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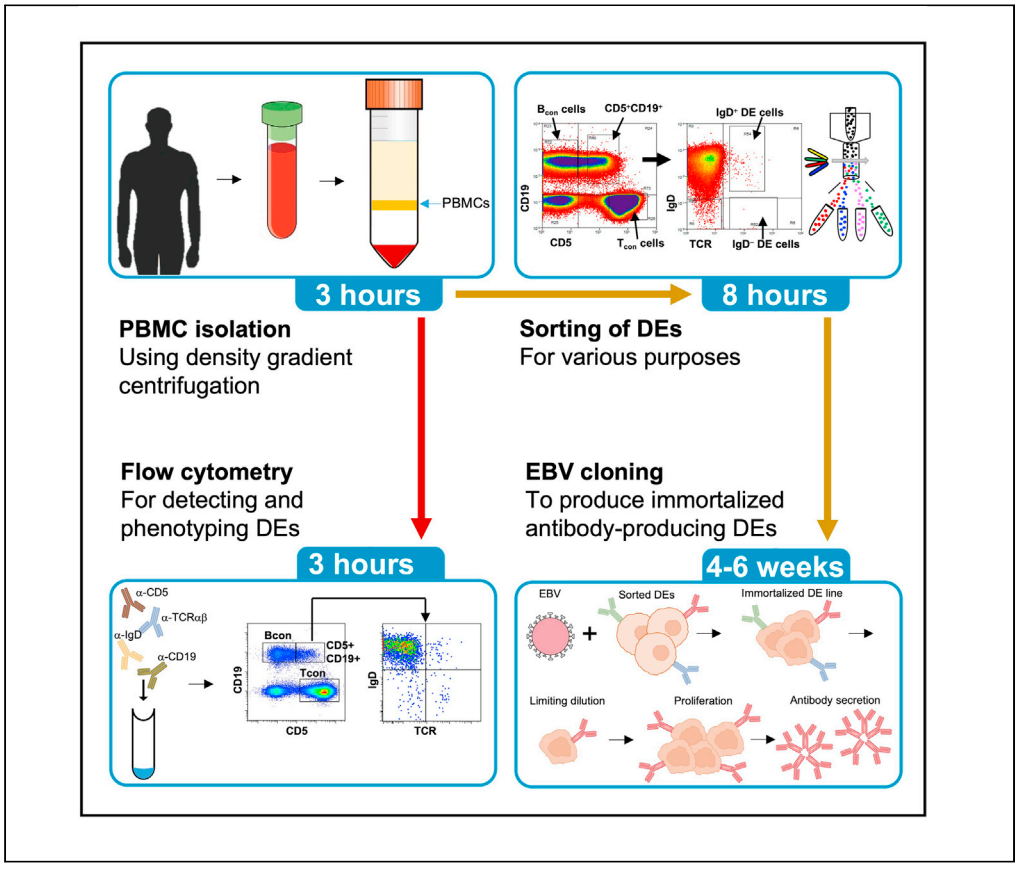
Protocol

Detection, sorting, and immortalization of dual expresser lymphocytes from human peripheral blood samples



This protocol describes how to identify Dual Expressers, a rare type of lymphocyte that co-expresses B-cell receptors and T-cell receptors, by flow cytometry using a cocktail of four antibodies. It also shows the subsequent gating strategy for detecting and sorting DEs and the generation of EBV-immortalized DE lymphoblastoid cell lines and clones for antibody production and cloning antigen receptors. Use of this protocol maximizes detection of DEs and minimizes inclusion of doublets.

Rizwan Ahmed, Kusuma Ananth, Zahra Omidian, Neha Majety, Hao Zhang, Abdel Rahim A. Hamad

rahmed14@jhu.edu (R.A.)
ahamad@jhu.edu (A.R.A.H.)

Highlights

Protocol to detect DEs and distinguish from T and B cells by flow cytometry

Sequential gating strategy to maximize and optimize detection of DEs

Strategy for efficient FACS-sorting and checking purity of sorted DEs

Protocol for generating EBV-immortalized DE lines and clones

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Rizwan Ahmed,[1,3,4,*] Kusuma Ananth,[1,3] Zahra Omidian,[1] Neha Majety,[1] Hao Zhang,[2] and Abdel Rahim A. Hamad[1,5,*]

[1]Department of Pathology, Johns Hopkins University School of Medicine, Baltimore, MD 21205, USA

[2]Department of Molecular Microbiology and Immunology, Johns Hopkins Bloomberg School of Public Health, Baltimore, MD 21205, USA

[3]These authors contributed equally

[4]Technical contact

[5]Lead contact

*Correspondence: rahmed14@jhu.edu (R.A.), ahamad@jhu.edu (A.R.A.H.)
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SUMMARY

This protocol describes how to identify Dual Expressers (DEs), a rare type of lymphocyte that co-expresses B-cell receptors and T-cell receptors, by flow cytometry using a cocktail of four antibodies. It also shows the subsequent gating strategy for detecting and sorting DEs and the generation of EBV-immortalized DE lymphoblastoid cell lines and clones for antibody production and cloning antigen receptors. Use of this protocol maximizes detection of DEs and minimizes inclusion of doublets.

For complete details on the use and execution of this protocol, please refer to Ahmed et al. (2019).

BEFORE YOU BEGIN

Isolation and aliquoting of PBMCs

Timing: 3 h

This procedure includes the following: (a) Collecting peripheral blood samples from donors (patients with an autoimmune disease or healthy control subjects) using an approved IRB protocol, (b) Isolating peripheral blood mononuclear cells (PBMCs) by performing density gradient centrifugation using Ficoll-paque density gradient method according to manufacturer's instructions: https://www.sigmaaldrich.com/US/en/technical-documents/protocol/clinical-testing-and-diagnostics-manufacturing/hematology/recommended-standard-method. (c) Aliquoting PBMCs (5–10 $\times 10^6$ cells in 1 mL freezing medium) per cryovial and store at −80°C for short terms (up to 6 months) and in liquid nitrogen for long term storage (up to I year).

Thawing cryopreserved PBMCs

Timing: 20 min

1. Thaw cryopreserved PBMCs by placing cryovials (one at a time) in a 37°C water bath and shake the vial gently until about 95% of the freezing medium has melted and only a small chunk of

ice remains. This should take about 1 min. Immediately place the cryovial on ice and quickly move to the next step to minimize the amount of time thawed cells stay in freezing medium.

2. Wash PBMCs with wash medium (see materials and equipment).
   a. Invert the cryovial twice to resuspend PBMCs.
   b. Open the vial and slowly transfer cells from each cryovial to a 15 mL polyethylene tube containing 10 mL of prewarmed (37°C) wash medium.

   Note: We find that the use of wash medium ensures maximum viability, stability and maintains integrity of thawed-PBMCs. The wash buffer also reduces cell loss through aggregation.

   c. Centrifuge cells at 400 × *g* for 5 min at room temperature (RT; 22°C–25°C).
   d. Remove the supernatant carefully with a pipette and perform an additional wash with complete tissue culture medium (CTM) (see materials and equipment).
   e. Resuspend the pellet and filter the cell suspension using a 70 μm cell strainer, and place in a new 15 mL polyethylene tube.
   f. Count viable cells using the Trypan Blue exclusion method. We routinely obtain ~ 90%–95% viability.
   g. If needed, use CTM to adjust cell density to the desired concentration (e.g., 1×10^6 cells / mL).

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
APC anti-human CD5 (L17F12)	BioLegend	Cat#364016; RRID: AB_2565726
BV421 anti-human CD19 (HIB19)	BD Biosciences	Cat#562440; RRID: AB_11153299
AF-488 anti-human TCRαβ (IP26)	BioLegend	Cat#306712; RRID: AB_528967
PE anti-human IgD (IA6-2)	BioLegend	Cat#348204; RRID: AB_10553900
Bacterial and virus strains		
Epstein-Barr Virus B95-8	American Type Cell Culture (ATCC)	Cat#ATCC VR-1492
Biological samples		
Human peripheral blood samples (8- to 80-year-old males and females)	Patients with autoimmune disease or healthy controls	N/A
Chemicals, peptides, and recombinant proteins		
2-mercaptoethanol	Thermo Fisher Scientific	Cat#21985023
Antibiotic Antimycotic Solution (AAS)	Sigma Aldrich	Cat#A5955-100ML
Benzonase nuclease	Sigma Aldrich	Cat#E8263
Bovine serum albumin (BSA)	Sigma Aldrich	Cat##A1933-100G
CpG ODN 2006 (ODN 7909)	InvivoGen	Cat#tlrl-2006-1
Dimethyl Sulfoxide (DMSO)	Fisher Scientific	Cat#BP231-100
Fetal Bovine Serum (FBS)	Sigma Aldrich	Cat#F2442-500ML
Ficoll Paque Plus	MilliporeSigma	Cat#GE17-1440-02
Hanks' Balanced Salt Solution (HBSS)	MilliporeSigma	Cat#55037C
HEPES	Thermo Fisher Scientific	Cat#15630-080
Live/Dead Fixable Aqua	Thermo Fisher Scientific	Cat#L34957
Non-essential amino acids (NEAA)	Quality Biological	Cat#116-078-721
Phosphate Buffered Saline (PBS)	Corning	Cat#46-013-CM
RPMI with L-glutamine	Corning	Cat#10-040-CV
Sodium Azide	Sigma Aldrich	Cat# S2002
Sodium Pyruvate	Thermo Fisher Scientific	Cat#11360070
Propidium Iodide	BD Biosciences	Cat#556463
Experimental models: Cell lines		
IRR-MRC-5 irradiated Fibroblast	American Type Cell Culture (ATCC)	Cat#ATCC 55-X

(*Continued on next page*)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
Software and algorithms		
FlowJo	TreeStar	https://www.flowjo.com/solutions/flowjo
Others		
0.22 μm vacuum filtration flask	Corning	Cat#431097
70 μm cell strainers	Falcon	Cat#352350
FACS tubes	MTC Bio	Cat#T9014
FACS tubes with 35 μm cell strainer caps	Falcon	Cat#352235
Multi-color Flow cytometer	Of your choice	N/A
MoFlo Legacy High Speed Cell Sorter	Beckman Coulter (or a sorter of your choice)	beckmancoulter.com

MATERIALS AND EQUIPMENT

Freezing medium		
Reagents	Final concentration	Amount
RPMI with L-glutamine	n/a	200 mL
DMSO	10%	50 mL
FBS	50%	250 mL
Total		500 mL

Prepare freezing medium under sterile conditions using a biosafety cabinet. Combine all the reagents, then filter the freezing media using a 0.2 μm filter (vacuum filtration flask). Aliquot filtered medium into 50 mL falcon tubes and store at −20°C for up to three weeks.

Wash medium		
Reagents	Final concentration	Amount
Complete tissue culture medium (CTM)	n/a	10 mL
Benzonase (250,000 U/mL)	0.02%	2 μL
Total		10 mL

Prepare wash medium under sterile conditions using a biosafety cabinet and store at 4°C for up to three weeks.

Complete tissue culture medium (CTM)		
Reagents	Final concentration	Amount
RPMI with L-glutamine	n/a	500 mL
Heat-inactivated FBS	8.7%	50 mL
Anti-mycotic and antibiotic solution (AAS)	1%	6 mL
HEPES buffer	1%	6 mL
Non-essential amino acids (NEAA)	1%	6 mL
Sodium pyruvate	1%	6 mL
Beta mercaptoethanol	0.08%	450 μL
Total		574.45 mL

Prepare CTM under sterile conditions in a biosafety cabinet. After mixing all reagents, filter the CTM using a 0.22 μm filter (vacuum filtration flask) and store at 4°C until use for about three weeks.

FACS buffer		
Reagents	Final concentration	Amount
1× Phosphate buffered saline (PBS)	n/a	500 mL
Bovine serum albumin (BSA)	1%	5 g
Sodium azide	0.05%	250 mg
Total		500 mL

Store at 4°C and use within three weeks.

Lymphocyte collection medium		
Reagents	Final concentration	Amount
RPMI with L-glutamine	n/a	229.6 mL
Heat-inactivated FBS	50%	250 mL
Anti-mycotic and antibiotic solution (AAS)	1%	5 mL
HEPES buffer	1%	5 mL
Non-essential amino acids (NEAA)	1%	5 mL
Sodium pyruvate	1%	5 mL
Beta mercaptoethanol	0.08%	400 µL
Total		500 mL

Prepare the collecting medium under sterile conditions using a biosafety cabinet. After combining all reagents, filter the medium using a 0.22 µm filter (vacuum filtration flask) and store at 4°C until use within three weeks.

STEP-BY STEP METHOD DETAILS

Part I. Detection of DEs by flow cytometry

This section details the methods for: (A) Staining PBMCs with our standard cocktail of 4-fluorophore-conjugated antibodies specific for CD19, CD5, IgD, and TCRαβ. (B) Sequential gating strategy to simultaneously detect DE cells, conventional T cells (Tcon) and conventional B cells (Bcon) cells in the same PBMC sample and compare their phenotypes – See below for details.

Identifying DEs by flow cytometry

Timing: 2–3 h

Note: Use fresh PBMCs for flow cytometry whenever possible. However, when using cryopreserved PBMCs, additional attention should be paid to exclude debri and doublets.

Note: To properly detect DEs, you will need to stain sample(s) with the cocktail of 4 antibodies (See Table 1). In addition, you will need 5 compensation control tubes (4 tubes for single fluorophore staining, and 1 tube for unstained sample).

Optional: You may stain your sample(s) with Live/Dead dye, such as PI or Live/Dead Aqua. It is highly recommended to use Live/Dead in your initial experiments until you become familiar with analyzing DEs or when you are expecting high numbers of dead cells in your samples.

1. Count live cells using the Trypan blue dye exclusion method.
2. Label your FACS tubes with permanent marker.
3. Add 1 to 2 million cells in 100 µL to each FACS tube. If you do not have enough cells, you may use fewer cells for compensation (200,000 cells per control tube).

   Alternatives: you may use compensation beads instead of cells. However, we prefer to use cells from the same sample because beads are usually more homogeneous and emit fluorescent intensity that does not necessarily match the fluorescent intensity of the molecule on the surface of cells of your interest.

4. Stain your sample(s) and controls:
   a. If you would like, stain your sample(s) first with the Live/Dead dye of your choice. We generally use PI (1 µg/mL) or Live/Dead Aqua (1 µL of dye as prepared in kit in 1 mL PBS). If you choose to stain your samples with Live/Dead, make sure you make a single-color compensation control for it and follow manufacturer's instructions.
   b. Stain your sample(s) with the cocktail of fluorophore-conjugated antibodies specific for TCRαβ, IgD, CD5, and CD19. We use 5 µL of each antibody and stain in FACS buffer, at a final volume of 100 µL. See Table 1 for the fluorophores we use and suggested dilution factors.

Table 1. Antibody cocktail used for detecting DEs

Surface marker	Fluorophore	[a]Suggested dilution factor
CD5	APC	1:20
CD19	Brilliant Violent 421	1:20
TCRαβ	Alexa Fluor 488	1:20
IgD	PE	1:20

[a]We suggest you determine optimal concentrations of your antibodies by doing your own titrations.

Note: if you are staining multiple samples, you may prepare a cocktail sufficient for all of them.

c. Stain your single-color compensation controls.
d. Vortex tubes and incubate on ice for 20–30 min, protected from light.

5. Wash your sample(s) and controls with 2–3 mL of FACS buffer. Centrifuge at 525 xg (1500 rpm) and discard supernatants.
6. Resuspend samples in 600 μL of FACS buffer.
7. Vortex tubes. They are now ready for flow cytometric analysis.
8. Move to your FACS instrument and perform compensation setup using your compensation controls.
9. Run your samples and acquire 500,000–1,000,000 events per sample when possible.
10. Export the FSC files.

Sequential gating strategy to identify DEs and analyze their phenotypes

This section details the sequential gating strategy we employ for identifying and analyzing DEs in acquired samples using the FlowJo software. The protocol is excellent for analyzing samples for the presence and determining the frequency of DEs.

Note: When desired, stain samples for appropriate markers for additional phenotypic and functional characterization of DEs and compare their properties to those of autologous B_{con} and T_{con}.

Timing: 30–60 min

11. Open FlowJo and drag your FCS files into the application
12. Open your sample files.
13. Gate total lymphocytes using the FSC-Area vs SSC-Area plot.
14. Gate on single cells and exclude doublets using the FSC-Area vs FSC-Height followed by SSC-Area vs SSC-Height plots. Alternatively, doublets can be excluded by using FSC-Height vs FSC-Width and SSC-Height vs SSC-Width plots.
15. If you stained your samples with Live/Dead, gate on live cells using a histogram of the Live/Dead staining as recommended by the manufacturer.
16. Draw a 2-parameter dot plot using CD5 vs CD19. Gate on B_{con} ($CD5^-$ $CD19^+$), T_{con} ($CD5^+$ $CD19^-$) and the $CD5^+$ $CD19^+$ subpopulations (we often use rectangle gates).

Note: When gating for DEs, avoid $CD5^{high}$ $CD19^{high}$ cells because they are almost always highly enriched for doublets. Thus, it is recommended that when gating on the $CD5^+$ $CD19^+$ population limit gating to $CD19^+CD5^{low}$ cells (see Figures 1 and 2).

17. Analyze each subpopulation for the presence of $TCR\alpha\beta^+IgD^+$ DEs. Use the B_{con} population as a reference to place the gates that distinguish between $TCR\alpha\beta^+$ and $TCR\alpha\beta^-$ and IgD^+ and IgD^- subpopulations.

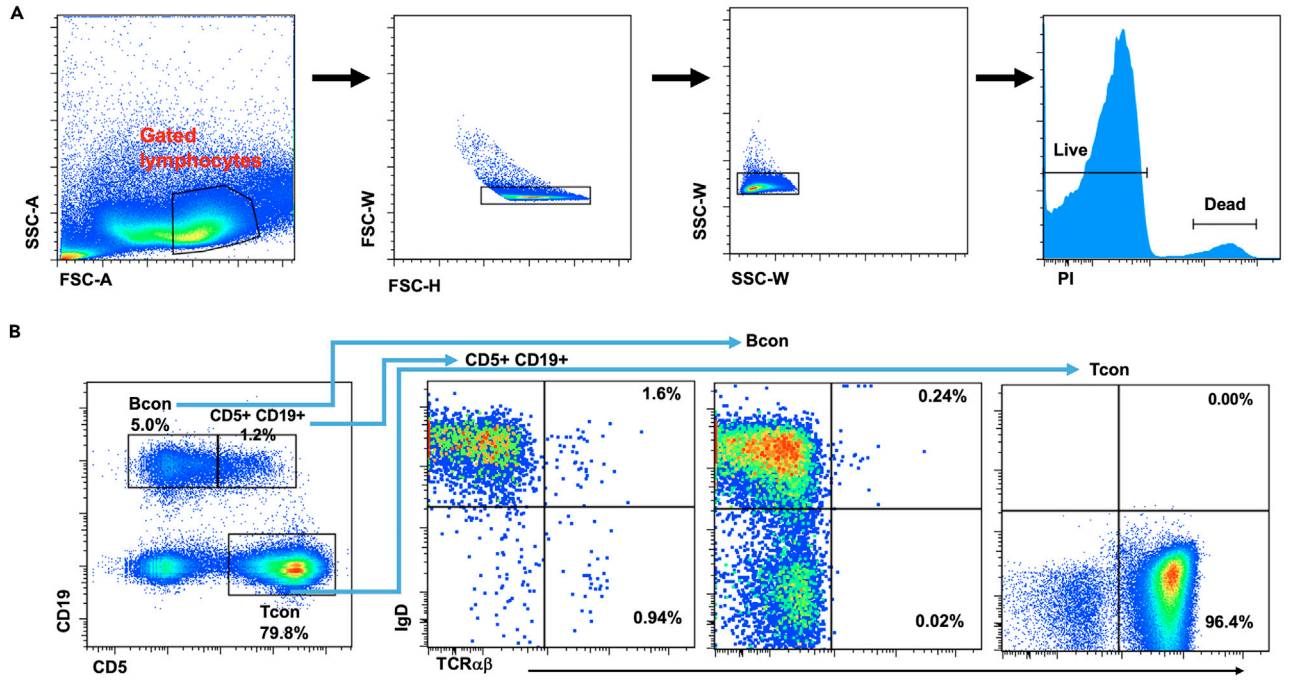

Figure 1. Consequential gating strategy for acquiring samples and detecting DEs, Bcon, and Tcon by using FACS and FlowJo

(A) Dot plots show how to sequentially gate live single lymphocytes and exclude doublets and dead cells in your PBMC sample.

(B) Left dot plot shows how to use a 2-parameter plot of CD19 versus CD5 to divide gated lymphocytes into B_{con} ($CD5^{-}CD19^{+}$); T_{con} ($CD5^{+}CD19^{-}$), and $CD5^{+}CD19^{+}$ populations. Right dot plots show how to analyze each gated subpopulation for DEs as identified by the coexpression of TCRαβ and BCR. Note: most DEs are located within the $CD5^{+}CD19^{+}$ compartments, rare among B_{con}, and virtually absent among T_{con}. Consequently, analysis and sorting of DEs, is limited to DEs localized within the $CD5^{+}$ $CD19^{+}$ population. Numbers indicate percentages in each quadrant. This figure is adopted from the manuscript by Ahmed and colleagues (Ahmed et al., 2019).

Note: DEs are mainly found within the $CD5^{+}$ $CD19^{+}$ population and are defined as lymphocytes that coexpress TCRαβ and surface immunoglobulin (BCR). According to the surface immunoglobulin expressed, DEs are divided into $TCR\alpha\beta^{+}$ IgD^{+} or TCR^{+} IgD^{-} subpopulation. The latter are often class-switched to either IgG or IgA. The phenotype of $TCR^{+}IgD^{-}$ cells should be confirmed by staining for the expression of IgG or IgA. For more details about the gating strategy, please refer to Figures 1 and 2 and supplementary data in the original manuscript (Ahmed et al., 2021).

⚠ CRITICAL: While gating DEs, an important step is to exclude doublets. We do so in 2 ways: 1) as mentioned in step 14, and 2) by ensuring that DEs that are gated are $CD5^{low}$ and TCR^{low} (See Figure 3). We believe that the $CD5^{high}$ and $TCR\alpha\beta^{high}$ cells are mostly doublets and not DEs.

Part II. Strategy for sorting DE cells

Preparation of single-cell suspensions and antibody staining

Timing: 2–3 h

18. We sort DEs using about 50 $\times 10^{6}$ fresh PBMCs initially suspended in 500 μL FACS buffer.
    a. About 50 μL (~ 5 $\times 10^{6}$ cells) will be used for preparing the single color compensation and unstained controls.
    b. The remaining 45 $\times 10^{6}$ cells in about 450 μL will be used for sorting.

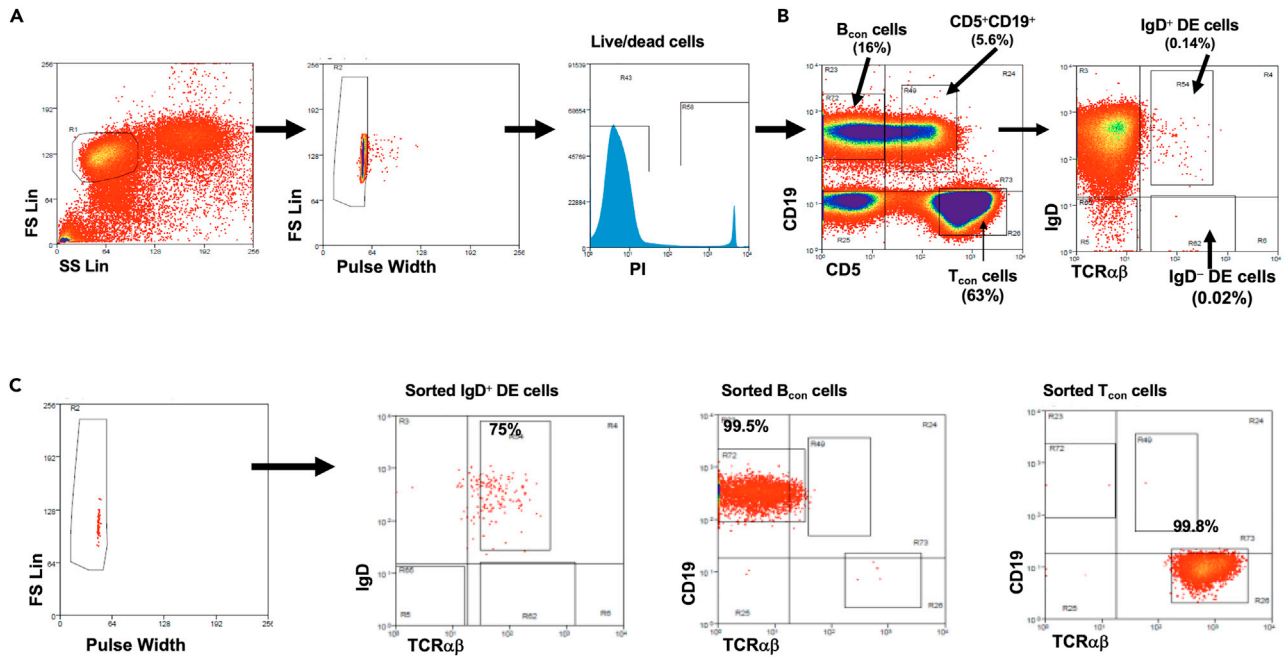

Figure 2. Steps used to sort and verify identity of DEs, Bcon, and Tcon

(A) In this series of dot plots, consecutive gating was used to identify lymphocytes and exclude doublets and dead cells, respectively, among PBMCs stained with the four-antibody cocktail.

(B) Left dot plot (CD5 vs CD19) shows how gated live lymphocyte singlets are divided into $CD5^{-}CD19^{+}$ Bcon, $CD5^{+}CD19^{-}$ Tcon, and $CD5^{+}CD19^{+}$ subpopulations – The identities of $CD5^{-}CD19^{+}$ cells and $CD5^{+}CD19^{-}$ cells as Bcon and Tcon cells are confirmed by analysis each subpopulation for the lack or expression of the TCRαβ, respectively (not shown). Right dot plot (IgD vs TCRαβ) shows how gated $CD5^{+}CD19^{+}$ cells are used to identify DE subsets. DEs are divided into a major $CD5^{+}CD19^{+}TCR\alpha\beta^{+}IgD^{+}$ subset and a minor $CD5^{+}CD19^{+}TCR\alpha\beta^{+}IgD^{-}$ cells (contains class-switched DEs, see Ahmed et al. Ahmed et al. (2019) for details).

(C) This series of plots shows the steps used to verify identity of sorted DEs by re-running an aliquot of sorted DEs. Bcon, and Tcon cells. Numbers in quadrants indicate percentages.

19. We use the same staining strategy and the antibody cocktail described in Part I: Identifying DEs by flow cytometry. See Table 1 for the antibody panel and dilution factors.
    a. Prepare and label single-color compensation control tubes and unstained control tube by pipetting 10 μL of PBMC suspension (5×10^5 cells) from the "master tube" into each control tube. Then add 90 μL FACS buffer for a total volume of 100 μL in the compensation control tubes.
    b. Stain the sorting sample using 20 μL of each fluorophore-conjugated antibody and stain the compensation control tubes using 5 μL of antibody.

Note: It is always recommended that you identify and use appropriate antibody concentrations.

Note: Importantly, we use propodeum iodide (PI) – however, you can use your preferred viability dye – for detection and exclusion of dead cells. We perform this step after staining and immediately before running samples and data acquisition (see step 21 in Part II: Sorting of DEs and autologous Tcon and Bcon cells).

    c. Gently vortex each FACS tube for mixing cells and homogeneous staining.
    d. Incubate tubes for 20 min on ice or at 4°C, protected from light.
    e. Wash samples at least three times using 2–3 mL of FACS buffer each time.
    f. Finally, resuspend the sorting sample into a total volume of 3 mL and each of the compensation control samples into a total volume of 500 μL of FACS buffer. Now, the samples are ready for sorting.

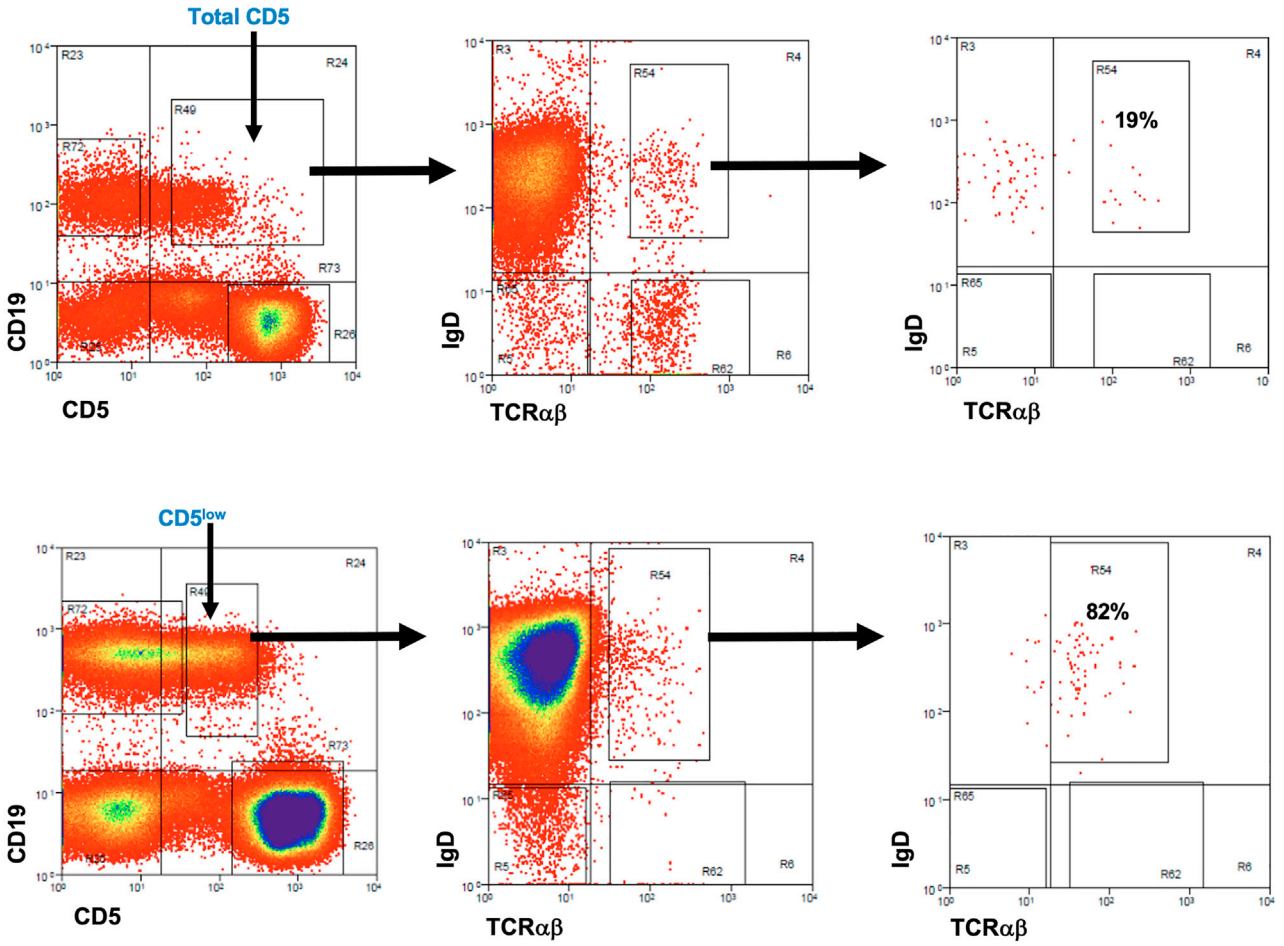

Figure 3. Exclusion of CD5^{hi} and TCRαβ^{hi} cells from analysis enhances purity of sorted DEs

In this experiment, a PBMC sample from T1D patients was stained using our four-antibody cocktail, acquired and two gating strategies used to identify and sort DEs as shown below. Purity was determined by rerunning sorted DEs. **Top panel.** The plots show how inclusion of all CD5^{+} cells (left plot) and TCRαβ^{+} cells (middle plot) significantly lowers purity of sorted DEs (right plot). The number in the right plot shows percentage of bona fide DEs (19%) among sorted cells. **Bottom panel**. The plots show how restricting gating to CD5^{low}CD19+ (left plot) and TCRαβ^{low} cells (middle plot) cells significantly increases purity of sorted DEs (right plot). The number in the right plot shows percentage of bona fide DEs (82%) among sorted cells.

Sorting of DEs and autologous Tcon and Bcon cells

We use High Speed MoFlo Legacy Cell Sorter - Beckman Coulter – However, you can use a sorter of your choice.

Timing: 6–8 h

20. Immediately before sorting, filter the "sort sample" using a FACS tube with 35 μm cell strainer cap to eliminate residual cell clumps.
21. Add PI to identify and exclude dead cells using stock solution of 1 mg/mL and final dilution of 1–2 μg / mL.
22. Setting up the compensation and gating strategy
    a. Acquire approximately 10,000 events from each compensation tube to perform compensation setup.
    b. Finalize the compensation using the fully-stained sample and make final adjustments as necessary (see Figure 2).

23. Collection of sorted subpopulations
    a. Set up your sorter for sorting. We use a 70 μm nozzle, sheath pressure of 60 psi, drop drive frequency of 95–98 kHz. The flow rate for sorting is approximately 1000 events/second.
    b. Sort your sample into three or four subpopulations, depending on whether DEs are sorted as separate (IgD^+ and IgD^-) subsets or as a one mixed population. The other two subpopulations are B_{con} and T_{con} cells.
    c. Sort DEs (IgD^+ and IgD^-) into 1.5 mL microcentrifuge tubes each containing about 700–800 μL of lymphocyte collection medium (See materials and equipment).
    d. Sorted B_{con} and T_{con} cells into 5 mL polypropylene tubes each containing 1 mL of lymphocyte collection medium.

Note: You can sort cells into your own preferred medium that is appropriate with the goal of your experiment.

    e. **Purity Check.** Checking the purity of sorted cells is an extremely crucial step in validating identity of sorted DEs and whether they are contaminated by doublets (which usually fall apart during a sample re-run).
        i. To check purity of sorted DE cells, re-run an aliquot of sorted DE cells (100–150 μL) on the sorter using the original gating strategy.

Note: Because of the low number of collected events please do not wash, re-stain or centrifuge samples to avoid cell loss; also note that the flow rate rarely exceeds 1000 event/sec during a re-run.

Note: If necessary, you can dedicate a full sorting session for checking the validity of your gating strategy and use the information gained to inform future sorting strategies.

        ii. Determine the percentage of DE cells in sorted sample by using the same gating strategy.

Note: We generally achieve 70%–90% purity for sorted DE cells and 99% for B and T cell populations.

24. Sorted cells are ready to be used for specific purposes.

Part III. Generation of Epstein-Barr Virus (EBV)-immortalized DE lymphoblastoid cell lines (LCLs) and single DE clones

This section details our method for immortalizing DEs using EBV to generate DE lymphoblastoid cell lines and clones using an established method (Traggiai et al., 2004). All steps should be performed under sterile conditions in a biosafety cabinet.

Timing: 4–6 weeks

The procedure requires culturing sorted DEs onto fibroblast feeder layers in the presence of infectious EBV as outlined below:

25. Day 1: Preparation of fibroblast feeder layers
    a. Thaw 1 vial of irradiated-fibroblasts (ATCC 55-X) using the same method described in the "thawing cryopreserved PBMCs" section. Each vial usually contains 1×10^6 cells in 1 mL.
    b. Resuspend fibroblasts to a cell density of 2.5×10^5 / mL using CTM.
    c. Gently pipette your sample up and down with a serological pipette to thoroughly mix the cells.
    d. Plate 200 μL of irradiated-fibroblasts onto wells of a 96-well flat bottom tissue-culture plate (50,000 cells / well).

e. Incubate the tissue culture plate overnight (18 h) at 37°C in a humidified incubator with 5% CO_2. It is recommended that you subculture at least once on fibroblast-coated wells to ensure robust growth.

26. Day 2: Sorting and culturing DEs on fibroblast layers in the presence of EBV-infectious medium
    a. Sort DEs, using the method described in Part II, using freshly isolated PBMCs (See Figure 2).
    b. Prepare infectious medium by diluting 1 mL EBV solution (B95-8 cells - ATCC VR-1492) to 30% in CTM containing 2.5 μg/mL CpG ODN 2006 (ODN7909)
    c. Suspend sorted DEs in the EBV-infectious medium
    d. Seed DEs onto the fibroblast feeder layers using 50 or 100 DE cells / well.

27. Day 3–7: Check cultures for DE growth
    a. Check cultures on a daily basis under light microscope for cell growth and any sign of contamination.
    b. After about one week, clusters of dividing cells become visible in culture wells under light microscopy. As time progresses, dividing cells will make clumps that are visible macroscopically and culture medium begins to turn yellow.
    c. Maintain the culture medium by replacing half of the spent medium with fresh infectious medium (CTM containing 2.5 μg/mL CpG ODN, 2006) usually when cell density reaches to 80%–90%.
    d. Typically, culture medium will need replenishment every 5–7 days.
    e. EBV-transformed DEs gradually expand and cultures need to be scaled up by transferring contents into wells of 24-well tissue culture plate and then onto wells of 6-well plates.

    Optional: you can subculture on fibroblast feeding layers to enhance viability and growth.

    f. Expand cultures to 75–100 mL using flasks over the next few weeks. These lines are referred to as DE lymphoblastoid cell lines (LCLs). Store generated LCLs at −80°C or liquid nitrogen.

    Optional: you can subculture on fibroblast feeding layers to enhance viability and growth.

28. Generation of single clones from immortalized DE LCLs using limiting dilution: Use LCL to generate single DE clones, using standard limiting dilution methods (Hamad et al., 1994). Serially dilute and seed 10, 3.3, 1.1, and 0.33 DEs onto fibroblast coated 96-well microplates using 100 μL of CpG 2006 containing CTM as described above. Using clones from 0.33 cell / well ensures a maximum of one cell per well.

    Note: Potential uses of clones include **1)** Antibody production, and **2)** Cloning, expression and generation of recombinant BCR and TCR from the same DE cell.

    Note: **Cryopreservation of DE LCLs and clones:** When freezing down cells, centrifuge cells at 500 × *g* for 5–6 minutes at room temperature. Remove supernatants and re-suspend cell pellets in cold freezing media at 0.5–1 × 10^7 cells / mL. Place tubes in liquid nitrogen for long term or −80°C for short term storage.

EXPECTED OUTCOMES

This protocol allows you to detect DEs in PBMC samples using flow cytometry and to simultaneously analyze DE, Tcon, and Bcon in the same sample. The protocol also outlines the steps for sorting DEs along with autologous Tcon and Bcon cells. Furthermore, the protocol describes how to generate immortalized DE lymphoblastoid cell lines and single clones that can be used for production of recombinant BCRs and TCRs and analysis of their TCR and BCR repertoires. Some key steps are shown in Figures 1, 2, and 3.

LIMITATIONS

DEs are a rare population of lymphocyte so any flow cytometric analysis should include acquisition of sufficient numbers of PBMCs (500,000–1,000,000 events) in order to ensure detection of sufficient numbers of DEs. The frequency of DEs varies between patients and healthy controls, and they usually comprise between 1%–3% of $CD5^{+}CD19^{+}$ cells.

When gating DEs, it is crucial to exclude doublets. To do this, gate on single cells using the FSC-Area vs FSC-Height and SSC-Area and SSC-Height plots. In addition, when gating the CD5+ CD19+ population, gate on $CD5^{low}$ but not $CD5^{high}$ cells and avoid $TCR\alpha\beta^{hi}$ cells (mostly or all doublets).

When sorted DEs are to be used for repertoire analysis, samples should be examined for both TCRαβ and BCR repertoires if cell number permits (detection of rearranged genes of both receptors confirms identity of sorted cells). Also, it is recommended to simultaneously analyze autologous T_{con} and B_{con} cells as controls.

When working with DEs from human PBMCs, it is important to remember that there are many variables outside of your control that can affect your analysis. For example, if your patients are taking an immunosuppressive medication, it may affect the way that the DEs respond to stimulation in your experiments and may dampen their effector functions. Our unpublished data show that DEs are highly sensitive to steroids that significantly reduce their frequencies.

TROUBLESHOOTING

Problem 1

Unable to observe distinct B_{con}, T_{con}, and CD5+ CD19+ populations (step 16 in Part I: Sequential gating strategy to identify DEs and analyze their phenotypes).

Potential solution

To troubleshoot this problem, you may try using fresh PBMCs, as opposed to cryopreserved PBMCs, because we find that DEs are prone to undergoing apoptosis if they are cryopreserved. In addition, you may titrate your antibodies to determine the optimal concentration of each antibody. Remember that with more colors used, detecting rare populations becomes more challenging.

Problem 2

Since DE cells are rare events, it is difficult to sort sufficient numbers of cells to work with for subsequent experiments (like antigen receptor repertoire analysis or cell culture related experiments) (step 23c in Part II).

Potential solution

We find that the frequency of DEs is rare and varies with different donors. To maximize the recovery of sorted DE cells, we suggest performing preliminary screening of DEs in samples from different donors using regular FACS analysis (as described above) to identify and select samples with higher frequencies of DEs for sorting experiments.

Note: Generally, if we start with 5×10^7 PBMCs, we often sort 800 to 1000 DE cells.

Problem 3

Poor EBV transformation rate (Part III).

Potential solution

To improve the transformation efficiency, we suggest considering the following steps:

Try different numbers (10, 50, 100, 500 cells per well) for immortalization to generate LCLs.

Check the viability of fibroblast cells before preparing feeder layers (especially if cells are frozen). Viability should be more than 95%–98%.

Use fresh PBMCs as the recovery of live sorted DE cells is often better compared to using cryopreserved PBMCs.

Problem 4

Slow or poor growth of immortalized DE clones (Part III).

Potential solution

We observed that adding human plasma to CTM significantly improves growth of EBV immortalized single DE cells after performing the limiting dilution. We would also suggest testing mycoplasma contamination if the cultures have slow or poor growth for a long period of time.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Abdel Rahim Hamad (ahamad@jhmi.edu).

Materials availability

This study did not generate new unique reagents.

Data and code availability

This study did not generate new unique reagents than published in the original manuscript Ahmed et al. (2019).

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AUTHOR CONTRIBUTIONS

R.A., K.A., and Z.O. conducted the experiments, interpreted data, and wrote the paper; H.Z. conducted sorting of DEs; N.M. critically read and offered constructive edits to the manuscript; A.R.A.H. supervised the study and edited the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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