High-fidelity detection of IFN-γ secreting CD4 and CD8 cells in response to soluble or Ad5 transduced influenza antigens using ELISPOT and cell sorting

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Abstract

ELISPOT allows detection of single IFN-γ-secreting cell, but the use of unseparated spleen cells removes the advantages of the method due to a high back ground of IFN-γ-production and uncertainty of the phenotype of secreting cells (CD4, CD8 or NK).

We used high purity sorted CD4 and CD8 cells.

For in vitro restimulation of CD8 T-cells, we used dendritic cells that present antigenic epitopes of M2, NP or of adenovirus in the context of MHC class I. In this instance, dendritic cells were transduced with Ad5-tet-M2, Ad5-tet-NP or Ad5-null, respectively.

For restimulation of CD4 T-cells in vitro, we used dendritic cells that present antigenic M2 or NP epitopes in the context of MHC class II. For this purpose, dendritic cells were additionally activated by lipopolysaccharide E. coli (1 μg/ml) and were loaded with recombinant protein NP or M2e peptide..

Citation: Aleksey Pichugin,Alexander Bagaev,Ravshan Ataullakhanov High-fidelity detection of IFN-γ secreting CD4 and CD8 cells in response to soluble or Ad5 transduced influenza antigens using ELISPOT and cell sorting. **protocols.io** dx.doi.org/10.17504/protocols.io.jn4cmgw

Published: 11 Sep 2017

Guidelines

Ref.

1. http://onlinelibrary.wiley.com./doi/10.1002/0471142735.im0301s39/pdf#

2. example of sorting https://community.cytobank.org/cytobank/experiments/66457

3. DOI: http://dx.doi.org/10.14440/jbm.2014.12

4. http://www.bdbiosciences.com/ds/ab/others/552569_552138_Book_Website.pdf

Protocol

. CD4 and CD8 sorting from splenocytes

Step 1.

Prepare FACSAriaII sorter for aseptic sorting according to User Manual

Step 2.

Install 70ul nozzle and Check AriaII performance by CST protocol

REAGENTS

✓ Cytometer Setup & Tracking Beads Kit (use with BD FACSDiva™ software v 6.x) cat. 642412 by Contributed by users

Step 3.

Set up sorting parameters at 87 kHz, adjust drop creating and drop delay using AccuDrop beads

NOTES

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Accudrop Bead BD Biosciences cat. 345249

Step 4.

Prepare cell suspensions from the spleen of the experimental mouse according to protocol [1]. In brief, add 6 ml PBS-cell buffer in 60 mm Petri dish, put spleen from 1 mouse into the dish. Crush spleen by back side of 5 ml syringe plunge. Carefully break up cell aggregates by intensive pipetting.

NOTES

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PBS-cell buffer: PBS (10 mM sodium phosphate, 137 mM sodium chloride, 2.7 mM potassium chloride, Amresco) enriched by 1% glucose, 10 mM HEPES and 0.5% of BSA), filtered 0.22 u

Step 5.

Add 4 ml of Ficoll-Paque solution in 15 ml centrifuge tube, carefully layer 6 ml of the spleen cell suspension, centrifuge at 450 g and 18oC for 20 min.

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Ficoll—Hypaque 1.09 g/cm3

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!! Set brakes rates of centrifuge to zero

Step 6.

Carefully move cell layer to new 15 ml tube, add PBS-cell buffer up to 15 ml, centrifuge 10 min at 300 g and 4oC discard supernatant, resuspend pellet in 1 ml PBS-cyto buffer and break up cell aggregates by pipetting.

Step 7.
Count cell concentration and viability: put 10 ul of cell suspension in 1,2 ml titer tube and add 200 ul PBS-count buffer. Run sample by AriaII, stop acquisition by counting 400 beads. Set lymphocytes gate within live (DAPI negative) events. Calculate splenocytes concentration from ratio of cell counts to calibration bead counts. Adjust cell concentration to 100 mln/ml PBS-cell buffer .

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PBS-count buffer: PBS (10 mM sodium phosphate, 137 mM sodium chloride, 2.7 mM potassium chloride, Amresco) enriched by 10 mM HEPES and 0.5% of BSA, , 0,01% sodium azide, 0,35 mM EDTA, pH 7,4 filtered 0.22 u. With addition of 1 ug/ml DAPI and Precision Count Beads™ Biolegends cat. 424902 diluted 1:100.

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Cell suspension and PBS-count buffer must be carefully vortexed before pipetting.

Step 8.
Move 0.6 ml of splenocytes suspension to new 15 ml tube, add 2 ul of each antibody: CD19 FITC, CD4 PerCP Cy5.5, CD8a APC vortex, incubate 20 min in the dark at 4oC.10. Add PBS-cell buffer up to 15 ml, centrifuge 10 min at 300 g and 4oC discard supernatant. Resuspend pellet in 3 ml PBS-cyto buffer and break up cells aggregates by pipetting. Add DAPI 1 ug/ml and pass the cell suspension through 40 um filter into the 4.5 ml tube.

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CD19 FITC clone 1D3 (BD Biosciences cat 552785), CD4 PerCP Cy5.5 clone RM4-5 (BD Biosciences, cat.N 550954) CD8a APC clone 53-6.7 (BD Biosciences cat. 553035)

Step 9.
Add 1 ml of FBS to two 15 ml tubes and install them in 2-way sorting holder of FACSAriaII. Vortex stained cell suspension and run acquisition.

Step 10.

Acquire sample by FACSAriaII. Set gates: exclude doublets by FCS-H vs. FSC-A dot blot, exclude dead cell by DAPI fluorescence, set lymphocytes size by FSC vs. SSC dot blot. Set CD4 as CD19 and CD8 negative events, set CD8 as CD19 and CD4 negative events. Set up sorting layout, set up sorting precision to "purity".

turn on 'sweet spot' mode and start sorting. Example of sorting [2]

Step 11.

Stop sorting at desired cells counts, add PBS-cyto buffer up to 15 ml to sorted cells and centrifugate 10 min at 300 g and 4oC. Discard supernatant and resuspend cells pellet in 1 ml DMEM-full and break up cell aggregates by pipetting. Count cell concentration as in the step 8 and adjust CD4 cell concentration to 2 mln/ml and CD8 to 1 mln/ml with DMEM-full.

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DMEM-full: DMEM, enriched by 10% FCS, 2 mM L-glutamine, Non essential AA, 1 mM sodium pyruvate, 50 uM β-ME, 100 U/mlп Pen Strep

Generation of autologous BM derived GM-CSF DC (based on [3])

Step 12.

Flush the bone marrow out of the hind leg femurs of two Balb/c mice (Six-week old female) with 2 ml of PBS-cell buffer using a 2-ml insulin syringe with a 25G × ½ needle into a sterile 50-ml centrifuge tube.

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8 days before experiment start.

Step 13.

Add 20 ml PBS-cyto and pellet cell by centrifugation 10 min at 300 g and 4oC. Discard supernatant and lyse RBC by osmotic shock: add 9 ml sterile deionized water and after 15 sec add 1 ml 10x HBSS and 20 ml PBS-cell buffer. Pellet cell by centrifugation 10 min at 300 g and resuspend in 1 ml PBS-cell buffer, count cell concentrations as described in step 8 and adjust cell concentration to 10 × 10 6 cells/ml by DMEM-full.

Step 14.

Place into 4 Petri dish 9 ml of the culture medium DMEM-full, add 1ml of cell suspension (10 × 10 6 cells/ml) into each Petri dish to achieve the final cell density of 1 × 10 6 cells/ml. Add rmGM-CSF from rmGM-CSF stocks (4000 ng/ml) into above Petri dishes so that final concentration of rmGM-CSF is 10 ng/ml. Gently swirl the Petri dishes to ensure uniform mixing of contents. Incubate the cells at 37°C, 5% CO2 and 95% humidity in CO2 incubator for 3 days.

NOTES

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rmGM-CSF (Gibco) stock solution 400 ng/100ul aliquots -80oC

Step 15.

At day 3 add 10 ml of fresh culture medium and rmGM-CSF to achieve 10 ng/ml rmGM-CSF into each Petri dish with BMDC cultures. Incubate the cultures at 37°C, 5% CO for additional 4 days.

DC transduction with Ad5 for MHCI presentation

Step 16.

On 7 day of generation of BMDC, harvest non-adhesive dendritic cells by careful washing from Petri dish. Use serological pipette and the lowest pipetting speed.

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1 day before experiment start

Step 17.

Transfer the cell suspension from Petri dishes into two 50ml tube, centrifuge at 300g/10min, resuspend cell pellet of each tube in 1 ml of PBS-cell buffer and combine them.

Step 18.

Estimate the purity and viability of DC cells by Flow Cyt as described in step 8 and adjust cells concentration to 1mln/ml with DMEM-full.

Step 19.
Dispense 3.5 ml cells suspension per each of five sterile 60-mm Petri dishes, pre-labeled as DC, DC-LPS, DC-Ad5-tet-M2, DC-Ad5-tet-NP or DC- Ad5-null

Step 20.
Stimulate part of DC cells dedicated to peptide antigen presentation with LPS (1 ug/ml).

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LPS Escherichia coli 055:B5(L-2880) Sigma 100ug/ml -80oC

Step 21.
Add appropriate Ad5 (100 pfu per cell) to the Petri dishes pre-labeled "Ad5-tet-M2", "Ad5-tet-NP" or "Ad5-null" .

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Ad5-null 10E8 PFU/ml
Ad5-tet-M2 6*10E9 PFU/ml
Ad5-tet-NP 7*10E9 PFU/ml

Step 22.
Incubate cell cultures at 37°C, 5% CO2 and 95% humidity in CO2 overnight.

Step 23.

Next day harvest BMDCs from each Petri dish by collecting non-adherent cells by gentle pipetting them with the culture medium. Place cells in 15 ml centrifuge tubes, add 10 ml PBS-cell buffer and pellet cells by centrifugation at 300g for 10min. Resuspend cells in 0.5 ml DMEM-full, count cell concentration as in step 8 and adjust cell concentration to 0.067 mln/ml with DMEM-full.

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In the day of the experiment

Step 24.
Cells from tube "" DC-LPS,"" divide to three tubes: ""LPS"", ""LPS-NP"" and ""LPS-M2"". Add to tubes

with labels ""LPS-NP"" and ""LPS-M2"" recombinant protein NP (end concentration 5 µg/ml) and M2e peptide (end concentration 50 µg/ml), respectively

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NP (Sinobiological, China) chemically synthesized M2 ectodomain (MSLLTEVETPIRNEWGCRCNDSSD) (Almabion, Russia).

ELISPOT procedure (based on [4])

Step 25.

Put 150 ul (10000 cells) of each type of DC's in appropriate number of wells (two wells for each experimental sample) of ELISPOT plate. Let cells to fall to the bottom at least for 1 hour before adding lymphocytes.

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ELISPOT Mouse IFN-γ Kit BD Biosciences, cat.N. 552569

Step 26.
In case of unseparated spleen cells add 50 ul of 10 mln/ml suspension (500000 cells per well).

Step 27.
In case of sorted CD4 (2mln/ml) and CD8 (1 mln/ml) add 50 ul of cells suspensions (100000 and 50000 cells per well, respectively).

Step 28.
Incubate plate at 37ºC in a 5% CO2 humidified atmosphere for 20 hours strictly in the horizontal position avoiding shaking.

Step 29.
Aspirate cell suspension. Wash wells 2× with deionized (DI) water. Wash wells 3× with 200 µL/well with Wash Buffer. Discard Wash Buffer.

Step 30.
Add Detection Antibody Solution at 100 µL per well. Replace lid and incubate for 2 hr at room temperature. Discard Detection Antibody solution. Wash wells 3× with 200 µL/well Wash Buffer

Step 31.
Add Streptavidin-HRP Solution at 100 µL/well. Replace lid; incubate for 1 hr at room temperature. Discard Streptavidin-HRP solution. Wash wells 4× with 200 µL/well Wash Buffer.

Step 32.
Wash wells 2× with 200 µL/well PBS. Add 100 µL of AEC Substrate Solution to each. Monitor spot development from 5 – 25 minutes. Do not let color overdevelop as this will lead to high background

Step 33.
Stop substrate reaction by washing wells with DI water. Air-dry plate at room temperature overnight

Step 34.

Image each well using a binocular microscope MBS-10 (magnification x32) and digital camera (Levenhuk DCM800 with 1280x960 pixel resolution). Quantificate the spots by using spot counting software package ImageJ

Determination of T-cells which synthesize IFN-γ by flow cytometry

Step 35.

Adjust the concentration of each type ot DC (transduced by Ad5-tet-M2 or Ad5 tet-NP or Ad5-null) prepared as described in the step 23 up to 0.1 mln/ml by DMEM-full. Place 1 ml of each type ot DC suspension (100,000 cells) in the appropriate number of wells of 24-well Nunclon culture plate. The number of wells is equal to the number of experimental samples multiplied by the number of replicates (usually two replicates).

Step 36.

Adjust the concentration of each type of experimental splenocytes suspension prepared as described in the step 7 up to 50 mln/ml by PBS-cell buffer. Place 100 µl each suspension of spleen cells (5 million cells) into all type of DC.

Step 37.

Incubate the plate for 12 hours in a CO2-incubator, then add brefeldin-A at a final concentration of 5 µg/ml.

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Brefeldin A Solution (1,000X) BioLegend cat. 420601

Step 38.

After 6 hours of incubation with brefeldin A, add 2 ml of cold PBS-cyto buffer to each wells. Pippet cells and transfer them into 4.5 mm-test-tubes (BD Biosciences).

Step 39.

Then, pellete cells by centrifugation (300g/10min/4°C), discarde supernatant, and add to cells 1 ml of PBS- cyto buffer. Resuspende the cells and transfer them to a 1.2 ml microtube.

Step 40.

Pellete cells by centrifugation (300g/10min/4°C), discarde supernatant, and add to the cells 50 ul of the following antibody mixture in the PBS- cyto buffer:

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CD4 PerCP Cy5.5 clone RM4-5 (BD Biosciencess, cat.N 550954) Final dilution 1:200

CD8 Alexa Fluor 700 clone 53-6.7 (Biolegend, cat.N 100730) Final dilution 1:100

CD44 PE-Cy7 clone IM7 (Biolegend, cat.N 103030) Final dilution 1:200

CD62L Alexa Fluor 488 clone MEL-14 (Biolegend, cat.N 104420) Final dilution 1:200

Step 41.

Incubate the cells for 20 min in the dark at 4°C, add 1 ml of PBS-cyto buffer, pellete cells (300g/10min/4°C) and discarde the supernatant

Step 42.

Vortex the cell pellet and add 100 µl of FixPerm solution (Kit BD CytStirreofix/Cytoperm™ 554722)

http://www.bdbiosciences.com/ds/pm/tds/554722.pdf.

Note: Cell aggregation can be avoided by vortexing prior to the addition of the BD Cytofix/Cytoperm™ solution

Step 43.

Incubate the tubes for 20 min. at 4°C in the dark. Wash cells twice with 0.5 ml of PermWash solution (300g/10min/4°C) and resuspende cells n 50 ul of PermWash solution. Divide each tube into two aliquots per 20 ul.

Step 44.

Add 20 µl of Anti-Mouse IFN-γ PE (BD Biosciencess Pharmingen, cat.N 554412) previously diluted 1:10 in PermWash solution into one aliquot. Add isotype antibody PE Rat IgG1, κ Isotype Control (554685) previously diluted 1:10 in a PermWash solution.into second aliquot.

Step 45.

Vortex the tubes and Incubate them at 4°C for 30 minutes in the dark.

Wash cells twice with 0.5 ml of PermWash solution (300g/10min/4°C) and resuspend cells pellet in 300 ul of PBS-cyto buffer.

Step 46.

When analyzing cells with FACSAriaII, acquire at least 1 million events