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Use of Chlamydial Elementary Bodies as Probes to Isolate Pathogen-Specific Human Monoclonal Antibodies

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Abstract

Chlamydia trachomatis is one of the most prevalent sexually transmitted infections in the world and the leading cause of infectious blindness. The role of antibodies in the prevention and clearance of infection is still not fully understood, but the analysis of the immunoglobulin response to novel vaccine candidates is an important part of many of these studies. In this chapter, we describe a novel method to identify and isolate *Chlamydia*-specific memory B cells by fluorescence-activated cell sorting (FACS) using fluorescently-labeled whole bacteria from cryopreserved human PBMC samples. This method allows for live single cells to be sorted for cell culture, in vitro assays, single-cell RNA sequencing, and cloning of paired heavy and light chains for recombinant monoclonal antibody production.

Keywords

Antigen-specific, B Cell, Antibody, Chlamydia, Elementary Body, Immunoglobulin, IgG

1. Introduction

Chlamydia trachomatis is a Gram-negative intracellular bacterial pathogen, responsible for over 100 million sexually transmitted infections a year (1) and nearly 40 million active cases of trachoma, an ocular chlamydial infection and the leading cause of infectious blindness (2). While protective immunity can be developed in the host by infection, damage caused by the inflammatory response to the bacteria can cause scarring in infected tissues, resulting in infertility or blindness (3).

There has therefore been substantial effort to develop a vaccine to *C. trachomatis* that would prevent infection and clear the bacteria before damage can occur. Initial work focused on live or attenuated whole-organism vaccination, and while capable of inducing a protective immune response, this

protection was often short-lived and highly serovar-specific (4, 5). More recent vaccine designs have used purified or engineered protein antigens, primarily the immunodominant major outer membrane protein (MOMP), which accounts for over 60% of the surface of the elementary body (EB), the extracellular, infectious form of *Chlamydia* (6–9).

The immune response to *Chlamydia* has been equally well studied, mainly using the related *C. muridarum* in mouse models. It has been shown that primary immunity to *Chlamydia* is reliant on CD4 T lymphocytes (10, 11), data which is supported by the observation that CD4-deficient HIV patients are more likely to develop *Chlamydia* infection and be affected by long-term sequelae of infection (12). The role of antibodies and B lymphocytes in immunity to *Chlamydia* is less well defined, although it has been shown that the presence of specific antibodies at the site of exposure can prevent infection to the same degree as CD4 T cells (13, 14).

For these reasons, analysing the antibody response to *Chlamydia*, either from infection with the bacteria or immunisation with a novel vaccine candidate, is of crucial importance. Immunoglobulin repertoire analysis has been significantly advanced in the last two decades with new developments in single-cell sorting and sequencing techniques. It is now possible to analyse the antibody response on a monoclonal basis, identifying individual antibodies against a specific immunogen that can be useful for research, diagnostics, or design of new and more immunogenic vaccines (Figure 1). Fluorescent antigen probes have been of particular use in pre-screening and enriching for antigen-specific B cells during the cell sorting process (15–20).

This chapter will outline the use of whole fluorescently-labeled elementary bodies to isolate human memory B cells specific to *C. trachomatis* serovars, derived from exposure to both natural infection with trachoma and to a novel MOMP-based vaccine antigen.

2. Materials

2.1. PBMC Isolation and Cryopreservation

1. Phosphate-buffered saline (PBS).

2. Histopaque-1077 [Sigma-Aldrich].
3. PBS containing 1% bovine serum albumin (PBS/BSA).
4. Pasteur pipettes.
5. Freezing medium: 90% foetal bovine serum (FBS), 10% dimethyl sulfoxide (DMSO).
6. Cell freezing chamber.

2.2. EB Staining

1. SPG buffer: 200 mM sucrose, 20 mM sodium phosphate, 5 mM glutamic acid.
2. Fixation buffer: 3.7% formaldehyde in sterile purified water.
3. Vybrant™ DiO Cell-Labeling solution [Thermo Fisher Scientific].
4. 1 mL syringe with 27G needle.
5. *C. trachomatis* elementary bodies, stored at -80°C in SPG buffer.

2.3. Cell Sorting

1. RPMI medium (R0).
2. Amine-reactive viability dye [e.g. Zombie Fixable Viability Dye from Biolegend].
3. Fluorescent antibodies against CD3, CD14, CD19, CD27, CD38, IgG, IgM, IgD, and IgA.
4. 5 mL Polystyrene tubes for FACS.
5. 35 µm nylon mesh.
6. Compensation beads for antibodies [e.g. CompBeads from BD Biosciences]
7. Compensation beads for viability dye [e.g. ArC Amine Reactive Compensation Bead Kit from Thermo Fisher Scientific].
8. Cell sorter with suitable number of channels, using a 70 µm nozzle [e.g. FACS Aria III from BD Biosciences]

3. Methods

3.1. PBMC Isolation and Cryopreservation

Due to the nature of human studies, the collection of blood samples is subject to the availability of the individuals to be sampled and trained phlebotomy personnel. Blood sampling may also take place a considerable distance from the researchers. Therefore, blood samples are often processed by isolation and cryopreservation of peripheral blood mononuclear cells (PBMCs), which can then be thawed for downstream analysis. This protocol was designed using cryopreserved PBMC samples ranging in storage time from several months to over 10 years, but could easily be adapted for use on freshly isolated PBMCs.

- 1) Transfer blood samples from collection tubes to 50 mL centrifuge tubes and dilute with an equal amount of PBS (*see Note 1*).
- 2) Pipette 15 mL of room temperature Histopaque into a 50 mL centrifuge tube. Carefully layer no more than 30 mL of the diluted blood on to the Histopaque layer. Repeat for as many tubes as needed for the complete diluted blood sample.
- 3) Centrifuge the layered blood for 20 minutes at 800 x g, ensuring that the brake is disabled.
- 4) Carefully remove the tubes from the centrifuge. Using a serological pipette, remove as much of the top layer of plasma as possible without disturbing the thin band of PBMCs below. The plasma can be discarded or stored for further analysis.
- 5) Using a Pasteur pipette, transfer the thin PBMC layer below the plasma to a new 50 mL centrifuge tube.
- 6) Top up the tube to 45 mL with 1% PBS/BSA and centrifuge for 10 minutes at 200 x g at room temperature, with the brake turned on.
- 7) Remove the tube from the centrifuge and pour off the supernatant. Resuspend the cell pellet in 45 mL of 1% PBS/BSA and centrifuge for 10 minutes at 200 x g at room temperature, with the brake turned on.

- 8) Pour off the supernatant and resuspend the cells in 1 mL of 1% PBS/BSA. Count the cells using any preferred method.
- 9) Centrifuge the cells for 5 minutes at 350 x g at room temperature. Remove the supernatant and resuspend in freezing medium to a concentration of 1×10^7 cells/mL.
- 10) Aliquot the cell suspension into cryovials at a volume of 1 mL per tube. Place the tubes in a cell freezing chamber and transfer the chamber to a -80°C freezer. After 24 hours, the tubes can be moved to a -150°C freezer or a liquid nitrogen storage vessel. This allows samples to be stored and shipped prior to downstream analysis.

3.2. Preparation of Labeled EBs

Elementary bodies (EBs) of *C. trachomatis* should be purified and quantified (*see Note 2*) using a preferred in-house method, which will not be covered here. For the purpose of this protocol, aliquots containing 10^7 purified EBs in SPG buffer should be prepared from a stock and stored at -80°C.

Labeled EBs should be prepared fresh from these aliquots and used for cell staining on the same day.

- 1) Thaw an aliquot of 10^7 EBs on wet ice for 5 – 10 minutes or until liquid.
- 2) Add 1 mL of PBS to the EB sample and transfer to a 1.5 mL microcentrifuge tube. Centrifuge for 20 minutes at $>20,000 \times g$ at 4°C (*see Notes 3 and 4*).
- 3) Carefully aspirate the supernatant using a 200 μ L pipette without disturbing the EB pellet area.
- 4) Add 500 μ L of prepared fixation buffer to the tube and vortex to resuspend the EBs. Incubate for 20 minutes at room temperature.
- 5) Add 500 μ L of PBS to the tube and vortex. Centrifuge for 20 minutes at $>20,000 \times g$ at 4°C.
- 6) Carefully aspirate the supernatant using a 200 μ L pipette without disturbing the EB pellet.
- 7) Add 500 μ L of PBS containing 1 μ L of Vybrant DiO staining solution to the EBs. Vortex to resuspend them and incubate for 20 minutes at 37°C protected from light.

- 8) Add 500 μ L of 1% PBS/BSA and transfer the EB suspension to a new 1.5 mL microcentrifuge tube (*see Note 5*).
- 9) Centrifuge for 20 minutes at $>20,000 \times g$ at 4°C. Carefully aspirate the supernatant using a 200 μ L pipette without disturbing the EB pellet.
- 10) Add 100 μ L of 1% PBS/BSA to the tube and vortex to resuspend the EBs. Keep this suspension on ice protected from light.
- 11) Immediately prior to use, pass the EB suspension through a 27G needle 5-10 times to break up any large aggregates.

3.3. Staining of Memory B Cells

- 1) Thaw cryopreserved PBMCs by placing the frozen tube directly into a 37°C water bath until almost all of the sample has thawed.
- 2) Transfer the entire sample to a 50 mL centrifuge tube and top up to 30 mL with room temperature R0 media. Add the media dropwise to the cell suspension using a serological pipette while gently rocking the tube to mix the contents.
- 3) Centrifuge the cell suspension for 5 minutes at $500 \times g$ at 4°C.
- 4) Pour off the supernatant and resuspend the cell pellet in 45 mL of R0. Centrifuge the cell suspension for 5 minutes at $500 \times g$ at 4°C.
- 5) Pour off the supernatant and resuspend the cell pellet in 1 mL of PBS. Count the cells using any preferred method.
- 6) Top up the cell suspension to 45 mL with PBS. Centrifuge the cell suspension for 5 minutes at $500 \times g$ at 4°C.
- 7) Aspirate the supernatant and resuspend the pellet in 100 μ L of PBS. Transfer the cell suspension to a 5 mL FACS tube.

- 8) Add the recommended volume of viability dye based on the cell count from step 5. Incubate the cells at room temperature for 20 minutes in the dark.
- 9) Add 1 mL of 1% PBS/BSA to the cells. Centrifuge for 5 minutes at 350 x g at 4°C and pour off the supernatant.
- 10) Resuspend the cell pellet in the 100 µL of labelled EB suspension from section 3.2. Incubate the cells at room temperature for 20 minutes in the dark.
- 11) Add 1 mL of 1% PBS/BSA to the cells and centrifuge for 5 minutes at 350 x g at 4°C.
- 12) Prepare a master mix containing titrated volumes of fluorescent antibodies against CD3, CD14, CD19, CD27, CD38, IgM, IgD, IgG, and IgA, to a total volume of 100 µL, in 1% PBS/BSA (*see Notes 6 and 7*).
- 13) Pour off the supernatant from the cell pellet and resuspend in 100 µL of the antibody master mix. Incubate for 20 minutes on wet ice.
- 14) Add 1 mL of 1% PBS/BSA to the cells. Centrifuge for 5 minutes at 350 x g at 4°C and pour off the supernatant.
- 15) Resuspend in 500 µL of 1% PBS/BSA. Pass the cell suspension through a 35 µm mesh to remove cell aggregates prior to sorting. Keep the cells on ice and in the dark until loading into the sorter.

3.4. Sorting of Chlamydia-specific Cells

- 1) The sorter should be fitted with a 70 µm nozzle for optimal sorting and viability of human immune cells.
- 2) Set the compensation values for the sort using single-stained compensation beads for each of the fluorescent antibodies and the viability dye.
- 3) Use a fluorescence minus one (FMO) control stained with viability dye and fluorescent antibodies but lacking the DiO-labeled EBs to set the gates for the sort. Acquire at least 10⁶ events on the cell sorter from the FMO sample (*see Note 8*).

- 4) To gate for the cells of interest (see Figure 2), first create a plot examining FSC-A and SSC-A. Adjust the threshold value of FSC-A to remove small debris from the analysis, and set a gate around the lymphocyte population.
- 5) From the lymphocytes, create a plot with FSC-A and FSC-H in order to define singlets and remove aggregates from the analysis.
- 6) From the singlet gate, create a plot of CD3/14 and CD19. Set a gate around the CD19⁺ CD3/14⁻ population to identify the B cells.
- 7) Within the B cell population, create a plot of CD27 and CD38. Set a gate around the CD27⁺ CD38⁻ population to identify memory B cells (MBCs).
- 8) Within the MBC population, create a plot of IgG and IgA. Set a gate around the IgG⁺ IgA⁻ population (*see Note 9*).
- 9) To remove any mistakenly-gated unswitched memory B cells, create a plot within the IgG⁺ IgA⁻ population of IgM and IgD. Set a gate around the IgM⁻ IgD⁻ population to identify the IgG⁺ MBCs.
- 10) Within the IgG⁺ MBC population, create a plot to show DiO. Set the DiO⁺ gate immediately above the negative population shown in the FMO control.
- 11) Using these gates, sort the DiO⁺ IgG⁺ MBC population into a tube or plate containing a buffer dependent on downstream analysis, such as culture media or RNA preservation buffer (*see Note 10*).

4. Notes

1. Dilution of blood is needed to reduce the density of the sample so as to not ‘overload’ the Histopaque layer and reduce PBMC recovery. If the blood sample has been kept for several hours before PBMC isolation, it may be necessary to increase the dilution with PBS to 2:1 to counter potential aggregation.

2. Purified EB samples are commonly quantified in inclusion forming units (IFU) by in vitro assays (21). However this method does not count the many non-infectious EBs that will still be stained by this protocol and will bind to and label cells. Therefore it is advised to quantify the EBs prior to this protocol using a method to measure absolute particle number or total protein.
3. When pelleting EBs during the staining procedure, a microcentrifuge with a maximum speed of >20,000xg should be used. However a higher speed rotor with a maximum speed of >30,000xg can also be used for a higher recovery of EBs.
4. Due to the small size of the EBs, a pellet will not be visible with the numbers used here. Before loading the tube into the centrifuge, use a marker pen to indicate the side of the tube that will be facing the outside edge of the rotor where the EBs will pellet.
5. DiO is a hydrophobic dye and requires strong vortexing to mix into an aqueous solution. It will also adhere to the walls of microcentrifuge tubes, so after incubation of the EBs with DiO it is necessary to transfer the EB suspension to a fresh tube to limit contamination of the labelled EBs with free DiO.
6. Both CD3 and CD14 are used to identify abundant non-B cells in the PBMC sample and as such can be conjugated to the same fluorochrome in a 'dump' channel.
7. An IgG antibody should be identified that does not bind to the antigen-binding portion of the IgG molecule and would inhibit binding of the EB to the B cell.
8. The FMO should be an identically-processed PBMC sample, thawed and stained concurrently with the experimental sample(s). However this sample does not need to contain B cells reactive against *C. trachomatis* EBs and thus can be isolated from a control sample, removing the need to use a valuable experimental sample.
9. *C. trachomatis*-reactive MBCs can also be identified and sorted from the IgA⁺ population using a similar gating strategy that includes removal of IgM⁺ and IgD⁺ MBCs.

10. If *C. trachomatis*-reactive B cells are to be sorted for the purpose of cloning and /or sequencing of the Ig mRNA, the sorting procedure should take place under RNA-sterile conditions.

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Figure Captions

Figure 1 – Schematic Showing Process of Antibody Isolation and Vaccine Development

Following vaccination with a novel immunogen, PBMCs are isolated from blood taken from the vaccine recipients and are stained using fluorescent elementary bodies (EBs) that are prepared concurrently with the PBMCs. This staining allows for antigen-specific B cells to be sorted and the immunoglobulin genes cloned and produced recombinantly, where they can be analysed for affinity, specificity, and neutralization potential. This data can then be used to design a new immunogen better able to push the humoral immune response towards epitopes that were shown to be beneficial.

Figure 2 – Gating Strategy for Identification of EB-specific IgG Memory B Cells

Lymphocytes are first gated on an FSC-A vs SSC-A plot, followed by gating of single cells on an FSC-A vs FSC-H plot. Live cells are then gated followed by B cells, identified as CD19⁺ CD3/14⁻. Within the B cell population, memory B cells are gated as CD27⁺ CD38⁻. Memory B cells are then selected by Ig isotype of interest, in this case IgG or IgA. IgM/D⁺ memory B cells are then excluded before gating of the EB-DiO⁺ cells.