TECHNICAL BULLETIN

Dendritic Cell/CD8$^+$ T Cell Co-Culture to Assess Antigen-Specific T Cell Functionality

Introduction

Dendritic cells (DCs) are powerful antigen-presenting cells (APCs) that process exogenous antigens and present them to CD4$^+$ and CD8$^+$ T cells to generate immune responses. The presentation of exogenous antigens on major histocompatibility complex (MHC) class I molecules to CD8$^+$ T cells is known as cross-presentation and is essential to induce antigen-specific responses of CD8$^+$ T cells to fight infections and disease. As such, co-culturing DCs and CD8$^+$ T cells is a useful method for studying the mechanisms of antigen presentation as well as T cell activation and proliferation. T cells expanded from the co-culture system can be further used to study the presentation of tumor antigens, T cell-mediated cytotoxicity, and memory T cell formation. Results from such studies may be used to develop T cell-mediated cancer treatments or vaccines that induce long-lasting immune memory and protection.

This technical bulletin describes, in detail, how to set up DC and CD8$^+$ T cell co-culture experiments that generate antigen-specific CD8$^+$ T cells, as well as how to assess CD8$^+$ T cell proliferation, functionality, and killing activity. The protocols within the technical bulletin have undergone in-house testing and validation, and provide a reliable, comprehensive guide to support your entire DC/T cell research workflow.

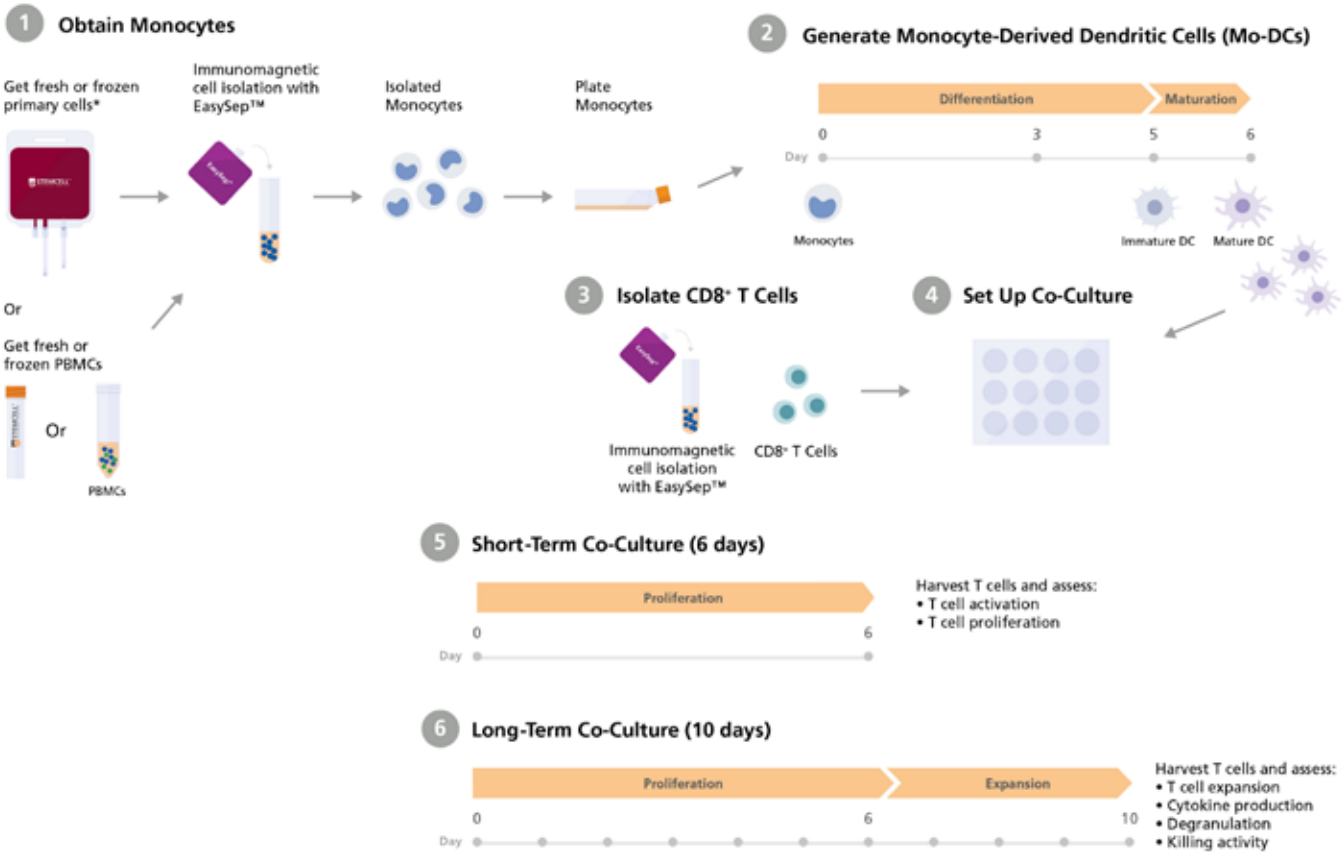

Figure 1. Experimental Workflow: DC/T Cell Co-Culture Protocol for the Activation and Expansion of Antigen-Induced CD8$^+$ T Cells

(1) Isolate monocytes from Human Peripheral Blood Leukopak, Fresh or from Human Peripheral Blood Mononuclear Cells (PBMCs), Fresh or Frozen, using EasySep™ Human Monocyte Isolation Kit. (2) Culture monocytes to generate monocyte-derived dendritic cells (Mo-DCs) using ImmunoCult™ Dendritic Cell Culture Kit and the peptide(s) of interest. (3) Isolate CD8$^+$ T cells from the same donor's blood or PBMCs using EasySep™ Human CD8$^+$ Cell Isolation Kit. (4) Co-culture DCs and CD8$^+$ T cells in ImmunoCult™-XF T Cell Expansion Medium. (5) For short-term co-culture, assess CD8$^+$ T cell proliferation and activation markers after 6 days. (6) For long-term co-culture, expand antigen-specific CD8$^+$ T cells with additional supplements. Analyze the phenotype and function of expanded CD8$^+$ T cells by assessing surface markers or cytokine production. Alternatively, enrich antigen-specific CD8$^+$ T cells with EasySep™, rest the cells for 2 days, and then assess killing activity.

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Protocols

The following protocols describe how to set up a co-culture with dendritic cells (DCs) and CD8+ T cells, as well as options for downstream functional analysis (see Figure 1). To study DC priming activity, the recommendation is to set up a short-term co-culture of 6 days. To study CD8+ T cell activation, proliferation, immune memory, and effects of re-exposure to the antigen, the recommendation is to set up a long-term, i.e. 10 - 12 days, co-culture system.

Isolate Human Monocytes and T Cells

1. Obtain peripheral blood mononuclear cells (PBMCs) from leukopaks (Catalog #70500)* or Human Whole Peripheral Blood (Catalog #70504)*. For more details, refer to our leukopak processing and PBMC isolation protocols, respectively.

2. Use one fraction of PBMCs to isolate monocytes and cryopreserve the remaining PBMCs to use at a later time for CD8+ T cell isolation and, if required, for antigen-presenting cell (APCs) enrichment. This will allow the establishment of an autologous co-culture system.

3. Isolate human $CD14^+CD16^-$ monocytes from PBMCs using EasySep™ Human Monocyte Isolation Kit (Catalog #19359). Refer to the Product Information Sheet for details.

4. When ready to start the co-culture system, thaw autologous cryopreserved PBMCs, culture overnight, and then isolate CD8+ T cells from the PBMCs using EasySep™ Human CD8+ T Cell Isolation Kit (Catalog #17953), following the instructions on the Product Information Sheet.

Note: *PBMCs can be obtained from large-volume sources, including fresh leukopaks* or human whole blood*. Additionally, you can save time by using pre-isolated fresh or frozen PBMCs* for your experiments.*

Note: *Dendritic cells and T cells used in the culture system can either be autologous (from the same donor) or allogeneic (derived from different donors). For co-cultures creating antigen-presenting conditions, it is recommended to use autologous cells. This means that the DCs are fully MHC-matched to the T cells because they come from the same donor, thereby maximizing the breadth of their antigen presentation to potentially reactive T cells.*

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Generate Mo-DCs and Pulse with Peptide Pool

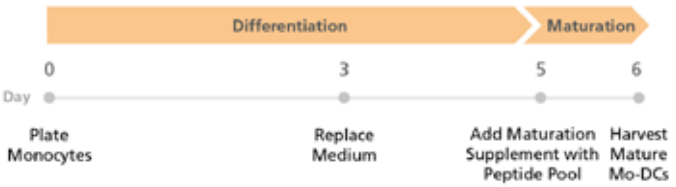

Figure 2. Protocol Diagram: Generation and Differentiation of Mo-DCs

Generate mature Mo-DCs from isolated monocytes. For optimal cell yield in this application, we recommend using EasySep™ Human Monocyte Isolation Kit. Culture the cells in ImmunoCult™-ACF Dendritic Cell Medium with added ImmunoCult™-ACF Dendritic Cell Differentiation Supplement for three days at 37°C. On Day 3, remove the medium and add fresh medium with added differentiation supplement, and incubate at 37°C for 2 more days. At Day 5, without changing the medium, add ImmunoCult™ Dendritic Cell Maturation Supplement and desired peptide pool(s) to the culture. On Day 6, harvest fully mature DCs for downstream applications.

The steps below describe how to generate mature Mo-DCs using ImmunoCult™ Dendritic Cell Culture Kit (Catalog #10985) following the Product Information Sheet, with modifications to pulse Mo-DCs with a peptide pool on Day 5 of culture (see Figure 2). Refer to the ImmunoCult™ Dendritic Cell Culture Kit Product Information Sheet for details omitted in this summary. For a list of suggested materials and products used in this protocol, see Table 1.

1. On Day 0, seed monocytes at 1 x 10^6 cells/mL in appropriate tissue cultureware.

2. On Day 3, replace the medium with fresh ImmunoCult™-ACF Dendritic Cell Medium (Catalog #10987).

3. On Day 5, add ImmunoCult™ Dendritic Cell Maturation Supplement (Catalog #10989) directly to the cultures at a 1 in 100 dilution. Add specific desired peptides (e.g. CMV (pp65) Peptide Pool (Catalog #100-0668), Influenza (HLA Class I Control) Peptide Pool (Catalog #100-0672), MART 1(26-35) (peptides&elephants GmbH, Catalog #EP09832_1), or CMV Sub Peptide Pool (peptides&elephants GmbH, Catalog #LB01713) at a concentration of 1 µg/mL per peptide).

4. On Day 6, harvest mature Mo-DCs by gently pipetting up and down to ensure all cells are in suspension, then transfer to an appropriate tube.

5. Wash the cells by adding an equivalent volume of ImmunoCult™-XF T Cell Expansion Medium (Catalog #10981) and gently pipetting up and down to make sure the cells are resuspended in the medium. Centrifuge at 300 x *g* for 10 minutes at room temperature. Remove and discard the supernatant.

6. Resuspend the cells in ImmunoCult™-XF T Cell Expansion Medium. Repeat step 5. After centrifugation, remove and discard the supernatant and resuspend the cells with ImmunoCult™-XF T Cell Expansion Medium at a concentration of 5 x 10^5 cells/mL.

Note: Selecting Peptide Pools

A peptide pool is a mixture of short peptide fragments of a specific protein or antigen that can stimulate T cells with a specific T cell receptor (TCR) specificity. Peptide pools allow researchers to screen and identify T cells that can recognize and respond to specific antigens in a complex mixture, such as those found in pathogens or cancer cells. The peptide chosen will depend on the research interest and question being addressed. The peptide pools used here are presented by human leukocyte antigen (HLA) class I molecules on DCs and interact with TCRs on CD8⁺ T cells to induce activation and proliferation. The CMV (pp65) Peptide Pool, Influenza (HLA Class I Control) Peptide Pool, and MART 1 (26-35) peptides are often good positive controls, as most individuals will have circulating CD8⁺ T cells that recognize these antigens.

Mo-DC and T Cell Co-Cultures

The length for co-culture of Mo-DCs and CD8⁺ T cells may vary depending on the specific scientific questions being addressed. Short-term co-culture of 6 days (see Figure 3) is typically performed to study the activation of dendritic cells and their priming of CD8⁺ T cells. However, longer-term co-culture of 10 - 12 days (see Figure 4) will generate a pool of expanded antigen-specific CD8⁺ T cells that can be used in downstream functional assays, such as CD8⁺ T cell-mediated cytotoxicity assays. Optimal cell yields depend on maintenance of cell health, which in turn will depend on following the recommended schedule of feeding and medium changes.

Option 1: Short-Term Co-Culture

This protocol is designed to promote the activation and proliferation of antigen-specific CD8⁺ T cells for 6 days within a co-culture system with autologous antigen presenting Mo-DCs (see Figure 3). Optimal cell yields depend on maintenance of cell health, which largely depends on following the recommended schedule of feeding and medium changes. For a list of suggested materials and products used in this protocol, see Table 1.

Figure 3. Protocol Diagram: Peptide-Pulsed Mo-DCs and CD8⁺ T Cell Co-Culture for 6 Days

Isolate CD8⁺ T cells from donor-matched PBMCs. For optimal cell yield in this application, we recommend using EasySep™ Human CD8⁺ T Cell Isolation Kit. Label the isolated CD8⁺ T cells with a cell proliferation tracking dye. Set up co-culture by seeding peptide-pulsed dendritic cell suspension with the CD8⁺ T cell suspension at a 1:4 ratio. Harvest the co-cultures after 5 to 6 days for downstream analysis.

1. Thaw autologous and cryopreserved PBMCs and culture overnight (to let the freshly thawed frozen cells rest) in ImmunoCult™-XF T Cell Expansion Medium, supplemented with 5 ng/mL Human Recombinant IL-7 (Catalog #78053).
2. On Day 0, isolate CD8⁺ T cells from PBMCs in step 1 using EasySep™ Human CD8⁺ T Cell Isolation Kit and label the cells with 5 µM of cell proliferation tracking dye CellTrace™ Violet Proliferation Dye (Thermo Fisher Scientific, Catalog #C34557), as per manufacturer's instructions. Add quench solution with 10% FBS and then wash cells by adding an equivalent volume of ImmunoCult™-XF T cell Expansion Medium and centrifuging at 300 x *g* for 10 minutes at room temperature. Repeat the wash. Resuspend labeled CD8⁺ T cells at a concentration of 2 x 10^6 cells/mL in ImmunoCult™-XF T Cell Expansion Medium.
3. Set up co-cultures by seeding 0.5 mL of the peptide-pulsed DC suspension (5 x 10^5 cells/mL) with 0.5 mL of the CD8⁺ T cell suspension (2 x 10^6 cells/mL) into each well of a 24-well tissue culture plate to obtain a 1:4 ratio of Mo-DCs to CD8⁺ T cells.
4. On Day 5 or 6, harvest the co-culture by gently pipetting up and down to ensure all cells are in suspension, then transfer to an appropriate centrifuge tube.
5. Wash cells by adding an equivalent volume of ImmunoCult™-XF T Cell Expansion Medium and centrifuge at 300 x *g* for 10 minutes at room temperature. Remove and discard the supernatant.
6. Repeat step 5 by resuspending cells in medium and centrifuge at room temperature. Remove the supernatant and resuspend the cell pellet in ImmunoCult™-XF T Cell Expansion Medium.
7. Determine the cell concentration and dilute by adding medium to obtain the desired final cell concentration for downstream applications and analysis.
8. Cells are ready for downstream analyses, including proliferation and phenotypic analyses, and quantification of antigen-specific T cells using tetramer staining.

For phenotypic analysis, the recommendation is to stain the cells with corresponding HLA class I tetramers, and fluorescent antibodies specific for human CD8 (clone RPA-T8), CD3 (clone SK7), CD25 (clone BC96), and CD279 (PD1; clone EH12.2H7), and viability dye DRAQ7™ (BioLegend, Catalog #424001). Stain the cells following the recommended protocol as per manufacturer's instructions, and analyze using flow cytometry (see Figure 5). To quantify the number of antigen-specific CD8⁺ T cells, multiply the frequency of viable tetramer CD8⁺ T cells by the cell concentration determined in step 7.

Note: *Tetramer staining is a powerful technique for analyzing antigen-specific T cells due to its high specificity and sensitivity in identifying and quantifying these cells and allowing researchers to study the immune response to specific antigens. The tetramers are bioengineered with four MHC-peptide molecules and provide stable binding to T cells that recognize a specific antigen. By labeling tetramers with fluorescent dyes, e.g. phycoerythrin (PE), researchers can use flow cytometry to identify and analyze antigen-specific T cell functionality in a sample, which allows accurate and precise quantification of antigen-specific T cells. The tetramer is selected depending on the HLA alleles of the donor and the specific peptide used to activate the cells, e.g. if the cells are stimulated with CMV (pp65) Peptide Pool, then stain with iTAg Tetramer/PE – HLA-A*02:01 CMV pp65 (NLVPMVATV) (MBL International Corporation, Catalog #TB-0010-1; see experiment data shown in Figure 6); if the cells are stimulated with MART 1(26-35) peptides, then stain with iTAg Tetramer/PE – HLA-A*02:01 Mart-1 (ELAGIGILTV) (MBL International Corporation, Catalog #TB-0009-1).*

Option 2: Long-Term Co-Culture

This protocol is designed to promote the proliferation/activation of antigen-specific CD8$^+$ T cells for 10 - 14 days within a co-culture system with autologous antigen-presenting Mo-DCs (Figure 4). Optimal cell yields depend on maintenance of cell health, which largely depends on following the recommended schedule of feeding and medium changes. For a list of suggested materials and products used in this protocol, see Table 1.

1. Thaw autologous and cryopreserved PBMCs and culture overnight in ImmunoCult™-XF T Cell Expansion Medium, supplemented with 5 ng/mL Human Recombinant IL-7.
2. On Day 0, isolate CD8$^+$ T cells from PBMCs in step 1 using EasySep™ Human CD8$^+$ T Cell Isolation Kit. Resuspend isolated cells at a concentration of 2 x 10^6 cells/mL in ImmunoCult™-XF T Cell Expansion Medium supplemented with 60 ng/mL of Human Recombinant IL-21 (Catalog #78193).
3. Set up co-cultures by seeding 0.5 mL of the peptide-pulsed dendritic cell suspension (5 x 10^5 cells/mL) with 0.5 mL of the CD8$^+$ T cell suspension (2 x 10^6 cells/mL) into each well of a 24-well tissue culture plate to obtain a 1:4 ratio of Mo-DCs to CD8$^+$ T cells.
4. On Day 3, add another 1 mL of fresh ImmunoCult™-XF T Cell Expansion Medium with Human Recombinant IL-7 and Human Recombinant IL-15 (Catalog #78031) to each well, to bring the final IL-7 and IL-15 concentrations to 5 ng/mL for each cytokine.
5. On Day 5, transfer the contents of each co-culture well from the 24-well tissue culture plate to a 6-well tissue culture plate. Add an additional 2 mL of fresh medium, supplemented with 5 ng/mL of IL-7 and 5 ng/mL of IL-15, to top up the existing 2 mL volume of cell culture.
6. On Day 7, split each co-culture well into 2 wells by removing 2 mL of cell suspension from the 6-well tissue culture plates and seed the cells into a fresh well of a 6-well tissue culture plate. Add an additional 2 mL of fresh medium, supplemented with 10 ng/mL of IL-7 and 10 ng/mL of IL-15, to each well and culture the cells for another 3 days.
7. On Day 10, harvest the cells by gently pipetting up and down to ensure all cells are in suspension, and transfer the cells to an appropriate tube for downstream analysis, functional assays, or to be seeded onto tissue culture plates for further expansion.

Note: *By Day 10 of the co-culture, nearly all of the cells constitute expanded T cells (see Figure 6) with very few Mo-DCs still present in the culture.*

See below for protocols and data examples to:

- Quantify antigen-specific T cells with tetramer staining (see Figure 6)
- Assess degranulation (CD107) and production of interferon-γ (IFN-γ) and CD137 expression (Figure 7)
- Isolate antigen-specific T cells (Figure 8A)
- Assess T cell killing activity (Figure 8B)

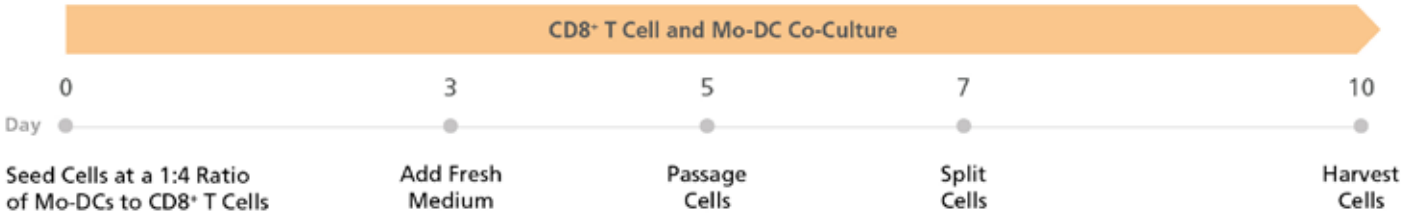

Figure 4. Protocol Diagram: Peptide-Pulsed Mo-DCs and CD8$^+$ T Cell Co-Culture for 10 - 12 Days

Isolate CD8$^+$ T cells from donor-matched PBMCs. For optimal cell yield in this application, we recommend using EasySep™ Human CD8$^+$ T Cell Isolation Kit. Set up co-culture by seeding peptide-pulsed dendritic cell suspension with the CD8$^+$ T cell suspension at a 1:4 ratio. Add supplementary cytokines and passage every 2 - 3 days. Harvest cells at Day 10 for downstream analysis or applications that include phenotyping by flow cytometry, cytokine quantification, degranulation assessment, and measurement of killing activity of effector CD8$^+$ T cells using a killing assay.

Assess T Cell Functions (Degranulation, IFN-γ Production, and 4-1BB (CD137) Expression)

T cell activation can be measured by detecting degranulation, interferon-γ (IFN-γ) production, and 4-1BB (CD137) expression, which provide valuable insights into the immune response and can help researchers understand the efficacy of potential immunotherapies. Measuring CD107 surface expression is a common method to quantify degranulation. When activated by a specific antigen, $CD8^+$ T cells begin to degranulate, transport CD107 to the cell surface, and deliver cytotoxic proteins to target cells (Betts et al., 2003). The exposure of antigens to $CD8^+$ T cells also induces the production of IFN-γ that can be measured by intracellular cytokine staining. The surface glycoprotein CD137 is also induced by antigen exposure and is a potent co-stimulatory signal that promotes T cell proliferation and formation of memory cells, enhances survival, and increases the production of IFN-γ (Fröhlich et al., 2020). For a list of suggested materials and products used in this protocol, see Table 1.

Part 1: Set Up Antigen-Presenting Cells for $CD8^+$ T Cell Recall Immune Responses

The DC/$CD8^+$ T cell co-culture is required to expand antigen-specific $CD8^+$ T cells. After the first 3 or 4 days of Mo-DC and $CD8^+$ T cell co-culture, most of the T cell expansion is driven by cytokines (IL-15 and IL-7). Therefore, T cells need to be reactivated with an autologous source of antigen-presenting cells (APCs) and fresh peptides to create a memory-like recall response to antigen, and to induce detectable levels of IFN-γ production and other activation markers. The steps below describe how to set up APCs to add to the expanded $CD8^+$ T cells to measure cytokine production.

1. On Day 10, thaw autologous PBMCs to use as a source of APCs for $CD8^+$ T cell reactivation.

2. Enrich APCs from autologous PBMCs by depleting $CD3^+$ and $CD2^+$ cells to remove T cells and NK cells. We recommend using EasySep™ Human CD3 Positive Selection Kit II (Catalog #17851) and EasySep™ Human CD2 Positive Selection Kit II (Catalog #17833) with the purple EasySep™ Magnet (Catalog #18000) as described below:

    a. Dilute PBMCs to 5 x 10^7 cells/mL and transfer to a 5 mL polystyrene round-bottom tube.

    b. Add EasySep™ Human CD3 Positive Selection Cocktail II and EasySep™ Human CD2 Positive Selection Cocktail at 150 µL/mL each, and incubate for 5 minutes.

    c. Add 150 µL/mL EasySep™ Dextran RapidSpheres™ (Component #50100) to the sample, mix, and incubate at room temperature for 5 minutes.

    d. Add EasySep™ Buffer (Catalog #20144) to top up to 2.5 mL, place the tube into the purple EasySep™ Magnet, and incubate at room temperature for 5 minutes.

    e. Pick up the magnet and, in one continuous motion, invert the magnet and tube, pouring off the supernatant to a new 5 mL polystyrene round-bottom tube. This new tube contains the enriched cells.

    f. Add 75 µL/mL EasySep™ Dextran RapidSpheres™ (Component #50100) to the enriched fraction collected from the pour-off. Mix and incubate at room temperature for 1 minute.

    g. Place the tube into the purple EasySep™ Magnet and incubate for 5 minutes.

    h. Pick up the magnet, and in one continuous motion, invert the magnet and tube, pouring off the supernatant to a new 5 mL polystyrene round-bottom tube.

    i. Once again, place the tube into the purple EasySep™ Magnet and incubate for another 5 minutes.

    j. Pick up the magnet, and in one continuous motion, invert the magnet and tube, pouring off the supernatant to a new 5 mL polystyrene round-bottom tube. The pour-off fraction will contain the enriched APCs.

3. Centrifuge the enriched APCs at 300 x *g* for 10 minutes. Resuspend in 2.5 mL phosphate-buffered saline (PBS) and centrifuge at 300 x *g* for 10 minutes.

4. Remove the supernatant and resuspend the cells in 1 mL PBS supplemented with cell proliferation tracking dye (5 µM of CellTrace™ Violet Proliferation Dye; Thermo Fisher Scientific, Catalog #34557), as per manufacturer's instructions. This step will help distinguish the APCs from the responder T cells when analyzing the cells by flow cytometry. Add a quench solution with 10% FBS and then wash cells by adding an equivalent volume of ImmunoCult™-XF T cell Expansion Medium and centrifuging at 300 x *g* for 10 minutes.

5. Resuspend fluorescently labeled APCs in 1 mL ImmunoCult™-XF T Cell Expansion Medium.

6. Pulse the APCs in the 5 mL polystyrene round-bottom tube used for APC enrichment with the same peptide pool that was previously used to activate Mo-DCs in the Mo-DCs maturation step, at 1 µg/mL per peptide(s). Leave a fraction of the APCs untreated as a negative control. Incubate the APCs with peptide at 37°C for 2 -16 hours. Add 2 mL of ImmunoCult™-XF T Cell Expansion Medium to the APCs and centrifuge APCs at 300 x *g* for 10 minutes to remove excess peptides.

7. Decant the supernatant, resuspend APCs in 3 mL ImmunoCult™-XF T Cell Expansion Medium, and centrifuge at 300 x *g* for 10 minutes. Decant the supernatant again, resuspend the APCs in ImmunoCult™-XF T Cell Expansion Medium, and dilute the cells to 1 x 10^5 cells/mL. The APCs are now ready to be used to assess $CD8^+$ T cell degranulation and IFN-γ production, described below.

Part 2: Assess Degranulation and IFN-γ Production (Following 10 Days of Co-Culture and CD8+ T Cell Expansion)

1. Dilute expanded CD8+ T cells (from "Long-Term Co-Culture" protocol) to 1 x 10^5 cells/mL. Add 100 µL of expanded CD8+ T cells to 100 µL of peptide-pulsed and fluorescently labeled APCs (at 1 x 10^5 cells/mL; from "Set-Up of Antigen-Presenting Cells for CD8+ T Cell Recall Immune Responses") in a round-bottom 96-well tissue culture plate and incubate at 37°C. The final concentration for each cell type should be 1 x 10^4 cells/well.

2. Add PE anti-human CD107a (LAMP-1) antibody (clone H4A3; Biolgend, Catalog #328607) to APC/CD8+ T cell co-culture at 0.2 µL/well. Incubate for 1 hour.

3. Add BD GolgiStop™ protein transport inhibitor (Monensin) (BD Biosciences, Catalog #554724) to the co-culture, as per the manufacturer's instructions.

4. Incubate for 5 hours.

5. Harvest the cells from the plates to analyze by flow cytometry.

Note: *The type of fluorochrome-conjugated antibodies should be chosen based on your experimental design, to ensure they work with your panel. We recommend avoiding fluorochromes that have been used already for the panel e.g. CellTrace™ Violet Proliferation Dye.*

For flow cytometry analysis, we recommend performing cell-surface antigen staining followed by intracellular staining to measure IFN-γ.

For surface antigen staining, we recommend using:

- Anti-Human CD32 Antibody, Clone FLI8.26 (Catalog #60135) or clone IV.3 (Catalog #60012) as a blocking antibody to reduce non-specific binding.
- Fluorochrome-conjugated anti-human CD8 antibody, clone RPA-T8
- Fluorochrome-conjugated anti human CD3 antibody, clone SK7

6. Add surface-staining antibodies to the samples as per manufacturer's instructions, and incubate for 15 minutes at room temperature.

7. Wash the cells twice with PBS (or antibody manufacturer-provided staining buffer) by gently pipetting up and down and then centrifuging twice at 500 x *g* for 3 minutes.

8. Add 1 µL GloCell™ Fixable Viability Dye Red 780 (Catalog #75007) per 1 mL of cell suspension, and follow manufacturer's recommended protocol.

9. Wash the cells twice with PBS (or antibody manufacturer-provided staining buffer) by gently pipetting up and down and then centrifuging twice at 500 x *g* for 3 minutes.

10. Fix and then permeabilize the cells with BD Cytofix/Cytoperm™ Fixation/Permeabilization Kit (BD Biosciences, Catalog #554714) as per manufacturer's instructions.

11. Add APC-conjugated anti-human IFN-γ (clone 4S.B3; BioLegend, Catalog #502511) and incubate for 20 minutes at room temperature.

12. Wash the cells twice with PBS (or antibody manufacturer-provided staining buffer) by gently pipetting up and down and then centrifuge twice at 500 x *g* for 3 minutes. Analyze cells by flow cytometry (see Figure 7A).

Part 3: Assess CD8+ T Cell Activation by Measuring 4-1BB (CD137) Expression

1. Add expanded CD8+ T cells (from the "Long-Term Co-Culture" protocol) and APCs with tracking dye (from the "Set-Up of Antigen-Presenting Cells for CD8+ T Cell Re-Exposure" protocol) at a 1:1 ratio in a round-bottom 96-well tissue culture plate and incubate at 37°C. The final concentration for both cell types should be 1 x 10^4 cells/well. Incubate at 37°C for 16 to 20 hours.

2. For flow cytometry analysis, we recommend staining the cells with fluorochrome-conjugated anti-human CD8 (clone RPA-T8), CD3 (clone SK7) and 4-1BB (clone 4B4-1), and viability dye DRAQ7™, all supplied by Biolegend, and as per manufacturer's instructions.

3. Wash the cells twice before performing flow cytometry analysis (Figure 7B).

Killing Assay with Enriched Antigen-Specific T Cells

Antigen-specific CD8+ T cells from the DC/CD8+ T cell co-culture can be isolated and then assessed for their activity by conducting CD8+ T cell killing activity assays. The following protocols first provide guidance for obtaining the antigen-specific CD8+ T cells from the long-term DC/CD8+ T cell co-culture. Prior to enrichment, the DC/CD8+ T cell co-culture is stimulated with MART 1 (26 - 35); the antigen-specific CD8+ T cells expanded from the co-culture are then detected by a PE-conjugated tetramer (iTAg Tetramer/PE – HLA-A*02:01 Mart-1 (ELAGIGILTV)) and isolated using the EasySep™ Release Human PE Positive Isolation Kit (Catalog #17654) and the purple EasySep™ Magnet (see Figure 8A). We then describe the use of enriched antigen-specific CD8+ T cells in a killing assay to measure cytotoxicity against a HLA-A*02:01 tumor cell line, U266 (see Figure 8B). For a list of suggested materials and products used in this protocol, see Table 1.

Part 1: Enrichment of Antigen-Specific CD8+ T Cells with EasySep™

1. Harvest the expanded CD8+ T cells at Day 10 from the "Long-Term Co-Culture" protocol.
2. Prepare cell suspension at 2 x 10^7 cells/mL in EasySep™ Buffer (Catalog #20144).
3. Add EasySep™ Anti-Human CD32 (Fc gamma RII) Blocker (Component of EasySep™ Release Human PE Positive Selection Kit), as a blocking antibody, to the cells at 200 µL/mL.
4. Add PE-conjugated tetramer such as MART 1(26-35)-specific PE-conjugated tetramer (iTAg Tetramer/PE – HLA-A*02:01 Mart-1 (ELAGIGILTV) at a concentration of 10 µL/mL and incubate for 30 minutes at room temperature, protected from light.

Note: *The tetramer is selected depending on the HLA alleles of the donor and the specific peptide used to activate and expand the cells, e.g. if the cells are stimulated with CMV (pp65) Peptide Pool, then use iTAg Tetramer/PE – HLA-A*02:01 CMV pp65 (NLVPMVATV) (see experiment data shown in Figure 6); if the cells are stimulated with MART 1(26-35) peptides, then use iTAg Tetramer/PE – HLA-A*02:01 Mart-1 (ELAGIGILTV).*

5. Add three volumes of EasySep™ Buffer, and centrifuge the cells at 300 x *g* for 10 minutes (low brake).
6. Remove the supernatant and resuspend the cells in 0.5 mL EasySep™ Buffer and transfer to a 5 mL polystyrene round-bottom tube.
7. Add EasySep™ Release Human PE Positive Selection cocktail to sample at 100 µL/mL and incubate for 10 minutes at room temperature.
8. Add EasySep™ Releasable Rapidspheres™ to sample at 75 µL/mL and incubate for 5 minutes at room temperature.
9. Add EasySep™ Buffer to top up the sample to 2.5 mL. Mix by gently pipetting up and down 2 - 3 times.
10. For the magnetic separations, place the tube without a lid into the purple EasySep™ Magnet and incubate for 10 minutes (first magnet incubation step).
11. Pick up the magnet and, in one continuous motion, invert the magnet and tube, pouring off the supernatant. Remove the tube from the magnet; this tube contains the desired positively selected cells.
12. Resuspend cells in 2.5 mL of EasySep™ Buffer.
13. Place the tube without a lid into the purple EasySep™ Magnet and incubate for 5 minutes (second magnet incubation step).
14. Pick up the magnet and, in one continuous motion, invert the magnet and tube, pouring off the supernatant. Remove the tube from the magnet; this tube contains the desired positively selected cells.
15. Resuspend cells in 2.5 mL of EasySep™ Buffer.
16. Repeat steps 13 and 14 for a third separation step. Optional: an additional separation step can be added to increase purity.
17. Resuspend positively selected cells in 2.5 mL of EasySep™ Release Buffer for 3 minutes.
18. Place the tube into the magnet and incubate for 5 minutes. This step will remove the magnetic particles.
19. Pick up the magnet and, in one continuous motion, invert the magnet and tube, pouring off the supernatant with enriched cells into a new tube.
20. Centrifuge enriched cells at 300 x *g* for 10 minutes and resuspend the cell pellets at 5 x 10^5 cells/mL in ImmunoCult™-XF T Cell Expansion Medium, supplemented with 5 ng/mL IL-7 and IL-15. Culture in U-bottom 96-well tissue culture plates at 37°C for 2 days.
21. After 2 days of rest, assess the killing activity of enriched antigen-specific CD8+ T cells by performing a killing assay as described below (see Figure 8B for results).

Part 2: Assess T Cell Killing Activity

1. Use the expanded antigen-specific $CD8^+$ T cells enriched from culture using a PE-conjugated tetramer and the EasySep™ Release Human PE Positive Selection Kit.

2. Harvest the tetramer-enriched $CD8^+$ T cells and transfer them to a 5 mL polystyrene round-bottom tube and centrifuge at 300 x *g* for 10 minutes. Decant the supernatant and resuspend the cells in fresh ImmunoCult™-XF T Cell Expansion Medium at a final concentration of 3 x 10^5 cells/mL. Prepare antigen-specific $CD8^+$ T cell suspensions at dilutions of 3 x 10^5 cells/mL, 1 x 10^5 cells/mL, and 0.3 x 10^5 cells/mL.

3. Prepare target cells: e.g. an HLA-A*02:01 tumor cell line, U266.

    a. Briefly, label the U266 cells with 5 µM eBioscience™ Cell Proliferation Dye eFluor™ 670 (Thermo Fisher Scientific, Catalog #65-0840-85), as per product instructions. Add a quench solution with 10% FBS and then wash cells by adding an equivalent volume of ImmunoCult™-XF T Cell Expansion Medium and centrifuge at 300 x *g* for 10 minutes.

    b. Resuspend fluorescently labeled target cells in 1 mL ImmunoCult™-XF T Cell Expansion Medium.

    c. Add the same desired peptide used for Mo-DC activation (see section "Generation and Differentiation of Mo-DCs," step 3 at Day 5) at 1 µg/mL per peptide for at least 2 hours at 37°C. Set up a negative control group of U266 cells with no peptide treatment.

    d. Wash excess peptides by adding 2 mL of ImmunoCult™-XF T cell Expansion Medium. Centrifuge the cells at 300 x *g* for 10 minutes. Resuspend in 1 mL ImmunoCult™-XF T Cell Expansion Medium.

    e. Dilute peptide-pulsed or negative control U266 cell line targets with ImmunoCult™-XF T Cell Expansion Medium to 1 x 10^5 cells/mL.

4. Add target cells to U-bottom 96-well tissue culture plates, at 1 x 10^4 cells per well, and add effector cells ($CD8^+$ T cells) at specific concentrations to establish a concentration curve of E/T ratios of 3:1, 1:1, and 0.3:1.

5. Targets and effector $CD8^+$ T cells are incubated for 4 - 6 hours to induce killing.

6. Centrifuge the 96-well plates at 500 x *g* for 3 minutes. Decant 150 µL of supernatant from each well. Resuspend the cells in 150 µL ice-cold PBS. Centrifuge the 96-well plates at 500 x *g* for 3 minutes. Decant 150 µL of supernatant from each well. Once again, resuspend the cells by adding 150 µL of fresh PBS to each well and repeat the centrifuge step, at 500 x *g* for 3 minutes. Decant 150 µL of supernatant from each well.

7. Prepare an Annexin V staining solution containing 80 µL/well of Annexin V Binding Buffer (Catalog #100-0334), supplemented with 0.2 µL/well PE-conjugated Annexin V (Catalog #100-0331) and 1/400 7-AAD dye (Catalog #75001). Resuspend the PBS-washed cells in 80 µL/well of Annexin V staining solution. Incubate the cells with Annexin V solution for 20 minutes at room temperature, in the dark.

8. Analyze cells using flow cytometry. Assess target-cell killing by gating on the fluorescently labeled (eFluor™ 670) target cells, and assess the frequency of apoptotic U266 target cells (Annexin V^+/7-AAD^-) or dead target cells (total Annexin V^+).

Case Study: Antigen-Specific T Cells That Show Cytotoxic Activity

In this case study, we present results showing that Mo-DCs generated with ImmunoCult™ Dendritic Cell Culture Kit and pulsed with desired peptides, when co-cultured with $CD8^+$ T cells, promote proliferation and expansion of antigen-specific $CD8^+$ T cells. Mo-DCs promoted proliferation of peptide-specific $CD8^+$ T cells in a short-term, 6-day co-culture system (Figure 5) and 10-day co-culture system (Figure 6). Expanded $CD8^+$ T cells showed antigen-specific responses when assessed for CD107 expression to measure degranulation (Figure 7A, 7C), IFN-γ production (Figure 7A, 7C), expression of activation marker 4-1BB (CD137) (Figure 7B, 7C), and cytotoxic killing activity (Figure 8) using flow cytometry.

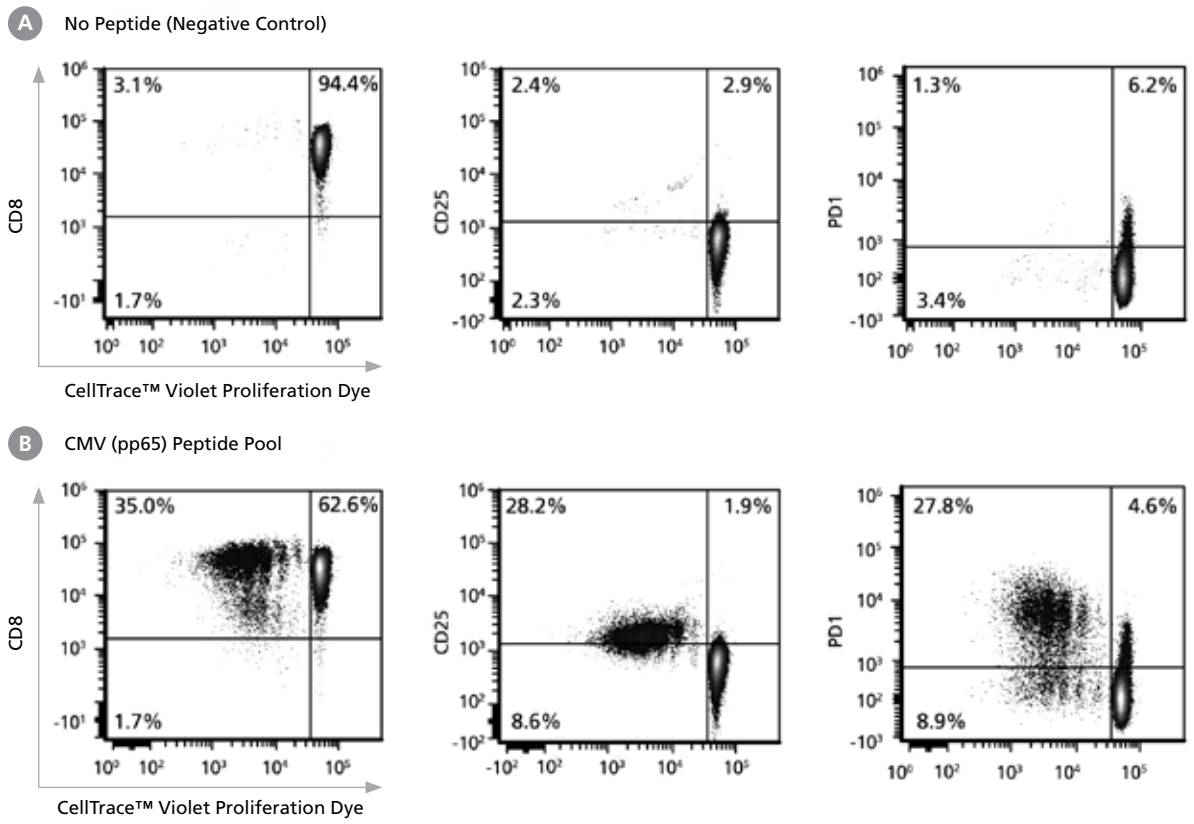

Figure 5. Mo-DCs Promote Proliferation of CMV Peptide-Specific CD8+ T Cells After 6 Days of Co-Culture

Mo-DCs were generated with ImmunoCult™ Dendritic Cell Culture Kit and cultured (A) without peptide or (B) with CMV (pp65) Peptide Pool. Enriched, autologous CD8+ T cells were isolated with EasySep™ Human CD8+ T Cell Isolation Kit, labeled with a cell proliferation tracking dye (450nm), and then co-cultured with peptide-loaded mature Mo-DCs at a 4:1 (CD8+ T cell/dendritic cell) ratio. Cells were harvested after 5 - 6 days of DC/CD8+ T cell co-culture and stained with anti-human CD8 and CD3 antibodies, DRAQ7™ viability dye, and antibodies for activation marker(s), human CD25 and CD279 (PD1). Cells were analyzed by flow cytometry, and the proliferation and activation of CMV peptide-stimulated CD8+ T cells was gated on viable T cells.

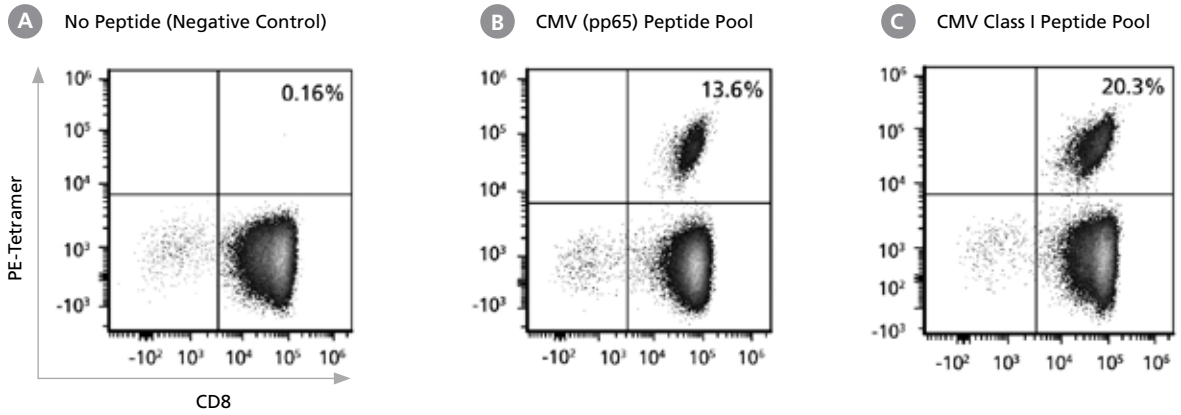

Figure 6. Mo-DCs Promote Expansion of CMV Peptide-Specific CD8+ T Cells After 10 Days of Co-Culture

Mo-DCs pulsed with CMV Class I peptide pool and co-cultured with CD8+ T cells promoted expansion of CMV peptide-specific CD8+ T cells. Mo-DCs were generated with ImmunoCult™ Dendritic Cell Culture Kit and loaded with CMV (pp65) peptide pool or a CMV Class I peptide pool. Enriched, autologous CD8+ T cells were isolated with EasySep™ Human CD8+ T Cell Isolation Kit and were added to peptide-loaded mature dendritic cells at a 4:1 (CD8+ T cell/dendritic cell) ratio. The co-culture was supplemented with recombinant human IL-21 for the first three days, then further supplemented with recombinant human IL-7 and IL-15 for the next seven days. Cell cultures were split every 2 - 3 days to promote CD8+ T cell expansion and harvested at Day 10. Cells were stained with fluorochrome-conjugated anti-human CD8 and anti-human CD3 antibodies and the HLA-A*02:01 CMV pp65 (NLVPMVATV) class I tetramer, DRAQ7™ viability dye and analyzed by flow cytometry. All samples were gated on viable T cells. A) Control co-cultures without peptide, B) Mo-DCs pulsed with CMV pp65 peptide pool, and C) Mo-DCs pulsed with CMV Class I peptide pool co-cultures generated 0.16%, 13.6%, and 20.3% viable Tetramer+CD8+ T cells, respectively, and a > 200-fold expansion of CMV-specific CD8+ T cells (data not shown). Data shown here are representative of one CD8+ T cell donor with each viral peptide pool in the DC/T cell co-culture.

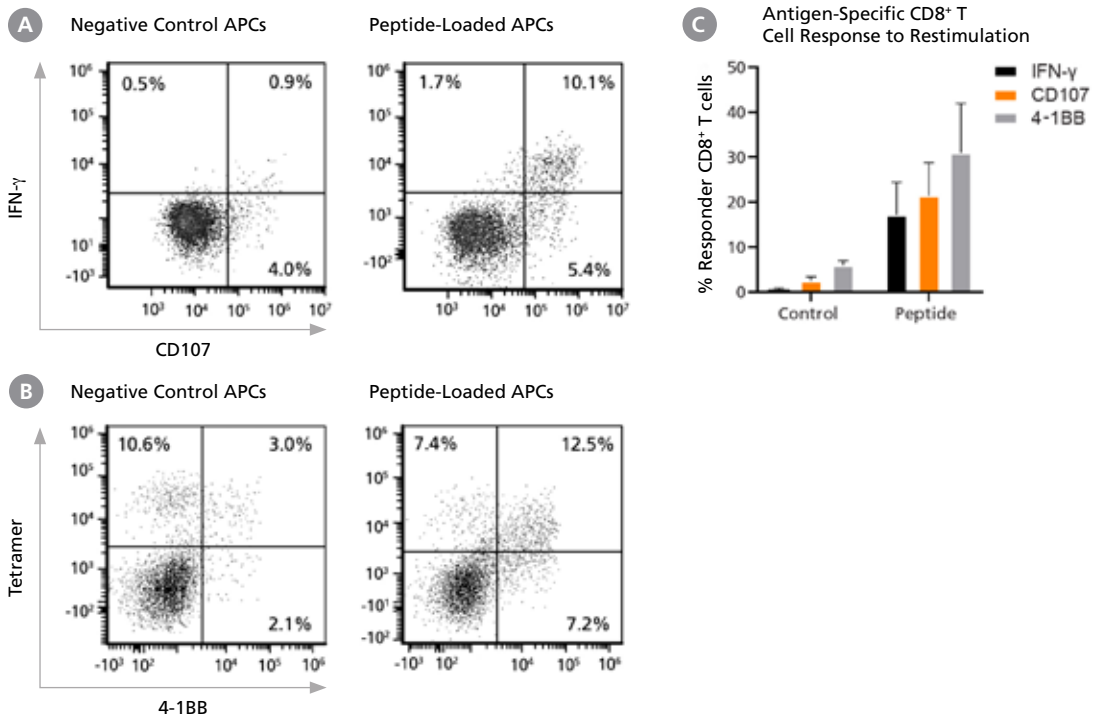

Figure 7. CD8⁺ T Cells Expanded in Co-Culture Generate Antigen-Specific Responses

Autologous antigen-presenting cells (APCs) were prepared from cryopreserved PBMCs by depleting CD2⁺ and CD3⁺ cells with EasySep™ Cell Isolation Kits following a modified protocol for depletion. Enriched APCs were then labeled with a fluorescent dye and loaded with a CMV viral peptide pool or were left untreated (negative control). A) Degranulation and IFN-γ responses by expanded CD8⁺ T cells were measured. Expanded CD8⁺ T cells were incubated with APCs at a 1:1 ratio for 4 - 6 hours. Degranulation (CD107) was assessed by adding PE-conjugated anti-human CD107 at T=0 of co-culture. Monensin was added at T=1hr, cultures were harvested at T= 6hrs, and cells were stained with surface markers anti-human CD3 and anti-human CD8, and fixable viability dye (780nm). An anti-human IFN-γ antibody was added following fixation and permeabilization steps. B) The expression of 4-1BB (CD137) by expanded CD8⁺ T cells was measured. Expanded CD8⁺ T cells were cultured with APCs at a ratio of 1:1 for 18 hours. Cells were stained with PE-conjugated Tetramer HLA-A*02:01 CMV pp65 (NLVPMVATV) class I tetramer, anti-human CD8, and anti-human CD137, followed by the viability stain Draq7™, and analyzed by flow cytometry. C) Summary of IFN-γ, CD107, and 4-1BB (CD137) expression by expanded CD8⁺ T cells. The graph shows a summary of degranulation (CD107), IFN-γ production, and 4-1BB (CD137) activation by expanded CD8⁺ T cells, as measured by flow cytometry. All functional responses were evaluated following acquisition by flow cytometry and gating on viable, CD8⁺CD3⁺ responder cells (n = 3 independent cultures from 2 donors; bars represent mean ± SEM).

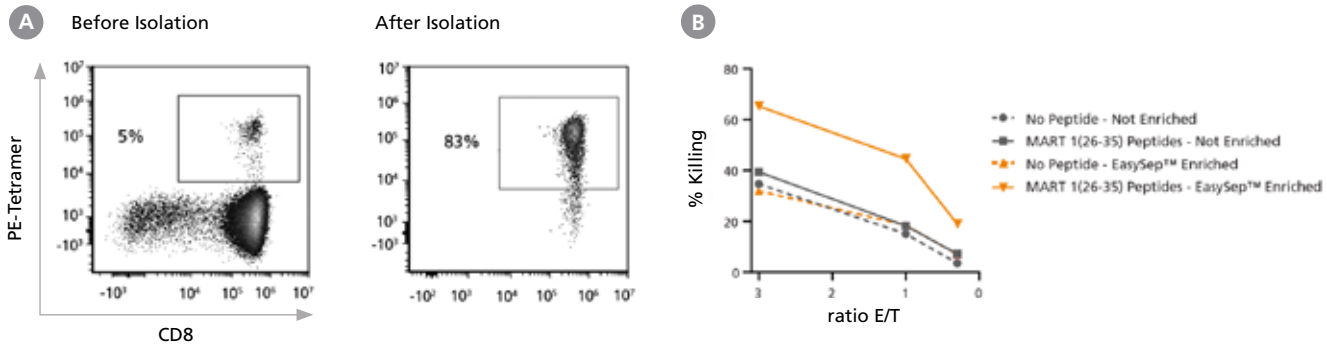

Figure 8. Enrichment of Expanded Antigen-Specific Cells Using Tetramer and EasySep™ Improves Antigen-Specific Killing Activity

A) Expanded CD8⁺ T cells were labeled with PE-conjugated HLA-A*02:01 MART 1(26-35) (ELAGIGILTV) Tetramer and then enriched with EasySep™ Release Human PE Positive Selection Kit. Starting with tetramer-positive expanded CD8⁺ T cells, the purities of the start and enriched samples were 5% and 83%, respectively. Isolated antigen-specific effector CD8⁺ T cells were cultured for 2 days in 5 ng/mL IL-7 and IL-15 before use in the killing assay. B) Killing activity of expanded CD8⁺ T cells primed by Mo-DCs was measured. An HLA-A*02:01-expressing tumor cell line was used as target cells. Target cells were labeled with a fluorescent dye (670 nm) and loaded with MART 1(26-35) peptide or left untreated as a negative control (dashed lines). Unenriched (gray lines) and tetramer and EasySep™-enriched (red lines) MART 1(26-35)-expanded CD8⁺ T cells were incubated with tumor target cells at various effector/target (E/T) ratios for 4 hours, then stained with fluorescently conjugated Annexin V and 7-AAD dye prior to analysis by flow cytometry. Annexin V and 7-AAD staining were analyzed on cell fluorescent dye-positive (670 nm) target cells. Data is representative of two independent donors.

Products

Table 1. List of Suggested Products Named in Protocols

Workflow	Product	STEMCELL Catalog # or Supplier Name
Cell Sourcing*	Human Whole Peripheral Blood	70504
	Human Peripheral Blood Leukopak, Frozen	200-0130
	Human Peripheral Blood Leukopak, Fresh	70500
	Human Peripheral Blood Mononuclear Cells, Frozen	70025
	Human Peripheral Blood Mononuclear Cells, Fresh	200-0077
Cell Isolation	Lymphoprep™	07801
	EasySep™ Magnet	18000
	EasySep™ Human Monocyte Isolation Kit	19359
	EasySep™ Human $CD8^+$ T Cell Isolation Kit	17953
	EasySep™ Human CD2 Positive Selection Kit II	17883
	EasySep™ Human CD3 Positive Selection Kit II	17851
	EasySep™ Release Human PE Positive Selection Kit	17654
Cell Culture	ImmunoCult™ Dendritic Cell Culture Kit	10985
	ImmunoCult™-ACF Dendritic Cell Medium	10987
	ImmunoCult™-XF T Cell Expansion Medium	10981
	CMV (pp65) Peptide Pool	100-0668
	CMV Sub Peptide Pool	peptides&elephants GmbH | LB01713
	Influenza (HLA Class I Control) Peptide Pool	100-0672
	MART 1 (26 - 35)	peptides&elephants GmbH | EP09832_1
	Human Recombinant IL-7	78053
	Human Recombinant IL-15	78031
	Human Recombinant IL-21, ACF	78193
Cell Analysis	eBioscience™ Cell Proliferation Dye eFluor™ 670	Thermo Fisher Scientific | 65-0840-85
	CellTrace™ Violet Proliferation Kit	Thermo Fisher Scientific | C34557
	7-AAD (7-Aminoactinomycin D)	75001
	GloCell™ Fixable Viability Dye Red 780	75007
	DRAQ7™	BioLegend | 424001
	Annexin V, PE	100-0331
	Annexin V Binding Buffer	100-0334
	Anti-Human CD32 Antibody, Clone IV.3	60012
	Anti-Human CD32 Antibody, Clone FLI8.26	60135
	EasySep™ Anti-Human CD32 (Fc gamma RII) Blocker	Component of Catalog #17654
	iTAg Tetramer/PE – HLA-A*02:01 Mart-1 (ELAGIGILTV)	MBL International Corporation | TB-0009-1
	iTAg Tetramer/PE – HLA-A*02:01 CMV pp65 (NLVPMVATV)	MBL International Corporation | TB-0010-1
	Fluorochrome-Conjugated Anti-Human CD3 Antibody, Clone SK7	BioLegend
	Fluorochrome-Conjugated Anti-Human CD8 Antibody, Clone RPA-T8	BioLegend
	Fluorochrome-Conjugated Anti-Human CD25 Antibody, Clone BC96	BioLegend
	Fluorochrome-Conjugated Anti-Human CD279 (PD1) Antibody, Clone EH12.2H7	BioLegend
	Fluorochrome-Conjugated Anti-Human 4-1BB (CD137) Antibody, Clone 4B4-1)	BioLegend
	PE anti-human CD107a (LAMP-1) Antibody, Clone H4A3	BioLegend | 328607
	APC anti-human IFN-γ Antibody, Clone 4S.B3	BioLegend | 502511
	BD GolgiStop™ Protein Transport Inhibitor (Containing Monensin)	BD Biosciences | 554724
	BD Cytofix/Cytoperm™ Fixation/Permeabilization Kit	BD Biosciences | 554714

Summary

The Mo-DC and CD8+ T cell co-culture system is a powerful tool for investigating antigen presentation and T cell activation, which has significant implications for the development of immunotherapies, such as cancer vaccines and T cell therapies.

Below are some takeaways from this case study, which uses a co-culture system to generate antigen-specific T cell responses in vitro.

- EasySep™ Human Monocyte Isolation Kit can be used to isolate monocytes, which may be further differentiated into Mo-DCs and used in co-culture systems to study T cell responses.
- ImmunoCult™ Dendritic Cell Culture Kit enables generation of Mo-DCs, which can be used to induce memory and effector CD8+ T cells in the above described co-culture system.
- EasySep™ Human CD8+ T Cell Isolation Kit can be used to isolate CD8+ T cells, which are an appropriate cell source for DC/CD8+ T cell co-culture.
- Co-culturing Mo-DCs and CD8+ T cells in ImmunoCult™-XF T Cell Expansion Medium with cytokines induces T cell proliferation and expansion, and generation of CD8+ T effector cells with cytotoxic activity.
- The co-culture can be optimized by adjusting the duration of the culture. Harvested CD8+ T cells are then ready to be analyzed, and analysis can include expression of activation markers, production of cytokines, and cytotoxic activity.
- PE-conjugated tetramer and EasySep™ Release Human PE Positive Selection Kit can be used to isolate expanded antigen-specific CD8+ T cells, and enrichment of antigen-specific CD8+ T cells with EasySep™ enhances killing activity.

Why Use ImmunoCult™ to Generate DCs?

- Activate, expand, or differentiate DCs in culture conditions optimized to promote high yield and frequency
- Minimize variation by using serum-free and animal-component free culture conditions
- Support DC differentiation and maturation with ready-to-use formulations
- Consistently achieve high yields of mature DCs with the desired phenotype and function
- Mix and match media, activators, and supplements to suit your specific research needs

Why Use EasySep™ Release to Isolate Antigen-Specific Cells?

- Isolate monocytes in 12.5 minutes, and CD8+ T cells in as little as 8 minutes, with a simple pour
- Achieve up to 94% purity for monocytes, and 91% purity for CD8+ T cells, with high recoveries
- Obtain viable, functional cells without the need for columns and washes
- Isolate cells from virtually any sample source, including whole blood and leukopaks

References

1. Betts MR et al. (2003). Sensitive and viable identification of antigen-specific CD8+ T cells by a flow cytometric assay for degranulation. J Immunol Methods 281(1-2): 65–78.
2. Fröhlich A et al. (2020). Comprehensive analysis of tumor necrosis factor receptor TNFRSF9 (4-1BB) DNA methylation with regard to molecular and clinicopathological features, immune infiltrates, and response prediction to immunotherapy in melanoma. EBioMedicine 52:102647.

E-BOOK

Cell Separation Techniques
www.stemcell.com/cell-separation

VIDEO

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