Metabolic Profiling of Regulatory T-cells from Umbilical Cord Blood and Adult Peripheral Blood for the Use in Adoptive Cell Transfer Therapies

Ali A. Mohamed

Todd M. Brusko Laboratory, Department of Pathology and Immunology and Laboratory Medicine, University of Florida, Gainesville, FL

Abstract:

Traditional methods of adoptive cell transfer therapy in Type I Diabetes involves the introduction of disease-modulating cells into a patient’s body; Regulatory T cells isolated from adult peripheral blood. In this context, little is known of the effects of the use of regulatory T cells derived from umbilical cord blood for the use of restoring immune tolerance in autoimmune diseases. The facilitated research is designed to address these effects by creating a better understanding of how the transcriptional profile of umbilical cord blood derived regulatory T cells differs from that of regulatory T cells isolated from adult peripheral blood. Moreover, these findings will have important implications for how these cells will traffic and engraft following adoptive cell therapy. In addition, convention T-cells derived from umbilical cord blood and blood from the periphery were included in the facilitated research and analysis. Establishing a comprehensive metabolic profile of these convention T-cells will have countless implications in expanding the overall understanding of immune function and autoimmune disease response regarding these cells, as well as progressing the development of clinical and therapeutic solutions to disease.

Significant interactions were found for basal respiration between umbilical cord blood convention T-cells & umbilical cord blood regulatory T-cells, spare respiratory capacity % between unacitvated adult peripheral blood convention T-cells & unactivated adult peripheral blood regulatory T-cells, and spare respiratory capacity % between activated adult peripheral blood regulatory T-cells & activated umbilical cord blood regulatory T-cells. Calculations regarding all other parameters for the area under the curve showed no significance. Fluctuation in graph form is apparent due to high variation between samples.

Although some significance was found across some parameters, this study showed that the transcriptional profile of regulatory T-cells from umbilical cord blood and adult peripheral blood is similar, therefore suggesting that these cells will traffic and engraft, following adoptive cell transfer therapy, in a similar fashion. This is however, based solely on the extracellular flux analysis conducted; other factors beyond cellular metabolic
performance may prove otherwise. Similarly, this study also showed some significant differences across some parameters between conventional T-cells and regulatory T-cells. These differences, however, do not elaborate enough on the metabolic differences between these two cell types because of the lack of significant differences observed across all other parameters including oxygen consumption rate and extracellular acidification rate.

1. Introduction:

Type 1 diabetes (T1D) is characterized by an autoimmune response, directed against the insulin producing pancreatic beta cells in children and young adults (1). As a result, beta cells die, rendering the diseased individual unable to produce insulin. Because of this, these individuals are required to monitor their blood glucose levels and maintain them via insulin injections for the rest of their lives. As T1D manifests at a relatively early age, therapeutic intervention has the potential to improve patients’ quality of life for an extended period of time.

Immune reactions, such as T1D, can be suppressed by the bystander activity of naturally occurring regulatory T cells (Tregs). These cells, CD4+, CD25+, CD127lo, and FOXP3+, are capable of repressing the immune response through T-cell receptor mediated cellular interactions and the production of repressive cytokines such as IL-10 and TGF-beta. Because of their immune suppressing capacities, Tregs have been considered potential therapeutic effectors in autoimmune diseases (2). In addition to stimulating Tregs inside the body, the idea has emerged to make use of Tregs’ anti immunity properties through an adoptive cell transfer.

Adoptive cell transfer has yet been used in a variety of diseases and is commonly used as immunotherapy for the treatment of cancer (3). Adoptive cell therapy proposes the use of cells rather than molecules as medication. The desired type and number of cells are usually obtained through isolation and ex vivo stimulation before infusing the cells back into the patient’s body where they can exert their desired immunological effect. In the context of type 1 diabetes, adoptive Treg cell transfer into non-obese diabetic mice suffering from T1D has shown to result in prevention or even reversal of the disease progression, providing promising results for the use of adoptive Treg cell therapy in humans (4).

Adoptive cell transfer therapies that introduce disease modulating cells of a specific phenotype into a patient’s body are going to be part of the clinician’s toolbox in the coming decades. To develop these treatments, minimally manipulated products must be designed that are as safe as what is feasibly possible. To this end, the facilitated research is designed to address the effects of the use of regulatory T cells
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derived from umbilical cord blood for the use of restoring immune tolerance in autoimmune diseases. The ability for cord blood Tregs to transition between glycolysis and oxidative metabolism is likely to impact their capacity for engraftment and long-term persistence upon adoptive cell transfer.

The metabolic program of UCB Tregs in comparison to peripheral blood Tregs was assessed using an extracellular flux analyzer (Seahorse Biosciences) and an established metabolic assay. The instrument calculates the extracellular acidification rate (ECAR) and the oxygen consumption rate (OCR) through the detection of O$_2$ and pH levels in real time. In this way, real time activation induced metabolic shifts can be observed (Fig. 1). In specific regard to ECAR and OCR levels, these are directly indicative of glycolytic and oxidative metabolism respectively (6). In addition, the following other parameters were assessed: basal respiration, ATP production, maximal respiration, spare capacity, non-mitochondrial respiration, coupling efficiency (%), spare respiratory capacity (%), and acute response. This was possible through the assessment of the impact of the applied agents (Fig. 2). These parameters further elaborate on cell metabolism, specifically in the context of quiescence and activation, both of which are extremely relevant in adoptive cell transfer therapies (7).

It is hypothesized that UCB Tregs will exhibit a transcriptional and metabolic program that will facilitate optimal engraftment, function, and trafficking when compared to Tregs isolated from adult peripheral blood.

**Seahorse XF Cell Mito Stress Test Profile**

<table>
<thead>
<tr>
<th>Agent</th>
<th>Effect on OCR</th>
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<tbody>
<tr>
<td>Oligomycin</td>
<td>Maximal Respiration</td>
</tr>
<tr>
<td>FCCP</td>
<td>Maximal Respiration</td>
</tr>
<tr>
<td>Rotenone &amp; antimycin A</td>
<td>Maximal Respiration</td>
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**Fig 1.** Schematic representation of the Agilent Seahorse XF Mito Stress assay. The Agilent Seahorse XF96 analyzer measures real time changes in pH & O$_2$ levels, metabolic impacts of applied agents at specific time points, and activation induced metabolic shifts. The following figure is demonstrative in the context of OCAR analysis, however, ECAR analysis was conducted simultaneously.

**Fig 2.** Schematic representation of the target of action of the applied agents on the complexes of the ETC.
2. **Methods:**

**Sample Collection and Mononuclear Cell Isolation**

“Fresh” CB (processed within 24 hr of cord blood draw) was collected by LifeSouth Community Blood Centers Corporate Headquarters, Gainesville, FL 32607 into CBUs containing 35 mL of citrate phosphate dextrose (CPD) anticoagulant. CBUs (n = 7) were then delivered to the University of Florida Diabetes Institute laboratories and immediately processed for isolation of cord blood mononuclear cells (CBMCs) after CD4+ enrichment by density gradient centrifugation (Ficoll-Paque PLUS, GE Healthcare).

IRB exempt LeukoPaks from adult peripheral blood were collected by LifeSouth Community Blood Center Peripheral, Gainesville, FL 32601. Blood was collected from healthy control subjects (males, mean age, 23.8 years; range, 20 – 29 years; n = 6) and processed within 24 hr for isolation of peripheral blood mononuclear cells (PBMCs) after CD4+ enrichment by density gradient centrifugation (Ficoll-Paque PLUS, GE Healthcare).

**Sample Processing and Isolation of T Cells by FACS**

All blood samples, PBS, 2% FBS, density medium (Ficoll-Paque PLUS, GE Healthcare) and centrifuge were at room temperature.

RosetteSep® Human CD4+ T Cell Enrichment Cocktail, STEMCELL Technologies Catalog #15022 was added at 50 μL/mL of whole blood (e.g. for 2 mL of whole blood, add 100 μL of cocktail). Then mixed well and Incubated 20 minutes at room temperature. Samples were diluted with an equal volume of PBS and mixed gently. Generally, 15 ml of whole blood, 15 ml PBS, and 15 ml density medium were added to a 50 ml conical centrifuge tube. Tubes were centrifuged at 1200 x g for 20 minutes for LeukoPaks and at 400 x g for 20 minutes for CB at room temperature, with the brake off.

**Sample conditions**

Regulatory and Conventional CD4+ T cells from CB and APB were expanded with anti-CD3/CD28 beads following protocol 3 as described in previous literature (5).

**Extracellular Flux Analysis**

OCR and ECAR measurements were performed on a Seahorse XF96 Analyzer (Seahorse Bioscience), using a modified version of the Cell Mito Stress Test Kit. Manufacturers’ procedures were followed unless stated otherwise. Briefly, samples were divided in activated (injection of 2.0μg/ml anti-CD3 and 1.0μg/mL anti-CD28) and non-activated (unactivated) groups. Addition of F(ab)2 antibody linkers are required for antibody effectivity (described shortly). Subsequent to three basal OCR and ECAR measurements, the activation cocktail was injected followed by five additional measurements. Next, three measurements during glycolysis induced
ATP production were acquired following the injection of the ATP synthase inhibitor oligomycin (2.5uM). Subsequently, three measurements were taken during maximal oxidative phosphorylation due to addition of the electron transport chain uncoupler FCCP (1.0uM). Finally, three measurements were taken during inhibition of both oxidative phosphorylation and glycolysis through the addition of Rot/AA and 2DG (1.0 uM and X uM respectively).

Cells were suspended in complete Seahorse medium to achieve 200k cells per well. Following cell seeding (Fig. 3),

the cell culture microplate was spun to 40g, rotated 180°, and spun to 300g (acceleration and break 1) and incubated in a non-CO2 incubator for 15min for optimal cell adhesion. Following, 100ul of complete seahorse medium was added without disturbing the adhered cells to bring the final well volume to 180ul, including IgG F(ab') 2 (final concentration of 11.12ug/ml) for half of the wells, dividing the samples in an activated and an un-activated group. Thereafter, the cell microplate was incubated for an additional 25 minutes prior to it replacing the calibration plate and being analyzed by the Seahorse XF analyzer.

Fig 3. All cells were seeded/plated using the demonstrated setup. Random selection was adopted to reduce the influence of batch effect.
3. Analysis:

The data was exported using Agilent Seahorse Wave Desktop and analyzed in Prism using a One-way ANOVA and a Tukey post-test with a significance level Alpha= 0.05 (95% confidence interval).

4. Results:

Significant interactions were found for basal respiration between umbilical cord blood convention T-cells & umbilical cord blood regulatory T-cells, spare respiratory capacity % between unacitvated adult peripheral blood convention T-cells & unactivated adult peripheral blood regulatory T-cells, and spare respiratory capacity % between activated adult peripheral blood regulatory T-cells & activated umbilical cord blood regulatory T-cells (Fig. 4). Calculations regarding all other parameters for the area under the curve showed no significance. Fluctuation in graph form is apparent due to high variation between samples (Fig. 5, 6, and 7).

Fig 4. Significant interactions were found for basal respiration between UCB Tconv & UCB Tregs, spare respiratory capacity % between APB TConv & APB Tregs (unactivated), and spare respiratory capacity % between APB Tregs & UCB Tregs (activated).
Fig. 5 Graphs depicting OCR and ECAR data across time and all groups. Apparent differences in OCR and ECAR values occur at specific time points in which different agents were applied. Refer to Fig. 1 for a schematic overview. Fluctuation in graph form is apparent due to high variation between samples and is not representative of a significant difference between interested groups. Refer to Fig. 6 & 7 for a more specific visual representation of OCR and ECAR data between interested groups.

Fig 6. Graphs depicting OCR and ECAR data across time in a comparative context between activated APB Tregs & activated UCB Tregs.
Fig 7. Graphs depicting OCR and ECAR data across time in a comparative context between (from top to bottom) activated UCB Tregs & unactivated UCB Tregs, activated APB Tregs & unactivated APB Tregs, unactivated APB Tregs & unactivated UCB Tregs, activated APB & activated APB Tregs, unactivated APB Tconv & unactivated Tregs, unactivated UCB Tconv & unactivated UCB Tregs, and activated UCB Tconv & activated UCB Tregs.
5. Discussion:

The observed significant interactions in metabolic behavior indicate slight differences in cellular metabolic function between the compared groups (Fig 4.). Differences observed in spare respiratory capacity % between unacitvated adult peripheral blood convention T-cells & unactivated adult peripheral blood regulatory T-cells, and between activated adult peripheral blood regulatory T-cells & activated umbilical cord blood regulatory T-cells signify differences in these cell’s overall capacity for energetic demand responses. In addition, this parameter measures how closely cells are to respiring at their theoretical maximum. This is relevant because the cell’s ability to respond to energetic demand responses can be indicative of cell fitness and/or flexibility (10). In the context of adoptive cell therapy, these characteristics are synonymous of quiescent cells, making this parameter a favorable one and a distinguishing factor in cell type selection for these therapies (11).

Spare respiratory capacity %, was greater in activated adult peripheral regulatory T-cells when compared to activated umbilical cord blood T-cells and in unactivated adult peripheral blood when compared to unactivated adult peripheral blood conventional T-cells. This indicates that the cells with a greater respiratory capacity %, when compared to their respective counterparts in which significance was found, are more oxidative and therefore more capable of producing additional ATP in cases of sudden increases in energy demand. This observation challenges the hypothesis, indicating that regulatory T-cells from adult peripheral blood are more suitable for adoptive cell transfer therapies (10).

An additional observation, basal respiration, signifies differences between regulatory T-cells and the conventional T-cells that were included in the facilitated research and analysis. Umbilical cord blood regulatory T-cells demonstrated lower levels of basal respiration when compared to umbilical cord blood regulatory T-cells. This indicates that these T-cells consume lower levels of oxygen to meet cellular ATP demands; under baseline conditions, these cells exhibit less of an energetic demand (12).

Overall, the results of this study are highly specific. The significant interactions observed cannot be generalized to the same cells of unactivated/activated populations or to cells from umbilical cord blood/adult peripheral blood. Because of this, it is difficult to compose an overall metabolic profile of each cell type since this study depends on comparative distinctions that could not be drawn because of the lack of overall significant differences across all groups.

Despite the significant interactions observed and based solely on the extracellular flux analysis conducted, it is suggestive that the transcriptional profile of regulatory T-cells from umbilical cord
blood and adult peripheral blood is similar in the context of metabolism. Because these two cells showed no significant differences in metabolic behavior across all parameters before and after activation, with the exception of spare respiratory capacity %, it is also suggestive that these cells will traffic and engraft, following adoptive cell transfer therapy, in a similar fashion. Other factors beyond cellular metabolic performance may prove otherwise.

Similarly, conventional T-cells showed no additional significant differences in metabolic behavior across all parameters before and after activation, besides basal respiration and spare respiratory capacity %, signifying an overall insignificant interaction between the metabolic profiles of both cells. Again, these indications are based exclusively on the conducted extracellular flux analysis and do not account for other factors beyond the assessed cellular metabolic performance observed by the study. If this is the case however, regulatory T-cells and conventional T-cells may be more similar than expected.

Although the facilitated research does not holistically address the effects of the use of regulatory T cells derived from umbilical cord blood for the use of restoring immune tolerance in autoimmune diseases, it challenges a generalized theory in the world of immunology. Ultimately, the lack of a significant difference between regulatory T-cells from umbilical cord blood and peripheral blood undermines the idea that cells derived from umbilical cord blood are superior in the context of adoptive cell therapy; demonstrate a greater capacity to transition between glycolysis and oxidative metabolism. Several studies delve deeper into this theory, specifically proposing the use of hematopoietic cells derived from umbilical cord blood (8). Other studies, with the same interest in stem cells, examine the differences between blood derived from the bone marrow in addition to the umbilical cord and the periphery (9). Clearly, the idea of cells behaving differently based on their source is an idea that has been studied for a long time. Respectively, data from these studies indicate that this is the case, however the results from this study narrow these conclusions and make them less generalizable. The differences in cell behavior demonstrated by the discussed studies are exclusive to stem cells and all proceeding data from other relevant studies may follow this trend of exclusivity across all cell types.
Star methods:

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References:


