ment was rapid. Median time to achieve an absolute neutrophil count >500 cells/μL was post-transplant day (D+) 9 (range, 8–11). Full donor lymphoid chimerism (≥95% by VNTR-PCR on CD3 sorted peripheral blood) was achieved in all pts (18/21 D + 14; 21/21 D + 28). Immune recovery was brisk and sustained. CD4, CD8, and NK cell numbers exceeded those at study entry by D + 28. Serum IL-7 levels were inversely correlated with CD4 counts. B-cell counts approached entry values by D + 100. Substantial numbers of naive (CD45RA+/CD62L+) CD4+ and CD8+ T cells were detected on D + 28, suggestive of early recovery of thymic function. **Conclusions:** Targeted pre-transplant immune depletion and NMSCT results in rapid, sustained donor-derived engraftment and immune reconstitution in pediatric pts with malignancy. Early immune recovery includes naive T cell subsets and B cells. Despite potential obstacles in pediatric pts, NMSCT conditioning can induce rapid engraftment and immune reconstitution, which may allow application of this novel platform to direct allogeneic anti-tumor immune responses in high-risk childhood cancers (Table 1).

Table 1. *Immune Depletion and Recovery (Mean Cell Counts/μL)*

	Pre	Post	D + 0	D + 28	D + 60	D + 100	D + 180
CD4	304	156	27	394	376	313	413
CD8	344	226	4	561	684	635	544
Naive CD4	69	35	20	123	64	100	118
Naive CD8	130	49	87	192	137	206	169
B cell	115	3	4	12	94	106	200
NK cell	87	111	1	274	222	154	130

Data represent mean cell counts/μL; Pre: entry; Post: after induction; D+: post-transplant day.

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PERIPHERAL T CELL APOPTOSIS AFFECTS IMMUNE RECONSTITUTION AFTER ALLOGENEIC BMT

Alpdogan, O.[1], McGoldrick, S.[1], Lu, S.[1], Patel, N.[1], Suh, D.[1], Smith, O.M.[1], van den Brink, M.R.M.[1] Memorial Sloan-Kettering Cancer Center, New York, NY.

Post-transplant T cell deficiency (especially in adults) is related to impaired thymus-dependent lymphopoiesis. However, others and we have previously found in human and murine studies that peripheral T cell apoptosis is increased in recipients of an allogeneic bone marrow transplantation (allo BMT). We used murine BMT models to further analyze peripheral T cell apoptosis in recipients of a T cell-depleted (TCD) or T cell-replete BMT. In recipients of a TCD BMT, we found that donor derived T cells had a high percentage of apoptotic cells (Annexin-V+) in both syngeneic and allogeneic hosts when analyzed at days 14 and 28 after BMT. Cleaved caspase-3 levels in these T cells were also increased in comparison with non-transplanted controls. Both CD4+ and CD8+ memory (CD44hi) T cells had a higher percentage of apoptotic cells compared to naive (CD44lo) cells. This was associated with decreased levels of intracellular Bcl-2 in de novo generated T cells from both young and old recipients of an allo TCD-BMT. We then transplanted allogeneic hosts with bone marrow (BM) from RAG-2-eGFP transgenic mice to examine apoptosis in newly generated T cells (recent thymic emigrants [RTE]) and found increased apoptotic cells in both eGFPhi and eGFPlo T cells. This demonstrates that both non-divided (eGFPhi) and proliferating (eGFPlo) donor BM derived T cells can undergo apoptosis in the periphery after TCD-BMT. In recipients with GVHD, we observed a severe loss of thymic cellularity and low numbers of BM derived de novo generated T cells with a high fraction of apoptotic cells. These had very low Bcl-2 expression and increased caspase 8 and 9 activation. Conversely, alloreactive T cells (from the mature donor T cells in the allograft) showed upregulation of Fas expression, higher Bcl-2 levels, less caspase activity, and contained lower numbers of apoptotic T cells compared to BM derived T cells. Allogeneic recipients receiving donor BM deficient for Fas, Fas Ligand and TRAIL, or over-expressing Bcl-2 and Akt had no decrease in donor derived apoptotic T cells, suggesting that these molecules were not absolutely required for post-transplant peripheral T cell apoptosis. We conclude that newly generated donor BM derived T cells have increased peripheral T cell apoptosis, especially in older recipients and in recipients with GVHD, which could be an important factor in the delay of post-transplant T cell reconstitution.

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IMMUNE RECONSTITUTION AFTER CD8-DEPLETED OR UNMANIPULATED PERIPHERAL BLOOD STEM CELLS TRANSPLANTATION FOLLOWING NONMYELOABLATIVE CONDITIONING: RESULTS FROM A PROSPECTIVE RANDOMIZED TRIAL

Castermans, E.[1], Baron, F.[1], Willems, E.[1], Meuris, N.[1], Schaaf-Lafontaine, N.[1], Cheynier, R.[2], Geenen, V.[1], Sekaly, R.-P.[3], Beguin, Y.[1] 1. CHU de Liege, Liege, Belgium; 2. Institut Pasteur, Paris, France; 3. Centre de Recherches du CHUM, Hotel-Dieu, Montreal, QC, Canada.

Aim of the study: We assessed the impact of CD8-depletion (performed in an effort to reduce the incidence of acute GVHD) on immune reconstitution after peripheral blood stem cell transplantation (PBSCT) with nonmyeloablative conditioning consisting of 2 Gy TBI with (n = 39) or without (n = 11) fludarabine. **Patients and methods:** 50 patients were randomised between CD8-depletion (n = 22) and lack of any manipulation (n = 28). Median patient age was 57 (range 36–69) years. Twenty patients received grafts from HLA-matched related donors and 30 from unrelated donors. CD8-depletion was performed with the Eligix system. GvHD prophylaxis consisted of mycophenomate mofetil (MMF) and cyclosporine (CSP). The different peripheral blood subpopulations were analyzed by 3-color flow-cytometry on days 28, 40, 60, 80, 100, 120, 180, and 365 after PBSCT. The diversity of the Vb repertoire in CD4+ and CD8+ T cells was assessed by rt-PCR on day 180 after PBSCT. Thymic output was determined through analyses of sjTRECS by quantitative PCR on days 100 and 365 after PBSCT. The expression of transcription factor Foxp3 was measured by quantitative RT-PCR on days 100 and 365 after PBSCT in order to assess regulatory T cells population. The impact of PBSC manipulation on T cell subsets recovery was analyzed by the Mann-Whitney rank sum test. **Results:** The median number of CD34+, CD3+, CD4+, and CD8+ cells × 106/kg infused were 4.1, 315, 185, and 131 in the unmanipulated group versus 3.7 (NS), 110 ($P < .001$), 94 ($P < .002$), and 4 ($P < .001$) in the CD8-depleted group, respectively.CD8 depletion significantly impaired recovery of CD3+CD8+ T cells on days 100, 120, and 180 after HCT ($P = .02$, $P = .02$ and $P = .03$, respectively), but not those of CD3+ T cells, CD3+CD4+ T cells, CD45RA+ T cells, CD19+B-cells, or CD56+ NK cells. CD3+T cell chimerism levels from day 28 to day 80 were also significantly decreased in the CD8-depleted group. The levels of sjTRECS after HCT correlated with those of CD3+CD4+CD45RA+ T cells (Spearman R = 0.41, $P = .0034$).The diversity of VB repertoire among CD4+ and CD8+ T cells on day 180 was similar among the 2 groups, as were the sjTRECS and Foxp3 levels. **Conclusions:** CD8-depletion of PBSC decreased T cell chimerism levels and recovery of CD8+ T cells, but did not affect recovery of other cell subsets, thymic output, nor Vb repertoire diversity, suggesting that immune reconstitution the first year after HCT was mainly driven by expansion of mature T cells contained in the graft.

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OPTIMIZATION OF EXPANSION OF CORD BLOOD T CELLS WITH ANTI-CD3/ANTI-CD28 COATED BEADS

Rieber, A.G.[1], Parmar, S.[2], Robinson, S.N.[1], Decker, W.[1], Xing, D.[1], Komanduri, K.V.[1], Bollard, C.[3], McMannis, J.D.[1], Yang, H.[1], Berenson, R.[4], Bonyhadi, M.[4], Shpall, E.J.[1] 1. Department of Blood and Marrow Transplantation, The University of Texas M. D. Anderson Cancer Center, Houston, TX; Department of Hematology/Oncology, University of Texas Southwestern Medical Center, Dallas, TX; Cell and

Gene Therapy, Baylor College of Medicine, Houston, TX; Xcyte Therapies, Inc, Seattle, WA.

Cord blood (CB) transplantation in adults is limited by low cell dose. This leads to delayed engraftment and delayed immune reconstitution with susceptibility to infections a major cause of morbidity and mortality. We hypothesize that the infusion of ex vivo CB T cells expanded using an anti-CD3/anti-CD28 bead may provide more rapid immune reconstitution, thereby reducing infectious complications. Prior to initiating a clinical trial, conditions for ex vivo CB T cell expansion need to be optimized. Variables investigated include addition of N-acetylcysteine (NAC) to culture (potentially reducing apoptosis), optimizing levels of interleukin 2 (IL-2), and continuous addition of anti-CD3/anti-CD28 beads to maintain a bead to cell ratio of 3:1. **Methods:** CB T cells were isolated from CB sample using anti-CD3/anti-CD28 paramagnetic beads and co-cultured with beads for a total of 14–28 days. Comparisons included: (1) 0 versus 10 mM NAC; (2) maintained 3:1 bead:cell ratio versus dilution with culture; (3) IL-2 concentration (0, 20, 200, 2000, and 20,000 IU/mL). Phenotypes were investigated using flow cytometry and T cell receptor diversity measured by spectratyping. **Results:** Overall, 300–1200-fold increases in T cell numbers were achieved. (1) While NAC did not significantly impact cell numbers, flow cytometry suggested cell death was reduced. (2) Maintaining a constant bead to cell ratio throughout culture did not improve proliferation. (3) Greatest T cell proliferation was obtained when 2000 and 20,000 IU/mL IL-2 were used. At the time of maximal expansion, the majority of the $CD4^+$ cells retained a naive phenotype ($CD27^+CD28^+$). A small proportion of late stage "effector" cells were present ($CD27^-CD28^-$ or $CCR7^-CD45RA^+$). $CD8^+$ T cell differentiation toward a "memory" phenotype ($CD27^+CD28^-$ or $CD27^-CD28^+$, or $CCR7^-CD45RA^-$ or $CCR7^+CD45RA^-$) was observed. Significantly, the expanded CB T cells retained a polyclonal TCR diversity. **Conclusions:** These preliminary data suggest that the ex vivo expansion of CB T cells may yield sufficient numbers of T cells to potentially improve immune reconstitution in CB recipients and reduce post-transplant mortality. Studies assessing the functionality of the expanded T cells are currently underway.

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ADOPTIVE TRANSFER OF IN VITRO GENERATED T CELL PRECURSORS IMPROVES T CELL RECONSTITUTION AND MEDIATES GRAFT-VERSUS-TUMOR ACTIVITY WITHOUT GRAFT-VERSUS-HOST DISEASE IN ALLOGENEIC HEMATOPOIETIC STEM CELL TRANSPLANTATION RECIPIENTS

Zakrzewski, J.L.[1], Kochman, A.A.[1], Lu, S.X.[1], Terwey, T.[1], Kim, T.D.[1], Hubbard, V.M.[1], Muriglan, S.J.[1], Suh, D.[1], Cabrera-Perez, J.[1], Heller, G.[1], Zuniga-Pflucker, J.-C.[2], Alpdogan, O.[1], van den Brink, M.R.M.[1] 1. Memorial Sloan Kettering Cancer Center, New York, NY; 2. University of Toronto, Sunnybrook & Women's College Health Sciences Centre, Toronto, ON, Canada. seqaence

Immunoincompetence after allogeneic hematopoietic stem cell transplantation (HSCT) particularly affects the T cell lineage resulting in significant morbidity and mortality from opportunistic infections. Recent studies have shown that murine T cells and their precursors can be generated from hematopoietic stem cells (HSC) in vitro using OP9 bone marrow stromal cells (H2Kk/H2Kb) expressing the Notch 1 ligand Delta-like 1 and growth factors. In this study we determined the effects of adoptively transferred in vitro generated T cell precursors on T cell reconstitution after allogeneic HSCT. We selected HSC (Lin-Sca-1hi c-kit+) from mouse bone marrow (BM) of various strains and cultured these cells on a monolayer of OP9-DL1 cells in the presence of growth factors. T lineage cell development proved to be strain independent and is therefore not MHC restricted in this culture system. C57BL/6 dervied HSC expanded 2000–5000-fold within 3–4 weeks and consisted of 90–95% CD4-CD8-double negative (DN) T cell precursors after 16–28 days of culture. We infused these cells (8×10^6) with T cell depleted (TCD) BM (5×10^6) or purified HSC into allogeneic recipients using minor antigen mismatched and MHC class I/II mismatched transplant models. Progeny of OP9-DL1 derived T cell precursors were found in the thymus and the periphery significantly improving thymic cellularity, and thymic and splenic donor T cell chimerism. Combination of T cell precursor administration and pre-conditioning with keratinocyte growth factor (KGF) further improved thymic engraftment of OP9-DL1 derived T cell precursors. T cell receptor repertoire and proliferative response to foreign antigen of OP9-DL1 derived mature T cells were intact. Moreover, Th1-type cytokine secretion of OP9-DL1 derived splenic T cells after stimulation with PMA/ionomycin was better than that of BM or HSC derived donor T cells. Progeny of OP9-DL1-derived T cell precursors remained detectable for at least 60 days after transplant with intact cytokine production. Administration of in vitro generated T cell precursors did not induce GVHD but mediated significant graft-versus-tumor (GVT) activity (determined by in vivo bioluminescence imaging) resulting in a subsequent significant survival benefit. We conclude that the adoptive transfer of OP9-DL1 derived T cell precursors significantly enhances donor T cell reconstitution in allogeneic HSCT recipients and results in GVT activity in the absence of GVHD.

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RECONSTITUTION OF IMMUNITY TO ADENOVIRUS (Ad) AFTER PEDIATRIC BONE MARROW TRANSPLANT

Myers, G.D.[1], Leen, A.M.[1], Buza, E.[1], Weiss, H.[1], Rooney, C.[1], Heslop, H., Bollard, C.M.[1] Center for Cell and Gene Therapy, Baylor College of Medicine, Houston, TX.

Adenovirus is a significant cause of morbidity and mortality in pediatric HSCT patients. Severity of immune suppression appears to be the most important risk factor for serious Ad disease. Without proven antiviral therapy, cellular immunity is the most effective protection against Ad disease. To better understand Ad immune recovery post-BMT, we prospectively monitored viral load, Ad subtype infection rates, and recovery of Ad cellular immunity in 22 pediatric HSCT patients (ages 14 months–20 years) who received matched-related (MRD n = 6), mismatched related (haplo n = 6), or unrelated donor (MUD n = 10) grafts. Real-time PCR (Q-PCR) provided quantitative measure of Ad viral load and detected Ad in blood samples from 68% (15/22) of patients on at least one occasion post SCT. 2/6 MRD recipients had Ad detected in their blood vs 8/10 MUD and 5/6 haplo patients. 13/16 (81%) of patients who provided stool samples were +ve for Ad on at least one occasion. Ad subgroup C predominated in blood (93%), while subgroups varied in stool: A(62%), B(12%), C(62%), D(6%), E(6%), F/G(44%). 8/16 patients were positive for multiple subtypes in their stool. Mean Ad-specific spot forming cells (SFC)/1×10^6 PBMC as measured by IFNg ELISPOT assay was not significantly different in the MRD and MUD groups at 3, 6, and 12 months post HSCT ($P \geq .1$). At 3 months, MUD patients had mean 29 SFC/10^6cells (range: 0–190) versus 27 SFC/10^6 cells (range: 0–100) in the MRD group. By 12months, MUD patients had 63 (0–225) versus 78(17–201) SFC/10^6 cells in MRD group. However, immune response to adenovirus was significantly delayed ($P = .005$) in Haplo patients at 3months (4 SFC/10^6cells, range:0–18) and 12months (23 SFC/10^6cells, range:0–60). Mortality from adenovirus was 4.5% (1/22). Mean ALC in MUD, MRD, and Haplo recipients at 12 months were:1901/ul (772–3728), 2153/ul (948–3280), 1713/ul (1128–2406), respectively ($P > .1$). Mean CD4 and CD8 counts/μl in MUD, MRD, and Haplo recipients at 12months were: 835 (range:37–1656) and 589 (range:18–2404), 1373 (560–2169) and 530 (332–706), and 1055 (451– 1659) and 398 (178–620), respectively ($P > .1$). In conclusion, our data shows recipients of T cell depleted HSCT products from haploidentical donors have significantly delayed Ad-specific cellular immune recovery. Further, ALC does not predict specific immune recovery to adenovirus. We have initiated a clinical study of donor-derived adenovirus-specific CTL infusions for the prophylaxis and treatment of Ad infection post SCT that will target this patient population.