APC-INDEPENDENT EXPANSION OF IMMUNOREGULATORY TR1 CELLS FROM STEM CELL SOURCES FOR ADAPTIVE IMMUNOTHERAPY

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Immunotherapy with both adaptive and innate regulatory T cells facilitates induction of transplant tolerance, including the prevention and treatment of GVHD. Current strategies to expand regulatory Tr1 cells from CD3+CD4+CD25negFoxp3lo to CD25int/Foxp3lo cells include culture with IL-10 and APCs (Groux, et al., Nature, 1997; Gregori et al., Methods Mol. Biol., 2007) and a non-APC-dependent protocol utilizing anti-CD3 and IL-2 combined with antibody cross-linking of the innate complement inhibitor I co-factor protein (CD46) (Kemper et al., Nature, 2003). Optimizing a robust APC-independent means of Tr1 cell generation is of particular utility to hematopoietic cell transplant (HCT) immunotherapy, as it offers potential for large-scale Tr1 cell expansion from autologous or allogeneic cell sources without need for re-separation of Tr1 cells from host or donor APCs prior to infusion. To determine the optimal methods and cellular products for non-APC-dependent expansion of Tr1 suppressors, we performed antibody-crosslinking of CD46, without addition of IL-10 or APCs, to derive a uniform population of CD3+CD4+CD25negFoxp3lo cells (>95% conversion of CD25negFoxp3lo to CD25int/Foxp3lo cells, regardless of cellular product type). Differential levels of expansion of Tr1 cells were similar according to the source of cell product, with greatest fold-expansion in PB-Tr1 (mean absolute numbers at day 21 ranging from 101 to 103 starting CD3+CD4+CD25negFoxp3lo cells: PB-Tr1 (n = 3): 2.5 ± 0.8 × 106; BM-Tr1 (n = 2): 7.2 ± 105; CB-Tr1 (n = 4): 5.6 ± 3.3 × 105). By day 14-21 of expansion, PB-Tr1 cells secrete IL-10 and IL-5, very low levels of IL-2 and IFN-γ, and no IL-4 or IL-17 by anti-CD2/CD3/CD28 bead stimulation and Luminex® supernatant assay, and they demonstrate suppressor function against sorted autologous CFSE-labeled CD3+CD4+CD25negFoxp3lo responders in 72-hour MLR using irradiated allogeneic APC stimulators (Table 1). We are currently optimizing similar protocols for expansion of BM-Tr1 and CB-Tr1 to allow application in tolerance induction and immunotherapy after HCT from each of these cellular therapy sources.

Table 1. Allo-suppressor MLR (n = 4) using day 21 PB-Tr1

<table>
<thead>
<tr>
<th>Responder:</th>
<th>Suppressor Ratio (± Stim*)</th>
<th>Mean ± SD % Proliferation</th>
<th>P value vs R only (t-test)</th>
<th>P value vs 1:0 (+ Stim) (t-test)</th>
</tr>
</thead>
<tbody>
<tr>
<td>R only</td>
<td>14.8 ± 3.1</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>1:0 (+ Stim)</td>
<td>38.0 ± 8.8</td>
<td>0.002</td>
<td>0.002</td>
<td></td>
</tr>
<tr>
<td>1:1 (+ Stim)</td>
<td>21.3 ± 3.5</td>
<td>0.044</td>
<td>0.010</td>
<td></td>
</tr>
<tr>
<td>1:5 (+ Stim)</td>
<td>13.8 ± 2.8</td>
<td>0.674</td>
<td>0.003</td>
<td></td>
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</tbody>
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*Stim = allogeneic 3300 cGy-irradiated APC stimulators

MULTIVIRUS-SPECIFIC CTL FOR ADAPTIVE TRANSFER USING IN VITRO PEMPIX STIMULATION

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We and others have shown that viral infections in allo-HSCT recipients can be prevented/treated by the adoptive transfer of virus-specific CTL. However, conventional CTL manufacture has taken >3 months. We recently developed a protocol to rapidly generate CTLs (rCTLs) using DCs nucleofected with viral antigen-encoding DNA plasmids as APCs and expansion in a gas permeable bioreactor (G-Rex) in IL7+IL14, which reduces manufacturing time to 16 days. To further streamline production without affecting potency, we are now assessing whether multivirus CTL can be generated by directly stimulating donor PBMCs with clinically applicable pepmixes (15mer peptide libraries) spanning viral antigens.

We isolated PBMCs from 4 healthy donors and generated rCTL using plasmid-nucleofected DCs or by directly stimulating bulk PBMCs using pepmixes spanning the same antigens encoded by the plasmids (EBV-EBNA1, LMP2, BZLF1; CMV-IE1, pp65 and Adv-Hexon, Pentagon) (pCTL). Subsequently the activated cells were expanded as outlined above. We compared the phenotype, proliferation, specificity and function of each cell population. There was no significant difference in the rate of expansion (rCTL vs pCTL, 8.6 vs 7.1 fold). Similarly, both were phenotypically indistinguishable, with a dominance of CD4+ T cells (70.5 vs 74.2%) expressing CD62L (77 vs 61%). Both rCTL and pCTL were specific for the stimulating antigens as measured by IFNγ ELispot with a median of 100 vs 55 EBNA1, 45 vs 47 LMP2, 55 vs 32 BZLF1, 195 vs 232 IE1, 3927 vs 4472 pp65, 525 vs 535 Hexon, 372 vs 782 Pentagon SFC/1x102 CTL. rCTL and pCTL lysed EBV (16 vs 19%), Adv (27 vs 34%) and CMV targets (42 vs 49%; E:T 80:1). Finally, to evaluate whether stimulation using 15mer peptides, which contain all CD4 but possibly not all CD4+ epitopes, results in loss of CD4+ specificity we generated pCTL using either 15- or 30mer peptides spanning a region of Hexon predominately recognized by CD4+ T cells. Phenotypically the CTL were similar (CD4+ = 56 vs 60%; CD8+ = 21 vs 16%) and epitope specificity, as evaluated by stimulation with peptide pools, demonstrated slightly broader specificity using 15- versus 30mer peptides with a mean of 10 vs 7 pools recognized.

In summary, we can efficiently generate polyclonal multivirus CTLs by directly stimulating PBMCs with pepmixes. This approach is clinically applicable, requires a starting blood volume of only 15mL, and reduces manufacturing time to < 10 days, with a consequent reduction in cost and complexity.

SENCESCENCE OF CULTURED BONE MARROW STROMAL CELLS

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Bone marrow stromal cell (BMSC) therapy is effective for treating acute-Graft versus Host Disease. Clinical BMSCs are produced by serial passage of adherent cells from marrow aspirates. Multiple passages allow for the production of large quantities of human BMSCs, but expansion is limited by cell senescence. We assessed the nature of BMSC changes associated with multiple passages in order to better understand the nature and impact of senescence on human BMSCs and to identify potential senescence associated biomarkers. BMSCs from marrow aspirates of 5 healthy subjects were cultured in flasks and passed serially until cell growth stopped between the 8th to 12th passages. Early (passage 1 and 2, n10) and late passage cells (passages 5 through 11, n15) were selected based on their replicative lifespan and compared. Senescence was associated with a change to a flattened morphology and increased senescence associated beta-galactosidase (SA-b-gal) staining. The BMSCs were analyzed by global transcriptome analysis with an oligonucleotide microarray with 44,000 probes. Unsupervised hierarchical clustering analysis separated the 25 samples into two clusters, one with all early and a second with all late passage BMSCs and a total of 1,739 genes were differentially expressed. Ingenuity pathway analysis revealed that genes highly expressed in the early passage BMSCs belonged to several immune pathways: cell-mediated immune response, humoral immune responses, hypersensitive responses, and lymphoid structure and development. Genes highly expressed in the late passage BMSCs belonged
to the DNA replicative, recombination and repair and nucleic acid metabolism pathways. Hierarchical clustering analysis of 147 differentially expressed cell cycle and apoptosis genes again separated the early and late passage cells into two groups and a node of genes up-regulated in late passages cells included two genes involved with replication senescence, cyclin-dependent kinase inhibitor 1A (p21, Cip1) (CDKN1A) and cyclin-dependent kinase inhibitor 2A (melanoma, p16, inhibits CDK4) (CDKN2A) and one that inhibits TRL, mediated apoptosis, tumor necrosis factor receptor superfamily, member 10d (TNFRSF10D, DCR2, CD2D24). Flow cytometry analysis confirmed greater expression of TNFRSF10D on late passage cells. BMSC senescence is associated with distinct molecular changes. Early passage BMSCs may have more immune modulatory properties.

**I72 THERAPEUTIC POTENTIAL OF GAMMADELTA T-CELLS IN CONTROLLING CMV AFTER ALLOGENIC STEM CELL TRANSPLANTATION**

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**Introduction:** Allogeneic stem cell transplantation (allo-SCT) is hampered by GvHD, infections like CMV and relapse of disease. Gammadelta T-cells (gdT-cells) seem to be important in virus control but also in malignancy control. CMV infections are associated with an increased expansion of Vδ1+T cells. Therefore, we investigated frequency and function of gdT-cells after allo-SCT in order to assess their therapeutic potential.

**Methods:** PBMCs at time points within 3 months after allo-SCT of 17 patients were sampled. CMV viral load was monitored by PCR. Phenotype and frequency of gdT-cells and alpha beta T-cells (αβT-cells) were analyzed by flow cytometry. GdT-cells of 2 patients with CMV reactivation were isolated and expanded. Frequency and clonality of Vδ1+, Vδ2+ and Vδ3+T cells were measured with spectratyping. The reactivity against CMV infected fibroblasts of Vδ2- and Vδ2+ T-cells was tested using an IFN-γ ELISPOT.

**Results:** We observed an increased expansion of Vδ1+T cells after allo-SCT in patients during CMV reactivation as assessed by flow cytometry and spectratyping. Furthermore Vδ1+ but not Vδ2+T-cells from these patients reacted against CMV infected fibroblasts. Moreover, following pp65-reactive αβT-cells in HLA-A2+ patients indicated that Vδ1+T cells precede an αβT-cell response. Finally Vδ1+T cells were able to induce antigen independent maturation of dendritic cells (DC) via CD1c.

**Conclusion:** GdT-cells are present in patients after allo-SCT, have the potential to eradicate CMV infected fibroblasts and possibly the potential to spread an immune response to αβT-cells via DC maturation. This strongly supports the idea to explore gdT-cells as cell population for immune interventions after allo-SCT.

**I73 DEVELOPMENT OF CD19-SPECIFIC CENTRAL MEMORY DERIVED T CELL PRODUCTS FOR THE TREATMENT OF CD19+ HEMATOLOGIC MALIGNANCIES**

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A key parameter affecting the therapeutic potency of adoptive T cell transfer is the extent to which infused cells persist and expand in vivo. Ex vivo expanded CD8* effector T cells derived from CD45RO– CD62L+ central memory (TCM) precursors are epigenetically programmed to persist and revert to memory T cells following adoptive transfer. TCM are a rare subpopulation of lymphocytes in peripheral blood, therefore we sought to develop a clinical-scale immunomagnetic purification method to isolate CD8+ TCM for subsequent genetic modification to express CD19-specific chimeric antigen receptors (CAR). Using a two step process by which monocytes, naïve and CD4+ T cells are first depleted from PBMC, followed by positive selection of CD62L+ TCM, we have found that CD8+ TCM can be enriched from 2-8% to 80-90% purity. Following isolation, CD8+ TCM can be activated with CD3/CD28 Dynal beads, transduced two days later with eGFP grade self-inactivating lentivirus (MOI 1.5), then expanded with 50U/ml IL-2 and 0.5ng/ml IL-15. This process results in the procurement of cell numbers for clinical use within 10-14 days without further ex vivo stimulation (i.e., 1-3x10^6 final product from 7-15x10^6 CD8+ TCM). These TCM-derived effector cell products (TE(CM)) are 80-90% CAR+ and exhibit CD19-specific cytolytic function and IFN-γ production. Additionally, these products consist of a broad repertoire of T(CM) based on TCR Vβ usage, retain expression of central memory markers such as CD62L/CD28, and, upon adoptive transfer into immuno-deficient NOD mice, exhibit in vivo engraftment fitness in a huLL-15 dependent manner. This process has undergone a series of full scale qualification runs under cGMP and our group will be applying CD19-specific CD8+ T(CM) for post autologous HSCT adoptive therapy of high risk lymphoma as an innovative strategy to deliver engineered GVL effectors for eradication of minimal residual disease.

**I74 BIOTherapy ORDER ENTRY (BOE) – ELECTRONIC PHYSICIAN ORDERS FOR CELLULAR THERAPY PRODUCTS**

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Electronic systems for physician orders have largely focused on medications but orders for cellular products are also a potential source of communication errors that may affect patient safety. As part of a quality improvement effort, we developed and implemented an electronic order entry system for therapeutic cellular products of various types. Biotherapy Order Entry (BOE) was built to include three types of physician orders: 1) cell collection, 2) cell processing and 3) product release for administration. For each order type, electronic templates are created based on requirements of specific treatment plans or IRB-approved clinical research protocols. Each order set imports patient demographics from the hospital registration system and patient registration information from the Quality Assurance for Clinical Trials (QACT) information system. Individual templates for each treatment plan or protocol are reviewed and approved before activation and orders can only be placed after consent to a treatment plan or protocol has been confirmed. For example, template orders for collection of hematopoietic stem cells specify cellular product type and the target number (or range) of cells requested. Processing orders for hematopoietic stem cells specify manufacturing steps such as CD34 selection, cryopreservation or thawing and desired cell dose. Release orders specify the cell type and cell dose needed for administration. When cryopreserved products are available for administration, physicians can select individual products for release. Since templates are specific for individual treatment plans or protocols, physician order choices are restricted by the requirements of the protocol each patient has been registered to. This system was implemented in June 2010 after extensive in-house planning, development, validation and end-user training. This quality improvement process gave us the opportunity to review and improve the workflow processes in all areas (clinical, collections, registration, processing, information management) related to cellular therapy.