Selective Targeting of Human Alloresponsive CD8+ Effector Memory T Cells Based on CD2 Expression

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Selective Targeting of Human Alloresponsive CD8⁺ Effector Memory T Cells Based on CD2 Expression

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Abstract

Costimulation blockade, specifically CD28/B7 inhibition with belatacept, is an emerging clinical replacement for calcineurin inhibitor-based immunosuppression in allotransplantation. However, there is accumulating evidence that belatacept incompletely controls alloreactive T cells that lose CD28 expression during terminal differentiation. We recently have shown that the CD2-specific fusion protein alefacept controls costimulation blockade-resistant allograft rejection in non-human primates. Here, we have investigated the relationship between human alloreactive T cells, costimulation blockade sensitivity and CD2 expression to determine whether these findings warrant potential clinical translation. Using polychromatic flow cytometry, we found that CD8⁺ effector memory T cells are distinctly high CD2 and low CD28 expressors. Alloresponsive CD8⁺CD2⁺CD28⁻ T cells contained the highest proportion of cells with polyfunctional cytokine (IFNγ, TNF and IL-2) and cytotoxic effector molecule (CD107a and granzyme B) expression capability. Treatment with belatacept in vitro incompletely attenuated allospecific proliferation, but alefacept inhibited belatacept-resistant proliferation. These results suggest that highly alloreactive effector T cells exert their late stage functions without reliance on ongoing CD28/B7 costimulation. Their high CD2 expression increases their susceptibility to alefacept. These studies combined with in vivo non-human primate data provide a rationale for translation of an immunosuppression regimen pairing alefacept and belatacept to human renal transplantation.

Introduction

Alloreactive T cells play a pivotal role in the immune response against a transplanted organ. Prior alloantigen exposure is known to expand the size of the primed memory T cell repertoire, increasing the likelihood that a clinically relevant allospecific effector response will be generated. Recent evidence suggests that T cells responding to environmental antigens can exhibit cross-reactivity with donor alloantigens through various mechanisms collectively known as heterologous immunity, and thereby mediate allograft rejection with kinetics similar to a bona fide allosensitized population (1). Thus, memory T cells represent an important barrier to allotransplantation, even in alloantigen naïve individuals.

Compared to their naïve counterparts, memory T cells are long-lived and have rapid recall effector function with reduced activation requirements (2,3). Individuals with a higher precursor frequency of donor-reactive memory T cells are at increased risk of developing acute allograft rejection after transplantation (4). Memory T cells are known to be sensitive to calcineurin inhibitors (CNIs) (5), and CNIs have proven themselves effective in controlling T cell mediated rejection in the vast majority of cases. However, CNIs mediate
many undesirable effects that have stimulated a continuing search for efficacious but less toxic replacements.

Costimulation blockade (6), particularly blockade of the CD28/B7 pathway with the B7-specific fusion protein belatacept, has emerged as a promising replacement for CNIs (7). Interruption of the CD28/B7 pathway inhibits naïve T cell activation and confers the theoretical advantage of selective inhibition of T cell responses to specific antigens rather than broad polyclonal inhibition. Recently published results of the multicenter phase III BENEFIT study have shown that belatacept is associated with superior renal function, similar graft and patient survival rates, and favorable side effect profiles compared to the CNI cyclosporine 1 year after renal transplantation (7–11). Importantly, however, belatacept treated patients experienced a higher rate of early aggressive (although reversible) T cell mediated rejections compared to cyclosporine treated patients suggesting that a significant number of patients, even when selected to be non-sensitized as they were in these trials, have alloresponsive belatacept-resistant T cells.

Multiple studies have shown that primed donor-reactive T cells are resistant to the effects of CD28/B7 blockade (12,13). Effector memory T cells (TEM) are less dependent on CD28 costimulation for activation and are able to provide immediate cytokine and cytotoxic effector recall response in an antigen specific manner (14–20). Therefore, antigen experienced T cells that are equipped with the effector functions necessary to trigger alloimmune-mediated rejection despite CD28/B7 blockade warrant further investigation.

We recently showed that treatment with the CD28/B7-specific fusion protein abatacept, alefacept (LFA3-Ig), and sirolimus significantly prolonged renal allograft survival in non-human primates (21). Alefacept is a recombinant LFA-3/IgG1 fusion protein designed to bind to CD2 via the LFA-3 domain. Through steric hindrance, Fc portion complement activation, and Fc-dependent interactions with FcγRIII receptors on NK cells, alefacept is known to inhibit T cell function, evoke T cell lysis and induce T cell apoptosis, respectively (22,23). Alefacept has proven to be a safe and effective drug and is clinically approved for the treatment of the T cell mediated condition psoriasis (6,24). Importantly, treatment with alefacept was shown to significantly reduce the number of TEM cells in psoriatic lesions and preferentially eliminate peripherally circulating TEM cells in vivo in psoriasis patients (25,26). Similarly, we demonstrated that alefacept selectively depletes TEM cells while preserving naïve and central memory T cell compartments after renal transplantation in non-human primates, and in doing so, eliminated CD28/B7 blockade-resistant rejection (21).

Alefacept’s target antigen, CD2, serves as both an adhesion and costimulatory molecule through interaction with its ligand, LFA-3 (CD58), on antigen presenting cells (27–29). CD2 is up-regulated on activated and memory T cells (30–32), and ligation of CD2 lowers the threshold for T cell activation (28,33), activates NK (34) and dendritic cells (35), and enhances IL-12 responsiveness of activated T cells (36).

Given the pre-clinical efficacy of alefacept treatment in non-human primates and its availability for clinical use, we examined the relationship between memory T cells and CD2 expression in humans in vitro. We found that CD8+ TEM cells concomitantly gained CD2 expression and effector capabilities while losing CD28, commensurate with alloantigen induced activation and differentiation. These data demonstrate a reciprocal relationship between allospecific T cell differentiation and the progressive loss of the molecular pathway targeted by belatacept. We posit that these cells are the most likely effectors responsible for mediating belatacept-resistant rejection, and that they can be targeted selectively by alefacept. This provides a rationale for the use of alefacept as an adjuvant therapy in
belatacept-based regimens to prevent early T cell mediated rejections in human renal transplantation.

**Methods**

**PBMC isolation and monoclonal antibodies**

Fresh peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll density gradient centrifugation (BD Biosciences, Franklin Lakes, NJ). PBMCs were stained with the following monoclonal antibodies (mAbs): anti-CD3-Alexa 700, anti-CD2-PerCP-Cy5.5, anti-CD28-FITC, anti-CD28-PE, anti-CCR7-PE-Cy7, anti-CD4-V450 (BD Biosciences or BD Pharmingen), anti-CD28-PE-Cy7 and anti-CD8-APC-Cy7 (Ebioscience, San Diego, CA), and anti-CD45RA-Qdot655 (Invitrogen, Carlsbad, CA).

**Polychromatic flow cytometric analysis**

PBMCs (2.0–5.0 × 10^5) were incubated with appropriately titered directly conjugated mAbs for 15 minutes at 4°C, followed by washings using phosphate buffered saline (PBS) supplemented with 2% fetal bovine serum (FBS). All stained samples were kept at 4°C and protected from light. Flow cytometric analysis was performed using a BD Biosciences LSR II multicolor flow cytometer, and data were analyzed using FlowJo software (Tree Star, San Carlos, CA).

**Intracellular Cytokine Staining**

Fresh PBMCs were isolated from 10 stimulator-responder pairs. Stimulators were depleted of CD3^+^ cells using magnetic CD3 MicroBeads according to the manufacturer’s instructions (MACS, Miltenyi Biotec, Auburn, CA). Irradiated stimulators (4 × 10^5^) and responders (2 × 10^5^) were incubated in the presence of 1 μl/mL GolgiPlug containing brefeldin A (BD Biosciences). After 6 hours in culture, cells were surface stained with mAbs, permeabilized and fixed using an intracellular cytokine staining kit (BD Biosciences). Intracellular cytokine staining (ICCS) was performed with the following mAbs: anti-CD107a–APC, anti-TNF-APC, anti-IL-2-PE, anti-IFNγ-FITC (BD Biosciences), and anti-Granzyme B-PE (Invitrogen). Cells were washed, stored at 4°C and acquired within 24 hours.

**Mixed Lymphocyte Reactions**

One-way mixed lymphocyte reactions (MLRs) were performed using human PBMCs from 7 stimulator-responder pairs. Responder PBMCs (2x10^5) were labeled with carboxyfluorescein succinimidyl ester (CFSE, Invitrogen) with 1 μl of 10mM CFSE per 10^7^ cells at 37°C for 10 minutes, and then cultured with irradiated stimulator PBMCs (4 × 10^5^) in RPMI 1640 supplemented with 10% plasma from the responder at 37°C. Cells were treated with PBS, belatacept (100 μg/mL, Bristol-Myers Squibb, NYC, NY), alefacept (0.1 μg/mL-10 μg/mL, Astellas Pharma, Deerfield, IL), or a combination of belatacept and alefacept at the start of incubation. After 5 days in culture, cells were surface stained, washed and acquired immediately.

**Statistical analysis**

For statistical analysis of CD2 expression in T cell subsets, one-way ANOVA with Bonferroni post-test analysis was used. To determine the statistical significance of cytokine production, we used a non-parametric Wilcoxon ranked sum test to compare two subsets and a non-parametric Friedman test to compare three subsets. Proliferation in one-way MLRs was analyzed using a non-parametric Friedman test, and CD2 MFI in one-way MLRs was analyzed using a non-parametric Wilcoxon ranked sum test. All calculated p values were two-tailed analyses.
**Results**

**Effector memory T cells express the highest levels of CD2 among memory subsets**

We have previously reported that among CD4\(^+\) and CD8\(^+\) memory T cell subsets in rhesus macaques, effector memory T cells exhibit the highest levels of CD2 expression (21). However, there are multiple species-specific characteristics that differentiate rhesus monkey from human T cells, leaving a significant question as to whether these results directly translate to humans (37). In humans, but not in rhesus monkeys, CD4\(^+\) and CD8\(^+\) T cells can be divided into four distinct memory phenotypes based on CCR7 and CD45RA expression: naïve (T\(_N\); CCR7\(^+\)CD45RA\(^+\)), central memory (T\(_{CM}\); CCR7\(^+\)CD45RA\(^−\)), effector memory (T\(_{EM}\); CCR7\(^−\)CD45RA\(^−\)), and terminally differentiated effectors (T\(_{EMRA}\); CCR7\(^−\)CD45RA\(^+\)) (19). To define the relationship between CD2 expression and memory T cell subsets in humans, peripheral blood mononuclear cells (PBMCs) from 12 healthy individuals, ranging in age from 27 to 50, were analyzed. Representative samples of CD4\(^+\) and CD8\(^+\) memory T cell subsets are shown (Figure 1A). Consistent with our previous data in other primates, CD4\(^+\) and CD8\(^+\) T\(_{EM}\) cells consistently expressed the highest levels of CD2 as measured by mean fluorescence intensity (p<0.001, Figure 1B).

To further characterize the relationship between CD2 and memory T cells, we divided T cells into CD2\(^{hi}\) and CD2\(^{lo}\) populations and analyzed these subsets independently. Naïve T cells comprised the majority of the CD8\(^+\)CD2\(^{lo}\) population, whereas the overwhelming majority of CD8\(^+\)CD2\(^{hi}\) cells segregated into the T\(_{EM}\) and T\(_{EMRA}\) subsets (Figure 1C). Among CD4\(^+\) T cells, almost all CD4\(^+\)CD2\(^{lo}\) cells were naïve T cells. In contrast, the CD4\(^+\)CD2\(^{hi}\) subset was composed predominantly of T\(_{CM}\) and T\(_{EM}\) subsets (Figure 1D). Thus, the relative expression of CD2 co-segregated with memory T cell phenotypes, with highest CD2 expression being characteristic of T\(_{EM}\) cells.

**CD8\(^+\)CD2\(^{hi}\) T cells rapidly express cytokines and cytotoxic effectors after alloantigen stimulation**

In previous studies in rhesus macaques, we have shown that CD8\(^+\) T cells that differentiated into multi-cytokine producers (cells producing a combination of IFN\(_γ\), TNF and IL-2) also possessed the highest CD2 surface expression (21). These multi-cytokine producers have been shown to be functionally more robust compared to single cytokine producers or cytokine non-producers (18,38). To specifically evaluate the effector function of CD2\(^{hi}\) and CD2\(^{lo}\) T cells in response to alloantigen in humans, PBMCs from 10 stimulator-responder pairs were incubated for 6 hours directly ex vivo and intracellular staining for IFN\(_γ\), TNF and IL-2 was performed. CD8\(^+\)CD2\(^{hi}\) T cells yielded a dramatically increased proportion of dual IFN\(_γ\)/TNF producers compared to CD8\(^+\)CD2\(^{lo}\) cells (p=0.002, Figure 2A). In half of the stimulator-responder pairs tested, there was a substantial number of triple cytokine producers (IFN\(_γ\), TNF and IL-2), and among this group CD8\(^+\)CD2\(^{hi}\) cells displayed a higher percentage of triple cytokine producers compared to the CD8\(^+\)CD2\(^{lo}\) subset, (p=0.06; data not shown). Unstimulated samples yielded little to no cytokine production (data not shown).

Cytotoxic effector function, important in both allograft destruction (39) and antiviral immunity (40), is mediated in part through the release of granzymes and perforin. Degranulation of lytic granules also results in the transient cell surface expression of CD107a (LAMP-1) on cytotoxic T lymphocytes (41). Therefore, we assessed the expression of granzyme B and CD107a on human CD2\(^{hi}\) and CD2\(^{lo}\) T cells after alloantigen stimulation. Again, CD8\(^+\)CD2\(^{hi}\) cells were the most functionally active population, displaying a markedly increased expression of both granzyme B and CD107a (p=0.007; Figure 2B). CD4\(^+\)CD2\(^{hi}\) cells also exhibited increased cytokine and cytotoxic effector expression compared to CD4\(^+\)CD2\(^{lo}\) cells, albeit to a lesser extent than the CD8\(^+\) cohort.
(data not shown). Thus, T cells that expressed the highest levels of CD2 demonstrated the most robust effector function. These findings suggest that a T cell subset highly functionally relevant to the alloimmune response can potentially be targeted through elevated CD2 expression.

**CD8^+CD2^{hi}CD28^{-} T cells are the most functionally active subset of alloresponsive T cells**

Since CD8^+CD2^{hi} cells were the most functionally active effectors and therefore the most likely to mediate rejection episodes, we hypothesized that high CD2 expressors might also have down-regulated surface expression of CD28, and thereby the relevance of B7 costimulation for a recall response. Therefore, CD4^+ and CD8^+ T cells were segregated based on CD2 and CD28 expression. Within CD8^+ T cells, three distinct populations emerged: CD2^{lo}CD28^+, CD2^{hi}CD28^+ and CD2^{hi}CD28^- (Figure 3A). These subsets were subsequently gated based on CCR7 and CD45RA expression. CD8^+CD2^{lo}CD28^+ cells were primarily naïve T cells. Within the CD2^{hi} group, the majority of CD2^{hi}CD28^+ cells were TEM while the CD2^{hi}CD28^- population included both TEM and TEMRA subsets (Figure 3A), suggesting that loss of CD28 was characteristic of distally committed effectors. In most individuals, CD4^+ cells were split between CD2^{lo}CD28^+ and CD2^{hi}CD28^+ (Figure 3B). A CD4^+CD2^{hi}CD28^- population was not detected in almost half of those studied, with the average being 2% over all samples analyzed.

Since CD8^+CD28^- cells have lost dependence on the CD28/B7 pathway, the functionality of this population was evaluated in vitro via intracellular cytokine and cytotoxic effector molecule detection after allostimulation. Cells that acquired a distinctive phenotype characterized by high CD2 and low CD28 expression produced the most cytokines and cytotoxic effector molecules upon allostimulation (Figure 4, A and B). We found that among CD8^+ T cells, the proportion of dual and triple cytokine producers (Figure 5A and 5B) and dual cytotoxic effector molecule expressors (Figure 5C) was significantly increased in the CD2^{hi}CD28^- subset (p<0.001, p<0.05, p<0.001 respectively) compared to CD2^{lo}CD28^+ cells. Taken together, these data suggest that highly alloresponsive cells, which are strikingly CD28^- and therefore likely to be resistant to CD28/B7 blockade, are armed for immediate effector function.

**Belatacept-resistant alloresponsive proliferating CD8^+ T cells are phenotypically CD2^{hi}CD28^- effector T cells**

To correlate the proliferative capacity, memory phenotype and CD2 expression of alloreactive CD8^+ T cells, we performed mixed lymphocyte reactions (MLRs) using CFSE-labeled responder and irradiated stimulator PBMCs. CFSE-labeling enables the detection and analysis of successive generations of dividing cells. Cells were analyzed after 5 days in culture. Compared to non-dividing cells, CD8^+ alloreactive proliferating cells were overwhelmingly CD2^{hi}CD28^- (Figure 6A). Cells that divided were CCR7^- and CD45RA^-, consistent with an activated effector T cell phenotype.

To assess the in vitro effects of CD28/B7 blockade on alloantigen-driven proliferation, PBMCs in one-way MLRs were treated with 100 µg/mL of belatacept, a concentration that correlates with levels achieved in recent clinical trials (Bristol-Meyer-Squibb, unpublished data), at the start of incubation. There was 1.9- to 5-fold reduction in the percentage of CD8^+ proliferating cells treated with belatacept compared to untreated controls, indicating that a substantial number of proliferating cells were belatacept-sensitive (Figure 6B). In some individuals, a small proportion of alloreactive cells did proliferate despite belatacept treatment. Belatacept-resistant cells were largely CD2^{hi}CD28^- cells. CD2 expression was significantly increased in dividing cells as compared to nondondividing cells in both untreated and belatacept treated samples (both p=0.01; Figure 6C, 6D). Interestingly, there was a
substantial range of belatacept responsiveness amongst individuals suggesting that individual repertoires had variable propensities to contain belatacept-resistant T cell populations.

**Alefacept inhibits belatacept-resistant alloresponsive proliferating CD8**^+^ **T cells**

Since proliferating cells expressed the highest levels of CD2, we hypothesized that this population could be targeted using alefacept. To test this hypothesis, cells were treated with increasing doses of alefacept (0.1 to 10 µg/mL) alone or in combination with belatacept (100 µg/mL). Cells treated with alefacept alone demonstrated a dose-dependent decrease in both the number of total cell divisions as well as the overall percentage of cells divided, with an average 3-fold reduction in percent proliferation in cells treated with the highest dose of alefacept compared to the untreated group (Figure 7A, 7B). The combination of belatacept and alefacept had an additive effect, inhibiting proliferation to the level of unstimulated controls (p<0.001, Figure 7B).

**Discussion**

The advent of costimulation blockade offers a new class of immunomodulating agents for use in transplantation. Although the tolerogenic effects of costimulation blockade are documented in naïve animal models (42), heterologous immunity and memory T cells pose a formidable barrier to allograft tolerance in antigen experienced recipients (43,44). Though memory T cells are traditionally thought to function relatively independent of CD28/B7 costimulation for recall responses (45), those that have lost CD28 expression can be surmised to be completely indifferent to belatacept treatment. Indeed, a population of belatacept-resistant cells in 20–25% of patients undergoing renal transplantation is strongly suggested by recently published results from the BENEFIT study. Compared to the cyclosporine group, patients in the belatacept-treated groups had higher rates and histologically more severe rejection episodes (11,46). Despite the invasive nature on biopsy, these cells were readily mollified by standard rescue therapies suggesting they were late phase effectors with limited capacity for additional division. The data reported herein lead us to hypothesize that the observed increase rate of belatacept-resistant rejection can be attributed to alloreactive memory T cells, indicating the need for an adjuvant therapy that selectively targets these armed effectors.

The aim of this study was to characterize alloreactive TEM cells that are capable of mediating belatacept-resistant rejection and to identify a means specifically to neutralize them in humans. We showed that CD8^+^ TEM cells are characterized by high CD2 and low CD28 expression, and CD8^+^CD2^hi^CD28^-^ T cells are the most functionally active T cell subset. Since memory T cells are capable of rapid trafficking into allografts (47), CD8^+^CD2^hi^CD28^-^ T cells may home to allografts where they can release cytokines and cytotoxic effectors, recruit inflammatory cells, and mediate early rejection responses. This alloreactive CD8^+^CD2^hi^CD28^-^ TEM subset represents a potent barrier to successful CoB therapy, and adjunct treatment modalities are needed to control this rapidly responding, highly functional population.

In contrast to the significant CD28^-^ population found within CD8^+^ T cell populations, CD4^+^ T cells maintained their CD28 expression and should be adequately targeted by CD28/B7 blockade (48). Treatment with CTLA-4-Ig has been shown to inhibit naïve T cell activation and proliferation (49) and prolong graft survival in some animal models (21,50). Studies have shown that under certain conditions CD4^+^ help during the initial priming phase is necessary for cytotoxic T lymphocytes to mount an effective secondary response upon antigen re-encounter in the settings of both infection and transplantation (51–53). Additionally, evidence suggests a critical precursor frequency of CD4^+^ T cells is required.
for effective anti-donor CD8+ T cell responses (54). Thus, belatacept may inhibit the generation of a competent memory T cell population by limiting the initial activation and expansion of CD4+ T cells as well as curtailing CD4+ help during CD8+ T cell programming. This differential between CD4+ and CD8+ cells also may provide a rationale for the exceptional efficacy that CD28/B7 blockade has in preventing alloantibody formation.

We specifically studied the effect of belatacept and alefacept treatment on alloantigen-induced proliferation in one-way MLRs. In allostimulated samples without drug inhibition, there was a marked proliferation of CD8+ T cells that were CCR7−CD45RA− and CD2hiCD28− by day 5, consistent with a differentiated effector phenotype. Belatacept-resistant, alefacept-sensitive proliferating cells were also CD2hiCD28− by day 5. Two potential sources of these CD8+CD2hiCD28− T cells include alloreactive naïve T cells that have proliferated and differentiated into effector T cells, or alloreactive memory T cells that have undergone recall proliferation after antigen recognition. Recent reports have demonstrated that alloresponsive T cells are present in sorted naïve and memory T cells in approximately equal frequencies (55). We hypothesize that a large subset of these CD2hiCD28− proliferating effectors in fact were derived from naïve alloreactive precursors, since a large component of the response was inhibited following belatacept treatment.

One limitation of our study is the inability to determine the relative contributions or precursor frequencies of alloreactive naïve and memory T cells to the proliferative response. The ideal experiment would be to sort cells into naïve and memory phenotypes and test their individual proliferative capacity in a CFSE-MLR. However, based on our previous experience, once T cells are sorted and taken out of context, they are not capable of responding in the same manner as when the complete cellular and cytokine milieu is present (56). These findings may reflect the fact that terminally differentiated effectors have limited proliferative capacity, and that naïve CD8+ T cells proliferate poorly without CD4+ help.

In this study we have investigated two types of alloresponses: cytokine production and proliferation. It is unclear whether cells that are capable of cytokine production and those capable of proliferation are, in fact, generated from the same precursors. Since alloresponsive populations are derived stochastically, the type of alloresponse manifested may be dependent on the antigen experience of the responding populations. It is reasonable to hypothesize that antigen experienced, highly differentiated cells may have limited proliferative capacity but ample cytokine and cytotoxic effector function, while more naïve, antigen inexperienced cells are not armed for immediate cytokine release but are able to proliferate. We favor a model in which alloresponsive cytokine-producing cells are highly relevant in early, aggressive rejections, such as those observed in the BENEFIT trial, while proliferative responses may be more important in later rejections.

While TEm cells have been shown to be detrimental to allograft survival (57), the ability to recognize and rapidly respond to previously encountered pathogens is a critical feature of immune memory. Specifically targeting TEm cells may threaten the protective mechanisms necessary to guard against viral infections, particularly latent viruses such as CMV and EBV that are prone to reactivation in immunosuppressed patients. Studies in mice have shown that in the setting of chronic viral infection, antigen-specific CD8+ T cell responses are down-modulated in the presence of CoB (58,59). However, in clinical trials patients treated with belatacept have had similar rates of infection compared to patients on standard CNI therapy (8–10,11). Additionally, psoriasis patients treated with alefacept have not experienced increased infectious complications, although these patients have not been concomitantly treated with other immunosuppressive agents (60,61). Thus, more studies are
needed to explore fully the relationship between selectively depleting memory T cells and protective immunity in the context of transplantation.

In this study, there was an expected variation of CD2 and CD28 expression between healthy volunteers, commensurate with a spectrum of immunologic history. The few individuals with evidence of the highest degree of antigen experience, as reflected by larger \( T_{EM} \) and \( T_{EMRA} \) cell populations, were found to have a defined \( CD8^+CD2^+CD28^- \) subset, which has not previously been described but may be functionally important. These cells potentially could harbor resistance to both belatacept and alefacept and warrant further investigation. Indeed, in vitro phenotyping studies may assist in selection of patients responsive to less morbid CoB-based therapies, guide adjuvant therapy selection, and identify those who might be better served by CNI-based therapy from the start.

The aim of the current study was to define the relationship between CD2 expression, memory T cells and CoB-resistant rejection. We found that alloreactive \( CD8^+ T_{EM} \) cells were characterized by high CD2 expression, loss of CD28 expression, and rapid cytokine and cytotoxic effector function. Alloresponsive proliferating cells were \( CD2^hiCD28^- \). We have identified an alloreactive T cell population that is capable of escaping CoB inhibition and functionally armed to mediate allograft rejection. However, treatment with alefacept, a drug that is already available for clinical use, was able to attenuate CoB-resistant proliferation. This evidence suggests that alefacept selectively targets alloreactive, costimulation blockade-resistant cells and would be a good adjuvant therapeutic agent for use in a costimulation blockade-based immunosuppression regimen. The efficacy of alefacept and costimulation blockade in a non-human primate model of renal transplantation in conjunction with the in vitro findings in humans reported herein provide a clear rationale for a trial of alefacept and belatacept in human renal transplantation.

**Supplementary Material**

Refer to Web version on PubMed Central for supplementary material.

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**References**


Figure 1.
Characterization of memory T cell subsets by polychromatic flow cytometry. (A) PBMCs were isolated from healthy volunteers and surface staining was performed. Lymphocytes were identified using forward and side scatter. CD3+ T cells were then subdivided based on CD4 and CD8 expression. Cells were segregated into memory subsets (T_N, T_CM, T_EM, T_EMRA) according to CCR7 and CD45RA expression. (B) CD2 mean fluorescence intensity (MFI) of T cell subsets as measured by polychromatic flow cytometry was performed in triplicate, and a representative sample is shown. (C) CD8+ and (D) CD4+ T cells were divided into CD2_hi and CD2_lo populations and then evaluated for memory phenotypes based on CCR7 and CD45RA expression.
Figure 2.
Cytokine and cytotoxic effector molecule expression in alloreactive CD8⁺ cells. Responder PBMCs were incubated with irradiated, CD3⁺-depleted stimulator PBMCs for 6 hours directly ex vivo. Gating was performed by taking the nonaggregate population and selecting CD3⁺ cells. CD8⁺CD2⁺ T cells were subdivided into CD2⁺hi and CD2⁺lo subsets and analyzed for expression of (A) IFNγ, TNF and IL-2 or (B) IFNγ, CD107a and granzyme B, via intracellular cytokine staining.
Figure 3.
Characterization of memory T cell subsets based on CD2 and CD28 expression. (A) CD8^+ and (B) CD4^+ T cells were gated based on CD2 and CD28 expression and divided into 3 subsets: CD2^{lo}CD28^+, CD2^{hi}CD28^+ and CD2^{hi}CD28^- . All three subsets were subsequently evaluated for memory T cell phenotypes by CCR7 and CD45RA expression. Representative results from one individual are shown.
Figure 4.
Expression of intracellular cytokines and cytotoxic effector molecules by CD8+ T cells after alloantigen stimulation. CD8+ T cells were divided into subsets based on CD2 and CD28 expression. (A) Expression of IFNγ, TNF and IL-2 or (B) IFNγ, CD107a and granzyme B by CD8+ T cell subsets after 6 hour allostimulation.
Figure 5.
Percentage of CD8+ T cells expressing multiple cytokine or cytotoxic effector molecules after alloantigen stimulation. (A) Percentage of dual (IFNγ and TNF) and (B) triple (IFNγ, TNF and IL-2) cytokine producers, and (C) percentage of dual cytotoxic effector molecule expressors (CD107a and granzyme B) among CD8+ CD2/CD28 T cell subsets.
Figure 6.
Proliferation of alloreactive CD8$^+$ T cells in one-way mixed lymphocyte reactions (MLRs). (A and B) CFSE-labeled responder PBMCs were allostimulated for 5 days in one-way MLRs. CD8$^+$ T cells were divided based on proliferation and analyzed for memory subsets (CCR7 and CD45RA, left) or CD2 and CD28 expression (right). Percentage of divided CD8$^+$ T cells is shown. PBMCs were treated with (A) PBS or (B) belatacept (100 µg/ml) at the start of incubation. CD2 MFI of nondividing and dividing populations in both (C) untreated and (D) belatacept treated samples was measured. Results of 7 experiments are shown.
Figure 7.
Inhibition of proliferation of alloreactive CD8+ T cells in one-way MLRs. (A) CFSE-labeled PBMCs were allostimulated in one-way MLRs and treated with increasing doses of alefacept (0 to 10.0 µg/ml) alone (top row) or increasing doses of alefacept in combination with belatacept (100 µg/ml, bottom row). Percentage of divided CD8+ T cells is shown. (B) Percent of divided CD8+ T cells in untreated, belatacept, alefacept, and combined belatacept and alefacept treated samples from 7 unique responders is shown. Compared to untreated samples, * p<0.05, ** p<0.001.