Chapter 16

Quantitative Multiparameter Assays to Measure the Effect of Adjuvants on Human Antigen-Specific CD8 T-Cell Responses

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Abstract

Large numbers and functionally competent T cells are required to protect from diseases for which antibody-based vaccines have consistently failed (1), which is the case for many chronic viral infections and solid tumors. Therefore, therapeutic vaccines aim at the induction of strong antigen-specific T-cell responses. Novel adjuvants have considerably improved the capacity of synthetic vaccines to activate T cells, but more research is necessary to identify optimal compositions of potent vaccine formulations. Consequently, there is a great need to develop accurate methods for the efficient identification of antigen-specific T cells and the assessment of their functional characteristics directly ex vivo. In this regard, hundreds of clinical vaccination trials have been implemented during the last 15 years, and monitoring techniques become more and more standardized.

Key words: direct ex vivo monitoring, CD8 T lymphocytes, staining, cytokines, proliferation, adjuvants.

1. Introduction

Considerable efforts have been directed during the last years toward the development of new, defined antigen-based cancer vaccines (2, 3). A T-cell vaccine is composed of at least three major components, i.e., antigen(s), adjuvant(s), and a delivery system. Vaccine optimization requires careful step-by-step development, whereby each component needs to be investigated with respect to its capacity to induce protective immune responses (4).

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Antigens need to recruit high-avidity T cells capable of efficient recognition of antigen-bearing cells. Adjuvants are required to activate dendritic cells that optimally promote and sustain T-cell activation. Finally, delivery systems may be optimized such that they provide the vaccine components in a targeted fashion and during prolonged time to lymphoid tissues where immune responses are generated. Induction of specific antitumor T-cell responses has been observed and monitored using several techniques (5). However, one of the current major limitations for a valid measurement of naturally acquired as well as vaccine-induced tumor antigen-specific T-cell responses remains the difficulty of ex vivo detection of specific T cells using accurate and reproducible methods. In this regard, one major breakthrough has been the development of fluorescent peptide-MHC-I multimers (6). This technique allows direct identification, enumeration, and phenotyping, as well as isolation of antigen-specific T cells. Additional approaches can be applied for the direct ex vivo assessment of effector functions of antigen-specific T cells and include the use of ELISPOT assays for enumeration of cytokine-secreting cells (7) or intracellular cytokine staining of cell suspensions (8). The latter further enables the direct visualization and quantification of single cytokine$^+$ cells, with the concomitant phenotyping in multiparameter, multicolor flow cytometry format. Our current monitoring strategy is therefore based on the use of a combination of standardized monitoring methods consisting in the direct ex vivo quantification of specific T cells together with the assessment of their functionality, in order to gather a comprehensive picture of the specific T-cell response in the patients, avoiding the bias introduced by the in vitro cell expansion (9, 10). In this regard, the initial handling of patients' samples, both peripheral blood as well as tissue material, is of paramount importance to the accuracy and reproducibility of quantitative and direct T-cell assays (11). Thus, only high-quality processing and preservation of the sampled material will enable reproducible analyses and will allow to draw conclusions on the efficacy of newly developed cancer vaccines.

This chapter represents a detailed description of materials, methods, and notes of laboratory techniques, with the aim to promote common procedures leading to standardization of assays, ultimately allowing wide application and improved understanding of the role of CD8 T cells in infection, cancer, autoimmune disease, and transplantation. Methods for the isolation and cryopreservation of human CD8 T cells from peripheral blood as well as from lymph nodes and tumor tissues are described first. Subsequently, we show ex vivo multimer and intracellular cytokine staining. Finally, we discuss limiting dilution analysis, ELISPOT assay, and carboxyfluorescein succinimidyl ester proliferation assays.

2. Materials

2.1. Isolation of Peripheral Blood Mononuclear Cells (PBMCs) from Human Blood

1. Heparinized human blood.
2. 50 mL tubes (Falcon, Becton Dickinson).
3. Lymphoprep™ (Axis-Shield PoC AS), store at 4°C.
4. PBS store at room temperature.
5. RPMI 1640+Glutamax™-I medium (Gibco), store at 4°C.
6. 10 and 2 mL sterile pipettes (Falcon, Becton Dickinson).

2.2. Isolation of Human Lymphocytes from Tumor-Infiltrated Lymph Nodes (TILNs) or Primary Tumor Material (TILs)

1. Pair of sterile scissors.
2. Petri dishes.
3. Autoclavable metallic "tea strainer."
4. Ethanol 70% in water.
5. 50 mL tubes (Falcon, Becton Dickinson).
6. 50 mL sterile syringe.
7. 10 mL sterile pipette.
8. CTL culture medium: RPMI 1640+Glutamax™-I supplemented with 8% human serum (recommended is the use of pooled A+ healthy donors' serum), 1% penicillin/streptomycin (Amimed), L-glutamine (1.5 mM, Gibco), L-asparagine (0.24 mM, Sigma,), L-arginine (0.55 mM, Sigma), 1% HEPES buffer (Amimed), 1% sodium pyruvate (100 mM, Gibco), and 0.1% 2-mercaptoethanol (5×10^{-2} M 100 mL, Sigma-Aldrich). Store at 4°C.
9. Tumor cell culture medium: RPMI 1640+Glutamax™-I supplemented with 10% FCS, 1% penicillin/streptomycin (Amimed), 1% L-glutamine (200 mM, Amimed), 1% HEPES buffer (1, Amimed), 1% nonessential amino acids (Gibco), 1% sodium pyruvate (100 mM, Gibco). Store at 4°C.
10. Cytokines: 150 UI/mL of IL-2 (Proleukine®, Roche) and 10 ng/mL of IL-7 (R&D). Store stock dilutions at –20°C.
11. 6-well plates (Corning Incorporated, Costar).

2.3. Cryopreservation of Human Cells

1. Round-bottom 1.8 mL Nunc CryoTubes™ (Nunc).
2. Freezing solution: pure FCS supplemented with 20% dimethyl sulfoxide (DMSO). Make fresh as required.
3. RPMI 1640+Glutamax™-I medium (Gibco), store at 4°C.

2.4. Thawing of Human Cells

1. Water bath at 37°C.
2. RPMI 1640+Glutamax™-I medium (Gibco), store at 4°C.

3. DNAse I (Sigma) reconstituted at 10 mg/mL in buffer according to manufacturer's instructions. Store at –20°C. Prepare aliquots at 1 mg/mL by diluting the stock in RPMI 1640 medium. Store aliquots at 4°C.
4. 15 mL tubes (Sarstedt).

2.5. Ex Vivo Multimer Staining and Phenotyping of Human PBMCs or TILNs and Limiting Dilution Analysis (LDA)

1. Staining buffer: PBS containing 0.2% BSA (Merck Fraction V for biochemical use) and 5 mM EDTA. Store at 4°C.
2. CD8 microbeads (Miltenyi Biotec). Store at 4°C.
3. MiniMACS^{TM} MS columns (Miltenyi Biotec).
4. MiniMACS^{TM} separation unit (Miltenyi Biotec).
5. 15 mL tubes (Sarstedt).
6. 4 mL V-bottom tubes (Greiner).
7. 4 mL U-bottom tubes (Falcon).
8. HLA-A2/peptide multimer-PE (internal production facility). Avoid exposure to light, store at 4°C in humid chamber.
9. PBS.
10. Monoclonal antibodies: our standard format successfully used for ex vivo monitoring of tumor antigen-specific CD8 T cells consists in the use of CD8-PercP (Becton Dickinson), CCR7-PECy7 (Becton Dickinson), CD45RA-ECD (Beckman Coulter), CD28-APC (Becton Dickinson), CD27-APC-Alexa780 (ebioscience), CD127-Pacific Blue (ebioscience), PD1– FITC (Becton Dickinson). Avoid exposure to light, store at 4°C.
11. LIVE/DEAD® Fixable Aqua Dead Cell Stain Kit (Invitrogen).
12. RPMI 1640+Glutamax^{TM}-I medium (Gibco), store at 4°C.
13. Cytokine: 150 UI/mL of IL-2 (Proleukine®, Roche). Store stock dilutions at 4°C.
14. Synthetic peptides of the specificity of interest (internal production facility). Store stock dilutions at –20°C.
15. CTL medium (*see* **Section 2.2**).
16. Round-bottom 96-well plates (Sarstedt).

2.6. ELISPOT Assay for Detection of IFN-γ-Producing Human CD8 T Cells

1. CTL culture medium (*see* **Section 2.2**).
2. 24-well plates (Corning Incorporated, Costar).
3. IFN-γ ELISPOT PVDF—Enzymatic kit (Diaclone) containing antihuman-IFN-γ capture and detection antibodies, streptavidin alkaline phosphatase conjugate, and ready-to-use BCIP/NBT substrate.

4. Microtiter plates Multiscreen^{TM}-HA (Millipore).
5. PBS containing 5% FCS.
6. 15 mL tubes (Sarstedt).
7. Phytohemagglutinin-L (PHA) (Remel). Store stock dilutions at –20°C.
8. Synthetic peptides of the specificity of interest (internal production facility). Store stock dilutions at –20°C.
9. Tween-20. Store at room temperature.
10. Automatic plate reader: Bioreader® 5000 (BioSys GmbH, Karben-Frankfurt Germany).

2.7. Intracellular Cytokine Staining

1. CTL culture medium (*see* **Section 2.2**).
2. 24-well plates (Corning Incorporated, Costar).
3. Synthetic peptides of the specificity of interest (internal production facility). Store stock dilutions at –20°C.
4. Phorbol 12-myristate 13-acetate (PMA) (Sigma), ionomycin (Sigma). Store stock dilutions at –20°C.
5. Brefeldin A (Sigma). Store stock dilutions at –20°C.
6. 4 mL V-bottom tubes (Greiner).
7. Staining buffer: PBS containing 0.2% BSA (Merck Fraction V for biochemical use), and 5 mM EDTA (Gibco). Store at 4°C.
8. Fixing buffer: PBS supplemented with 1% formaldehyde (Fluka), 2% glucose (Fluka), and 5 nM sodium azide (Merck). Store at 4°C.
9. Saponin (Sigma). Store stock dilutions at –20°C.
10. 4 mL U-bottom tubes (Falcon).
11. Monoclonal antibodies: INFγ-PECy7 (Becton Dickinson), TNFα-Alexa700 (Becton Dickinson), IL-2-APC (Becton Dickinson), CD107a-FITC (Becton Dickinson).

2.8. Carboxyfluorescein Succinimidyl Ester (CFSE) Proliferation Assay

1. CFSE (Molecular Probes/Invitrogen). Store stock dilution at –80°C.
2. Labeling buffer: PBS containing 2% FCS. Store at 4°C.
3. Washing buffer: PBS containing 5% FCS. Store at 4°C.
4. 24-well plates (Costar).
5. CTL medium (*see* **Section 2.2**).
6. Synthetic peptides of the specificity of interest (internal production facility). Store stock dilutions at –20°C.
7. 150 UI/mL of IL-2 (Proleukine®, Roche). Store stock dilutions at 4°C.

3. Methods

3.1. Isolation of Peripheral Blood Mononuclear Cells (PBMCs) from Human Blood

1. Dilute freshly collected heparinized human peripheral blood in 1:1 or 1:2 ratio with PBS or RPMI 1640+Glutamax™-I medium in 50 mL tubes (*see* **Note 1**).
2. Distribute, using a 10 mL sterile pipette, 15 mL of Lymphoprep™ (at room temperature) in 50 mL tubes and carefully overlay 35 mL of the diluted blood on the Lymphoprep™ layer, creating a sharp blood–Lymphoprep™ interface (*see* **Note 2**). Cap the tube in order to prevent accidental contamination.
3. Centrifuge the tube at 800 × *g* for 20 min without brake, in a swing-out rotor, at room temperature (*see* **Note 3**). Centrifugation will result in sedimentation of erythrocytes and polymorphonuclear leukocytes to the bottom of the tube; thus, peripheral blood mononuclear cells will form a distinct white layer at the Lymphoprep™/medium interface, with the appearance of an opalescent ring.
4. Carefully harvest the PBMC layer, together with about half of the Lymphoprep™ solution laying directly below the cells, using a 2 mL sterile pipette. Transfer the cells into a new 50 mL tube. Fill up the tube with PBS or RPMI 1640+Glutamax™-I, mix well, and centrifuge for 5 min, at 600 × *g*, with break, at room temperature.
5. Discard the supernatant, add PBS or RPMI 1640+Glutamax™-I, then centrifuge the PBMCs at 200 × *g* for 10 min at room temperature without brake (*see* **Note 4**).
6. Wash the cells once more in PBS or RPMI 1640+Glutamax™-I (5 min at 600 × *g*).
7. Resuspend the cell pellet in RPMI 1640+Glutamax™-I and count them before use.

3.2. Isolation of Human Lymphocytes from Tumor-Infiltrated Lymph Nodes (TILNs) or Primary Tumor Material (TILs)

1. Put 3 mL of RPMI 1640+Glutamax™-I in a Petri dish together with the TILNs or the tumor sample (*see* **Note 1**).
2. Cut the tissue sample into small pieces using sterile scissors (*see* **Note 5**).
3. Transfer into a sterile strainer and grind with a syringe plunger above the Petri dish (*see* **Note 6**). Rinse the strainer with additional 5 mL of RPMI 1640+Glutamax™-I.
4. Collect with a sterile 10 mL pipette the dissociated tissue together with the medium from the Petri dish, into a 50 mL tube. Centrifuge for 5 min at 600 × *g*.

5. Discard the supernatant and resuspend the pellet in 10–20 mL of RPMI 1640+Glutamax^{TM}-I. Mix gently using a 10 mL pipette and count both the lymphocytes and the tumor cells.
6. Put part of the cells in culture, in 6-well plates (1–2 × 10^6 cells/2 mL/well), either with tumor cell culture medium in order to derive a tumor cell line (*see* **Note 7**) or with CTL culture medium supplemented with IL-2 (150 UI/mL) and IL-7 (10 ng/mL) in order to derive TILNs lines (*see* **Note 8**). Place the plates in an incubator at 37°C, with 5% CO_2 and add fresh medium every 3–4 days.
7. Freeze remaining cells.

3.3. Cryopreservation of Human Cells

1. Prepare the freezing solution and put it on ice.
2. Label cryotubes and put them on ice.
3. Count cells, wash them with pure medium, and resuspend them in 500 µL of ice-cold culture medium per vial to be frozen. Distribute 500 µL of cell suspension into cryotubes on ice (*see* **Note 9**).
4. Add 500 µL of ice-cold freezing solution dropwise on the cell suspension in each vial. Final DMSO concentration will be 10% per vial and final total volume will be of 1 mL (*see* **Note 10**).
5. Close the cryotubes and mix the suspension gently.
6. Immediately transfer the vials into –80°C precooled polystyrene box (*see* **Note 11**).
7. Cryotubes can be transferred to liquid nitrogen 24 h after freezing at the earliest.

3.4. Thawing of Human Cells

1. Heat water bath at 37°C and keep RPMI 1640+Glutamax^{TM}-I medium at room temperature.
2. Distribute 10 mL of culture medium into 15 mL tubes, add 100 µL of DNAse I to give a final concentration of 10 µg/mL.
3. Thaw frozen cell vials (no more than two frozen cell vials at a time) in the water bath until a small piece of frozen medium is still left.
4. Immediately transfer the cells dropwise into the tubes containing the RPMI 1640+Glutamax^{TM}-I medium and the DNAse I. It is critical to dispense the cell suspension dropwise as the changes in osmotic pressure may be important and only a gradual adjustment may preserve cell viability and ensure high recovery after thawing. Centrifuge for 5 min at 600 × *g*.

5. Resuspend the cell pellet in RPMI 1640+Glutamax^{TM}-I medium and count the cells using trypan blue.

3.5. Ex Vivo Multimer Staining and Phenotyping of Human PBMCs and TILNs and Limiting Dilution Analysis (LDA)

The use of peptide MHC-I multimers allows direct identification and, when combined with other markers, the phenotyping of single antigen-specific T cells in ex vivo samples by flow cytometry. The current limit of detection of specific cell populations using MHC-I/peptide multimers is set at 0.01%, rendering impossible the direct monitoring of populations at lower frequencies. In order to do that, a limiting dilution analysis (LDA) (12, 13) that uses multimers to count individual microcultures can be applied and gives as result an estimation of the precursor frequency of the cells of the specificity of interest. Both methods will be described hereafter.

3.5.1. Ex Vivo Multimer Staining and Phenotyping of Human PBMCs and TILNs

1. Thaw, wash, and count PBMCs or TILNs following the methods explained in **Section 3.4.**
2. Resuspend PBMCs/TILNs (up to 10×10^6) in 85 µL ice-cold staining buffer. Add 15 µL of CD8 microbeads (*see* **Note 12**). Incubate for 20 min at 4°C.
3. During this incubation period, hang the magnet on its support and place the column on the magnet. Equilibrate the column with 0.5 mL of ice-cold staining buffer.
4. After the incubation period, add 5 mL of ice-cold staining buffer to the cell suspension and centrifuge for 5 min at $600 \times g$.
5. Resuspend the cell pellet in 0.5 mL of ice-cold staining buffer. Place a 15 mL tube under the column to collect the CD8-negative fraction. Apply the sample into the column, and let the negative fraction pass through. Then rinse the column three times with 0.5 mL ice-cold staining buffer.
6. When all the staining buffer has passed through, remove the column from the magnet and place it on a 4 mL V-bottom tube. Pipette 2 mL of ice-cold staining buffer onto the column and firmly flush out the positive fraction by using the plunger supplied with the column.
7. Count the cells from the CD8-positive fraction. Spin down and resuspend up to 10^6 cells/tube in 50 µL of ice-cold staining buffer containing the optimal concentration of PE-labeled HLA-A2/peptide multimer (*see* **Note 13**). Sample is then incubated for 45 min at 4°C in the dark (*see* **Note 14**).
8. Add the appropriate amounts of antibody conjugates and incubate for 20 min at 4°C (*see* **Note 15**) in the dark.
9. Wash the cell with 1 mL of ice-cold PBS buffer (*see* **Note 16**). In the meantime, prepare Aqua solution according to the manufacturer's instructions. Remove the

supernatant and add 200 µL of Aqua solution on pelleted cells. Incubate for 30 min at 4°C in the dark.

10. Wash the cells with 1 mL of ice-cold PBS buffer. After removal of the supernatant, cell pellet (up to 10^6 cells) is resuspended in 300 µL of ice-cold staining buffer and transferred into 4 mL U-bottom tube. Samples are analyzed on a flow cytometer (LSR II, using DIVA software, Becton Dickinson, San Diego, CA). In order to have a reliable picture of the multimer-positive CD8 T-cell population, we recommend to acquire at least 3–5 × 10^5 total events (*see* **Note 17**) (**Fig. 16.1**).

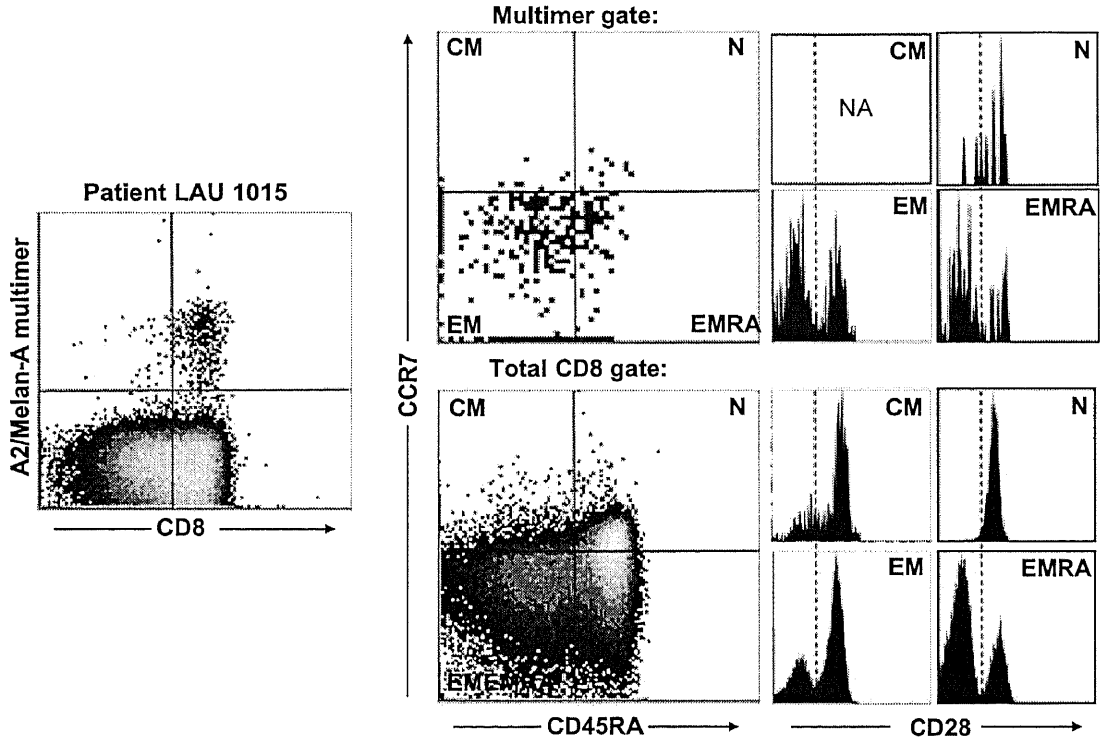

Fig. 16.1. Representative example of Melan-A^{MART-1}-specific CD8 T cells monitoring from a vaccinated melanoma patient using HLA-A2/Melan-A^{MART-1} multimers (*leftmost dot plot*). Naïve, central memory, effector memory, and effector subsets are represented according to CD45RA and CCR7 expression (*middle panels*) from total CD8 or multimer-specific populations. CD28 expression in each subset is depicted in the *right panels*.

3.5.2. Limiting Dilution Analysis (LDA)

1. Purify CD8-positive cells and collect the CD8-negative fraction as described in **Section 3.5.1** from Steps 1–6.
2. Count both cell fractions.
3. Pipette 100 µL of CTL culture medium into 50 wells in a round-bottom 96-well plate (five times 10 wells in each row). Add 100 µL of the CD8 T-cell suspension at a 1 × 10^6 cells into the first 10 wells. Proceed with a serial dilution of the CD8 T cells by pipetting 100 µL from the first 10 wells (remaining volume 100 µL, containing 50 × 10^3

CD8-positive cells) into the following 10 wells in the second row, mix well by pipetting up and down five consecutive times, and so on. Finally 10 replicates of five cell doses will be obtained (50×10^3; 25×10^3; 12.5×10^3; 6.25×10^3; 3.125×10^3 cells).

4. Irradiate the CD8-negative fraction at 3,000 rad and resuspend cells at a concentration of 2×10^6 cells/mL in CTL culture medium. Pipette 50 µL of this suspension to all the 50 wells (each well gets a constant number of 1×10^5 "feeder" cells).
5. Add to each well 50 µL of CTL culture medium containing the peptide of interest at a final concentration of 2 µM. Incubate for 14 days at 37°C.
6. At day 2, replace 100 µL of CTL culture medium with medium containing IL-2 at a final concentration of 150 IU/mL. Change medium every 2–3 days, split the cells if necessary.
7. At day 14, each well is stained individually with multimers and CD8 antibody, according to the methods described in the **Section 3.5.1** from point 7–10.
8. For each cell dose, the number of multimer-negative wells out of the 10 replicates is counted and the percentage of negative wells versus the dose of CD8 T cells is plotted in a graph. If the dots are aligned within a confidence interval of 95% the results follow a single-hit curve and the single-hit Poisson analysis can be applied, which says that 37% of negative wells correspond to the estimated precursor frequency (*see* **Note 18**) (**Fig. 16.2**).

3.6. ELISPOT Assay for Detection of IFN-γ-Producing Human CD8 T Cells

1. Thaw, wash, and count PBMCs following the methods explained in **Section 3.4**. PBMCs are then kept overnight in CTL culture medium (*see* **Note 19**) in a 24-well plate (2×10^6 cells/well, in 2 mL of CTL culture medium).
2. Anti-IFN-γ capture antibody (IFN-γ ELISPOT PVDF—Enzymatic kit) is coated on ELISPOT plates. In detail, antibody is diluted in PBS (10 µL antibody in 1 mL of PBS, for a final antibody concentration of 10 µg/mL) and 100 µL are distributed into each well. Plates are then incubated overnight at 4°C.
3. The capture antibody solution is removed from wells and plates are washed six times with 200 µL of PBS/well (*see* **Note 20**).
4. Antibodies' free sites are blocked by adding 200 µL of PBS supplemented with 5% FCS into each well, followed by an incubation of 1 h at 37°C.

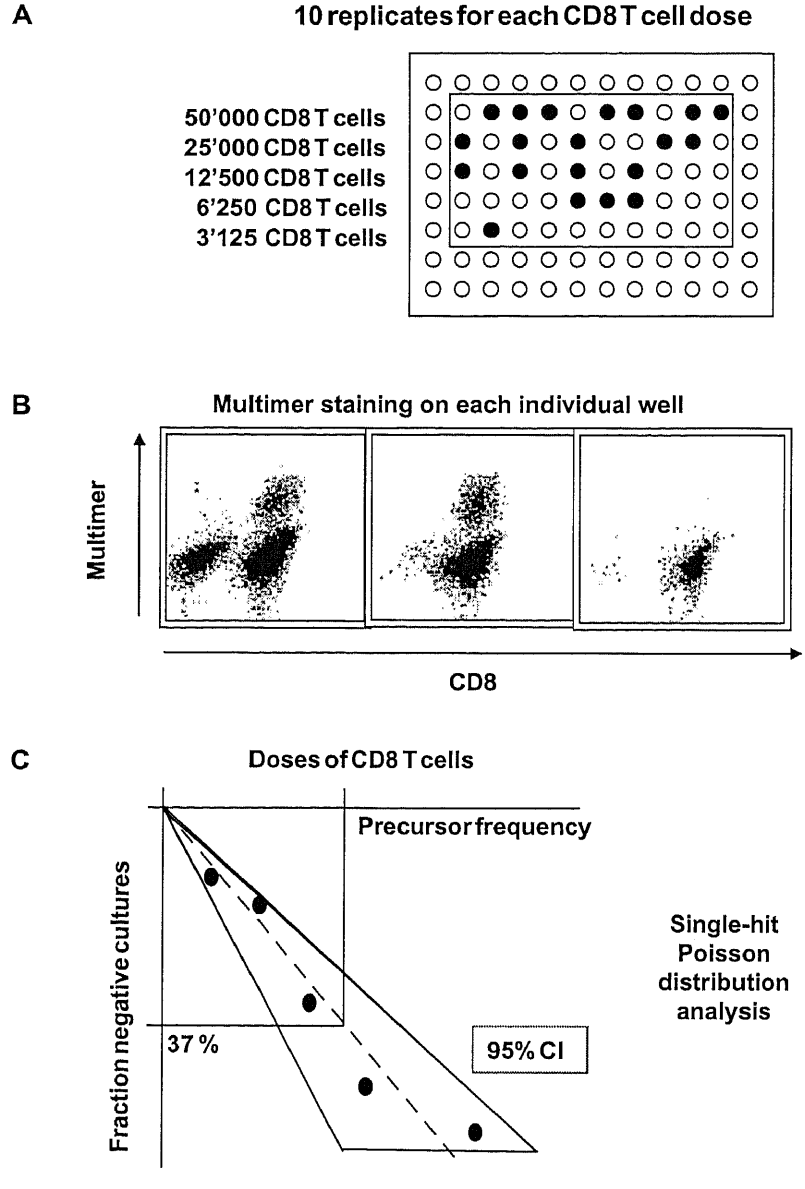

Fig. 16.2. Limiting dilution analysis of antigen-specific CD8 T cells. (**a**) Representative diagram of a 96-well plate loaded with 10 replicates of each CD8 T-cell dose. *Black dots* indicate multimer positive wells ($CD8^+$ $multimer^+ > 1\%$), defined according to multimer staining on each individual well as shown in (**b**). (**c**) Estimation of the precursor frequency of antigen-specific CD8 T cells based on the plotted fraction of multimer-negative wells (*Y*-axis) versus the different CD8 T-cell doses (*X*-axis). The *dashed line* represents the calculated fitting line with the 95% CI shown by the two continuous lines. Precursor frequency is then calculated using the single-hit Poisson distribution analysis, which says that the CD8 T-cell dose required for 37% of negative wells corresponds to the estimated precursor frequency.

5. During this incubation, PBMCs are collected in a 15 mL tube, counted, and washed once. Each condition (i.e., tested peptides, negative and positive controls) will be performed in triplicates and 5×10^5 PBMCs will be used per condition. PBMCs are therefore resuspended in CTL

culture medium in order to have 1.67×10^5 cells per 180 µL of CTL culture medium (corresponding to 5×10^5 cells per triplicates).

6. After the incubation period, remove the blocking solution from the plates and add 180 µL of medium containing PBMCs into each well.
7. Peptides of interest are added to the desired wells at the final concentration of 10 µg/mL. Thus 20 µL of a stock solution at 100 µg/mL are added to the PBMCs. For the negative control, add 20 µL of CTL culture medium alone or an irrelevant peptide (at 100 µg/mL). For the positive control, add 20 µL of PHA at 10 µg/mL (final concentration 1 µg/mL). Plates are incubated at least for 20 h at 37°C.
8. Plates are washed three times with 200 µL/well of PBS supplemented with 0.05% of Tween-20 and three additional times with 200 µL of pure PBS per well.
9. Dilute antihuman IFN-γ detection antibody (IFN-γ ELISPOT PVDF—Enzymatic kit) by adding 10 µL of antibody into 1 mL of PBS. 100 µL of diluted antibody is thereafter distributed in each well. Plates are wrapped with aluminum paper and incubated at room temperature for 2 h.
10. Plates are washed three times with 200 µL/well of PBS 0.05% Tween-20 and then three times with 200 µL/well of pure PBS.
11. Dilute 1 µL of streptavidin alkaline phosphatase (IFN-γ ELISPOT PVDF—Enzymatic kit) into 1 mL of PBS and add 100 µL to each well. Plates are incubated for 1 h at room temperature.
12. Wash the plates three times with PBS 0.05% Tween-20 and three times with pure PBS.
13. Add 100 µL of substrate solution/well (ready-to-use BCIP/NBT substrate from IFN-γ ELISPOT PVDF—Enzymatic kit) (*see* **Note 21**).
14. Plates are incubated in the darkness at room temperature until distinct dark spots develop and entirely fill the positive control wells (around 15 min) (*see* **Note 22**).
15. Stop the color development by rinsing plates three times with tap water.
16. Remove the base of the plates and dry them overnight at room temperature or for 2 h at 40°C. Then count the spots with the automatic reader (**Fig. 16.3a**).

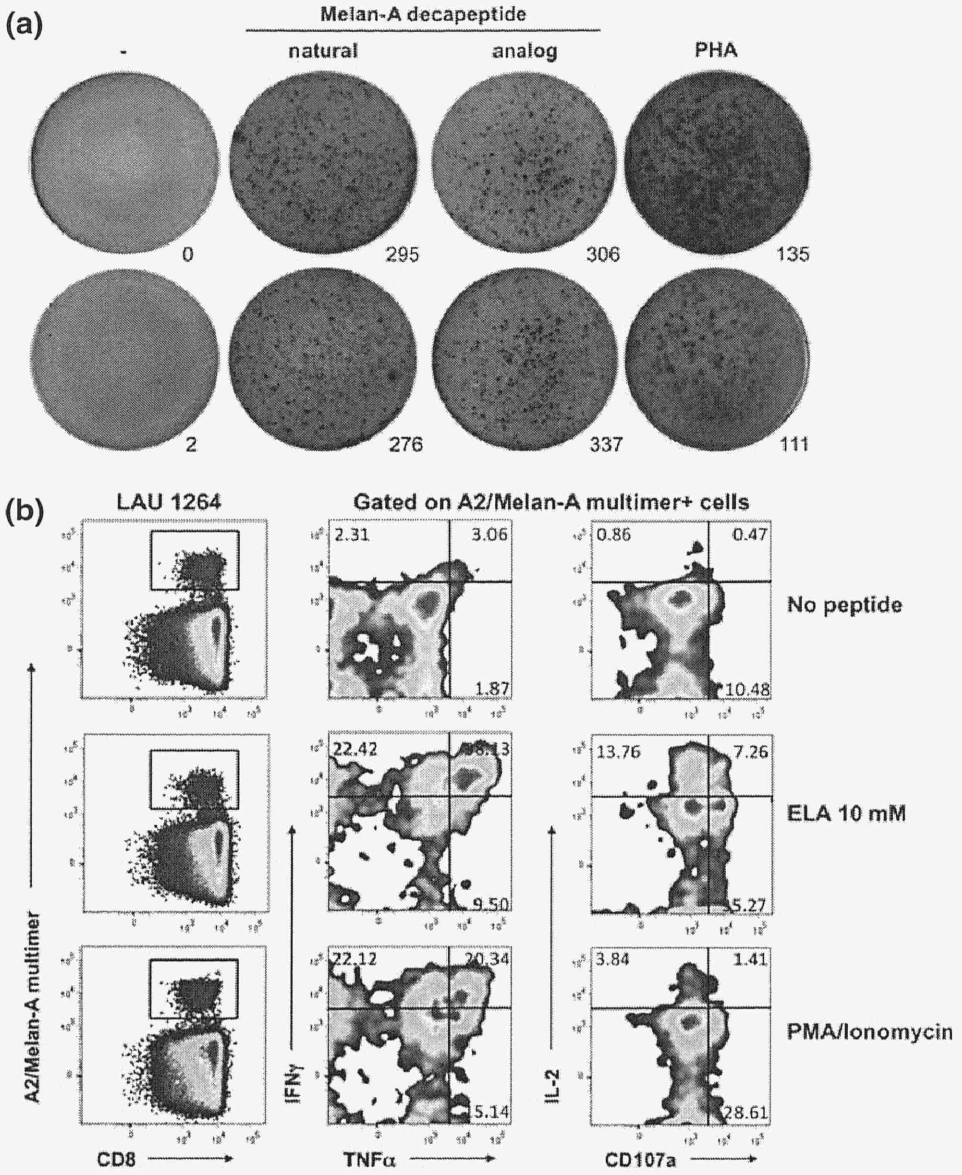

Fig. 16.3. Direct ex vivo analysis of Melan-A^{MART-1}-specific T-cell function. (**a**) PBMCs from patient LAU 1015 taken after four vaccine injections were tested ex vivo in IFN-γ ELSIPOT assays without peptide (*left panel*), Melan-A^{MART-1} natural and analog peptides (respectively EAAGIGILTV and ELAGIGILTV, *middle panels*), and PHA (*right panel*). The number of spots is indicated at the bottom right of each well. For the PHA condition, since the spots are numerous and confluent, counting of spots is not accurate which is actually not necessary as long as results reflect a valid positive control. (**b**) Intracellular staining of PBMCs from patient LAU 1164 after 4 h of stimulation without peptide (*top panels*), with Melan-A^{MART-1} analog (*middle panels*) or PMA/ionomycin (*bottom panels*). In each condition, IFN-γ, TNF-α, IL-2, and CD107a expression are represented from A2/Melan-A^{MART-1} multimer-positive cells.

3.7. Intracellular Cytokine Staining

1. Thaw, wash, and count PBMCs following the methods explained in **Section 3.4**.
2. Perform a multimer staining as described in **Section 3.1** (*see* **Note 23**).

3. Resuspend cells in culture medium in order to have 2×10^6 cells/mL. Distribute 1 mL of cell suspension/well in 24-well plates.
4. Add 1 mL of culture medium containing either specific or irrelevant peptides at 2–20 μg/mL, together with Brefeldin A at 10 μg/mL. As positive control, use PMA/ionomycin at a final concentration of 1 and 0.5 μg/mL, respectively. Incubate for 4–6 h at 37°C (*see* **Note 24**).
5. After incubation, collect the cells of each condition in a 4 mL V-bottom tube. Perform a multimer staining as described in **Section 3.5.1**, and if desired additional extracellular stainings can be performed followed by staining with Aqua solution in order to discriminate dead cells from living cells, as describe in **Section 3.5.1**.
6. Wash the cells with 1 mL staining buffer/tube and resuspend the pellet in 300 μL of fixing buffer. Mix gently and incubate for 20 min at room temperature, in the dark.
7. Wash with 1 mL/tube of staining buffer (*see* **Note 25**).
8. Prepare a mix of fluorescent antibody specific for cytokines, diluted in staining buffer supplemented with 0.1% of Saponin. Incubate for 30 min at room temperature in the dark (*see* **Note 26**).
9. Wash samples with 1 mL of staining buffer and resuspend them in 200–300 μL of staining buffer/tube. Transfer cells into 4 mL-U-bottom tube and analyze on a flow cytometer (**Fig. 16.3b**).

3.8. Carboxyfluorescein Succinimidyl Ester (CFSE) Proliferation Assay

1. Thaw, wash, and count PBMCs following the methods explained in **Section 3.4**. Resuspend them at a maximal concentration of 20×10^6 cells/mL in labeling buffer, at room temperature.
2. Prepare CFSE dilution in pure PBS in order to have a CFSE concentration of 2–4 μM.
3. Add the CFSE dilution on the cell suspension (1:1 v:v) (*see* **Note 27**), mix gently, and incubate for 4–5 min at room temperature. Do not exceed this labeling time.
4. Stop labeling by adding 10-fold volume of ice-cold washing buffer. Wash twice by centrifugation at $600 \times g$ using washing buffer.
5. Count the cells and resuspend them in order to have 2×10^6 cells/mL of CTL culture medium. Distribute 1 mL/well in a 24-well plate.
6. Dilute the cognate and irrelevant peptides at 1–10 μg/mL in CTL culture medium supplemented with 150 UI

IL-2/mL. Add 1 mL of peptide dilution on the CFSE-labeled cells. Incubate at 37°C.

7. Measure cell proliferation by flow cytometric analysis after 3–14 days upon stimulation (*see* **Note 28**). In addition, multimer as well as extracellular and/or intracellular staining can be performed in combination with the CFSE proliferation assay, on the day of analysis. A caveat is that profound TCR downregulation may follow 1 day after stimulation with the cognate antigen, which is antigen dose dependent. Cell surface expression of TCR is restored to the pre-stimulation levels gradually over the first week of in vitro stimulation.

4. Notes

1. Always wear gloves when handling human tissues and perform PBMCs isolation in a sterile environment, under a clean laminar flow hood.
2. High-quality separation of PBMCs after density gradient centrifugation depends upon a sharp interface between lymphocytes and the separation solution after the layering step. This step can be achieved by a slow overlay of the diluted peripheral blood onto the Lymphoprep™ solution using a 10 mL pipette. The second method involves inclining the two tubes (one containing the Lymphoprep solution and the other the blood) in an upside down "V" position with the rims firmly touching each other, such that the blood can be carefully and continuously poured onto the solution along the side of the tube. Avoid any shaking of the tubes to avoid mixing of the two solutions and progressively straighten the receiving tube as it becomes filled.
3. If the blood is stored for more than 2 h, increase the centrifugation time to 30 min.
4. This centrifugation step at lower speed allows elimination of contaminating platelets.
5. All the instruments used for tissue dissociation should be sterilized either by autoclaving them or by using 70% alcohol. If the tumor tissue is hard and compact, perform a collagenase digestion. Transfer small pieces of tumor into a tube containing tumor cell culture medium with 0.1% collagenase type I (Sigma), 0.02% DNase I (Sigma) and incubate at 37°C. Mix regularly by inverting the tube. Depending on the sample, the digestion may take incubation times ranging from 2 h up to overnight. After few hours,

check the digestion extent by pipetting and counting viable cells in suspension. If enough cells have been released from the tissue sample, let large undigested pieces settle down by gravity, transfer supernatant to a new tube (keep original tube with undigested pieces), and centrifuge at 600 × *g* for 5 min to collect the dissociated cells. Recover the supernatant (collagenase solution) and reuse it to continue digestion of large pieces in the original tube until needed. Wash the single cell suspension two times with medium and distribute into plates.

6. Obtained dissociated sample should be small enough to be aspirated using a 10 mL pipette and as close as possible to a single cell suspension. To obtain, in some cases, deep tumor infiltrating lymphocytes it is advised to set separate cultures with the remaining small tumor pieces in CTL medium supplemented with rIL-2 (150 U/mL) and rIL-7 (10 ng/mL). Sometimes, activated lymphocytes come out of tumor fragments after a few days of culture.
7. If the size of the tumor sample is too small, do not pass the teased tissue fragments through the strainer. After teasing, wash the sample directly and culture the cells in tumor cell culture medium. After 1–2 days, collect only the cells in suspension (most melanoma tumor cells adhere firmly to tissue culture plates), wash them, and put them back in culture in CTL culture medium supplemented with IL-2 (150 UI/mL) and IL-7 (10 ng/mL). Add 2 mL of tumor cell culture medium onto the remaining adherent tumor cells.
8. In order to obtain short-term cultured TILNs, avoid culturing them for more than 15–20 days.
9. DMSO is a toxic compound, therefore all the manipulation of the freezing process should be performed on ice to slow down the DMSO entrance into the cells.
10. A total of 2–30 × 10^6 cells can be frozen in a final volume of 1 mL/cryotubes. Total volume should be increased up to 1.5–1.8 mL, if more than 30 × 10^6 cells are frozen per vials.
11. Additional options for successful cell cryopreservation are either the use of the Nalgene freezing containers (Nalgene) which allow repeatable −1°C/min cooling rate required for successful cell recovery or the use of a controlled rate freezing device (Thermo Scientific).
12. When using more than 10 × 10^6 cells, scale up the staining volumes accordingly.

13. Multimers as well as antibodies should be titrated prior to routine use in flow cytometry to determine the optimal dilution to be used for staining. In the case of multimers, it is useful to have large cryopreserved stocks of the appropriate T-cell clones or lines. The usual working concentrations for HLA-A2/peptide tetramers are, in our experience, in the range of 5–20 μg/mL.
14. Optimal incubation times and temperatures for efficient multimer staining have been determined by comparison of signals obtained using various protocols, e.g., incubation for variable times at 4°C, at room temperature, or at 37°C. In our current experience, the best compromise for a limited background signal concomitant with highly efficient specific multimer staining can be achieved either by incubation of the cells for 1 h at room temperature or for 45 min at 4°C (10, 14).
15. Antibodies should be kept on ice during the preparation of the mix and rapidly stored at 4°C after use, in order to avoid fluorochromes dissociation. Staining should be performed avoiding exposure to direct light.
16. Aqua dye employs an amine-reactive fluorescent dye to evaluate mammalian cell viability by flow cytometry. The dye reacts with free amines both in the cell interior and on the cell surface in case of dead cells, yielding intense fluorescent staining. In viable cells, the dye's reactivity is restricted to the cell-surface proteins, resulting in less intense fluorescence. In order to avoid quenching of staining by the BSA contained in the buffer, work at this step with plain PBS.
17. Compensation settings are performed using beads: CompBeads anti-mouse or anti-rat Ig (Becton Dickinson Biosciences) and CompBeads negative control are used according to the manufacturer's instructions. For compensation of Aqua, use some Aqua-labeled cells.
18. An important difference between the direct ex vivo multimer staining and the LDA analysis resides in the fact that the direct staining allows the quantification of a real frequency of positive cells, while the LDA can only give an estimation of the precursor frequency of specific cells. This estimation depends on the capacity of the specific cells to proliferate during the culture period; therefore an underestimation of the effective frequency in the sample is possible.
19. No IL-2 or other cytokines are added to the CTL culture medium in order to avoid unspecific growing of T cells during the overnight incubation.
20. After every washing step, residual buffer in the wells should be removed by tapping on absorbent paper.

21. Substrate solution should be at room temperature before use, since cold substrate slows down the enzymatic reaction.
22. Ideally, spot formation should be monitored by eye.
23. In order to avoid loss of multimer signal and therefore detection of false-negative too low frequencies of specific cells due to TCR downregulation after peptide stimulation, two multimer stainings should be performed: one before and one after incubation with the peptide (15, 16).
24. Degranulation can be visualized by flow cytometry during a short period of time following lytic effector activation by detection of the lysosomal CD107a protein on the effector cell surface (17). Therefore, the CD107a mobilization assay can be used as a surrogate assay of lytic activity. Because of the transient surface expression and rapid internalization of CD107a by the endocytic pathway (18), staining for CD107a is maximized by addition of anti-CD107a antibody during cell stimulation. By virtue of their parallel kinetics, CD107a and intracellular cytokines can be assessed at the same time using 4–6 h of stimulation.
25. After fixation and washing, cells can be kept in staining buffer at 4°C in the dark for 1–3 days before intracellular staining.
26. Antibodies should be previously titrated in order to define the optimal dilution to use. Intracellular staining can be performed with the mix of antibodies specific for the different cytokines at the same time. Isotype-matched control antibodies should be used to enable precise electronic gating of positive and negative events during analysis of flow cytometry data.
27. Final labeling conditions are thus 1–2 μM CFSE in PBS 1% FCS and cells at 10×10^6/mL. However, it must be kept in mind that optimal concentrations of CFSE for efficient labeling of cells may vary for different commercial batches. Thus, it is recommended to titrate new batches of CFSE as to empirically identify the optimal concentrations for labeling.
28. This protocol enables a "3.5 log shift" in fluorescent signal intensity optimally detected at 525 nm wavelength. For compensation, use some CFSE-labeled cells. In this way, only slight adjustments in the settings are needed and still allow to discriminate up to seven cell generations (19, 20).

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