ARTICLE

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

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Haematologica 2018
Volume 103(3):531-539

ABSTRACT

T-helper 1 and T-helper 17 lymphocytes mediate acute graft-*versus*-host disease (GvHD). Interleukin 12 is critical for T-helper 1 differentiation and interleukin 23 for T-helper 17 maintenance. Interleukin 12 and 23 are heterodimeric cytokines that share the p40 subunit (IL-12/IL-23p40). In a randomized, blinded, placebo-controlled trial, we examined the biological impact and clinical outcomes following IL-12/IL-23p40 neutralization using ustekinumab. Thirty patients received peripheral blood mobilized hematopoietic cell transplantation (HCT) from HLA-matched sibling or unrelated donors, received sirolimus plus tacrolimus as GvHD prophylaxis, and were randomized to ustekinumab *versus* placebo with 1:1 allocation after stratification by donor type. The primary end point of the trial was the mean percentage (%) T-regulatory (Treg) cells on day 30 post HCT. Ustekinumab was delivered by subcutaneous injection on day -1 and day +20 after transplantation. On day 30 post transplant, no significant difference in % Treg was observed. Ustekinumab suppressed serum IL-12/IL-23p40 levels. Host-reactive donor alloresponse at days 30 and 90 after transplantation was polarized with significant reduction in IL-17 and IFN-γ production and increase in IL-4. No toxicity attributed to ustekinumab was observed. Overall survival and National Institute of Health moderate/severe chronic GvHD-free, relapse-free survival were significantly improved among ustekinumab-treated patients. No significant improvements were observed in acute or chronic GvHD, relapse, or non-relapse mortality. These data provide first evidence that IL-12/IL-23p40 neutralization can polarize donor anti-host alloresponse *in vivo* and provide initial clinical efficacy evidence to be tested in subsequent trials. (Trial registered at *clinicaltrials.gov identifier: 01713400*.)

Introduction

Differentiation of CD4+ T cells into distinct lineages [Th1, Th2, Th17, T-regulatory (Tregs)] is co-ordinated by specific cytokine programs.[1-3] IL-12 plays a key role in Th1 differentiation, and IL-23 stabilizes the Th17 phenotype. These cytokines share a common p40 subunit. Previous data have implicated Th1 and Th17 in acute graft-*versus*-host disease (GvHD) pathogenesis,[4-6] and demonstrated that regulatory Tregs control alloreactivity.[7] In experimental systems, disruption of Tbet and ROR t transcription factors,[8] or neutralization of IL-12/IL-23p40, reduces Th1 and Th17 differentiation, increases Th2 and Tregs, and reduces GvHD mortality.

Clinical translation of this work is made possible through neutralization of IL-12/IL-23p40 using the monoclonal antibody ustekinumab (Stelara, Janssen Biotech Inc.), which is approved for therapy of plaque psoriasis, psoriatic arthritis, and

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Received: April 20, 2017.
Accepted: December 6 2017.
Pre-published: December 14, 2017.

doi:10.3324/haematol.2017.171199

Check the online version for the most updated information on this article, online supplements, and information on authorship & disclosures: www.haematologica.org/content/103/3/531

Crohn disease. In humans, ustekinumab has clinical activity in several immune-mediated disorders, including steroid-refractory acute GvHD.[10] A sirolimus (SIR)-based pharmacological immune suppression program provides an ideal platform for examining the biological effect of IL-12/IL-23p40 neutralization: SIR inhibits differentiation of Th1 and Th17 cells, and promotes generation of Tregs from naïve T cells. Tregs proliferate and survive in the presence of SIR, as they do not activate the phosphatidyl inositol 3-kinase (PI3-K)/AKT pathway.[11,12]

The primary objective of the trial was to examine the biological impact of IL-12/IL-23p40 neutralization. We anticipated disruption of Th1 and Th17 with sparing of Th2 and Tregs. Secondary objectives were to determine safety of this investigational GvHD prevention approach and examine initial evidence of clinical efficacy.

Methods

See *Online Supplementary Appendix* for full details.

Inclusion criteria

Patients were aged 18-70 years with adequate vital organ function and Karnofsky performance status. Exclusion criteria were: uncontrolled infection, active HIV, hepatitis B or C infection, or

Table 1. Patient, disease, and transplantation variables.

Variable	Ustekinumab	Placebo	P
Patient age (median, range)	53 (22-69)	59 (38-69)	P=0.16
Donor age (median, range)	33 (22-72)	29 (22-66)	P=0.64
Donor:recipient sex			
F:M	4	4	P=0.88
Others	11	11	
Donor:recipient CMV			
N:N	3	5	P=0.22
N:P	7	2	
P:N	2	2	
P:P	3	6	
Disease			
ALL	3	1	P=0.67
AML	6	6	
CLL	1	2	
HD	2	1	
MDS	2	1	
MPN	0	2	
NHL	1	2	
Remission status			
CR	9	9	P=0.18
PIF	1	0	
PR	3	0	
REL	0	2	
SD	2	4	
Disease risk index[29]			
Low	2	0	P=0.22
Intermediate	12	12	
High	1	3	
Graft type			
PBSC	15	15	
BM	0	0	
Conditioning regimen			
FluBu (6.4 mg/kg)	3	1	P=0.57
FluBu (AUC 3500)	1	3	
FluBu (AUC 5300)	8	9	
FluMel	3	2	
Donor relation			
MRD	7	6	P=1
MUD	8	9	
DQB1-mismatch			
No	13	15	P=0.12
Yes	2	0	

F: female; M: male; CMV: cytomegalovirus; N: negative; P: positive; ALL: acute lymphoblastic leukemia; AML: acute myelogenous leukemia; CLL: chronic lymphocytic leukemia; HD: Hodgkin lymphoma; MDS: myelodysplastic syndrome; MPN: myeloproliferative neoplasm; NHL: non-Hodgkin lymphoma; CR: complete remission; PIF: primary induction failure; PR: partial response; REL: relapsed/refractory disease; SD: stable disease. PBSC: peripheral blood mobilized stem cells; BM: bone marrow; Flu: fludarabine; Bu: busulfan; Mel: melphalan; AUC: average daily exposure of busulfan in µMol*minute (min); MRD: matched related donor; MUD: matched unrelated donor.

hematopoietic stem cell transplantation (HCT) comorbidity (HCT-CI) ≥ 3.[13] 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 according to standard practices. All patients received tacrolimus and sirolimus for GvHD prophylaxis. The trial was conducted under the approval of the University of South Florida Institutional Review Board.

Trial design and treatment plan

Both HCT recipients and their donors provided consent for participation in the study. Eligible patients were randomized (1:1 allocation, stratified by donor relation) to ustekinumab *versus* placebo (*clinicaltrials.gov identifier: 01713400*). Study investigators, clinical staff, and patients were blinded throughout to study arm assignment. Ustekinumab was delivered as a subcutaneous injection on day -1, and again on day +20 post HCT at a dose of 45 mg (for those with weight ≤ 100 kg) or 90 mg (weight > 100 kg). Placebo was a subcutaneous injection of sterile saline of identical volume, character, and packaging as the investigational agent administered on the same schedule.

Clinical outcomes

Neutrophil engraftment was defined by an absolute neutrophil count over 500 per microliter of blood sustained for at least three days, and platelet engraftment was defined by platelet count over 20 per microliter of blood sustained for at least seven days without transfusion support. Mucositis was graded per Common Toxicity Criteria (CTC) v.4.0. Diagnosis and severity grading of thrombotic microangiopathy (TMA) adhered to Bone Marrow Transplantation Clinical Trials Network consensus.[14] Hepatic veno-occlusive disease (VOD) was diagnosed according to standard clinical criteria.[15] Acute GvHD was scored weekly from HCT to day 100 according to consensus guidelines.[16] Chronic GvHD scoring used National Institutes of Health (NIH) Consensus Criteria for diagnosis and staging.[17]

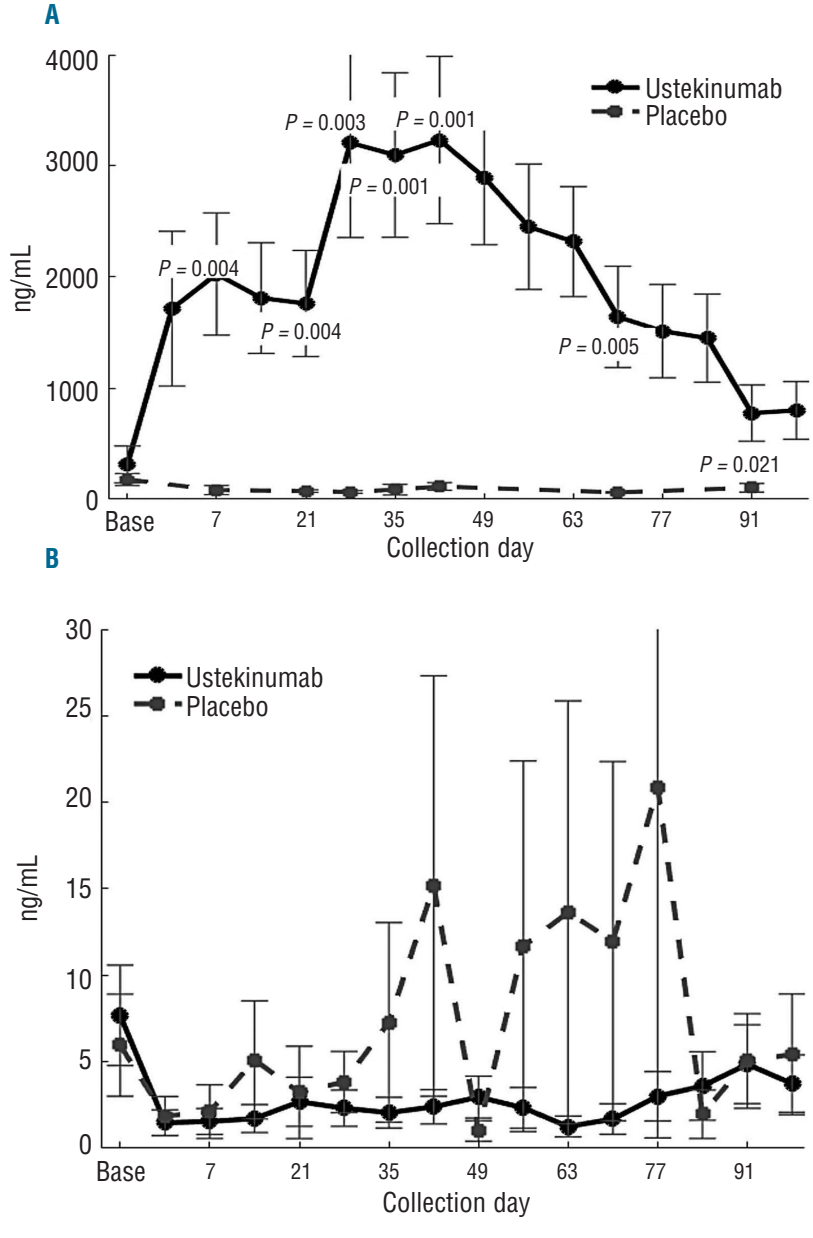

Figure 1. Pharmacokinetic and pharmacodynamics measurements. (A) Concentration of anti-IL-12/IL-23p40 antibody over time post hematopoietic stem cell transplantation (HCT). (B) Concentration of circulating IL-12/IL-23p40 over time post HCT.

Statistical analysis

The primary end point 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.[18] 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.

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 biological correlative data and Quality of Life over the study period, adjusting for the stratification variable donor type: matched sibling *versus* matched unrelated donor. The difference in cumulative incidence of grade II-IV acute GvHD was estimated using the stratified Gray test.[19] Survival data were analyzed using the Kaplan-Meier method, and stratified comparisons used the log-rank test.

Results

Patients' characteristics

Randomization resulted in a balanced distribution of patients', disease, and HCT variables (Table 1). Included patients were adults with an anticipated representation of hematologic malignancies. No significant differences were observed between groups for the studied variables.

Pharmacokinetic and pharmacodynamic studies

The mean serum concentration-time plots for ustekinumab are shown in Figure 1A. Sustained levels of ustek-

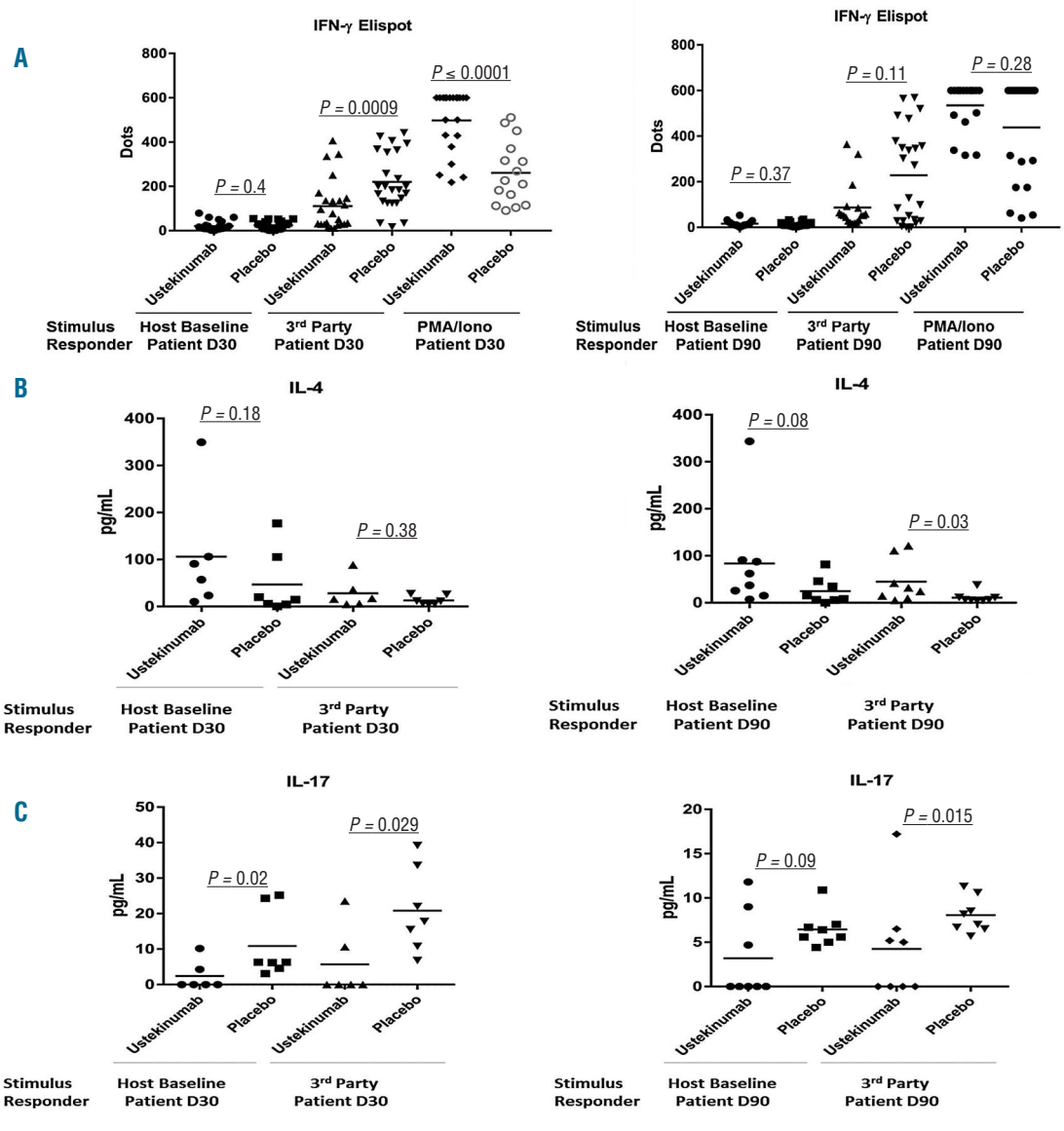

Figure 2. Donor responder cell cytokine production after stimulation with host or 3rd-party stimulators. *IFN-γ ELISPOT and supernatant cytokines (IL-4, IL-17) shown as in (A), (B) and (C). *P*-values for comparison by Mann-Whitney test.

inumab above 1 μg/mL were observed throughout the treatment period, and were associated with neutralization of circulating IL-12/IL-23p40 (Figure 1B). From day 0 to peak level post HCT, placebo-treated patients had a 14.1-fold increase in IL-12/IL-23p40, while ustekinumab-treated patients had only a 2.7-fold increase.

Donor alloreactive T-cell polarization

When stimulated with third-party alloantigen, donor T cells collected on day 30 from blood of ustekinumab-treated patients produced less IFN-γ (P=0.001) and IL-17 (P=0.03), and similar amounts of IL-4 (P=0.38) compared to donor T cells from blood of placebo-treated patients (Figure 2). Other Th2 cytokines (IL-5, IL-13) were not tested. The sensitivity to detect host-specific alloresponses was low because the transplants were from HLA compatible donors. However, lower production of IL-17 by host-specific donor T cells was also observed in ustekinumab-treated patients compared to placebo at day 30 (P=0.02). Findings on day 30 and day 90 samples were similar (Figure 2). IFN-γ production in response to PMA/ionomycin was higher among the ustekinumab group at day 30, yet similar at day 90. Among other tested supernatant cytokines, there were no significant differences in TNF-α, TGF-β, IL-10, IL-2, IL-6, IL-12p40, or IL-23. We did not observe significant differences in Th1/Th2/Th17 phenotype by flow cytometry at days 30 and 90 post HCT, and did not detect significant differences in serum cytokines at days 7 and 28 post HCT, except a significant reduction in IL-12/IL-23p40 in the ustekinumab-treated group at day 28 (*Online Supplementary Figures S2-S4*). No significant differences in gene expression were observed among peripheral blood mononuclear cells assayed with the NanoString nCounter GX Human Immunology V2 Kit (*data not shown*).

Regulatory T-cell frequency and suppressive function

There was no significant difference in Treg/total CD4 at day 30 (ustekinumab: median 13, range 2-22; placebo: median 11, range 3-20), both without (P=0.4) and with (P=0.4) adjustment for donor type (Figure 3). There was also no significant difference in absolute number of Treg or Treg suppressive function between groups (Figure 3).

Engraftment and early toxicity

There were no significant differences in neutrophil (P=0.2) or platelet (P=0.5) engraftment between the ustekinumab- and placebo-treated patients, and all cases achieved these milestones promptly. No differences in thrombotic microangiopathy (TMA) (n=1 for each arm), TMA grade, or median time to TMA onset (48 *vs.* 62 days) were observed. No difference was observed in hepatic VOD (n=3 *vs.* n=2), VOD severity (moderate in all cases), or median time to VOD onset (28 *vs.* 31 days). In all cases, the syndrome resolved without intervention beyond diuresis, and no deaths occurred from VOD.

Acute and chronic GvHD

Acute GvHD organ staging and overall grade are presented in Table 2. The day 100 cumulative incidence of grade II-IV acute GvHD was 33% for the ustekinumab arm *versus* 40% for the placebo arm (Figure 4A). Median time to acute GvHD onset was significantly longer among ustekinumab-treated patients compared to placebo (56 days *vs.* 28 days; P=0.02). Examining validated serum biomarkers (REG3α, ST2) of lethal GvHD and non-relapse mortality (NRM) at day 7 post HCT (analyzed by ELISA using methods previously described),[21,22] we found ustekinumab-treated subjects had significantly lower REG3α compared to the placebo group [median: 55 (range 8-234) *vs.* 135 (21-387) ng/mL; P=0.014]; ST2 was not significantly different [median: 11,817 (range 3493-40,898) *vs.* 19,923 (range 3791-97,662) pg/mL; P=0.68] (Figure 5). There was no significant difference in cumulative incidence of any grade chronic GvHD or NIH moderate/severe chronic GvHD (Figure 4B) between groups. In addition, no significant differences were observed between groups for individual organ involvement and severity or overall NIH 0-3

Table 2. Acute graft-*versus*-host disease (GvHD) organ staging and overall grade.

	Study arm		
Overall acute GvHD grade	Ustekinumab	Placebo	P=0.51
0	9	6	
I	1	3	
II	5	5	
III	0	1	
Skin stage			
0	12	8	P=0.35
1	1	4	
2	0	1	
3	2	2	
GI stage			
0	12	10	P=0.57
1	3	4	
2	0	1	
Liver stage			
0	15	15	

GI: gastrointestinal tract.

score at both chronic GvHD onset and maximal severity (*Online Supplementary Table S2*).

Infectious complications

Standard institutional infectious prophylaxis and monitoring was performed in all cases. There was no significant difference between ustekinumab and placebo groups for time from HCT to first systemic infection (median 27 *vs.* 26 days; *P*=0.7), total number of infections (n=14 *vs.* n=10; *P*=0.9), infection sites or organisms identified, or infection density (defined as infections per survival time, median 0.21 *vs.* 0.22; *P*=0.8). There was no significant difference in number with cytomegalovirus (CMV) reactivation more than 1000 copies (n=2 *vs.* n=1) and median time to CMV reactivation (57 *vs.* 24 days; *P*=0.3), Epstein-Barr virus (EBV) reactivation more than 1000 copies (n=0 *vs.* n=2) and median time to EBV reactivation (45 *vs.* 80 days; *P*=1), or HHV-6 reactivation more than 1000 copies (n=1 *vs.* n=2) and median time to HHV-6 reactivation (83 *vs.* 73 days; *P*=1.00).

Patient-reported outcomes

There was no significant difference in change in Quality of Life between study arms (*P*=0.15) (*Online Supplementary Figure S1*). The Functional Assessment of Cancer Therapy-Bone Marrow Transplant (FACT-BMT) Trial Outcome Index (TOI) scores demonstrated transient worsening in both groups (*P*<0.001). Between-group comparisons at each time point revealed no significant group differences in Quality of Life pre-HCT (*P*=0.32) or 30 days post HCT (*P*=0.98), although the placebo arm demonstrated better Quality of Life at 90 days post HCT (*P*=0.04). Mean Standard of Error (SE) FACT-BMT TOI scores at 90 days post HCT were 58.47 (3.19) in the ustekinumab arm and 68.51 (3.43) in the placebo arm.

Discussion

Th1 and Th17 lymphocytes have been implicated in acute GvHD pathogenesis, and prior pre-clinical work has demonstrated that targeted disruption of key lineage-defining transcription factors (Tbet, RORy) or IL-12/IL-23p40 cytokine neutralization polarizes T-cell differentiation and mitigates GvHD lethality. In an original translational effort, targeted IL-12/IL-23p40 neutralization was tested in a blinded randomized trial to discern the biological impact of this intervention and to examine the clinical safety and initial efficacy in acute GvHD prevention. With the examined dose and schedule of ustekinumab delivered, IL-12/IL-23p40 was neutralized through the early post-HCT period *in vivo*. Consistent with murine experimental data, donor alloresponse was polarized. Specifically, IFN-y production was significantly reduced among ustekinumab-treated patients in response to third-party stimulus at day 30, while response to HLA-matched host was low, as expected. IL-4

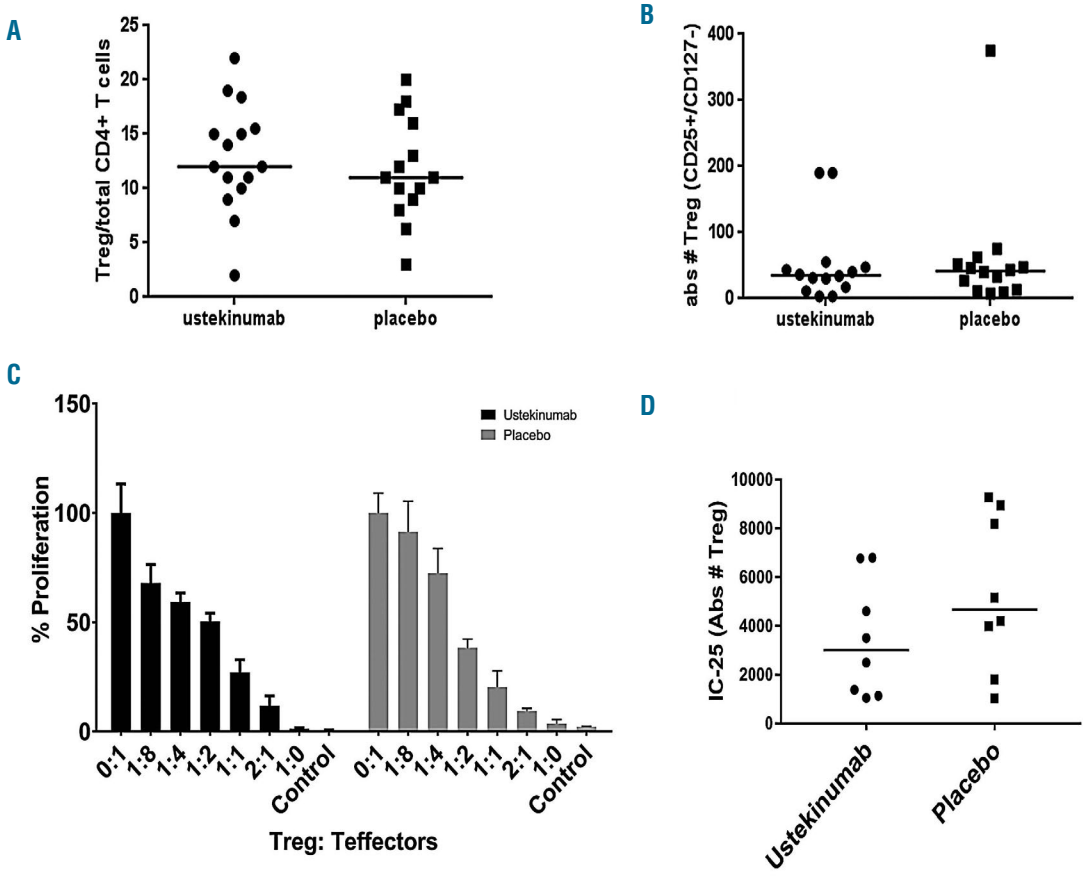

Figure 3. Regulatory T cell (Treg) numbers and suppressive function at 30 days post HCT. (A) number of peripheral blood Treg/total CD4+ T cells at day 30 post HCT; (B) absolute number of Treg/μL at day 30 post-HCT. (C) Representative Treg suppression assay, demonstrating reduced proliferation of T-responder cells in presence of escalating Treg:T-effector ratio; D) absolute Treg numbers to achieve IC-25.

production was significantly increased among ustekinumab-treated patients in response to third-party stimulus at day 90. IL-17 production was decreased among ustekinumab-treated patients in response to host at day 30, and to third-party at both days 30 and 90. These findings were likely detected due to the sensitivity of this functional assay, in contrast to that of circulating serum cytokine or peripheral blood phenotype assays.

In contrast, this intervention failed to increase peripheral blood Treg. This could be due to several factors. In contrast to murine models, patients in both arms of this trial received sirolimus and tacrolimus throughout. The effect of this pharmacological immune suppression platform may have exerted effects on Treg reconstitution post HCT that overwhelmed any alteration from IL-12/IL-23p40 neutralization. In addition, cytokines relevant to Treg homeostasis were not targeted by the study intervention, and IL-6 (central to Th17/Treg balance) was not targeted by this approach. While anti-IL-6 receptor antibody (tocilizumab) therapy (together with cyclosporine and methotrexate) produced a low incidence of grade II-IV acute GvHD in a previous trial, peripheral blood Tregs were similarly not altered.[23] In total, these data support the concept that targeted neutralization of IL-12/IL-23p40 or inhibition of IL-6 receptor signaling in the context of conventional pharmacological immune suppression fails to augment peripheral blood Tregs, and suggests that clinical benefit of these approaches is not dependent upon increased Tregs.

These data support an acceptable safety profile for combined IL-12/IL-23p40 neutralization among HCT recipients. Compared to the randomized placebo control in this trial, we observed no evidence of increased toxicity, impaired donor engraftment, infectious morbidity, or death. In contrast to particular infectious risks observed among patients with genetic deficiency in IL-12/IL-23p40 or IL-12Rβ,[1,24] no cases of Mycobacterium tuberculosis or Salmonella were seen, and all *Candida albicans* infections (oral n=4, esophageal n=1) on trial were seen among the placebo arm. In addition, there was no evidence that IL-12/23p40 depletion increased disease relapse. This finding is important, given the relevance of IL-12 to natural killer (NK) and $CD8^+$ cytotoxic T lymphocytes and tumor control,[25,26] and anti-tumor effects of IL-23 (although tumor-promoting effects of IL-23 have been demonstrated).[24,27] A recent analysis of 3117 patients (with 8998 person-years of follow up) from 4 major randomized phase II and III ustekinumab trials supports the overall safety profile of this therapy with no evidence for opportunistic infections, or increased rates of malignancies or mortality above those of the general US population.[28]

The trial was not powered for clinical end points, and provides only initial estimates to be formally tested in a subsequent randomized trial. Specific analysis of acute GvHD target-organ differences is restricted by limited (skin) or no (liver) involvement of sites other than gas-

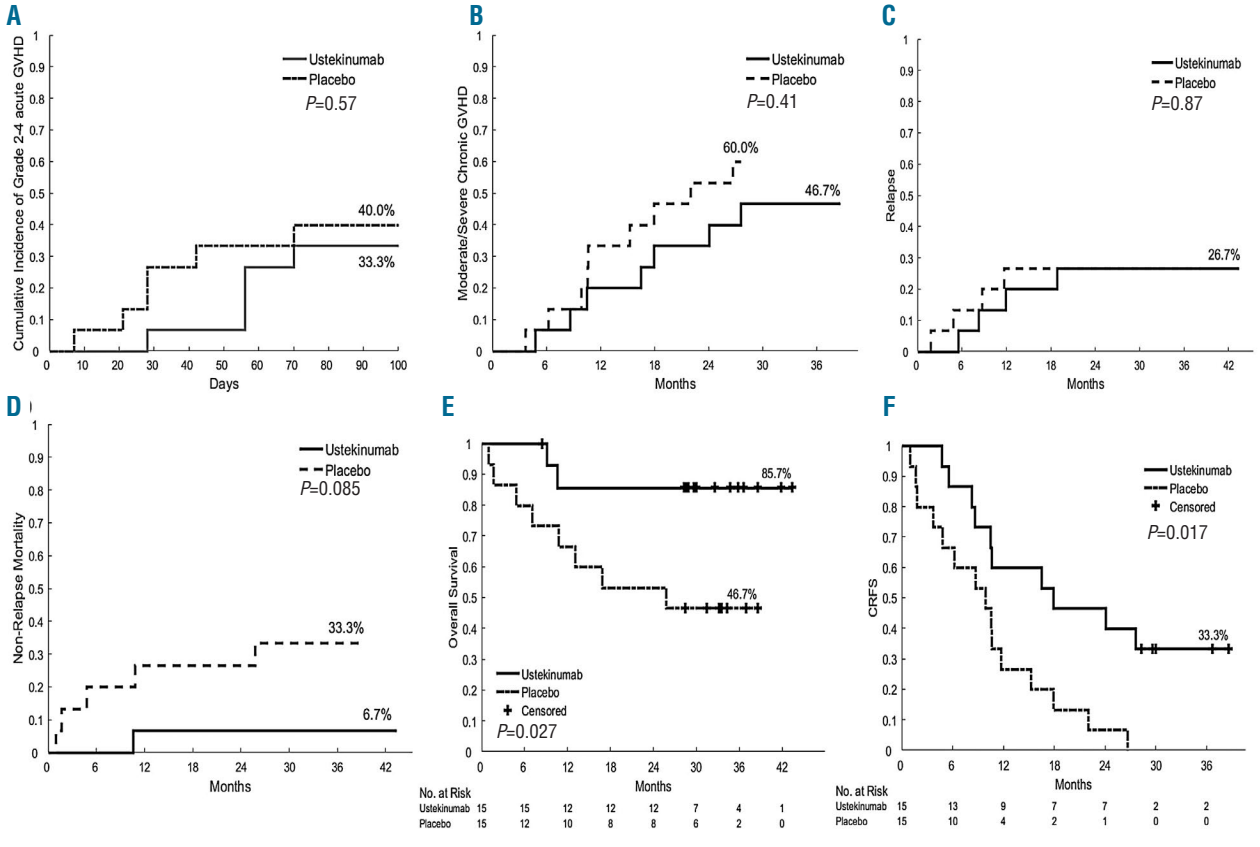

Figure 4. Comparison of clinical outcomes for ustekinumab *versus* placebo-treated subjects. (A) Cumulative incidence of grade II-IV acute graft-*versus*-host disease (GvHD). (B) Cumulative incidence of Naitonal Institutes of Health (NIH) moderate/severe chronic GvHD. (C) Cumulative incidence of malignancy relapse. (D) Cumulative incidence of non-relapse mortality. (E) Overall survival. (F) NIH moderate/severe chronic GvHD-, relapse-free survival (Conditional Random Fields Score, CRFS). **P*-value for comparisons of cumulative incidence by Gray method; *P*-value for survival plots by log-rank test.

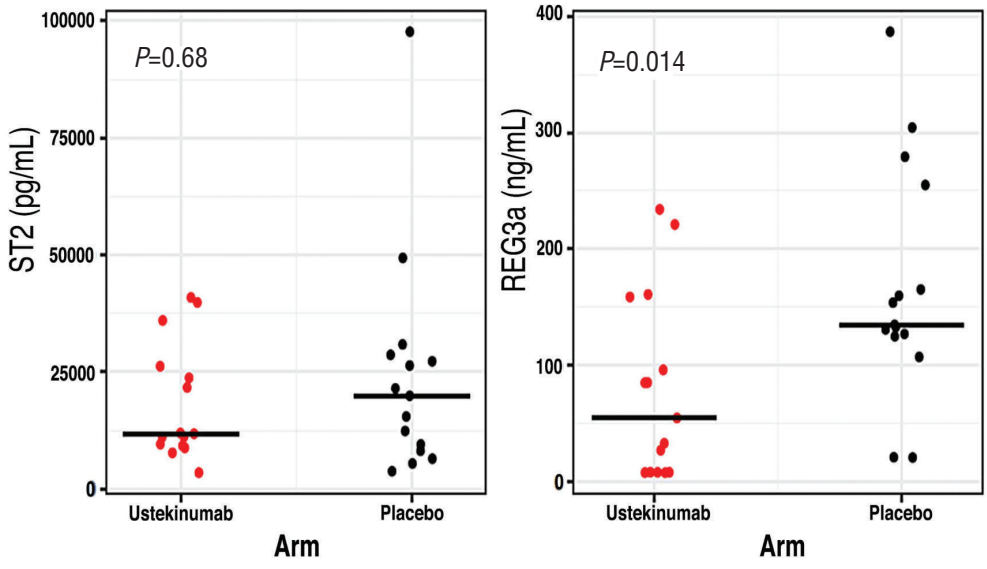

Figure 5. Serum concentration of ST2 and REG3α at day 7 post-HCT.

trointestinal tract (GI). REG3α was significantly reduced in the ustekinumab-treated subjects at day 7 post HCT, suggesting that neutralization of IL-12/IL-23p40 may ameliorate early damage to the GI tract that ultimately cascades into GvHD lethality. This finding also requires confirmation in a subsequent trial. While there are conflicting data regarding the relative contribution of Th2 (*vs.* other Th subsets) to chronic GvHD development, our study demonstrates no evidence of worsened chronic GvHD after IL-12/IL-23p40 neutralization.

We acknowledge as a limitation that IL-12/IL-23p40 neutralization together with sirolimus/tacrolimus did not offer patients complete protection from acute GvHD. Additional dosing of ustekinumab beyond the studied approach may offer benefit, as anti-IL-12/IL-23p40 antibody levels declined and IL-12/IL-23p40 cytokine levels increased in the range of 50-60 days post HCT onward. Future trials could incorporate prolonged maintenance dosing modeled after approved maintenance therapy in psoriasis and Crohn disease.

Funding

We acknowledge the following funding support: Gateway for Cancer Research (to JP) G12-900 (inclusive of trial costs and study drug costs), American Cancer Society MRSG-11-149-01-LIB, Moffitt Cancer Center Support Grant P30 CA076292 (flow cytometry, analytical pharmacology, biostatistics, and analytic microscopy cores).

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