Pacritinib Combined with Sirolimus and Low-Dose Tacrolimus for GVHD Prevention after Allogeneic Hematopoietic Cell Transplantation: Preclinical and Phase I Trial Results

Joseph Pidala1,2,3, Kelly Walton4, Hany Elmariah1,3, Jongphil Kim5, Asmita Mishra1,3, Nelli Bejanyan1,3, Taiga Nishihori1,3, Farhad Khimani1,3, Lia Perez1,3, Rawan G. Faramand1,3, Marco L. Davila1,2,3, Michael L. Nieder1,3, Elizabeth M. Sagatys6, Shernan G. Holtan4, Nicholas J. Lawrence7, Harshani R. Lawrence7, Bruce R. Blazar8, Claudio Anasetti1,2,3, Said M. Sebti9, and Brian C. Betts4

ABSTRACT

Purpose: In this first-in-human, phase I, GVHD prevention trial (NCT02891603), we combine pacritinib (PAC), a JAK2 inhibitor, with sirolimus to concurrently reduce T-cell costimulation via mTOR and IL6 activity. We evaluate the safety of pacritinib when administered with sirolimus plus low-dose tacrolimus (PAC/SIR/TAC) after allogeneic hematopoietic cell transplantation.

Patients and Methods: The preclinical efficacy and immune modulation of PAC/SIR were investigated in xenogeneic GVHD. Our phase I trial followed a 3+3 dose-escalation design, including dose level 1 (pacritinib 100 mg daily), level 2 (pacritinib 100 mg twice daily), and level 3 (pacritinib 200 mg twice daily). The primary endpoint was to identify the lowest biologically active and safe dose of pacritinib with SIR/TAC (n = 12). Acute GVHD was scored through day +100. Allografts included 8/8 HLA-matched related or unrelated donor peripheral blood stem cells.

Results: In mice, we show that dual JAK2/mTOR inhibition significantly reduces xenogeneic GVHD and increases peripheral regulatory T cell (Treg) potency as well as Treg induction from conventional CD4+ T cells. Pacritinib 100 mg twice a day was identified as the minimum biologically active and safe dose for further study. JAK2/mTOR inhibition suppresses pathogenic Th1 and Th17 cells, spares Tregs and antileukemia effector cells, and exhibits preliminary activity in preventing GVHD. PAC/SIR/TAC preserves donor cytomegalovirus (CMV) immunity and permits timely engraftment without cytopenias.

Conclusions: We demonstrate that PAC/SIR/TAC is safe and preliminarily limits acute GVHD, preserves donor CMV immunity, and permits timely engraftment. The efficacy of PAC/SIR/TAC will be tested in our ongoing phase II GVHD prevention trial.

Introduction

Nearly a decade has passed since JAK2 was identified as a therapeutic target to control human alloreactive T cells (1). Since that time, major gains in clinical translation have occurred. The first JAK inhibitor, ruxolitinib, was recently approved for use in steroid-refractory GVHD (2). Distinct from selective JAK2 blockade (1), ruxolitinib is a dual JAK1/2 inhibitor that simultaneously targets a broad range of cytokines, inflammatory and otherwise (3–6). While relapse of underlying hematologic malignancies does not appear to be problematic with ruxolitinib, cytomegalovirus (CMV) reactivation and cytopenias do pose a challenge when treating recipients of allogeneic hematopoietic cell transplantation (alloHCT; ref. 2). Furthermore, the durability of responses by ruxolitinib do wane over time and could be a result of Treg impairment (2).

We have shown that pacritinib (PAC), a selective JAK2 inhibitor, reduces alloreactivity mediated by murine or human T cells (6). Unlike ruxolitinib, pacritinib spares IL2 signal transduction along with Tregs and their suppressive function (6). Conversely, pacritinib readily eliminates IL6 activity and reduces pathogenic T effectors of GVHD (6). Pacritinib offers a degree of finesse in its selective immune suppression, lending its use to GVHD prevention. In this setting, pressure against pathogenic T cells is needed, yet key mediators of antiviral immunity, graft-versus-leukemia (GVL), and allotolerance must be left unperturbed. Our premise is that pacritinib is well positioned for GVHD prevention based on these unique immune effects.

To enhance the immune-suppressive potency afforded by pacritinib, and leverage its beneficial effects toward Tregs, we combined pacritinib with our sirolimus (target range, 5–14 ng/mL) and low-dose tacrolimus (NCT02891603), we combine pacritinib (PAC), a JAK2 inhibitor, with sirolimus to concurrently reduce T-cell costimulation via mTOR and IL6 activity. We evaluate the safety of pacritinib when administered with sirolimus plus low-dose tacrolimus (PAC/SIR/TAC) after allogeneic hematopoietic cell transplantation.

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GVHD Prevention with Combined JAK2/mTOR Inhibition

Translational Relevance
GVHD remains an important cause of morbidity and mortality after allogeneic hematopoietic cell transplantation (alloHCT). Distinct from broadly acting GVHD prophylaxis, JAK2 inhibition suppresses alloreactive T cells, while sparing regulatory T cells (Treg) and graft-versus-leukemia. IL6 activity via JAK2 and phosphorylated STAT3 in CD4+ T cells is associated with acute GVHD onset. T-cell costimulation by CD28 and mTOR activation is also implicated in GVHD pathogenesis. We demonstrate the safety and preliminary efficacy of combined JAK2/mTOR inhibition in a phase I trial of pacritinib, sirolimus, and low-dose tacrolimus (PAC/SIR/TAC). Importantly, we show PAC/SIR/TAC effectively blocks pathogenic IL6 and CD28 signal transduction and reduces critical T effectors of GVHD. Extensive correlative studies and data from mice show combined JAK2/mTOR blockade mechanistically increases the ratio of STAT5 to STAT3 in CD4+ T cells and promotes the induction of potent Tregs. The favorable safety profile and its impact on immune reconstitution after alloHCT make PAC/SIR/TAC a promising strategy to prevent GVHD.

tacrolimus (3–7 ng/mL; SIR/TAC) GVHD prophylaxis regimen (7, 8). Exposure to SIR and specifically limiting the dose of TAC (3–7 ng/mL) assists in the reconstitution and function of Tregs across conditioning regimens and donor sources (7, 9). The addition of JAK2 inhibition to mTOR blockade also parallels our prior study in mice where we demonstrated that concurrent suppression of T-cell costimulation, and cytokine activation prevents GVHD while maintaining GVL (10). In that past study, we used a bispecific inhibitor of JAK2 and Aurora kinase A, AII-100 (10). Conceptually, PAC/SIR/TAC parallels that approach as CD28 signals through mTOR as well as Aurora kinase A (10). Thus, PAC/SIR/TAC provides concurrent inhibition of CD28-mediated T-cell costimulation plus JAK2 cytokine activation.

In this phase I clinical trial, we demonstrate that PAC/SIR/TAC is safe and provides evidence of early activity in GVHD prevention. PAC/SIR/TAC leads to an immune phenotype that mirrors our prior observations in mice transplanted with CD4+ JAK2-deficient T cells (6). PAC/SIR/TAC promotes Tregs and substantially improves the ratio of Tregs to pathogenic effectors of GVHD. Importantly, PAC/SIR/TAC permits timely donor engraftment, avoids cytopenias, and is not associated with excess CMV reactivation. We provide supporting data in mice, demonstrating the long-term efficacy of concurrent JAK2/mTOR inhibition in xenogenic GVHD as well as key benefits regarding Treg biology and function. Our ongoing phase II trial is testing the efficacy of PAC/SIR/TAC in GVHD prevention.

Patients and Methods

Included subjects

Included were subjects ≥ age 18. HLA-A-, HLA-B-, HLA-C-, and HLA-DRB1–matched related or unrelated donors were allowed. The graft source was mobilized peripheral blood stem cells (5–10 × 10^6 CD34+ cells/kg). Remission criteria included: acute leukemia (complete remission with < 5% marrow blasts, no peripheral blasts, and absolute neutrophil count (ANC) > 1,000/µL), myelodysplastic syndrome and chronic myeloid leukemia (<5% marrow blasts), myeloproliferative neoplasms (<5% marrow and peripheral blood blasts, and no JAK2 inhibitor therapy within 4 weeks preceding HCT date), and Hodgkin or non-Hodgkin lymphoma (complete or partial response).

Outcome measures

The phase I trial had a composite primary endpoint, which considered both safety and the biologic effect of pacritinib therapy, with the objective of identifying the lowest biologically active safe dose. Clinical DLT events included: left ventricular systolic dysfunction ≥ grade 3, myocardial infarction ≥ grade 3, atrial fibrillation or flutter ≥ grade 3, supraventricular tachycardia ≥ grade 3, gastrointestinal hemorrhage ≥ grade 3, intracranial hemorrhage ≥ grade 3, or any other ≥ grade 3 AEs considered at least possibly related to pacritinib therapy. Management of sirolimus and tacrolimus followed usual program standard operating procedures, and included guidelines for drug level targets (SIR 5–14 ng/mL, TAC 3–7 ng/mL) and management of drug interactions. Surveillance for signs, symptoms, and laboratory findings of thrombotic microangiopathy (TMA) was followed, and the protocol included guidance in how to manage and mitigate TMA if observed. The protocol did not mandate the duration of tacrolimus or sirolimus administration after HCT.

Adequate vital organ function [LVEF ≥ 50%; FEV1 (forced expired volume in one second), FVC (forced vital capacity), and adjusted DLCO (diffusing capacity from carbon monoxide) ≥ 50% of predicted values; transaminases < 2 times upper limit of normal values; creatinine clearance ≥ 50 cc/minute] and adequate Karnofsky performance status (≥80%) were required. The following were exclusion criteria: uncontrolled infection, history of HIV or hepatitis B or C infection, HCT-CI > 4 (11), QTcF > 450 ms, coagulation studies [PT (prothrombin time), PTT (partial thromboplastin time), thrombin time] > 2 × upper limit of normal values, ≥ grade 3 cardiac or bleeding complications within the preceding 6 months, uncontrolled current myocardial infarction/angina, or class III–IV congestive heart failure. To reduce treatment heterogeneity, the following restrictions were enforced: conditioning regimens could include only myeloablative pharmacokinetic-targeted intravenous busulfan/fludarabine, or a reduced intensity fludarabine/melphalan regimen (per program standard procedures). Any planned use of post-HCT cyclophosphamide, anti-thymocyte globulin, alemtuzumab, or bortezomib was prohibited. Finally, planned use of post-HCT maintenance (e.g., tyrosine kinase inhibitors, JAK1/2 inhibitors) was exclusionary to avoid drug interactions and confounding of toxicity assessment for pacritinib.

Treatment program

Phase 1 dose-finding procedures followed a standard 3+3 dose-escalation design and included dose level 1 (pacritinib 100 mg daily), level 2 (100 mg twice daily), and level 3 (200 mg twice daily). Dose level 1 represented the lowest possible dose capable of inhibiting pSTAT3 in CD4+ T cells, based on prior data (12). Dose finding was governed by a composite of clinical safety [dose-limiting toxicity (DLT) events] and key pharmacodynamic assessment [day +21 pSTAT3 in blood CD4 T cells after HCT, with a goal of achieving <35% CD4+, pSTAT3+ T cells (13, 14)]. Pacritinib was given at the full intended dose (per dose level) from day 0 onward, followed by treatment at 50% of original dose from day +70 through +83, then at 25% of original dose from day +84 through +100, and then stopped. Concurrent therapy with strong CYP3A4 inhibitors required 50% dose reduction in pacritinib. Specific drug interruption and discontinuation rules were based on QTcF prolongation, left ventricular systolic dysfunction, diarrhea, and any other ≥ grade 3 adverse events (AE) deemed at least possibly related to pacritinib therapy. Management of sirolimus and tacrolimus followed usual program standard operating procedures, and included guidelines for drug level targets [SIR 5–14 ng/mL, TAC 3–7 ng/mL] and management of drug interactions. Surveillance for signs, symptoms, and laboratory findings of thrombotic microangiopathy (TMA) was followed, and the protocol included guidance in how to manage and mitigate TMA if observed. The protocol did not mandate the duration of tacrolimus or sirolimus administration after HCT.
chimerism, acute and chronic GVHD per consensus criteria (15, 16), GVHD therapy requirements, TMA (17), hepatic veno-occlusive disease (VOD; ref. 18), discontinuation of immune suppression, infectious complications including CMV reactivation, and HCT outcomes including malignancy relapse, nonrelapse mortality, overall survival, and causes of death.

Detailed methods regarding flow cytometry, allogeneic mixed leukocyte reactions, Treg suppression assays, and Institutional Animal Care and Use Committee (IACUC)-approved xenogeneic GVHD mouse experiments are included as Supplementary Materials and Methods.

Study approval

Written informed consent was obtained from eligible patients on our Institutional Review Board–approved study, A Phase I/II GVHD Prevention Trial Combining Pacritinib with Sirolimus-Based Immune Suppression, at Moffitt Cancer Center (NCT02891603). This was done in accordance with the principles set forth within the Declaration of Helsinki. NSG mice (male or female, age 6 to 24 weeks old) were purchased from The Jackson Laboratory and housed within American Association for Laboratory Animal Care–accredited Animal Resource Centers at Moffitt Cancer Center or the University of Minnesota (Minneapolis, MN). All mice were treated in adherence with the NIH Guide for the Care and Use of Laboratory Animals according to protocols approved by IACUC.

Statistical methods

The phase I dose-finding procedure followed a 3+3 design; however, it incorporated both the described clinical DLT events and the key pharmacodynamic measure. Dose-escalation proceeded until a safe dose level was determined (i.e., ≤1/6 subjects experiencing a DLT event) where the key pharmacodynamic measure was met. Baseline characteristics and major outcome variables were summarized using descriptive statistics. Correlative and murine data are reported as mean values ± SEM. ANOVA was used for group comparisons, including a Dunnett test for correction of multiple comparisons with a control. For comparison of murine survival curves, a log-rank test was used. The statistical analysis was conducted using Prism software version 5.04 (GraphPad) for correlative and murine data. Statistical significance was defined by a two-tailed P value less than 0.05.

Results

Xenogeneic GVHD

Concurrent JAK2 or STAT3/mTOR blockade synergistically reduces xenogeneic GVHD mediated by human T cells

To test the effect of dual JAK2 or STAT3/mTOR inhibition on alloreactive human T cells, mixed leukocyte reactions [T cell to dendritic cell (DC) ratio 30:1] were incubated with varying doses of pacritinib alone or with a fixed, clinically relevant concentration of sirolimus (10 ng/mL; Fig. 1A). Consistent with clinical experience (19), sirolimus alone moderately reduced T-cell proliferation against allogeneic monocyte-derived DCs (Fig. 1A). Pacritinib at 2.5 μmol/L and greater also inhibited alloreactive T effectors. We previously demonstrated that this minimum concentration of pacritinib (2.5 μmol/L) decreases the frequency of CD4+ pSTAT3+ T cells, permits CD4+ T-cell STAT5 phosphorylation, and suppresses the proliferation of alloreactive T cells in vitro (6). Importantly, the combination of pacritinib plus sirolimus significantly suppressed T-cell proliferation even at low nanomolar concentrations of the JAK2 inhibitor (Fig. 1A).

The efficacy of JAK2/mTOR inhibition was investigated in vivo using our established model of xenogeneic GVHD (10). Immunodeficient NSG mice were transplanted with 25 × 10^6 human peripheral blood mononuclear cells (PBMC) and then treated with vehicle, pacritinib, S3I-201 (S3I; an inhibitor of downstream STAT3), sirolimus, PAC/SIR, or S3I/SIR for 14 days. We previously demonstrated that pacritinib administered at 100 mg/kg twice a day significantly suppressed human alloreactive T cells targeting skin grafts in vivo (6). As we were interested in testing the potential synergy of PAC/SIR, a lower but biologically active dose of pacritinib (50 mg/kg) was used in these experiments. The combination of JAK2 or downstream STAT3 inhibition plus sirolimus significantly improved survival from xenogeneic GVHD in mice, compared with either inhibitor alone (Fig. 1B). GVHD clinical scores were significantly reduced by sirolimus and the combination treatment groups, with S3I/SIR performing greater than sirolimus (Fig. 1C).

STAT3 and S6 phosphorylation are biologic readouts for JAK2 and mTOR activation, respectively, in T cells (1, 13, 14). Mice treated with pacritinib, S3I-201, PAC/SIR, or S3I/SIR exhibited a significantly decreased frequency of CD4+, pSTAT3+ human T cells in the mouse spleens at day +14 (Fig. 1D). Conversely, mice treated with the mTOR inhibitor, sirolimus, alone or in combination with pacritinib or S3I-201, demonstrated significantly less CD4+, pS6+ T cells (Fig. 1E). Only the mice treated with concurrent mTOR blockade plus pacritinib or S3I-201 had a significant reduction in both the frequency of CD4+, pSTAT3+ and pS6+ T cells (Fig. 1E), thus providing evidence that concurrent JAK2 or pSTAT3 plus mTOR inhibition successfully removes relevant downstream signaling pathways in vivo. Furthermore, these data confirm that a reduced dose of pacritinib at 50 mg/kg exhibits on-target pathway inhibition of JAK2 (Fig. 1D). IL2/pSTAT5 responses were largely intact among mice in the control and treatment groups, though the frequency of CD4+, pSTAT5+ T cells was significantly reduced among mice treated with sirolimus (Fig. 1F). The similar survival outcomes of PAC/SIR and S3I/SIR strengthen the hypothesis that targeting IL6 receptor signal transduction, either at the level of JAK2 or downstream STAT3, plus mTOR inhibition significantly reduces the clinical impact of GVHD.

Targeting JAK2 or STAT3/mTOR suppresses Th1 and Th17 cells

While NSG mice treated with S3I, sirolimus, PAC/SIR, or S3I/SIR exhibited a significant decrease in frequency of pathogenic Th1 cells in the spleen on day +14, the greatest reductions trended toward the dual pathway blockade groups (Fig. 2A and B). However, when DC- allostimulated T cells were cultured with vehicle, pacritinib, S3I-201, sirolimus, PAC/SIR, or S3I/SIR in vitro, production of the inflammatory IFNγ, IL13, and GMCSF cytokines was only significantly suppressed with PAC/SIR or S3I/SIR (Supplementary Fig. S1). In addition, only PAC/SIR- and S3I/SIR-treated mice had a significant reduction in human Th17 cells (Fig. 2A and C). The frequency of human Th2 cells in the recipient NSG spleens were similar across all experimental groups (Fig. 2A and D).

Concurrent JAK2 or STAT3/mTOR blockade maintains peripheral Tregs, but significantly improves Treg conversion from CD4+ Tconv

Similar to our previous data with single-agent pacritinib (6), JAK2 inhibition alone significantly increased the amount of Tregs in the mouse spleen at day +14 (Fig. 3A and B). However, this was not observed with the S3I-201, sirolimus, or either of the combination treatments (Fig. 3A and B). Importantly, exposing peripheral Tregs to...
PAC/SIR in vitro significantly improved their suppressive potency against alloreactive T cells compared with vehicle or single-agent controls (Fig. 3C). This suggests that while the frequency of Tregs may be similar to controls when blocking JAK2/mTOR, the beneficial effects on Treg function may contribute toward the observed reduction in GVHD. In addition, we have previously demonstrated that dual inhibition of T-cell costimulation and IL6 signal transduction substantially improved inducible Treg generation and potency, by way of concurrently targeting CD28 via Aurora kinase A and IL6 by JAK2 using a bispecific drug, AJI-100 (10). Further supporting that concept, we now show that dual inhibition of mTOR plus JAK2 or STAT3 leads to a significant gain in the frequency of Tregs when the mice are transplanted with Treg-depleted PBMCs at the outset (Fig. 3D and E).

Concurrent inhibition of JAK2 or STAT3/mTOR preserves donor antileukemia immunity

To test donor antileukemia immunity, human antitumor CTLs were generated in vivo and then their specific killing of tumor targets was evaluated in vitro. NSG mice received human PBMCs and were inoculated with irradiated U937 cells on days 0 and +7. Mice were treated with vehicle, pacritinib, S3I-201, sirolimus, PAC/SIR, or S3I/SIR from day 0 until day +14. Mice were monitored for GVHD survival (B) and severity (C; mean ± SEM). Transplanted NSG mice were humanely euthanized at day +14 and human T cells were acquired from the spleens and phosphoproteins were analyzed by flow cytometry. Graph shows the frequency (mean ± SEM) of human CD4⁺, pSTAT3⁺ T cells (D), CD4⁺, pS6⁺ T cells (E), and CD4⁺, pSTAT5⁺ T cells (F). Pooled data from three independent experiments is shown, 12 mice per group. ANOVA (A, C, D, E, and F) and log-rank test (B). *, P < 0.05; **, P = 0.01–0.001; ***, P = 0.001–0.0001; ****, P < 0.0001. NS, not significant.

Baseline patient characteristics of PAC/SIR/TAC phase I trial

The positive resultant data from our xenograft experiments served as proof of concept that dual JAK2/mTOR inhibition can reduce...
GVHD and spare antileukemia activity. As such, we investigated the safety and preliminary activity of PAC/SIR/TAC as GVHD prophylaxis following HLA-matched related or unrelated alloHCT. The primary aim of this phase I trial was to identify the lowest biologically active dose of pacritinib, defined as achieving <35% circulating CD4⁺, pSTAT3⁺ T cells at day +21, that is also safe when combined with sirolimus-based immune suppression (13). This pSTAT3 threshold was determined by our prior work showing that the risk for grade 2–4 acute GVHD was increased among patients with >35% circulating CD4⁺, pSTAT3⁺ T cells at day +21 (13, 14). A total of 12 evaluable patients were treated on this phase I trial (NCT02891603), with 6 patients each included in pacritinib dose levels 1 (100 mg/day) and 2 (100 mg twice a day). Pacritinib was administered orally from day 0 to day +70, then tapered to off on day +100 to mitigate the risk of JAK inhibitor withdrawal syndrome (21, 22). Overall, the patients comprising the two dose level cohorts were similar in regard to age, gender, race, and ethnicity (Supplementary Table S1). In addition, disease and pretransplant response status, functional status, comorbidities, conditioning regimens, donor characteristics, and allograft source were comparable between the pacritinib dose level groups (Supplementary Table S1).

Safety and DLTs
Median follow-up time for surviving patients at the time of this analysis was 18 months (range, 3.7–29.6 months). A single DLT was observed in dose level 1 only and consisted of angioedema possibly related to pacritinib. No patients treated on dose level 2 experienced any DLTs. Neither CMV reactivation nor disease was observed among patients treated at dose level 2, with only a single case of CMV reactivation among dose level 1 (Supplementary Table S2). Complete data on AEs including infectious complications are summarized in Supplementary Table S3. No patients treated with PAC/SIR/TAC developed VOD. Grade 1–2 TMA was observed among two dose level 2 subjects (one grade 1 resolved without intervention and one grade 2 resolved after tacrolimus discontinuation). Additional safety data (QTcF, cardiac ejection fraction monitoring, and coagulation studies) are presented in Supplementary Fig. S2. Two deaths occurred during the conduct of the study. One patient died of relapsed disease in dose level 1. In dose level 2, 1 patient died of steroid-refractory grade 4 acute GVHD after prematurely discontinuing tacrolimus for non-TMA acute kidney injury and electively stopping pacritinib. Importantly, PAC/SIR/TAC allowed for timely neutrophil (median 15 days) and platelet (median 10 days) engraftment (Fig. 5A; Supplementary Fig. S3) and permitted full donor chimerism by day +30 (Fig. 5A and B; Supplementary Table S4).

Pharmacokinetics and pharmacodynamics
The frequency of CD4⁺, pSTAT3⁺ T cells at day +21 among patients treated at dose level 1 were not reduced compared with baseline values (Fig. 5C). Importantly, PAC/SIR/TAC at dose level 2 significantly decreased the amount of CD4⁺, pSTAT3⁺ T cells (Fig. 5C). This pharmacodynamic data, along with the favorable safety profile, identified pacritinib 100 mg twice a day as the recommended phase II dose (RP2D). In addition, dose level 2 of PAC/SIR/TAC significantly reduced the fluorescence intensity of pSTAT3 among circulating CD4⁺ T cells at day +21 (Fig. 5D and E). Study drug adherence and pharmacokinetic studies for pacritinib 100 mg daily and twice a day are detailed in Supplementary Tables S5 and S6. Among those cases that stopped pacritinib before full planned duration of study therapy, a withdrawal syndrome was not observed despite...
not tapering the drug first or using steroid therapy to address this possibility. Sirolimus suppresses mTOR signal transduction, and we confirmed that PAC/SIR/TAC at dose level 2 also significantly decreased downstream protein S6 phosphorylation in peripheral CD4+ T cells (Fig. 5F). Given that pacritinib inhibits JAK2 and subsequent STAT3 phosphorylation, without impairing common...
gamma chain receptor cytokine signal transduction, we confirmed that CD4+ T cells from patients treated at dose level 2 exhibited intact pSTAT5 (Fig. 5G). Furthermore, dose level 2 of PAC/SIR/TAC significantly increased the ratio of pSTAT5 to pSTAT3 among CD4+ T cells (Fig. 5H). A high CD4+ T cell pSTAT5/pSTAT3 ratio favors Tregs and opposes pathogenic T-cell subsets, like Th1 and Th17 (13, 14, 23). Consistent with the central hypothesis of this interventional trial, dose level 2 of PAC/SIR/TAC concurrently inhibited T-cell costimulation via mTOR blockade and cytokine activation by JAK2.

Acute and chronic GVHD

Overall grade 2–4 acute GVHD occurred in 2 subjects in dose level 1 [both gastrointestinal (GI) involvement treated with beclomethasone and budesonide with no systemic prednisone], and 1 subject in dose level 2 (steroid-refractory skin and GI involvement starting day +20 after alloHCT after early discontinuation of tacrolimus for non-TMA acute kidney injury at day +13). Additional stage 1–2 skin (overall grade 1) acute GVHD was observed in 1 subject in dose level 1 (resolved after topical steroid only), and 3 subjects in dose level 2 (resolved with topical agents only in two cases, and resolved after topical agents and 0.5 mg/kg/day prednisone in one case). NIH chronic GVHD was uncommon, with 2 subjects affected by overall score 1 (mild) chronic GVHD in dose level 1, and 1 subject affected by overall score 1 (mild) chronic GVHD in dose level 2. None of these required...
systemic immune-suppressive therapy for chronic GVHD. Full acute and chronic GVHD staging tables are presented in Supplementary Tables S7 and S8. With current follow-up time, 4 total subjects have successfully stopped tacrolimus after intentional taper (median, 8.8 months after HCT; range, 5.6–10.4), and 2 subjects stopped sirolimus (at 14.7 and 8.7 months after HCT, respectively) following successful taper. Separately, 4 subjects stopped tacrolimus for other reasons (suspected allergy $n = 1$, relapse $n = 1$, TMA $n = 1$, acute kidney injury $n = 1$), and 1 patient stopped sirolimus (relapse).

**Combined JAK2/mTOR inhibition polarizes Tregs over pathogenic Th1/Th17 cells**

PAC/SIR/TAC at dose level 2 did not impair the reconstitution of CD4$^+$ T cells, but as observed in our xenograft experiments it did significantly reduce the frequency of pathogenic Th1 and Th17 cells at day $+21$, compared with baseline values or subtherapeutic doses of pacritinib in dose level 1 (Fig. 6A–C). Like mice transplanted with allogeneic, CD4$^+$ JAK2-deficient T cells (6), PAC/SIR/TAC dose level 2 resulted in a significant increase in the frequency of Th2 cells (Fig. 6D). Furthermore, the percentage of CD4$^+$ Tregs was maintained among patients treated at both dose levels of PAC/SIR/TAC (Fig. 6E). Thus, the ratio of immune-suppressive Tregs to pathogenic Th1 and Th17 cells was significantly increased among those treated with PAC/SIR/TAC dose level 2, compared with baseline controls (Fig. 6F). While the frequency of Tregs at day $+21$ is similar among patients receiving dose level 1 or 2 of PAC/SIR/TAC, there was trend toward greater Tregs later at day $+100$ among dose level 2 (Fig. 6G).

**PAC/SIR/TAC maintains the reconstitution of effector T and NK cells**

Importantly, dose level 2 of PAC/SIR/TAC preserves the number of circulating CD8$^-$ T cells and NK cells at day $+21$, compared with baseline and dose level 1 (Fig. 6H and I). PAC/SIR permitted NK-cell cytolytic function against K562 target cells in vitro, albeit reduced compared with vehicle or ruxolitinib (JAK1/2 inhibitor) controls (Supplementary Fig. S4A). However, PAC/SIR fully maintained NK-cell proliferation in response to JAK1-dependent IL2 and IL15 (Supplementary Fig. S4B), while ruxolitinib significantly curtailed NK-cell expansion in vitro as observed previously (5, 6). The absolute number of peripheral B cells at day $+21$ from patients treated at PAC/SIR/TAC dose level 2 was not significantly reduced compared with baseline or dose level 1 values (Fig. 6J). Thus, while dose level 2 of PAC/SIR/TAC decreased the amount of pathogenic Th1 and Th17s, important effectors needed for GVL and antiviral immunity remained.

Figure 6. PAC/SIR/TAC favors Tregs over pathogenic Th1 and Th17 cells. Bar graphs show the total number (mean ± SEM) of CD4$^+$ T cells (A) at day $+21$ and frequency (mean ± SEM) of CD4$^+$, IFN$^+$ Th1 (B), CD4$^+$, IL17$^+$ Th17 (C), CD4$^+$, IL4$^+$ Th2 (D), and CD4$^+$, CD127$^+$, CD25$^+$ Foxp3$^+$ Treg (E) cells at day $+21$ among patients treated on dose level 1 or dose level 2 of the phase I trial. F, Graph depicts the ratio (mean ± SEM) of Treg to Th1 and Th17s at day $+21$. G, Graph shows Treg reconstitution at day $+21$ versus day $+100$ after alloHCT. Bar graphs show the absolute numbers (mean ± SEM) of CD8$^-$ T cells (H), NK cells (I), and B cells (J) at day $+21$ among patients treated on dose level 1 or dose level 2 of the phase I trial ANOVA (B–F, J). ‘‘*, $P = 0.001–0.01$; ‘‘‘‘, $P = 0.0001–0.001$, and ‘‘‘‘‘‘, $P < 0.0001$. Baseline data were acquired pretransplant between days −30 and −5.
achieve a low incidence of VOD or TMA (7–10) using the SIR/TAC in GVHD prevention and developed a strategy to decrease the amount of CD4+ T cells through therapeutic dosing of sirolimus (20, 24, 25). While we observed a significant decrease in the amount of CD4+, p35ser10+ T cells between dose levels 1 and 2 of PAC/SIR/TAC, the percentage of Aurora kinase-activated T cells was still over 50% (Supplementary Fig. S6A). This suggests that sufficient JAK2 and mTOR inhibition may limit the overall activation of the CD4+ T cells but does little to counter aberrant Aurora kinase A activity. Similarly, NSG mice transplanted with human PBMCs still demonstrated high frequencies of CD4+, p35ser10+ T cells among groups of animals treated with PAC/SIR or S3I/SIR, compared with controls (Supplementary Fig. S6B). This identifies the potential for unchecked T-cell costimulation via Aurora kinase A as a mechanism for GVHD resistance despite potent inhibition of JAK2 and mTOR.

Discussion

This phase I clinical trial is the first investigation to demonstrate that combining a selective JAK2 inhibitor, pacritinib, with sirolimus-based GVHD prophylaxis is safe. Importantly, PAC/SIR/TAC exhibits preliminary activity in GVHD prevention, without posttransplant cytopenias, CMV reactivation, or relapse. In part, pacritinib selectively inhibits pathogenic IL6, without impairing common gamma chain receptor cytokines, IL2 and IL15, which are critical for immune tolerance, antiviral immunity, and GVL (6). This is distinct from JAK1/2 inhibitors, where ruxolitinib exposure was linked to cytopenias and high rates of CMV reactivation among patients treated for steroid-refractory GVHD (2). Others and we have shown that ruxolitinib broadly impairs the signal transduction of IL2 and IL15, reducing the numbers and function of human NK cells, CTLs, and Tregs (3–6). Here we demonstrate that the immune reconstitution of NK cells, effector T cells, and Tregs are unperturbed by pacritinib. Furthermore, the combination of pacritinib and sirolimus significantly increases peripheral Treg suppressive potency.

The decision to combine pacritinib with SIR/TAC is supported by extensive translational investigations (1, 6–10). We have refined the use of SIR/TAC in GVHD prevention and developed a strategy to achieve a low incidence of VOD or TMA (7–9). Of the 2 patients that developed TMA with PAC/SIR/TAC, the severity was mild with no major adverse consequences. In both cases of TMA, the patients improved with either no intervention or discontinuation of tacrolimus. Furthermore, SIR/TAC enhances the immune reconstitution of Tregs and limits severe mucositis compared with tacrolimus plus methotrexate (TAC/MTX; refs. 7–9, 26). This is important as our published data in mice show that JAK2-deficient T cells favor a Treg phenotype over pathogenic Th17 cells (6). Our use of SIR/TAC includes a proportionally higher amount of sirolimus to tacrolimus, and that approach has demonstrated efficacy in reducing acute and chronic GVHD compared with TAC/MTX in a randomized controlled trial (7). While not a tacrolimus-free regimen, the limited exposure to tacrolimus in our SIR/TAC (target range, 3–7 ng/mL) compared with standard TAC/MTX (10–15 ng/mL) further assists in Treg accumulation after alloHCT (7). Although CTX 0.402, a multicenter randomized comparison of SIR/TAC to TAC/MTX as GVHD prophylaxis, resulted in equivalence with regard to the incidence of chronic GVHD, TAC/SIR was associated with a beneficial increase in the ratio of Tregs to conventional T cells after alloHCT (26, 27).

The rationale to combine pacritinib with SIR/TAC was also based on extensive preclinical data showing concurrent inhibition of T-cell costimulation via CD28 and cytokine activation from IL6 synergistically reduced xenogeneic GVHD in mice (10). PAC/SIR/TAC represents our first clinical iteration of this approach, merging mTOR blockade with JAK2 inhibition. The data from this phase I trial provides proof of concept that dual JAK2/mTOR inhibition is safe and shows preliminary activity in preventing GVHD. Interestingly, aberrant Aurora kinase A activity appears to be a possible route for resistance to PAC/SIR/TAC, primarily by bypassing mTOR inhibition (10, 24, 28). Aurora kinase A and mTOR share a common signal transduction pathway, mediated by CD28 activation (10, 24, 28). We have previously shown that dual JAK2/Aurora kinase A inhibition is synergistic using a novel bispecific inhibitor (10). Thus, clinical studies adding small molecule inhibitors of Aurora kinase A and JAK2 may overcome any potential resistance to mTOR blockade and warrant consideration.

There are important distinctions that require discussion to appreciate the nuanced implications of JAK1/2 versus JAK2 inhibition on the immune system after alloHCT. Starting with ruxolitinib, its broad inhibition of common gamma chain receptor cytokines, IL6, and the p40 cytokines, IL12 and IL23, provide very potent suppression over alloreactive T cells (3–6, 29). While this successfully achieves initial responses in GVHD therapy, the unbridled activity of ruxolitinib against beneficial Tregs, NK cells, and antitumor effectors poses a challenge toward durable allotolerance, antiviral immunity, and disease control. So far, these concerns derived from preclinical studies have primarily emerged as an increased risk for CMV reactivation (2). Importantly, waning complete responses by day 56 of ruxolitinib therapy may be secondary to the cumulative loss of Tregs deprived of IL2 (2). We previously demonstrated pacritinib alone offers partial protection from alloreactive T cells, compared with ruxolitinib (6). However, the combination of PAC/SIR has enhanced suppression over human T cells in xenogeneic GVHD models and in vitro. We show the combination of PAC/SIR successfully inhibits mTOR and IL6 signal transduction, reduces pathogenic Th1 and Th17 cells, maintains IL2 activity in T cells, and enhances peripheral Treg function as well as Treg induction from Tconv. Furthermore, PAC/SIR does not impair donor T-cell antileukemia activity.

In conclusion, we demonstrate that PAC/SIR/TAC is safe in GVHD prevention and have identified pacritinib 100 mg twice a day as the RP2D. Our phase II GVHD prevention trial of PAC/SIR/TAC is underway and actively accruing patients. This pending investigation
is dedicated to studying the efficacy of PAC/SIR/TAC in GVHD prevention after HLA-matched related or unrelated donor allografts for hematologic malignancies. The phase II experience will more completely address efficacy overall in GVHD prevention and provide an opportunity to examine the impact of individual factors (patient-level differences in variables associated with GVHD risk, impact of serial sirolimus and tacrolimus levels) on GVHD outcomes. On the basis of the successful completion of the PAC/SIR/TAC phase I and II trial, we look forward to future investigations aimed at eliminating tacrolimus from the combination of PAC/SIR, as we demonstrated in mice. We anticipate that uncoupling PAC/SIR from the calcineurin inhibitor will permit unconstrained allotolerance by optimizing Treg populations and function.

Authors' Disclosures

J. Pidala reports personal fees from CTI Biopharma during the conduct of the study, as well as personal fees from Syndax, Angen, and Regeneron outside the submitted work. K. Walton reports grants from R01 HL133823 during the conduct of the study. N. Bejanyan reports personal fees from Magenta therapeutics outside the submitted work. T. Nishihori reports other from Karyopharm and Novartis outside the submitted work. F. Khimani reports grants from Bristol Myers Squibb outside the submitted work. L. Perea reports grants from R01 HL133823 (to B.C. Betts) during the conduct of the study. R.G. Faramand reports grants from Kite/Gilead outside the submitted work. E.M. Sagatys reports grants from NIH/NHLBI during the conduct of the study. S.G. Holtan reports personal fees from Incyte, Generon, CSL Behring, and Bristol Myers Squibb outside the submitted work. N.J. Lawrence reports a patent for WO2017/156527A1 pending and a patent for U.S. Patent 7960434 issued and licensed to GLG Pharma. H.R. Lawrence reports a patent for U.S. Patent 7960434 issued and licensed to GLG Pharma and a patent for WO2017/156527A1 issued to none. C. Anasetti reports a patent for WO 2017/058950 A1 issued. S.M. Sebti reports grants from NIH/NHLBI during the conduct of the study. B.C. Betts reports grants from NHLBI, nonfinancial support from CTE BioPharma, and other from a patent for WO2017/058950A1 during the conduct of the study. B.C. Betts also reports personal fees from Incyte outside the submitted work, as well as a patent for WO2017/0512046A2 pending. No disclosures were reported by the other authors.

Authors' Contributions

J. Pidala: Conceptualization, data curation, formal analysis, validation, investigation, methodology, writing–original draft, writing–review and editing. K. Walton: Data curation, software, formal analysis, methodology, writing–review and editing. H. Elmariah: Resources, data curation, methodology, writing–review and editing. J. Kims: Conceptualization, formal analysis, validation, investigation, methodology, writing–review and editing. A. Mishra: Resources, data curation, methodology, writing–review and editing. N. Bejanyan: Resources, data curation, methodology, writing–review and editing. T. Nishihori: Resources, data curation, methodology, writing–review and editing. F. Khimani: Resources, data curation, methodology, writing–review and editing. L. Perea: Resources, data curation, methodology, writing–review and editing. R.G. Faramand: Resources, data curation, methodology, writing–review and editing. E.M. Sagatys: Resources, data curation, software, investigation, methodology, writing–review and editing. S.G. Holtan: Methodology, project administration, writing–review and editing. N.J. Lawrence: Resources, data curation, formal analysis, investigation, writing–review and editing. H.R. Lawrence: Resources, data curation, formal analysis, visualization, writing–review and editing. B.R. Blazar: Investigation, methodology, writing–review and editing. C. Anasetti: Conceptualization, investigation, methodology, writing–review and editing. S.M. Sebti: Conceptualization, resources, investigation, methodology, writing–review and editing. B.C. Betts: Conceptualization, resources, data curation, software, formal analysis, supervision, funding acquisition, validation, investigation, visualization, methodology, writing–original draft, project administration, writing–review and editing.

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