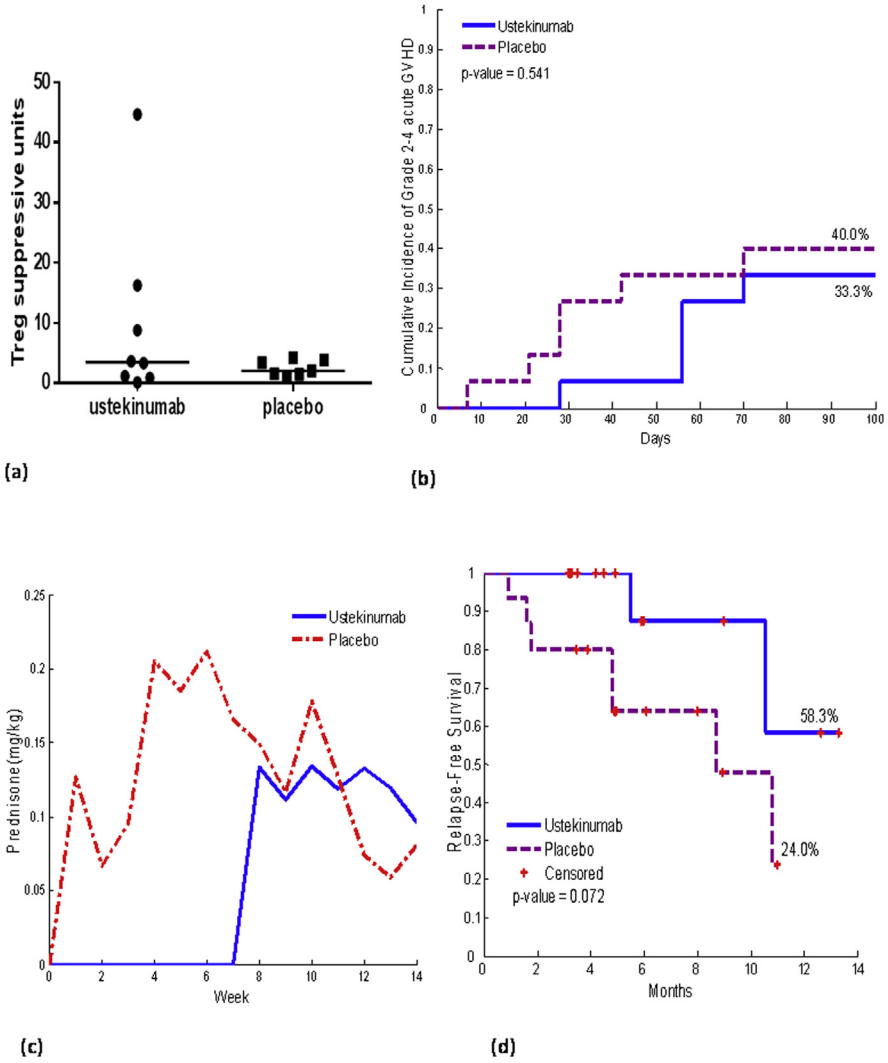

Figure 1. (a) Treg suppressive units, defined as (absolute Treg)/(absolute Treg capable of achieving 50% suppression of CD4+CD25- T responders with CD3/CD28 stimulus) at day 30 post-HCT, (b) cumulative incidence of grade II-IV acute GVHD, (c) mean weekly prednisone dose/kg recipient body weight among patients without death or malignancy relapse, and (d) relapse-free survival from time of HCT

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CD3/CD28 Co-Stimulated Activation of PI3K-mTORC2-AKT Metabolic Programming Augments Granzyme B Expression and Direct Cytotoxicity in Expanded Human iNKT Cells

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Invariant natural killer T (iNKT) lymphocytes are rare but potent effectors of immune regulation and tumor immunosurveillance. iNKT cells are MHC-non-restricted and thus ideal candidates for separation of GVHD and GVT after either MHC-matched or mismatched allogeneic hematopoietic cell transplantation (HCT). We have optimized a reproducible therapeutic expansion protocol for human iNKT cells from peripheral blood pheresis units and used this platform to delineate targetable pathways to augment cytokine secretion and direct cytotoxicity of expanded $CD3^+V\alpha24^+V\beta11^+$ iNKT cells against lymphoid malignancies. Expanded, anti-CD2/CD3/CD28-stimulated iNKT cells expressed significantly higher (> 1500 pg/mL) levels of Th2 cytokines IL-4, IL-5, and IL-13, and GM-CSF, and substantially higher intracellular granzyme B (GrB) and PI3-kinase downstream phospho-AKT (p-AKT) at both the mRNA and protein levels as compared to iNKT cells treated with vehicle ($P < 0.01$), α-galactosylceramide (α-GalCer) ($P < 0.01$), or anti-Vα24 CDR3 loop stimulating antibody clone 6B11 ($P < 0.01$) (n = 4-6 serial expansions). *In vitro* iNKT cytotoxicity against NALM/6 Ph+ B-lineage acute lymphoblastic leukemia (B-ALL) was significantly augmented by CD2/CD3/CD28 activation (mean ± SEM % cytotoxicity in BADTA® assay at 1:1 iNKT: target ratio 60.8 ± 2.8, $P < 0.001$, n = 12) but not α-GalCer stimulation (11.5 ± 5.0, $P = 0.98$, n = 12) or 6B11 treatment (11.0 ± 4.1, $P = 0.99$, n = 12) as compared to vehicle control. Potent augmentation of tumor clearance *in vivo* after CD2/CD3/CD28 but not α-GalCer or 6B11 stimulation was confirmed in

luciferase+ NALM/6 xenograft-bearing C.B17 SCID mice (n = 25 mice/group; $P < 0.001$). Specific GrB inhibition by iNKT pre-treatment with the non-competitive inhibitor Z-AAD-CMK abrogated CD2/CD3/CD28 augmented iNKT cytotoxicity, both *in vitro* and *in vivo*. Using permutations of CD2, CD3, and CD28 stimulation combined with specific inhibitors of the PI3K-AKT-mTORC pathway, we defined that CD3/CD28-co-stimulated, PI3K-downstream, mTORC2-driven AKT activation directly up-regulates GrB protein expression and augments GrB-dependent cytotoxicity in expanded human iNKT cells. Notably, CD2/CD3/CD28-induced augmented iNKT killing was not inhibited by cyclosporin A, rapamycin, or tacrolimus. These data demonstrate the novel potential for augmentation of cytokine secretion and cytotoxicity in human iNKT cells through AKT signaling independent of mTORC1 or NF-AT, defining a key pathway for control of iNKT effector functions with broad implications for immunotherapy, vaccine augmentation, and anti-infective therapies. As our studies utilized random donor human iNKT cells expanded using a clinically applicable protocol, these data are directly relevant to therapeutic human iNKT cell manipulation to separate GVHD and GVT in the peri-transplant or non-transplant setting of human iNKT immunotherapy.

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Non-Myeloablative TLI/ATG + Alkylator Conditioning Augments Bidirectional Immune Tolerance Via Regulatory MDSC in a Robust Murine Model of MHC-Mismatched BMT for Beta-Thalassemia

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Allogeneic hematopoietic cell transplantation (allo-HCT) for hemoglobinopathies is restricted by limited matched donors and by graft rejection and graft-versus-host disease (GVHD) after alternative donor HCT. Based upon our clinical data in aplastic anemia (Pillai *et al*, ASBMT 2011), we pre-clinically assessed non-myeloablative Total Lymphoid Irradiation (TLI) + Anti-Thymocyte Serum (ATS) +/- cyclophosphamide (CTX) vs. total body irradiation (TBI)/ATS +/- CTX in an MHC-mismatched BALB/c (H-2^d) into HW-80 (H-2^b) β-thalassemia (HW-80 β-thal$^{+/-}$) model.

Methods: Wild-type (WT) and β-thal$^{+/-}$ HW-80 mice received TLI (240 cGy x 10) + ATS (TLI/ATS, n = 10), +/- CTX (200 mg/kg) (TLI/ATS/CTX, n = 15), ATS/CTX alone (n = 10), or ATS/CTX with 1200 cGy, 600 cGy, or 300 cGy TBI (1200 T/A/C, 600 T/A/C, 300 T/A/C; n = 10 each), and 50 x 10^6 bone marrow + 60 x 10^6 splenocytes (BMT) from WT BALB/c donors. Survival, weekly weight, day 28 and day 100 tri-lineage engraftment and CBC, and histopathologic GVHD were assessed.

Results: WT HW-80 recipients of either TLI/ATS or TLI/ATS/CTX engrafted through day 100; HW-80 β-thal$^{+/-}$ mice had > 40% graft rejection after TLI/ATS + BMT, but 100% graft retention after TLI/ATS/CTX + BMT, indicating a disease-associated engraftment barrier overcome by addition of CTX to TLI/ATS. All engrafting β-thal$^{+/-}$ mice showed stable mixed hematopoietic chimerism and day 100 hematocrit (Hct) correction ($P = 0.01$ vs untreated **β-thal$^{+/-}$**). However, TLI/ATS/CTX recipients had no GVHD, whereas engrafting TLI/ATS mice developed lethal GVHD by day 100 [cumulative scores: TLI/ATS/CTX: 0.3 +/- 0.2; TLI/ATS: 6.8 +/- 0.9; $P < 0.01$]. 100% of 1200 T/A/C, 60% of 600 T/A/C, and 40% of 300 T/A/C mice engrafted. In all T/A/C groups, > 80% engrafters had lethal GVHD, whereas > 70% of 100-day survivors showed non-engraftment. We delineated that recipient regulatory MDSC induce tolerance via MHC-independent *in vivo* expansion of donor Foxp3+ nTreg after TLI/ATS + BMT (van der Merwe *et al*, J Immunol 2013). We found significant ($P < 0.001$) increase in recipient CD11b$^+$Gr-1(G)^{hi} MDSC in TLI/ATS/CTX- versus TLI/ATS-conditioned **β-thal$^{+/-}$** mice, with increased number ($P < 0.01$) and proliferation ($P < 0.05$) of donor Foxp3+ nTreg and decreased CD8+ T cell number and proliferation ($P < 0.01$) in spleens at day 6. In adoptive transfers, TLI/ATS/CTX-derived recipient CD11b$^+$Gr-1G^{hi} MDSC regulated otherwise lethal GVHD through day 100 post-BMT in 1200 cGy TBI-conditioned WT B6 recipients of WT BALB/c BMT. Our data indicate that TLI/ATS/CTX facilitates robust recipient <–> donor tolerance, provide pre-clinical support for application of TLI/ATS/CTX prior to (potentially MHC-mismatched/haploidentical) allo-HCT for hemoglobinopathies, and give the first *direct* evidence for an innate immune mechanism of alkylator-associated transplantation tolerance, which we propose to validate in the context of a clinical trial.

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In Landmark Analysis the Severity of Day 100 Acute Graft-Versus-Host Disease (aGVHD) Has No Impact on Long-Term Progression-Free Survival (PFS) after Double-Unit Cord Blood Transplantation (DCBT)

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Introduction: While DCBT has been associated with high PFS in both children & adults, rates of grade II-IV aGVHD are significant in patients transplanted with calcineurin inhibitor/ mycophenolate mofetil (CNI/MMF) immunosuppression (IS) without anti-thymocyte globulin. However, GVHD biology in DCBT recipients appears distinct from that of adult donor allografts with a high incidence of corticosteroid responsiveness & low rates of chronic GVHD. Consequently, the impact of aGVHD upon long-term PFS in DCBT may be different to that of adult donor allografts & is not established. Methods: We analyzed the time to systemic (sys.) IS cessation, transplant-related mortality (TRM), relapse, & PFS in a day 100 landmark analysis of DCBT recipients transplanted for hematologic malignancies who were engrafted & progression-free according to the incidence & severity of their aGVHD by day 100.

Results: 129 day 100 progression-free DCBT survivors (median age 36 years, range 0.9-70) were evaluated. Of this cohort, 80 (62%) had grade II-IV aGVHD (55 grade II, 21 grade III, & 4 grade IV disease), & all 129 patients were on sys.IS at day 100. With a median follow-up of 4.8 years (range 1.2-9.0) in this cohort, the cumulative incidence of achieving sustained sys.IS cessation was 33% (95%CI: 25-42) at 1-year, 55% (95%CI: 45-63) at 2-years, & 61% (95%CI: 51-69) at 3-years