Current management strategies are suboptimal and associated with significant adverse effects. Patients undergoing alloSCT are profoundly immunocompromised and do not respond to vaccination with CMV antigens. Immunotherapy (IT) with CMV specific donor-derived cytotoxic T lymphocytes (CTL) is effective after alloSCT, but is expensive, labor intensive, and difficult to replicate. Non-toxic strategies are needed to improve outcomes. In earlier studies, we showed that *ex vivo* expanded activated T cells (ATC) armed with anti-CD3 × anti-Her2/neu bispecific antibody (Her2Bi) exhibit high levels of specific cytotoxicity directed at breast cancer cell lines. Using this strategy, we tested ATC armed with anti-CD3 (OKT3) × anti-CMV (Cytogam®) bispecific antibodies (CMVBi) to target and lyse CMV-infected fibroblasts. Normal donor ATC were generated and armed with CMVBi. Specific cytotoxicity was tested by ^{51}Cr release using CMV-infected or uninfected human fibroblasts. CMVBi alone, CMVBi-armed ATC (aATC), and ATC alone were tested for their ability to kill. ATC were armed with 0.001 to 500 ng of CMVBi/10^6ATC at effector:target (E:T) from 3.125:1 to 25:1. IFN_γ EliSpot was used to assess IFN_γ secreting cells in ATC and aATC after exposure to CMV-infected and uninfected fibroblasts. ATC armed with CMVBi as little as 0.001 ng/10^6 ATC was significantly more cytotoxic than unarmed ATC. There was higher cytotoxicity mediated by aATC as the multiplicity of infection (MOI) increased in target cells. At all E:T, CMVBi arming at a dose of 50 ng/10^6 ATC exhibited dramatically higher lysis of CMV-infected targets (MOI 1) than that exhibited by ATC alone. Cytotoxicity mediated by unarmed and armed ATC was background in uninfected targets. Immunofluorescent studies showed that aATC aggregated around GFP fluorescence-marked CMV-infected fibroblasts. Cytokine secretion analyzed by IFN_γ EliSpot confirmed immune responses. The lack of MHC restriction in the mediation of cytotoxicity, polyclonal targeting of multiple CMV antigens and the simplicity of expanding donor T cells makes it easy to adapt this effective strategy against CMV infection post alloSCT.

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NAÏVE T CELLS FROM CORD BLOOD AND CMV-SERONEGATIVE DONORS RECOGNIZE ATYPICAL EPITOPES OF CMVpp65 AND CAN BE USED FOR ADOPTIVE IMMUNOTHERAPY

Hanley, P.J.[1,2,4], Lam, S.[1,2,4], Cruz, C.R.Y.[1,4], Savoldo, B.[1,4], Benjamin, T.[1,4], Stephanie, K.[1,4], Lilleri, D.[3], Gerna, G.[3], Demmler-Harrison, G.[4], Dotti, G.[1,2,4], Heslop, H.E.[1,4], Brenner, M.K.[1,4], Rooney, C.M.[1,2,4], Shpall, E.J.[5], Rodgers, J.R.[2], Bollard, C.M.[1,2,4] [1] Baylor College of Medicine, Houston, TX; [2] Baylor College of Medicine, Houston, TX; [3] IRCCS Policlinico San Matteo, Pavia, Italy; [4] Baylor College of Medicine, Houston, TX; [5] University of Texas MD Anderson Cancer Center, Houston, TX

Adoptive transfer of virus-specific T cells can effectively restore antiviral immunity after stem cell transplantation. However, protocols for eliciting virus-specific T cells from existing memory T cells of CMV-seropositive adult (CMVpos) donors have been ineffective when applied to naïve cord blood (CB) transplant (CBT) recipients due to absence of antigen-specific memory cells in these grafts. We previously demonstrated that CMVpp65-specific T cells could be generated from CB using a modified protocol and that 15/15 CB T cell lines recognized atypical epitopes of pp65. To test if naïve T cells from CMV-seronegative donors (CMVnegs) also recognize atypical epitopes, we again optimized our previous method. CD45RA+ naïve T cells were selected from peripheral blood and stimulated with pp65-pepmix-pulsed DC with IL7, IL12, and IL15. For subsequent stimulations T cells were stimulated with pp65-pepmix-pulsed EBV-LCL and IL15 or IL2. CMVpp65-specific T cells (CMV-CTL) expanded from 8 of 11 CMVnegs and were primarily CD8+ T cells (mean 71%). These T cells secreted markedly more IFN-γ in response to pp65 peptides (mean 224; range: 38-611 SFC/1x10^5 cells) than to irrelevant peptides (mean 12;Range 3-37) as measured in Elispot assays. These T cells lysed pp65-pulsed target cells (mean:48;Range:15-70%) but not negative controls (mean 22;Range 4-40%) in cytotoxicity assays. As with CB, CMVneg CMV-CTL were derived from naïve T cells and recognized only atypical pp65 epitopes. These findings raised the concern that the viral epitopes presented by endothelial cells infected with CMV in patients might not present the atypical peptides recognized by our CMV-CTL. We hypothesized that the epitopes presented to T cells by endothelial cells or other APCs after CMV infection are different to those presented in our in vitro system. We tested if different epitopes would dominate the naïve T cell response to CMV AD169-infected fibroblasts and CMV VR1814-infected DC. The responding naïve T cells were again specific for atypical epitopes showing that these epitopes were indeed presented by cells naturally infected with CMV. This reassured us that despite their unusual repertoire, T cells derived from CB or CMVnegs should control CMV infections. We are now evaluating their clinical efficacy in recipients of CBT and soon Hematopoietic Stem Cell Transplantation. These studies should determine if naïve T cells primed in vitro are able to persist and establish memory in vivo.

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EFFECT OF A CO-STIMULATORY ENDODOMAIN ON THE PERFORMANCE OF T CELLS EXPRESSING A CHIMERIC ANTIGEN RECEPTOR DIRECTED AT CD19 IN PATIENTS WITH RELAPSED/REFRACTORY B-CELL MALIGNANCIES

Ramos, C.A.[1], Savoldo, B.[1], Liu, E.[1], Bollard, C.M.[1], Carrum, G.[1], Kamble, R.T.[1], Mims, M.P.[2], Keating, M.[3], Gee, A.P.[1], Mei, Z.[1], Rooney, C.M.[1], Heslop, H.E.[1], Brenner, M.K.[1], Dotti, G.[1] [1] Baylor College of Medicine, Houston, TX; [2] Baylor College of Medicine, Houston, TX; [3] The University of Texas M. D. Anderson Cancer Center, Houston, TX

First generation chimeric antigen receptors (CAR) usually combine the antigen binding domain of a monoclonal antibody with the ζ signaling domain of the T cell receptor/CD3 complex. When expressed in T cells, those CAR provide potent antigen-specific, non MHC-restricted effector function against tumor cells in vitro, but clinical trials have shown limited expansion and persistence of these lymphocytes in vivo. This limitation is likely attributable to the failure of these CAR to fully activate T cells following target antigen engagement on tumor cells, since tumors lack expression of costimulatory molecules required for sustained T cell activation. Incorporation of costimulatory endodomains within the CAR, such as CD28, increases proliferation and activity of the modified T cells in vitro and may enhance their benefit in vivo but, as yet, the relevance and value of these modifications in human subjects remains speculative.

We now present results from a phase I trial of T cells redirected to CD19 given to patients with refractory/relapsed B-cell malignancies. Patients were simultaneously infused with 2 autologous T cell products expressing CAR with identical CD19-specific exodomains. In one product, the endodomain contained only the ζ sequence (CAR.19ζ) while, in the second product, we added a costimulatory CD28 domain (CAR.19-28ζ). We could thus directly measure and compare the expansion, persistence and effectiveness of each cell population in every patient. Six subjects have been treated at 3 cell dose levels (Table). Persistence of CAR+ T cells was assessed in blood by Q-PCR assays specific for each population.

Table 1. Characteristics of the infused CD19-redirected CAR-bearing T cell products (N=6)

	CD4+	CD8+	Naïve (CD45RA+)	Memory (CD45RO+ CD62L+)	Specific lysis (20:1 E:T ratio)
CAR.19ζ T cells	44 ± 23	49 ± 22	6 ± 5	50 ± 24	53 ± 10
CAR.18-28ζ T cells	47 ± 26	48 ± 22	6 ± 6	47 ± 66	65 ± 19

All numbers are shown as mean percentage ± standard deviation. T cell products were generated by activation of autologous peripheral blood mononuclear cells with immobilized OKT3, gene modification with retroviral vectors encoding either CAR.19ζ or CAR.19-28ζ and ex vivo expansion for a median of 14 days (range 6-18) in the presence of IL-2. Three dose levels have been administered: 2×10^7, 1×10^8 and 2×10^8 cells of each product/m^2.

All infusions were well tolerated. CAR.19-28ζ cells were detected at a low level after infusion, but progressively increased 7- to 63-fold, peaking at 1-2 wk, before declining to background levels over the

ensuing 3 mo. By contrast, CAR.19ζ cells were barely detectable after infusion, showed no expansion and disappeared rapidly. Following treatment, 2 patients had stable disease for up to 6 mo and 4 had progressive disease.

In conclusion, infusion of both CAR.19ζ and CD19-28ζ T cells is safe at the doses used. Direct comparison of each cell product in individual patients showed that the CD28 endodomain enhances expansion and persistence of the CAR T cells. The limited clinical benefits suggest that additional modifications will be required and our approach will allow these changes to be systematically evaluated even in small-scale clinical studies.

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BK VIRUS SPECIFIC T CELLS EXPANDED EX VIVO FOR USE IN CELLULAR THERAPY SHOW MULTIPLE ANTIGEN SPECIFICITY AND POLYFUNCTIONAL TH1 RESPONSES

Blyth, E.[1], Clancy, L.E.[2], Simms, R.[1], Gottlieb, D.J.[1,2,3,4] [1] University of Sydney, Westmead, NSW, Australia; [2] Westmead Hospital, Westmead, NSW, Australia; [3] Westmead Hospital, Westmead, NSW, Australia; [4] University of Sydney, Westmead, NSW, Australia

There is increasing evidence for the role of BK virus in the aetiology of haemorrhagic cystitis and renal impairment post haemopoietic stem cell transplant (HSCT). Cellular therapy for immune reconstitution post HSCT has been used clinically for CMV, EBV and adenovirus. Broadening the scope of viral targets in this type of therapy is desirable to reduce the burden of opportunistic infections in this patient group.

Methods: Monocyte derived dendritic cells or peripheral blood mononuclear cells were pulsed with mixes of overlapping peptides covering the 5 BKV proteins (VP1, VP2, VP3, LTA and STA). These were used to stimulate T cells on days 1 and 7 and cells were cultured for 21 days with increasing doses of IL-2 from day 7. The cellular product was then analysed for phenotype, BKV specificity and functionality by examining cytokine production and cytotoxicity.

Results: Cellular proliferation was seen in all donors, mean fold increase in total viable cells was 5.9 fold. All cell products were mainly CD3 cells (mean 94.1%) with individual variation in CD4:CD8 ratio (ranges: CD4 9.7 to 97.5%; CD8 0.9 to 77%). T cell subsets analysis showed the majority of cells to be Tem (mean 71.2%), with a sizable minority of Tcm (mean 21.5%). Data on antigen specificity was available in 11 of 15 cultures. Within cultures, BKV responsive cells varied (CD3 mean 12.7%, CD8 mean 15.2%, CD4 mean 12.5%,). There was heterogeneity in the specificity of cells to different BKV proteins. Most responses were directed to VP1, LTA and STA with smaller magnitude responses seen to VP2 and VP3. The quality of the cytokine response was assessed by multiparameter flow cytometry for IFN-γ, TNF and IL-2. In all cases, the percentage of cells producing multiple cytokines to stimulation with BKV proteins was high (for CD3 mean triple cytokine 37.5%, double 34.3% and single 28.2%). Cytotoxicity was assessed using CD107a/b expression and the CARE-LASS cell lysis assay. CD107 expression was present in both CD8 and CD4. This was higher in CD8 cells and lysis of antigen coated target cells correlated with the presence of CD107 expression on CD8 cells.

Discussion: The clinical utility of this product will be determined in clinical trials of adoptive immunotherapy following HSCT or renal transplantation. This method for large-scale expansion of BKV specific CTL could also be utilised for analysis of BKV targeted immune responses and epitope identification.

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EXPANDED HUMAN INKT CELLS EXHIBIT TH2 POLARIZATION AND DIRECT CYTOTOXICITY AGAINST HEMATOLYMPHOID TUMOR TARGETS

Luszczek, W.[1], Morales-Tirado, V.[1], Woolard, S.N.[1], Van Der Merwe, M.[1], Shook, D.[1,2], Campana, D.[2], Pillai, A.[1] [1] St. Jude Children's Research Hospital, Memphis, TN; [2] St. Jude Children's Research Hospital, Memphis, TN

CD1d-restricted invariant NKT (iNKT) cells are rare but potent innate regulatory cells capable of immune modulation after transplantation via robust production of Th1/Th2 cytokines, as well as tumor immunosurveillance via direct cytotoxicity. Protocols to expand iNKT cells and tailor their cytokine secretion would broaden their application in transplant immunotherapy. We have optimized a protocol for ex vivo expansion of highly purified human $CD3^+V\alpha24^+$ iNKT cells from a variety of cell sources including human peripheral blood (PB), bone marrow, and umbilical cord blood. PB $CD3^+V\alpha24^+$ iNKT cells (> 98% pure by sort) were expanded using PB-derived autologous APCs, Vα24-specific TCR stimulation without added glycolipids, and low-dose IL-2 and IL-7. This results in mean $> 10^3$-fold expansion, with peak yields at day 14-21 (range, 3×10^6 - 7×10^7 iNKT cells from 10^3 - 10^4 starting $CD3^+V\alpha24^+$ cells). Expanded iNKT cells are $CD3^+CD4^{neg}V\alpha24^+$ and retain viability in culture through day 49. At 21 days, these iNKT cells secrete high IL-10 and IL-5, moderate IL-4 and IFN-γ, and low IL-2 and IL-13 in anti-CD2/CD3/CD28 bead-stimulated Luminex® supernatant assay. Day 21 expanded iNKT cells maintained an $IL\text{-}4^{hi}IFN\text{-}\gamma^{lo}$ phenotype even with potent Th1-polarizing stimuli [100 ng/mL of the glycolipid ligand alpha-galactosyl ceramide (α-GalCer)], and are dose-dependent suppressors of sorted autologous $CD3^+CD4^{neg}V\alpha24^{neg}$ (> 95% $CD3^+CD8^+$) responders in 72-hr CFSE MLR. Non-glycolipid activation of day 21 iNKT cells induced high levels of cytolytic effector molecules, including granzyme B. We measured cytotoxicity of activated day 21 iNKT cells following co-incubation of iNKT cells versus control effector populations with firefly luciferase-transduced RS4,11 and Nalm6 (B-ALL), U937 (monocytic) and K562 (CML) targets. iNKT cell effectors (E) demonstrated dose-dependent cytotoxicity against B-ALL targets (T) (e.g. Nalm6: 31.2 ±9.1% at E:T 0.1:1, 32.6 ±4.4% at E:T 0.5:1, 48.5 ±5.7% at E:T 1:1), with no significant cytotoxicity against myeloid targets (e.g. K562: 12.3 ±1.6% at E:T 0.1:1, 14.9 ±3.0% at E:T 0.5:1, 14.2 ±2.6% at E:T 1:1). Our results indicate that human iNKT cells expressing high levels of Th2 and regulatory cytokines can be potently expanded ex vivo without exogenous glycolipid stimulation and exert significant cytotoxicity against B-ALL targets. This supports their potential for application in anti-tumor or regulatory immunotherapy in the pre- and post-transplant setting.

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ADOPTIVE TRANSFER THERAPY USING EXPANDED MELANOMA-SPECIFIC T CELLS PROGRAMMED *EX VIVO* FOR IMPROVED EFFICACY *IN VIVO*

Andersson, H.A.[1], Hernandez, J.A.[2], Maiti, S.[1], Huls, H.[1], Radvanyi, L.[2], Cooper, L.J.N.[1] [1] University of Texas M.D. Anderson Cancer Center, Houston, TX; [2] University of Texas M.D. Anderson Cancer Center, Houston, TX

Adoptive cell transfer (ACT) of autologous tumor-infiltrating lymphocytes (TIL) mediates tumor regression in 50% of Stage IV melanoma patients previously refractory to all other types of therapy. Further improvement of this therapy based on current technology (using OKT3 and allogeneic PBMC) to propagate T cells has reached a point of diminishing returns due to the technically cumbersome and resource-intensive production of TIL for clinical administration. The extended culture times needed to generate sufficient numbers of TIL typically results in acquisition of terminally-differentiated T cells with loss of effector memory (EM) function and reduced antigen specificity, resulting in poor *in vivo* persistence and reduced therapeutic potential. Compounding this problem is that TIL cannot be expanded from many melanoma patients, leaving them without an option for cellular therapy. To improve ACT, here we show that K562 cells can function as artificial antigen-presenting cells (aAPC) for propagating melanoma-specific T cells from both TIL and peripheral blood. K562 were genetically modified to function as "generic" aAPC for *in vitro* propagation of T cells with central/effector memory phenotypes by enforced expression of the costimulatory molecules CD86 and 41BB-L in addition to membrane-bound versions of the cytokines IL7/IL15/IL21. As K562 do not express endogenous HLA A and B molecules engendering allogeneic responses, they were genetically modified as "specialized" aAPC using the *Sleeping*