ORIGINAL ARTICLE

Evidence of the immunomodulatory role of dual PI3K/mTOR inhibitors in transplantation: an experimental study in mice

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SUMMARY

The PI3K/mTOR signaling cascade is fundamental in T-cell activation and fate decisions. We showed the distinct regulation of PI3K/mTOR in regulatory and effector T-cells and proposed the potential therapeutic benefit of targeting this pathway to control the balance between effector and regulatory T-cell activities. Substantial adverse effects in long-term clinical usage of rapamycin suggest the use of alternative treatments in restraining effector T-cell function in transplant patients. We hypothesize that dual PI3K/mTOR inhibitors may represent an immunosuppressant alternative. Here we show that dual PI3K/mTOR PI-103 and PKI-587 inhibitors interfered IL-2-dependent responses in T-cells. However, in contrast to the inhibitory effects in non-Treg T-cell proliferation and effector functions, dual inhibitors increased the differentiation, preferential expansion, and suppressor activity of iTregs. Rapamycin, PI-103, and PKI-587 targeted different signaling events and induced different metabolic patterns in primary T-cells. Similar to rapamycin, *in vivo* administration of PI-103 and PKI-587 controlled effectively the immunological response against allogeneic skin graft. These results characterize specific regulatory mechanisms of dual PI3K/mTOR inhibitors in T-cells and support their potential as a novel therapeutic option in transplantation.

Transplant International 2017; 30: 1061–1074

Key words
dual PI3K/mTOR inhibitors, human T cells, immunosuppression, regulatory T cells, tolerance, transplantation

Received: 14 March 2017; Revision requested: 7 April 2017; Accepted: 17 May 2017;
Published online: 31 July 2017

Introduction

Standard therapeutic strategies in preventing allograft rejection include immunosuppressive regimens that rely on the nonspecific depletion or inhibition of T-cell responses. Despite dramatic increases in first-year graft survival rates, chronic allograft dysfunction remains a major challenge in transplantation [1–3]. In this context, a growing body of evidence recognizes the suppressive activity of Treg cells [4–7] and, specifically, the balance between graft-reactive effector cells and graft-protective suppressor Tregs, as the ultimate determinant of long-term allograft survival [8–10].

doi:10.1111/tri.12989

Clinical and experimental studies support the therapeutic potential of targeting the mTOR pathway in transplant patients as a critical regulator of the balance between regulatory and effector T-cells [11–14]. However, serious adverse effects of long-term use of rapamycin [15,16], the prototypical mTOR inhibitor in clinical transplantation, have fueled the development of alternative options for the efficient and safe targeting of mTOR-dependent activity in T-cells. The development of pharmacological inhibitors of multiple targets within the PI3K/AKT/mTOR pathway may represent a valid alternative, but their clinical implementation in the transplant field requires further investigation.

In the present study, we explored the effects and mechanisms of action of dual PI3K/mTOR inhibitors PI-103, a pyridinyl-furano-pyrimidine with a high degree of selectivity for class-IA PI3K and mTORC1 [17], and PKI-587, a novel inhibitor of class-IA PI3K, mTORC1 and mTORC2. Our group and others have reported the inhibitory properties of these two compounds against cancer cell growth [18–22] but, to date, the immunoregulatory potential of these drugs has not yet been explored. In this study, we have compared the effects of these dual PI3K/mTOR inhibitors to rapamycin *in vitro* and *in vivo* on the regulation of T-cell phenotype, function and fate choices in primary human and mouse T-cells. The results establish a solid experimental base to support the potential therapeutic benefit of dual PI3K/mTOR inhibitors in clinical transplantation.

Materials and methods

The description of materials methods and statistical analyses is detailed in (Appendix S1).

Results

Dose-limiting toxicity of dual PI3K/mTOR inhibitors on primary human T-cell culture

The goal of the initial set of experiments was to identify the highest effective dose of dual inhibitors PKI-587 and PI-103 that did not cause significant loss of cell viability in 4-day T-cell culture with anti-CD3 (OKT3) and IL-2. The results demonstrated that 400 nM of PI-103 and 80 nM of PKI-587 were the highest doses tolerated by primary T-cells in culture without altering the cell viability (Fig. 1a). Rapamycin at 100 nM and 20 nM as standard optimal and low concentration ranges, respectively, did not produce any significant decrease in cell viability either. We therefore established the low and high working doses of the drugs throughout the study as 20 nM and 100 nM for rapamycin, 80 nM and 400 nM for PI-103, and 16 and 80 nM for PKI-587, respectively. Addition of these concentrations of drugs in the cell culture did not induce any significant increase on the expression levels of active Caspase-3 or Annexin V in T-cells (Fig. 1b and c).

Reduction of early T-cell activation by PI3K/mTOR inhibition

We assessed the effects of rapamycin, PI-103, and PKI-587 on the expression of the early activation markers CD69 and CD25 (the IL-2-R-alpha subunit). After 18-h activation of primary naïve human $CD4^+$ T-cells with OKT3 and anti-CD28, approximately 20% of cells expressed high levels of CD69. Addition of inhibitors did not induce any significant change in the percentages

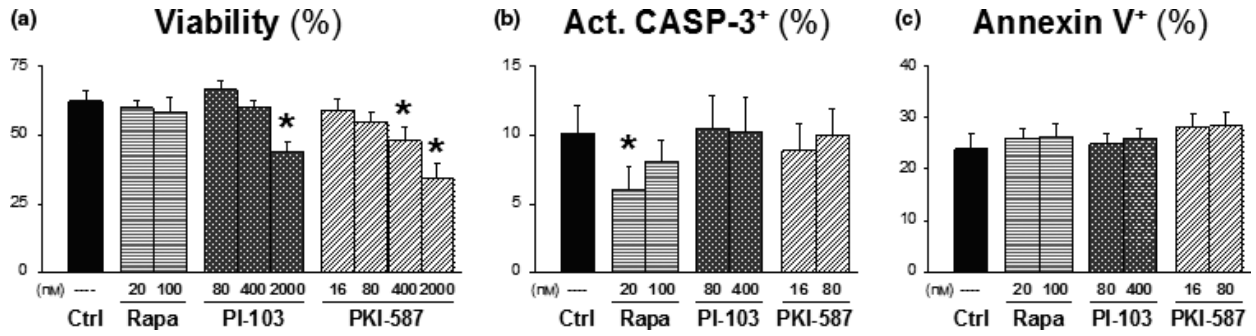

Figure 1 Cell viability, active Caspase-3, and Annexin-V assay in human naïve $CD4^+$ T-cells cultured in OKT3 + IL2 medium in the absence (Control-Ctrl-) or in the presence of rapamycin (Rapa), PI-103, or PKI-587 at the indicated doses. (a) Percentages of viable cells were measured by trypan blue exclusion after 4-day culture. From the conditions that demonstrate no significant changes in cell viability, we evaluated: (b) Percentages of cells expressing active-Caspase 3 and (c) cell surface Annexin-V by flow cytometry after 18 h-culture. Data are shown as mean ± SD pooled from at least three independent experiments. (*) Significant ($P < 0.05$) differences with respect to untreated (Control) cells measured by *Student's t* test.

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of CD69$^+$ cells (Fig. 2a). However, unlike CD69, the percentages of T-cells expressing high levels of CD25 were consistently reduced in the presence of either rapamycin or dual inhibitors (Fig. 2a and b).

Reduced IL-2 production in human T-cells by PI-103

We next measured the IL-2 produced by naïve T-cells cultured in OKT3/anti-CD28-containing medium for 40 h in the presence or not of rapamycin, PI-103, and PKI-587. The addition of drugs at high doses inhibited IL-2 production (Fig. 2c). When we examined the functional link between IL-2 producing and responding cells, we observed a significant reduction in CD25 expression on IL-2$^+$ cells in all pharmacologically treated T-cell cultures compared to untreated-control cells (Fig. 2d), supporting the notion that drug-treated, IL-2-producing cells cannot efficiently respond to IL-2.

Decreased human T-cell proliferation caused by PI3K/mTOR inhibition

We next assessed the impact of rapamycin, PI-103, and PKI-587 on the proliferation of CFSE-labeled CD4$^+$ T-cells activated with OKT3 and anti-CD28 for 5 days. As expected, all compounds produced a substantial reduction in the proliferative response (Fig. 2e), although quantitative and qualitative differences were observed among the drugs. The effect of rapamycin was not dose-related, as low (20 nM) and high (100 nM)

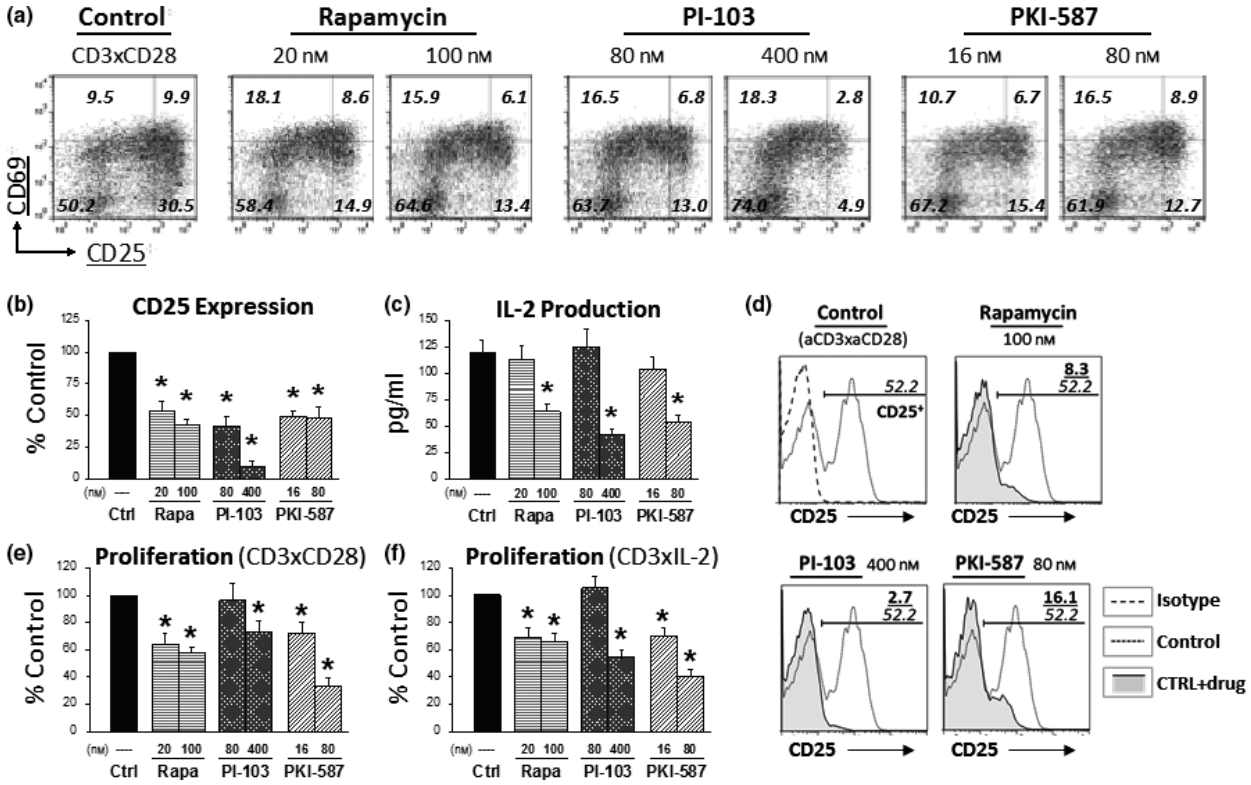

Figure 2 Activation, IL-2 production, and proliferation in human naïve CD4$^+$ T-cells. (a) Dot plots represent the expression of CD69 and CD25 in T-cells after 18-h culture with OKT3 and anti-CD28 in the absence (Control) or in the presence of rapamycin, PI-103, or PKI-587 at the indicated doses. Distribution in percentages of CD69- and CD25-expressing cells is indicated inside the panels. (b) Mean ± SD of pooled data collected from three independent experiments for CD25 expression are depicted with respect to control conditions. (c) Supernatants from T-cells cultured for 40 h in antiCD3 + anti-CD28-media in the absence (Control) or in the presence of rapamycin, PI-103, or PKI-587 were collected, and the IL-2 produced was measured by ELISA. (d) The expression of CD25 was evaluated in IL-2-producing T-cells after 18-h culture with OKT3 and anti-CD28 in the absence (Control) or in the presence of rapamycin, PI-103, or PKI-587 at the indicated doses. The percentage of CD25$^+$ cells in drug-treated samples are indicated in bold and compared with the control (untreated) cells indicated in *italic*. (e-f) CSFE-labeled CD4$^+$ T-cells were activated with OKT3 + anti-CD28 (e) or with OKT3 + IL-2 (f) for 5 days in absence (Control) or in the presence of rapamycin, PI-103, and PKI-587 at the indicated doses. The results depict the mean ± SD of pooled data from three experiments. (*) indicates significant differences ($P < 0.05$) compared with medium alone as measured by Student's *t* test.

concentrations induced similar responses, although lower doses (5 nM) were ineffective (not shown). In contrast, PKI-587 and PI-103 inhibited T-cell proliferation in a dose-dependent manner, with PKI-587 showing stronger effect than PI-103. These results, and the fact that proliferation induced by OKT3 with anti-CD28 or with exogenous IL-2 exhibited similar sensitivity patterns (Fig. 2f and S1a), support the IL-2-dependent expansion of T-cells driven by the prolonged OKT3 and anti-CD28 engagement as suggested in other reports [23,24].

PI-103 and PKI-587 promote differentiation into $CD25^+$/$FoxP3^+$ Tregs

We demonstrated in early studies that primary human naïve $CD4^+$ T-cells differentiate into suppressor Tregs in the presence of TGF-ß and IL-2 only after undergoing multiple rounds of cell division [25–27]. In light of our results, we questioned whether the antiproliferative action of rapamycin and dual inhibitors was compatible with the IL-2-dependent differentiation of naïve T-cells into iTregs. Our results confirmed the ability of rapamycin to improve the differentiation of Tregs as previously reported [11–13] and revealed that both PI-103 and PKI-587 exert similar increasing effects on iTreg differentiation and that the resulting Treg cells display enhanced suppressive function (Fig. 3a-c, and S1b). The acquisition of Treg phenotype by T-cells cultured in the presence of rapamycin or dual inhibitors started at earlier cell divisions than homologous cells in iTreg medium alone (Fig. 3b). Therefore, the sequence of cell expansion followed by cell differentiation during iTreg fate determination is abrogated, and the presence of these drugs eliminates the requirement of multiple rounds of cell divisions before acquiring the Treg phenotype and promotes the early accumulation of $FoxP3^+$-iTreg cells. In addition, Treg cells are more resistant to the antiproliferative effects of drugs compared to activated ($CD25^+$) non-Treg T-cells (Fig. 3d), which further contributes to the predominance of the Treg population among the drug-treated T-cells. The *FOXP3* gene is the master regulator of the Treg gene expression program and its constitutive expression is required for Treg-suppressive function [28]. Methylation of CpG dinucleotides at the highly conserved TSDR region of the *FOXP3* locus represses FoxP3 expression, and their demethylation correlates with stable FoxP3 expression [29]. The methylation levels across the nine CpG sites of the TSDR in freshly sorted natural Tregs were 30.2 ± 3.6% ($n = 9$) and in $CD4^+$ naïve T-cells increased to 89.3 ± 0.9% ($n = 12$). Our data analysis on iTreg cells generated from naïve $CD4^+$ T-cells and maintained in culture for 8 days showed methylation levels of 50.3 ± 4.8% ($n = 5$). The presence of rapamycin (41.5 ± 4.6, $n = 5$), PI-103 (43.8 ± 5.7, $n = 5$), and PKI-587 (37.4 ± 4.4, $n = 4$) showed a similar trend toward higher demethylation compared to control iTreg conditions, although the effects of PI-103 were not significant. Addition of drugs to the cell culture resulted in the substantial reduction of IL-17-producing/$ROR\gamma t^+$ [30] cells (Fig. 3e and S1c). Taken together, our results support the notion that rapamycin and dual inhibitors drive the plasticity of T-cell responses toward the prevalence of the regulatory activity by increasing iTreg differentiation, function and stability, and by reducing the expansion and differentiation of the effector T-cell population.

PI3K/mTOR signaling modulated by PI-103 and PKI-587

To investigate specific signaling events by which rapamycin or dual inhibitors may regulate iTreg differentiation, we assessed the expression of several key components of the AKT-mTOR signaling pathway by Western Blot analysis. The inclusion of rapamycin induced a robust dose-dependent attenuation of the phosphorylation of AKT at Serine-473, a requirement to achieve the stable, full activated state of AKT [31] (Fig. 4). The results across the different conditions tested also showed a strong positive correlation between phospho-AKT levels and phospho-GSK3β at Serine-9, a downstream target of AKT [32]. There is a general consensus that Serine-473 of AKT is specifically phosphorylated by mTORC2 [33]. Accordingly, the low levels of phosphorylated AKT would imply the inhibitory action of prolonged rapamycin treatment to mTORC2 activity as reported [16]. Rapamycin-treated cells also displayed reduced mTORC1 signaling as evidenced by low phosphorylation levels of the translational repressor 4EBP1 at Threonines 37 and 46. A similar pattern resulted from the action of PI-103 at low dose (80 nM), with reduced phospho-AKT and phospho-GSK3β levels and a limited decline in phospho-4EBP1 levels. However, at higher doses, PI-103 elicited a substantial increase in the phosphorylated forms of AKT and GSK3β above control levels. In contrast, PKI-587, even at the lowest concentrations (16 nM) completely abrogated the phosphorylation of 4EBP1 and induced a concurrent large increase in phospho-AKT and phosho-GSK3β levels. Unlike rapamycin and PI-103, PKI-587 treatment also

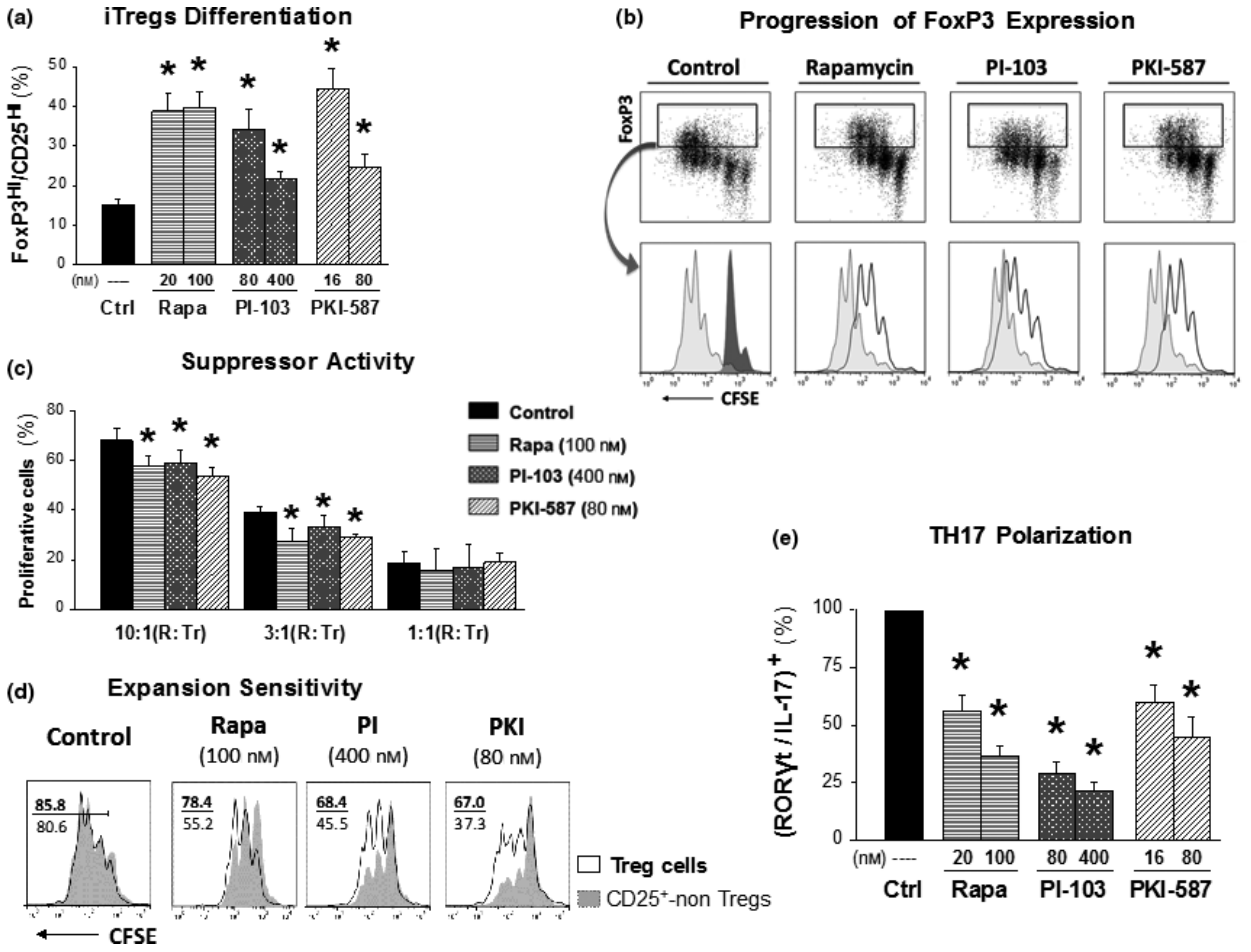

Figure 3 Differentiation of $CD25^+/FoxP3^+$ Tregs and effector TH17 from human naïve $CD4^+$ T-cells. (a) Expression of Treg phenotype ($FoxP3^{HIGH}/CD25^{HIGH}$) in T-cells after 6-day culture in iTreg medium (OKT3 + IL-2 + TGF-β) in the absence (Control) or in the presence of rapamycin, PI-103, or PKI-587 at the indicated doses. Data represent mean ± SD of pooled percentages of Treg differentiation from at least three independent experiments performed with cells isolated from different individuals. (b) $CD4^+$ T-cells undergo several rounds of proliferation before acquiring iTreg phenotype (shown as $FoxP3^{HIGH}$, square window) during 5-day culture in iTreg medium as assessed by CSFE. Accumulation of $FoxP3^{HIGH}$ iTregs in untreated (Control) cells occurs only after a strong proliferative phase. In contrast, FoxP3 expression in rapamycin (100 nM)-, PI-103 (400 nM)-, and PKI-857 (80 nM)-treated cells starts at earlier cell division cycles (top panels). The bottom panels display the sequential CFSE dilutions of $FoxP3^+$ cells in the absence (control medium, gray filled, dotted histogram) or in the presence of drugs (empty, dark-lined histogram); dark-filled histogram shows the CFSE staining in non-activated cells. (c) Mean ± SD of suppressor Treg activity measured as the percentage of proliferating CFSE-labeled T-cells resulting from the coculture with different concentrations of sorted Treg cells from three independent experiments. (d) Distinct sensitivity of Treg and non-Treg T-cells to the effects of rapamycin, PI-103, or PKI-587. Sorted Treg ($CD25^{HIGH}/CD127^{DIM}$) and activated ($CD25^+$) non-Treg cells were sorted, CFSE-labeled and cultured separately in anti-CD3/IL-2-containing medium in the absence (Control) or in the presence of rapamycin, PI-103, or PKI-587 for 4 days. The resulting percentages of proliferating Tregs cells (empty histograms) are shown inside the panels in bold and non-Tregs (gray-filled histograms) in regular font. (e) Expression of effector TH17 phenotype (dual $ROR\gamma_t^+$ and $IL\text{-}17^+$ expression) in T-cells after 6 days in culture in TH17-polarizing medium in the absence (Control) or presence of different doses of rapamycin, PI-103, or PKI-587 after 6-day culture. Results show the mean ± SD pooled data from three independent experiments (three different donors) compared to control (untreated) cells. In all panels, (*) indicates significant ($P < 0.05$) differences compared with control medium alone as measured by *Student's t* test.

altered ERK1/2 signaling. The phosphorylation pattern of mTOR at Serines 2448 and 2481 did not directly correlate with mTORC1 and mTORC2 activities (Fig. S2) as previously proposed in studies of tumor cells lines [34]. These results are more in line with subsequent manuscripts depicting the inconsistent functional link between downstream events and specific phosphorylation states of mTOR [35,36]. Collectively the Western Blot analysis demonstrated that rapamycin, PI-103, and PKI-587 are targeting distinct signaling events and reflect the complex plasticity of the PI3K-AKT-mTOR pathway in T-cells.

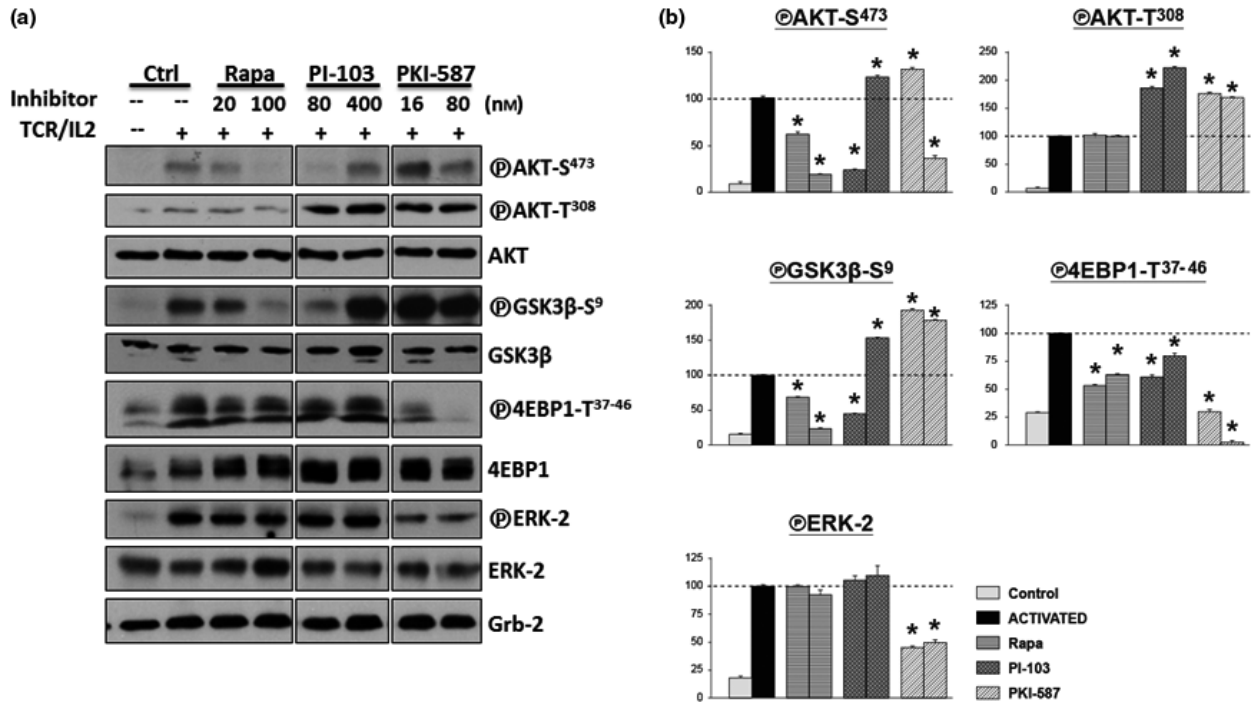

Figure 4 Effects of rapamycin and dual inhibitors on the expression of PI3K/mTOR target proteins. (a) Total cell lysates were prepared from 10^6 primary human $CD4^+$ T-cells cultured 48 h in OKT3 + IL2 medium in the absence or the presence of rapamycin, PI-103, or PKI-587 at the indicated concentrations. The results show western blotting analysis for total AKT and phosphorylated forms at Serine-473 and Threonine-308, total GSK3β and Serine-9 phosphorylated form, total and phosphorylated 4EBP1 at Threonines 37 and 46, and total and phosphorylated ERK-2 expression; Grb-2 expression was used to demonstrate equally loading. The results are from one representative experiment of three performed with comparable results. (b) Quantification of band densities corresponding to the pooled average ± SD of experiments indicated in a. Relative expression of phosphorylated proteins is indicated as a rate with the total protein levels and measured by the percentage relative to the activated control conditions. (*) indicates significant ($P < 0.05$) differences compared with control medium alone as measured by Student's *t* test.

PI3K/mTOR inhibition modulates IL-2 dependent metabolic profile

Our group and others have reported the cell metabolic switch during the transition from naïve to iTreg cells and the fundamental role of the PI3K-AKT-mTOR signaling cluster in this process [26,37–39].To evaluate the effects of PI3K/mTOR inhibitors on the bioenergetic status of the T-cells, we measured oxygen consumption rates (OCR, a measure of mitochondrial respiration) and extracellular acidification rates (ECAR, an indicator of glycolytic cell function). As expected in the early phase of iTreg differentiation (2 day-culture), T-cells in iTreg medium still demonstrated a predominant aerobic glycolytic metabolism and thus ECAR measurements were more sensitive to the metabolic stress induced by drugs. Rapamycin-treated T-cells exhibited the most prominent reductions in ECAR, whereas the effects induced by PI-103 and PKI-587, although less prominent, did also caused significant reduction in the glycolytic rates compared to untreated (control) cells (Fig. 5 panels a, c, d, and g). After 7 days in iTreg medium, all cells in culture exhibited a metabolic switch toward a less glycolytic profile (Fig. 5b, c, and g), which is known to be a common metabolic signature of Tregs [38,39]. In this late phase of iTreg differentiation, we found a significant reduction of OCR in all drug-treated T-cells (Fig. 5, panels e, f, and g). However, qualitative differences emerged among treatments regarding the type of substrate used to fuel mitochondrial respiration. In fact, addition of Etomoxir (a specific inhibitor of fatty acid oxidation –FAO-) demonstrated that while all drug treatments lowered FAO-related OCR, only rapamycin-treated cells improved significantly non-FAO to partially compensate the reduced utilization of fatty acids. In contrast, only PI-103-treatment caused also a decrease in non-FAO rates. Our results suggest that the distinct signaling events targeted by rapamycin, PI-103, or PKI-587 may influence different kinetics and pathways implicated in the metabolic reprogramming of T-cells. However, after 7 days in culture, the resultant metabolic profile is similar in all drug treatments.

Therefore, in spite of the specific mechanisms of action, the bioenergetic changes induced by rapamycin, PI-103, or PKI-587 share a common shift toward a cell metabolic state optimally suited to differentiate into functional Treg cells.

Administration of PI3K/mTOR inhibitors *in vivo* alters T-cell subset distribution and activation

Because the development, regulation, and function of T-cells differ between mice and human [40–43], we first verified the suppressive potential of rapamycin, PI-103, and PKI-587 *in vitro* in mouse T-cells on CD25 expression, IL-2 production and proliferative responses (Fig. S3). However, unlike the response in human primary T-cells, we did not detect any significant alteration in the expression of CD69 (Fig. S3a). Our next set of experiments was aimed at the evaluation of the effects of *in vivo* administration of dual PI3K/mTOR inhibitors compared to those evoked by rapamycin or vehicle control. Fourteen days after the first treatment, the animals were sacrificed, spleens removed and $CD4^+$ T-cells isolated for analysis. The total number of lymphocytes in the spleens of drug-treated mice were unchanged ($1.37 \times 10^7 \pm 0.27$, $n = 7$, in control; $1.31 \times 10^7 \pm 0.18$, $n = 8$, in rapamycin, $1.22 \times 10^7 \pm 0.21$, $n = 6$, in

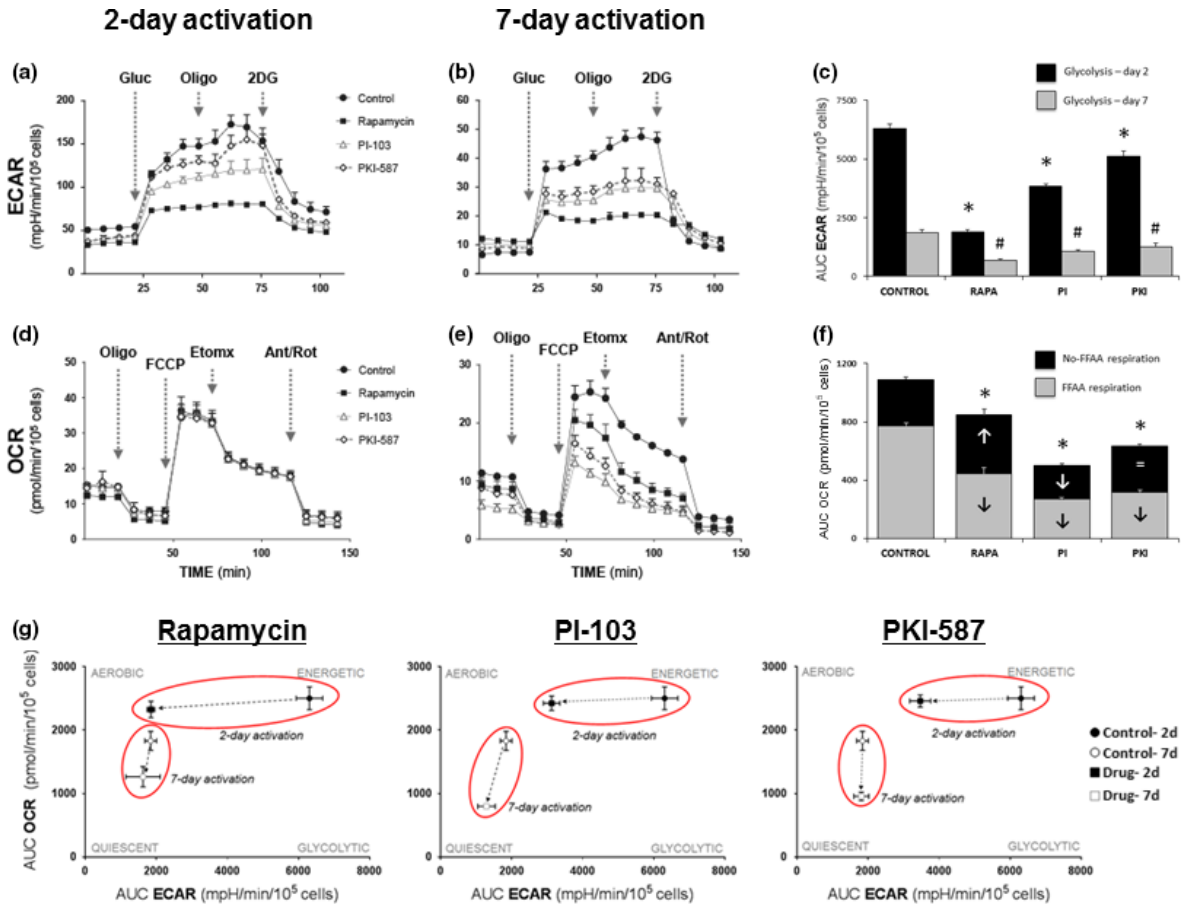

Figure 5 Effects of rapamycin and dual inhibitors on the bioenergetic profile of human T-cells. (a-c) Extracellular acidification rate (ECAR) profiles of human naïve $CD4^+$ T-cells cultured for 48 h (a) or 7 days (b) in iTreg medium in the absence (Control) or in the presence of rapamycin (100 nM), PI-103 (400 nM), or PKI-587 (80 nM). (c) Area under the curve (AUC) measurements of ECAR values corresponding to the experiments shown in a and b. (d-f) Oxygen consumption rate (OCR) profiles of human naïve $CD4^+$ T-cells under the same conditions as in a-c. (f) AUC values of Maximal mitochondrial respiratory capacity corresponding to 7-day culture differentiated between FFAA-dependent (etomoxir-sensitive, gray bars) and FFAA-independent (etomoxir-resistant, black bars) respiration. Symbols (*) and (#) identify $P < 0.05$ with respect to untreated (Control) cells as measured by *Student's t* test. In F, the symbols inside the bar identify differences in FFAA-dependent or-independent OCR. (g) The total AUC values of OCR and ECAR in a, b, d, and e are plotted against one another to illustrate the metabolic changes of the cells in response to the rapamycin, PI-103, or PKI-587 with respect to the untreated cells (Control) after 2 and 7 days in culture. Dotted arrows depict the metabolic transitions induced by drug treatments. Data are shown as mean ± SD pooled from at least three independent experiments.

PI-103, and 1.28 x 10^7 ± 0.21, n = 7, in PKI-587). The analyses of T-cells showed also similar CD4/CD8 ratios, although the frequency of $CD4^+$ and $CD8^+$ T-cells was higher in PI-103 mice compared to control (Fig. 6a). No differences were noted on the percentages of $FoxP3^{HIGH}/CD25^{HIGH}/CD127^{-/dim}$ Treg cells, ranging from 7.9 to 12.6% of $CD4^+$ T-cells. Staining for CD44 and CD62L expression revealed the elevated percentages of $CD62L^{hi}CD44^{lo}$ $CD25^-$ naive T-cells and the corresponding low pool of activated and memory-type T-cells among the CD4 and CD8 subsets in rapamycin-, PI-103-, and PKI-treated mice (Fig. S4). Next, we asked whether the reduced population of antigen-experienced T-cells was associated with the inability of *in vivo* drug-treated cells to respond to the TCR/CD28 costimulation. To the same extent as the *in vitro*-treated T-cells, the *in vivo* treatment with rapamycin, PI-103, or PKI-587 rendered T-cells unable to adequately upregulate the early expression of CD25 (Fig. 6b, top panel) and to induce a proliferative response (Fig. 6c). However, unlike the *in vitro* results, the expression of CD69 was also significantly reduced, suggesting that the *in vivo* effects of drugs may partially rely on indirect environmental variables that alter the capacity of T-cells to achieve full activation. Interestingly, although, the inhibitory effects induced by rapamycin and PI-103 on the expression of CD25 and CD69 were only transient, as an extended period of activation (30 h) normalized the expression of both activation markers to the levels of the control untreated group. In contrast, the *in vivo* treatment with PKI-857 poised the T-cell population to a less-responsive state (Fig. 6b, bottom panels). IL-2 production also showed substantial differences from the *in vitro* culture with the drugs, and only the T-cells isolated from animals treated with PKI-587 showed a significant reduction in IL-2 production (Fig. 6d). We next examined whether the *in vivo* treatments skewed the susceptibility of $CD4^+$ T-cells to differentiate toward a regulatory or an inflammatory phenotype. Rapamycin and PKI-587 treatments promoted the differentiation of T-cells into iTregs and inhibited the progression to effector TH17 (Fig. 6e-g). However, the PI-103 treatment was ineffective altering the generation of iTregs and only reduced the polarization into effector TH17. Similar to *in vitro* experiments, these results demonstrated that the *in vivo* treatments with rapamycin, PI-103, and PKI-587 can prime $CD4^+$ T-cell lineage differentiation by altering the sensitivity of the cells to express FoxP3 or RORγt and determining whether $CD4^+$ T-cells adopt the Treg or the effector TH17 fate, respectively.

Administration of PI3K/mTOR inhibitors *in vivo* modulates the response against allograft

We used MHC-mismatched skin from BALBc mouse grafted onto C57BL/6 recipients to evaluate the effect of dual PI3K/mTOR inhibition *in vivo*. Kaplan–Meier plots illustrated the significant increase in allograft survival in all group of mice treated with drugs (Fig. 7, top panels). Histology of the skin allografts in control mice analyzed on day 12 post-transplant (Fig. 7, bottom panels) revealed a prominent necrotic cuticular layer of the skin graft and abundant inflammatory cell infiltration, ranked as grade IV according to the Zdichavsky classification [44]. In contrast, the rapamycin, PI-103, and PKI-587 groups did not exhibit any necrotic tissue and the skin graft was well integrated. Only mild dermal inflammation without vasculitis, folliculitis, or epidermal degeneration was seen, compatible with grade I allograft rejection.

Discussion

Our results with primary human naïve $CD4^+$ T-cells illustrate a potential mechanism of action of rapamycin and dual inhibitors in early stages of T-cell activation by interrupting the positive feedback loop between IL-2 and IL-2-R signaling at two different levels: One through the very sensitive regulation of CD25 expression, and a second through the regulation of IL-2 production. In control conditions cells that produced IL-2 also co-expressed high levels of CD25, but this ligand–receptor co-expression was lost in the presence of drugs. In addition, the proliferative response of T-cells in response to CD3/TCR engagement demonstrated similar patterns of sensitivity to the drugs regardless the addition of exogenous IL-2 or the costimulation with anti-CD28. These findings suggest the prevalence of a common IL-2-dependent control of T-cell expansion as proposed in other studies [23,45], although we cannot rule out the contribution of mTOR-mediated IL-2-independent proliferation in TCR/CD28-engaged T-cells [23,24]. The fact that the IL-2-R of intermediate affinity expressed in the absence of CD25 is still fully functional to respond to IL-2 (albeit less efficient) may explain the reduced but not abrogated proliferative response in drug-treated cells.

Like rapamycin [11–13], dual PI3K/mTOR inhibitors PI-103 and PKI-587 promoted the differentiation of primary human naïve T-cells into $FoxP3^+$-iTreg suppressor cells. Early work from our group characterized the transition into functional iTregs as a tightly regulated and

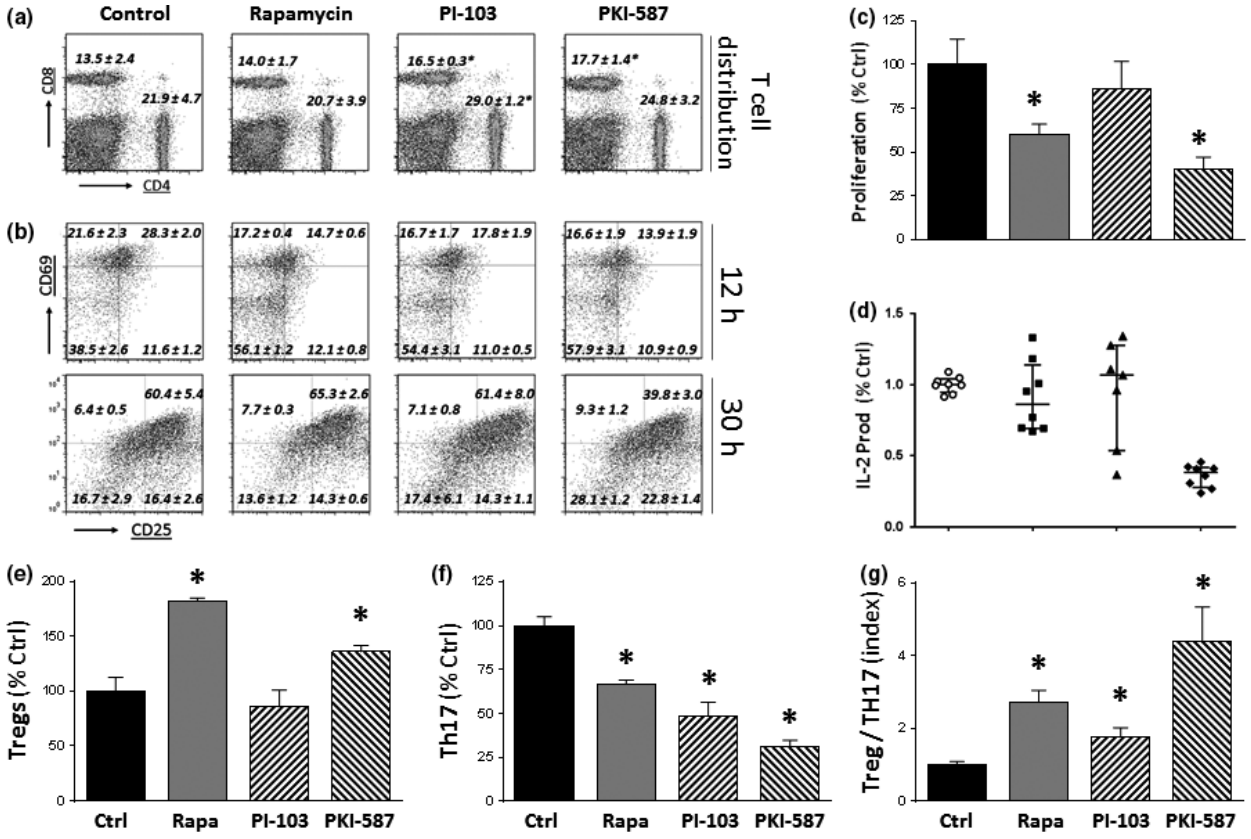

Figure 6 *In vivo*-treated mice: Distribution of T cells, activation, IL-2 production, proliferation, iTreg generation, and TH17 polarization. Spleens were collected from untreated (Control) or 14-days drug-treated mice and the distribution of splenic $CD4^+$ and $CD8^+$ T cells determined by flow cytometry (a) or $CD4^+$ T cells were isolated and cultured *in vitro* with no further addition of drugs (panels b-g). (b) CD69 and CD25 expression was analyzed after 12 and 30 h in culture with anti-CD3 and anti-CD28 antibodies. Panels show the dot plots of $CD4^+$-gated T-cells, and the mean ± SD values of the percentages of positive cells pooled from four different mice are indicated inside the panels. (c) Proliferation was assessed in T-cells cultured in anti-CD3 and anti-CD28 medium for 40 h and ^{3}H-Thymidine was added for additional 14 h. Data represent the mean ± SD of the percentage of d.p.m. with respect to the control values obtained from triplicate measurements of at least four different mice. (d) IL-2 was measured from the 40-h culture supernatants of T-cells stimulated with anti-CD3/anti-CD28. The median and interquartile ranges for each group are overlaid on the dot plots. Each dot represents the value of an individual mouse calculated as the rate of the mean of IL-2 produced by untreated (control) mice. (e) Expression of Treg ($FoxP3^{HIGH}/CD25^{HIGH}$) and (f) TH17 ($ROR\gamma_t{}^+/IL\text{-}17^+$) phenotypes generated after 6-day culture in (anti-CD3 + IL-2 + TGF-β)- or (anti-CD3 + anti-CD28 + TGF-β+IL-6+anti-IFNγ+anti-IL-4)-containing medium, respectively. Data show the mean ± SD of pooled percentages of at least four mice. (g). Treg/TH17 ratios from experiments (e) and (f) relative to those in untreated (Control) mice levels. (*) is indicative of significant differences ($P < 0.05$) with respect to untreated mice as measured by *Student's t* test, with the exception of the *Wilcoxon's rank-sum* test used in the IL-2 production experiments.

sequential process. This occurs in response to weak TCR stimulation in the presence of IL-2 and TGF-β only after the cell undergoes several rounds of cell division, and it entails a fine-tuned reconfiguration of the PI3K/AKT/mTOR pathway [25–27]. This is consistent with the "two-step differentiation model" proposed by Guo *et al.* [46]. According to this model the initial TCR-dependent conditioning induces the expression of CD25 and "primes" the cell to efficiently respond to the "cytokine-dependent" acquisition of the Treg phenotype. In this study, the presence of rapamycin, PI-103, or PKI-587 promoted the differentiation of iTregs without requiring multiple rounds of cell division. Under suboptimal TCR stimulation, IL-2 may transduce STAT5-dependent signals needed to induce FoxP3 expression [47]. In combination with STAT-5, TGF-β signaling through Smad3 plays a major role in the regulation of FoxP3 and CD25 expression [48,49]. We can speculate that, in the presence of rapamycin and dual inhibitors, the weak IL-2-R signaling in conjunction with Smad3 would be sufficient to sustain iTreg differentiation and expansion but not to support the optimal IL-2-dependent expansion of conventional non-Treg cells. Consequently, in agreement with our results

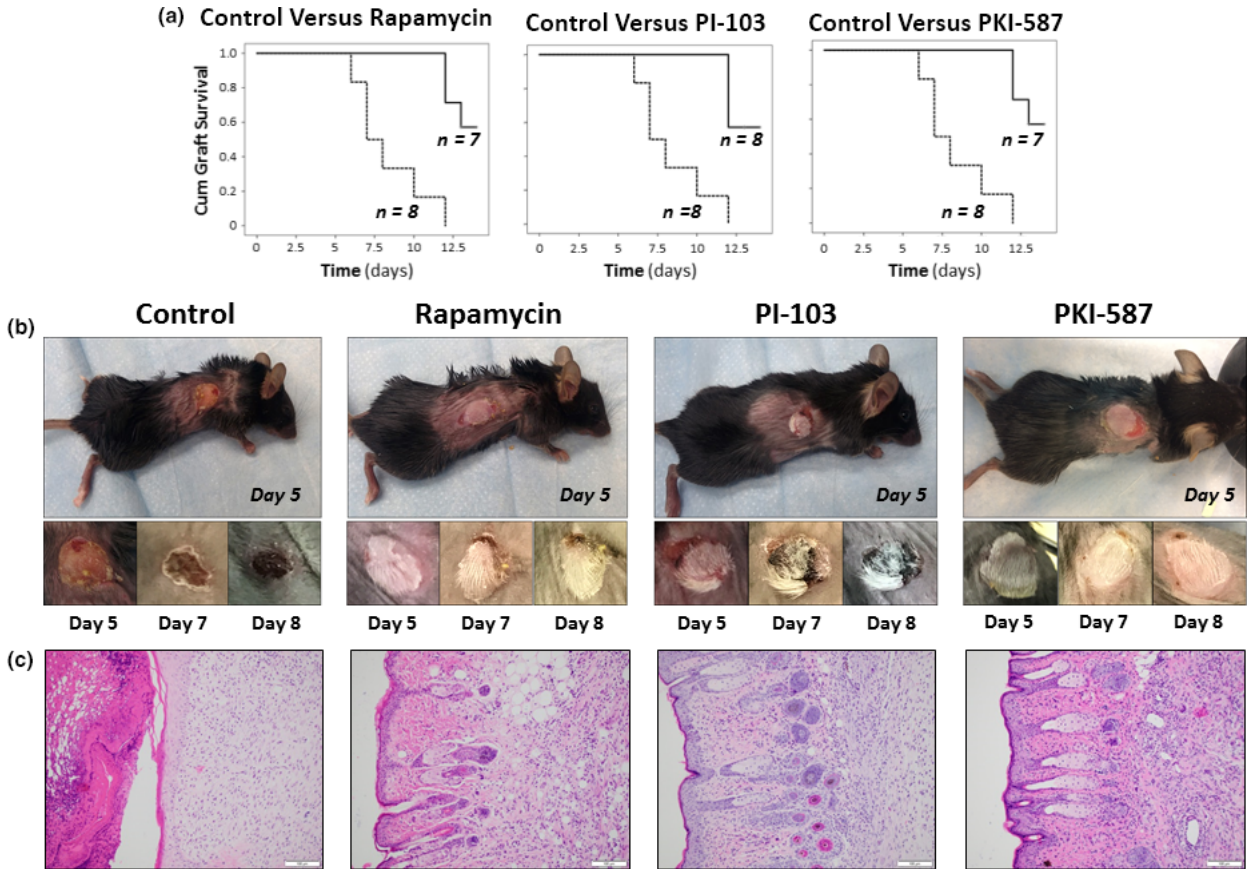

Figure 7 *In vivo*-treated mice: Histology and survival rates of allogeneic skin grafts. Skin transplants were performed using BALB/c mice as donors of tail skin in C57BL/6 receptors. Vehicle-control or drug treatments to C57BL/6 mice were initiated 48 h before transplants and continued until the end of the experiments. (a) Kaplan–Meier survival curves show prolonged allogeneic skin graft survival in rapamycin-, PI-103-, and PKI-587-treated compared to vehicle (control)-treated mice as demonstrated by log rank test, with *P*-values < 0.002 in all treatments. (b) Photographs of recipient mice and evolution of skin allografts at indicated days post-transplant. (c) Histology. Representative images of H&E stained skin grafts harvested at day 12 after transplant (20× magnification). Control skin biopsy shows a necrotic graft with underneath reepithelization and marked cellular infiltration in the dermis consistent with severe rejection (left). The biopsies from rapamycin-, PI-103-, and PKI-587-treated mice show an integrated graft with mild infiltration in the dermis.

(Fig. 3d), addition of rapamycin or dual PI3K/mTOR inhibitors in a mixed population of activated T-cells would result in the accumulation of functionally suppressive iTreg cells.

mTOR delivers an obligatory signal for proper activation and differentiation of T-cells through two functionally distinct signaling complexes, mTORC1, and mTORC2 [50]. Rapamycin is well known to selectively inhibit mTORC1 in short course treatments, but prolonged exposure leads to reduced mTORC2 activity and a partial rescue of mTORC1 [16,51]. Our group and others have reported functional mTOR as a crucial regulator of iTreg cell differentiation and suppressor activity [26,39]. Here, we demonstrated the distinct influence of rapamycin and dual-PI3K/mTOR inhibitors on the mTORC1 and mTORC2 balance during the transition of naïve T-cells to iTregs. Despite inducing similar outcomes in promoting iTreg differentiation and opposing pro-inflammatory effector function, our results clearly establish that single mTOR (rapamycin) and dual PI3K/mTOR inhibitors target different signaling events of the same pathway. The Western Blot results fit with the complex plasticity of the AKT/mTOR signaling reported in other studies [52–56], including cross-talks, feedback loops and compensatory mechanisms within the PI3K/AKT/mTOR signaling-cluster and with several other pathways.

The association between cell signaling and metabolism is especially relevant in the regulation of T-cell function. Pro-inflammatory effector responses rely mostly on glycolysis to meet their energy demands; conversely, functional Treg cells exhibit higher dependency on

mitochondrial oxidative phosphorylation [38,57,58]. Therefore, cellular metabolic control is currently envisioned as a novel therapeutic strategy to redirect T-cell function and restore immune homeostasis. Since mTOR is a critical sensor and driver of cell metabolism, the metabolic modulation through the pharmacological targeting of the PI3K/AKT/mTOR pathway may constitute an attractive immunotherapeutic approach to redirect T-cell function, as it is in cancer cells. In our study, addition of rapamycin and dual PI3K/mTOR inhibitors in the culture media resulted in the alteration of the glycolytic pathway. This early antiglycolytic effect and the limited effect of rapamycin and dual inhibitors on mitochondrial respiration would facilitate the metabolic reprogramming toward the prevalence of mitochondrial respiration over glycolysis, a metabolic state that favors iTreg cell differentiation. Our results also revealed the different substrate utilization in mitochondrial respiratory metabolism in rapamycin-treated and dual PI3K/mTOR inhibitor-treated cells as illustrated by their distinct Etomoxir-sensitivity. Overall, our findings uncovered the different metabolic programs induced by rapamycin and dual inhibitors, although all of them drive T-cells toward similar Treg-compatible bioenergetic profile. Additional studies are underway to elucidate the specific signaling events associated with the distinct metabolic alterations and particular effector responses modulated by rapamycin and dual inhibitors. The characterization of fundamental differences between activation of naïve and memory T-cells may also offer new opportunities to tailor more efficient therapeutic interventions. While naïve T-cells require a rapid mTORC1-dependent readjustment of the metabolic machinery toward glycolysis, the glycolytic switch in antigen-experienced cells is more mTORC2-dependent [57–59] In this context, the mTORC2 prevalence of PI-103-treated cells may be particularly effective in the activation of memory T-cell recall responses. The selective control of these mTORC-specific metabolic T-cell responses may be of the utmost relevance not only in preventing allograft rejection, but also in mediating a protective response against pathogens and tumor growth.

In the field of transplantation, there is an emerging interest in enhancing the suppressor immune response as an alternative strategy to reach a clinical tolerogenic state and preserve long-term graft function [60–64]. The immunosuppressive action of rapamycin has been already described in different mouse models of transplantation [65–68] and, in this study, we have characterized the immunomodulatory effects of PI-103 and PKI-587 in T-cells *in vitro* and *in vivo*. Our analyses of the skin grafts demonstrated that the *in vivo* treatment with PI-103 and PKI-587 induced an effective control of the immunological response against allogeneic skin graft, likely by harnessing the activation and effector function of T-cells and, at least for PKI-587, by also priming their differentiation into protective Tregs. Indeed, all *in vivo* drug treatments reduced the susceptibility of $CD4^+$ T-cells to differentiate toward a pro-inflammatory TH17 effector phenotype. These results are in line with the growing body of literature recognizing the involvement of effector TH17 cells in transplant rejection [69–75]. We have noted substantial differences on the effects promoted by PI-103 *in vitro* and *in vivo*, which may be attributed to the rapid plasma and tissue clearance exhibited by this compound [18]. This study provides the first evidence supporting the potential of dual PI3K/mTOR inhibitors to generate a successful tolerogenic state essential in the prevention of allograft rejection The *in vivo* treatments with dual PI3K/mTOR inhibitors as well as with rapamycin promoted the skewed differentiation of $CD4^+$ T-cells toward a noninflammatory response, which may define the outcome of the immune reaction toward the increased rates of allograft survival. In addition, we characterized specific cellular, signaling and metabolic mechanisms triggered by dual PI3K/mTOR inhibitors that may control T-cell activation and lineage fate decisions. Further studies are warranted to assess the immunosuppressant efficiency and toxic profile of this family of compounds to confirm their clinical utility in transplantation.

Authorship

RG and FM: designed the research. VV, LT, MIM, CLQ, JAB, and FM: performed research. VV, LT, MIM, VC, and FM: collected data. VV, DAB, MIM, VC, and FM: analyzed data. RG and FM: wrote the manuscript. VV, LT, DAB, MIM, CLQ, VC, RG, and FM: involved in the critical editing of content. Approval of final version: all authors.

Funding

This work is supported in part through the National Cancer Institute Cancer Center Support Grant P30CA177558 to the University of Kentucky Markey Cancer Center to support the Redox Metabolism Shared Resource Facility, the Markey Biospecimen Procurement and Translational Pathology Shared Resource Facility (BPTP SRF), and the University of Kentucky Flow Cytometry and Cell Sorting Core Facility (FCCS).

Conflict of interest

The authors declare no commercial or financial conflicts of interest.

Acknowledgements

The authors wish to thank Greg Bauman and Jennifer Strange for their assistance with flow cytometry analysis, Dana Napier for handling the tissue and preparing the histologic sections, and Maria Moliner for her rendering of figures. The content the manuscript is solely the responsibility of the authors and does not necessarily represent the official views of the NIH.

SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article:

Appendix S1. Materials and Methods.

Figure S1. Effects of rapamycin and dual inhibitors on the proliferation and differentiation of human naïve $CD4^+$ T cells.

Figure S2. Effects of rapamycin and dual inhibitors on the expression of mTOR.

Figure S3. Altered activation, proliferation and IL-2 production in in vitro drug-treated mouse T cells.

Figure S4. Phenotype of splenocytes from in vivo drug-treated and control mice.

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