Successful support of longer T cell expansion workflows with CTS Dynabeads CD3/CD28 and CTS OpTmizer T Cell Expansion SFM, no phenol red

Introduction

T cell therapy displays immense promise in a multitude of disease states. The fundamental process behind manufacturing these therapies involves isolating cells from the patient (autologous) or a donor (allogeneic), then genetically modifying the cells *ex vivo* for therapeutic targeting. Once modified, the cell population must be expanded, harvested, preserved, qualified, and finally infused into the patient. The expansion phase consistently represents the longest phase in the workflow. Currently, typical autologous T cell therapy expansion phases last up to 12 days from activation to harvest. Allogeneic workflows are distinct from autologous workflows in that allogeneic manufacturing is designed to produce enough cells to administer many doses. As a result, these workflows tend to be longer, usually lasting more than 14 days. The additional culture time can negatively impact differentiation and the function of the cellular product, so thoughtful consideration needs to be given to the workflow details, such as cell density maintenance and supplementation. To avoid these potential issues in the longer workflows, the expansion process and products utilized need to be tailored.

T cells are activated via two signals, the primary signal being agonistic ligation of the T cell receptor and simultaneous costimulatory signals such as CD28. Gibco™ CTS™ Dynabeads™ CD3/CD28 are magnetic microbeads coated with CD3 and CD28 antibodies that, respectively, provide the primary and costimulatory signals required for activation and expansion of T cells. These beads have long been applied in the successful *ex vivo* expansion of primary T cells in both research and clinical settings. Until recently, most of this work was done with a traditional shorter workflow associated with autologous therapies. However, the ongoing shift toward longer workflows associated with allogeneic manufacturing protocols has created interest in understanding the durability of the response elicited by these beads over time.

We were specifically interested in whether a secondary or "restimulation" was necessary or beneficial to the cells. To this end, the primary T cells from three independent donors were activated with CTS Dynabeads CD3/CD28 and expanded with Gibco™ CTS™ OpTmizer™ T Cell Expansion Serum-Free Medium (SFM), with no phenol red, in longer workflows of 18–20 days. In addition, the impacts of the longer workflow process relating to the maintenance cell density and supplementation schedule were evaluated.

Materials and methods

T cell isolation

Primary human T cells from healthy donors were negatively isolated from PBMCs with the Invitrogen™ Dynabeads™ Untouched™ Human T Cells Kit. Alternatively, for process development and clinical use, please see the application note titled "One-step isolation and activation of naive and early memory T cells with CTS Dynabeads CD3/CD28" (Pub. No. COL23050).

Medium

CTS OpTmizer T Cell Expansion SFM, no phenol red, contains two parts: 1 L CTS OpTmizer Expansion Basal Medium, and 26 mL CTS OpTmizer Expansion Supplement. The components are combined per the user manual to create the complete culture medium. We additionally supplement the medium with 4 mM Gibco GlutaMAX™ Supplement and 2.5% Gibco CTS Immune Cell Serum Replacement (ICSR).

Activation and stimulation

T cells were seeded in culture dishes at 1 x 10^6 cells/mL in the indicated medium and activated with Gibco Dynabeads Human T-Expander CD3/CD28 at a ratio of 3 beads per T cell in the presence of 100 IU/mL of rIL-2.

Routine maintenance

T cells were counted every 2–3 days using a Beckman Coulter™ Vi-CELL™ Cell Viability Analyzer. Viable cell density was maintained at 0.5 x 10^6 cells/mL unless otherwise specified, and rIL-2 was added to the culture to a concentration of 100 IU/mL.

Flow cytometry

Cellular phenotype was assessed on the days indicated by staining T cells with Invitrogen CD3 Pacific Orange™, CD4 FITC, CD8 Pacific Blue™, CD62L APC, and CCR7 PE antibodies.

Results

A single round of T cell activation with CTS Dynabeads CD3/CD28 is sufficient

The growth and viability results not only demonstrated that a single round of activation with the beads was sufficient to induce robust cell proliferation and high viability over the entire 20-day workflow, but also provided evidence that restimulation with the beads actually causes a temporary growth lag and plunge in viability during the immediately following days (Figure 1A, 1B). Restimulation with CTS Dynabeads CD3/CD28 was also shown to influence the CD8:CD4 ratio and differentiation status. Cells that were subjected to secondary activation by the beads displayed a lower CD8:CD4 ratio (Figure 1C) and a sharp downregulation of biomarkers for central memory (Figure 1D). In both cases, with and without restimulation, there is an evident decrease in the central memory population ($CD62L^+$ $CCR7^+$) and a reciprocal increase in the double-negative effector population ($CD62L^-$ $CCR7^-$) as the culture is carried from day 10 to day 19. Both trends are amplified in the "restimulation" group (Figure 1D).

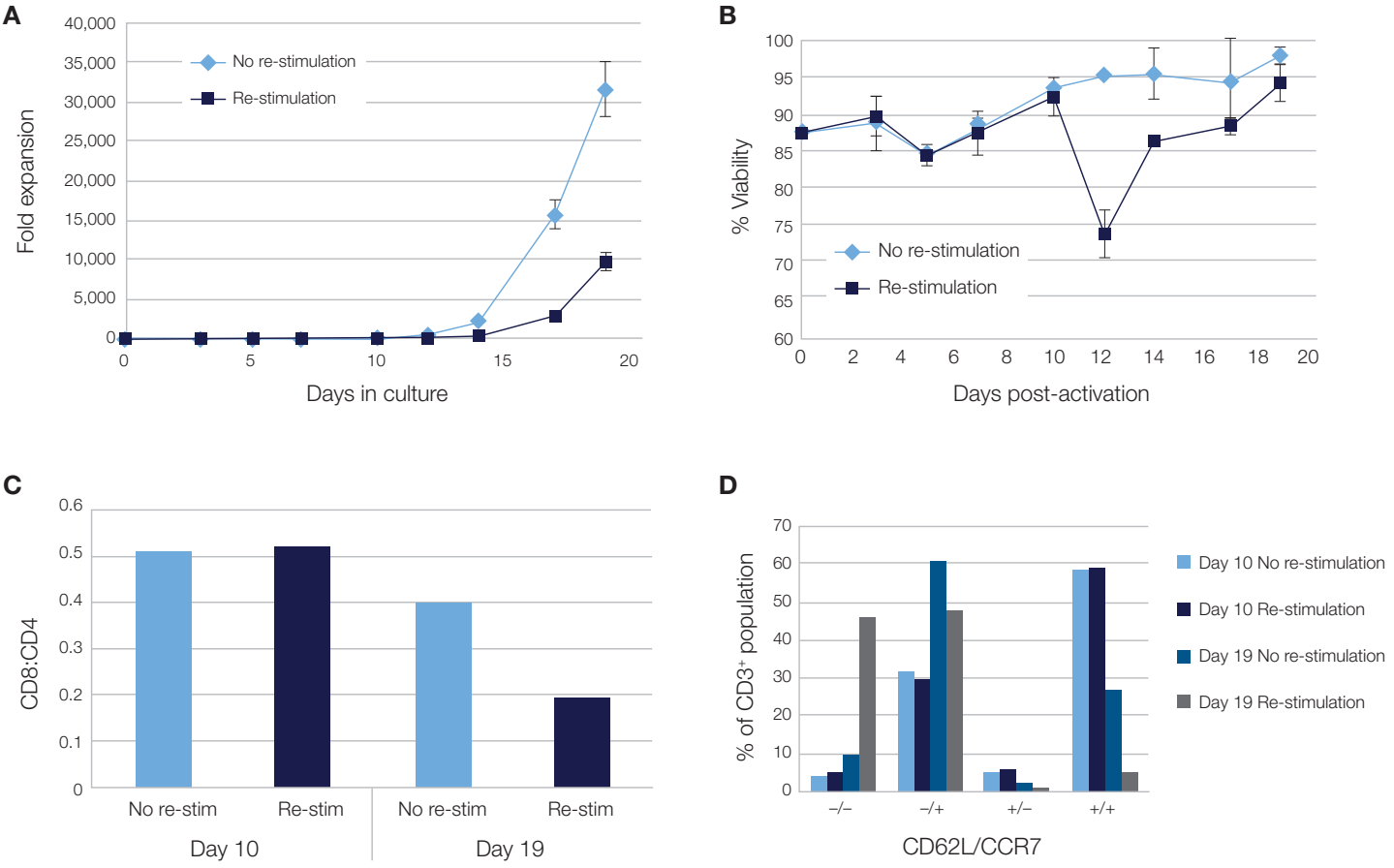

Figure 1. Effect of restimulation on T cell growth and phenotype. A second round of stimulation with CTS Dynabeads CD3/CD28 negatively impacts **(A)** cell expansion, **(B)** cell viability, **(C)** the CD8:CD4 ratio, and **(D)** cell differentiation during a longer workflow. Error bars represent the standard deviation of 3 donors performed in triplicate.

Process details impact the cellular output following longer expansion

Investigation into the longer workflow processes identified relatively simple process modifications that help preserve the desirable central memory population. Specifically, we found that adjusting the maintenance cell density and the supplementation schedule during expansion resulted in considerable cell-related improvements in the longer workflows.

Cell density

The classical T cell therapy expansion protocol calls for the maintenance of the cells at 0.5 x 10^6 cells/mL; however, when cell densities above and below 0.5 x 10^6 cells/mL were evaluated, several direct effects on the quantity and quality of the cellular product were observed. Maintaining a lower cell density of 0.25 x 10^6 cells/mL correlated with a higher fold expansion and improved viability (Figure 2A, 2B). In accordance with this trend, increasing the maintenance cell density from 0.5 x 10^6 cells/mL to 0.75 x 10^6 cells/mL led to lower fold expansion and slightly lower cell viability (Figure 2A, 2B).

The relationship between the maintenance cell density and cellular phenotypes, specifically the CD8:CD4 ratio and the differentiation status, were also investigated. A rise in the CD8:CD4 ratio and a larger central memory population were demonstrated when the culture was maintained at the lower density of 0.25 x 10^6 cells/mL. In contrast, maintaining the culture at 0.75 x 10^6 cells/mL led to a lower CD8:CD4 ratio and a shift in the differentiation status of the population away from memory cells (Figures 2C and 2D).

Together, these results show that the density at which you maintain the culture during expansion is highly influential on the quantity and quality of the cellular output. A lower cell density at 0.25 x 10^6 cells/mL instead of the typical 0.5 x 10^6 cells/mL resulted in higher fold expansion, improved viability, cellular phenotype, and a larger central memory population.

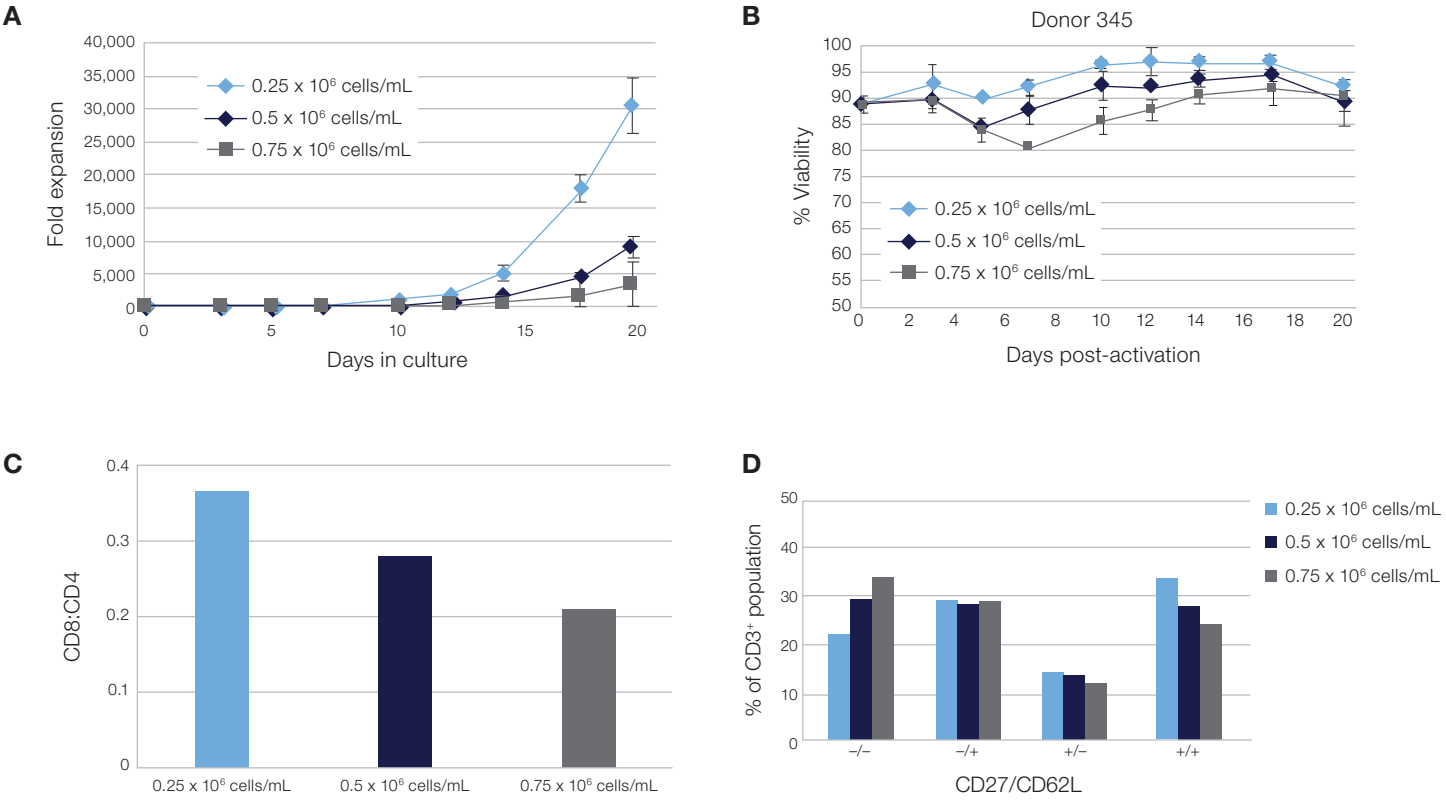

Figure 2. Effect of cell density on T cell growth and phenotype. The cell density at which the culture is maintained impacts **(A)** cell expansion, **(B)** cell viability, **(C)** the CD8:CD4 ratio, and **(D)** cell differentiation. Error bars represent the standard deviation of 3 donors performed in triplicate.

Supplementation schedule

Supplementation of the culture medium was also found to significantly impact the cellular outcome. It is well documented that components such as cytokines, supplements, and growth factors have differential impacts on cellular expansion and the quality of cellular output. However, the timing of supplementation is often less appreciated in T cell therapy manufacturing.

An example is the application of CTS ICSR over the course of longer manufacturing workflows. Traditionally, CTS ICSR is added starting at the time of T cell activation and refreshed for the entire manufacturing course. We investigated whether delaying the addition of CTS ICSR until later in the workflow or not adding it at all would affect the final cellular product of the primary T cells from three donors. The results presented in Figure 3 highlight the differences between adding CTS ICSR at day 0 or day 5, or never adding it. A day 10 addition was also evaluated, but the results were very similar to those associated with no CTS ICSR addition and were not reported for ease of graphical interpretation.

Cultures grown in the presence of CTS ICSR demonstrate a clear growth advantage. Here, we observed an average growth increase of 3- to 4-fold compared to those grown without CTS ICSR. In addition, there is a slight growth advantage for cells given CTS ICSR at day 0 over those given it at day 5 (Figure 3A). The percentage of viable cells throughout the workflow is high for all three groups, and there is almost no disparity between the individual results (Figure 3B). However, the length of exposure to CTS ICSR or the timing of addition was shown to influence the expression of cell surface markers. The results demonstrated that the CD8:CD4 ratios were similar between the groups when CTS ICSR was added on either day 0 or day 5. The CD8:CD4 ratios for the day 0 and day 5 groups were about 25% higher than the ratio for the cell population not exposed to CTS ICSR (Figure 3C). In Figure 3D, when CTS ICSR was added at day 5 instead of day 0, there was about a 20% increase in the amount of central memory cells as indicated by the expression of markers CD27 and CD62L. In agreement with this, there was a positive correlation between CTS ICSR exposure time and the number of effector cells, as indicated by double-negative staining of the central memory markers.

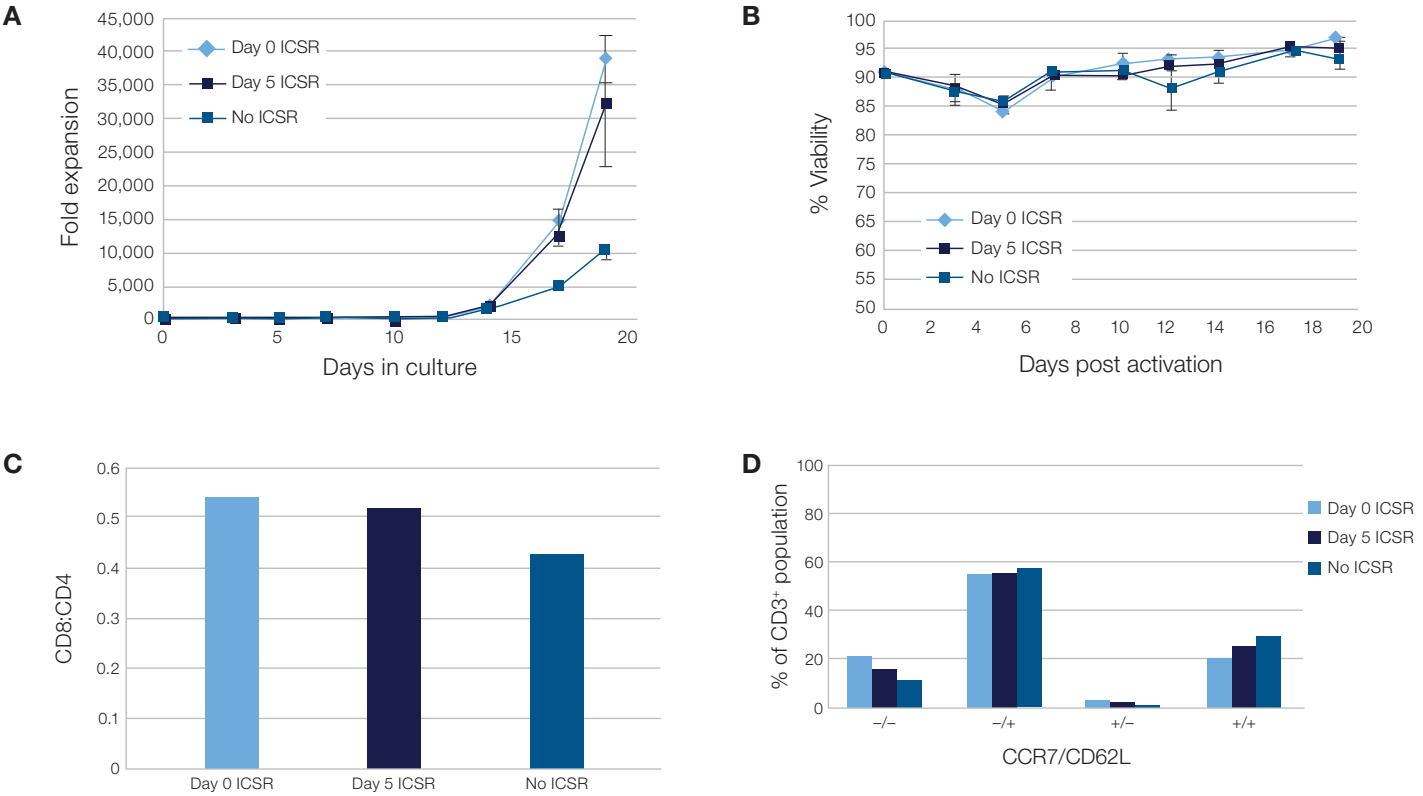

Figure 3. Effect of CTS ICSR supplementation timing on T cell growth and phenotype. (A) Cell expansion, **(B)** cell viability, **(C)** the CD8:CD4 ratio, and **(D)** cell differentiation were evaluated. Error bars represent the standard deviation of 3 donors performed in triplicate.

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Conclusion

One consequence of longer workflows is that T cells tend to rapidly differentiate when they are stimulated and expanded over longer periods of time. Slowing this transition is important to developing and fine-tuning T cell manufacturing processes lasting up to 2 or 3 weeks. Taken together, these results reveal that activating and culturing cells with CTS Dynabeads CD3/CD28 and CTS OpTmizer T Cell Expansion SFM, no phenol red, can support robust expansion of T cells for longer workflows without the need for restimulation. In addition, it was determined that relatively simple adjustments to the workflow processes, involving decreasing the maintenance cell density from 0.5×10^6 cells/mL to 0.25×10^6 cells/mL and adjusting the timing of supplementation, are examples of modifications that preserve a “younger” phenotype. In addition, type and amount of supplementation has long been known to influence the phenotype of the cellular product. It is also important to note that although the specific data shown here were generated using workflows in culture dishes, these results and relationships have been verified at a larger scale in rocking bioreactors.

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