American Journal of Transplantation 23 (2023) 1603–1611

Contents lists available at ScienceDirect

American Journal of Transplantation

journal homepage: www.amjtransplant.org

Brief Communication

Siplizumab combination therapy with belatacept or abatacept broadly inhibits human T cell alloreactivity in vitro

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ARTICLE INFO

Keywords:
dual costimulation blockade
CD2/CD58
CD28/B7
siplizumab
belatacept
abatacept
mixed lymphocyte reaction

ABSTRACT

Combined antigen-specific T cell receptor stimulation and costimulation are needed for complete T cell activation. Belatacept and abatacept are nondepleting fusion proteins blocking CD28/B7 costimulation, whereas siplizumab is a depleting antiCD2 immunoglobulin G1 monoclonal antibody targeting CD2/CD58 costimulation. Herein, the effect of siplizumab combination therapy with abatacept or belatacept on T cell alloreactivity in mixed lymphocyte reactions was investigated. In contrast to monotherapy, the combination of siplizumab with belatacept or abatacept induced near-complete suppression of T cell proliferation and increased the potency of siplizumab-mediated T cell inhibition. Furthermore, dual targeting of CD2 and CD28 costimulation enhanced the selective depletion of memory T cells compared with monotherapy. Although siplizumab monotherapy leads to significant regulatory T cell enrichment, high doses of cytotoxic T-lymphocyte-associated antigen 4 and a human IgG1 Fc fragment in the combination therapy reduced this effect. These results support the clinical evaluation of dual costimulation blockade, combining siplizumab with abatacept or belatacept, for the prophylaxis of organ transplant rejection and improvement of long-term outcomes following transplantation. Ongoing investigative research will elucidate when other forms of siplizumab-based dual costimulatory blockade may be able to induce similarly strong inhibition of T cell activation although still allowing for enrichment of regulatory T cells.

Abbreviations: CNI, calcineurin inhibitor; CTLA4-Ig, cytotoxic T-lymphocyte-associated antigen 4 and a human IgG1Fc fragment; IC50, half maximum inhibitory concentration; MLR, mixed lymphocyte reaction; PBMC, peripheral blood mononuclear cells; Treg, regulatory T cell; VPD450, violet proliferation dye 450.

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https://doi.org/10.1016/j.ajt.2023.05.032
Received 9 January 2023; Received in revised form 9 May 2023; Accepted 25 May 2023
Available online 1 June 2023

1. Introduction

Upon antigen recognition, T cell responses are regulated by signaling through complementary receptors expressed on T cells, a process known as costimulation. The development of selective costimulation-blocking biologics could improve long-term outcomes in transplant recipients that currently rely on broadly immunosuppressive small molecule maintenance therapy. Although potent in the prevention of graft rejection, calcineurin inhibitors (CNIs), such as cyclosporine and tacrolimus, are associated with a negative impact on patient morbidity and mortality when administered chronically.[1] Costimulation blockers abatacept and belatacept are fusion proteins of the extracellular domain of cytotoxic T-lymphocyte-associated antigen 4 and a human IgG1Fc-fragment (CTLA4-Ig).[2] Both bind B7 (CD80/CD86) on antigen-presenting cells and thus prevent costimulation through the B7/CD28 pathway. In a large phase III de novo renal transplant trial, belatacept improved patient/graft survival, kidney function, and decreased the incidence of donor-specific antibodies when compared with a cyclosporine-based regimen.[3] However, the benefits of belatacept use are counterbalanced by the increased risk and severity of acute rejection compared with CNIs.[3,4] Thus, despite the approval of belatacept in 2011 for use in kidney transplant recipients, most patients today receive traditional tacrolimus-based maintenance immunosuppression regimens.[5]

Although costimulatory blockade of a single pathway strongly reduces T cell activation, heterogeneous expression of costimulatory receptors on different subpopulations leaves some T cells unaffected. This may contribute to the relatively high rates of acute cellular rejection in clinical studies investigating belatacept-based immunosuppression. However, preclinical in vitro and in vivo evidence suggests that a combination of costimulation blockers, ie, dual costimulatory blockade, may be an effective modality to achieve more complete inhibition of T cell activation.[6,7] Namely, blocking independent costimulatory pathways that tend to display inverse expression patterns may adequately target the alloreactive T cell subpopulations. One example is the combined blockade of CD28/B7 costimulation and CD2/CD58 costimulation. CD2 plays an important role in the formation and organization of the immunological synapse between T cells and antigen-presenting cells.[8] Siplizumab is an investigational humanized antiCD2 IgG1 monoclonal antibody that is currently undergoing multiple phase II clinical trials. Siplizumab has shown potent immune modulatory properties in vivo, affecting both T and NK cells.[9-17] Herein, we investigated the effect of combining siplizumab with abatacept or belatacept on human T alloreactivity in mixed lymphocyte reactions (MLRs). Our results show that low doses of siplizumab suppress the proliferation of abatacept/belatacept-resistant T cells and enhance the selective depletion of memory T cells. Therefore, we propose a novel approach to combine blockade of CD28/B7 and CD2/CD58 costimulation as a potential strategy to replace standard CNI-based maintenance immunosuppression and improve long-term outcomes following transplantation.

2. Methods

2.1. Test substances

Abatacept and belatacept (Bristol Myers Squibb) were purchased from Apoteket Hjärtat AB. Siplizumab (humanized antiCD2 IgG1κ; ITB-MED), an investigational drug, was provided by ITB-MED AB.

2.2. Allogeneic mixed lymphocyte reaction

Peripheral blood mononuclear cells (PBMC) were isolated via Ficoll Paque Plus (Cytiva) density gradient centrifugation from buffy coats. Buffy coats were obtained from anonymous healthy adult donors from the Karolinska University Hospital blood bank, and PBMC isolation was performed within 24 hours of blood collection. In line with Swedish legislation section code 4§ 3p SFS 2003:460 (Lag om etikprövning av forskning som avser människor), an ethics approval was not needed since the blood bank provides buffy coats for research purposes only from anonymous donors that have given prior written informed consent, and biological material cannot be traced back to a specific individual. Human PBMC from 15 individual donors in total were isolated in 5 batches of 3 donors and mixed in pairs (donor 1 + 2, donor 1 + 3, and donor 2 + 3) resulting in 15 different MLR combinations. Specifically, PBMC from 2 donors were mixed in PBS at a concentration of 1.5 to 2.0 $\times$ 10^7 cells per mL and stained with violet proliferation dye 450 (VPD450; BD Biosciences) following the manufacturer's instructions. This study used a two-way allogeneic MLR, ie, PBMC from neither donor were inactivated via irradiation/chemical treatment. Thus, PBMC from both donors functioned as responders and stimulators. As this study investigated the effect of antibodies/fusion proteins on T cell responses in general and not the responsiveness of a specific donor, this setup was deemed satisfactory as a measure of alloreactivity.

VPD450-stained PBMC were washed and resuspended in 10% heat-inactivated FBS (Gibco) in AIM V medium (Gibco, Thermo Fisher Scientific Inc). Resuspended PBMC were dispensed into round-bottom 96-well cell culture plates, and pure medium or medium supplemented with siplizumab and/or abatacept or belatacept was added to a final concentration of 2 $\times$ 10^6 cells per mL in a final volume of 200 μL. MLRs were incubated at 37 °C, 5% CO_2 for 7 and 10 days, respectively. On days 5 or 6, 100 μL fresh culture medium (no additional antibody; final volume = 300 μL) was added to each well. On day 8, 100 μL medium was aspirated from each well without disturbing the cell pellet and replaced with 100 μL fresh culture medium.

Antibody concentrations used for siplizumab titrations were between 1 μg/mL and 50.8 pg/mL (10-step 3-fold serial dilution in culture medium). Siplizumab titrations with added CTLA4-Ig were supplemented with 100 μg/mL abatacept or belatacept throughout all serial dilution steps. Fusion protein concentrations used for abatacept/belatacept titrations were between 100 μg/mL and 0.1 pg/mL (10-step 10-fold serial dilution in culture medium). Abatacept/belatacept titrations with added siplizumab were supplemented with 1 μg/mL siplizumab throughout all serial dilution steps.

2.3. Flow cytometry

After 7 days of MLR, samples were blocked with an Fc-receptor binding inhibitor (Invitrogen; Thermo Fisher Scientific Inc) and then stained with antibodies against cell surface antigens. Full list of antibodies can be found in the respective Supplementary Tables. T cells were gated as $CD3^{+}CD56^{-}$. T cell subpopulations were defined as follows: naïve T cells: $CCR7^{+}CD45RA^{+}$; central memory T cells: $CCR7^{+}CD45RA^{-}$; effector memory T cells: $CCR7^{-}CD45RA^{-}$; terminally differentiated effector memory T cells: $CCR7^{-}CD45RA^{+}$. After 10 days of MLRs, intracellular FoxP3 staining was performed using the eBioscience FoxP3/transcription factor staining buffer set (Invitrogen; Thermo Fisher Scientific Inc) according to the manufacturer's instructions. T cells were gated as $CD3^{+}$. Regulatory T cells (Tregs) were identified as $CD4^{+}CD127^{-}CD25^{+}FoxP3^{+}$. Cell proliferation was assessed using VPD450 (VPD450high: nonproliferated; VPD450low: proliferated). Samples were stained in the dark at 4 °C and washed twice in saline solution, followed by analysis using a BD Celesta flow cytometer (BD Biosciences).

2.4. Graphs and statistical analysis

Visualization of results and statistical analysis of underlying data were carried out using GraphPad Prism 8 software (GraphPad Software). Data displayed in graphs can be found in the respective Supplementary Tables. Data were analyzed using one-way analysis of variance followed by Dunnett's multiple comparison test, with no antibody controls serving as the comparison data set.

3. Results

3.1. T cell proliferation

To assess the inhibitory effects of combining siplizumab with abatacept or belatacept, T cell proliferation was assessed using flow cytometry after 7 and 10 days of MLR (n = 6; Supplementary

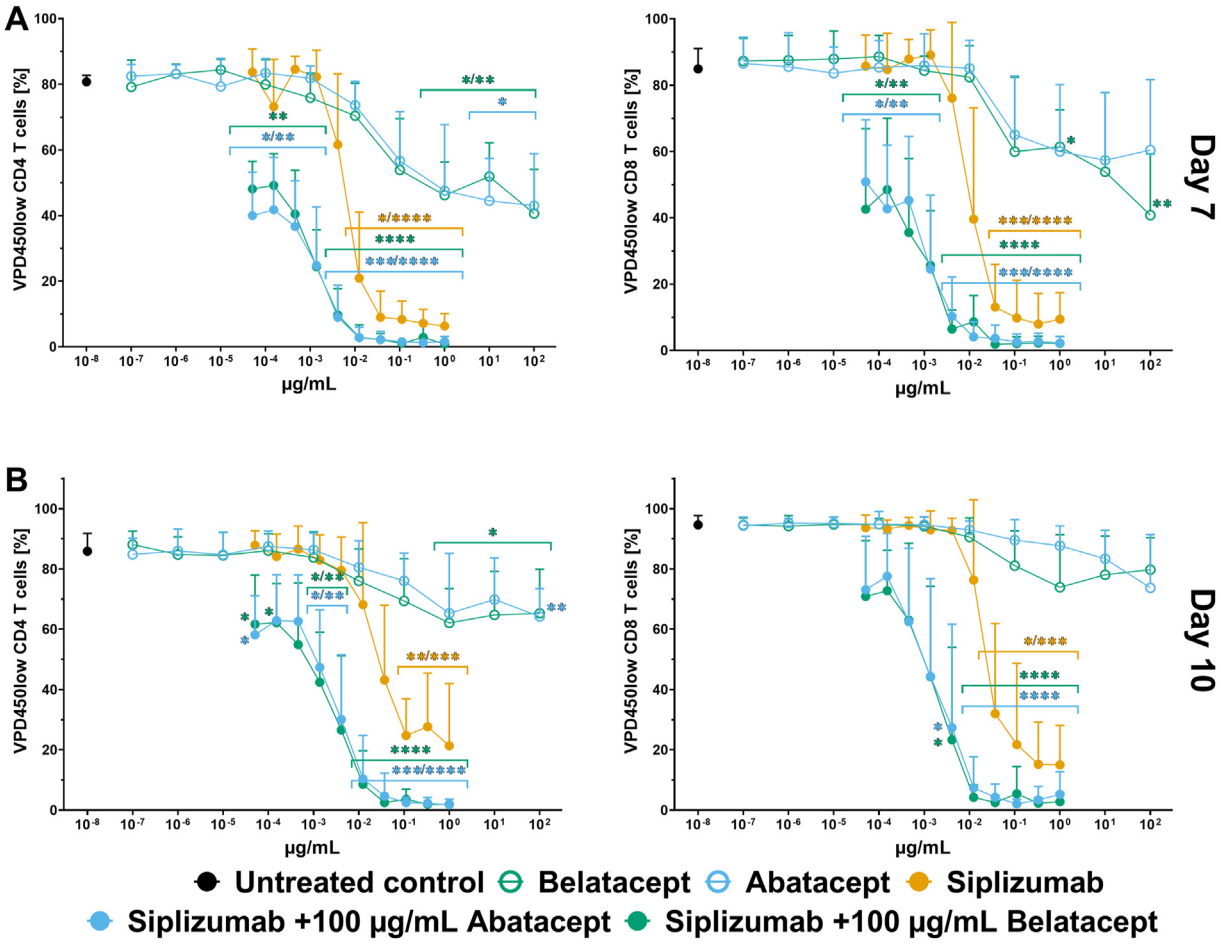

Figure 1. Inhibition of T cell proliferation by siplizumab and CTLA4-Ig combination therapy. The mean percentage of proliferated T cells in mixed lymphocyte reactions (MLRs) ± SD (n = 6). MLRs were supplemented with increasing concentrations of abatacept (1×10^{-7} to 100 µg/mL; blue open circle), belatacept ($1 \times$ 10-7 to 100 µg/mL; green open circle), and siplizumab (5.08×10^{-5} to 1 µg/mL; yellow). Further, additional titration series of siplizumab (5.08×10^{-5} to 1 µg/mL) were supplemented with 100 µg/mL of abatacept (blue filled circle) or 100 µg/mL of belatacept (green filled circle), respectively, throughout the entire titration series. Statistical analysis was conducted via one-way analysis of variance followed by Dunnett's multiple comparison test with untreated controls (no antibody; black) as the comparison data set (* $P < .05$, ** $P < .01$, *** $P < .001$, **** $P < .0001$). (A) Mean CD4 (left) and CD8 (right) T cell proliferation after 7 days of MLR. (B) Mean CD4 (left) and CD8 (right) T cell proliferation after 10 days of MLR. CTLA4-Ig, cytotoxic T-lymphocyte-associated antigen 4 and a human IgG1Fc-fragment; SD, standard deviation.

Tables 1–4). Relative to abatacept, belatacept has a higher affinity for B7 (especially CD86) due to 2 amino acid substitutions (A29Y and L104E).[2] As shown in Figure 1A, abatacept (10-100 μg/mL, $P \leq .0303$), belatacept (1-100 μg/mL, $P \leq .0156$), and siplizumab (0.012-1 μg/mL, $P \leq .0105$) induced a significant reduction in CD4 T cell proliferation after 7 days of MLRs relative to untreated controls. Further, abatacept (100 μg/mL, $P \leq .0028$), belatacept (1-100 μg/mL, $P \leq .041$), and siplizumab (0.11-1 μg/mL, $P \leq .0071$) induced a significant reduction in CD4 T cell proliferation after 10 days of MLRs (Fig. 1B).

Notably, monotherapy with each agent induced incomplete inhibition of CD4 T cell proliferation. In contrast, significant and near-complete inhibition of CD4 T cell proliferation was mediated by the combination of different doses of siplizumab with abatacept (100 μg/mL, $P \leq .0183$) or belatacept (100 μg/mL, $P \leq .0061$) (Fig. 1). Interestingly, combination therapy with abatacept or belatacept decreased the half maximum inhibitory concentration (IC50) for the suppressive effect of siplizumab on day 7 of MLRs from 0.0068 μg/mL (monotherapy) to 0.0015 μg/mL and 0.0011 μg/mL, respectively. Following 10 days of MLRs, the IC50 of siplizumab monotherapy was 0.0235 μg/mL and 0.0035 μg/mL or 0.0025 μg/mL for combination with 100 μg/mL Abatacept or 100 μg/mL Belatacept, respectively. As displayed in Figure 1 (right panels), a similar pattern was observed for inhibition of CD8 T cell proliferation. After 7 days of MLR, inhibition of CD8 T cell proliferation by siplizumab monotherapy had an IC50 of 0.0105 μg/mL whereas combination therapy with abatacept or belatacept lowered the IC50 of siplizumab to 0.0012 μg/mL in both combinations. Following 10 days of MLRs, siplizumab monotherapy had an IC50 of 0.024 μg/mL, which was lowered to 0.0017 μg/mL when combined with abatacept or belatacept.

It was further tested to determine which concentrations of abatacept or belatacept were required to reach maximum efficacy in combination with siplizumab. Concentrations of abatacept or belatacept were titrated although keeping the concentration of siplizumab fixed (n = 9; Supplementary Tables 5–8). Maximum inhibition of CD4 and CD8 T cell proliferation after both 7 and 10 days was reached when combining

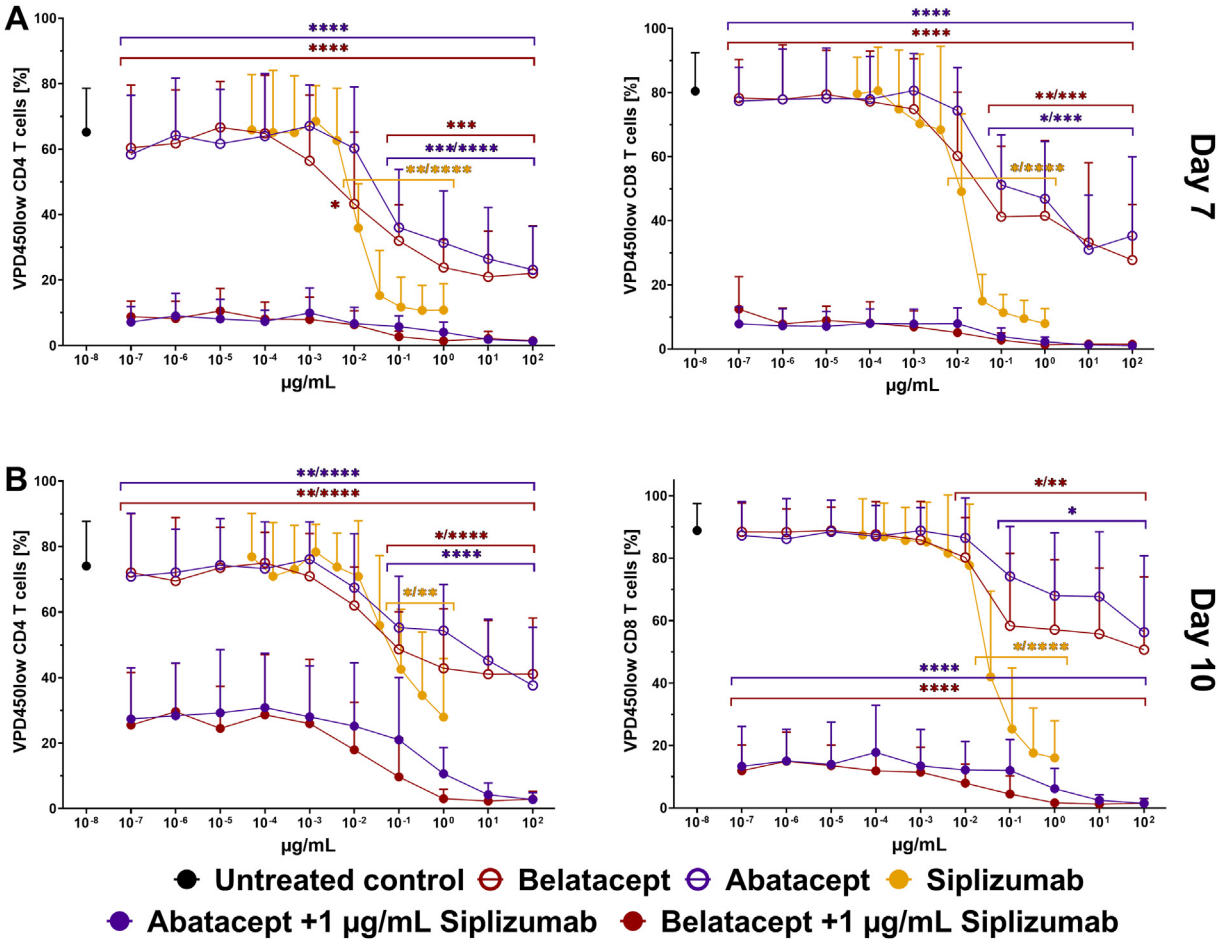

Figure 2. Inhibition of T cell proliferation by CTLA4-Ig and siplizumab combination therapy. The mean ± SD percentage of proliferated T cells in mixed lymphocyte reactions (MLRs) (n = 9). MLRs were supplemented with increasing concentrations of abatacept (1×10^{-7} to 100 μg/mL; purple open circle), belatacept (1×10^{-7} to 100 μg/mL; burgundy open circle), and siplizumab (5.08×10^{-5} to 1 μg/mL; yellow). Further, additional titration series of abatacept (purple filled circle) or belatacept (1×10^{-7} to 100 μg/mL; burgundy filled circle) were supplemented with 1 μg/mL siplizumab throughout the entire titration series. Statistical analysis was conducted via one-way analysis of variance followed by Dunnett's multiple comparison test with untreated controls (no antibody; black) as the comparison data set (* $P < .05$, ** $P < .01$, *** $P < .001$, **** $P < .0001$). (A) Mean CD4 (left) and CD8 (right) T cell proliferation after 7 days of MLR. (B) Mean CD4 (left) and CD8 (right) T cell proliferation after 10 days of MLR. CTLA4-Ig, cytotoxic T-lymphocyte-associated antigen 4 and a human IgG1Fc-fragment; SD, standard deviation.

abatacept (10-100 μg/mL) or belatacept (1-100 μg/mL) with siplizumab (1 μg/mL) (Fig. 2).

3.2. T cell depletion

To assess the effects of combining siplizumab (a T cell depleting agent[9]) and CTLA4-Ig (a nondepleting agent; Nulojix Public Assessment Report, European Medicines Agency, published July 7, 2011), T cell counts were determined using flow cytometry after 10 days of MLRs (n = 9; Supplementary Tables 9 and 10). As shown in Figure 3 (left panel), high doses (10-100 μg/mL) of either abatacept ($P \leq .0278$) or belatacept ($P \leq .0187$) reduced CD4 T cell counts compared with untreated controls. Relative to nondepleting CTLA4-Ig, siplizumab (0.037-1 μg/mL; $P \leq .0142$) monotherapy induced a stronger reductions in CD4 T cell counts. Similarly effective reduction in CD4 T cell counts were achieved across all combination therapies that paired siplizumab (1 μg/mL) with either abatacept ($P \leq .0264$) or belatacept ($P \leq .0132$).

As shown in Figure 3 (right panel), CD8 T cell counts were significantly reduced by siplizumab (0.037-1 μg/mL, $P \leq .0215$), abatacept ($P \leq .0391$) or belatacept ($P \leq .0154$) when dosed as CTLA4-Ig (0.1 100 μg/mL) monotherapies. Although investigating the combination therapies, the addition of siplizumab (1 μg/mL) to belatacept (1×10^{-7} to 100 μg/mL) attained CD8 T cell count reductions between 5- and 7-fold ($P \leq .0173$) compared with the untreated control. Interestingly, the combination of CD2 therapy with abatacept further reduced CD8 T cell counts, between 7- and 20-fold compared with the untreated control. In particular, the paring of siplizumab (1 μg/mL) with abatacept (100 μg/mL) was maximally effective in reducing CD8 T cell counts after 10 days of MLRs ($P = .0090$).

3.3. Naïve T cell enrichment

We have previously reported that siplizumab altered the relative abundance of specific T cell subsets by reducing the percentage of memory T cells in an MLR.[9] Here we assessed the effects of combining siplizumab and CTLA4-Ig on the percentage of naïve ($CCR7^+$ $CD45RA^+$) T cells using flow cytometry after 7 days of MLRs (n = 9; Supplementary Tables 11 and 12). As shown in Figure 4 (left panel), abatacept (0.1-100 μg/mL, $P \leq .0069$), belatacept (0.1–100 μg/mL, $P \leq .0050$) and siplizumab (0.012-0.11 μg/mL or 1 μg/mL, $P \leq .0437$) induced a significant enrichment in naïve CD4 T cells relative to untreated controls. The addition of siplizumab (1 μg/mL) to either abatacept ($P \leq .0391$) or belatacept ($P \leq .0408$) replicated the effect of high-dose siplizumab monotherapy and resulted in a 2-fold increase in the percentage of naïve CD4 T cells compared with the untreated control after 7 days of MLR.

As displayed in Figure 4 (right panel), a similar pattern was observed for inhibition of CD8 T cell proliferation. Namely, abatacept (0.1–100 μg/mL, $P \leq .0080$), belatacept (0.1-100 μg/mL, $P \leq .0091$), and siplizumab (0.012-1μg/mL, $P \leq .0385$) induced a significant enrichment in naïve CD8 T cells after 7 days of MLRs relative to the untreated controls. Finally, the maximal 3.5-fold enrichment in naïve CD8 T cells compared with the untreated controls was achieved when siplizumab (1 μg/mL) was added to either abatacept ($P \leq .0035$) or belatacept ($P \leq .0067$).

3.4. Treg enrichment

Additionally, we investigated how combination therapy with siplizumab and abatacept or belatacept affects Treg enrichment in MLRs treated with siplizumab. Following 10 days of MLRs, the percentage of Tregs among proliferated CD4 T cells was

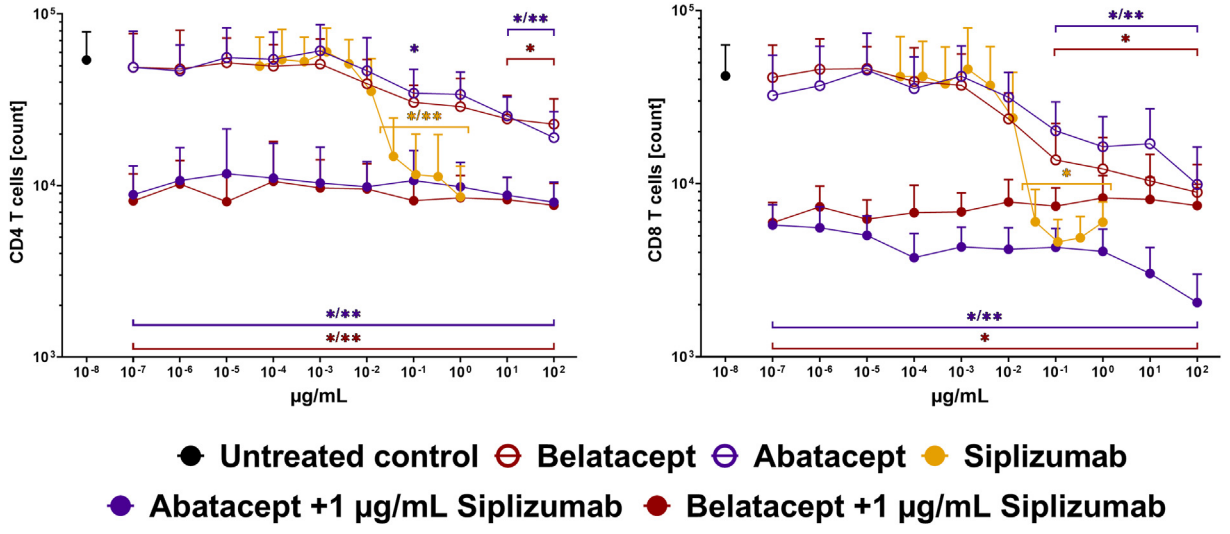

Figure 3. T cell depletion by CTLA4-Ig and siplizumab combination therapy. The mean ± SD T cell counts in mixed lymphocyte reactions (MLRs) (n = 9). MLRs were supplemented with increasing concentrations of abatacept (1×10^{-7} to 100 μg/mL; purple open circle), belatacept (1×10^{-7} to 100 μg/mL; burgundy open circle), and siplizumab (5.08×10^{-5} to 1 μg/mL; yellow). Further, additional titration series of abatacept (purple filled circle) or belatacept (1×10^{-7} to 100 μg/mL; burgundy filled circle) were supplemented with 1 μg/mL siplizumab throughout the entire titration series. Statistical analysis was conducted via one-way analysis of variance followed by Dunnett's multiple comparison test with untreated controls (no antibody; black) as the comparison data set (* $P < .05$, ** $P < .01$, *** $P < .001$, **** $P < .0001$). Mean CD4 (left) and CD8 (right) T cell counts after 10 days of MLRs. CTLA4-Ig, cytotoxic T-lymphocyte-associated antigen 4 and a human IgG1Fc-fragment; SD, standard deviation.

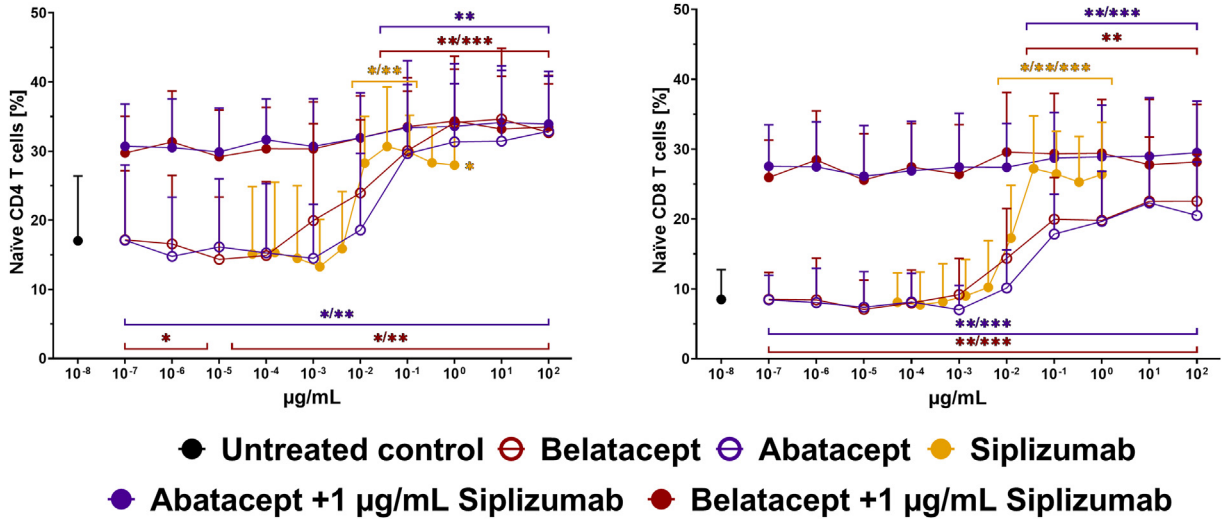

Figure 4. Naïve T cell enrichment by CTLA4-Ig and siplizumab combination therapy. The mean ± SD percentage of naïve T cells in mixed lymphocyte reactions (MLRs) (n = 9). MLRs were supplemented with increasing concentrations of abatacept (1×10^{-7} to 100 µg/mL; purple open circle), belatacept (1×10^{-7} to 100 µg/mL; burgundy open circle) and siplizumab (5.08×10^{-5} to 1 µg/mL; yellow). Further, additional titration series of abatacept (purple filled circle) or belatacept (1×10^{-7} to 100 µg/mL; burgundy filled circle) were supplemented with 1 µg/mL siplizumab throughout the entire titration series. Statistical analysis was conducted via one-way analysis of variance followed by Dunnett's multiple comparison test with untreated controls (no antibody; black) as the comparison data set (* $P < .05$, ** $P < .01$, *** $P < .001$, **** $P < .0001$). The mean ± SD percentage of naïve CD4 (left) and CD8 (right) T cells after 7 days of MLRs. CTLA4-Ig, cytotoxic T-lymphocyte-associated antigen 4 and a human IgG1Fc-fragment; SD, standard deviation.

measured using flow cytometry (n = 9; Supplementary Tables 13 and 14).

As previously reported and shown here in Figure 5 (left panel), siplizumab (0.037-1 µg/mL, $P \leq .0338$) induced significant enrichment of Tregs among proliferated CD4 T cells.[9,10] Whereas low doses of abatacept (10^{-7} to 1 µg/mL) or belatacept (10^{-7} to 10^{-2} µg/mL) combined with siplizumab (1 µg/mL) still resulted in significant Treg enrichment, the effect was lost (comparable to the untreated control) once the concentration of abatacept or belatacept increased to the point where maximum inhibition of T cell proliferation was reached (10-100 µg/mL abatacept or 1-100 µg/mL belatacept). In addition, given the strong reduction in

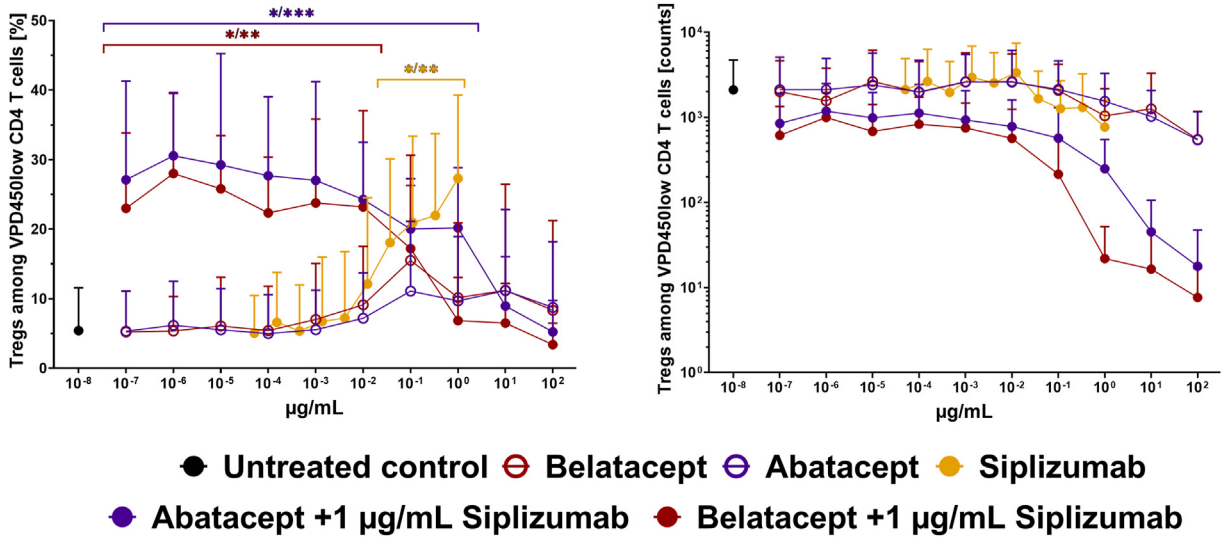

Figure 5. Regulatory T cell (Treg) enrichment. The mean ± SD percentage and cell counts of Tregs among proliferated ($VPD450^{low}$) CD4 T cells (n = 9). Mixed lymphocyte reactions (MLRs) were supplemented with increasing concentrations of abatacept (1×10^{-7} to 100 µg/mL; purple open circle), belatacept (1×10^{-7} to 100 µg/mL; burgundy open circle), and siplizumab (5.08×10^{-5} to 1 µg/mL; yellow). Further, additional titration series of abatacept (purple filled circle) or belatacept (1×10^{-7} to 100 µg/mL; burgundy filled circle) were supplemented with 1 µg/mL siplizumab throughout the entire titration series. Statistical analysis was conducted via one-way analysis of variance followed by Dunnett's multiple comparison test with untreated controls (no antibody; black) as the comparison data set (* $P < .05$, ** $P < .01$, *** $P < .001$). The mean ± SD percentage (left) and cell counts (right) of Tregs among proliferated (VPD450low) CD4 T cells after 10 days of MLRs. CTLA4-Ig, cytotoxic T-lymphocyte-associated antigen 4 and a human IgG1Fc-fragment; SD, standard deviation.

proliferation and total T cell counts presented in Figures 1-3, we investigated Treg cell counts among proliferated CD4 T cells using flow cytometry after 10 days of MLRs (Fig. 5; right panel). Neither CTLA4-Ig nor CD2 monotherapy resulted in significantly different Treg cell counts when compared with the untreated control. Interestingly, siplizumab (1 μg/mL) combinations with high-dose CTLA4-Ig therapy trended toward decreased Treg cell counts, but the reduction was not statistically significant after 10 days of MLRs among the subjects tested (n = 9).

4. Discussion

This study demonstrates that dual costimulation blockade with siplizumab and CTLA4-Ig (abatacept or belatacept) results in potent synergistic inhibition of both alloreactive CD4 and CD8 T cells. An increase in the potency of MLR inhibition of up to 14-fold was observed with the combination compared with siplizumab alone. Dual CD2/CD28 costimulation blockade additionally induced potent selective depletion of memory T cells. Specifically, the combination of siplizumab with abatacept reduced total CD8 T cell counts 20-fold, although it enriched the naïve CD8 T subset 3.5-fold, when compared with the untreated control in the MLRs setting.

The adoption of belatacept as first-line immunosuppression in kidney transplant recipients has been hindered by the increased risk of acute rejection, mostly within the first year posttransplant, when compared with CNI-based therapy.[18] Investigations into the mechanisms of belatacept-resistant rejection suggested the presence of alloreactive T cell clones that can escape CTLA4-Ig-mediated immune control. Specifically, belatacept-treated renal transplant recipients possessing a higher pretransplant frequency of $CD28^{+}CD4^{+}$ T cells (that could rapidly downregulate CD28 upon ex vivo stimulation) were more likely to experience acute rejection.[19,20] Additional immunophenotyping of kidney transplant recipients highlighted a multifunctional population of $CD57^{+}CD28^{-}CD2^{+}CD4^{+}$ T cells, associated with belatacept-resistant rejection and capable of infiltrating the graft.[21] The heterogeneous and inverse expression of CD2 and CD28 on human T cells generated the hypothesis that dual pathway targeting should provide coverage against all alloreactive T cells when they are dependent on at least 1 of these 2 costimulatory pathways. Encouragingly, belatacept-resistant alloreactive T cells ($CD2^{hi}CD28^{-}CD8^{+}$) have been shown to be susceptible to the treatment with the antiCD2 fusion protein alefacept in vitro.[6] The approach was extended into a preclinical nonhuman primate kidney transplantation model with promising tolerance induction results.[7] Alefacept is a fusion protein of the extracellular domain of CD58 (LFA-3) and a human IgG1 Fc fragment, that depletes CD2-expressing cells through an FcγRIII-dependent mechanism.[22] Notably, by constituting the natural ligand of CD2, alefacept can function as an agonist and loses its inhibitory function when Fc-silenced.[22,23] Although it was previously marketed for the treatment of psoriasis, alefacept has been voluntarily withdrawn from the market, and there are currently no marketed CD2-targeting treatments available. The investigational antiCD2 antibody siplizumab, currently in multiple phase 2 clinical trials, depletes effector memory, although sparing naïve conventional T cells, and promotes the expansion of alloreactive Tregs in vitro.[9,10] In vivo, as part of a conditioning regimen for hematopoietic stem cell or combined kidney-bone marrow transplantation, siplizumab enriched for functional donor-specific Tregs.[14-17] Given its unique binding and mechanisms of action, siplizumab may offer a more effective rejection prevention option than alefacept when combined with CTLA4-Ig.

Alternative de novo kidney transplant regimens have been designed to lower acute rejection rates by combining belatacept with a T cell-depleting agent. Two belatacept-based regimens, one involving alemtuzumab (a lymphocyte-depleting antiCD52 monoclonal antibody) and a second using rabbit antithymocyte globulin induction, demonstrated the potential benefits of CNI-free regimens.[18] However, acute rejection rates remained high, suggesting the need for broader control of alloreactive T cells. We have recently demonstrated in vitro that siplizumab may provide a superior tolerogenic effect compared with alemtuzumab and rabbit antithymocyte globulin, providing the rationale for the evaluation of a siplizumab + belatacept kidney transplant induction regimen.[1]

When concentrations of CTLA4-Ig exerting the maximum inhibitory effect together with siplizumab were used, the Treg enrichment seen with siplizumab monotherapy was diminished.[9,10] Our results are congruent with previous in vitro studies that describe a dose-dependent inhibition of Treg generation in MLRs by belatacept.[24] Tregs are dependent on CD28 costimulation, with CD86 acting as the dominant ligand that supports the utilization of IL-2 secreted by activated conventional T cells.[25] The strong antiproliferative effect of the combination of CTLA4-Ig and siplizumab in vitro could have reduced interleukin (IL)-2 concentrations below the threshold needed for Treg generation/survival. In a murine transplant model, high doses of CTLA4-Ig therapy conferred graft protection even after Treg depletion, suggesting that a strong net suppressive effect can compensate for the role of Treg cells.[26] Moreover, the use of IL-2/antiIL2-complexes improved the efficacy of CTLA4-Ig by counterbalancing its unfavorable effect on Tregs.[26] The effect of belatacept on Treg frequency and suppressive function remains inconclusive in the clinic, with limited numbers of patients immunophenotyped for this cell type.[27,28] Thus, one potential mechanistic drawback of the approach tested herein is that the addition of high-dose CTLA4-Ig appears to counteract the Treg enrichment properties normally exerted by siplizumab. Further investigations of other costimulatory pathways or alternative methods of targeting CD28 may identify a siplizumab pairing strategy where similarly broad suppression of T cell activation can be achieved without suppressing Treg survival or function. One possible approach includes antibody-mediated direct blockade of CD28, although sparing the CTLA-4 and PD-L1 coinhibitory signals, that is under investigation in the clinic.[29]

Although MLRs using primary human PBMCs provide a good mechanistic understanding of the therapeutics tested, they also constitute this study's largest inherent limitation. Further in vivo preclinical testing is impeded by siplizumab's phylogenetic restricted binding to human and chimpanzee CD2.[30] Siplizumab,

however, follows an expected biodistribution for IgG1 monoclonal antibodies and has been confirmed to exert its effector functions in both circulation and secondary lymphoid organs,[13] thereby increasing the clinical translatability of these in vitro findings.

Overall, our results demonstrate that even low doses of siplizumab combined with CTLA4-Ig are sufficient to enhance the selective depletion of memory T cells and completely attenuate any allospecific proliferation. The combined understanding of siplizumab's unique tolerogenic mechanisms of action and the in vitro findings presented herein provide a clear rationale for the clinical translation of an immunosuppression regimen combining siplizumab and CTLA4-Ig to human kidney transplantation.

Funding

The authors received no specific funding for this work.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: David Berglund reports financial support was provided by ITB-MED. David Berglund reports a relationship with ITB-MED that includes: employment, equity or stocks, and funding grants. David Berglund has patent pending to ITB-MED.Erik Berglund reports financial support was provided by ITB-MED. Erik Berglund reports a relationship with ITB-MED that includes: employment and equity or stocks. Erik Berglund has patent pending to ITB-MED. Filip Cvetkovski reports financial support was provided by ITB-MED. Filip Cvetkovski reports a relationship with ITB-MED that includes: employment. Filip Cvetkovski has patent pending to ITB-MED. The other authors have nothing to declare.

The authors of this manuscript have conflicts of interest to disclose as described by the *American Journal of Transplantation*. F. Cvetkovski, R. Razavi, E. Berglund, and D. Berglund are employees of ITB-MED. E. Berglund and D. Berglund own shares in ITB-MED. F. Sellberg is a former employee of ITB-MED. The ITB-MED Group has applied for patent protection for the use of siplizumab in combination with CTLA4-Ig treatment.

Disclosure

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Acknowledgments

The authors would like to thank J. Mark Hexham for reviewing the manuscript and providing thoughtful feedback.

Data availability

The data that support the findings of this study are available on request from the corresponding author.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ajt.2023.05.032.

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