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Short Communication

A new workflow combining magnetic cell separation and impedance-based cell dispensing for gentle, simple and reliable cloning of specific CD8+ T cells

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ABSTRACT

Reverse immunology has open the door to innovative cancer immunotherapy strategies such as immunogenic antigen-based vaccination and transgenic T cell receptor (TCR)-based adoptive cell transfer. This approach enables the identification of immunogenic tumor specific antigen derived peptides. One of the major challenges is the rapid selection of antigen-specific CD8+ T cell clones. Thus, IFNγ-producing CD8+ T cells magnetic sorting combined with limiting dilution cloning approach represents the most common method of specific T cell cloning. However, during plate setup several wells will not contain T cells whereas others will contain mixed population of T cells. In this case, a re-cloning step is required which make limiting dilution based cloning a laborious, inefficient, expensive and a time-consuming method. To address these obstacles, here we present a novel 2-step workflow combining simple, affordable and gentle magnetic cell separation followed by single cell isolation using a device called DispenCell-S1. We aimed to compare this new workflow with the traditional limiting dilution method using *in vitro* generated antigen-specific CD8+ T cells. Herein, we reported the reliability of DispenCell-S1 method and its efficiency in T cell clones isolation.

Introduction

Antigen-specific T cell clones are required for the development of several cancer immunotherapy strategies such as therapeutic vaccines and transgenic TCR-based adoptive cell transfer (ACT). However, one of the main challenges of these approaches is the identification of highly immunogenic tumor antigens. Reverse immunology is a procedure allowing the prediction and identification of immunogenic peptides derived from targeted antigen gene sequence [1–5]. This high-throughput method is particularly efficient for antigen-derived peptides identification [6–8] as well as cancer immunotherapies development as we previously used to start phase I immunotherapy vaccine protocols UCPVax', 'UCPVax-Glio' and 'VolATIL' registered on the clinical trials.gov website and identified respectively by the National Clinical Trial (NCT) (NCT02818426), (NCT04280848) and (NCT03946358).

Reverse immunology is a several steps approach starting from tumor-specific transcripts identification, prediction and determination of proteasomal cleavage sites, peptide-binding prediction to HLA molecules and experimental validation, assessment of the *in vitro* and *in vivo* immunogenic potential of selected peptide antigens to isolation of specific cytolytic T cell clones and validation of their tumor cell recognition.

Thus, the isolation of T cell clones specific to immunogenic peptides is required for the development of cancer immunotherapy strategies. Indeed, T cell clones are used to provide evidence for tumor cells immunogenicity and therefore show that the antigen processing and presentation machinery generates immunogenic antigen-derived peptides. Therefore, limiting dilution represents the most common method of T cell cloning. It relies on statistical probability of isolating a monoclonal proliferating T cell per well. Although up-to-date limiting dilution is widely used for T cell cloning, the main encountered limit when it comes

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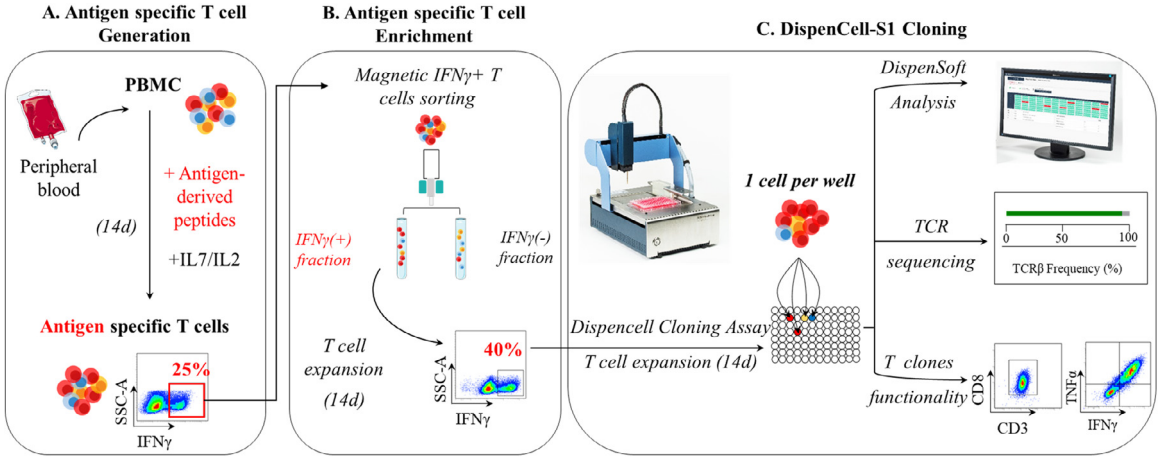

Fig. 1. Overview of antigen specific T clones isolation using DispenCell-S1. A. Peripheral blood mononuclear cells (PBMC) are isolated from healthy donor blood by density gradient separation and cultured *in vitro* with candidate peptides and IL7, IL2 cytokines. By day 14, IFNγ production is evaluated by *in vitro* stimulation and intracellular flow cytometry. **B.** IFNγ-secreting T cells are magnetically sorted and expanded for 14 days on irradiated feeder cells, PHA and IL2. **C.** Enriched T cells are cloned by DispenCell-S1 instrument. One cell per well is dispensed on round-bottom 96 wells plate and expanded on irradiated feeder cells, PHA and IL2 for 14 days. Single cell quality control is performed using DispenSoft and TCRβ sequencing and production of IFNγ and TNFα linked to T clones functionality is evaluated by flow cytometry.

to this method is the inefficiency of T cell dispensing. Consequently, re-cloning steps are warranted which comes as money and time consuming. Therefore, we developed a novel 2-step workflow combining simple, affordable and gentle magnetic cell separation followed by single cell isolation using a device called DispenCell-S1[9,10]. Moreover, this device provides an immediate proof of single cell isolation, which guarantees the monoclonality of selected T cell candidates and reduce the need for checking cell clonality.

Over two decades after reverse immunology concept was initiated, the evolution of T cell cloning steps remains rudimentary. Here, we report the development of a high efficient, reproducible and scalable method for T cell cloning using this single cell cloning system called DispenCell-S1. Antigen-specific CD8+ T cell clones were gently isolated and TCR sequencing was performed for clonality validation. In addition, we demonstrated that *in vitro* expanded CD8+ T cell clones functionality was preserved after DispenCell-S1 cloning method.

Materials and methods

Samples

Anonymized healthy donors' blood was collected in the Etablissement Francais du Sang (EFS, Besancon, France). Apheresis kits were obtained after written informed consent. Blood was diluted 1:2 in PBS (Gibco) and layered on a density gradient (lymphocyte separation medium Eurobio). After centrifugation, peripheral blood mononuclear cells (PBMC) were collected from the interface and washed twice in PBS then resuspended in RPMI 1640 medium (Gibco) supplemented with 10% HS (Human Serum) and 1% PS (Penicillin 10000 UI/mL, Streptomycin 10000 μg/mL).

In vitro *CEF specific T cell generation*

Specific T cells were generated *in vitro* from PBMCs of two healthy donors. Briefly, PBMCs were resuspended in RPMI 1640 medium with 10% HS, PS at 4.106 PBMC/mL. Cells were plated at 1 mL per well in 24 well plate and pulsed with 1ug/mL of CEF peptides pool (CTL Europe GmbH), derived from cytomegalovirus, Epstein-Barr virus (EBV), and influenza virus and able to bind to MHC-I molecules. IL7 (5ng/mL) (Peprotech) and IL2 (Peprotech) (20UI/mL) cytokines were then added to the culture at day 1, day 3, 6 and 12 respectively. At day 14, intracellular IFNγ staining was performed to evaluate T cell specificity.

DispenCell-S1 based single cell cloning

DispenCell-S1 procedure relies on impedance-based pipetting technology allowing the detection and the record of single cell impedance signal during cell dispensing. This technology principle is well detailed elsewhere [9,10]. The commercial device DispenCell-S1 was used according to standard protocol (SEED Biosciences, Switzerland). The device comes with a disposable kit containing a sensing tip and an optimized methylcellulose-based medium. DispenSoft is a dedicated software allowing for post-processing quality control of the single cell isolation. Each time a single cell cloning experiment is performed, cells were first filtered using a 10 µm cell strainer (Miltenyi Biotec, Germany), counted and diluted in the optimized medium to a working concentration of 2.10^4 cells/mL. Then, DispenCell-S1 device was used to load the cell suspension in the sensing tip and instructed to dispense the single cells individually into wells of one or more 96-well plates. A single-cell quality control using DispenSoft was then performed. DispenSoft provides a simple readout of the impedance-based quality control: each well of the plate is represented in a color-coded matrix. Wells with single cells are marked in green while the other onces are red and should be discarded. Using the same software, it's possible to access the raw impedance data for full traceability. A single sharp peak is the signature of a single T cell while multiple peaks result from multiple cells. To discriminate between debris and single cells, a threshold is set. Only the peaks which are higher than the threshold are counted as cells.

Limiting dilution assay

CEF specific T cells were cloned by limiting dilution method as previously described [11] In brief, counted T cells were seeded in 96-well plates at a ratio of one cell per well and a ratio of 0.3 cell per well. To each well, 100 000 irradiated PBMCs derived from 2 healthy donors (irradiated to 25Gy), 10 000 irradited EBV-transformed feeder lymphoid cells (B-EBV) (irradited to 50Gy), 150UI/mL of IL2 and 1µg/mL of PhytoHemaGlutinnine (PHA) were added in a total culture volume of 200µL per well. Cultures were maintained 14 days in RPMI 1640 medium

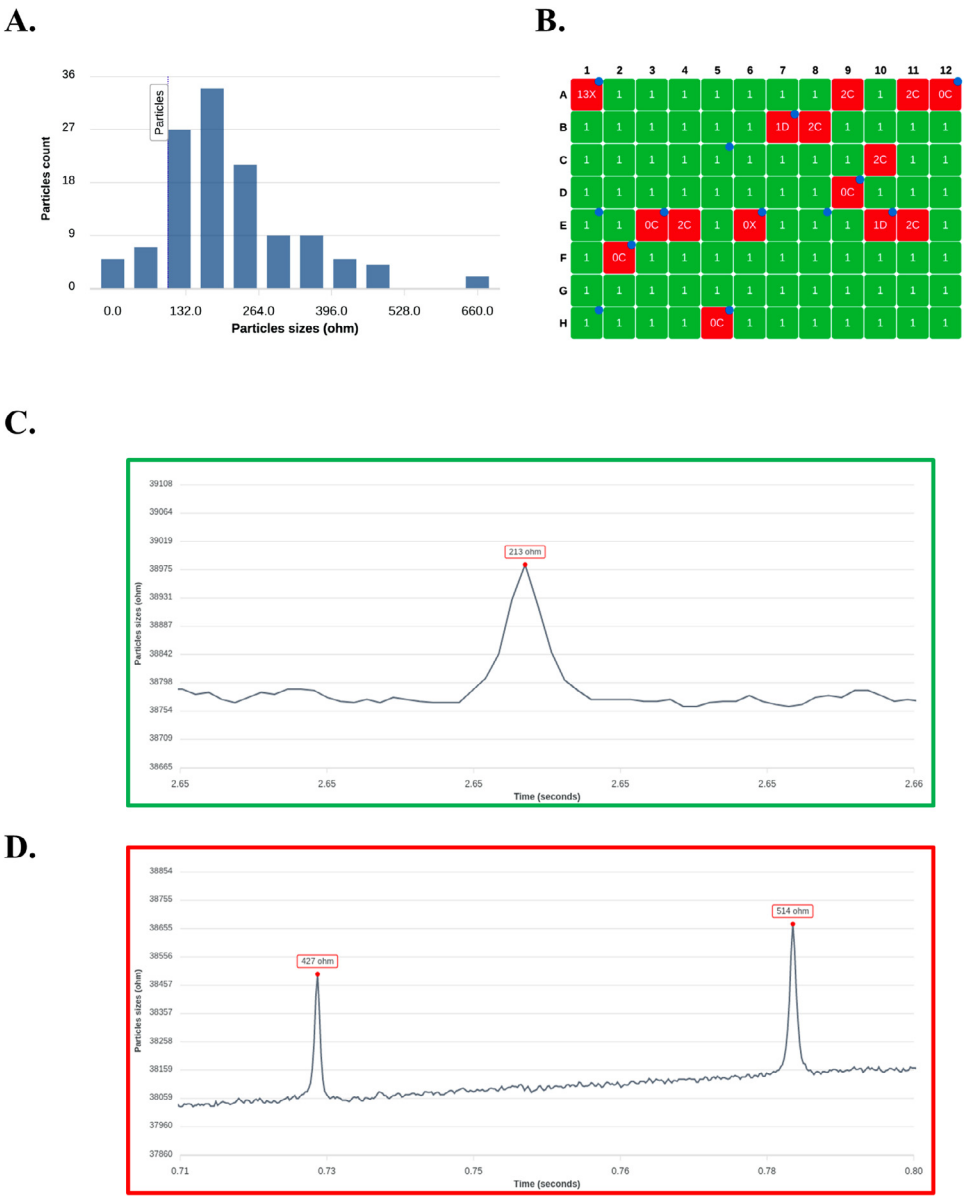

Fig. 2. Single T cell isolated by DispenCell-S1. A. Size-based histogram of the cell population analyzed by DispenCell-S1. To discriminate small debris from cells, the detection threshold is set at 100 ohm. **B.** Representative plate profile provided by DispenSoft. The color code is as follows: a green well contains a single cell, a red well should be discarded because it is empty (0X) or because it contains more than one cell (1D = doublet; 2C = 2 cells). **C.** Retained peak signature present a single and sharp peak with amplitude above the detction threshold (Well D12). **D.** Peak signature presenting multiple peaks. This well (B8) contains two cells and should be discarded.

(Gibco) supplemented with 10% HS (Human Serum) and 1% PS (Penicillin 10000 UI/mL, Streptomycin 10000 μg/mL) at 37°C in 5% CO2. Half of media and IL2 were replaced every 2-3 days.

Intracellular cytokine staining

IFNγ intracellular staining was performed to evaluate T cells specificity to CEF peptides pool. One million cells were pulsed with 1µg/mL of CEF peptides pool incubated at 37°C in 5% CO2 for 5h in RPMI medium containing GolgiPlug (BD Biosciences) according to the manufacturer's instructions. Cells were stained for 30 min with Live/Dead fixable dead cell stain (eBioscience^{TM} Fixable Viability Dye eFluor^{TM} 506 Invitrogen), and anti-CD3 (PB, BD Pharmingen), anti-CD4 (APC-H7, BD Pharmingen), anti-CD8 (PE, Diaclone). For intracellular staining, cells were incubated with Cytofix/CytopermTM (BD Biosciences) for 30 min, before staining with anti IFNγ (APC, BD Pharmingen) and anti TNFα (FITC, BD Pharmingen). Cells were then resuspended in PBS for BD FACS Canto II (BD Biosciences) acquisition. Data were analyzed using Diva Software.

CEF specific T cell sorting and expansion

CEF specific T cells were enriched using magnetic IFNγ-release guided T cell sorting (MACS Miltenyi Biotec) according to the manufacturer's instructions. Sorted cells were then *in vitro* expanded using a classic protocol for polyclonal T cell expansion. Briefly, irradiated feeder cells (allogenic PBMC from 2 healthy donors and B-EBV cell lines) were added to sorted cells cultured in 96-well plate with 150UI/mL IL2 and 1µg/mL PHA. Plates were incubated at 37°C in 5% CO_2 for 14 days. T cell specificity was evaluated by intracellular IFNγ staining.

TCR sequencing

TCR sequencing was performed on T cell clones isolated by DispenCell-S1 and limiting dilution methods as previously described [12] Briefly, mRNA was extracted using the Dynabeads mRNA DIRECT purification kit according to the manufacturer's instructions (Thermo Fisher Scientific). mRNA was reverse transcribed using oligo dT and M-MuLV reverse transcriptase (NEB). Second strand cDNA was synthetized with a collection of TRAV/TRBV specific primers and 1 amplification cycle with the Phusion DNA polymerase (NEB) TCRs were

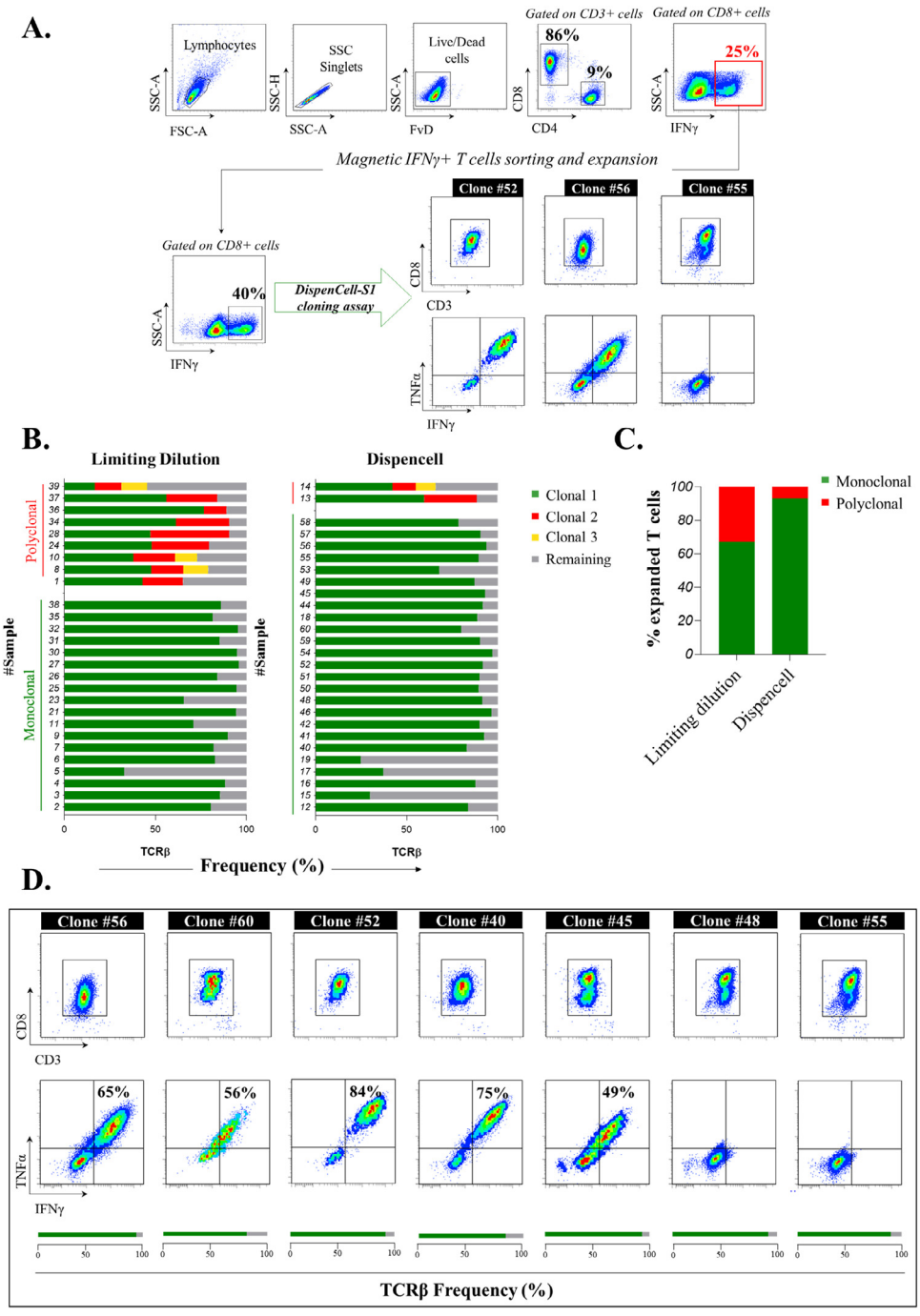

Fig. 3. T cell clonality and functionality. A. Representative flow cytometry gating strategy used to define *in vitro* generated specific CD8+ T cells and isolated CD8+ T clones. PBMC drived from healthy donor were pulsed with CEF peptides pool. By day 14 of the culture, generated IFNγ secreting CEF-specific CD8+ T cells (upper plot on the right) were enriched by magnetic sorting followed by cell expansion on irradiated feeder cells in the presence of IL2 and PHA (lower plot on the left). Expanded enriched T cells were used for DispenCell-S1 cloning assay and specific CD8+ T clones were isolated (lower plots on the right). **B.** TCRβ sequencing performed on proliferating CD8+ T cells isolated by DispenCell-S1 or by limiting dilution cloning method. Each bar corresponds to the frequency of TCRβ sequences detected in the same sample. Bars in green, red or yellow color indicate clonotypes repeatedly detected in the same sample (clonal). Gray bars indicate the remaining clonotypes. **C.** Comparison of monoclonal (green) and polyclonal (red) expanded T cells proportion derived from limiting dilution (n=27) and DispenCell-S1 (n=27) cloning methods. **D.** Representative flow cytometry plots presenting frequencies of IFNγ and TNFα-producing CD8+ T clones isolated by DispenCell-S1 cloning procedure. TCRβ sequence frequencies corresponding to each clone are indicated.

then amplified by PCR (20 cycles with the Phusion from NEB) with a single primer pair binding to the constant region and the adapter linked to the TRAV/TRBV primers added during the reverse transcription. A second round of PCR (25 cycles with the Phusion from NEB) was performed to add the Illumina adapters containing the different indexes. The TCR products were purified with AMPure XP beads (Beckman Coulter), quantified and loaded on the MiniSeq instrument (Illumina) for deep sequencing of the TCRα/TCRβ chain. The TCR sequences were further processed using ad hoc Perl scripts to: (i) pool all TCR sequences coding for the same protein sequence; (ii) filter out all out-frame sequences; (iii) determine the abundance of each distinct TCR sequence. TCR with a single read were not considered for the analysis.

Results and discussion

General outline of antigen-specific T cell clones isolation by DispenCell-S1 cloning procedure

According to reverse immunology strategy, the identification of an immunogenic peptide epitopes is followed by the isolation of specific T cell clones. To begin with, a total PBMC from healthy donors samples were pulsed *in vitro* with candidate antigenic peptides. Here we used CEF antigens, an MHC-I peptides pool for specific CD8+ T cell activation. Cells were maintained in culture with IL7 and IL2 cytokines. By day 14, IFNγ-secreting CEF specific CD8+ T cells were enriched by magnetic cytokine release-guided T cell sorting and expanded in the presence of

irradiated feeder cells, IL2 and PHA for 14 days. Next, single cell cloning of enriched CEF specific CD8+ T cells was performed using DispenCell-S1 (Fig. 1). Thus, Dispencell technology provides single T cell clones isolation and here we aimed to first validate its cloning efficiency and reproducibility and then the clonality and functionality of Dispencell isolated T clones.

Dispencell cloning efficiency and reproducibility

We tested the capacity of DispenCell-S1 to isolate single human T cells individually into wells of a 96-well plate (N=3 plates). Immediately after, we used DispenSoft to perform an impedance-based quality control of the single cell isolation. Based on the size-based histogram (Fig. 2 A), we set a threshold at 100 ohm to discriminate single T cells from debris. The software provided a color-coded map that we used as quality control (Fig. 2 B). In this map, only the wells that contain a single cell are marked in green while the others are marked in red. Using the same software, we can access the impedance profile of each well for additional quality control or for documenting the procedure. For example, the impedance showed the peak signature for single cells (Fig. 2 C) and doublets (Fig. 2 D)

On average over the three plates, the software indicated that the single cell efficiency (percentage of green wells) was 83%. The average of dispensing time per plate was 8 min. These results clearly demonstrate a high efficiency of single-cell dispense which provides an immediate theoretical proof of monoclonality based on impedance profile. Altogether, these results witness the practicality of impedance-based pipetting technology and the rapidity of DispenCell-S1 single T cell cloning and its efficiency and reproducibility.

DispenCell-S1 predict single cell TCR and preserve T cell functionality

To further determine whether DispenCell-S1 isolated T cells were clonal, CEF specific CD8+ T cells were initially generated as previously described (Fig. 1). By day 14 of the cultured CEF pulsed PBMC, 25% of CD8+ T cells produced significant amounts of IFNγ in response to CEF stimulation as measured by intracellular staining. Thus, CEF-specific IFNγ-producing CD8+ T cells were then enriched by a magnetic IFN-γ-positive T cell sorting and expanded for 14 days. Single cell cloning of enriched CEF specific CD8+ T cells (40% of CD8+ IFNγ + T cells) was further performed using Dispencell-S1 instrument procedure (Fig. 3 A.) and limiting dilution method and dispensed T cells were re-expanded for 14 days.

A total of 27 proliferating T cells were obtained after limiting dilution and Dispencell-S1 cloning methods by day 14. To next confirm the reliability of DispenCell-S1, TCRβ chain sequencing of expanded CD8+ T cell clones isolated either by DispenCell-S1 or limiting dilution cloning method was performed. The monoclonality of tested samples is demonstrated by the detection of single clonal rearrangement of TCRβ locus from the same sample, whereas, polyclonality corresponds to the detection of distinct TCRβ locus rearrangements within the same sample. TCR sequencing analysis have shown the presence of single dominant rearrangement of TCRβ chains in samples derived from both cloning methods (Fig. 3 B). Out of the 27 growing colonies generated using DispenCell-S1, 25 of them presented a single dominant TCRβ chain sequence corresponding to T cell clones (Fig. 3 C). To the contrary, only 18 T cell clones over 27 growing colonies were generated by limiting dilution. While the reliability of DispenCell-S1 could be further improved, notably by using T cell adapted specific tips, it already provides clones with much more efficiency and reliability than limiting dilution.

Given the importance of functional characteristics of CD8+ T cells, we aimed to study the impact of DispenCell-S1 cloning method on T cell functionality associated to IFNγ and TNFα production in response to CEF stimulation. Thus, CD8+ T cell clones isolated by DispenCell-S1 and presenting single dominant TCRβ sequence were selected for *in vitro* functional testing. Upon *in vitro* stimulation with CEF peptides pool, intracellular flow cytometry analysis have shown that expanded CD8+ T clones were able to produce significant amounts of IFNγ and TNFα (Fig. 3 D.). These results show that DispenCell-S1 cloning approach do not alter specific CD8+ T cell clones functionality. Altogether, magnetic cell sorting combined with DispenCell-S1 single cell cloning provides gentle efficient isolation of living single CD8+ T clones. The use of DispenCell technology could make it easier T cell clones generation and thus, could optimize reverse immunology strategy for cancer immunotherapy development.

Perspectives

This new workflow offers a remarkably simple and affordable approach for routine cloning experiment in the booming field of immunology and beyond. Several paths are under investigation to increase the rapidity and reliability of the DispenCell-S1 even further (Hannart H. *et al.*, submitted in the same issue). The sensing tips used in this study were developed for applications in CHO cell line development where cells are two to three times bigger than T cells. As demonstrated previously [9], a simple way to increase signal-to-noise ratio is to adapt the size of the Coulter aperture to the size of the cells. We expect that new sensing tips with reduced aperture size (around 20 µm) should give higher dispensing reliability when used with T cells.

Conflicts of interest

G.M. and L.A. have financial interests in SEED Biosciences SA and intellectual property described herein.

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