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Identification of new cytokine combinations for antigen-specific T-cell therapy products via a high-throughput multi-parameter assay

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ABSTRACT

Infusion of viral-specific T cells (VSTs) is an effective treatment for viral infection after stem cell transplant. Current manufacturing approaches are rapid, but growth conditions can still be further improved. To optimize VST cell products, the authors designed a high-throughput flow cytometry-based assay using 40 cytokine combinations in a 96-well plate to fully characterize T-cell viability, function, growth and differentiation. Peripheral blood mononuclear cells (PBMCs) from six consenting donors were seeded at 100 000 cells per well with pools of cytomegalovirus peptides from IE1 and pp65 and combinations of IL-15, IL-6, IL-21, interferon alpha, IL-12, IL-18, IL-4 and IL-7. Ten-day cultures were tested by 13-color flow cytometry to evaluate viable cell count, lymphocyte phenotype, memory markers and interferon gamma (IFNγ) and tumor necrosis factor alpha (TNFα) expression. Combinations of IL-15/IL-6 and IL-4/IL-7 were optimal for the expansion of viral-specific CD3+ T cells, (18-fold and 14-fold, respectively, compared with unstimulated controls). CD8+ T cells expanded 24-fold in IL-15/IL-6 and 9-fold in IL-4/IL-7 cultures ($P < 0.0001$). CD4+ T cells expanded 27-fold in IL-4/IL-7 and 15-fold in IL-15/IL-6 ($P < 0.0001$). CD45RO+ CCR7− effector memory (CD45RO+ CCR7− CD3+), central memory (CD45RO+ CCR7+ CD3+), terminal effector (CD45RO− CCR7− CD3+), and naive (CD45RO− CCR7+ CD3+). T cells were the preponderant cells (76.8% and 72.3% in IL-15/IL-6 and IL-15/IL-7 cultures, respectively). Cells cultured in both cytokine conditions were potent, with 19.4% of CD3+ cells cultured in IL-15/IL-6 producing IFNγ (7.6% producing both TNFα and IFNγ) and 18.5% of CD3+ cells grown in IL-4/IL-7 producing IFNγ (9% producing both TNFα and IFNγ). This study shows the utility of this single-plate assay to rapidly identify optimal growth conditions for VST manufacture using only 10^7 PBMCs.

Introduction

Adoptive T-cell immunotherapies are increasingly used to treat infection and malignant disease. A common technique involves culturing T cells with antigen-presenting cells (APCs) exposed to peptide antigens in the presence of a cytokine cocktail. Several immunomodulatory cytokines are currently used to promote T-cell division and differentiation, but optimal conditions for the growth and function of peptide-stimulated T-cell products have yet to be fully defined. Cell products used in clinical trials have typically supplemented T-cell cultures with the growth-promoting cytokines IL-2, IL-15, IL-4 and IL-7 [1–3]. However, the search to optimize culture conditions is limited by the time and labor needed to screen multiple cytokine combinations. Therefore, the authors established a flow cytometry-based approach to rapidly evaluate many cytokine combinations in a single 96-well plate to measure T-cell phenotype and potency for a limited number of cells.

Manufacture of viral-specific T-cell products expands a heterogeneous pool of pre-existing memory T cells from peripheral donor blood, with the final product containing a polyclonal mixture of CD4+ helper and CD8+ cytotoxic T cells [4]. This diversity increases the complexity of manufacture, as CD4+ T cells and CD8+ T cells respond differently to cytokine stimulation. For example, IL-4 enhances the survival of resting T cells and induces CD4+ Th2 helper differentiation [5–7], whereas IL-15 promotes survival and diversity of CD8+ memory T cells [8,9]. IL-2 is a canonical T-cell growth cytokine that continues to be used in clinical trials because of its effectiveness in expanding T cells derived from tumor-infiltrating lymphocytes [10]. However, other cytokines also have essential functions:

(i) IL-6 may enhance Th17 development [11].
(ii) IL-7 promotes T-cell homeostatic survival [12–14].
(iii) IL-21 promotes the activity of CD8+ T cells [15–17].

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The authors' manufacturing methods have transitioned from culturing T cells in 24-well plates with IL-2 and APCs transduced with viral antigens [18–20] to a simplified culture containing a combination of IL-4 and IL-7 in G-Rex gas permeable devices (Wilson Wolf Manufacturing, St Paul, MN, USA) with soluble mixes of peptides [3,21,22]. This system rapidly expands functionally competent T cells specific for multiple viruses. Ten-fold expansions of 1.5×10^7 donor peripheral blood mononuclear cells (PBMCs) cultured in a G-Rex (Wilson Wolf Manufacturing) can provide sufficient CD3+ viral-specific T cells (VSTs) to treat multiple patients in the third-party "off the shelf" setting [3,23–26]. Manufactured T cells are currently characterized for safety, phenotype and potency in three separate assays: (i) enzyme-linked immune absorbent spot (ELISpot) for interferon gamma (IFNγ) release, (ii) ^{51}Cr release to measure cytotoxicity and (iii) flow cytometry to identify cellular phenotype. However, routinely available 13-color flow cytometry panels make it possible to measure intracellular cytokines, surface marker phenotype and correlates of cytotoxicity and alloreactivity in a single assay to define product quality. Flow cytometric assays minimize culture volume, reducing the number of cells needed for validation while increasing the number of testable conditions that can be applied to donor PBMCs. Using this approach, the authors identified new cytokine combinations, controlling phenotypic diversity, growth and function of viral-specific T-cell products. The authors found that high-throughput screening by multicolor flow cytometry is affordable and practical for product development.

Methods

Blood collection

Peripheral blood was collected from de-identified platelet transfusion filters from donors according to institutional review board-approved protocol.

Cell culture

PBMCs were separated by Ficoll and spun at 800 × g for 25 min to purify lymphocytes. Cells were washed twice with complete RPMI, and 2×10^7 cells were resuspended in 5 mL with 10 μL of 200 μg/mL peptide libraries encompassing IE1 and pp65. Cells were incubated at 37°C for 1 h. Next, 5 mL of complete media was added, and 100 000 cells per well were plated in 96-well round-bottom plates. Cytokines were added at the indicated concentration at a final volume of 200 μL. Cells were cultured for 7 days. Plates were then spun down at 400 × g and the cells resuspended in 200 μL of complete media. Samples were split into two plates of 100 μL each, and fresh media and cytokines were added at the indicated concentration at a final volume of 200 μL. Cells were cultured for 3 additional days before antigen restimulation and antibody staining.

For culture within G-Rex 10 gas permeable chambers (Wilson Wolf Manufacturing), $1–1.5 \times 10^7$ cells were isolated from PBMCs and resuspended in 1 mL of complete media with 2 μL of 200 μg/mL peptide libraries encompassing IE1 and pp65 and cultured for 1 h at 37°C. Next, 29 mL of complete media was added, along with either 400 U/mL IL-4 plus 10 ng/mL IL-7 or 10 ng/mL IL-15 plus 100 ng/mL IL-6. Cells were cultured for 7 days, after which 15 mL of media was removed and replaced with fresh complete media and cytokines. Cells were cultured for 3 additional days before antigen restimulation in ELISpot assays.

Intracellular cytokine staining

Plates were spun down at 400 × g for 5 min and resuspended in 100 μL of complete media containing the mix of IE1 and pp65 peptide libraries at 1.0 μg/mL final concentration with no peptide controls. Cells were incubated with peptides at 37°C for 1 h. Next, 100 μL of complete media containing brefeldin A or monensin plus brefeldin A was added. Cells were cultured for an additional 5 h, after which cells were stained with antibodies for phenotyping.

^{51}Cr cytotoxicity assay

VSTs were collected and resuspended in complete media to a concentration of 2×10^6 cells per mL. Autologous target cells (phytohemagglutinin blasts) were collected, washed once with phosphate-buffered saline (PBS) and resuspended in PBS to a concentration of 2.5×10^6 cells per mL. Next, 10 μL of ^{51}Cr was added, along with 1 μL of either actin or IE1 plus pp65 peptide pools per 100 μL target suspension. Targets were incubated with chromium and peptides for 1 h at 37°C, after which targets were washed three times with complete media. Effectors were added in serial dilution in triplicate, with 2×10^5 effectors as the top condition. After 1 h, targets were resuspended in complete media at a concentration of 5×10^4 cells per mL, and 100 μL of targets (5000 cells) was added per well in a 96-well U-bottom plate. Conditions included targets alone (spontaneous release), targets plus 1% Triton X-100 (maximum release) and 40:1, 20:1 and 10:1 E:T combinations using no peptide, actin peptide or IE1 plus pp65 peptide pools. Cells were cultured for 4 h, after which supernatant was collected for detection of ^{51}Cr release. Replicates were averaged together, and cytotoxicity was calculated according to the formula {[(specific release) − (spontaneous release)]/[(max release) − (spontaneous release)]}.

ELISpot assay

ELISpot plates were coated with 100 μL of 1 μg/mL final concentration anti-IFNγ monoclonal antibodies (clone 1-D1K; Mabtech, Cincinnati, OH, USA) in sterile ELISpot carbonate coating buffer (1.59 g sodium carbonate, 2.93 g sodium bicarbonate per liter of sterile water) overnight at 4°C. Plates were washed twice with 150 μL of coating buffer, and 100 μL of complete media was added to wells and incubated for 1 h at 37°C. Cells from G-Rex 10 culture vessels (Wilson Wolf Manufacturing) were plated at the indicated concentrations in 200 μL total volume with actin (1 μg/mL), IE1 and pp65 peptide pools (1 μg/mL), staphylococcal enterotoxin B (0.5 μg/mL) or media alone. Plates were incubated for 16 h at 37°C, after which cells were decanted and plates were washed six times with ×1 PBS/0.05% Tween 20.

Next, 100 μL of 1 μg/mL biotinylated anti-IFNγ monoclonal antibodies (clone 7-B6-1; Mabtech) in biotin buffer (2.5 g biotin in 500 mL ×1 PBS) was added to each well and incubated at 37°C for 1 h. Plates were washed six times with ×1 PBS/0.05% Tween 20. Then, 100 μL of an avidin-peroxidase solution in ×1 PBS/Tween 20 (Vectastain Elite ABC-HRP; Vector Labs, Burlingam, CA, USA) was added per well and incubated for 1 h at room temperature. Plates were washed three times with ×1 PBS/0.05% Tween 20 and three times with ×1 PBS. Spots were developed using 100 μL of 3-amino-9-ethylcarbazole substrate (Vector Labs, Burlingame, CA, USA) for 4 min at room temperature and rinsing with tap water. Plates were dried overnight, and individual wells were punched out onto adherent film for enumeration by an independent third party. Corrected spot counts were derived according to the formula {raw spot value + 2 × [(raw spot value × %confluence) / (100% − %confluence)]}.

Antibody staining

Cells were spun down at 400 × g for 5 min and washed once in 100 μL ×1 PBS. Cells were spun down again and resuspended in 50 μL ×1 PBS containing Live/Dead aqua at a dilution of 1:500 and then stained for 20 min at 4°C and washed with 100 μL ×1 PBS containing 2% fetal calf serum. Cells were fixed in 50 μL ×1 Cytofix/Cytoperm (BD Biosciences, San Jose, CA, USA) for 30 min at 4°C and washed twice in ×1 Perm/Wash (BD Biosciences), then resuspended in 25 μL of staining

solution containing 12.5 μL ×1 Perm/Wash (BD Biosciences) and 12.5 μL Brilliant Violet staining solution (BioLegend, San Diego, CA, USA). Markers for staining included CD62L V450 (clone DREG-56; BioLegend), CD4 BV570 (clone RPA-T4; BioLegend), CD45RO BV605 (clone UCHL1; BioLegend), CD8 BV711 (clone SK1; BioLegend), CD56 BV785 (clone 5.1H11; BioLegend), CCR7 FITC (clone G043H7; BioLegend), CD28 PE (clone REA612; Miltenyi Biotec, San Diego, CA, USA), CD95 PE Dazzle CF594 (clone DX2; BioLegend), CD3 PerCP Cy5.5 (clone OKT3; BioLegend), tumor necrosis factor alpha (TNFα) PE-Vio770 (clone cA2; Miltenyi Biotec), IFNγ APCs (clone RS.B3; BioLegend), CD107a APC H7 (clone REA 792; Miltenyi Biotec) and CD45RA APC H7 (clone HI100; BioLegend). Cells were stained for at least 30 min at 4°C, washed twice with 100 μL ×1 Perm/Wash (BD Biosciences) and resuspended in 55 μL ×1 Perm/Wash (BD Biosciences). Cells were loaded onto an iQue Screener Plus (Sartorius, Göttingen, Germany) to collect all samples. Data were analyzed using ForeCyt and FlowJo software (FlowJo, Ashland, OR, USA), and statistics were analyzed using Prism software (GraphPad, San Diego, CA, USA).

High-throughput screening of alternative cytokine combinations

The method allowed for up to 40 cytokine culture conditions to be tested on 1×10^7 PBMCs in a 96-well plate using the iQue Screener Plus high-throughput screener for all flow cytometry. This method combined initial culturing and antigen-specific expansion with staining and analysis in a single culture plate (see supplementary Figure 1). The authors selected flow cytometry as the method for analysis to combine measurements of cellular phenotype, viability, expansion and effector function in a single 13-color panel. To test for antigen specificity, cells were split on day 7 into two identical plates with fresh media and cytokines, and plates were subsequently challenged with or without peptide pools on day 10. The authors initially tested combinations of IL-15, IL-6, IL-21 and interferon alpha against IL-4 and IL-7 as a reference standard for four samples (plate layouts 1 and 2; also see supplementary Figure 2). The authors also tested modified layouts, which added IL-12 and IL-18, and combinations intermixing IL-15, IL-6, IL-4 and IL-7. Furthermore, the authors tested layouts with additional replicates of IL-15 plus IL-6 and IL-4 plus IL-7 and replicates challenged with irrelevant peptide pools as an additional measure of antigen specificity (plate layouts 3 and 4; also see supplementary Figure 2).

Overall, the use of 96-well plates as culture and staining vessels for flow cytometric analysis allowed the authors to test 92 different combinations of cytokines using four different plate layouts. The workflow for the analysis of samples utilized a hierarchical gating strategy that categorized positive and negative gates based on initial fluorescence minus one staining controls analyzed within FlowJo (see supplementary Figure 3). For phenotyping, the authors measured the frequency of living CD3+, CD3+ CD4+ and CD3+ CD8+ T cells present within the culture, along with CD3− CD56+ natural killer (NK) cells. Viability was measured by vital dye staining using Live/Dead aqua, whereas cytotoxic function was compared by measuring IFNγ and TNFα intracellular cytokine production in viral wells pulsed with peptide pools over antigen-non-specific background wells. Finally, T-cell memory marker surface expression was used to judge the differentiation status of cells, including CD45RA, CD45RO, CCR7, CD28, CD95 and CD62L.

Results

High-throughput screening of cytokine combinations

The authors first screened the growth and function of cells in all cytokine conditions. Representative heat map data are shown in Figure 1A and summarized in supplementary Figure 4. The control culture of PBMCs without added cytokine did not support T-cell growth. CD3+ T-cell expansion in the standard cytokine mix of IL-4 and IL-7 (optimum results with 400 U/mL and 10 ng/mL, respectively) achieved a 12.3-fold increase over control. IL-15 alone or in combination with other cytokines achieved the best expansions at the highest dose of 10 ng/mL, representing a 22.5-fold increase over control. Mixtures containing IL-6 and I-L21 produced only a modest expansion of 2.9-fold.

The authors then selected the combination of IL-15 (10 ng/mL) and IL-6 (100 ng/mL) for further investigation based on the favorable expansion of CD3+ T cells and their cytokine production in four experiments (Figure 1B). The authors confirmed that the addition of IL-15 or IL-7 was sufficient for the expansion of CD3+ T cells and that the original selection of IL-15/IL-6 was superior to all other combinations. Overall, culture in a combination of IL-15/IL-6 consistently promoted CD3+ T-cell expansion and IFNγ production to levels similar to culture in IL-4/IL-7.

Selective cytokine culture imparts bias on the ratio of CD4 versus CD8 cells in viral-specific T-cell products

Six cytomegalovirus (CMV)-reactive patient samples in IL-15 alone, IL-15 and IL-6 and IL-4 and IL-7 were compared in replicate testing (Figure 2). Culture in IL-15/IL-6 expanded a median of 17.1-fold more CD3+ cells compared with no cytokine controls ($P < 0.0001$). Culture in IL-4/IL-7 expanded a median of 13.8-fold more compared with no cytokine control ($P < 0.0001$) (Figure 3A). The viability of CD3+ cells on average was not significantly different between wells containing IL-15/IL-6 or IL-4/IL-7, with a median of 90% and 89%, respectively ($P = 0.966$). CD3− CD56+ NK cells were present in cultures expanded with IL-15/IL-6, with a median of 6.6% of total cells recovered (4106 cells) (Figure 2A). Less than 300 NK cells were recovered on average from wells containing IL-4/IL-7, representing 0.6% of total cells recovered.

Interestingly, the authors identified a strong bias in the ratio of CD4+ to CD8+ cells in the final product, depending on the initial culture's cytokines. Culture in IL-15/IL-6 conditions favored outgrowth of CD8+ T cells compared with culture in IL-4/IL-7, which favored outgrowth of CD4+ T cells. Culturing cells in IL-4/IL-7 expanded 2.1-fold more CD4+ T cells than cells cultured in IL-15/IL-6 ($P < 0.0001$)(Figure 2B), whereas culturing cells in IL-15/IL-6 more than doubled the expansion of CD8+ cells compared with IL-4/IL-7, with 2.8-fold more CD8+ cells on average in IL-15/IL-6 versus IL-4/IL-7 ($P < 0.0001$) (Figure 2B). The viability of CD4+ and CD8+ cells was not significantly different when comparing culture in IL-15/IL-6 and IL-4/IL-7 (CD4 viability, $P = 0.4381$, CD8 viability, $P = 0.1033$; data not shown), suggesting the different cytokine combinations were stimulating outgrowth of either CD4+ or CD8+ cells, rather than preserving the selective survival of individual subsets.

Both culture conditions expanded CD3+ T cells producing IFNγ in response to CMV peptide pool restimulation. The highest concentration of IL-15 plus IL-6 induced a median of 19.3% IFNγ-producing CD3+ T cells (range, 0.8–55.3%), whereas IL-4 plus IL-7 induced a median of 16.3% IFNγ-producing CD3+ T cells in response to CMV peptides (range, 3.8–49.6%, $P = 0.99$) (Figure 2C). The authors also investigated the proportion of multi-cytokine-producing cells, as evidence suggests these cells offer superior protection against viral infection compared with cells producing a single cytokine. A median of 6.1% (range, 0.6–35.4%) of cells cultured in IL-15/IL-6 and 6.9% (range, 2.3–25.0%) of cells cultured in IL-4/IL-7 produced both IFNγ and TNFα in response to CMV peptides ($P = 0.22$) (Figure 2C). The authors observed no significant difference in the total number of CMV-reactive CD3+ cells when comparing IL-15/IL-6 with IL-4/IL-7 cytokine conditions ($P = 0.45$) (Figure 2D).

Limited production of cytokines by IL-15-expanded CD4+ T cells has been previously reported. Therefore, the authors compared the

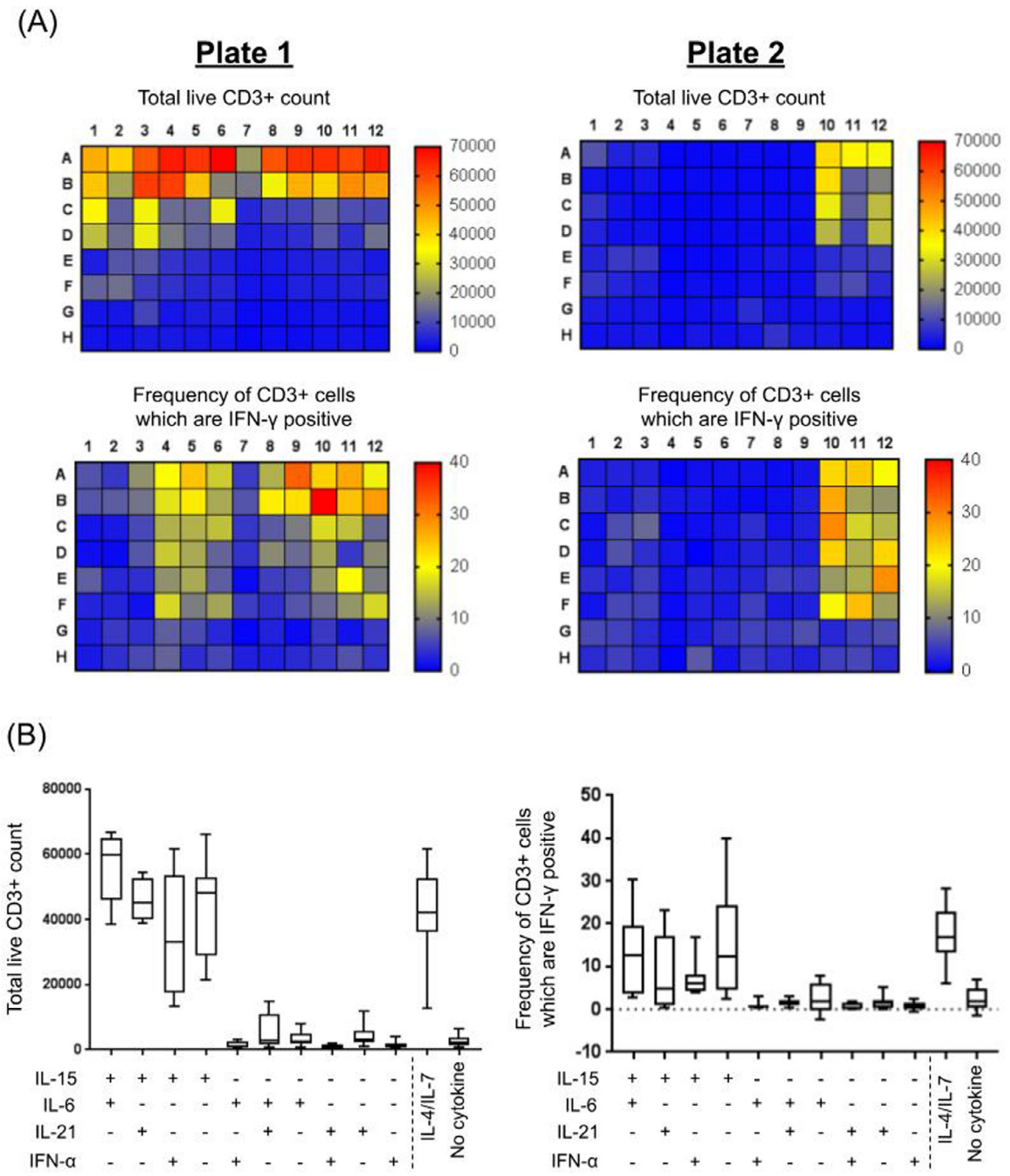

Fig. 1. High-throughput flow cytometry analysis identifies superior cytokine combinations. (A) Quantifications of phenotype and function of two plates from sample 4 were analyzed by flow cytometry on day 10, with entire contents of wells collected and visualized by heat map. The total count of viable CD3+ T cells was quantified from wells restimulated with media alone. The frequency of IFNγ+ CD3+ cells was derived from the frequency of IFNγ+ CD3+ cells after restimulation with IE1 and pp65 peptide pools and subtracted from media alone control wells. (B) Wells containing the highest concentration of cytokines were compared between samples 1–4 when cultured using plate layouts 1 and 2. The total recovered viable CD3+ count and frequency of CMV-specific CD3+ IFNγ+ cells ($n \geq 8$) were compared across each sample.

production of IFNγ by CD4+ and CD8+ subsets within the CD3+ T-cell population and found culture in IL-15/IL-6 was sufficient to expand CMV-specific CD8+ and CD4+ cells in a manner equivalent to culture in IL-4/IL-7 (Figure 2E). In addition, 6.6% (range, 0–34.2%) of CD8+ cells cultured in IL-4/IL-7 and 11.1% (range, 0–45.7%) of CD8+ cells cultured in IL-15/IL-6 produced IFNγ in response to CMV ($P = 0.46$). Similarly, a median of 17.3% (range, 0–49.4%) and 16.6% (range, 0–62.7%) of CD4+ cells produced IFNγ in response to CMV peptides when cultured in IL-4/IL-7 and IL-15/IL-6, respectively ($P = 0.46$). In summary, compared with IL-4/IL-7, cultures in IL-15/IL-6 produced a comparable number of CMV-reactive CD3+ VSTs that were more skewed toward a CD8+ phenotype.

VSTs grown in IL-15/IL-6 show CMV-specific cytotoxicity

The authors compared the CMV-specific cytotoxicity of cells grown in IL-15/IL-6 by traditional ^{51}Cr release assays and CD107a expression in the flow-based assay in Figure 3. VSTs were expanded in IL-15/IL-6 as effectors and split between assays after 10 days of expansion for donors 3 and 4. Neither donor was reactive against autologous targets up to a 10:1 E:T ratio. VST effectors were also challenged with autologous targets loaded with either actin peptide (negative control) or IE1 plus pp65 peptide pools. Both donors demonstrated CMV-specific reactivity, with VSTs from donor 4 reaching 26% CMV-specific killing and VSTs from donor 3 reaching 18%

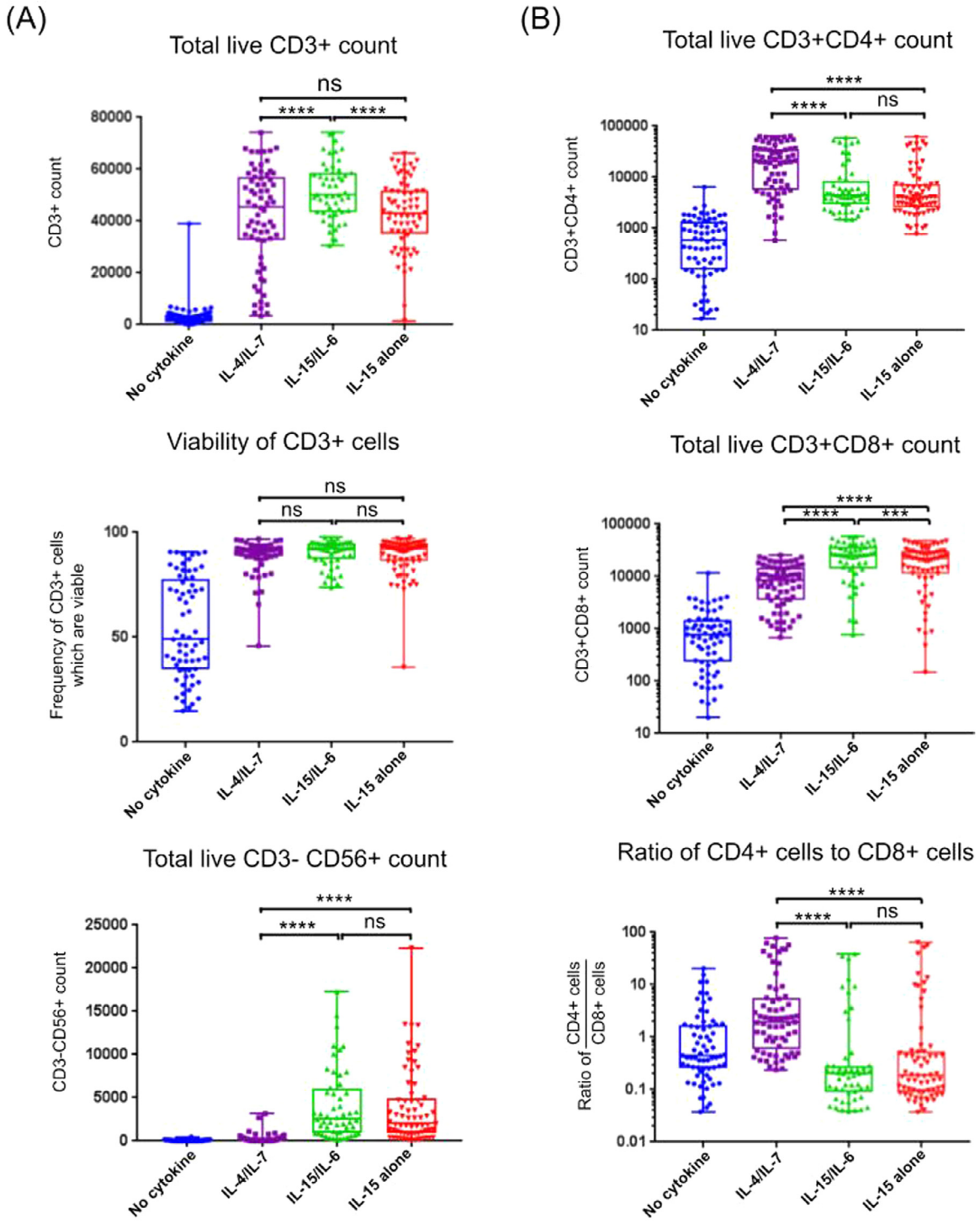

Fig. 2. Culture in IL-15 and IL-6 stimulates expansion of CMV-specific CD3+ T cells equal to or better than IL-4 and IL-7. Cells were restimulated with CMV peptide pools for 6 h and evaluated for phenotype and function by flow cytometry, with the entire well contents analyzed for the top dilutions of IL-15 and IL-6, IL-4 and IL-7, IL-15 alone and no cytokine controls. The median of individual replicates ($n \geq 8$) was analyzed across experiments, and two-way ANOVA analyzed individual patient samples ($n = 6$) and statistics with Tukey's correction. From wells restimulated with media alone, the total count of viable CD3+ T cells, percentage of viability of all CD3+ cells and viable CD56+ CD3− NK cell count were calculated from wells (A). The median total count of viable CD3+ CD4+ cells and CD3+ CD8+ cells and the ratio of CD4+ to CD8+ cells were calculated from wells (B). From wells restimulated with IE1 and pp65 peptide pools, the median frequency of CD3+ IFNγ+ cells and CD3+ IFNγ+ TNFα+ cells was calculated from wells (C), along with the total number of CMV-specific CD3+ IFNγ+ cells (D), and the frequency of IFNγ+ cells within CD4 + CD3+ and CD8+ CD3+ subtypes was analyzed (E). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$. ANOVA, analysis of variance; ns, not significant ($P > 0.05$).

CMV-specific killing at a 40:1 E:T ratio with 1% actin-specific killing (Figure 3A). VSTs from these cultures were also restimulated with actin or IE1 plus pp65 peptide pools and analyzed by flow cytometry for CD107a expression. CD107a (LAMP1) can potentially be used as a surrogate marker for cytotoxicity, as it is transiently expressed on the cell surface during degranulation [27]. A median of 8.3% of CD3+ VSTs derived from donor 3 expressed CD107a in response to IE1 plus pp65 peptide restimulation, whereas a median of 17.6% of CD3+ VSTs

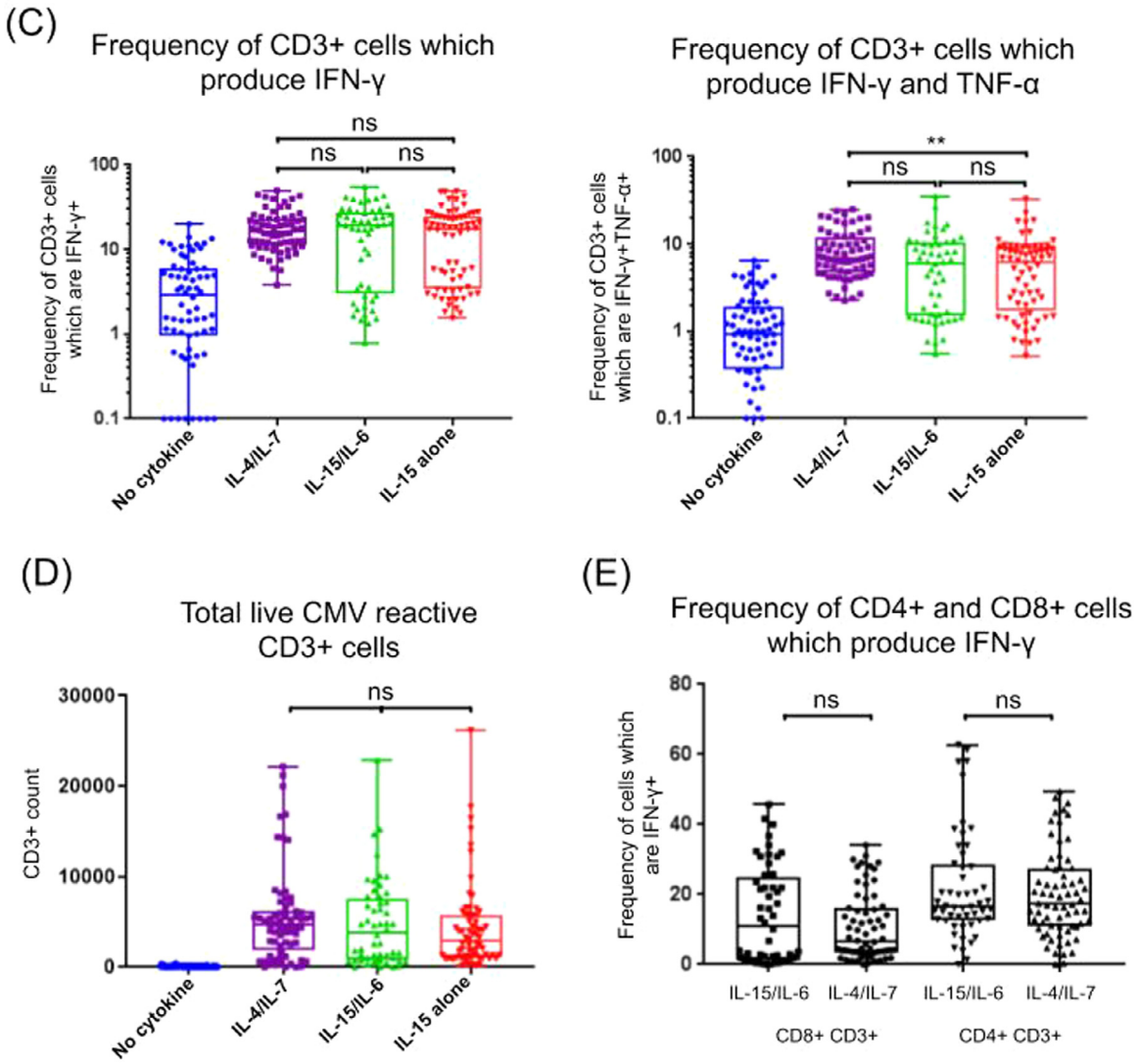

Fig. 2 Continued.

derived from donor 4 expressed CD107a after restimulation (Figure 3B). This demonstrates that CD107a is a potential alternative marker for cytotoxicity as part of the assay.

VSTs are effector memory in phenotype

The extent of T-cell differentiation has been suggested to influence the persistence of adoptively transferred T cells [28,29]. The authors characterized the surface phenotype of VSTs expanded by *in vitro* culture in IL-15/IL-6 and IL-4/IL-7 to identify the proportion of cells expressing different combinations of T-cell memory markers in Figure 4. Pre-culture CD3+ T cells comprised on average 32% naive/stem cell memory cells, 30.4% effector memory cells, 22% central memory cells and 15.4% terminal effectors. Ten-day VST products had a preponderance of effector memory cells (CD45RO+ CCR7- CD3+), representing 72.3% and 76.9% of cells grown in IL-4/IL-7 and IL-15/IL-6, respectively. Terminal effector cells lacking both CCR7 and CD45RO represented 11.3% of cells grown in IL-4/IL-7 and 14.3% of cells grown in IL-15/IL-6. Central memory cells (CD45RO+ CCR7+ CD3+) represented a minority of cells after culture: 9.3% and 6.6% in IL-4/IL-7 and IL-15/IL-6 cultures, respectively. There was a greater frequency of naive cells (CD45RO- CCR7+ CD3+) in IL-4/IL-7 cultures (7.0%) than in IL-15/IL-6 cultures (2.3%). Both culture conditions substantially reduced the frequency of naive cells compared with pre-culture frequencies (32%). The authors also compared the memory phenotype of antigen-specific cells with antigen-non-responsive cells. CD3+ IFNγ+ cells had a predominantly effector memory phenotype, whereas a small number of naive cells (4.2% in IL-4/IL-7 and 1.7% in IL-15/IL-6) remained within the IFNγ− (antigen-non-responsive) fraction, suggesting that culture in IL-4/IL-7 was preserving a subset of naive cells within the final product.

Process development time substantially reduced by using iQue

Process development of antigen-specific T cells such as VSTs has mostly been limited to testing specific conditions in 24-well plates or G-Rex 10 devices, limiting the systematic testing of an array of cytokines and growth conditions. Expanding and testing VSTs using such methods require approximately 20 h of work and 34 h of incubation per cytokine condition. By contrast, process development in 96-well plates and a 13-color flow cytometry panel required only 12 h of work and 8 h of incubation. Therefore, evaluating 40 cytokine conditions takes 2160 h per sample using existing methods but only 20 h using the authors' improved approach (Table 1).

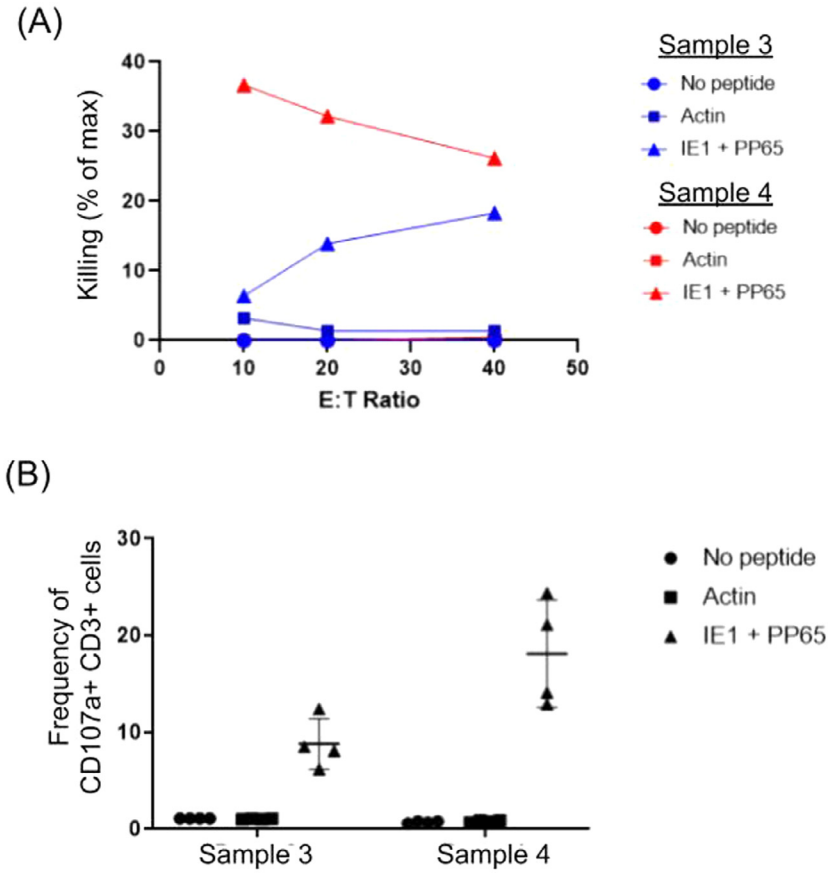

Fig. 3. VSTs cultured in IL-15/IL-6 show CMV-specific cytotoxicity. VSTs cultured in the presence of IL-15/IL-6 were investigated for cytolytic activity as measured by ^{51}Cr release assay (A) and by expression of CD107a (B). VSTs were measured for cytotoxicity against autologous PHA blasts pulsed with no peptide, actin or IE1 and pp65 peptide pools (A). VSTs were measured for cytolytic activity by flow cytometry of CD107a expression after restimulation for 6 h with no peptide, actin or IE1 and pp65 peptide pools (B). PHA, phytohemagglutinin.

Optimized cytokine conditions identified in the 96-well plate translate to clinical scale manufacturing

Miniaturized cell cultures may not reliably scale up in a linear fashion. To test whether the authors' system could predict the phenotype and function of clinical-sized products, we investigated whether IL-15/IL-6 cultures in G-Rex 10 culture vessels would recapitulate the data from our experiments using 96-well plates. 1-1.5 $\times$ 10^7 cells were seeded with IE1 and pp65 peptide pools in G-Rex 10 culture vessels with medium and either IL-4/IL-7 at 400 U/mL IL-4/ 100 ng/mL IL-7 or 10 ng/mL IL-15/ 100 ng/mL IL-6 (Figure 5). Cells grown in IL-15/IL-6 produced 638 $\pm$ 297 spots per 100 000 cells in response to CMV peptides, whereas cells cultured in IL-4 and IL-7 produced a mean of 555 $\pm$ 230 spots per 100 000 added cells in response to CMV peptide pool restimulation. The CMV response was antigen-specific, as cells produced less than 10 spots on average in response to either actin or no peptide controls per 100 000 added cells. This demonstrates that cells grown in IL-15/IL-6 were functionally equivalent to cells grown in IL-4/IL-7 when cultured to clinical scale and that the high-throughput screening method can reliably optimize product development.

Discussion

Here the authors describe a high-throughput flow cytometric assay to rapidly and efficiently evaluate the growth of viral-specific T cells from donor PBMCs in multiple cytokine combinations. Among the combinations tested, the authors identified superior and comparable expansion and T-cell effector function for cells cultured in IL-4/IL-7 and IL-15/IL-6. Culture with IL-4/IL-7 favored an expansion of CD4+ T cells at the expense of CD8+ T cells, whereas culture with IL-15/IL-6 expanded both CD8+ and CD4+ T cells. The authors subsequently confirmed that the IL-15/IL-6 cytokine growth condition was equivalent to IL-4/IL-7 by IFNγ at clinical scales.

This flow cytometry approach allowed the authors to evaluate 40 cytokine combinations per plate using only 1 $\times$ 10^7 cells. Promising cytokine combinations were reinvestigated with additional replicates in subsequent experiments, ultimately using only 3 $\times$ 10^7 total PBMCs to measure 90 total cytokine combinations. Importantly, both the culture conditions and the functional assay were modular by design, allowing for simple exchange of new cytokine combinations into the culture layout as inferior conditions were removed and introducing new flow cytometric markers into the functional assay. This flexibility helps process development by increasing speed, replicating reproducibility and efficiently using a limited starting product. The approach generates a large amount of data, necessitating some restrictions on the parameters selected to study. Therefore, the authors selected specific cytokine combinations that were optimal for T-cell expansion, secretion of IFNγ and TNFα, maintenance of cytotoxicity and central/effector memory status. Cytokine combinations of IL-6, interferon alpha and IL-21 (not including IL-15) were excluded because they induced little CD3+ proliferation compared with combinations of IL-4 and IL-7. When comparing IL-7 and IL-4 combined with other cytokines, the authors found that IL-7 but not IL-4 promoted VST growth. This is consistent with observations that IL-4 promotes cell survival but only supports the growth of naive T cells [5,6,30].

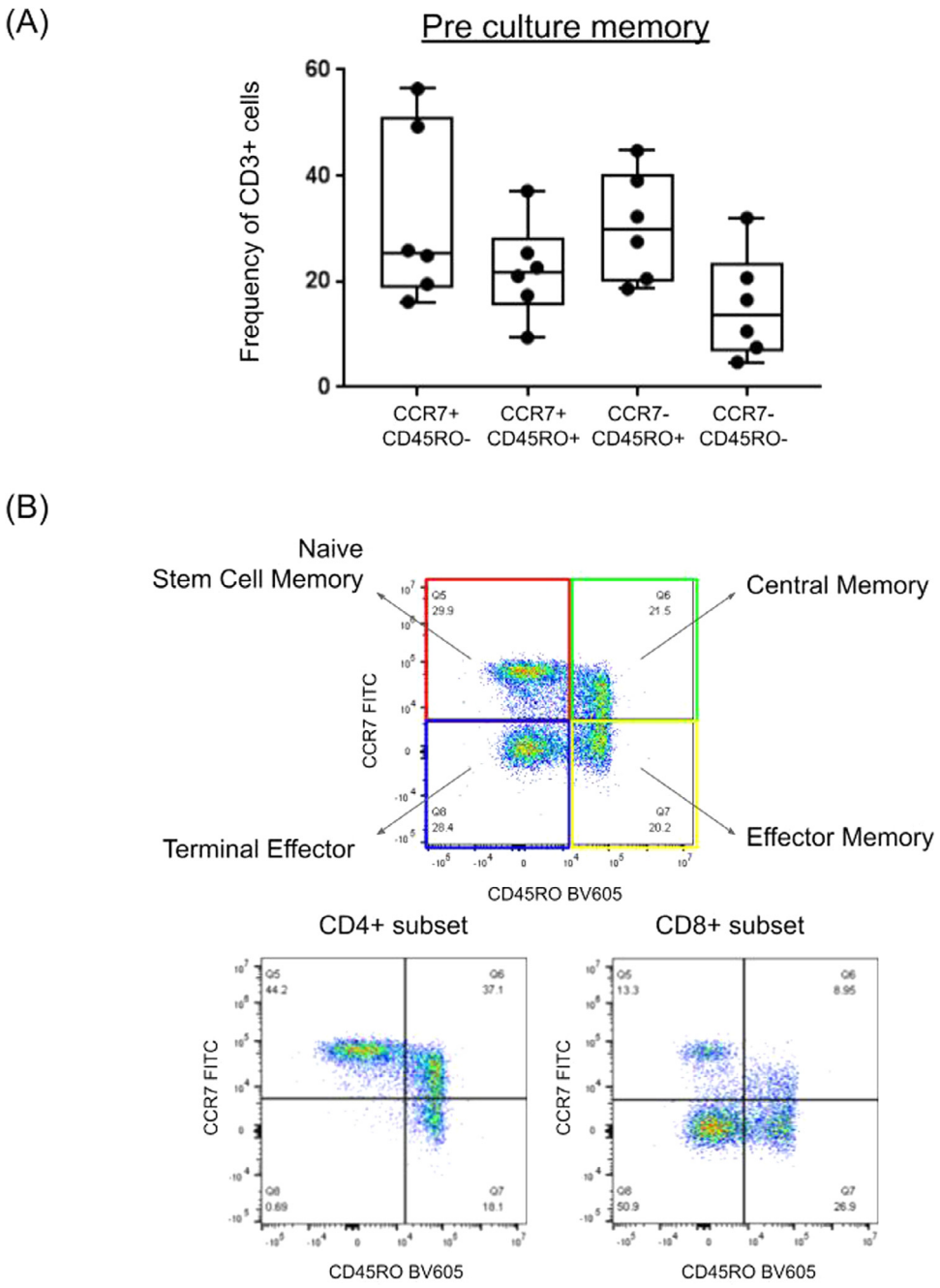

Fig. 4. T-cell therapy products are effector memory in phenotype (CCR7− CD45RO+). Cells were analyzed for the expression of memory markers CCR7 and CD45RO and divided into four populations, both pre- and post-culture, with cytokines. The pre-culture memory phenotype of viable cells was quantified for all samples (A) according to the layout presented for representative sample 1 (B). After 10-day culture, samples were analyzed again for memory markers CCR7 and CD45RO and averaged samples cultured in IL-15/IL-6 and IL-4/IL-7 growth conditions (C) and analyzed using two-way ANOVA with Tukey's correction. One representative sample was examined for the memory phenotype of CD3+ cells that were positive or negative for IFNγ after restimulation with CMV-specific peptides. $^{*}P < 0.05$. ANOVA, analysis of variance; pep, peptide.

The authors also found that IL-6 improved cell expansion in combination with IL-15 without modifying effector function. These results are consistent with knockout mouse experiments showing that IL-6 reduces the threshold for T-cell receptor signaling in CD8+ T cells [31], promoting memory T-cell expansion in response to antigen-specific peptide restimulation. The requirement for including IL-15 or IL-7 for memory T-cell expansion is expected, as both receptors share homology with IL-2 and use the common gamma chain and its associated JAK/STAT signaling proteins [32–34]. Recombinant IL-7 has been used clinically to expand T-cell subsets in cases of lymphopenia [35,36] and was included with IL-4 for its pro-survival benefits for T cells [37].

The authors show that culture in IL-15 and IL-6 supports robust antigen-specific CD4+ T-cell expansion. This is in contrast to earlier studies suggesting that culture in IL-15 was inferior to culture in IL-4/IL-7 because of a lack of antigen-specific CD4+ T-cell expansion—despite superior total cell expansion—and excessive CD56+ NK cell growth [37]). The authors identified only a small (median, 6.6%) growth of NK cells in culture with either IL-15/IL-6 or IL-15 alone. Production of an efficacious mix of viral-specific CD4+ and CD8+ is needed for T-cell therapy products to enhance the cytotoxic CD8+ T-cell response, with "help" provided by anti-viral CD4+ T cells in the form of immune activation, recruitment and inhibition of viral replication [38]. For CMV infections, CD8+ T-cell

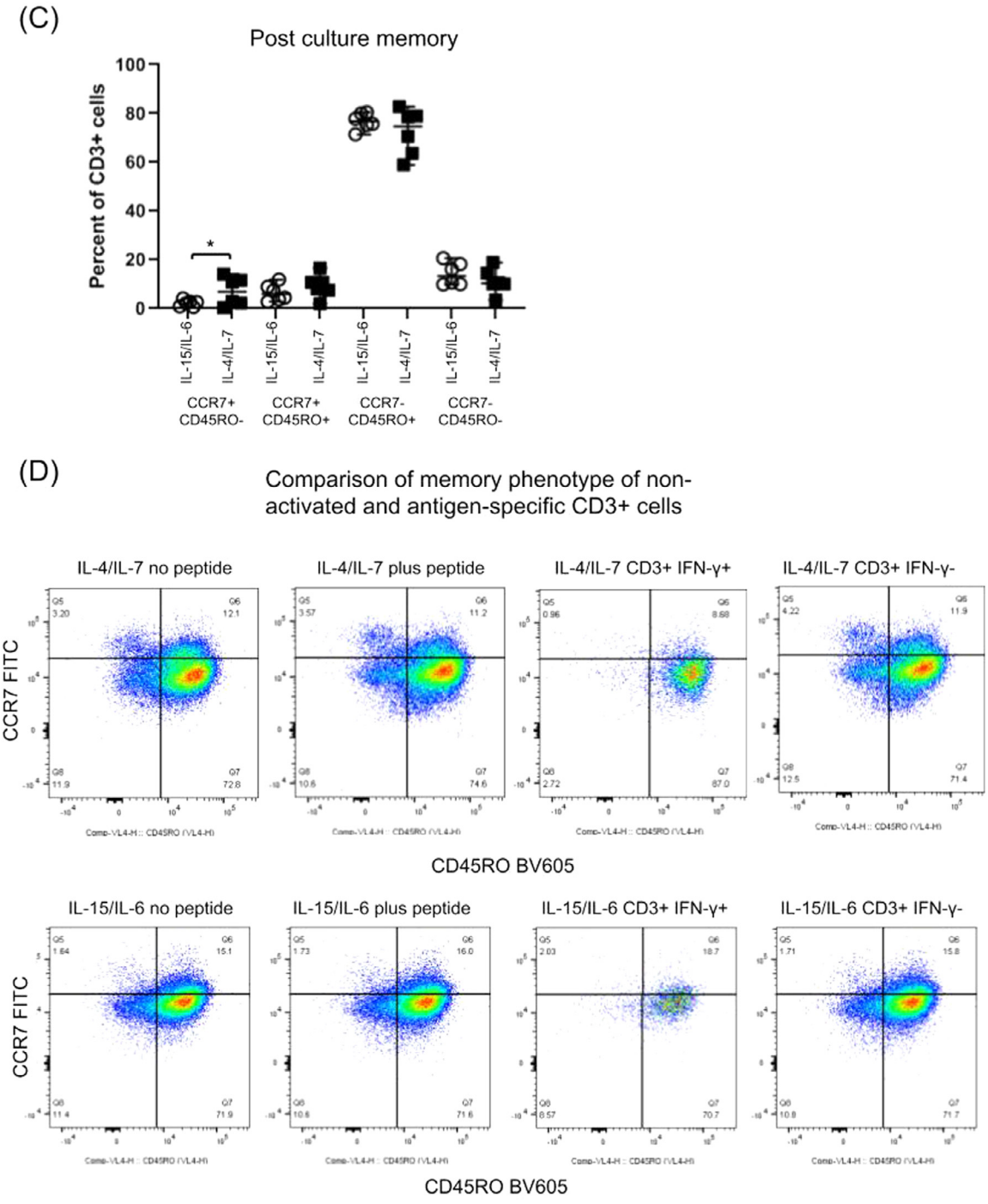

Fig. 4 Continued.

responses correlate with the resolution of disease after hematopoietic stem cell transplantation [39], whereas the addition of CMV-specific CD4+ T cells has also been demonstrated to help CD8+ T-cell responses for some hematopoietic stem cell transplantation patients [40] and has been suggested to support CD8+ cell persistence [41]. The authors also discovered that some donors appeared to have a preference for expansion of CD3+ cells in IL-4/IL-7, whereas other donors favored expansion in IL-15/IL-6 (see supplementary Figure 5). Individual donors may present with a pre-existing bias favoring CD4+ CMV-specific T cells, whereas other donors may have a bias favoring CD8+ CMV-specific T cells.

Importantly, the authors' experiments demonstrated CMV-specific VSTs expanded in both culture conditions, and multiple clinical trials have used VST cells cultured in IL-4/IL-7 to treat ongoing viral infections, including Epstein-Barr virus-related post-transplant lymphoproliferative disorder [42–45]. These successes may represent the relative abundance of antigen-specific memory T cells expanded by the memory VST protocol. Nevertheless, the combination of IL-15/IL-6 may provide a more balanced ratio of antigen-specific CD4+ to CD8+ T cells during the polyclonal expansion of T-cell products against not only viral-specific antigens but also other targets, including tumor-associated antigens. Specifically, T cells expanded against tumor-associated antigens and antigen-specific T cells derived from naive cord blood require APCs as stimulators to promote differentiation and expansion of naive (e.g., cord blood) T cells and/or to improve stimulation of potentially exhausted T cells (e.g., from cancer patients).

Table 1

	Traditional PD in culture vessels	Traditional PD in plates	Microassay using flow cytometry
Culture vessel	G-Rex 10	24-well plate	96-well plate
Sample usage	15×10^6	50×10^6	10×10^6
Conditions per culture vessel	1	24	48 duplicates
Time to Ficoll, culture and feed	8 h culture	10 h culture	8 h culture
Time for analysis, ELISpot	4 h setup, 8 h incubation	4 h setup, 18 h incubation	Included in intracellular flow
Phenotype	2 h setup, 1 h analysis	2 h setup, 1 h analysis	Included in intracellular flow
Cytotoxicity	4 h setup, 8 h incubation	4 h setup, 8 h incubation	Included in intracellular flow
Viable count	1 h setup	1 h setup	Included in intracellular flow
Intracellular flow	NA	NA	4 h setup, 6 h incubation
Total time investment for all conditions	8 h culture, 38 h analysis (4416 h for 96 conditions)	10 h culture, 38 h analysis (192 h for 96 conditions)	8 h culture, 10 h analysis (36 h for 96 conditions)

NA, not applicable; PD, process development.

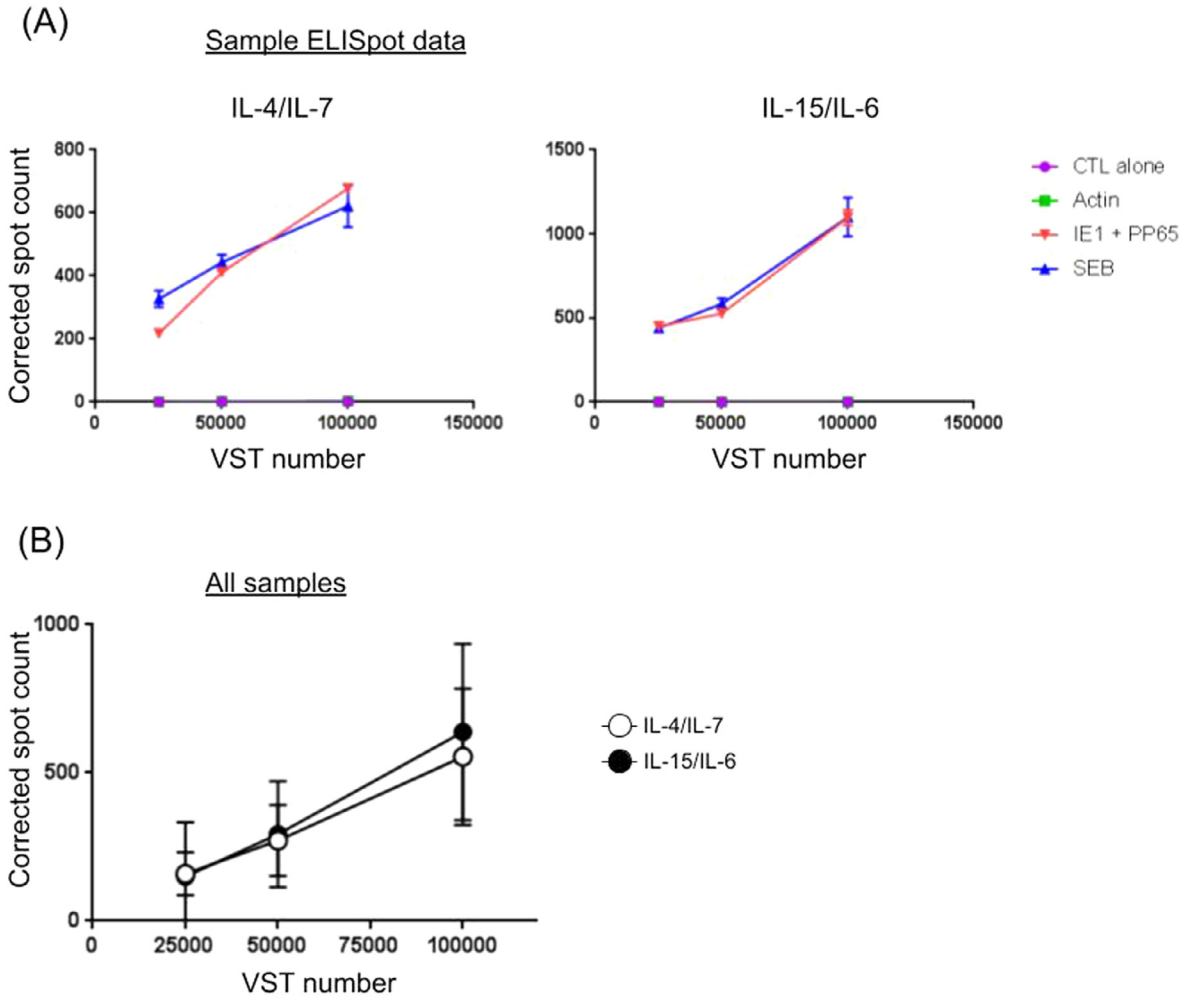

Fig. 5. Cells cultured in G-Rex 10 vessels with IL-15/IL-6 produce equivalent levels of IFN-γ compared with culture in IL-4/IL-7. Cells were grown in G-Rex 10 culture vessels with IL-15 and IL-6 or IL-4 and IL-7 for 10 days and tested in ELISpot assays for IFN-γ production when restimulated with media alone, actin (1 μg/mL), IE1 and pp65 peptide pools (1 μg/mL) or SEB (0.5 μg/mL). A representative sample is given (A), and the mean of four different samples (B) was compared using two-way ANOVA with Tukey's correction. ANOVA, analysis of variance; SEB, staphylococcal enterotoxin B; VST, virus specific T cell.

The authors' published clinical trials targeting lymphomas [46] and leukemias [47,48] demonstrate that we can expand tumor-specific T cells from the peripheral blood of healthy donors and cancer patients using peptide-pulsed antigen-presenting cells co-cultured with IL-15, IL-6, IL-7 and IL-12 as growth factors. Subsequently, tumor-associated antigen-specific T cells are further expanded using antigen-presenting cells cultured with IL-7 (10 ng/mL) and IL-2 (100 U/mL). Hence, all of these steps (dendritic cell generation; first, second, third stimulations) present unit operations that could be tested using a high-throughput approach, such as the one presented here.

In conclusion, the authors have shown that this high-throughput plate-based flow cytometric assay can effectively and reliably measure T-cell growth, function and phenotype to optimize VST product development. The authors have shown that IL-15/IL-6 is equivalent to IL-4/IL-7 in Good Manufacturing Practice culture conditions. The

assay's modular nature facilitates future investigations to optimize culture conditions with three or four cytokine combinations using IL-15/IL-6 and IL-4/IL-7 as a baseline.

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Declaration of Competing Interest

Sartorius loaned the iQue Screener Plus. CMB serves as an advisory board member for Cellectis, is a co-founder of Mana Therapeutics, owns stock in Torque Therapeutics and NexImmune and serves on the board of directors for Cabaletta Bio. PJH is a co-founder and serves on the board of directors of Mana Therapeutics as well as the scientific advisory board of Cellevolve. MDK serves as an advisory board member for Gilead Sciences.

Author Contributions

Conception and design of the study: CAL, MDK and PJH. Acquisition of data: CAL, AAD and EKR. Analysis and interpretation of data: CAL, AAD and EKR. Drafting or revising the manuscript: CAL, MDK, CMB and PJH. All authors have approved the final article.

Supplementary materials

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.jcyt.2020.08.006.

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