Curate BIOSCIENCES

Deterministic Cell Separation™ Microfluidics

Manufacturing Challenges in Cell and Gene Therapy

Significant progress in understanding the immune system and our ability to engineer increasingly sophisticated synthetic gene constructs has led to the regulatory approval and commercialization of T-cell–based chimeric antigen receptor (CAR) therapies for the treatment of various hematological cancers. However, clinical data increasingly show that the original multiweek manufacturing processes are suboptimal from a therapeutic perspective,[1,2] in addition to being extremely costly. Key clinical learnings have evolved: (1) better patient outcomes are obtained with shorter cell culture times,[1] (2) the presence of regulatory T cells in the final product adversely impacts clinical benefit,[2] and (3) less exhausted T cells are associated with improved CAR T-cell efficacy.[3]

Deterministic Cell Separation™ (DCS) Microfluidics

Making therapeutic doses in a shorter time obligates a more efficient process, as reliance on multiple cell divisions to achieve "dose" is now known to be less desirable. The use of density gradient separation, such as Ficoll; counterflow elutriation (CFE); and direct magnetic selection for cell recoveries is inefficient and suffers to varying degrees from impurities, such as platelets, or requires T-cell–damaging ammonium chloride to eliminate erythrocytes.[4] In contrast, microfluidic Deterministic Cell Separation™ (DCS) provided by the Curate® Cell Processing System (Figure 1) has uniformly high recovery and purity and does not require the introduction of density media or ammonium chloride. Further, DCS's unbiased and exceptionally efficient size-based separation delivers leukocytes that are the most "fit" of all of these methods for the purpose of T-cell engineering. The DCS process delivers consistent starting material with plentiful T cells, high viability, and wanted phenotypes, all factors within CAR T-cell manufacturing that contribute to making dose.

DCS separates cells much like a pachinko or coin sorter, which separates on the basis of size as the particle (cell) passes through an obstacle array that is sized and positionally defined so that particles and cells that appear smaller pass through the device and cells larger than that specific size are repeatedly deflected. For cell therapies, a mononuclear apheresis collection containing platelets, erythrocytes, and leukocytes is fluidically processed through the highly parallel 3.5-million-micropost array. The micropost array is oriented to separate larger leukocytes, or white blood cells (WBCs) >4 um, from platelets and erythrocytes (red blood cells [RBCs]) that appear smaller than <4 um. Additionally, the DCS consumable integrates a 20-um prefilter to remove gross clumps protecting the micropost array, with the added benefit of eliminating pro-inflammatory cell aggregates from the separation.

To move the cells gently through the array, fluidically stable (nonturbulent) co-flowing sample and buffer streams are established and fed into a manifold system and the parallel array, resulting in a stable interface boundary that the larger leukocytes must cross as they "size" into the clean buffer in a precise and controlled direction (Figure 2). The result is leukopak fractions of waste RBCs being separated from targeted leukocyte/WBCs, with viability *upwards of 96%.*

Key challenges for CAR T-cell manufacturing

1. Shorter cell culture times are associated with better patient outcomes[1]
2. The presence of regulatory T cells in the final product adversely impacts clinical benefit[2]
3. Less exhausted T cells are associated with improved CAR T-cell efficacy[3]

DCS to dose advantages

1. **Shorter** cell culture time
2. **Less** regulatory T cells
3. **More** naïve T cells

DCS-processed cells have better:

- T-cell phenotype
- Health and viability
- Activation/expansion
- Transduction
- Yield
- Consistency
- Time to dose

Figure 1. Image of Curate® Cell Processing System and list of advantages for chimeric antigen receptor (CAR) T-cell manufacturing.

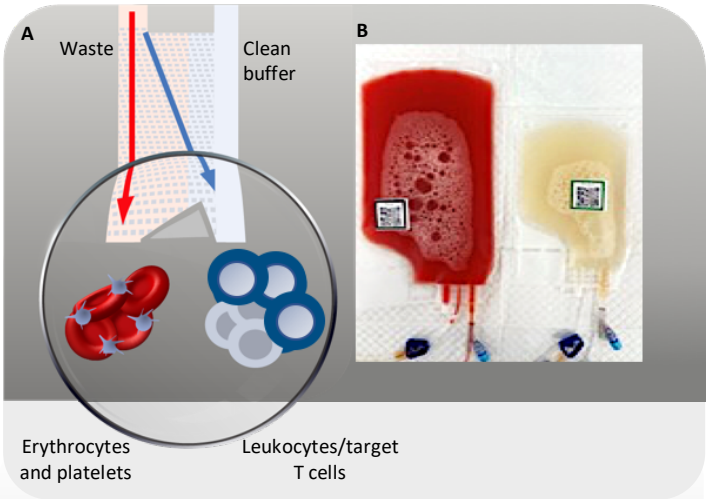

Figure 2. (A) Simple schematic showing how sample enters the array and large cells (leucocytes) are individually moved into the clean buffer stream and separated from waste. (B) Image of Deterministic Cell Separation™ (DCS)-processed leukopak fractions: waste (left), targeted leukocytes/product (right).

CAR T-Cell Manufacturing Process

The CAR T-cell manufacturing process requires multiple steps, including (1) apheresis and debulking, (2) T-cell selection, (3) activation, (4) transduction, (5) expansion, (6) formulation, (7) cryopreservation, and (8) accomplishing dose and treating patients with the final CAR T-cell product (Figure 3).[5] Each step from vein to vein can impact the therapeutic dose of CAR T cells.

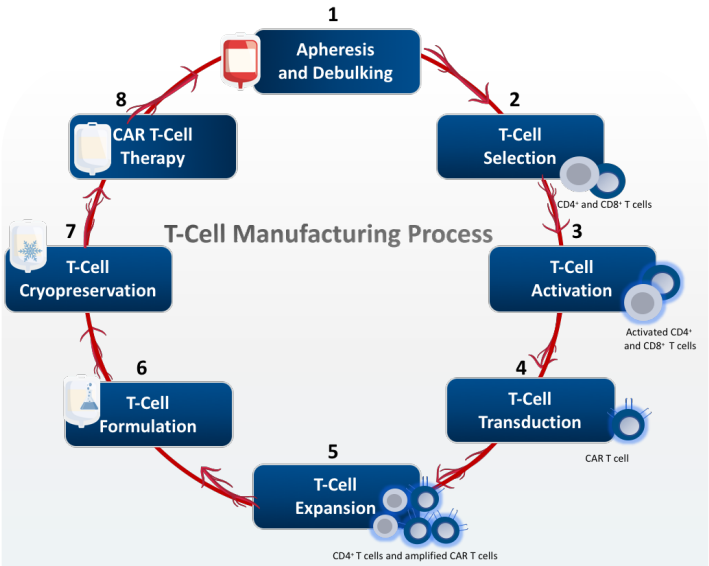

Figure 3. CAR T-cell manufacturing process at a glance.[5]

Apheresis and Debulking With DCS Microfluidics Prior to T-Cell Selection

Immediately following apheresis, the Curate cell processing system is capable of sorting/debulking cells on an individual basis at unprecedented rates. An average development run processes $3.4x10^{11}$ cells (leukocytes, erythrocytes, and platelets) in ~20 minutes following the system's priming cycle. A maximum throughput consists of $2.5x10^{12}$ cells per hour, while individually sorting $1.8x10^{10}$ leukocyte sets, a throughput benchmark that is *>3000-fold faster* than typical commercial microfluidic cell sorters. The end result of DCS processing is more T cells and less erythrocytes and platelets as compared to other methods, such as Ficoll (Figure 4). The debulking accomplished by DCS improves the starting material needed for T-cell selection and maintaining the fidelity across the entire manufacturing process.

In contrast to DCS, which has *a >95% removal of erythrocytes*, other techniques may require lysis using ammonium chloride. Ammonium chloride has been shown to induce mitochondrial dysfunction,[6] likely impacting T-cell activation and expansion,[7] enhancing T-cell exhaustion,[8] and causing functional T-cell

DCS recovers more T cells while removing more erythrocytes and platelets

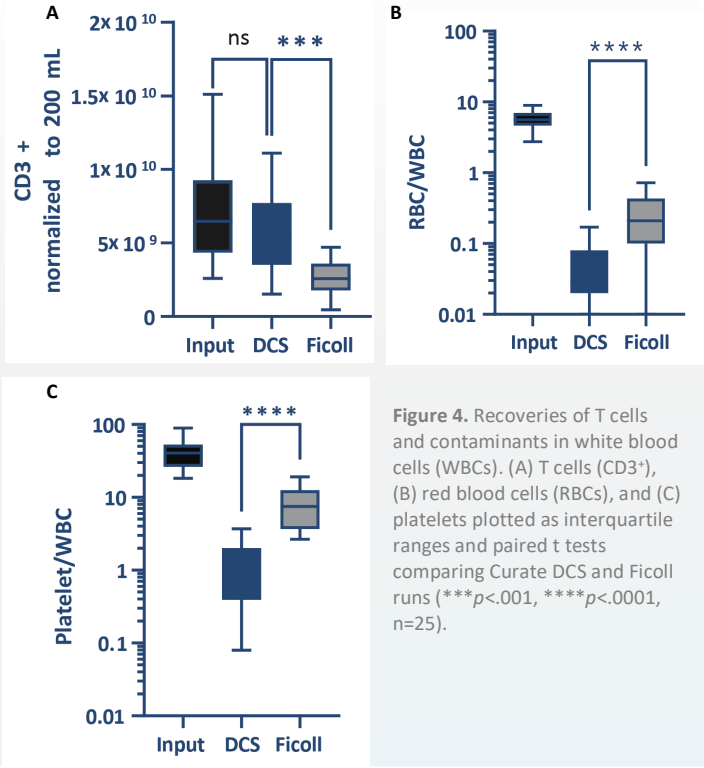

Figure 4. Recoveries of T cells and contaminants in white blood cells (WBCs). (A) T cells ($CD3^+$), (B) red blood cells (RBCs), and (C) platelets plotted as interquartile ranges and paired t tests comparing Curate DCS and Ficoll runs (***p<.001, ****p<.0001, n=25).

DCS removes harmful platelets

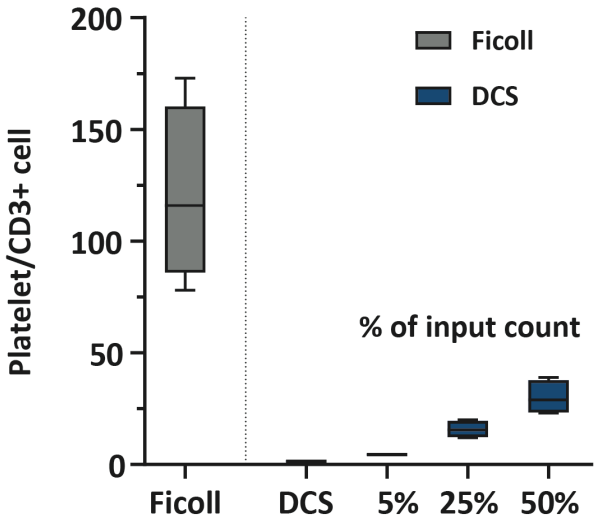

Figure 5. Platelet to T cell ratios in peripheral blood mononuclear cells (PBMCs) following "2-wash" Ficoll protocol versus DCS and addition of "dissociable" ethylenediaminetetraacetic acid (EDTA) into DCS product. N=4. Tukey plot.

response to lose consistency.[9] Other separation techniques carry over platelets that are prone to subsequent interaction with damaged immune cells, amplifying the potential for cell aggregation. Platelets and their soluble factors are recognized

as major hindrances to T-cell activation and proliferative expansion.[10] Thus, *DCS's near instantaneous removal of platelets* (Figure 5) provides a unique opportunity to manage the detrimental effects of platelets regarding cell therapy applications.

In addition to platelet impurities, numerous cytokines and chemokines are present during the typical apheresis process and can act as stressors and activators to immune cells.[11] To better characterize this, a 52-plex cytokine analysis was conducted 4 hours after processing by the Ficoll and DCS to determine the cellular environment/milieu post separation (Figure 6). The residual matrix shows that Ficoll-processed cells are being exposed to a mixture of strong activators and promotors of regulatory T-cell differentiation, such as platelet-derived growth factor (PDGF)[12] and transforming growth factor β (TGF-β).[13] An ~8-fold higher amount of the pro-inflammatory chemokine RANTES (regulated upon activation, normal T cell expressed and secreted), known for blocking viral infection,[14,15] was also observed. In addition, there are higher levels of known drivers of senescence, such as plasminogen activator inhibitor-1 (PAI-1),[16] and factors associated with exhaustion, such as C-X-C motif chemokine ligand 1/growth-related oncogene α (GRO-α).[17] The impact of a potent cytokine such as TGF-β on all cells targeted for engineering is broad given that TGF-β is responsible for the induction of regulatory T cells, inhibition of helper T type 1 (T_H1) and cytotoxic responses, subversion of dendritic cell function, suppression of natural killer cells, and regulation of macrophages.[18]

DCS averts a detrimental inflammatory milieu

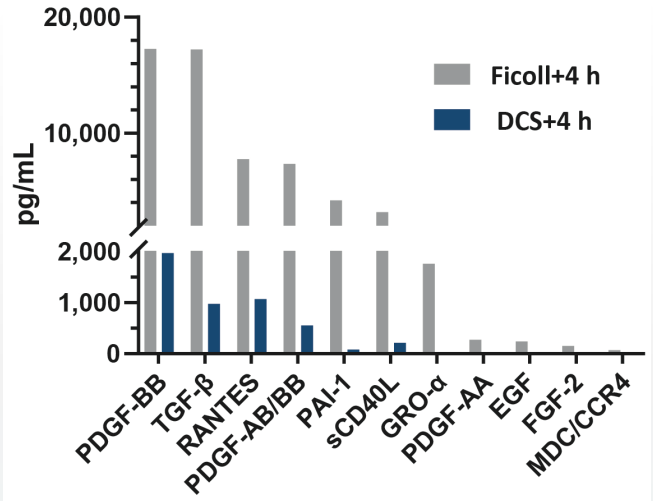

Figure 6. Cytokines/chemokines present post processing of a 24-hour apheresis. Samples were measured 4 hours after separation. Only samples >5-fold different and >50 pg/mL are presented. N=4. EGF, epidermal growth factor; FGF-2, fibroblast growth factor 2; GRO-α, growth-related oncogene α; MDC, macrophage-derived chemokine; PAI-1, plasminogen activator inhibitor-1; PDGF-AA, platelet-derived growth factor a subunits; PDGF-AB, platelet-derived growth factor a b subunits; PDGF-BB, platelet-derived growth factor b subunits; RANTES, regulated upon activation, normal T cell expressed and secreted; sCD40L, soluble CD40 ligand; TGF-β, transforming growth factor β.

Factors promoting T-cell exhaustion and regulatory T-cell differentiation are >5-fold higher with Ficoll compared to DCS

T-Cell Activation Induced Expansion With DCS Microfluidics

The net effect of cytokines and pro-exhaustion agents highlights a comparative differential in the potential susceptibility toward an anergic profile. These factors are important in that T cells are less responsive to direct activation attempts that are critical during the CAR T-cell manufacturing process, with the typical result being that only a portion of cells will activate and an additional portion will go on to die.

Incomplete activation can lead to an anergic phenotype, while persistent activation and/or inflammatory signals can lead to an exhausted T-cell phenotype.[19] Nonproductive T-cell activation in the peripheral blood mononuclear cell (PBMC) fraction immediately following apheresis alone is 2-fold greater for Ficoll processing versus DCS, as observed by expression of the early activation marker CD69 (data not shown). It is likely that the numerous soluble factors present in a typical apheresis process act as stressors to influence the initial status of T cells.

Productive activation of T cells (T-cell receptor stimulation) is a necessary step for transduction and expansion in T-cell manufacturing. Thus, naïve T-cell populations are

Expansion with DCS is 1.3-fold greater

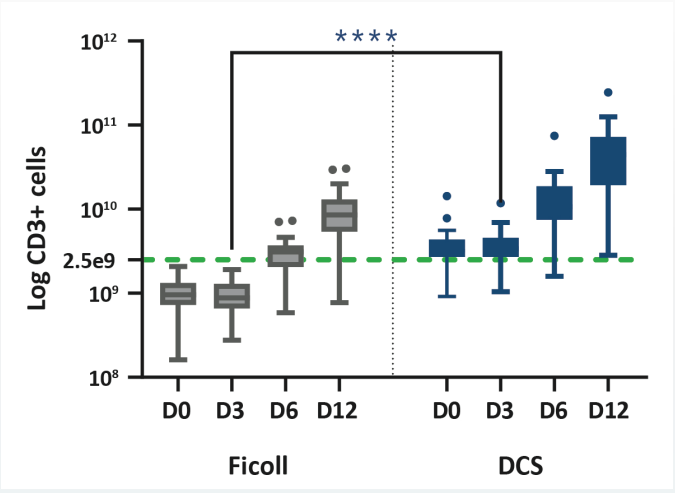

Figure 7. T cells from 20 donors were isolated and activated using CD3/CD28 beads and cultured in TexMACS supplemented with 5 ng/mL of both interleukin 7 (IL-7) and interleukin 15 (IL-15) using a 500-mL G-Rex chamber (****p<.0001, n=20). CD3 counts were determined by Coulter count and flow cytometry, and viability was determined using CD3 and 7-aminoactinomycin D (7-AAD) at each time point.

advantageous as starting material. Activation-induced expansion is greater with DCS than with Ficoll (Figure 7). Within the Ficoll arm (gray), at Day 3 the commonly experienced "dip" in total T-cell counts was found. In contrast, in the DCS arm (blue), at Day 3 the average proliferative expansion was equal or modestly ahead of Day 0. By Day 12, the average fold *proliferative expansion for the DCS arm was 1.3-fold that of the Ficoll arm.*

Viability during the proliferative expansion was aligned with the expansion profile (data not shown) and the lack of "dip or lag" found in the Ficoll preparation. This finding is also consistent with the DCS preparation experiencing reduced cell death from anergy and/or nonproductive cell activation. This is also more aligned with the desire to shorten overall processes and *minimize culture time*, a correlative factor of better patient outcomes.[1]

The gentle processing of DCS provides T cells that are most "fit" in respect to T-cell subset and activation status. An ideal starting population would consist of both naïve CD8⁺ cytotoxic T cells and CD4⁺ helper T cells that go on to promote killing capacity while preserving as many memory T cells as possible to provide a cell preparation with durable efficacy. In contrast, a poor starting population would consist of inhibitory CD4⁺ regulatory T cells and exhausted/anergic CD8⁺ T cells. The presence of anergic or nonresponsive T cells in a starting population reduces the coherence of any activation process. Furthermore, it will likely delay any cell doublings and integration of gene construct, however delivered.

Cells from DCS and Ficoll processing were assessed for CD4⁺ and CD8⁺ T-cell subsets and lineage including naïve T (Tn) cells, central memory T (Tcm) cells, effector memory T (Tem) cells, and CD45RA-expressing memory T (Temra) cells. Initial characterization of these subsets revealed a slight bias toward more CD4⁺ T-cell recovery and was accompanied by a bias toward recovering more Tn and Tcm cells (Figure 8). Importantly, the presence of Tn and Tcm cells was greater with DCS than with Ficoll. While the profile of desired cells is important, the presence of undesired cells is as well. In this regard, regulatory T cells can have a profound impact on T-cell manufacturing. First, as regulatory T cells initially expand, they secrete TGF-β, which causes even more naïve CD4⁺ cells to differentiate into T regulatory cells. Second, as they expand, their immunosuppressive function impairs the expansion of nonregulatory T cells, making it more difficult to make "dose" (data not shown). Third, targeting regulatory T cells dilutes the functional efficacy of an effector T-cell preparation, as the now targeted regulatory T cells suppress and compete with the cytotoxic effector T cells at the site of the tumor. This latter problem has been identified to correlate with the poorer clinical outcomes in CAR T-cell recipients.[2]

With the clinical relevance of regulatory T cells and their effect on CAR T-cell preparations, we assessed their generation in both fresh and frozen/thawed DCS-prepared cells. In both cases, the number of regulatory T cells generated was less for DCS as compared to Ficoll preparations. Furthermore, we assessed the effect of platelet add-backs on fresh DCS product to confirm that only a 30-minute exposure to a small amount

DCS recovers more naïve T-cell populations

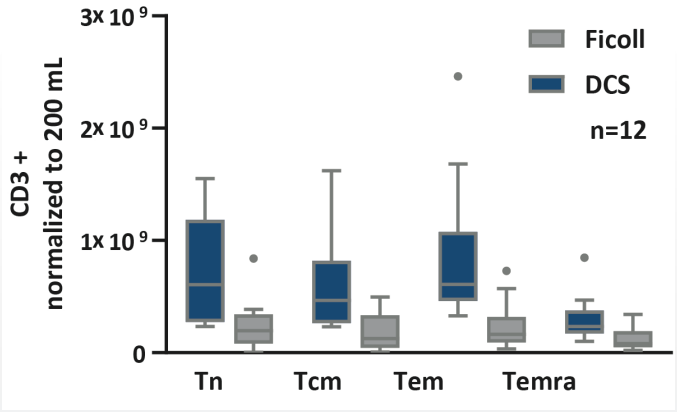

Figure 8. Recovery and subset analysis of CD3⁺ T cells from 12 normal donor apheresis samples that were split 80% to DCS, 20% to Ficoll. Data normalized to reflect a 200-mL apheresis volume. WBC counts were determined by Coulter counter, and T-cell counts were multiplied by the CD3 as a percent of CD45⁺ PerCP cells. CD3⁺ T-cell subpopulations were determined using antibodies: CD3, BV421-CD45RA, PE-Cy7-CCR7, PE/APC-CD4, and FITC-CD8. The recovery advantage is described as a ratio, and the within-T-cell-subpopulation bias is expressed relative to the T-cell average (ie, 3.96/3.16 for Tcm population=1.25). Tcm, central memory T cells; Tem, effector memory T cells; Temra, CD45RA-expressing memory T cells; Tn, naïve T cells.

Just **30 minutes** of incubation with platelets can lead to a regulatory T-cell phenotype

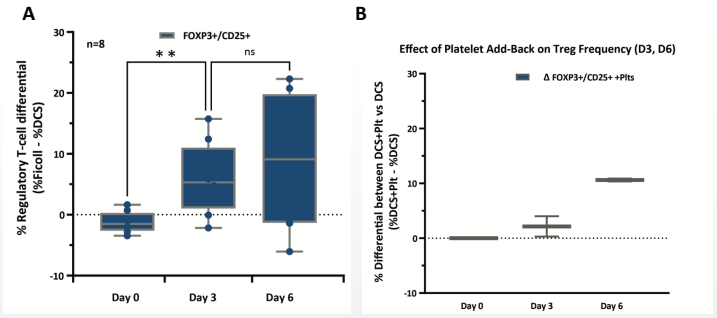

Figure 9. (A) PBMCs from either DCS or Ficoll were processed and frozen in CS-10 cryopreservation media. Post thaw, PBMCs were washed into media and following overnight rest were activated with TransAct CD3/CD28 nanomatrix to induce expansion. Cells were stained for CD3, CD4, CD8, and FoxP3 and CD25 and CD127 to identify and confirm regulatory T-cell (Treg) phenotype. Data plotted are a difference plot (% Treg in Ficoll - % Treg in DCS). ***p*<.01, n=8. (B) Fresh PBMCs were supplemented with 5% of autologous platelet burden on a platelet/T-cell basis, incubated for 30 minutes, and then activated, expanded, and immunostained using the method in A. n=2. Plt, platelet.

of platelet milieu was sufficient to generate regulatory T cells (Figure 9).

Based on this data, it should be apparent that microfluidic DCS size-based processing will significantly address several of the key challenges that are seen as limiting for the success of CAR

T-cell therapy. A process that robustly maximizes the recovery of younger naïve cells combined with best-in-class cell health will be important for all patients, but it is likely to be especially important for immunosuppressed individuals with leukopenia who may struggle to make dose when less time in culture processes becomes more widely adopted.

T-Cell Transduction and Expansion With DCS Microfluidics

An unanticipated benefit of DCS processing is that DCS-prepared cells, whether fresh or frozen/thawed, show a *~50% relative transduction advantage* (Day 3). Freshly prepared material shows an approximately 50% integration advantage for a lentiviral-green fluorescent protein construct in the first days of expansion (n=8) (Figure 10), which aligns well with the ability to manufacture in a short time frame with stable integration. This advantage may be attributed to the fact that DCS-prepared cells are exposed to ~8-fold less RANTES (Figure 6), a chemokine known to inhibit the integration of lentivirus.

DCS cells have a **50%** transduction advantage by Day 3

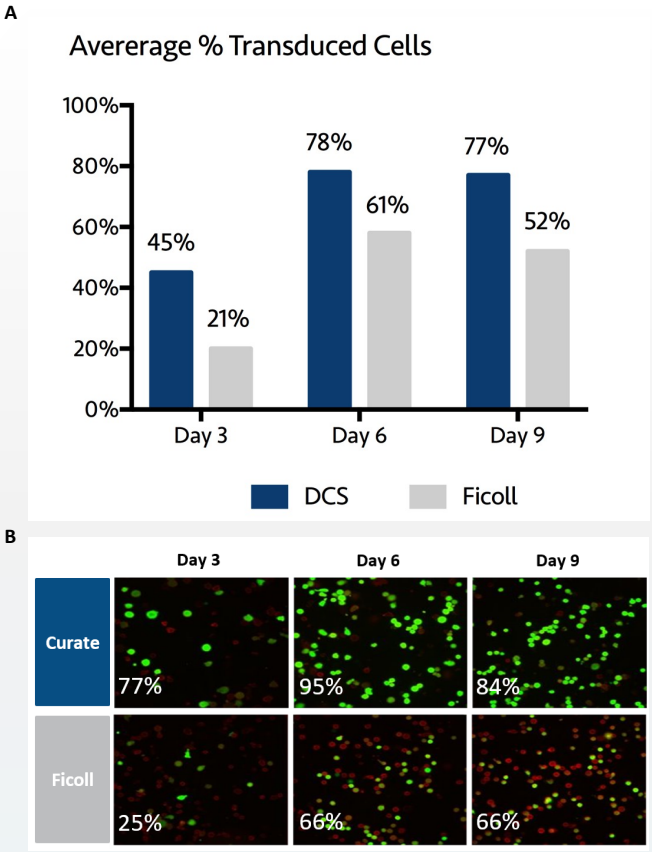

Figure 10. Lentiviral-green fluorescent protein transduction into CD3⁺ T cells activated with CD3/CD28 Dynabeads and supplemented with 5 ng/mL IL-7 and IL-15. A multiplicity of infection 5 was used to transduce at 24 hours post selection/activation. The recovery advantage is described as a ratio, and the within-T-cell-subpopulation bias is expressed relative to the T-cell average (ie, 3.96/3.16 for Tcm population=1.25).

This relative advantage is maintained through steps in cryopreservation where DCS-derived cells following the "cell journey" of CAR T-cell engineering show a memory phenotype and cytotoxicity (data not shown).

Across different platforms, from recovery through transduction and expansion, DCS provides a greater relative yield of transduced T cells (Figure 11). The faster and higher yield of Curate-prepared cells is a result of superior health and fitness and a lack of a lag phase in expansion enabled by the DCS process. The DCS-processed cells performed best or near best at each step of the manufacturing journey and at the end showed a potential yield of minimally *50% more cells than the nearest competitor and as much as 300% more cells*. DCS-prepared cells demonstrate a superior vitality and functional profile analytically as compared to other leading separation methodologies.

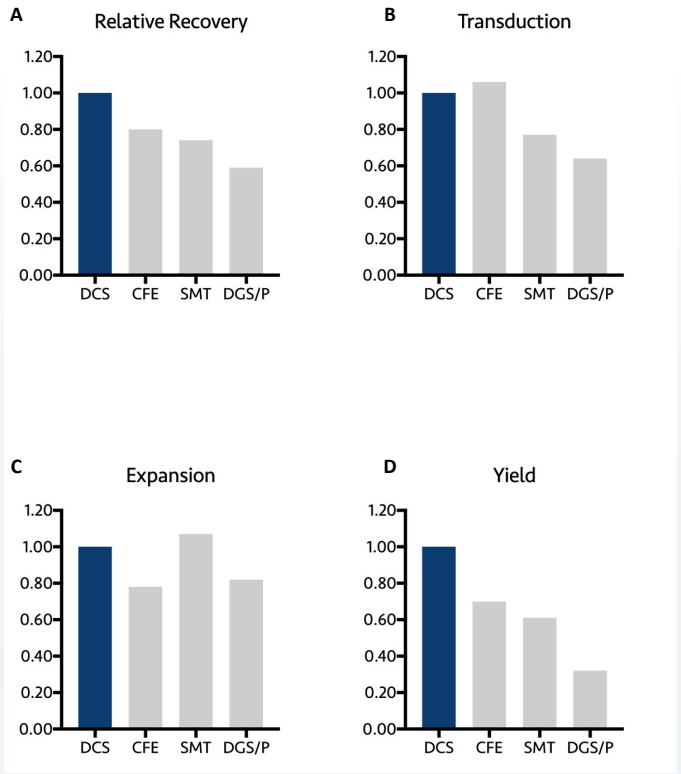

Figure 11. The benefits of DCS processing extend cell recovery metrics, enabling confidence in making a dose of the highest quality with the minimum amount of time. CFE, counterflow elutriation; DGS/P, density gradient separation/plunger; SMT, spinning membrane technology.

Operational Gains of DCS Microfluidics Across All Steps

The fitness of T cells derived from DCS processing likely results from gentle processing and separation from platelets and impurities that influence T-cell phenotype (Figure 12). The benefits of DCS processing and selection accumulate and propagate throughout the prototypical CAR T-cell manufacturing process. DCS-processed cells lead to better selected cell phenotypes, shorter time to activation-induced expansion, greater transduction efficiency, lower variability, and greater yield, which permits less time spent in pursuit of

DCS equals quicker time to dose

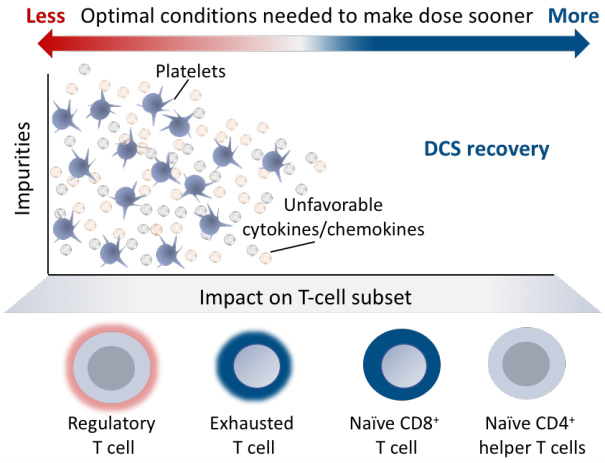

Figure 12. Platelets and impurities' impact on T-cell phenotype and relationship with DCS processing.

dose. DCS therefore addresses the need for streamlining the manufacturing process for gene/cellular therapies. An added benefit to DCS is the *reduction in cost* associated with shorter expansion times. Most importantly, DCS is ideal for accomplishing shorter vein-to-vein times that are directly associated with better patient outcomes.[20]

References:

1. Panch SR, Srivastava SK, Elavia N, et al. Effect of cryopreservation on autologous chimeric antigen receptor T cell characteristics. *Mol Ther*. 2019;27(7):1275-1285. doi:10.1016/j.ymthe.2019.05.015
2. Good Z, Spiegel JY, Sahaf B, et al. Post-infusion CAR T(Reg) cells identify patients resistant to CD19-CAR therapy. *Nat Med*. 2022;28(9):1860-1871. doi:10.1038/s41591-022-01960-7
3. Long AH, Haso WM, Shern JF, et al. 4-1BB costimulation ameliorates T cell exhaustion induced by tonic signaling of chimeric antigen receptors. *Nat Med*. 2015;21(6):581-590. doi:10.1038/nm.3838
4. Plank K, Dorn C, Krause SW. The effect of erythrocyte lysing reagents on enumeration of leukocyte subpopulations compared with a no-lyse-no-wash protocol. *Int J Lab Hematol*. 2021;43(5):939-947. doi:10.1111/ijlh.13484
5. Wang X, Rivière I. Clinical manufacturing of CAR T cells: foundation of a promising therapy. *Mol Ther Oncolytics*. 2016;3:16015. doi:10.1038/mto.2016.15
6. Luo C, Shen G, Liu N, et al. Ammonia drives dendritic cells into dysfunction. *J Immunol*. 2014;193(3):1080-1089. doi:10.4049/jimmunol.1303218
7. Sena LA, Li S, Jairaman A, et al. Mitochondria are required for antigen-specific T cell activation through reactive oxygen species signaling. *Immunity*. 2013;38(2):225-236. doi:10.1016/j.immuni.2012.10.020
8. Bell HN, Huber AK, Singhal R, et al. Microenvironmental ammonia enhances T cell exhaustion in colorectal cancer. *Cell Metab*. 2023;35(1):134-149.e6. doi:10.1016/j.cmet.2022.11.013
9. Simon MR, Roi LD, Desai S, Salberg DJ, Rose NR. Comparison of cultures of human lymphocytes obtained following NH4Cl induced red blood cell lysis and Ficoll-Hypaque density gradient centrifugation. *Immunol Commun*. 1983;12(3):301-314. doi:10.3109/08820138309050752
10. Polasky C, Wendt F, Pries R, Wollenberg B. Platelet induced functional alteration of CD4+ and CD8+ T cells in HNSCC. *Int J Mol Sci*. 2020;21(20):7507. doi:10.3390/ijms21207507
11. Bacal CJO, Maina JW, Nandurkar HH, et al. Blood apheresis technologies–a critical review on challenges towards efficient blood separation and treatment. *Mater Adv*. 2021;2(22):7210-7236. doi:10.1039/d1ma00859e
12. Agrawal S, Ganguly S, Hajian P, Cao JN, Agrawal A. PDGF upregulates CLEC-2 to induce T regulatory cells. *Oncotarget*. 2015;6(30):28621-28632. doi:10.18632/oncotarget.5765
13. Schmidt A, Eriksson M, Shang MM, Weyd H, Tegnér J. Comparative analysis of protocols to induce human CD4+Foxp3+ regulatory T cells by combinations of IL-2, TGF-beta, retinoic acid, rapamycin and butyrate. *PLoS One*. 2016;11(2):e0148474. doi:10.1371/journal.pone.0148474
14. Challita-Eid PM, Klimatcheva E, Day BT, et al. Inhibition of HIV type 1 infection with a RANTES-IgG3 fusion protein. *AIDS Res Hum Retroviruses*. 1998;14(18):1617-1624. doi:10.1089/aid.1998.14.1617
15. Capobianchi MR, Abbate I, Antonelli G, Turriziani O, Dolei A, Dianzani F. Inhibition of HIV type 1 BaL replication by MIP-1alpha, MIP-1beta, and RANTES in macrophages. *AIDS Res Hum Retroviruses*. 1998;14(3):233-240. doi:10.1089/aid.1998.14.233
16. Vaughan DE, Rai R, Khan SS, Eren M, Ghosh AK. Plasminogen activator inhibitor-1 is a marker and a mediator of senescence. *Arterioscler Thromb Vasc Biol*. 2017;37(8):1446-1452. doi:10.1161/atvbaha.117.309451
17. Zhang Z, Liu S, Zhang B, Qiao L, Zhang Y, Zhang Y. T cell dysfunction and exhaustion in cancer. *Front Cell Dev Biol*. 2020;8:17. doi:10.3389/fcell.2020.00017
18. Batlle E, Massagué J. Transforming growth factor-β signaling in immunity and cancer. *Immunity*. 2019;50(4):924-940. doi:10.1016/j.immuni.2019.03.024
19. Crespo J, Sun H, Welling TH, Tian Z, Zou W. T cell anergy, exhaustion, senescence, and stemness in the tumor microenvironment. *Curr Opin Immunol*. 2013;25(2):214-221. doi:10.1016/j.coi.2012.12.003
20. Locke FL, Hu ZH, Siddiqi T, et al. Real-world impact of time from leukapheresis to infusion (vein-to-vein time) in patients with relapsed or refractory (r/r) large B-cell lymphoma (LBCL) treated with axicabtagene ciloleucel. *Blood*. 2022;140(Supplement 1):7512-7515. doi:10.1182/blood-2022-155603