T CELL COSTIMULATION IN ANTI-TUMOR IMMUNITY AND AUTOIMMUNITY

DISSERTATION

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ABSTRACT

Costimulatory molecules, including 4-1BB, CTLA-4, and B7, play a critical role in the activation, sustenance, and regulation of T cell immune responses. Manipulation of these pathways holds promise for the development of therapies for cancer and autoimmunity. Administration of anti-4-1BB monoclonal antibody (mAb) has been demonstrated to boost anti-tumor immunity in animal models, with varying conclusions regarding mechanism of action. Using a model of tumor-specific CD8 T cell adoptive immunotherapy, we demonstrate that anti-4-1BB mAb can mediate the rejection of large established tumors in the absence of CD4 T cell help. Anti-4-1BB mAb increases populations of tumor-specific CD8 T cells in peripheral blood by reduction of activation-induced cell death, but not increased T cell proliferation.

The use of anti-CTLA-4 mAb has also been shown to enhance anti-tumor immunity. Here we describe the use of two novel “humanized” mouse models to screen anti-human CTLA-4 mAb for translation to human cancer therapy. Using the hu-PBL-SCID model of Epstein-Barr virus (EBV)-associated lymphoproliferative disease, we show that anti-human CTLA-4 mAb promotes the in vivo expansion of human CD8 and CD4 T cells, and the generation of antigen specific CD8 T cell responses to EBV lymphoma. This correlates with reduced levels of the oncogenic EBV protein LMP-1,
and increased survival and delay of lymphoproliferative disease in these mice. We also characterize the creation of a knock-in mouse model in which mouse T cells express the human CTLA-4 molecule. Preliminary testing in this mouse model supports the use of this model to screen anti-human CTLA-4 mAb for clinical use.

Immunological function of B7/CD28/CTLA-4 interaction in immune activation and tolerance is fundamental to successful immune intervention targeted at costimulatory molecules. As an integrated part of this dissertation, I have devoted considerable effort to investigating the basic immunobiology of T cell costimulation. We describe the spontaneous development of whole-body alopecia, lymphadenopathy, and skin disease in mice lacking B7 molecules. This disease is mediated by autoimmune CD4 T cells, which induce multi-organ inflammation when transferred to mice expressing B7 molecules. This disease may result from impaired development of CD4^+CD25^+ regulatory T cells (Treg) in B7-deficient mice. Since provision of Treg can abrogate the multi-organ inflammation despite lack of B7 molecules on the auto-pathogenic T cells, interaction between CTLA-4 on Treg and B7-1/2 on effector T cells is not essential for Treg function.
Dedicated to my family
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CHAPTER 1

INTRODUCTION

1.1 T lymphocytes

T lymphocytes, or T cells, are major effectors of the cellular adaptive immune response. Most T cells express a T cell antigen receptor (TCR) composed of an alpha and a beta chain. Alpha/beta T cells are categorized into two major subsets, called CD8 T lymphocytes and CD4 T lymphocytes, based on distinct cell surface proteins that play a role in specific recognition of stimuli and targets by the immune system. CD8 T cells express the CD8 co-receptor which associates with the T cell receptor (TCR) to interact with major histocompatibility complex class I (MHC I) molecules expressed by all nucleated cells. CD8 T cells are often referred to as cytotoxic T lymphocytes (CTL) because their major function is to kill abnormal cells (such as virally-infected cells or tumor cells) that express abnormal peptides within their MHC Class I molecules. CD4 T cells express the CD4 co-receptor which associates with the TCR to interact with major histocompatibility complex class II (MHC II) molecules expressed mainly by professional antigen presenting cells (APC), such as dendritic cells, macrophages, and B
CD4 T cells, or helper T cells, secrete proteins called cytokines which orchestrate the adaptive immune response by “helping” other cells such as CD8 T cells to function properly [1].

1.2 T cell activation and costimulation

To perform their effector functions, T cells must become activated. T cell activation is a carefully coordinated sequence of molecular events that begins with the recognition by the T cell receptor of cognate peptide presented in the context of an MHC molecule (Figure 1.1, top panel). This recognition of pMHC by TCR initiates the formation of an immunological synapse, which is an area of close sustained contact between the T cell and pMHC-expressing cell. This synapse facilitates signal transduction through CD3, a complex of cell surface molecules associated with the T cell receptor, and various other associated signaling molecules. Signals transduced by the TCR trigger a cascade of intracellular pathways that result in gene expression and protein synthesis of cytokines (IL-2) and cell surface molecules (IL-2 receptor, CD25), rearrangement of cytoskeletal proteins, and the enabling of effector functions (cytokine secretion, cytolytic granule secretion) [2].

While TCR ligation with peptide:MHC confers specificity to T cell interactions and initiates T cell activation, a second set of signals is required to regulate the outcome of activation. This critical second signal is provided by costimulatory molecules (Figure 1.1, middle panel). Costimulatory molecules represent a large array of cell surface molecules that are differentially expressed by many different cell types, including T cells and APC [3]. The term “costimulatory” is actually confusing when one considers the
diverse actions that these molecules perform. Some costimulatory molecules, such as CD28, function to enhance the activating signal initiated by the TCR [4]. Others, such as PD-1, are negative regulators that dampen T cell activation when ligated [5]. Still other costimulatory molecules, such as B7-1, B7-2, and CTLA-4 may induce different responses depending on the nature and setting of ligation [6]. As new costimulatory molecules are discovered, the picture of how costimulation influences T cell activation becomes more complex.

1.2.1 CD28/B7 costimulation

The prototypical costimulatory event in T cell activation involves the interaction of CD28 [7, 8] on the T cell with B7-1 (CD80) [9] or B7-2 (CD86) [10, 11] (collectively known as B7) on the APC. In conjunction with a stimulatory TCR:pMHC interaction, binding of CD28 with its ligand B7 provides the proverbial second signal required for optimal T cell activation [4, 12]. CD28 transduces signals into the cell, which mediate a number of events that promote and sustain T cell activation and proliferation, including expression of cytokines and cytokine receptors (especially IL-2 and IL-2 receptor) [4, 12] and upregulation of anti-apoptotic molecules such as bcl-XL [13]. CD28 costimulation also reduces the threshold required for T cell activation by permitting activation to occur with fewer TCR:pMHC interactions [14] and facilitating the formation of the immunological synapse between T cell and APC [15-17]. Studies using knockout mice lacking these costimulatory molecules have illustrated their role in T cell activation. CD28-deficient mice displayed impaired T cell proliferation and cytokine production to many, but not all stimuli [18, 19]. Mice deficient in both B7-1 and B7-2
have impaired T cell-dependent antibody production [20], and APCs from these mice are not as efficient in priming T cell proliferation and cytokine production in vitro compared with wild-type APC [21]. Furthermore, B7-deficient mice display resistance to induction of autoimmune diseases, such as experimental autoimmune encephalomyelitis [22].

1.2.2 CTLA-4/B7 costimulation

While costimulation of T cell activation via CD28/B7 interaction has been clearly elucidated, the role of a second ligand for B7, called cytotoxic lymphocyte antigen 4 (or CTLA-4) [23, 24], is more controversial. CTLA-4 has a much higher affinity for B7 binding than does CD28 [25-27]. However, unlike CD28 which is constitutively expressed on the T cell surface [12], CTLA-4 is expressed following T cell activation and is found at much lower levels on the T cell surface [25, 28]. Current consensus considers CTLA-4 to be the inhibitory counterpart to the stimulatory CD28. Early studies with anti-CTLA-4 monoclonal antibodies (mAb) by several groups demonstrated that soluble anti-CTLA-4 mAb appeared to block CTLA-4/B7 interaction, resulting in an enhanced T cell proliferation, while cross-linking CTLA-4 with CD28 and T cell receptor inhibited proliferation [29, 30]. Further characterization indicated that CTLA-4 expression is optimal in the presence of CD28 [28], and that anti-CTLA-4 mAb is most effective under conditions of optimal CD28 costimulation. In this model, anti-CTLA-4 mAb inhibited anti-CD28 mAb-costimulated IL-2 production, IL-2 receptor expression, and cell cycle progression [31]. Perhaps the strongest piece of evidence supporting CTLA-4 as an inhibitor of immunity is the massive lymphoproliferative disorder observed in CTLA-4 knockout mice [32, 33]. CTLA-4(-/-) mice develop multi-organ
inflammatory infiltration by activated lymphocytes in the liver, pancreas, heart, lung, and bone marrow. These activated lymphocytes are capable of spontaneous proliferation in media alone and increased proliferation with anti-CD3 mAb stimulation compared with CTLA-4(+/+) cells in vitro. CTLA-4(-/-) mice also have increased numbers of B cells and increased serum immunoglobulin of all isotypes. These findings indicate that CTLA-4/B7 interaction might be responsible for dampening immune responses in vivo. A third line of evidence supporting CTLA-4 as a negative regulator is from studies of intracellular signaling events. The cytoplasmic domain of CTLA-4 is associated with the tyrosine phosphatase SHP-2, and the interaction of CTLA-4 and associated SHP-2 with the T cell receptor prevents phosphorylation of the TCR and inhibits T cell activation [34, 35]. Furthermore, CTLA-4 ligation with mAb inhibited IL-2 gene transcription and production of cyclins and cyclin-dependent kinases to induce cell-cycle arrest [36].

While a majority of studies refer to CTLA-4 as a negative regulator of the immune response, other studies provide data that challenge this interpretation and suggest that CTLA-4 may function in a stimulatory role in certain circumstances. For example, it has been demonstrated that CTLA-4 “blockade” can either promote or inhibit T cell proliferation depending on the activation status and strength of TCR signaling during stimulation [37]. Furthermore, the development of lymphoproliferative disorder in CTLA-4(-/-) mice can be eliminated or significantly delayed and diminished in severity when the T cell repertoire is restricted to a single transgenic TCR in a RAG-1(-/-) or RAG-2(-/-) background [38, 39]. CTLA-4(-/-) cells from these TCR-restricted mice show no increased activation phenotype and respond to antigen in an indistinguishable manner from CTLA-4(+/+) cells [38]. RAG-2(-/-) mice reconstituted with CTLA-4(-/-)
bone marrow do not develop lymphoproliferative disorder, although they exhibit some lymphocytic infiltration in several organs [40]. RAG-2(-/-) mice reconstituted with 50% CTLA-4(-/-) and 50% CTLA-4(+/+) bone marrow exhibit no signs of lymphoproliferation and have unaltered T cell responses to several different types of infections [40, 41]. These studies indicate that an unregulated activation phenotype is not strictly correlated with lack of CTLA-4. Furthermore, development of autoimmunity cannot be used to delineate whether a receptor is a positive or negative regulator, since mice lacking the costimulatory molecules CD28, B7-2, or B7-1/B7-2 also display increased autoimmunity, as described below and in Chapter 5. Regarding the association of CTLA-4 with the tyrosine phosphatase SHP-2, it has been shown that the proliferative effect mediated by anti-CTLA-4 mAb does not require this interaction, as CTLA-4 mutants lacking cytoplasmic tyrosine motifs or even the entire cytoplasmic domain still proliferate in response to anti-CTLA-4 mAb [42]. Furthermore, a recent report showed that a bispecific tandem single chain Fv ligand of human CTLA-4 can mediate an activating signal through CTLA-4 in the absence of TCR ligation [43]. Additionally, CTLA-4 confers resistance to activation-induced cell death in Th2 cells [44].

Experiments by our lab have demonstrated that T cell proliferation can be stimulated by CTLA-4/B7 interaction in the absence of CD28/B7 interaction using CD28(-/-) T cells or a mutant B7 molecule that can bind CTLA-4 but not CD28 [45]. These findings of positive costimulation by CTLA-4/B7 interaction have been extended to studies of anti-tumor immunity by demonstrating that rejection of B^7^ tumors occurs in CD28(-/-) mice [46]. Using a model of tumor-specific transgenic T cells, CTLA-4 costimulation appears to function at the effector phase rather than at the proliferative
phase of the anti-tumor immune response [47]. Taken together, these studies bring into question the definitive function of CTLA-4 as a negative regulator of immunity. It has been suggested that CTLA-4 actually functions as an enhancer of TCR signal transduction, which plays opposing roles during different stages of T cell development [6]. During thymic selection, CTLA-4 might increase avidity to facilitate negative selection and elimination of potentially autoreactive T cell clones, while during peripheral T cell stimulation CTLA-4 ligation might enhance or inhibit activation, depending on the context.

1.2.3 4-1BB/4-1BB ligand costimulation

4-1BB (CD137) and 4-1BB ligand (4-1BBL) are another pair of costimulatory molecules that have been the subject of recent intensive investigation. Unlike CD28, CTLA-4, and B7, 4-1BB and 4-1BBL are members of the tumor necrosis factor receptor (TNFR) and tumor necrosis factor (TNF) ligand superfamily, respectively. 4-1BB is expressed mainly by activated CD8 and CD4 T cells [48], and natural killer (NK) cells [49]. 4-1BBL is expressed by antigen-presenting cells, including dendritic cells, macrophages and B cells [50-52]. Expression of 4-1BB by T cells begins only after activation and peaks at 2-3 days [53], and may require prior stimulation by TCR and CD28 for maximal expression [54]. While CD28 costimulation appears to be important in the initial activation of T cell responses, 4-1BB costimulation plays a critical role in sustaining ongoing T cell responses and generating memory T cell populations [55-57]. Conflicting evidence exists regarding the effect of 4-1BB costimulation on the CD8 or CD4 T cell subsets. Many reports show a preferential effect of 4-1BB ligation on CD8 T
cells in a variety of settings, including responses to allografts [58], virus [55, 59], or superantigen [60], while several studies demonstrate equal effects on both CD8 and CD4 T cells [61-63]. As detailed in Chapter 2, we have demonstrated that 4-1BB ligation on tumor-specific CD8 T cells can promote their survival and persistence in vivo in the absence of CD4 T cells [64].

Another area of interest is the manner in which 4-1BB costimulation promotes T cell responses. The increased numbers of T cells observed with 4-1BB costimulation may be due to promotion of T cell proliferation, increased T cell survival, or both. Using stimuli such as anti-CD3 mAb, alloantigens, or artificial APC to investigate T cell responses in vitro, some studies showed that 4-1BB enhanced T cell proliferation [48, 58, 65], while others showed both increased proliferation and survival in vitro [57, 62, 66, 67]. In vivo experiments also present a confusing picture, attributing increased numbers of T cells to increased proliferation [58] or enhanced survival [60], without actually confirming the mechanism behind the T cell accumulation. Using more sensitive techniques to monitor cell division of adoptively transferred transgenic T cells in vivo, more recent studies have attempted to elucidate the mechanism of action of 4-1BB ligation. One group reported a small increase in division rate of CD4 T cells to specific antigen with anti-4-1BB mAb treatment [68], while another group reported decreased division of CD8 T cells with blockade of 4-1BBL [56]. Interestingly, this latter study also reported decreased survival of CD8 T cells, especially those in later stages of division, with 4-1BBL blockade [56]. In the setting of anti-tumor immunity, we saw no increase in tumor-specific CD8 T cell division either in vitro or in vivo with anti-4-1BB treatment.
mAb treatment [64]. Conversely, we did observe a promotion of CD8 T cell survival that could account for increased numbers of CD8 T cells that accumulate with anti-4-1BB mAb therapy. These findings are presented in Chapter 2.

Analysis of the intracellular events following 4-1BB costimulation provide potential molecular mechanisms for the role of this molecule in T cell proliferation and survival. Kwon and colleagues have demonstrated in vitro that agonist anti-4-1BB mAb, in conjunction with anti-CD3 mAb, can promote CD8 and CD4 T cell proliferation by upregulating expression of cyclins and inhibiting expression of cyclin dependent kinase inhibitors [69, 70]. Furthermore, 4-1BB ligation promotes survival by upregulating the anti-apoptotic proteins Bcl-xL and Bfl-1 in CD8 T cells [57, 67, 71], and Bcl-xL and Bcl-2 in CD4 T cells [70]. Taken together, it appears that 4-1BB costimulation may promote both proliferation and survival of T cells, with the variation observed depending on the form of stimulation and in vitro or in vivo setting.

1.3 Costimulation and anti-tumor immunity

Normal T cell responses are stimulated by recognition of abnormal or foreign peptides presented by antigen-presenting cells. In this situation, APCs also express the requisite costimulatory molecules critical for T cell activation (Figure 1.1, middle panel). Tumor cells represent a special situation because of their derivation from normal cells. In many cases, tumor cells express the same or very similar peptides as non-tumor cells, making them hard to distinguish as abnormal by T cells. T cell populations may be tolerated by tumors, with the elimination of T cell clones with high-affinity for tumor antigen [72]. Additionally, most tumors do not express costimulatory molecules [73, 74],
or may downregulate costimulatory molecules [75], and therefore may not be capable of fully activating T cell responses. In order to overcome these barriers, many groups have attempted to manipulate costimulatory pathways to provide T cells the required boost to mount an effective anti-tumor immune response [73, 74]. One successful strategy is the introduction of costimulatory molecules onto the tumor cell itself, usually by transfection or viral transduction. The seminal studies using this strategy observed that tumor cells transfected with the costimulatory molecule B7 are more easily rejected than non-transfected counterparts [76-78], revealing an important role for B7 costimulation in the induction phase of anti-tumor immunity.

Studies from our lab have shown that B7 expression is also important for recruitment to the tumor [79] and effector function of T cells [47, 79, 80]. Tumor cells transfected with B7 are more efficiently lysed by tumor-infiltrating lymphocytes in vitro than tumor cells transfected with a control vector [79]. Mice challenged with mixtures of B7+ and B7− tumor cells or B7+ and B7− tumor cells in opposite flanks show selective rejection of B7+ tumor cells by tumor-specific CD8 T cells [47, 80]. These data suggest that local B7 costimulation is required for the effector phase of T cell-mediated tumor rejection. Since delivery of B7 molecules to all tumor cells in an individual represents a difficult if not impossible task, the requisite costimulation may be provided to effector T cells by alternative strategies.

One such strategy is the use of monoclonal antibodies targeting costimulatory molecules that can bypass the requirement for expression of costimulatory molecules by tumor cells (Figure 1.1, bottom panel), or enhance endogenous costimulation by antigen-presenting cells [74]. These agents have the potential for wide clinical application since
their use is not restricted to any particular cancer type or patient population, and can be combined with other chemotherapeutic or immunotherapeutic modalities. While a number of monoclonal antibodies have shown significant effect in murine systems, the translation of these treatments to human cancer patients has been less successful due to several factors. One critical factor is that anti-human mAbs may not have the same functional properties as their murine counterparts. Individual antibodies may bind to different epitopes of the same costimulatory molecule or have different mechanisms of action (agonist or antagonist). Furthermore, lack of correlation between in vitro and in vivo effects on T cells by the same antibody require the use of more extensive preclinical screening techniques to adequately assess actual function of these molecules [81]. The current dearth of adequate models with which to screen large numbers of candidate antibodies presents a frustrating challenge to determining the most effective molecules for clinical translation. Chapters 2, 3, and 4 discuss the characterization of several monoclonal antibodies directed against two costimulatory molecules, 4-1BB and CTLA-4, and the development of two models to facilitate preclinical screening of these agents.

1.3.1 Anti-CTLA-4 antibodies in anti-tumor immunity

With the abundance of evidence revealing that ligation/blockade of CTLA-4 could enhance T cell activation, the application of anti-CTLA-4 monoclonal antibodies to tumor immunotherapy quickly followed. A number of studies have investigated the use of anti-CTLA-4 mAb in boosting anti-tumor immunity. The initial study performed by Leach et al. [82] showed that anti-CTLA-4 mAb could stimulate rejection or reduce the
growth of colon carcinoma and fibrosarcoma. Significantly, this mAb was able to reduce the growth of established tumors and protect against a second tumor challenge. Subsequent studies extended the use of anti-CTLA-4 mAb to the treatment of prostate cancer [83-85], melanoma [86-88], ovarian carcinoma [89], mammary carcinoma [90], and plasmacytoma [91]. A number of these groups combined anti-CTLA-4 mAb with a GM-CSF producing tumor cell vaccine [86, 87, 90], depletion of CD4⁺CD25⁺ regulatory T cells [88], or low-dose chemotherapy [91] to enhance elimination of tumors normally resistant to an anti-tumor response. These studies showed the requirement of CD8⁺, but not CD4⁺, T cells in the protective response [86-88]. Conflicting evidence exists as to whether anti-CTLA-4 mAb is capable of reversing T cell tolerance to tumor antigen [92, 93]. This preponderance of data from mouse models provides a convincing rationale for the creation and testing of monoclonal antibodies directed against human CTLA-4.

Recently the use of anti-CTLA-4 mAb has been translated to the clinical setting for treatment of human cancer patients. Two small clinical trials testing the same anti-human CTLA-4 mAb have been reported in the literature. One study involved patients with metastatic melanoma and ovarian carcinoma that had been previously immunized with several types of immunomodulating vaccines [94]. Treatment with anti-human CTLA-4 mAb induced tumor necrosis and lymphocytic and granulocytic infiltrate into the tumor in melanoma patients, and reduction or stabilization of tumor antigen (CA-125) levels in the blood in ovarian cancer patients, all previously immunized with GM-CSF-secreting tumor cells. Other patients immunized with dendritic cell or peptide vaccines exhibited no tumor necrosis. Low level autoimmunity, including rashes and depigmentation of the eye (both associated with T cell infiltrates), was observed with no
major toxicities. A second study in metastatic melanoma patients using the same anti-human CTLA-4 mAb achieved much better clinical efficacy, with 3 of 14 patients having partial or complete regressions of tumor [95]. Unfortunately, 6 of 14 patients in this study also experienced severe autoimmune toxicities, such as dermatitis, hypophysitis, enterocolitis, and hepatitis. Further studies are now being done in non-human primates to more carefully assess the safety of anti-human CTLA-4 mAb [96]. These studies clearly show the therapeutic potential of anti-CTLA-4 mAb for use in human cancer patients, while at the same time warn that serious side effects may be encountered with these agents. New methods for preclinical screening of anti-human CTLA-4 mAb are warranted and necessary to select mAb that may be most efficacious with the least toxicity. Chapters 3 and 4 discuss the development of two new models to achieve this goal.

1.3.2 4-1BB costimulation in anti-tumor immunity

As with CTLA-4, the demonstration that 4-1BB costimulation could dramatically enhance and sustain T cell responses led to the targeting of this pathway in tumor immunotherapy. Most of the studies investigating the function of 4-1BB costimulation in tumor immunity employ one of two strategies, namely, agonist anti-4-1BB monoclonal antibodies to mimic receptor-ligand interaction or transfection of 4-1BBL into antigen presenting cells or tumor cells. The first study showing the benefit of anti-4-1BB mAb in boosting antitumor immunity described the eradication of large established sarcoma and mastocytoma tumors, and increased survival in mice with disseminated metastases [97]. The effects of anti-4-1BB mAb required both CD8 and CD4 T cell subsets. Further
studies using anti-4-1BB mAb demonstrated its variable efficacy against different tumor types. Anti-tumor effects were observed against fibrosarcoma, mastocytoma, renal cell carcinoma, and glioma, but not melanoma [98, 99]. Site of tumor challenge also may play a role in the efficacy of anti-4-1BB mAb [98]. In poorly immunogenic tumors, including melanoma and lung carcinoma, combination of anti-4-1BB mAb with tumor antigen immunization is able to break tolerance to these tumors and mediate regression, where mAb or immunization alone have little effect [100]. Transfection of 4-1BBL [101-103] or single chain Fv fragments specific for 4-1BB [104] into tumor cells has produced comparable effects to mAb in stimulating anti-tumor immunity.

These studies also reflect the confusing picture of which T cell subset is preferentially effected by 4-1BB ligation. Several groups demonstrated that CD8 T cells but not CD4 T cells are required for anti-tumor immunity [99-101], while others also show a requirement for CD4 T cells [98, 104] or natural killer cells [49, 102, 104]. As presented in Chapter 2, we extend the analysis of anti-4-1BB mAb to the treatment of large established tumors with adoptive immunotherapy of tumor-specific CD8 T cells [64]. We demonstrate that adoptively-transferred tumor-specific CD8 T cells, in conjunction with anti-4-1BB mAb, can mediate reject large established tumors in the absence of CD4 T cells.

1.4 Costimulation in autoimmunity

Autoimmune disease has many parallels with anti-tumor immunity. Cancer is an uncontrolled growth of self cells, and in mounting an anti-tumor immune response, T cells must distinguish tumor cells from normal cells. This often presents a difficult
challenge since many of the antigens expressed by tumor cells are in fact self antigens that are slightly altered or aberrantly expressed. As such, an immune response against tumor antigens is not very different from an autoimmune response, in which the immune system responds inappropriately to normal self antigens, causing destruction of normal tissues. This parallel is well illustrated in melanoma patients whose clinical response to immunotherapy is often accompanied by autoimmune depigmentation, or vitiligo, in which melanoma-reactive T cells kill normal melanocytes in the skin [95, 105, 106]. Therefore, in many ways the role of costimulation in anti-tumor immunity and autoimmunity can be considered as looking at the same problem from two different angles [107]. In anti-tumor immunity, the goal is to boost T cell responses to tumor antigens, while in autoimmunity one tries to dampen harmful T cell responses against self antigens. As costimulation can boost anti-tumor immune responses, so can it exacerbate autoimmune responses [95].

1.4.1 CD28/CTLA-4/B7 costimulation in autoimmunity

The CD28/CTLA-4/B7 costimulatory pathway plays an important role in tolerance to self-tissues and the development of autoimmunity. When CTLA-4 is viewed as a negative regulator of immune responses, CTLA-4 knockout mice display the most dramatic demonstration of this by developing profound lymphoproliferation that is lethal by 4 weeks of age [32, 33]. On the other hand, CD28/B7 costimulation normally provides a required second signal for T cell activation, lowering the threshold of TCR signaling at which activation may occur. It follows that elimination or blockade of these molecules would dampen ongoing immune responses, such as those displayed in
autoimmunity. Indeed, blockade of the CD28/B7-1/B7-2 pathway, or genetic knockouts of CD28 or B7, show increased resistance to disease development or decreased disease severity in a variety of autoimmunity models, including experimental autoimmune encephalomyelitis (EAE) [22, 108], arthritis [109], and myocarditis [110]. Furthermore, B7 blockade using CTLA-4-Ig has been shown in humans to reduce the severity of the autoimmune skin disease psoriasis vulgaris [111, 112]. However, nature is never so straightforward, particularly in the world of costimulatory molecules. Both B7-deficient and CD28-deficient mice in the non-obese diabetes (NOD) background exhibit an increased incidence, earlier age of onset, and more rapid progression of spontaneous diabetes than normal counterparts [113]. Treatment of NOD mice with CTLA-4-Ig or anti-B7-2 mAb prevented diabetes development, while anti-B7-1 mAb with or without anti-B7-2 mAb hastened diabetes development [114]. B7-2 knockout mice in the NOD background developed spontaneous polyneuropathy rather than diabetes [115]. In models of EAE, B7-deficient mice of certain strains were resistant to disease while other strains were not [116]. Additionally, B7-1 blockade decreased the incidence of EAE, while B7-2 blockade increased EAE severity [117].

These paradoxical findings relating to the role of CD28/CTLA-4/B7 costimulation in autoimmunity may be reconcile by considering the contribution of costimulation to other processes besides peripheral activation. One emerging role for CD28/CTLA-4/B7 costimulation occurs during T cell development in the thymus. Provision of CD28 [118] or B7 costimulation [119] in conjunction with TCR stimulation increased negative selection of CD4⁺CD8⁺ thymocytes in vitro. In fetal thymic organ cultures, B7 blockade with monoclonal antibodies rescued CD4 thymocytes from clonal
deletion [120], while CD28-deficient mice displayed impaired clonal deletion of
superantigen-reactive thymocytes [121]. Most recently, our lab has demonstrated that
blockade of B7-1 and B7-2 during the perinatal period leads to the rescue from clonal
deletion of autoreactive T cells that cause fatal multi-organ inflammation [122]. Our
observation of spontaneous alopecia and skin disease in B7-deficient mice detailed in
Chapter 5 represents another example of the importance of CD28/CTLA-4/B7
costimulation in the clonal deletion of autoreactive T cells and the suppression of
autoimmunity.

1.4.2 B7 costimulation in the generation and function of regulatory T cells

A second mechanism through which CD28/CTLA-4/B7 costimulation helps to
suppress autoimmunity is in the generation of CD4⁺CD25⁺ regulatory T cells (Treg).
Treg are a naturally-occurring subpopulation of T cells that have been shown to suppress
effector T cell responses in a variety of in vitro [123, 124] and in vivo settings, including
anti-tumor immunity [88, 125, 126] and autoimmunity [113, 127-130]. Treg are
generated in the thymus, possibly in response to self-antigen [131], and costimulatory
molecules play an important role in their development and survival. B7-deficient and
CD28-deficient mice display decreased numbers of CD4⁺CD25⁺ regulatory T cells in the
spleen and thymus [113, 132, 133], and both CD28 [133] and B7 [132] are purported to
be important for survival of Treg in the periphery. The role of costimulation in Treg
function remains controversial. CD4⁺CD25⁺ Treg are known to constitutively express
high levels of intracellular CTLA-4 [113, 134, 135], and some studies show a requirement for CTLA-4 in suppression by Treg [134, 135], while others do not [88, 123, 136, 137].

A recent review article by Gavin and Rudensky proposed that suppression by Treg may occur via “reverse signaling” delivered by CTLA-4 on Treg to B7 molecules expressed by effector T cells [138]. In a graft versus host disease model, T cell-T cell interactions via CTLA-4/B7 binding have been shown to mediate suppression of the B7-expressing alloreactive T cells by other T cells expressing CTLA-4 [139]. However, in opposition to Gavin and Rudensky’s hypothesis, this suppression was not mediated by CD4+CD25+ regulatory T cells. This study also demonstrated that B7-deficient cells could not be suppressed, presumably due to lack of B7 molecules required for suppression [139]. Another possible mechanism for Treg suppression via CTLA-4 may involve the dendritic cell. Evidence for reverse signaling through B7 on dendritic cells has been demonstrated using CTLA-4-Ig, which upregulates tryptophan catabolism in dendritic cells [140]. Tryptophan catabolism by antigen-presenting cells via the upregulation of the enzyme indoleamine 2,3-dioxygenase has been shown to suppress T cell responses [141-143], and presents a potential mechanism for Treg suppression. This has recently been confirmed for both mouse CD4+CD25+ regulatory T cells [144] and human CD4+ T cells [145], in which CTLA-4 expressed by these T cells interacts with B7 on dendritic cells to induce tryptophan catabolism and subsequent T cell suppression. Our results presented in Chapter 6 provide evidence that B7-deficient T cells are indeed susceptible to suppression by CD4+CD25+ regulatory T cells both in vitro and in vivo.
1.4.3 4-1BB costimulation in autoimmunity

While the role of 4-1BB costimulation in T cell responses, and anti-tumor immunity in particular, is becoming fairly clear, the effects of 4-1BB in autoimmunity are quite confounding. Fu and colleagues have published several intriguing reports in two different in vivo models of autoimmunity that demonstrate the efficacy of anti-4-1BB mAb as a therapy for autoimmune disease [68, 146]. Using the same agonist anti-4-1BB antibody clone that boosted CD8 T cell responses in tumor models [64, 97, 100], they showed that mAb treatment decreased the incidence, severity, and relapse of experimental autoimmune encephalomyelitis (EAE) [68]. 4-1BB ligation affected the induction phase, but not the effector phase, of the autoimmune CD4 T cell response by causing initial CD4 T cell division, followed by increased activation-induced cell death. This was followed by a second study examining the effects of anti-4-1BB mAb in a spontaneous model of lupus-like autoimmune disease, characterized by lymphoproliferation, auto-antibody production, and immune complex deposition in the kidney [146]. In this model, agonist anti-4-1BB mAb diminished lymphadenopathy and autoimmune pathology, and increased mouse survival. The mAb appeared to reduce both CD4 T cell, double-negative T cell, and B cell populations by inducing apoptosis. This finding was corroborated by another group that showed similar results in a different lupus-like autoimmune mouse model [147]. These results present a confusing picture as to how ligation of the same molecule can appear to profoundly boost T cell responses in one setting and diminish them in another. The involvement of 4-1BB signaling in suppressor or regulatory T cell function has been offered as an explanation by some [148, 149], but contradicted by others [150, 151].
In summation, costimulation via CD28/CTLA-4/B7 and 4-1BB/4-1BBL plays a critical role in the normal activation and function of T cell immune responses. Despite the large body of work on the function of these molecules, many areas of study remain unresolved. The effort to understand these molecules in the setting of diseases such as cancer and autoimmunity has accentuated the importance of more clearly elucidating the mechanism of action, cell types targeted, and side effects that may be associated with manipulating these pathways. The following chapters present an effort to more clearly discern mechanism of action and therapeutic application of these costimulatory molecules in tumor immunotherapy, and their role in the induction and suppression of autoimmune disease.
Figure 1.1 **Provision of costimulation by antigen-presenting cells or monoclonal antibody.** T cells recognize self peptide presented by normal tissue or antigen-presenting cells (signal 1) in the absence of costimulation, leading to no T cell activation (top panel). Foreign peptide, such as viral peptide, is presented to T cells by antigen-presenting cells expressing costimulatory molecules (signal 2), which can initiate T cell activation (middle panel). Tumor cells present abnormal peptides but lack the costimulatory molecules needed to activate T cells. Provision of costimulation to T cells using monoclonal antibodies is one strategy to overcome the lack of costimulatory molecules on tumor cells (bottom panel).
Figure 1.1

Signal 1 = TCR recognition of Peptide:MHC

Signal 2 = Costimulation

Monoclonal antibody provides costimulation
CHAPTER 2

ANTTI-4-1BB MONOCLONAL ANTIBODY ENHANCES REJECTION OF LARGE TUMOR BURDEN BY PROMOTING SURVIVAL, BUT NOT CLONAL EXPANSION OF TUMOR-SPECIFIC CD8$^+$ T CELLS

2.1 Abstract

Anti-4-1BB monoclonal antibody (mAb) has been shown to induce anti-tumor immunity by a CD4/CD8-dependent mechanism, but its direct effect on tumor-specific CD8 T cells in tumor rejection is unclear. Here we used transgenic CD8 T cells against the unmutated tumor rejection antigen P1A to analyze whether this mAb can promote CD8 T cell function against large tumors in the absence of CD4 T helper cells. RAG-2(-/-) mice were challenged with P1A-expressing plasmacytoma J558. Once tumor size reached a diameter of 0.85-1.75 cm, mice were treated with P1A-specific CD8 CTL (P1CTL) in conjunction with anti-4-1BB mAb or control IgG. All mice showed a partial regression of tumor, but mice treated with anti-4-1BB mAb exhibited markedly enhanced tumor rejection, delayed tumor progression, and prolonged survival. Correspondingly, we observed a substantial increase in the number of P1CTL in anti-4-1BB mAb treated mice. Surprisingly, anti-4-1BB mAb did not accelerate division of the tumor-specific CD8 T cells, and the increase in tumor-specific T cell number was due to reduced
activation-induced cell death. These results indicate that anti-4-1BB mAb can promote CD8 T cell-mediated protection against large tumors in the absence of CD4 T cell help by promoting P1CTL survival without increasing initial clonal expansion.

2.2 Introduction

4-1BB is an inducible costimulatory molecule expressed on activated T cells [48] and NK1.1 cells [49]. 4-1BB ligand is expressed on antigen-presenting cells, such as dendritic cells [51], B cells, and macrophages [50]. The costimulatory signal provided by 4-1BB has been shown to play an important role in CD8 T cell responses in a variety of systems, including viral infection [59, 152, 153], allograft rejection [58, 154], and tumor immunity [49, 97, 98, 100-103]. Several studies have also demonstrated the efficacy of using anti-4-1BB mAb as an agonist to bypass or enhance the natural costimulation provided by APC. It has been reported that mice treated with anti-4-1BB mAb show increased survival or enhanced regression of tumor [49, 97, 98]. These effects were both CD8 and CD4 T cell dependent, as depletion of either subpopulation eliminated the anti-tumor effect. Since the CD8 T cell response can be enhanced by CD4 T helper cells, the direct effect of anti-4-1BB mAb on CD8 T cells in vivo is less clear. Here we used transgenic CD8 T cells against the unmutated tumor rejection antigen P1A to more clearly elucidate the effect of anti-4-1BB mAb therapy on the anti-tumor CTL immune response. By adoptively transferring transgenic CD8 T cells into tumor-bearing RAG-2(-/-) mice, we are able to show that the enhanced tumor rejection, delayed tumor progression, and prolonged survival seen with anti-4-1BB mAb therapy can be mediated by CD8 T cells in the absence of CD4 T cells.
A second issue addressed in this study is the mechanism behind the enhanced anti-tumor immunity due to anti-4-1BB mAb. Costimulation through 4-1BB has been reputed to enhance proliferation and promote survival of T cells [48, 58, 60, 62, 66]. Most of these studies have used in vitro stimulation models, and the relative contribution of these two mechanisms has not been clearly established in vivo, especially in tumor models. Here we used a well-defined tumor model to dissect the contribution of proliferation and survival to CD8 T cell immunity. Our results demonstrate that anti-4-1BB mAb can enhance T cell immunity by promoting survival without affecting proliferation of CTL.

2.3 Materials and Methods

Experimental animals

Transgenic mice expressing a T cell receptor (TCR) specific for tumor antigen P1A35-43:Lf complex have been described [155]. BALB/c mice with a targeted mutation of the RAG-2 gene were purchased from Taconic (Germantown, NY).

Cell lines and tumorigenicity assay

BALB/c plasmacytoma J558 transfected with Neo vector (J558-Neo) has been described [79]. 5x10^6 J558-Neo tumor cells were injected subcutaneously in the flanks of mice. Tumor size and incidence were determined every 2-4 days by physical examination.
Antibodies

Anti-4-1BB mAb producing hybridoma, 2A, has been described [100]. Anti-4-1BB mAb was purified from supernatant by a Protein G column. Rat IgG was purchased from Rockland (Gilbertsville, PA) and Sigma (St. Louis, MO).

Flow cytometry

Cell surface expression of 4-1BB, peripheral blood P1CTL numbers, T cell division kinetics, and cell survival markers were visualized by flow cytometry. Transgenic T cells were visualized by surface expression of CD8 and TCR-α chain Vα8. All antibodies used for flow cytometry were purchased from BD Pharmingen (San Diego, CA).

Adoptive transfer of purified transgenic T cells

For some experiments, unpurified spleen cells from RAG-2(-/-)xP1CTL were adoptively transferred directly after RBC lysis with ammonium chloride (Sigma, St. Louis, MO). For other experiments, pooled spleen and lymph node cells from BALB/c P1CTL-transgenic mice were incubated with a mAb cocktail (anti-CD4 mAb GK1.5, anti-FcR mAb 2.4G2, and anti-CD11c mAb N418). Unbound mAb was removed and cells were incubated with anti-Ig-coated magnetic beads. Antibody coated cells were removed with a magnet. Enriched CD8 T cells with no detectable CD4 T cells were adoptively transferred into tumor-bearing mice via intraperitoneal or intravenous injection. For some experiments, enriched CD8 T cells were labeled with CFSE as described [156] before adoptive transfer.
In vitro cell division

In one type of experiment, CD8 T cells were purified from spleens of transgenic P1CTL mice using magnetic separation and labeled with CFSE as described above. Labeled T cells were cultured with irradiated J558-Neo cells and harvested at 24 hour intervals and stained with anti-CD8-CyChrome and anti-Vα8 TCR-PE. In another type of experiment, unpurified CFSE-labeled P1CTL spleen cells were cultured with P1A peptide and harvested and stained as above.

BrdU incorporation

Tumor-bearing mice were adoptively transferred with transgenic P1CTL and treated with either Rat IgG or anti-4-1BB mAb on days 4 and 6 after adoptive transfer. On days 6, 9, and 11, mice were given an intraperitoneal injection of 1mg BrdU (Sigma, St. Louis, MO). On day 12, mice were sacrificed and splenic cells were stained with anti-BrdU antibody using a BD Pharmingen kit.

2.4 Results

Anti-4-1BB mAb therapy increases resistance to large tumor burden.

To test whether anti-4-1BB mAb could enhance rejection of a P1A-expressing tumor by P1CTL, RAG-2(-/-) mice were challenged with a subcutaneous inoculation of the plasmacytoma J558. When mean tumor diameter reached 0.85-1.75 cm, mice were adoptively transferred with 4x10^6 spleen cells from RAG-2(-/-)xP1CTL transgenic mice. Because of lack of endogenous TCR rearrangement, T cells from these mice are CD8^+ and CD4^- . On days 4, 6, 24 and 31 after adoptive transfer, tumor-bearing mice received
either 100 µg anti-4-1BB mAb or control IgG via intraperitoneal injection. As shown in Figure 2.1, both control IgG-treated and anti-4-1BB Ab-treated mice that received P1CTL showed an initial regression in tumor size. However, 4 of 5 mice treated with anti-4-1BB mAb completely rejected their tumors while only 1 of 5 control mice rejected tumors. Among those control mice that did not reject tumor, several mice exhibited a rapid regrowth of tumor and were sacrificed when moribund. The enhanced immunity by anti-4-1BB mAb has been reproduced by two other experiments with RAG-2(+/+) and RAG-2(-/-) transgenic P1CTL. Thus, anti-4-1BB mAb enhances tumor rejection by CD8+ P1CTL and extends the survival of those mice that do not completely reject tumor in a CD4 T cell-independent manner.

**Anti-4-1BB mAb therapy increases percentage of tumor-specific T cells in peripheral blood.**

To observe the effect of anti-4-1BB mAb therapy on adoptively transferred tumor-specific CD8 T cells, the peripheral blood of tumor-bearing mice from Figure 2.1 was analyzed at various time points after adoptive transfer by flow cytometry (Figure 2.2). During the first 3 weeks after adoptive transfer, mean P1CTL percentages within the peripheral blood lymphocyte gate were similar in both anti-4-1BB mAb and control groups at days 13 and 23, though slightly higher in anti-4-1BB mAb treated mice. At 31 days after adoptive transfer, the mean percentages of P1CTL in the anti-4-1BB mAb group (64.8%) remained stable, while the mean percentage of P1CTL in the control group (32.2%) had dropped to less than half of the anti-4-1BB-treated group. By 38 days, 2.2 fold greater mean percentage of P1CTL were found in the anti-4-1BB group than in the control IgG group. This difference was extremely significant (p = 0.001). To
determine if the high percentage of P1CTL could be maintained for an extended period of time, we ceased anti-4-1BB mAb treatments after day 31 and took a final blood sample 42 days after the last mAb treatment and 73 days after adoptive transfer. At this late time point, the large difference in mean percentage of P1CTL was maintained (65% with anti-4-1BB vs. 33.2% with control IgG) and was extremely significant (p<0.0001). P1CTL percentage was also increased among splenocytes in anti-4-1BB mAb treated mice, while recruitment of tumor-infiltrating lymphocytes was not enhanced by anti-4-1BB mAb (data not shown).

**Anti-4-1BB mAb does not cause clonal expansion of P1CTL by increasing initial division rate.**

Several groups have demonstrated varying patterns of 4-1BB cell surface expression depending on the method of T cell activation [60, 62, 66]. To establish the cell surface expression kinetics of 4-1BB on our tumor specific P1CTL, we activated spleen cells from transgenic P1CTL mice with P1A peptide. 4-1BB expression was analyzed by flow cytometry at 24 hour intervals for 4 days after stimulation (Figure 2.3). We observed no 4-1BB expression by unstimulated T cells or on day 1 after stimulation, but did see a marked increase in 4-1BB expression beginning at day 2 that was sustained through day 4.

The significant expression of 4-1BB on days 2 and 3 prompted us to test if anti-4-1BB mAb could increase in vitro clonal expansion of P1CTL at these time points. When spleen cells were cultured with P1A peptide for 48 hours in vitro, most T cells divided 2 to 3 times (Figure 2.4a). By 72 hours, most T cells divided 4 to 5 times. However, division rates of P1CTL treated with anti-4-1BB mAb or control IgG were very
similar. Additionally, we tested the division rate of P1CTL stimulated in vitro by irradiated J558 tumor cells in the absence of antigen-presenting cells (APC) (Figure 2.4b). At 48 hours, few cells had divided, but by 72 hours the proportion of divided cells had increased significantly. Nevertheless, anti-4-1BB mAb did not appreciably enhance the division of P1CTL. Thus anti-4-1BB mAb did not promote T cell division regardless of whether P1CTL were stimulated by tumor cells, or by APC presenting P1A antigen. A comparison of Figures 2.4a and 2.4b reveal that direct stimulation of P1CTL by tumor cells is less efficient than stimulation by peptide and APC. Even in this suboptimal setting with no APC, anti-4-1BB mAb had no effect on T cell proliferation. It is worth noting that cross-linked anti-4-1BB can promote T cell division in vitro [100].

To determine if our in vitro observations of P1CTL division rate could be extended to in vivo P1CTL, we analyzed the early division rate of P1CTL in tumor-bearing mice (Figure 2.5). RAG-2(-/-) mice with a large tumor burden of 1.5-2.0 cm were adoptively transferred with 5x10^6 CD8-enriched CFSE-labeled P1CTL spleen cells and treated with anti-4-1BB mAb or control IgG on the same day. Mice from each treatment group were sacrificed at one day intervals and spleen cells were harvested and stained to visualize dividing P1CTL. We observed similar division rates among P1CTL from anti-4-1BB mAb-treated and control mice at time points ranging from 38 to 95 hours using intraperitoneal or intravenous modes of adoptive transfer. These data suggest that the higher number of P1CTL observed in mice that received anti-4-1BB mAb therapy does not result from an increase in early division rate.

The limit of detecting cell division rate extends to only about 7 or 8 divisions, and thereafter the mean fluorescence levels are negligible. For in vivo detection of P1CTL,
this limit is reached after about 4 days (Figure 4.5). Since it is possible that anti-4-1BB mAb does not begin to affect P1CTL division until after 4 days, CFSE labeling of P1CTL may not detect a delayed increase in division rate. To test if anti-4-1BB mAb increased P1CTL proliferation at a later time point after adoptive transfer, we treated tumor-bearing RAG-2(-/-) mice that had been given an adoptive transfer of P1CTL with BrdU. BrdU is a nucleotide analogue that is incorporated during DNA synthesis and therefore can be used as an indicator of cell proliferation. Mice were given three intraperitoneal injections of BrdU (1mg/injection) during the 7 days preceding sacrifice. On day 12 after adoptive transfer, mice were sacrificed and CD8+ P1CTL were analyzed for BrdU incorporation. We observed that the percentage of CD8+ cells with a high level of BrdU incorporation (as determined by intracellular staining with FITC-labeled anti-BrdU Ab) might be somewhat lower in anti-4-1BB mAb-treated mice than in control mice (Figure 4.6), though this difference was not significant (p = 0.18). These results are consistent with our CFSE data showing that anti-4-1BB mAb does not appear to increase proliferation of P1CTL in vivo.

**Anti-4-1BB mAb reduces activation-induced cell death of P1CTL**

If anti-4-1BB mAb does not increase proliferation of P1CTL, then the increased percentages of P1CTL observed in peripheral blood may be due to an effect of anti-4-1BB mAb on cell survival. To test this potential role of anti-4-1BB mAb on P1CTL survival, we adoptively transferred tumor-bearing RAG-2(-/-) mice with P1CTL and subsequently treated the mice with either anti-4-1BB mAb or control IgG. At day 12 or 13, mice were sacrificed and P1CTL from spleens were tested for their binding to
Annexin V, which interacts with inner cell membrane molecules that translocate to the outer cell membrane during early stages of apoptosis. In one experiment in which spleen cells were stained on the same day of harvest (Figure 4.7a), we observed a two-fold increase in Annexin V positive cells among CD8+ cells from control IgG-treated mice compared with anti-4-1BB mAb treated mice (16.2% vs. 8.3%, p-value = 0.02). In another experiment, pooled spleen cells from adoptively transferred tumor-bearing mice were harvested and incubated overnight in culture before staining the following day (Figure 4.7b). We observed that the fraction of CD8+ cells staining positive for Annexin V was again greater in control IgG-treated mice (13.3%) compared with anti-4-1BB mAb treated mice (8.7%). Since the decline of T cells occurred over a long period, the difference in death rate adequately accounts for the difference in the number of cancer-specific T cells. Taken together, these results suggest that anti-4-1BB mAb treatment can enhance accumulation of P1CTL in vivo by decreasing activation-induced cell death.

2.5 Discussion

A critical issue in the field of anti-tumor immunity is the co-existence of both established tumor and tumor-specific T cells in the same patient [157, 158]. In animal studies, it has been observed that the presence of tumor-specific CTL does not always lead to tumor rejection [155, 159, 160]. Thus a major challenge to successful immunotherapy of established tumors is the relative lack of efficacy of the tumor-specific CTL response once tumor burden has reached a large size. Adoptive transfer of tumor-specific CTL can consistently mediate rejection of concurrent challenge of tumor cells or
even small established tumors [159]. However, once tumor burden passes a certain threshold (0.5-0.7cm), then adoptively transferred CTL may cause some tumor shrinkage but rarely can cause a complete rejection [80].

In order to overcome this barrier, we and others have attempted to modulate costimulatory pathways as a means to boost the anti-tumor T cell response. In this study, we analyzed the mechanisms behind the enhanced tumor immunity observed with anti-4-1BB mAb therapy. In RAG-2(-/-) mice with large J558 tumor burden (> 0.85 cm), we observed enhanced tumor rejection, delayed regrowth of tumor in mice not completely rejecting tumor, and prolonged survival with adoptive transfer of tumor-specific CD8 T cells and treatment with anti-4-1BB mAb. This finding has added clinical significance for human immunotherapy since most human tumors are discovered only after they have reached a large enough size to be detected. Identifying strategies that can be effective against large tumor burdens is thus a key goal in improving immunotherapy.

Because the recipient RAG-2(-/-) mice used in our adoptive transfer model lack endogenous T and B cells and the P1CTL donor mice are also RAG-2(-/-), the anti-4-1BB mAb enhanced rejection that we observed is mediated by a CD4 T cell-independent mechanism. Previous studies have shown conflicting data regarding the requirement for specific T cell subsets in anti-4-1BB mAb mediated tumor immunity using wild type mice. Several reports have shown a requirement for both CD8 and CD4 T cells in anti-4-1BB mAb enhanced tumor immunity [97, 98], in contrast to the data presented here. Other studies have demonstrated a CD4 T cell-independent mechanism for anti-4-1BB mAb-mediated tumor immunity, when either anti-4-1BB mAb [100] or
4-1BB ligand-transfected tumor cells were used [101-103]. However, the mechanism of CD8 T cell stimulation by anti-4-1BB mAb has not been studied in the absence of CD4 T cells. It is therefore somewhat unclear if anti-4-1BB mAb can activate CD8 T cells directly. Our results observing transgenic CD8 T cells in a RAG-2(-/-) mouse model clearly showed that this is likely to be the case.

We have chosen this transgenic model to clearly discern the mechanism of anti-4-1BB mAb-mediated CD8 T cell immunity. Our findings presented here are consistent with those seen in nontransgenic models. One of us (L. Chen) has observed an increase in tetramer-positive CD8 T cells from tumor draining lymph nodes in anti-4-1BB mAb treated mice after in vitro restimulation [100]. Others have shown increased CD8 T cell numbers in animal models of graft vs. host disease [58] or superantigen challenge [60]. Correspondingly, decreased CD8 T cell expansion has also been observed in LCMV-infected 4-1BB ligand knockout mice [59, 153]. Thus the mechanism uncovered here should have general implications for other settings.

The published data on antigen-specific CD8 T cells involves acute T cell responses. However, most human cancer involves a chronic interaction between T cells and cancer cells. It is generally accepted that chronic interaction with antigen often leads to malfunction of T cells [161], which has indeed been documented in the cancer patient [157]. Thus, a major challenge in tumor immunotherapy is to enhance function of cancer-specific T cells in a chronic setting. Here we showed that treatment with anti-4-1BB mAb had a long-lasting effect on the survival of cancer-specific T cells concomitant with a better immunity.
An interesting question is whether 4-1BB ligation maintains cell proliferation, enhances cell survival, or both. Most evidence for 4-1BB mediated enhancement of proliferation has come from in vitro experiments, using thymidine incorporation in allogeneic mixed lymphocyte reaction or with simultaneous anti-CD3 mAb administration [48, 51, 58, 62, 66, 154]. While these studies do give information about the conditions required for 4-1BB mediated proliferation, they may not completely reflect what occurs to T cells in vivo. Sun, et al. showed a slight increase in CD4 T cell division rate at 55 hours with anti-4-1BB mAb treatment after adoptive transfer of CFSE-labeled transgenic T cells into wild type BALB/c recipients immunized with specific antigen [68]. While several groups have shown an overall increase in CD8 T cells in vivo with anti-4-1BB mAb treatment [58, 60, 100], these increases can be attributed to either increased proliferation or to reduced cell death. None of the publications on the subject have clearly distinguished the two. Using adoptive transfer of CFSE-labeled P1CTL and in vivo BrdU incorporation, we observed that anti-4-1BB mAb does not increase P1CTL proliferation in tumor-bearing mice. Division rates of CFSE-labeled P1CTL from anti-4-1BB mAb treated and control mice were very similar, and BrdU incorporation was actually somewhat higher in control mice. These surprising findings led us to examine tumor-specific T cells for indications that anti-4-1BB mAb treatment may reduce T cell death, which has been demonstrated in vitro by Hurtado, et al. [66]. In vivo, Takahashi, et al. [60] have shown that normal decline of staphylococcal enterotoxin A (SEA)-stimulated CD8 T cells can be prevented by anti-4-1BB mAb. Although the authors have suggested that 4-1BB ligation enhanced CD8 T cell survival, the effect anti-4-1BB mAb on T cell proliferation was not studied. Here we showed that anti-4-1BB mAb did not
increase T cell proliferation, and yet the mAb decreased Annexin V staining on P1 CTL harvested from tumor-bearing mice. Thus, anti-4-1BB mAb therapy can increase the number of cancer-specific T cells by reducing cell death without increasing cell division. However, our results do not rule out that anti-4-1BB mAb can enhance T cell proliferation in other settings. The lack of effect on T cell proliferation could be due to absence of CD4 T cell help. Regardless of the immunological basis for the split effect of the mAb, our data provide clear-cut evidence that anti-4-1BB mAb can provide a “bona fide” survival signal, as has been proposed before [60].

It has become increasingly clear that cancer-specific T lymphocytes are expanded in patients with a large tumor burden. However, with one notable exception [162], the existence of these T cells has not been demonstrated to promote immunity to cancer [157, 158]. Our results clearly demonstrate that anti-4-1BB mAb can increase the efficacy of T cells specific for tumor antigen, even when it has no effect on T cell proliferation. The long-lasting effect of anti-4-1BB mAb suggests the potential therapeutic value of this approach.
Figure 2.1  Anti-4-1BB mAb therapy enhances rejection of large tumor burden by tumor-specific CD8+ P1CTL.  BALB/c RAG-2(-/-) mice were subcutaneously challenged with 5x10^6 P1A-expressing J558-Neo cells.  When tumors reached 0.85-1.75 cm in diameter, mice were adoptively transferred with 4x10^6 spleen cells from RAG-2(-/-) P1CTL transgenic mice via intraperitoneal injection.  Mice were treated with 100μg of anti-4-1BB mAb or Rat IgG via intraperitoneal injection on days 4, 6, 24, and 31 after adoptive transfer.  The increased resistance conferred by anti-4-1BB mAb has been observed in three independent experiments.
Figure 2.2 Anti-4-1BB mAb increases percentage of tumor-specific P1CTL in peripheral blood of tumor-bearing mice. Peripheral blood samples were taken from J558-Neo tumor-bearing mice from Figure 2.1 at indicated day after adoptive transfer of 4X10^6 spleen cells from RAG-2(-/-)P1CTL. Bars represent mean percentage of P1CTL within the peripheral blood lymphocyte gate from 5 mice. P-values were determined by two-sample t-test. Data shown are representative of three independent experiments.
Figure 2.3  Cell surface expression of 4-1BB by tumor-specific transgenic P1CTL. Spleen cells harvested from a transgenic P1CTL mouse were cultured and activated with P1A peptide (0.1 µg/ml). At 24 hour intervals after beginning activation, cells were harvested and stained CD8, Vα8.3 TCR, and 4-1BB. Solid lines represent staining with anti-4-1BB antibody, while dotted lines represent staining with isotype control antibody. Histograms are gated from CD8⁺ Vα8.3 TCR⁺ cells.
Figure 2.4  Anti-4-1BB mAb does not increase in vitro division rate of P1CTL induced by either P1A peptide presented by spleen cells or by intact tumor cells. a). Unpurified P1CTL spleen cells were labeled with CFSE and cultured with P1A peptide (0.1 µg/ml) and either 10 µg/ml anti-4-1BB mAb or Rat IgG. b). CD8-enriched P1CTL spleen cells were labeled with CFSE and cultured with irradiated J558-Neo cells and either 10 µg/ml anti-4-1BB mAb or Rat IgG. Cells were harvested at 24 hour intervals and stained for CD8 and Vα8 TCR. All panels show cells within the CD8⁺ Vα8 TCR⁺ gate.
Figure 2.5 Anti-4-1BB mAb does not increase in vivo division rate of P1CTL. Mice with 1.0-1.5 cm J558-Neo tumors were given an adoptive transfer of 5x10^6 CFSE-labeled CD8-enriched P1CTL spleen cells and treated with 100 µg of either anti-4-1BB mAb or Rat IgG on the same day. Route of administration of adoptively transferred T cells was either a) intraperitoneal or b) intravenous. Mice were sacrificed at indicated time points after adoptive transfer and spleen cells were stained for CD8^+ Vα8 TCR^+ cells. Panels represent 2 of 3 independent experiments.
Figure 2.6  Anti-4-1BB mAb does not increase BrdU incorporation by P1CTL in vivo. J558-Neo tumor-bearing mice were given an adoptive transfer of $5 \times 10^6$ CD8-enriched P1CTL spleen cells when tumors were at least 1.0 cm in diameter. 100 µg of either anti-4-1BB mAb or Rat IgG was given on days 4 and 6 after adoptive transfer. 1mg BrdU was given intraperitoneally on days 6, 9, and 11 after adoptive transfer. Mice were sacrificed on day 12 and spleen cells were stained with anti-CD8-PE, anti-BrdU-Fitc, and 7AAD. Panels are representative of two independent experiments.
Figure 2.7  **Anti-4-1BB mAb reduces T cell death.** J558-Neo tumor-bearing mice were given an adoptive transfer of 5x10^6 CD8-enriched P1CTL spleen cells when tumors were at least 1.0 cm in diameter. 100 µg of either anti-4-1BB mAb or Rat IgG was given on days 4 and 6 after adoptive transfer. a) At day 12 after adoptive transfer, mice were sacrificed and spleen cells were harvested and stained with anti-CD8-Fitc and Annexin V-PE. Data shown are the profiles of gated P1CTL from: i) Anti-4-1BB mAb-treated, or ii) control Ig-treated mice. Mean and data range of the % of Annexin V^+ cells are shown in iii). A p-value was determined using a two-sample t-test. b) At day 13 after adoptive transfer, mice were sacrificed and spleen cells were harvested and placed in culture overnight. The following day, cells were stained with anti-CD8-Fitc and Annexin V-PE. Data shown represent spleen cells pooled from 5 Rat IgG-treated mice or 6 anti-4-1BB mAb-treated mice.
a. i. Anti-4-1BB mAb

ii. Rat IgG

iii. Mean % Annexin V+ Cells

b. Rat IgG

Anti-4-1BB mAb

Figure 2.7
CHAPTER 3

ANTI-HUMAN CTLA-4 MONOCLONAL ANTIBODY PROMOTES T CELL EXPANSION AND IMMUNITY IN A HU-PBL-SCID MODEL: A NEW METHOD FOR PRECLINICAL SCREENING OF COSTIMULATORY MONOCLONAL ANTIBODIES

3.1 Abstract

When adopting basic principles learned in mice to clinical application in humans, it is often difficult to distinguish whether a failed “translation” is due to an invalid target in the human disease or because the therapeutic agents are not optimal for the human target. It is therefore desirable to develop preclinical models to optimize therapies for human targets using in vivo settings when in vitro functional correlates are not available. Although anti-mouse CTLA-4 monoclonal antibodies (mAbs) are known to enhance immune responses in vivo, their effect on T cell activation in vitro ranges from enhancement to inhibition. Here we use the hu-PBL-SCID mouse model of EBV-associated lymphoma development to screen a panel of anti-human CTLA-4 mAbs for their effect on human lymphocytes in an in vivo “humanized” environment. We report significant heterogeneity of anti-human CTLA-4 mAb in enhancing expansion of human
T cells in mice. These data validate the development of additional screening tools such as the one described, to further characterize functional activity of anti-human antibodies before proceeding with clinical translation to human studies.

3.2 Introduction

A major challenge in translating basic principles learned in mice to clinical application in humans is that both diseases and therapeutic agents are changed at the same time. This is particularly relevant for cancer immunotherapy involving monoclonal antibodies that are species-specific. Monoclonal antibodies directed against costimulatory molecules are a promising treatment modality that can bypass the requirement for or enhance endogenous costimulation by antigen-presenting cells. While a number of monoclonal antibodies have shown significant effect in murine systems, the translation of these treatments has been less successful due to several factors. One critical factor is that anti-human mAbs may not have the same functional properties as their murine counterparts. Individual antibodies may bind to different epitopes of the same costimulatory molecule with varying binding affinities and mechanisms of action (agonist or antagonist). Another key issue is the lack of adequate in vivo models with which to prescreen large numbers of candidate antibodies to determine the most effective molecules for clinical translation.

The costimulatory molecule cytotoxic T lymphocyte antigen 4 (CTLA-4) has been widely investigated as a target of monoclonal antibody therapy to boost anti-tumor immunity. The initial study performed by Leach et al. [82] showed that anti-CTLA-4 mAb could stimulate rejection or reduce the growth of colon carcinoma and fibrosarcoma
in mice. Significantly, this mAb was able to reduce the growth of established tumors and protect against a second tumor challenge. Subsequent studies extended the use of anti-CTLA-4 mAb to the treatment of prostate cancer [83-85], melanoma [86-88], ovarian carcinoma [89], and mammary carcinoma [90]. A number of these groups combined anti-CTLA-4 mAb with a GM-CSF producing tumor cell vaccine [86, 87, 90] or depletion of regulatory T cells [88] to enhance elimination of tumors normally resistant to an anti-tumor response. These studies showed the requirement of CD8⁺, but not CD4⁺, T cells in the protective response [86-88]. Conflicting evidence exists as to whether anti-CTLA-4 mAb is capable of reversing T cell tolerance to tumor antigen [92, 93].

More recently, anti-human CTLA-4 mAb has been tested in phase I clinical trials with human cancer patients, showing mixed results. In one study, patients with metastatic melanoma or ovarian carcinoma, showed evidence of tumor necrosis and T cell infiltration into tumors, but with no significant clinical benefit [94]. Another study involving patients with metastatic melanoma demonstrated significant clinical activity, with objective tumor regression in 3 of 14 patients [95]. However, 6 of 14 patients in this same study developed grade III/IV autoimmune toxicities. This variability of responses in different settings suggests that more extensive preclinical screening of different clones of monoclonal antibody may prove beneficial in selecting clones that induce more potent anti-tumor immunity while at the same time minimizing autoimmune side effects.

Preclinical screening of anti-human CTLA-4 mAbs is fraught with difficulty because in vitro immunological correlates are sometimes of little value, as demonstrated by experience with anti-mouse CTLA-4 mAb. The same anti-mouse CTLA-4 antibodies
that induced potent anti-tumor immunity in the above studies had variable effects on T
cells in vitro. Anti-CTLA-4 mAb enhanced T cell proliferation in response to
alloantigen, but suppressed T cell proliferation in response to costimulation by anti-CD28
mAb [29, 30]. Also, CTLA-4 engagement with antibody could either promote or inhibit
T cell proliferation to different subsets of T cells in the same culture [37].

This complication can be overcome if one can study human T cell responses in a
rodent model. A widely used model is the hu-SCID mouse [163]. One approach
involves establishment of human thymus or stem cells in these mice [163, 164], which
then produce human T cells. This model has been used for studies of HIV infection [165]
and development of human T cells [163]. At the same time, less demanding chimera
have been produced involving SCID mice engrafted with either human peripheral blood
leukocytes (PBL) [166], or antigen-specific T cells [167]. In the area of cancer
immunotherapy, hu-SCID mice have been used to measure the effector function of
tumor-specific T cells [167] and cloned tumor-specific T cell lines [168, 169].

Despite numerous attempts, varying success has been reported using hu-SCID
mice to study the immune response of human T cells. A notable example was reported
by Carballido et al., who demonstrated that grafting fetal human bone, thymus, skin and
lymph nodes into SCID mice allows the induction of immune responses by human
lymphocytes [170]. The requirement of multiple surgeries, however, makes it impractical
to use this model to pre-screen immune modulators for activation of T cells.
Interestingly, peptide-loaded dendritic cells were found to be capable of priming CD4 T
cells in hu-SCID mice [171]. We have recently reported the boosting of Epstein-Barr
virus (EBV)-specific cytotoxic T lymphocyte (CTL) responses in hu-PBL-SCID mice
using a combination of IL-2 and GM-CSF [172]. Remarkably, up to 20% of the T cells recovered from spleens of the engrafted mice can be specific for a single EBV viral peptide. Administration of these cytokines also leads to a significant protection from EBV-associated lymphoproliferative disorder, which is CD8 T cell-dependent. This makes it possible to study human CTL responses in mice, at least for antigens to which T cells have been primed. Here we report that in a similar hu-PBL-SCID model, five different clones of anti-human CTLA-4 mAb promoted enhanced T cell responses, with significant variability in efficacy between clones. The functional heterogeneity suggests that this model can be used to screen for therapeutic antibodies targeted at the CTLA-4 molecule.

3.3 Materials and Methods

Experimental animals

BALB/c mice were purchased from Charles River Laboratories under contract with the National Cancer Institute. CB.17 SCID mice and BALB/c RAG-2(-/-) mice were purchased from Taconic (Germantown, NY). All mice were maintained in the University Laboratory Animal Research Facility at the Ohio State University under specific pathogen-free conditions.

Monoclonal antibody production

BALB/c mice were immunized two times with a fusion protein consisting of the extracellular domain of the human CTLA-4 protein and the Fc fragment of human IgG1 (huCTLA-4Ig). Spleen cells were harvested from immunized mice and fused with myeloma cell line XAg8.653 using polyethylene glycol (MW 1000) (Sigma, St. Louis,
MO). Hybridomas were selected in HAT media and further cultured in HT media. Culture supernatant was screened for the presence of anti-human CTLA-4 mAb by ELISA. Clones producing mAb that bound to human CTLA-4Ig but not mouse CD28Ig fusion protein were rescreened by ELISA and further subcloned and expanded. Large scale antibody production of selected clones was achieved by purifying mAb from culture media using a Protein G column or by intraperitoneal injection of 5x10^6 hybridoma cells into BALB/c RAG-2(-/-) mice to produce ascites. Isotyping of mAb was performed using a kit purchased from BD Pharningen (San Diego, CA).

**Engraftment of human peripheral blood leukocytes**

PBL were obtained from normal healthy donors that were consented under an IRB-approved protocol for leukapheresis performed by The Ohio State University Hospitals apheresis unit. Selected donors were EBV-seropositive and Hepatitis B and HIV-seronegative. These PBL were previously shown to generate EBV lymphoproliferative disorder in greater than 90% of engrafted hu-PBL-SCID mice. PBL were separated from other cell types using a Ficoll gradient (Sigma, St. Louis, MO). 50x10^6 PBL were injected intraperitoneally in 0.5mL PBS into CB.17 SCID mice.

**Monoclonal antibody and cytokine treatment**

Mice were given intraperitoneal injections of 100 µg of TMβ1 mAb to deplete murine NK cells on the day preceding or the day of engraftment. In the experiments analyzing T cell expansion and LMP-1 expression, this initial treatment was followed by two additional treatments of 100 µg of TMβ1 mAb every other day. Mice received intraperitoneal injections of 300 µg purified anti-human CTLA-4 mAb or 100 µL ascites containing anti-human CTLA-4 mAb, or 100-300 µg control mouse IgG (Sigma, St.
Louis, MO) on days 1, 5, 9, and 13 after PBL engraftment. Mice also received intraperitoneal injections of 3 µg human GM-CSF every other day for 3 weeks. In the experiment assessing IFNγ production, mice received a single dose of 100 µg TMβ1 mAb, followed by 300 µg purified anti-human CTLA-4 mAb or control mouse IgG and 3 µg human GM-CSF on days 1, 5, and 9.

**Flow cytometry**

All antibodies used for staining of cell surface and intracellular proteins, such as CD3, CD4, CD8, CD45, LMP-1, IFNγ, and were purchased from BD Pharmingen (San Diego, CA). Intracellular staining for LMP-1 and IFNγ was performed using a Cytofix/CytoPerm kit (BD Pharmingen, San Diego, CA).

**IFNγ production assay**

Hu-PBL-SCID spleen cells were stimulated with an autologous EBV⁺ lymphoblastoid cell line (LCL) or an allogeneic EBV⁻ Burkitt’s lymphoma cell line at a 4:1 ratio for 6 hours in the presence of GolgiStop (BD Pharmingen, San Diego, CA). After stimulation, cells were washed and stained for extracellular CD45, CD8, and CD4, followed by intracellular staining with IFNγ or isotype IgG1.

**Survival experiment**

CB.17 SCID mice were engrafted with 50x10⁶ PBL and treated with 100 µg of TMβ1 mAb on the same day, followed by 100 µL ascites containing anti-human CTLA-4 mAb or 100 µg mouse IgG and 3 µg human GM-CSF on days 1, 5, 9, and 13. Mice were monitored for signs of illness and sacrificed when moribund. Necropsy was performed to determine the presence of lymphoproliferative disorder or graft-versus-host disease. The
mean survival time and standard error of the mean survival time were calculated for each
group using the Kaplan-Meier estimate. The survival times of the groups were compared
using the log rank test [173].

3.4 Results

Generation of a panel of mouse anti-human CTLA-4 monoclonal antibodies.

BALB/c mice were immunized two times with human CTLA-4Ig fusion protein,
consisting of the extracellular domain of human CTLA-4 and the Fc fragment of human
IgG1. Spleen cells from these mice were fused with the myeloma cell line XAg8.653.
After several fusions, we have generated a panel of more than 20 hybridomas producing
significant amounts of monoclonal antibody against the human CTLA-4 molecule. Five
of these clones were selected for experimentation upon demonstration of significant
binding to human CTLA-4 by ELISA (Table 3.1). All five of the antibodies were
determined to be IgG1,κ isotype, which facilitates direct comparison of any immunologic
response that may be mediated by these antibodies.

Anti-human CTLA-4 mAb promotes a profound expansion of T cells in a hu-PBL-
SCID model.

To test whether our anti-human CTLA-4 mAb had any biological activity in vivo,
we employed the hu-PBL-SCID mouse model. This model provides a unique setting in
which we can observe the interaction of a functional human immune system with EBV-
generated lymphoproliferative disorder [166]. SCID mice were engrafted with human
PBL and treated with different clones of anti-human CTLA-4 mAb, plus human GM-CSF
to promote the generation and maturation of antigen-presenting cells [174]. As shown in
Figure 3.1a, at 12 days after injection of human PBL, all three anti-human CTLA-4 antibodies increased the total number of splenocytes by more than 3-fold compared with control mice (p=0.0002). In addition, a selective expansion of human leukocytes, as marked by expression of human CD45, was observed among all antibody-treated mice (Figure 3.1b top panel, 3.1c and 3.1d left panels). The total number of CD4 and CD8 T cells was also increased (Figure 3.1b lower panel and 3.1c). However, at this time point, the antibodies differ in their ability to selectively expand human T cell subsets. First, in mice that received L3D10 and YL2, the proportion of CD4 T cells expanded significantly at the expense of CD8 T cells. In contrast, KM10 did not cause such preferential expansion (Figure 3.1b lower panels and 3.1d). The proportion of CD8 T cells among human leukocytes decreased significantly even as the total numbers increased (Figure 3.1c and 3.1d).

At the third week after reconstitution, we analyzed all five anti-CTLA-4 antibodies for their effect on the number of human CD4 and CD8 T cells in the spleen. An example is given in Figure 3.2a, and the comparison of the different antibodies is presented in Figure 3.2b. As shown in Figure 3.2a, L3D10 caused a more than 10-fold expansion of CD4 and CD8 T cells. Interestingly, the five clones of anti-human CTLA-4 mAb displayed differential effects not only on the amount of T cell expansion, but also on the relative effect on CD4 versus CD8 T cells subsets. Most clones of anti-human CTLA-4 mAb showed a preferential expansion of CD8 T cells at this time point, while one clone of mAb showed a slightly preferential increase in CD4 T cells. These data clearly demonstrate that our anti-human CTLA-4 mAb promotes the expansion of human T cells and increases the engraftment of total human PBL in the hu-PBL-SCID
mouse model. Since anti-human CTLA-4 mAb clone L3D10 showed the greatest effect in expanding human T cells, this clone was chosen for further characterization.

**Anti-human CTLA-4 mAb decreases EBV-mediated transformation of human cells.**

When PBL from EBV-seropositive donors are used in the hu-PBL-SCID mouse model, transformation of PBL by EBV leads to the development of lymphoproliferative disorder. Latent membrane protein 1 (LMP-1) is an EBV oncoprotein involved in the immortalization of B cells leading to this transformation [175-179], and LMP-1 has been shown to be a potential target of T cell responses [180, 181]. Hence, the number of cells expressing LMP-1 can be taken as a reflection of the number of cells that have been transformed by EBV and could undergo oncogenesis. One way to determine whether the expansion of T cells mediated by anti-human CTLA-4 mAb treatment has any therapeutic effect is to examine the level of LMP-1 being expressed within engrafted cells. To test this, SCID mice were engrafted with PBL and treated with anti-human CTLA-4 mAb L3D10 or mouse IgG. Mice were sacrificed 22 days after engraftment, and spleen cells were analyzed. Similarly to the experiment shown in Figure 3.2, we observed a substantial expansion in both CD8 and CD4 T cell subsets (data not shown). As shown in Figure 3.3a and 3.3b, the percentage of LMP-1⁺ cells was 2-3 fold lower in mice treated with L3D10. These results suggest that the percentage of EBV-infected cells can be reduced as a result of anti-CTLA-4 mAb treatment.

**Anti-human CTLA-4 mAb promotes expansion of LCL-reactive CD8 T cells.**

To test whether antigen-specific T cells were induced in our model, we stimulated spleen cells harvested from hu-PBL-SCID mice with an autologous EBV-positive lymphoblastoid cell line (LCL) or allogeneic EBV-negative Burkitt’s lymphoma for 6
hours in vitro, and evaluated IFNγ production by CD8 T cells. The LCL was generated from a tumor harvested from a hu-PBL-SCID mouse previously engrafted with the same donor’s PBL. To verify the expression level of EBV protein, we stained these stimulator cell types for intracellular LMP-1 expression. As shown in Figure 3.4a, almost all the LCL cells express high levels of intracellular LMP-1, while the Burkitt’s lymphoma cells show minimal or no LMP-1 expression. After 6 hours of stimulation with these cells, we observed nearly a 3-fold increase in the percentage of IFNγ-producing CD8 T cells in L3D10-treated mice compared with control mice (Figure 3.4b). This indicates that anti-human CTLA-4 mAb can promote the preferential expansion of antigen-specific CD8 T cell responses, as well as promoting overall expansion of T cells.

**Anti-human CTLA-4 mAb delays the development of lymphoproliferative disease in hu-PBL-SCID mice.**

To determine whether this reduction in LMP-1 translates into reduced development of lymphoproliferative disease associated with the hu-PBL-SCID model, we performed a long-term survival experiment in which engrafted mice were treated with anti-human CTLA-4 mAb and human GM-CSF for two weeks after engraftment, and then observed for signs of illness. Figure 4.5 shows the survival curve of control mice and the most effective anti-human CTLA-4 mAb L3D10. L3D10 mediated an almost two-fold extension in the mean survival time of engrafted mice compared with control mice (100.3 +/- 17.5 days with L3D10 versus 53.0 +/- 6.2 days with mouse IgG), which was statistically significant (p = 0.0195).
3.5 Discussion

The manipulation of costimulatory pathways using monoclonal antibodies has proven to be a highly effective strategy for boosting anti-tumor immunity in mice. Unfortunately, very few of these achievements have been successfully translated to the treatment of human cancer patients. Since the first use of monoclonal antibodies targeting CTLA-4 to treat mouse tumors [82], CTLA-4 has shown great promise for clinical translation. The two recent human trials using mAbs to target the human CTLA-4 molecule showed promising effects [94, 95], but also highlighted several difficulties in translating results observed in murine models to human patients. For example, in one study, the anti-human CTLA-4 mAb showed no toxicities but also minimal clinical benefit in patients with metastatic melanoma and ovarian cancer [94]. Another study with the same mAb showed several dramatic clinical responses, accompanied by serious autoimmune toxicities [95]. While these studies validate the potential of anti-human CTLA-4 mAb as a therapy for human cancer patients, further preclinical testing of this mAb is in order to address these issues [96].

Here we describe the use of a hu-PBL-SCID mouse model to obtain a more thorough preclinical screening of anti-human CTLA-4 mAb to identify the most efficacious clones from a panel of mAbs. The hu-PBL-SCID mouse model was first described by Mosier et al. as a method to reconstitute a functional human immune system in SCID mice by intraperitoneal injection of human peripheral blood leukocytes [166]. This report described the long term engraftment of all cellular components on the human immune system, and also observed the spontaneous development of human B cell lymphomas when PBL from Epstein Barr virus (EBV)-seropositive donors were used.
These lymphomas were subsequently characterized as being similar to the large cell lymphomas observed in immunosuppressed transplant patients [182], also known as post-transplant lymphoproliferative disorder (PTLD). Since the initial reports, numerous groups have utilized this model to test various aspects of immune function and lymphomagenesis, and in the process, discovered a number of limitations of this model, including xenograft-versus-host disease, variations in PBL engraftment, and leakiness of the SCID phenotype [183-187]. Despite these caveats, the hu-PBL-SCID model remains one of the few mouse models with which to assess spontaneous human tumor development and the resultant anti-tumor immune response. More recently, evidence has accumulated that the control of EBV-lymphoproliferative disorder is mediated by CD8+ cytotoxic T lymphocytes both in patients with PTLD [188, 189] and hu-PBL-SCID mice [172, 190]. With the identification of EBV latent and lytic antigens, it has been demonstrated that specific CD8 T cell responses to these EBV antigens can be detected in seropositive human patients [180, 191, 192] and in hu-PBL-SCID mice [172]. Correlation of CD8 T cell responses to protection against EBV-lymphoproliferative disease in hu-PBL-SCID mice makes this model valuable for the study of anti-human CTLA-4 mAb.

In this study, we have clearly demonstrated the ability of anti-human CTLA-4 mAb to mediate dramatic expansion of CD8 and CD4 T cell populations. In conjunction, mAb promotes the overall engraftment or survival of human PBL in the SCID mouse. Interestingly, each clone of anti-human CTLA-4 mAb possessed varying ability to promote T cell expansion and PBL engraftment. Since all antibodies were of the same isotype, these variations cannot be attributed to isotype difference. The variability of
response could be due to different binding affinities of the clones to the human CTLA-4 molecule. If binding affinities vary greatly among clones of mAb against the same molecule, a more stringent preclinical screening regimen is indeed warranted to select clones of mAb that can elicit the most dramatic T cell response.

It has been reported that human T cells engrafted in SCID mice represent an anergic phenotype and that once anergy is broken, most reactivity of CD4 T cells is directed against mouse antigens [193]. It is possible that the lack of T cell expansion in our control mice was due to an anergic state of the T cells, and that treatment with anti-human CTLA-4 mAb was sufficient to reverse this anergy and permit T cell expansion. This has important implications for a tumor setting in which T cells might be tolerized to tumor antigen, as been demonstrated in at least one mouse model [93].

Not only did anti-human CTLA-4 mAb promote overall T cell expansion, but several different parameters suggested that the robust T cell expansion with anti-human CTLA-4 mAb treatment had therapeutic value. The first was a significant decrease in the percentage of cells that expressed intracellular LMP-1. As an EBV oncoprotein that is critical for the generation of lymphoproliferative disease, LMP-1 may be viewed as a surrogate marker for the potential formation of tumor within the mice. Reduced levels at an early time point before lymphoproliferative disease normally appears, reveals the impact of mAb treatment in reducing the oncogenic source of tumor.

Secondly, we observed the preferential expansion of antigen-specific CD8 T cells with anti-human CTLA-4 mAb treatment. This enhanced expansion was elicited in mice treated with a more limited GM-CSF regimen than that used in experiments showing overall T cell expansion. Interestingly, the mice treated with L3D10 under the limited
GM-CSF regimen did not show overall expansion of T cells compared with control mice, despite their preferential increase in antigen-specific CD8 T cells. Additional experiments using frozen spleen cells from mice treated with L3D10 and a more extensive GM-CSF protocol (every other day) showed enhanced overall T cell expansion but not antigen-specific expansion when compared with control mice. It is difficult to directly compare the use of fresh and frozen cells for in vitro stimulation due to the decreased viability and increased background staining associated with thawed cells, but perhaps the interaction of anti-CTLA-4 mAb and GM-CSF is more complicated than predicted in the hu-PBL-SCID mouse model.

Thirdly, in a long-term survival experiment we observed a prolongation of survival with anti-human CTLA-4 mAb treatment, providing another piece of evidence that mAb can promote anti-tumor immune responses. This result must be taken with caution, as this experiment and other attempts to reproduce the survival advantage were complicated by the development of severe illness, most likely xenograft-versus-host disease, which sometimes caused death before lymphoma formation in a substantial fraction of mice involved. One mouse treated with L3D10 was excluded from the analysis due to early death at day 15. However, in mice that escaped this early death, the trend of prolonged survival is intriguing. Taken together our data show an important role for anti-human CTLA-4 mAb in the expansion of human T cells and the promotion of immunity against a spontaneous virally-induced tumor. Furthermore, variability in the efficacy of different clones of mAb warrants the use of novel models such as this one, to provide more thorough preclinical screening of candidate mAb for clinical translation.
Table 3.1 Five clones of anti-human CTLA-4 mAb bind to huCTLA-4Ig fusion protein. Supernatant from hybridoma cultures was tested by ELISA against a human CTLA-4Ig fusion protein for specific binding, and a mouse CD28Ig fusion protein for non-specific binding. OD values were read at 495 nm.

<table>
<thead>
<tr>
<th>Clone name</th>
<th>huCTLA-4Ig binding (OD value)</th>
<th>moCD28Ig binding (OD value)</th>
</tr>
</thead>
<tbody>
<tr>
<td>L3D10</td>
<td>2.625</td>
<td>0.405</td>
</tr>
<tr>
<td>L1B11</td>
<td>1.831</td>
<td>0.216</td>
</tr>
<tr>
<td>K4G4</td>
<td>1.575</td>
<td>0.662</td>
</tr>
<tr>
<td>KM10</td>
<td>1.322</td>
<td>0.288</td>
</tr>
<tr>
<td>YL2</td>
<td>1.773</td>
<td>0.093</td>
</tr>
</tbody>
</table>
Figure 3.1 Anti-human CTLA-4 mAb promotes the engraftment of PBL and expansion of human T cells within 12 days. CB.17 SCID mice were engrafted with 50x10^6 human PBL, and treated with 100 µg TMB1 mAb on day 0, 2, and 4, followed by 300 µg anti-human CTLA-4 mAb or mouse IgG on days 1, 5, and 9, and 3 µg human GM-CSF every other day. At 12 days after engraftment, mice were sacrificed and spleens were harvested for staining. a) Total cellularity within spleens. b) Representative FACS plot showing expanded percentage of CD45^+ CD4^+, and CD8^+ cells. CD4^+ and CD8^+ are gated from among CD45^+ cells. c) Total cell numbers of CD45^+ (left panel), and CD4^+ and CD8^+ cells (right panel). d) Percentage of CD45^+, CD4^+, and CD8^+ cells within live cell gate. Bars represent mean plus SEM. All panels are representative of 4-5 mice per treatment group.
Figure 3.1

(a) Bar graph showing total spleen cells (10^6) for different treatment groups:
- Mouse IgG
- L3D10
- KM10
- YL2

(b) Flow cytometry plots comparing Mouse IgG and L3D10:
- Forward scatter vs. CD45
- Forward scatter vs. CD8

Values for selected regions:
- Mouse IgG: 6.5, 19.3, 18.0, 11.7
- L3D10: 22.8, 38.1

(continued)
Figure 3.2  Anti-human CTLA-4 mAb promotes the engraftment of PBL and expansion of human T cells at 24 days. CB.17 SCID mice were engrafted with 50x10^6 human PBL, and treated with 100 µg TMβ1 mAb on days -1, 1, and 3, 100 µL ascites containing anti-human CTLA-4 mAb or 100 µg mouse IgG on days 1, 5, 9, and 13, and 3 µg human GM-CSF every other day. At 24 days after engraftment, mice were sacrificed and spleens were harvested and pooled for staining. a) Representative dot plot showing expansion of CD8 and CD4 T cells with anti-human CTLA-4 mAb clone L3D10 treatment. b) Variable expansion of CD8 and CD4 T cells with treatment by different clones of anti-human CTLA-4 mAb. Bars represent cells from pooled spleens from two to three mice per treatment group. Data is representative of two independent experiments, testing various clones of anti-human CTLA-4 mAb.
a.

![Flow cytometry plots for Mouse IgG and L3D10](image)

- **CD4**
  - Mouse IgG: 3.4
  - L3D10: 31.1

- **CD8**
  - Mouse IgG: 3.4
  - L3D10: 53.3

*b.*

![Bar charts for IgG and L3D10](image)

- **% of lymphocyte gate**
  - CD8
  - CD4

- **Cell number (10^6)**
  - IgG
  - L3D10
  - L1B11
  - K4G4
  - KM10
  - YL2

*Figure 3.2*
Figure 3.3 Anti-human CTLA-4 mAb L3D10 decreases percentage of engrafted cells expressing EBV latent membrane protein 1 (LMP-1). Mice were treated as described in Figure 3 legend, except TMβ1 mAb was given on days 0, 2 and 4. Spleens were harvested at day 22 after engraftment and stained for intracellular LMP-1 or isotype IgG2a. a) Representative FACS plot of LMP-1 staining, depicting cells from lymphocyte gate. b) Summary graph showing LMP-1 staining for 3 to 4 individual mice per treatment group. Data is representative of two independent experiments. A p-value was determined using a two-sample t-test.
Figure 3.4  Anti-human CTLA-4 mAb L3D10 promotes preferential expansion of lymphoblastoid cell line-reactive CD8 T cells. a) LMP-1 expression by an autologous EBV-positive lymphoblastoid cell line (LCL) and an allogeneic EBV-negative Burkitt’s lymphoma cell line, used as stimulators for IFNγ production by hu-PBL-SCID spleen cells. b) CB.17 SCID mice were engrafted with 50x10^6 human PBL, and treated with 100 µg TMβ1 mAb on the same day, followed by 300 µg anti-human CTLA-4 mAb L3D10 or mouse IgG and 3 µg human GM-CSF on days 1, 5, and 9. Spleen cells were harvested at day 29 after engraftment and stimulated for 6 hours with autologous LCL or allogeneic Burkitt’s lymphoma as a control. Samples were then stained for IFNγ-producing CD8 T cells. L3D10-treated mice show an almost 3-fold increase in percentage of IFNγ-producing CD8 T cells with LCL stimulation compared with control mice. Neither treatment group showed reactivity to Burkitt’s lymphoma. FACS plots represent pooled spleens from nine mouse IgG-treated and five L3D10-treated mice. Plots represent cells within the CD45⁺CD8⁺ gate.
Figure 3.4

a. Lymphoblastoid cell line | Burkitt’s lymphoma
---|---
LMP-1 | 86.9 | 1.59
IgG2a | 2.4 | 0.09
huCD19 | 0.14 | 0.069

b. Lymphoblastoid cell line (EBV+) | Burkitt’s lymphoma (EBV-)
---|---
Mouse IgG | L3D10 | Mouse IgG | L3D10
IFNγ | 0.35 | 0.041 | 0.26 | 0.069
IgG1 | 0.14 | 0.088 | 0.088 | 0.041
Figure 3.5  Anti-human CTLA-4 mAb L3D10 prolongs survival and delays onset of lymphoproliferative disorder in hu-PBL-SCID.  CB.17 SCID mice were engrafted with 50x10^6 human PBL and treated with 100 µg TMβ1 mAb the same day, followed by 100 µL ascites containing anti-human CTLA-4 mAb L3D10 or 100 µg mouse IgG, and 3 µg human GM-CSF on days 1, 5, and 9, and 13 following engraftment.  Mice were monitored for signs of illness and sacrificed when moribund.  Necropsy was performed to evaluate presence or absence of lymphoproliferative disorder.  Survival curve for the most effective clone L3D10 is shown.  Other clones of anti-human CTLA-4 mAb increased survival compared with control mice but not to the extent of L3D10.  One L3D10 mouse with early death at day 15 was excluded from the survival analysis based on our experience that no lymphoma-related death is possible at this point.  The survival times of the groups were compared using the log rank test.
CHAPTER 4

NOVEL HUMAN CTLA-4 KNOCK-IN MICE AS A MODEL TO STUDY ANTI-HUMAN CTLA-4 MONOCLONAL ANTIBODY INDUCTION OF ANTI-TUMOR IMMUNITY

4.1 Abstract

CTLA-4 is a costimulatory molecule expressed by activated T cells and is the high affinity ligand for B7-1 and B7-2. Monoclonal antibodies (mAbs) targeting mouse CTLA-4 have the ability to promote anti-tumor immunity against a variety of murine tumor models. In order to extend these findings to a human setting, we have created a panel of anti-human CTLA-4 monoclonal antibodies for potential translation to human cancer patients. In order to study the candidate antibodies, we have constructed a knock-in mouse model in which the gene encoding mouse CTLA-4 has been replaced with a gene encoding human CTLA-4. Human CTLA-4 is expressed by mouse T cells at both the RNA and protein levels upon activation. Additionally, mice with homozygous expression of human CTLA-4 show no signs of the lymphoproliferative disorder associated with CTLA-4 knockout mice. Using several different tumor models, we demonstrate a promotion of anti-tumor immunity with anti-human CTLA-4 antibody
treatment in heterozygous human CTLA-4(+/-) mice. These results support the use of our human CTLA-4 knock-in mouse model as a tool for preclinical screening of anti-human CTLA-4 monoclonal antibodies.

4.2 Introduction

Since the discovery of the costimulatory molecule CTLA-4 in mouse [23] and man [194], and its demonstration as a second receptor for B7 [24], this molecule has been the subject of extensive study. CTLA-4 has been suggested by some groups to be important for the inhibition of T cell responses as a counterpart to the activating costimulatory molecule CD28 [29, 30]. In contrast, our lab has suggested that CTLA-4 may possess agonist activity during the effector phase of certain T cell responses [45-47]. Generation of monoclonal antibodies targeting CTLA-4 provided a new manner in which to test the mechanism of action of CTLA-4 [25], as to whether the antibody functioned via CTLA-4/B7 blockade or as an agonist. Furthermore, evidence that transfection of tumor cells with B7, the ligand for CTLA-4, could induce potent anti-tumor immunity [76] revealed therapeutic possibilities for anti-CTLA-4 monoclonal antibody. Subsequent studies showed that indeed anti-CTLA-4 mAb could promote anti-tumor immunity against a variety of tumors including colon carcinoma [82], fibrosarcoma [82], prostate cancer [83-85], melanoma [86-88], ovarian carcinoma [89], mammary carcinoma [90], and plasmacytoma [91].

More recently, an anti-human CTLA-4 mAb has been generated and tested in several trials of advanced ovarian cancer and melanoma patients [94, 95]. In one of these trials, anti-CTLA-4 mAb induced severe autoimmune toxicities [95], and consequently,
further safety and toxicity studies are being performed [96]. To facilitate the preclinical screening of new anti-human CTLA-4 mAbs that have been produced in our lab, we have created a novel human CTLA-4 knock-in mouse model. In this mouse, the extracellular and transmembrane domains of mouse CTLA-4 have been replaced with their human counterparts. We show that human CTLA-4 is expressed by T cells upon antigen stimulation at both the RNA and protein levels. Furthermore, treating heterozygous human CTLA-4 knock-in with anti-human CTLA-4 mAb can promote anti-tumor immunity and delay tumor growth in two different tumor models.

4.3 Materials and Methods

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 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 is a 6.5 kb plasmid containing a neomycin (Neo) resistance gene/herpes simplex virus (HSV) thymidine kinase (TK) gene cassette flanked by loxP sites.

DNA containing a 14 kb fragment of the human CTLA-4 gene was prepared from a lamda phage clone [194, 195], 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 BamHI to obtain a 3.2 kb BamHI 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 BamHI. The 9.5 kb fragment was purified and ligated with the 3.2 kb fragment of human CTLA-4 exons 2 and 3.

A 2.4 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 ATCCTCTAGAAGCTTCAAAGCAGGTTATCA, 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 4.1.

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

Embryonic stem (ES) cell line R1 was transfected with the DNA construct described above by electroporation. Specifically, the plasmid was linearized by Sal I digestion. Approximately 2 x 10^7 ES cells were suspended in culture media (DMEM) without serum and electroporated in the presence of 30 µg of the humanized CTLA-4 transgene construct using a BioRad Gene Pulser. The electroporated ES cells were then resuspended in growth media and plated onto 100mm plastic dishes containing fibroblasts treated with mitomycin C. After 24 hours the media was replaced with
selection media containing 150 µg/ml neomycin (G418) and changed daily. ES colonies growing after 10 days of selection were trypsinized, and individual colonies were transferred to 24-well culture plates with mitomycin C-treated fibroblasts. After a week of culture one half of the individual ES clones was used to prepare DNA for analysis and the other half was frozen in the presence of 10% DMSO and stored at –80°C for further use.

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 (CCTCTGAGCATCCTTAGC) 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 is 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 was isolated, digested with EcoR I, 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 Hind III sites, using primers CTGCAGTGACACCCCTCTC and ACGTCTCCAGTTCCTCAGAG. The probe was labeled with 32P 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 the 3.2 kb 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 with the pCre-Pac plasmid described by Taniguchi M., et al. [196] by electroporation. After two days of selection with 1µg/ml puromycin in the growth media, the majority of cells died. The cells that survived expressed transiently both the puromycin resistance gene and the Cre-recombinase. Cre-recombinase induces a re-arrangement to delete the lox P flanked Neo-TK cassette. We then continued the selection with gancyclovir, a drug that is converted by TK into a toxic metabolite. ES cells that lost the TK gene after Cre-mediated excision were not affected by the gancyclovir and grew into colonies. Twenty colonies were individually isolated and tested for the presence of Neo and TK genes. In several such colonies, the loss of Neo and TK genes from the humanized CTLA-4 mouse transgene was confirmed and the cells were further analyzed.

Two set of PCR reactions were carried out to detect the floxed and deleted alleles of the CTLA-4 locus. The first PCR (D) used 5'-TCCCTCTCAGACACCTGC-3' as the forward primer and 5'-GTCATAAACATCTCTCAGGTAA-3' as the reverse primer. This reaction amplifies the alleles in which Neo/TK have been deleted with a product of 1.1 kb. While this reaction should theoretically also amplify the endogenous murine CTLA-4 alleles, the PCR conditions used did not allow amplification of a large product.
of 4 kb. ES cell clones 2, 5, 7.1, 7.2, 18, 20, 22 were found to have deleted Neo/TK alleles. The second PCR (F) used 5'-TCCCTCTCAGACACCTCTGC-3' as the forward primer and 5'-CGACCTGTCCGGTGC-3' as the reverse primer. This PCR only amplified the floxed (Neo-TK containing) alleles. Only clone 7.2 had significant amount of cells with floxed alleles.

**Expression of the humanized CTLA-4 gene in ES cells.**

It has been reported that ES cells can express the CTLA-4 gene at low levels [197]. To analyze the expression of the humanized CTLA-4 alleles in the ES cells with an uninterrupted humanized CTLA-4 gene, we designed two sets of RT-PCR reactions. Reaction H used 5'-GAGGCATCGCCAGCTTTGTG-3' as the forward primer, and 5'-CACATAGACCCCTGTTGTAAGA-3' as the reverse primer. This reaction did not amplify murine CTLA-4 as cDNA prepared from mouse thymus did not yield any product. In the majority of the clones tested (clones 2, 7.1, 7.2, 20 and 22), this reaction detected two forms of humanized CTLA-4 gene product: one comprising exons 2 and 3 of the human CTLA-4 gene and exon 4 of the murine CTLA-4 gene, and the other comprising exon 2 of the human CTLA-4 gene and exon 4 of the murine CTLA-4 gene. Reaction M used the same reverse primer, but the forward primer (5'-TGTGCCACGACATTCAAGA-3') corresponded to a unique sequence on murine exon 2. This reaction amplified murine CTLA-4.

**Production of chimeric and transgenic mice**

Chimeric mice were prepared by an aggregation method essentially as described [198]. Morula stage embryos of the C57BL/6 mouse strain were aggregated and
cocultured for 24 hours with 8-16 cells of the ES cell clones. After 24 hours the embryos developed to the blastocyst stage and were transplanted into the uteruses of pseudopregnant female mice. After 3 weeks of pregnancy chimeric pups were born and identified by chimerism of the skin which resulted in areas of agouti (brown) and black fur. The C57BL/6 mouse strain is black and the 129 mouse strain from which the ES cells were derived is agouti (brown). When the ES genotype is passed to the progeny it can be readily identified by the brown coat color. 50% of such animals carry the humanized CTLA-4 transgene and can be positively identified by PCR detecting the human exons.

**Experimental animals and tumor cell lines**

P1CTL transgenic mice expressing a T cell receptor specific for P1A35-43:Ld complex have been previously described [155]. BALB/c and C57BL/6j mice were purchased from Charles River Laboratories under contract from the National Cancer Institute. BALB/c mice with a targeted mutation in the RAG-2 gene were purchased from Taconic (Germantown, NY) and bred in our facility. B7-1(-/-) B7-2(-/-) mice [20], which have been backcrossed to the C57BL/6j strain for at least 10 generations, were purchased from the Jackson Laboratory (Bar Harbor, ME). All mice were maintained in the University Laboratory Animal Research Facility at the Ohio State University under specific pathogen-free conditions. Plasmacytoma J558 cells transfected with Neo vector or B7-1 have been previously described [79]. MC38 colon carcinoma cells were purchased from American Type Culture Collection.
Analysis of human CTLA-4 RNA and protein expression

Spleen cells were obtained from F1 offspring of heterozygous human
CTLA-4(+/−) mice crossed with BALB/c P1CTL mice, and stimulated in culture with
0.1 µg/ml P1A peptide. To analyze RNA expression, cells were harvested after 24 hours.
RNA was extracted from these cells with TRIzol (Gibco Invitrogen, Carlsbad, CA)
according to manufacturer’s protocol, and cDNA was produced using reverse
transcriptase. An RT-PCR was performed with cDNA 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’-CTTGTCTTGGACTCCGGAGGTAC-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’-
GAGGCATCGCCAGCTTTGTG-3’) and a common reverse primer for mouse exon 4
(5’-CACATAGACCCCTGTTGTGAAGA-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.

To analyze human CTLA-4 protein expression, spleen cells 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 (cell surface), PE-conjugated anti-mouse
CTLA-4 (intracellular), and CyChrome-conjugated anti-human CTLA-4 (intracellular). Conjugated antibodies and CytoFix/CytoPerm intracellular staining kit were purchased from BD Pharmingen (San Diego, CA).

Tumorigenicity experiments

Mice were subcutaneously inoculated with 5x10^6 J558Neo plasmacytoma cells or 5x10^5 MC38 colon carcinoma cells, and tumor size and incidence were evaluated.

Adoptive transfer experiments

Spleens and lymph nodes from BALB/c P1CTL huCTLA4 (+/+ or BALB/c P1CTL huCTLA4(+/-) mice were harvested and ground into a single cell suspension. Red blood cells were lysed with ammonium chloride (Sigma, St. Louis, MO) and total cells were injected intraperitoneally. Peripheral blood T cells were stained with conjugated antibodies against CD8, CD4, and Vα8 TCR (BD Pharmingen, San Diego, CA).

Antibodies

Anti-human CTLA-4 monoclonal antibody L3D10 (Chapter 3) and anti-4-1BB monoclonal antibody 2A [100] have been described previously. Antibody was purified from hybridoma culture supernatant using a Protein G column. Mouse IgG was purchased from Sigma (St. Louis, MO). Conjugated antibodies for staining cell surface and intracellular molecules were purchased from BD Pharmingen (San Diego, CA).
4.4 Results

Creation of a human CTLa-4 knock-in mouse.

The gene encoding for 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 [194, 195]. Due to the conservation of the cytoplasmic domain between both species, it is reasonable to assume that both molecules have similar signaling functions. Since human CTLa-4 is able to bind to murine B7-1 and B7-2 [199], it is likely that interaction of human CTLa-4 and murine B7 would maintain normal signal transduction by CTLa-4. We have created a chimeric DNA construct replacing the exons encoding for the extracellular (exon 2) and transmembrane (exon 3) domains of murine CTLa-4 with those of human CTLa-4 (Figure 4.1a). The gene product of exon 1 is a signal peptide not expressed in the mature protein, and the cytoplasmic domain (exon 4) is completely conserved.

We transfected an embryonic stem (ES) cell line R1 with the human CTLa-4 DNA construct in Figure 4.1a by electroporation. After selection with neomycin, DNA was isolated from the drug-resistant ES cell clones and screened for homologous recombination by Southern blot (Figure 4.1b). 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 described by Taniguchi M. et al. [196]. This plasmid expresses both the Cre-recombinase and puromycin resistance gene, which allows for very fast selection of cells that contain the plasmid. The Cre-recombinase is an enzyme that
recombines specific DNA sequences called loxP. The Neo/TK gene that is situated between two loxP sites is excised by the action of the Cre-recombinase. 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 had not been excised. Analysis of DNA from several surviving colonies by PCR indicated that the Neo/TK cassette was excised from the knock-in locus (Figure 4.2a). Analysis of RNA by RT-PCR followed by Southern blot demonstrated that the locus is functional in expressing a humanized CTLA-4 gene (Figure 4.2b). Interestingly, except clone 2, none of the other ES cell clones appeared to express significant amounts of the murine CTLA-4 gene, perhaps due to a lower efficacy of PCR amplification. Based on the analysis of DNA and RNA, ES cell clone 20 was chosen for the production of chimera mice by a standard aggregation method. Chimera mice have been bred with C57BL/6j and BALB/c mice to obtain germline transmission of the human CTLA-4 gene.

**RNA expression and splicing of human CTLA-4 in knock-in mouse.**

To determine whether human CTLA-4 is properly expressed and spliced at the RNA level, mice with a single copy of human CTLA-4 were bred with P1CTL transgenic mice that express a T cell receptor recognizing the tumor antigen P1A in the BALB/c background. Spleen cells from a huCTLA-4(+-) P1CTL mouse were stimulated in culture with P1A peptide for 24 hours. 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 and 4 were used to amplify a band of 648 base pairs. As shown in Figure 4.3, basically all CTLA-4 is properly spliced and expressed as full length RNA containing exons 1 to 4, with no alternative splicing products.
Additionally, to test whether the human and mouse CTLA-4 alleles are co-dominantly expressed after antigenic stimulation, cDNA was amplified with forward primers specific for mouse or human exon 2 and a common reverse primer for mouse exon 4. 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. As shown in Figure 4.3, RT-PCR of RNA from heterozygous human CTLA-4 knock-in mice revealed a co-dominant expression of both mouse and human CTLA-4 RNA with amplified fragments of the predicted size with no alternative splicing products.

**Expression and function of human CTLA-4 protein in the knock-in mouse.**

To determine whether human CTLA-4 protein is properly expressed, spleen cells from huCTLA-4(+/−) P1CTL and huCTLA4(-/-) P1CTL littermate mice were stimulated in culture with P1A peptide for 66 hours. Cells were harvested and stained for intracellular mouse and human CTLA-4 expression. Figure 4.4 (upper panel) shows the co-dominant expression of both mouse and human CTLA-4 protein on the same cells. Additionally, diagonal distribution of the human and mouse CTLA-4 molecules reveal that the two alleles are regulated by same mechanism. Figure 4.4 (lower panel) shows the lack of human CTLA-4 expression in a littermate that does not contain the human CTLA-4 knock-in gene. Also shown are stains with isotype control antibodies to demonstrate specificity of staining. This coordinate expression of human and mouse CTLA-4 was also observed in spleen cells from huCTLA-4(+/-) mice after activation with anti-CD3 antibody (data not shown).

One of the striking observations in CTLA-4 research was the lymphoproliferative disorder observed in CTLA-4 knockout mice [32, 33]. A concern in the production of
human CTLA-4 knock-in mice is the ability of human CTLA-4 to functionally replace mouse CTLA-4. While heterozygous human CTLA-4 knock-in mice still retain one copy of the mouse CTLA-4 gene, homozygous huCTLA-4 knock-in mice represent a kind of mouse CTLA-4 knockout. To determine if human CTLA-4 could functionally replace mouse CTLA-4, two huCTLA-4(+/-) P1CTL mice were bred to produce huCTLA-4(+/+) offspring. From several litters comprising 32 mice, 7 (22%) were huCTLA-4(-/-), 19 (59%) were huCTLA-4(+/-), and 6 (19%) were huCTLA-4(+/+) (Table 4.1). Mice were observed to more than seven weeks of age with all human CTLA-4 knock-in mice remaining healthy and exhibiting no signs of the lethal lymphoproliferative disease observed in CTLA-4 knockout mice by 4 weeks of age. The healthy survival of these huCTLA-4(+/+) mice suggests that the human CTLA-4 protein can adequately replace the function of mouse CTLA-4 protein.

**Promotion of anti-tumor immunity in heterozygous human CTLA-4(+/-) knock-in mice by anti-human CTLA-4 monoclonal antibody.**

A number of studies have shown the efficacy of anti-mouse CTLA-4 mAb in promoting anti-tumor immunity. In order to determine if we could demonstrate a similar enhancement with anti-human CTLA-4 mAb, two different tumor models were tested. In one model, heterozygous huCTLA-4(+/-) x BALB/c F1 mice were challenged with the plasmacytoma J558. Mice were treated with 200 µg L3D10 or mouse IgG on days 0, 2, 6, and 23 after tumor challenge. Figure 4.5 shows one of two independent experiments, in which a combined 5 of 15 mice (38.5%) of mice treated with L3D10 rejected their tumor challenge, while only 2 of 9 control mice (22.2%) remained tumor-free. In one of two experiments, mice that did not reject their tumors displayed delayed tumor growth
kinetics when treated with L3D10 compared with mouse IgG. Taken together, anti-human CTLA-4 mAb conveys a survival advantage and delay of tumor growth in human CTLA-4 (+/-) mice. To determine if anti-human CTLA-4 mAb promoted the expansion of tumor-specific T cells, spleen cells from mice in both groups, including those that had rejected established tumors, were analyzed for the presence of P1A-specific CD8 T cells. No difference in numbers of P1A-specific cells, as determined by staining with H-2Ld:P1A dimer, was observed in either anti-human CTLA-4 mAb or mouse IgG treated mice (data not shown). It is possible that costimulation is required at the site of T cell:tumor cell interaction to elicit a detectable P1A-specific T cell response. To test if this was the case, huCTLA-4(+/-) x BALB/c F1 mice were inoculated with either J558Neo or J558B7 and treated with L3D10 or mouse IgG. At 16 days when tumors were a small size or not palpable, mice were sacrificed and spleens were analyzed for P1A-specific CD8 T cells. Again, no difference in P1A-specific CD8 T cell numbers was seen between any group (data not shown).

The second tumor model that we tested utilized the MC38 colon carcinoma. Heterozygous human CTLA-4(+/-) mice backcrossed into the C57BL/6j background for 5 generations where challenged subcutaneously with 5x10^5 MC38 cells. Mice were subsequently treated with 500 µg anti-human CTLA-4 mAb L3D10 or mouse IgG on days 2, 9, and 16 after tumor challenge, and 200 µg anti-4-1BB mAb 2A on day 20. Treatment with L3D10 and 2A mediated a delay in tumor growth in 3 of 8 mice compared with 0 of 8 mice treated with mouse IgG and 2A (Figure 4.6).
Adoptive immunotherapy with huCTLA-(+/-) P1CTL or huCTLA-4(+/+) P1CTL and anti-human CTLA-4 mAb.

Treatment with anti-CTLA-4 mAb on the same day of tumor challenge represents a minimal disease or prevention model. To test the effect of anti-human CTLA-4 mAb in a therapeutic setting, we employed an adoptive transfer model to treat established tumors (Figure 4.7). RAG2(-/-) mice were challenged with J558Neo cells and tumors were permitted to grow for 9 days to sizes ranging from 4 to 12.5 mm in mean diameter. At day 9, 4.6x10^6 spleen and lymph node cells from a heterozygous huCTLA-4(+/-) x P1CTL or homozygous huCTLA-4(+/+) x P1CTL F1 mouse were transferred along with 200 µg L3D10 or mouse IgG. Additional antibody treatments were given at days 11, 13, 23, and 32. All mice received 100 µg anti-4-1BB mAb on day 32. As observed with our wildtype P1CTL mice, all tumors exhibited some degree of regression around one week following P1CTL transfer. However, tumors in all groups eventually regressed at similar rates, indicating that anti-human CTLA-4 mAb therapy in this setting did not provide a detectable improvement in the rejection of tumor or survival of tumor-bearing mice.

Despite a lack of effect on tumor growth, we analyzed peripheral blood to determine if anti-human CTLA-4 mAb mediated any change in levels of transgenic tumor-specific T cells. Both CD8 and CD4 T cells were analyzed from peripheral blood of all mice at days 14, 23, and 31 after adoptive transfer (Figure 4.8). In both homozygous and heterozygous huCTLA-4 P1CTL recipients, CD4 T cells were found in higher numbers than CD8 T cells in the peripheral blood at all time points. However, no
statistically significant differences were observed between L3D10-treated and control mice, except for a modest significant difference between CD4 T cell levels at day 23 in huCTLA-4(+/+) P1CTL recipients.

**Effect of anti-human CTLA-4 mAb in the absence of B7 molecules.**

The mechanism of action of anti-CTLA-4 mAb remains controversial. While many groups consider this antibody to mediate a “blockade” of CTLA-4/B7 interaction, data from our lab suggests that CTLA-4 may have agonist function. Consequently, monoclonal antibodies raised against CTLA-4 may possess agonist properties that could mimic the natural CTLA-4/B7 interaction. To test this possibility using our huCTLA-4 knock-in mice, C57BL/6j huCTLA-4(+/-) mice were crossed for two generations with C57BL/6j B7-1(-/-) B7-2(-/-) double knockout mice to produce offspring of the genotype huCTLA-4(+/-) B7(-/-). To confirm that human CTLA-4 molecules could be functionally expressed in the absence of B7, we activated spleen cells from huCTLA-4(+/-) B7(-/-), huCTLA-4(+/-) B7(+/-), huCTLA-4(-/-) B7(+/-) with anti-CD3 mAb and stained for the presence of intracellular human and mouse CTLA-4. As shown in Figure 4.9, huCTLA-4(+/-) B7(-/-) are capable of expressing human CTLA-4 in the absence of B7 costimulation, although the level of CTLA-4 was lower than what was observed in the presence of B7. This is consistent with previous reports that suggest that expression of CD28 is required for optimal expression of CTLA-4 [28, 200], although CTLA-4 can be expressed in the absence of CD28 [47].

These mice were used to test the effect the anti-human CTLA-4 mAb in the absence of any natural ligand for CTLA-4. Any effect observed would therefore be due to direct agonism of the antibody via CTLA-4, rather than a blockade of CTLA-4/B7
interaction. HuCTLA-4(+/-) B7(-/-) mice were challenged with $5 \times 10^5$ MC38 cells and treated with 500 µg anti-human CTLA-4 mAb L3D10 or mouse IgG on days 2, 9, and 16 after tumor challenge, and 200 µg anti-4-1BB mAb 2A on day 20. In the absence of B7 molecules, mice treated with L3D10 (n=9) or mouse IgG (n=8) grew tumors at similar rates and 100% incidence (Figure 4.10).

4.5 Discussion

The manipulation of costimulatory pathways using monoclonal antibodies has proved valuable to the dissection of the role of these molecules in immune responses. Much of this investigation by our lab and others has been directed towards the strategy of using mAbs targeting costimulatory molecules such as CTLA-4 [82-91], 4-1BB [64, 97, 100], and OX-40 [201, 202] to enhance anti-tumor immunity. Only one monoclonal antibody targeting human CTLA-4 has been translated into clinical trials of cancer patients [94, 95], and this showed promising anti-tumor effects, but also severe toxicities. We have produced a panel of monoclonal antibodies targeting human CTLA-4, and have concurrently developed a novel humanized mouse model with which to screen these mAbs for clinical translation. Here we describe the creation of a mouse in which the exons encoding the extracellular and transmembrane domains of mouse CTLA-4 have been replaced by the corresponding exons from the human CTLA-4 gene. This mouse expresses human CTLA-4 molecules upon activation of its T cell receptor, both at the RNA and protein levels. With identical cytoplasmic domains, it is reasonable to assume that the mouse and human CTLA-4 molecules will transduce signals in a similar, if not identical manner. In this study, we have chosen to focus on a single clone (L3D10) of
anti-human CTLA-4 mAb to demonstrate the use of this mouse model for the preclinical screening of effective anti-tumor agents. Using two different tumor models (plasmacytoma and colon carcinoma) from two different backgrounds (BALB/c and C57BL/6j), we have demonstrated an enhancement of anti-tumor immunity and prolonged survival in mice containing a single copy of the human CTLA-4 gene.

The mechanism of action of anti-CTLA-4 mAb in tumor therapy has traditionally been viewed as a “blockade” since its initial description [82]. In all studies using anti-mouse CTLA-4 mAb, a theoretically complete blockade of CTLA-4 molecules could occur. It is unknown whether a complete blockade of CTLA-4 is actually required to induce anti-tumor immunity, or whether a partial blockade might be sufficient to induce a response. Alternatively, if anti-CTLA-4 mAb can mediate agonist effects, it could be argued that partial engagement of CTLA-4 may be sufficient to elicit detectable enhancement of immunity. Our humanized CTLA-4 mouse model provides a vehicle with which to test the requirements of complete versus partial CTLA-4 blockade or engagement in anti-tumor immunity. The heterozygous human CTLA-4 mice which we use in this study express both mouse and human CTLA-4 molecules upon stimulation, as shown in Figure 4.4. Furthermore, the expression is co-dominant and both molecules are present on the same cells, which indicates that the expression of human and mouse CTLA-4 are regulated in the same manner. It is unknown how mouse and human CTLA-4 molecules interact with each other in our humanized mouse model, since both mouse [203] and human [204, 205] CTLA-4 molecules have been shown to exist as homodimers at the T cell surface. However, it is reasonable to assume that treatment
with anti-human CTLA-4 mAb as a single agent will not engage all CTLA-4 molecules on the T cell surface in mice expressing both human and mouse proteins.

The anti-tumor effects we observed in several tumor models provide support for the idea that incomplete blockade or partial agonist activity provided by use of anti-human CTLA4 mAb alone is sufficient to elicit anti-tumor immunity. In the J558 and MC38 tumor models we demonstrated that engaging only some of the cell surface CTLA-4 was necessary to show enhanced rejection or delayed growth of tumor. One may speculate that engaging all CTLA-4 molecules, using either a combination of anti-mouse and anti-human CTLA-4 mAbs, or by testing our anti-human CTLA-4 mAb in human CTLA-4 homozygous mice, would further potentiate the anti-tumor effects observed in this study. The lack of effect of anti-human CTLA-4 mAb therapy with adoptive transfer of tumor-specific T cells (P1CTL) cannot be explained by lack of complete engagement of CTLA-4, as mAb was tested with both homozygous and heterozygous human CTLA-4 P1CTL. Of course, large tumor burden provides a much more difficult challenge for immunotherapeutic interventions to overcome. Due to incomplete backcross at the time the experiments were performed, the necessity of using only a single P1CTL donor mouse limited the number of T cells we were able to transfer. This T cell dose may have been below the threshold necessary to mediate complete rejection of tumor. However, in our experience with adoptive immunotherapy of established tumors using P1CTL, the addition of anti-mouse CTLA-4 mAb (clone 4F10) has never provided substantial benefit (unpublished observation) compared with the striking results we observed with addition of anti-mouse 4-1BB mAb [64].
The use of B7-deficient human CTLA-4(+/−) mice provided an opportunity to test the mechanism of action of anti-human CTLA-4 mAb. A demonstration of enhanced anti-tumor immunity in the group treated with mAb would provide evidence that our anti-human CTLA-4 mAb could act as an agonist, since no blockade could occur in the absence of B7. As it stands, the lack of difference with anti-human CTLA-4 mAb suggests two possibilities. One is that anti-CTLA-4 mAb provides a blockade of CTLA-4/B7 interaction, and in the absence of B7 has no effect. Another possibility that remains to be tested is that the anti-tumor immune response is so blunted in B7-deficient mice that any effect of anti-CTLA-4 mAb is undetectable. It is well-known that B7-deficient mice have dampened immune responses [20], and our data shows that MC38 tumors grew much faster in B7-deficient mice compared with their B7+ littermates (Figures 4.6 and 4.10). The addition of anti-CD28 mAb to this model may boost the immune response sufficiently to overcome B7-deficiency and allow for a more fair test of the mechanism of action of anti-CTLA-4 mAb in the absence of B7.

Another final point of interest is that the application of our novel human CTLA-4 knock-in mouse need not be limited to studies of tumor immunology. CTLA-4/B7 costimulation has been shown to play an important role in the development of autoimmune diseases, such as experimental autoimmune encephalomyelitis (EAE) and diabetes [206]. Studies using anti-CTLA-4 mAb during EAE induction have demonstrated accelerated and exacerbated disease progression in EAE-susceptible mouse strains [207, 208], and increased induction of disease in EAE-resistant strains [209]. In a transgenic mouse model of diabetes, anti-CTLA-4 mAb induced a much more aggressive insulitis and earlier onset of diabetes [210]. Polymorphisms in the human CTLA-4 gene
have been associated with increased susceptibility to autoimmune disease in humans [211-215]. Our humanized CTLA-4 mouse model provides a means to extend many of these immunological observations made with mouse CTLA-4 in the setting of autoimmunity to the human CTLA-4 molecule, as well as to test clinical therapies targeting the human CTLA-4/B7 costimulatory pathway in autoimmune disease. In conclusion, we have developed a novel mouse model expressing a human costimulatory molecule, CTLA-4, that is critical to proper immune function. This model affords the opportunity for preclinical screening of therapies for human patients, as demonstrated here with the enhancement of anti-tumor immunity using anti-human CTLA-4 mAb, and for further elucidation of the mechanism of action of this molecule.
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<tr>
<th>Genotype</th>
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<tr>
<td>huCTLA-4(-/-)</td>
<td>7</td>
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<tr>
<td>huCTLA-4(+/-)</td>
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<td>huCTLA-4(+/+)</td>
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Table 4.1  Genotypic distribution of offspring from breeding of heterozygous human CTLA-4 knock-in mice.
Figure 4.1  Creation of a human CTLA-4 knock-in DNA construct.  a) Schematic diagram of structure of construct.  Exons 2 and 3 of the mouse CTLA-4 gene are replaced with exons 2 and 3 of the human CTLA-4 gene, plus a loxP-flanked Neo/TK sequence used for selection purposes.  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 the intact mouse CTLA-4 gene (no recombination).
Figure 4.2  
**Intact expression of the uninterrupted human CTLA-4 gene in transfected ES cells.** a) Excision of Neo/TK by Cre-recombinase. ES cells were transiently transfected with pCre/Pac plasmid to excise Neo/TK gene via Cre-recombinase-mediated excision of DNA sequence between flanking loxP sites shown in Figure 4.1. To confirm excision, DNA from ES cells underwent PCR followed by Southern blot. DNA from PCR Reaction D used primers outside the loxP sites spanning the Neo/TK gene. A successful excision of Neo/TK (deleted) produced a 1.1 kb fragment, while undeleted Neo/TK (floxed) did not produce a fragment due to PCR conditions used. PCR Reaction F used a forward primer outside of and a reverse primer within the Neo/TK gene. Successful excision of Neo/TK (deleted) produced no fragment, as the site of reverse primer hybridization is eliminated. Unsuccessful deletion of Neo/TK (floxed) produced a 1.3 kb fragment. 7 of 8 ES cell clones screened showed complete excision of Neo/TK while one clone (7.2) showed some fraction of cells retaining Neo/TK. b) Expression of intact human CTLA-4 RNA by ES cells as detected by RT-PCR followed by Southern blot of RNA prepared from ES cells clones shown in a). RT-PCR Reaction M used a forward primer on mouse CTLA-4 exon 2 and a reverse primer on exon 4 to amplify a 345 bp fragment, identifying the expression of mouse CTLA-4 RNA. RT-PCR Reaction H used a forward primer on human CTLA-4 exon 2 and the same reverse primer on mouse CTLA-4 exon 4 to amplify a 455 bp fragment, identifying the expression of the knock-in sequence. Most clones also expressed a 354 bp spliced variant of the knock-in gene. Clone 2 shows expression of both human and mouse CTLA-4 RNA. The RT-PCR reactions are specific as no fragments were amplified from RNA prepared from mouse thymus (Mo Thy).
Figure 4.2
Figure 4.3  Expression of human and mouse CTLA-4 RNA in heterozygous knock-in mice. Spleen cells from human CTLA-4(+-) x P1CTL F1 mice were stimulated for 24 hours in vitro with 0.1 µg/mL P1A peptide. 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 unspliced RNA of the knock-in gene was being expressed (left reaction). The same set of primers detailed in Figure 4.2b were used to amplify mouse (middle reaction) or human CTLA-4 RNA (right reaction). Expression of both mouse and human CTLA-4 genes in the same cells suggests a co-dominant expression pattern.
Figure 4.4 Co-dominant expression of human and mouse CTLA-4 protein by T cells from heterozygous human CTLA-4 knock-in mice. Spleen cells from human CTLA-4(+/-) \times P1CTL F1 mice were stimulated for 66 hours in vitro with 0.1 \mu g/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, compared with a complete lack of human CTLA-4 expression by non-knock-in littermates (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 stimulation of T cells.
Figure 4.5  Anti-human CTLA-4 mAb promotes anti-tumor immunity against plasmacytoma J558. Human CTLA-4(+/−) x BALB/c F1 mice were subcutaneously challenged with 5x10⁶ J558 cells, and treated with 200 µg anti-human CTLA-4 mAb clone L3D10 or mouse IgG on days 0, 2, 6, and 23. Tumor sizes represent the mean of two perpendicular directions of tumor growth in each mouse. 3 of 8 mice treated with L3D10 mAb rejected tumor challenge compared with only 1 of 5 mice treated with mouse IgG. This experiment is representative of two independent experiments. In the second experiment, 2 of 5 mice treated with L3D10 mAb rejected tumor compared with 1 of 4 mice treated with mouse IgG.
Figure 4.6  Anti-human CTLA-4 mAb promotes anti-tumor immunity against MC38 colon carcinoma. Human CTLA-4(+-) mice backcrossed into the C57BL/6 strain for 5 generations were subcutaneously challenged with $5 \times 10^5$ MC38 cells, and treated with 500 µg anti-human CTLA-4 mAb L3D10 or mouse IgG on days 2, 9, and 16, and 200 µg anti-4-1BB mAb 2A on day 20 after tumor challenge. Treatment with L3D10 and 2A mediated a delay in tumor growth for 3 of 8 mice compared with treatment with mouse IgG and 2A. Lines represent tumor kinetics for individual mice.
Figure 4.7  Anti-human CTLA4 mAb does not enhance rejection of tumors by adoptively transferred tumor-specific T cells. BALB/c RAG-2(-/-) containing no endogenous T or B cells were challenged with 5x10⁶ plasmacytoma J558Neo cells. After tumors reached a mean tumor diameter of 9.2 mm by day 9, mice were given an adoptive transfer of 4.6x10⁶ homozygous huCTLA-4(+/+) or heterozygous huCTLA-4(+/-) P1CTL spleen and lymph node cells. Mice were treated with 200 µg anti-human CTLA-4 mAb clone L3D10 or mouse IgG on days 9, 11, 13, 23, and 32, plus 100 µg anti-mouse 4-1BB mAb on day 32.
Figure 4.8  Anti-human CTLA-4 mAb does not promote the expansion of tumor-specific T cells in vivo. Peripheral blood samples were taken from mice shown in Figure 4.7 on days 14, 23, and 31, and stained for CD8 and T cell receptor Vα8.3 to detect P1A-specific P1CTL. CD4 T cells were gated from the CD8 Vα8.3 region. Bars represent the mean percentage and SEM of CD8 and CD4 T cells within the peripheral blood lymphocyte gate from four mice per treatment group. Differences between CD8 and CD4 T cells from mouse IgG and L3D10-treated groups are not statistically significant, with one exception.
Figure 4.9  T cells from human CTLA-4(+/-) B7(-/-) mice are capable of expressing mouse and human CTLA-4 molecules. Spleen cells from huCTLA-4(+/-) B7(+/-), huCTLA-4(+/-) B7(-/-), and huCTLA-4(-/-) B7(+/-) mice were stimulated for 64 hours with anti-CD3 mAb in vitro. Cells were harvested and stained with for cell surface mouse CD3, followed by intracellular mouse and human CTLA-4. Similar to Figure 4.4, co-dominant expression of human and mouse CTLA-4 is observed in both B7(+/-) (top panel) and B7(-/-) (middle panel) human CTLA-4 heterozygous mice. Littermates without the human CTLA-4 gene show the expected lack of human CTLA-4 staining (bottom panel). Interestingly, B7(-/-) mice display lower levels of both mouse and human CTLA-4 protein compared with B7(+/-) mice.
Figure 4.10  Anti-human CTLA-4 mAb does not promote anti-tumor immunity against MC38 colon carcinoma in B7(-/-) mice. Human CTLA-4(+/−) mice deficient for B7-1 and B7-2 were subcutaneously challenged with $5 \times 10^5$ MC38 cells, and treated with 500 µg anti-human CTLA-4 mAb L3D10 or mouse IgG on days 2, 9, and 16, and 200 µg anti-4-1BB mAb 2A on day 20 after tumor challenge. Lines represent tumor kinetics for individual mice.
CHAPTER 5

SPONTANEOUS ALOPECIA, LYMPHADENOPATHY, AND SKIN DISEASE IN B7-DEFICIENT MICE

5.1 Abstract

The role of the costimulatory molecules B7-1 and B7-2 in the induction and effector function of a T cell response is well established. Paradoxically, mice with targeted mutations of CD28, and B7-2 were reported to have exacerbated autoimmune disease in non-obese diabetic mice that are prone to autoimmunity. In this study, we report that targeted mutations of B7-1 and B7-2 were associated with spontaneous alopecia and dermatitis in C57BL/6j mice that are otherwise not known to have a predisposition to autoimmune disease. The alopecia and dermatitis were significantly more pronounced in female mice at an earlier age. Development of skin disease was associated with lymphoproliferation and lymphocyte activation despite the fact that the mice lack the major costimulatory molecules. Adoptive transfer of the spleen cells from the B7-1/2-deficient mice to B7-1/2(+/+)RAG1(-/-) recipients induced a broad spectrum of autoimmune inflammation, and the pathogenesis is mediated primarily by CD4 T cells.
Thus, targeted mutation of B7-1 and B7-2 increased the production of autoreactive T cells that are pathogenic both in the presence and absence of costimulatory molecules B7-1 and B7-2.

5.2 Introduction

It is well established that costimulatory molecules, of which B7-1 and B7-2 are prototypes, play an important role in the induction and effector function of T cell responses [4, 79, 216]. Further studies demonstrate that CD28 co-localizes with the TCR in the super-molecular activation complex and serves to reduce the threshold for T cell activation [14-17]. Consistent with the important role for costimulatory molecules in the activation and effector function of T cells, blockade of B7-1 and B7-2 prevents rejection of transplants, and reduces the severity of autoimmune disease [111, 112, 217-219].

Paradoxically, mice with targeted mutation of components of the B7-1/2:CD28/CTLA-4 pathway show enhanced rather than reduced risk of autoimmunity. While this is most drastic in mice with targeted mutations of CTLA-4 [32, 33], those that have mutations of CD28 also show significantly higher risk of autoimmunity. In the non-obese diabetic (NOD) background, targeted mutations of CD28 drastically accelerated the onset of diabetes and erased the gender difference between male and female mice [113]. Interestingly, in NOD mice with a targeted mutation of B7-2, polyneuropathy rather than type I diabetes developed [115]. It is unclear whether targeted mutation of the B7 family members is sufficient to increase the risk of autoimmune disease in mice that are not prone to autoimmune disease.
In the process of studying mice with targeted mutations of B7-1 and B7-2, we observed a drastic increase of alopecia with regards to incidence, onset and severity in comparison to C57BL6/j mice. This is followed by lymphoadenopathy and the development of multifocal skin ulcerations with extensive epidermal hyperplasia, mainly among female mice.

5.3 Materials and Methods

Experimental animals

B7-1(-/-) B7-2(-/-) mice [20] and RAG-1(-/-) [220], which have been backcrossed to the C57BL/6j strain for at least 10 generations, were purchased from the Jackson Laboratory (Bar Harbor, ME). C57BL/6j mice were purchased from the Charles River Animal Facility (Wilmington, MA). All mice were maintained in the University Laboratory Animal Research Facility at the Ohio State University under specific pathogen-free conditions.

Flow cytometry

Cell surface markers, including CD4, CD8, CD25, CD44, and CD62L, and intracellular cytokines, including IFNγ, IL-4, and IL-10, were analyzed with conjugated mAbs purchased from BD PharMingen (San Diego, CA).

Adoptive transfer

20 x10^6 spleen cells from age-matched wild-type and B7-1(-/-) B7-2(-/-) mice were injected intraperitoneally into RAG-1(-/-) C57BL/6j mice. In other experiments, the CD4 or CD8 subsets of T cells were purified from spleens and lymph nodes of
B7-1(−/−) B7-2(−/−) mice by negative selection using a cocktail of antibodies that react to FcR, Mac-1, CD11c, B220, and either CD4, CD8, or both, followed by anti-Rat IgG coated magnetic Dynabeads (Dynal Biotech, Oslo, Norway), according to manufacturer’s instructions. 2x10^6 CD4 T cells, 3x10^6 CD8 T cells, or 5x10^6 CD4+CD8 T cells combined were injected intraperitoneally as above. Mice were sacrificed for histological analysis when clinical symptoms appeared.

**Intracellular cytokine production**

To assess intracellular cytokine production, spleen or lymph node cells were cultured for 4-6 hours with 50 ng/ml PMA, 500 ng/ml ionomycin, and 2 µM GolgiStop (BD Pharmingen). Cells were stained for cell surface markers CD4 and CD8 followed by intracellular staining for IFNγ and IL-4 using a CytoFix/CytoPerm kit (BD Pharmingen).

**Histological analysis**

Mouse organs were fixed with 10% buffered formalin and paraffin-embedded. Tissue sections were stained with hematoxylin and eosin (H & E), and examined under a microscope. PCNA, CD4, and CD8 stains were carried out using tissue frozen with OCT. PCNA staining kit was purchased from Zymed Laboratories, Inc. (San Francisco, CA).
5.3 Results

Mice with targeted mutation of B7-1 and B7-2 develop whole body hair loss and skin disease

In the process of studying mice with targeted mutations of B7-1 and B7-2, we observed a high incidence of cutaneous abnormalities. Interestingly, the skin abnormalities were observed primarily among the female mice. As shown in Figure 5.1a and b (upper panel), among the female mice, whole-body alopecia was first observed at around two to three months of age. By the sixth month, an overwhelming majority of the female mice demonstrated whole-body hair loss (33 of 51), while only one of 57 male mice showed significant hair loss. After more than one year, male mice began to exhibit whole body hair loss at an increased frequency. It should be emphasized that the hair loss observed in female mice differed from classic alopecia observed in wild-type C57BL/6j mice both in terms of the incidence (usually less than 0.9%) (http://jaxmice.jax.org/) and pattern (whole body loss with sparse hair in the B7-deficient mice versus patchy hair-loss in wild-type mice). Histologically, the hair loss observed in the B7-deficient mice was consistent with non-scarring alopecia, exhibiting miniaturization of the hair follicles. Hairshafts were dystrophic with incomplete keratinization and nuclear retention in the inner root sheath (data not shown).

Subsequent to the hair loss, mice often developed severe skin lesions (Figure 5.1a and b lower panel). Again, this was observed predominantly among the B7(-/-) female mice, but rarely in the male littermates (Figure 5.1c) until after one year of age. Grossly, there were two types of skin lesions. One was characterized by progressively expanding sharply demarcated ulcers caused by external trauma from scratching, while other lesions
were erythematous scaly plaques with an intact hyperplastic epidermal surface. Compared to normal skin shown in Figure 5.2a, histological analysis of the scaly erythematous plaques (Figure 5.2b) revealed several features consistent with a psoriaform dermatitis. There was marked attenuation of the suprapapillary plates and elongation of the rete ridges. The epidermis was surmounted by a neutrophil-imbued parakeratotic scale (Figure 5.2b). In addition, there were foci of spongiform pustulation as manifested by neutrophils permeating slightly widened intercellular spaces of the spinous layer of the epidermis. The dermal papillae capillaries were dilated, lying in intimate apposition to the suprapapillary plates (Figure 5.2c).

Phenotypic studies of the lymphoid populace revealed a dearth of CD8 lymphocytes (data not shown), while the majority of lymphoid forms were of CD4 phenotype manifesting dermal localization (Figure 5.2d), compared with few detectable T cells in skin from healthy wild-type C57BL/6j mice. Moreover, staining for proliferating cell nuclear antigen (PCNA) revealed prominent staining of the suprabasilar spinous layer although largely confined to the lower third of the epidermis in B7(-/-) mice (Figure 5.2e, bottom panel). In contrast, the healthy wild-type controls showed PCNA staining exclusively confined to the basal layer of the epidermis (Figure 5.2e, top panel).

Internal organs from B7-deficient mice with alopecia and dermatitis, including lung, liver, pancreas, intestine, salivary glands, heart, and kidney, showed no evidence of pathology. This indicates that the pathology associated with absence of B7 molecules is restricted to the skin.
Lymphoproliferation preceding the development of skin lesions.

We observed a progressive enlargement of spleen and lymph nodes that preceded the development of the skin lesions. In mice with skin lesions, there was more than a 30-fold increase in the cellularity of lymph nodes (with a corresponding increase of size of the lymph nodes) (Figure 5.3a). Both spleens and lymph nodes displayed a remarkable increase among various subsets of myeloid cells with increased forward and side scatter, including GR-1^''^F4/80^{low} neutrophils, and F4/80^{high} macrophages with various levels of GR-1 (Figure 5.3b).

In moribund female B7-deficient mice, the overall activation of CD4 and CD8 T cells, as judged by their cell surface expression of CD44 and CD62L, was comparable to wild-type mice. The lymphocytes from age-matched B7-deficient male mice had somewhat lower levels of CD44 expression, which could be expected in healthy mice with impaired costimulatory pathways (Figure 5.4a). Additionally, the ratio of CD8 to CD4 T cells was increased in enlarged lymph nodes in B7-deficient mice with skin disease (data not shown). To measure the amount of antigen-reactive cells and their cytokine expression profiles, spleen cells from diseased B7(-/-) mice and healthy wild-type mice were stimulated with ionomycin and PMA. As shown in Figure 5.4b, despite lack of B7-1 and B7-2, there were high numbers of cytokine-producing cells in female B7-deficient mice. Approximately 3% of CD4 T cells from both wild-type and B7-deficient mice produce IL-4. CD8 T cells from neither group produced IL-4 upon stimulation by ionomycin and PMA, while nearly 60% of CD8 T cells rapidly synthesized IFN\gamma. There were, however, 2-3 fold more CD4 T cells in the wild-type
mice than those in the B7-deficient mice that were programmed to synthesize IFN$\gamma$. This difference is not surprising as B7-1 and B7-2 can promote CD4 T cell responses to environmental antigens.

To determine if the autoimmune phenomena observed in B7-deficient mice are caused by increased production of auto-antibodies, serum from healthy female C57BL/6j mice, healthy male B7-deficient mice, and diseased female B7-deficient mice was tested for the presence of anti-double strand DNA antibodies by ELISA. Both diseased and healthy B7-deficient mice exhibited decreased amounts of anti-double strand DNA antibodies compared to healthy wild-type mice (data not shown).

**T cells from B7-1(-/-) B7-2(-/-) female mice with autoimmune pathology induce multi-organ inflammation in RAG-1-deficient mice with normal B7-1 and B7-2 genes**

To evaluate the immunological basis of the autoimmune disease observed in our B7-deficient mice, we isolated and transferred spleen cells from diseased B7-deficient and sex-matched C57BL/6j mice into RAG-1(-/-) B7(+/+) C57BL6/j recipients. At about 7 weeks after the adoptive transfer when some recipients started to show clinical signs of illness, including weight loss, hair-loss, and wobbly-gait, all recipient mice were sacrificed for immunological and histological analyses. With the exception of sporadic low-grade hair loss, which is seen occasionally in RAG-1(-/-) C57BL6/j mice that received no adoptive transfer, the recipients of the normal spleen cells show no clinical signs during the entire 7 week period observed.

In comparison to the spleen cells prior to the adoptive transfer (Figure 5.5a), T cells from both wild-type and B7-deficient mice underwent further activation after
adoptive transfer, as demonstrated by the increase of CD44^{+}CD62L^{+} and CD44^{+}CD62L^{-} T cells (Fig. 5.5b). However, the CD8 T cells from B7-deficient donors underwent more extensive activation as judged by the increased accumulation of CD44^{+}CD62L^{-} T cells. We also compared T cells recovered from recipients for their cytokine production after short-term stimulation in vitro. As shown in Figure 5.5c, the CD4 T cells from wild-type and B7-deficient donors consisted of comparable proportions of IFN\(\gamma\)-producing cells. However, a high proportion of CD4 T cells from B7-deficient mice gained the ability to produce IL-4.

Given the signs of morbidity observed in several mice, we examined whether internal organs were affected by the transferred spleen cells. As shown in Figure 5.6 and Table 5.1, mice that received spleen cells from wild-type mice had essentially no inflammation in any organs examined, including lung, liver, kidney, pancreas, heart, salivary glands, spinal cord, and intestine. In contrast, in mice that received spleen cells from B7-1(-/-) B7-2(-/-) mice, extensive inflammation was found in lung, liver, pancreas, and intestine, although no inflammation was observed in heart, salivary gland, or spinal cord tissues (data not shown). In the lung, there was an accumulation of large numbers of mononuclear cells, including lymphocytes and macrophages surrounding blood vessels and bronchi with variable permeation of the interalveolar septae. In the liver, there was mononuclear cell infiltration in the portal tracts, and also surrounding the central venules, accompanied by piecemeal necrosis. The pancreas showed interstitial and islet lymphohistiocytic inflammation surrounding blood vessels and secretory ducts. The inflammation within the small intestine closely simulated those changes found in autoimmune colitis, namely transmural inflammation comprised of lymphocytes and
neutrophils with foci of crypt abscess formation. Skin from recipients of B7(-/-) cells had more extensive epidermal hyperplasia and increased numbers of inflammatory cells than the skin from recipients of wild-type cells (Table 5.1). These recipient mice did not display overt skin lesions as seen in the donor mice, most likely due to the fact that clinical signs of morbidity prevented us from extending our observation period to a length of time required for more advanced skin disease to manifest.

**CD4 T cells are primarily responsible for multi-organ inflammation.**

To determine the subset of T cells involved in the development of multi-organ inflammation, CD4 and CD8 T cells isolated from either wild-type mice or diseased B7-deficient mice were transferred into RAG-1(-/-) B7(+/+) mice. At 7 weeks after the adoptive transfer when some of the recipient mice became moribund, all mice were sacrificed and spleen and other organs were harvested. Despite the potential for homeostatic proliferation, mice that received CD4 T cells alone had very few CD8 T cells, and vice versa. These results verified the purity of the T cells used for the adoptive transfer (Figure 5.7a). Moreover, compared to the phenotype of the starting populations (Figure 5.5a), both CD4 and CD8 T cell subsets had undergone further activation in the recipients as revealed by the increase of the CD44+CD62L- population regardless of whether the two subsets were co-transferred (Figure 5.7b). Likewise, cytokine synthesis after short-term in vitro culture was not affected by co-transfer of the two subsets (Figure 5.7c). Interestingly, only the group that received CD8 T cells exhibited pronounced lymphadenopathy (data not shown). These results demonstrated that CD8 T cells can be activated regardless of the presence of CD4 T cells, and vice versa.
Histologically, mice that received CD4 T cells alone and CD4+CD8 T cells developed inflammation in multiple organs, including lung, liver, pancreas, and intestine. The severity and pattern of inflammation were comparable between the two groups (Figure 5.8 and Table 5.1). In contrast, despite their enhanced activation phenotype (Figure 5.7b), the CD8 T cell subset alone induced very limited inflammation in the RAG-1(-/-) recipients with notable absence of inflammation in the pancreas and intestine (Figure 5.8 and Table 5.1). The inflammation in the liver was very focal. These results demonstrate that the inflammation is mediated primarily by CD4 T cells, although some inflammation can be found in the group that received CD8 T cells alone.

### 5.5 Discussion

In a variety of experimental autoimmune models, blockade of B7-1 and B7-2 can prevent the development and progression of spontaneous autoimmune disease [206]. Likewise, targeted mutation of B7-1 and B7-2 prevents the induction of experimental autoimmune encephalomyelitis [22]. These studies established that in mice with ongoing autoimmune disease, costimulatory molecules act as positive regulators. A largely unexpected observation in the study of the costimulatory molecules is the apparent predisposition of mice with defective costimulatory pathways to develop autoimmune diseases. While mice with a targeted mutation of CTLA-4 are the most prominent example [32, 33], those with mutations in B7-2 and CD28 are also found to increase the risk and types of autoimmune diseases, respectively [113, 115]. Thus, our observation reported here may reflect a rule rather than an exception. However, it is worth pointing out that even in a larger context, there are several notable features of our observation.
First, the autoimmune disease was observed in the C57BL/6j background, which is not known to be prone to autoimmune diseases. Second, this alopecia/skin disease model recapitulates a major feature of autoimmunity, namely, the substantially higher risk of autoimmune disease in females compared to males [221]. Since conversion from a resistant (C57BL6/j) to a susceptible strain is due to the mutation of two related genes, this model offers some advantage in studying the gender bias in autoimmunity.

There are several mechanisms, which are not mutually exclusive, that can be proposed to explain these paradoxical observations. First, since costimulatory molecules enhance immunity, mice with defective costimulatory molecules may have higher levels of infectious insult, which has been suggested as an important environmental factor for the development of autoimmunity in general [222, 223], and psoriatic skin disease in particular [224-227]. In this regard, it is worth pointing out that although B7-deficient mice have been reported [20] and widely used for several years, a severe skin disease, as described in the current study, has not been reported. This can be due to several factors, including environmental factors in our animal facility, the length of observation, and number of mice observed. It should be emphasized that in our specific pathogen-free facility housing multiple strains of immunodeficient mice, this phenotype of such intensity is unique to mice with the B7-deficiency. Therefore, environmental factors, if any, must work in conjunction with B7-deficiency to cause autoimmune disease as described here. Our communication with the Jackson Laboratory from which these mice were obtained suggests that the B7-deficient mice there also seem to have skin defects, although they have not carried out a systematic analysis on the issue.
Secondly, we have recently reported an important role for costimulatory molecules in the clonal deletion of highly pathogenic autoreactive T cells [122]. It is therefore likely that mice with targeted mutations of B7-1 and B7-2 will have an increased burden of autoreactive T cells, some of which may contribute to the development of skin disease as reported here. Our data demonstrated that both CD4 and CD8 T cells from B7-deficient mice are pathogenic upon adoptive transfer into B7(+/+) RAG1(-/-) mice. Although some contribution of homeostatic proliferation to the pathogenesis mediated by the adoptively transferred T cells cannot be ruled out, such proliferation is insufficient to cause the multiple organ inflammation documented here, as spleen cells from wild-type mice did not cause significant pathology.

Thirdly, CD4^+CD25^+ regulatory T cells (Treg) appear to play a major role in self-nonself discrimination. Treg are produced in the thymus, possibly resulting from exposure to self antigen [131]. A number of studies suggest that CD28 and CTLA-4 may be involved in the function and/or the development of Treg [113, 133-135, 228]. It has been shown that mice with targeted mutations of B7-1 and B7-2 have decreased numbers of CD4^+CD25^+ T cells in the spleen [113, 132]. Our work shows that Treg numbers are also decreased in the thymuses of B7-deficient mice (Chapter 6). In addition to serving as a survival signal in the periphery, as suggested by Lohr et al. [132], B7 may play its most critical role in the thymic development of Treg, as the difference in the number of Treg produced in the thymus is sufficient to account for all reduction observed in Treg numbers in the spleen. The reduced number of Treg and an increased burden of autoreactive T cells may explain the lymphoproliferation and spontaneous skin lesions observed in our B7-deficient mice.
Taken together, we have demonstrated that mice with targeted mutations of B7-1 and B7-2 in the germ-line developed alopecia, lymphadenopathy, and skin disease. By enhancing signal transduction of the TCR, B7-1 and B7-2 may have opposite roles in the induction of autoimmunity depending on the stage of TCR engagement. During T cell development, costimulation can result in more thorough clonal deletion [122] and production of Treg [113], thus lowering the risk of autoimmunity. On the other hand, if autoreactive T cells escape clonal deletion and are undergoing activation by self antigen, costimulation may exacerbate autoimmunity. It is perhaps too simplistic to completely attribute exacerbated autoimmunity to a negative regulatory function of the B7 molecules and their receptors.
**Table 5.1 Multi-organ inflammation induced by mononuclear cells from B7(-/-) mice.** Mononuclear spleen cells or purified T cells from diseased B7(-/-) mice were transferred into RAG-1(-/-) B7(+/+) recipients, which were sacrificed at 7 weeks after adoptive transfer. Mouse tissues were fixed in 10% formalin and examined for inflammation after H&E staining. - = no infiltrates; + = 1-3 inflammatory aggregates; ++ = 4-6 inflammatory aggregates; +++ = >6 inflammatory aggregates, or a diffuse pattern of inflammation covering more than 50% of the organ section.

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Figure 5.1 Female B7(-/-) mice display progressive alopecia and development of skin lesions.  a) Gross comparison of age-matched male and female B7(-/-) (B7KO) mice.  b) Male (M) and female (F) B7(-/-) mice were observed for severe hair loss and development of skin lesions.  Graph represents cumulative incidence over time observed.  Mice with localized or no hair loss were considered negative for hair loss and skin disease at time points before initial observation.  Mice exhibiting severe hair loss and skin disease at any time during observation period were counted as positive for the remainder of the observation period.  Cumulative incidence was calculated retrospectively from 36-77 mice per sex during the 0 to 52 week observation period, and 14-64 mice during the 52 to 76 week observation period.  c) Gross comparison of age-matched B7(-/-) female and male mice with wild-type (WT) C57BL/6j female mouse.
Figure 5.1
Figure 5.2  B7(-/-) mice with skin lesions exhibit a psoriasis-like dermatitis. H&E stain of healthy skin from a wild-type (WT) C57BL/6j mouse (a), showing a thin epidermis, and diseased skin from a B7(-/-) (KO) mouse (b), showing several characteristic features of psoriasis, including epidermal hyperplasia with rete ridge elongation, suprapapillary plate attenuation, and neutrophil-imbued parakeratosis. c) The dermal papillae capillary (arrow) is dilated and lying in intimate apposition to the basal layer of the epidermis, defining the classic morphology of the "psoriatic" capillary, in skin from a diseased female B7(-/-) mouse. d) Immunohistochemical stain for H&E and CD4, showing increased numbers of CD4 T cells within the dermis of B7(-/-) mice (KO) compared with wild-type (WT) mice. Few or no CD8 T cells were observed. e) Immunohistochemical stain for PCNA in healthy skin from a wild-type mouse showing single layer of proliferating cells restricted to the basal layer of epidermis (top panel); and in diseased skin from a B7(-/-) mouse showing increased proliferation of keratinocytes involving suprabasilar epidermis (bottom panel).
Figure 5.2
Figure 5.3  Splenomegaly and lymphadenopathy in B7(-/-) female mice with skin disease. a) Representative spleen and lymph nodes (left panel). Columns represent average cell number per lymph node of four matched lymph nodes per mouse (n=3) (right panel). Error bars represent SEM. P-values were determined using two-sample t-tests. b) Increased percentage of cells from granulocyte and monocyte lineages in spleens and lymph nodes of diseased B7(-/-) mice (KO) compared with healthy wild-type mice (WT).
Figure 5.4  Activation phenotype and cytokine production by T cells from B7(-/-) mice.  a) Activation phenotype of T cells from spleen and lymph nodes from healthy C57BL/6 female (WT F) and B7(-/-) male (B7KO M) mice, and a B7(-/-) female mouse with skin disease (B7KO F).  b) A significant proportion of T cells from B7(-/-) mice produce cytokines upon short-term stimulation in vitro.  Spleen cells from female C57BL/6 (WT) and B7(-/-) (KO) mice were stimulated in vitro with PMA and ionomycin for 6 hours and stained for intracellular IFNγ and IL-4.  Solid lines in histogram depict stain with cytokine-specific monoclonal antibody while dotted lines represent isotype controls.  The numbers in the panels are % of cells within the gate after subtracting those stained by isotype control.
Figure 5.4

a. B6 F

b. B7KO M

CD4 CD8

Spleen

Lymph node

WT

KO

Intracellular cytokine

Cell No.

γ

IFN
Figure 5.5  Enhanced activation of B7(-/-) spleen cells after transfer to RAG-1(-/-) B7(+/+) recipients. Cell surface staining of spleen cells from healthy wild-type (WT) and diseased B7(-/-) (KO) mice before (a) and after (b) adoptive transfer into RAG-1(-/-) B7(+/+) recipients. Both CD4 and CD8 subsets display increased activation after adoptive transfer, as indicated by the CD44^CD62L^ population. However, CD8 T cells from recipients of B7(-/-) spleen cells have undergone more activation than CD8 T cells from recipients receiving wild-type spleen cells. c) Spleen cells from recipient RAG-1(-/-) mice were stimulated in vitro with PMA and ionomycin for 4 hours and stained for intracellular cytokines. The numbers in the panels are % of cells within the gate after subtracting those stained by isotype control.
Figure 5.6  **Spleen cells from diseased B7(-/-) mice cause multi-organ inflammation.**  20x10^6 spleen cells from diseased B7(-/-) mice (KO) or healthy wild type mice (WT) were transferred into RAG-1(-/-) B7(+/+) recipients. Seven weeks after transfer when mice began to demonstrate clinical symptoms, all mice were sacrificed. Organs were H&E-stained and examined for inflammation. B7(-/-) spleen cells caused substantial inflammation in multiple organs, especially lung, liver, pancreas, and intestine, whereas wild-type spleen cells caused minimal or no inflammation.
Figure 5.7  T cells from diseased B7(-/-) mice display an activated effector phenotype in RAG-1(-/-) B7(+/+) recipients. Seven weeks after transfer of 2x10^6 CD4, 3x10^6 CD8, or 5x10^6 CD4+CD8 T cells, spleens were harvested and stained for activation markers and intracellular cytokines. a) Purity of transferred T cells was maintained after seven weeks in vivo. As observed with total spleen cell transfer, most CD4 and CD8 T cells display an ex vivo activated phenotype of CD44+CD62L- (b), and substantial fractions of these cells produce IFNγ after PMA and ionomycin stimulation in vitro for 4 hours (c). Solid lines in histogram depict stain with cytokine-specific monoclonal antibody while dotted lines represent isotype controls. The numbers in the panels are % of cells within the gate after subtracting those stained by isotype control.
Figure 5.8  Purified CD4 T cells from diseased B7(-/-) mice cause multi-organ inflammation. T cells purified from spleen and lymph nodes were transferred into RAG-1(-/-) B7(+/+) recipients, and organs were fixed and H&E-stained seven weeks later. CD4 and CD4+CD8 T cell transfers caused substantial inflammation in lung, liver, pancreas, and intestine, while CD8 T cells alone caused no inflammation in pancreas and intestine, and minimal inflammation in the lung.
CHAPTER 6

EXPRESSION OF B7-1/2 ON AUTO-PATHOGENIC EFFECTOR T CELLS IS NOT REQUIRED FOR THEIR SUPPRESSION BY CD4⁺CD25⁺ REGULATORY T CELLS IN VIVO

6.1 Abstract

CD4⁺CD25⁺ regulatory T cells (Treg) suppress immunity to infections and tumors as well as autoimmunity and graft versus host disease. Since Treg constitutively express CTLA-4 and activated T cells express B7-1 and B7-2, it has been suggested that the interaction between CTLA-4 on the Treg and B7-1/2 on the effector T cells may be required for immune suppression. We have observed a spontaneous autoimmune skin disease in mice with targeted mutations of B7-1 and B7-2 (Chapter 5). The availability of auto-pathogenic B7-deficient T cells in these mice gives us an opportunity to test if B7-1/2 expression on effector T cells is required for their suppression by regulatory T cells. Our results revealed that Treg from wild-type mice suppress lymphoproliferation and immune destruction mediated by the B7-1/2 deficient auto-pathogenic T cells in vivo. Thus, Treg function in vivo does not require B7 expression by the auto-pathogenic T cells.
6.2 Introduction

CD4⁺CD25⁺ regulatory T cells (Treg) are able to suppress the function of autoimmune T cells that have escaped thymic deletion [229]. Both in vitro [123, 124] and in vivo [127, 129, 130], Treg have been shown to suppress proliferation, cytokine production, and inflammation by effector T cells, and thereby autoimmunity [113, 124, 127-130]. The costimulatory molecules B7-1 and B7-2 have been implicated in the development, survival, and effector function of Treg. Mice lacking B7 molecules or wild-type mice treated with CTLA-4Ig to block B7 have reduced numbers of Treg in the spleen [113, 132]. Anti-B7-1 and anti-B7-2 monoclonal antibodies reduce Treg numbers in the thymuses and spleens of wild-type mice [133]. It has been suggested that basal B7 expression in the periphery is required for Treg survival [132].

Treg express high levels of the costimulatory molecule CTLA-4 and it has been suggested that CTLA-4 may play an essential role in the suppressor function of Treg [134, 135]. It has been hypothesized that CTLA-4 on Treg may interact with B7-1 and B7-2 molecules expressed by activated effector T cells to suppress their function through a reverse signaling mechanism [138]. This hypothesis predicts that the expression of B7-1/2 by the effector T cell is required for adequate suppression by Treg. A recent study has shown that B7 expression by activated T cells in a graft-versus-host disease model can downregulate alloresponses by interaction with CTLA-4 expressed by other T cells [139]. However, this downregulation by B7/CTLA-4 ligation occurred independently of CD4⁺CD25⁺ Treg. In order to evaluate these conflicting hypotheses, we used auto-pathogenic T cells from B7-deficient mice that develop spontaneous
alopecia, lymphadenopathy, and progressive dermatitis. Our results demonstrate that autoimmune T cell function can be suppressed by CD4⁺CD25⁺ Treg in the complete absence of B7 on the pathogenic T cells.

6.3 Materials and methods

Experimental animals

B7-1(-/-) B7-2(-/-) mice [20] and RAG-1(-/-) [220], which have been backcrossed to the C57BL/6j strain for at least 10 generations, were purchased from the Jackson Laboratory (Bar Harbor, ME). C57BL/6j mice were purchased from the Charles River Animal Facility (Wilmington, MA). All mice were maintained in the University Laboratory Animal Research Facility at the Ohio State University under specific pathogen-free conditions.

Flow cytometry

Cell surface markers, including CD4, CD8, CD25, CD44, and CD62L, and intracellular cytokines, including IFNγ, IL-4, and IL-10, were analyzed with conjugated mAbs purchased from BD PharMingen (San Diego, CA).

CD4⁺CD25⁺ regulatory T cell purification

CD4⁺CD25⁺ cells were purified from pooled spleens and lymph nodes of normal C57BL/6j mice in two steps. First, CD4⁺ cells were isolated by negative selection using a cocktail of antibodies that react to CD8, FcR, Mac-1, CD11c, and B220, followed by anti-Rat IgG coated Dynabeads (Dynal Biotech, Oslo, Norway), according to manufacturer’s instruction. CD25⁺ cells were then isolated from the CD4⁺ population by positive selection using PE-conjugated or APC-conjugated anti-CD25 antibody
(BD Pharmingen, San Diego, CA) followed by anti-PE or anti-APC MACS beads (Miltenyi Biotec, Auburn, CA). In some cases, the CD4⁺CD25⁻ fraction was used for proliferation assays.

**Proliferation assay**

CD4⁺CD25⁺ cells were purified from pooled spleens and lymph nodes of normal C57BL/6j mice as described above to use as suppressor cells. The CD4⁺CD25⁻ fraction was used as responder cells. CD4⁺CD25⁻ cells from healthy B7-deficient mice showing no signs of alopecia or skin disease were purified in the same manner. CD4⁺CD25⁻ responder cells from either wild-type or B7-1(-/-) B7-2(-/-) mice were cultured at 5x10⁴ cells per well with 1 µg/mL soluble anti-CD3 mAb. CD4⁺CD25⁺ cells were added at two-fold titrations. Cells were cultured for 69-72 hours, and ³H-TdR was added at 1 µCi/well for the last six hours of culture. Cells were harvested and counted on a beta-counter. Suppressive activity was calculated using the formula: 100% x (CPM\text{responder} – CPM\text{responder + suppressor})/CPM\text{responder}

**Adoptive transfer**

20 x10⁶ spleen and lymph node cells from age-matched wild-type and B7-1(-/-) B7-2(-/-) mice were injected intraperitoneally into RAG-1(-/-) B7(+/-) C57BL/6j mice. Purified Treg were injected at 2.7x10⁶ cells per mouse in conjunction with 20x10⁶ spleen and lymph node cells from B7-deficient mice into RAG-1(-/-) B7(+/-) C57BL/6j mice. Recipient mice were sacrificed for histological analysis when clinical symptoms appeared.
Intracellular cytokine production

To assess intracellular cytokine production, spleen cells were cultured for 4 hours with 50 ng/ml PMA, 500 ng/ml ionomycin, and 2 µM GolgiStop (BD Pharmingen). Cells were stained for cell surface markers CD4 and CD8 followed by intracellular staining for IFNγ, IL-4, or IL-10 using a CytoFix/CytoPerm kit (BD Pharmingen).

Histological analysis

Mouse organs were fixed with 10% buffered formalin and paraffin-embedded. Tissue sections were stained with hematoxylin and eosin (H & E), and examined under a microscope.

6.4 Results

B7-deficient mice possess decreased numbers of CD4⁺CD25⁺ regulatory T cells in both thymus and spleen

It has been reported that B7-deficient mice have decreased numbers of CD4⁺CD25⁺ regulatory T cells in the spleen, which was postulated to be due to a lack of basal B7 expression required to maintain Treg survival [132]. We found a similar 6.4 fold reduction of Treg (CD4⁺CD25⁺CD62L⁺) in the spleens of C57BL/6 B7(-/-) mice compared with wild-type mice (Figure 6.1). However, we also found a nearly identical 6.6 fold decrease in thymic CD4⁺CD25⁺ Treg (Figure 6.1), suggesting that B7 plays a critical role in the development of Treg.
**CD4^{+}CD25^{+} regulatory T cells can suppress the proliferation of B7-deficient T cells in vitro**

To determine if B7 molecules are required for suppression mediated by Treg, we isolated CD4^{+} T cells from spleens and lymph nodes from wild-type and B7-deficient mice, as well as CD4^{+}CD25^{+} regulatory T cells from wild-type mice. Figure 6.2a shows the basal expression of B7-1 and B7-2 on wild-type T cells or lack thereof on B7-deficient T cells, while Figure 6.2b confirms the constitutive expression of intracellular CTLA-4 by unstimulated Treg. We tested the proliferation of B7(+/+) and B7(-/-) CD4^{+}CD25^{-} T cells with anti-CD3 mAb stimulation in vitro and observed that both wild-type and B7-deficient cells were susceptible to suppression by Treg (Figure 6.2c). Interestingly, B7-deficient cells demonstrated higher levels of proliferation with and without suppression than wild-type cells (data not shown), which has been reported by others [139].

**CD4^{+}CD25^{+} regulatory T cells suppress multi-organ inflammation mediated by pathogenic B7-deficient T cells**

We have demonstrated the spontaneous development of alopecia, lymphadenopathy, and skin disease in B7-1/2-deficient mice, and that T cells from the spleens of these mice are highly pathogenic when transferred into RAG-1(-/-) B7(+/+) mice. To extend our in vitro findings and determine if the addition of Treg could blunt the autoimmune multi-organ inflammation caused by B7(-/-) cells, we co-transferred CD4^{+}CD25^{+} T cells purified from naïve wild-type C57BL/6j mice (Figure 6.3a) with B7-1/2(-/-) spleen and lymph node cells into RAG-1(-/-) mice. At 6 weeks after transfer,
when mice receiving B7-deficient cells began to show clinical symptoms of illness (weight loss, wobbly gait), all mice were sacrificed for analysis of T cell function and for the presence of inflammation in various organs.

As shown in Table 6.1 and Figure 6.3b, all mice receiving spleen and lymph node cells from diseased B7-deficient mice had extensive inflammation in multiple organs, particularly in the lung, liver, and intestine. Mice receiving B7(-/-) cells plus CD4+CD25+ Treg had minimal organ inflammation, similar to the amount observed in control mice receiving spleen and lymph node cells from healthy wild-type mice.

**CD4+CD25+ regulatory T cells blunt the lymphoproliferation, activation, and cytokine production by pathogenic B7-deficient T cells**

To evaluate the relative proportions and activation status of the transferred cells after 6 weeks in recipients, lymph nodes and spleens were harvested and pooled from all mice in each group. The lymph nodes from mice treated with B7(-/-) cells were grossly larger and had 5-fold greater total cellularity on average than nodes from mice receiving B7(-/-) cells plus Treg. Lymph nodes from B7(-/-) recipients had 7-fold more CD4 and 3.5-fold more CD8 T cells on average than Treg recipients (Figure 6.3c), with a reversal of CD4/CD8 ratio from 1.3/1 in B7(-/-) recipients to 0.65/1 in Treg recipients. Spleens from Treg recipients exhibited a similar reduction in CD4 T cell numbers compared with B7(-/-) recipients (data not shown). This greater reduction of CD4 T cell numbers suggests that Treg are more efficient in suppressing the expansion and proliferation of the CD4 T cell subset than the CD8 T cell subset.

Lymph node cells from B7(-/-) cell recipients showed increased activation among both CD4 and CD8 T cells subsets compared with mice receiving B7(-/-) cells plus Treg
(as judged by percentages of CD44+CD62L- cells) (Figure 6.4a). Interestingly, a major population of CD4 T cells express intermediate levels of CD44 but high levels of CD62L in the group that received both B7(-/-) cells and Treg. Spleen cells showed a similar reduction in activation status among CD4 and CD8 T cells with Treg transfer, but with all cells exhibiting higher activation levels overall (Figure 6.4b).

To assess cytokine production by the cells, pooled spleen cells from RAG-1(-/-) recipients were subjected to short-term stimulation with PMA and ionomycin and stained for intracellular cytokines. A significant proportion of CD4 T cells from B7(-/-) donors produced IFNγ and this is reduced by about 25% by the co-transferred Treg (Figure 6.5a, top panel). However, CD4 T cells from mice receiving B7(-/-) cells displayed substantially increased IL-4 and IL-10 production compared with mice receiving wild-type cells, which showed minimal IL-4 and IL-10 production (Figure 6.5a, middle and bottom panels). This increase is completely eliminated by Treg. Although Treg had no effect on the IFNγ production by CD8 T cells, they did decrease the generation of IL-4 and IL-10 by CD8 T cells (Figure 6.5b). Clearly, co-transfer of Treg substantially abrogated the increased IL-4/IL-10 production by the B7-1/2(-/-) T cells.

6.5 Discussion

The results presented here demonstrate that CD4+CD25+ regulatory T cells are capable of suppressing the auto-pathogenic inflammation caused by B7-deficient cells upon transfer into RAG-1(-/-) B7(+/-) hosts. This suppression by Treg occurs despite a lack of B7 on the autoimmune effector T cells. Accumulating evidence demonstrate that much like effector T cells, the development, activation and effector function of Treg are
regulated by both antigen and costimulatory molecules. Treg are generated in the thymus, perhaps as a result of exposure to self antigen [131]. Several studies suggest that CD28 and CTLA-4 may be involved in the development and/or function of Treg [113, 133-135, 228]. Developmentally, it has been shown that mice with targeted mutations of B7-1 and B7-2 have decreased numbers of CD4^+CD25^+ T cells in the spleen [113, 132]. Our data shows that Treg numbers are also decreased in the thymuses of B7-deficient mice, which indicates that the decreased number of Treg in the periphery is likely due to defective development in the absence of B7, in addition to any role B7 may have on Treg survival [132].

Treg constitutively express CTLA-4, and several groups have suggested that CTLA-4 plays an essential role in the regulatory function of Treg [134, 135]. However, the mechanism for such function has not been revealed. Another mystery in the field is why activated T cells express significant levels of B7-1 and/or B7-2 [230]. A number of possibilities have been suggested to account for the role of these molecules. Lanier and others have suggested that B7 functions to costimulate T cell-T cell interaction [230]. Alternatively, Gavin and Rudensky suggested that CTLA-4 might be the suppressor effector molecule that interacts with B7 molecules on the surface of the autoimmune T cells utilizing reverse signaling through B7 [138]. A third scenario implicates reverse signaling through B7 expressed on dendritic cells by ligation with CTLA-4 on Treg [140, 144]. This interaction upregulates tryptophan catabolism in dendritic cells, which has been shown to suppress T cells [143, 145]. A fourth possibility proposed by Taylor et al. is that B7 expression by effector T cells, in this case alloreactive T cells in a GVHD
model, mediates suppression by CTLA-4 expressed by other T cells, but independently of traditional CD4+CD25+ regulatory T cells [139].

Here we employed an in vivo model to test these interesting hypotheses. In our model, auto-pathogenic T cells from diseased B7-deficient mice induce multi-organ inflammation when adoptively transferred into syngeneic RAG-1(-/-) recipients. We showed that co-transfer of B7-1/2(+/+) Treg decreases the activation, cytokine production, and inflammatory infiltration of the auto-pathogenic B7-1/2-deficient T cells. This clear suppression occurs despite the lack of B7-1/2 on the effector cells with which CTLA-4 on the Treg can interact. Since the recipient mice lack endogenous T cells, all T cell-T cell interactions must occur between transferred cells. This was corroborated by our in vitro experiments showing efficient suppression of B7-deficient CD4 cells by wild-type Treg. As such, our data do not support the hypotheses reported by Gavin and Rudensky [138] or Taylor and colleagues [139]. Our data are consistent with two possibilities. First, CTLA-4 on Treg may target B7-1/2 on other cell types, such as dendritic cells [144]. Second, B7/CTLA-4 interaction may not be essential for Treg function, as suggested by Shevach [231].
Table 6.1 CD4⁺CD25⁺ regulatory T cells protect RAG-1(-/-) recipients from multi-organ inflammation mediated by B7(-/-) spleen and lymph node cells.

Mononuclear spleen/lymph node cells from diseased B7(-/-) mice plus or minus wild-type Treg were transferred into RAG-1(-/-) B7(+/-) recipients, which were sacrificed at 6 weeks after adoptive transfer. Mouse tissues were fixed in 10% formalin and examined for inflammation after H&E staining. - = no infiltrates; + = 1-3 inflammatory aggregates; ++ = 4-6 inflammatory aggregates; +++ = >6 inflammatory aggregates, or a diffuse pattern of inflammation covering more than 50% of the organ section.

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Figure 6.1  
**B7(-/-) mice have reduced numbers of CD4^+CD25^+ regulatory T cells in the spleen and thymus.** Spleens and thymuses from 5-7 week old female wild-type (WT) and B7(-/-) (KO) C57BL/6 were harvested and stained for the presence of regulatory T cells. Representative FACS plot of a) CD25^+CD62L^+ cells within the CD4^+ gate of small splenocytes and b) CD4^+CD25^+ cells within the CD4^+ single positive gate of thymocytes from wild-type and B7(-/-) mice. c) Columns represent mean percentage of CD4^+CD25^+ cells within gated small cells in spleens or thymuses (n=4). Error bars represent SEM. P-values were determined using a two-sample t-test.
Figure 6.2  CD4<sup>+</sup>CD25<sup>+</sup> regulatory T cells can suppress the proliferation of B7(-/-) T cells in vitro.  a) Basal expression of B7-1 and B7-2 by naive T cells from wild-type (WT), but not B7-1/2(-/-) (B7KO) mice.  Solid lines in histograms represent B7-1 or B7-2 staining, while dashed lines represent isotype control stains.  Plots represent spleens pooled from 4 mice per group.  b) Expression of intracellular CTLA-4 by naive CD4<sup>+</sup>CD25<sup>+</sup> regulatory T cells.  Solid line in the histogram represents intracellular CTLA-4 staining, while the dashed line represents isotype control stain.  Plot represents Treg purified from spleens and lymph nodes of four C57BL/6j mice.  c) CD4<sup>+</sup>CD25<sup>-</sup> T cells from wild-type (WT) or B7(-/-) (B7KO) mice were used as responders and stimulated with anti-CD3 mAb (1.0 µg/mL) in the presence of wild-type CD4<sup>+</sup>CD25<sup>+</sup> regulatory T cells.  Treg were titrated to responder cells at 1:2, 1:4, 1:8, and 1:16 ratios.  Cells were cultured for 69 hours with tritiated thymidine (1 µCi/well) added for the last 6 hours of culture.  Wells were performed in triplicate.  Suppressive activity was determined by the following formula:

$$100\% \times \frac{\text{CPM}_{\text{responder}} - \text{CPM}_{\text{responder + suppressor}}}{\text{CPM}_{\text{responder}}}$$
Figure 6.2
Figure 6.3  

**CD4^+CD25^+ regulatory T cells suppress multi-organ inflammation and lymphadenopathy mediated by pathogenic B7(-/-) T cells.**  

a) Purity of CD4^+CD25^+ Treg used for adoptive transfer. b) CD4^+CD25^+ regulatory T cells suppress multi-organ inflammation mediated by pathogenic B7(-/-) T cells. 20x10^6 spleen and lymph node cells from diseased B7(-/-)mice or healthy wild-type mice were transferred into RAG1(-/-) B7 (+/+ ) recipients. One group of mice received 2.7x10^6 CD4^+CD25^+ T cells (Treg) isolated from spleens and lymph nodes of wild-type B7(+/+) C57BL/6j mice. Six weeks after transfer when mice began to exhibit clinical symptoms, all mice were sacrificed. Organs were H&E-stained and examined for inflammation. Lung, liver, and intestine from B7(-/-) (B7KO)cell recipients had numerous inflammatory foci compared with wild-type (WT) cell recipients. Inflammatory infiltration was observed mainly surrounding bronchi and blood vessels of lung, the portal tracts and central venules of the liver, and in the submucosa of the intestine. Mice receiving Treg in addition to B7(-/-) cells showed minimal inflammation, similar to wild-type cell recipients.  

C) CD4^+CD25^+ Treg decrease lymphoproliferation of B7(-/-) (KO) spleen and lymph node cells transferred into RAG-1(-/-) recipients. Bars represent number of CD4 and CD8 T cells per lymph node. Data shown are derived from pools of 8-10 anatomically-matched lymph nodes from 4-5 mice per group.
Figure 6.3

- **a.** CD25 levels of CD4+ CD8+ T cells in WT, KO, and KO+Treg mice.

- **b.** Histological images of lung, intestine, and liver from B7KO and B7KO+Treg mice compared to WT.

- **c.** Bar chart showing cell numbers x 10^5 in lymph node for WT, KO, and KO+Treg groups for CD4 and CD8 cells.
Figure 6.4  CD4⁺CD25⁺ Treg diminish the enhanced activation status of B7(−/−) T cells transferred into B7(+/+) RAG-1(−/−) recipients. Cell surface staining of a) lymph node and b) spleen cells from B7(+/+) RAG-1(−/−) recipients six weeks after receiving either wild-type (WT) spleen and lymph node cells, or B7(−/−) (B7KO) spleen and lymph node cells with or without co-transfer of CD4⁺CD25⁺ Treg.
Figure 6.5  **CD4^+CD25^+ Treg diminish the enhanced cytokine production by B7(-/-) T cells transferred into B7(+/+) RAG-1(-/-) recipients.** Spleen cells from recipient B7(+/+) RAG-1(-/-) mice were stimulated in vitro with PMA and ionomycin for 4 hours and stained for intracellular cytokines.  a) CD4 T cells from RAG-1(-/-) mice receiving B7(-/-) cells (B7KO) show significant IL-4 production (11%), which is virtually absent in wild-type (WT) mice and in mice receiving Treg in addition to B7(-/-) cells. IL-10 production by CD4 T cells from recipients of B7(-/-) cells was also increased compared to wild-type and Treg recipients.  b) CD8 T cells from wild-type, B7(-/-), and B7(-/-) plus Treg cell recipients show comparable levels of IFNγ production. However, CD8 T cells from recipients of B7(-/-) cells have increased IL-4 and IL-10 production, which is reduced to wild-type levels by the presence of Treg. Solid lines in histograms depict stain with cytokine-specific monoclonal antibody while dotted lines represent isotype controls. Numbers shown in the panels represent % of positive cells after subtracting those stained with isotype controls.
Figure 6.5
CHAPTER 7

CONCLUDING REMARKS

The findings presented here further the understanding of the role of costimulatory molecules in the settings of anti-tumor immunity and autoimmunity. These two types of immune responses represent similar modes of attack by T cells, one with a desired and the other with an undesired target. Therapeutically, the T cells that require boosting to fight tumors are the very same T cells that need to be dampened to prevent autoimmune destruction. In this way, insights gained from the study of one response may be applied to the study of the other. The goals of this study have been: 1) to more clearly elucidate the mechanism of action of several key costimulatory molecules involved in anti-tumor immunity and regulation of autoimmunity, and 2) to further the translation of agents targeting these molecules to a therapeutic setting for human patients.

Manipulation of 4-1BB with monoclonal antibody has been demonstrated to be efficacious in the treatment of murine tumors [97-100]. These studies analyzed the effect of 4-1BB ligation in an intact immune system. Rosenberg and others have pioneered the use of adoptive cellular immunotherapy as a method to treat advanced cancer patients [232]. A recent study demonstrated dramatic effect of treating metastatic melanoma patients with myeloablation followed by infusion of ex vivo expanded tumor-infiltrating
lymphocytes [106]. One of the major challenges with adoptive cellular therapy has been the lack of persistence of transferred cells in the patient [233]. Our experiments with anti-4-1BB mAb in the setting of adoptive cellular immunotherapy for large established tumors detail a method to optimize this clinical strategy. We demonstrate that anti-4-1BB mAb is able to sustain high levels of tumor-specific CD8 T cells in the peripheral blood of tumor-bearing mice. This effect is mediated by increased survival, but not increased proliferation, of the T cells. The mechanism of increasing T cell survival has been recently confirmed by analysis of the molecular effects of 4-1BB ligation [57, 67, 71]. Using a RAG-2(-/-) model in which tumor-bearing mice have no T cell immunity aside from the transferred cells, we verify that the effect of mAb on CD8 T cells can occur in the absence of CD4 T cell help. This immuno-deficient mouse model also parallels the immuno-compromised state of many patients treated with immunotherapy and the myeloablative state induced in recent clinical adoptive cellular therapy protocols [106].

An area of further study is effect of anti-4-1BB mAb on CD4 T cells included in the adoptive therapy regimen. The effective clinical protocol employed by Rosenberg and colleagues revealed that the addition of CD4 tumor infiltrating T cells to the traditional CD8 T cell adoptive transfer enhanced the persistence of CD8 T cells in the patient [106]. Considering the sometimes divergent effects of 4-1BB ligation on CD4 versus CD8 T cells, it will be important to know whether anti-4-1BB mAb can further enhance T cell survival or whether it will diminish CD4 T cells, as demonstrated in
settings of autoimmune disease [68, 146]. The ability of anti-4-1BB mAb to blunt autoimmunity may also provide an additional benefit to patients who often develop autoimmune side effects with adoptive cellular therapy [105, 106].

The investigation of anti-CTLA-4 mAb therapy for cancer has made more rapid progress. As with anti-4-1BB mAb, numerous studies in mouse models have shown the benefit of CTLA-4 ligation for boosting tumor immunity [82-93]. Translation of these findings to human cancer patients has been detailed in two recent clinical trials testing the effect of anti-human CTLA-4 mAb in ovarian cancer and metastatic melanoma [94, 95]. The outcomes using the same clone of monoclonal antibody revealed minimal effect on tumor with few side effects [94], or dramatic tumor responses accompanied by severe autoimmune toxicities [95]. This illustrates a critical problem in the translation of monoclonal antibodies targeting costimulatory molecules from mouse to man. To facilitate the preclinical screening of anti-human CTLA-4 mAb, we describe two humanized mouse models that provide more extensive in vivo characterization of potentially therapeutic clones of antibody.

The hu-PBL-SCID mouse model of EBV-lymphoproliferative disease has been used for a number of years to test various manipulations of human immunity in an in vivo setting [185, 186]. We apply this model to the screening of anti-human CTLA-4 mAb by demonstrating the dramatic expansion of human CD8 and CD4 T cells, as well as the generation of tumor-specific CD8 T cell responses with several clones of mAb. Furthermore, this enhanced immunity causes a decrease in the EBV oncogenic protein LMP-1, and promotes increased survival and delay in lymphoma development. Our work reveals that different clones of anti-human CTLA-4 mAb can have striking differences in
their ability to mediate these immune effects. We have identified one clone, L3D10, that appears to be more efficacious than others in boosting immune responses. Further characterization of such properties as binding affinity and location of binding epitopes will facilitate correlation of structural and molecular properties of monoclonal antibody clones with functional differences observed in vivo. While screening of mAb in the hu-PBL-SCID model may lead to therapies that are widely applicable to many types of cancer, these specific studies also highlight the potential use of anti-human CTLA-4 mAb in the clinical setting of post-transplant lymphoproliferative disorder (PTLD). CD8 T cell responses have been associated with regression of PTLD in humans [188, 189], and cytokine therapy enhances EBV-specific CD8 T cell expansion and lymphoma prevention in the hu-PBL-SCID model [172]. Anti-CTLA-4 mAb may be able to further enhance these CD8 T cell responses and promote clearance of PTLD in these patients.

The second model developed to evaluate anti-human CTLA-4 mAb is a novel human CTLA-4 knock-in mouse, in which human CTLA-4 is expressed by murine T cells. The advantages of this model are complementary to those offered by the hu-PBL-SCID model. While the hu-PBL-SCID model allows for direct observation of the effect of anti-CTLA-4 mAb on human T cells, the xenogeneic setting and associated complications of this model may not reflect an entirely natural immune response [185, 186]. Comparatively, the human CTLA-4 knock-in mouse model provides a completely intact and normally functioning immune system, albeit the targeting of a human molecule on murine T cells. The studies presented here have focused on the creation and characterization of proper expression of human CTLA-4 by murine T cells. Due to the necessity of extensive backcrossing into purebred strains of mice, analysis of anti-human
CTLA-4 mAb has been performed almost exclusively in heterozygous mice expressing a single copy of the human CTLA-4 gene. In this regard, the induction of modest antitumor immunity with mAb is intriguing but must be tested in homozygous mice in which human CTLA-4 has completely replaced mouse CTLA-4. In heterozygous mice, only half of the CTLA-4 molecules may be engaged by mAb, while in homozygous mice a complete engagement or blockade can be achieved. Additional studies using both heterozygous and homozygous human CTLA-4 mice will be valuable in more clearly dissecting out whether the effect of anti-CTLA-4 mAb engagement is actually a blockade, as is widely claimed, or can mediate positive signaling through the targeted molecule. This mouse model may also be used for future studies analyzing the role of human CTLA-4 costimulation in other disease models, such as autoimmunity.

During the course of studying mice with targeted mutations of B7-1 and B7-2, we observed a spontaneous disease manifested by whole-body alopecia, lymphadenopathy, and severe skin disease in the mice. This disease model adds to the growing list of autoimmune diseases reported with B7-deficiency [113, 115], and highlights several important roles for B7 in T cell development and function. B7-deficiency in the thymus may lead to impaired deletion of autoreactive T cells, which can mediate autoimmune pathology in the periphery, as evidenced by work in our lab [122]. Furthermore, B7-deficiency dramatically reduces the generation of CD4^+CD25^+ regulatory T cells (Treg), as shown here and by others [113, 132], which play an important role in the suppression of autoimmunity. In an adoptive transfer model, we show that pathogenic CD4 T cells from diseased B7-deficient mice can induce inflammation in multiple organs when B7 expression is restored, though disease may be restricted to the skin when B7 is absent.
Therefore, our model exemplifies the dual role of B7 in autoimmunity. Presence of B7 facilitates proper thymic deletion of autoreactive T cell clones, but exacerbates autoimmune disease in the periphery, while absence of B7 increases the autoreactive T cell burden and reduces Treg, but restricts peripheral autoimmune manifestations.

This model of autoimmune skin disease in B7-deficient mice will facilitate the further investigation of B7 requirements for various aspects of autoimmune pathology. We illustrate one example in which this model provides us with the opportunity to test the role of B7 molecules in suppression by Treg, an area that has been the subject of recent speculation and conflicting evidence [138, 139, 144]. We show that Treg are capable of suppressing in vitro proliferation and in vivo multi-organ inflammation of B7-deficient T cells, revealing that B7 expression by the target cell is not required for suppression by Treg. This model can also be useful in the testing of new therapies, both immunological and pharmacological, for the prevention or treatment of autoimmune skin diseases. Of particular interest with regards to the discussions presented herein is the potential role of anti-4-1BB mAb in abrogating this CD4 T cell-mediated disease.

As presented in the introduction to this work, T cell costimulation is a field of constant expansion and ever-increasing complexity. Extensive research has revealed many insights into the nature of these molecules critical to T cell responses, but has also left many questions unresolved. Our goal has been to investigate the function and application of several key costimulatory pathways, 4-1BB/4-1BBL and CD28/CTLA-4/B7, in the fields of anti-tumor immunity and autoimmunity. Hopefully, this work will advance the efforts to translate understanding of these molecules in animal models into therapeutic interventions which can ease the suffering of human disease.
BIBLIOGRAPHY


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