Amino Acid Analysis Indicates Metabolic Differences in Multi-Cytokine Backpack - Manufactured CAR T-Cells

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Overview

Novel reagents that aid in the consistent manufacturing of CAR T-cells with well-defined phenotypes are needed to improve treatment outcomes in patients. Traditional *ex vivo* expansion reagents provide only TCR activation and co-stimulation. Recently developed multi-cytokine backpacks (MCBs, **Fig 1**; Ref 1) have been shown to provide CAR T-cell manufacturing with additional cytokine support, critical for cell expansion, differentiation, and the selection of potent, long-lived phenotypes. A commonly used method for *ex vivo* activation is using the cytokine IL-2 with Dynabeads™. In this project (Ref 1) different combinations of immunomodulating proteins were used to create an MCB library. The activated cells were tested for viability, CAR expression, phenotype and *in vivo* activity (Ref 1). Here we describe the amino acid (AA) analysis done with the REBEL device (908 Devices) to assess differences in the metabolic activity in these activated cells. The differences in metabolism were measured for all groups with Dynabead + exogenous IL-2 as the control and a reference point.

Figure 1

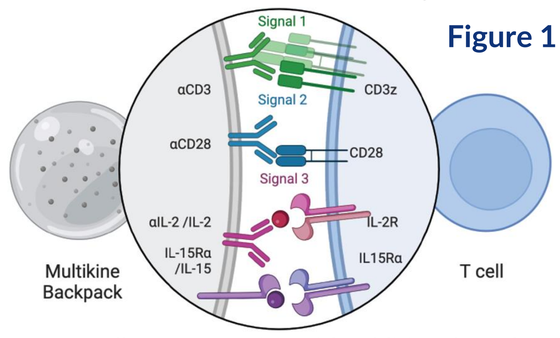


***Figure 1**: Multi-cytokine backpacks induce activation in CAR T-cell manufacturing with additional cytokine support, which is critical for cell expansion, differentiation, and the selection of potent, long-lived phenotypes.*

Methods

The T-cells were isolated and transduced as described in Ref 1; **Fig 2**. After activation, the cells were expanded in RPMI-1640 + 10 % FBS in a total volume of 200µL. AA concentrations were measured from the fresh and spent (end-point) media using the REBEL (908 Devices Inc.).
The nutrient compositions of the final CAR T-cell product spent media were analyzed to identify differences in their cellular metabolism. The ratio of the mean AA concentrations from the microparticle backpack library samples to the mean concentrations from the control samples (Dynabead + IL-2) were plotted using 908 Devices add-in for JMP software.

Figure 2

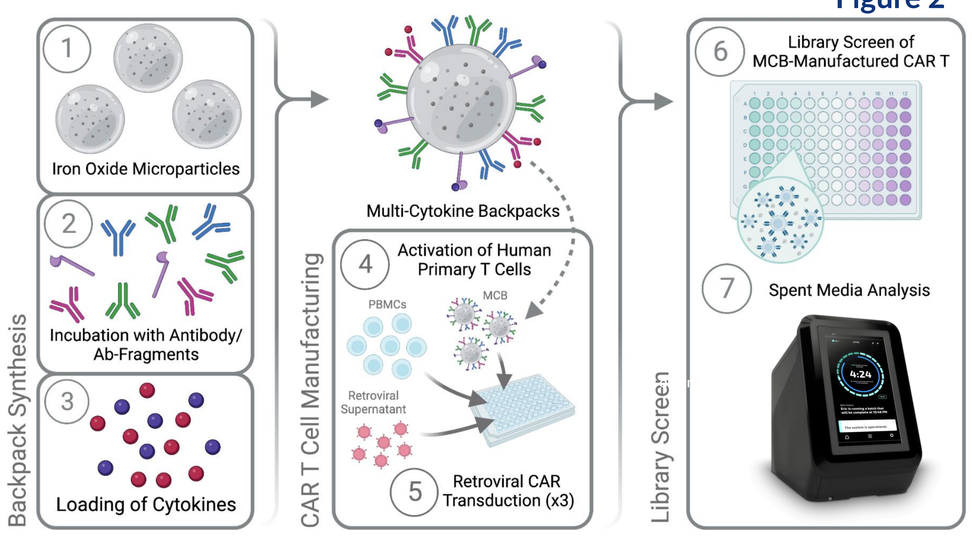
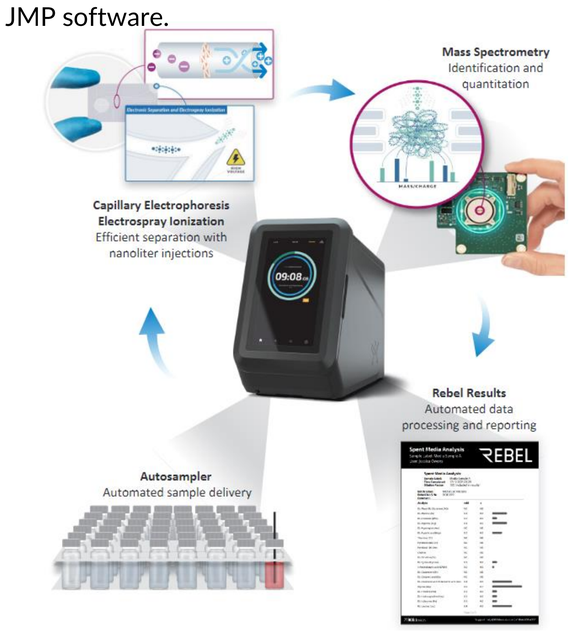


***Figure 2**: CAR T-cell manufacturing using the multi-cytokine backpacks for activation. The resulting T-cells were tested for viability, CAR expression, phenotype and in vivo activity (Ref 1).*

The REBEL at-line cell culture media analyzer: Actionable information of your bioprocess at the point of need

AA concentrations were measured from the fresh and spent (end-point) media using the REBEL (908 Devices Inc.), a capillary-electrophoresis (CE) - mass spectrometry (MS) -based device for AA analysis (**Fig 3**). CAR T-cell products were isolated via centrifugation / 400xg for 5 minutes, and the cell-free supernatant was diluted 1:10 with manufacturer-provided diluent. Automated quantitation of AAs for each sample was achieved using embedded calibrations. Two biological replicates of each sample were analyzed in triplicate using the REBEL.
The nutrient compositions of the final CAR T-cell product spent media were analyzed to identify differences in the cell metabolism, and the measured levels were plotted using 908 Devices add-in for JMP software.

Figure 3



REBEL Spent Media Analysis Kit

- Minimal sample requirement as low as 10 µL
- Simple sample prep: spin or filter and dilute
- Integrated analyzer includes autosampler, separation, detection, analysis and reporting
- Analysis run-time ~10 min per sample
- Consumable kit optimized for 200 analyses

REBEL analyte panel

Amino Acids						
Alanine	Asparagine	Glutamic Acid	Histidine	Lysine	Proline	Tryptophan
Alanyl-Glutamine	Aspartic Acid	Glutamine	Isoleucine	Methionine	Serine	Tyrosine
Arginine	Cystine	Glycine	Leucine	Phenylalanine	Threonine	Valine
Vitamins etc.						
Choline	Nicotinamide	Pyridoxal	Pyridoxine	Thiamine	Betaine	
Amines						
ß-Alanine	Citrulline	GABA	Hydroxy-proline	Methyl-Histidine	Sarcosine	

Results

Based on several biological assays, the MCB library compounds were clustered into "high", "intermediate", and "low" groups with respect to proportions of CD8+ and CD4+ less-differentiated CAR T-cell phenotypes (T central memory cells (T_{CM}) and T stem cell memory cells (T_{SCM}); please see Ref 1). A focus on the top candidates (#09, #16, #27, and #43) spent media samples interestingly showed differences as compared to control in concentration for the AAs Ala, Gln, Gly, Pro, Ser, Thr (selection shown in Fig 6). It was observed that some AAs (ie. Ala, Gly, Pro) were accumulating in the media during culture in all samples, whereas some (Arg, Ser) were consumed (Fig 5).

Amino acid levels in the fresh medium

Figure 4

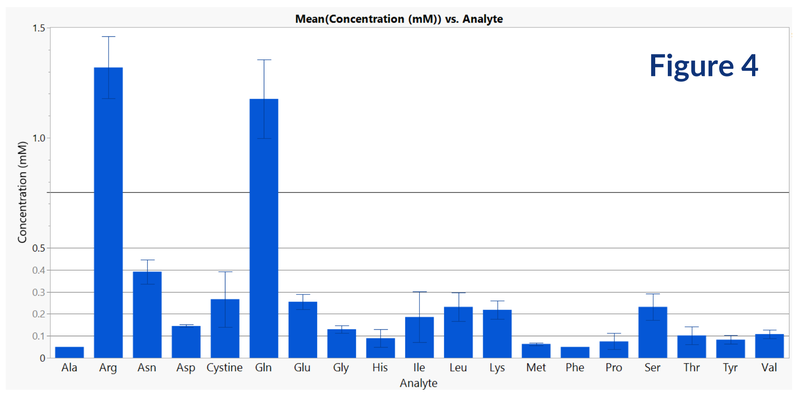


Figure 4: The amino acid levels in the cell culture medium used in the multi-cytokine backpack T-cell activation (RPMI-1640 with L-glutamine (VWR) with 10% FBS (Corning), 100IU/ml penicillin and 100 ug/ml streptomycin (Gibco)). Cystine (dipeptide) is measured instead of Cysteine, and Tryptophan was below the detection range. The cell culture medium contains low levels of amino acids, including low levels of essential amino acids.

Control samples for activated CAR T-cells –fresh and spent media analysis

Figure 5

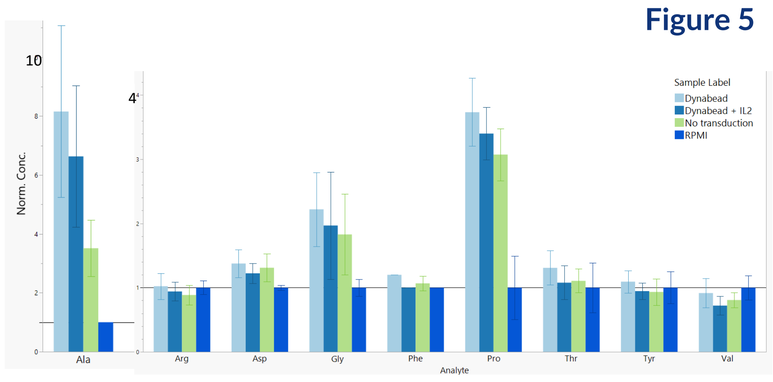


Figure 5: AA levels of the spent media from the control samples were compared to the fresh medium used for CAR T-cell manufacturing. Please note that the AA levels have been normalized to the fresh media concentration ("RPMI" – bright blue), as well as the difference in scale for the AA Ala. It was observed that some AAs (ie. Ala, Gly, Pro) were accumulating in the media during culture in samples, whereas some (Arg, Val) were consumed.

This analysis may give an indication of certain metabolic pathways specific to amino acids (such as Proline) being active in T-cells, as also indicated in Ref 3.

Multi-cytokine backpack – activated CAR T-cell spent media analysis

The nutrient compositions of the final CAR T-cell product spent media were analyzed to identify differences in their cellular metabolism. The AA levels of all spent media samples were compared with Dynabead + exogenous IL-2 –activated T-cell spent media as the control and a reference point. Compounds from the multi-cytokine backpack library were categorized using cluster analysis. Compounds resulting in different phenotypes, with respect to CD8+ and CD4+ levels for less-differentiated CAR T-cell phenotypes as were clustered as "high", "intermediate", and "low" performance (Ref 1).

Figure 6

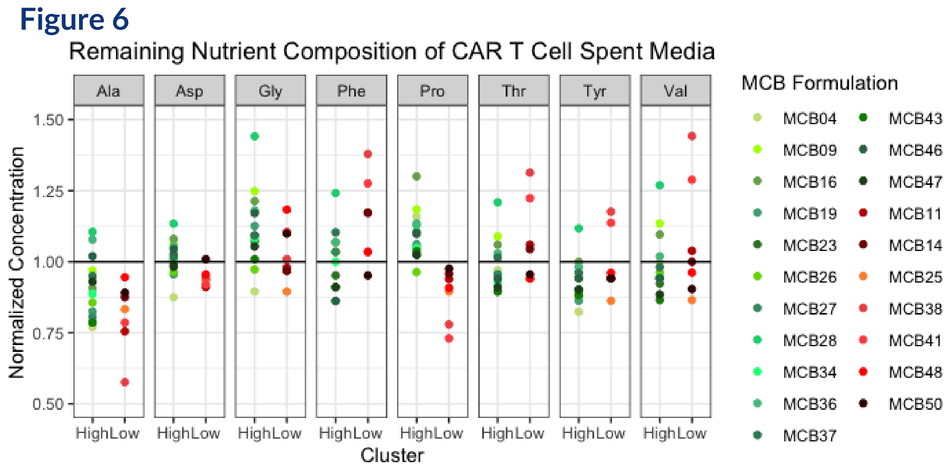


Figure 6: Eight amino acids selected here showed some differences in spent media levels;
1) as compared to the control Dynabead + IL-2 activated T-cell spent media (concentrations used for normalization, black line);

2) between the spent media samples from the "High" and "Low" MCB library compounds. For example, Ala was accumulating less in Dynabead+IL-2 than in the MCB activated T-cells. Pro was accumulating more in the "High" group (less differentiated T-cells) than in the "Low" group.

This may indicate different metabolic pathways being active in less differentiated CAR T-cell products.

Conclusions

AA analysis is not regularly performed as part of CAR T-cell experiments due to the complexity of many traditional analysis methods and sample volume limitations. With automated quantitation and low sample volume requirements, the REBEL device enables rapid analysis at the bench side with no requirement for prior MS expertise.
Studies to define relationships between AA dynamics, T-cell metabolism, and CAR T-cell biology are currently in progress; however previous findings by Ref 2 indicate that AAs are critical for efficient T-cell activation and proliferative responses.

With this work presented here, and in Ref 1, we demonstrate a possible correlation between CAR T-cell activation, nutrient metabolism, and efficacy. The "high", and "low" activity MCB compounds with respect to CD8+ and CD4+ less-differentiated CAR T-cell phenotypes (T_{CM} and T_{SCM}) showcased some differences in post-manufacturing spent media amino acid analysis. This also highlights the opportunity to optimize cell culture media for the *ex-vivo* manufacturing of CAR T-cell therapies.

References

1 - Lin H and Uricoli B *et al*. Adv Healthc Mater. 2024 Jan 21:e2302425.
2 - Cobbold SP, *et al*. Proc Natl Acad Sci USA (2009) 106:12055-60
3 – Ye L, *et al*. Cell Metab (2022) Apr 5;34(4):595-614