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CD28 Blockade Induces Division-Dependent Down-Regulation of Interleukin-2 Receptor Alpha

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Abstract

Background—Blockade of T cell costimulatory molecules represents a promising new method of attenuating donor-reactive T cell responses to promote graft survival following transplantation. However, recent studies in murine models have shown the presence of an initial high frequency of naïve donor-reactive T cells may render this strategy ineffective.

Methods—In this report, we examined the phenotypic changes associated with CD28 blockade on T cells stimulated at increasing precursor frequencies *in vitro*.

Results—We found that treatment with the CD28 blocker CTLA-4 Ig resulted in downregulation of the alpha chain of the IL-2 receptor (CD25) in a division-dependent manner. Significantly, blockade of the CD28 pathway was more effective in down-regulating CD25 when the donor-reactive T cell population was present at low as compared to high precursor frequency.

Conclusions—These results imply that treatment with CD28 blockers and anti-CD25 mAbs may cooperate in promoting graft survival under conditions of low MHC matching where the donor-reactive T cell precursor frequency is high.

Keywords

costimulation blockade; interleukin-2; precursor frequency; T cell activation

1. Introduction

Blockade of T cell costimulatory molecules represents a promising new method of attenuating donor-reactive T cell responses to promote graft survival following transplantation [1]. The CD28 pathway is one the earliest and beststudied T cell costimulatory pathways [2], and attempts to block this pathway using CTLA-4 Ig fusion proteins met with success in animal models [3]. A second-generation CD28 inhibitor, LEA29Y (belatacept) showed promising results in non-human primate studies [4;5;6] as well as recent phase II and phase III clinical trials [7], both in terms of inhibiting acute rejection and minimizing non-immune toxicities commonly associated with current immunosuppressive regimens. Almost two decades of research on the mechanisms by which blockade of the CD28 pathway inhibits alloimmune responses suggests that failure to

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MLF designed the research, performed the research, and analyzed the data, and wrote the paper, LLS designed research and performed research, and CPL designed research, analyzed the data, and wrote the paper.

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receive CD28- mediated signals during T cell activation results in abortive activation, incomplete differentiation, and enhanced death of alloreactive T cell populations [2]. Despite these successes, recent data suggests that use of a calcineurin-inhibitor sparing belatacept-based regimen led to slightly increased risk and severity of acute rejection following renal transplantation [8]. Thus, identifying the parameters which stratify acute rejection risk in patients receiving belatacept-based treatment remains an important goal.

The programmed differentiation model of T cell activation emerged from studies which indicated that, after a brief period of antigenic stimulation, naïve CD8⁺ T cells become committed to a program of autonomous clonal expansion of at least seven cell divisions and differentiation into effector and memory cells [9]. Subsequent studies have shown T cell programs are flexible and can be altered by the initial priming conditions and by extrinsic factors (e.g., cytokines) during the execution of the program. Importantly, considerable evidence suggests that IL-2 is a critical factor for the execution of the antigen-independent phase of the proliferation program [9;10]. Early T cell cycling is antigen-dependent, but studies using IL-2^{-/-} or CD25^{-/-} TCR transgenic T cells indicate that IL-2 is important for sustaining T cell expansion, in part by inducing bcl-2 and allowing re-expression of IL-7R α [11;12]. Moreover, transient exposure to IL-2 early after priming conferred a robust and long-standing survival advantage upon T cell populations *in vivo* [11].

Despite the potential concerns about possible negative effects on T_{reg} function by targeting the IL-2 pathway [13], blocking IL-2 signaling through the use of anti-CD25 mAbs has proven to be remarkably safe and reasonably effective as an immunosuppressive strategy in transplantation [14]. Moreover, there is increasing interest and encouraging reports regarding the use of prolonged or chronic therapy the anti-CD25 antibodies in autoimmunity and transplantation [15]. We have previously shown that the IL-2 pathway plays an important role in the costimulation blockade-resistant response in murine models of transplantation [16], and previous work from Wells and colleagues suggested that CD28 blockade altered *in vitro* expression of CD25 following antigenic stimulation [17].

An additional modifying factor of both programmed T cell expansion and the relative efficacy of costimulation blockade-based treatment in transplantation is the initial precursor frequency of the responding donor-reactive T cell population [18;19;20]. We have previously shown that naïve CD4⁺ and CD8⁺ T cell precursor frequency plays a critical role in determining the quantity and quality of the donor-reactive T cell response following transplantation, and thus in mediating costimulation blockade-resistant rejection [18;19;20]. Specifically, we reported that high frequency populations of naïve graft-specific CD8⁺ T cells expanded and differentiated into competent effectors even in the presence of costimulation blockade, thus precipitating graft rejection [18]. In contrast, low-frequency populations of naïve graft-specific CD8⁺ T cells failed to significantly expand in the presence of costimulation blockade and did not differentiate into high quality effectors that were capable of rejecting a skin graft. These studies demonstrated that high-frequency naïve T cell populations may obviate the requirement for costimulation during priming and play a significant role in mediating costimulation blockade-resistant allograft rejection.

In this study we addressed the ability of blockade of the CD28 pathway to impact expression of the IL-2 receptor alpha chain (CD25) during T cell activation under conditions where the initial anti-donor frequency is either high or low. Measuring the magnitude and kinetics of this effect, we found that blockade of the CD28 pathway resulted in division-dependent downregulation of CD25. Due to decreased numbers of cell divisions in cells stimulated at an initial high frequency, CD25 expression levels were maintained on a subset of cells within this population, suggesting that these cells may be responsible for mediating costimulation blockade-resistant rejection *in vivo*.

2. Hypothesis

CD25 is preferentially down-regulated on cells that were induced to undergo extensive cell division in the presence of CD28 blockade.

3. Materials and Methods

3.1 Mice

TCR transgenic OT-I and OT-II mice were purchased from Taconic, Inc. and were bred onto Thy1.1⁺ backgrounds at Emory. C57BL/6 mice were purchased from Jackson Laboratories (Bar Harbor, ME). Animals received humane care and treatment in accordance with Emory University Institutional Animal Care and Use Committee guidelines.

3.2 In Vitro T Cell Stimulation and Treatment with Costimulation Blockade

Splenocytes were harvested from OT-I and OT-II mice, and OT-I and OT-II T cells were stimulated with OVA peptide (OT-I: OVA 257–264, sequence SIINFEKL, and OT-II: OVA323–339, sequence ISQAAHAVHAEINEAGR, Emory University Microchemical Core Facility) (3×10^5 OT-I or II T cells/well plus 2.5×10^6 B6 splenocytes) in a 24-well plate in the presence of CTLA-4 Ig (Bristol-Myers Squibb Company, 100 µg/ml), and/ or anti-CD154 (MR-1, BioXcell, 100 µg/ml). Culture media consisted of RPMI medium 1640 supplemented with 10% FBS (Mediatech, Herndon, VA), 2mM L-glutamine, 0.01 M Hepes buffer, 100 µg/ml gentamycin (Mediatech), and 5×10^{-5} M 2-mercaptoethanol (Sigma, St. Louis, MO). Where indicated, recombinant murine IL-2 (Pharmingen) was added at time 0 to cultures at a concentration of 300 pg/ml. Also where indicated, anti-CD25 monoclonal antibody (PC.61, BioXCell) was added to in vitro cultures at a concentration of 10 µg/ml.

3.3 Flow Cytometric Analyses for CD25 Expression

At the indicated timepoints, cell cultures were harvested, counted, and stained with Thy1.1-PerCP, CD8 or CD4-FITC, and CD25-APC (all BD Pharmingen) for flow cytometric analysis on a BD FACS Calibur flow cytometer. Flow cytometric data were analyzed using FlowJo Software (Treestar, San Carlos, CA).

3.4 CFSE-Based Proliferation Assay

Splenocytes were harvested from OT-I mice, and OT-I T cells were stimulated with OVA peptide at high (3×10^6 cells/well) or low frequency (3×10^5 cells/well) in a 24-well plate in the presence of CTLA-4 Ig (Bristol-Myers Squibb Company, 100 µg/ml), and/or anti-CD154 (MR-1, BioXcell, 100 µg/ml). Naïve B6 splenocytes were added to the low frequency cultures to keep the total number of cells per well consistent. In the indicated experiments, cells were labeled prior to culture with 1 µM CFSE (Molecular Probes) for 5 min at 25 degrees.

3.5 In vivo mixed lymphocyte reaction

5×10^6 CFSE-labeled (5 µM) H-2^b B6 splenocytes were injected into an irradiated (400 rads) fully allogeneic H-2^d BALB/c responder. Mice were treated with nothing, CTLA-4 Ig alone (500 µg/dose), or the combination of CTLA-4 Ig (500 µg/dose) and anti-CD25 mAb (250 µg/dose). All reagents were administered on days 0 and 2. Recipients were sacrificed at 72h and CFSE-labeled H-2K^b B6 T cells were analyzed by flow cytometry for the frequency of proliferating cells as measured by a decrease in CFSE fluorescence. Data shown are representative examples of three independent animals per group.

3.6 Statistical Analyses

Correlation between number of cell divisions and CD25 MFI was calculated using Spearman's non-parametric correlation test (two-tailed, 95% confidence interval). Comparison of frequency of divided T cells was done using a Mann Whitney U-test. Analysis was done using GraphPad Prism software.

4. Results

4.1 Blockade of CD28 signaling through CTLA-4 Ig results in downregulation of CD25 on antigen-specific CD8⁺ T cells

We sought to address the effect of blockade of the CD28 and CD154 costimulatory pathways on the expression of the IL-2 receptor alpha chain (CD25) following CD8⁺ T cell activation at high versus low frequency. We focused on CD8⁺ T cells as these cells have been shown to be responsible for mediating costimulation blockade-resistant rejection [21;22]. In order to accomplish this, we established an *in vitro* system in which naïve monoclonal CD8⁺ TCR transgenic T cells (OT-I) were stimulated with cognate peptide antigen in the presence or absence of blockade of the CD28 pathway through CTLA-4 Ig, blockade of the CD40/CD154 pathway using anti-CD154 (MR-1), or a combination of the two. *In vitro* stimulation with cognate OVA peptide resulted in the generation of activated CD8⁺ Thy1.1⁺ antigen-specific effector T cells, which expressed CD69, granzyme B, and CD107 on the cell surface following incubation with OVA peptide-loaded splenocytes cells (data not shown). These data indicate that the antigen-specific T cells were activated following *in vitro* stimulation with OVA peptide. As shown in Figure 1, effector T cells receiving antigen stimulation also exhibited dramatic upregulation of CD25 by 24 hours post-stimulation, whereas those T cells not receiving antigenic stimulation did not upregulate CD25. However, results from the different treatment conditions revealed that in the presence of CD28 blockade, activated T cells first upregulated (at 24 hours) and then rapidly downregulated their CD25 expression by 48 hours post stimulation. Antigen-specific CD8⁺ T cells stimulated in the presence of CTLA-4 Ig continued to further down-regulate this molecule with increasing time, such that by 96 hours post-stimulation it had returned to baseline levels similar to unstimulated controls. In contrast, antigen-specific CD8⁺ T cells in untreated samples maintained a high level of CD25 expression even to 96 hours post-stimulation. CD40/CD154 blockade-treated T cells exhibited a delayed reduction in CD25 expression, such that expression was similar to untreated controls at 48 and 72 hours but was significantly decreased at 96 hours post-transplant. Cultures treated with a combination of the two treatments did not exhibit a synergistic or additive reduction in CD25 expression levels, but instead mimicked the magnitude and kinetics of downregulation observed in samples treated with CTLA-4 Ig alone.

4.2 CD28 blockade results in division-dependent downregulation of CD25

Given the observation that CD25 is first upregulated and then subsequently downregulated in antigen-activated T cell cultures treated with CD28 blockade, we sought to determine whether this downregulation occurred in concert with cell division following stimulation. In order to address this, antigen-specific T cells were labeled with CFSE prior to stimulation with peptide antigen and treatment with CTLA-4 Ig to block CD28 signaling. As shown in Figure 2A (right panel), we observed a division-dependent downregulation of CD25 in the CTLA-4 Ig treated samples. This was in stark contrast to cells stimulated under conditions of sufficient CD28 signaling, which maintained high expression levels even after several rounds of cell division (Figure 2A, left panel). Analysis of the results of four independent cultures revealed that that increasing number of divisions significantly correlated with decreasing CD25 expression (Spearman $r = -0.7756$, $p < 0.001$), signifying that, under

conditions of CD28 blockade, cells progressively lose CD25 expression as they progress through the cell cycle (Figure 2B).

4.3 High precursor frequencies render CD28 blockade unable to downregulate CD25 following cell division

Thus far we have demonstrated that blockade of CD28 signaling dramatically reduced CD25 expression levels, especially in cells that had undergone several rounds of division. However, previous studies have shown that CTLA-4 Ig synergized with anti-CD25 monoclonal antibodies in inhibiting allograft rejection in BALB/c→B6 model [16]. Based on these findings, we hypothesized that there must be some circumstance under which CD28 blockade alone does not result in CD25 downregulation, such that the addition of anti-CD25 mAbs provides additional benefit in terms of graft survival. One such circumstance might be the high precursor frequencies that would be observed in fully allogeneic settings. Therefore, we compared high- versus low- frequency T cell responses *in vitro* by modifying the ratios of T cells to APCs during peptide stimulation. As depicted in Figure 3A, stimulation of T cells with peptide antigen under conditions of low frequency resulted in increased rounds of cell proliferation as compared to stimulation of T cells under high frequency conditions. These differences are quantified in Figure 3B, where it is apparent that the majority of T cells stimulated under conditions of low frequency had undergone >4 divisions (Figure 3B), whereas the majority of the T cells stimulated under conditions of high frequency had undergone <4 divisions (Figure 3C). These results are consistent with our previously published *in vivo* findings [18;19]. This difference in the number of rounds of division seen at high vs. low frequency was observed even under conditions of CD28 or CD40 pathway blockade as well as combined blockade (Figure 3A).

Next, we examined the level of CD25 expression on cells in each round of division in the high versus low T cell frequency cultures. As shown in Figure 3D, we observed high levels of CD25 expression on antigen-stimulated T cells in both high- and low T cell frequency cultures (left panels). However, when cells were treated with CTLA-4 Ig (as well as combined blockade), we again observed a division-dependent decrease in CD25 expression level. Importantly, this decrease was much more striking in the low T cell frequency cultures. The high T cell frequency cultures, in contrast, contained subsets of antigen-specific T cells that had only undergone 1–3 divisions, and thus had not downregulated their CD25 as much as the cells that had undergone >5 divisions in the low T cell frequency cultures. Thus, we conclude that precursor frequency indirectly impacted the ability of CD28 blockade to result in CD25 downregulation, through its ability to regulate the number of ensuing cell divisions following antigenic stimulation of T cell populations.

4.4 Exogenous IL-2 rescues CD25 expression on CD8⁺ T cells under conditions of CD28 blockade

Given the above results that CD8⁺ T cells stimulated under conditions of high T cell precursor frequencies retained CD25 expression, we speculated that higher precursor frequencies might increase the local concentration of IL-2 and this might be one mechanism by which CD25 expression is restored. In order to test this hypothesis, we stimulated naïve antigen-specific CD8⁺ T cells *in vitro* under low frequency conditions in the presence of exogenous IL-2 and asked whether this could restore the expression of CD25. As shown in Figure 4A, the addition of exogenous IL-2 resulted in the maintenance of CD25 expression at 48h, 72h, and 96h post-stimulation, even under conditions of CD28 and/or CD40/CD154 pathway blockade. Thus, the provision of exogenous IL-2 was able to override the lack of CD28 and/or CD40-mediated costimulatory signals and restored CD25 expression to levels observed in untreated controls (Figure 4B).

We also tested the impact of CD28 blockade on antigen-specific CD4⁺ OT-II T cell responses, and found a differential effect as compared to that observed in CD8⁺ T cells. Specifically, we found that antigen-specific CD4⁺ OT-II T cells that were stimulated with cognate OVA peptide in the presence of CTLA-4 Ig did not appreciably down-regulate their CD25 expression (Figure 5A). This is in contrast to what we observed in CD8⁺ T cells (Figure 1). However, treatment with anti-CD154 (MR-1) did result in an approximate 50% reduction in the level of CD25 surface expression. Also in contrast to our observations in antigen-specific CD8⁺ T cells, addition of exogenous IL-2 to the cell cultures was not able to “rescue” the downregulation of CD25 expression following CD154 blockade (Figure 5B). Thus, CD28 blockade has differential effects on CD8⁺ versus CD4⁺ antigen-specific T cell responses.

4.5 Blockade of CD25 *in vitro* further attenuates antigen-specific CD8⁺ T cell proliferation

The results presented above suggest that maintained expression of CD25 is one mechanism by which CD8⁺ T cells stimulated under conditions of high precursor frequency might escape the effects of CD28 blockade through CTLA-4 Ig. Thus, we hypothesized that simultaneous blockade of CD28 and CD25, using CTLA-4 Ig and an anti-CD25 monoclonal antibody (PC.61), would further reduce CD8⁺ T cell proliferation as compared to CD28 blockade alone. We stimulated CFSE-labeled antigen-specific CD8⁺ OT-I T cells with OVA peptide as described above either alone or in the presence of CTLA-4 Ig or CTLA-4 Ig plus anti-CD25 and measured proliferation by CFSE dilution 72 hours later. As shown in Figure 6A and 6B, results indicated that while CD28 blockade alone significantly attenuated CD8⁺ T cell proliferation, the addition of CD25 blockade further reduced the amount of proliferation. This reduction in proliferation, as measured by an increase in the CFSE MFI, was statistically significant between the CD28 blockade and CD28/CD25 blockade treated cells ($p=0.0133$).

In order to explore whether CD25 blockade would have similar effects on the activation and expansion of high-frequency donor-reactive T cell responses *in vivo*, we performed an experiment in which CFSE-labeled cells from a B6 animal were injected into an irradiated fully allogeneic BALB/c responder. Mice were treated with nothing, CTLA-4 Ig alone, or the combination of CTLA-4 Ig and anti-CD25 mAb. Three days later, recipients were sacrificed and CFSE-labeled H-2K^b B6 T cells were analyzed for the frequency of proliferating cells as measured by a decrease in CFSE fluorescence. Results indicated that while *in vivo* treatment with CTLA-4 Ig resulted in a decrease in the frequency of proliferating cells, the combination of CTLA-4 Ig and anti-CD25 led to a further reduction in the frequency of CFSE^{lo} dividing cells. Thus, these data provide *in vivo* evidence that blockade of CD25 cooperates with CD28 blockade to reduce the activation and expansion of donor-reactive T cell responses.

5. Discussion and References

In this study we demonstrated that blockade of the CD28 pathway resulted in a division-dependent down-regulation of the IL-2 receptor alpha chain (CD25) during T cell activation. This effect was apparent after 48 hours-post stimulation, when cells had begun cycling. Thus, we conclude that one mechanism by which CD28 blockade may exert its inhibitory effects is by decreasing the potential for signaling through the high affinity IL-2R (CD25) in responding cells, potentially limiting the survival of these cells. Importantly, this study has demonstrated that this effect was less pronounced on cells that had undergone fewer cell divisions, a situation which arises in T cell populations stimulated at an initial high frequency. In these instances, CD25 expression levels were maintained on subsets of cells having undergone fewer divisions, suggesting that these cells may be responsible for mediating costimulation blockade-resistant rejection *in vivo*.

These data and our previously published results [18] raise the question of the mechanism underlying the observed reduction in average number of cell divisions when T cells are stimulated at high versus low precursor frequency. We speculate that this is due to increased competition for antigen among antigen-specific T cells stimulated at high frequency; indeed, preliminary in vitro studies analyzing proliferation of antigen-specific CD8⁺ T cells with decreasing concentrations of cognate peptide revealed that as the amount of available antigen decreased, so did the degree of proliferation of the responding T cells (data not shown). This conclusion is further supported in the literature by studies demonstrating that increasing the precursor frequency of antigen-specific T cells results in increased competition for antigen both in vitro and in vivo [23;24].

These results have direct clinical implications in that they suggest a potential mechanism of synergy between CD28 blockade (belatacept) and CD25 blockade (basiliximab) under conditions of high CD8⁺ T cell precursor frequency. In cases of high degree of MHC matching, allospecific T cells would be expected to be present at low frequency and therefore CD28 blockade (belatacept) alone might be sufficient to inhibit IL-2 signaling through CD25, via downregulation of this molecule. Thus, additional induction therapy with basiliximab may not be necessary in these instances. In contrast, in cases of low degree of MHC matching, allospecific CD8⁺ T cells would be expected to be present at high frequencies and therefore CD25 expression may be maintained on subsets of cells following belatacept treatment. Indeed, our in vitro experiments combining CD28 and CD25 blockade demonstrated a statistically significant reduction in degree of antigen-specific CD8⁺ T cell proliferation as compared to CD28 blockade alone (Figure 6). Results from our study therefore provide an experimental foundation for the testing of basiliximab as an adjunct therapy for inhibiting costimulation blockade-resistant rejection in these instances. In support of this, previous results have demonstrated that belatacept and basiliximab synergized in promoting long-term graft survival in a renal transplant model in MHC disparate rhesus macaques [6]. A second major clinical implication of our study is that in settings of high MHC disparity where donor-reactive T cell precursor frequencies are predicted to be high, agents designed to globally reduce the initial frequency of T cells, such as anti-CD52 [25;26] or anti-CD3 mAbs [27], might synergize with CD28 costimulatory blockade. Specifically, these agents would be predicted to effectively lower the initial frequency of graft-specific T cells and thereby increase the ability of costimulatory blockade to result in CD25 downregulation on donor-reactive CD8⁺ T cell populations.

We hypothesized that the mechanism of CD28 blockade-induced CD25 downregulation on CD8⁺ T cells could be an autocrine feedback loop dependent on the presence or absence of IL-2. Several published studies as well as our unpublished results suggest that blockade of CD28 signaling dramatically decreases the amount of IL-2 produced by naïve T cells following antigenic stimulation [28;29;30;31]. Thus, we hypothesized that CD25 expression may be regulated in an autocrine positive feedback loop, such that when IL-2 is devoid from the microenvironment, the lack of CD25 signaling further downregulates CD25 on the cell surface. In order to test this possibility we conducted experiments wherein we added back exogenous IL-2 to the culture conditions, and observed a rescue in the surface expression of CD25 on CD8⁺, but not CD4⁺ T cells. These data suggest that the mechanism by which CD28 blockade reduced surface CD25 expression in CD8⁺ T cells is through inhibition of IL-2 production in an autocrine or paracrine manner.

Decreased CD25 expression on effector T cells during the course of an antidonor immune response could have important implications for the function of T_{reg} populations. High levels of expression of CD25 on effector T cells could act as a sink for IL-2 in the microenvironment, thus lowering the local concentration of this cytokine and decreasing T_{reg} function [13]. Thus, it is interesting to speculate that an additional effect of CD28 blockade-

induced downregulation of CD25 on responding effector cells could be to increase the activation and function of T_{regs}, and thereby further enhance graft survival.

In conclusion, our results suggest blockade of the CD28 pathway has division-dependent effects on the expression of CD25 on CD8⁺ T cells. Factors that impact the number of divisions of a responding T cell population, including naïve donor-reactive T cell precursor frequency, are likely to impact the relative strength of the effect of CD28 blockade on CD25 expression.

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Figure 1. Blockade of CD28 signaling through CTLA-4 Ig results in downregulation of CD25 on antigen-specific T cells

OT-I T cells were stimulated with OVA peptide (3×10^5 cells/well) in a 24-well plate in the presence of CTLA-4 Ig (100 $\mu\text{g/ml}$), and/or anti-CD154 (100 $\mu\text{g/ml}$) as indicated. A, At the indicated timepoints, cell cultures were harvested, counted, and stained with Thy1.1-PerCP, CD8-FITC, and CD25-APC for flow cytometric analysis. Data shown are gated on CD8⁺ Thy1.1⁺ OT-I T cells. B, Median fluorescence intensity (MFI) of CD25 is shown plotted over time post-stimulation. Data shown are representative of three independent experiments.

Figure 2. CD28 blockade results in division-dependent downregulation of CD25

OT-I T cells were CFSE-labeled and stimulated with OVA peptide (3×10^6 cells/well) in a 24-well plate in the presence of CTLA-4 Ig (100 $\mu\text{g}/\text{ml}$) where indicated. A, At 72 hours post-stimulation, harvested and stained with CD8, Thy1.1, and CD25. Data shown are gated on $\text{CD8}^+ \text{Thy1.1}^+$ cells. B, $\text{CD8}^+ \text{Thy1.1}^+$ were gated into subpopulations based on number of CFSE divisions. The results of four independent cultures are summarized in B, and show that increasing number of divisions significantly correlated with decreasing CD25 expression (Spearman $r = -0.7756$, $p < 0.001$).

Figure 3. High precursor frequencies render CD28 blockade unable to downregulate CD25 following cell division

OT-I T cells were CFSE-labeled and stimulated with OVA peptide at high (3×10^6 cells/well) or low frequency (3×10^5 cells/well) in a 24-well plate in the presence of CTLA-4 Ig (100 $\mu\text{g/ml}$) and/or anti-CD154 (100 $\mu\text{g/ml}$) as indicated. At 72 hours post-stimulation, cells were harvested and prepared for flow cytometric analysis. A, B, Data shown are gated on CD8⁺ Thy1.1⁺ T cells. Data shown are representative of four independent cultures. C, Results were averaged from four independent experiments demonstrated that the preponderance of T cells stimulated at low frequency had divided >4 times, whereas the preponderance of T cells stimulated at high frequency had divided <4 times ($p < 0.001$ by Mann-Whitney U-test).

Figure 4. Exogenous IL-2 rescues CD25 expression under conditions of CD28 blockade
 OT-I T cells were stimulated with OVA peptide (3×10^5 cells/well) in a 24-well plate in the presence of CTLA-4 Ig (100 $\mu\text{g/ml}$), and/or anti-CD154 (100 $\mu\text{g/ml}$) as indicated. Cultures were supplemented with 300 pg/ml of IL-2 at the time of stimulation. A, At the indicated timepoints, cell cultures were harvested, counted, and stained with Thy1.1-PerCP, CD8-FITC, and CD25-APC for flow cytometric analysis. Data shown are gated on CD8⁺ Thy1.1⁺ OT-I T cells. B, Median fluorescence intensity (MFI) of CD25 is shown plotted over time post-stimulation. Data shown are representative of three independent experiments.

Figure 5. Impact of CD28 blockade on antigen-specific CD4⁺ T cell responses

CD4⁺ OT-II T cells were stimulated with OVA 323–331 peptide (3×10^5 cells/well) in a 24-well plate in the presence of (100 $\mu\text{g/ml}$), and/or anti-CD154 (100 $\mu\text{g/ml}$) as indicated. A, At 72 hours post-stimulation, cell cultures were harvested, counted, and stained with Thy1.1-PerCP, CD4-FITC, and CD25-APC for flow cytometric analysis. Data shown are CD25 MFI of CD4⁺ Thy1.1⁺ OT-II T cells. Data shown are representative of two independent experiments.

Figure 6. *In vitro* CD25 blockade further attenuates antigen-specific CD8⁺ T cell proliferation CFSE-labeled antigen-specific CD8⁺ OT-I T cells (3×10^6 cells/well) with stimulated OVA peptide in a 24-well plate as described in Materials and Methods either alone or in the presence of CTLA-4 Ig (100 μ g/ml) or CTLA-4 Ig (100 μ g/ml) plus anti-CD25 mAb (P6.61, 10 μ g/ml). At 72 hours post-stimulation, cells were harvested and prepared for flow cytometric analysis. Data shown are gated on CD8⁺ Thy1.1⁺ cells. **A**, While CD28 blockade alone significantly attenuated CD8⁺ T cell proliferation, the addition of CD25 blockade further reduced the amount of proliferation. **B**, This reduction in proliferation, as measured by an increase in the CFSE MFI, was statistically significant between the CD28 blockade and CD28/CD25 blockade treated cells ($p=0.0133$ by Mann-Whitney U-test). Data are representative of two independent experiments performed in triplicate. **C**. 5×10^6 CFSE-

labeled B6 splenocytes were injected into an irradiated fully allogeneic BALB/c responder. Mice were treated with nothing, CTLA-4 Ig alone, or the combination of CTLA-4 Ig and anti-CD25 mAb (days 0 and 2, as described in Materials and Methods). Recipients were sacrificed at 72h and CFSE-labeled H-2K^b B6 T cells were analyzed by flow cytometry for the frequency of proliferating cells as measured by a decrease in CFSE fluorescence. Data shown are representative examples of three independent animals per group.