Deterministic cell separation revolutionizes cell engineering for advanced therapies.

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NOTE

Curates "Modernizing Cell Therapy Manufacturing with Microfluidics" white paper describes benefits of DCS processing in the context of known biological pathways and is available at www.curatebio.com/presentations. This article extends that work, linking new epigenetic understandings, and new insights on mechanistic differences. Relevant summary data are included here to provide context.

Overview

Curate's Deterministic Cell Separation (DCS) and mechanism of action has shown considerable biological advantage compared to existing cell selection approaches. These benefits can be explained in part by the integration of a highly efficient wash that largely eliminates exposure to soluble signaling factors such as cytokines and chemokines, and platelets. DCS provides a unique ability to isolate PBMCs using a non-centrifugal, low cell-cell contact, or low tonic signaling, technology. By contrast, we show that cells using industry standard strategies including centrifugation, and positive or negative immunoselection induce substantial changes in the epigenetic profile of T cells, and in particular naïve T cells, which are specifically important in T-cell therapy efficacy[1,2]. This is a critically important benefit as the trend towards higher number of gene edits and ever shorter processes coincides with the drive toward a more effective cell therapy manufacturing solution.

During the development of the Curate Cell Processing System, we observed that the gentle, uniform size-based separation and washing process, aka Deterministic Cell Separation or "DCS", consistently recovered leukocytes without reduction or loss of specific cell subsets (such as naïve cells) when compared to centrifugal techniques[3,4]. In addition, we found that DCS cells visually appeared "healthier" or "vital" under brightfield microscopic examination. We observed that a higher percentage of T cells recovered by DCS were less differentiated, more readily transduced, and activated and expanded in a more synchronized or coherent manner[3,5,6].

Initially, the superior vitality of DCS processed cells was ascribed to benefits coming from DCS' intrinsic 3-log cell wash which quickly removes erythrocytes, platelets and soluble cytokines causing cell activation and differentiation as well as the avoidance of metabolically destructive chemicals such as ammonium chloride lysis[7-12]. Unexpectedly however, we found that a less disrupted mitochondrial state persists through multi-divisional cell expansion, raising the possibility that additional epigenetic changes might be behind the durably observed differences. For clarity, in this context "epigenetics" is defined as changes in gene activity that occur without altering the underlying DNA sequence, and critically that those changes persist across multiple cell divisions.

Engineering Haematopoietically derived Cells

Effective transcription of DNA by ribosomal apparatus into RNA more readily occurs when the chromatin complex is in an "open" or non-condensed state[13]. Chromatin condensation is controlled by a highly conserved methyltransferase/acetylation system involving histone proteins residing within the chromatin. In particular the transcription "off" switch is achieved by the EZH2 methyltransferase, which methylates a lysine residue on Histone H3 (H3K27) causing chromatin condensation[14-17].

In the case of haematopoietically derived cells, Ji et al. generated the first comprehensive hematopoietic DNA methylomic map and identified changes in DNA methylation associated with myeloid and lymphoid lineage commitment[18]. Subsequent work by Komori, Gray and Laugsen pinpointed the central role of EZH2 in normal CD4 and CD8 T cell differentiation from memory to effector state, and also in oncology related aberrancies[19-21], leading to acceptance of the methylation status of H3K27 as a measure of epigenetic availability, wherein hypermethylation indicates the transcription "off" state[22-25]. As shown in Figure 1, cells with condensed chromosomes driven by methylation of H3K27 will be driven away from their naïve state down a differentiation path to a variety of mature cell types.

The broad impact (and potential reversibility) of the EZH2 activity on T-cell differentiation has made it an attractive target for inhibitor development. As a result, inhibitors such as Dasatinib which acts

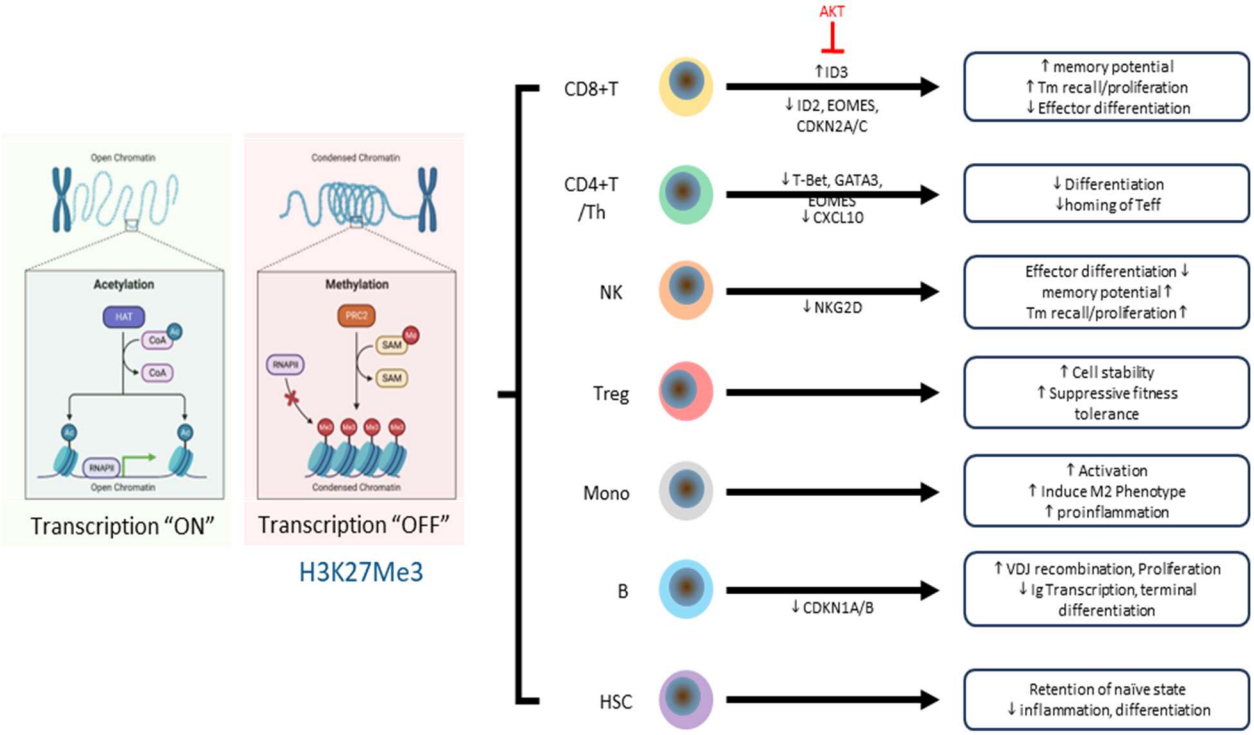


Figure 1 shows some of the haematopoietically derived cells that are relevant to cell and gene therapy, and the function of EZH2 on their development and differentiation, which involve multiple pathways of significance within the immune system. (Figure adapted from Biorender, The functions of EZH2 in immune cells: Principles for novel immunotherapies, J Leukocyte Bio. 10.1002/JLB.1RU0520-311R)

on downstream T-Cell Receptor signaling kinases, and Tazemetostat, a direct inhibitor of EZH2 have been developed.

This is relevant for chimeric antigen receptor (CAR) T cell therapeutic developers because terminal T-effector cell differentiation and associated exhaustion limits immune response to cancer, and is a major cause of resistance and relapse to CAR-T therapeutics[26-28]. To combat this issue, recent studies demonstrated[27,29] that Dasatinib and Tazemetostat[30] abrogate the tendency of T cells towards exhaustion during CAR-T production, enhancing CAR-T product efficacy[31-33]. In parallel, the use of direct cellular engineering to re-direct and actively control cell activation and differentiation[34], implementing earlier harvest time points prior to cells differentiating [35,36], and even the utilization of shorter processes using *in-vivo* expansion[37-38] have also shown consistent pre-clinical promise. However, these strategies also bring additional cost, engineering, and/or validation/safety assessment complexity. DCS unique processing approach should provide a better way.

The power of a uniquely better mechanism of action

Curate's DCS is the world's fastest microfluidic device, bringing a fundamentally different approach to cell separation. Separating cells using their size, the system operates much like a pachinko machine or coin sorter, only at the scale of a few microns. Cells separate based on size as they pass through an obstacle array that is sized and positionally defined such that cells smaller than the design criteria pass straight through the device, and cells that are larger are gently and rapidly deflected into a clean buffer stream, ***a rapid, continuous, non-cell co-locating process*** (Fig. 2)**.**

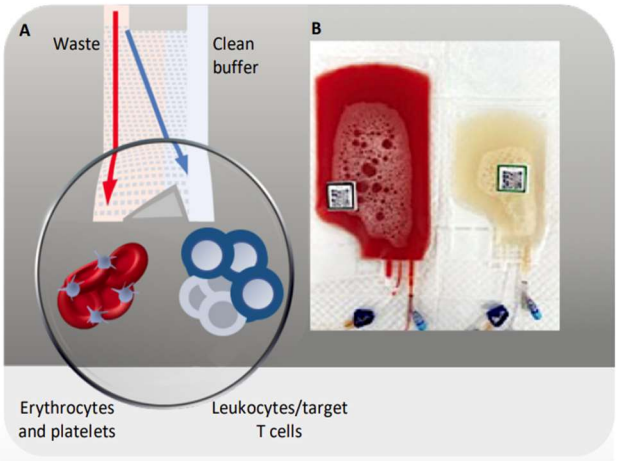


Figure 2. (A) Simple schematic showing how sample enters the array and large cells (leucocytes) are individually moved into the clean buffer stream and separated from waste. (B) Image of Deterministic Cell Separation™ (DCS)-processed leukopak fractions: waste (left), targeted leukocytes/product (right).

In contrast, other commercial technologies (Fig. 3) require a physical co-location of cells in an dense interface during "batch" processing that occurs for a minimum of 15 minutes, and a discrete extraction phase to remove biologically active components, such as platelet aggregates and soluble cytokines/chemokines. Following processing, cells are diluted to reduce the highest cell density that occurs during processing. The challenge with these approaches is twofold. First, cell:cell interactions are exacerbated during processing steps that concentrate cells by centrifugal force or against a membrane. And second, technologies in common use today are relatively ineffective at removing platelets and soluble factors driving cell signaling (I,II). To make matters worse, multiple steps in the process create these high cell concentrations (III), and frequently require used of red cell lysis agents as part of the process (IV). For example, density gradient separation requires 3-4 cell pelleting events to obtain an acceptable apheresis scale PBMC prep for an immunostaining reaction. Even a simple immunoselection requires 3 high cell density washing steps. In sharp contrast, DCS processing does not force cells to co-locate

either at an interface or via centrifugation during the short second that it takes to individually process the cells into a clean buffer stream.

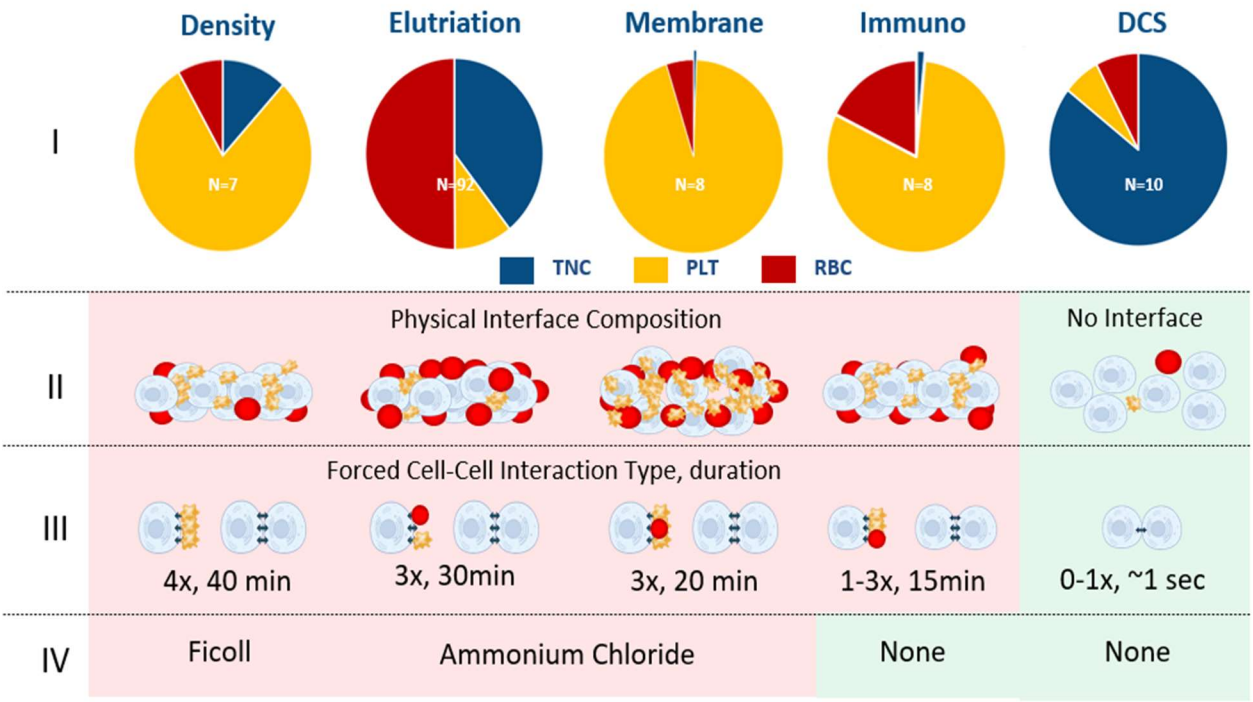


Figure 3. Schematic showing the fundamental differences in purified product composition (I), interface generation, and composition at the interface (II), expected forced cell:cell interactions at the interface, including typical number of interactions and potential interaction partners, and their typical interaction time (III).and typical chemical exposure further required (ex: Ammonium Chloride for red cell lysis), Data in Pie charts in row (I) was obtained from 3rd party collaborations, using cell counts determined by hematology counter. Elutriation data includes a 500mL Ammonium Chloride lysis step Membrane data is published manufacturer data. Immunoselection is membrane mfg. data separated using a 99% depletion assumption.

Figure 4 shows comparisons of DCS as compared to Ficoll based separations. Panels a, b provide a graphic overview of showing the range and robust nature of WBC recoveries and viability resulting from DCS processing. Figure 4 c, d show the positive bias towards, showing the DCS processing recovering more of the naïve and central memory T cells on an absolute and relative basis compared to a Ficoll process; which is known to lose naïve cells during processing to rosette formation with eryhtrocytes[39]. Figure 5 shows DCS' superior naïve T-cell retention compared to elutriation, affirming that systematic bias for density based approaches is present. Data from a 3rd party collaborator.

***Figure 4**. Recovery and viability metrics of DCS (Blue) vs. Ficoll (Grey) methodologies. Data presented as %Average (Std. Dev)*

- ***a*** *Recovery of total nucleated cells compared to density gradient approaches: DCS ~89.3% (5.9) vs. Ficoll 56.4% (17%). N=48*
- ***b*** *WBC Viability as determined by 7-AAD negativity of CD45+ gated WBC cells measured ~2Hrs post processing. DCS 95.8 (3.0) vs Ficoll 94.7 (3.8%) n=48, N=65 (DCS)*
- ***c,d*** *Recovery and subset analysis of CD3+ T cells from 12 normal donor apheresis samples that were split 80% to DCS, 20% to Ficoll. Data normalized to reflect a 200mL apheresis volume. White blood cell counts were determined by coulter counter and T cell counts were multiplied by the CD3 as a percent of CD45+ PerCP cells. T-subsets used CD3/CD45RA/CCR7/CD4/ CD8.*

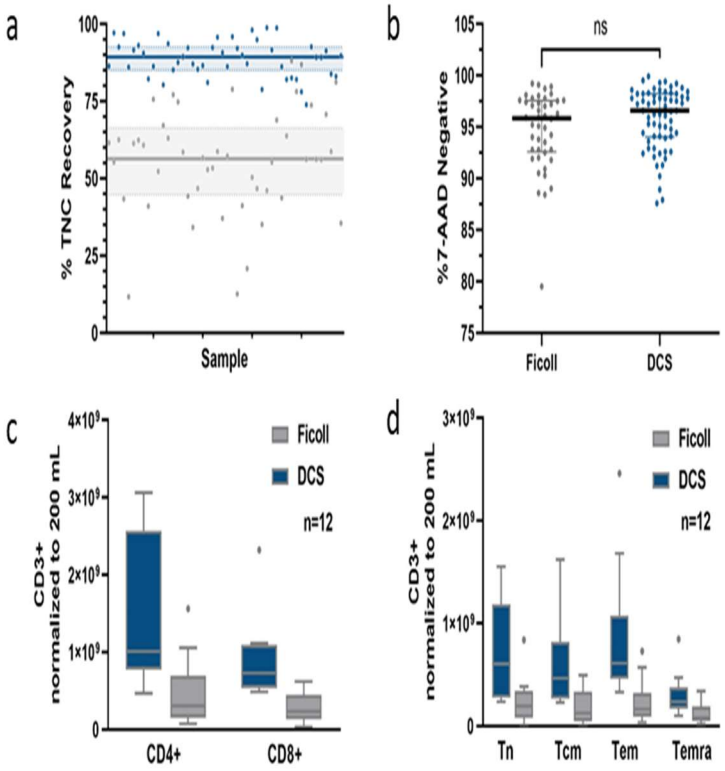
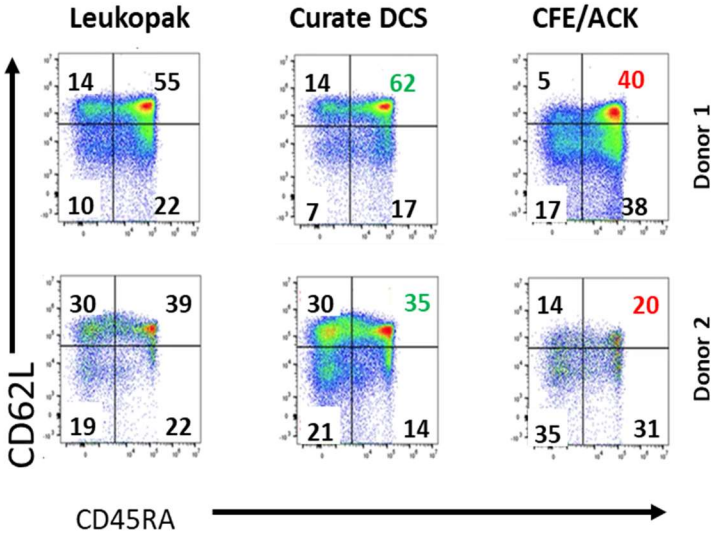


***Figure 5**. 2 Donors were split and processed either by DCS or by counterflow elutriation using ammonium chloride lysis protocol (CFE/ACK). Flow cytometric analysis using and alternate T cell memory compartment using CD62L in conjunction with CD45RA reveals that DCS retained approximately 60% more Naïve T cells (green) vs. the counterflow elutriation process (red). 3rd Party data.*



Pathway-specific benefits of a 3-log wash in under a second

In our previous white paper, graphically summarized in Figure 6 below, we identified multiple overlapping benefits of DCS processing including higher initial recovery and wash performance that better retains desired phenotypes and delivery of a better overall T cell starting material and process for CAR-T. Specifically, physical recovery of at least 40% more leukoctyes that were ~50% less pre-activated, due in part, to the 3 log wash which significantly reduces platelet and T-cell derived cytokines (1/18x TGF-β, 1/10x PDGF, 1/50x PAI-1 and 1/114x Gro-α) and chemokines (1/8x RANTES level) in the sample. Further, it showed that just 30 minutes of exposure to centrifugal milieu was enough to double the amount of inhibitory regulatory T cells that were generated in culture. Intentional activation using CD3/CD28 microbeads, and further expansion using IL-7/ IL-15 resulted in ~30% more cells by day 12. In addition, cells prepared this way show a 30-50% increased integration of lentivirus, a finding that occurs irrespective of cells being fresh or frozen/thawed. In combination, these gains show that synergies can contribute to a robust manufacturing and engineering path and be wholly compatible with a shorter process, potentially as short as 2 days, and also infer higher per cell functional efficacy may be available.

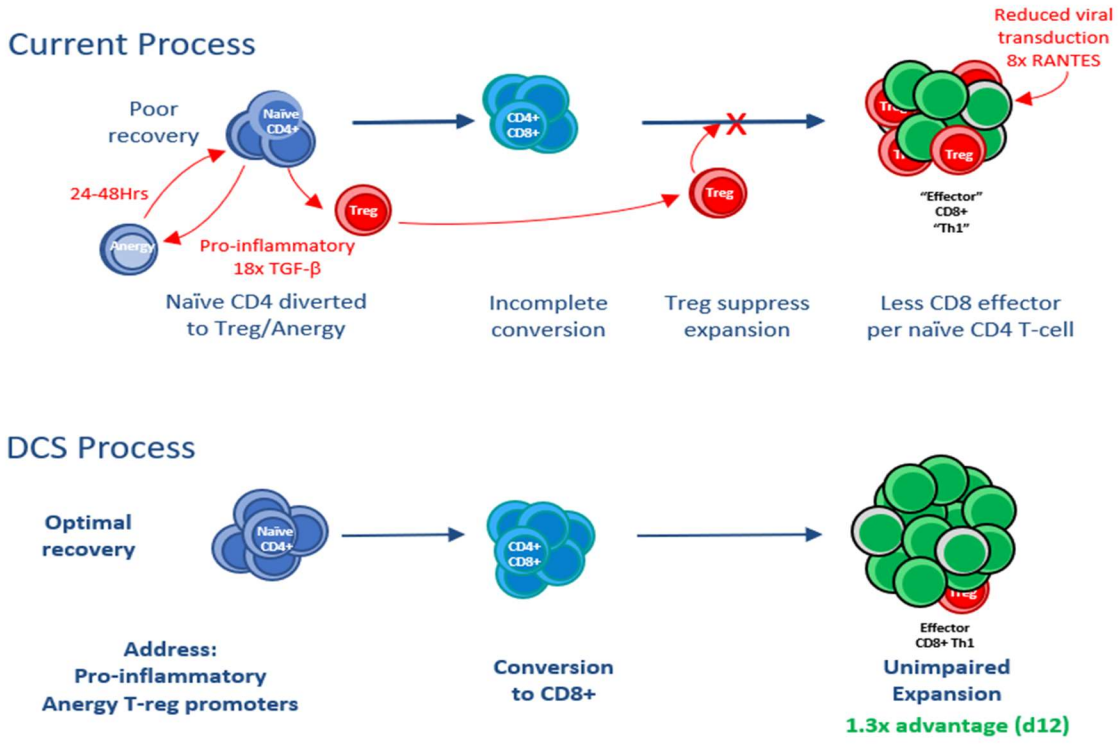
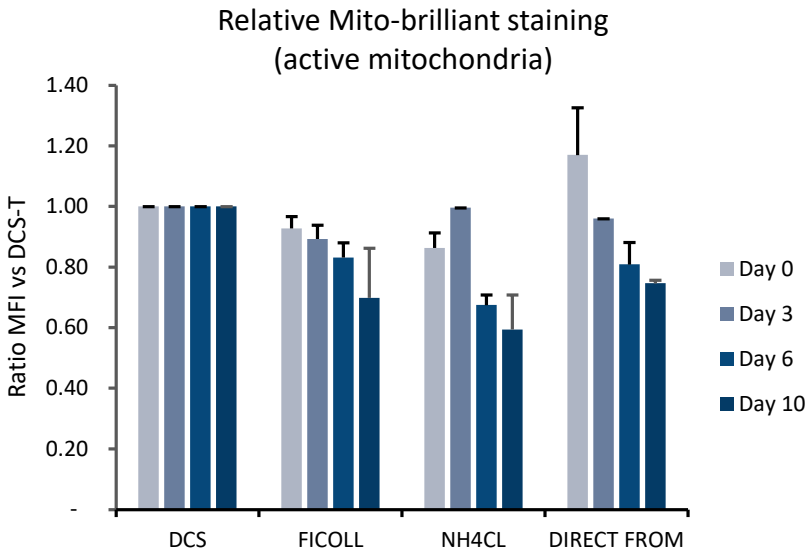


***Figure 6**. Graphical Summary of DCS prototypic known pathway advantage for a CAR-T process. High recovery of the right cells, with elimination of activation, anergy and reduced pathway specific biology from cytokines and chemokines allow a more effective T cell expansion and retention of a generally younger T cell phenotype and less exhausted profile without addition of drugs.*

DCS Processing Preserves Metabolic and Epigenetic State

To better understand the nature of the ~30% expansion benefit we evaluated active mitochondrial mass in either fresh, or frozen/thawed PBMCs. Reasoning that cells prepared by DCS had not been subjected to co-location or chemical processing, we used DCS cells to test methods and buffers commonly used in cell and gene therapy. As shown in Figure 7, we observed up to 40% relative reduction in the amount of active mitochondrial mass. We also found that the relative impairment of the mitochondrial state was durable and accentuated over the course of a 10-day expansion, inferring that epigenetic factors could be at play.

***Figure 7**. Mitochondrial mass of cells prepared using a variety of debulking and selection techniques prior to expansion. DCS prepared PBMCs were exposed to routine processing with Ficoll, Ammonium Chloride, and magnetic negative selection from the start matrix ("direct from"). Following selection, T cells were activated with TransAct and supplemented with IL-7, IL-15 during expansion. Mitotracker/Mito-Brilliant stains performed. Data expressed as ratio of MFI-Test T/MFI-T DCS preparation. (5 donors, 6 experiments, 3 fresh, 3 frozen).*



To assess cell signaling impairment, we evaluated phosphorylation states (pan Phospho-Tyrosine) in similarly treated cells. We found modest activation with a Ficoll protocol (Fig 8a), aligned with our earlier work showing similar rates of CD69 activation. Following 10' of ammonium chloride exposure, a substantial reduction (>30%) of phosphorylation was observed. This is likely due oxidative stress response (Fig 8b), and consistent with the literature showing both metabolic and known tumor promoting effects of ammonium chloride[40-43].

To understand if this phenomenon persisted through a freeze/thaw cycle, DCS prepared PBMC were frozen in CS-10 and thawed 1 month later (Fig. 9). Cells were either processed with Ficoll or left untouched prior to activation and culture. T-cells were stained on Day 6 to assess the phosphorylation and tri-methylation state of Histone H3K27 using the same staining protocol as used in Figure 8. Cells that were not further processed after thawing showed a slightly higher level of phosphorylation compared to the cells further re-processed with Ficoll, indicating that the DCS cells were slightly more responsive (Fig 9a). Interestingly the methylation staining of unselected, activated T cell cultures showed almost twice the amount of tri-methylation in cells that were processed with Ficoll relative to those not further processed after thawing (Fig 9b). These data suggest that cell separation processes that force high-density cell co-location can drive previously unseen and persistent changes in cell response to activation.

Figure 8. *DCS-prepared PBMC were thawed and exposed to cell separation reagents. Samples were fixed within 10 min and differential phosphorylation was measured using a pan-Phospho-Tyrosine FITC. Samples were stained with CD3/CD4/CD8, and a Far-red fixable viability dye prior to fixation and permeabilization for intracellular staining with pan-Phospho-Tyrosine FITC. Singlet live CD3 cells were gated. a) In both CD4+ and CD8+ cells we observed an increase of phosphorylation in the Ficoll-processed cells of 8.57% and 16.9% respectively relative to non Ficoll processed cells, due to Ficoll initiated activation. b) After processing with ammonium chloride (as would be performed to remove erythrocytes), CD4+ and CD8+ cells exhibited a 43.16% and 32.62% reduction in phosphorylation relative to DCS cells due to metabolic damage. Representative Data, n=3*

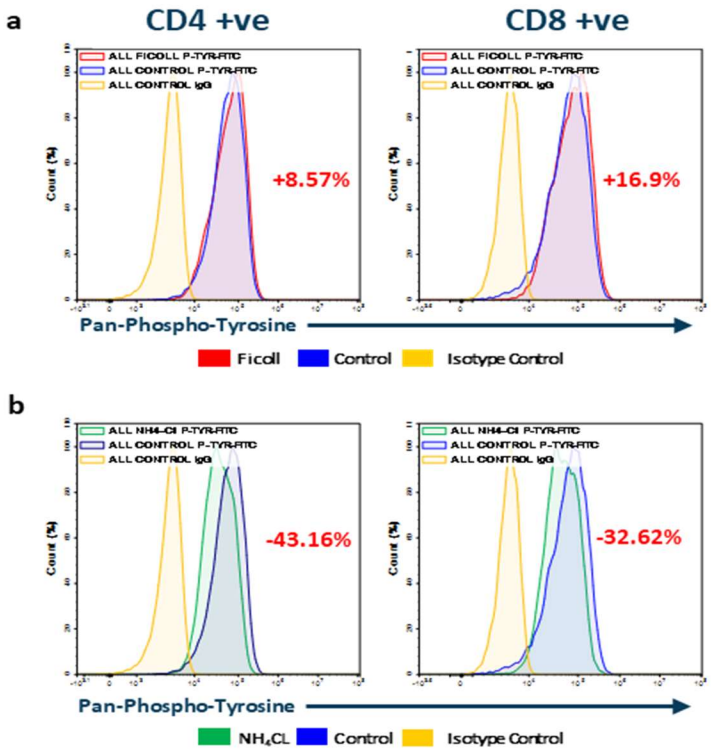
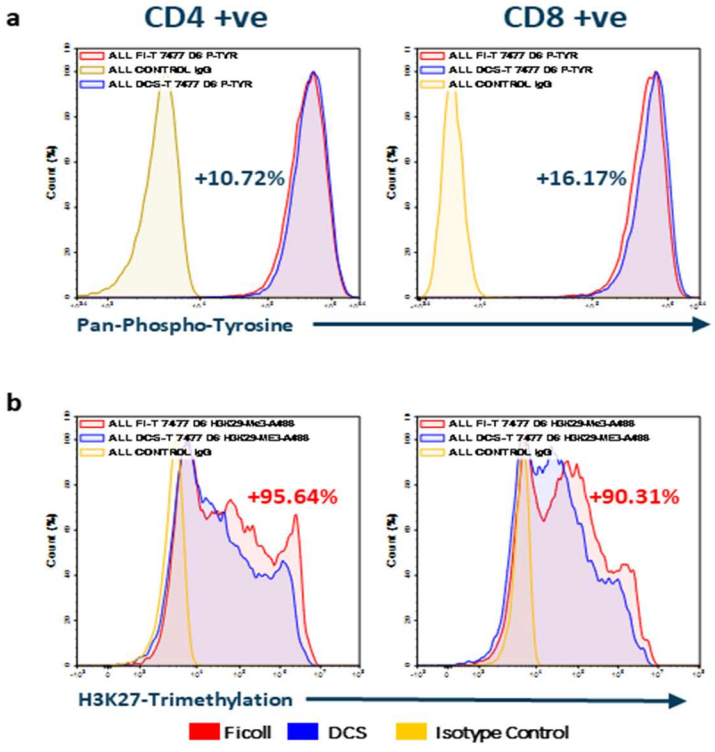


Figure 9. *DCS cells were frozen in CS-10, thawed after 1 Mo. Cells were either left untouched or subjected to standard Ficoll separation and then activated/expanded without T selection (Transact, IL-7/-15). Samples were taken at day 6 and stained as in figure 10, with the substitution of an antibody for tri-methylated H3K27 FITC, and were gated on singlet Live CD3 T cells. a) Processing with DCS leaves cells with an increase in phosphorylation potential in both CD4+ (+10.72%) and CD8+ (+16.17%) cells. b) The same test conditions resulted in a >90% increase in hypermethylation in Ficoll processed cells. Representative Data, n=3*



To determine if this change in tri-methylation was due to cell-cell/tonic signaling interaction, Jurkat T cells were expanded and then either mixed with Ficoll (no gradient) or layered on Ficoll to create a gradient (Fig. 10). In this experiment both groups were centrifuged, but the layered Ficoll condition forced cells into high-density proximity. Surprisingly, a substantial shift in the methylation state occurred in the gradient condition, whereas the control exhibited very little shift in methylation. This indicates that tonic signaling interactions that occur during processing could play a significant role in the DNA accessibility.

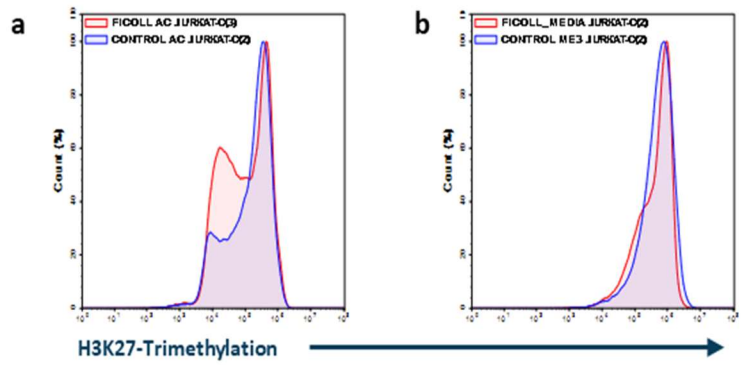


Figure 10. Jurkat T cell line was expanded for 4 days and then either processed by a standard Ficoll procedure or were mixed 1:1 with Ficoll and Media and incubated for 30 min at 37C, and then fixed and stained using CD3, fixable viability and for tri-methylated H3K27 (FITC), cells were gated on live CD3+ singlets.

a) Cells processed on a Ficoll gradient.
b) Cells in 50% Ficoll without a gradient.

Centrifugal processes impair DNA accessibility relative to DCS

To assess the effect of centrifugation on isolated primary T cells using a typical centralized manufacturing CAR-T process, an experiment was designed as shown in Fig 11a. A thawed apheresis donation was split into three and processed either using 1) a DCS separation/wash protocol, 2) a Ficoll protocol to remove DMSO, or 3) a centrifuge protocol to wash out DMSO twice. T-cells were then either positively or negatively selected from each DMSO removed preparations using Miltenyi MACS clinical scale columns, activated with CD3/CD28 (Transact) with IL-7/15 in TexMACS media. Aliquots were taken on Day 3 to mimic the shorter 2–4-day expansions of new manufacturing paradigms, such as T-charge. The overlay in Fig 11b demonstrates that DCS prepared (blue) contains substantially fewer highly methylated (closed DNA) cells compared to cells processed via other methods involving centrifugal processing. Ficoll prepared cells, which experience more centrifugal steps show the most hypermethylation, but negatively immunoselected cells, chosen as the best-case scenario (immuno), is almost as high. We also observed that positive immunoselected cells have ~30% higher trimethylation at day 3 vs negative selected T cells (Data not shown).

Memory subset analysis (Fig 11c) shows that DCS prepared cells (blue, blue arrows) have a substantially different, more accessible histone trimethylation profile compared to Ficoll and negative immunoselected T cells (green/red, red arrows). CD4+ T-cells have more methylation differences than CD8+ T cells. The most notable differences in memory subsets are in naïve/stem central memory. Compared to DCS cells almost ~3 fold more hypermethylated cells are present in centrifuged or negatively immunoselected T cells. Modest differences in methylation in central memory cells, and a similar profile in CD197- T-effector cells. In CD45RA+ effector cells (TEMRA) the CD4+ population show

a marked reduction in relative hypermethylation, which may be a significant finding given establishment of long term memory in T-cells.

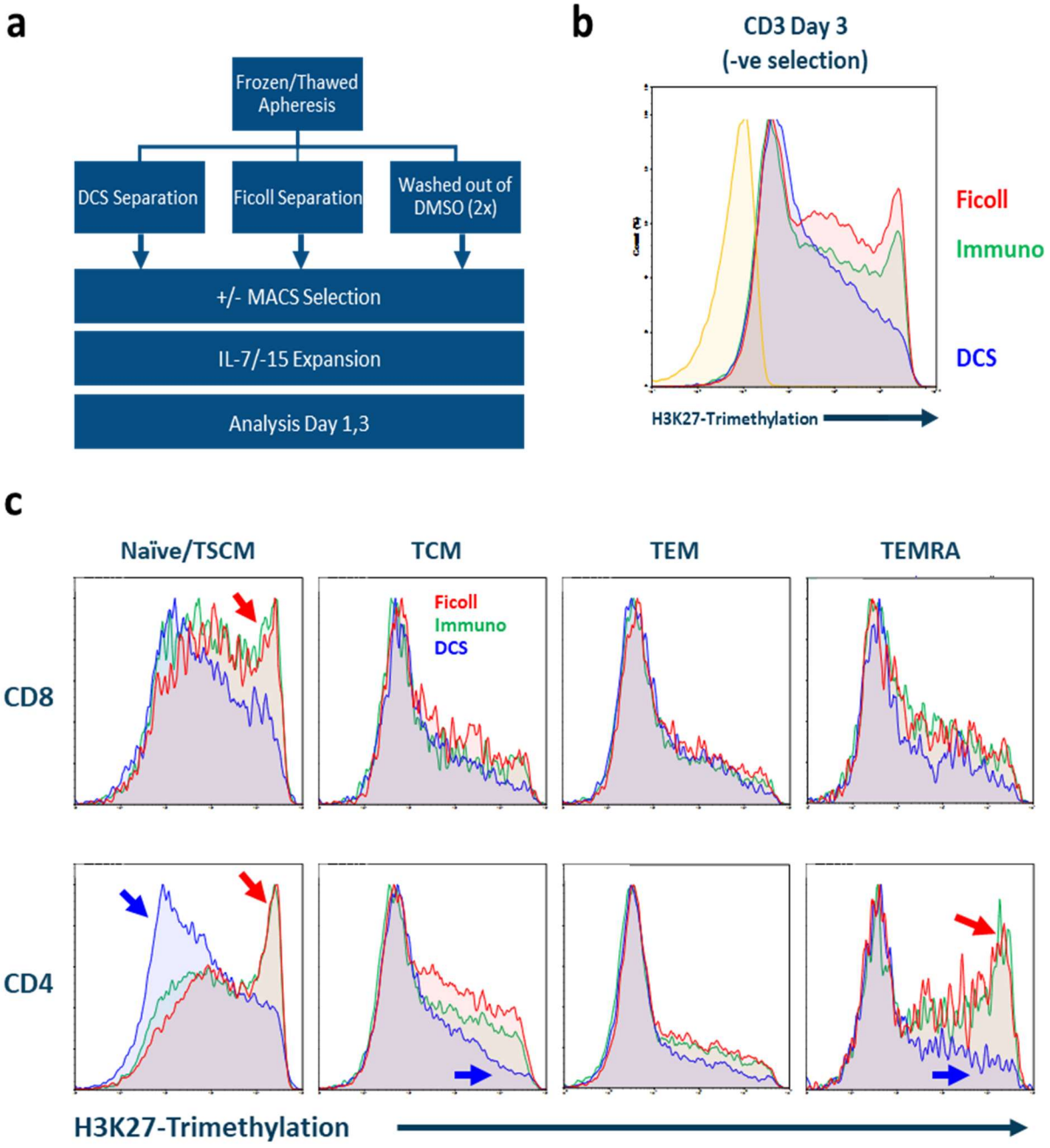


Figure 11. a: Experimental Design. b: Overlay of CD3 negatively selected T cells stained as in Figure 11, with the addition of CD45RA and CD197 to facilitate memory compartment analysis. c. T cell memory subset analysis of same donor as in b, showing CD4 and CD8 naïve and stem central memory (CD45RA+CD197+), central memory, (CD45RA-,CD197+), effector memory (CD45RA-, CD197-) and terminally differentiated effectors (TEMRA CD45RA+CD197-). Data in b,c is normalized. Representative donor shown.

Summary and Discussion

The data presented here provide strong evidence that cell-cell contact/tonic signaling driven by centrifugation or forced interface is likely to result in epigenetic and metabolic changes that meaningfully impact key cell populations used for CAR-T therapies. These epigenetic and metabolic findings are fully aligned with prior published data examining the specific effects of "soluble" cell signaling. Together, the data strongly indicates that current processing approaches are **negatively impacting** effective T-cell engineering.

The Table below consolidates the key learnings regarding cells prepared using DCS:

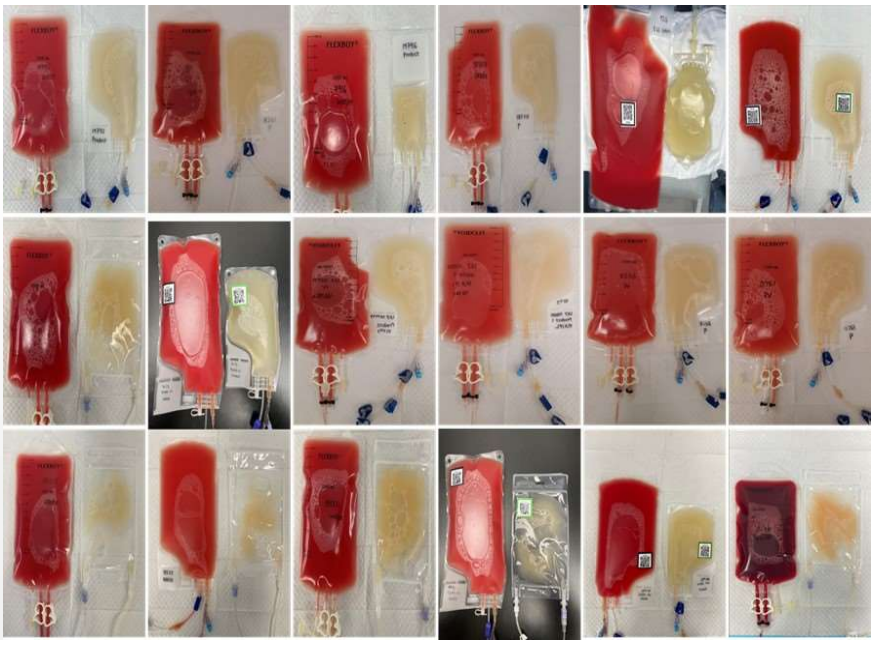
Attribute	Separation	Wash	Source of DCS Advantage
Cell Recovery	✓	✓	Consistent recovery of critical naïve cells with full metabolic potential and epigenetic availability
Platelet Removal	✓	✓	Best in class removal of platelets responsible for unintended propagation of T-regulatory cells.
T-regulatory cell generation		✓	Average 50% fewer T-regulatory cells due to immediate elimination of platelet derived cytokines (i.e. TGF-β)
Specific T-cell activation	✓		Fewer pre-activated and anergic cells prior to intentional activation allows for more coherent activation, expansion.
T-Cell Expansion	✓	✓	Highest quality input; metabolic potential, fewer inhibitory T-regs allows 30% expansion advantage in normal donors .
Lentiviral Transduction	✓	✓	Consistent and durable integration advantage derived from better DNA accessibility and elimination of processing-induced inhibitory T cell related factors (i.e. RANTES)
Improved Process	✓	✓	Consistent maximal recovery of naïve cells combined with highest editability and vitality to withstand multi-step engineering steps to produce highest yield in least amount of time.

As might be expected with such a clean separation and improved biology, leukopacks processed by DCS result in visually clean leukocyte products. The robust and efficient processing is especially evident even when looking at a gross product level as seen in Figure 12.

Figure 12. Composite image of 18 processed Leukopack fractions – red waste (left), purified white cell product (right) per panel.

Samples were run at customer sites or at Curate Biosciences.

No significant differences observed across sites.



It is clear from the data, and supported by the literature, that DCS preparation is superior to all other cell preparation technologies, delivering the full potential of a patient's starting material. DCS recovers the most critical subset for CAR-T engineering (naïve T cells) and has superior DNA access and metabolic fitness to withstand increasingly complex cell-engineering steps. **DCS processing is a major step forwards in the ability to maximize the potential of cell and gene therapy writ large**.

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