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Research paper

FACS isolation of low percentage human antigen-specific CD8$^+$ T cells based on activation-induced CD3 and CD8 downregulation

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ABSTRACT

As T cell activation leads to downregulation of T cell receptor (TCR) and coreceptor CD8, we developed a novel FACS-based sorting method to enrich activated antigen-specific CD8$^+$ T cells. Using multiple established or low percentage T cell cultures, with either single antigen specificity or multiple influenza A virus antigen specificities, we have optimized the sorting method for T cell activation time and stimulating antigen dose. We have also sorted various numbers of antigen-specific CD8$^+$ T cells into 96-well plates to demonstrate these T cells are capable of expanding into nearly pure CD8$^+$ T cell lines. Our approach has the advantage of sorting antigen-specific T cells without knowing their specific antigenic epitopes or restricting HLA. We believe this method can be very helpful for successfully establishing CD8$^+$ T cell lines for various purpose, including immunotherapy.

1. Introduction

CD8$^+$ cytotoxic T lymphocytes (CTLs) play key roles in viral clearance and tumor surveillance. CD8$^+$ T cells recognize specific peptide-MHC class I complexes (pMHC-I) on the surface of antigen presenting cells (APCs) with T cell receptors (TCRs). The TCR-CD3 is a multi-subunit cell surface protein complex composed of an antigen-binding TCR disulfide-linked αβ heterodimer and CD3 complex (Davis and Bjorkman, 1988; Wegener et al., 1992; Krogsgaard et al., 2005); the CD3 complex is formed by CD3δε and CD3γε heterodimers, and CD3ζζ homodimers (Clevers et al., 1988; Smith-Garvin et al., 2009). Unlike the TCR αβ chains, CD3 components possess large intracytoplasmic domains responsible for coupling antigen recognition to various signal transduction pathways (Wegener et al., 1992). CD3γ, δ and ε each possesses an intracellular immunoreceptor tyrosine-based activation motifs (ITAM), while CD3ζ possesses three ITAMs in a tandem arrangement. Phosphorylation of these ITAMs leads to signal transduction and ultimately T cell activation (Kane et al., 2000; Kuhns and Davis, 2012). It has been demonstrated that CD3-γ phosphorylation mediates the downregulation of TCR/CD3 complex after T cell activation (Cantrell et al., 1985; Krangel, 1987; Valitutti et al., 1995).

CD8 is known as the coreceptor on cytotoxic T cells. It is a transmembrane glycoprotein which facilitates and amplifies pMHC-I triggered T cell activation (Bierer et al., 1989; Janeway, 1992; Gao and Jakobsen, 2000; Holler and Kranz, 2003). It binds to the α3 domain of the MHC that the TCR interacts with for optimal signal transduction during T cell activation (Salter et al., 1990; Janeway Jr., 1992). When CD8 molecule is blocked or in absence, such T cell activation is either blocked or requires longer TCR engagement time (Arcaro et al., 2001). Most importantly, CD8 engagement enhances and stabilizes the interaction of TCR/pMHC-I with lower affinity (Schott and Ploegh, 2002; Laugel et al., 2007). Upon activation CD8$^+$ T cells not only downregulate their TCR (CD3) complexes (Valitutti et al., 1995) but also its coreceptor CD8 (Xiao et al., 2007).

Establishing CD8$^+$ T cell lines or clones *in vitro* has been an important technique and has played very important role in understanding T cell biology and, more recently, T cell-based immunotherapy. For instance, *in vitro* propagated CD8$^+$ T cells have been used as essential tools for discovering CD8$^+$ T cell epitopes and studying the mechanisms of immunodominance hierarchies during virus infection (Wu et al., 2011; Grant et al., 2013; Clemens et al., 2016) and they have been used to treat cancer patients in adoptive cell therapy (Rosenberg et al., 2004). They can also be used to study mechanisms and kinetics of antigen processing and presentation (Pang et al., 2006a). However, successfully establishing CD8$^+$ T cell cultures is still largely an empirical and lab-based phenomenon. The major difficulty of such T cell cultures is that limited antigen-specific CD8$^+$ T cell precursors are often overwhelmed by the nonspecific proliferation of T cells with irrelevant specificities during early culture stage. It is often the case that although some antigen-specific T cells are detected in many primary cultures,

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these T cells are eventually lost during the next round of antigen re-stimulation. Hence, the key solution for successful *in vitro* $CD8^+$ T cell expansion is to isolate or enrich these antigen-specific $CD8^+$ T cells as early as possible to avoid being over grown by irrelevant T cell populations.

Our group evaluated T cell culturing conditions previously including culture sera, cytokines and feeder cells and developed a robust human T cell culture method for monitoring both antigen-specific $CD4^+$ and $CD8^+$ T cells during short-term cultures (Jackson et al., 2004). We also optimized culture conditions for establishing murine $CD8^+$ T cells (Zanker et al., 2013). In this study, we successfully established a method to enrich antigen-specific $CD8^+$ T cell during primary T cell culture stage to dramatically boost antigen-specific T cell purity and enhance the success rate of $CD8^+$ T cell cultures with minimal knowledge of T cell epitope and its HLA restriction. Our novel approach is based on the phenomenon that upon activation $CD8^+$ T cells down-regulate their TCR (CD3) complexes and its coreceptor CD8. Therefore, antigen-specific $CD8^+$ T cells can be detected and sorted as a $CD3^{low}CD8^{low}$ population by fluorescence activated cell sorting (FACS). These features were utilized by our group to reveal low frequency antigen-specific human $CD4^+$ Tregs previously (Ebert et al., 2012). Using this approach, we successfully enriched human antigen-specific $CD8^+$ T cell populations, sometimes < 1% of total $CD8^+$ T cells.

2. Materials and methods

2.1. Human Peripheral Blood Mononuclear cells (PBMCs)

PBMCs of healthy donors used in this study were obtained from Australian Red Cross Blood Service (ARCBS) in Melbourne under the agreement 12-07VIC-17. PBMCs were isolated from blood buffy coats using Ficoll-Hypaque gradient centrifugation, then resuspended in FCS (Hyclone, South Logan, Utah) containing 10% dimethyl sulfoxide (DMSO) and stored in liquid nitrogen until use.

2.2. Culture medium, synthetic peptides and antibodies

Unless otherwise stated, antigen presenting cells (APCs) were cultured in RF-10 (RPMI 1640 supplemented with 10% FCS (Hyclone), 50 μM 2-ME, 2 mM L-glutamine and antibodies (penicillin 100 U/mL and streptomycin 100 μg/mL) (all from Gibco, NY, USA)). Peptides used in this study were synthesized by Mimotopes (Clayton, Victoria, Australia). Influenza A virus (IAV) peptides $M1_{58–66}$ (GILGFVFTL), $NP_{172–181}$ (LPRRSGAAGA), $NP_{44–52}$ (CTELKLSDY), and a melanoma peptide Melan $A_{26–35}$ (EAAGIGILTV) were synthesized as crude, desalted peptides with a purity > 75%. Peptides were reconstituted to a concentration of 1 mM in DMSO. APC-labelled anti-CD8 was purchased from BD Biosciences, e450-labelled anti-CD3 was purchased from eBioscience (San Diego, CA, USA). FITC-labelled anti-CD3 and PE-cy7 labelled anti-IFNγ were purchased from Invitrogen (San Diego, CA, USA).

2.3. Viruses and antigen presenting cell

The influenza A virus strain A/Puerto Rico/8/1934 H1N1 (PR8) was propagated in 10 days old embryonated chicken eggs. Two days after infection, the allantoic fluids were collected, aliquoted and stored at −80 °C freezer until use. Recombinant vaccinia viruses (rVV) encoding individual IAV proteins HA, NA, NP, NS1, NS2, M1, M2, PB1, PB2, PB1F2, PA, and empty vector rVV-CR19 were kind gifts from Drs Jonathan Yewdell and Jack Bennink (National Institutes of Health, Bethesda, MD). Autologous B lymphocyte cell line (BLCL) was made from donor PBMCs using EBV transformation (Wu et al., 2011) and cultured in RF-10.

2.4. Generation of poly-specificity or single peptide-specific $CD8^+$ T cell lines in vitro

For generating poly-specificity $CD8^+$ T cell lines, 1×10^6 PBMCs were resuspended in 200 μL acidic RPMI-1640 (pH adjusted to 6.8) and infected with PR8 at a MOI of 10 for 1 h in a 37 °C water bath. Later, 2 mL of prewarmed RF-10 was added to the cells and incubated for a further 4 h. Cells were pelleted and subsequently co-cultured with 9×10^6 PBMCs for 12–15 days in RF-10 contains 20 IU/mL of human recombinant interleukin 2 (hrIL-2) (Peprotech Inc., Rocky Hill, NJ, USA). For generating peptide-specific $CD8^+$ T cell lines, 1×10^6 PBMCs were resuspended in 200 μL RF-10 and pulsed with 10^{-7} M peptides for 1 h in a 37 °C water bath. Next, the peptide pulsed PBMCs were washed and co-cultured with 9×10^6 PBMCs for 12–15 days in RF-10 contains 20 IU/mL of hrIL-2 (Jackson et al., 2004; Wu et al., 2011; Grant et al., 2013). Unless otherwise stated, $CD8^+$ T cells were cultured with RF-10 supplemented with Sodium Pyruvate (1×) and Non-essential amino acid (1×) (Gibco, NY, USA).

2.5. Intracellular cytokine staining (ICS) and antigen presentation kinetic assay

The IFN-γ ICS assay was performed as previously described (Wu et al., 2011). For antigen presentation kinetic assay, the detailed method was previously published (Pang et al., 2006b). Briefly, the antigen-presentation of PR8-infected autologous BLCLs was blocked by addition of BFA (Sigma-Aldrich, St Louis, USA) at different time points, peptide-specific $CD8^+$ T cells were added for 4 h to assess the antigen-presentation at that time point; the T cells were then harvested and synchronized on ice before being stained together in a standard ICS. Samples were analyzed using BD FACS Canto II machine (BD Biosciences) and FlowJo software (Tree Star Inc., Ashland, USA).

2.6. Antigen-specific $CD8^+$ T cell enrichment by sorting the $CD3^{low}CD8^{low}$ population

Autologous BLCLs were pulsed with 10^{-7} M peptides for 1 h then cocultured with the $CD8^+$ T cells in the presence of BFA for 4 h. In addition, same T cells cocultured with un-pulsed BLCLs served as a negative control and were used to guide $CD3^{low}CD8^{low}$ population gating. In cysteine-containing peptide, for example $NP_{44–52}$, BLCLs were pulsed with $NP_{44–52}$ in the presence of 500 μM TCEP (tris(2-carboxyethyl)phosphine, Pierce™), washed out excess peptides, then incubated with $NP_{44–52}$-specific $CD8^+$ T cells again in the presence of 500 μM TCEP and BFA for 4 h. After 4 h activation, the cells were stained with anti-CD3 (e450) and anti-CD8 (APC) for 30mins at 4 °C; washed and resuspended in sorting buffer (5% FCS, 0.5 μM EDTA in PBS) and sorted for $CD3^{low}CD8^{low}$ (downregulated) population using a FACS ARIA III (BD Biosciences).

For T cell cloning, the $CD3^{low}CD8^{low}$ population were sorted and various cell numbers (1 cell/well, 3 cells/well, 10 cells/well, 100 cells/well, 1000 cells/well, 5000 cells/well, 10,000 cells/well) were directly deposited into U-bottom 96 wells containing 3×10^5 irradiated allogeneic PBMC feeder cells (1:1:1 mixed feeders from 3 different donors' PBMC) in RF-5 (RPMI-1640 supplemented with 5% pooled human AB sera, L-glutamine, 2-ME, Non-essential amino acids, Sodium pyruvate, antibiotics, 300 IU/mL hrIL-2 and 1 μg/mL phytohaemagglutinin (PHA, Invitrogen, San Diego, USA,) (Potter and Moore, 1975). Sorted cells were cultured in the central wells of the plate to avoid medium evaporation. Only the central 60 wells from each 96-well plate were used to culture the sorted $CD8^+$ T cells. The outer wells of 96 well plates were filled up with sterile PBS to act as a buffer zone to minimized medium evaporation over long periods of incubation. Half medium was replaced with fresh one 24 h after sorting to reduce PHA concentration. The cells were checked every second day and medium changed as required. Growing wells were first split in 2 × 96-well before being

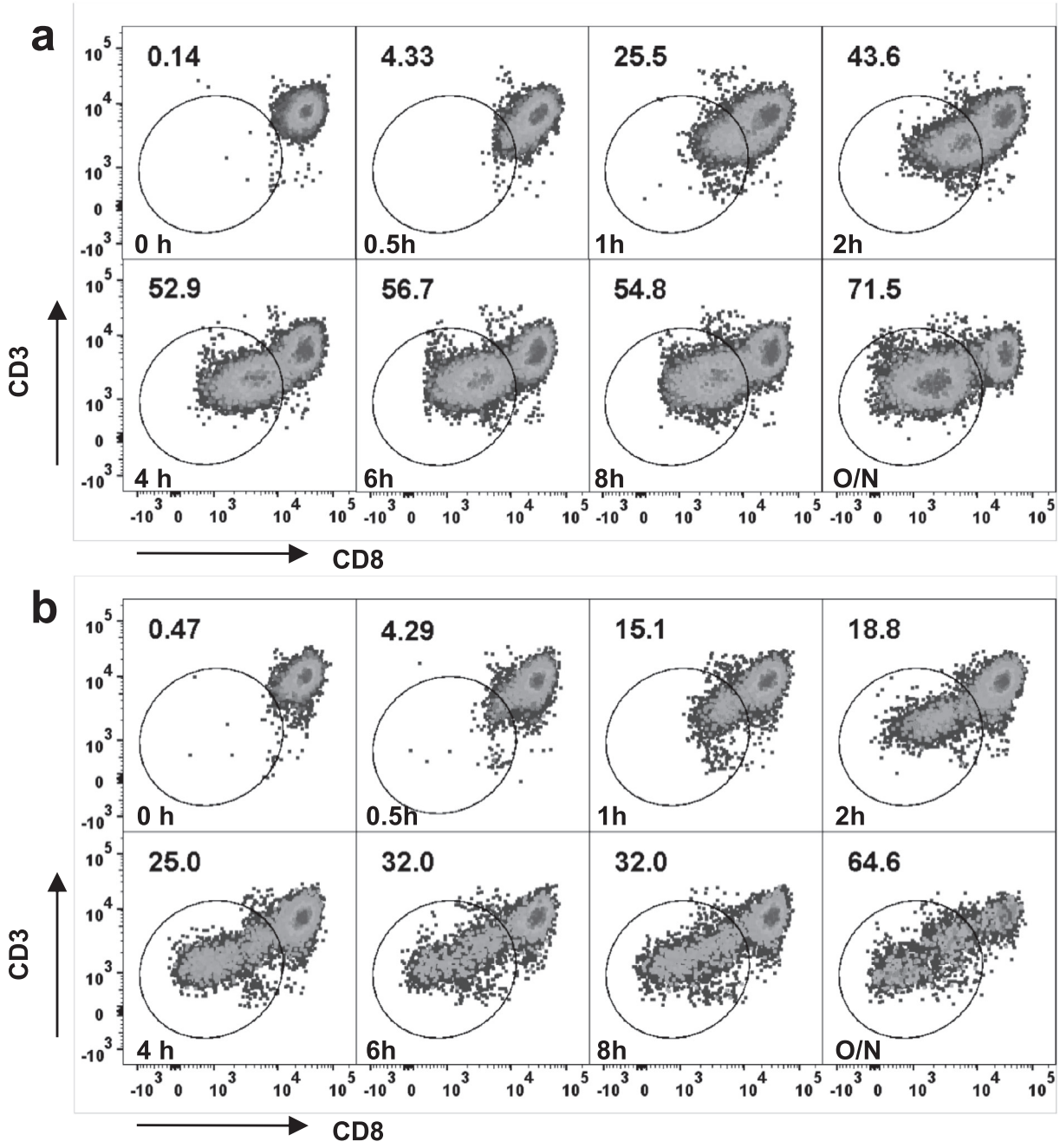


Fig. 1. 4-h activation provides optimal CD3CD8 downregulation peptide-specific $CD8^+$ T cell lines.
T cells were activated by $M1_{58–66}$ or $NP_{172–181}$ peptide; at different time points (0 h, 0.5 h, 1 h, 2 h, 4 h, 6 h, 8 h, and 16 h) the T cell activation-induced CD3CD8 downregulation was assessed by flow cytometry. **a)** the kinetic of CD3CD8 downregulation in $M1_{58–66}$ specific $CD8^+$ T cells; **b)** CD3CD8 downregulation kinetics of $NP_{172–181}$-specific $CD8^+$ T cells. Data shown are representative of two independent experiments.

transferred into 48-well plate when cell numbers reached to about 2 million per well.

3. Results

3.1. CD3 and CD8 are quickly down-regulated on antigen-specific $CD8^+$ T cells after activation

To simplify the method development process, two $CD8^+$ T cell lines specific to influenza A virus Matrix 1 protein ($M1_{58–66}$ restricted to HLA-A*02:01) and Nuclear Protein ($NP_{172–181}$ restricted to HLA-A*55:01) (Grant et al., 2013) were initially established. We first investigated the kinetics of TCR and CD8 downregulation. The two $CD8^+$ T cell lines were activated by 10^{-6} M peptide-pulsed APC and their activation-induced TCR and CD8 downregulation, at various time points (0 h, 0.5 h, 1 h, 2 h, 4 h, 6 h, 8 h, and 16 h) were monitored. As shown in Fig. 1, a population of the $M1_{58–66}$ specific T cells (25.5%) and the $NP_{172–181}$ specific line (15.1%) responded within an hour after stimulation.

The activation-induced TCR and CD8 downregulation gradually increased over time, peaking at 4 h after activation. Although the later time points, especially the overnight (16 h) point, showed even more significant downregulation, some T cells were clearly overstimulated and underwent apoptosis as previously reported (Alexander-Miller et al., 1998). We, therefore, concluded that a 4-h activation of these $CD8^+$ T cells provided optimal CD3 and CD8 downregulation.

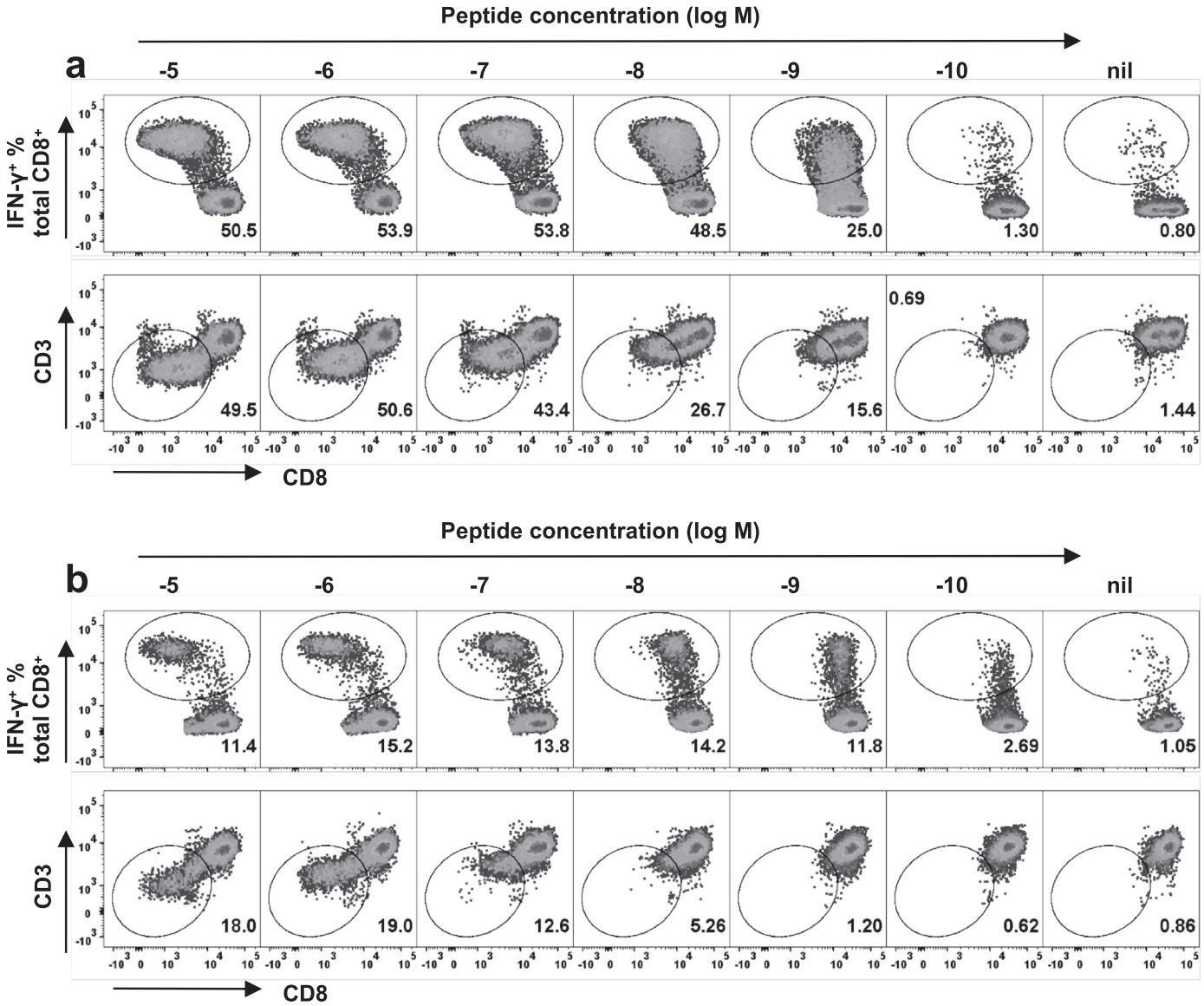


Fig. 2. 10^{-7} M peptides induce optimal CD3CD8 downregulation in antigen-specific $CD8^+$ T cells.
APCs were pulsed with serially diluted peptides for 1 h in complete medium then washed and incubated with T cells for assessing $M1_{58–66}$- and $NP_{172–181}$-specific $CD8^+$ T cell percentage at various peptide concentrations by a standard ICS. **a)** $M1_{58–66}$-specific T cell IFN-γ expression and CD3CD8 downregulation; **b)** $NP_{172–181}$-specific T cell IFN-γ response and CD3CD8 downregulation. Data shown are representative of two independent experiments.

3.2. Sub-micromolar antigen doses induce sufficient T cell activation-induced CD3CD8 downregulation

It is well established that over stimulating T cells could lead to T cell exhaustion (Wherry and Kurachi, 2015). To avoid T cell over-stimulation and yet to find out the optimal antigen dose range for activating $CD8^+$ T cells to sufficiently downregulate CD3 and CD8, we performed peptide titration experiments and monitored T cell lines' CD3 and CD8 downregulation stimulated by various peptide concentrations. Fig. 2 shows the two $CD8^+$ T cell lines' IFN-γ response and CD3CD8 downregulation at 4 h after stimulation by various peptide concentrations. It was obvious that IFN-γ response required less antigen stimulation than that required for CD3CD8 downregulation. For example, 10^{-8} M of $M1_{58–66}$ peptide activated almost all specific T cells (48.5%) to produce IFN-γ, but only about half of these cells (26.7%) showed obvious CD3 and CD8 downregulation (Fig. 2a). Such difference seemed to be T cell specificity dependent, because lower $NP_{172–181}$ peptide concentration was able to stimulate its specific T cells to produce IFN-γ (10^{-9} M activated 80%); while much higher $NP_{172–181}$ peptide concentration (10^{-7} M) was required to stimulate the same T cells to downregulate CD3 and CD8 (Fig. 2b). Taken together from these data, we concluded T cell stimulation with 10^{-7} M peptide should induce optimal CD3 and CD8 downregulation in antigen-specific $CD8^+$ T cells while avoiding significant T cell over stimulation.

3.3. Sorting $CD3^{low}CD8^{low}$ T cells after activation enriches antigen-specific $CD8^+$ T cells

Having worked out the optimal antigen dose (10^{-7} M) and stimulation time (4 h), we wanted to test the sensitivity of our T cell enriching approach. To achieve this, a T cell culture with relatively low antigen-specific T cell purity (around 2%) was activated and enriched by sorting $CD3^{low}CD8^{low}$ T cells. The sorting gate was set according to the control group that was cocultured with un-pulsed BLCLs. The gating example is shown in Fig. 3a. Only a $CD3^{high}CD8^{high}$ population can be observed from the un-stimulated group (Fig. 3a, upper panel); while a $CD3^{low}CD8^{low}$ subpopulation was obviously found in the stimulated group (Fig. 3a, lower panel). $M1_{58–66}$-specific $CD8^+$ T cells were sorted

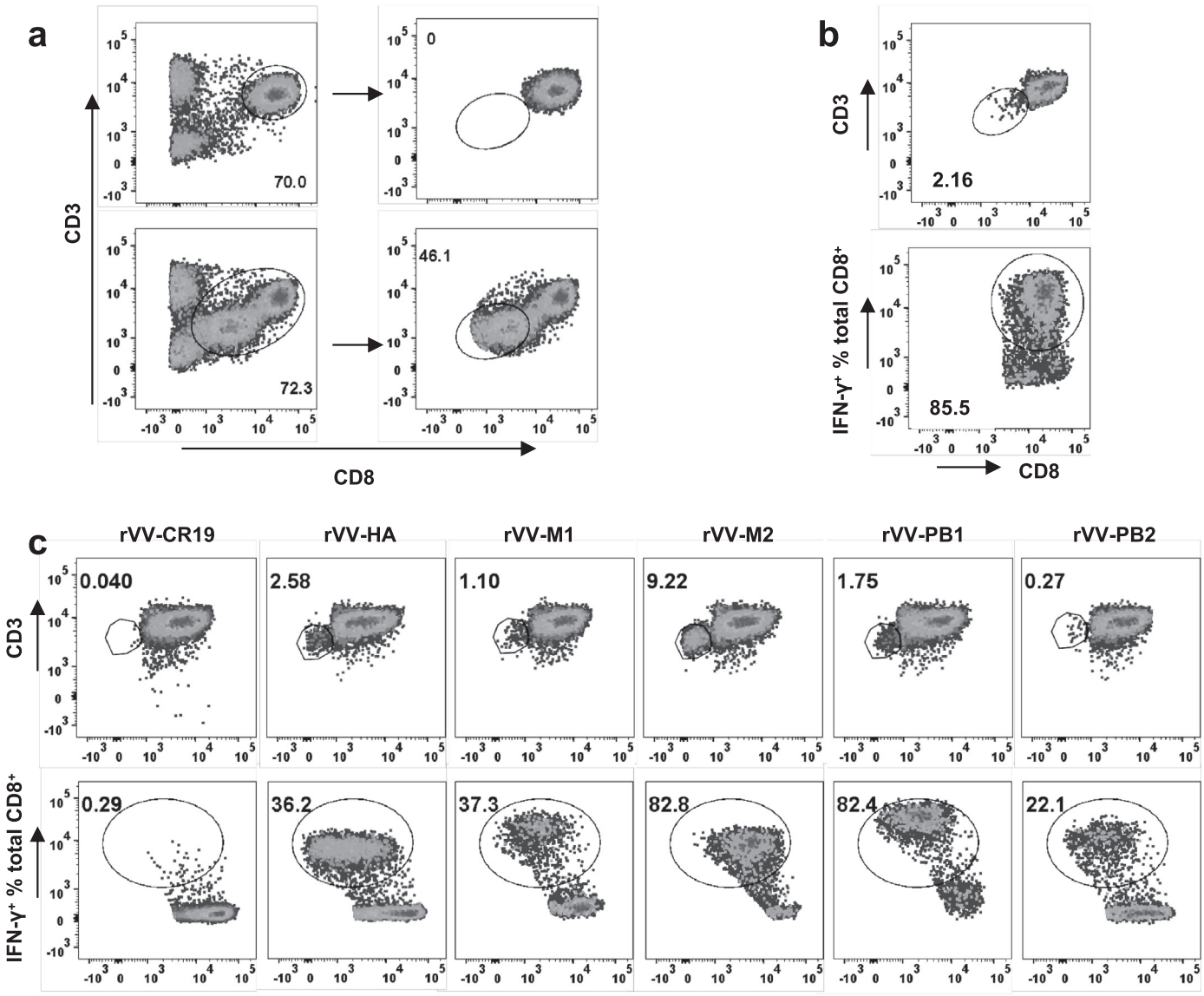


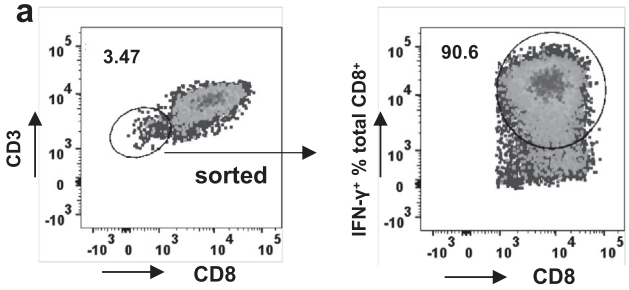
Fig. 3. Sorting $CD3^{low}CD8^{low}$ T cells after activation enriches peptide- or virus-specific $CD8^+$ T cells.
a) An example for gating CD3CD8 downregulated $M1_{58–66}$-specific T cells responding to peptide-pulsed or un-pulsed APCs. $CD8^+$ T cell culture was established as described in the M&M using PR8 infected PBMCs. $CD8^+$ T cells were stimulated with $M1_{58–66}$ peptide-pulsed (10^{-7} M) APCs for 4 h, stained with anti-CD3 and anti-CD8 and analyzed by flow cytometry. Gates were set according to T cell group co-cultured with un-pulsed BLCLs. First, gated on $CD8^+$ T cells then displayed as CD3 (Y-axis) and CD8 (X-axis). An oval gate was then applied to $CD3^{low}CD8^{low}$ population (Fig. 3a, right panel). **b)** BLCLs pulsed with $M1_{58–66}$ peptide (10^{-7} M) were used to stimulate low purity $M1_{58–66}$-specific $CD8^+$ T cells; then the $CD3^{low}CD8^{low}$ population was sorted and assessed by ICS. **c)** The rVV-infected BLCLs were used as APCs to stimulate a bulk IAV-specific $CD8^+$ T cell culture, then the $CD3^{low}CD8^{low}$ T cells were sorted and assessed by ICS. rVV-CR19 infected APCs served as a negative control.

and cultured in 48-well plate; an ICS was performed to assess the purity of antigen-specific $CD8^+$ T cells 72 h after sorting. As shown in Fig. 3b, T cells were enriched about 40 times (from 2.16% to 85.5%) after sorting $CD3^{low}CD8^{low}$ T cells. This result indicated that sorting CD3CD8 downregulated T cells after antigen stimulation could efficiently increase the purity of antigen-specific $CD8^+$ T cell population.

Generally, working out unknown antigenic epitopes is not only a time-consuming process it also often requires a large number of epitope-specific T cells to screen synthetic overlapping peptides. To achieve quality data, T cell lines with good purities are highly desired. However, it is often the case that lower purity T cell cultures are difficult to maintain for its specificity. As the above sorting was conducted using T cell lines with known peptide-specificities, we were curious to know whether our novel method would allow us to enrich $CD8^+$ T cells with unknown specificity.

We therefore established a bulk T cell culture using autologous BLCLs infected with IAV as stimulating APCs. 15 days later, $CD8^+$ T cells with various IAV specificities and purities (as a percentage of the total $CD8^+$ T cells in the culture) were expected. At this stage, autologous BLCLs were infected with recombinant vaccinia viruses (rVV) expressing individual IAV antigen, such as HA, M1, M2, PB1 or PB2, for 16 h, then used as APCs to stimulate each IAV antigen-specific T cell population in the bulk culture. 4 h later, activated T cells as a $CD3^{low}CD8^{low}$ population were sorted into 96-well plate. 72 h later the purities of sorted antigen-specific T cells were assessed by ICS. As shown in Fig. 3c, the M2-specific T cell population was 9.22% before sorting and it reached to 82.8% after sorting indicating nearly 10-fold enrichment; similarly, the T cell population specific to PB2 was 0.27% before sorting and it reached 22.1% after sorting, nearly 100-fold enrichment. Other T cell populations specific to HA, M1, and PB1 also

a



b

Cells/96-well plate	Cells expansion rate Average%(SD%)
1 cell	NA
3 Cells	12 (2)
10 Cells	19 (4)
100 Cells	32 (2)
1,000 Cells	90 (3)
5,000 Cells	100 (0)
10,000 Cells	100 (0)

Note: 1. only 60 wells are used per 96-well plate
2. Data are summarised from three independent experiments

Fig. 4. CD3CD8 downregulation-guided sorting show good $CD8^+$ T cell survival and expansion.
a) Activated $M1_{58–66}$-specific $CD8^+$ T cells were sorted into 96-well plate with ranged numbers including 1, 3, 10, 100, 1000, 5000, 10,000 cells per well; and each well contained 3×10^5 irradiated allogeneic feeder cells and was cultured in RF-5 containing 300UI/mL hrIL-2 and 1 μg/mL PHA. Cells were carefully maintained for two weeks and the specificities and purity were assessed by ICS at day 15 for IFN-γ production. NA represents not expanded wells after two week's culture. **b)** A summary of various cell numbers sorted based on CD3CD8 downregulation in three independent experiments.

reached 10–40-fold enrichment. Taken together, these results indicate that sorting according to antigen-specific CD3CD8 downregulation can efficiently enhance virus-specific $CD8^+$ T cell purity.

3.4. High purity $CD8^+$ T cell lines can be established from sorted $CD3^{low}CD8^{low}$ antigen-specific T cells

To further investigate the potential of this approach, various number of cells (1 cell/well, 3 cells/well, 10 cells/well, 100 cells/well, 1000 cells/well, 5000 cells/well, 10,000 cells/well) were sorted and deposited into U-bottom 96-well plate with 3×10^5 allogeneic feeder cells and cultured in RF-5. Two weeks later, an ICS was conducted to assess the purity of these T cells. All the expanded antigen specific T cells achieved 90% purity after CD3CD8 downregulation-guided sorting (Fig. 4a). However, no single T cell clone was successfully established from these experiments, although high purity T cell cultures (> 90%) were derived from 3 cells/well group. Fig. 4b summarises cell expansion results from the above-mentioned groups in three independent experiments. For example, from 3 cells/well group, an average of 12% wells expanded and became high purity T cell lines. However, in the 1000 cells/well group 90% expanded successfully. Furthermore, all the expanded wells eventually contained high purity T cell lines. Taken together, CD3CD8 downregulation-guided sorting was not only able to increase T cell purity but also has the potential to derive pure T cell line from very limited cell numbers (3 cells per well was demonstrated in these experiments).

3.5. CD3CD8 downregulation-guided sorting enriches multiple small antigen-specific $CD8^+$ T cell populations

To determine the reproducibility and sensitivity of this approach, a primary bulk T cell culture with multiple small IAV-specific $CD8^+$ T cell responses was established from a known donor according to our published method (Wu et al., 2011; Grant et al., 2013). The IAV specificity was then assessed using a panel of recombinant vaccinia viruses (rVV) encoding 11 individual IAV proteins (Chen et al., 2001). As shown in Fig. 5a, among all $CD8^+$ T cells only 2.85% IAV-specific $CD8^+$ T cells were identified by obvious CD3CD8 downregulation. Importantly, these IAV-specific T cells responded to 9 different IAV proteins (HA, M1, M2, NA, NP, NS1, PA, PB1, and PB2) and all the specific T cell populations were smaller than 1% of the total $CD8^+$ T cells (Fig. 5b). The IAV-specific T cells were then activated by IAV-infected BLCLs and about 1 million $CD3^{low}CD8^{low}$ cells were sorted into a well in a 48-well plate. These T cells formed clusters 24 h after sorting, suggesting that they were healthy and activated (Observed by phase contrast microscopy daily, data not shown). 72 h later, 10^4 cells were used to assess specificity and purity for each IAV antigen. The total IAV specific T cell purity increased from 2.85% to 45.8% (Fig. 5c). To further confirm the specificity of these $CD3^{low}CD8^{low}$ sorted cells, BLCLs were again infected with the rVV panel encoding 11 single IAV proteins and used to stimulate the enriched T cells in a standard ICS. As shown in Fig. 5d, specificities of every single protein were increased in the sorted $CD3^{low}CD8^{low}$ group, indicating that CD3CD8 downregulation-guided sorting was able to greatly enrich a low percentage, poly-specificity IAV-specific T cell population.

3.6. $CD8^+$ T cells that downregulate CD3CD8 less dramatically can also be enriched

Some T cells are very sensitive to antigen stimulation and subsequently show a dramatic CD3CD8 downregulation pattern after T cell activation, as shown above. However, some $CD8^+$ T cells may not downregulate their CD3 and CD8 so dramatically after antigen stimulation. We therefore sought to investigate whether our novel sorting method was also capable of enriching such $CD8^+$ T cells and, if so, whether similar purity could be achieved after a single sort. For example, it has been demonstrated that posttranslational modification of cysteine could affect immunogenicity and antigenicity of cysteine-containing peptides (Chen et al., 1999), leading to reduced T cell activation. We first selected a viral epitope that contains a cysteine in its first amino acid position (NP44–52 CTELKLSDY) (Wu et al., 2011) to test our novel enrichment method. As the peptide stock was dissolved in DMSO which is an oxidant, cysteines could easily be oxidized to form dimer peptides through disulfide bonds (Chen et al., 1999). $NP_{44–52}$-specific $CD8^+$ T cells were stimulated by peptide-pulsed APCs in the presence of 500 μM Tris(2-carboxyethyl)phosphine (TCEP) to reduce dimer peptide formation. As shown in Fig. 6a, $NP_{44–52}$-specific $CD8^+$ T cells failed to show dramatic CD3CD8 downregulation after activation. 2.43% of $NP_{44–52}$-specific $CD8^+$ T cells were detected and sorted according to the unstimulated group. Impressively, T cell purity increased by 10-fold assessed at 72 h after sorting.

We then investigated $MelanA_{26–35}$ specific $CD8^+$ T cells as this peptide binds HLA-A*02:01 weakly (Valmori et al., 1999). As shown in Fig. 6b, the CD3CD8 downregulation was rather subtle. Again, our sorting gates were set according to the unstimulated group, only 1.2% antigen-specific $CD3^{low}CD8^{low}$ T cells were detected and then sorted. Interestingly, an ICS assay conducted 72 h after the sorting again indicated a 10-fold enrichment was acheived for this $MelanA_{26–35}$-specific $CD8^+$ T cells. Taken together, the above results indicate that CD3CD8 downregulation-guided sorting is able to enrich antigen-specific $CD8^+$ T cells that show much less dramatic CD3CD8 downregulation and also capable of purifying activated $CD8^+$ T cells specific to a cysteine-containing peptide or a peptide with weak HLA-binding ability.

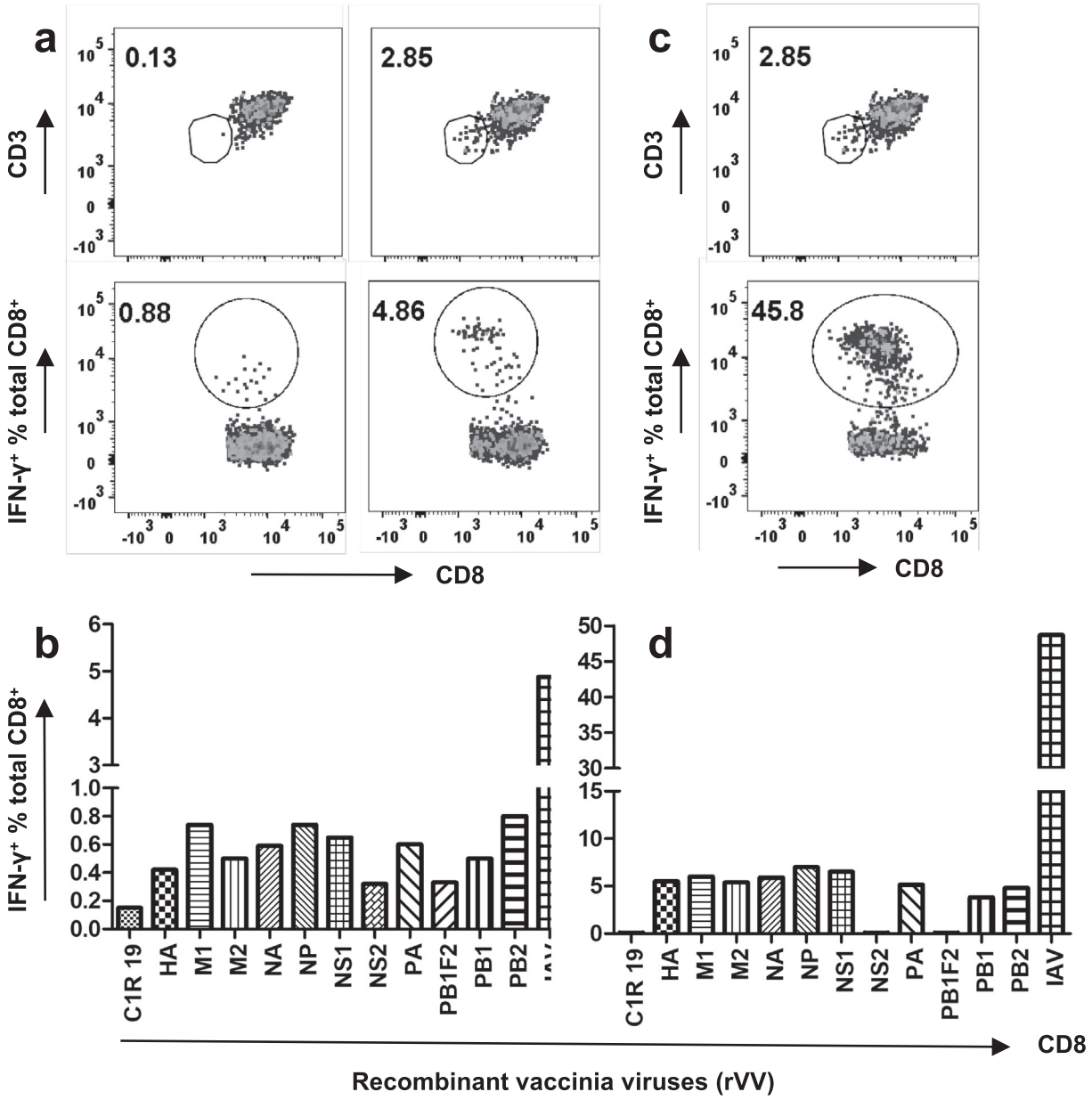


Fig. 5. Multiple small IAV-specific CD8$^+$T cell specificities were simultaneously enriched by CD3CD8 downregulation-guided sorting.
Primary T cell culture was established using IAV PR8-infected PBMC as described in the method. **a-b)** After 2 weeks' culture, IAV-specific CD8$^+$ T cell responses were assessed by their reactivity to the 11 individual IAV proteins expressed by rVV infected autologous BLCLs in an ICS assay. The IAV infected BLCLs served as a positive control and the rVV-CR19 vector infected BLCLs served as a negative control (Fig. 5b). TCR and CD8 were also stained and gated the CD3^{low}CD8^{low} population according to the unstimulated group (Fig. 5a, upper left panel). **c-d)** CD3^{low}CD8^{low} population was sorted and cultured for 3 days before being assessed for the overall IAV response and responses to the individual IAV antigens by ICS for IFN-γ as described above.

4. Discussion and conclusion

In this study, we have established and optimized a novel method for enriching antigen-specific CD8$^+$ T cells. We have demonstrated that using CD3CD8 downregulation-guided sorting can greatly enrich antigen-specific CD8$^+$ T cells. We have successfully employed this method first to enrich established immunodominant CD8$^+$ T cell line (Fig. 3b), later single IAV protein-specific T cells (Fig. 3c); and finally, a poly-specificity, low purity IAV-specific bulk CD8$^+$ T cell population. We have further demonstrated that enriched CD8$^+$ T cell populations using this method maintained broad T cell specificities (Fig. 5) and they are capable of expansion from very low numbers.

This method could potentially be widely applicable to any antigen-specific CD8$^+$ T cell enrichment, especially at the early stage of T cell culture which is the most problematic time for most, if not all T cell cultures in worldwide laboratories. Comparing to the mostly utilized tetramer-guided antigen-specific T cell sorting, our novel antigen-specific T cell enriching method has the following major advantages: first, our novel approach enriches any antigen-specific CD8$^+$ T cells without the need to know HLA restriction or minimum epitope sequence, as both are critical for the tetramer-guided T cell sorting (Altman et al., 1996; Sims et al., 2010; Hunsucker et al., 2015). In this regard, our approach should be more convenient with much wider application; second, tetramers are expensive to make, and they often have limited shelve life. So, our approach will be cheaper and always ready to run; and finally, tetramer-guided sorting is mostly only applicable to individual T cell population specific for a single pMHC. However, our approach, as demonstrated in this study, is able to enrich many antigen-

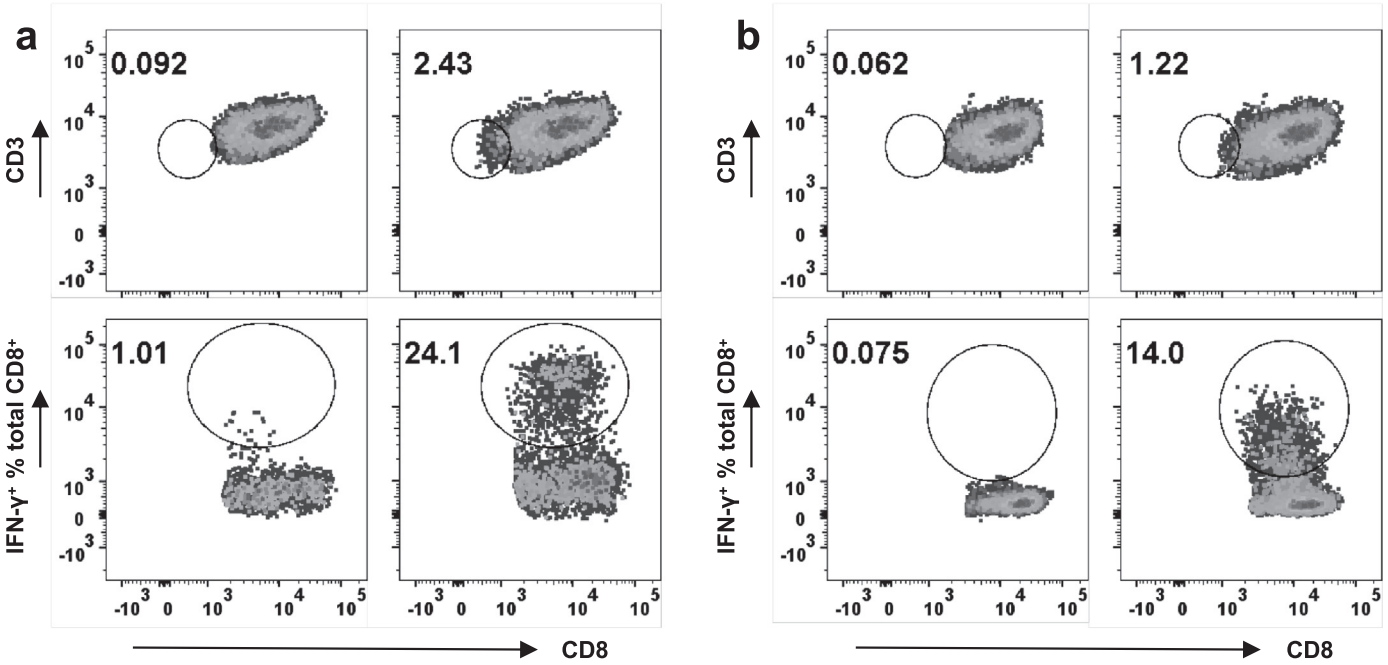


Fig. 6. CD3CD8 downregulation-guided sorting can enrich $CD8^+$ T cells with limited CD3CD8 downregulation after antigen activation.
a) Autologous BLCLs were pulsed with cysteine-containing $NP_{44–52}$ (CTELKLSDY HLA-A*01:01) then incubated with $NP_{44–52}$-specific $CD8^+$ T cells in the presence of TCEP. After activation a small population of $CD3^{low}CD8^{low}$ T cells were sorted and assessed for IFN-γ production as described in the method. **b)** Melan $A_{26–35}$ specific $CD8^+$ T cells were stimulated by peptide-pulsed HLA-A*02:01^+ BLCLs. T cell activation induced $CD3^{low}CD8^{low}$ T cells were sorted and assessed *via* ICS.

specific T cell populations by a single run, which may allow our method be used for antigen discovery in potentially complicated epitope discovery experiments or quick assessments to rank major T cell response to a particular antigen or pathogen.

In this study, we have not performed a side-by-side comparison study with tetramer-guided sorting. As a result, we are not sure about which method is more sensitive and/or more accurate. However, several studies have reported that pMHC tetramer staining fail to detect fully functional T cells as the TCR-affinity threshold for tetramer staining is higher than that required for T cell activation (Derby et al., 2001; Dolton et al., 2014). Furthermore, a more recent study has demonstrated that standard pMHC tetramer staining fail to detect relevant functional T cell clonotypes and underestimated antigen-reactive T cell populations (Rius et al., 2018) stressing the importance of using optimized protocols for T cell study.

Although we trialled T cell cloning using this novel method, we failed to acquire any antigen-specific T cell clone originated from single cell culture. Judging from the results we have so far, especially those from 3 cells per well cultures, we have every reason to believe this approach should be suitable for T cell cloning. We believe it is likely a cell culture related issue rather than a sorting related one, which is also reflected by a relatively low success rate of 3 cells/well group. If it was a sorting related issue, for example, cell damage during activation and/or sorting, we would have expected the same negative outcome from 3 cells/well, or even higher number wells. The second possibility could be a low rate related issue as we only trialled 60 culture for each cell number although three trials were conducted. It might be possible that should we have trialled a few hundred each time, we would have had a few clones successfully.

During this study, we observed that a T cell line did not show any CD3CD8 downregulation after being stimulated by 10^{-7} M peptide. This was a long-term cultured $CD8^+$ T cell line and had been stimulated for > 10 times and cultured for 6 months continuously. We then increased the stimulating peptide concentration to 10^{-6} M or 10^{-5} M, also enhanced T cell activation time to 6–8 h. After such activation, a small portion of $CD3^{low}CD8^{low}$ T cells could be detected in the 10^{-5} M peptide stimulated group. We also observed that after CD3CD8 downregulation-guided sorting, EBV-specific $CD8^+$ T cells were dramatically enriched. This could be due to the fact that autologous BLCL line was used as APCs. Hence, the best approach to reduce such unwanted response is to avoid using autologous BLCL. Instead, using an APC that shares only the peptide presenting HLA allele(s). For example, T2 cell line expresses HLA-A*02:01 can be used to replace HLA-A*02:01 positive autologous BLCLs. By doing this, all the EBV-specific $CD8^+$ T cells restricted to other HLA alleles, rather than HLA-A*02:01 will be avoided.

In conclusion, we have developed a novel method which utilizes the basic T cell property of CD3CD8 downregulation after antigen-triggered activation to enrich antigen-specific T cells. We have shown that this method can be utilized to enrich various T cell populations with known HLA-restrictions and peptide specificities. We have also shown that such a method can be used to enrich T cell populations with diverse specificities, unknown HLA restriction and unknown epitope sequence. Most importantly, we expect this method to be used to enrich low antigen-specific T cell populations at early, not yet established T cell culture stage, at which culture failure seems inevitable, to go on to establish relatively high purity T cell lines, or even clones without the need for tetramers or even the critical information relating to HLA restriction and T cell fine epitope sequence. As once a T cell line, or a clone is established, all this information can be easily derived. We also hope this approach may contribute to future T cell immunotherapy.

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References

Alexander-Miller, M.A., Derby, M.A., Sarin, A., Henkart, P.A., Berzofsky, J.A., 1998. Supraoptimal peptide-major histocompatibility complex causes a decrease in bc1-2 levels and allows tumor necrosis factor alpha receptor II-mediated apoptosis of

cytotoxic T lymphocytes. J. Exp. Med. 188, 1391–1399.
Altman, J.D., Moss, P.A., Goulder, P.J., Barouch, D.H., McHeyzer-Williams, M.G., Bell, J.I., McMichael, A.J., Davis, M.M., 1996. Phenotypic analysis of antigen-specific T lymphocytes. Science (New York, N.Y.) 274, 94–96.
Arcaro, A., Gregoire, C., Bakker, T.R., Baldi, L., Jordan, M., Goffin, L., Boucheron, N., Wurm, F., van der Merwe, P.A., Malissen, B., Luescher, I.F., 2001. CD8beta endows CD8 with efficient coreceptor function by coupling T cell receptor/CD3 to raft-associated CD8/p56(lck) complexes. J. Exp. Med. 194, 1485–1495.
Bierer, B.E., Sleckman, B.P., Ratnofsky, S.E., Burakoff, S.J., 1989. The biologic roles of CD2, CD4, and CD8 in T-cell activation. Annu. Rev. Immunol. 7, 579–599.
Cantrell, D.A., Davies, A.A., Crumpton, M.J., 1985. Activators of protein kinase C down-regulate and phosphorylate the T3/T-cell antigen receptor complex of human T lymphocytes. Proc. Natl. Acad. Sci. U. S. A. 82, 8158–8162.
Chen, W., Yewdell, J.W., Levine, R.L., Bennink, J.R., 1999. Modification of cysteine residues in vitro and in vivo affects the immunogenicity and antigenicity of major histocompatibility complex class I-restricted viral determinants. J. Exp. Med. 189, 1757–1764.
Chen, W., Calvo, P.A., Malide, D., Gibbs, J., Schubert, U., Bacik, I., Basta, S., O'Neill, R., Schickli, J., Palese, P., Henklein, P., Bennink, J.R., Yewdell, J.W., 2001. A novel influenza a virus mitochondrial protein that induces cell death. Nat. Med. 7, 1306–1312.
Clemens, E.B., Grant, E.J., Wang, Z., Gras, S., Tipping, P., Rossjohn, J., Miller, A., Tong, S.Y., Kedzierska, K., 2016. Towards identification of immune and genetic correlates of severe influenza disease in indigenous Australians. Immunol. Cell Biol. 94, 367–377.
Clevers, H., Alarcon, B., Wileman, T., Terhorst, C., 1988. The T cell receptor/CD3 complex: a dynamic protein ensemble. Annu. Rev. Immunol. 6, 629–662.
Davis, M.M., Bjorkman, P.J., 1988. T-cell antigen receptor genes and T-cell recognition. Nature 334, 395–402.
Derby, M.A., Wang, J., Margulies, D.H., Berzofsky, J.A., 2001. Two intermediate-avidity cytotoxic T lymphocyte clones with a disparity between functional avidity and MHC tetramer staining. Int. Immunol. 13, 817–824.
Dolton, G., Lissina, A., Skowera, A., Ladell, K., Tungatt, K., Jones, E., Kronenberg-Versteeg, D., Akpovwa, H., Pentier, J.M., Holland, C.J., Godkin, A.J., Cole, D.K., Neller, M.A., Miles, J.J., Price, D.A., Peakman, M., Sewell, A.K., 2014. Comparison of peptide-major histocompatibility complex tetramers and dextramers for the identification of antigen-specific T cells. Clin. Exp. Immunol. 177, 47–63.
Ebert, L.M., MacRaild, S.E., Davis, I.D., Cebon, J., Chen, W., 2012. A novel method for detecting antigen-specific human regulatory T cells. J. Immunol. Methods 377, 56–61.
Gao, G.F., Jakobsen, B.K., 2000. Molecular interactions of coreceptor CD8 and MHC class I: the molecular basis for functional coordination with the T-cell receptor. Immunol. Today 21, 630–636.
Grant, E., Wu, C., Chan, K.F., Eckle, S., Bharadwaj, M., Zou, Q.M., Kedzierska, K., Chen, W., 2013. Nucleoprotein of influenza a virus is a major target of immunodominant CD8+ T-cell responses. Immunol. Cell Biol. 91, 184–194.
Holler, P.D., Kranz, D.M., 2003. Quantitative analysis of the contribution of TCR/pepMHC affinity and CD8 to T cell activation. Immunity 18, 255–264.
Hunsucker, S.A., McGary, C.S., Vincent, B.G., Enyenihi, A.A., Waugh, J.P., McKinnon, K.P., Bixby, L.M., Ropp, P.A., Coghill, J.M., Wood, W.A., Gabriel, D.A., Sarantopoulos, S., Shea, T.C., Serody, J.S., Alatrash, G., Rodriguez-Cruz, T., Lizee, G., Buntzman, A.S., Frelinger, J.A., Glish, G.L., Armistead, P.M., 2015. Peptide/MHC tetramer-based sorting of CD8(+) T cells to a leukemia antigen yields clonotypes drawn nonspecifically from an underlying restricted repertoire. Cancer immunology research 3, 228–235.
Jackson, H.M., Dimopoulos, N., Chen, Q., Luke, T., Yee Tai, T., Maraskovsky, E., Old, L.J., Davis, I.D., Cebon, J., Chen, W., 2004. A robust human T-cell culture method suitable for monitoring CD8+ and CD4+ T-cell responses from cancer clinical trial samples. J. Immunol. Methods 291, 51–62.
Janeway Jr., C.A., 1992. The T cell receptor as a multicomponent signalling machine: CD4/CD8 coreceptors and CD45 in T cell activation. Annu. Rev. Immunol. 10, 645–674.
Kane, L.P., Lin, J., Weiss, A., 2000. Signal transduction by the TCR for antigen. Curr. Opin. Immunol. 12, 242–249.
Krangel, M.S., 1987. Endocytosis and recycling of the T3-T cell receptor complex. The role of T3 phosphorylation. J. Exp. Med. 165, 1141–1159.
Krogsgaard, M., Li, Q.J., Sumen, C., Huppa, J.B., Huse, M., Davis, M.M., 2005. Agonist/endogenous peptide-MHC heterodimers drive T cell activation and sensitivity. Nature 434, 238–243.
Kuhns, M.S., Davis, M.M., 2012. TCR signaling emerges from the sum of many parts. Front. Immunol. 3, 159.
Laugel, B., van den Berg, H.A., Gostick, E., Cole, D.K., Wooldridge, L., Boulter, J., Milicic, A., Price, D.A., Sewell, A.K., 2007. Different T cell receptor affinity thresholds and CD8 coreceptor dependence govern cytotoxic T lymphocyte activation and tetramer binding properties. J. Biol. Chem. 282, 23799–23810.
Pang, K.C., Sanders, M.T., Monaco, J.J., Doherty, P.C., Turner, S.J., Chen, W., 2006a. Immunoproteasome subunit deficiencies impact differentially on two immunodominant influenza virus-specific CD8+ T cell responses. Journal of immunology (Baltimore, Md. : 1950) 177, 7680–7688.
Pang, K.C., Wei, J.Q., Chen, W., 2006b. Dynamic quantification of MHC class I-peptide presentation to CD8+ T cells via intracellular cytokine staining. J. Immunol. Methods 311, 12–18.
Potter, M.R., Moore, M., 1975. PHA stimulation of separated human lymphocyte populations. Clin. Exp. Immunol. 21, 456–467.
Rius, C., Attaf, M., Tungatt, K., Bianchi, V., Legut, M., Bovay, A., Donia, M., Thor Straten, P., Peakman, M., Svane, I.M., Ott, S., Connor, T., Szomolay, B., Dolton, G., Sewell, A.K., 2018. Peptide-MHC class I tetramers can fail to detect relevant functional T cell Clonotypes and underestimate antigen-reactive T cell populations. Journal of immunology (Baltimore, Md. : 1950) 200, 2263–2279.
Rosenberg, S.A., Yang, J.C., Restifo, N.P., 2004. Cancer immunotherapy: moving beyond current vaccines. Nat. Med. 10, 909–915.
Salter, R.D., Benjamin, R.J., Wesley, P.K., Buxton, S.E., Garrett, T.P., Clayberger, C., Krensky, A.M., Norment, A.M., Littman, D.R., Parham, P., 1990. A binding site for the T-cell co-receptor CD8 on the alpha 3 domain of HLA-A2. Nature 345, 41–46.
Schott, E., Ploegh, H.L., 2002. Mouse MHC class I tetramers that are unable to bind to CD8 reveal the need for CD8 engagement in order to activate naive CD8 T cells. Eur. J. Immunol. 32, 3425–3434.
Sims, S., Willberg, C., Klenerman, P., 2010. MHC-peptide tetramers for the analysis of antigen-specific T cells. Expert review of vaccines 9, 765–774.
Smith-Garvin, J.E., Koretzky, G.A., Jordan, M.S., 2009. T cell activation. Annu. Rev. Immunol. 27, 591–619.
Valitutti, S., Muller, S., Cella, M., Padovan, E., Lanzavecchia, A., 1995. Serial triggering of many T-cell receptors by a few peptide-MHC complexes. Nature 375, 148–151.
Valmori, D., Fonteneau, J.F., Valitutti, S., Gervois, N., Dunbar, R., Lienard, D., Rimoldi, D., Cerundolo, V., Jotereau, F., Cerottini, J.C., Speiser, D.E., Romero, P., 1999. Optimal activation of tumor-reactive T cells by selected antigenic peptide analogues. Int. Immunol. 11, 1971–1980.
Wegener, A.M., Letourneur, F., Hoeveler, A., Brocker, T., Luton, F., Malissen, B., 1992. The T cell receptor/CD3 complex is composed of at least two autonomous transduction modules. Cell 68, 83–95.
Wherry, E.J., Kurachi, M., 2015. Molecular and cellular insights into T cell exhaustion. Nat. Rev. Immunol. 15, 486–499.
Wu, C., Zanker, D., Valkenburg, S., Tan, B., Kedzierska, K., Zou, Q.M., Doherty, P.C., Chen, W., 2011. Systematic identification of immunodominant CD8+ T-cell responses to influenza a virus in HLA-A2 individuals. Proc. Natl. Acad. Sci. U. S. A. 108, 9178–9183.
Xiao, Z., Mescher, M.F., Jameson, S.C., 2007. Detuning CD8 T cells: down-regulation of CD8 expression, tetramer binding, and response during CTL activation. J. Exp. Med. 204, 2667–2677.
Zanker, D., Xiao, K., Oveissi, S., Guillaume, P., Luescher, I.F., Chen, W., 2013. An optimized method for establishing high purity murine CD8+ T cell cultures. J. Immunol. Methods 387, 173–180.