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WORKS FOR ME 1

Parallel detection of multiple effector functions in live T cells using a short coculture assay

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ABSTRACT

Assays measuring T cell effector function are powerful tools for determining the antigenic specificity of T cells. Conventional flow cytometric detection of cytokine production by T cells via intracellular staining necessarily compromises cell viability. In contrast, the use of a TNFa converting enzyme (TACE) inhibitor paired with monensin enables detection of both TNFa production and degranulation (CD107a/b) in viable T cells, enabling isolation of activated T cells by MACS or FACS for downstream applications such as cell culture or transcriptomic analysis. Herein, we describe the use of this assay to perform antigen rechallenge experiments in human T cells previously sensitized to antigen ex vivo.

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Introduction

1 Assays measuring T cell effector function are powerful tools for determining the antigen-specificity of T cells. Conventional methods for measuring effector cytokine production of T cells in response to antigen include ELISPOT, ELISA, and intracellular cytokine staining (ICS). ICS enables parallel detection of multiple effector cytokines (e.g. IFNg, IL2, TNFa), degranulation markers (CD107a/b), and activation markers (CD137, CD69, CD25) via flow cytometry; however, ICS necessarily compromises cell viability, precluding downstream cell culture of analyzed T cells. An alternative method to assay for T cell effector function while preserving cell viability is to use TAPI-0, a TNFa converting enzyme (TACE) inhibitor, in combination with monensin to trap TNFa and CD107a/b on the surface of activated T cells for detection and/or isolation by FACS.

Herein, we describe the use of this assay to perform antigen rechallenge experiments in human T cells previously sensitized to antigen ex vivo, similar to the use case described in Dolton et al. This assay has also been previously described for unfractionated PBMC, first by Haney et al. with further optimizations by Takahama et al. The coculture time for this assay is 4-5h when using T cells and separate target cells.

Materials

2
- BD GolgiStop Protein Transport Inhibitor (containing Monensin)

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- Flow cytometry reagents for detection of CD107a, CD107b, TNFa, relevant lineage markers, and live/dead dyes. We use: CD107a AF647, CD107b AF647, TNFa PE, CD8 Pacific Blue, and Zombie Green (all from BioLegend).
- FcR blocking reagent (optional)
- TAPI-0 (Calbiochem) reconstituted to 300 µM (10-20X) in cell culture media and frozen in aliquots at -20°C. Despite the low aqueous solubility of TAPI-0, we prefer to reconstitute it at a low concentration in culture media to avoid the use of DMSO.
- Phorbol-12-myristate-13-acetate (PMA) reconstituted in a 10X working stock at 500ng/mL
- Ionomycin reconstituted in a 10X working stock at 10ug/mL
- 96-well V bottom culture plates
- T cells of interest
- Appropriate target cells (for peptide antigen-specificity: DC, EBV-BLCL, monocytes, T2 cells, K562-based aAPC). PBMC or PHA blasts are not recommended for use as targets as they will complicate flow cytometric discrimination of the T cells of interest. Alternatively, targets can be labeled with dyes such as CFSE or CTV to enable discrimination from effector T cells.
- FACS buffer: PBS w/o Ca2+ or Mg2+ supplemented with 0.5% BSA and 0.1% sodium azide. If sorting cells, do not use azide.

Method

3 We describe the steps involved when assaying human T cells for activity against an HLA-presented peptide antigen. Relevant controls for peptide-specificity include targets pulsed with an irrelevant peptide as well as irrelevant HLA-expressing targets pulsed with peptide. As technical negative and positive controls, we include T cells without targets as well as PMA/Ionomycin-treated targets.

Target preparation:

1. Determine the adequate number of cells to be harvested. The assay involves coculturing T cells with targets at an effector-target (E:T) ratio of 1:1 - 1:2, with 1E5 T cells per well in a 96-well plate (Note 1).
2. Harvest target cells from culture and pulse with peptide of interest at 10-20 µg/mL in serum-free media at a concentration of 1E6 cells/mL at 37°C for 1-2h. Alternative methods not covered here to generate antigen-presenting targets include transfection of mRNA encoding peptide minigenes, infection with viral lysate of interest (e.g. EBV, CMV, HIV, AdV), or viral transduction of plasmid DNA to overexpress the gene of interest.
3. Wash target cells with PBS or culture medium and resuspend at 1E6 cells/mL in culture medium.

T cell preparation:

1. Harvest T cells from culture and resuspend in fresh culture medium at 1.1E6 cells per mL
2. Add the following reagents at 2x concentration to the resuspended T cells: anti-TNFa PE (1:100), anti-CD107a AF647 (0.5µg/mL), anti-CD107b AF647 (0.5µg/mL), TAPI-0 (30-60µM), and 1.4µL/mL GolgiStop (Table 1; Note 2)

Coculture initiation:

1. Add 100µL/well T cells to a 96-well V bottom plate.
2. Add 100µL/well targets to the relevant T cell-containing wells
3. Add 100µL/well T cells to designated "T cell only" wells along with 100µL/well culture media

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4. Add 100µL/well T cells to designated "PMA/Ionomycin" wells along with 100µL/well culture media and 20uL/well each of PMA and Ionomycin working stocks.
5. Mix all wells with a multichannel pipette, taking care to change pipette tips to avoid cross-well contamination
6. Spin plate at 350xg for 2 min at RT.
7. Incubate plate at 37°C for 4-5 hours.

Reagent	Final concentr	Sample groups
anti-TNFa PE	1:200 (v/v)	All wells
anti-CD107a A	0.25 µg/mL	All wells
anti-CD107b A	0.25 µg/mL	All wells
TAPI-0	15-30 µM	All wells
GolgiStop	0.7 µL/mL	All wells
PMA	50 ng/mL	PMA/Iono well
Ionomycin	1 µg/mL	PMA/Iono well
T cells	1E5 cells/well	All wells
Target cells	1E5 cells/well	Coculture wells

Table 1: Final concentration of reagents in culture

Staining:

1. Remove plate from incubator
2. Spin cells at 860xg, 4min at 4°C.
3. Remove supernatant by flicking plate onto a waste container and dabbing onto a stack of paper towels. Resuspend each well in 200µL FACS buffer.
4. Repeat spin step and FACS wash.
5. Remove supernatant and resuspend cells in 50µL/well of staining cocktail with live/dead dye and relevant lineage markers, e.g.: 1:50 CD8 Pac Blue + 1:1000 Zombie Green + 1:100 FcR blocking reagent in FACS buffer. Incubate 30min. at 4C. (Note 3)
6. Wash cells 2x with FACS buffer as above
7. Resuspend cells in 100-200uL FACS buffer

Flow cytometry:

1. Acquire the "T cell only" sample first to set appropriate gating for T cells
2. Acquire the "PMA/Ionomycin" sample to confirm expression of CD107a/b (Fig. 1). Note that PMA/Ionomycin cause significant alterations to FSC and SSC.
3. Acquire coculture samples, using the PMA/Ionomycin sample to guide gating for TNFa+ and CD10a/b+ T cells.

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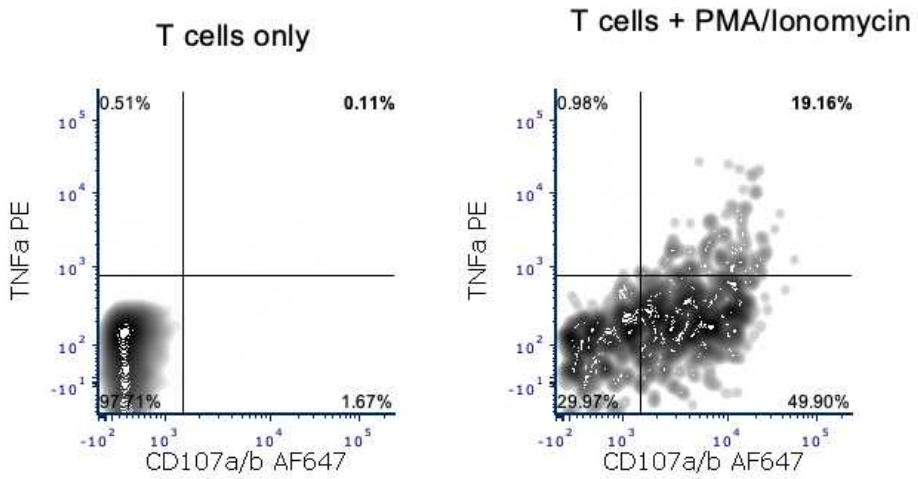

Fig 1. Representative results of unstimulated T cells (left) and PMA/Ionomycin-treated T cells (right). Plots are gated on live, single, FSClo/SSClo CD8+ cells.

Notes

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1. Fewer T cells can be used per well but will require more events to be acquired during flow cytometric analysis.
2. Agonistic antibodies can be added at this step to potentially enhance responses. Anti-CD28 and anti-CD49d are commonly used in combination at a final concentration of 0.5-1.0 µg/mL each. However, their efficacy is dependent on the differentiation state of the T cell. Generally, T cells isolated directly from PBMC benefit to a greater extent from these antibodies than T cells primed and differentiated ex vivo.
3. Other activation markers, such as CD137 (4-1BB), CD69, CD154 (CD40L), CD134 (OX40) and CD25 can be included during the surface staining step, but detection may require a longer incubation period to coincide with peak expression, such as for CD137 and CD154. Incubation periods with monensin longer than 18 h can compromise cell viability (Lamoreaux et al.).
4. In contrast to assays using unfractionated PBMCs and exogeneous antigen, monensin is added at the start of this assay since antigen processing and presentation is provided by peptide-pulsed target cells.
5. TAPI-0-mediated surface TNFa detection only detects 45-95% of TNFa+ cells compared to traditional ICS (Haney et al.; Fig. 3)
6. Background TNFa- and CD107a/b-expression may be reduced by resting T cells in fresh culture media with reduced serum (5%) overnight prior to the assay.

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