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Victor Tkachev, Seattle Children's Research Institute
Scott N. Furlan, Seattle Children's Research Institute
Benjamin Watkins, Emory University
Daniel J. Hunt, Seattle Children's Research Institute
Hengqi Betty Zheng, Seattle Children's Research Institute
Angela Panoskaltsis-Mortari, University of Minnesota
Kayla Betz, Seattle Children's Research Institute
Melanie Brown, Seattle Children's Research Institute
John B. Schell, Seattle Children's Research Institute
Katie Zeleski, Seattle Children's Research Institute

Only first 10 authors above; see publication for full author list.

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

Victor Tkachev1,*, Scott N. Furlan1, Benjamin Watkins1,3, Daniel J. Hunt1, Hengqi Betty Zheng1, Angela Panoskaltsis-Mortari2, Kayla Betz1, Melanie Brown1, John B. Schell1, Katie Zeleski1, Alison Yu1, Ian Kirby4, Sarah Cooley2, Jeffrey S. Miller2, Bruce R. Blazar2, Duncan Casson4, Phil Bland-Ward4, and Leslie S. Kean1,*

1Ben Towne Center for Childhood Cancer Research, Seattle Children's Research Institute, Seattle WA, the University of Washington, Seattle WA, and the Fred Hutchinson Cancer Research Center, Seattle WA 98101
2Department of Pediatrics, Division of Blood and Marrow Transplantation, University of Minnesota, Minneapolis, MN 55454
3Emory University School of Medicine, Atlanta GA 30322
4Kymab Ltd, Cambridge UK CB22 3AT

Abstract

One of the critical questions facing the field of transplantation is how to control effector T cell activation yet simultaneously preserve regulatory T cell (Treg) function. Thus, standard calcineurin inhibitor-based strategies can partially control effector T cells (Teffs), but breakthrough activation still occurs, and these agents are antagonistic to Treg function. Conversely, mTOR inhibition with sirolimus is more Treg-compatible, but is inadequate to fully control Teff activation. In contrast, blockade of OX40L signaling has the capacity to partially control Teff activation despite maintaining Treg function. Here we have used the non-human primate (NHP) 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 striking, synergistic control of GVHD with KY1005 + sirolimus (median survival time >100 days, p< 0.01 compared to all other regimens), which was associated with potent control of both Th/Tc1 and Th/Tc17 activation. Combined administration also maintained Treg reconstitution (resulting in an enhanced Treg:Tcon 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

*To whom correspondence should be addressed: leslie.kean@seattlechildrens.org, vtkach@uw.edu.

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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 allo-immunity 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 allo-immunity is the ability to simultaneously control the proliferation and activation of effector T cells (Teff) and still support regulatory T cell (Treg) homeostasis. This represents a particularly difficult challenge, as most non-targeted immunosuppressive agents have non-discriminatory inhibitory effects on both effector and regulatory populations. This is certainly true for calcineurin inhibitors (CNI), which are the mainstay of immunosuppression for both SOT and HCT. Both cyclosporine and tacrolimus CNIs have been shown to be detrimental to Treg 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 17 cell /Cytotoxic T 17 cells (Th/Tc17) pathways along with defects in Treg reconstitution and function, which results in breakthrough GVHD after HCT in non-human primates (NHP) (3). In contrast, 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 Treg function and homeostasis (1, 2, 4). However, although sirolimus has several pro-tolerogenic 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: First, post-transplant monotherapy with sirolimus, in the absence of adjunctive pre-transplant GVHD prevention (7, 8) is unable to sufficiently control Teff activation and, thus cannot in itself prevent GVHD (3, 9). Further, combination strategies that pair sirolimus with CNI 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 Treg 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 Teffs and, at the same time, permit Treg 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 Tregs on these pathways (14, 15) and resistance of Teff and memory T cells (Tmem) 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 Tmem and Teff function and nonetheless permit Treg homeostasis and function (18-20).
Moreover, transplant with OX40- T cells has been shown to mitigate GVHD despite preserving graft-versus leukemia (GVL) and anti-viral T cell responses (21). Here we show that, when combined with sirolimus, OX40L blockade with a novel IgG4 anti-CD252 antibody, ‘KY1005’, provides potent control of both Th/Tc1 and Th/Tc17 activation as well as synergistically preserving Treg 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+ mDC 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 following 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 (Figure 1A). The increased expression of OX40 could be measured on CD4 T cells isolated from the peripheral blood (Figure 1A), as well as from lymphoid and non-lymphoid organs during unprophylaxed aGVHD, and also during aGVHD that occurred in the setting of Tac/MTX prophylaxis (Figure S1A), suggesting that up-regulation of OX40 might be a hallmark of alloreactive CD4 T cells regardless of immunoprophylaxis regimen. The majority of OX40 expression was found in the CD4+ central memory (T_{CM}) compartment, whereas naïve and effector memory CD4 T cells remained largely OX40-negative prior to and after HCT (Figure 1B). Consistent with this, OX40+ CD4 T cells bore hallmarks of a differentiated cell population, expressing more effector cytokines (including IL-2, TNFα, and IL-17A), and with a higher proportion of polyfunctional T cells (simultaneously expressing >2 cytokines) than their OX40- counterparts (Figure 1C and Figures S1B-C). This observation was consistent among T cells isolated from HC and those from recipients diagnosed with aGVHD following unprophylaxed allo-HCT. Transcriptional studies demonstrated that expression of the OX40-encoding transcript, TNFRSF4, was increased in peripheral blood CD3+CD20- T cells isolated from the No Rx cohort, as well as from NHP recipients prophylaxed with either Tac/MTX or with sirolimus monotherapy. (Figure 1D). Importantly, this observation was also made in clinical samples (3) from patients diagnosed with aGVHD within the first month of transplant compared to those that did not develop GVHD (Figure 1E). Moreover, OX40L blockade using the novel human anti-OX40L antibody KY1005, could inhibit allo-proliferation of human cells in vitro, (Figure 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 (APC), including myeloid dendritic cells (mDC; 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-CD56-DC from HC and from the No Rx cohort, which were subdivided into CD123+ plasmacytoid (pDC) and CD11c+ mDC subsets (Figure S2). As shown in Figure 1G, the percentage of OX40L+ mDC was increased during aGVHD, whereas there was no parallel increase in OX40L+ pDC.

**OX40 blockade controls the expansion of conventional CD4 T cells after HCT and preserves Treg reconstitution**

To determine the impact of isolated OX40L blockade on T cell reconstitution and aGVHD, we performed monotherapy experiments, wherein transplant recipients were prophylaxed with the KY1005 antibody alone in the peri-transplant period, beginning on Day -2 and continuing weekly thereafter, using 10mg/kg KY1005 per dose (Figure 2A). This dosing regimen resulted in a peak KY1005 concentration of 320.1 ± 18.3 μg/mL and a trough of 107.9 ± 11.7 μg/mL (Figure 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 (Figure 2B). Thus, as shown in the figure, CD4 T cell proliferation in the presence of KY1005 persisted during the first week post-transplant, after which there was a decrease in the number of proliferating cells that correlated with a decrease in the accumulation of OX40+ T cells in these animals (Figure 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 (Figure 2B).

To determine which CD4 T cell subpopulations were most prominently affected by OX40L blockade, we measured the relative impact of KY1005 on naïve (T_N), central memory (T_CM) and effector memory (T_EM) T cells. We have previously shown that NHP aGVHD is associated with the expansion of CD4+ T_CM and CD8+ T_CM/T_EM, (17) and in the current study we observed that CD4 T_CM cells represent the major reservoir of OX40+ lymphocytes (Figure 1B). Figure 2D and Figure S3A-C document that KY1005’s predominant impact on conventional T cells was within the CD4 T_CM compartment, leading to significant reductions (p<0.05) in both the relative and absolute numbers of CD4 T_CM cells, without similar impact on CD8 T_CM cells (Figure S3B, D) after HCT. In contrast, the naïve T cell compartment of both CD8 and CD4 cells was preserved after transplant with KY1005 prophylaxis (Figure 2D and Figure S3B, D). Notably, monophylaxis with KY1005 did not impair CD8 T_EM cell 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 IFNγ, TNFα or IL-2 (Figure 2E).

In addition to determining its effect on conventional T cell populations, we also interrogated the impact of KY1005 on CD4+ Tregs, given the fact that these cells express OX40, both in steady-state and during unprophylaxed GVHD (Figure S3E). Of note, and pertinent to the control of GVHD, although KY1005 reduced the relative numbers of OX40+ T cells after transplant, this effect was restricted to conventional T cells with the relative numbers of

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OX40+ Tregs remaining stable after transplant, even during KY1005 prophylaxis (Figure 2F and Figure S3E).

OX40L Blockade Extends GVHD-Free Survival

KY1005’s ability to restrain CD4+ T_{CM} proliferation and expansion was associated with clinical benefit. Thus, even as a monotherapy, KY1005 delayed clinical signs of aGVHD and extended GVHD-free survival in NHP (Figure 2G-H). However, likely due to its predominant impact on CD4+ T cells without notable effects on CD8+ T cells, the clinical outcome differential of KY1005 monotherapy was relatively modest, leading to an improvement in GVHD-free survival from 8 days in the No Rx cohort to 19.5 days (p<0.05), and with histopathologic evidence for severe disease at terminal analysis (Figure S4).

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 inhibition using sirolimus as a monotherapy (transplant schema shown in Figure 3A) modestly prolongs survival following allo-HCT in NHP (MST = 14 days vs. 8 days without GVHD prophylaxis, p < 0.001). However, despite maintaining therapeutic serum concentrations (5-15 ng/mL) and successfully blocking mTOR signaling pathways as measured by gene set enrichment analysis (Figure S5 and Table S1), sirolimus alone was insufficient to fully control T cell proliferation (Figure 3B) and recipients ultimately developed severe disease with clinical and immunopathologic features similar to unprophylaxed aGVHD (Figure S4). We next combined sirolimus with weekly dosing of 10mg/kg KY1005 from Day -2 to Day 54 post-transplant (KY1005 peak concentration = 297.5 ± 10.9 μg/mL and trough concentration =113.8 ± 7.9 μg/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 ± 17.5 μg/mL and troughs of 204.9 ± 9.6 μg/mL between days 31 and 60, with an estimated mean terminal half-life of 20 ± 8 days (Figure S6A) This combined immunoprophylaxis resulted in significant control of both CD4+ and CD8+ T cell proliferation (p<0.05; Figure 3B and Figure S6B, C). Importantly, KY1005/sirolimus sustained the reconstitution of naïve CD4+ and CD8+ T cells following allo-HCT, despite controlling the expansion of both CD4 T_{CM} and CD8 T_{CM}/T_{EM} cells (Figure 3C-D and Figures S6D, E). Consistent with this flow cytometric analysis, transcriptional analysis using Gene Set Enrichment Analysis (GSEA) (25) also demonstrated enrichment of naïve cells, with over-representation 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 (Figure 3E and Table S1).

Importantly, all recipients prophylaxed with KY1005/sirolimus exhibited successful donor engraftment, with robust T cell chimerism (Figure 4A) and with effective hematologic reconstitution post-transplant, with all recipients demonstrating prompt multilineage hematologic recovery (Figure 4B-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 post-transplant, without viral, fungal or symptomatic bacterial disease.

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(Table S2). In particular, CMV reactivation and disease were monitored prospectively, with no recipients developing viral disease. As expected after myeloablative HCT, CMV reactivation did occur in some (3 of 5) recipients, similar to autologous controls (Figure 4F), but all episodes of viral reactivation were successfully controlled with cidofovir prophylaxis and standard gancyclovir therapy.

KY1005/sirolimus preserved Treg reconstitution following allo-HCT

To determine the impact of HCT on Tregs, 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 Treg cell numbers in the peripheral blood (30.0 ± 11.0 cells/μL before HCT vs. 2.9 ± 0.9 cells/μL at terminal analysis; P<0.05) as well as in a significant decrease in the Treg:100 Tconv ratio (2.0 ± 0.4 before HCT vs. 0.6 ± 0.1, p <0.001, Figure 5A). As monoprophylactic regimens, both KY1005 and sirolimus protected against this drop in the Treg to Tconv ratio (Figure 5A). Importantly, combined KY1005/sirolimus prophylaxis was able to not only preserve, but to significantly (1.30 ± 0.30 before HCT vs. 1.82 ± 0.43; p<0.05) augment the Treg to Tconv ratio in all transplant recipients, an effect that remained stable for the duration of analysis (Figures 5A,B) and was associated with preservation of the absolute number of Tregs in the peripheral blood for the duration of the experiment (Figure 5C). Transcriptomic analysis confirmed these flow cytomteric findings, with enrichment for Treg-associated gene sets in the KY1005/sirolimus cohort compared to KY1005 and sirolimus monotherapies (Figure 5D and Table S1).

KY1005/Sirolimus combination prophylaxis synergistically protects against clinical and pathologic aGVHD

The ability of KY1005/sirolimus to preserve Treg reconstitution despite controlling Teff activation resulted in the synergistic protection against aGVHD in this cohort. The combined therapy provided potent control of the clinical signs of aGVHD (Figure 6A), which resulted in prolonged (>100 day) GVHD-free survival following transplantation (Figure 6B) even after discontinuation of KY1005 at Day 54 post-transplant (Figure S6A). This survival was significantly different than all comparator cohorts (p<0.01). Thus, although both sirolimus and KY1005 as monotherapies 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 (Figure 6A) resulting in an MST >100 days, with p <0.001 compared to No Rx, sirolimus and KY1005, Figure 6B) and which was confirmed by histopathologic analysis (Figure S4). KY1005/sirolimus also displayed superior control of aGVHD and survival compared to previously published (3, 9) Tac/MTX and CTLA4-Ig/Sirolimus cohorts (Figure 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 allo-proliferate against either recipient or third-party stimulator cells (Figure 6C-E). As expected, donor PBMC samples minimally responded to autologous stimulator PBMC, both before and after HCT (Figure 6C-D, left column and Figure 6E). However, in the pre-HCT setting, these
cells demonstrated more proliferation when stimulated by either recipient or third-party cells (Figure 6C, **middle and right columns** and Figure 6E) than when stimulated by autologous donor cells. Importantly, when responder PBMC were harvested from recipients at the terminal post-transplant time-point, in the setting of high donor T cell chimerism, a smaller amount of allo-proliferation 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 3 of 5 recipients (Figure 6D, **middle and right columns** and Figure 6E).

**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 following allo-HCT, we interrogated the transcriptomes of these T cells, in order 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 immunoprophylaxis 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 were, nonetheless, distinct from those in untransplanted animals. Thus, as documented with Principal Component Analysis (PCA, Figure 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, pathway analysis was performed, using a strategy designed to identify the most stable enriched pathways in KY1005/sirolimus recipients, defined as those that were enriched at all three post-transplant time-points analyzed (Day +14, +28, +100). This analysis identified 101 transcripts that were stably enriched (Figure 7B, Table S3). Analysis of the resulting dataset using DAVID (26, 27) revealed statistically significant enrichment for two pathways: the KEGG hsa04630: Jak-STAT signaling pathway and the Reactome HSA-909733: Interferon α/β signaling pathway. Enrichment in the Jak-STAT pathway was identified with both DAVID-based pathway analysis and GSEA (Figure 7C, **top panel**, and Table S1) and revealed a complex enrichment pattern, that included transcripts encoding both pro- and anti-inflammatory cytokines/cytokine receptors (pro-inflammatory transcripts included IL6, IL6R and IL6ST (28); IL26 (29); anti-inflammatory transcripts included OSM (30), IL2RA and IL-10RB (31), CSF3R (32), and IL13RA1 (33)). In addition, both positive and negative regulators of Jak-STAT and TCR signaling cascades were enriched (positive regulators included STAT1, STAT2 and STAT3; negative regulators included SOCS1 and, SOCS2, PIM1, SOS1 and SOS2, CISH, PIAS1, SPRY1 (34-36)).

This analysis also uncovered enrichment for Interferon α/β signaling pathways in the comparison of KY1005/sirolimus with HC (Figure 7C, **middle panel**, and Table S1). The identification of these pathways was consistent across three independent enrichment measures, including the pathway analysis described above, as well as by GSEA and Class Neighbor (CN) analysis. Importantly, GSEA also demonstrated enrichment of these pathways in the KY1005/sirolimus cohort as compared to both KY1005 and sirolimus monotherapies, as well as compared to autologous transplant controls (Figure 7C, **bottom panel**). Likewise, CN analysis, which identifies transcripts whose expression predominates
the signature of one cohort relative to others, followed by pathway analysis (37) also uncovered the Reactome Interferon α/β signaling pathway as significantly enriched in a comparison of KY1005/sirolimus versus HC, as well as versus the Autologous, No Rx, sirolimus, KY1005, CTLA4-Ig/sirolimus and Tac/MTX cohorts (Table S4). These analyses identified several transcripts that have been previously recognized to serve a critical function in IFNα/β sensing or signal transduction (including IGS15, IFIT3, RSAD2) or in the effector anti-viral response (IFIT1, IFIT2, IFIT3, MX1, OASL, OAS2, RSAD2) (38, 39).

As a final test of the pathways associated with the ability of KY1005/sirolimus to control aGVHD, we interrogated whether their transcriptomes provided evidence for control of the Th/Tc1 and Th/Tc17 pathways that we have previously demonstrated to be activated during aGVHD, in both NHP and human T cells (3, 9). With respect to Th/Tc1-driven Hyperacute GVHD, we observed that T cells in the KY1005/sirolimus cohort significantly down-regulated canonical Hyperacute GVHD transcripts (Ki67 and Granzyme A, Figure 8A) as well as Th1-associated gene sets (including the canonical Th/Tc1 genes CCR5, IL12RB2, and IFNG; Figure 8B; Table S1). Of note, despite down-regulation of the above Th/Tc1-associated transcripts, the expression of TBX21 (encoding Tbet) itself was not significantly changed during GVHD compared to healthy controls (Figure S7). With respect to Th/Tc17-driven Breakthrough Acute GVHD, we found that KY1005/sirolimus successfully normalized expression of the Th17-defining transcription factor, RORC (Figure 8C), and successfully inhibited Th/Tc17-associated gene sets (Figure 8D; 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 Th/Tc1- and Th/Tc17-mediated immune pathology.

Discussion

In this study, 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 co-signaling derives from the fact that it represents one of the few pathways for which there are data to suggest a potentially dichotomous effect on conventional versus regulatory T cells. It is well-documented that OX40 is up-regulated on CD4+CD8 conventional T cells 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 conventional T cells, OX40 ligation on Tregs can have the opposite effect, i.e. inhibition of Treg survival (18) and an inhibition of their ability to suppress Teff activation (18, 19, 44). This suggests that blocking the OX40:OX40L pathway might, paradoxically, have salutary effects on Treg function. There has been controversy concerning this effect, however, and several studies have argued that OX40 signaling enhances Treg 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 impact 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 now in Phase I testing in healthy volunteers and patients with psoriasis (Clinicaltrials.gov # 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, most importantly including graft-versus-leukemia (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 anti-viral immunity (21), there is no leukemia model in NHP, and thus this issue cannot be directly evaluated in primates prior to clinical evaluation. As with all new immunomodulatory strategies, early phase clinical trials with KY1005/sirolimus will thus need to employ 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 bio-activity and mechanism-of-action of novel agents through monotherapy experiments. Our results demonstrate that OX40L blockade with KY1005 significantly controlled conventional CD4+ T cell proliferation and effector maturation, and, importantly, could stabilize the Treg:Tconv ratio, that was otherwise seriously degraded after transplant. However, despite the clear biologic effect of KY1005 as a monotherapy, and likely due to its predominant impact on CD4\(\gg\)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 post-transplant. Transplant recipients prophylaxed with this combination remained clinically healthy, and free from signs of GVHD, even with weaning of KY1005 after Day 54 post-transplant. Importantly, 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 \(\geq 100\) days post-transplant in NHP undergoing high-risk T-cell replete 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 Tregs were donor-specific, this result nonetheless supports the hypothesis that in aGVHD, OX40L blockade does indeed have dichotomous effects on Treg compared to Teff, 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 Teff activation and simultaneously support Treg reconstitution.

In addition to identifying the naïve T cell 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 1 interferon (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) as well as the promising results of clinical trials utilizing Jak-STAT inhibitors for GVHD prevention (49, 50). However, the detailed analysis of this Jak-STAT signature revealed that enrichment in transcripts encoding pro-inflammatory 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 1 IFN pathways, canonically associated with anti-viral 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 anti-viral 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 Th17-driven inflammation in autoimmune models (55, 56) and in patients with IBD (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 graft-versus leukemia responses and overcome tumor-driven T cell tolerance (61), thus potentially augmenting, rather than controlling, aGVHD (53, 54). Our dataset underscores the influence of canonical innate signaling 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 (Th/Tc1) and Breakthrough Acute (Th/Tc17) GVHD (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 NHP designed to determine the clinical, immunologic and molecular outcomes of OX40L blockade during allogeneic HCT. Two cohorts of transplant recipients were studied: (1) Allogeneic transplants using the OX40L-blocking human IgG4 antibody ‘KY1005’ (Kymab Ltd, Cambridge, UK, antibody design described in detail below) as monotherapy for GVHD prophylaxis (abbreviated as ‘KY1005’; n=4). KY1005 was given at a dose of 10mg/kg starting on Days -2 and then once weekly until planned discontinuation on Days 54 (Figure 2A). (2) Allogeneic transplants using KY1005 in combination with sirolimus (with the target range of 5-15 ng/mL) for GVHD prophylaxis (abbreviated as ‘KY1005/Sirolimus’; n=5). KY1005 was given at a dose of 10mg/kg starting on Days -2 and then once weekly until discontinuation on Days 54 (Figure 3A). These two OX40L-blockade groups were compared to the following cohorts, aspects of which have been described previously (3, 9, 17): (1) Autologous transplants (abbreviated as ‘Auto’, n = 6 for clinical and flow cytometric data and n = 4 for transcriptomic analysis); (2) Allogeneic transplants with no GVHD prophylaxis (abbreviated as ‘No Rx’, n = 11 for clinical and flow cytometric data and n = 4 for transcriptomic analysis); (3) Allogeneic transplants using Sirolimus monotherapy for GVHD prophylaxis (LC laboratories, ‘Sirolimus’, n = 11 for flow cytometric analysis, n = 4 for transcriptomic analysis). Sirolimus was given daily for the length of analysis as an intramuscular formulation as previously described with doses adjusted to achieve a serum trough of 5-15 ng/mL (3, 9, 17). This cohort contains a sub-group of animals prophylaxed with sirolimus alone (n = 5) which were only evaluated until Day +9 post-transplant, and for which flow cytometry data were included in this study; (4) Allogeneic transplants using CTLA4-Ig plus sirolimus, reported previously (3) (n = 7); (5) Allogeneic transplants using tacrolimus plus methotrexate for GVHD prophylaxis (3, 9) (abbreviated as ‘Tac/MTX’, n =3).

Transplant recipients and donors were chosen from breeding colonies based on their MHC-genotypes. For these studies, we utilized 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 USDA 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 peripheral blood mononuclear cell (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 IRB-approved clinical trials: (1) The Bone Marrow Immune Monitoring

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Protocol and (2) 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 anti-proliferative 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 manuscript were enrolled in clinical trials that were conducted according to the principles set forth in the Declaration of Helsinki, and which were approved by the institutional review boards (IRB). Written informed consent was received from all participants.

Transplant Strategy—We utilized our previously-described strategy for allogeneic HCT in rhesus macaques, described in detail in Supplementary Materials (17).

Immune Analysis

Flow cytometry

Longitudinal flow cytometric analysis: Multicolor flow cytometric analysis was performed using an LSRII or LSR Fortessa flow cytometers (BD Biosciences) on all transplant recipients. Details of this analysis are provided in the Supplementary Methods.

Flow Cytometric CD3+ T cell sorting and microarray cohort designation—Details of sorting and microarray cohort designation are provided in the Supplementary Methods.

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

Transcriptional studies of patient samples—Transcriptional studies on patient samples are described in detail in the Supplementary 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. ANOVA analysis 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 (DE) was 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 > 1.4 with the limma package (67).

**Supplementary Material**

Refer to Web version on PubMed Central for supplementary material.

**Acknowledgments**

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**References**


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Figure 1. OX40 and OX40L are up-regulated during aGVHD

(A) Representative flow cytometry plots showing OX40 expression on peripheral blood CD4 and CD8 T cells obtained from healthy, immunologically naïve Rhesus macaques (Healthy Ctrl) or from allo-HCT recipients transplanted without immunoprophylaxis, performed at terminal analysis (No Rx).

(B) The percentages of OX40+ T cells in CD28+CD95- (Naïve), CD28+CD95+ (CM) and CD28-CD95+ (EM) CD4 T cells were quantified in healthy immunologically naïve animals (Healthy Ctrl, n=6; Black) and at terminal analysis in recipients transplanted without GVHD.
immunoprophylaxis (No Rx, n=3; Red) from a single experiment shown in (A). Data represent one of 2 similar experiments. Statistical analysis was performed using the Student’s t-test ****p<0.001.

(C) Peripheral blood from the healthy immunologically naïve animals (n=5) and after allo-HCT recipients transplanted without GVHD immunoprophylaxis, performed at terminal analysis (n=4) were activated with PMA/Ionomycin and cultured 4 hours. Numbers of T cells co-producing ≥2 cytokines simultaneously were quantified in OX40- and OX40+ CD4 T cells subsets. Statistical analysis was performed using a paired t-test (between OX40- and OX40+ CD4 T cells belonging to the same animal) or an unpaired Student’s t-test (between different experimental groups). *p<0.05, **p<0.01, NS – not significant.

(D) Relative expression (Log$_2$ normalized fluorescent intensity signal) of the TNFRSF4 gene transcript (encoding OX40) in CD3+CD20- T cells, flow cytometrically sorted from the peripheral blood of healthy immunologically naïve animals (Healthy Ctrl, n=46; Black), following autologous HCT at day 100 post-transplant (Auto, n=4; Gray), following allogeneic HCT at the time of terminal analysis in experimental groups without GVHD immunoprophylaxis (No Rx, n=4; Red), prophylaxed with Tacrolimus/Methotrexate (Tac/MTX, n=3; Purple), or with sirolimus (n=4; Orange). Horizontal significance bars denote comparisons with a moderated t-statistic <0.05 corrected for multiple hypotheses testing using the Benjamini-Hochberg procedure.

(E) Relative expression (Log$_2$ normalized fluorescent intensity signal) of the TNFRSF4 gene transcript (encoding OX40) in human T cells, flow cytometrically sorted from the peripheral blood of HCT recipients with (n=7; red symbols) or without (n=7; black symbols) GVHD on day 28 post-transplant. Gene expression was measured using the Human Transcriptome 2.0 Array (Affymetrix) as detailed in Methods. Statistical analysis was performed using Student’s t-test, *p<0.05.

(F) The percentage of human CD3 T cells, isolated from healthy controls and labeled with CellTrace Violet dye, which underwent proliferation in an allogeneic mixed lymphocyte reaction in the absence (No antibodies, n=9; Red) or the presence (n=9; Cyan) of 150 μg/mL KY1005. ***p<0.001 using a paired t-test.

(G) Representative flow cytometry plots showing anti-OX40L staining on CD11c+ myeloid dendritic cell (mDC) and CD123+ plasmacytoid dendritic cells (pDC), both gated on HLA-DR+CD3-CD56-CD20- cells (left panel) and the relative number of OX40L+ mDC and pDC (right panel), as shown in Figure S2. Flow cytometry was performed on lymph node cells from HC (n=5) and from No Rx animals (n=4). Staining is shown for mDC (top panel) and pDC (bottom panel) and is representative of 2 independent experiments. Statistical analysis was performed using Student’s t-test, *p<0.05.
Figure 2. Impact of KY1005 on T cell expansion and cytokine secretion after HCT
(A) Experimental schema detailing the HCT protocol and KY1005-based GVHD immunoprophylaxis regimen used in this study.
(B) Ki67 expression in peripheral blood CD4 (left panel) and CD8 (right panel) T cells before after allogeneic HCT in recipients without GVHD immunoprophylaxis (No Rx, number of animals included to this analysis (n)=7; Red) or prophylaxed with KY1005 (n=4; Cyan). Statistical analysis was performed using Holm-Sidak multiple-comparison t-test, *p<0.05.
(C) OX40 expression on peripheral blood CD4 T cells was quantified before and following allogeneic HCT in experiments shown in (B), and the percentages of OX40+ CD4 T cells (left panel) and the absolute numbers of OX40+ and OX40- CD4 T cells (right panel) were quantified on day 7 after allo-HCT in untreated (No Rx, n=7; Red) and prophylaxed with KY1005 (KY1005, n=4; Cyan) cohorts. Statistical analysis was performed using Holm-Sidak multiple-comparison t-test (on left panel) or Student t-test (on right panel), *p<0.05.
(D) The relative numbers of CD28+CD95- Naïve (T_N), CD28+CD95+ central memory (T_CM) CD4 T cells in peripheral blood before and at different time points following allo-
HCT in recipients without immunoprophylaxis (No Rx, n=7; Red) or prophylaxed with KY1005 (n=4; Cyan). Statistical analysis was performed using Holm-Sidak multiple-comparison t-test, *p<0.05, NS – not significant.

(E) Peripheral blood mononuclear cells isolated at the time of terminal analysis from allo-HCT recipients without immunoprophylaxis (No Rx, n=7; Red) or prophylaxed with KY1005 (n=4; Cyan) were activated with PMA/Ionomycin and cultured 4 hours as detailed in Methods, then were stained for CD3, CD4, CD8, CD14, CD20, OX40, IFNγ, TNFα, IL-2 and IL-17A. Numbers of T cells producing the indicated cytokines were quantified in OX40+ CD4 T cells subset. Statistical analysis was performed using Student's t-test, *p<0.05.

(F) Spleen cells were isolated from healthy, immunologically-naïve Rhesus macaques (Healthy Ctrl, n=5; Black) or from allo-HCT recipients either without immunoprophylaxis (No Rx, n=7; Red) or prophylaxed with KY1005 (n=4; Cyan) at the time of terminal analysis. The percentage of OX40+ cells was quantified in Treg (CD25+CD127-FoxP3+) and Tconv (CD25-CD127+) CD4 T cell subsets and presented as the percentage of total CD4 T cells. Statistical analysis was performed using Student's t-test. **p<0.01.

(G) Survival curves for recipients after allo-HCT in the unprophylaxed cohort (No Rx, n=11; Red) and in cohort prophylaxed with KY1005 (n=4; Cyan). *p<0.05 using the long-rank method.

(H) The clinical score of recipients after allo-HCT in the unprophylaxed cohort (No Rx, n=11; Red) and in cohort prophylaxed with KY1005 (n=4; Cyan). Differences between clinical scores were analyzed using the Holm-Sidak multiple comparisons t-test, *p<0.05.
Figure 3. KY1005/Sirolimus combination prophylaxis synergistically controls T cell activation after HCT

(A) Experimental schema detailing the HCT protocol, sirolimus and KY1005/Sirolimus-based GVHD immunoprophylaxis regimens used in this study.

(B) Ki67 expression in NHP peripheral blood CD4 (top panel) and CD8 (bottom panel) T cells after allo-HCT in recipients prophylaxed with sirolimus (n=10; Orange) and KY1005/Sirolimus (n=5; Green). Statistical analysis was performed using the Holm-Sidak multiple-comparison t-test, *p<0.05.
(C) The relative (left panels) and absolute (right panels) numbers of CD28+CD95- naïve (TN) CD4 (top) and CD8 (bottom) T cells in the peripheral blood measured longitudinally after allo-HCT in recipients prophylaxed with sirolimus (n=10; Orange) or KY1005/Sirolimus (n=5; Green). Statistical analysis was performed using the Holm-Sidak multiple-comparison t-test, *p<0.05.

(D) The relative numbers of CD28+CD95+ central memory (TCM) CD4 (top panel) and CD8 (bottom panel) T cells in the peripheral blood of allo-HCT recipients prophylaxed with sirolimus (n=10; Orange) or KY1005/Sirolimus (n=5; Green).

(E) Representative GSEA plots performed as previously described (25) showing naïve T cell-related gene sets over-represented in KY1005/Sirolimus (n=4) versus KY1005 (n=3; left panels) and KY1005/Sirolimus versus sirolimus (n=4; Right panels). All depicted gene sets are enriched in KY1005/Sirolimus cohort with FDR<0.05.
Figure 4. KY1005/Sirolimus combination prophylaxis permits robust hematopoietic reconstitution after allogeneic HCT

(A) Donor chimerism, determined using microsatellite analysis, in flow-cytometrically-sorted CD3+CD20- T cells following allo-HCT. Chimerism for the No Rx (n=7), KY1005 (n=4) and sirolimus (n=5) cohorts are shown at terminal analysis. Chimerism for the KY1005/Sirolimus cohort (n=5) is shown at Day 28, 60 and 100 after transplant.

(B-F) Absolute blood cell counts: absolute CD3+CD14-CD20- T cell counts (T cells; B), absolute neutrophil counts (ANC; C), absolute lymphocyte counts (ALC; D), 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 shown as mean (line with symbols) with SEM (shaded area around the line).

(G) CMV viral load in the peripheral blood of auto-HCT recipients (Gray lines) and KY1005/Sirolimus-prophylaxed allo-HCT recipients (Green lines). Each line represents an individual recipient.
Figure 5. KY1005/Sirolimus prophylaxis enables reconstitution of Treg cells after allogeneic HCT
(A) The ratio of CD25+CD127-FoxP3+ Tregs to 100 Tconv cells in the peripheral blood is shown before HCT (Pre) and at terminal analysis (Nx). Each line represents a single transplant recipient. Individual graphs indicate the different GVHD prophylaxis regimens: No immunoprophylaxis (No Rx, n=7; Red), KY1005 (n=4; Cyan), sirolimus (n=4; Orange) and KY1005/Sirolimus (n=5; Green). Paired t-test was used for statistical analysis. *p<0.05, ** p <0.01 NS – not significant.
(B) The ratio of CD25+CD127-FoxP3+ Treg to 100 Tconv cells in the peripheral blood tracked longitudinally in the KY1005/Sirolimus cohort (n=5).

(C) The absolute numbers of CD25+CD127-FoxP3+ Treg cells in the peripheral blood tracked longitudinally by flow cytometry in No Rx (n=7), KY1005 (n=4), Sirolimus (n=9), and KY1005/Sirolimus (n=5) cohorts.

(D) Representative GSEA plots performed as previously described(25) showing Treg-related gene sets over-represented with FDR q<0.05 in KY1005/Sirolimus (n=4) versus KY1005 (n=3; Left) or versus sirolimus (n=4; Right) cohorts.
Figure 6. KY1005/Sirolimus prophylaxis results in long-term GVHD-free survival following allogeneic HCT

(A, B) The combined clinical score (A), as detailed in Methods, and survival (B) of recipients after allo-HCT in cohorts prophylaxed with KY1005 (n=4; Cyan), sirolimus (n=6; Orange) and KY1005/Sirolimus (n=5; Green). Differences between clinical scores were analyzed using the Holm-Sidak multiple comparisons t-test, *p<0.05. Differences in survival between experimental cohorts were analyzed using the long-rank method **p<0.01.

(C, D) Representative flow cytometry plots showing CellTrace Dilution profiles of CD3+ (either CD4+ or CD8+) CD14/CD20- T cells from donors (C) and recipients at terminal analysis (D) stimulated by donor PBMC (left), recipient PBMC (center) or third-party healthy control PBMC (right) after ex-vivo culture for 7 days. Inset numbers in Figure 6 C,D indicate the percentage of cells undergoing ≥ 1 divisions.

(E) The calculated Proliferation Indexes from recipient MLR cultures (Green symbols) were normalized to the values of the Proliferation Indexes of the corresponding donor samples (Black symbols), and then plotted in pair-wise fashion. The paired t-test was used for statistical analysis. *p<0.05, NS – not significant.
Figure 7. Enrichment of Jak/STAT and Type 1 IFN pathways in T cells from the KY1005/Sirolimus immunoprophylaxis cohort

(A) First, second and third principal component (PC) projections reveal clustering of transplanted animals by immunoprophylactic regimens: Healthy controls (n=46; Black), No immunoprophylaxis (No Rx, n=4; Red), Sirolimus (n=4; Orange), KY1005 (n=3; Cyan) and KY1005/Sirolimus (n=4; Green). Each dot represents an individual array sample.

(B) Weighted Venn diagram showing the number of transcripts over-represented in the KY1005/Sirolimus cohort at different time points after transplant compared to HC (top table). Pathway analysis includes:

<table>
<thead>
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<th>Pathway</th>
<th>Benjamin p value</th>
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</thead>
<tbody>
<tr>
<td>IFN-STAT alpha/beta signaling</td>
<td>7.0*10^{-3}</td>
</tr>
<tr>
<td>JAK/STAT signaling</td>
<td>3.6*10^{-2}</td>
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</tbody>
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(C) Comparison of running enrichment scores for specific pathways:
- KEGG_JAK_STAT_SIGNALING_PATHWAY
- HECKER_IFNB1_TARGETS

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Overlapping areas indicate over-represented transcripts shared between multiple comparisons. 101 transcripts, over-represented in the KY1005/Sirolimus cohort at all time-points, were then used for pathway analysis using DAVID (26), (bottom table).

(C) Representative GSEA plots performed as previously described (25) showing JAK/STAT pathway-related gene sets (top panels) and type I Interferon signaling-related gene set over-represented in gene arrays from the KY1005/Sirolimus cohort at Day 14 (n=5), 28 (n=5) and 100 (n=4) post-transplants versus HC (n=46; middle panels), or showing type I Interferon signaling-related gene sets over-represented in KY1005/Sirolimus (n=4) versus T cells purified from autologous HCT recipients at day 100 (n=4), from the sirolimus cohort (n=4) and from the KY1005 cohort (n=3; bottom panels).
Figure 8. KY1005/Sirolimus prophylaxis efficiently controls both Th/Tc1- and Th/Tc17-driven alloimmunity

(A) Relative expression (Log₂ normalized fluorescent 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 (Affimetrix) as detailed in Methods. * denotes comparisons with a moderated t-statistic <0.05 corrected for multiple hypotheses testing using the Benjamini-Hochberg procedure between the indicated experimental groups.
(B) Representative GSEA plots performed as previously described (25) showing Th/Tc1-related gene sets under-represented in KY1005/Sirolimus on day 14 (n=5) versus KY1005 (n=3; Left panels) or versus sirolimus (n=4; Right panels) cohorts at the time of necropsy. (C) Relative expression (Log$_2$ normalized fluorescent 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 using moderated t-test with Benjamini-Hochberg correction. (D) Representative GSEA plots performed as previously described (25) showing Th/Tc17-related gene sets under-represented in KY1005/Sirolimus on day 100 (n=4) versus CTLA4-Ig/Sirolimus (n=7; Left panels) or versus Tacrolimus/Methotrexate (Tac/MTX, n=3; Right panels) cohorts at the time of necropsy.