In vivo IL-12/IL-23p40 neutralization blocks Th1/Th17 response after allogeneic hematopoietic cell transplantation

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Supplemental Data

Supplemental table 1: Summary of severe adverse events according to study arm

Ustekinumab				Placebo		
SAE	grade	attribution		SAE	grade	attribution
upper GI bleed	3	unrelated		hepatic veno-occlusive disease	3	unlikely
hepatic veno-occlusive disease	3	unrelated		confusion	2	unlikely
acute cholecystitis	3	unrelated		skin infection	3	unlikely
hepatic veno-occlusive disease	3	unrelated		respiratory failure	5	unrelated
hepatic veno-occlusive disease	3	unlikely		febrile neutropenia	3	unrelated
BK virus infection	3	unrelated		hypotension	3	unrelated
hallucinations	4	unrelated		acute kidney injury	3	unrelated
				thrombotic microangiopathy	4	unrelated
				HHV6 infection (CNS)	4	possible
				acute kidney injury	4	unrelated

**Supplemental table 2:   Chronic GVHD maximum organ involvement**

|	Ustekinumab	Placebo	p-value
Skin			0.48
0	15	13	
1	0	1	
2	0	1	
3	0	0	
Mouth			1
0	11	10	
1	4	4	
2	0	1	
3	0	0	
Eye			0.26
0	9	10	
1	6	3	
2	0	2	
3	0	0	
GI			0.33
0	14	11	
1	1	3	
2	0	0	
3	0	1	
Liver			0.2
0	11	12	
1	0	2	

2	1	1	
3	3	0	
Lung			
0	15	15	
1	0	0	
2	0	0	
3	0	0	
Joint/fascia			1
0	14	15	
1	1	0	
2	0	0	
3	0	0	
Overall			0.3
0	9	8	
1	2	1	
2	1	5	
3	3	1	

Supplemental figure 1: Changes in FACT-BMT TOI by Study Arm
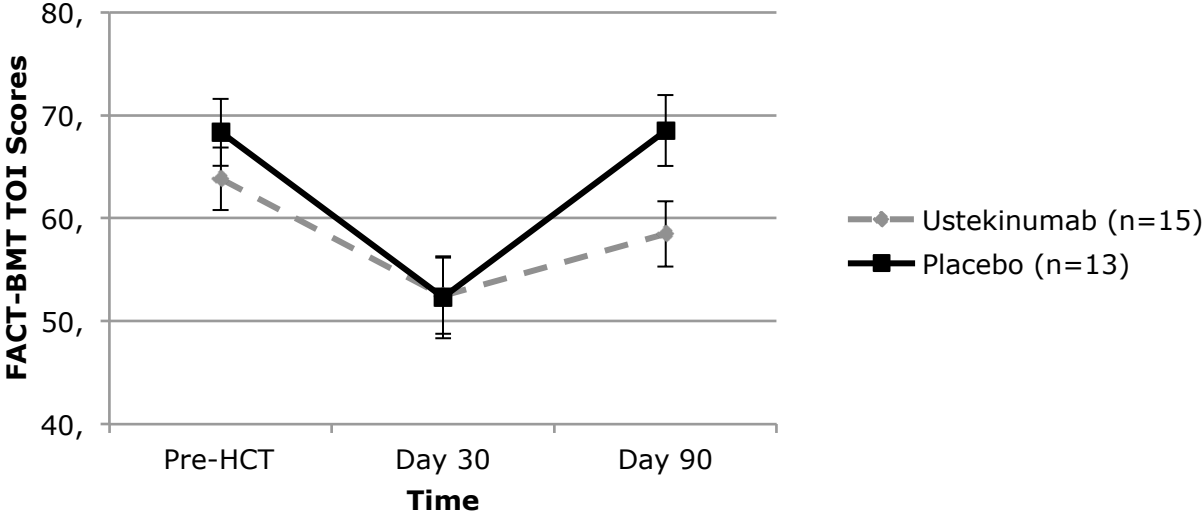
Supplemental figure 2 : Serum cytokines measured day 7 and 28 post-HCT.

IL-10

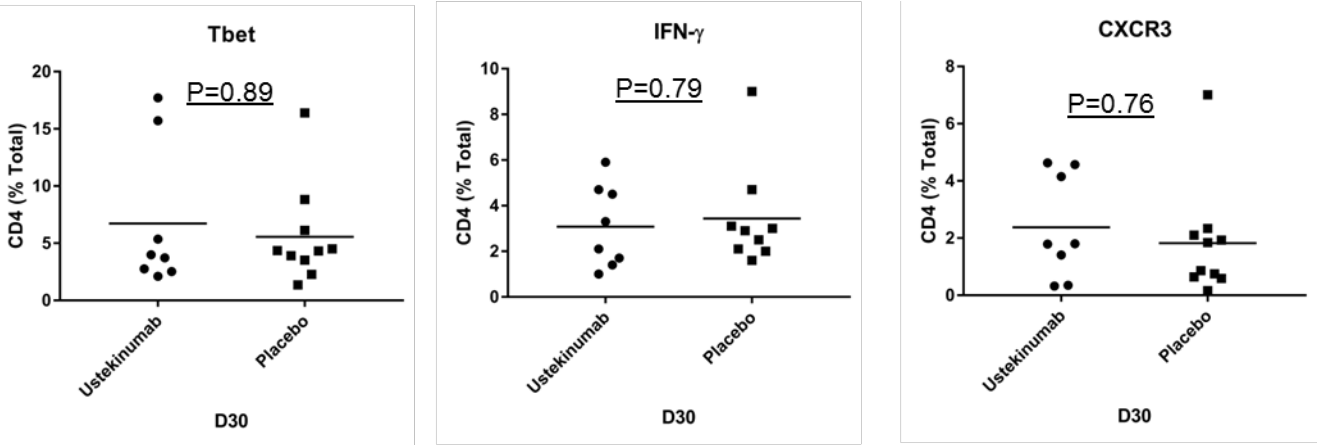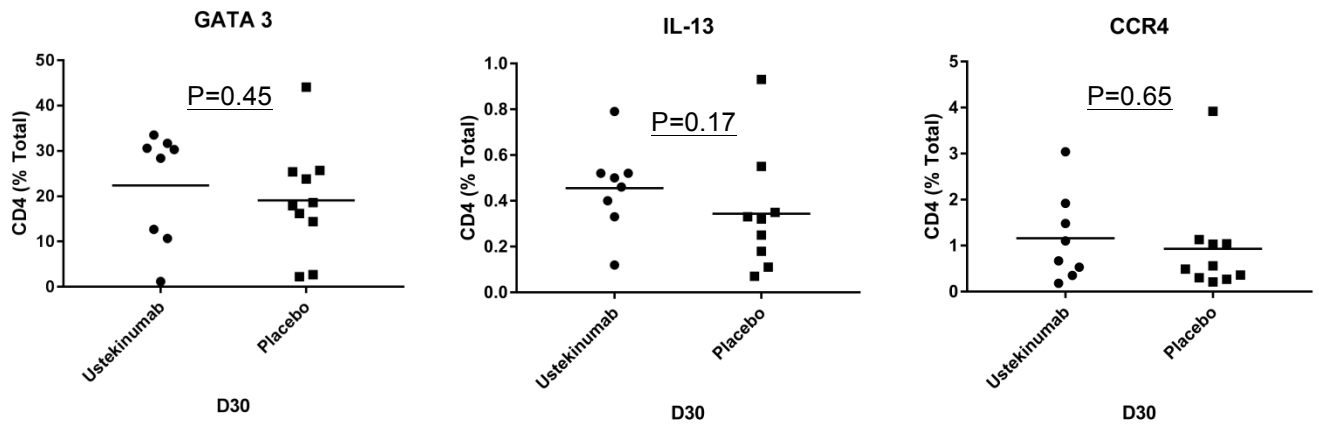**IL-12/IL-23p40**
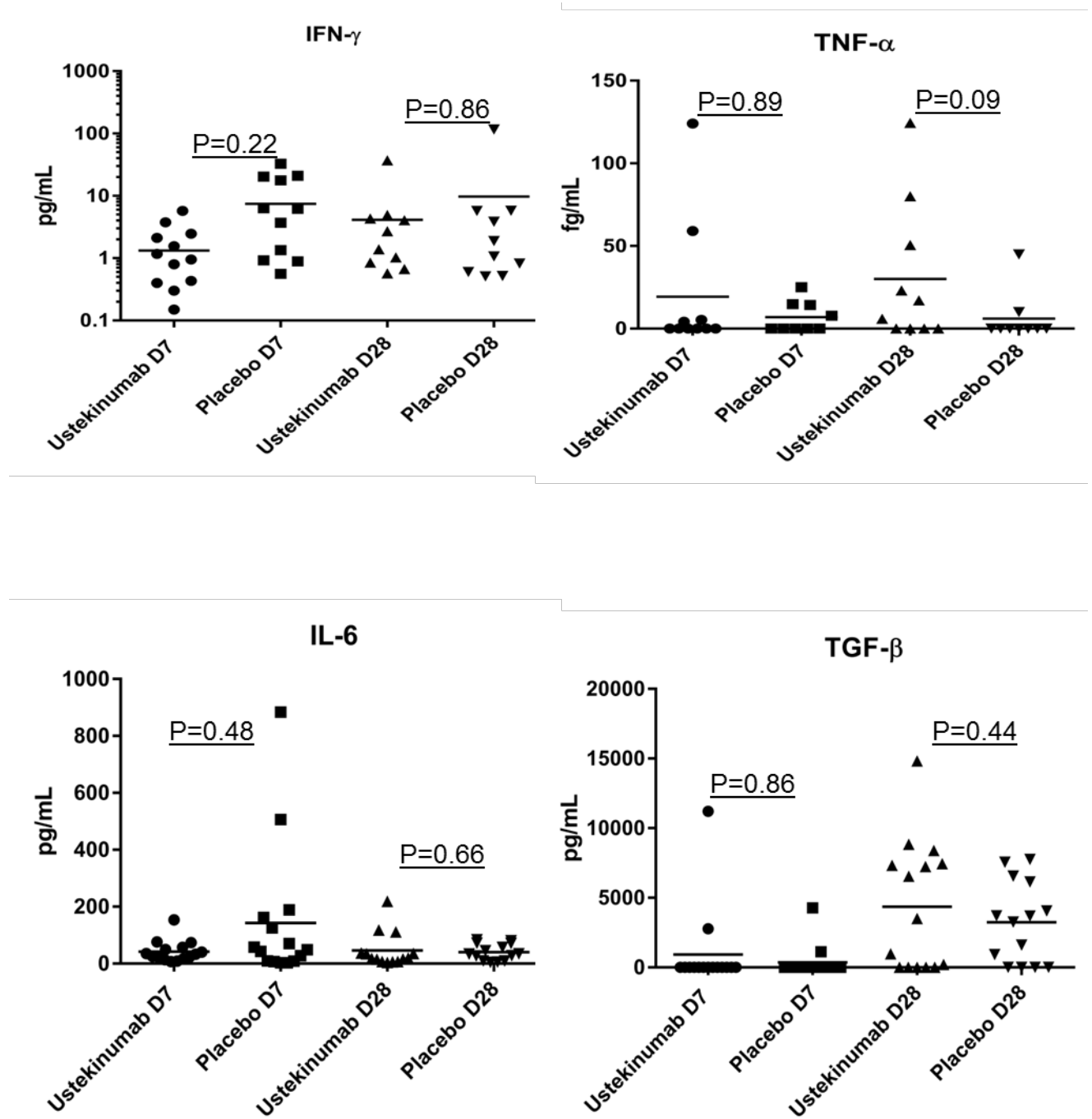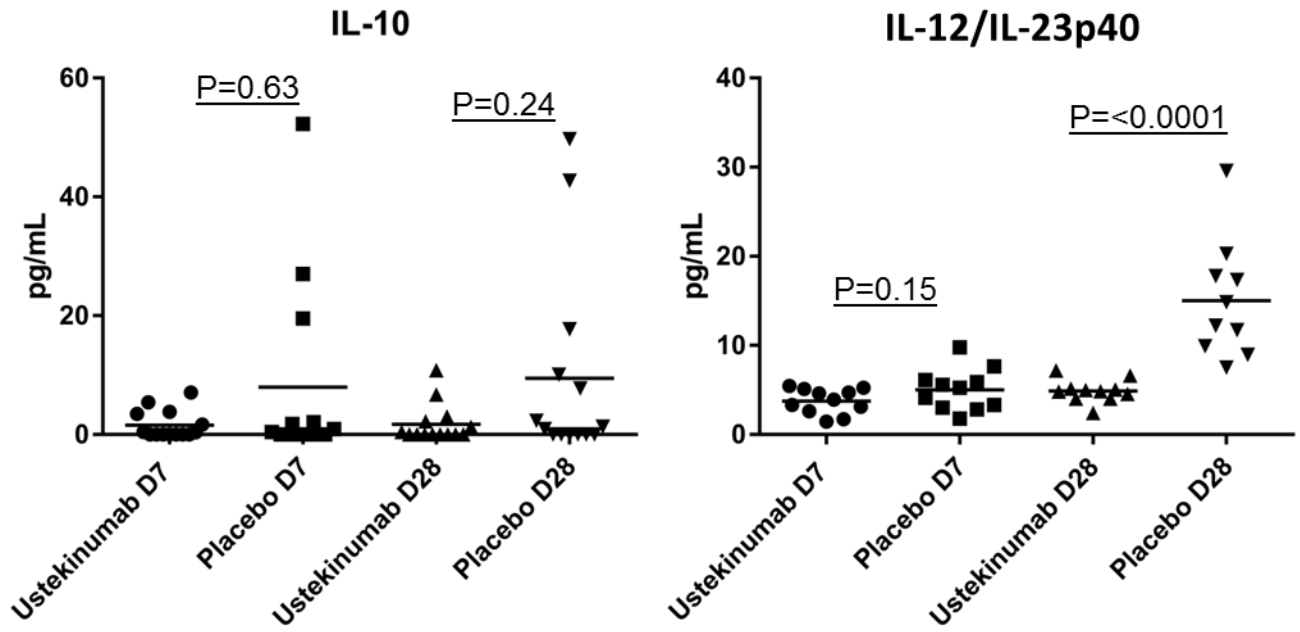
*p value for comparison by Mann-Whitney test for each.

Supplemental figure 3: (a) Th1, (b) Th2, and (c) Th17 phenotype at day 30 post-HCT.
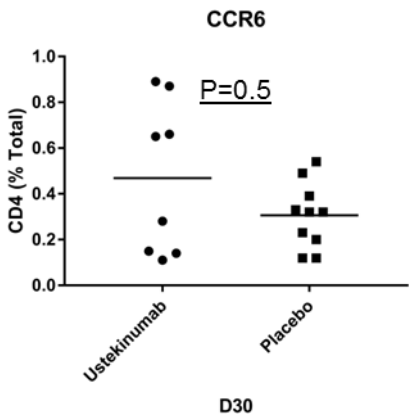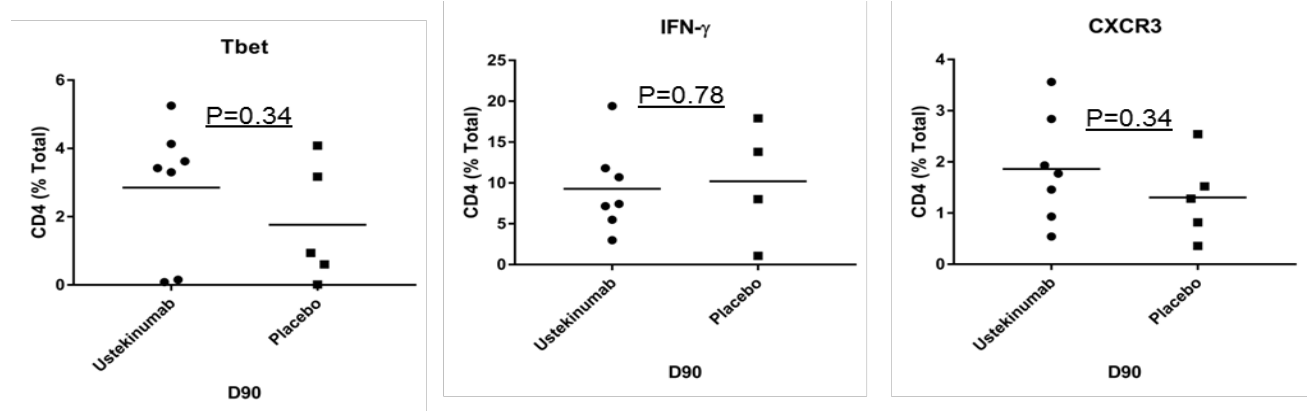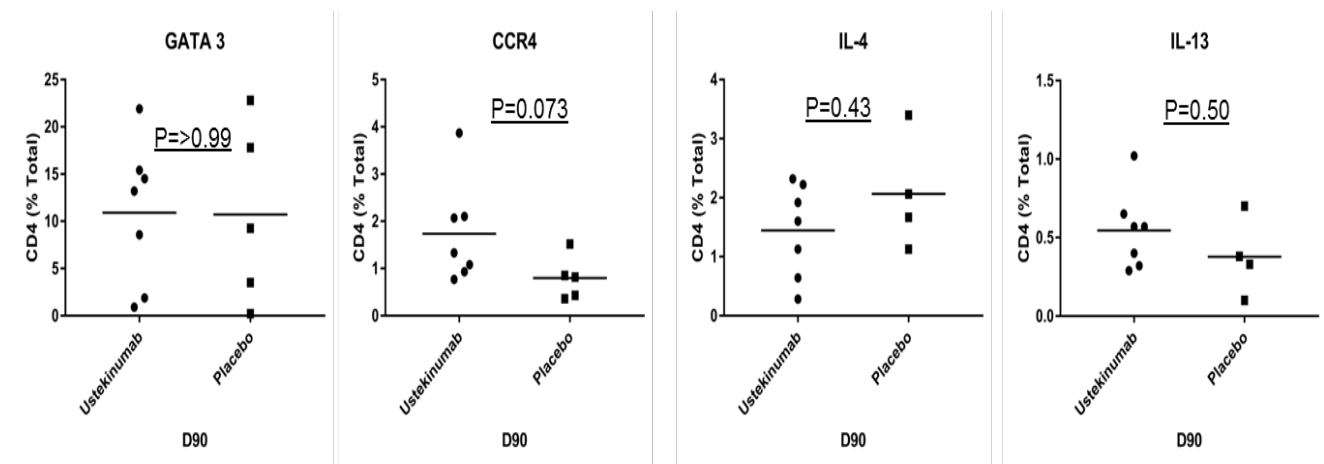
a)

b)

c)

*p value for comparison by Mann-Whitney test for each.

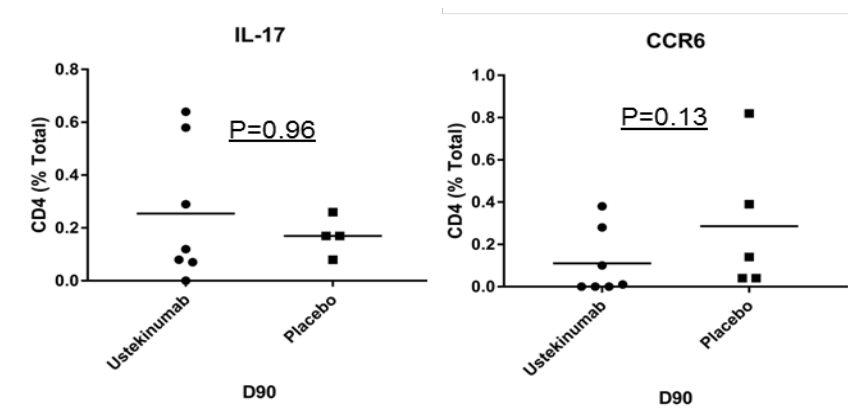**Supplemental figure 4: (a) Th1, (b) Th2, and (c) Th17 phenotype at day 90 post-HCT.**

a)

b)

c)

*p value for comparison by Mann-Whitney test for each.

Supplemental methods:

Included patients

Included patients were adults age 18-70 with hematologic malignancy or disorder requiring HCT. Additional inclusion criteria were adequate vital organ function (LVEF ≥ 45%; FEV1, FVC, and DLCO ≥ 50% of predicted values; AST, ALT < 3 times upper limit of normal values; creatinine clearance ≥ 50 cc/min) and Karnofsky performance status (≥ 60%). Exclusion criteria were uncontrolled infection, active HIV, hepatitis B or C infection, or HCT comorbidity (HCT-CI) ≥ 3.[1]

Transplantation protocol

Conditioning regimens including cyclophosphamide, busulfan targeted to an average daily AUC of greater than 5,300μM/L*min/day, and use of anti-thymocyte globulin were not permitted. Other restrictions beyond these were not placed on selection of conditioning regimen, thus these reflected commonly utilized regimens at our center (table 1). Eligible related or unrelated donors matched with the patient at HLA-A, B, C, and DRB1 by high resolution typing provided filgrastim-mobilized peripheral blood stem cells (PBSC) for transplantation per standard practices. All patients received tacrolimus and sirolimus for GVHD prophylaxis. TAC was administered according to program standards, starting day -3 as an IV formulation, then converted to oral therapy when tolerated. TAC levels were maintained at a target range of 3-7ng/mL. SIR was administered as an oral loading dose on day -1, followed by daily oral maintenance dosing to maintain levels of 5-14ng/mL. Total duration of TAC and SIR therapy was not mandated by the trial.

Trial design and treatment plan

Both HCT recipients and their respective donors provided consent for participation in the study; donors provided a single pre-mobilization research peripheral blood sample for use in the biologic correlative studies (24 of 30 HCT donors consented to this pre-mobilization research sample). Eligible patients were randomized (1:1 allocation, stratified by donor relation) to Ustekinumab vs. placebo (*NCT01713400*). Study investigators, clinical staff, and patients were blinded throughout to study arm assignment. Data were un-blinded after completion of the final analyses. Ustekinumab was delivered as a subcutaneous injection on day -1, and again on day +20 post-HCT at a dose of 45mg (for those with weight ≤ 100kg) or 90mg (weight > 100kg). The dosing schedule was based upon anticipated half-life, not based on known IL-12/23p40 cytokine levels post-HCT. Placebo was a subcutaneous injection of sterile saline of identical volume, character, and packaging as the investigational agent administered on the same schedule. Injection site reactions were not observed, and thus did not pose risk of un-blinding.

Regulatory T cell reconstitution, and frequency and cytokine profile of alloreactive donor cells

Absolute Treg count

FACS analysis was performed on lysed whole blood samples. Absolute cell counts were enumerated using BD TruCOUNT tubes (BD Biosciences, San Jose, CA USA) before, and on days 30 and 90 post-HCT. Fluorescent-conjugated CD45-FITC, CD3-PacBlue, CD4-Percp-CY5.5, CD8-APC-Cy7, CD25-PE-Cy7, CD127-Alexa647 (BD Biosciences) were added to the TruCOUNT tube, followed by 50 μl of EDTA whole blood, vortexed and incubated in dark at room temperature for 15 min. Cells were lysed by adding 450 μL of 1x BD Facs lysing solution (BD Biosciences), vortexed and incubated in dark at room temperature for 15 min. Samples were analyzed using the LSRII flow cytometer (BD Biosciences).

Treg Suppressive Function

The suppressive potency of Treg at days 30 and 90 post-HCT was determined in a mixed leukocyte culture suppression assay. Patient peripheral blood mononuclear cells (PBMC) were sorted for viable $CD4^+ CD25^+ CD127^-$ and $CD4^+ CD25^- CD127^+$ using a FACSAria II cell sorter (BD Biosciences). Treg were added at 1:1, 1:2, 1:4, 1:8 and 1:16 ratio to autologous $CD4^+CD25^-$ T responder cells ($1x10^4$) with 1:1 Human T-Activator CD3/CD28 Dynabeads (Life technologies, Grand Island, NY USA) in a 96-well, round bottom plate. Cells were cultured at 37^oC, 5% CO_2 for 3 days and pulsed with 1 μCi/well [^{3}H] thymidine for the last 18 hours. Proliferation was analyzed by [3H] thymidine incorporation using a gas scintillation counter (Matrix 96 counter; Camberra Pakard). Results are expressed in counts per minutes (CPM) of at least triplicates measurements. IC25 values are presented, representing the absolute number of Treg capable of suppressing 25% T responder cells.

Frequency and cytokine profile of alloreactive donor cells

PBMC from patients at days 30 and 90 post-HCT were co-cultured with γ-irradiated monocyte-derived DC from host before transplant, donor before transplant, and an unrelated 3rd party at a 1:1 ratio for 6 days. Monocyte-derived DCs were cultured in X-VIVO 15 serum-free medium (Lonza) for 6 days in 1000U/mL of GM-CSF (R&D Systems) and 1000U/mL of IL-4 (R&D Systems). On day 6, DC were washed and incubated with 1000U/mL of GM-CSF, 1000U/mL of IL-4 and 10ng/mL of TNF-α for 48hrs for maturation. DC are then washed and irradiated. Third party PBMC were used as controls, and PMA (50ng/mL) and Ionomycin (500ng/mL) as positive experimental control. Differences in recipient DC and third-party DC phenotype were not analyzed. After 6 days, supernatant was collected for cytokine (IL-4, IL-17, TNF-α, IL-10, TGF-β, IL-2, IL-6, IL-12p40, IL-23) analysis (Q-Plex Multiplex ELISA, Quansis Biosceinces, Logan, Utah) and IFN-γ production was read using an ELISPOT reader (BD Biosciences). Briefly, ELISPOT plates were coated with 100ml of capture antibody (BD Biosciences) at 1:200 and stored at 4^oC overnight. Plates were washed and blocked for 2 hours at room temperature. Plates were washed, 100ml responder and stimulator cells were added at $5x10^4$ cells/well, and then incubated at 37^oC, 5% CO_2 for 6 days. After incubation, supernatant was collected and plates were washed followed by addition of 100ml of detection antibody at 1:250. Plates were incubated at room temperature for two hours and washed. Then, 100ml of Enzyme Conjugate

Streptavidin-HRP at 1:100 was added, and plates were incubated at room temperature. After 1 hour, plates were washed and substrate solution was added for spot development. Spots were enumerated in an AID Elispot high Resolution Reader System (AID, San Diego, CA US).

Pharmacokinetic and pharmacodynamic studies

Pharmacokinetics

Peripheral blood samples were obtained from all patients at baseline, 24 hours after first administration of ustekinumab/placebo, weekly through day 20, 24 hours after second ustekinumab/placebo, and then weekly through day 100 post-HCT. Serum anti-IL-12p40 concentration was measured by a capture antigen ELISA: Recombinant human IL-12p40 (BD Biosciences) was diluted in coating buffer (BD Biosciences) and 100μL was added to a 96-well EIA/RIA flat bottom plate (Fisher scientific, Pittsburgh, PA). The plate was incubated at 4°C overnight then washed and blocked for two hours at room temperature and then washed. To create a standard curve, ustekinumab was diluted in PBS containing 0.5% normal human serum, and then 100μL was added to plate at 20, 10, 5, 2.5, 1.25, 0.612ng. Patient samples were diluted (to 0.5% human serum) and 100μL was added to plate, incubated for two hours at room temperature, and then washed. A secondary biotin mouse anti-human IgG antibody was diluted at 1:250 in assay diluent and added to the plate 100 μL/well. Plates were incubated at room temperature for two hours, washed, and 100 μL of Streptavidin-HRP (BD Biosciences) was added to the plate for one hour at room temperature. Plates were washed, and substrate solution (BD Biosciences) was added for 5 minutes in the dark followed by stop solution. Absorbance was determined at 450nM.

Pharmacodynamics

For all time points outlined above, levels of IL-12/IL-23p40 were analyzed by ELISA (BD Biosciences) following manufacturer's instructions. Briefly, microwells were coated with 100μL of capture antibody diluted in coating buffer, and incubated overnight at 4^{0}C. Plates were aspirated and washed 3 times, then blocked with 200μL assay diluent and incubated at room temperature for 1 hour. Plates were again aspirated and washed 3 times. Following addition of 100μL of standard or sample to corresponding wells, plates were incubated for 2 hours at room temperature, and then aspirated and washed 5 times. Working detector (Detection Ab + SAv-HRP) - 100μL - was added to each well, incubated for 1 hour at room temperature, then aspirated and washed 7 times. Then, 100μL of substrate solution was added to each well, incubated 30 minutes at room temperature in the dark, followed by 50 μL of stop solution to each well. Samples were read at 450nm within 30 minutes with λ correction 570 nm.

Clinical outcomes

Neutrophil engraftment was defined by an absolute neutrophil count over 500 per microliter of blood sustained for at least 3 days, and platelet engraftment was defined by platelet count over 20 per microliter of blood sustained for at least 7 days without transfusion support. Mucositis was graded per CTC version 4.0. Diagnosis and severity grading of thrombotic microangiopathy

(TMA) adhered to BMT Clinical Trials Network consensus.[2] Hepatic veno-occlusive disease (VOD) was diagnosed according to standard clinical criteria.[3] Acute GVHD was scored weekly from HCT to day 100 according to consensus guidelines.[4] Biopsies were obtained according to usual clinical practice, and GVHD biopsies were reviewed by Pathologists blinded to the study arm assignment. Chronic GVHD scoring used NIH Consensus Criteria for diagnosis and staging.[5] Peripheral blood sorted (CD3 and CD33) and bone marrow donor chimerism were assessed by PCR. Relapse was defined as hematologic relapse or any unplanned intervention (including withdrawal of immune suppression) to prevent progression of disease in patients with evidence (molecular, cytogenetic, flow cytometric, radiographic) of malignant disease. Non-relapse death was defined as death in continuous remission. The composite endpoint CRFS was defined as time to first event of any of the following: NIH moderate/severe chronic GVHD, relapse, or death.

Patient-reported outcomes

Quality of life was assessed with the Functional Assessment of Cancer Therapy – Bone Marrow Transplant (FACT-BMT)[6] at baseline, day 30, and day 90 post-HCT. The FACT-BMT is composed of subscales assessing physical well-being (PWB), functional well-being (FWB), social/family well-being (SWB), emotional well-being (EWB), and BMT-specific concerns (BMTS). A trial outcome index (TOI) was calculated by summing the PWB, FWB, and BMTS subscales. TOI was selected as the QOL outcome of interest due to its sensitivity to GVHD.[7,8] Consistent with previous research,[9] a difference of 5–9 points on the TOI was considered clinically meaningful.

Safety assessment

A comprehensive data and safety monitoring plan adhered to the standard procedures of the H. Lee Moffitt Cancer Center & Research Institute. The trial was conducted with the approval of the University of South Florida IRB. The study principal investigator and study team members monitored toxicity, and reported adverse and serious adverse events (scored per CTCAE version 4.0) to the IRB, the Moffitt Protocol Monitoring Committee (PMC), and FDA. Three or greater unexpected adverse events served as a toxicity stopping boundary that required PMC review. For summary of severe adverse events, see supplemental table 1.

Statistical methods

The primary endpoint of the study was the peripheral blood mean Treg/total CD4+ ratio at day 30 following HCT. The expected Treg/total CD4+ was 19% at 30 days among SIR/TAC/placebo patients based on observed data in SIR/TAC treated patients in a previous trial.[10] With 15 patients per study arm, standard deviation of 11, and type I error of 0.05, the study had 80% power to detect an increase in Treg/total CD4+ to 31% among ustekinumab-treated patients using a two-sided Mann Whitney test.

Patient baseline characteristics were summarized using descriptive statistics including mean, median, standard deviation and range for continuous measures and counts and frequencies for categorical measures. Comparisons between study arms were made by the Cochran-Mantel-

Haenszel test for categorical measures and by the two-way analysis of variance (ANOVA) or the Friedman two-way ANOVA for continuous measures including biologic correlative data (Treg/total CD4+ cells, absolute Treg, ELISPOT assay cytokine levels, serum cytokine levels) and quality of life over the study period, adjusting for the stratification variable donor type: matched sibling vs. matched unrelated donor. The difference in cumulative incidence of grade II-IV acute GVHD was estimated using the stratified Gray test.[11] Survival data was analyzed using the Kaplan-Meier method, and stratified comparisons used the log-rank test.

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