COSTIMULATION AND TOLERANCE IN T CELL IMMUNOTHERAPY

DISSEPTION

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ABSTRACT

The results of numerous basic studies support the use of anti-CTLA-4 antibodies in human cancer therapy. To assist in the translation of this concept to the clinic it would be helpful to establish preclinical models to identify anti-human CTLA-4 antibodies that can induce anti-cancer immunity with acceptable autoimmune side effects. To that end we have produced a human CTLA-4 knock-in mouse in which murine CTLA-4 has been replaced with its human homolog. We used our knock-in mouse to screen a panel of anti-human CTLA-4 antibodies to determine whether our model was useful in discriminating the antibodies' therapeutic effects and autoimmune side-effects. Surprisingly, while all of the antibodies induced protection against cancer and demonstrated some autoimmune side effects, the antibody that induced the strongest protection also induced the least autoimmune side effects. These results suggest that anti-tumor immunity is not necessarily linked to autoimmunity, and that it may be possible to uncouple the two.

Studies of neonatal tolerance have suggested that pre-existing antigens are tolerogenic to subsequently generated T cells. However whether the same is true in the adult host is not known. As the effect of tumor on developing T cells has not been tested, we analyzed the impact of resident tumor on the development of tumor-reactive T cells in the thymus and their immune
competence in the periphery. Our results suggest that newly produced T cells with specificity for pre-existing tumor cells are activated rather than inactivated by tumor antigens in the host.

The importance of B7:CD28 costimulation in Treg development is a widely accepted, yet poorly understood concept. As Treg have been shown to be produced in the periphery as well as intrathymically, it remains unclear as to whether costimulation plays a similar role and produces equivalent effects at each location. To further explore this matter we characterized the expansion of regulatory T cells within Treg-deficient mice following agonistic anti-CD28 antibody treatment. We show here, in the thymus costimulation promotes Treg expansion by licensing developing thymocytes, as Treg are generated de novo, in the absence of proliferation. In the periphery costimulation promotes Treg expansion primarily through enhanced proliferation.
Dedicated to Angela and Abigail
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CHAPTER 1

INTRODUCTION

1.1 T cells

T cells represent a unique class of lymphocytes functioning in the cellular adaptive immune response. As a class, T cells share one unique feature in that all T cells express a T cell receptor (TCR). T cells may express one of two types of TCR, these being either alpha:beta or gamma:delta. T cells can also be categorized on the basis of their function, which in large part can be distinguished by their expression of the cell surface receptors CD4 and CD8. CD4 and CD8 are TCR co-receptors and play two vital roles critical for T cell function. First, co-receptor engagement augments TCR signaling by reducing the amount of antigen required for T cell activation. For example, when the TCR and CD4 co-receptor are simultaneously engaged, T cell activation requires 100-fold less antigen [1]. Second, antigen recognition depends upon the type of co-receptor present at the T cell surface. Thus T cells which express the CD4 co-receptor recognize antigen in the context of major histocompatibility antigen class II (MHC class II). These cells are referred to as T helper cells (Th) and are particularly important in assisting in the function of other immune effector cells.
CD8 T cells function as cytotoxic T lymphocytes (CTL), killing virally infected or transformed cells [2], and thereby providing protection from further infection or growth. Invariably the decision to become a helper or a cytotoxic T cell is rendered during T cell development in the thymus.

1.2 T cell development and tolerance

T cell development occurs through a highly ordered process involving multiple stages as well as locations [3]. The earliest phase of development involves bone marrow-derived lymphoid-committed progenitor cells migrating into the thymus through cognate interactions with ligands expressed on post-capillary venules. Once these cells have gained access to the thymus they begin to interact with thymic stomal cells and this interaction provides the first signal to T-cell lineage commitment. Having received this signal the thymocytes proliferate for several days and begin expressing their first T cell lineage marker, Thy1.1.

At the end of this proliferative cycle the thymocytes bear other T-cell lineage markers, but are yet to express either TCR or co-receptors, and are thus referred to as double negative thymocytes (DNT). The next major step in development involves thymocytes transitioning from double negative to double positive (DP). This transition is marked by DNT expressing their TCR along with the co-receptors CD4 and CD8 (DP). Progression from DN to DP occurs through a structured series of events which can be identified based on surface expression of CD44 and CD25. At DN1 the cells express CD44 but not CD25, and the genes encoding the TCR are still in their germ-line configuration. During
DN2 thymocytes acquire CD25 expression, but TCR β-chain rearrangement does not commence until the cells begin to transition from DN2 to DN3 (i.e. CD44lowCD25+). While cells which have produced successful rearrangements progress into DN3, those that have failed to do so undergo apoptosis. Pairing of the β-chain with the surrogate α-chain marks the complete assembly of the pre-TCR. Cell surface expression of the pre-TCR and CD3 initiates signaling events leading to cell proliferation, the arresting of further β-chain rearrangement and finally expression of the CD4 and CD8 co-receptors. Once the cells stop proliferating they rearrange their α-chains and upon successful rearrangement begin thymic selection.

The need for thymic selection stems from two important properties of T cells. First, because T cells are MHC-restricted, only T cells capable of recognizing antigen presented in the context of self-MHC will be of value to the organism. Therefore survival signals are only granted to those T cells capable of self-MHC recognition, and this process is termed positive-selection. Positive-selection ensures that the immune system will operate under the most efficient conditions by preventing what would otherwise be an unnecessary waste of resources. Second, because T cell receptors are generated randomly there is potential for strong self-MHC recognition, which left unchecked could lead to autoimmune disease. Thus auto-reactive T cells are eliminated in the thymus by negative-selection whereby T cells strongly activated by self-MHC are clonally deleted. Concurrently with thymic selection, DP thymocytes progress to the single positive stage and exit the thymus as either mature CD4 or CD8 T cells.
Once mature T cells enter the periphery they begin to migrate throughout the body in search of antigen, cycling through the vascular and lymphatic systems. Despite their complex and extensive education in the thymus, central tolerance is not sufficient to eliminate all autoreactive T cells. Among other things, this may be due to the fact that not all self-antigens are presented in the thymus. Regardless of how or why autoreactive T cells escape clonal deletion in the thymus there are several mechanisms which have evolved to maintain tolerance in the periphery. These include immunological ignorance, anergy, activation induced cell death and suppression by third party regulatory cells. Whether and how T cells respond to antigen, be it self or non-self, depends upon additional signals they receive.

1.3 B7:CD28/CTLA-4 costimulation in T cell activation and function

The two-signal hypothesis predicts that the outcome of the T cell response is governed by the net effect of signals received through antigen recognition via the TCR, as well as by additional signals generated through accessory or costimulatory receptors [4, 5]. Whereas signal one provides specificity to the response, signal two is antigen independent and occurs through germ-line encoded receptors. Of these costimulatory receptors CD28 and CTLA-4 are among the most widely studied. Because these two genes map to the same chromosome [6-9], are structurally homologous [10] and bind to the same ligands [11-14], it was at first proposed that they may be functionally related. However subsequent studies have suggested otherwise and now a clearer picture has
emerged as to the function of these receptors. While CD28 is regarded as a positive regulator of T cell activation, CTLA-4 appears in most circumstances to function by negatively regulating T cell responses.

The effects of CD28 costimulation in promoting T cell activation have been well characterized [15-18]. Much like the CD4 and CD8 co-receptors, signals delivered through CD28 promote T cell activation by integrating with and augmenting TCR signaling. Moreover, CD28 signaling promotes T cell activation and survival by inducing IL-2 [16, 17], IL-2R [19] and the anti-apoptotic protein Bcl-XL [20]. In the absence of sufficient CD28 costimulation T cells fail to become activated or may be rendered anergic [21].

Unfortunately the mechanism by which CTLA-4 attenuates T cell responses is less clear, and the sheer number of models proposed to explain CTLA-4 inhibition serves to underscore the complexity surrounding its function. Broadly, these models can be divided into two categories based upon whether the inhibitory effect is an indirect consequence of ligand competition or a direct effect of initiating a signaling cascade. The latter category may be further divided into cell autonomous and non-autonomous events.

The first model proposed to explain the inhibitory effect of CTLA-4 was based upon information obtained from binding studies comparing the affinities of CD28 and CTLA-4 to their shared ligands B7.1 and B7.2. It was determined that CTLA-4 binds to both B7.1 and B7.2 with much higher avidity than does CD28 [12, 22]. This finding was later supported by studies suggesting that unlike CD28, CTLA-4 is bivalent and therefore capable of forming oligomeric lattices
with B7.1 [23, 24], its preferred binding partner [25]. CD28 which is thought to interact primarily with B7.2 during T cell priming, is itself monovalent and associates with B7.2 monomers forming a much weaker network [24]. Moreover, CD4+CD25− T cells from CTLA-4 tail-less mutants were found to be as effective as traditional CD4+CD25+ Treg in suppressing the proliferation of wild type responder T cells [26], presumably through their high surface expression of CTLA-4 owing to the loss of AP-2 mediated endocytosis. Taken together these results provide support for the idea that ligand competition is at least a component of the inhibitory effect of CTLA-4.

In addition to direct competition for available ligands, CTLA-4 is also believed to function by initiating negative signaling within the cell itself. Support for this model comes from early experiments demonstrating that CTLA-4 ligation results in reduced IL-2 expression and cell cycle arrest [27]. Furthermore, numerous studies have demonstrated that treatment with anti-CTLA-4 antibodies capable of blocking CTLA-4:B7 interactions enhance T cell proliferation and effector functions [28]. Interestingly, CTLA-4 mutants lacking a cytoplasmic tail demonstrated enhanced proliferation when treated with CTLA-4 blocking antibodies, but proliferation was inhibited when the antibodies were cross-linked by FcR+ APC [29]. This would suggest that the transmembrane region may be sufficient for signaling. Other reports suggest that CTLA-4 may negatively regulate T cell activation by the interruption of TCR signaling via the disruption of lipid rafts [30, 31]. These studies demonstrated that although only a small portion of CTLA-4 was found to be contained within rafts, the level of CTLA-4
partitioning into lipid rafts increased in an APC-dependent TCR/CTLA-4 co-ligation manner. Finally, Lee et al determined that CTLA-4 was able to associate with the TCR ζ chain in primary T cells and that cells transfected with the CTLA-4-associated phosphatase SHP-2 resulted in ζ chain dephosphorylation [32]. These findings suggest an apparent role of CTLA-4 negative signaling in a cell autonomous manner.

Aside from its ability to sequester B7 ligands and directly initiate negative signaling in effector cells, CTLA-4 has recently been implicated in T cell suppression via a non-autonomous mechanism referred to as reverse signaling. Reverse signaling refers to a situation in which CTLA-4 binds to B7 ligand and initiates a signaling event through B7 which subsequently leads to negative T cell signaling. Several lines of evidence support the role of reverse signaling in down-regulating immune responses. First, CTLA-4 reverse signaling has been reported in B7-expressing APC [33-35]. It was shown that both soluble CTLA-4 and CD4⁺CD25⁺ regulatory T cells (Treg) bearing surface bound CTLA-4 were able to initiate a signaling cascade which led to an increase in IFN-γ production in B7-expressing DC. The increased IFN-γ production led to the subsequent activation of the enzyme indoleamine 2,3-dioxygenase (IDO), which catabolizes tryptophan leading to T cell suppression [34]. Secondly, it has been reported that although only B7.2 is expressed on resting T cells, both B7.1 and B7.2 can be upregulated on activated T cells [36]. Though the functional significance of B7 expression on activated T cells remains uncertain, CTLA-4 reverse signaling has been reported in B7-expressing T cells [37]. Here they demonstrate that
whereas CD4^+CD25^- cells from B7-deficient mice are resistant to Treg mediated suppression, expression of full-length B7, but not mutant B7 molecules lacking the cytoplasmic domain, restores Treg mediated suppression in these cells. Thus, it appears that CTLA-4 may be a component of Treg mediated suppression.

1.4 CD28 costimulation in Treg development and function

Successful cellular immunity is predicated upon the development of a diverse immune repertoire composed of clonal cells each capable of recognizing specific antigen. However the mechanism by which immunological diversity is born carries with it incredible risk, for in the process of random gene arrangements necessary for receptor diversity, receptors capable of self-recognition are also created. Therefore, regulatory mechanisms have co-evolved to select only those cells bearing receptors that may be beneficial but not harmful to the host. This task is initially carried out during T cell development in the thymus (central tolerance) and is later reinforced in the periphery (peripheral tolerance). Thus immunity is shaped by regulation occurring both initially during T cell development, as well as at times subsequent to activation.

With regards to this latter form of regulation, classic work by Sakaguchi et al [38] lead to the realization that peripheral tolerance was maintained in large part by a unique subset of T cells with potent immunosuppressive function. These cells were defined by their co-expression of CD4 and CD25, and have since been referred to as CD4^+CD25^+ regulatory T cells or Treg. It is interesting
to note that Sakaguchi himself had postulated the existence of these cells five years prior to their discovery [39]. Although his discovery was initially met with great enthusiasm, interest in this idea eventually subsided and for several years the importance of the discovery dismissed. A reemerging interest in Treg has developed in recent years with the discovery of the gene responsible for initiating Treg development, Foxp3 [40-42].

Although the suppressive function of Treg is well established much remains to be determined concerning the ontogeny of Treg as well as their maintenance in the periphery. Several recent reports have demonstrated the importance of CD28:B7 costimulation in Treg development. First, both CD28(-/-) and B7.1/2(-/-) mice are known to have reduced numbers of Treg and a higher susceptibility to autoimmune disease [43]. Interestingly, mice engineered to express both a transgenic TCR and cognate antigen in the thymus express higher levels of thymic and peripheral Treg [44]. This would suggest that Treg represents a population of cells, which despite high avidity for self, escape negative selection. With the use of knock-out animals as well as antibody-blockade Tang et al demonstrated that CD28 is crucial for the thymic development of Treg as well as their homeostasis in the periphery [45]. In their model CD28 promoted Treg maintenance in the periphery by sustaining CD25 expression. Though CD28-mediated IL-2 production has been implicated in Treg development, Tai et al showed that signaling through CD28 induces Treg differentiation independently of IL-2 through the induction of the ‘master T regula-
tory gene’ *Foxp3* [46]. These findings are surprising in light of previous microarray data which suggested that the primary effect of CD28 costimulation was to augment CD3 signaling rather than initiate a unique gene program [47].
CHAPTER 2

HUMAN CTLA-4 KNOCK-IN MICE UNRAVEL THE QUANTITATIVE LINK BETWEEN TUMOR IMMUNITY AND AUTOIMMUNITY INDUCED BY ANTI-CTLA-4 ANTIBODIES

2.1 Abstract

Although results from preclinical studies in animal models have proven the concept for use of anti-CTLA-4 antibodies in cancer immunotherapy, two major obstacles have hindered their successful application for human cancer therapy. First, the lack of in vitro correlates of the anti-tumor effect of the antibodies makes it difficult to screen for the most efficacious antibody by in vitro analysis. Second, significant autoimmune side-effects have been observed in recent clinical trials. In order to address these two issues, we have generated human CTLA-4 gene knock-in mice and used them to compare a panel of anti-human CTLA-4 antibodies for their ability to induce tumor rejection and autoimmunity. Surprisingly, while all antibodies induced protection against cancer and demonstrated some autoimmune side effects, the antibody that induced the strongest protection also invoked the least autoimmune side effects. These re-
results demonstrate that autoimmune disease does not quantitatively correlate with cancer immunity. Our approach may be generally applicable to the development of other human therapeutic antibodies.

2.2 Introduction

Antibodies have emerged as one of the most valuable immunotherapeutics for cancer [48]. Therapeutic antibodies can be divided into two categories. The first category of antibodies directly bind to cancer cells [48-50]. This binding results in the death of cancer cells by immune-dependent and/or independent mechanisms [51]. The second category of antibodies cause tumor rejection by binding to and activating cells of the immune system, such as T lymphocytes [52, 53]. Because this category of antibodies targets lymphocytes regardless of antigen specificity, a major concern of immunotherapy based on this category of antibody is the risk of severe autoimmune side effects [54].

A prominent example of a category II therapeutic antibody is the anti-CTLA-4 antibody [53]. CTLA-4 is the high affinity receptor for B7-1 and B7-2 [13, 55]. Anti-CTLA-4 mAbs have been shown to promote anti-tumor immunity against a variety of tumors including colon carcinoma [56], fibrosarcoma [56], prostate cancer [57-59], melanoma [60-62], ovarian carcinoma [63], mammary carcinoma [64], and myeloma [65]. These observations have led to enthusiasm for the translation of CTLA-4 antibody therapy to human cancer. More recently, an anti-human CTLA-4 mAb has been generated and tested in clinical trials of
advanced ovarian cancer and melanoma patients [66, 67]. In one of these trials, despite some promising anti-cancer effects, anti-CTLA-4 mAb induced grades 3 and 4 autoimmune toxicities [66].

To facilitate translation of this concept, it would be helpful to establish preclinical models to identify anti-human CTLA-4 antibodies that can induce anti-cancer immunity with acceptable autoimmune side effects. Unfortunately, in vitro cultures of human T cells have proven to be an unsuitable model as the same antibody can have opposite effects on different clones of T cells in the same culture [68]. We have recently reported the use of the human PBL-SCID model to screen for therapeutic anti-CTLA-4 antibodies in vivo [69]. While this model allows us to demonstrate the protective effects of the antibody against human EBV lymphoma, it does not afford us the opportunity to evaluate autoimmune side effects. Taking advantage of the fact that human CTLA4 is capable of interacting with mouse B7-1 and B7-2 [55, 70], we created a mouse with a knock-in of the human CTLA-4 gene. Using this model we compared the autoimmune side-effects and cancer immunity of three anti-human CTLA-4 antibodies. Surprisingly, the antibody that induced the most potent cancer immunity provoked the least autoimmune side effects. These results demonstrate that autoimmunity does not quantitatively correlate with cancer immunity and that selective tuning of cancer immunity over autoimmunity may be possible with careful choice of antibody.
2.3 Materials and Methods

Antibodies

Anti-human CTLA-4 monoclonal antibodies L3D10, K4G4 and L1B11 have been described previously [69]. Antibody was purified from hybridoma culture supernatant using a Protein G column. Mouse IgG was purchased from Sigma (St. Louis, MO).

Creation of a human CTLA-4 knock-in construct

The P1 clone containing a 100 Kb murine CTLA-4 gene was purchased from Genomic Systems Inc. (St. Louis, MO). A 3.8 kb DNA fragment containing the 5’ promoter region, exon 1 and part of intron 1 of the murine CTLA-4 gene was amplified using two primers: CTGAAGCTTCAGTTTCAAGTTGAG which corresponded to a sequence starting at base 734 of the 5’ promoter region, and TTGGATGGTGAGGTTCACTC which corresponded to base 4524 of the exon 2 region. The PCR product was digested with Hind III and the 3.0 Kb fragment was cloned into a Hind III-digested pFlox vector (from Dr. Raj Muthusamy, Children’s Hospital, Columbus OH). The vector was a 6.5 Kb plasmid containing a neomycin resistance gene/HSV thymidine kinase gene cassette flanked by loxP sites.

DNA containing a 14 Kb fragment of the human CTLA-4 gene was prepared from a lambda phage clone [6, 9], and digested with the restriction enzyme Hind III. A 3.2 kb Hind III fragment containing part of intron 1, exon 2,
intron 2 and exon 3 of the human CTLA-4 gene was purified and inserted into a *Hind* III-digested pBluescript plasmid. Plasmid DNA with the insert in the correct orientation was linearized by *Xho* I digestion and partially digested with *Bam* HI to obtain a 3.2 Kb *Bam* HI fragment for use in further cloning. The pFlox plasmid containing a 3 Kb exon 1 of mouse CTLA-4 was linearized by *Xho* I digestion and partially digested with *Bam* HI. The 9.5 kb fragment was purified and ligated with a 3.2 Kb fragment of human CTLA-4 exons 2 and 3.

A 2.9 Kb DNA fragment containing part of intron 3, exon 4 and part of the 3’ sequence of the murine CTLA-4 gene was cloned from the P1 clone using primers ATCCTCTAGAAGCTTCAAAGGTTATCA, corresponding to base 6160 through base 6181 of intron 3 and TCTAGTCGACCACAGAGTCAAGGCCCTG, corresponding to base 8617 through base 8588 of the 3’ region. The PCR product was digested by *Xba* I and *Sal* I and inserted into the pFlox clone containing mouse CTLA-4 exon 1 and human CTLA-4 exons 2 and 3. The final construct is illustrated in Figure 2.1a.

**Preparation of embryonic stem cells with a disrupted humanized CTLA-4 transgene**

Embryonic stem cell line R1 was transfected with the DNA construct described above by electroporation, and drug-resistant ES cell colonies were obtained as previously described [71]. To verify that homologous recombination had occurred, DNA was extracted from ES clones for analysis by PCR.
Fragments were amplified using a forward primer CCAAGACTCCACGTCTCCAG corresponding to a region upstream of exon 1 of the mouse CTLA-4 gene that is outside of the region used in the transgene construct, and a reverse primer CCTCTGAGCATCCTTAGCAC corresponding to a region in exon 2 of the human CTLA-4 gene. These two primers gave rise to a PCR product of 3.3 kb only when the human exon was inserted into the mouse CTLA-4 gene by homologous recombination. Eight of 153 DNA samples screened were positive for this product. The positive clones were analyzed by Southern blot to further confirm homologous recombination. Briefly, the genomic DNA from PCR positive and negative ES clones were isolated, digested with EcoRI, and transferred to a Nylon membrane (Osmonics, Westborough, MA). A 0.9 Kb probe was generated by PCR targeting the region upstream from exon 1 between the EcoRI and HindIII sites using primers CTGCAGTGAACACCCCTCTC and ACGTCTCCAGGTCTCAGAG. The probe was labeled with $^{32}$P using DECAprime DNA labeling kit (Ambion, Austin TX), and hybridized to the membrane. The blot was exposed to BIOMAX MS film (Kodak, Rochester NY) with a Kodak HE intensifying screen for 2 days at –70°C. The endogenous murine CTLA-4 gene yielded a band of 4.7 Kb, while homologous recombination yielded a band of 7.0 Kb by the replacement of the 0.9 Kb murine exon 2, with 3.2 Kb of human exons 2 and 3.
Generation of ES cells with a functional humanized CTLA-4 locus by Cre-mediated excision of the Neo-TK cassette

To remove the Neo-TK selection cassette, we transfected ES cells of clone #63 by electroporation with the pCre-Pac plasmid described by Taniguchi M., et al. [72]. Two sets of PCR reactions were carried out to detect the floxed and deleted alleles of the CTLA-4 locus. The first PCR reaction (A) used 5'-TCCCTCTCAGACACCTCTGC-3' as the forward primer and 5'-GTCATAAACATCTCTCAGGTAA-3' as the reverse primer. This reaction amplified the alleles in which Neo/TK had been deleted, giving rise to a 1.1 Kb product. While this reaction could theoretically also amplify the endogenous murine CTLA-4 allele, PCR conditions were used that would not allow amplification of a large 4 kb product. The second PCR (B) used 5'-TCCCTCTCAGACACCTCTGC-3' as the forward primer and 5'-CGACCTGTCCGGTGCG-3' as the reverse primer.

Production of chimeric and transgenic mice

Chimeric mice were prepared by an aggregation method essentially as previously described [71]. The chimera mice were bred to C57BL/6 mice to obtain founders with germ-line transmission of the human CTLA-4 knock-in allele. The founders were then backcrossed to either C57BL/6 or BALB/c background for at least six generations. Homozygous knock-in mice were used for screening anti-human CTLA-4 antibodies.
Experimental animals and tumor cell lines

P1CTL transgenic mice expressing a T cell receptor specific for P1A35-43:Ld\textsuperscript{d} complex have been previously described \cite{73}. BALB/c and C57BL/6 mice were purchased from Charles River Laboratories under contract from the National Cancer Institute. All mice were maintained in the University Laboratory Animal Research Facility at the Ohio State University under specific pathogen-free conditions. MC38 colon carcinoma cells were purchased from American Type Culture Collection (Manassas, VA).

Analysis of human CTLA-4 RNA and protein expression

Spleen cells were obtained from human CTLA-4 (+/-) and human CTLA-4 (+/+\textsuperscript{E}) C57BL/6 mice and stimulated with anti-CD3 (2C11, 0.1 µg/ml) for 30 hrs in culture. Following culture, total RNA was isolated and an RT-PCR was performed with cDNA from stimulated splenocytes to amplify the full length CTLA-4 sequence from exons 1 to 4. The forward primer began at base pair 5 on mouse exon 1 (5'-CTTGTCTTGGACTCCCGGAGGTAC-3') and the reverse primer at base pair 652 on mouse exon 4 (5'-AAGGCTGAAATTGCTTTTCACATTC-3') for a total amplified fragment size of 648 base pairs. To determine the coordinate expression of both mouse and human CTLA-4 genes, cDNA was amplified with forward primers specific for mouse (5'-TGTGCCACGACATTCACAGA-3') or human exon 2 (5'-GAGGCATCGCCAGCTTTGT-3') and a common reverse primer for mouse
exon 4 (5’-CACATAGACCCTGTTGTAAGA-3’). The amplified fragment using forward and reverse primers for mouse CTLA-4 was 354 base pairs, while the fragment using a forward primer for human CTLA-4 was 455 base pairs. Forward and reverse primers for HPRT were used as an internal control and gave rise to a 100bp product.

To determine if huCTLA-4 protein was expressed properly, we bred P1CTL transgenic mice with huCTLA-4 (+/-) mice to create P1CTL(+)+huCTLA-4(+-) mice. Freshly harvested spleens from these mice were stimulated with 0.1 µg/ml P1A peptide and harvested after 66 hours in culture. Spleen cells were stained with FITC-conjugated anti-mouse CD3, PE-conjugated anti-mouse CTLA-4 (intracellular) and CyChromax-conjugated anti-human CTLA-4 (intracellular). To further confirm that CTLA-4 protein was appropriately regulated we also stained naïve spleens from WT, human CTLA-4 (+/-) and human CTLA-4 (+/+) C57BL/6 mice. Spleen cells were stained with PerCP-conjugated anti-mouse CD4, FITC-conjugated anti-mouse CD25, PE-conjugated anti-mouse CTLA-4 (intracellular), and APC-conjugated anti-human CTLA-4 (intracellular). Conjugated antibodies and CytoFix/CytoPerm intracellular staining kit were purchased from BD Pharmingen.

**Tumorigenicity Assay**

Mice used for tumorigenicity studies have been backcrossed to C57BL/6 for at least 6 generations. MC38 cells (5 X 10^5) suspended in serum free RPMI
(100 µL) were injected s.c. in the lower abdomen of mice. In the minimal disease model mice were treated once a week beginning on day two. In the established disease model, mice were treated every four days with treatments beginning 10-14 days post-challenge. In both models the tumor-bearing mice received identical doses of either anti-human CTLA-4 mAb or mouse IgG control (200 µg/mouse/injection). Tumor size and incidence were determined every 2-5 days by physical examination. Tumor volumes were calculated using an established formula of volume = \frac{1}{2} (\text{long} \times \text{short}^2). All mice were sacrificed when the tumor volume reached 4000 mm$^3$. The number of days required for tumors to reach this endpoint was used for survival analysis.

**Detection of anti-double stranded DNA antibodies**

Anti-DNA antibodies were measured by ELISA according to published procedure [74].

**Immunofluorescence for antibody and complement deposition in the kidney glomerulus**

Frozen sections of kidney were prepared from euthanized mice and fixed in acetone. After blocking with 10% normal goat serum, the sections were stained with Rhodamine-conjugated goat anti-mouse IgG and FITC-conjugated goat anti-mouse C3 antibodies (ICN Biomedicals, Inc.)
2.4 Results

Functional replacement of mouse CTLA-4 gene with its human homologue.

The gene encoding CTLA-4 is composed of four exons in both mice and humans, with 76% percent overall homology between murine and human CTLA-4 proteins and 100% homology between their cytoplasmic domains [6, 9]. Since human CTLA-4 is able to bind to murine B7-1 and B7-2 [55, 70], it is likely that the interaction of human CTLA-4 and murine B7 would maintain normal signal transduction through CTLA-4. We have created a chimeric DNA construct in which the exons coding for the extracellular and transmembrane domains of murine CTLA-4 (exons 2 and 3, respectively) have been replaced with those of human CTLA-4 (Figure 2.1a). As the product of exon 1 is a signal peptide not expressed in the mature protein, and the cytoplasmic domain (exon 4) is completely conserved between human and mouse, only replacement of only exons two and three was required to create a humanized CTLA-4 knock-in mouse.

We transfected an embryonic stem (ES) cell line R1 with the human CTLA-4 DNA construct in Figure 2.1a by electroporation. After selection with G418, DNA was isolated from the drug-resistant ES cell clones and screened by PCR for homologous recombination, which was then confirmed by Southern blot (Figure 2.1b). Probing for a sequence at the 5’ end of the CTLA-4 gene, homologous recombination of the human CTLA-4 knock-in gene yielded a band of 7.0 Kb, while the endogenous mouse CTLA-4 gene yielded a band of 4.7 Kb. ES cell clone 63, which had undergone homologous recombination, was
transfected with the plasmid pCre-Pac that expresses both the Cre-recombinase and puromycin resistance gene. After selection with puromycin and potential excision of Neo/TK by Cre-recombinase, we further selected with gancyclovir which eliminated all cells in which the Neo/TK gene was not excised. PCR analysis of DNA from several surviving colonies indicated that the Neo-TK cassette was excised from the knock-in locus (Figure 2.1c). Based on the analysis of DNA and RNA, ES cell clone 20 was chosen for the production of chimera mice, which were bred with C57BL/6 and BALB/c mice to obtain germline transmission of the human CTLA-4 gene. Those mice that have been backcrossed to C57BL/6 for at least 6 generations and homozygous for huCTLA-4 were used for tumorigenicity studies.

To determine if human CTLA-4 was properly expressed and spliced at the RNA level, spleen cells were obtained from human CTLA-4 (+/-) and human CTLA-4 (+/+) C57BL/6 mice and stimulated with anti-CD3 (2C11) for 30 hrs in culture. RNA was extracted from these cells and an RT-PCR was performed with cDNA to amplify the full length CTLA-4 sequence from exons 1 to 4. Primers spanning mouse exons 1 to 4 amplified a band of 648 base pairs. As shown in Figure 2.1d, the overwhelming majority of CTLA-4 contained exons 1 to 4. In addition, by using primers specific for mouse and human CTLA-4, we were able to observe expression of both mouse and human CTLA-4 alleles. As shown in Figure 2.1d, primers designed to amplify mouse CTLA4 exons 2 to 4 fail to produce a product in the homozygous knock-in mice, further confirming homologous recombination.
To determine whether human CTLA-4 protein was properly expressed, spleen cells from P1CTL(+)huCTLA-4 (+/-) and P1CTL(+)huCTLA-4(-/-) littermate mice were stimulated in culture with P1A peptide for 66 hours. Cells were harvested and stained for both mouse and human intracellular CTLA-4 expression. As shown in Figure 2.2a (upper panels), both mouse and human CTLA-4 proteins are detected in the human/mouse CTLA-4 heterozygous mice. In addition, diagonal distribution of the human and mouse CTLA-4 molecules reveals that the two alleles are regulated by the same mechanism (upper left). The specificity of the staining was confirmed by both isotype control staining as well as by the lack of binding of anti-human CTLA-4 antibodies in WT (mo/mo) littermates. To test whether the mouse and human CTLA-4 alleles were similarly regulated in vivo, we analyzed freshly isolated spleen cells from WT mice or those that were either heterozygous or homozygous for the human CTLA-4 alleles. It has been reported that the only subset of cells that constitutively expresses CTLA-4 in the peripheral lymphoid organs are the CD4+CD25+ regulatory T cells (Treg) [43]. As shown in Figure 2.2b (bottom middle panel), among Treg there is a diagonal distribution of human and mouse CTLA-4 proteins in heterozygous mice. As expected homozygous knock-in mice did not express murine CTLA-4 protein. A minute non-Treg population in all three strains of mice expressed appreciable levels of CTLA-4 protein (top row). As with the Treg population observed in the heterozygous mice, these cells also expressed mouse and human CTLA-4 at similar levels, again as revealed by their diagonal distribution (Figure 2.2b, top middle panel).
CTLA-4 knockout mice are known to develop profound lymphoproliferative disorder and die within 4 weeks of birth [75]. Our extensive observations have indicated that the homozygous human CTLA-4 knock-in mice have a normal life span and show no apparent signs of autoimmune disease over a greater than one year period of observation. As shown in Figure 2.3a, no enlargement of the lymphoid organs was observed in the human CTLA-4 knock-in mice. Moreover, the extent of T cell activation \textit{in vivo} was essentially the same as that observed for WT T cells (Figure 2.3b). Therefore, the human CTLA-4 allele has functionally replaced the mouse CTLA-4 gene. As such the knock-in mice may be used to study antibodies targeting the human CTLA-4 molecule \textit{in vivo}.

The human CTLA-4 knock-in mice discriminate therapeutic effects of anti-CTLA-4 antibodies with essentially identical affinity and isotype.

We have recently described a panel of anti-human CTLA-4 antibodies that promote expansion of human T cells in the human PBL-SCID mouse model. Moreover, the antibody-treated mice survived longer than the control Ig-treated mice [69]. To test whether human CTLA-4 knock-in mice are useful in discriminating the therapeutic effect of the anti-CTLA-4 antibodies, we injected colon cancer cell line MC38 subcutaneously into the CTLA-4 knock-in mice. Two days later, the tumor cell-bearing mice received either control IgG or one of three isotype-matched anti-CTLA-4 antibodies. Among them, L3D10 and K4G4 have the same affinity and binding kinetics, while L1B11 has approximately 3-fold lower affinity. As shown in Figure 2.4a, all three antibodies demonstrated a
statistically significant delay in tumor growth compared with mouse IgG control antibody. In addition, L3D10 proved to be the most potent antibody when compared to the other two treatment antibodies (Figure 2.4a and b). As seen in Figure 2.4c all three antibodies led to enhanced survival compared to control Ig-treated mice. A survival advantage of L3D10-treated mice was also observed over those treated with L1B11 and K4G4 (Figure 2.4c).

To explore the therapeutic potential of the L3D10 antibody for large established tumors, we delayed treatment until approximately 2 weeks after tumor cell challenge. As shown in Figure 2.5, in comparison to control IgG, L3D10 antibody delayed tumor growth, and prolonged survival of tumor-bearing mice. Nevertheless, it should be noted that L3D10 alone did not cause complete tumor rejection. Therefore, it is likely that even the most efficient anti-CTLA-4 antibody will need to be used in combination with other reagents in order to achieve complete rejection of established tumors.

The human CTLA-4 knock-in mice unravel the link between cancer immunity and autoimmunity.

Given the tendency of anti-CTLA-4 antibodies to exacerbate autoimmune diseases in experimental autoimmune models, it is of interest to determine whether the autoimmune side effects quantitatively correlate with anti-tumor immunity. Our analyses in wild-type mice revealed that, anti-mouse CTLA-4 antibody 4F10 suppressed tumor growth, but enhanced anti-double stranded DNA antibodies. In contrast, anti-4-1BB antibody 2A induced cancer immunity
without triggering anti-DNA antibody response (Supplemental Fig. 2.1). Thus, the presence of anti-DNA antibodies can serve as a useful marker for autoimmunity associated with anti-CTLA-4 antibody. Using this marker we first compared mice treated with three different anti-CTLA-4 antibodies for their production of anti-double stranded (ds) DNA antibodies. As shown in Figure 2.6a and b, although anti-dsDNA antibodies were detected in all tumor bearing mice-treated with anti-CTLA-4 antibodies the levels were significantly different among the treatment groups. Mice that received K4G4 and L1B11 had 3-5-fold higher levels of anti-dsDNA antibodies than mice treated with L3D10. The difference was stable over the course of the treatment, as similar results were obtained at earlier and later timepoints. Consistent with this variability in anti-dsDNA antibody induction, we observed more IgG deposition in kidney glomeruli of K4G4 or L1B11-treated mice than in those treated with L3D10 (Table 2.1).

Results from comparing the amount of the anti-dsDNA antibody and tumor size suggests that for mice that received control Ig, L1B11 or K4G4, tumor size correlated inversely with the amount of anti-dsDNA antibodies. This observation suggests that among these 3 groups, the intensity of the anti-tumor immune response correlates with that of the anti-DNA antibody response (Figure 2.6c). However, the group that received L3D10-treatment had the smallest tumor size with the lowest anti-dsDNA antibody levels. Thus, stronger cancer immunity does not have to be coupled with more severe autoimmune side-effects.
Anti-CTLA-4 antibodies that induce different potencies in anti-tumor and autoimmune response bind to an overlapping site on CTLA-4.

As measured by Biacore, L3D10 and K4G4 have essentially identical affinity for human CTLA-4 [69]. In addition, these antibodies have identical isotype (IgG1, κ). To determine whether the antibodies have overlapping binding sites, we tested whether they compete with each other in binding to human CTLA-4. As shown in Figure 2.7a-c, all three antibodies cross-blocked each other’s binding to CTLA-4, with efficiency that grossly correlates with their affinity to CTLA-4 [69]. Moreover, all antibodies were capable of blocking the binding of CTLA-4 to its natural ligand B7-1 (Figure 2.7d). The similarity of the immunochemical properties of these antibodies highlights the need for preclinical models to screen for anti-CTLA4 antibodies with favorable therapeutic activity and acceptable autoimmune side effect.

2.5 Discussion

We have demonstrated that human CTLA-4 gene knock-in mice can serve as a valuable model for the preclinical screening of cancer therapeutic antibodies targeting the human CTLA-4 protein. The utility of this approach is based on three factors. First, human CTLA-4 has natural ligands in the mouse, as previously reported [14, 55, 70, 76]. Second, the signaling pathways used by mouse and human CTLA-4 are similar. Although this is difficult to verify as the mechanism of signal transduction for CTLA-4 is still unclear, the fact that the cytoplasmic domain of mouse and human CTLA-4 is 100% identical [13]
suggests that the signaling pathway may likely be the same. Third and most importantly, human and mouse CTLA-4 must have the same biological function. In support of this notion, we have demonstrated that the homozygous knock-in mice do not develop lethal autoimmune disease such as was observed in CTLA-4 knockout mice [75]. Moreover the fact that polymorphisms of both mouse and human CTLA-4 genes affect genetic susceptibility to autoimmune diseases [77] further supports the idea that these genes play similar roles in their respective hosts.

Previously we utilized the hu-PBL-SCID mouse model to explore the potential efficacy of anti-CTLA-4 antibody treatment [69]. In this model SCID mice are reconstituted with human peripheral blood thereby creating a functional human immune system. Though this model is useful in screening antibodies for their potential anti-cancer effect it is somewhat limited in evaluating other clinical parameters such as autoimmunity. By comparison huCTLA-4 gene knock-in mice offer several important advantages, foremost of which is the fact that the immune response takes place in a natural setting. In contrast to knock-in mice, SCID animals require significant intervention to promote and maintain responses. Indeed, in the SCID model human T cell survival is predicated on repeated injections of anti-NK cell antibodies as well as cytokines such as GM-CSF. These factors must be taken into consideration when the therapeutic effects are interpreted. Nevertheless, it is of interest to note that in both models L3D10 treatment led to the most efficacious response. In the SCID model mice undergoing L3D10 treatment exhibited the most substantial T-cell expansion, as
well as the longest survival. Similarly, L3D10-treated huCTLA-4 knock-in mice displayed the most significant reduction in tumor growth, which in turn provided the greatest survival benefit.

Perhaps the most important advantage with the knock-in model is our ability to evaluate autoimmune side effects associated with potential human therapeutic antibodies. A previous trial with a humanized anti-CTLA-4 antibody reported considerable side effects with 43% of patients presenting with grades 3-4 autoimmune toxicity, including dermatitis, colitis/enterocolitis, hypophysitis and hepatitis [66]. In another trial, reactivity to melanocytes in skin and retina was associated with T cell infiltration and necrosis of tumors [67]. Autoimmune reactivity in anti-CTLA-4 treated mice has not been systematically analyzed, although depigmentation has been reported [60, 78]. Perhaps because of the relative short course of transplanted tumors, the autoimmune side effect in the mouse tumor model is relatively mild. However, a model that recapitulates autoimmune side-effects will not only allow us to select antibodies with fewer side effects, but also develop approaches to abrogate remaining side effects. In this regard, our quantitative comparison of anti-dsDNA antibody titers and concurrent kidney pathology have revealed considerable heterogeneity among different antibodies in terms of their autoimmune side effects. Surprisingly, L3D10, which induced the strongest therapeutic effect, provoked the least autoimmune side effects. This discordance between cancer immunity and autoimmunity reveals that autoimmune side-effects and cancer therapeutic effects are not quantitatively linked. Such uncoupling provides a theoretical basis for selecting
therapeutic agents with the most desirable balance between cancer immunity and autoimmunity. Nevertheless, it should be pointed out that our extensive search for pathological changes, including blood chemistry and histological examination of major organs, has failed to reveal severe autoimmune disease in tumor bearing mice attributable to anti-CTLA-4 antibodies (data not shown). This is in contrast to results obtained in the clinic with a fully humanized anti-human CTLA-4 antibody [66].

Several different mechanisms may be responsible for the differential effects of anti-CTLA4 antibodies. For instance, cancer immunity and autoimmunity may involve different effector cells. Alternatively, cancer targets and normal tissues may differ in their resistance to immune attack. In this context, previous studies by others have revealed that even when the antigen is shared between tumor and normal tissue, the antibody doses required for tumor rejection and autoimmune side effects differ [79-81]. Regardless of the immunological basis, the uncoupling of autoimmunity and cancer immunity demonstrated here suggests that autoimmunity may not be a necessary price for cancer immunity. These findings provide a theoretical basis for the selective modulation of cancer immunity over autoimmunity.
Table 2.1. Antibody and complement C3 deposition in kidney glomeruli.

Frozen section of kidney were analyzed after the mice were euthanized when they reach early removal criteria (tumors reach 4000 mm³), with exception of 2 mice in the L3D10-treated group in which tumors never reached the criteria for early removal. The incidences of IgG deposition in mice treated K4G4 (P=0.029) and L1B11 (P=0.029), but not L3D10 (P=0.47), are significantly higher than the control group.

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Figure 2.1 Creation of human CTLA-4 knock-in mice.  a) Schematic diagram of the structure of construct. The primer positions for screening the floxed and deleted genotypes are also illustrated. PCR Reaction A used primers outside the loxP sites spanning the Neo/TK gene. A successful excision (deleted) of Neo/TK produced a 1.1 Kb fragment, while undeleted (floxed) Neo/TK did not produce a fragment due to the PCR conditions used. PCR Reaction B used a forward primer outside of and a reverse primer within the Neo/TK gene. b) Southern blot of DNA from ES cells transfected with the human CTLA-4 construct. A 7 Kb band represents successful homologous recombination with the human CTLA-4 construct, while a 4.7 Kb band represents an unaltered mouse CTLA-4 gene. c) Excision of Neo/TK by Cre-recombinase. As depicted schematically in a), reaction A produced the expected 1.1 Kb fragment, while reaction B amplified no fragment, consistent with successful deletion of Neo/TK. d) Expression of human and mouse CTLA-4 RNA in homozygous (left panel) and heterozygous (right panel) knock-in mice. Spleen cells from human CTLA-4(+/-) and human CTLA4(+/+) mice were stimulated for 30 hours in vitro with 0.1 µg/mL anti-CD3 mAb 2C11. RNA was extracted and an RT-PCR was performed. Primers spanning the full length CTLA-4 RNA sequence were used to confirm that full length RNA of the knock-in gene was being expressed (left reaction), while those that were specific for either mouse (mE2) or human (hE2) CTLA-4 exon 2 were used to identify mouse and human CTLA-4, respectively.
Figure 2.1

a) Diagram showing the locations of EcoRI sites and the exons of mCTLA4 and hCTLA4 knock-in allele.

b) Gel showing bands at 7 kb and 4.7 kb.

c) Southern blot with probes A and B, showing bands at 7 kb and 4.7 kb, and a deleted band at 1.3 kb.

d) PCR gel showing bands at 700 bp, 500 bp, 300 bp, and 100 bp for Human CTLA4 +/+ and Human CTLA4 +/-.

Legend: mCTLA4, hCTLA4, EcoRI, Exons 2&3, Neo, TK, Floxed, Deleted.
Figure 2.2  Co-dominant expression of human and mouse CTLA-4 protein by T cells from human CTLA-4 knock-in heterozygotes.  a. Co-dominant expression of human and mouse CTLA-4 in T cells after antigen stimulation. Spleen cells from human CTLA-4(+/−) x P1CTL F1 mice were stimulated for 66 hours in vitro with 0.1 µg/mL P1A peptide. Cells were harvested and stained for cell surface mouse CD3, followed by intracellular mouse and human CTLA-4. The top left panel shows the co-dominant expression of human and mouse CTLA-4 protein on the same cells as indicated by the diagonal staining pattern. Non-knockout littermates demonstrated a complete lack of human CTLA-4 expression (lower left panel). Middle and right panels show isotype controls for each intracellular antibody. All profiles represent cells within the CD3+ gate. The same staining pattern has been observed with anti-CD3 mAb stimulated T cells (unpublished observations). b. Expression of mouse and human CTLA-4 molecules in unstimulated spleen CD4 T cells. Spleen cells from WT (CTLA-4 mo/mo), homozygous human (CTLA-4 hu/hu) and heterozygous human (CTLA-4 mo/hu) CTLA-4 mice were surfaced-stained with anti-CD4 and anti-CD25, and then stained for intracellular mouse and human CTLA-4 protein. Data shown were gated CD4+CD25− (upper panels) and CD4+CD25+ subsets (lower panels).
Figure 2.2

(a) WT C57Bl/6     Human CTLA-4 +/- Human CTLA-4 +/-

(b)  moCTLA-4-PE moCTLA-4-PE moCTLA-4-PE

moCTLA-4-PE

moCTLA-4-PE

moCTLA-4-PE

Isotype-PE

Isotype-PE

Isotype-PE

huCTLA-4-CyC

huCTLA-4-CyC

huCTLA-4-CyC

huCTLA-4-APC

huCTLA-4-APC

huCTLA-4-APC

hu/mo CTLA-4

hu/mo CTLA-4

hu/mo CTLA-4

mo/mo CTLA-4 mo/mo CTLA-4 mo/mo CTLA-4

WT C57Bl/6 Human CTLA-4 +/- Human CTLA-4 +/+
Figure 2.3 Functional replacement of mouse CTLA-4 with the human CTLA-4 gene in vivo.  

a. Normal appearance of secondary lymphoid organs in one year old homozygous human CTLA-4 knock-in mice.  
b. Normal expression of activation markers among spleen CD4 and CD8 T cells. Data shown are dot plots of gated CD8 (top panels) and CD4 (lower panels) T cells.
Figure 2.4  Anti-human CTLA-4 antibodies with different potency in delaying tumor growth.  a. Growth kinetics of MC38 tumors in minimal disease model. CTLA-4(hu/hu) mice were challenged s.c. with MC38 (5x10^6/mouse) in the lower abdomen. Two days later, the mice received either control mouse IgG or anti-CTLA-4 antibodies K4G4, L1B11 or L3D10 and the tumors were measured every 3-4 days. Data shown represent means and SEM of tumor volumes until day 55 when some mice in antibody treated groups reached their tumor burden endpoint.  b. Log transformation of tumor volume. The tumor growth over time was analyzed using Stata’s XTGEE (cross sectional generalized estimating equations) model. Six tests were done to compare the exponential slopes. All anti-CTLA-4 mAbs significantly delayed the growth kinetics of tumors (P<0.001). In addition, significant delay of tumor growth was observed in mice that received L3D10 in comparison to those that received either L1B11 or K4G4 (P<0.001).  c. Kaplan-Meier survival curves of mice that received either control IgG or one of the anti-CTLA-4 antibodies. Complete rejection of tumors was observed in 2 out of 9 mice in the L3D10-treated group. A log-rank test revealed that the three mAbs significantly prolonged mouse survival (p=0.0000-.0038). Data shown in a and b are representative of two independent experiments involving a total of 8-9 mice per group, while data in c summarizes the survival benefit from two independent experiments.
Figure 2.4

(a) Tumor volume (mm$^3$)

(b) Survival

(c) Days
Figure 2.5 L3D10 treatment delays growth of established tumors in human CTLA-4 knock-in mice. MC38 tumor cells were injected subcutaneously into human CTLA-4 knock-in mice. At 10-14 days after tumor injection, when the tumors reached a mean diameter of 8 mm, the mice were injected with either L3D10 or control Ig every four days for 4 weeks. a. Growth kinetics of established tumors in mice treated with either control IgG or L3D10 (n=9). Data shown are means and SEM of tumor volumes. The volumes of large holes caused by necrosis in some mice were subtracted. Student t tests were used to compare the tumor size at each time point, those with P<0.05 were indicated with *, while those with P<0.01 were indicated with **. b. Kaplan-Meier survival curves of mice that received control IgG or L3D10. A log-rank test revealed that L3D10 significantly prolonged mouse survival (p=0.011).
Figure 2.6 Autoimmune side effects associated with different anti-CTLA-4 antibodies. Serum samples from mice that received anti-CTLA-4 treatment, as detailed in Figure 2.4 legends, were collected on days 30 (a) and 55 (b) and tested for anti-dsDNA antibodies. Data shown are means and S.D. of O.D. at 490. c. Correlation between tumor growth suppression and anti-DNA antibodies in control IgG, L1B11 and K4G4, but not in L3D10-treated mice. Data shown are the means and SEM of tumor sizes and O.D.490 of ELISA tests using 1:270 dilution of sera from tumor bearing mice. Tumor size and anti-DNA antibody levels shown in (c) reflect data collected at 30 days post tumor challenge. The relative strength of anti-cancer immunity and autoimmunity has been repeated in two independent experiments involving 8-9 mice per group.
Figure 2.7 Anti-CTLA-4 antibodies with distinct anti-tumor and autoimmune effects bind to an overlapping site on CTLA-4 and block B7-1/CTLA-4 interaction. a-c. Cross-competition of binding. 100 µg/mL of unlabeled anti-CTLA-4 antibodies were added to plates coated with CTLA-4Ig. Given concentrations of biotinylated antibodies were added to the wells after 10 min. The amounts of biotinylated antibodies bound were determined by adsorption of HRP-labeled streptavidin to the plates. Data shown were means and SEM of O.D.490. d. All anti-CTLA-4 antibodies used in the study block B7-1-CTLA-4 interaction. CHO cells transfected with human B7-1 were incubated with a mixture of CTLA-4Ig and given anti-CTLA-4 antibodies. After washing away the unbound antibodies, the binding of CTLA-4Ig was determined by flow cytometry using APC-labeled goat anti-human CTLA-4 antibody. Data shown are histograms depicting CTLA-4Ig binding to human B7-1-transfected CHO cells.
Figure 2.7

(a) O.D. 490 nm vs Concentration (μg/ml) for mIgG, L3D10, K4G4, and L1B11.

(b) O.D. 490 nm vs Concentration (μg/ml) for L3D10, K4G4, and L1B11.

(c) O.D. 490 nm vs Concentration (μg/ml) for L1B11.

(d) CTLA4-Ig binding of L3D10, K4G4, or L1B11.
Figure 2.8  Anti-ds DNA antibodies are associated with anti-CTLA-4 but not with anti-4-1BB antibody treatment of tumor bearing mice.  a. Therapeutic effect of anti-mouse CTLA-4 antibody 4F10 and anti-mouse 4-1BB antibody 2A. Data shown are means and SE of tumor sizes (n=5/grp).  b. Anti-ds DNA antibodies as measured by ELISA. Data shown in both a. and b. are representative of 3 or more experiments.
CHAPTER 3

IMMUNE COMPETENCE OF CANCER-REACTIVE T CELLS GENERATED DE NOVO IN ADULT TUMOR-BEARING MICE

3.1 Abstract

The impact of timing of antigen introduction during fetal and neonatal development has lead to the suggestion that pre-existing antigens are tolerogenic to immune competent cells generated thereafter. This hypothesis predicts that in cancer patients undergoing bone marrow transplantation, newly produced T cells with specificity for pre-existing tumor cells will be inactivated by the tumor antigens in the host. As the effect of tumor cells on developing cancer-reactive T cells has not previously been investigated, we set out to systematically analyze the impact of tumor cells in the periphery on the development of tumor-reactive T cells in the thymus and their immune competence in the periphery. Our data demonstrate that in hosts with established tumor, cancer-reactive T cells develop normally, remain fully immune competent, become activated in the periphery and cause regression of large est-
ablished tumor. Moreover we show that these findings are not unique to tumor antigens because similar results were obtained toward alloantigens in a skin graft transplantation model.

3.2 Introduction

Classic studies by Medawar et al. established a fundamental principle that antigens presented to an immature immune system are tolerogenic [82]. This discovery forms the cornerstone of immunology, as it is the basic tenet of the clonal deletion theory proposed by Burnet [83], Talmage [84] and Lederberg [85]. Brestcher and Cohn formulated their two-signal theory in part based on consideration that this process is continuous throughout the life-span of the animal [4]. Although the tolerogenic effect of pre-existing antigens on immune competent cells has been demonstrated in neonates [86-88] what is less clear is the fate of self-reactive T cells produced de novo in adults. This is an intriguing question with regard to understanding the limits of immune competence following immune reconstitution in the adult. Interestingly, studies with chimera mice have suggested that for the same antigen and T cell combination, transgenic T cells specific for allogeneic antigen expressed in the skin are tolerized in neonatal, but not in adult mice [89].

In recent years bone marrow transplantation has become a promising cancer immunotherapy [90-92]. Thus T cell tolerance in adult animals to antigens not expressed in the thymus is a fundamental issue considering the implications for immunity generated subsequent to bone marrow transplantation.
In addition to a wealth of clinical data showing increased patient survival following bone marrow transplantation, it has also been demonstrated that tumor antigen-specific T cells have been produced after bone marrow transplantation [93]. Likewise, donor cells transplanted into virally-infected baboons generated virus-specific CTL [94]. Unfortunately it is unclear as to whether the antigen-specific T cells were derived from the carry-over of mature T cells in the bone marrow or from T cells produced \textit{de novo} in the antigen-bearing host.

In order to definitively address whether developing T cells in adult animals remain competent to peripheral tumor antigens, we investigated the development of transgenic T cells specific for tumor antigen P1A in tumor-bearing adult mice and analyzed their immune competence, antigen-induced activation and effector function. Here we report that cancer-specific cells produced \textit{de novo} in tumor-bearing mice survive thymic deletion and remain competent when encountering tumor cells causing the regression of large established tumors.

3.3 Materials and methods

Experimental animals and cell lines

BALB/c and C57BL/6 mice were purchased from Charles River Labs (Wilmington, MA) under contract with the National Cancer Institute. BALB/c RAG-2(-/-) and BALB/c nude mice were purchased from Taconic (Germantown, NY) and bred in our facility. Mice containing a transgenic T cell receptor for the tumor antigen P1A (P1CTL mice) have been described previously [95]. Mice were housed in a specific-pathogen free environment.
Bone marrow transplantation

Bone marrow from donor mice was obtained by flushing out marrow from the femur, tibia, and humerus. Red blood cells were lysed with ammonium chloride (Sigma, St. Louis, MO). Mature T cells were depleted by incubation with rat anti-mouse CD4 and CD8 mAbs, followed by goat anti-rat IgG Dynabeads (Dynal, Brown Deer, WI). T cells bound by Dynabeads were removed by magnetic separation, and T cell-depleted bone marrow was injected intravenously. For some treatment groups, T-depleted bone marrow from P1CTL mice was mixed with T-depleted bone marrow from BALB/c mice at a 3:2 ratio before injection.

Nude P1CTL bone marrow transplantation

Athymic nude mice were bred with P1CTL mice to obtain F1 mice. F1 mice were screened for the presence of the TCR transgene by staining peripheral blood for the Vα8.3 TCR. F1 mice were bred together to produce F2 mice. Nude pups were screened for the presence or absence of the TCR transgene by PCR of tail DNA. Bone marrow from TCR transgene-positive or transgene-negative mice was harvested as above. Red blood cells were lysed and bone marrow was injected intravenously.

Tumorigenicity experiments

1-5x10^6 J558 cells were injected subcutaneously in the lower abdomen. In experiments using mixed T-depleted bone marrow from BALB/c and P1CTL
donors, bone marrow was given at days 6 and 13 after tumor challenge. In the Nude P1CTL experiment, bone marrow was given in a single dose on day 2 after tumor challenge. In some experiments, mice were sacrificed after one month to analyze spleen and thymus. Tumor size was measured in two perpendicular dimensions and volume calculated as previously described [96].

**Flow cytometry**

Fluorochrome-labeled antibodies against cell surface markers and H-2L<sup>d</sup> dimeric fusion proteins were purchased from BD Pharmingen (San Diego, CA).

**Allogeneic skin graft experiments**

BALB/c mice were anesthetized and a 1-2 cm patch of flank skin was removed and replaced with a similar size patch of C57BL/6 skin. Grafts were bandaged for approximately one week, and mice possessing healthy intact grafts after at least 2 weeks were used for experiments. T-depleted bone marrow (prepared as above) or total spleen cells from BALB/c mice were injected intravenously (bone marrow) or intraperitoneally (spleen cells) in two doses at one week intervals. Mice were observed for signs of graft rejection, and grafts were considered rejected when completely removed from mice.

### 3.4 Results

Since mice with a targeted mutation of RAG-2 lack endogenous T and B cells, tumors can be easily established in these mice in their absence. Taking
advantage of this, we began by injecting plasmacytoma J558 into syngeneic RAG-2(-/-) mice. Once the tumors became palpable, the tumor-bearing mice were reconstituted with a mixture of T cell-depleted bone marrow from both syngeneic wild-type mice and transgenic mice expressing a TCR specific for the unmutated tumor antigen P1A[95]. As a control for the effect of tumor, we also generated chimera mice with no tumor challenge.

We have previously demonstrated that more than 95% of transgenic T cells express high levels of transgenic TCR [95, 97], and therefore have the ability to bind to their cognate antigen. Based on this, we used the L\textsuperscript{d}:P1A dimer to identify transgenic T cells [98]. As shown in Fig. 3.1a (upper panels), despite a 3:2 advantage of bone marrow from transgenic mice, the majority of T cells produced are from WT bone marrow as revealed by the fact that most of the thymocytes are L\textsuperscript{d}:P1A\textsuperscript{−}. To determine whether established tumors caused deletion of tumor-specific T cells, we compared the composition of cells derived from TCR-transgenic bone marrow in mice with or without pre-existing tumor. As shown in Fig. 3.1a (lower panels), thymocytes from the non-transgenic donors have developed normally regardless of whether the recipients have tumor. Perhaps owing to deletion of P1CTL as a result of expression of P1A antigen in the thymic medullary epithelial cells [99], the phenotype of transgenic T cells is consistent with that of a partial clonal deletion, characterized by an increased proportion of CD4\textsuperscript{−}CD8\textsuperscript{−} T cells and a decreased proportion of CD4\textsuperscript{+}CD8\textsuperscript{+} T cells (Fig. 3.1a, lower panels). However, the profiles of thymocytes from tumor-free and tumor-bearing mice are indistinguishable (Fig. 3.1a and b). Moreover, in
contrast to non-transgenic mature T cells, which consist of more CD4+CD8- subset, there is a significant skewing toward the CD8+CD4- subset in both groups. This is consistent with normal positive selection in both groups. Therefore it appears that established tumors in the periphery have little impact on the development of cancer-reactive T cells in the thymus.

It has been established that self-reactive T cells that exit the thymus are also controlled by peripheral mechanisms, including functional inactivation and activation-induced cell death [86, 99, 100]. We compared the peripheral T cells from tumor-bearing and non-tumor bearing mice in terms of their frequency and phenotype. As shown in Fig. 3.2a, the number of P1A-reactive T cells is significantly increased in tumor bearing mice. Since the number of transgenic T cells in the thymus is similar between tumor and non-tumor bearing hosts, it is likely that the T cells have expanded in the periphery. Consistent with this notion, essentially all splenic transgenic T cells in tumor-bearing mice exhibit a phenotype of activated T cells, as revealed by up-regulation of CD44 and down-regulation of CD62L (Fig. 3.2b).

Although we have depleted mature T cells from the bone marrow to undetectable levels (data not shown), it remains possible that the cells responding to tumor antigens in the periphery are expanded from undepleted mature T cells. To rule out this possibility, we bred the P1CTL-transgene into the nude background (nu/nu) and used the P1CTL+nu/nu bone marrow cells to reconstitute tumor-bearing RAG-2(-/-) hosts. Since both donor and recipient mice were incapable of producing mature T cells, all T cells in the chimera mice
were produced \textit{de novo} after bone marrow reconstitution of the tumor-bearing host. As expected, in mice that received nu/nu bone marrow, there were scarcely detectable levels of P1A-reactive T cells. However it is interesting to note that a high level of P1A-reactive T cells was found among tumor-infiltrating cells, although considerable variation was observed in different recipients (Fig. 3.3a). These data indicate that the P1A-reactive T cells generated \textit{de novo} in tumor-bearing hosts were expanded. In order to study the immune competence of P1A-reactive T cells, we analyzed the expression of cell surface markers and cytokine response of P1A-reactive T cells derived from P1CTL\(^+\)nu/nu bone marrow. As shown in Fig. 3.3b, in mice that received a mixture of both transgenic and non-transgenic nu/nu bone marrow, the priming of P1A reactive T cells is suggested by the down-regulation of CD62L and up-regulation of CD44. To test whether transgenic T cells have been primed in tumor-bearing hosts, we stimulated spleen cells from tumor-bearing recipients with tumor antigenic peptide P1A35-43 and analyzed the synthesis of IFN\(\gamma\) following a short \textit{in vitro} stimulation. As shown in Fig. 3.3c, transgenic T cells within the spleens were highly responsive to antigenic peptide as judged by the considerable production of IFN\(\gamma\).

The fact that tumor-specific T cells are being primed \textit{in vivo} indicates that they have come into contact with tumor antigens. To determine the functionality of these T cells, we analyzed tumor-infiltrating lymphocytes (TIL) from mice that received P1CTL\(^+\) nu/nu bone marrow cells. As shown in Fig. 3.3d (top, right), essentially all P1A-specific T cells exhibit an activated phenotype. Moreover,
after P1A peptide stimulation, a high percentage of tumor-infiltrating T cells, were capable of producing IFNγ (Fig. 3.3d, bottom). Since TIL appear to remain immune competent in an antigen-rich milieu, contact with pre-existing antigen does not tolerize newly produced T cells.

An important issue is whether tumor-specific T cells generated de novo in tumor-bearing hosts are capable of causing the rejection of tumor cells. As shown in Fig. 3.4a, with a single dose of bone marrow we observed a substantial survival advantage and delay in tumor growth in mice receiving P1CTL+ nu/nu bone marrow, as compared to mice receiving non-transgenic nu/nu bone marrow. The efficacy of the bone marrow cells is comparable to that of mature T cells, as we and others have reported [98, 101-103]. Moreover, as shown in Fig. 3.4b, repeated treatment of tumor-bearing mice with T cell-depleted bone marrow cells from transgenic mice resulted in a substantial rejection of tumor cells.

Previous work by Alferink et al. [89] demonstrated that transgenic T cells were neither tolerized nor activated in adult hosts that exclusively expressed specific antigen in the skin. To test whether polyclonal T cells developed in hosts bearing established allogeneic grafts are immune competent, we transferred T-depleted bone marrow cells from immune competent BALB/c mice into syngeneic BALB/c RAG-2/- mice bearing an established skin graft from C57BL/6 mice. As shown in Fig. 3.5, graft rejection was observed in all chimera mice that received T-depleted bone marrow cells. The significant delay in rejection in comparison to mice that received allogeneic spleen cells is consistent with the time frame
required for the maturation of T cells. Thus, immune competence of the T cells generated in an antigen-bearing host is not restricted to those that are specific for tumor antigen.

3.5 Discussion

Our data demonstrate that antigen-specific T cells generated de novo in antigen-bearing hosts are activated rather than inactivated. Our results provide a new explanation for the substantial clinical benefit of bone marrow transplantation into cancer patients [93, 104]. Our results are supported by the earlier studies of others [93, 94], although it less clear in those earlier studies whether the antigen-specific T cells were derived from mature T cells in the bone marrow or from T cells produced de novo in the antigen-bearing host. Using bone marrow from T cell-deficient hosts, we have demonstrated unequivocally the immune competence of antigen-specific CD8 T cells generated in tumor-bearing hosts.

Our data also indicated that it is necessary to achieve a high frequency of antigen-specific T cells to reject rapidly growing tumors. Since the technical barrier of genetic reprogramming has been overcome recently [105], antigen-specific T cells can be produced en masse in tumor-bearing hosts to convey protection against established tumors.

Since neonatal tolerance often involves clonal deletion in the thymus [106, 107], one may consider the possibility that the antigens in the peripheral tissues in neonates may be expressed or presented in the thymus. Previous work by
Aferink et al. [89] demonstrated that T cells produced in the adult host are ignorant to allogeneic antigens expressed as a transgene. On the other hand, our data demonstrated that tumor antigens are capable of priming T cells. The fact that substantial numbers of tumor-reactive T cells (around 7% of CD8 TIL) can be detected in tumor-bearing mice reconstituted with \textit{nu/nu} bone marrow indicate that the T cells have undergone substantial clonal expansion. Moreover, in a non-transgenic system, we have shown that newly generated T cells cause complete rejection of allogeneic skin grafts. The difference between our results and those reported by Alferink et al. [89] are likely the result of differences in how these antigens are presented (i.e. whereas alloantigen cannot be cross-presented, tumor antigens can [108]) and/or to differences in the cells expressing alloantigens. Perhaps Langerhans cells in the graft are migrating into lymphoid organs to cause T cell priming. This explanation is supported by an elegant study by Barker and Billingham [109], which established a critical role for afferent lymphatics in skin graft rejection. Regardless of what the ultimate explanation may be, our results demonstrate the utility of stem-cell based immunotherapy. This approach differs from T-cell adoptive therapy in an important way, as stem cells can continuously supply immune competent cells in cancer-bearing hosts, cells that are educated, but not tolerized.
Figure 3.1 Development of P1A-reactive T cells in RAG-2-deficient chimera mice reconstituted with a mixture of bone marrow cells from BALB/c and BALB/c P1CTL. RAG-2(-/-) BALB/c mice were injected with either PBS or J558 tumor cells subcutaneously. Six days later, when the tumors were palpable, a 3:2 mixture of T-depleted BALB/c P1CTL and WT BALB/c bone marrow cells were injected intravenously in two doses at a one week interval. At three weeks after the second injection, when tumor rejection was observed in the periphery, the mice were sacrificed and the T cell subsets in the thymus were analyzed by four-color flow cytometry using Ld:P1A dimer, anti-CD3, CD4 and CD8. a. Representative FACS profiles of thymocytes from control (left panels) and tumor-bearing (right panels) mice are shown. The profiles of non-transgenic T cells are shown on the left of each group, while that of the transgenic T cells, as revealed by their specific binding to the P1A:Ld dimer, are shown on the right. b. Summary of the T cell subsets among polyclonal (top panel) and transgenic P1A-reactive T cells (bottom panel) in tumor-bearing and unchallenged mice. Graphs depict the mean ± SEM of 6-7 mice per group, and are representative of two independent experiments.
Figure 3.1

(a) Thymus-No Tumor

CD3

Ld:P1A dimer

CD4

CD8

Thymus-Tumor

CD3

Ld:P1A dimer

CD4

CD8

(b) CD3⁺/Ld:P1A⁻ summary

% of gated cells

No tumor

Tumor

CD8⁺ CD4⁺

CD8⁻ CD4⁺

CD8⁺ CD4⁻

CD8⁻ CD4⁻

CD3⁺/Ld:P1A⁺ summary

% of gated cells

No tumor

Tumor

CD8⁺ CD4⁺

CD8⁻ CD4⁺

CD8⁺ CD4⁻

CD8⁻ CD4⁻
Figure 3.2  Activation of P1A-reactive T cells in the periphery of tumor-bearing mice. Spleen cells from the chimera mice reconstituted with bone marrow from BALB/c P1CTL\(^+\) and BALB/c mice as described in the Figure 3.1 legend were analyzed for their phenotype and number of P1A-specific T cells.  
a. Expansion of P1A-specific T cells in the spleens of tumor-bearing mice. Spleens from chimera mice were stained with anti-CD8, and L\(^d\):P1A dimer or anti-\(\alpha\)8 TCR. FACS plots are representative profiles of cells within the lymphocyte gate. Graph depicts mean ± SEM of 6-7 mice per group. This trend of expanded P1A-specific T cells in tumor-bearing mice was observed in two independent experiments.  
b. Phenotype of P1A-specific T cells from tumor-bearing and non-bearing mice. Data shown are CD44 and CD62L profiles of gated CD8\(^+\)\(\alpha\)8 TCR\(^+\) T cells. A representative profile from each group is presented on the left, while the graph on the right depicts the mean ± SEM of 3 mice per group, and is representative of two independent experiments.
No Tumor

CD8

L$^d$:P1A dimer

CD8

Tumor

% P1A-specific CD8$^+$ T cells in splenic lymphocyte gate

p = 0.013

Figure 3.2

b

No Tumor

Tumor

CD44$^{\text{high}}$ cells  CD62L$^{\text{low}}$ cells

CD44

FSC

CD62L

% of splenic Vα8.3 CD8$^+$ cells

p = 0.017

p = 0.01
Figure 3.3 Immune competence of T cells produced *de novo* in tumor bearing mice. RAG-2-deficient BALB/c mice were challenged with $5 \times 10^6$ J558 tumor cells or PBS as control. Two days later, the mice received 500 Rad of irradiation and infusion of nu/nu bone marrow cells or a 4:3 mixture of bone marrow cells from P1CTL$^+$ nu/nu and nu/nu mice. The recipient mice were sacrificed at the fourth week and tumor-specific T cells analyzed for their phenotype and response to P1A antigenic peptide.  
a. High frequency of P1A-specific T cells among the tumor-infiltrating lymphocytes (TIL) of nu/nu bone marrow recipients. Left panels show representative FACS profiles depicting highly expanded tumor-specific T cells among the TIL (top left panel) but not in the spleen (bottom left panel). Right panel FACS profiles (upper and lower) depict control stainings. A summary graph of P1A-reactive CD8 T cells among the splenocytes and TIL of nu/nu recipients is shown in the far right panel.  
b. Enhanced activation of P1A-specific CD8 T cells. Cell surface phenotype of P1A-reactive and non-reactive CD8 T cells in the spleens of tumor-bearing mice reconstituted with a 4:3 mixture of bone marrow from nu/nu and P1CTL$^+$nu/nu mice.  
c. Cytokine response of spleen cells in tumor-bearing mice. Spleen cells from tumor-bearing mice reconstituted with a mixture of nu/nu and P1CTL$^+$nu/nu bone marrow were stimulated with P1A (left panels) or control peptides (right panels) in the presence of Golgi blocker for 6 hours and stained with anti-IFN$\gamma$ antibodies after fixation and membrane permeablization. Data shown are representative of 3 independent experiments.  
d. Accumulation and immune competence of P1A-specific T cells in the tumors. Top panels show
accumulation (left) and activation status (far right) of tumor reactive T cells, while the lower panels show cytokine response to tumor antigenic peptide P1A (left panel pair) or control peptide (right panel pair). Tumor single-cell suspension from chimera mice reconstituted with P1CTL+nu/nu bone marrow were stimulated with P1A or control peptides in the presence of Golgi blocker for 6 hours and stained with anti-IFNγ antibodies after fixation and membrane permeabilization. Irradiated T-depleted splenocytes from syngeneic wild-type mice were used as APC. All data presented in this figure has been repeated 2-3 times.
Figure 3.3

- **nu/nu bone marrow**
  - TIL
  - Spleen

- **CD8**
  - Ld:P1A dimer
  - Ld:control dimer

**%Ld:P1A⁺ among CD8⁺ T cells**

- **Spleen**
  - 24.1 23.1
  - 5.01 0.33

- **Tumor**
  - 49.6 0.19
  - 5.29 0.026

- **No Tumor**
  - 0

- **p=.0020**

- **n=11 n=12 n=6**

- **Tumor**
  - 1% 0% 0.57%

- **Spleen**
  - 6.9%
Figure 3.3 cont’d

nu/nu + P1CTL+ nu/nu bone marrow

Spleen

CD8

L^d:P1A dimer

CD62L

CD44

67.8 15.1
6.31 10.8

24.2 43.3
7.69 24.8
Figure 3.3 cont’d

c nu/nu + P1CTL<sup>+</sup> nu/nu bone marrow

![Graphs showing IFN-γ expression and CD8, CD62L, CD44 markers for P1A and control groups.](image)

![Graphs showing FSC and CD8, CD62L, CD44 markers for P1A and control groups.](image)

d P1CTL<sup>+</sup> nu/nu bone marrow

![Graphs showing IFN-γ expression and CD8, CD62L, CD44 markers for P1A and control groups.](image)

![Graphs showing FSC and CD8, CD62L, CD44 markers for P1A and control groups.](image)
Figure 3.4 Bone marrow therapy of established tumors.  a. Delayed tumor growth in RAG-2-deficient mice receiving bone marrow from P1CTL$^+$ nu/nu mice compared to those that receiving bone marrow from nu/nu mice. The growth kinetics of tumors is shown in the left panel while the survival of tumor-bearing mice (as defined by time to reach tumor size of 4,000 mm$^3$) is shown in the right panel. Growth kinetics are representative of 3 independent experiments, while the survival data of tumor-bearing mice are cumulative data from two independent experiments. b. Mixture of T-depleted bone marrow cells from P1CTL transgenic mice and WT mice (3:2 ratio) causes regression of large established tumors. Mice shown are those described in Figure 3.1 legend. In both a and b, the schedule of bone marrow cell injection are indicated by arrows.
Figure 3.5  T-depleted allogeneic bone marrow cells cause rejection of pre-established skin grafts. RAG-2-deficient mice were transplanted with skin grafts from C57BL/6j mice. Survival of the skin grafts was monitored over a 2-3 month period. Kaplan-Meier curves depict skin graft survival in mice treated with either T-depleted bone marrow (n=6, closed squares) or undepleted spleen cells (n=7, open circles). The rejection of allogeneic skin grafts after treatment with T-depleted bone marrow has been observed in two independent experiments.
4.1 Abstract

Despite the fact that there is significant evidence demonstrating the importance of B7:CD28 costimulation in the development and maintenance of CD4⁺CD25⁺ regulatory T cells, the specifics of how this occurs has remained largely a matter of conjecture. Central to this argument is the need to understand what costimulation does to influence Treg development within the thymus and throughout the periphery. Though Treg have been shown to be produced within the periphery as well as intrathymically, it remains unclear whether costimulation plays a similar role and produces equivalent effects at each location. To explore this matter further we characterized the expansion of regulatory T cells within Treg-deficient mice following agonistic anti-CD28 antibody treatment. We show here that the effects of costimulation on Treg development are fundamentally different in the thymus and periphery. In the thymus Treg are generated de novo from developing thymocytes receiving costimulatory signals delivered through CD28. In the periphery CD28 costimulation results in Treg expansion primarily through increased proliferation, but may also be involved in the conversion of
conventional CD4 cells to regulatory T cells. Treg induction was CD28-dependent, but TGF-β-independent as neutralizing TGF-β1,2,3 did not abrogate Treg expansion. The requirement for B7 in Treg-mediated suppression is not Treg-intrinsic as Treg induced and isolated from anti-CD28 treated B7-deficient mice were capable of potent suppression *in vivo*. Finally, in contrast with a previous report our results indicate that expression of B7 on effector cells is not an absolute requirement for Treg-mediated suppression provided that regulatory cells are generated from the effector population.

4.2 Introduction

Numerous reports have demonstrated the importance of B7:CD28 interactions in normal immune function. This idea is substantiated in part by the fact that mice deficient in B7 or CD28 respond weakly to a variety of immune challenges, including challenges from pathogen [110-112] and tumor [113] (and unpublished observations). Paradoxically, these mice exhibit increased susceptibility to spontaneous autoimmune disease [43], yet are remarkably more resistant to disease in models of experimentally-induced autoimmunity [114, 115]. These confounding results are now reconcilable in view of recent observations demonstrating the importance of B7:CD28 costimulation in the development and maintenance of CD4+CD25+ regulatory T cells [45, 46] Such evidence now provides reasonable account as to why mice deficient in either B7 or CD28 have dramatically reduced levels of Treg, and are subsequently more susceptible to autoimmune disease.
CD4⁺CD25⁺ regulatory T cells, or Treg, comprise a unique subset of CD4⁺ cells with potent immunosuppressive function. These cells appear to be critical for maintaining peripheral tolerance, as reduction in their number or function have been shown to correlate with various autoimmune conditions [40, 116, 117]. Similarly, elevated levels of Treg have been implicated in T cell anergy and tolerance to tumor [118-120] and infection [121, 122]. Given the importance of regulatory T cells in mediating tolerance to peripheral-antigens, both self and non-self, it is not surprising then that treatments capable of expanding or reducing Treg number or modifying their function are being pursued for therapeutic use.

Recently it was reported that CD4⁺CD25⁻ T cells could be converted to CD4⁺CD25⁺ Treg in sub-lethally irradiated mice without thymic involvement, but that such conversion required B7 costimulation [123]. Their conclusions were based upon observations showing that conversion occurred in thymectomized, but not B7-deficient mice. Surprisingly, a similar though somewhat less dramatic effect was observed in non-irradiated mice, suggesting the effect was not entirely due to homeostatic proliferation. Given that B7:CD28 costimulation is a necessary requirement for successful Treg development and function, an interesting corollary is whether costimulation plays a similar role and produces equivalent effects during thymic or peripheral Treg development. As B7-deficient mice exhibit global Treg deficiency owing to insufficient CD28 costimulation, we hypothesized that reinstating costimulation with agonistic anti-CD28 antibody would be sufficient to restore Treg in these mice.
We report here that the requirement for B7 in Treg development can be circumvented upon provision of anti-CD28 antibody, as antibody treatment lead to a prolific expansion of Treg in both the thymus and periphery. Moreover, our results suggest that that the effects of costimulation are fundamentally different in the thymus and periphery. In the thymus CD28 costimulation functions in licensing developing thymocytes, as Treg are generated de novo, in the absence of proliferation. In the periphery CD28 costimulation contributes to Treg homeostasis through the expansion of resident Treg, but may also generate Treg de novo from conventional non-regulatory CD4 cells.

4.3 Materials and Methods

Mice

C57BL/6 mice with targeted mutations of B7.1 and B7.2 or RAG-1 (hereafter referred to as B7-deficient and RAG-deficient, respectively) were purchased from the Jackson Laboratory (Bar Harbor, ME). Wild type C57BL/6 mice were purchased from Charles River Laboratories (Wilmington, MA) under contract with the National Cancer Institute. Mice were age and sex-matched within each experiment and were typically used at 6-12 weeks of age. Mice were maintained under specific pathogen-free conditions at the Ohio State University Laboratory Animal Research Facility. Mice with targeted gene mutations have been backcrossed at least 10 generations.
Antibody and ascites production and characterization

Hamster control IgG antibody was purchased from Sigma (St. Louis, MO) dialyzed and filter-sterilized prior to use. TGF-β1,2,3 neutralizing mAb (1D11) and hamster anti-mouse CD28 hybridoma (37N) were kind gifts from Drs. M.A. Caliguri (The Ohio State University, Columbus, OH) and J.P. Allison (Memorial Sloan-Kettering Cancer Center, N.Y.C., N.Y.), respectively. 37N ascites were prepared essentially as described by Wong et al[124]. In brief, RAG-deficient mice were primed by i.p. injection with 0.5mL of pristine (2,6,10,14-tetra methyl-pentadecane) (Sigma, St. Louis, MO) one week prior to hybridoma injection. Each mouse received 2x10^6 hamster anti-mouse CD28-producing hybridoma cells (37N) by i.p. injection with serum-free media. Ascites were removed aseptically by paracentesis 14-21 days later with a 19-ga needle. Ascites were pooled and centrifuged at 1500 rpm for 10 min to remove cells and debris and then aliquoted and stored at -70° C until used.

Ascitic fluid was characterized by ELISA and FACS prior to experimentation. To determine the concentration of 37N antibody within the ascites preparation a sandwich ELISA was performed. In brief, unconjugated anti-hamster IgG was used for antibody capture and HRP-conjugated anti-hamster IgG antibody was used for detection. Serial dilutions of 37N ascites were performed and the concentration of antibody within the ascitic fluid determined by extrapolation from a standard curve generated with purified hamster IgG.
FACS was used to confirm the affinity of 37N ascites to murine CD28. In brief, naïve eight-week old C57BL/6 mice were sacrificed and splenocytes analyzed by two-color flow cytometry. Titrations were performed with 37N ascites and PE-conjugated anti-hamster IgG was used as the secondary reagent.

**De novo induction of Treg in B7-deficient mice**

On day 0, 6-10 week old B7-deficient mice received a single dose of 30µg of anti-CD28 antibody (37N) by i.p. injection. Mice were sacrificed at various time points post-injection and blood, spleen, LN, and thymus analyzed by flow cytometry. Hamster IgG-treated animals were also analyzed in all experiments and time points as a control. Similar results were obtained when evaluating Treg based on co-expression of CD4^+CD25^+, CD4^+Foxp3^+ or CD4^+CD25^+Foxp3^+. The effects of 37N-treatment in B7-deficient mice have been confirmed in more than five independent experiments involving 3-5 mice per group.

**FACS analysis**

To block non-specific Fc-receptor binding, cells were suspended in 200µL of 2.4G2 supernatant and incubated on ice for 20 min. Thereafter, cells were spun down and resuspended in 50µL of surface staining cocktail, which consisted of fluorochrome-conjugated antibodies diluted 1:50 in staining buffer (PBS/0.1%BSA/0.02%NaN₃). Cells were incubated on ice for 30 min and then washed three times with staining buffer and fixed with 1% PFA. For intracellular staining of Foxp3 and BrdU, cells were surface stained as described above and
then washed, permeabilized and stained with eBioscience’s Mouse Regulatory T cell Staining Kit according to the manufacturer's protocol (eBioscience, San Diego, CA). Anti-mouse CD4 (GK1.5), -CD8 (Ly-2), -CD25 (PC61), and Foxp3 (FJK-16s) antibodies and corresponding isotype controls were obtained from eBioscience (eBioscience, San Diego, CA). Anti-mouse CD44 (IM7), -CD62L (MEL-14), -CD28 (37.51), BrdU antibodies and corresponding isotype controls were obtained from BD Pharmingen (BD Biosciences Pharmingen, San Diego, CA). Samples were run on a FACSCalibur flow cytometer (BD Biosciences, Franklin Lakes, NJ) and data analyzed with FlowJo Software 6.3 (Tree Star, Inc., Ashland, OR).

Assessment of Treg proliferation in vivo

To determine whether Treg proliferate in vivo in response to anti-CD28 antibody 37N, a BrdU incorporation assay was performed. Groups of B7-deficient mice received 30µg of 37N or hamster IgG antibody intraperitoneally on day 0. Three mice from each treatment group were sacrificed on days 1, 3, and 5 post-injection for FACS analysis. Three hours prior to sacrifice mice received 1mg of the nucleotide analog bromodeoxyuridine by i.p. injection (1mg of BrdU in 100µL PBS). BrdU incorporation was determined by flow cytometry using a BrdU Flow Cytometry Kit as described by the manufacturer (BD Pharmingen, San Diego, CA).
**Cell isolation**

For functional assays CD4+CD25+ T cells were isolated by magnetic bead separation from untreated- wild type and 37N-treated B7-deficient mice. In brief, spleens and lymph nodes (mandibular, axillary, inguinal, and mesenteric) were ground into single cell suspensions, RBC lysed, and then the cells were incubated for 30 min in a CD4+ T cell enrichment cocktail composed of rat hybridoma supernatants 2.4G2 (anti-FcR), B220 (anti-B220), MAC-1 (anti-CD11b), HB224 (anti-CD11c) and mAb TIB210 (anti-CD8). Rat Ig-treated cells were then incubated with anti-rat Ig Dyna beads (Dyna Beads, Dynal Biotech, Oslo, Norway) and CD4+ T cells isolated per the manufacturer’s instructions. In some experiments CD4+ B7-deficient cells were used for T cell transfer. In other experiments CD4+ T cells from wild type and B7-deficient mice were further separated into CD4+CD25+ and CD4+CD25- subsets by staining the cells with FITC-conjugated anti-mouse CD25 and then passing the cells through a MACS anti-FITC column (Miltenyi Biotec, Lake Success, N.Y.). Purity of cell isolation was >90% as assessed by flow cytometry.

**In vivo suppression assays**

In vivo suppression assays [37, 38] were used to evaluate Treg function. In some experiments Treg were isolated from 37N-treated B7-deficient mice and tested for their ability to suppress WT CD4+CD25- responders and subsequent wasting disease. Briefly, groups of RAG-deficient mice were reconstituted by tail vein injection with 1x10^6 WT CD4+CD25- responder cells or received 1x10^6
responder cells in conjunction with 1x10^6 Treg isolated from untreated-WT or 37N-treated B7-deficient mice (n= 4, 2, and 4, respectively). The mice were observed and weighed every 3-9 days and monitored for signs of wasting disease.

Previously it was reported that the transfer of CD4^+ B7-deficient T cells into RAG-deficient hosts results in a fatal multi-organ inflammatory wasting disease [37]. To test whether 37N-treatment could rescue mice from disease, groups of RAG-deficient mice were reconstituted with 1x10^6 CD4^+ B7-deficient cells and then treated with 30µg of hamster control Ig or anti-CD28 (37N) by i.p. injection. The mice were observed and weighed every 3-9 days and monitored for signs of wasting disease.

**Histology**

At sacrifice, organs were removed and fixed in 10% formalin (lung, liver, kidney, colon and pancreas). Tissues were paraffin embedded and H&E stained. Samples were reviewed by a pathologist in blinded fashion and scored for cellular infiltration.

**4.4 Results**

Provision of anti-CD28 antibody 37N is sufficient to restore the Treg pool in B7-deficient mice.

Recently we reported that a panel of anti-human-CTLA-4 antibodies with similar binding kinetics, affinities and therapeutic effect differed significantly in
terms of their ability to invoke autoimmune side effects in human-CTLA-4 knock-in mice [96]. To more fully characterize these antibodies we developed a system that would allow us to determine whether the antibodies behaved as receptor agonist or antagonists. We tested the antibodies in B7-deficient mice so as to allow us to evaluate them in a ligand-free system. As CTLA-4 expression is maximum under conditions of optimal costimulation [12, 27] we used anti-CD28 Ab (37N) to reinstate CD28 costimulation in these mice. As proof of principle that anti-CD28 antibody could activate T cells in these mice, we treated B7-deficient mice with or without anti-CTLA-4 and anti–CD28. The mice were sacrificed one week after treatment and freshly harvested spleens analyzed by flow cytometry to determine the extent of T cell activation. Treatment with anti-CD28 antibody greatly enhanced activation as both CD4 and CD8 cells demonstrated a significant upregulation of CD44^{high}CD62L^{low}, CD69 and CD25 cells (data not shown). The extent of activation was significantly less in mice that received both anti-CD28 and anti-CTLA-4 (data not shown), further confirming previous reports that CTLA-4 ligation antagonizes CD28-signaling [27, 125-127]. However, what was most intriguing was the effect anti-CD28 treatment had on CD4^+CD25^+ cells. In numerous experiments anti-CD28 treatment led to a 10-fold increase in CD4^+CD25^+ expression, in both percent and absolute number (data not shown).

As CD4^+CD25^+ co-expression has previously been identified as a marker for T regulatory cells we reasoned that anti-CD28 antibody treatment could be expanding Treg in these mice. Owing to the fact that this observation was made in B7-deficient human-CTLA-4 mice we decided to determine whether this result
was reproducible in wild type B7-deficient mice or an artifact of our knock-in mouse. Therefore we treated wild type B7-deficient mice with anti-CD28 antibody or hamster Ig and analyzed spleens and thymii from mice collected seven days later. Due to the fact that activated T cells transiently express CD25, the use of this marker to identify Treg could lead us to potentially overestimate the number of regulatory T cells. To avoid this problem we also evaluated the expression of Foxp3, a marker recently identified as the 'master T regulatory gene' [42, 128, 129]. Our results indicate that Foxp3 induction was primarily restricted to CD4⁺ cells in the spleen and CD4SP cells in the thymus, although there was a modest induction of Foxp3 among CD8⁺ splenocytes (data not shown). Representative FACS profiles of antibody-treated animals depicted in Figure 4.1 demonstrate that a single low dose of 37N (30ug) results in a prolific increase of Treg in both the spleen and thymus of treated animals (Figure 4.1a. and c., respectively.) The results of one individual experiment involving 4 mice per group are summarized for CD4⁺ splenocytes and CD4SP thymocytes in Figure 4.1b. and d., respectively. Similar results have been obtained in more than five-independent experiments involving 3-5 mice per group.

**TGF-β is expendable for 37N-mediated Treg induction.**

Emerging evidence has suggested that TGF-β may play a vital role in Treg development and function. First it has been reported that conversion of naïve peripheral CD4⁺CD25⁻ to CD4⁺CD25⁺ regulatory T cells can be achieved *in vitro* and *in vivo* through TCR costimulation and TGF-β [130]. Transition to a
regulatory phenotype was found to be mediated by TGF-β-induced Foxp3 expression. In a related study, Huber et al used a transgenic mouse model with impaired TGF-β signaling to demonstrate that intact TGF-β signaling is required for Treg expansion as well as Treg-mediated suppression in vivo [131]. With regard to our observations it is interesting to note that Fu et al have reported that the conversion of conventional CD4+CD25- cells to regulatory T cells requires stimulation of the TCR, IL-2R and TGF-β receptor, yet strong costimulation through CD28 prevents Foxp3 expression and development of suppressive function in vitro [132].

To confirm whether TGF-β plays a role in anti-CD28-mediated induction of Treg we treated groups of B7-deficient mice with 30µg of 37N or hamster Ig along with 125µg of TGF-β1,2,3-neutralizing antibody (1D11) or mouse IgG. FACS analysis of spleens and thymii from antibody-treated mice revealed that TGF-β was not required for Treg induction in either organ (Figure 4.2). Unexpectedly, neutralizing TGF-β in the thymus led to a significant increase in both the total number and percentage of CD4SP (data not shown), and Foxp3+CD4SP subsets.

**Induction of Treg in the thymus precedes that in the spleen.**

Taking into account that Treg appear to be expanding in both the spleen and thymus an interesting question is whether the increased number of Treg seen in the spleen are due to expansion of residual Treg, de novo induction from conventional T cells, or if increases in the spleen are merely the result of
increased thymic output. To address this issue we treated groups of mice with 30µg of 37N or hamster Ig and then sacrificed these mice one, three and five days after treatment to analyze Treg frequency within the spleen and thymus. As expected there was no increase of Treg in the spleens or thymii of hamster control Ig-treated mice (Figure 4.3a. and c., respectively). However there was a consistent enhancement of Treg among CD4+ splenocytes and CD4SP thymocytes over time in response to 37N-treatment (Figure 4.3b. and d., respectively). The kinetics of Treg induction within the spleen and thymus were evaluated based on folds of increase over corresponding control Ig-treated animals at a given time point and the results are presented in Figure 4.3e. These results clearly illustrate that the induction of thymic Treg precedes that in the spleen and raises the possibility that the increase of peripheral Treg is in large part the result of increased thymic output.

**Splenic but not thymic Treg proliferate in response to 37N.**

Though the data is suggestive, accelerated Treg kinetics in the thymus do not prove that Treg increases in the spleen are purely the result of thymic output. Moreover it remains unclear as to whether increases in either the spleen or thymus are the result of de novo induction or enhanced proliferation. To address this issue we analyzed the proliferation of Treg within the spleen and thymus of antibody-treated animals by BrdU incorporation. BrdU incorporation was determined among splenic and thymic Treg at one, three and five days post-treatment. The results of these studies clearly demonstrate that splenic, but not
thymic Treg proliferate in response to 37N (Figure 4.4). Consistent with our observation that hamster Ig does not increase Treg frequency in either the spleen or thymus; we observed no Treg proliferation in the organs of control Ig-treated animals (Figure 4.4b. and c., respectively). It is intriguing to note that whereas we observed progressive proliferation of splenic Treg over time following 37N-treatment (Figure 4.4a. and summary in b.), thymic Treg do not appear to proliferate (Figure 4.4c.). These results provide conclusive evidence that thymic Treg are generated de novo in response to 37N-treatment.

37N-induced Treg persist in donor animals and are as effective as WT Treg in mediating suppression.

The ability to mediate suppression of conventional T cells is a defining characteristic of regulatory T cells. To determine whether 37N-induced CD4^+CD25^+ cells were bona fide Treg, we assessed their capacity to suppress CD4^+CD25^- responders in a wasting disease model as first described by Sakaguchi [38]. Our results indicate that 37N-induced CD4^+CD25^+ cells are functional in vivo, as their ability to suppress wasting disease was equal to that observed for wild type Treg (Figure 4.5a. and b.). Protection from wasting disease correlated with decreased cellular infiltration in target organs, as well as a reduced ratio of CD4^+CD25^-:CD4^+CD25^+ cells in the blood (Figure 4.5c. and d., respectively). Moreover our results suggest that although there may be a requirement for B7 in Treg-mediated suppression [37], that requirement is not Treg-intrinsic.
37N is effective in preventing wasting disease.

Recently it was reported that Treg-mediated suppression requires Treg engagement of B7 on effector T cells [37]. This finding is based on the observation that transfer of B7-deficient CD4\(^{+}\) cells into RAG-deficient mice results in a multi-organ inflammatory wasting disease which is not susceptible to Treg-mediated suppression. Based on our earlier proliferation studies we wondered whether Treg could be induced or expanded in such a system, and moreover whether generating Treg de novo from the effector population might prevent disease. As seen in Figure 4.6a. and b. a single low dose of 37N (30µg) was sufficient to prevent the development of wasting disease in treated mice. Conversely, control Ig-treated mice began to show signs of weight loss by d30 and were moribund by about d60, with one mouse succumbing to wasting disease at d61. Protection from wasting disease correlated with a reduction of cellular infiltration in the lung, liver, kidney and pancreas, but rather surprisingly both treatment groups had similar levels of infiltrates in the colon (Figure 4.6c.). By comparison, 37N-treated mice had a higher percentage of Treg in the blood though there was considerable variation in this regard among each group (Figure 4.6d.). Although there seems to be some variation in Treg number among antibody treated animals it may be the case that Treg function is enhanced by anti-CD28. Consistent with this idea, 37N-treated animals had a 3-fold decrease in the percent of CD4\(^{+}\) cells (4.64±0.49% v. 1.53±0.44%) and a six-fold decrease in total number (3603±310 \(\text{v}\) 551±198). With respect to the work of Paust et al,
our data suggests that expression of B7 on effector cells may not be an absolute requirement for Treg-mediated suppression provided that regulatory cells are generated from the effector population.

4.5 Discussion

With regard to the importance of CD4⁺CD25⁺ regulatory T cells in mediating immunity to self, pathogen and tumor, the key to unlocking their therapeutic potential lies in understanding the factors controlling their development and function. To this end considerable progress has been made as evidenced by the findings of two recent reports. First Masteller et al have reported that in vitro expanded Treg adoptively transferred to NOD mice were effective in preventing diabetes [133]. Secondly, Beyersdorf et al reported that Treg could be preferentially expanded over conventional T cells with the use of a superagonistic anti-CD28 antibody [134]. More importantly, such treatment proved to be highly efficacious as both a preventive, as well as therapeutic agent in a rat model of EAE.

Though we have observed that treatment with anti-CD28 antibody (37N) similarly expands Treg in mice, 37N does not fall into the category of superagonistic antibodies described by others [135, 136]. Foremost is the fact that superagonistic antibodies appear to be unique in that they are capable of activating naïve T cells in the absence of TCR ligation, both in vitro and in vivo [135]. Though we observed significant activation of T cells in vivo, similar effects were not observed in in vitro assays (data not shown), suggesting that 37N-
mediated activation is dependent upon TCR occupancy. Moreover conventional and superagonistic antibodies appear to recognize CD28 through distinct epitopes [136] and bind with different kinetics [135]. While conventional anti-CD28 antibodies have been shown to bind at or near the B7-binding motif, superagonistic antibodies are unique in that they recognize the laterally exposed C"D loop of rat CD28 [136]. The fact that this epitope is more accessible following TCR stimulation [137] is thought to explain the slower binding kinetics of superagonistic antibodies, and suggests the complexity of CD28 clustering may have been previously underappreciated [136]. Finally, conventional and superagonistic antibodies appear to differ in terms of how they effect Treg development. Whereas superagonistic antibodies preferentially expand Treg in the periphery [134], our data suggests that conventional anti-CD28 antibodies play a dual role in Treg homeostasis by producing Treg de novo in the thymus and then promoting their expansion in the periphery.

The ability of regulatory T cells to mediate peripheral tolerance is likely dependent upon many factors including their frequency, functional capacity and ability to traffic. As we and others have now offered evidence as to the role of CD28-costimulation in controlling the size of the Treg pool, it remains to be seen how this pathway impacts Treg-suppressor function and trafficking. With regard to these points, it is interesting to note that we observed significant increases in granzyme B and CCL5 expression among whole-blood lysates following 37N-treatment (personal communication, G. Zheng, Genospectra, Fremont, CA). These findings are relevant in light of recent reports demonstrating the
importance of granzyme B and chemokine signaling in Treg-mediated suppression. First, Gondek et al demonstrated that Treg-mediated suppression occurs through a granzyme B-dependent pathway whereby Treg were shown to induce effector cells to undergo apoptosis [138]. These results are consistent with the idea that 37N-treatment enhances Treg-mediated suppression, as we observed a significant decrease in the percent and total number of CD4 cells among 37N-treated mice protected from wasting disease. Secondly, with regards to the importance of Treg trafficking in maintaining peripheral tolerance Wysocki et al have recently reported that Treg-mediated protection from GVHD correlated with expression of CCR5 on Treg cells [139]. In their work they demonstrated that CCR5 is dramatically upregulated in Treg following activation and that upregulation of CCR5 correlated with increased Treg chemotaxis in response to the CCR5 ligands CCL3, CCL4 and CCL5.

In summary, our results demonstrate the importance of CD28-costimulation in Treg development and homeostasis. Moreover, we show here that the effects of costimulation are fundamentally different in the thymus and periphery. In the thymus costimulation is involved in programming Treg from developing thymocytes, as Treg are generated de novo without proliferation. Once in the periphery Treg numbers are maintained primarily by proliferation induced through CD28 ligation.
Figure 4.1 Anti-CD28 mAb 37N expands the Treg pool in B7-deficient mice.

B7-deficient C57BL/6 mice received 30µg of 37N or hamster control Ig by i.p. injection (n=4/grp). Six days later the mice were sacrificed and the spleens and thymii were removed and analyzed by four-color flow cytometry using fluorochrome-conjugated anti-CD4, CD8, CD25, and Foxp3 antibodies.  

a. Representative FACS profile of splenocytes isolated from control Ig and 37N-treated mice (middle and right panels, respectively). Foxp3 isotype control staining is included to demonstrate specificity of staining (left panel).  

b. Summary of Foxp3 expression in the spleens of antibody-treated animals. The graph depicts the individual percentages of Foxp3+ cells within the CD4+ gate for each mouse and the horizontal bar represents the mean within each group.  

c. Representative FACS profile of CD4SP thymocytes isolated from control Ig and 37N-treated mice (middle and right, respectively). Again an isotype control stain is included to demonstrate staining specificity (left panel).  

d. Summary of thymic Treg frequency among the CD4SP subset. Results are reported for individual mice and the horizontal bar represents the group mean. The effects of 37N-treatment in B7-deficient mice have been confirmed in more than five independent experiments involving 3-5 mice per group.
Figure 4.1

a. Spleen

b. CD4+ splenocytes

c. CD4SP thymocytes

d. CD4SP thymocytes
Figure 4.2 TGF-β is not required for 37N-mediated Treg induction. B7-deficient mice were co-injected with 30µg of hamster control Ig or 37N and 125µg of TGF-β1,2,3-depleting antibody (1D11) or mouse control Ig. Six days after injection the mice were sacrificed and spleens and thymii isolated for four-color flow cytometric analysis. a. Summary of Treg frequency within the spleen. Graph depicts the mean percentage (±SEM) of Treg among CD4+ splenocytes isolated from each of the three groups (n=4/grp). Similar results were obtained when evaluating Treg based on CD4+CD25+ or CD4+Foxp3+ co-expression. b. Summary of the frequency of Treg among CD4SP thymocytes. Graph depicts the mean percentage (±SEM) of Treg among CD4+ splenocytes isolated from each of the three groups (n=4/grp).
Figure 4.3  Treg induction kinetics within spleen and among thymic subsets. On day 0, groups of B7-deficient mice received 30µg of 37N or hamster control Ig by i.p. injection (n=3 grp). Three mice per group were sacrificed on days 1, 3 and 5 and their spleens and thymi harvested for four-color flow cytometric analysis. Treg were defined on the basis of co-expression of CD4+CD25+, as well as by CD4+Foxp3+, and both methods gave similar results. a. and b. Splenic Treg induction kinetics of hamster control Ig-treated and 37N-treated mice (a. and b., respectively). Treg frequency among CD4+ splenocytes was evaluated for each group of antibody-treated mice. Graphs depict the mean percentage (±SEM) of Treg within the CD4+ gate. c. and d. Thymic Treg induction kinetics of hamster control Ig-treated and 37N-treated mice (c. and d., respectively). Treg frequency among CD4SP thymocytes was evaluated for each group of antibody-treated mice. Graphs depict the mean percentage (±SEM) of Treg among the various gated thymic subsets. e. Thymic Treg induction precedes splenic Treg induction. Graph depicts the induction of Foxp3 among CD4+ splenocytes as well as CD4SP thymocytes reported as folds of increase as compared to corresponding hamster Ig-treated mice.
Figure 4.3

(a) hlg

(b) 37N

(c) hlg

(d) 37N

(e) Foxp3 Induction

Days post-treatment

Folds of Induction

CD4 SP thymocytes
CD4 splenocytes
Figure 4.4 Splenic but not thymic Treg proliferate in response to 37N. On day 0, groups of B7-deficient mice received 30µg of 37N or hamster control Ig by i.p. injection (n=3/group). Three mice per group were sacrificed on days 1, 3 and 5 and their spleens and thymii harvested for four-color flow cytometric analysis using fluorochrome-conjugated anti-CD4, CD8, CD25, Foxp3 and BrdU antibodies. a. and b. Progressive proliferation of splenic Tregs in response to anti-CD28 antibody 37N. a. Representative FACS profiles showing BrdU incorporation among gated splenocytes isolated from individual 37N-treated mice on days 1, 3 and 5 post-treatment (left, middle and right, respectively). b. Summary of the percentage of BrdU incorporation among CD4⁺Foxp3⁺CD25⁺ Treg isolated from control hamster Ig and 37N-treated mice. Results are reported as mean percentage (±SEM) of BrdU incorporation within the indicated gate. c. Lack of proliferation among thymic Treg. Representative FACS profiles showing both CD25⁺ expression and BrdU incorporation among gated CD4SP thymocytes isolated from antibody-treated mice on days 1 and 5 post-treatment (left panel pair and right panel pair, respectively).
Figure 4.4

a) Treg Proliferation in Spleen (gated FoxP3+CD25+)

b) Spleen

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Spleen

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Treg Proliferation in d1 Thymus (gated CD4SP)

Treg Proliferation in d5 Thymus (gated CD4SP)
Figure 4.5 37N-induced Treg are as functional as WT Treg. On day 0 B7-deficient C57BL/6 mice received 30µg of anti-CD28 antibody 37N by i.p. injection. The mice were sacrificed six days later and CD4⁺CD25⁺ cells were isolated from spleens by magnetic bead separation. Wild-type C57BL/6 mice were also sacrificed and spleens harvested for the isolation of CD4⁺CD25⁺ Treg and CD4⁺CD25⁻ responder cells. Thereafter, groups of RAG-deficient C57BL/6 mice were reconstituted by tail vein injection with 1x10⁶ WT CD4⁺CD25⁻ responder cells or received 1x10⁶ responder cells in conjunction with 1x10⁶ CD4⁺CD25⁺ Treg isolated from untreated-WT or 37N-treated B7-deficient mice (n= 4, 2, and 4, respectively). The mice were observed and weighed every 3-9 days and monitored for signs of wasting disease. At d60 the mice were bled and their blood stained and analyzed by flow cytometry. At d64 the mice were sacrificed and their organs harvested and evaluated for histopathology. a.- c. Provision of Treg from 37N-treated B7-deficient donor animals protects mice from wasting disease as effectively as WT Treg. a. Graph depicts mean (±SEM) weights by group over time. b. Graph depicts mean percent of weight change among various groups (±SEM) relative to starting weight. c. Summary of histopathological findings. On d64 the mice were sacrificed and their organs harvested to determine cellular infiltration by H&E staining. d. Protection from wasting disease correlates with Treg prevalence. On d61 mice were retro-orbitally bled and their blood analyzed by four-color flow cytometry. Graph shows the ratio of CD4⁺Foxp3⁻ to CD4⁺Foxp3⁺ cells within individual mice for each treatment group and the horizontal bars represent the group means.
Figure 4.5

(a) Weight (g) vs. Days Post Transfer

(b) % Weight Change vs. Days Post Transfer

(c) Distribution of Treg across organs:

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(d) CD4/Foxp3 vs. Days Post Transfer:

- Treg: 33.24
- WT Treg: 5.35
- 37N Treg: 2.68
Figure 4.6  Anti-CD28 antibody 37N protects mice from wasting disease.

B7-deficient mice were sacrificed and CD4⁺ donor cells isolated by magnetic bead separation. Thereafter, groups of RAG-1-deficient mice were reconstituted with 1x10⁶ donor cells by tail vein injection. On the same day mice received either 30µg of hamster control Ig or 37N by i.p. injection. The mice were observed and weighed every 3-9 days and monitored for signs of wasting disease. A sharp decline in health was observed among control-Ig treated mice beginning around day d45 and one mouse succumbed to wasting disease on d63. At d61 the mice were bled and their blood stained and analyzed by flow cytometry. By d64 the mice had become moribund and were sacrificed. At sacrifice organs were harvested and evaluated for histopathology. a.-c. 37N treatment protects mice from wasting disease. a. Graph depicts mean (±SEM) weights by group over time. b. Graph depicts mean percent of weight change among various groups (±SEM) relative to starting weight. c. Summary of histopathological findings. On d64 the mice were sacrificed and their organs harvested to determine cellular infiltration by H&E staining. d. Protective effects of anti-CD28 may be due to both Treg-dependent and Treg-independent events. On d61 mice were retro-orbitally bled and their blood analyzed by four-color flow cytometry. Graph shows the ratio of CD4⁺Foxp3⁻ to CD4⁺Foxp3⁺ cells within individual mice for each treatment group and the horizontal bars represent the group means.
Figure 4.6

(a) Weight (g) over days post transfer for hIg and 37N groups.
(b) % Weight Change over days post transfer for hIg and 37N groups.
(c) Organ availability:
   
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*Organ unavailable for one animal that succumbed to wasting disease.
CHAPTER 5
CONCLUDING REMARKS

An overwhelming number of diseases may be attributed to immune dysfunction [140-145]. As such, a great deal of effort has been put forth to develop therapies that aim at modulating the immune system. The importance of T cell costimulation in promoting, sustaining or inhibiting T cell responses makes it a powerful point for disease intervention, particularly as it pertains to cancer immunity. Consistent with this idea, antibodies have emerged as a promising tool for augmenting anti-cancer responses [50, 57, 146-149]. Chief among these are anti-receptor antibodies targeting T cell costimulatory receptors. Though numerous receptors have been proposed, antibodies targeting Cytotoxic T Lymphocyte Antigen-4 (CTLA-4) are among the most promising.

Despite a growing body of evidence supporting the use of anti-CTLA-4 antibodies in cancer therapy [59, 67, 150], two obstacles have hampered their translation to human cancer therapy. First, in vitro assays have proven to be an unreliable means by which to predict an in vivo response [68]. Secondly, numerous studies have demonstrated that the use of anti-CTLA-4 antibodies appears to carry with it the risk of invoking autoimmune disease [151]. Thus the
focus of our attention must be the development of suitable *in vivo* models, for in their absence it will be difficult to select the most effective agent while avoiding potential risks.

In response to these obstacles we created a human CTLA-4 knock-in mouse [96] along with several anti-human CTLA-4 antibodies [152] to facilitate the translation of this concept to the clinic. Our findings here have two important implications. First, we have demonstrated that the CTLA-4 knock-in mouse is a suitable model for preclinical screening efforts, in that it affords the opportunity to evaluate both desired therapeutic effect as well as unwanted side-effects. Second and perhaps more importantly, is our observation that anti-tumor immunity and autoimmunity are not necessarily linked, suggesting that stronger therapeutic effect need not occur at the expense of greater autoimmune side-effect. This result is both surprising and encouraging in light of previous clinical data which seemed to suggest that the strength of the anti-tumor response correlates with that of the autoimmune response [151, 153].

Since its first successful use 45 years ago, clinically administered bone marrow or peripheral blood stem cell transplantation has drawn increasing attention as a treatment for a variety of diseases and conditions [154-159]. When used as a treatment modality for hematological malignancies the graft-v-leukemia/lymphoma effect (GVL) is well documented [104, 160, 161], but whether the same is true for solid or metastatic malignancies is less certain [155, 162]. Interestingly Kalina et al recently reported that donor cells transplanted into
virally-infected baboons generated virus-specific CTL [94]. Understanding whether an analogous graft-v-tumor (GVT) effect occurs has important long term implications for host immunity in individuals undergoing BM therapy.

To further address this issue we set up a system that would allow us to follow the development of tumor-reactive T cells within a tumor-bearing host. Our central aim was to determine not only whether tumor-specific T cells can develop in a host with established tumor, but whether they would remain immunologically competent, and thus capable of responding to existing tumor. Previous studies of neonatal tolerance have suggested that pre-existing antigens are tolerogenic to subsequently generated T cells [86-88]; thus one possibility is that the tumor-reactive T cells might be eliminated in the thymus by clonal deletion. However, as self-tolerance is also maintained by mechanisms operating in the periphery including T cell anergy, activation induced cell death and regulation in trans by regulatory T cells [163-165], the cells must also pass this checkpoint. Surprisingly, in a host with established tumor, tumor-specific T cells develop normally in the thymus; remain fully immune competent in periphery, infiltrating and causing the destruction of large established tumors. Our results further demonstrate the utility of stem-cell based immunotherapy.

CD4^+CD25^+ regulatory T cells, or Treg, comprise a unique subset of CD4^+ cells with potent immunosuppressive function. Ever emerging evidence has implicated these cells in various forms of immunity, including immunity to self, tumor and pathogen. Recently it was reported that CD4^+CD25^- T cells could be converted to CD4^+CD25^+ Treg in sub-lethally irradiated mice without thymic
involvement, but that such conversion required B7:CD28 costimulation [123].

With the use of knock-out animals as well as antibody-blockade Tang et al demonstrated that CD28 is crucial for the thymic development of Treg as well as their homeostasis in the periphery [45]. In their model CD28 promoted Treg maintenance in the periphery by sustaining CD25 expression. Though CD28-mediated IL-2 production has been implicated in Treg development, Tai et al showed that signaling through CD28 induces Treg differentiation independently of IL-2 through the induction of the ‘master T regulatory gene’ Foxp3 [46]. These findings are surprising in light of previous microarray data which suggested that the primary effect of CD28 costimulation was to augment CD3 signaling rather than initiate a unique gene program [47].

Given that B7:CD28 costimulation is a necessary requirement for successful Treg development and function, an interesting corollary is whether costimulation plays a similar role and produces equivalent effects during thymic and peripheral Treg development. To address this question we followed the development of Treg within the spleen and thymus of Treg deficient mice. As B7-deficient mice exhibit global Treg deficiency owing to insufficient CD28 costimulation, we hypothesized that reinstating costimulation with agonistic anti-CD28 antibody would be sufficient to restore Treg in these mice. Indeed this appears to be the case for we observed that a single low dose of anti-CD28 antibody was adequate to restore the Treg pool in these mice. Furthermore our results indicate that the effects of CD28 costimulation on Treg development in the thymus and periphery are fundamentally different. In the thymus
costimulation is involved in programming Treg from developing thymocytes, as Treg are generated de novo without proliferation. Once in the periphery Treg numbers are maintained primarily by proliferation induced through CD28 ligation.

It is interesting to note that we also observed a significant expansion of Treg (~2-fold) in wild type C57BL/6 mice following low dose anti-CD28 antibody treatment, suggesting this approach may be useful in the treatment of human autoimmune disorders. This notion is supported by two recent reports. First, in vitro expanded Treg remained functional following their antibody-induced expansion and were effective in preventing diabetes when adoptively transferred into NOD mice [133]. Secondly, Beyersdorf et al reported that Treg could be preferentially expanded over conventional T cells with the use of a superagonistic anti-CD28 antibody [134]. More importantly, such treatment proved to be highly efficacious as both a preventive, as well as therapeutic agent in a rat model of EAE.

Immunotherapy offers great promise in our fight against human disease. It is difficult to image a more perfect weapon to combat disease than our own immune system, a system crafted and refined through millions of years of evolution. Unfortunately a lack of understanding has prevented us from exploiting its strengths, correcting its deficiencies and realizing the potential of immunotherapy. This work marks our attempt to address some basic issues pertaining to T cell and stem cell-based therapies. With regard to the former our objective was to understand the role of costimulation in anti-tumor and autoimmunity and how these pathways might be exploited for desired immune
effect. Concerning our work in stem cell-based tumor therapy our goal was to determine the impact of tumor on newly developing tumor-specific T cells. It is our sincere hope that these findings will serve to advance the field, providing insight and future direction.
BIBLIOGRAPHY


