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## Siplizumab selectively depletes effector memory T-cells and promotes a relative expansion of alloreactive regulatory T-cells *in vitro*

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### Abstract

Siplizumab, a humanized anti-CD2 monoclonal antibody, has been used in conditioning regimens for hematopoietic cell transplantation and tolerance induction with combined kidney-bone marrow transplantation. Siplizumab-based tolerance induction regimens deplete T cells globally while enriching regulatory T cells (Tregs) early post-transplantation. Siplizumab inhibits allogeneic mixed-lymphocyte reactions (MLRs) *in vitro*. We compared the impact of siplizumab on Tregs versus other T-cell subsets in HLA-mismatched allogeneic MLRs using PBMCs. Siplizumab predominantly reduced the percentage of CD4<sup>+</sup> and CD8<sup>+</sup> effector memory T cells, which express higher CD2 levels than naïve T cells or resting Tregs. Conversely, siplizumab enriched proliferating CD45RA<sup>-</sup> FoxP3<sup>HI</sup> cells in MLRs. FoxP3 expression was stable over time in siplizumab-containing cultures, consistent with enrichment for *bona fide* Tregs. Consistently, high-throughput TCRβ CDR3 sequencing of sorted unstimulated and proliferating T cells in MLRs revealed selective expansion of donor-reactive Tregs along with depletion of donor-reactive CD4<sup>+</sup> effector/memory T cells in siplizumab-containing MLRs. These results indicate that siplizumab

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#### Disclosure

The authors of this manuscript have conflicts of interest to disclose as described by the *American Journal of Transplantation*. Dr. Sykes is the head of the Scientific Advisory Board of ITB-Med AB. Dr. Berglund is the Chief Scientific Officer of ITB-Med AB. Dr. Sellberg and C. Binder are both employees of ITB-Med AB. The other authors have no conflicts of interest to disclose.

#### Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

may have immunomodulatory functions that may contribute to its success in tolerance-inducing regimens. Our studies also confirm that naïve in addition to effector/memory T cells contribute to the allogeneic MLR and mandate further investigation of the impact of sipilizumab on alloreactive naïve T cells.

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## Introduction

Siplizumab is a humanized IgG1 $\kappa$  monoclonal antibody directed against the human CD2 glycoprotein, a receptor expressed on virtually all mature human T cells and on the vast majority of thymocytes<sup>1</sup>. CD2 promotes the adhesion of T cells to antigen-presenting cells through its interaction with the ligand LFA-3 (CD58)<sup>2</sup>. Binding of CD2 to LFA-3 also leads to a cascade of intracellular signals necessary for T-cell activation, conferring an important costimulatory function to this molecule<sup>3–5</sup>, and blockade of CD2 has been shown to promote islet allograft tolerance induction in a murine model<sup>6,7</sup>.

Ligation of either sipilizumab or its parent antibody (BTI-322/Lo-CD2a) to human CD2 promotes T-cell depletion through antibody-dependent cell-mediated cytotoxicity (ADCC), an effect that is notably more pronounced on activated T cells<sup>8,9</sup>. In addition, these drugs efficiently inhibit the *in vitro* allogeneic mixed-lymphocyte reaction (MLR), with subsequent hyporesponsiveness upon allogeneic restimulation, while preserving reactivity to non-specific stimulation. This effect was found to be mediated, at least in part, by specific activation-associated T-cell depletion<sup>10,11</sup>.

We have previously used sipilizumab as part of the conditioning regimen for HLA-mismatched, haploidentical hematopoietic cell transplantation alone<sup>12</sup> or for combined HLA-mismatched kidney-bone marrow transplantation (CKBMT), a protocol that achieved allograft tolerance in association with transient mixed chimerism induction<sup>13,14</sup>. In recipients of hematopoietic cell transplantation to treat malignancies<sup>15</sup> and CKBMT recipients<sup>16</sup>, a marked early enrichment in regulatory T cells (Tregs) was observed during the T-cell reconstitution phase. Phenotypic analysis suggested that the increase in Tregs was due to a combination of de novo generation in the thymus and lymphopenia-driven expansion of residual T cells, occurring in the presence of antigenic pressure from the graft<sup>17</sup>. These observations were consistent with previous evidence indicating a suppressive/regulatory mechanism of tolerance in the first year after transplantation in CKBMT patients<sup>16</sup>. Consistently, we have used a TCR sequencing approach to demonstrate preferentially expanded donor-specific Tregs at 6 months post-transplant in the circulation of tolerant patients but not in a patient who failed to achieve tolerance<sup>18</sup>.

Taken together, these data point towards a possible novel, additional effect of sipilizumab as a selective modulator of alloimmunity towards the graft, i.e. the selective expansion of donor-specific suppressive Tregs. For these reasons, we studied CD2 expression on different T-cell subsets and tested the hypothesis that sipilizumab may selectively spare Tregs from depletion *in vitro* in HLA-mismatched allogeneic MLRs. We also analyzed TCR $\beta$  CDR3 repertoires to determine whether: (1) donor-specific Treg clones would be selectively expanded by allostimulation in the presence of sipilizumab; and (2) donor-specific effector/

memory T cells would be selectively depleted. Our data demonstrate these potential tolerance-promoting effects of sipilizumab.

## Materials and Methods

Detailed methods, including cell isolation, mixed lymphocyte reactions, flow cytometry analyses, cell sorting, genomic DNA isolation and sequencing, clonal and statistical analysis may be found in Supplementary Material.

### Allogeneic CFSE-Violet Dye MLRs

All experiments were performed with the approval of the local institutional review board (protocols IRB-AAAF0548 and IRB-AAAR0324). Human PBMCs from ten HLA-typed healthy donors were isolated from fresh whole blood. PBMCs from responder-stimulator pairs, selected to have the greatest number of HLA-mismatches available, were labelled with CFSE and Violet-Dye respectively, as described<sup>17</sup>, and combined in a 1:1 ratio. Sipilizumab (produced by Medimmune Oncology Inc, Gaithersburg, MD USA – Lot#: 01AZ19B-2 for ITB-Med AB, kindly provided by Dr. David H.Sachs) was added to cell suspensions at several concentrations (Supplementary Figure 1). Cells were cocultured at 37°C for 6 days. For prolonged MLR cultures, 500 µl of fresh MLR medium was added to each well at day 6 and day 9. In some experiments, modified MLRs were used, as described in Supplementary Methods.

### Clonal Analysis

Analysis of TCRβ CDR3 sequencing data was performed with R programming language, as detailed in the Supplementary Methods. CD4<sup>+</sup> and CD8<sup>+</sup> clones were defined as alloreactive based on the comparison between unstimulated and post-MLR CD4<sup>+</sup>/CD8<sup>+</sup> CFSE<sup>LO</sup> samples, as previously described<sup>19</sup>. Alloreactive CD4<sup>+</sup> clone sequences were compared with unstimulated CD4 Treg and CD4 Non-Treg sequences to determine to which subset these alloreactive clones belonged at the time of input, thus defining the alloreactive Treg and alloreactive Non-Treg subsets. A similar approach was applied to the analysis of alloreactive CD8<sup>+</sup> T cells (Supplementary Figure 3).

### Statistical Analysis

Continuous variables from experimental data were reported as mean ± SD or median [IQR], as appropriate. Parametric and non-parametric tests were employed to analyze unpaired and matched data, as fully detailed in the supplementary material. P-values for ANOVA and non-parametric equivalents are reported in the text, while significant differences in the follow-up comparisons are represented in figures. The relative change of the mean or median (range across drug concentrations) is provided for significant differences. Statistically significant differences were assumed at 5% level of probability. All analyses were performed with GraphPad Prism version 7 (GraphPad Software Inc, La Jolla, CA USA).

## Results

### CD2 expression in unstimulated T-cell subsets

Using PBMCs, we analyzed the relative surface expression of CD2 on unstimulated CD4<sup>+</sup> and CD8<sup>+</sup> Naive, Central Memory, Effector Memory and TEMRA subsets and on Treg subsets defined as resting (rTreg), non-suppressive (nsTreg) and activated (aTreg) according to Miyara et al<sup>20</sup> (Figure 1A). CD2 expression levels were significantly different among the T-cell subsets analyzed ( $p < 0.0001$  with both siplizumab and RPA-2.10 mAbs). Both of the anti-CD2 antibodies employed (Figures 1B and 1C) returned similar results in terms of relative CD2 density, with memory T cells showing the highest expression of the molecule.

Our studies with both mAbs confirmed previous studies<sup>21</sup> indicating that naïve T cells express lower levels of CD2 compared to central and effector memory T cells. This observation applied to both CD4<sup>+</sup> and CD8<sup>+</sup> subsets. Interestingly, Tregs appeared to have lower CD2 expression than their effector counterparts: the rTreg subset consistently showed the lowest CD2 expression among the subsets investigated, with statistically significant differences compared to most of the other subsets (Figure 1B,C). The level of CD2 on nsTregs was lower than that on the memory subsets and the level on aTreg was lower than that on CD4<sup>+</sup>effector memory T cells.

### Effect of Siplizumab on T-cell proliferation and CD2 expression in the allogeneic MLR

Addition of siplizumab at all concentrations tested (0.1 – 50 µg/mL) to the allogeneic HLA-mismatched MLR resulted in a significant reduction of total CD4<sup>+</sup> and CD8<sup>+</sup> T-cell absolute numbers (Supplementary Table 1). This reduction was much more pronounced for proliferating T cells (CFSE<sup>LO</sup> CD4<sup>+</sup>: [-98.5%;-99.3%],  $p < 0.0001$ ; CFSE<sup>LO</sup> CD8<sup>+</sup>: [-98.3%;-99.3%],  $p < 0.0001$ ), but was significant even for non-proliferating T cells (CFSE<sup>HI</sup> CD4<sup>+</sup>: [-46.5%;-58.8%],  $p = 0.0008$ ; CFSE<sup>HI</sup> CD8<sup>+</sup>: [-66.3%;-74.6%],  $p = 0.0004$ ) (Figure 2A and B, Supplementary Figure 4). These findings validate the previous suggestion that the depleting effect of siplizumab is preferentially directed toward activated T cells<sup>9</sup>. Additionally, a dose effect was evident when the ratio of CFSE<sup>HI</sup> to CFSE<sup>LO</sup> T cells was considered, showing that increasing drug concentrations had a greater effect on proliferating than non-proliferating cells in both the CD4<sup>+</sup> and CD8<sup>+</sup> subsets ( $p < 0.0001$  for both) (Supplementary Figure 5).

No difference in CD69 or HLA-DR surface expression was observed between control and siplizumab-containing cultures on days 1, 2 or 4 of MLR, whereas at day 7 the expression of both markers was significantly lower in siplizumab-treated samples compared to control (Supplementary Figure 6). Of note, CD69 and HLA-DR expression post-MLR was directly correlated to the frequency of proliferating T cells, which expressed higher levels of both markers compared to resting T cells (data not shown). No dose-response relationship could be observed in the tested concentration range.

Analysis of CD2 expression by indirect staining (Siplizumab + anti-human IgG Fc) on CD3<sup>+</sup> cells revealed significant surface CD2 down-regulation post-MLR in cultures containing siplizumab compared to controls ([-93.5%;-95.0%],  $p = 0.0089$ ) (Figure 2C and D). This effect was apparent in both CFSE<sup>HI</sup> and CFSE<sup>LO</sup> CD3<sup>+</sup> cells ([-87.2%;-90.1%],  $p = 0.0052$

and [-93.4%;-94.8%],  $p = 0.0162$ , respectively). Proliferating cells showed higher CD2 expression than non-proliferating cells in control MLRs (+358.9%,  $p = 0.0184$ ) and to a lesser extent in cultures containing sipilizumab ([+123-3%;+138.6%],  $p < 0.05$  at every drug concentration). Levels of surface-bound sipilizumab over time during the MLR were also tracked with a secondary antibody (Supplementary Figure 7).

### Effect of sipilizumab on CD8<sup>+</sup> and CD4<sup>+</sup> FoxP3<sup>-</sup> subsets in the allogeneic MLR

Sipilizumab reduced the absolute number of cells in all subsets recovered from the allogeneic MLR at day 6 of culture (Supplementary Table 1), and also consistently modified the relative abundance of specific T-cell subsets compared to the control MLR (Figure 3 and Supplementary Figure 8). Overall, the percentage of both CD4<sup>+</sup> and CD8<sup>+</sup> naive T cells increased in sipilizumab-containing cultures compared to the control MLR ([+105.9%; +118.5%],  $p < 0.0001$  and [+52.4%;+60.3%],  $p = 0.0002$ , respectively). In addition, the TEMRA fraction of CD8<sup>+</sup> T-cells was significantly increased with sipilizumab ([+40.4%; +59.6%],  $p < 0.0001$ ). On the other hand, T-cell memory subsets were reduced by sipilizumab addition to the culture, with a significant reduction of CD8<sup>+</sup> central memory ([-73.4%;-74.6%],  $p = 0.0018$ ) and CD4<sup>+</sup> and CD8<sup>+</sup> effector memory T cells ([-63.3%; -65.1%],  $p < 0.0004$  and [-52.7%;-59.3%],  $p = 0.0013$ , respectively). Most of these changes were already evident with the lowest concentration of the drug.

Separate analysis of non-proliferating (CFSE<sup>HI</sup>) subsets (Supplementary Figure 8) revealed a significant albeit marginal reduction of CD8<sup>+</sup> central memory ([-37.4%;-42-4%],  $p = 0.0005$ ), as well as a small decline in CD4<sup>+</sup> and CD8<sup>+</sup> effector memory T cells ([-23.1%; -26.5%],  $p = 0.0034$  and [-29.1%;-36.1%],  $p = 0.0007$ , respectively) in sipilizumab-containing cultures. Proliferating (CFSE<sup>LO</sup>) subsets (Supplementary Figure 8) showed significant reductions in CD4<sup>+</sup> and CD8<sup>+</sup> effector memory T cells ([-57.5%;-66.8%],  $p = 0.0019$  and [-34.9%;-52.0],  $p < 0.0001$ , respectively) in the presence of sipilizumab.

### Effect of sipilizumab on CD4<sup>+</sup>FoxP3<sup>+</sup> T cells in the allogeneic MLR

The absolute numbers and frequencies of CD4<sup>+</sup> CD25<sup>+</sup> CD127<sup>-</sup> FoxP3<sup>+</sup> cells among total (CFSE<sup>HI</sup> plus CFSE<sup>LO</sup>) CD3<sup>+</sup> T cells (Figure 4A and 4C, Supplementary Figure 4) decreased significantly ([-55.0%;-59.4%],  $p < 0.0001$ ) in sipilizumab-containing cultures compared to controls, as did the percentages of CD45RA<sup>-</sup> FoxP3<sup>LO</sup> (nsTregs) and CD45RA<sup>-</sup> FoxP3<sup>HI</sup> (aTregs) cells ([-75.7%;-78.4%],  $p < 0.0001$  and [-63.2%;-72.0%],  $p = 0.0002$ , respectively). Consistent with their lower CD2 expression, however, CD45RA<sup>+</sup> FoxP3<sup>LO</sup> cells (resting phenotype) showed a significant frequency increase ([+98.9%;+109.2%],  $p = 0.0067$ ) in sipilizumab-containing MLR compared with control. No significant differences in these FoxP3<sup>+</sup> subsets were detected among the non-proliferating cells (CFSE<sup>HI</sup>; Figure 4C). However, when proliferating cells (CFSE<sup>LO</sup>) were analyzed separately (Figure 4B and 4C), we observed a significant increase in the percentage of CD4<sup>+</sup>FoxP3<sup>+</sup> cells ( $p = 0.0163$ ), with borderline significance at high drug concentrations on the post-hoc test (10 µg/mL +70.0%,  $p = 0.05$  and 50 µg/mL +72.3%,  $p = 0.06$ ). This difference was clearly due to a marked enrichment in CD45RA<sup>-</sup> FoxP3<sup>HI</sup> Tregs (i.e. those with an activated phenotype) compared to control ([+250.9%;+262.6%],  $p = 0.0002$ ), while CD45RA<sup>-</sup> FoxP3<sup>LO</sup> Tregs (i.e. those with a non-suppressive phenotype) showed a non-significant tendency to decline ( $p = 0.10$ ).

in siplizumab-containing cultures. Overall, ratios of CFSE<sup>LO</sup> CD4<sup>+</sup> FoxP3<sup>+</sup> cells to CFSE<sup>LO</sup> CD4<sup>+</sup> or CFSE<sup>LO</sup> CD8<sup>+</sup> T cells (Figure 4D) were significantly increased by siplizumab ([+70.5%;+75.0%],  $p = 0.0034$  and [+42.2%;+79.3%],  $p = 0.0073$ , respectively), with a significant increase also observed in ratios of CFSE<sup>LO</sup> CD45RA<sup>-</sup> FoxP3<sup>HI</sup> to both CFSE<sup>LO</sup> CD4<sup>+</sup> and CFSE<sup>LO</sup> CD8<sup>+</sup> cells ([+235.2%;+247.5%],  $p < 0.0001$  and [+201.9%;+356.7%],  $p < 0.0001$ , respectively).

### Stability of FoxP3 expression over time in Siplizumab-containing and control cultures

FoxP3 not only identifies Tregs, but is also expressed by activated human conventional T cells that lack suppressive function. However, FoxP3 expression in these cells tends to be lower and more transient compared to that of Tregs<sup>22–24</sup>. In order to differentiate between transient activation-dependent and stable Treg-associated FoxP3 expression, we extended four allogeneic HLA-mismatched MLRs until day 13. Phenotypic data were obtained by applying the same gating to the analysis at every time point in a given MLR, and results from days 6, 9 and 13 were compared (Figure 5, Supplementary Figures 9–10). We identified Tregs according to expression of CD4, FoxP3, CD25 and CD127 and assessed their frequency in total T cells as well as in proliferating (CFSE<sup>LO</sup>) and in non-proliferating (CFSE<sup>HI</sup>) T cells separately. As expected, the frequency of FoxP3<sup>+</sup> cells among total T cells showed a progressive decrease from day 6 to day 13 in the control MLR, likely due to progressive loss of FoxP3 expression by activated conventional T cells. On the other hand, the percentage of FoxP3<sup>+</sup> cells in siplizumab-containing cultures showed an opposite trend over time, increasing by approximately 2- to 4-fold on day 13 compared to day 6. When linear regression of FoxP3 expression over time was performed, a statistically significant difference between the slopes was observed between siplizumab-containing and control MLRs ( $p < 0.0001$ ) (Supplementary Table 2), which was most pronounced at the lowest drug concentration. At day 13 post-culture, the total CD4<sup>+</sup> FoxP3<sup>+</sup> CD25<sup>+</sup> CD127<sup>-</sup> Treg percentage was significantly greater in siplizumab-containing compared to control MLRs ([+120.6%;+255.9%],  $p = 0.0233$ ) (Figure 5C). Similar trends were observed when the same analysis was performed on proliferating T-cells (Figure 5C), for which linear regression analysis of FoxP3 expression over time also revealed a statistically significant difference between siplizumab-containing and control MLRs ( $p = 0.0042$ ). The borderline significant difference in percentage of FoxP3<sup>+</sup> cells observed at day 6 between siplizumab-containing and control MLRs progressively increased over time, reaching statistical significance at both day 9 ([+411.6%;+474.8%],  $p = 0.0177$ ) and day 13 ([+537.3%;+770.4%],  $p = 0.0103$ ). Conversely, the percentage of FoxP3<sup>+</sup> cells among non-proliferating T cells in siplizumab-containing MLRs did not differ significantly from that in control MLRs at days 9 and 13 (Figure 5C).

To gain further insight into the commitment of post-MLR FoxP3<sup>+</sup> T cells, we assessed the FoxP3 promoter methylation status of CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup> cells sorted after a 14 days MLR, cultured with and without the addition of siplizumab, and compared it with that of sorted unstimulated Tregs isolated from the same donors. The FoxP3 promoter region, known as the Treg-specific demethylated region (TSDR) is heavily methylated in activated human conventional T cells, whereas suppressive Tregs display complete FoxP3 promoter demethylation<sup>25</sup>. The TSDR methylation status did not vary significantly over time in

cultures to which sipilizumab was added ( $-13.7\%$ ,  $p = 0.58$ ), whereas the TSDR in FoxP3<sup>+</sup> cells had a more methylated phenotype compared to unstimulated Tregs in control MLRs ( $+92.2\%$ ,  $p = 0.0096$ ) (Supplementary Figure 11).

### Clonal Analysis

The relative and sustained enrichment of FoxP3<sup>+</sup> cells among proliferating T-cells in sipilizumab-containing MLRs strongly supported the hypothesis that these were alloreactive Tregs. This enrichment could theoretically be due to a combination of three different mechanisms, including selective depletion of alloreactive non-Treg cells, expansion of pre-existing alloreactive Tregs and/or the induction of alloreactive Tregs from CD4<sup>+</sup> effector T cells during the culture period. In order to obtain additional insight into this phenomenon, we performed high-throughput TCR $\beta$  CDR3 sequencing on genomic DNA extracted from unstimulated CD4<sup>+</sup> and post-MLR CFSE<sup>LO</sup> CD4<sup>+</sup> sorted T cells. Using this method, alloreactive CD4<sup>+</sup> T cell sequences were identified in eight pairs of Sipilizumab-containing (1  $\mu\text{g}/\text{mL}$ ) and control MLRs (Supplementary Table 3). By additionally interrogating FACS-sorted, unstimulated CD4<sup>+</sup> Treg and CD4<sup>+</sup> Non-Treg sequences for these alloreactive CD4<sup>+</sup> sequences, we were able to map their origin to the Treg or Non-Treg populations in the unstimulated sample.

The fraction of unique alloreactive CD4<sup>+</sup> sequences that mapped to the Treg fraction of the unstimulated population was significantly higher in sipilizumab-containing MLRs compared to control cultures ( $+110.9\%$ ,  $p = 0.0078$ ) (Figure 6A). Consistently, the cumulative frequency of sequences identified as alloreactive Treg in the alloreactive CD4<sup>+</sup> subset increased significantly with sipilizumab compared to control ( $+132.7\%$ ,  $p = 0.0078$ ) (Figure 6B). On the other hand, both the clone fraction and the cumulative frequency of identifiable Non-Treg among alloreactive CD4<sup>+</sup> cells was significantly reduced in sipilizumab-containing cultures compared to control ( $-71.2\%$ ,  $p = 0.0002$  and  $-86.2\%$ ,  $p < 0.0001$ , respectively).

Because Adaptive's deep sequencing platform does not readily capture sequences present at frequencies below  $10^{-5}$  and most alloreactive T cells detected after expansion in an MLR are present in the circulation at very low frequencies and are therefore not detected in the unstimulated T cell pool<sup>26</sup>, only a minor fraction of alloreactive CD4<sup>+</sup> clones could be mapped to the sorted unstimulated Treg or Non-Treg population. Consequently, the data in Figure 6A and B underestimates the clone fraction and the cumulative frequency of each subset in the CFSE<sup>LO</sup> population, as most sequences could not be assigned to (i.e. was not "mappable" to) either subset. To overcome this limitation, we also analyzed the clone fractions and cumulative frequencies of each subset in the alloreactive population using only the mappable clones (to either Treg or Non-Treg) as the denominator (Figures 6C and 6D, Supplementary Figure 12). The Treg clone fraction in sipilizumab-containing MLRs again increased significantly, with an average of 68.8% of sequences identifiable as Tregs compared to 26.9% of sequences in control cultures ( $p < 0.0001$ ). Conversely, the mappable Non-Treg clone fraction decreased significantly to a mean of 31.2% in sipilizumab-containing cultures compared to 73.1% in control cultures ( $p < 0.0001$ ). Likewise, the cumulative frequency of mappable Tregs in sipilizumab-containing MLRs was markedly increased to an average of 68.2% compared to 14.4% in control cultures ( $p < 0.0001$ ), while

the cumulative frequency of Non-Tregs decreased in the sipilizumab-containing cultures (31.9% vs 85.6 in control cultures,  $p < 0.0001$ ).

In three pairs of samples the analysis was extended to allow identification of alloreactive clones that mapped to the CD4<sup>+</sup> Naive, CD4<sup>+</sup> Memory, CD8<sup>+</sup> Naive, CD8<sup>+</sup> Effector Memory and CD8<sup>+</sup> TEMRA populations in the unstimulated circulating PBMCs (Supplementary Figure 13). The cumulative frequency of alloreactive sequences identifiable as CD4<sup>+</sup> Memory in the unstimulated repertoire decreased significantly in sipilizumab-containing MLRs compared to controls ( $-68.7\%$ ,  $p = 0.0169$ ), whereas the alloreactive clone fraction identifiable as CD4<sup>+</sup> Memory did not change significantly ( $p = 0.11$ ), though each individual showed a downward shift in the sipilizumab cultures. In contrast, the contribution of CD8<sup>+</sup> Effector Memory and TEMRA subsets to the alloreactive population did not change consistently in either direction in the presence of sipilizumab (Supplementary Figure 13).

The above study provided an opportunity to examine the relative contribution of naïve and memory T cells to the human MLR. While only small numbers of clones identifiable as naïve CD4<sup>+</sup> or CD8<sup>+</sup> T-cells contributed to the alloreactive cell populations in this assay, when only mappable (to naïve or memory subsets in unstimulated populations) clones were used as the denominator in the analysis, the contribution of naïve CD4<sup>+</sup> and CD8<sup>+</sup> cells to the response was readily apparent (Supplementary Figure 14A–D). The greater diversity of naïve compared to memory T cells in unstimulated human PBMCs (Supplementary Figure 14E and F) is associated with a lower frequency of each naïve clone in a given sample, resulting in a bias against their detection in the unstimulated repertoire and hence against the identification of a naïve T cell contribution to the CFSE<sup>LO</sup> population. The inability to assign a naïve or memory phenotype to about 80–95% of CFSE<sup>LO</sup> CD4<sup>+</sup> or CD8<sup>+</sup> clones in the MLR (Supplementary Figure 13) is consistent with this interpretation.

To further corroborate this point, we assessed the alloreactivity of sorted naïve and memory T cells in a modified MLR, in which the two subsets were stained with different proliferation dyes: our experiments confirmed that both naïve and memory T cells participate in the alloresponse, both when responder cells are cultured separately or are recombined in the same MLR (Supplementary Figure 15).

## Discussion

Siplizumab is a T cell-depleting agent that binds to a specific region of the human CD2 molecule, which differs from the classic CD2 epitopes described in earlier work and that is not shared with most non-human primates<sup>27</sup>. Even though the exact role of the CD2 molecule in human T-cell immunology is still incompletely understood, perturbation of specific regions of this receptor can inhibit T-cell proliferation<sup>28</sup>. In addition to this inhibitory effect, sipilizumab also mediates a robust T-cell depletion through ADCC, which is in turn dependent on the activity of monocytes and NK-cells<sup>9</sup>. Interestingly, this action is more pronounced against activated T lymphocytes, likely because these cells express CD2 at higher density compared to resting T cells, and because NK-cell activity is enhanced by the high IL-2 concentrations that follow T-cell activation<sup>29</sup>.



Our *in vitro* experiments showed that sipilizumab may have additional features of potential interest for solid organ transplantation: when added to the allogeneic MLR, sipilizumab predominantly reduced the frequency of CD4<sup>+</sup> and CD8<sup>+</sup> effector memory T cells compared to other subsets. This effect likely reflects both reduced T-cell proliferation and selective depletion of effector memory T cells. Indeed, our data show that both CD4<sup>+</sup> and CD8<sup>+</sup> effector memory T cells express CD2 at high density, which likely renders them a preferential target for the drug. Similar results have been described for alefacept, a CD2-specific LFA-3-Ig fusion protein that was shown to selectively deplete memory T cells<sup>30,31</sup>. In contrast, T cell-depleting agents currently available, such as polyclonal anti-thymocyte globulin (ATG) and alemtuzumab, are predominantly active against naïve T cells and do not efficiently deplete alloantigen-primed T cells<sup>32–34</sup>. Considering the relevance of alloreactive effector memory T cells in graft rejection<sup>35</sup>, sipilizumab may confer additional protection compared to other T cell-depleting agents, especially in recipients at high immunological risk.

Our data also demonstrate that sipilizumab selectively increases the percentage of proliferating Tregs in the allogeneic MLR compared to control. Since we found that resting Tregs express the lowest surface CD2 levels among T-cell subsets, they may be selectively spared from sipilizumab-mediated T-cell depletion and inhibition of proliferation, leading in turn to a relative expansion of regulatory T cells during the alloresponse. This effect differs from that originally described for ATG, which featured FoxP3 induction from conventional T cells that was not associated with suppressive activity<sup>36,37</sup>. Notably, in the published experiments, FoxP3 upregulation was completely lost after ten days of culture, in contrast to our observations of sipilizumab-containing MLRs, in which FoxP3 expression tended to increase over time.

Despite efficiently inhibiting T-cell proliferation, sipilizumab did not affect early T-cell activation as assessed by CD69 and HLA-DR expression. This lack of downregulation is probably important in permitting expansion of alloreactive Tregs, since the expression of these molecules has been shown to play a central role in the development and suppressive function of this regulatory subset<sup>38,39</sup>.

While we did not functionally confirm the suppressive ability of CFSE<sup>LO</sup> Tregs in sipilizumab cultures, the stability and increasing enrichment of FoxP3 expression over time in sipilizumab-containing compared to control MLRs is inconsistent with the transient upregulation of FoxP3 that is typical of activated conventional T cells. Moreover, FoxP3<sup>+</sup> T cells harvested from sipilizumab-containing cultures retained a high degree of FoxP3 promoter demethylation, which was comparable to that assessed in unstimulated Tregs. Most importantly, the TCR repertoire analysis we performed demonstrated that the enrichment in alloreactive FoxP3<sup>+</sup> T cells observed post-MLR derives from the expansion of Tregs pre-existing in the unstimulated T-cell pool. Indeed, the striking predominance of Treg-derived sequences among total alloreactive sequences in sipilizumab MLRs that could be mapped to the unstimulated pool suggests that sipilizumab MLRs may provide a means of expanding donor-specific Tregs *in vitro*. Expansion of donor-specific Tregs has been reported to occur in MLRs to which belatacept has been added<sup>40</sup>. However, in our hands, this method also expands large numbers of non-donor-specific Tregs<sup>18</sup> and it would be of interest to compare

these two methodologies side by side. Since sipilizumab greatly reduces the number of proliferating T-cells, as shown by the steep decrease in absolute T-cell counts observed in sipilizumab MLRs, additional expansion of this population would be needed to obtain sufficient quantities for infusion.

Our mapping of MLR-expanded T cells to sorted unstimulated T cell subpopulations also allowed us to compare the contributions of naïve and memory T cells to the allogeneic HLA-mismatched MLR, a type of analysis that has not been previously reported. We were able to map markedly greater numbers of clones to the memory T-cell pool than to the naïve subset for both CD4<sup>+</sup> and CD8<sup>+</sup> cells. While one interpretation of this result would be that most of the T-cells responding to alloantigens during alloreaction are cross-reactive memory T-cells, we believe the result is biased to detection of memory cells due to sampling effect. Similar to previous studies<sup>41</sup>, we observed that unstimulated circulating memory T cells have a lower diversity (higher clonality and Simpson's index) compared to their naïve counterparts, resulting in a low frequency of each naïve T cell in the circulation and a reduced chance of detecting it in a pool of 10<sup>5</sup> TCR templates, which is the order of magnitude used for Adaptive's deep sequencing method. Consistent with this interpretation, we have shown that the vast majority of alloreactive T-cell clones detected with this MLR method are undetectable in the unstimulated population due to their low frequency in the circulation<sup>26</sup>. Thus, we interpret the ability to map even a small fraction of alloreactive T cells in the MLR to the naïve population in a relatively small sample as an indication that naïve T cells make a major contribution to the alloreactive pool. These conclusions are consistent with our experiments and previous data obtained from both CFSE-based proliferation and limiting dilution assays, indicating that the alloreactive T-cell repertoire is not restricted to a specific subset, and that both naïve and memory T cells contribute to the MLR<sup>42,43</sup>.

In view of the relatively low CD2 expression we detected on naïve CD4<sup>+</sup> and CD8<sup>+</sup> T-cell populations, it is possible that sipilizumab may be relatively ineffective at inhibiting this component of the allograft response if used as induction therapy. Since naïve human T-cells convert to the memory phenotype in a T-cell-deficient environment<sup>44</sup>, however, further studies are needed to assess CD2 expression on naïve T cells undergoing this lymphopenia-driven activation.

The experiments described herein were designed to evaluate within-subject differences. Hypothesis testing with a matched-pair design increases the power to detect significant differences. Therefore, particular caution should be paid when interpreting results from small changes. The major effects of sipilizumab on Tregs and effector memory cells, however, were large.

In conclusion, we have shown that the T-cell depleting effect of sipilizumab targets mainly the effector memory subset and that the drug promotes a relative expansion of alloreactive regulatory T-cells *in vitro*. These activities help to explain the marked enrichment of Tregs observed in hematopoietic stem cell transplant recipients and tolerant CKBMT recipients conditioned with a sipilizumab-based regimen, which recent studies have shown to reflect an expansion of donor-specific Tregs<sup>18</sup>. Further studies will be needed to assess the mechanisms by which these effects occur *in vivo*.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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## Abbreviations:

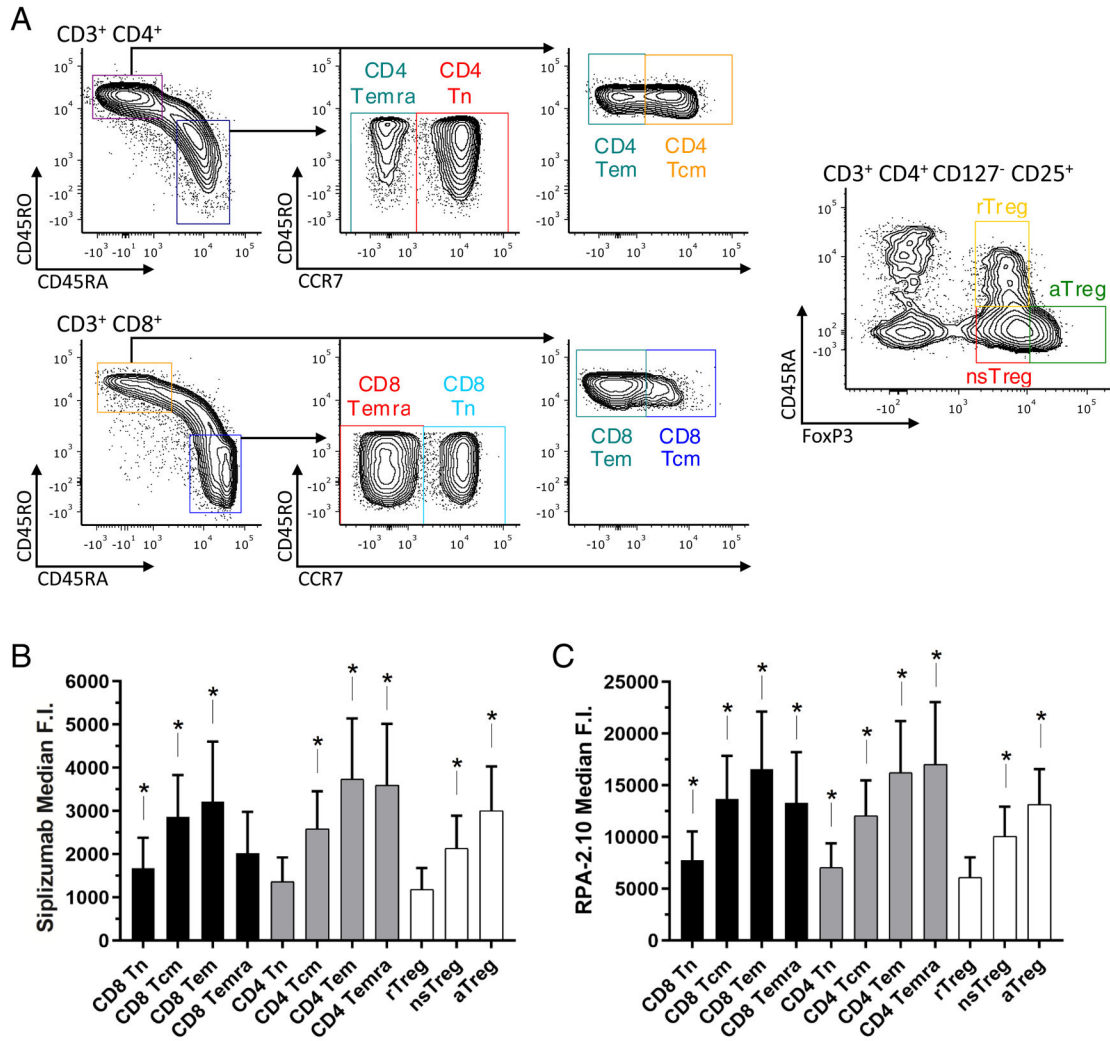
<b>ADCC</b>	antibody-dependent cell-mediated cytotoxicity
<b>CKBMT</b>	combined kidney-bone marrow transplantation
<b>MLR</b>	mixed-lymphocyte reaction
<b>Tregs</b>	regulatory T-cells

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**Figure 1. CD2 expression among unstimulated T-cell subsets.**

(A) Gating strategy for the subsets of interest on which CD2 expression was assessed. (B,C) Median fluorescence intensity of CD2 (using Siplizumab and the commercially available clone RPA-2.10) among T-cell subsets (mean ± SD); \* p < 0.05 compared to rTreg, n = 10 unique donors (repeated-measures ANOVA and Tukey’s multiple comparisons test).

Significant differences in selected additional comparisons:

- [Siplizumab] CD4<sup>+</sup> Naïve vs Central Memory: p < 0.0001 – CD4<sup>+</sup> Naïve vs Effortory Memory p = 0.0004 – CD8<sup>+</sup> Naïve vs Central Memory: p < 0.0001 – CD8<sup>+</sup> Naïve vs Effortory Memory p = 0.0047 – nsTregs vs CD4<sup>+</sup> Central Memory: p = 0.0116 – nsTregs vs CD4<sup>+</sup> Effortory Memory: p = 0.0016 – nsTregs vs CD8<sup>+</sup> Central Memory: p = 0.0003 – nsTregs vs CD8<sup>+</sup> Effortory Memory: p = 0.0459 – aTregs vs CD4<sup>+</sup> Effortory Memory: p = 0.0249.

- [RPA-2.10] CD4<sup>+</sup> Naïve vs Central Memory: p < 0.0001 – CD4<sup>+</sup> Naïve vs Effortory Memory p < 0.0001 – CD8<sup>+</sup> Naïve vs Central Memory: p < 0.0001 – CD8<sup>+</sup> Naïve vs Effortory Memory p = 0.0003 – nsTregs vs CD4<sup>+</sup> Central Memory: p = 0.0025 – nsTregs vs CD4<sup>+</sup> Effortory Memory: p = 0.0006 – nsTregs vs CD8<sup>+</sup> Central Memory: p = 0.0019 –

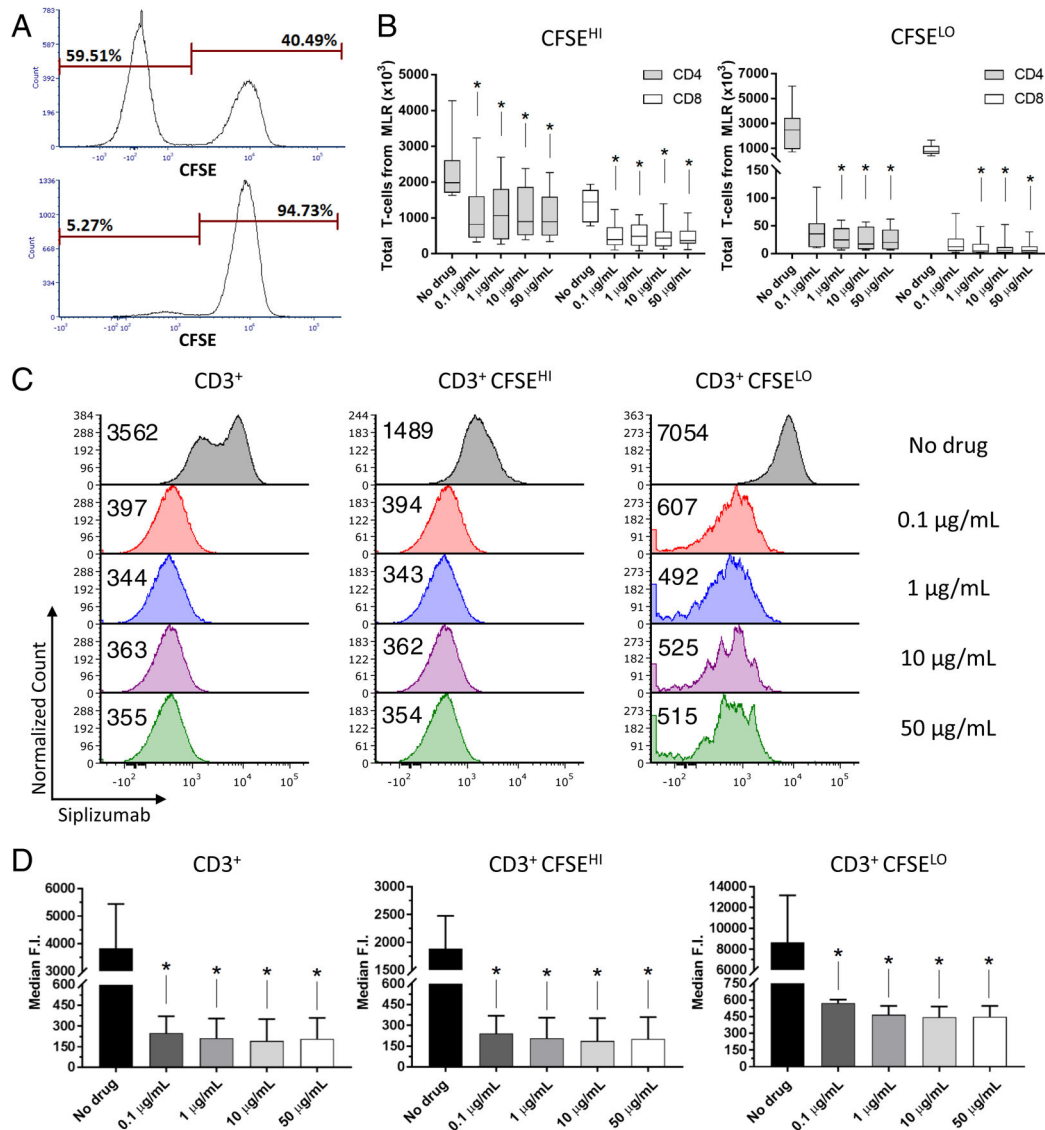
nsTregs vs CD8<sup>+</sup> Effector Memory:  $p = 0.0092$  – aTregs vs CD4<sup>+</sup> Effector Memory:  $p = 0.0154$ .

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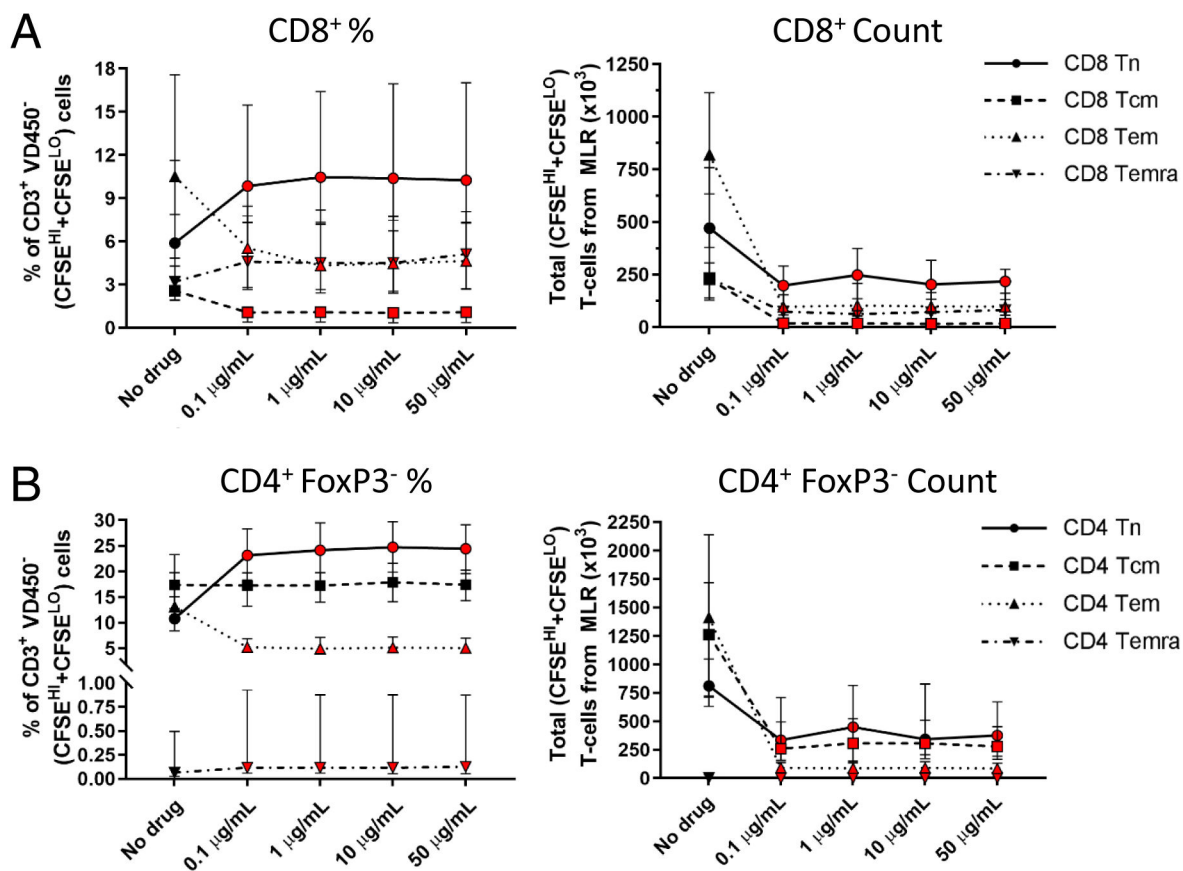
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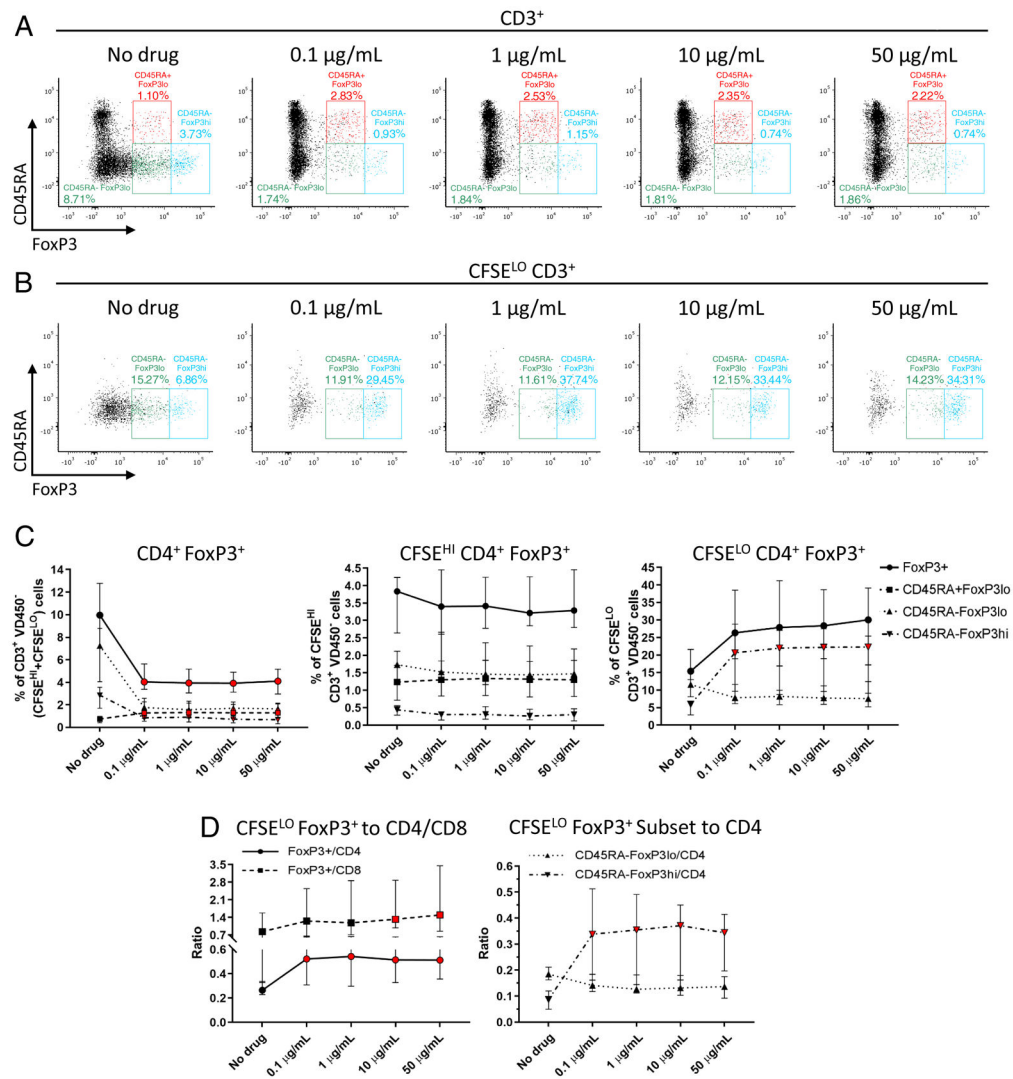
**Figure 2. Cell proliferation and CD2 expression in both proliferating and non-proliferating T cells are reduced in MLRs containing siplizumab at day 6.**

(A) Proliferation of CD3<sup>+</sup> T cells in MLRs containing Siplizumab (bottom panel, 1 µg/mL) compared to control MLRs (top panel). (B) Numbers of non-proliferating (CFSE<sup>HI</sup>) and proliferating (CFSE<sup>LO</sup>) T cells recovered from allogeneic MLRs at increasing drug concentrations (median [IQR]); n = 10 unique donor pairs (Friedman's test and Dunn's multiple comparisons tests). (C) Siplizumab median fluorescence intensity (with PE-Dazzle594 anti-human IgG Fc) in a representative sample of total, non-proliferating and proliferating T cells at increasing drug concentration, with (D) summary of the analysis (mean ± SD); n = 5 unique donor pairs. \* p < 0.05 compared to No drug MLR (repeated-measures ANOVA and Dunnett's multiple comparisons test).



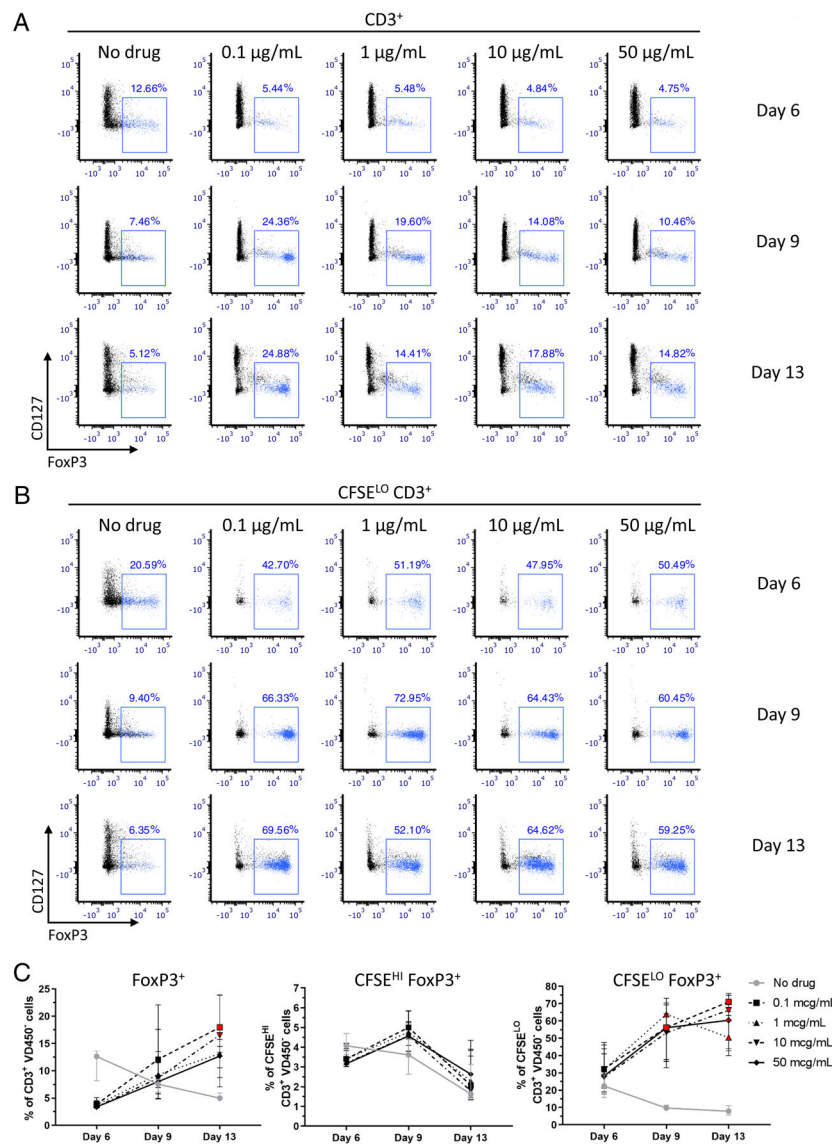


**Figure 3. Siplizumab lymphodepleting effect varies among different CD8<sup>+</sup> and CD4<sup>+</sup> FoxP3<sup>-</sup> T-cell subsets in allogeneic HLA-mismatched MLRs.** CD8<sup>+</sup> (A) and CD4<sup>+</sup> FoxP3<sup>-</sup> (B) T-cell subsets recovered from the HLA-mismatched MLRs expressed both as percentage of total CD3<sup>+</sup> responders and as absolute cell numbers (median [IQR]); n = 10 unique donor pairs. Red-colored symbol: p < 0.05 compared to No drug MLR (repeated-measures ANOVA and Dunnet’s multiple comparisons test, or Friedman’s test and Dunn’s multiple comparisons tests, as appropriate).



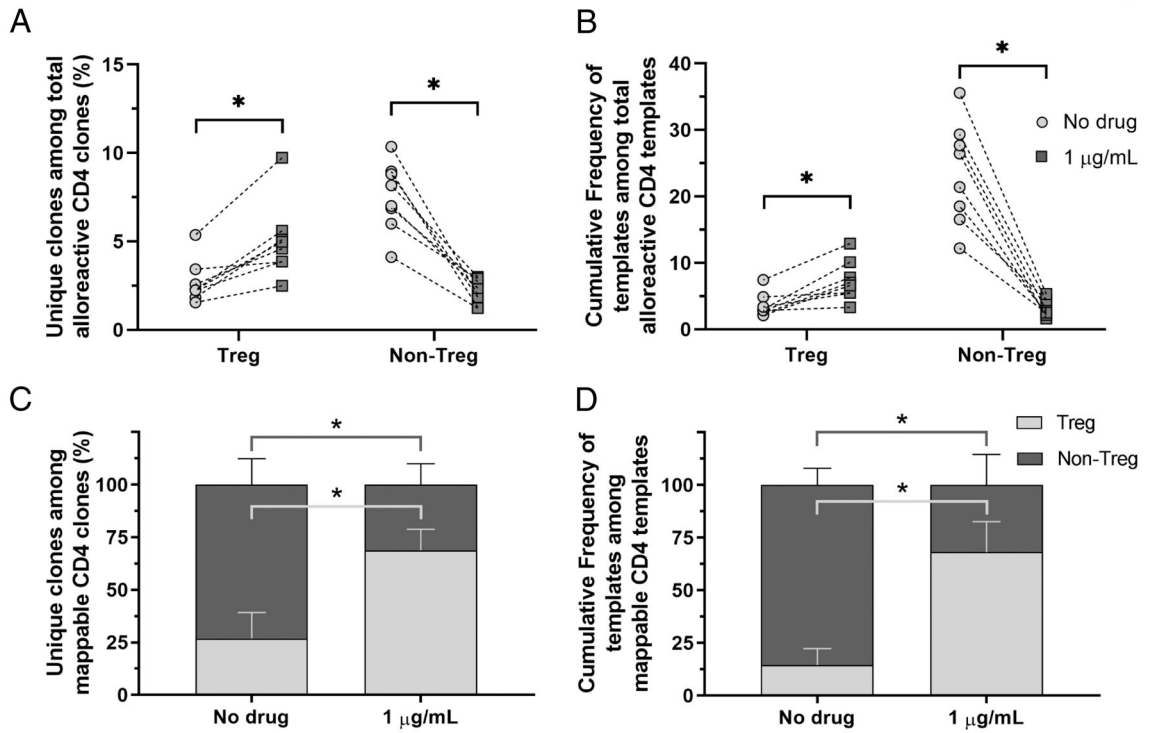
**Figure 4. Siplizumab reduces the percentage of total FoxP3<sup>+</sup> cells, but expands the fraction of proliferating FoxP3<sup>HI</sup> cells and the ratio of proliferating FoxP3<sup>+</sup> to CD4<sup>+</sup> and CD8<sup>+</sup> cells in allogeneic HLA-mismatched MLRs.**

Total (A) and proliferating (B) CD4<sup>+</sup> CD25<sup>+</sup> CD127<sup>-</sup> FoxP3<sup>+</sup> subsets post-MLR at different drug concentrations, back-gated on responder total and proliferating CD3<sup>+</sup> T cells respectively. FoxP3<sup>+</sup> cells were further characterized on the basis of CD45RA (+/-) and FoxP3 (hi/lo) expression. (C) Total, non-proliferating (CFSE<sup>HI</sup>) and proliferating (CFSE<sup>LO</sup>) CD4<sup>+</sup> CD25<sup>+</sup> CD127<sup>-</sup> FoxP3<sup>+</sup> cells represented as percentage of total, non-proliferating and proliferating CD3<sup>+</sup> T cells respectively (median [IQR]); n = 10 unique donor pairs. (D) Ratios of proliferating (CFSE<sup>LO</sup>) CD4<sup>+</sup> CD25<sup>+</sup> CD127<sup>-</sup> FoxP3<sup>+</sup> cells to either CFSE<sup>LO</sup> CD4<sup>+</sup> or CFSE<sup>LO</sup> CD8<sup>+</sup> cells, and ratio of CFSE<sup>LO</sup> CD45RA<sup>-</sup> FoxP3<sup>HI/LO</sup> subsets to CFSE<sup>LO</sup> CD4<sup>+</sup> cells (median [IQR]); n = 10 unique donor pairs. Red-colored symbol: p < 0.05 compared to No drug MLR (repeated-measures ANOVA and Dunnet's multiple comparisons test, or Friedman's test and Dunn's multiple comparisons tests, as appropriate).



**Figure 5. FoxP3 expression in total and proliferating (CFSE<sup>LO</sup>) T cells declines over time in control allogeneic HLA-mismatched MLRs, but shows an opposite trend in allogeneic HLA-mismatched MLRs containing siplizumab.**

Total (A) and proliferating (B) CD4<sup>+</sup> CD25<sup>+</sup> CD127<sup>-</sup> FoxP3<sup>+</sup> cells post-MLR at different drug concentrations and at different timepoints, back-gated on responder total and proliferating CD3<sup>+</sup> T cells respectively. (C) Total, non-proliferating (CFSE<sup>HI</sup>) and proliferating (CFSE<sup>LO</sup>) CD4<sup>+</sup> CD25<sup>+</sup> CD127<sup>-</sup> FoxP3<sup>+</sup> cells over time represented as percentage of responder total, non-proliferating and proliferating CD3<sup>+</sup> T-cells respectively (median [IQR]); n = 4 unique donor pairs. Red-colored symbol: p < 0.05 compared to No drug MLR (repeated-measures ANOVA and Dunnet's multiple comparisons test).



**Figure 6. Siplizumab promotes a relative expansion of alloreactive Treg clones over CD4<sup>+</sup> Non-Treg clones.**

(A) Percentage of unique clones identified either as alloreactive Treg or alloreactive Non-Treg, based on detection in CFSE<sup>LO</sup> populations and sorted unstimulated Tregs or non-Tregs, among total CD4<sup>+</sup> clones identified as alloreactive in the CFSE<sup>LO</sup> population (median [IQR]). (B) Cumulative frequency of sequences identified either as alloreactive Treg or alloreactive Non-Treg among total CD4<sup>+</sup> sequences identified as alloreactive in the CFSE<sup>LO</sup> population (median [IQR]). (C) Fraction of unique clones identified as either alloreactive Treg or alloreactive Non-Treg among total CD4<sup>+</sup> mappable clones (i.e. those identifiable as alloreactive and detectable in either unstimulated Treg or unstimulated Non-Treg PBMCs) (mean ± SD). (D) Cumulative frequency of sequences identified either as alloreactive Treg or alloreactive Non-Treg among mappable CD4<sup>+</sup> sequences (i.e. those identifiable as alloreactive and detectable in either unstimulated Treg or unstimulated Non-Treg PBMCs) (mean ± SD). n = 8 unique donor pairs for all experiments. \* p < 0.01 compared to No drug MLR (paired t-test or Wilcoxon’s matched-pairs signed rank test, as appropriate).