Supplement

VST Manufacturing Process

Mononuclear Cell Separation

Mononuclear cell (MNC) enrichment from whole blood occurred using either a manual or an automated density gradient centrifugation procedure (Ficoll), depending on the volume of blood received. The blood was collected at a blood center convenient for the donor and sent via courier to Hoxworth. Small volume blood samples collected from either related or unrelated donors were diluted with phosphate-buffered saline (PBS), manually layered over Lymphocyte Separation Medium (LSM), and centrifuged at 400 xG to isolate the mononuclear cells. Larger volume blood samples were diluted with Plasmalyte prior to performing the density gradient separation on a Sepax™ automated cell processing device. After MNC enrichment, the MNC were suspended in PBS or RPMI 1640 and counted with an automatic cell counter (Coulter) prior to being placed in culture for VST generation.

VST Generation

The MNC were pelleted in a conical centrifuge tube and incubated for 30 minutes at 37^{o}C with at least 2, and up to 9 commercially available peptide pools (2 CMV, 3 EBV, 2 AdV, and 2 BK virus) that span the immunogenic portions of 4 different viruses, as a combination of all 4. In order to stimulate antigen-specific T cell activation and proliferation, the MNC were simultaneously stimulated with 10ng per 10^6 cells of EBV PepMixes LMP-2,EBNA-1 and BZLF-1, Adenoviral PepMixes HAdV3 & HAdV5 (hexon and penton), BKPyV (LTS & VP1) and CMV PepMixes PP65-2 & IE1. Without washing, the stimulated MNC population was then diluted to approx. 0.5 x 10^6 cells/mL and cultured for approximately12 days in culture media supplemented with 400U/mL IL-4, 10ng/mLIL-7, and 10% fetal calf serum, thus enabling the EBV- Adv- BK and CMV-specific T-lymphocyte clones to proliferate and the non-specific allo-reactive clones to die off. The cultures were initiated at 30mL per G-Rex 10 culture device, with cytokine replenishment at day 4, and media + cytokine replenishment at day 7, as the cultures are transferred to a larger G-Rex 100 culture device.

When the desired VST number was achieved, most often on day 11 or 12, the VSTs were pooled into a large centrifuge tube and sampled for cell count, sterility, cytotoxic activity, and phenotype as described below. VSTs were then washed with plasmalyte containing 0.5% human serum albumin either by centrifugation, or by using a Sepax automated cell washer, to remove unwanted components. The precise dilution factor obtained with these wash steps varied slightly with the volume of the culture being harvested, but all media components and cytokines in the culture at the time of harvest were diluted at least 100,000-fold prior to infusion or cryopreservation. Washed VSTs were suspended in CryoStor 5 for cryopreservation in cryovials.

Cytotoxicity

The cytotoxicity assay was performed by the Diagnostic Immunology Laboratory at CCHMC. Target cells that mimic virally-infected cells and target cells that should not be killed (autologous PHA-blasts or, if unable to generate autologous blasts from the recipient, third party PHA-blasts will be used in place) are loaded with radioactive Chromium51 then co-cultured for 6 hours with the effector cells (CTL) at four different effector to target cell ratios. Target cell killing

(cytotoxicity) by the CTL is detected by measuring the amount of radioactive Chromium51 that is released into the supernatant.

The percent cytotoxicity for each effector/target (E/T) ratio is determined according to the following formula:

$$\% \text{ cytotoxicity} = \frac{\text{mean cpm of dilution - mean cpm of spontaneous}}{\text{mean cpm of maximum- mean cpm of spontaneous}} \times 100$$

There is no specification for cytotoxic activity against the LCL targets, but there should be ≤ 10% cytotoxicity (at 25:1 E:T) against the autologous PHA-stimulated blasts.

VST Cryopreservation

Protocol-specified infusion doses were aliquoted into 5mL cryovials, pelleted, and resuspended in a commercially-available cryopreservation media (CryoStor 5; BioLife Solutions) to a volume of 2mL. The VSTs were frozen in an insulated container inside a -70°C mechanical freezer and then transferred to vapor phase LN_2 for storage within 72hrs. Retain vials containing small numbers of VSTs were cryopreserved and stored along with the product vials in case they are needed at a later date to investigate an adverse event or unexpected result.

Final Product Formulation

Once all release criteria were been met, the cryopreserved VST products were thawed rapidly in a 37°C waterbath and diluted slowly to 5X their original volume with Dextran and 2.5% HSA. Cells were then further diluted another 2X with plasmalyte containing 0.5% HSA and transferred to a labeled 30cc syringe, sealed in a plastic overwrap bag, and transported to the patient's bedside in a validated hard plastic cooler containing 4°C gelpacks. Upon arrival at the clinical site, the product was given to the patient's caregiver (nurse or physician) for immediate infusion. The infusion process occurs according to well established clinical site procedures.

The labeling complied with all ISBT 128 terminology and with all FACT and FDA guidelines for final product labeling, including warning labels as applicable.

VST line characterized by flow cytometry

Frozen VST lines were rapidly thawed in a 37°C water bath and then transferred into cell culture media RPMI-1640 supplemented with 10% fetal bovine serum and 2mM Glutamine. After centrifugation at 300g for 5 minutes at room temperature, cells were resuspended and counted under a microscope after 4% trypan blue solution staining. Viable cells were seeded into a 96-well plate at the density of 2.5×10^5 per well and stimulated with individual viral PepMixes. Following 5-6-hour culture in the incubator, cells were harvested, washed with PBS, and stained with an antibody cocktail of CD3-PE, CD4-APC-eFluor 780, CD8-FITC plus the fixable viability dye Zombie Violet. After fixation and permeabilization, cells were stained with the intracellular antibody IFN-γ-APC. Each sample subjected to flow cytometry needs to collect 1×10^5 events.

Supplemental Table S1: *VST Release Criteria and Assessment Methods*

Test	Purpose	Method(s)	Measurement	Release Criteria
Cell Count	Potency	Hemacytometer	Viable Cell #	Meets Protocol Requirements
Phenotype	Purity	Flow Cytometry	% $CD3^+$	≥80% $CD3^+$
Viability	Potency	Trypan Blue dye exclusion	Viability (%)	≥ 70%
Mycoplasma	Safety	PCR	Microbial contamination	PCR Negative
Endotoxin (If 14 day sterility result is unavailable)	Safety	LAL assay	Endotoxin Units/ml	≤5 EU/Kg
Sterility	Safety	Gram Stain	Microbial contamination	No organisms seen
Sterility	Safety	14 day culture.	Microbial contamination	No Growth
Cytotoxicity	Safety	Cr^{51} Release	% killing (Allogeneic donor PHA-blasts)	≤ 10% at 20:1 (E:T)
Chimerism	Identity	DNA Sequencing	% Donor	100% donor

Supplemental Table S2: VST response to additional viruses

	Total subjects	**Donor derived VST**	**Third-party VST**
	n=10	**n=3**	**n=7**
Response VTS			
CMV			
CR	100%	100%	100%
PR	NA	NA	NA
CR+PR	100%	100%	100%
NR	NA	NA	NA
ADV			
CR	100%	NA	100%
PR	NA	NA	NA
CR+PR	100%	NA	100%
NR	NA	NA	NA
EBV			
CR	38%	100%	33%
PR	53%	NA	58%
CR+PR	91%	100%	91%
NR	9%	NA	9%