figure 2.1 LT α deficiency prevents clonal deletion of tumor-reactive T cells in the TRAMP mice. *LT α ^{+/+}, LT α ^{+/-} and LT α ^{-/-} TCR-1TRAMP* mice were sacrificed at 6 weeks for analyses. Thymocytes (35) and splenocytes (d, e) were harvested and analyzed by flow cytometry, using antibodies specific for V β 7 (transgenic TCR β), CD4 and CD8. All the cells are gated on V β 7⁺ cells except for the right panel in figure b and d. a. Number of total V β 7⁺ cells in the thymus. Data shown are means and SEM of cell numbers (n=8). b, d. FACS plots depicting the distribution of CD4 and CD8 markers among the thymocytes (b) and splenocytes (d). Data shown are from one representative mouse per group and similar data were obtained in 2 independent experiments, involving a total of 8 mice per group. c. e. Number of different subsets of transgenic V β 7⁺ T cells in the thymi (c) and spleens (e). Data shown are means and SEM of cell numbers (n=8).

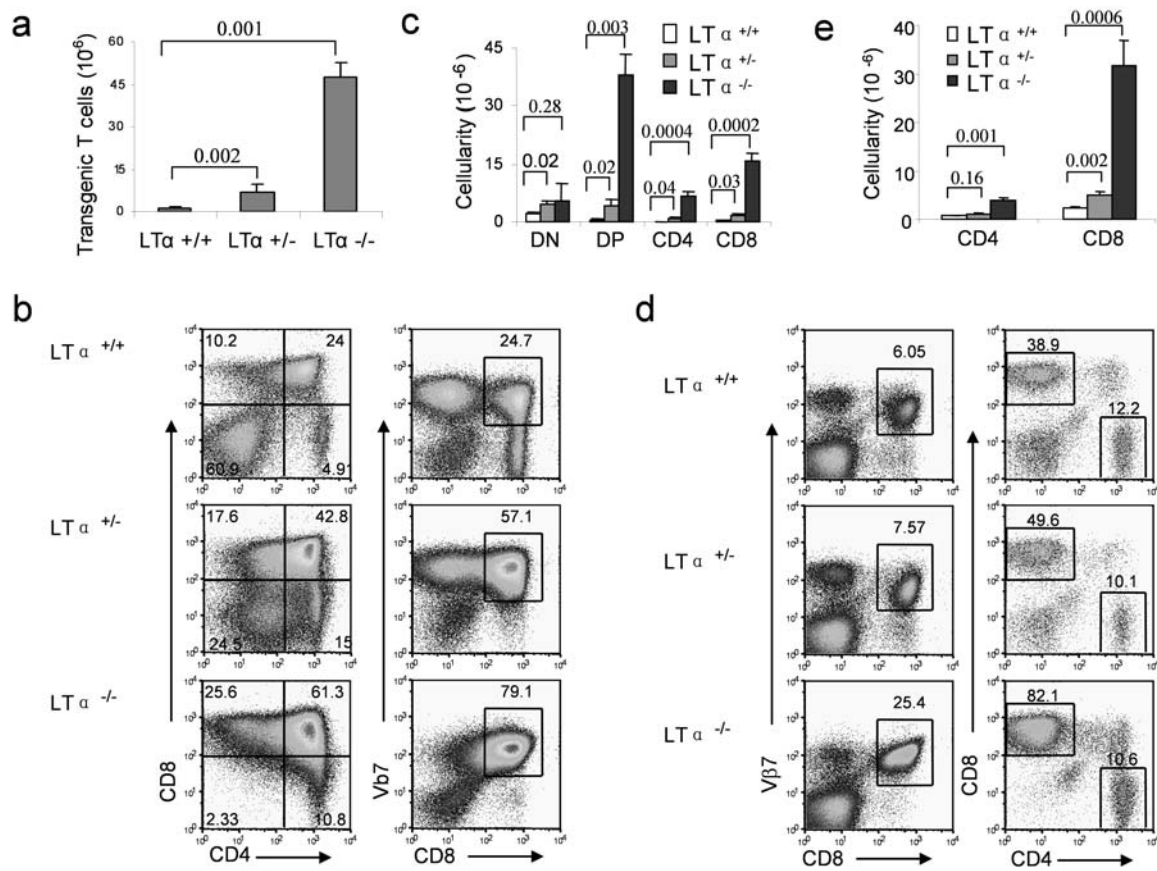


Fig. 2.1

Figure 2.2 LT α deficiency inhibits development of prostate cancer.

The tumor incidence of LT $\alpha^{+/+}$ TRAMP, LT $\alpha^{+/-}$ TRAMP and LT $\alpha^{-/-}$ TRAMP mice were diagnosed by double blind histology examination by two individuals at 34 weeks; while the prostate volumes were measured by MRI at 30 weeks. a. Representative local prostate images of LT $\alpha^{+/+}$ TRAMP, LT $\alpha^{+/-}$ TRAMP and LT $\alpha^{-/-}$ TRAMP mice. The prostate is identified with thick white outlines. b. The prostate sizes of LT $\alpha^{+/+}$ TRAMP, LT $\alpha^{+/-}$ TRAMP and LT $\alpha^{-/-}$ TRAMP mice at 30 weeks of age. c. Targeted mutation of LT α resulted in reduction of prostate cancer incidence and elimination of distal metastasis. The raw data for incidence are provided on top of the bars, while the P value shown in the panels was obtained by X² analyses for gene dose effects. The malignancy and metastasis were diagnosed by two independent and double blind evaluations of at least three slides per organ, including, heart, liver, lung, kidney, pancreas and intestine, 25 microns apart.

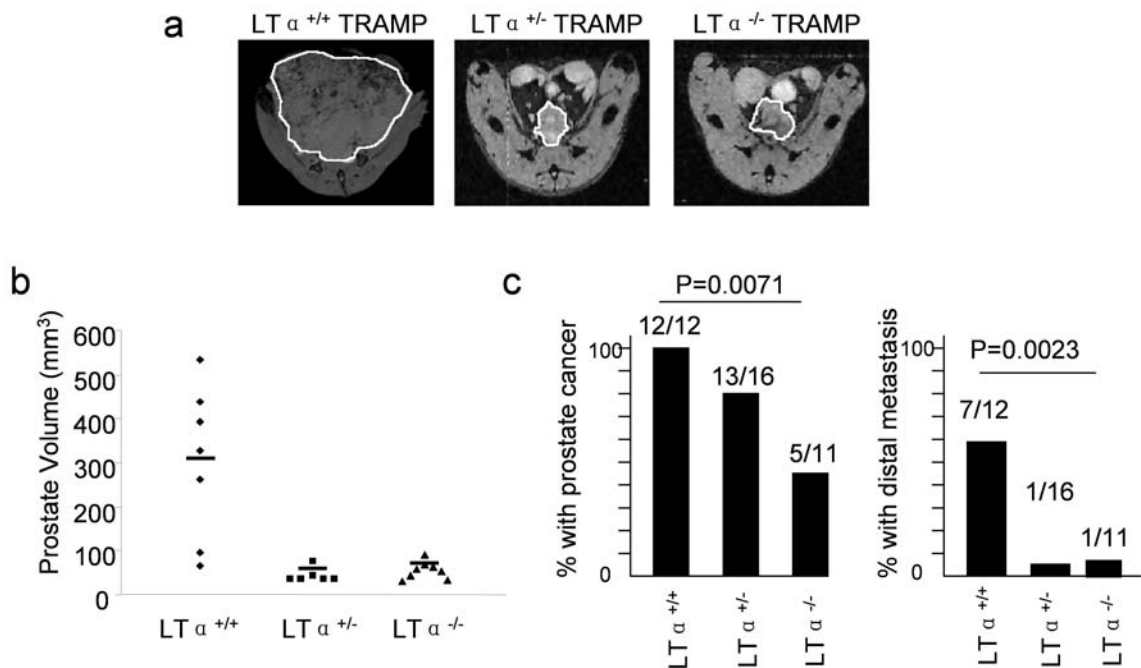


Figure 2.3 Identification of a time window to avoid lymphocyte infiltration associated with $LT\beta RIg$ treatment. 4, 6 and 11 week old C57B6 mice received 3 weekly i.p. injections with 100 μg of either soluble murine $LT\beta RIg$ or Human IgGFc. The mice were sacrificed 4 weeks after the last injection. Peripheral organs were collected for H&E staining. a. Lymphocyte infiltration into the liver was only observed when the treatment was initiated at 4 weeks of age, but not at 6 or 11 weeks. b. Infiltration into lung was only observed if the treatment was initiated at 4 weeks of age.

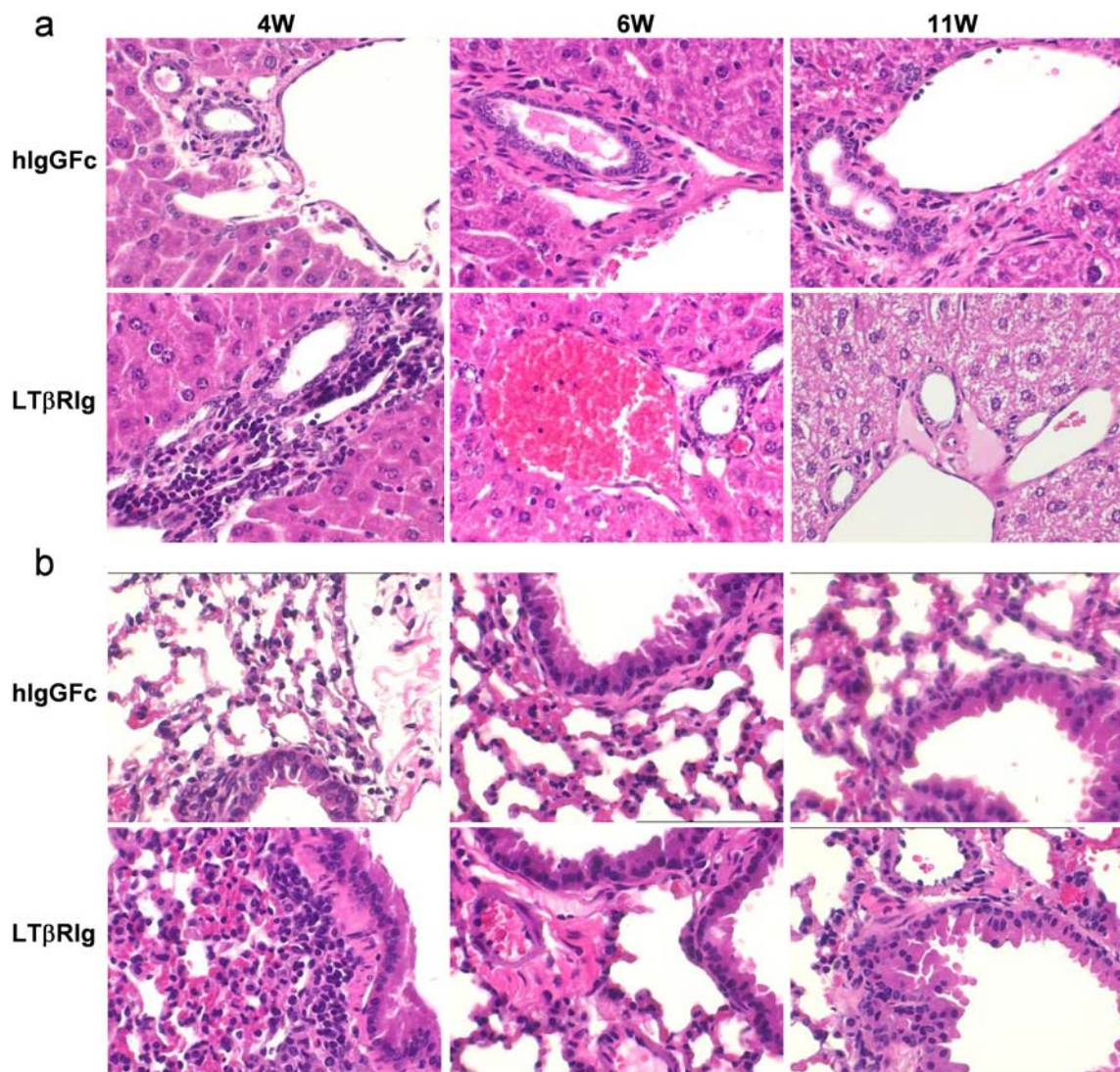


Figure 2.4 LT β Rlg treatment rescued tumor reactive T cells from clonal deletion in the thymus. TRAMP/TGB (a-e) or TGB (e-j) transgenic mice received 3 weekly injections (i.p.) of 100 μ g of either soluble LT β Rlg or Human IgGFc, starting at 6 weeks of age. The mice were sacrificed 2 weeks after the last injection. Thymocytes (a-c, f-h) and splenocytes (d, e, i, j) were harvested and analyzed by flow cytometry using antibodies specific for CD4, CD8 and V β 8. a, f. Number of V β 8⁺ thymocytes. b, d, g, i. Representative plots depicting distribution of CD4, CD8 and transgenic TCR β among thymocytes. Similar data were obtained from two independent experiments, each involving 4 mice per group. The numbers of different subsets of transgenic thymocytes (c, h) and splenocytes (e, j) are presented in bar graphs as means and SEM, involving 8 mice per group.

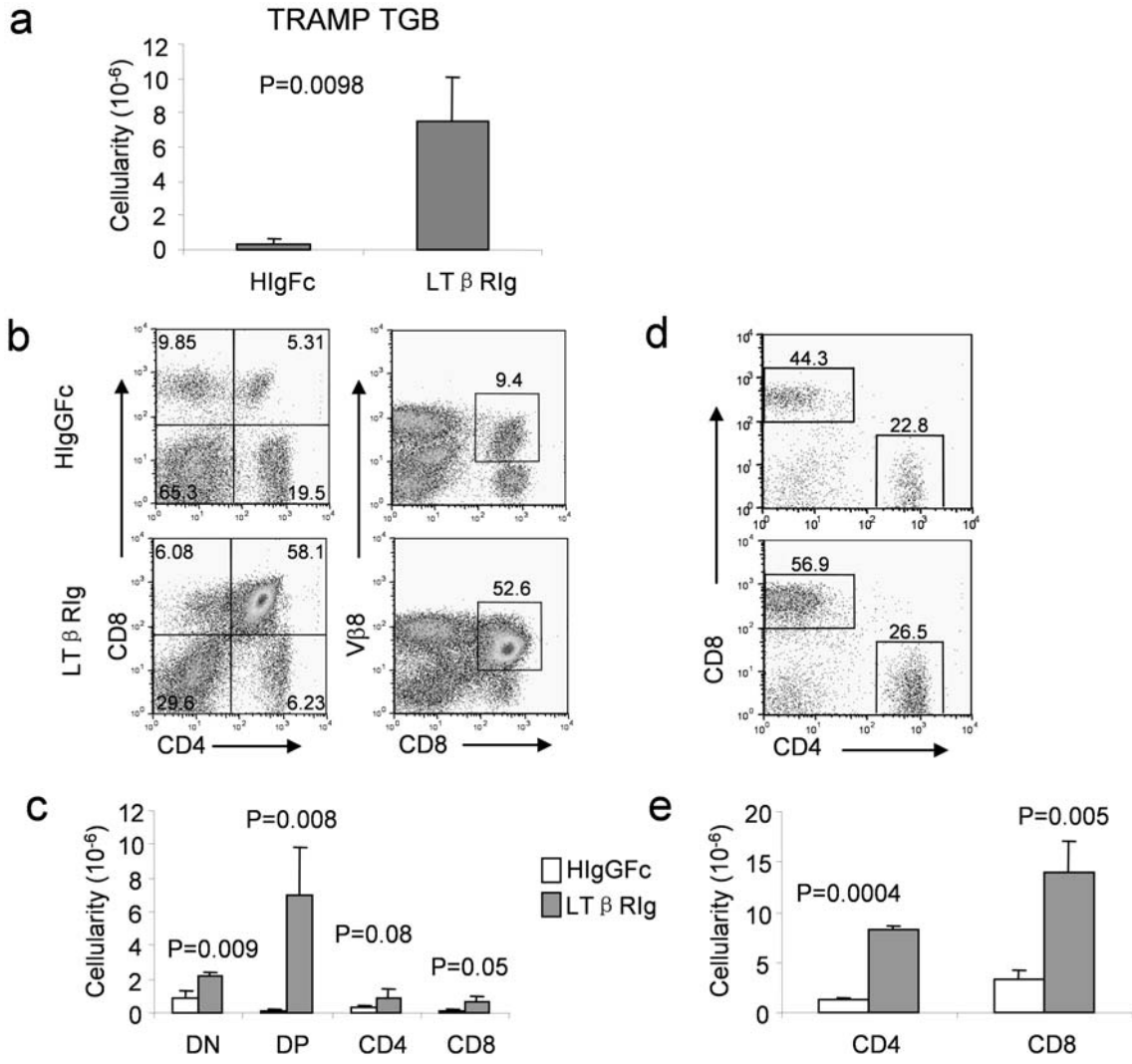


Fig. 2.4 a-e

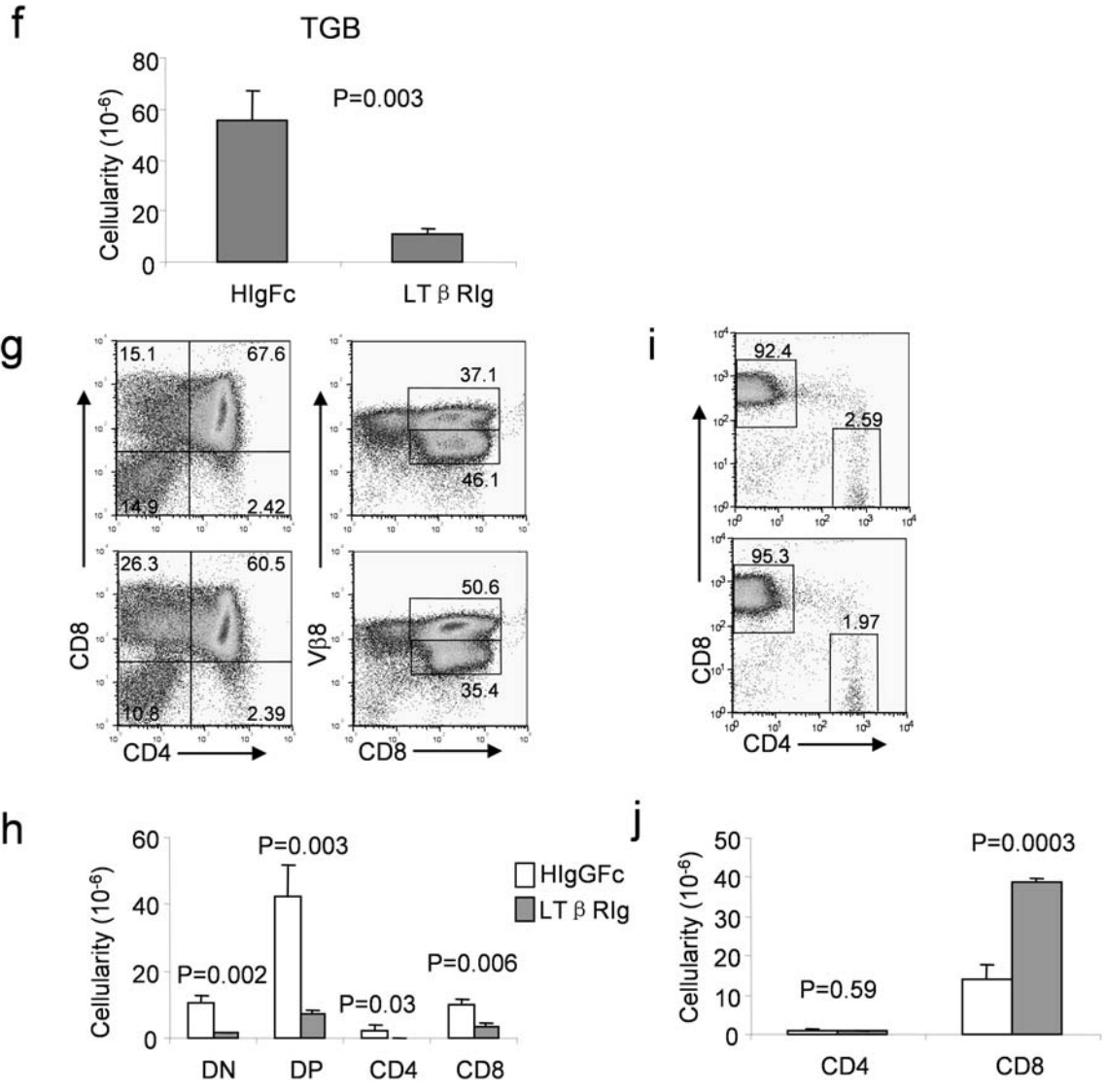


Fig. 2.4 f-j

Figure 2.5 LT β Rlg reduced apoptosis of transgenic T cells in the TRAMP/TGB transgenic mice. Thymocytes and splenocytes of the TRAMP/TGB mice as described in Fig. 4 legends were stained with antibodies against V β 8, CD4 and CD8 in conjunction with Annexin V. All the cells are gated on V β 8⁺ cells except for the right panel in figure b and g. a. LT β Rlg treatment on TRAMP/TGB mice reduced the percentage of apoptotic cells in the thymus, mainly at the DP stage. b. LT β Rlg had no impact on apoptosis of transgenic T cells in the spleen. Plots depict apoptotic cells among different subsets of thymocytes. The numbers in the panels are the means and SEM of the % of apoptotic cells, summarized from two independent experiments, each with 4 mice per group (n=8).

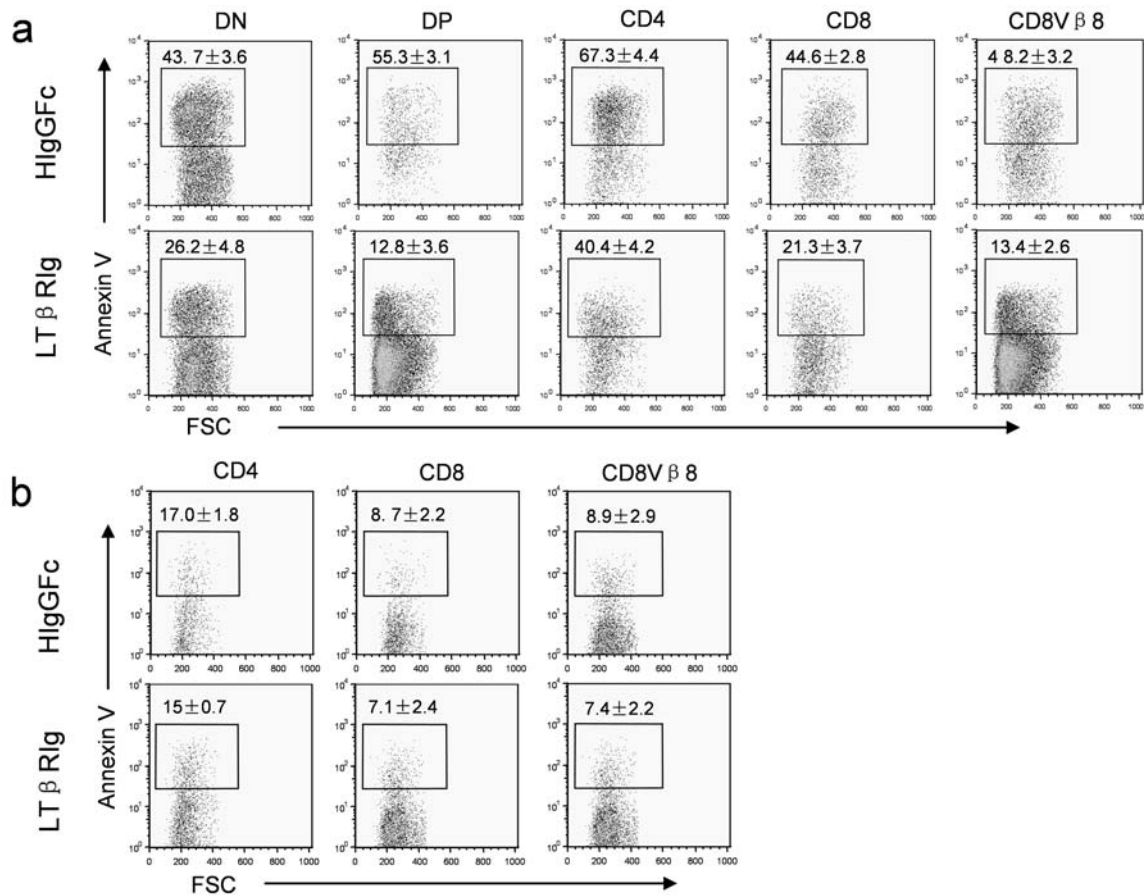


Figure 2.6 LT β Rlg treatment reduces size of prostate cancer and prevents metastasis. a. Prostate volumes as measured by MRI. Male TRAMP mice received 3 weekly i.p. injections with either 100 μ g of soluble murine LT β Rlg or Human IgGFc at 6 weeks of age. The prostate volume was measured at 30 weeks. The upper panels show representative local images of Human IgGFc treated and LT β Rlg treated TRAMP mice. The prostate is identified with thick white outlines. The lower panels depict the sizes of individual prostates (n=7). b. Histological analysis of tumor metastasis. TRAMP mice that received 3 weekly treatments of control Ig or LT β Rlg starting at 6 weeks were sacrificed at 33 weeks after MRI analysis at week 30. H&E sections were examined double blind by a pathologist for metastatic lesions in all internal organs, including liver, lung, kidney, colon, heart and pancreas. Metastases (to the lung and/or liver) were found in 4/7 control Ig treated mice and none in the LT β Rlg-treated mice. The differences in the rate of metastasis was statistically significant (P=0.012).

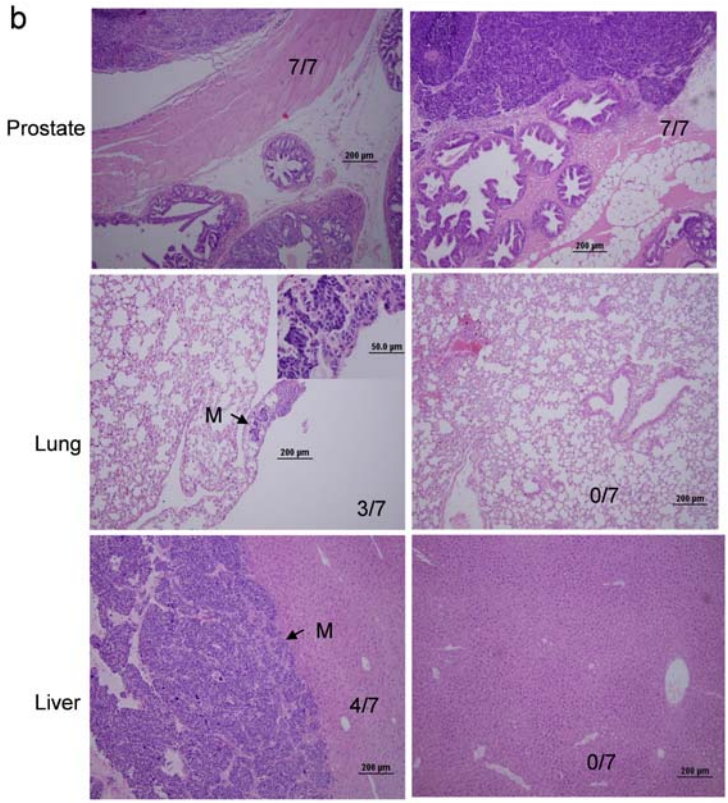
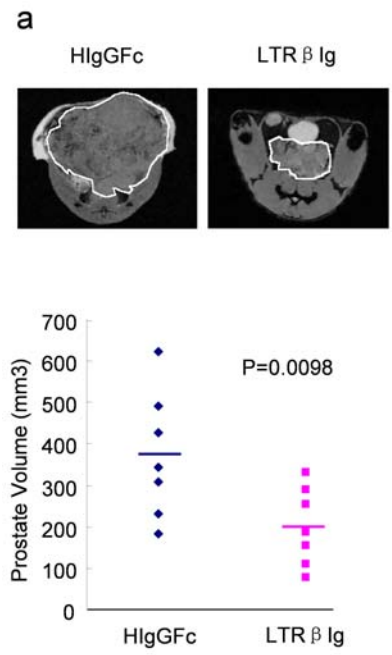


Fig. 2.6

Chapter 3

B7 Blockade Alters the Balance between Regulatory T cells and Tumor-reactive T Cells for Immunotherapy of Cancer

3.1 Statement of Clinical Relevance

Despite the conceptual advances in cancer immunotherapy, clinical development has been slow. Immunotherapy has so far failed to show a clear-cut effect once cancers are established in an advanced stage. Here in this paper, we demonstrated that temporary blockade of B7-1 and B7-2 reduced the number of regulatory T cells and conveyed considerable therapeutic effects in TRAMP mice with spontaneous prostate cancer. To our knowledge, we are the first to demonstrate that prostate cancer in TRAMP mice can be effectively treated after large tumors have been established. Mechanistically we showed that transient blockade of B7-1/2 resets the balance of Tregs and cancer-reactive T cells to confer prevention and therapy of prostate cancer. A second major advantage is that the data can be easily translated into human use as the drug that blocks B7-1 and B7-2 (FDA approved CTLA4Ig) has already been approved for the treatment of autoimmune diseases. It is therefore possible to dramatically shorten the path of clinical development for this novel immunotherapy.

3.2 Abstract

Purpose: In prostate cancer bearing hosts, regulatory T cells restrain the activity of tumor antigen specific T cells. Since B7:CD28 interactions are needed for both the function of CD4⁺CD25⁺ Treg cells and CD8⁺ effector T cells, targeting this pathway may help to overcome immunotherapy barriers.

Experimental design: Anti-B7-1/B7-2 mAbs were administrated to a transgenic mouse model of prostate cancer (TRAMP) ectopically expressing SV40 large T antigen (TAg) in different tumor development stages for prevention and therapy of prostate cancer. The treatment was also tested in transplanted MC38 colon adenocarcinoma in mice.

Results: Here we showed that short-term administration of anti-B7-1/B7-2 mAbs in TRAMP mice leads to a significant inhibition of primary tumor growth and a decrease in the size of metastatic lesions. The treatment is also effective in inhibiting MC38 colon cancer growth. Correspondingly, this treatment results in a transient reduction of Tregs in both the thymus and the periphery. In vivo cytotoxicity assay revealed TAg-specific CTL effectors in anti-B7 treated, but not in control IgG-treated TRAMP mice.

Conclusions: Transient blockade of B7-1/2 alters the balance between Tregs and cancer-reactive T cells to enhance cancer immunotherapy.

3.3 Introduction

Many of the tumor antigens identified so far are self-antigens (37, 157-159) and may therefore trigger immune tolerance. Logically, mechanisms that mediate self-tolerance may contribute to the inadequacy of tumor immunity. The

best characterized mechanism of self-tolerance is clonal deletion (160, 161). In this context, we have demonstrated that a tumor antigen controlled by a tissue-specific promoter is also expressed in the thymus to trigger clonal deletion (147).

In addition to clonal deletion, CD4⁺CD25⁺ regulatory T (Treg) cells play a pivotal role in the maintenance of peripheral self-tolerance (46-48, 107, 109). Accumulating evidence also supports a role for Tregs in restrained cancer immunity. Thus, cancer patients have elevated numbers of Treg cells in the blood of malignant effusions (49, 50, 110). Treg cells are also recruited and accumulated at tumor sites in animal models and in cancer patients (51, 52, 111). Correlation between the number of CD4⁺CD25⁺ Treg cells and clinical outcomes in some, although not all, cancer patients support the hypothesis that Tregs may suppress the effector function of tumor antigen-specific T cells, allowing tumor growth in the presence of tumor antigen-specific T cells (53, 162). Consistent with this concept, the removal of CD4⁺CD25⁺ Treg cells by an anti-CD25 antibody promoted rejection of transplanted tumor cells (54). However, this approach has showed little efficacy in animals with spontaneous tumors, which better reflect the challenge of cancer immunotherapy. In a recent study using a transgenic model of prostate dysplasia, anti-CD25 mAb treatment at 12 weeks of age caused only a 25% reduction in the prostate mass at 20 weeks, although extended observation has not been carried out to document long term effect (55).

Alternatively, it is worth considering conditions that are selectively required for the generation and maintenance of Tregs. CD28^{-/-} and B7-1/B7-2^{-/-} mice

have markedly decreased numbers of CD4⁺CD25⁺ Treg cells in the thymus as well as in the periphery (113, 114, 163). Meanwhile, we and others have reported a significant role for B7:CD28 interaction in clonal deletion of some, although not necessarily all self antigens (103, 164). As such, transient blockade of B7-1/2 may reduce Tregs while increasing the frequency of cancer-reactive T cells, thus overcoming the two major barriers to effective cancer immunity.

TRAMP is a well established mouse model for prostate cancer with clearly defined progression of prostate cancer that resembles the human disease (122). Metastasis to periaortic lymph nodes and lungs can be detected frequently (165). By the time the mice are 24-30 weeks old, the prostate cancer becomes palpable in the abdomen. We have adopted the TRAMP mouse model to test our hypothesis due to the challenge of treating established spontaneous tumors. We report here that transient blockade of B7-1/2 with monoclonal antibodies resulted in temporal deletion of Tregs and rescue of cancer-reactive T cells from clonal deletion. These effects were associated with an increased effector function of cytotoxic T lymphocytes. Remarkably, this relatively simple treatment conferred prevention and therapy of spontaneous prostate cancer and transplantable colon cancer. Since recombinant protein that blocks B7-1 and B7-2 has already been approved for human use, the path for translating our observation into patient care is considerably shorter than most therapeutic approaches.

3.4 Materials and Methods

Experimental animals

C57BL/6 mice and TRAMP mice expressing the SV40 Tag controlled by rat probasin regulatory elements in the C57BL/6 background were purchased from the Jackson Laboratory (Bar Harbor, ME). The mice were bred at the animal facilities of the Ohio State University (Columbus, OH) and the University of Michigan (Ann Arbor, MI). All animal experimental procedures were reviewed and approved by The Ohio State University and University of Michigan Institutional Animal Care and Use Committees. Mice were typed for SV40 Tag by isolation of mouse tail genomic DNA. The PCR-based screening assay was described previously(147). Transgenic mice expressing TCR specific for SV40 large T antigen (TGB) have been described (145). Generation of TRAMP mice expressing TGB TCR (TGB-TRAMP) was also described(147).

Antibody treatment of the TRAMP mice

TRAMP mice were treated with anti-B7-1 and anti-B7-2 antibodies at two different stages. In the first regiment, 4-6 week old TRAMP mice were injected intraperitoneally with 5 injections of anti-B7-1 (Rat anti-mouse CD80, clone 3A12, (166)) and anti-B7-2 (Hamster anti-mouse CD86, clone GL1, ATCC, (167)) antibodies or control hamster/rat IgG (Sigma) at 100 µg/antibody/injection every other day. Long term prostate cancer incidence was recorded by physical examination. In the second regiment, 25 week old TRAMP male mice without palpable prostate cancer were treated intraperitoneally with the anti-B7 or control IgG at 100µg/antibody/injection for 5 injections every other day. The MRI

examination was carried out before treatment and 8 weeks later at the age of 33 weeks. In a separate experiment, 25 week old mice were treated with one intraperitoneal injection of 1 mg anti-CD25 (PC61) or control rat IgG (168). The efficiency of anti-CD25 depletion was examined by flow cytometry with staining of PBL using conjugated anti-CD4, anti-CD25 (clone 7D4, ATCC) and anti-Foxp3. The MRI examination was carried out before treatment and 5 weeks later at the age of 30 weeks. For long term prostate cancer incidence study, anti-B7 and control IgG treated mice were examined at least weekly for palpable tumor in the lower abdomen, and were euthanized when they either become moribund or when the tumor size exceeded 5% of the body weight.

6-8 wk old TRAMP or TRAMP/TG-B mice were sub-lethally irradiated (500 Rad) on day 0 and the treatment started on day 1 with either anti-B7-1/2 mAbs (100 µg/each) or control rat/hamster IgG (100 µg/each) intraperitoneally. The mice were treated 6 times every other day. One week after the last treatment, the mice were sacrificed and the total thymocytes and splenocytes were harvested and stained with fluorochrome-conjugated antibodies anti-CD4 (RM4.5), anti-CD8 (53-6.7), anti-Vβ8.1+8.2 (MR5-2) (BD, San Diego).

For the transplantable tumor model, MC38 murine colon carcinoma cells were grown in RPMI medium with 5% FBS and subcutaneously injected to male C57BL/6 mice (5×10^5 /mouse). 10 day after injection, mice were divided evenly into two groups based on the tumor sizes, and administered either anti B7 or control IgG 3 times i.p. every other day. Peripheral blood was collected at 0, 6 days (0 day is the day before the administration of antibodies) and the

splenocytes were collected at 14 days and stained with anti-CD4, CD8, CD25 and Foxp3 antibodies (BD),

Proliferation of T cells to antigenic peptides

Total spleen cells (3×10^5 /well) from control Ig or anti-B7-treated TRAMP x TG-B (H-2^{b^xk}) F₁ mice were cultured with the given concentrations of SV40 Tag K560–568 peptide or control HSV gB peptide in Click's Eagle-Hank's amino acid medium for 72 h. The proliferation of T cells was determined by incorporation of [³H] thymidine (TdR) pulsed (1 μ Ci/well) during the last 6 h of culture. The data presented are means of triplicates with variation from the means <15%.

Peptide synthesis

All peptides used were synthesized by Research Genetics, Inc. (Huntsville, AL). The peptides were dissolved in dimethyl sulfoxide (DMSO) at a concentration of 10 mg/ml and diluted in PBS or culture medium before use. Peptides used were SV40 Tag 560-568 SEFLLEKRI (147) and HSV gB peptide gB498-505: SSIEFARL (169).

Immunohistochemistry

Mouse organs were fixed with 10% buffered formalin. Tissue sections were stained with hematoxylin and eosin (H&E), and examined under a microscope. Frozen sections were prepared and stained with 2 μ g/ml of antibodies specific for CD3 (2C11, hamster IgG). CD3 positive foci were counted using 20X microscope visual fields.

In vivo cytotoxicity assay

Spleen cells from C57BL/6 mice were pulsed with 10 μ g/ml of either SV40 Tag

560-568 SEFLLEKRI or a control peptide HSV gB 498-505 SSIEFARL in the presence of either 0.5 mM or 5 mM of CFSE, respectively. After mixing at a 1:1 ratio, the labeled cells were injected intravenously into recipients and spleen cells were harvested 20 hours later and analyzed by flow cytometry for the relative abundance of CFSE^{low} (SV40 Tag peptide) and CFSE^{hi} (HSV peptide) populations.

Detection of anti-dsDNA

Anti-DNA antibodies were measured by ELISA according to the published procedure (170).

Magnetic resonance imaging (MRI) of prostate

The progression of prostate cancer in the TRAMP model was measured by MRI as described (148). Briefly, MRI experiments were performed on a Varian system equipped with a 7.0-Tesla, 18.3-cm horizontal bore magnet (300-MHz proton frequency). For MRI examination, the mice were anesthetized with sodium pentobarbital (70 mg/kg intraperitoneally) and maintained at 37°C inside the magnet using a heated circulation water blanket, with pelvis motion (due to respiration) minimized by a small plastic support placed before insertion into a 3-cm diameter quadrature birdcage coil (USA Instruments). Multislice images were acquired using a T₁-weighted spin echo sequence (TR/TE = 880/13, field of view = 30 × 30 mm using a 128 × 128 matrix, slice thickness = 1.5 mm, and slice separation = 1.0 to 1.6 mm.). Each set contained 9 to 25 slices and enough sets were obtained to provide contiguous image data of the prostate tumor. Prostate volume was measured using the formula $V=4/3[(D_1+D_2)/4]^3\pi$, where D₁ and D₂

correspond to the longest and shortest (transverse and sagittal) diameter measured from the MRI image. The accuracy of this measurement was confirmed by comparing preneoplasia MRI volumes to postneoplasia actual prostate volumes in select cases.

3.5 Results

3.5.1 Anti B7-1/B7-2 antibody treatment of young TRAMP mice reduced Treg cells in both the thymus and periphery and delayed development of prostate cancer

We and others have reported that targeted mutation of CD28 and B7-1/2 abrogated generation of regulatory T cells (163). To test whether this pathway can be targeted for transient reduction of Tregs, we treated C57BL/6 mice with either anti B7-1/B7-2 mAbs or control IgG, 5 times every other day. Thymi and spleens were harvested 8 days after the last injection. Cells were stained for flow cytometry analysis. This treatment affected either the total cellularity (Fig. 1a) or the numbers of CD4 (Fig. 1b) and CD8 T cells (Fig. 1c). However, the number of CD4⁺FoxP3⁺CD25⁺ cells was reduced by 50% in the thymus and by 4 fold in the spleen. When gated on lymphocyte gate, all CD4⁺ T cells were CD3⁺ (supplemental Fig. 1). Therefore, all FoxP3⁺CD25⁺ cells analyzed in this study are Tregs. These data indicate that Treg cells can be significantly reduced in both the thymus and spleen by anti B7-1/B7-2 antibodies.

In order to investigate whether anti B7-1/B7-2 antibody treatment delays the development of prostate cancer, 4-week old male TRAMP mice were treated with either control IgG or anti-B7-1/B7-2 antibodies and the incidence of cancer

development was followed by physical examination. Using 50% of mice with palpable prostate cancer as a reference point, we observed that anti-B7 delayed the tumor development by more than 14 weeks (Fig. 1e). Therefore, anti-B7 treatment may be valuable for preventing prostate cancer development.

3.5.2 Enhanced tumor specific cytotoxicity after anti-B7-1/B7-2 antibody treatment.

To test tumor antigen-specific immunity following anti-B7-1/B7-2 treatment, we further investigated tumor specific cytotoxicity by an in vivo killing assay. Six-week old male TRAMP mice were injected i.p. with anti B7-1/B7-2 mAbs or control IgG 5 times every other day. Two weeks after the first injection, they received an i.v. injection of a 1:1 mixture of SV40 Tag peptide-pulsed (CFSE^{lo}) and control HSV gB peptide-pulsed (CFSE^{hi}) spleen cells. The spleens were harvested 20 hours later and analyzed by flow cytometry. As shown in Fig. 2a, in mice treated with anti-B7 antibodies, the SV40 TAg pulsed targets were preferentially eliminated while the CFSE^{lo} and CFSE^{hi} cells remained in the 1:1 ratio in control Ig treated mice. These data demonstrated anti-B7 treatment enhanced the CTL response against the SV40 large T antigen without intentional immunization.

3.5.3 Anti-B7 antibodies rescued SV40 large T-specific T cells from clonal deletion in the TRAMP mice

Our previous studies have demonstrated that SV40 large T antigen is expressed in the thymic peripheral antigen-expressing cells in the TRAMP mice and that such expression caused nearly complete deletion of transgenic T cells

expressing a TCR specific for a SV40 large T specific peptide presented by H-2K^k (147). Moreover, we reported that perinatal blockade of B7-1 and B7-2 reduced clonal deletion of autoreactive T cells (164). To test whether the anti-B7-treatment rescues SV40 T antigen-specific T cells from clonal deletion in the TRAMP mice, we produced TRAMP mice expressing the SV40 Tag specific for the TGB TCR and divided the double transgenic mice into either anti-B7 mAbs or control Ig G treatment groups.

As mice recover from irradiation, a new wave of bone marrow derived cells differentiate into mature T cells in the thymus. This de novo process increases the sensitivity of blocking studies (86) (our unpublished observations). In order to study the effect of anti-B7 treatment on newly formed T cells undergoing thymic development and clonal deletion, we gave sublethal irradiation (500 Rad) to TGB single transgenic and TRAMP/TGB double transgenic mice. At one week after 6 treatments, the thymic cellularity and mature CD8 T cells were measured by flow cytometry. As shown in Fig. 2b, due to clonal deletion, the numbers of reconstituted thymocytes were extremely low in the double transgenic TGB-TRAMP mice compared with the single transgenic TGB mice. Importantly, anti-B7 treatment increased thymic cellularity by approximately 10-fold (Fig. 2b). A corresponding increase in the CD8 T cells expressing high levels of V β 8⁺ transgenic TCR was observed in both spleen and thymus (Fig. 2c, left panel). When the spleen cells were analyzed for CD4/CD8 T cell ratios, it was clear that, perhaps due to clonal deletion, T cells in the control Ig-treated mice host the predominance of the CD8 subset due to expression of MHC class I-restricted

TCR. This is corrected to a large extent by anti-B7 treatment (Fig. 2c, right panel). Thus, anti-B7 treatment greatly reduced the efficiency of clonal deletion. However, the numbers of transgenic T cells in the anti-B7 treated TGB-TRAMP mice were still much more reduced compared to the numbers of transgenic T cells in TGB mice, which demonstrated that the rescue is only partial.

To test the whether the T cells rescued by anti-B7 treatment were responsive to tumor antigen, we stimulated spleen cells from control Ig or anti-B7 treated mice with different concentrations of the SV40 T antigenic peptide or control peptide from HS. As shown in Fig. 2d, anti-B7-treated spleen cells underwent a significant proliferation to SV40 T antigenic peptide. Based on the dose response, the anti-B7-treated spleen cells were at least 100-fold more responsive than the control Ig-treated spleen cells, which corresponded to an increased number of antigen-specific T cells. Therefore the anti-B7 rescued T cells are functional. However, after in vitro stimulation, the rescued T cells showed poor cytotoxicity (data not shown), which suggests that the rescued T cells may be functionally impaired to some extent.

3.5.4 Anti B7-1/B7-2 antibody treatment caused significant albeit transient reduction of Tregs in mice with established prostate cancer

One of the most difficult challenges in cancer immunotherapy is the treatment of established solid tumors. It has been shown that microscopic lesions of prostate cancer can be observed in the TRAMP mice between 18 and 24 weeks of age (165). To confirm the development of tumor in the 25 week old TRAMP mice in our colony, we used magnet resonance imaging (MRI) to

compare the size of the prostate at 25 weeks. As shown in Fig. 3a, all of the 12 TRAMP mice tested had considerably larger prostate organ sizes compared to non-TRAMP littermates. Thus, essentially all of the 25 week old TRAMP mice developed cancer in the prostate.

To determine the impact of anti-B7 antibodies for B7-1 and B7-2, we injected either control or anti-B7 mAbs every other day for 5 times. Blood samples were collected at 0, 1, 2 or 6 weeks after antibody treatment and stained for either anti-CD25 or anti-Foxp3 in conjunction with anti-CD4. As shown in Fig. 3b, in comparison to control Ig-treated mice, a significant reduction of Tregs can be observed in the peripheral blood at one and two weeks after completion of the treatment. Interestingly, the number of Tregs is restored to normal levels at 6 weeks after completion of the treatments. Thus, in mice bearing established prostate cancer, anti-B7-1 and anti-B7-2 antibodies caused a significant, albeit transient reduction of Tregs in tumor bearing mice.

3.5.5 Anti-B7 antibodies delayed growth of established prostate cancer without autoimmune side effects

In order to determine whether anti-B7 antibodies can confer a therapeutic effect in mice with established prostate cancer, we randomly divided 25 week TRAMP mice into two groups, and measured their tumor size prior to treatment with either control Ig or anti-B7 antibodies, starting at 25 weeks. After 5 injections, the mice were monitored for tumor progression by either palpation or MRI. As shown in Fig. 3c, at age 33 weeks (8 weeks after the first treatment), in the control IgG-treated group, the volume of prostate expanded by 2.5-9 fold with

an average of more than 4.5 fold. In contrast, all but one of the anti-B7 treated mice showed less than a two -fold expansion of the prostate volume. The Mann-Whitney test indicated that the difference was statistically significant ($P=0.04$). Since the tumors are not palpable at the beginning of the treatment, we also used the time when the mice developed palpable tumors as a second endpoint with a larger sample size (12 mice for each group). As shown in Fig. 3d, even when treated as late as 25 weeks of age, the anti-B7 antibodies delayed tumor development by approximately 7 weeks.

In the TRAMP model, lymph node metastasis has occurred at 25 weeks (165), we therefore tested the impact of anti-B7 treatment on metastatic lesions in other organs, including the lung, kidney, and liver. As shown in Fig. 4, 3/6 mice in the control Ig-treated group have substantially higher numbers of metastatic lesions in lung (2/6). In addition, massive metastatic lesions were found in the kidney (1/6) and liver (2/6) (data not shown). Only one metastasis was observed in the anti-B7 treated group, and the metastasis was limited to the lung. In addition, the metastatic lesions in the anti-B7 treated group were substantially smaller than those found in the control Ig treated group (Fig. 4).

Corresponding to reduced tumor growth, we have observed increased numbers of T-cells infiltrating into the tumors. Immunohistochemical staining revealed an increased number of T cell infiltrates (Fig. 5a). Quantitative analysis by flow cytometry indicated that the frequency of T cells among the mononuclear cells from the collagenase-treated prostate cancer tissue increased by 4-5 fold, with the majority of the T cells identified as CD8 subsets (Fig. 5b). In

both groups, a higher percentage of CD4⁺ T cells expressed Foxp3 than what was found in the lymphoid organ (Fig. 5b, left lower panel), similar to observations made by others (168). Nevertheless, the % of Tregs was significantly lower in the anti-B7 treated group. Moreover, the ratio of Tregs over effector CD4 and CD8 T cells significantly decreased in the anti-B7-treated group (Fig. 5b, lower right). Therefore, anti-B7 treatment alters the ratio of Tregs over effector T cells in the tumor, presumably in favor of a local immune response.

A general concern for immunotherapy of cancer is autoimmune side effects. In order to determine whether autoantibodies were induced in the tumor-bearing TRAMP mice, sera were collected at 1 week, 2 weeks and 6 weeks after the start of anti B7-1/B7-2 antibody treatments. The anti-dsDNA antibodies were detected by ELISA. As shown in Fig. 5c, while an increase in anti-DNA antibodies were detected at 6 weeks after control Ig treatment, presumably due to tumor growth, such increase was not observed in the anti-B7 treated mice. Histological analysis showed no inflammation of internal organs in either group (data not shown). Therefore, anti-B7 antibodies can induce significant protection against established tumor without eliciting an autoimmune side effect.

3.5.6 Anti-B7 antibodies inhibit MC38 colon carcinoma cell growth

To confirm the general anti-tumor effect of anti-B7 treatment, we tested it with the MC38 colon carcinoma tumor model. Male C57BL/6 mice were injected with 5×10^6 MC38 tumor cells subcutaneously. Ten days after injection, mice developed palpable tumors and were divided evenly into two groups based on the tumor sizes. MC38 tumor bearing mice were administered either anti B7-1/B7-2

mAbs (1:1 mixture of 100µg 3A12 and 100µg GL-1), or control IgG 3 times i.p. every other day. Peripheral blood samples were taken at 0 day, 6 day, and spleens were collected 14 days after completion of antibody treatments. As shown in Fig. 6a and 6b, at 6 days and 14 days after anti-B7 antibody treatment, the CD4⁺CD25⁺ and CD4⁺Foxp3⁺ Treg cells were significantly reduced. Correspondingly, anti-B7 treatment conferred a significant reduction in the growth rate of MC38 colon carcinoma (Fig. 6c, $p=0.035$).

3.5.7 Transient depletion of Tregs by anti-CD25 antibody delays established prostate cancer growth in TRAMP mice.

To test whether transient depletion of Tregs alone inhibits tumor growth, we treated 25 week old TRAMP mice with anti-CD25 antibody to deplete CD4⁺CD25⁺ cells. Male 25 week old TRAMP mice were examined by MRI to measure prostate size and divided into two groups. The mice were treated i.p. with either 1mg anti-CD25 mAbs (PC61) or 1mg control rat Ig. Peripheral blood samples were taken at 0 day, 3 day, and spleens were collected on day 35. 0 day is the day before injection. As shown in Fig. 7a, 99% of Foxp3⁺CD25⁺ cells were depleted three days after anti-CD25 treatment, however, the Foxp3⁺CD25⁺ cells were fully recovered to normal levels 5 weeks after the treatment. Five weeks after anti-CD25 treatment when mice reached 30 weeks old, two groups of TRAMP mice were re-examined by MRI. As shown in Fig. 7b, the prostate sizes were enlarged by 3-5 fold during the 5 week period due to aggressive prostate cancer growth. Compared with the control group, the prostate sizes were increased by 2-3 folds in the anti-CD25 treated group (Fig. 7c). The significant difference

revealed an effect of Treg depletion on tumor growth. However, this treatment is substantially less effective than transient B7 blockade (the average after/before treatment prostate size ratio in anti-CD25 treatment group was 2.55 after 5 weeks, compared with the anti-B7 treatment average ratio which was 1.72 after 8 weeks) (Fig. 3).

3.6 Discussion

Traditionally, blockade of costimulatory molecules B7-1 and B7-2 has been explored for treatment of autoimmune diseases and transplant rejection (171). Recent studies that reveal a critical role for B7-1/2 in the production and maintenance of Tregs (113, 114, 163) and in clonal deletion of self-reactive (164) as well as cancer-reactive T cells (147) suggest that this pathway may be targeted for overcoming the barrier of immune tolerance in the cancer setting. The data described herein demonstrate the unexpected efficacy of this new approach.

We chose the TRAMP mice, which develop malignant transformation of prostate epithelial cells as early as 12 weeks to test this notion. Our data demonstrated that a short-term anti-B7 blockade prior to the development of pathological lesions delays the development of palpable tumor for approximately 14 weeks. These data demonstrate that a short-term anti-B7 treatment may prevent the development of prostate cancer among individuals with a predisposition of prostate cancer.

It is generally agreed that immunotherapy is very inefficient for treatment of established tumors (172). This can be more challenging in transgenic tumor models where malignant tumor cells continue to arise due to transgenic

expression of oncogenes. Our data demonstrated that even when administered at a time when the TRAMP mice show more than a three-fold enlargement of prostate size, transient blockade of B7-1 and B7-2 dramatically reduced the rate of tumor growth. Thus, at eight weeks after initiation of the treatment, the prostate of the control Ig-treated expanded by five fold in volume. In contrast, those from anti-B7-treated mice expanded by less than two fold during the same period. When the palpable tumors were used as an endpoint, the anti-B7 treatment at 25 weeks reduced tumor development by 7 weeks. Nevertheless, perhaps because of the continuous production of new cancer cells from the germline insertion of SV40 large T antigen and waning of antibodies, short term treatment did not completely eradicate the tumors. Since the majority of tumors that develop in humans have clonal origin, the malignant transformation is likely less frequent than that which was observed in this transgenic model of spontaneous tumor development. Therefore, this relatively simple treatment may show greater efficacy in practice. Given the broad function of B7-1 and B7-2 in the host immune system, including T cell costimulation at both priming and effector phases, Treg generation and maintenance, and clonal deletion, it is unlikely that a single mechanism is responsible for the therapeutic efficacy reported herein.

First, we have demonstrated significant, albeit transient reduction of Tregs in both the thymus and in the peripheral blood. Since the treatment with anti-CD25 antibody also showed some efficacy in slowing prostate tumor growth in TRAMP mice, Treg depletion alone is sufficient to convey significant, although

less marked, protection. It is worth noting that anti-CD25 antibody depletes almost 95% of CD4⁺CD25⁺ cells in 6 days, however, 60% of CD4⁺ Foxp3⁺ cells still remained in the peripheral blood at the same time. Since the treated mice had more CD25⁻Foxp3⁺ cells than the untreated group, anti-CD25 ablated part of the CD25⁺Foxp3⁺ cells and down-regulated CD25 on others. On the other hand, anti-B7 treatment caused a similar extent of reduction in the CD4⁺Foxp3⁺ cells regardless of their CD25 phenotype. It is unclear whether the different depletion profile contributed to different efficacy.

Interestingly, the number of Tregs returns to normal levels at 6 weeks after reconstitution. It is therefore of interest why the anti-tumor effect appears to have lasted long after the frequency of Tregs is restored. In this regard, it should be emphasized that in vivo Treg reconstitution is almost universal for all methods of Treg depletion, including antibody elimination and treatment of toxin targeting Tregs that express the specific receptor for the toxin (173-175). In all cases, however, restoration of Tregs did not prevent the immune response against the antigen or pathogen. These studies suggested that numerical restoration of Tregs is usually not accompanied by immune suppression of ongoing immune responses and therefore makes it plausible that temporary reduction of Tregs can promote cancer immunity.

Second, in line with the function of B7 in clonal deletion of autoreactive T cells, including some tumor-reactive T cells, it is possible that anti-B7 treatment also rescues some tumor-reactive T cells that are otherwise deleted. In this regard, we showed that transient blockade of B7-1 and B7-2 reduced the clonal

deletion of SV40 T-reactive CTLs. Therefore, it is likely that anti-B7 blockade may also increase the frequency of tumor-reactive T cells. Taken together, by reducing the burden of Tregs and increasing the frequency of cancer-reactive T cells, B7 blockade resets the balance between regulatory burden and effector function. These two factors provide plausible explanation for the prevention described herein. Since the TGB mice do not survive long enough for us to study clonal deletion at 25 weeks, due to the insertional mutation by the TCR transgene (176), the impact of rescue of tumor-reactive T cells in this therapy setting remains to be demonstrated.

It is possible to argue that since the majority of cancer patients develop cancer late in their life when thymic function has deteriorated, the rescue of the TCR repertoire may be less relevant for cancer immunotherapy in humans. Nevertheless, we would like to point out that continuous production of T cells has been demonstrated throughout the life-span of an individual(177). Moreover, it is worth pointing out that hormone ablation is part of the standard therapy for prostate cancer. An unexpected benefit of this therapy is reinvigoration of thymic function (178). Therefore, it may be valuable to combine anti-B7 blockade with hormone ablation in human prostate cancer treatment.

Finally, it is worth pointing out that blockade of B7-1 and B7-2 with their soluble receptor CTLA4Ig has been approved for therapy of autoimmune disease with little side effect (171). In this study, we showed that despite the modulation of Tregs and rescue of potentially self-reactive T cells, anti-B7 blockade does not trigger an autoimmune side effect. The availability of a safe drug makes

blockade of B7-1 and B7-2 an attractive approach for cancer immunotherapy.

Figure legend

Figure 3.1 Anti B7-1/B7-2 antibody treatment reduced Tregs in both the thymus and periphery of normal mice and delayed the development of palpable tumors. C57BL/6 mice were injected i.p. with either anti B7-1/B7-2 mAbs (1:1 mixture of 100µg 3A12 and 100µg GL-1), or control IgG (1:1 mixture of 100µg hamster and 100µg rat IgG) at 6 weeks old, 5 times every other day. The mice were sacrificed 8 days after the last injection. Thymocytes and splenocytes were harvested and analyzed by flow cytometry. Plots shown are from CD4⁺ cells among the lymphocyte gates. a-c. Anti B7-1/B7-2 antibody treatment did not alter the number of thymocytes, spleen cells and CD4 and CD8 subsets. d. Anti B7-1/B7-2 antibody treatment reduced CD25⁺Foxp3⁺ cells both in thymus and spleen. Statistical analysis was done using the Student T test. *, P<0.05; **, P<0.01. e. Kaplan Meier analysis of tumor incidence. The experimental endpoint was 2 cm in tumor diameter as determined by palpation. Data shown have been repeated 3 times.

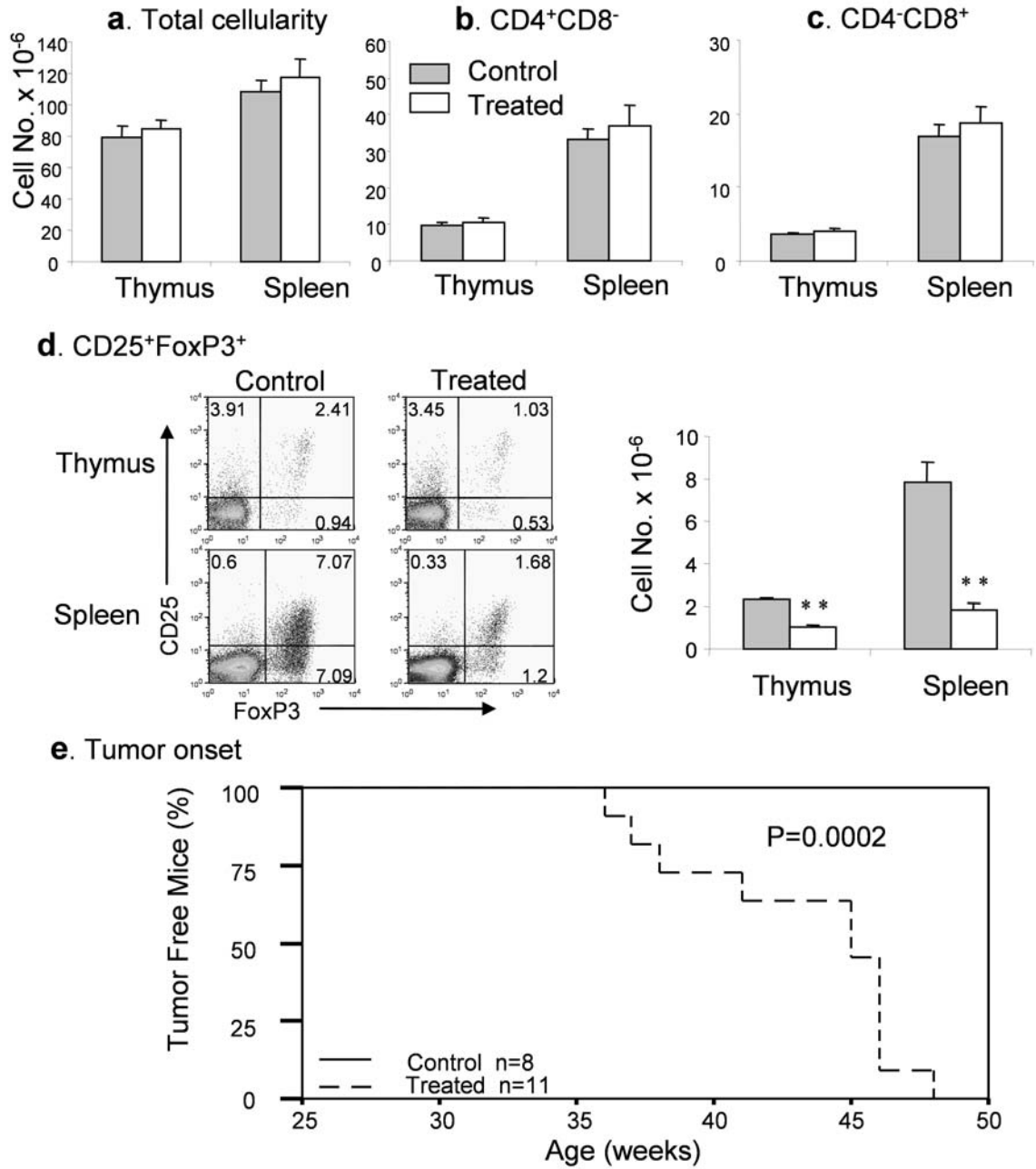


Fig. 3.1

Figure 3.2 Anti B7-1/B7-2 antibody treatment enhanced TAG-specific CTLs and partially rescued clonally deleted TAG-specific T cells in the TRAMP mice.

a. Six-week old male TRAMP mice were treated i.p. with either anti B7-1/B7-2 mAbs (1:1 mixture of 100µg 3A12 and 100µg GL-1), or control IgG (1:1 mixture of 100µg hamster and 100µg rat IgG) 5 times every other day. 2 weeks after the first injection, mice received an i.v. injection of a 1:1 mixture of TAG peptide-pulsed (CFSE^{lo}) and control HSV peptide-pulsed (CFSE^{hi}) spleen cells (5x10⁶ each). Twenty hours later, the spleens were harvested and analyzed by flow cytometry. The left panel shows representative profiles; while the right panel shows a summary of the two experiments involving a total of 6 mice per group. CFSE^{hi} and CFSE^{lo} cells are gated as indicated. The number shown in the gates are the % of gated cells. b-d. Anti-B7 treatment rescued tumor-reactive T cells from the force of clonal deletion. TRAMP/TGB double transgenic or TGB single transgenic mice that received sublethal irradiation (500 Rad) were treated with either control Ig or anti-B7 mAbs five times every other day. The thymocytes and splenocytes were harvested on day 7 after the final treatment and analyzed by flow cytometry. b. Thymocyte and splenocyte cellularities in TGB single transgenic and TRAMP/TGB double transgenic mice treated with control Ig or anti-B7 antibodies. c. Increase of CD8⁺Vβ8^{high} T cells in thymus and spleen. Left panel: Summary of CD8⁺Vβ8^{high} cell number change in thymus and spleen from TRAMP/TGB double transgenic mice. Right panels: Representative profiles of CD4 and CD8 T cells in the spleens of control Ig or anti-B7 treated mice. The left flow cytometry panels show those for total spleen cells, while the right flow

cytometry panels show those for gated $V\beta 8^{hi}$ cells. d. Antigen-reactivity of T cells rescued by anti-B7. The splenocytes from TRAMP/TGB double transgenic mice were stimulated with either SV40 T antigenic peptide or control HSV peptide for 72 hours and pulsed with $^3\text{HTdR}$ to determine the rate of T cell proliferation. Data shown in b, c and d are means \pm -SEM (n=3) and the conclusions have been confirmed with another independent experiment.

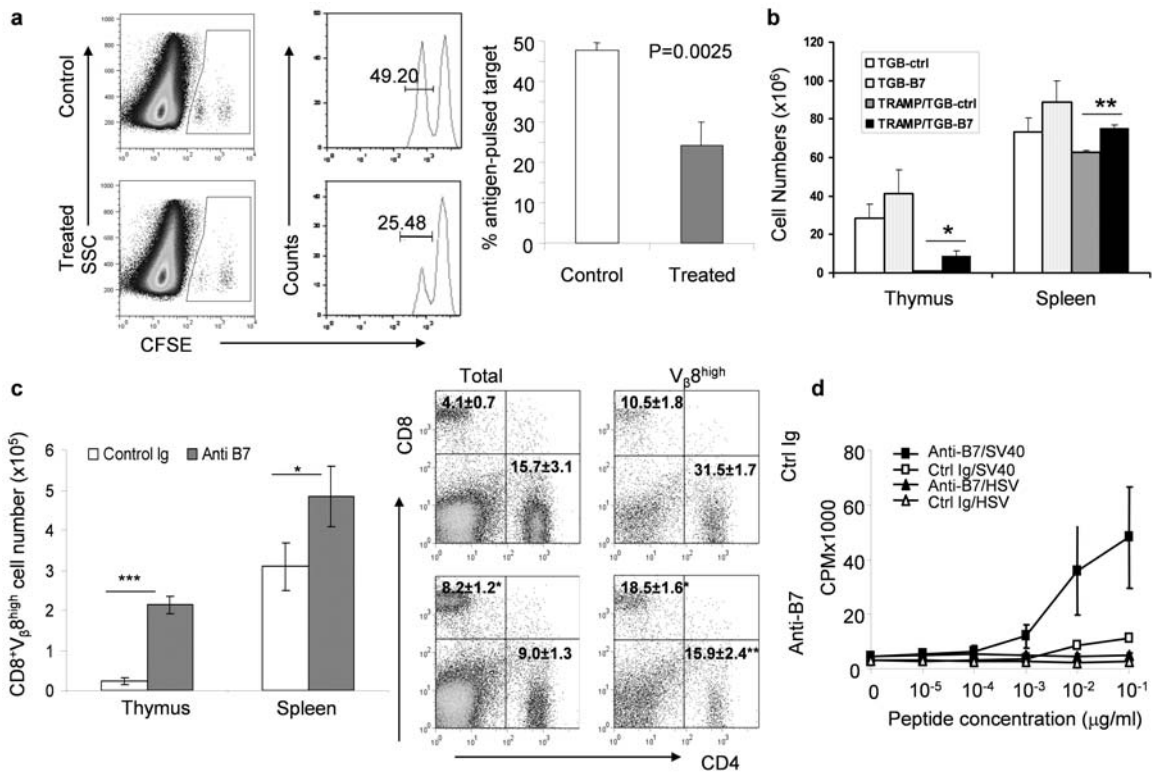


Figure 3.3 Anti B7-1/B7-2 mAbs treatment in mice with established prostate cancer inhibited cancer progression. a. MRI measurement of prostate volumes of 25 week old normal and TRAMP mice. Left panels: Representative local images of male B6 and TRAMP mice. The prostate is identified with thick white outlines. Right panel: Prostate sizes of three B6 and 12 TRAMP mice, all at 25 weeks of age. b and c. Anti-B7 treatment initiated in 25-week old TRAMP mice transiently depleted Tregs. Male TRAMP mice were administrated i.p. with either anti B7-1/B7-2 mAbs (1:1 mixture of 100µg 3A12 and 100µg GL-1), or control IgG (1:1 mixture of 100µg hamster and 100µg rat IgG) 5 times every other day. Peripheral blood was taken at 0 week, 1 week, 2 week and 6 week. 0 week is the day before injection. Cells were stained for flow cytometry. Plots are gated on CD4⁺ cells. b. CD25⁺FoxP3⁺ cell number started to wane following the first week of treatment, and almost recovered to normal levels one month after the treatment was stopped. Data shown have been repeated 2 times, involving a total of 12 mice per group. c. MRI image of TRAMP mice at 25 weeks and 33 weeks (8 weeks after starting treatments with either control Ig or anti-B7 mAbs). Summary data shown are ratios of prostate volumes at 33 weeks vs. 25 weeks when the treatments started. d. Kaplan Meier analysis for incidence of palpable tumors in TRAMP mice treated with either control Ig or anti-B7 antibodies at age of 25 weeks.

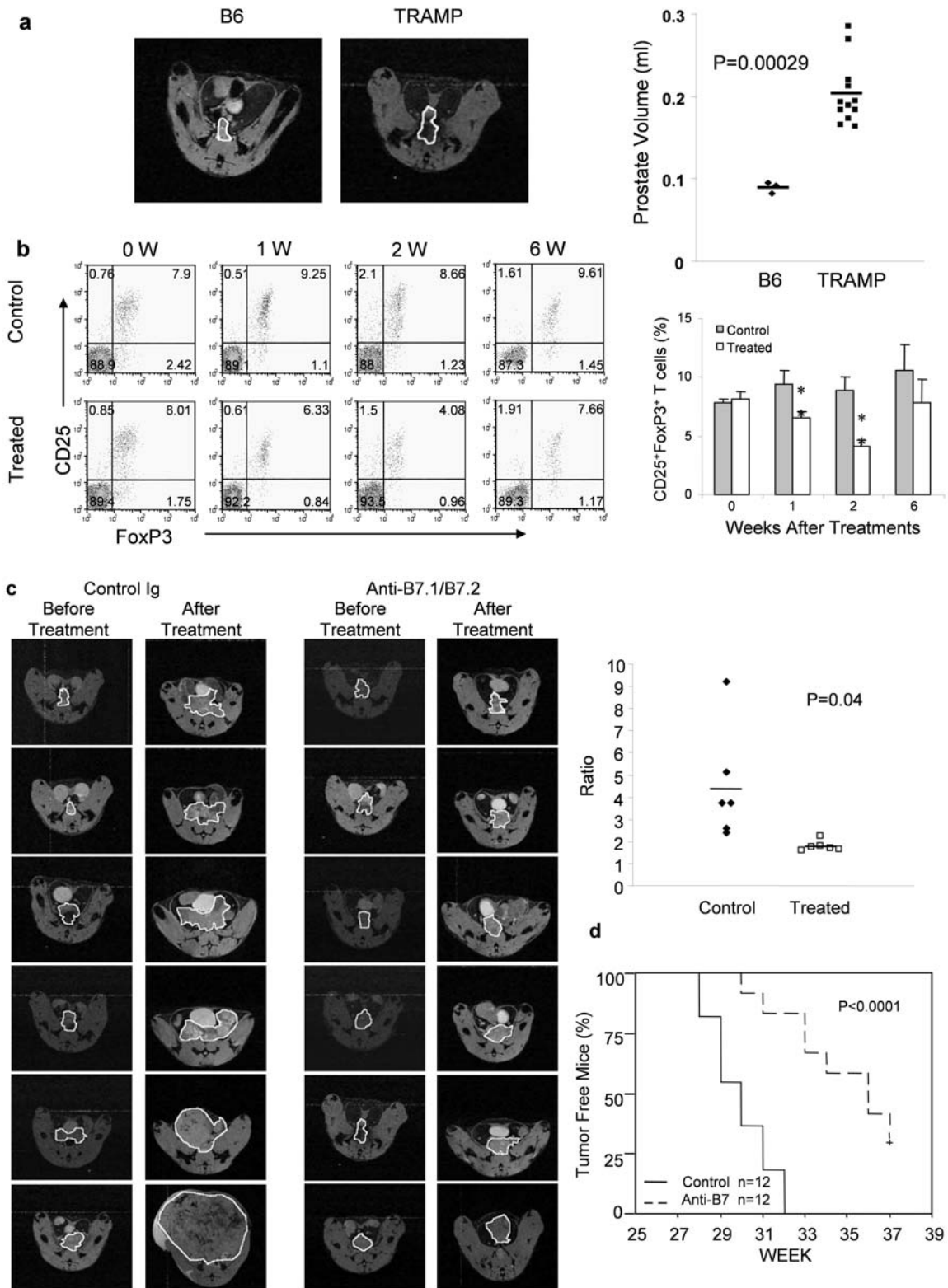


Fig. 3.3

Figure 3.4 Anti-B7 treatment reduces the number and size of metastatic lesions in the TRAMP mice. Internal organs from mice from Fig. 3 were analyzed for metastatic lesions. Three sections of liver, lung, kidney, intestine and heart, 30 microns apart, were examined double blind by a pathologist. A representative field of lung sections of control Ig-treated mice (3 of 6 mice analyzed had metastasis) and the only metastatic lesion in the anti-B7 treated group is shown. Metastatic lesions are marked with yellow arrows. In the control Ig treated group, massive metastases were also observed in the liver (2/6) and kidney (1/6).

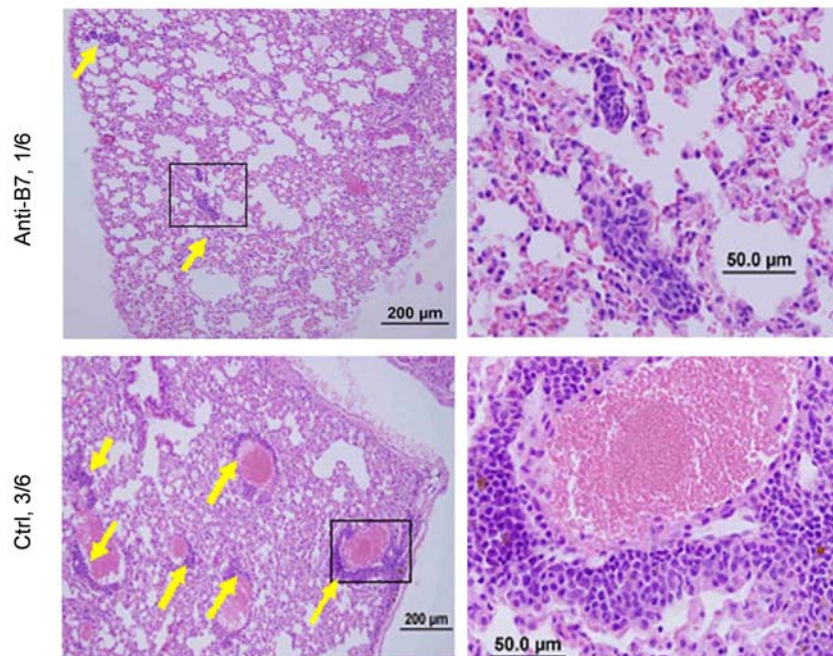


Figure 3.5 Anti-B7 blockade in tumor-bearing mice increased infiltration of T cells into tumors but did not cause autoimmunity. Mice from Fig. 3 were analyzed for infiltrating lymphocytes and autoimmune reactions. a. Representative sections stained with anti-CD3 mAb. b. FACS profiles showing representation of CD4, CD8 T cells and the CD4⁺CD25⁺Foxp3⁺ T cells. Left top panels shows profiles of mononuclear cells isolated from the tumor, while the left lower panels are profiles from the gated CD4 T cells. Data shown are from pooled cells from 6 mice per group. The top right panel shows frequencies of CD4 and CD8 T cells among mononuclear cells isolated from the prostate cancer, while the lower right panel shows the ratio of Tregs over CD4 or CD8 T cells. Data shown are means \pm SEM (n=6). c. Serum anti-double stranded DNA antibodies. Data shown are O.D. 490 from an ELISA, using 1:50 dilution of sera. Data shown are means and SEM, involving 6 mice per group.

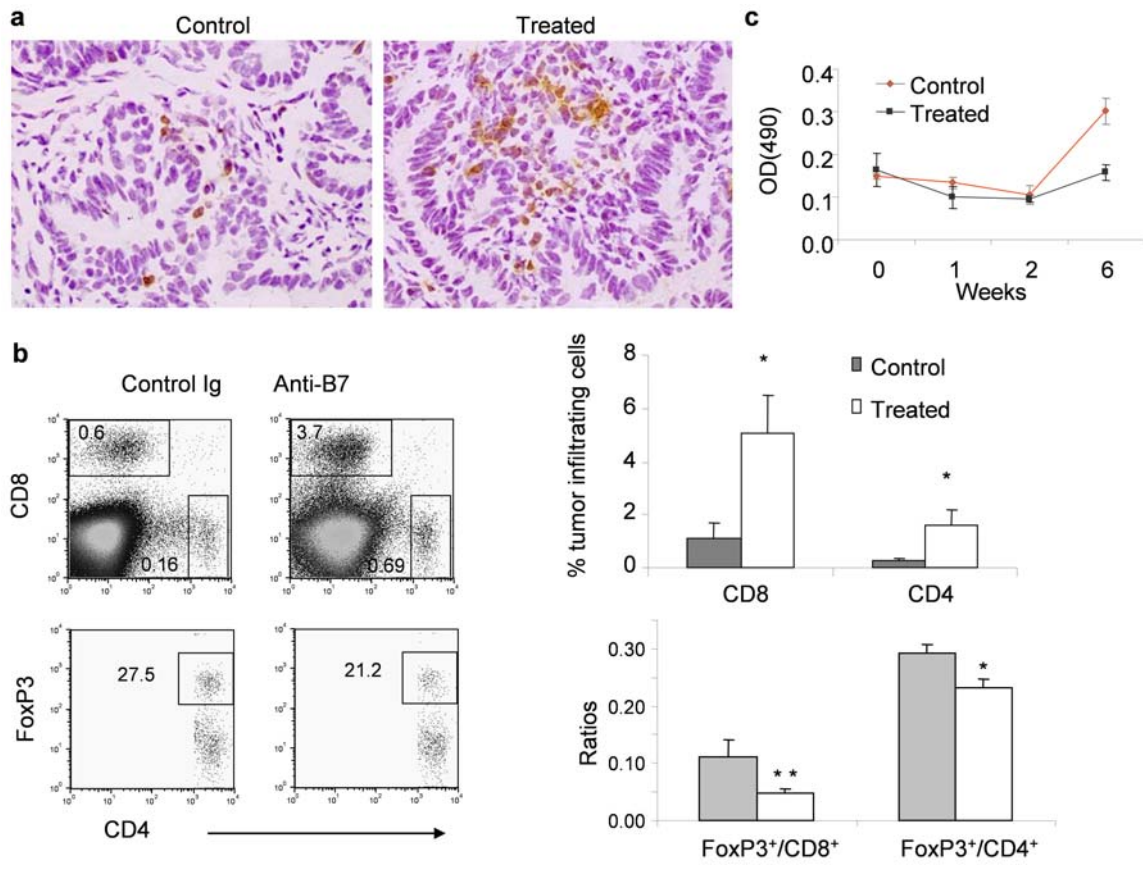


Fig. 3.5

Figure 3.6 Anti B7-1/B7-2 mAbs treatment in mice bearing MC38 colon carcinoma. Eight week old male C57BL/6 mice were injected s.c. with 5×10^5 MC38 tumor cells. Ten days after injection, mice were divided evenly into two groups based on their tumor sizes. The mice were i.p. administered either anti B7-1/B7-2 mAbs (1:1 mixture of 100 μ g 3A12 and 100 μ g GL-1), or control IgG (1:1 mixture of 100 μ g hamster and 100 μ g rat IgG) 3 times every other day. Peripheral blood was taken at 0 day, 6 day, and spleens were collected at 14 day. 0 day is the day before the administration of antibodies. Cells were stained for flow cytometry. Plots of gated CD4⁺ cells are presented. a. CD4⁺FoxP3⁺CD25⁺ cell number started to fall following the first week of treatment. Representative profiles are shown in the left and the summary of the data is shown in the right panel. b. Anti-B7 treatment delayed growth of MC38 tumors (six mice per group). Data shown are means and SEM of tumor diameters at different time points. Day 1 is defined as the first injection of antibody. The statistical significance was determined by Plos Fisher's test.

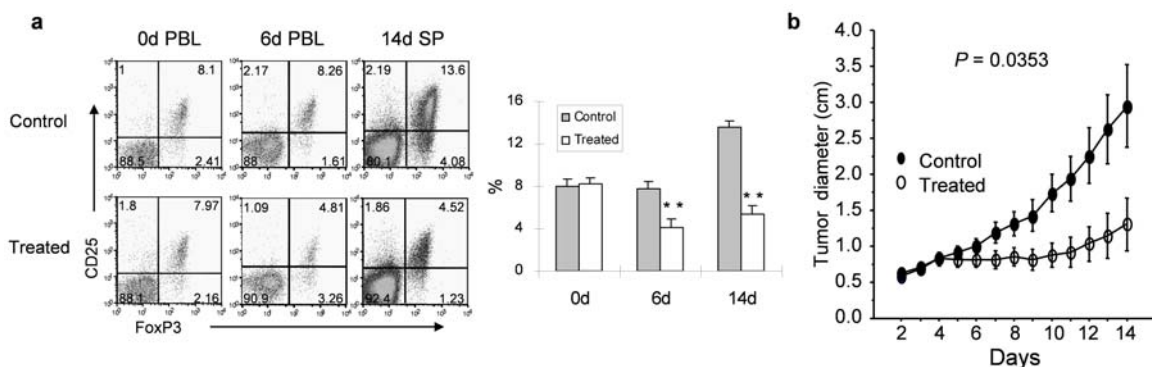
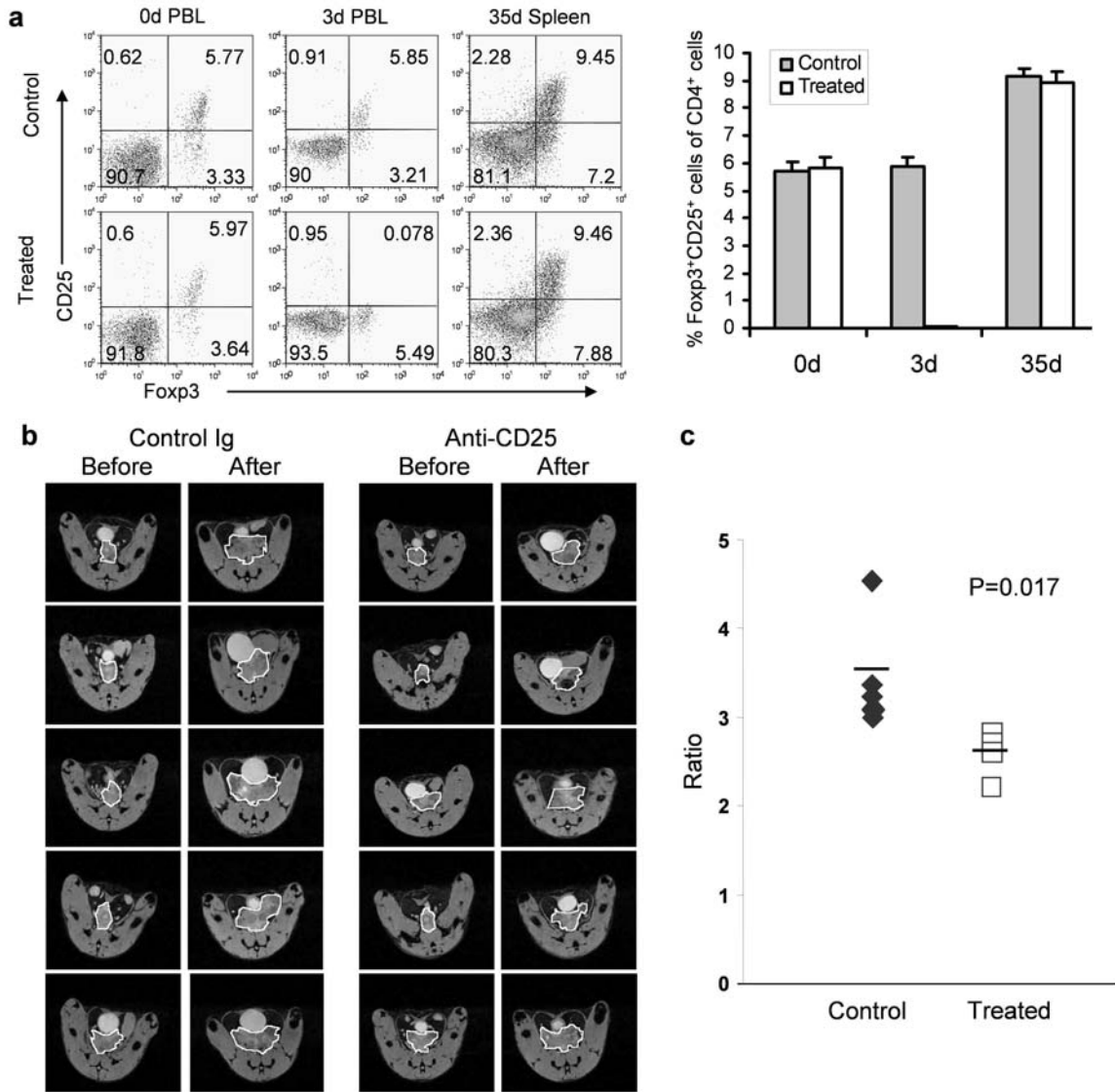


Figure 3.7 Anti-CD25 treatments of mice with established prostate cancer inhibited cancer progression. a. Anti-CD25 (clone PC61) treatment initiated in 25-week old TRAMP mice transiently depleted Tregs. Male TRAMP mice were i.p. administered one dose of anti-CD25 (1 mg/mouse) or control rat IgG (1 mg). Peripheral blood was taken at 0 week, 1 week, 2 week and 6 week. 0 week is the day before injection. Cells were stained for flow cytometry. Plots of gated CD4⁺ cells are shown. The conjugated anti-CD25 from a different clone 7D4 was used to avoid blocking by the depleting antibody. CD4⁺CD25⁺Foxp3⁺ cells numbers were reduced 6 days after anti-CD25 treatment but fully recovered at 35 days. b. MRI image of TRAMP mice at 25 weeks and 30 weeks (5 weeks after the treatments with either control Ig or anti-CD25 antibody, 5 mice per group). Summarized data show the ratio of prostate volumes at 30 weeks vs. 25 weeks when the treatments started. The difference was compared by a student t-test.



Supplemental Figure 3.1 CD4⁺ cells from the lymphocyte gate express CD3.

Profiles shown from left to right are those from sequential gating.

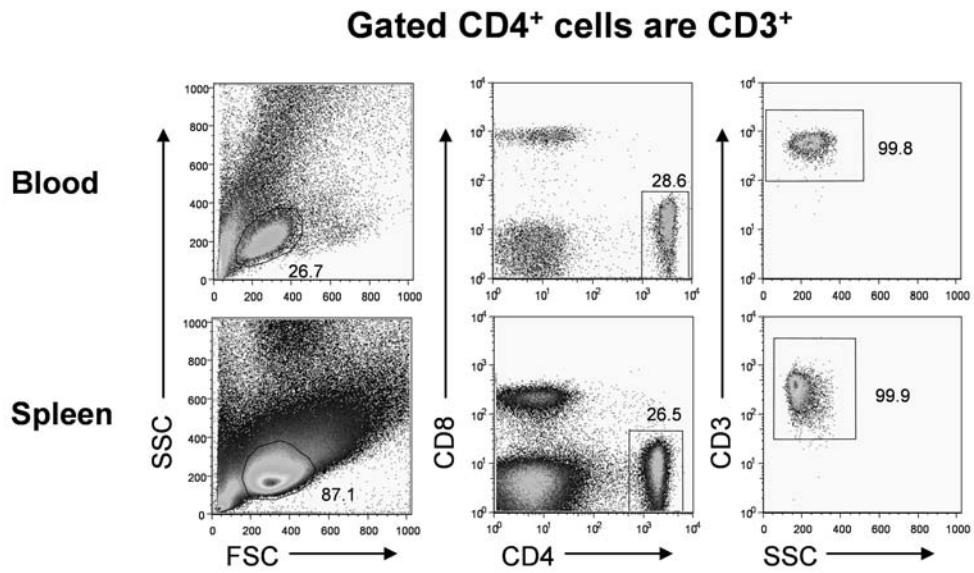


Fig. 3.S1

CHAPTER 4

The Role of Bone Marrow-derived APCs and Thymic Stroma-derived APCs in Regulatory T Cell (Treg) Development

4.1 Abstract

It is generally accepted that Treg development within thymus requires interaction of TCR with self-peptide/MHC complex expressed on antigen presenting cells in thymus. There are two major groups of APCs in thymus. One group is the thymic epithelial cells, which are thymic stroma-derived. The other group is the thymic dendritic cells, which are bone marrow-derived. The role of these two groups of APCs in Treg development in the thymus remains unclear. The B7-CD28 ligand/receptor system is involved in Treg development and maintenance in both the thymus and periphery. By creating B7KO chimeric mice with restricted expression of B7 on either bone marrow-derived cells or thymic stroma-derived cells, we showed bone marrow-derived cells are critical for producing a normal number of Tregs, while the thymic stroma-derived cells are critical for the functional development of Tregs. Therefore, both bone marrow-derived cells and thymic stroma-derived cells are critical for regulatory T cell development.

4.2 Introduction

The theory of central tolerance obtained by negative selection has been widely accepted. Thymocytes with T cell receptors having 'high affinity' for self antigen are potentially reactive to self-antigens. These hazardous self-reactive T cells are depleted via a mechanism known as negative selection that occurs in the thymic cortex. Those thymocytes with TCRs having low to medium affinity to self-antigens, but with potency to respond to non-self antigens are released into the periphery. The low-affinity T cells that leave the thymus, however, are not completely safe. For example, mice with neonatal thymectomy on day 3 develop autoimmune disease (179, 180). To avoid autoimmunity, the immune system has evolved another mechanism to check or delete these released autoreactive T cells. It was found that the autoimmune disease in mice with neonatal thymectomy on day 3 was mainly due to a deficiency in the production of Treg cells from the thymus and enriched autoreactive pathogenic CD4⁺ T cells escaped from the deletion in the neonatal thymus (179-181). Tregs are a group of CD4 T cells that constitutively express CD25 and FoxP3, and which functionally suppress self-reactive T cells (47). The key role of Treg in the control of responses to self and non-self has been demonstrated by the genetic disease IPEX (Immunodysregulation Polyendocrinopathy Enteropathy X-linked Syndrome) in humans and the Scurfy phenotype in mice, in which a mutation in FoxP3 results in severely impaired immune regulation (104-106).

Treg development within thymus requires a unique interaction of TCR with self-peptide/MHC complex expressed on antigen presenting cells in thymus (115,

116). Recent studies showed that Tregs are developed from T cells with 'high affinity' TCRs for self antigen during negative selection (47). Therefore, induction of Tregs during negative selection is another mechanism of central tolerance. Antigen presenting cells (APCs) play a critical role in negative selection. There are two major groups of APCs in thymus. One group is the thymic epithelial cells, which are thymic stroma-derived. The other group is the thymic dendritic cells, which are bone marrow-derived. The role of these two groups APCs in negative selection has been well demonstrated. However, their roles in Treg development remain unclear. Many studies showed that expression of antigen by the thymic epithelium is the most effective way of inducing Treg cells (115), which suggested that the thymic epithelium is essential for Treg development. However, controversial findings reported that antigen expression by hematopoietic cells can be sufficient to induce Treg cells (117). Mature dendritic cells and antigen-processing dendritic cells are also reported to be able to expand Treg cells in periphery (118). In addition, it was observed that a subpopulation of B7.1 and B7.2 expressing dendritic cells in the thymic medulla is critical for the positive selection of high-affinity autoreactive T cells to differentiate into Treg cells (119). Our recent study also supports an important role of DCs in the induction of Tregs. It is not surprising to have these controversial results given that these studies limited their focus to one group of APCs while ignoring the contribution of the other group. In order to fully understand the roles of these two groups APCs in Treg development, it is necessary to compare both of their contributions in one system.

It is now well-established that the B7-CD28 pathway is involved in the thymic development of Tregs. The B7-CD28 ligand/receptor system is a T cell co-stimulatory pathway which acts in concert with TCR signaling to enhance T cell activation and proliferation (182). Accumulating evidence showed that the B7-CD28 interaction is involved in the development and maintenance of Tregs in both the thymus and the periphery (112). CD28 deficient and B7 deficient NOD mice have markedly reduced numbers of Treg cells in thymus as well as in the periphery (113). The particular region of the CD28 cytoplasmic tail required for Treg development has also been identified as the C-terminal praline motif that binds LCK (183). B7.1 and B7.2 are mainly present at high levels on professional APCs such as dendritic cells, and thymic epithelial cells (120, 121)

In this study, we differentiate the roles of thymic stroma-derived cells and bone marrow-derived cells in the thymic development of Treg cells via reconstitution of bone marrow chimerical mice by using B7-deficient mice and wild-type B6 mice. Our data indicates that B7 expression on bone-marrow derived cells is sufficient to produce normal numbers of Tregs. However, impaired function of these Treg cells was also observed. Surprisingly, the thymic epithelium, which was thought to be the most important antigen-presenting cells for inducing Tregs, only induced $\frac{1}{2}$ of the Treg numbers in thymus. Our results demonstrate that both bone marrow-derived cells and thymic stroma-derived cells are critical in Treg development in the thymus. In order to produce a normal number of functional Tregs, both bone marrow-derived APCs and thymic stroma-derived APCs are required.

4.3 Materials and Methods

Mice

C57L/B6, CD45.1 C57L/B6, B7.1/B7.2 deficient and RAG1 deficient mice were purchased from Jackson Laboratories. FoxP3^{EGFP} mice that express both FoxP3 and EGFP under the endogenous regulatory sequence of the FoxP3 locus have been described(184). All mice were used at 6-8 weeks of age and maintained under specific pathogen-free conditions In accordance with the institutional guidelines for animal welfare.

Bone Marrow Transplantation

C57L/B6 and B7.1/B7.2 deficient mice were lethally irradiated with 8.0-Gy total body irradiation respectively by X-ray on day -1. On day 0, 5 million B6 WT or B7 KO donor BM cells were T cell depleted by incubation with anti-CD4, anti-CD8 plus rabbit complement and administered i.v. to recipients.

FACS and Abs

Both cell surface markers and intracellular staining were analyzed by flow cytometry (Becton Dickinson, Mountain View, CA). The fluorescence conjugated antibodies anti-CD4 (GK1.5), anti-CD8 (53-6.7), anti-B7.1 (3A12), anti-B7.2 (GL-1), anti-CD11C (N418), anti-CD45.1 (A20), anti-Annexin V, anti-BrdU and anti-CD25 (PC615) were purchased from BD PharMingen (San Diego, CA, USA). An anti-Foxp3 staining kit (Ebioscience, CA) was used for Foxp3 staining according to manufacturer's protocol. The samples were analyzed by flow cytometry. Data acquisition was performed on a FACSCalibur instrument with CELLQUEST software (BD Biosciences, Mountain View, CA), and data were

analyzed using FLOWJO software (Tree Star, Inc., Ashland, OR).

Immuno-fluorescence Staining

Thymi from the four groups of chimeric mice were embedded in optimal cutting temperature compound (Sakura) and were 'snap frozen'. Sections 8–10 μm in thickness were cut from the frozen blocks, fixed in acetone, rehydrated in PBS plus, and blocked at room temperature for 1 h with 10% goat serum in PBS. K5 (Covance), and Anti-B7.2 (GL-1) were incubated with tissue sections at room temperature for 1h and were detected with appropriate secondary fluorescence reagents.

Treg Function Assay

CD4 T cells were purified by negative selection in 1 X PBS/2% FBS. Single-cell suspensions were prepared from the spleen and incubated for 30 minutes with rat anti-mouse CD8, B220, MAC-1, GR-1 and NK1.1. After being washed, cells were incubated for 30 min with magnetic sheep anti-rat Ab-coated beads (Dynal, Great Neck, NY), and Ab-bound cells were removed by magnetic separation to isolate CD4 T cells. 1 million purified CD4 T cells from each group were IV injected into RAG1^{-/-} mice. Mice were weighed every week after injection.

4.4 Results

4.4.1 B7-1 and B7-2 are required for Treg generation

It has been reported that B7-deficient NOD mice contain very few CD4⁺CD25⁺ Treg cells. In order to confirm the reduction of Tregs in B7-deficient mice, we compared CD4⁺Foxp3⁺ cells in B7-deficient mice with those in wild-type B6 mice. The percentage of Foxp3⁺ cells in CD4⁺ T cells in the thymus was reduced from

3.89% in wild-type B6 mice to 0.25% in B7-deficient mice. In the spleen, it was reduced from 15.9% to 2.37% (Fig. 1a, b). These data confirmed that a B7 signal is required for the generation and maintenance of Tregs.

4.4.2 B7 expression on bone marrow-derived APCs is sufficient for production of CD4⁺FoxP3⁺ cells

We created four groups of chimeric mice with restricted expression of B7 either on bone marrow-derived cells or on thymic stroma-derived cells by irradiation and reconstitution with wild-type or B7 deficient bone marrow: I) B7^{+/+} → B7^{+/+}, II) B7^{+/+} → B7^{-/-}, III) B7^{-/-} → B7^{+/+} and IV) B7^{-/-} → B7^{-/-}. Table 1 shows the B7 expression on different APCs in the four groups of chimeric mice.

We used DCs to represent bone marrow-derived APCs. B7.1 and B7.2 expression was detected by flow cytometry. DCs were gated using the marker CD11c. To further confirm that the DCs were derived from transplanted bone marrow, CD45.1 congenic B6 mice were used as bone marrow donors in group I and group II chimeric mice. In the chimeric mice reconstituted with CD45.1 congenic bone marrow, more than 99% of the DCs were derived from the transplanted bone marrow (Figure 2a, b). As expected, the group I and group II chimeric mice, reconstituted with wild-type bone marrow, had normal expression of B7.1 and B7.2 on DCs (Fig. 2c, d). Conversely, the group III and group IV chimeric mice which were reconstituted with B7-deficient bone marrow had no detectable expression of B7.1 and B7.2 on DCs (Fig. 2c, d). These data indicate that the expression of B7 on bone marrow-derived APCs in these 4 groups of chimeric mice is dependent on whether the bone marrow donor expresses B7. To

investigate the expression of B7 on the thymic stroma-derived APCs of these 4 groups of chimeric mice, we studied B7 expression on thymic epithelial cells by Immuno-fluorescence staining on frozen thymic sections. K5 was used as the thymic epithelial cell marker. As expected, only K5⁺ cells in group I and III chimeric mice were detected with B7 expression (Fig. 2e). Thus, the expression of B7 on stroma derived APCs is dependent on whether the recipient expresses B7. Overall, these data demonstrated that B7 is expressed on bone marrow-derived APCs but not on thymic stroma-derived APCs in the thymus of the group II chimeric mice. While in the thymus of the group III chimeric mice, B7 is expressed on thymic stroma-derived APCs but not on bone marrow-derived APCs. Given the important role of B7 in Treg development, these two groups of chimeric mice will be able to differentiate the contribution of bone marrow-derived APCs and thymic stroma-derived APCs to Treg development.

We next investigated the contribution of B7 expression on bone marrow-derived APCs and on thymic stroma-derived APCs to Treg development. 8 weeks after bone marrow transfer, Tregs were detected in these 4 groups of chimeric mice by flow cytometry. As expected, the group I chimeric mice had a normal percentage of Tregs in the thymus. While the group IV chimeric mice have very few Tregs due to the lack of B7 signal. Interestingly, a normal percentage of Tregs was found in the thymus of group II chimeric mice (wild-type B6→B7KO), in which only bone marrow-derived APCs expressed B7 (Fig. 3a, b). This indicates that bone marrow-derived APCs are sufficient enough to induce normal numbers of Tregs. Surprisingly, a more significant reduction in the percentage of Tregs was

found in the thymus of group III chimeric mice (B7KO→wild-type B6), in which only the thymic stroma-derived cells expressed B7. However, the percentage of Tregs was higher than that in the group IV chimeric mice. These data suggest that B7 expressed on thymic stroma have a significant although reduced capacity to induce Tregs in the thymus. Similar results were found in spleen. A normal percentage of Tregs was observed in the spleen of group II chimeric mice, while a large reduction of the percentage of Tregs was found in the spleen of group III chimeric mice. Interestingly, the reduction of Tregs in the periphery was much more than that which was found in the thymus, raising the possibility that bone marrow-derived APCs may also play a role in the maintenance or homeostasis of Tregs in the periphery.

4.4.3 B7 signaling is critical for the development of Tregs

There are four possible reasons for the more severe reduction of Treg numbers found in the periphery: 1) reduced proliferation, 2) enhanced apoptosis or 3) reduced export from thymus or 4) increased migration into target tissue. To address this question, apoptosis and proliferation of Treg cells in the four groups of chimeric mice were analyzed by FACS. Annexin V was used as a marker of apoptosis. As shown in Fig. 4a, b, there was no significant difference in Annexin V staining among the four groups of Treg cells. This indicated that the reduced Treg number without B7 signaling is not a result of enhanced apoptosis. To detect the proliferation of these Treg cells, the chimeric mice were injected with Brdu twice. The first injection was performed one day before sacrifice. The second injection occurred 3 hours before sacrifice. Brdu staining didn't show a statistically

significant difference among the Treg cells from the four groups of chimeric mice (Fig. 4c, d). Thus, reduced proliferation was not the reason for reduced Treg numbers in the periphery in a system lacking B7 signaling. Therefore, reduced import or increased exit might be the mechanism responsible for the more severe reduction of Tregs found in the spleen compared to the thymus.

4.4.4 B7 expression on thymic stroma-derived APCs is critical to Treg function

Although the percent of Tregs was normal in the group II chimeric mice, we observed skin lesions in the mice from this group. To better assess the degree of autoimmunity in these mice, we evaluated the neck lesions in these chimeric mice 4 months after the bone marrow transfer. More than 70% (19 out of 26) of the group II chimeric mice developed skin lesions 4 months after bone marrow transfer (Fig. 5a). Enlarged intestines were also found in all of the mice with neck lesions (Fig. 5b). A smaller proportion of group IV mice also develop neck lesions, while no skin lesions and no evidence of autoimmune disease was found in the mice from group I and group III. These data suggest that the function of Tregs from group II chimeric mice might be impaired.

A classic assay for Treg function involves the protection it affords from inflammatory bowel disease (IBD). To evaluate the function of Tregs in these four groups of chimeric mice, 1×10^6 spleen CD4 cells from each of the four groups of chimeric mice were i.v. transferred into RAG-1 deficient mice. Interestingly, the recipients of CD4 cells from group II chimeric mice, which have a normal percent of Treg cells, developed wasting disease. In contrast, the recipients with CD4 cells

from group III chimeric mice which only had 1/3 of the normal Treg cell population did not develop wasting disease (Fig. 5c). We further analyzed the blood of RAG-1 deficient recipients 1 week after the transfer. Surprisingly, the percentage of Tregs in recipients with group II CD4 cells was decreased significantly with an increased percent of CD4 cells (Fig. 5d). These data indicate that Tregs from the group II chimeric mice are not able to control the expansion of CD4 cells. Interestingly, in the recipients with group III CD4 cells, both the percent of Tregs and CD4 cells were comparable to those recipients with group I CD4 cells. This suggests that the Tregs in the group III chimeric mice might have recovered to normal ratios via homeostasis. Consistent with our previous report, the recipients with group IV CD4 cells developed wasting disease (Fig. 5c). Analysis of the spleen cells of these recipients at 6 weeks after the transfer showed largely increased number of total spleen cell in the recipients with CD4 cell from group II chimeric mice (Fig. 5f). Consistent to the results in the blood, a decreased percent of Tregs and an increased percent of CD4 cells were found in the spleens of recipients with group II CD4 cells. In the spleens of recipients with group III CD4 cells, both the percent of Tregs and percent of CD4 cells were comparable to that found in the spleens of recipients with group I CD4 cells (Fig. 5e). Due to the largely increase number of spleen cells, largely increased CD4⁺ cell number was observed in the spleens of recipients with group II CD4 cells while Treg number was only slightly increased (Fig. 5g, h).

To confirm that the wasting disease resulted from impaired Tregs, we used FoxP3^{EGFP} mice as bone marrow donor to create group I and group II chimeric

mice. CD4⁺GFP⁺ Treg cells were sorted from the spleens and lymph nodes of group I and group II chimeric mice. 0.5 Million of sorted Tregs mixed with 1 million CD4⁺GFP⁻ cells from normal FoxP3^{EGFP} mice were i.v. transferred into RAG-1 deficient mice. Again, the recipients with group II Tregs developed wasting disease although the disease was not severe as transfer with CD4 cells in previous observation (Fig. 6a). Interestingly, the recipients with Treg cells from group II chimeric mice showed similar neck lesion. To better assess the autoimmune disease, we evaluate the neck lesion at 4 month after the adoptive transfer. 4 out of 5 recipients with Treg cells from group II chimeric mice showed neck lesion with enlarged intestine, while none of the recipients with Treg cells from group I chimeric mice showed neck lesion or enlarged intestine (Fig. 6b, c). Consistent with previous observation in the recipient adoptively transferred with CD4 cells, Treg percent was decreased in the spleens of recipients with Treg cells from group II chimeric mice. However, the percent of CD4⁺ cell was decreased, which might due to the migration of these CD4⁺ cell to peripheral organs (Fig. 6d).. Consistently, these recipients showed largely increased total number of spleen cells and the number of CD4⁺ cell, while the Treg cell number was only slightly increased (Fig. 6e, f, g). These data demonstrate the impaired function of Tregs from group II chimeric mice. Over all, these data suggest that bone marrow-derived APCs are important for the quantity of Tregs, while thymic stroma-derived APCs are important for the quality of Tregs. To produce a normal number of functional Tregs, both thymic stroma-derived APCs and bone marrow-derived APCs are required.

4.5 Discussion

Although the role of thymic APCs in negative selection has been well studied, the contribution of bone marrow-derived APCs and Thymic stroma-derived APCs in Treg development in thymus remains controversial (115, 117-119). Due to critical role of the thymic epithelium in central tolerance, it was thought that Treg cells could be induced by MHC class II molecules expressed by cortical epithelium. Accumulated findings also indicated that the thymic development of Treg cells requires unique interaction of their TCRs with self-peptide/MHC complexes expressed on the thymic stromal cells (115, 116). However, recent studies also support a role of DCs in the development of Tregs in thymus (118). In this study, we created bone marrow chimeric mice to limit the expression of B7 either on bone-marrow derived APCs or on thymic stroma. Due to the critical role of the B7 signal in the development of Tregs (112) and its main expression on dendritic cells and thymic epithelial cells(120, 121), we are able to differentiate the contribution of bone-marrow derived APCs and thymic stroma-derived APCs to Treg development. Our results demonstrated that B7 expression on thymic stroma-derived APCs only induced 1/2 of the normal number of Tregs, whereas B7 expression on bone marrow-derived APCs is enough to produce a normal number of Tregs. Surprisingly, the function of Tregs in the group II chimeric mice, in which B7 was expressed on bone marrow-derived APCs, was markedly reduced. This indicated that bone marrow-derived cells are critical for inducing the number but not the function of Tregs. For the thymic stroma-derived APCs, we found that B7 expression on these APCs was only able to induce half of Treg

number in the thymus although the function of the Tregs was normal. Therefore, both thymic stroma-derived APCs and bone marrow-derived APCs are required to produce a normal number of functional Tregs.

Studies showed that Foxp3 is an essential gene for the development and function of Treg cells(185, 186). In this study, we found that the function of Tregs in the group II chimeric mice was impaired although they did express Foxp3. This indicates that some other factors are essential for Treg function besides Foxp3. These factors may function downstream of Foxp3. It will be very interesting to identify these factors.

Table 4.1 B7 expression on different APCs in the four groups of chimeric mice

Group	I	II	III	IV
Donor	B6	B6	B7KO	B7KO
Recipient	B6	B7KO	B6	B7KO
DC	+	+	-	-
TEC	+	-	+	-

Figure Legend

Figure 4.1 B7 signal is required for Treg generation. a, b. 6 week old B7 deficient mice and C57BL/6 mice were sacrificed. Thymocytes and splenocytes were stained for flow cytometry. Reduction of CD4⁺Foxp3⁺ Treg cells in B7 deficient mice was observed both in the thymus and in the spleen. Plots are gated on CD4⁺ cells.

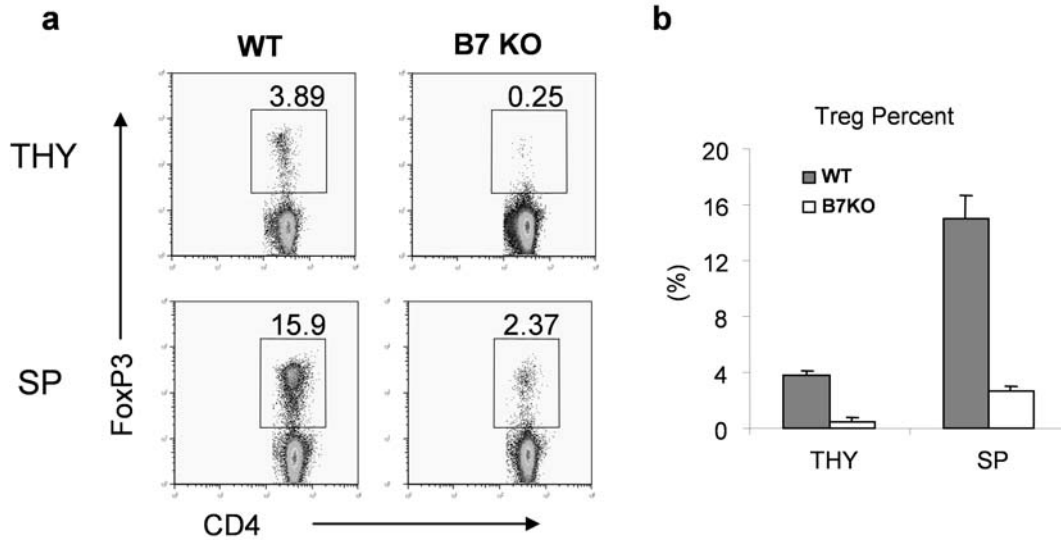


Figure 4.2 B7 expression on bone marrow-derived APCs and thymic stroma-derived APCs in the four groups of chimerical mice. C57L/B6 and B7 deficient mice were lethally irradiated with 8.0-Gy total body irradiation respectively by X-ray on day -1. On day 0, 5 million T cell depleted B6 WT or B7 KO donor BM cells were administered i.v. into recipients. Chimeric mice were sacrificed in 2 month after reconstitution. Thymus and spleen were harvested for FACS. Frozen section was collected for immuno-flouresence staining. a, b. B7 expression on dendritic cells. Plots are gated on CD11c⁺ cells. c, d. Dendritic cells are derived from the transplanted bone marrow in the four groups of chimeric mice. CD45.1 congenic C57BL/6 mice were used as bone marrow donors. Plots are gated on CD11c⁺ cells. e. B7 expression was restricted on bone marrow-derived cells in group II chimeric mice and on thymic stroma-derived cells in group III chimeric mice. Frozen thymic section was stained with B7.2 and K5 (for thymic medullar epithelial cells).

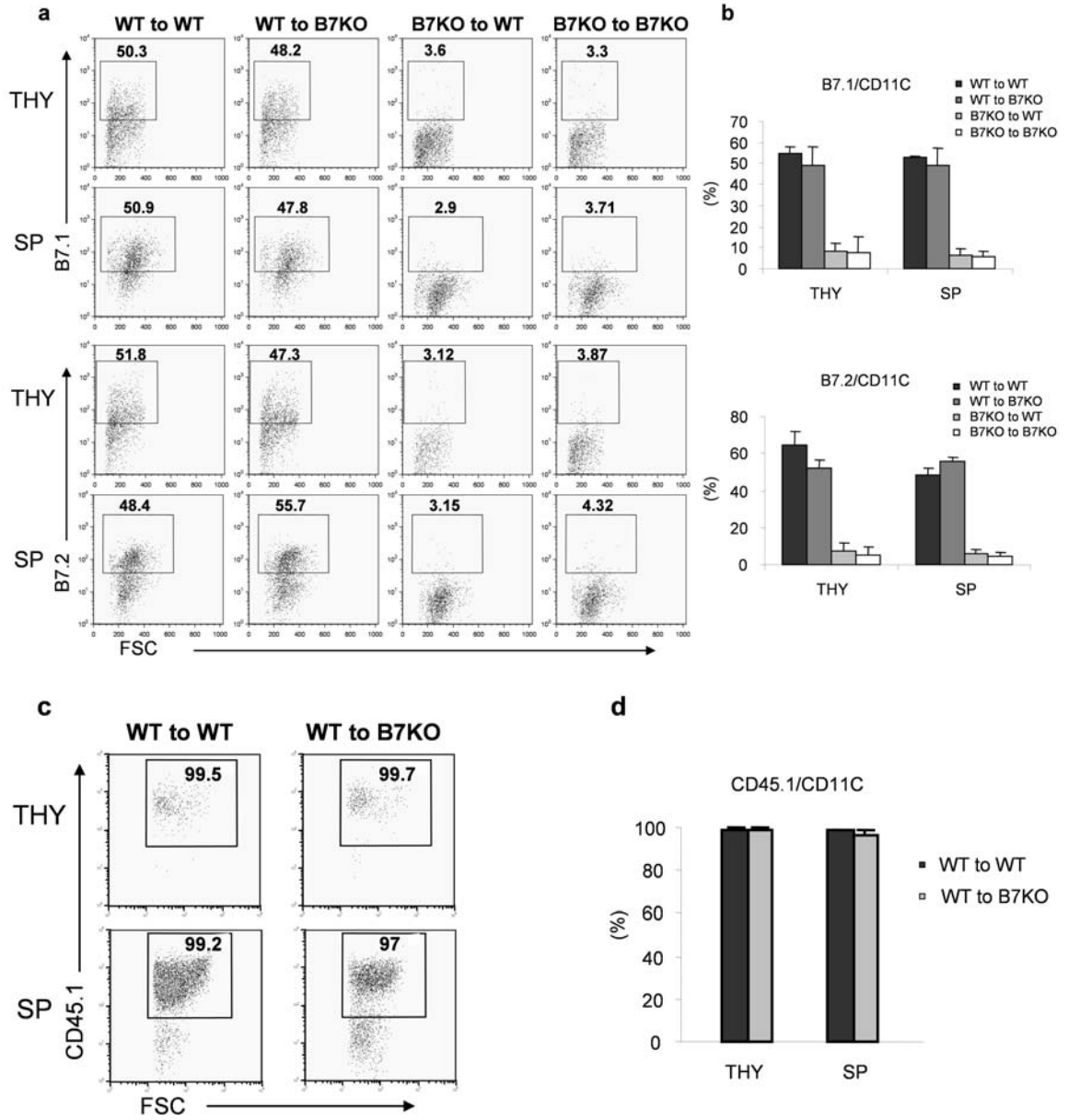


Fig. 4.2 a-d

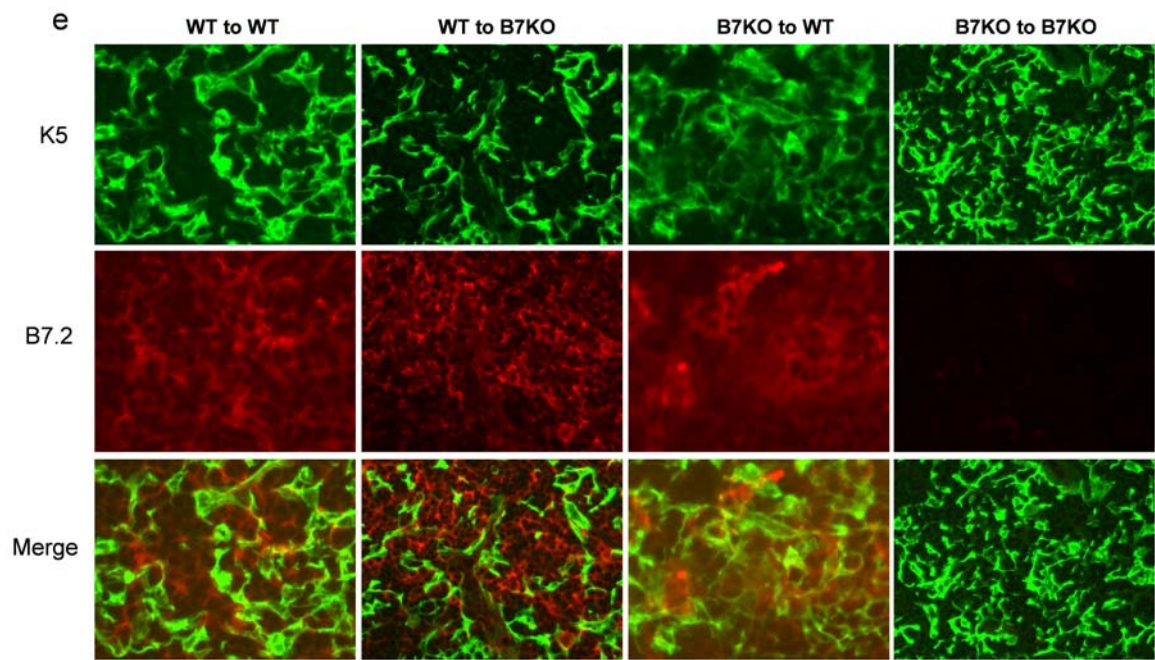


Figure 4.2 e

Figure 4.3 B7 expression on bone marrow-derived APCs is sufficient for production of CD4⁺FoxP3⁺ cells. Thymocytes and splenocytes harvested from the 4 groups of chimeric mice were stained with CD4, CD8 and FoxP3 for flow cytometry. a, b. The number of Treg in group II chimeric mice was recovered completely both in thymus and spleen. While the number of Treg in group III chimeric mice was only recovered to 1/2 in thymus and 1/3 in spleen. Plots are gated on CD4⁺ cells.

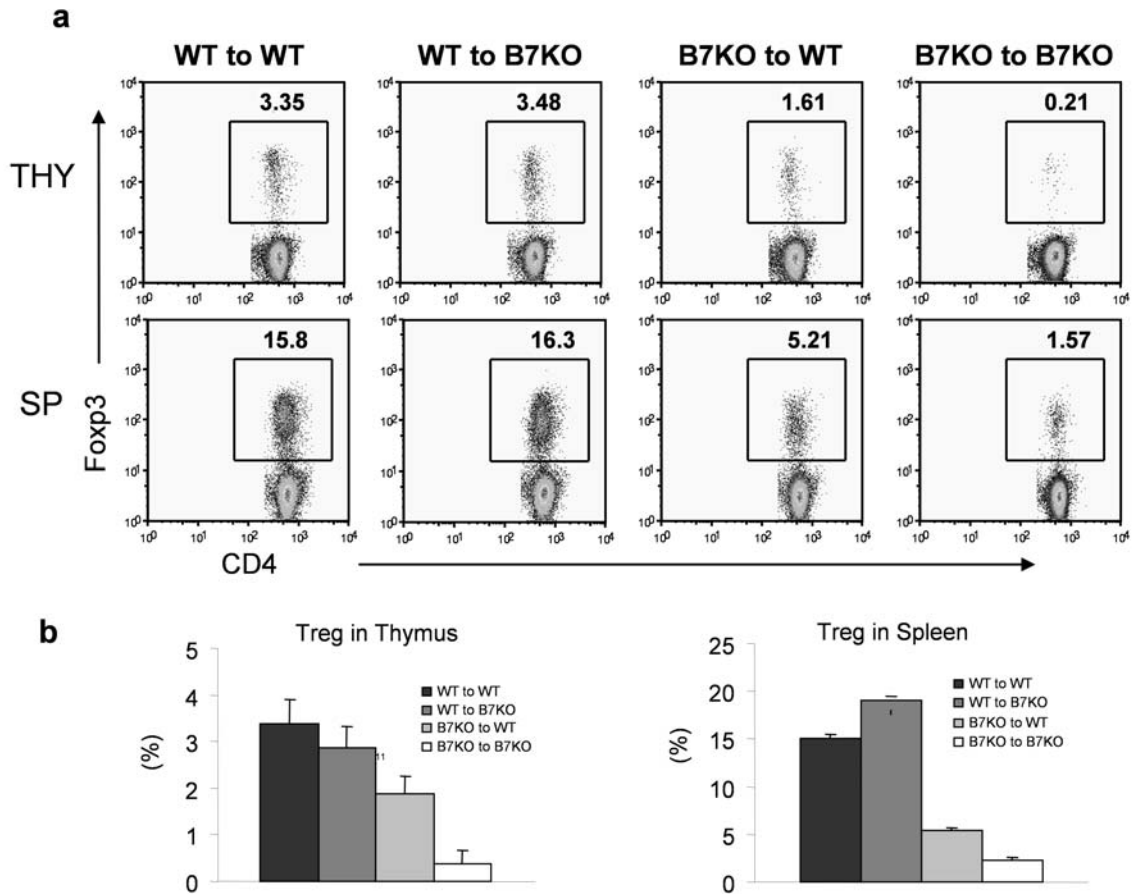


Figure 4.4 B7 signaling is critical for the development of Tregs. a, b. The reduced Treg number without B7 signal was not result from enhanced apoptosis. Thymocytes and splenocytes from the 4 groups of chimeric mice were stained with CD4, CD8, FoxP3 and Annexin V for FACS. Plots are gated on CD4⁺FoxP3⁺ cells. c, d. The reduced Treg number without B7 signal was not result from reduced proliferation. The 4 groups of chimeric mice were injected with Brdu twice. The first injection was performed one day before sacrifice. The second injection occurred 3 hours before sacrifice. Thymocytes and splenocytes were stained with CD4, CD8, FoxP3 and BrdU. Plots are gated on CD4⁺FoxP3⁺ cells.

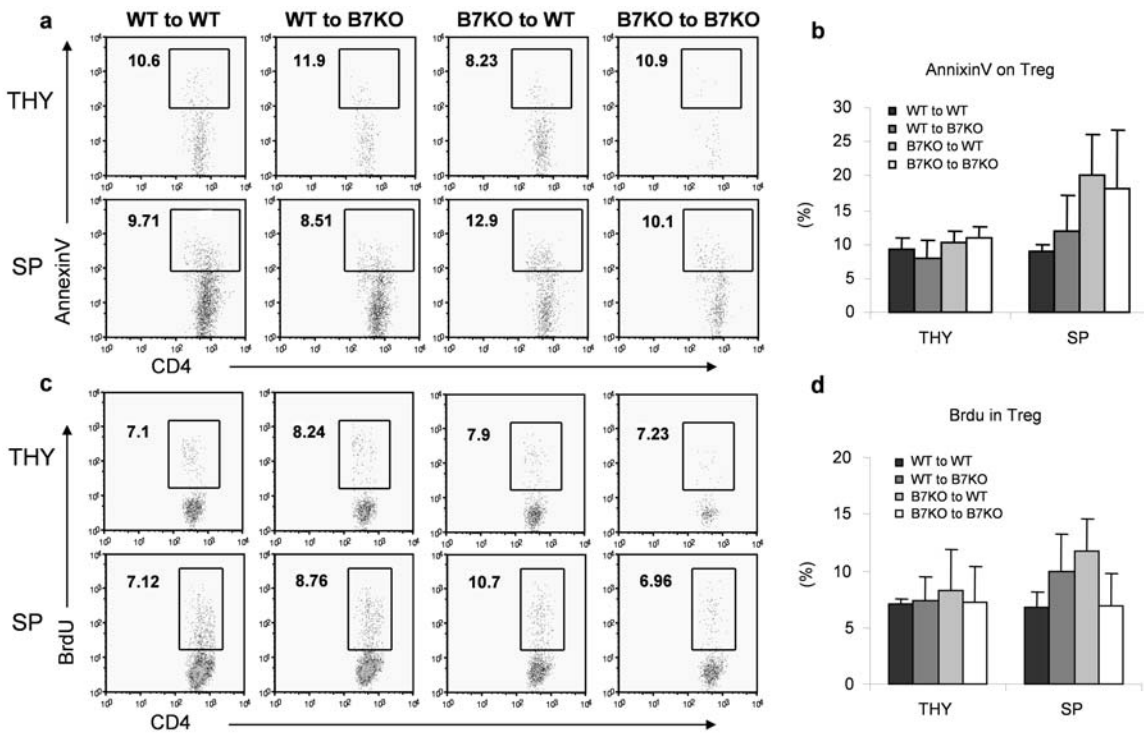


Figure 4.5 B7 expression on thymic stroma-derived APCs is critical to Treg function. To test function of recovered Treg cells, 1 million CD4 cells from each of the four groups of chimeric mice were i.v. transferred into RAG-1 deficient mice. Mice were weighted once per week after the adoptive transfer. The recipients of CD4 cells from group II chimeric mice appeared waste disease. While the recipients of CD4 cells from group III chimeric mice didn't appear waste disease. a, b. Neck lesion and Enlarged intestine was observed in group II chimeric mice. c. Recipients with CD4 cells from group II chimeric mice appeared waste disease. d, e. Recipients with CD4 cells from group II chimeric mice showed Increased CD4 cells percent and decreased Treg percent in peripheral blood and spleen. Peripheral blood was taken at 1 week after the adoptive transfer. Spleen was collected at 6 week after the adoptive transfer. Cells were stained for flow cytometry. f, g. Increased total cell number, CD4⁺ cells in spleen of recipients with CD4 cells from group II chimeric mice. h. slightly increased number of Treg in spleen of recipients with CD4 cells from group II chimeric mice.

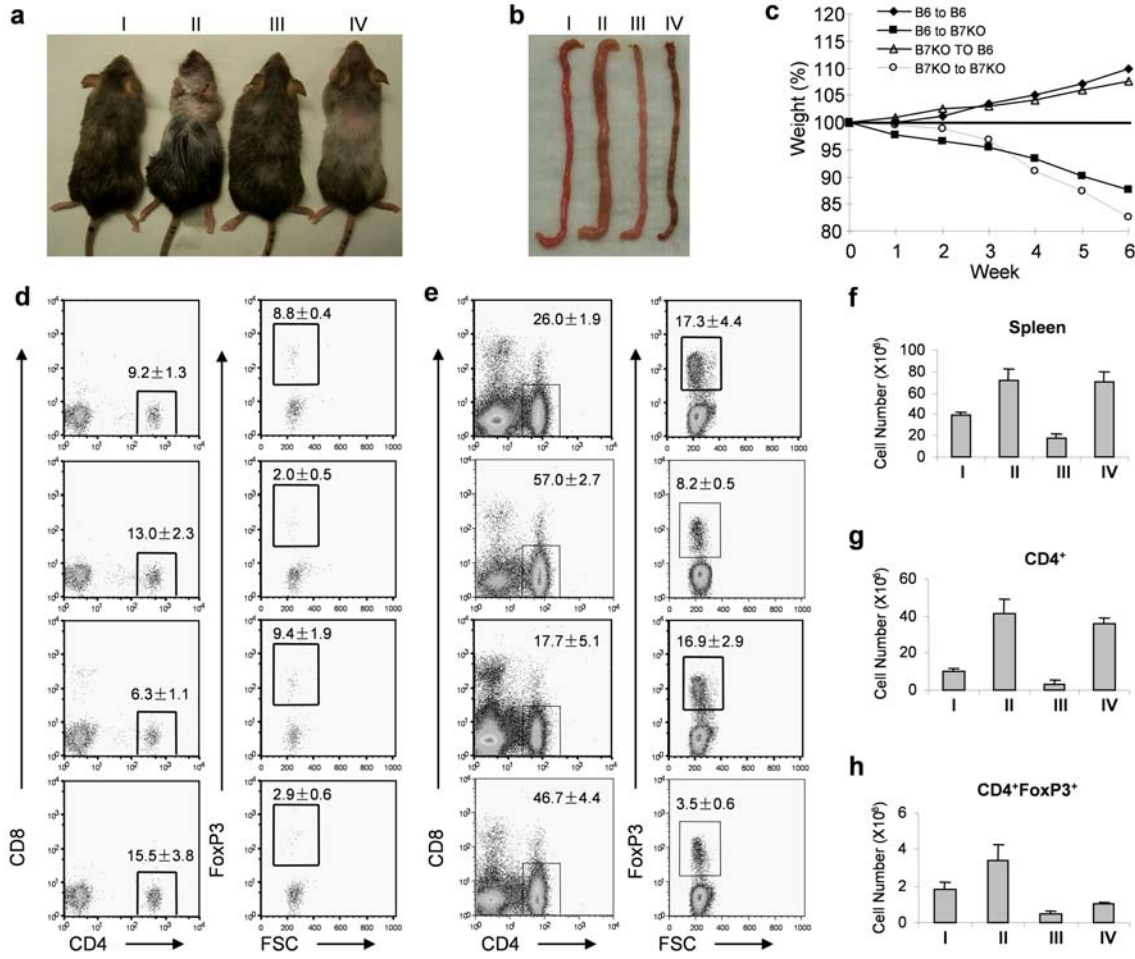


Fig. 4.5

Figure 4.6 Impaired function of Treg from group II chimeric mice To test the function of Treg cells in group II chimeric mice, FoxP3^{EGFP} mice were used as bone marrow donor to create group I and group II chimeric mice. CD4⁺GFP⁺ Treg cells were sorted from the spleens and lymph nodes of group I and group II chimeric mice. 0.5 Million of sorted Tregs mixed with 1 million CD4⁺GFP⁻ cells from normal FoxP3^{EGFP} mice were i.v. transferred into RAG-1 deficient mice. Mice were weighted once per week after the adoptive transfer. a. The recipients of Treg cells from group II chimeric mice appeared waste disease. While the recipients of Treg cells from group I chimeric mice didn't appear waste disease. b, c. The recipients of Treg cells from group II chimeric mice showed neck lesion and enlarged intestine. Mice were sacrificed at 4 month after adoptive transfer. Neck lesions were evaluated. Peripheral organs were collected to detect autoimmune disease. Spleen was collected and cells were stained for flow cytometry. d. Decreased CD4⁺ cell percent and Treg percent in the recipients with Treg cells from group II chimeric mice. e, f. largely Increased of spleen cellularity and number of spleen CD4⁺ cells in the recipients with Treg cells from group II chimeric mice. h. Slightly increased Treg number in the spleen of the recipients with Treg cells from group II chimeric mice.

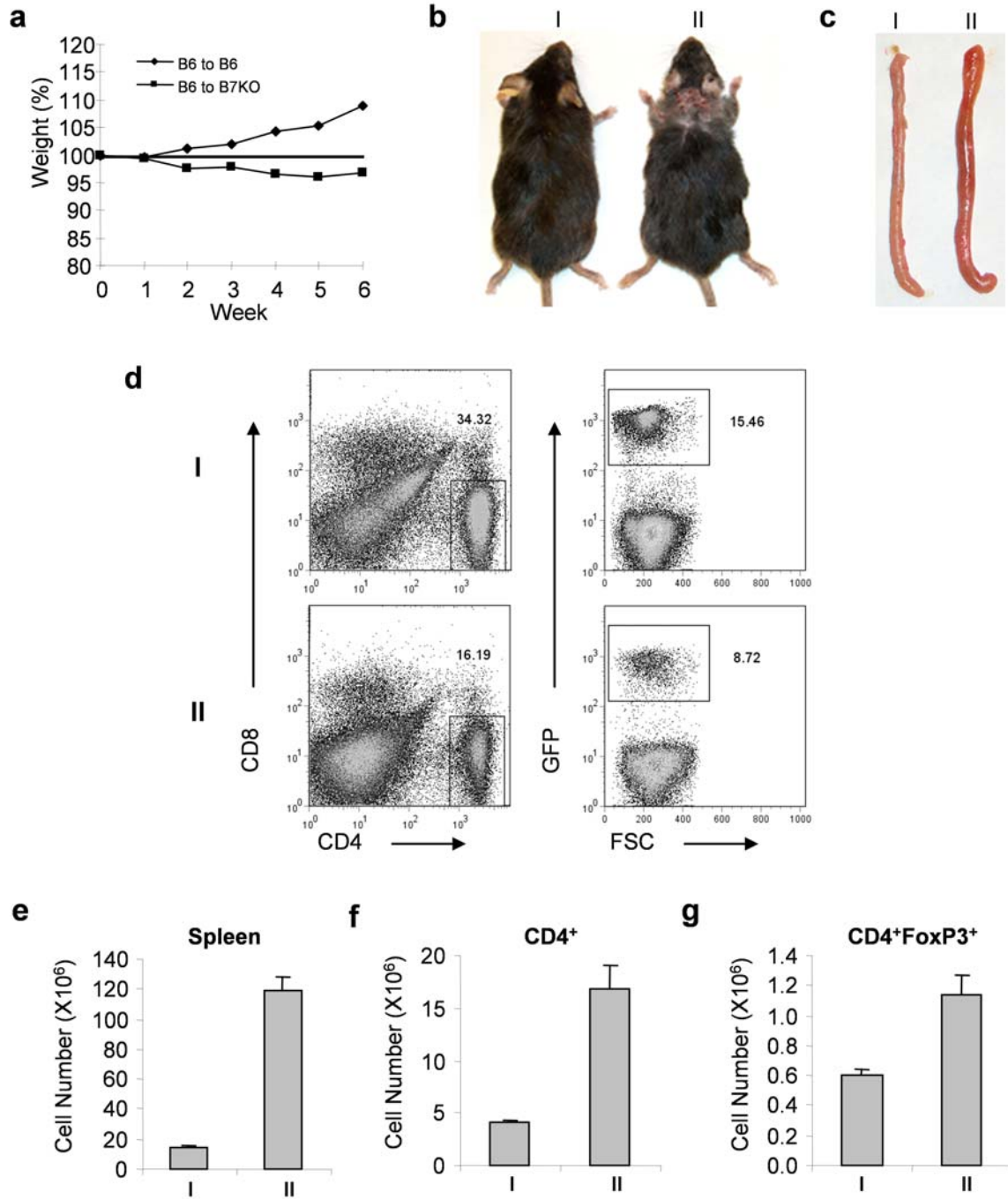


Fig. 4.6

Chapter 5

Concluding Remarks

With the rapid growth of our understanding of cancer development at the molecular level, targeting molecules involved in tumor development is becoming a feasible approach for developing new therapies. In this study, we observed enhanced anti-tumor immunity by targeting molecules involved in the induction of immune tolerance. The finding in this study expands cancer therapies and furthers the understanding of the mechanisms used by cancer cells to avoid anti-tumor immunity.

Immune tolerance is an immune mechanism to protect us from autoimmune disease. Since tumor cells are derived from transformed self cells, it is not surprising that tumor cells use immune tolerance to escape from immune surveillance. Cumulating evidence indicates that both central tolerance and peripheral tolerance are adapted by tumor cells to avoid attack from immune system. A common mechanism of central tolerance is negative selection, in which T cells with high avidity to self-antigens are deleted in the thymus. Since most tumor antigens identified so far are also self-antigens, the expression of these tumor antigens in thymus may result in the deletion of T cells with high avidity to

these antigens. Only those T cells with low avidity for cancer antigens are exported to periphery. Here we take advantage of the critical role for $LT\alpha$ in clonal deletion of T cells specific for peripheral antigen in order to rescue cancer-reactive T cells for the purpose of tumor immunotherapy. Our data showed that short-term treatments with soluble $LT\beta RIg$ rescue cancer-reactive T cells that would be otherwise deleted in the thymus and in the periphery. Correspondingly, we found that TRAMP mice that received soluble $LT\beta RIg$ at six weeks showed significantly reduced tumor growth. Moreover, the targeted mutation of $LT\alpha$ is sufficient to prevent clonal deletion of Tag-reactive T cells. This suggested that blocking $LT\alpha$ is responsible for the rescue of cancer-reactive T cells. These results suggest that breaking central tolerance helps to enhance anti-cancer immunity via rescuing T cells with high avidity to tumor antigens.

Tregs are a subgroup of T cells produced in the thymus, characterized by the expression of CD25 and FOXP3. Its critical role in the maintenance of peripheral self-tolerance has been well demonstrated (46-48, 107, 109). Accumulating evidence also supports a role for Tregs in restraining cancer immunity. Thus, reduction of Tregs in cancer patient may be a promising immunotherapy for cancer. Consistently, the removal of $CD4^+CD25^+$ Treg cells by an anti-CD25 antibody promoted rejection of transplanted tumor cells (54). However, this approach has showed little efficacy in animals with spontaneous tumors, which better reflect the challenge of cancer immunotherapy. In a recent study using a transgenic model of prostate dysplasia, anti-CD25 mAb treatment at 12 weeks of age caused only a 25% reduction in the prostate mass at 20 weeks,

although extended observation has not been carried out to document the long term effect (55). Recent studies that reveal a critical role for B7-1/2 in the production and maintenance of Tregs (113, 114, 163) and in clonal deletion of self-reactive (164) as well as cancer-reactive T cells (147) suggest that this pathway may be targeted for overcoming the barrier of immune tolerance in the cancer setting.

In chapter III, we found that anti-B7 blockade significantly reduced Tregs in both the thymus and in the peripheral blood. Interestingly, the number of Tregs returns to normal levels 6 weeks after reconstitution. Reduced clonal deletion of SV40 T-reactive CTL was also observed after a transient blockade of B7-1 and B7-2. These data suggest that anti-B7 blockade may be able to reduce Tregs and increase the frequency of tumor-reactive T cells at the same time. In terms of an effective immunotherapy, we found that a short-term anti-B7 blockade prior to the development of pathological lesions in TRAMP mice delays the development of a palpable tumor for approximately 14 weeks. These data demonstrate that short-term anti-B7 treatment may prevent the development of prostate cancer among individuals with a predisposition for prostate cancer. Most importantly, our data demonstrated that even when administered at a time when the TRAMP mice showed more than a three-fold enlargement of prostate size, transient blockade of B7-1 and B7-2 dramatically reduced the rate of tumor growth. Thus, at eight weeks after initiation of the treatment, the prostate of the control Ig-treated expanded five-fold in volume. In contrast, those from anti-B7-treated mice expanded less than two-fold during the same period. When the palpable tumors

were used as an endpoint, the anti-B7 treatment at 25 weeks reduced tumor development by 7 weeks. Nevertheless, perhaps because of the continuous production of new cancer cells from the germline insertion of SV40 large T antigen and waning of antibodies, short term treatment did not completely eradicate the tumors. These data indicate that anti-B7 treatment is also an effective immunotherapy for established tumors. In addition, evaluation of autoimmunity didn't reveal any significant autoimmune disease after short-term anti-B7 treatment. These results demonstrate that breaking immune tolerance is an effective immunotherapy to enhance anti-cancer immunity.

An interesting question that remains unclear is the role for different thymic APCs in Treg development. Treg development within thymus requires the unique interaction of TCR with self-peptide/MHC complex expressed on antigen presenting cells in the thymus (115, 116). There are two major groups of APCs in thymus. One group is the thymic epithelial cells, which are thymic stroma-derived. The other group is the thymic dendritic cells, which are bone marrow-derived. Controversial results on the roles of these two groups of APCs in Treg development were reported (115, 117-119). Due to the critical role of the thymic epithelium in central tolerance, it was thought that Treg cells could be induced by MHC class II molecules expressed by cortical epithelium. Accumulated findings also indicate that the thymic development of Treg cells requires the interaction of their TCRs with self-peptide/MHC complexes expressed on the thymic stromal cells (115, 116). However, recent studies also support a role of DCs in the development of Tregs in thymus (118). In chapter IV, we created bone marrow

transferred chimeric mice to limit the expression of B7 either on bone-marrow derived APCs or on thymic stroma-derived APCs. Given the critical role of B7 signaling in the development of Tregs (112) and its main expression on professional APCs such as dendritic cells and thymic epithelial cells (120, 121), we are able to differentiate the contribution of bone-marrow derived APCs and thymic stroma-derived APCs to Treg development. Our data showed that B7 expression on thymic stroma-derived APCs only induced 1/2 of the normal number of Tregs, whereas B7 expression on bone marrow-derived APCs is enough to produce normal numbers of Tregs. Surprisingly, the function of Tregs produced in the group II chimeric mice, in which B7 are expressed on bone marrow-derived APCs, is found to be impaired. This indicated that bone marrow-derived cells are critical in inducing the number but not the function of Treg. For the thymic stroma-derived APCs, we found that B7 expression on these APCs was only able to induce half of the Treg number in the thymus although the function of these Tregs was normal. Therefore, both thymic stroma-derived APCs and bone marrow-derived APCs are required to produce a normal number of functional Tregs.

Over all, our study demonstrated that breaking immune tolerance is an effective approach to enhance anti-tumor immunity. In addition, our study also revealed the contribution of thymic APCs to Treg development.

Our enhanced understanding of immune tolerance has provided novel immunotherapies for cancer.

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