CTLA4-Ig in combination with FTY720 promotes allograft survival in sensitized recipients

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Introduction

The relatively high incidence of early aggressive T cell–mediated acute rejection is a significant impediment to the widespread use of belatacept in clinical transplantation, despite recent reports of improved long-term outcomes (1–3). High frequencies of alloreactive memory T cells have emerged as the major hypothesis to explain this emergence of belatacept-resistant cellular rejection. Memory T cells differ from naive T cells at a cell-intrinsic level, by having a lower threshold for activation that allows them to divide after a shorter lag time and to more rapidly elaborate effector function (4–6). Memory T cells are also relatively resistant to cell death, which allows them to accumulate more rapidly and to higher numbers (7). Sensitized recipients harbor higher donor-specific T cell frequencies and circulating antibodies that can, in turn, promote more vigorous T cell responses in vivo (8, 9). In humans, previous transplantation, pregnancy, or blood transfusion are the most clinically important sensitizing events that result in increased donor-specific antibody (10), and presumably donor-specific T cells that provide help to the B cells producing these antibodies. In addition, exposure to environmental antigens and infections can result in heterologous immunity and the accumulation of memory alloreactive T cells in healthy individuals with no prior history of allosensitization (11). Indeed, Thome et al. reported that the frequencies of CD45RA–CCR7– effector memory CD4+ and CD8+ T cells within the lymphoid compartments, including blood, accumulate rapidly with age (12). These high frequencies of heterologous memory T cells, if donor reactive, are postulated to play key roles in belatacept-resistant rejection in both clinically sensitized as well as nonsensitized recipients.

Despite recent evidence of improved graft outcomes and safety, the high incidence of early acute cellular rejection with belatacept, a high-affinity CTLA4-Ig, has limited its use in clinical transplantation. Here we define how the incomplete control of endogenous donor-reactive memory T cells results in belatacept-resistant rejection in an experimental model of BALB/c.2W-OVA donor heart transplantation into C57BL/6 recipients presensitized to donor splenocytes. These sensitized mice harbored modestly elevated numbers of endogenous donor-specific memory T cells and alloantibodies compared with naive recipients. Continuous CTLA4-Ig treatment was unexpectedly efficacious at inhibiting endogenous graft-reactive T cell expansion but was unable to inhibit late CD4+ and CD8+ T cell infiltration into the allografts, and rejection was observed in 50% of recipients by day 35 after transplantation. When CTLA4-Ig was combined with the sphingosine 1-phosphate receptor-1 (S1PR1) functional antagonist FTY720, alloantibody production was inhibited and donor-specific IFN-γ-producing T cells were reduced to levels approaching nonsensitized tolerant recipients. Late T cell recruitment into the graft was also restrained, and graft survival improved with this combination therapy. These observations suggest that a rational strategy consisting of inhibiting memory T cell expansion and trafficking into the allograft with CTLA4-Ig and FTY720 can promote allograft survival in allosensitized recipients.
Belatacept, whereas Th17 cells were resistant due to their elevated expression of CTLA-4. In contrast, de Graa et al. (14) reported the downregulation of surface CD28 on both CD4+ and CD8+ T cells as a potential escape mechanism from belatacept-mediated immune suppression. More recently, Espinosa et al. (3) reported in a small study of 14 transplant recipients that the pretransplant frequency of a highly functional CD4+CD28–PD-1–CD57+ T cell subset was predictive of belatacept-resistant rejection. Currently, clinical experience with belatacept-resistant rejection is relatively limited, so why these rejection episodes occur in some recipients and not others, and how rejection can be prevented require further investigation.

We and others have reported that CTLA4-Ig inhibits early T cell–dependent alloantibody responses as well as recall responses (15–18). In this study, we extend those observations by characterizing the impact of extended CTLA4-Ig treatment on graft-reactive memory T cell responses. We tracked the fate of endogenous donor-specific memory T cells and found that CTLA4-Ig was unexpectedly efficacious at inhibiting the expansion of endogenous graft-reactive memory T cells, but was nevertheless unable to control their eventual migration into the graft to induce graft rejection. We further showed that when CTLA4-Ig was used in combination with the clinically approved fingolimod (FTY720), a sphingosine 1-phosphate receptor-1 (S1PR1) functional antagonist, T cell infiltration was reduced and allograft survival in sensitized recipients was significantly improved.

**Results**

**CTLA4-Ig prolongs allograft survival and inhibits donor-specific IgG production in sensitized recipients.** To investigate how memory T cells mediate CTLA4-Ig–resistant rejection, we generated allosensitized C57BL/6 (B/6) recipients by immunizing subcutaneously with splenocytes from BALB/c (B/c) mice that ubiquitously expressed the 2W-OVA transgene (Figure 1A). This immunization elicited B/c-specific and 2W-OVA–specific T cell and donor-specific antibody (DSA) responses, so in order to minimize antibody-mediated rejection, we monitored the levels of DSA monthly and waited for 14 or more months after transplantation for the DSA levels to be reduced (from ≥ 20-fold to ≤ 5-fold higher) relative to naive mice (Figure 1B). At these late time points after sensitization, we enumerated the numbers of donor-specific 2W:I-Ab–binding CD4+ and OVA:Kb–reactive CD8+ T cell subset was predictive of belatacept-resistant rejection. Currently, clinical experience with belatacept-resistant rejection is relatively limited, so why these rejection episodes occur in some recipients and not others, and how rejection can be prevented require further investigation.

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The total numbers of donor-specific OVA:Kb-binding central memory (TCM: CD44hiCD62Lhi) and effector memory (TEM: CD44hiCD62Llo) CD8+ T cells were significantly increased, albeit by only ~4-fold, over those in naive controls (Figure 1C). These observations are consistent with reports of a greater longevity of CD8+ over CD4+ memory T cells (19, 20).

Despite the relatively low frequencies of 2W:I-A^b-reactive CD4+ and OVA:Kb-reactive CD8+ memory cells, transplantation of B/c heart grafts into the allosensitized B/6 recipients treated with continuous CTLA4-Ig resulted in only approximately 50% of the grafts surviving for more than 60 days after transplantation, in contrast to 100% graft acceptance in comparably treated naive recipients (Figure 2, A and B). The rejection in CTLA4-Ig–treated sensitized recipients occurred even though alloantibody production was inhibited (Figure 2C), consistent with our previous observations (16). Because rejection in this transplant model is mediated primarily by T cells, we investigated the effects of CTLA4-Ig in sensitized recipients by quantifying the frequency of donor-reactive IFN-γ-producing cells. Surprisingly, we observed that continuous treatment with CTLA4-Ig significantly inhibited the increase in frequencies of IFN-γ-producing CD4+ and CD8+ T cells with TEM and TCM phenotypes compared with untreated sensitized heart recipients (Figure 2D). These observations suggest an unexpected efficacy of CTLA4-Ig at preventing the expansion of donor-specific IFN-γ-producing memory T cells upon antigen reencounter.
CTLA4-Ig inhibits the expansion of endogenous graft-specific T cell responses in sensitized recipients. A lack of increase in the numbers of donor-specific IFN-γ–producing T cells in recipients treated with CTLA4-Ig could be explained by suppression of IFN-γ production, skewing towards non–IFN-γ–producing T cells or inhibition of cell expansion/accumulation. To more fully investigate these possibilities, we quantified the total number of donor-specific T cells in B/6 mice sensitized with spleen cells and receiving hearts from B/c donors that expressed the 2W-OVA transgene 14 months or more later. The total number of 2W:I-Ab–binding CD4+ and OVA:Kb-binding CD8+ T cells were pooled from the spleen and 6 lymph nodes (axial, brachial, and inguinal). In sensitized mice receiving B/c-2W-OVA hearts, there was a 26-fold increase in 2W:I-Ab–binding CD4+ cells and a 5-fold increase in OVA:Kb-binding CD8+ T cells by days 7–10 after heart transplantation (Figure 3, A and B). This increase was primarily due to an increase in the CD44+CD62L– TEM subset, which reached ~80% of 2W:I-Ab–reactive CD4+ cells and ~65% of OVA:Kb-reactive CD8+ T cells (Figure 3, C and D). There was no significant increase in the percentages of donor-specific CD44+CD62L+ TCM cells, and a compensatory decrease in the percentages of naive-phenotype cells was observed after transplantation.

To assess the extent to which CTLA4-Ig was able to control the donor-reactive T cell responses, we compared sensitized recipients treated with CTLA4-Ig from days 0–30 after transplantation to comparably treated naive recipients. On day 30 after heart transplantation in CTLA4-Ig–treated sensitized recipients, the total numbers of 2W:I-A^b–binding CD4+ and OVA:Kb-binding CD8+ T cells were not significantly increased over pretransplant numbers, and in fact, the total numbers of graft-reactive CD4+ and CD8+ T cells in sensitized mice treated with CTLA4-Ig were not significantly different from naive recipients treated with CTLA4-Ig (Figure 3, A and B). Furthermore, because the percentage of graft-reactive TEM cells was increased in sensitized recipients following heart transplantation (Figure 3, C and D), we focused on this subset of 2W:I-A^b–binding CD4+ and OVA:Kb-binding CD8+ T cells. We again observed no significant increase in the total number of TEM cells in sensitized recipients treated with CTLA4-Ig compared with prior to transplantation (Figure 3, E and F), and confirmed that the lack of increase in total numbers of graft-reactive IFN-γ–producing T cells (Figure 2D) was not due to a skewing towards T cells producing other cytokines.

**CTLA4-Ig fails to inhibit the accumulation of T cells into the allografts of sensitized recipients.** Despite the efficacy of CTLA4-Ig treatment at inhibiting the expansion of donor-specific memory T cells in sensitized hosts,
we noted that the percentage and total numbers of donor-specific CD4+ and CD8+ TEMs prior to transplantation were already higher in sensitized than in naive recipients (Figure 1C). We therefore hypothesized that these higher frequencies of donor-specific TEMs may be sufficient to migrate into and mediate allograft rejection that occurred in ~50% of recipients (Figure 2B). Histologic examination of day 30 allografts from sensitized CTLA4-Ig–treated recipients revealed significantly more cellular infiltration than similarly treated naive recipients (Figure 4, A and B). Quantification of the total numbers of graft-infiltrating CD4+ and CD8+ T cells at day 30 after transplantation from CTLA4-Ig–treated sensitized recipients revealed 3.4- and 8.0-fold more cells, respectively, compared with grafts from similarly treated naive recipients (Figure 4C). Thus, while CTLA4-Ig was able to inhibit T cell expansion in sensitized recipients, T cells accumulated in the allograft over time, reaching numbers that approached those observed in untreated hosts. These observations raise the possibility that preventing T cell accumulation into the allograft might prevent CTLA4-Ig–resistant rejection.

CTLA4-Ig in combination with FTY720 prevents DSA development and reduces donor-specific IFN-γ–producing T cell frequencies in sensitized recipients. FTY720 causes lymphocyte sequestration into secondary lymphoid organs, thereby preventing antigen-activated T cells’ egress to sites of inflammation (21–25). FTY720 has been shown to be most effective for naive and TCM cells (26) but has also been reported to impact TEM migration in mice (27). To test whether FTY720 plus CTLA4-Ig in sensitized recipients would more effectively prevent alloreactive T cell infiltration into the allograft compared with CTLA4-Ig alone, sensitized recipients received a 30-day treatment with FTY720 alone or in combination with CTLA4-Ig.

One potentially undesired consequence of the sequestration of memory T cells in the lymphoid organs by FTY720 that has previously been reported is their ability to continue to provide help to B cells and promote antibody responses (28). Similarly, we observed that FTY720 monotherapy permitted an increase in alloantibody production in sensitized recipients after B/c heart transplantation (Figure 5A). However, the combination of FTY720 with CTLA4-Ig prevented this recall DSA response, comparable to CTLA4-Ig monotherapy, thus confirming that FTY720 did not abrogate the beneficial effects of CTLA4-Ig on memory B cell responses and alloantibody production (16).

Previous studies report that FTY720 trapped CD4+ and CD8+ T cells in the secondary lymphoid organs without inhibiting their activation, expansion, and differentiation into effector cells (21, 25, 28) raised the possibility that FTY720 might antagonize the ability of CTLA4-Ig to control memory T cell response. To address this possibility, we quantified the numbers of alloreactive T cells in the spleen of sensitized recipients of B/c hearts treated at day 30 after heart transplantation. CTLA4-Ig and FTY720 monotherapy resulted in similar, low frequencies of donor-specific IFN-γ–producing CD4+ (<1%) and CD8+ (~4%-6%), while the combination of FTY720 plus CTLA4-Ig further reduced the percentages of donor-specific IFN-γ–producing T cells (Figure 5B). Notably, the total numbers of donor-specific IFN-γ–producing CD4+ and CD8+ T cells were not significantly different than those observed in tolerant nonsensitized recipients (Figure 5B). A similar trend of reduced total numbers of bulk TEMs in the combination-treated recipients, compared with monotherapy, was also observed (Supplemental Figure 2). These observations that FTY720 plus CTLA4-Ig depleted alloreactive memory T cells in transplanted sensitized recipients suggest a potential novel approach for T cell desensitization.

CTLA4-Ig in combination with FTY720 prevents T cell infiltration into allografts and prolongs graft survival in sensitized recipients. The superior effects of the combination treatment of CTLA4-Ig plus FTY720 over
monotherapy on splenic donor-specific T cell responses (Figure 5) was not due to the relocation of these cells into the allograft. Indeed, the accumulation of CD4+ and CD8+ T cells in the allograft was further reduced in the combination therapy group compared with monotherapy groups, and approached numbers that were comparable to naive tolerant allografts (Figure 6A). We also tested whether FTY720 plus CTLA4-Ig reduced donor-specific infiltrating T cells by using sensitized mice that harbored, from the time of donor spleen cell sensitization, a tracer population of donor-reactive T cell receptor–transgenic TCR75 cells (1,000–2,000 cells/mouse) with specificity for donor-derived peptide (Kd 54–68) presented on recipient I-Ab. We similarly observed that the combination FTY720 plus CTLA4-Ig significantly decreased the numbers of TCR75 cells (which were predominantly CD44+CD62L–) infiltrating the allografts compared with CTLA4-Ig monotherapy (Figure 6B). These findings were confirmed with histology, where reduced cellular infiltrate was observed in allografts receiving combination compared with CTLA4-Ig monotherapy or FTY720 (Figure 6, C and D). Finally, we compared DSA titers in recipients sensitized with B/c splenocytes ~4 months and ~14 months after transplantation, and found significantly higher titers in the former group (Supplemental Figure 3). Allografts in these sensitized recipients succumbed to rejection under CTLA4-Ig or FTY720 monotherapy, while the combination of FTY720 and CTLA4-Ig promoted graft survival, with 10 of 12 recipients accepting their allografts for 30 or more days (Figure 6E). These data demonstrate that inhibition of T cell infiltration promotes graft acceptance in sensitized recipients treated with CTLA4-Ig and FTY720.

Discussion
Recent reports showing that patients on belatacept experience higher rates of severe acute cellular rejection (2, 3) are prompting new investigations into the mechanistic basis of belatacept-resistant rejection. A leading hypothesis is that this rejection is mediated by donor-specific memory T cells that become reactivated in a CD28-independent manner (3). Because prior transplantation has been shown to be the most robust allosensitizing event in clinical transplantation (10), we investigated the effect of CTLA4-Ig in recipients presensitized with fully mismatched donor splenocytes. This mode of sensitization results in alloantibody production and memory T cell generation (16) that persisted with modestly elevated numbers of memory

Figure 5. CTLA4-Ig plus FTY720 combination therapy prevents rejection in sensitized recipients. (A) Donor-specific antibody (DSA)-IgG and (B) IFN-γ T cell responses were assessed in ~4-month post-sensitization recipients (S) prior to transplantation, or on day 30 after transplantation (HTx) and treatment with CTLA4-Ig and/or FTY720. Nonsensitized tolerant recipients (N-Tol) received anti-CD154 (day 0, 7, and 14) plus B/c spleen cells (day 0) to induce tolerance to B/c heart grafts, and were analyzed on day 60 after transplantation. Data were pooled from 2 to 3 independent experiments (n = 6–8/group), with the minimum number of IFN-γ+ T cells set at 100. Data are presented as mean ± SEM, and statistical analyses were by ANOVA and Kruskal-Wallis tests. *P < 0.05; **P < 0.01, ***P < 0.005.
CD4+ and CD8+ T cells and DSA titers up to ~14 months after sensitization. Despite the modest increases in memory CD8+ T cells at 14 or more months after sensitization, CTLA4-Ig was only effective at preventing acute rejection in approximately 50% of allosensitized recipients, with the remaining allografts succumbing to rejection before day 35 after transplantation. We therefore reasoned that this model is appropriate for investigating how CTLA4-Ig curtails acute rejection in sensitized hosts, and why the allografts eventually succumb to CTLA4-Ig-resistant rejection. We acknowledge an important caveat of this mouse model, namely that CD28-negative terminally differentiated T cells that are present in significant frequencies in humans are absent in our immunized mice. CD28-negative cells have been found mostly within the CD8+ subset, and comprises T cells with suppressive, exhausted, or effector activity (29). Therefore, depending on the properties of CD28-negative cells present in the transplant recipient, it is possible that the efficacy of CTLA4-Ig observed in our mouse model may be an overestimate of its effects in humans.

Our observations that CTLA4-Ig prevents memory T cell expansion appear to contradict an extensive body of literature reporting that memory T cells are resistant to costimulation blockade of the CD28-B7 or CD40-CD154 pathways (11, 30–33). However, the majority of those studies focused on tolerance induction that used a short treatment course of costimulation blockade, whereas in this study we treated continuously with CTLA4-Ig, which is similar to its clinical use (1, 2, 34). Furthermore, most of those studies were based on the adoptive transfer of donor-reactive memory T cells that drove the costimulation blockade-resistant rejection process (11, 28, 35, 36). While that approach, especially when using graft-reactive TCR-transgenic T cell transfer, allows for elegant in-depth mechanistic analysis, their presence at nonphysiologically high TCR affinities and frequencies can lead to observations not replicated with endogenous T cells (37, 38), and can also profoundly enhance endogenous graft-specific responses (39). Furthermore, Ford et al. (9) reported that increasing the frequency of adoptively transferred CD4+ and CD8+ T cells to 0.5% and 5%, respectively, reduced the efficacy of CD28/CD154 blockade, and
shown that these cells retained an imprint of the frequency at which they were primed. In our model, presensitized mice received a B/c heart at 4 to 14 months or more after immunization, a time when the DSA titers and the numbers of memory CD4+ and CD8+ T cells were only very modestly elevated over naive controls. Nevertheless, sensitized mice were significantly less susceptible to CTLA4-Ig-mediated immunosuppression, thus underscoring the potency of this presensitization approach and utility for investigating the mechanisms of CTLA4-Ig–resistant rejection.

While some studies suggest that memory T cells do not require costimulation for reactivation, others have reported that costimulation remains necessary for their recall activation and expansion in vivo (40–45). By carefully tracking endogenous donor-specific T cells using the IFN-γ ELISPOT assay as well as with fluorescently labeled 2W.1-Aα and OVA:Kb multimers, we observed that CTLA4-Ig was able to prevent the expansion of donor-specific T cells in sensitized mice. These observations are consistent with the hypothesis that the increased sensitivity of memory T cells to elaborate effector function has to be balanced with tight control in order to limit their pathogenicity. Increased antigen threshold requirements have been reported for memory CD4+ and CD8+ T cells, resulting in preferential proliferation of naive T cells over memory cells (46–50). Thus, it is possible that the proliferation observed in our presensitized mice following B/c heart transplantation was actually driven predominantly by naive cells rather than by memory T cells, thereby explaining our observation that CTLA4-Ig inhibited donor-specific T cell expansion in sensitized recipients. Nevertheless, in either scenario of naive or memory T cell proliferation after B/c heart transplantation in sensitized recipients, CTLA4-Ig was not able to inhibit the accumulation of memory T cells in the allograft.

The observation that the frequency of CD8+ TEM and TEM cells was already elevated in presensitized mice compared with naive mice or naive recipients of B/c hearts treated with CTLA4-Ig raised the possibility that these memory T cells are capable of mediating rejection without requiring a robust expansion in the secondary lymphoid organs. We showed that CD4+ and CD8+ T cells accumulated within the allografts despite continuous CTLA4-Ig treatment, and achieved frequencies at day 30 after transplantation comparable to those observed in acutely rejecting allografts. Whether this was solely the result of migration and gradual accumulation into the allograft, or included further, albeit modest, expansion within the allograft, where the concentrations of CTLA4-Ig may be lower, was not determined in our studies. In support of the latter possibility, Lakkis and colleagues (51, 52) reported that allospecific memory but not naive T cells can home directly to the allograft to proliferate and mediate rejection independently of secondary lymphoid organs. Finally, the observation that the majority of the cells infiltrating the allografts are CD8+ TEMs, and of memory CD8+ TEMs being more resistant to costimulation blockade (11, 45, 53, 54), are consistent with CD8+ TEMs as the mediators of CTLA4-Ig–resistant rejection.

The accumulation of memory CD8+ T cells in the allograft suggested that inhibition of T cell infiltration into the allograft would promote graft survival. Consistent with the previously reported ability of FTY720 to inhibit lymphocyte egress from lymph nodes, but not alloantibody production (28), our results show that FTY720 does not inhibit alloantibody production but the addition of CTLA4-Ig was sufficient to do so, and to improve graft survival. Intriguingly, the total number of donor-specific IFN-γ–producing CD4+ and CD8+ T cells in the spleen was also reduced to levels that were comparable to tolerant naive recipients, as well as the total numbers of bulk TEMs. A depletion of naive, and effector/memory T cells with prolonged FTY720 treatment, has previously been reported, although the mechanism of this depletion was not elucidated (23). More recently, Han et al. (22) reported that FTY720 hinders DC migration into lymph nodes, while Schaper et al. (55) showed that it inhibited IL-12 and IL-23 secretion and enhanced IL-27 production in DCs. Collectively, these observations raise the possibility that a combination treatment with FTY720 and CTLA4-Ig may reduce the frequency of donor-reactive IFN-γ–producing TEM cells to levels that are lower than pretransplant frequencies in sensitized recipients, and we speculate that this may potentially serve as a T cell–desensitizing protocol.

There are a number of limitations to these studies. First, it will be important to test in future studies whether CTLA4-Ig in combination with immunosuppressive drugs used in the clinic, such as mycophenolic acid or steroids, improves graft outcomes in sensitized recipients, especially those that harbor circulating donor-specific antibodies. Secondly, this model does not define the effects of CTLA4-Ig on memory T cells the way that an adoptive transfer model would. However, we consider the sensitization of B and T cells and the presence of low levels of circulating antibodies a strength of this model, as we have previously shown that DSA can promote antigen uptake through opsonization, enhance antigen-presenting cell (APC)
intracellular staining, which was performed in an ice-water bath. (eBiosciences) was added and incubation continued for an additional 6 hours. Cells were then collected for cell incubation with 2W(EA WGALANW AVDSA):I-Ab tetramers (NIH Tetramer Core Facility) and γ557653), and IFN-γ(XMG1.2, catalog 505810) (all from BD Biosciences) were used to stain T cells. TCR αβ, catalog 553047), CD8 (53-6.7, catalog 100744), CD44 (IM7, catalog 563114), CD62L (Jo2, catalog 40-0393-82), CD90.2 (53-2-1, catalog 47-0902-82), CD4 (RM4-82), CD19 (1D3, catalog 48-0193-80), and Ter119 (TER-119, catalog 48-5921-82) (all from eBiosciences) antibodies (DX5, catalog 48-5971-82), CD11b (M1/70, catalog 101224), F4/80 (BM8, catalog 48-4801-82), and rabbit complement. Stimulators were then incubated overnight with 5 μg/mouse (i.v.) on day 0 and then 250 μg on days 7 and 14 (i.p.), in combination with 20 × 10^6 donor spleen cells (i.v.) on day 0 after heart transplantation. FTY720 in combination with cyclosporine was reported in phase IIb studies to provide adequate protection from acute rejection, but concerns about the safety profile halted its further development in transplantation. FTY720 was been approved by the Food and Drug Administration for the treatment of multiple sclerosis in 2010, and its efficacy and safety profile have been extensively described and its toxicities have proven to be manageable (60–62). We report here that CTLA4-Ig treatment of sensitized recipients significantly delayed T cell–mediated rejection by preventing T cell expansion and alloantibody production, and when combined with FTY720, prevented T cell infiltration and extended graft survival. By carefully tracking alloreactive memory CD4+ and CD8+ T cells in the spleen and graft, we have gained mechanistic insights into the efficacy of CTLA4-Ig alone and in combination with FTY720, thereby setting the stage for future investigations into T cell desensitization with FTY720 or related approaches that target S1PR1 and/or prevent T cell migration into allografts.

Methods

Animals and heart transplantation. Eight- to 10-week-old B/6 (only females were used as recipients) and 4- to 6-week-old B/c (both males and females were used as donors) mice were purchased from Envigo, and Act-2W-OVA transgenic mice on a B/6 background (B/6.2W-OVA) mice were a gift from James Moon (Massachusetts General Hospital, Harvard Medical School, Charlestown, Massachusetts, USA). Donor B/c.2W-OVA–transgenic mice were backcrossed from B/6-2W-OVA mice for 6 to 9 generations. Heart transplantation, flow cytometry, and H&E staining were performed as previously reported (18). In some experiments, B/6 mice sensitized with B/c-2W-OVA or B/c splenocytes, received, upon B/c-2W-OVA heart transplantation, 500 μg of CTLA4-Ig/mouse (abatacept; Bristol-Myers Squibb) on days –2, 0, and 2 (i.v.), and then 250 μg/mouse (i.p.) twice per week until the end of the experiment. In other groups, recipients were treated daily with FTY720 (Enzo Life Sciences) at 0.3 mg/kg, administered daily by gavage in a volume of 100 μl/10 g body weight (24). In some experiments, naïve B/6 mice were treated with 500 μg of anti-CD154 (i.v.; MR1; BioXCell) on day 0 and then 250 μg on days 7 and 14 (i.p.), in combination with 20 × 10^6 donor spleen cells (i.v.) on day 0 after heart transplantation to induce tolerance to B/c heart allografts. For tracking alloreactive CD4+ T cells, TCR75 (1 × 10^3 to 2 × 10^5/mouse) cells were adoptively transferred as an enriched CD4+ T cell population on day –1 or 0 of donor spleen cell sensitization.

Flow cytometry of donor-specific T cells. Splenocytes or graft-infiltrating cells were stained for flow cytometry using AquaFluor LiveDead (Life Technologies) solution to exclude dead cells, and a cocktail of dump antibodies (DX5, catalog 48-5971-82), CD11b (M1/70, catalog 101224), F4/80 (BM8, catalog 48-4801-82), CD19 (1D3, catalog 48-0193-80), and Ter119 (TER-119, catalog 48-5921-82) (all from eBiosciences) to exclude unwanted cells. Additional antibodies against CD90.2 (S3-2-1, catalog 47-0902-82), CD4 (RM4-5, catalog 553047), CD8 (S3-6.7, catalog 100744), CD44 (IM7, catalog 563114), CD62L (JJo2, catalog 557653), and IFN-γ (XMG1.2, catalog 505810) (all from BD Biosciences) were used to stain T cells. T cell incubation with 2W(EA WGALANW AVDSA):I-Ab tetramers (NIH Tetramer Core Facility) and OVA(SIINFEKL):Kb pentamers (Proimmune) was performed at room temperature for 30 minutes prior to staining with additional antibodies.

For the identification of IFN-γ–producing donor-specific CD4+ and CD8+ T cells, splenocyte stimulators from TCRαβ−/− B/6 mice, or from B/6XB/c F1 mice, which were depleted of T cells with anti-CD90 and rabbit complement. Stimulators were then incubated overnight with 5 μg/ml LPS (Sigma-Aldrich) in complete medium (RPMI media supplemented with 10% FBS, 1% sodium pyruvate, 1% penicillin, 1% MEM nonessential amino acids, 1% L-glutamine, and 1% HEPES). Responder cells (1 × 10^5) were incubated with 5 × 10^6 stimulators (200 μl per well) for 18 hours in complete medium, and then 1 μg of monensin (eBiosciences) was added and incubation continued for an additional 6 hours. Cells were then collected for intracellular staining, which was performed in an ice-water bath.
Isolation of graft-infiltrating cells. Heart allografts from transplanted animals were perfused with sterile HBSS with 1% heparin, cut into small fragments, and placed in digestion buffer (HBSS, 0.1% DNase I [MP Biomedicals], 400 U/ml collagenase IV [Sigma-Aldrich], and 50 mM HEPES) for 30 minutes at 37°C. The digested heart tissue was manually dissociated, and then filtered through a 70-μm strainer. The cells were then stained and analyzed by flow cytometry.

Histological analysis. Grafts were removed and placed in 10% formalin. Sections were then cut and stained by H&E. Slides were then scanned using the CRI Pannoramic Whole Slide Scanner (Perkin Elmer) at ×20 magnification.

DSA quantification. Fresh B/c splenocytes were harvested and their red blood cells lysed with 1 ml ACK Lysing Buffer (Quality Biological). Cells were washed, and then 1 × 10^6 cells were stained with 1 μl of serum from sensitized, transplanted, or naive recipients. After 2 washes, cells were stained with anti-CD19 (1D3, catalog 550992, BD Biosciences), anti-IgM (AF6-78, catalog 406208, Biolegend), and anti-IgG (goat anti–mouse IgG, catalog 1031-02, Southern Biotech) fluorescent antibodies. CD19+ cells were gated out due to their expression of IgM+, IgG+, and FcγR.

Statistics. Data are presented as mean ± SEM. Student’s t test (unpaired; 2-tailed) or Mann-Whitney (2-tailed) was used when 2 groups were compared, and ANOVA or Kruskal-Wallis when there were more than 2 groups, followed by post-hoc Tukey’s, Holm-Sidak’s, or Dunn’s multiple comparison tests. Survival curves were compared using the log-rank test. All analyses were performed using Prism (GraphPad), and P values less than or equal to 0.05 were considered statistically significant.

Study approval. All animal experiments were performed under protocols approved by the Institutional Animal Care and Use Committee of The University of Chicago.

Author contributions
SHK, JY, JSY, and JC designed and performed the research studies and data analysis. QW was responsible for mice husbandry. DPY performed the microsurgery. VV performed the immunohistochemistry. MM assisted in the analysis of the endogenous T cell responses. RS and MLA provided advice, discussion, and helped in the design of the research studies. ASC designed the research studies, analyzed the data, and wrote the manuscript. All authors reviewed the manuscript before submission.

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