

IL-2 promotes early Treg reconstitution after allogeneic hematopoietic cell transplantation

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Received: July 19, 2016.

Accepted: January 18, 2017.

Pre-published: January 19, 2017.

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SUPPLEMENTAL METHODS:

Eligibility Criteria

Five patients failed screening and were not enrolled or treated with IL-2 due to the following reasons: death during vital organ testing, lack of donor, cardiac ejection fraction not documented, or withdrew consent. Inclusion was limited to patients >18 years old with an ejection fraction $\geq 45\%$; forced vital capacity, forced expiratory volume in 1 second, and carbon dioxide diffusion capacity $\geq 50\%$ of predicted values; aspartate and alanine aminotransferase levels <2 times the upper limit of normal; creatinine clearance ≥ 50 cc/min; and a Karnofsky Performance Status $\geq 80\%$. Acute myeloid or lymphoblastic leukemia required <5% marrow blasts, no peripheral blasts, >20% marrow cellularity, and a peripheral absolute neutrophil count of >1000/ μ L. Myelodysplasia, chronic myeloid leukemia, and myeloproliferative neoplasms were included if marrow and peripheral blood blasts were <5%. Patients with an active infection, human immunodeficiency virus, hepatitis B or C infection, known hypersensitivity to IL-2, or an HCT-CI score of >4 were excluded. Chronic lymphocytic leukemia, Hodgkin lymphoma, and non-Hodgkin lymphoma were excluded as these malignancies may express the IL-2 receptor.

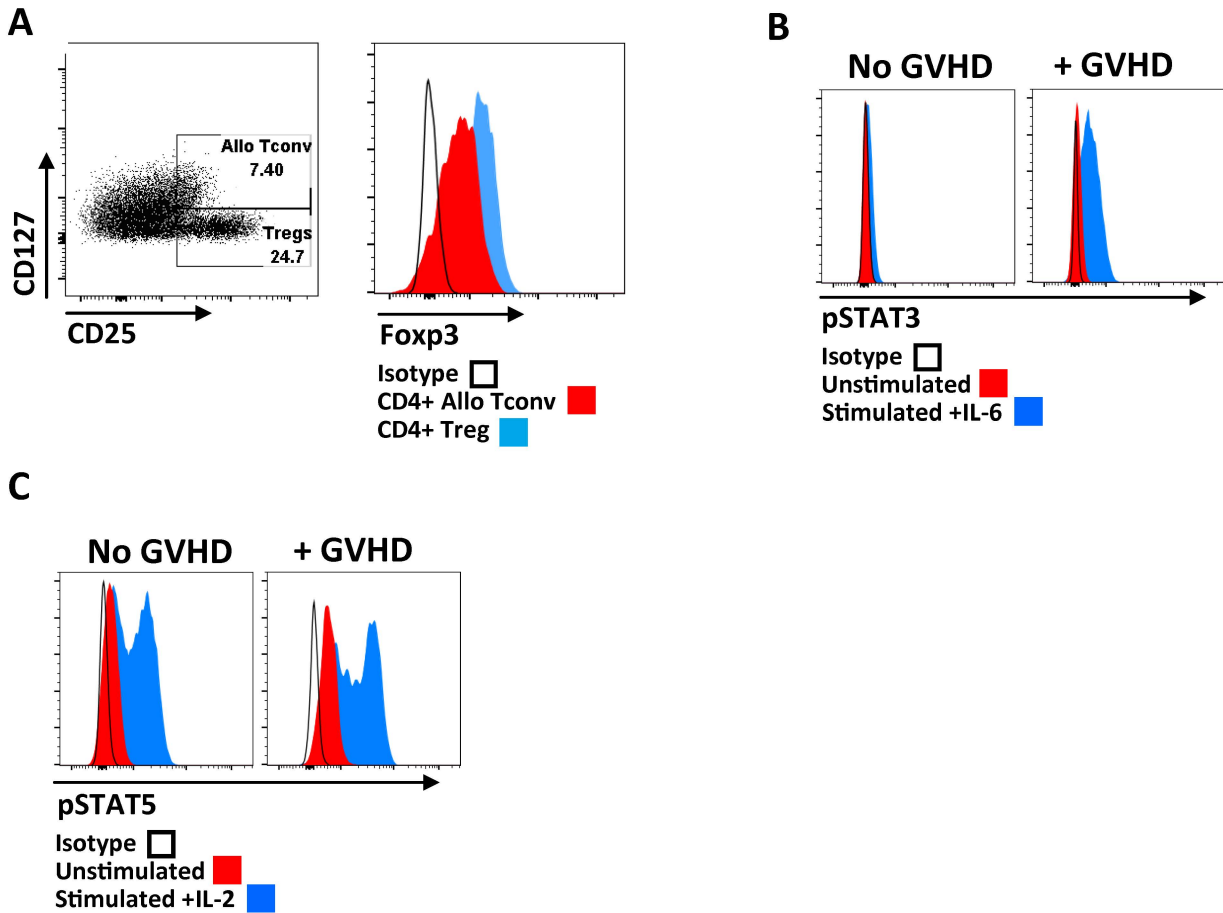
Monoclonal antibodies and flow cytometry

Fluorochrome-conjugated mouse anti-human monoclonal antibodies included anti-CD3, CD4, CD25, CD127, -Foxp3, phosphorylated STAT3 Y705, phosphorylated STAT5 Y694, and phosphorylated S6 (BD Biosciences, San Jose, CA; eBioscience San Jose, CA; Cell Signaling Technology, Boston, MA). LIVE/DEAD Fixable Yellow Dead Cell Stain (Life Technologies, Grand Island, NY) determine viability. Live events were acquired on a FACSCanto (FlowJo

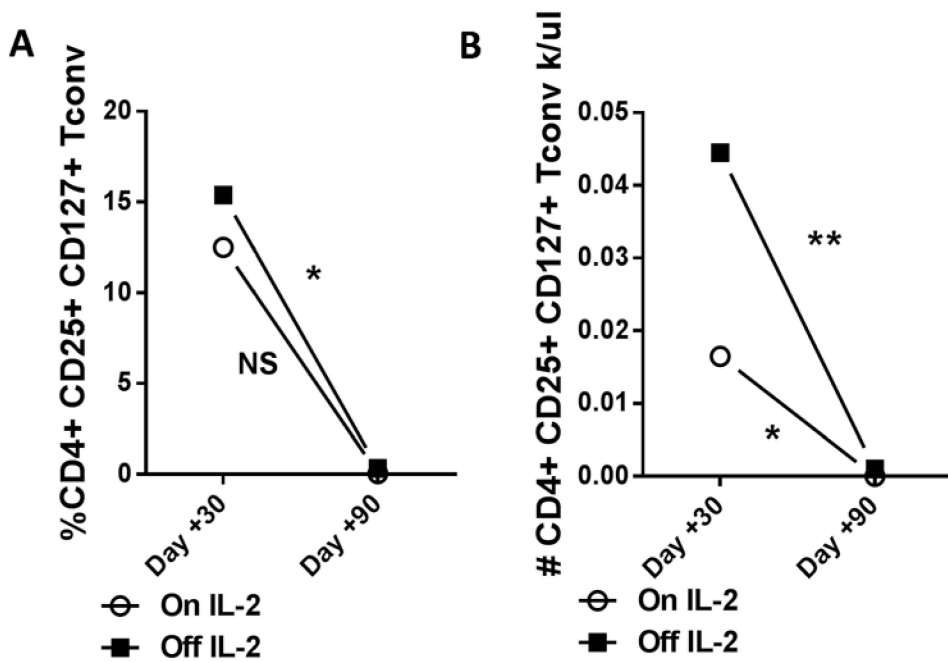
software, v10; TreeStar, Ashland, OR). The acquisition of flow data was batched when possible. Rainbow beads (Spherotech, Lake Forest, IL) were used to uniformly calibrate the flow cytometer prior to each acquisition.

CD4+ T-cell STAT3, STAT5, and S6 protein phosphorylation

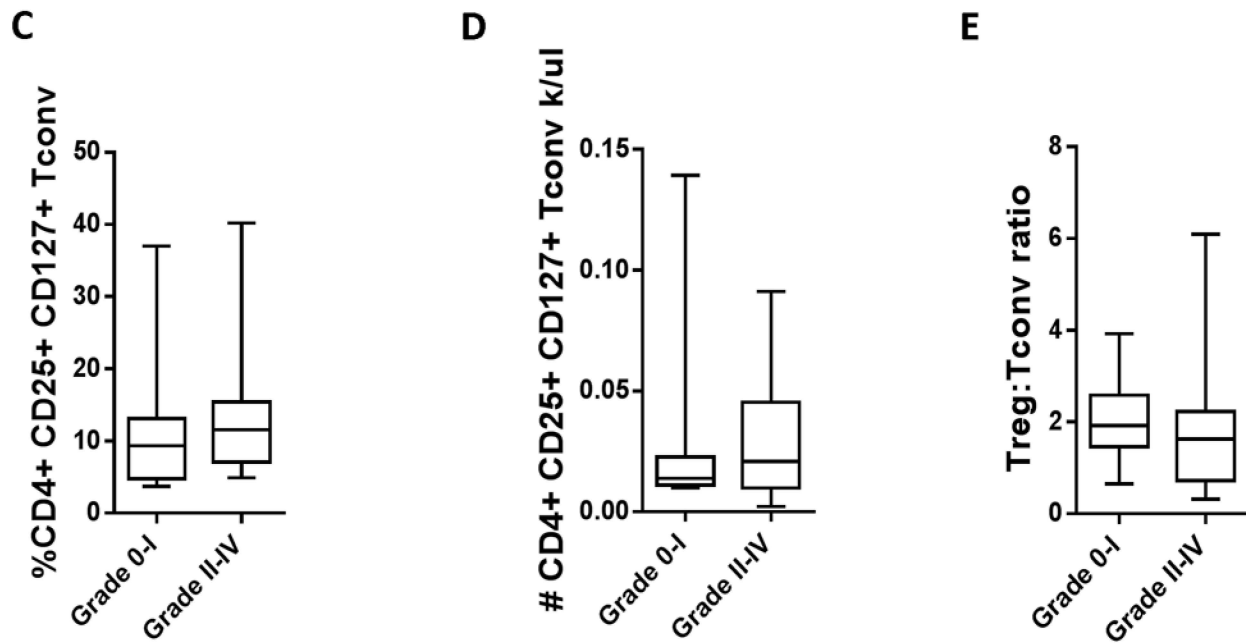
Pretransplant, day +30, and day +90 STAT3 and STAT5 phosphorylated CD4+ T-cell staining was performed using in vitro stimulation with IL-6 or IL-2 respectively (Supplemental Figure 1B,C)^{4,16}. S6 phosphorylation was also measured among CD4+ T-cells to evaluate mTOR inhibition by SIR. The staining protocol maximizes the resolution of phosphorylation by flow cytometry but degrades the CD25 and CD127 surface epitopes, precluding direct measurement of phosphoproteins in Tregs and non-Treg subsets²⁶.



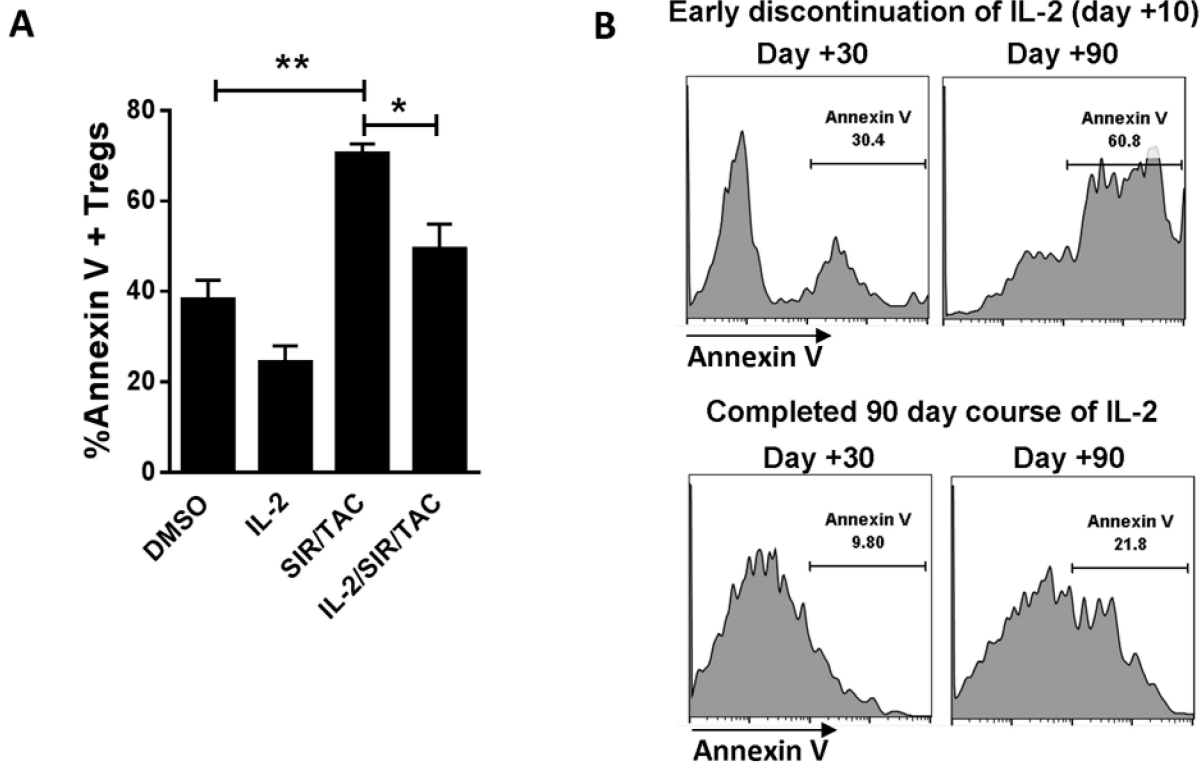
Supplemental Figure 1: Representative data for Treg, activated Tconv, and CD4+ T-cell STAT phosphorylation. A) representative dot plot shows gating strategy for CD4+ Treg versus allo Tconv. Treg are CD4+, CD25+, and CD127-. Activated Tconv are CD4+, CD25+, and CD127+. Histograms show differential expression of Fcγ3 among the CD4+ Tregs (blue) and activated Tconv (red). B) representative histograms show expression of pSTAT3 gating on CD4+ T-cells at day +30 in patients who did or did not develop grade II-IV acute GVHD by day +100. Isotype (black line), unstimulated (red), and IL-6 stimulated (blue) histograms are shown. C) representative histograms show expression of pSTAT5 gating on CD4+ T-cells at day +30 in patients who did or did not develop grade II-IV acute GVHD by day +100. Isotype (black line), unstimulated (red), and IL-2 stimulated (blue) histograms are shown.



Supplemental Figure 2. A,B) bar graphs show mean CD4+, CD25+, CD127+ activated Tconv (% and absolute #, \pm SE) for those receiving IL-2 or off cytokine at days +30 and +90, Mann-Whitney test. *P < 0.05, **P = 0.001–0.01. NS = not significant.



Supplemental Figure 2. C-E) Box and whisker plots show median (min to max) CD4+, CD25+, CD127+ activated Tconv or the Treg:Tconv ratio at day +30 among SIR/TAC-treated patients who developed acute GVHD or not by day +100. Data is based on our previously published trial (NCT00803010)(6).



Supplemental Figure 3. A) Healthy donor T-cells were cultured with cytokine-matured, allogeneic dendritic cells (DC:T ratio 1:30) for 6 days with DMSO, IL-2 (20IU/ml), or sirolimus (10ng/ml) plus tacrolimus (5ng/ml) alone or with IL-2. Sirolimus and tacrolimus (or DMSO vehicle) were added once on day 0 of the culture. IL-2 was replenished on days +2 and +4. The T-cells were harvested on day +6 and surface stained for CD4, CD25, and CD127. LIVE/DEAD Fixable Yellow Dead Cell Stain (Life Technologies, Grand Island, NY) and Annexin-V (Thermo-Fisher, Waltham, MA) staining were performed to identify apoptotic cells. Tregs were identified as CD4+, CD127-, and CD25+ by flow cytometry as described in the manuscript text. Bar graph shows the amount (mean and SD) of apoptotic Tregs. 1 representative experiment of 3, all performed in triplicate, is shown, paired t-test. B) Representative histograms show the amount of Annexin-V per Treg gate among a patient who stopped IL-2 early and a patient that completed the full course of IL-2, at days +30 and +90. *P < 0.05, **P = 0.001—0.01.