Extended Patients and Methods (Supplemental Material)

Monoclonal antibodies and flow cytometry

Fluorochrome-conjugated anti-human monoclonal antibodies used are as follows:

Antibodies				
Antigen:	**Fluor:**	**Manufacturer:**	**Clone:**	**Catalog #**
CD127	AF647	BD Biosciences	HIL-7R-M21	558598
CD4	FITC	ThermoFisher	OKT4	11-0048-42
CD25	PECy7	ThermoFisher	M-A251	557741
Ki67	PE	ThermoFisher	20Raj1	12-5699-42
CD4	PECy7	ThermoFisher	SK3	25-0047-42
IL-4	APC	ThermoFisher	8D4-8	17-7049-42
IFN-γ	FITC	BD Biosciences	B27	554700
IL-17	BV421	Biolegend	BL168	512322
Phospho- Histone H3 (Ser10)	AF647	Cell Signaling	D2C8	3458S
Anti-STAT3	PE	BD Biosciences	pY705	612569
CD3	FITC	ThermoFisher	OKT3	11-0037-42
CD4	PE	ThermoFisher	RPA-T4	12-0049-42
Anti-STAT5	AF647	BD Biosciences	pY694	612599
Phospho-S6 (SER235/236)	PE	Cell Signaling	D57.2.2E	5316S

CD107a	AF647	BD Bioscience	626622	H4A3
CD8	PE	BD Bioscience	555635	HIT8A
Foxp3	PE	BD Bioscience	560046	259D/C7

Key Reagents		
Reagent	**Manufacturer:**	**Catalog #**
IL-2 (CF)	Biolegend	589104
IL-15 (CF)	Biolegend	570302
IL-7 (CF)	Biolegend	581902
NK Isolation Kit, Hu	Miltenyi	130-092-657
Pierce LDH Cytotoxicity Assay Kit	ThermoFisher	88953

LIVE/DEAD Fixable Yellow or Aqua Dead Cell Stain (Life Technologies) was used to determine viability. Live events were acquired on a BD FACSCanto II or LSRII flow cytometer (FlowJo software, version 7.6.4, Tree Star).

Cell Lines

U937 (catalog # CRL-1593.2) and K562 (catalog # CCL-243) were purchased from ATCC. Passages were limited to maximum of 5 for cell lines used in the tumor lysis experiments. Frozen cell line aliquots were stored in LN freezers that were monitored for temperature control. Frozen cell line aliquots were periodically tested and consistently found to be free of Mycoplasma contamination (Mycoalert); last tested (negative) on October 12, 2020.

Allogeneic mixed leukocyte reaction

Human, DC-allostimulated T cell proliferation was tested in standard, 5-day mixed leukocyte reactions (MLRs) (DC:T cell ratio 1:30). DMSO, sirolimus (10ng/ml), pacritinib (156 nM-5 μM), or a combination of sirolimus and pacritinib was added once on day 0. T cell proliferation was determined by a colorimetric assay according to the manufacturer's instructions after 5 days of culture (CellTiter96 Aqueous One Solution Cell Proliferation Assay MTS; Promega) (6,10).

Treg Suppression Assay

Treg potency was determined using suppression assays well described by our lab (6,10). In brief, $CD4^+$, $CD25^+$ Tregs were isolated from healthy human donor leukocyte concentrates by magnetic bead purification. The Tregs were expanded with CD3/CD28 beads (Treg:bead ratio 1:1) for 5 days and supplemented with IL-2. DMSO (0.1%), pacritinib (1.25μM), sirolimus (10ng/ml), or PAC/SIR was only added to the initial culture to expand the Tregs. No drugs were added to the Treg suppression assay medium. Purified Tregs were titrated against alloMLRs consisting of 5×10^4 responder T cells from the Treg donor and 1.6×10^3 allogeneic monocyte-derived DCs and cultured for 5 days. Conventional, alloreactive T cell (Tconv) proliferation was measured by Ki-67 expression using flow cytometry.

Xenogeneic GVHD model

Recipient NSG mice received 25×10^6 fresh human PBMCs (Memorial Blood Center) once on day 0 of the transplant (6). As indicated, mice either received DMSO vehicle

control, pacritinib 50mg/kg, S3I-201 2.5mg/kg, sirolimus 0.5mg/kg, PAC/SIR, or S3I/SIR from day 0 until day +14. Each independent experiment was performed with a different human PBMC donor. Mice were monitored for GVHD clinical scores and premoribund status. Where indicated, short-term experiments were completed on day +14 via humane euthanasia to evaluate GVHD target organ pathology and the content of human T cell subsets within the murine spleens. To test the effect of dual JAK2 or STAT3 plus mTOR blockade on donor T cell–mediated responses against U937 leukemia cells, mice were transplanted with 25×10^6 human PBMCs and received an inoculum of irradiated U937 cells (1×10^7) on day 0 and +7 (6). Control mice received PBMCs alone without tumor. Mice were treated with vehicle, pacritinib, S3I-201, sirolimus, pacritinib plus sirolimus, or S3I-201 plus sirolimus for 2 weeks as described. On day +14, the mice were humanely euthanized, and the spleens were harvested. Human $CD8^+$ T cells within the spleens were purified by magnetic beads and cocultured with fresh U937 cells at varying T cell to Target ratios for 4 hours. Tumor lysis assays were performed in vitro using a colorimetric assay (6,10).