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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. Although anti-mouse CTLA-4 antibodies 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, and this heterogeneity cannot be attributed to Ig isotypes or affinity for CTLA-4. 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.
Abbreviations: PBL, peripheral blood leukocytes; EBV, Epstein-Barr virus; mAb, monoclonal antibody; CTLA-4, cytotoxic T lymphocyte antigen 4; LMP-1, latent membrane protein 1; LCL, lymphoblastoid cell line; xenograft-versus-host disease, XGVHD; PTLD, post-transplant lymphoproliferative disorder; CTL, cytotoxic T lymphocyte
Introduction

A major challenge in translating basic principles learned in mice to clinical application in humans is that both diseases and therapeutics 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. [1] 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 [2-4], melanoma [5-7], ovarian carcinoma [8], and mammary carcinoma [9]. A number of these groups combined anti-CTLA-4 mAb with a GM-CSF producing tumor cell vaccine [5, 6, 9] or depletion of regulatory T cells [7] 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 [5-7]. Conflicting evidence exists as to whether anti-CTLA4 mAb is capable of reversing T cell tolerance to tumor antigen [10, 11]. 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 [12]. Another study involving patients with metastatic melanoma demonstrated significant clinical activity, with objective tumor regression in 3 of 14 patients [13]. 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 [14, 15]. Also, CTLA-4 engagement with antibody could either promote or inhibit proliferation of different subsets of T cells in the same culture [16].

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 [17]. One approach involves establishment of human thymus or stem cells in these mice [17, 18], which then produce human T cells. This model has been extensively used for studies of HIV infection [19] and development of human T cells [17]. At the same time, less demanding chimera have been produced involving SCID mice grafted with either human peripheral blood leukocytes (PBL) [20], or antigen-specific T cells [21]. In the area of cancer immunotherapy, hu-SCID mice have been used to measure the effector function of tumor-specific T cells [21] and cloned tumor-specific T cell lines [22, 23].

Despite numerous attempts, varying success has been reported using hu-SCID mice to study the immune response of human T cells. A notable exception reported by Cabarllido et al. demonstrated that grafting fetal human bone, thymus, skin, and lymph nodes into SCID mice allows induction of immune responses of human lymphocytes [24]. The requirement of multiple surgeries, however, makes this an impractical 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 [25]. 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 [26]. 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 CD8 T cell-dependent protection from EBV-associated lymphoproliferative disease. 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 enhancement of 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 targeting the CTLA-4 molecule.

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 fusion protein 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 Pharmingen (San Diego, CA).

Binding kinetics and affinity of monoclonal antibodies  These experiments were performed by Biacore, Inc. through a contract service. Human CTLA-4Ig fusion protein was immobilized on a Biacore sensor chip using primary amine covalent chemistry. Briefly, N-hydroxysuccinimide esters were introduced on the chip surface by modification of the carboxymethyl groups on the chip surface with a mixture of N-hydroxysuccinimide (NHS) and N-ethyl-N’-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC) for 7 minutes. The
human CTLA-4Ig was diluted in 10mM sodium acetate (pH 5.5) at a concentration of 2.5 µg/mL and injected over the activated surface for approximately 1-3 minutes. The surface was then blocked for 7 minutes with ethanolamine to remove any remaining esters. An NHS/EDC activated and ethanolamine blocked surface was used as the reference surface. Anti-human CTLA-4 mAbs were injected at various concentrations in duplicate over the protein and reference surface for 3 minutes, followed by 10 minutes of dissociation time using an automated method. The running buffer was HBS-EP (0.01 HEPES pH 7.4, 0.15 M NaCl, 3mM EDTA, 0.005% Surfactant P20) pH 7.4 and the detection temperature was 25°C.

**Engraftment of human peripheral blood leukocytes** PBL were obtained from normal healthy donors that were consented under an Ohio State University IRB-approved protocol (2003A0041) for leukapheresis performed by The Ohio State University Hospitals apheresis unit according to the Declaration of Helsinki. 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. 50x10⁶ 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 anti-IL2Rβ (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). Samples were analyzed on a BD FACSCalibur flow cytometer.

**IFNγ production assay**  Hu-PBL-SCID spleen cells were stimulated with an autologous EBV+ lymphoblastoid cell line 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 $5 \times 10^6$ PBL and treated with $100 \, \mu g$ of TMβ1 mAb on the same day, followed by $100 \, \mu L$ ascites containing anti-human CTLA-4 mAb or $100 \, \mu g$ mouse IgG and $3 \, \mu g$ 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.

Statistical analysis  Statistical significance and p-values for T cell expansion experiments were determined using one-way ANOVA with Tukey's procedure for multiple comparisons. The p-value for difference in LMP-1 expression was determined by two-sample t-test. For the survival curve, 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 [27].
Results

1. 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. 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. The affinities of each antibody for human CTLA-4Ig fusion protein were measured using a Biacore instrument. As shown in Table 1, the $K_d$ of the antibodies ranged from 0.72 nM to 10 nM.

2. Anti-human CTLA-4 mAb promotes a profound expansion of T cells in a hu-PBL-SCID mouse 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 disease [20]. 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 [28]. As shown in Fig. 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<0.002). In addition, a selective expansion of human leukocytes, as marked by expression of human CD45, was observed among all antibody-treated mice (p<0.003) (Fig. 1b top panel, c & d left panels). The total number of CD8 T cells was increased in all antibody-treated groups (p<0.03), while total number of CD4 T cells was increased in L3D10 and YL2-treated groups (p<0.0003) (Fig. 1b, lower panel and Fig. 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 (p<0.0003) and YL2 (p<0.02), the proportion of CD4 T cells expanded significantly in comparison to those treated with control IgG. In contrast, KM10 did not cause any preferential expansion (p=0.52) (Fig. 1b lower panels and 1d). The proportion of CD8 T cells among human leukocytes decreased significantly (p<0.009) even as the total numbers increased (Fig. 1c and d). A comparison between Fig. 1a and 1c revealed that the numbers of mouse cells were also significantly increased, perhaps in response to cytokines induced by anti-CTLA-4 antibodies.

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 Fig. 2a, and the comparison of the different antibodies is presented in Fig. 2b. As shown in Fig 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 cell 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. Surprisingly, the extent of T cell expansion did not correlate with the binding affinities of the antibodies to human CTLA-4 (Table 1). The two antibodies with highest affinities, KM10 and YL2, did not induce the greatest T cell expansion, and even varied from one another in their ability to induce T cell expansion despite very similar $K_d$ values. Since anti-human CTLA-4 mAb clone L3D10 showed the greatest effect in expanding human T cells, this clone was chosen for further characterization.

3. 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 promotes the development of lymphoproliferative disease. Latent membrane protein 1 (LMP-1) is an EBV oncoprotein involved in the immortalization of B cells leading to this transformation [29-33], and LMP-1 has been shown to be a potential target of T cell responses [34, 35]. 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. Similar to the experiment shown in Figure 2, we observed a substantial expansion of both CD8 and CD4 T cell subsets (data not shown). As shown in Fig. 3a and b, the percentage of LMP-1+ cells was 3-4 fold lower in mice treated with L3D10 (p=0.0015). A similar magnitude of reduction was observed in the total number of LMP1+ cells in the spleens. However, large intra-group variation reduced the p-value to 0.0854. These results suggest that the percentage of EBV-infected cells can be reduced as a result of anti-CTLA-4 mAb treatment. Interestingly, while essentially all CD19+ cells expressed LMP1, the majority of the LMP1+ cells lacked the CD19 marker. The origin of these LMP1+CD19- cells is unclear at this stage.

4. 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 Fig. 4a, almost all the LCL cells expressed high levels of intracellular LMP-1, while the Burkitt’s lymphoma cells had 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 (Fig. 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.

5. Anti-human CTLA-4 mAb L3D10 delays the development of lymphoproliferative disease in hu-PBL-SCID mice. We performed a long-term survival experiment in which engrafted mice were treated with anti-human CTLA-4 mAbs and human GM-CSF for two weeks after engraftment, and then observed for signs of illness. Figure 5 shows the survival curve of mice that received control IgG or one of five different anti-CTLA-4 antibodies. Based on our previous experience that no lymphoproliferative disease can be observed within one month of engraftment, we have excluded from the final analysis two mice that died before 30 days. 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). In addition, KM10G11 also had a statistically significant impact (p=0.045) on survival. However, while it appears that other anti-CTLA-4 antibodies also extended the life-span of recipient mice somewhat,
pair-wise comparisons demonstrate no statistical difference between these antibodies and control IgG.

**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 [1], CTLA-4 has shown great promise for clinical translation. Two recent human trials using mAbs to target the human CTLA-4 molecule showed promising effects [12, 13], 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 [12]. Another study with the same mAb showed several dramatic clinical responses, accompanied by serious autoimmune toxicities [13]. 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 [36].

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 [20]. This report described the long-term engraftment of all cellular components of 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 [37], 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, have discovered a number of limitations of this model, including xenograft-versus-host disease (XGVHD), variations in PBL engraftment, and leakiness of the SCID phenotype [38-42].

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 [43, 44] and hu-PBL-SCID mice [26, 45]. 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 [34, 46, 47] and in hu-PBL-SCID mice [26]. Correlation of CD8 T cell responses to protection against EBV-
lymphoproliferative disorder 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. In addition, the affinity of the antibodies used does not adequately explain the functional heterogeneity. For instance, two pairs of antibodies with essentially identical affinity showed different function in vivo. This lack of in vitro and in vivo correlation warrants the use of more stringent preclinical screening regimens such as the one described here to select clones of mAb that can elicit the most dramatic T cell response in vivo.

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 [48]. 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 [11].
Not only did anti-human CTLA-4 mAb promote overall T cell expansion in vivo, 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 lymphoma, 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 and LMP-1 reduction. 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 the 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 longer-term 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 finding were complicated by the development of severe illness, most likely XGVHD, which sometimes caused death before lymphoma formation in a substantial fraction of mice involved. However, in mice that escaped GVHD, 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.

Acknowledgement

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Table 1: Binding kinetics and affinity of anti-human CTLA-4 mAb to
huCTLA-4Ig fusion protein (as determined by Biacore).

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Figure 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 TMβ1 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. All panels are representative of 4-5 mice per treatment group. Bars represent mean plus SEM. P-values were generated using one-way ANOVA with Tukey's procedure for multiple comparisons. Asterisks indicate a difference from the control mouse IgG treatment with a significance of p<0.05.
Figure 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.

**Figure 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,
Data are representative of two independent experiments. The p-value was depicting cells from lymphocyte gate. Data shown are LMP1 and CD19 profiles of gated human CD45* cells. b) Summary graph showing % (top) and number (bottom) of LMP-1 staining cells for 3 to 4 individual mice per treatment group. Data are representative of two independent experiments. The p-value was generated using a two-sample t-test.
Figure 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 50x10⁶ 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 shown are within the CD45⁺CD8⁺ gate.
Figure 5: Some anti-human CTLA-4 mAbs prolong survival and delay onset of lymphoproliferative disorder in hu-PBL-SCID mice. CB.17 SCID mice were engrafted with 5x10⁶ human PBL and treated with 100 μg TMβ1 mAb the same day, followed by 100 μL ascites containing anti-human CTLA-4 mAbs 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. One L3D10-treated mouse with early death at day 15 and one KM10G11-treated mouse with early death on day 13 were excluded from the survival analysis based on our experience that no lymphoma-related death is
possible at this point. The survival times of the antibody-treated groups were compared to the control IgG-treated group using the log rank test. P values are:

Mouse IgG versus L3D10 = 0.0195
Mouse IgG versus L1B11 = 0.481
Mouse IgG versus K4G4 = 0.323
Mouse IgG versus KM10G11 = 0.045
Mouse IgG versus YL2 = 0.324
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

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