Combined OX40L and mTOR blockade controls effector T cell activation while preserving T<sub>reg</sub> reconstitution after transplant

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A critical question facing the field of transplantation is how to control effector T cell (T<sub>eff</sub>) activation while preserving regulatory T cell (T<sub>reg</sub>) function. Standard calcineurin inhibitor–based strategies can partially control T<sub>eff</sub> activation, but breakthrough activation still occurs, and these agents are antagonistic to T<sub>reg</sub> function. Conversely, mechanistic target of rapamycin (mTOR) inhibition with sirolimus is more T<sub>reg</sub>-compatible but is inadequate to fully control T<sub>eff</sub> activation. In contrast, blockade of OX40L signaling has the capacity to partially control T<sub>eff</sub> activation despite maintaining T<sub>reg</sub> function. We used the nonhuman primate graft-versus-host disease (GVHD) model to probe the efficacy of combinatorial immunomodulation with sirolimus and the OX40L-blocking antibody KY1005. Our results demonstrate significant biologic activity of KY1005 alone (prolonging median GVHD-free survival from 8 to 19.5 days), as well as marked, synergistic control of GVHD with KY1005 + sirolimus (median survival > 100 days, P < 0.01 compared to all other regimens), which was associated with potent control of both T<sub>eff</sub> and T<sub>reg</sub> reconstitution [resulting in an enhanced T<sub>reg</sub>/T<sub>eff</sub> ratio (40% over baseline) in the KY1005/sirolimus cohort compared to a 2.9-fold decrease in the unprophylaxed GVHD cohort]. This unique immunologic signature resulted in transplant recipients that were able to control GVHD for the length of analysis and to down-regulate donor/recipient alloreactivity despite maintaining anti–third-party responses. These data indicate that combined OX40L blockade and sirolimus represents a promising strategy to induce immune balance after transplant and is an important candidate regimen for clinical translation.

INTRODUCTION

Despite an ever-increasing arsenal of clinically available immunomodulating agents, the ability to successfully control alloimmunity after solid organ (SOT) or hematopoietic stem cell transplant (HCT) is still significantly lacking. This results in graft rejection after SOT and graft-versus-host disease (GVHD) after HCT, which both occur despite the treatment of patients with multiple immunosuppressive agents. Central to controlling alloimmunity is the ability to simultaneously control the proliferation and activation of effector T cells (T<sub>eff</sub>) and still support regulatory T cell (T<sub>reg</sub>) homeostasis. This represents a particularly difficult challenge because most nontargeted immunosuppressive agents have nondiscriminatory inhibitory effects on both effector and regulatory populations. This is certainly true for calcineurin inhibitors (CNIs), which are the mainstay of immunosuppression for both SOT and HCT. Both cyclosporine and tacrolimus CNIs have been shown to be detrimental to T<sub>reg</sub> homeostasis, which contributes to their established antagonism to immune tolerance induction after transplant (1, 2). Moreover, we have recently shown that CNI-based immunosuppression is linked to breakthrough activation of T helper cell 17/cytotoxic T cell 17 (T<sub>H1</sub>/T<sub>C17</sub>) pathways along with defects in T<sub>reg</sub> reconstitution and function, which results in breakthrough GVHD after HCT in nonhuman primates (NHPs) (3). In contrast, mechanistic target of rapamycin (mTOR) inhibition with sirolimus represents a potentially more advantageous backbone immunomodulator compared to CNIs given that it has been shown to be significantly more permissive to both T<sub>reg</sub> function and homeostasis (1, 2, 4). However, although sirolimus has several protolerogenic mechanistic advantages, it is still not understood how best to deploy this agent, and it currently remains a second-line therapy that is not clinically superior to CNI (5, 6). This lack of clinical superiority is due to a number of factors: Posttransplant monotherapy with sirolimus, in the absence of adjunctive pretransplant GVHD prevention (7, 8), is unable to sufficiently control T<sub>eff</sub> activation and thus cannot in itself prevent GVHD (3, 9). Further, combination strategies that pair sirolimus with CNIs or inhibitors of proliferation [such as mycophenolate mofetil (MMF) or methotrexate] have not improved rates of GVHD (6, 10, 11), likely due to the antagonistic impact of these agents on T<sub>reg</sub> function. Thus, although sirolimus is likely a better immunomodulatory platform than CNI, the best agents with which to pair this drug remain undetermined.

Finding an ideal agent to pair with sirolimus requires the identification of the rare targeted agents that can simultaneously control T<sub>eff</sub> and, at the same time, permit T<sub>reg</sub> reconstitution and function. The work of our group and others has suggested that many of the clinically available costimulation agents, including those that target the CD28/CTLA4–CD80/86 pathway, may not be ideal (12, 13), given the reliance of T<sub>reg</sub> on these pathways (14, 15) and resistance of T<sub>eff</sub> and memory T cell (T<sub>mem</sub>) to CD28–targeting costimulatory blockade (16, 17). Of the potential pathways to target for combinatorial therapy with sirolimus, the OX40 (CD134)–OX40L (CD252) pathway is...
especially appealing, given that previous data have suggested that blockade of this pathway may have the capacity to control \( T_{\text{mem}} \) and \( T_{\text{eff}} \) function and nonetheless permit \( T_{\text{reg}} \) homeostasis and function (18–20). Moreover, transplant with OX40\(^{+} \) T cells has been shown to mitigate GVHD despite preserving graft-versus-leukemia (GVL) and antiviral T cell responses (21). Here, we show that, when combined with sirolimus, OX40L blockade with a novel immunoglobulin G4 (IgG4) anti-CD25 antibody, “KY1005,” provides potent control of both \( T_{\text{H}}/T_{\text{CL}} \) and \( T_{\text{H}}/T_{\text{C17}} \) activation, as well as synergistically preserves \( T_{\text{reg}} \) reconstitution. This control is associated with uniform long-term survival in a highly translational NHP model of acute GVHD (aGVHD). These results suggest that OX40L blockade plus sirolimus represents an important clinical candidate regimen for the prevention of alloreactivity after transplantation.

### RESULTS

**OX40 and OX40L are up-regulated on CD4\(^{+} \) T cells and CD11c\(^{+} \) myeloid dendritic cells during aGVHD**

To understand the biological role and potential therapeutic significance of OX40/OX40L signaling in aGVHD, we first measured the expression of OX40 and OX40L in healthy control NHP (HC) and compared this expression to that in NHP transplant recipients that developed aGVHD after T cell–replete haploidentical HCT in the absence of immunosuppression (referred to as the “No Rx” cohort) (3, 9, 17). We found that OX40 protein expression was up-regulated on the cell surface of peripheral blood CD4 T cells during aGVHD, whereas CD8 T cells expressed very low amounts of OX40 both in HC and in the No Rx cohort (Fig. 1A). The increased expression of OX40 could be measured on CD4 T cells isolated from the peripheral blood (Fig. 1A), as well as from lymphoid and nonlymphoid organs during unprophylaxed aGVHD, and also during aGVHD that occurred in the setting of Tac/MTX prophylaxis (fig. S1A), suggesting that up-regulation of OX40 might be a hallmark of alloreactive CD4 T cells regardless of immunosuppression regimen. Most of the OX40 expression was found in the CD4\(^{+} \) central memory (TCM) compartment, whereas CD4 naïve T cell (T\(_{\text{N}}\)) and CD4 effector memory T cell (T\(_{\text{EM}}\)) remained largely OX40\(^{-} \) before and after HCT (Fig. 1B). Consistent with this, OX40\(^{+} \) CD4 T cells bore hallmarks of a differentiated cell population, expressing more effector cytokines [including interleukin-2 (IL-2), tumor necrosis factor–α (TNF\(_{\alpha}\)), and IL-17A] and with a higher proportion of polyfunctional T cells (simultaneously expressing more than two cytokines) than their OX40\(^{-} \) counterparts (Fig. 1C and fig. S1, B and C). This observation was consistent among T cells isolated from HC and those from recipients diagnosed with aGVHD after unprophylaxed allogeneic HCT (allo-HCT). Transcriptional studies demonstrated that expression of the OX40-encoding transcript TNRFSF4 was increased in peripheral blood CD3\(^{+} \)CD20\(^{-} \) T cells isolated from the No Rx cohort and from NHP recipients prophylaxed with either Tac/MTX or sirolimus monotherapy (Fig. 1D). This observation was also made in clinical samples (3) from patients diagnosed with aGVHD within the first month of transplant compared to those who did not develop GVHD (Fig. 1E). Moreover, OX40L blockade using the novel human anti-OX40L antibody KY1005 could inhibit alloproliferation of human cells in vitro (Fig. 1F), suggesting that blockade of this pathway could be a target for in vivo GVHD prevention.

OX40L has previously been shown to be expressed on activated antigen-presenting cells (APCs), including myeloid dendritic cells [mDCs; reviewed in (22)], and this expression has been shown to play an important role in APC–T cell interactions (23, 24). Thus, we monitored the expression of OX40L on lymph node–derived HLA-DR\(^{+} \)CD3\(^{+} \)CD20\(^{-} \)DCs from HC and from the No Rx cohort, which were subdivided into CD123\(^{+} \)plasmacytoid DC (pDC) and CD11c\(^{+} \)mDC subsets (fig. S2). As shown in Fig. 1G, the percentage of OX40L\(^{+} \) mDCs was increased during aGVHD, whereas there was no parallel increase in OX40L\(^{+} \) pDCs.

**OX40L blockade controls the expansion of CD4 conventional T cells after HCT and preserves T\(_{\text{reg}} \) reconstitution**

To determine the impact of isolated OX40L blockade on T cell reconstitution and aGVHD, we performed monoprophylaxis experiments, wherein transplant recipients were prophylaxed with the KY1005 antibody alone in the peritransplant period, beginning on day –2 and continuing weekly thereafter, using KY1005 (10 mg/kg per dose; Fig. 2A). This dosing regimen resulted in a peak KY1005 concentration of 320.1 ± 18.3 \( \mu \)g/ml and a trough concentration of 107.9 ± 11.7 \( \mu \)g/ml (fig. S3A). Although prophylaxis with KY1005 did not affect the rapid initial burst of CD4 T cell proliferation that occurred after HCT in the absence of immunosuppression, it had an inhibitory impact on sustained CD4 T cell proliferation, as measured by Ki67 expression (Fig. 2B). Thus, as shown in the figure, CD4 T cell proliferation in the presence of KY1005 persisted during the first week after transplant, after which there was a decrease in the number of proliferating cells that correlated with a decrease in the accumulation of CD4\(^{+} \) T cells in these animals (Fig. 2C). KY1005 as a monotherapy had less effect on the proliferation of CD8\(^{+} \) T cells, which is consistent with the low OX40 expression of this T cell subset (Fig. 2B).

To determine which CD4 T cell subpopulations were most prominently affected by OX40L blockade, we measured the relative impact of KY1005 on T\(_{\text{N}}\) T\(_{\text{CM}}\) and T\(_{\text{EM}}\). We have previously shown that NHP aGVHD is associated with the expansion of CD4\(^{+} \) T\(_{\text{CM}}\) and CD8\(^{+} \) T\(_{\text{CM}}\)/T\(_{\text{EM}}\) (17), and in the current study, we observed that CD4 T\(_{\text{CM}}\)s represent the major reservoir of OX40\(^{+} \) lymphocytes (Fig. 1B). Figure 2D and fig. S3 (A to C) document that KY1005’s predominant impact on conventional T cells (T\(_{\text{conv}}\)) was within the CD4 T\(_{\text{CM}}\) compartment, leading to significant reductions (\( P < 0.05 \)) in both the relative and absolute numbers of CD4 T\(_{\text{CM}}\) without similar impact on CD8 T\(_{\text{CM}}\) (fig. S3, B and D) after HCT. In contrast, the T\(_{\text{N}}\) compartment of both CD8 and CD4 cells was preserved after transplant with KY1005 prophylaxis (Fig. 2D and fig. S3, B and D). Notably, monoprophylaxis with KY1005 did not impair CD8 T\(_{\text{EM}}\) expansion. OX40L blockade also produced a targeted impact on the ability of CD4\(^{+} \) T cells to produce cytokines after transplant, specifically reducing their ability to express IL-17A, without affecting their production of interferon-\( \gamma \) (IFN-\( \gamma \)), TNF\(_{\alpha}\), or IL-2 (Fig. 2E).

In addition to determining its effect on T\(_{\text{conv}}\) populations, we also interrogated the impact of KY1005 on CD4\(^{+} \) T\(_{\text{reg}}\)s given the fact that these cells express OX40, both in steady state and during unprophylaxed GVHD (fig. S3E). Notably, and pertinent to the control of GVHD, although KY1005 reduced the relative numbers of OX40\(^{+} \) T cells after transplant, this effect was restricted to T\(_{\text{conv}}\), with the relative numbers of OX40\(^{+} \) T\(_{\text{reg}}\)s remaining stable after transplant, even during KY1005 prophylaxis (Fig. 2F and fig. S3E).

**OX40L blockade extends GVHD-free survival**

KY1005’s ability to restrain CD4\(^{+} \) T\(_{\text{CM}}\) proliferation and expansion was associated with clinical benefit. Thus, even as a monotherapy, KY1005 delayed clinical signs of aGVHD and extended GVHD-free...
controls included in the present study, we have shown that mTOR inhibition using sirolimus as a monotherapy (transplant schema shown in Fig. 3A) modestly prolongs survival after allo-HCT in NHPs [median survival time (MST), 14 days versus 8 days without GVHD prophylaxis; *P < 0.001]. However, despite maintaining therapeutic serum concentrations (5 to 15 ng/ml) and successfully blocking mTOR signaling pathways, as measured by gene set enrichment analysis (GSEA) (fig. S5 and table S1), sirolimus alone was insufficient to fully control T cell proliferation (Fig. 3B), and recipients ultimately developed severe disease with clinical and immunopathologic features similar to unprophylaxed aGVHD (fig. S4). We next combined sirolimus with weekly dosing of KY1005 (10 mg/kg) from day −2 to day 54 after transplant [KY1005 peak concentration of 297.5 ± 10.9 μg/ml and

**Combination prophylaxis with KY1005 plus sirolimus synergistically controls T cell activation during post-HCT hematopoietic reconstitution**

In previously published results (9, 17) and in contemporaneous controls included in the present study, we have shown that mTOR

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trough concentration of $113.8 \pm 7.9 \mu g/ml$ between days 0 and 30, and (due to the long half-life of the antibody and resultant accumulation) increased peak serum concentration of $372.5 \pm 17.5 \mu g/ml$ and trough concentration of $204.9 \pm 9.6 \mu g/ml$ between days 31 and 60, with an estimated mean terminal half-life of $20 \pm 8$ days) (fig. S6A). This combined immunophrophylaxis resulted in significant control of both CD4$^+$ and CD8$^+$ T cell proliferation ($P < 0.05$; Fig. 3B and fig. S6, B and C). KY1005/sirolimus sustained the reconstitution of CD4$^+$ and CD8$^+$ T$N$ after allo-HCT, despite controlling the expansion of both CD4 T$C_M$ and CD8 T$C_M$/T$E_M$ (Fig. 3, C and D, and fig. S6, D and E). Consistent with this flow cytometric analysis, transcriptional analysis using GSEA (25) also demonstrated enrichment of naïve cells, with overrepresentation of gene sets associated with naïve CD4 and CD8 T lymphocytes in the KY1005/sirolimus cohort in comparison to both the KY1005 and the sirolimus monotherapy cohorts (Fig. 3E and table S1).

All recipients prophylaxed with KY1005/sirolimus exhibited successful donor engraftment, with robust T cell chimerism (Fig. 4A) and with effective hematologic reconstitution after transplant, with
all recipients demonstrating prompt multilineage hematologic recovery (Fig. 4, B to E). As shown in the figure, the hematologic recovery was similar to a control group of recipients of autologous HCT who did not receive immunoprophylaxis and who demonstrated long-term GVHD-free survival. Recipients were clinically healthy after transplant, without viral, fungal, or symptomatic bacterial disease (table S2). In particular, cytomegalovirus (CMV) reactivation and disease were monitored prospectively, with no recipients developing viral disease. As expected after myeloablative HCT, CMV reactivation did occur in some (three of five) recipients, similar to autologous controls (Fig. 4F), but all episodes of viral reactivation were successfully controlled with cidofovir prophylaxis and standard ganciclovir therapy.

**KY1005/sirolimus preserves T<sub>reg</sub> reconstitution after allo-HCT**

To determine the impact of HCT on T<sub>reg</sub>, we tracked these cells in the peripheral blood of transplant recipients. We found that the development of fulminant aGVHD in unprophylaxed transplant recipients was associated with a rapid decline of absolute T<sub>reg</sub> cell numbers in the peripheral blood (30.0 ± 11.0 cells/µl before HCT versus 2.9 ± 0.9 cells/µl at terminal analysis; P < 0.05) as well as in a significant decrease in the T<sub>reg</sub>/100 T<sub>conv</sub> ratio (2.0 ± 0.4 before HCT versus 0.6 ± 0.1; P < 0.001; Fig. 5A). As monoprophylactic regimens, both KY1005 and sirolimus protected against this drop in the T<sub>reg</sub>/T<sub>conv</sub> ratio (Fig. 5A). Combined KY1005/sirolimus prophylaxis was able to not only preserve but also significantly (1.30 ± 0.30 before HCT versus 1.82 ± 0.43; P < 0.05) augment the T<sub>reg</sub>/ T<sub>conv</sub> ratio in all transplant recipients, an effect that remained stable for the duration of analysis (Fig. 5, A and B) and was associated with preservation of the absolute number of T<sub>reg</sub> in the peripheral blood for the duration of the experiment (Fig. 5C). Transcriptomic analysis confirmed these flow cytometric findings, with enrichment for T<sub>reg</sub>-associated gene sets in the KY1005/sirolimus cohort compared to KY1005 and sirolimus monotherapies (Fig. 5D and table S1).

**KY1005/sirolimus combination prophylaxis synergistically protects against clinical and pathologic aGVHD**

The ability of KY1005/sirolimus to preserve T<sub>reg</sub> reconstitution despite controlling T<sub>eff</sub> activation resulted in the synergistic protection against aGVHD in this cohort. The combined therapy provided potent control of the clinical signs of aGVHD (Fig. 6A), which resulted in prolonged (>100 days) GVHD-free survival after transplantation (Fig. 6B) even after discontinuation of...
KY1005 at day 54 after transplant (fig. S6A). This survival was significantly different than all comparator cohorts (P < 0.01). Thus, although both sirolimus and KY1005 as monophylaxis extended survival compared to no prophylaxis (MST, 14 and 19.5 days, respectively; P < 0.05 for both), KY1005/sirolimus effectively controlled clinical aGVHD for the length of the planned analysis (Fig. 6A), resulting in an MST of >100 days, with P < 0.001 compared to No Rx, sirolimus, and KY1005 (Fig. 6B) and which was confirmed by histopathologic analysis (fig. S4). KY1005/sirolimus also displayed superior control of aGVHD and survival compared to previously published (3, 9) Tac/MTX and CTLA4-Ig/sirolimus cohorts (fig. S6F), underscoring the unique ability of this strategy to control alloreactivity.

To investigate the specificity in control of alloreactivity in these recipients, we performed ex vivo MLR studies to measure the ability of reconstituting donor T cells to alloproliferate against either recipient or third-party stimulator cells (Fig. 6, C to E). As expected, donor peripheral blood mononuclear cell (PBMC) samples minimally responded to autologous stimulator PBMCs, both before and after HCT [Fig. 6, C and D (left column) and E]. However, in the post-HCT setting, these cells demonstrated more proliferation when stimulated by either recipient or third-party cells [Fig. 6, C (middle and right columns) and E] than when stimulated by autologous donor cells. When responder PBMCs were harvested from recipients at the terminal posttransplant time point, in the setting of high donor T cell chimerism, a smaller amount of allopotent load was measured against recipient cells, whereas proliferation against third-party stimulators was better preserved, with equal or greater proliferation against third-party cells observed in three of five recipients [Fig. 6, D (middle and right columns) and E].

**Longitudinal transcriptional analysis identifies CD3+ T cell gene expression signatures associated with long-term control of aGVHD with KY1005/sirolimus**

Given that KY1005/sirolimus prophylaxis enabled aGVHD-free T cell reconstitution after allo-HCT, we interrogated the transcriptomes of these T cells to define the gene expression networks inherent in long-term aGVHD control. We identified several new pathways that were enriched in the KY1005/sirolimus cohort in comparison to other immunophylaxis regimens and HC. The comparison to HC was prompted by the fact that, although KY1005/sirolimus-prophylaxed recipients were healthy, the transcriptome of T cells that reconstituted in these recipients was, nonetheless, distinct from that in untransplanted animals. Thus, as documented with principal components analysis (Fig. 7A), the KY1005/sirolimus transcriptome profiles were distinct from all others, including those from HC. To elucidate transcriptional signals that distinguished KY1005/sirolimus from HC, we performed pathway analysis using a strategy designed to identify the most stable enriched pathways in KY1005/sirolimus recipients, defined as those that were enriched compared to HC at all three posttransplant time points analyzed (days +14, +28, and +100). This analysis identified 101 transcripts that were stably enriched (Fig. 7B and table S3). Analysis

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Fig. 4. KY1005/sirolimus combination prophylaxis permits robust hematopoietic reconstitution after allo-HCT. (A) Donor T cell chimerism, determined using microsatellite analysis, in flow cytometrically sorted CD3+CD20− T cells after allo-HCT. Chimerism for the No Rx (n = 7), KY1005 (n = 4), and sirolimus (n = 5) cohorts is shown at terminal analysis. Chimerism for the KY1005/sirolimus cohort (n = 5) is shown at days 28, 60, and 100 after transplant. (B to E) Absolute blood cell counts—absolute CD3+CD14−CD20− T cell counts (T cells; B), absolute neutrophil counts (ANC; C), absolute lymphocyte counts (ALC; D), and absolute platelet counts (PLT; E)—in allo-HCT recipients from the KY1005/sirolimus cohort (n = 5; green) and autologous HCT cohort (n = 6; gray). Data are means (line with symbols) with SEM (shaded area around the line). (F) CMV viral load in the peripheral blood of autologous HCT recipients (gray lines) and KY1005/sirolimus-prophylaxed allo-HCT recipients (green lines). Each line represents an individual recipient.
of the resulting data set using DAVID (Database for Annotation, Visualization and Integrated Discovery) (26, 27) revealed statistically significant enrichment for two pathways: the KEGG (Janus kinase/signal transducer and activator of transcription) signaling pathway and the reactome HSA-909733:IFN-α/β signaling pathway. Enrichment in the JAK-STAT pathway was identified with both DAVID-based pathway analysis and GSEA (Fig. 7C, top, and table S1) and demonstrated enrichment of these pathways in the KY1005/sirolimus cohort (n = 5). With respect to TH/TC1-driven hyperacute GVHD, we observed that T cells in the KY1005/sirolimus cohort significantly down-regulated canonical hyperacute GVHD, in both NHP and human T cells (30, 31), and Tac/MTX cohorts (table S4). These analyses identified several transcripts that have been previously recognized to serve a critical function in IFN-α/β sensing or signal transduction (including IGS15, IFIT3, and RSAD2) or in the effector antiviral response (IFIT1, IFIT2, IFIT3, MX1, OASL, OAS2, and RSAD2) (38, 39).

As a final test of the pathways associated with the ability of KY1005/sirolimus to control aGVHD, we interrogated whether their transcripts were provided evidence for control of the T1H/Tc1 and T1H/Tc17 pathways that we have previously demonstrated to be activated during aGVHD, in both NHP and human T cells (3, 9). With respect to T1H/Tc17-driven hyperacute GVHD, we observed that T cells in the KY1005/sirolimus cohort significantly down-regulated canonical hyperacute GVHD transcripts (Ki67 and granzyme A; Fig. 8A) and T1H/Tc17-associated gene sets (including the canonical T1H/Tc1 genes CCR5, IL12RB2, and IFNG; Fig. 8B and table S1). Notably, despite down-regulation of the above T1H/Tc17-associated transcripts, the expression of TBX21 (encoding Tbet) itself was not significantly changed during GVHD compared to HC (Fig. S7). With respect to T1H/Tc17-driven breakthrough acute GVHD, we found that KY1005/sirolimus successfully normalized
the expression of the TH17-defining transcription factor RORC (Fig. 8C) and successfully inhibited T_{H1}/T_{C17}-associated gene sets (Fig. 8D and table S1) when compared to Tac/MTX and CTLA4-Ig/sirolimus, both of which we have previously shown to be associated with breakthrough acute disease (3). Given the wealth of data that document the centrality of both these pathways to aGVHD pathology, the ability of KY1005/sirolimus to control them is striking. It suggests that we have established a transcriptional standard for the control of alloreactivity, which includes the positive signaling pathways described above, along with potent control of both T_{H1}/T_{C1} and T_{H1}/T_{C17}-mediated immune pathology.

DISCUSSION

Here, we have identified OX40L blockade as a synergistic combinatorial strategy with sirolimus to control aGVHD and have identified the immune pathways that associate with this control. Our interest in OX40-OX40L cosignaling derives from the fact that it represents one of the few pathways for which there are data to suggest a potential dichotomous effect on Tconv versus Tregs. It is well documented that OX40 is up-regulated on CD4>>CD8 Tconv during activation (18, 23, 40) and that OX40-OX40L blockade in murine aGVHD models can augment survival of allo-HCT recipients, with the major effects observed in CD4+ T cell–mediated aGVHD (41–43). In contrast to its positive impact on T_{conv} OX40 ligation on Treg can have the opposite effect, that is, inhibition of T_{reg} survival (18) and an inhibition of their ability to suppress T_{eff} activation (18, 19, 44). This suggests that blocking the OX40-OX40L pathway might, paradoxically, have salutary effects on T_{reg} function. There has been controversy concerning this effect, however, and several studies have argued that OX40 signaling enhances T_{reg} survival and expansion, particularly during lymphopenia and inflammation (45–47). However, these studies were all performed in mice, and until this study, it remained undetermined which effects of OX40-OX40L blockade would translate to primates or how the presence of combinatorial immunomodulation would alter these outcomes. This is especially important, given that combination therapies will certainly be necessary for successful clinical translation. Our results have the potential to affect clinical practice based on two key attributes: First, they combine OX40L blockade with sirolimus, an established immunomodulation platform. Second, the anti-OX40L antibody studied, KY1005, is poised for clinical implementation: It is a human antibody, developed for clinical use, and is based on two key attributes: First, they combine OX40L blockade with sirolimus, an established immunomodulation platform. Second, the anti-OX40L antibody studied, KY1005, is poised for clinical implementation: It is a human antibody, developed for clinical use, and is now in phase 1 testing in healthy volunteers and patients with psoriasis [ClinicalTrials.gov no. NCT03161288 (48)]. Trials of combinatorial prophylaxis with KY1005/sirolimus will be critical to establish the clinical safety and efficacy of this strategy, including its impact on other clinical outcomes, including GVL effects. Although previous murine studies demonstrated that the elimination of OX40+ T cells from an allograft could reduce aGVHD and simultaneously preserve both GVL and antiviral immunity (21), there is no leukemia model in NHPs, and thus, this issue cannot be directly evaluated in primates before clinical evaluation. As with all new immunomodulatory strategies, early-phase
clinical trials with KY1005/sirolimus will thus need to use strong stopping rules to mitigate any increased risk of relapse.

Although interrogating synergy in the setting of combination therapy is critical, one of the strengths of NHP models compared to clinical studies is their ability to also specifically probe bioactivity and mechanism of action of novel agents through monoprophylaxis experiments. Our results demonstrate that OX40L blockade with KY1005 significantly controlled conventional CD4^+ T cell proliferation and effector maturation and could stabilize the T_{reg}/T_{conv} ratio, which was otherwise seriously degraded after transplant. However, despite the clear biologic effect of KY1005 as a monotherapy, and likely because of its predominant impact on CD4^+CD8^+ T cell proliferation and activation, it was insufficient to fully protect from aGVHD.

In contrast, KY1005/sirolimus prophylaxis was able to potently control T cell activation and yet still support successful hematologic reconstitution and donor engraftment after transplant. Transplant
Fig. 8. KY1005/sirolimus prophylaxis efficiently controls both Th1- and Th17-driven alloimmunity. (A) Relative expression (log2-normalized fluorescence intensity signal) of GZMA and MKI67 gene transcripts (encoding granzyme A, Ki67, and RORγt, respectively) in CD3+CD20+ T cells, flow cytometrically sorted from the peripheral blood of animals from the indicated experimental cohorts. Gene expression was measured using GeneChip Rhesus Macaque Genome Array (Affymetrix), as detailed in Materials and Methods. ** denotes comparisons with a moderated t statistic of <0.05 corrected for multiple hypothesis testing using the Benjamini-Hochberg procedure between the indicated experimental groups. (B) Representative GSEA plots performed as previously described (25), showing Th1/Th17-related gene sets underrepresented in KY1005/sirolimus on day 14 (n = 5) versus KY1005 (n = 3; left) or versus sirolimus (n = 4; right) cohorts at the time of necropsy. (C) Relative expression (log2-normalized fluorescence intensity signal) of RORC gene transcript (encoding RORγt) in CD3+CD20+ T cells. T cell isolation, gene expression measurement, and statistical analysis were performed as in (A). *P < 0.05, moderated t test with Benjamini-Hochberg correction. (D) Representative GSEA plots performed as previously described (25), showing Th1/Th17-related gene sets underrepresented in KY1005/sirolimus on day 100 (n = 4) versus CTLA4-Ig/sirolimus (n = 7; left) or versus Tac/MTX (n = 3; right) cohorts at the time of necropsy.

Recipients prophylaxed with this combination remained clinically healthy and free from signs of GVHD, even with weaning of KY1005 at day 54 after transplant. They exhibited recipient-specific blunting of ex vivo alloreactivity, with better maintenance of anti–third-party proliferative responses. This is the first time that we have successfully controlled GVHD for ≥100 days after transplant in NHPs undergoing high-risk T cell–replete major histocompatibility complex (MHC) haploidentical HCT (3, 9, 17), representing a milestone for this model.

Flow cytometric and transcriptomic analyses allowed us to probe the mechanisms driving the control of alloreactivity in KY1005/sirolimus-prophylaxed animals. One of the most notable findings was the ability...
of this combination strategy to enhance the Treg/Tconv ratio after transplant, an effect that was durable for the length of analysis, even after weaning KY1005. Although these experiments did not allow us to specifically test whether the expanding Treg were donor-specific, this result nonetheless supports the hypothesis that in aGVHD, OX40L blockade does have dichotomous effects on Treg compared to T eff, an effect that is strengthened by combination with sirolimus. This critical observation supports the clinical translation of this therapeutic strategy, given its unique ability to potently suppress T eff activation and simultaneously support Treg reconstitution.

In addition to identifying the TN and Treg signatures in the KY1005/sirolimus-prophylaxed T cells, transcriptome analysis revealed other enriched pathways that deserve special discussion. These include the JAK-STAT signaling pathway and the type I IFN pathways, which were both robustly enriched in KY1005/sirolimus T cells compared to all other cohorts. The enrichment in JAK-STAT signaling is somewhat unexpected given the well-established role of cytokine signaling in promoting alloreactivity after HCT (31) and the promising results of clinical trials using JAK-STAT inhibitors for GVHD prevention (49, 50). However, the detailed analysis of this JAK-STAT signature revealed that enrichment in transcripts encoding proinflammatory cytokines/cytokine receptors was counterbalanced by the enrichment in genes encoding anti-inflammatory cytokines and their receptors, suggesting complex regulation of this pathway in the setting of combined mTOR/OX40L blockade.

Although the type I IFN pathways, canonically associated with antiviral and other pathogen-driven responses, are classically attributed to innate cells, they are also well documented in T cells (51, 52). Given that we observed CMV reactivation in recipients who were prophylaxed with KY1005/sirolimus, these pathways may have been activated as part of an antiviral response. However, whether incited by viral reactivation or induced directly during combined OX40L and mTOR blockade, the activation of these pathways may also have contributed to the potent control of aGVHD mediated by KY1005/sirolimus. Thus, previous studies have documented significant activity of type I IFN pathways against aGVHD (53, 54), in addition to evidence supporting the ability of these signaling pathways to restrain Treg17-driven inflammation in autoimmune models (55, 56) and in patients with inflammatory bowel disease (57). Moreover, type I IFNs have also been shown to enhance Treg survival and anti-inflammatory function in both mice and humans (58–60). However, the data surrounding IFNs are complex; some experiments indicate that these pathways can also potentiate CD8-dependent alloimmunity and, in so doing, can potentiate GVL responses and overcome tumor-driven T cell tolerance (61), thus potentially augmenting, rather than controlling, aGVHD (53, 54).

Our data set underscores the influence of canonical innate immune pathways on the adaptive immune response and indicates that these pathways may enhance rather than impede the control of aGVHD.

One of the most important observations in the current study is the degree to which previously established transcriptomic signatures of both hyperacute (T eff/Treg:1) and breakthrough acute (T eff/Treg:17) aGVHD (3, 9) were normalized in T cells emerging during KY1005/sirolimus prophylaxis. These pathways have been identified during aGVHD that occurs despite both Tac/MTX- and sirolimus-based combinatorial immunoprophylaxis and therefore define key, clinically relevant immunologic barriers to aGVHD control. Our results suggest that KY1005/sirolimus sets a transcriptomic standard for aGVHD prevention and for pathways leading to intact T cell homeostasis after transplant. The ability of KY1005/sirolimus to control pathology in the complex, clinically relevant NHP model of aGVHD suggests that this regimen may be a promising candidate for clinical translation.

**MATERIALS AND METHODS**

**NHP transplant study design**

This was a cohort study in NHPs designed to determine the clinical, immunologic, and molecular outcomes of OX40L blockade during allo-HCT. All primary data associated with this study are found in table S10. Two cohorts of transplant recipients were studied: (i) allogeneic transplants using the OX40L-blocking human IgG4 antibody KY1005 (Kymab Ltd.; antibody design described in detail below) as monotherapy for GVHD prophylaxis (abbreviated as KY1005; n = 4) (KY1005 was given at a dose of 10 mg/kg starting on day –2 and then once weekly until planned discontinuation on day 54; Fig. 2A) and (ii) allogeneic transplants using KY1005 in combination with sirolimus (with the target range of 5 to 15 ng/ml) for GVHD prophylaxis (abbreviated as KY1005/sirolimus; n = 5) (KY1005 was given at a dose of 10 mg/kg starting on day –2 and then once weekly until discontinuation on day 54; Fig. 3A). These two OX40L blockade groups were compared to the following cohorts, aspects of which have been described previously (3, 9, 17): (i) autologous transplants [abbreviated as Auto; n = 6 (clinical and flow cytometric data) and 4 (transcriptomic analysis)]; (ii) allogeneic transplants with no GVHD prophylaxis [abbreviated as No Rx; n = 11 (clinical and flow cytometric data) and 4 (transcriptomic analysis)]; (iii) allogeneic transplants using sirolimus monotherapy for GVHD prophylaxis [abbreviated as sirolimus; n = 11 (flow cytometric analysis) and 4 (transcriptomic analysis); LC Laboratories] [sirolimus was given daily for the length of analysis as an intramuscular formulation as previously described with doses adjusted to achieve a serum trough concentration of 5 to 15 ng/ml (3, 9, 17); this cohort contains a subgroup of animals prophylaxed with sirolimus alone (n = 5), which were only evaluated until day +9 after transplant, and for which flow cytometry data were included in this study]; (iv) allogeneic transplants using CTLA4-Ig plus sirolimus, reported previously (n = 7) (3); and (v) allogeneic transplants using tacrolimus plus methotrexate for GVHD prophylaxis (n = 3) (3, 9).

Transplant recipients and donors were chosen from breeding colonies based on their MHC genotypes. For these studies, we used both microsatellite and allele-specific MHC typing (62–64) to choose donor-recipient pairs. The vast majority of our recipients and donors were half-siblings and were MHC haploidentical, with a small number of pairs being unrelated and either haploidentical or otherwise MHC mismatched (table S5). Blinding was performed on all pathologic analysis and on the initial analysis of flow cytometry data, as well as on transcriptome sample handling and data processing.

**NHP ethics statement**

This study was conducted in strict accordance with U.S. Department of Agriculture regulations and the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. It was approved by the Emory University and the University of Washington Institutional Animal Care and Use Committees.

**Human study design**

This study was designed as a retrospective, case-control study. Available cryopreserved patient PBMC samples were obtained from patients enrolled in HCT clinical trials performed at Emory University and the University of Minnesota. Patients from Emory University were enrolled on two contemporaneous institutional review board (IRB)--approved


11 of 14

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clinical trials: (i) the Bone Marrow Immune Monitoring Protocol and (ii) the Abatacept Feasibility Study, as previously described (65, 66). The patients from the University of Minnesota were enrolled on an immune monitoring protocol approved by the University of Minnesota IRB. Samples were collected on day 28 from patients with confirmed aGVHD diagnosis or from HCT recipients without GVHD who were matched for sample collection day, preparative regimen intensity, disease, stem cell source, and GVHD prophylaxis. Patients were on GVHD prophylaxis with CNI (cyclosporin A or tacrolimus) plus antiproliferative agent (MMF or methotrexate). Clinical details pertaining to each of the samples included in the clinical gene array analysis are shown in table S9.

Human studies ethics statement
The patients and healthy participants described in this study were enrolled in clinical trials that were conducted according to the principles set forth in the Declaration of Helsinki and that were approved by the IRBs. Written informed consent was received from all participants.

Transplant strategy
We used our previously described strategy for allo-HCT in rhesus macaques, described in detail in Supplementary Materials and Methods (17).

Immunologic analysis
Flow cytometry: Longitudinal flow cytometric analysis
Multicolor flow cytometric analysis was performed using an LSRII or LSR Fortessa flow cytometer (BD Biosciences) on all transplant recipients. Details of this analysis are provided in Supplementary Materials and Methods.

Flow cytometric CD3+ T cell sorting and microarray cohort designation
Details of sorting and microarray cohort designation are provided in Supplementary Materials and Methods.

NHP microarray and data analysis
Details of the NHP microarray protocol used and data analysis paradigms followed are described in Supplementary Materials and Methods.

Transcriptional studies of patient samples
Transcriptional studies on patient samples are described in detail in Supplementary Materials and Methods.

Statistical analysis
Statistical analysis of histopathologic and flow data
Distributions of values within all groups were checked for Gaussian distribution using the D’Agostino-Pearson normality test. Both paired and unpaired Student’s t test (for normally distributed values) or the Mann-Whitney test (for non-normal data) was then used where appropriate. Analysis of variance (ANOVA) with Holm-Sidak multiple comparison t test was used for comparing multiple groups. Groups were considered as significantly different when $P < 0.05$

Statistical analysis of transcriptomic data
Analyses of gene differential expression were performed using an empirical Bayes moderated t statistic, with a cutoff of 0.05, corrected for multiple hypothesis testing using Benjamini–Hochberg procedure, and an absolute fold change cutoff of $>1.4$ with the limma package (67).

REFERENCES AND NOTES
prophylaxis for reduced-intensity conditioning umbilical cord blood transplantation.


Combined OX40L and mTOR blockade controls effector T cell activation while preserving Treg reconstitution after transplant


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Tackling T cells in GVHD

Graft-versus-host disease (GVHD) after stem cell transplantation is mediated by effector T cells derived from donor stem cells, but GVHD can also be abrogated by donor-derived regulatory T cells. GVHD prophylaxis ideally should then allow regulatory T cell responses while inhibiting effector T cells. Tkachev et al. now provide very promising results in a nonhuman primate model, which suggest that such therapy is possible. They used mTOR inhibition in combination with OX40L blockade, which resulted in reduced damaging T cell reconstitution but preserved regulatory T cell activity. The combination therapy also led to a considerable survival benefit. These findings support testing of this therapy in patients.