Chapter 7

Multiplex ImmunoSpot® Assays for the Study of Functional B Cell Subpopulations

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Abstract

B cells mediate humoral immunity by producing antibody molecules, but they also participate in innate and acquired immune functions via the secretion of effector molecules such as cytokines, chemokines, and granzyme. B cell subpopulations releasing such effector molecules have been implicated in immunobiology and a number of diseases.

Unlike antigen-specific T cells that can be identified by multimer staining, and then counter-stained to define T cell subpopulations, antigen-specific B cells cannot be detected by flow cytometry. Staining antigen-specific B cells with labeled antigen, in large, has been unsuccessful. Instead, antigen-specific B cells can be and are commonly studied by ELISPOT. In the ELISPOT approach, the B cell is identified via the antibody that it secretes being captured on a membrane by the antigen itself. Should it be feasible to measure simultaneously antibody production and the secretion of other secretory B cell products, it would then be possible to identify B cell subpopulations that co-express effector molecules. Here we introduce multiplex ELISPOT assays in which measurements of antibody secretion are combined with the detection of Granzyme B, IL-6, IL-10, IFN-γ, and TNF-α. Such multiplex assays will help define effector B cell subpopulations, as well as the understanding of their role in health and disease.

Key words ELISPOT, Fluorospot, B cell subpopulation, Effector B cell, Multiplexing, Granzyme B, B-reg, IL-10, Co-expression, Antibodies, Polyfunctional B cells, IL-10, IL-6, TNF-α, IFN-γ, Immune monitoring, Antigen-specific B cell, Be-1, Be-2, Effector plasma cells, Cytokine, Chemokine

1 Introduction

Each B cell has a unique antigen specificity endowed by its antigen receptor, a membrane-anchored immunoglobulin molecule. When a B cell engages in an immune response, its progeny, plasma cells, will secrete immunoglobulins with the same hypervariable region, that is, with the same antigen specificity. These hypervariable regions, however, will be combined with different constant regions defining the antibody class and subclass [1]. While each B cell can secrete only one class/subclass of antibody, the different classes/subclasses mediate various effector functions due to

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their differential ability to neutralize antigen, activate complement, promote phagocytosis, bind to mast cells, and be exported to mucosal surfaces [2]. When defining antibody-mediated B cell effector functions, therefore, mainstream interest has focused on the delineation of the class and subclass of the antigen-specific B cell repertoire. This can be readily done in the ELISPOT format (but not by flow cytometry) when seeding B cells over antigen-coated membranes [3]. If a B cell is specific for the test antigen, the antibody it secretes will bind to the antigen-coated membrane around the secreting B cell and will be retained as an antibody "spot." This type of membrane-bound antibody "spot" can then be identified by using immune globulin class- and subclass-specific detection reagents. In this way, not only the frequency of antigen-specific B cells within peripheral blood mononuclear cells (PBMC) can be established, that is, the clonal size/magnitude of B cell memory, but also its quality, as the frequencies of B cells will be determined for each immunoglobulin class and subclass. Multiplex ELISPOT assays permitting measurement of all antibody classes and subclasses simultaneously are described in a dedicated chapter of this book [4].

An alternative method for detecting antigen-specific B cells begins with coating the membrane with immunoglobulin-specific capture antibody, for example, antibodies specific for the κ/λ light chains. When B cells secrete antibodies, they first will be captured, irrespective of their antigen specificity. However, the addition of labeled antigen in a second step permits to identify those "spots" formed by B cells that produced antigen-specific antibodies [5].

In addition to secreting antibodies, B cells also exert immune functions via the secretion of cytokines, chemokines, and Granzyme B [6]. While antigen-specific B cells can be reliably detected in ELISPOT assays, as described above, they cannot be readily identified by flow cytometry. Unlike T cells, which can be stained with antigen-containing multimers [7], antigen-specific B cells typically do not stain with labeled antigen. Therefore, while the definition of functional T cell subpopulations can be achieved by counter-staining of multimer-positive T cells for other markers, defining subclasses of antigen-specific B cells cannot be done by flow cytometry. The point of reference for the multiplex B cell ELISPOT assays described here for identifying functional B cell subpopulations is therefore the identification of the antigen-specific B cell via the antibody it produces. B cells that secrete effector molecules other than that antibody will produce a double positive ELISPOT in an assay that captures both the antibody and the effector molecule of interest [8]. Such assays can be multiplexed by introducing an increasing number of analytes, presently up to six, next to the antibody to be captured as the seventh color. The principle of 7 color B cell ELISPOT assays is described in a chapter [4], and that of multicolor ELISPOT analysis in another chapter [9] of this book.

In this chapter, we focus on the detection of Granzyme B, IL-6, IL-10, IFN-γ, and TNF-α co-secretion along with immunoglobulin. Although, in essence, any secretory product of B cells should be measurable taking this approach, we narrowed in on these analytes because they have been the primary ones implicated in B cell biology and immune pathology [6]. Due to the extensive literature published on this topic, we provide just a few examples here illustrating the significance of studying B cell subpopulations in health and disease.

B cells that produce cytokines can be further subdivided into regulatory and effector subsets. The regulatory subset, B-regs, produce IL-10 and/or TGF-β, whereas the Be-1 effector subset secretes IFN-γ, IL-12, and TNF-α, and Be-2 cells produce IL-2, IL-4, and IL-6 [10]. In addition, B cells can engage in Granzyme B (GzB) production following stimulation with IL-21 alone, or with the combination of IL-4 and IL-10 (but not other cytokines) [11]. Additionally, B cell receptor triggering can result in Granzyme B secretion within less than 24 h. These B cells however do not secrete Perforin, Granzyme A, or TRAIL. The selective GzB response occurs simultaneously with the upregulation of antigen presentation on the B cell, including MHC class II and adhesion molecule (CD54) expression. It is not yet known what the physiological role of Granzyme B production by B cells is, in particular because it happens in the absence of Perforin and FAS secretion, but recent findings suggest it may play a role in early antiviral immunity, the regulation of autoimmunity, and in cancer immunosurveillance [12]. Increased Granzyme B secretion by B cells has also been linked with recent vaccination [11] and infectious disease [13].

B cells of patients with multiple sclerosis (MS) have been reported to secrete high levels of IL-6 [14], and this cytokine, when produced by B cells, aggravated disease severity in the animal model of MS, experimental allergic encephalomyelitis, EAE [15]. IL-15 production by B cells was also elevated in MS, and was linked to the ability of B cells to penetrate the blood brain barrier [14]. GM-CSF and IL-10 production by B cells was shown to be reciprocally regulated in MS patients [16], and B cell-derived IL-10 has been implied in controlling EAE [15].

In systemic lupus erythematosus (SLE), B cells produce increased amounts of IL-1α, IL-4, and IL-6 [6], and in B cell chronic lymphocytic leukemia, IL-8 production by B cells has been linked with disease progression [6]. B cell-derived TNF-α is required for lymphoid structure development, and for sustained antibody production [6]. IL-17 production by B cells was shown to exert a protective role in the immune control of the parasite *Trypanosoma cruzi* [17].

Much remains to be elucidated regarding the biological significance of B cell subsets in health and disease, but it has become clear that these subsets exert major contributions to immunity and

provide valuable biomarkers for disease and pathology. Multiplex B cell ELISPOT assays that detect the co-secretion of various effector molecules along with antibodies may facilitate substantial progress in this field.

2 Materials

2.1 Instrumentation and Software

1. ImmunoSpot® Series 6 Ultimate Analyzer (Cellular Technology Limited (CTL), Cat # S6UTM12).
2. ImmunoSpot® 7.0 Software, (CTL, Cat # IPS01).
3. ImmunoSpot® LDA 1.4 Software (CTL, Cat # LDA01).

2.2 ImmunoSpot® Kits (See Notes 1 and 2)

Human Granzyme B Detection Kit (CTL, Cat # hT59).

Human IL-10 Detection Kit (CTL, Cat # hT24).

Human IL-6 Detection Kit (CTL, Cat # hT62).

Human IFN-γ Detection Kit (CTL, Cat # hT03).

Human TNF-α Detection Kit (CTL, Cat # hT12).

Human IgG Detection Kit (CTL, Cat # hB09).

Human IgA Detection Kit (CTL, Cat # hB17).

Human IgM Detection Kit (CTL, Cat # hB01).

Human Granzyme B/IgG/IgA Three-Color FluoroSpot Capture Kit (CTL, Cat # hCA3002F).

Human Granzyme B/IgG/IgM Three-Color FluoroSpot Capture Kit (CTL, Cat # hCA3003F).

Human IL-10/IgG/IgA Three-Color FluoroSpot Capture Kit (CTL, Cat # hCA3004F).

Human IL-10/IgG/IgM Three-Color FluoroSpot Capture Kit (CTL, Cat # hCA3005F).

Human IL-6/IgG/IgA Three-Color FluoroSpot Capture Kit (CTL, Cat # hCA3006F).

Human IL-6/IgG/IgM Three-Color FluoroSpot Capture Kit (CTL, Cat # hCA3007F).

Human IFN-γ/IgG/IgA Three-Color FluoroSpot Capture Kit (CTL, Cat # hCA3000F).

Human IFN-γ/IgG/IgM Three-Color FluoroSpot Capture Kit (CTL, Cat # hCA3001F).

Human TNF-α/IgG/IgA Three-Color FluoroSpot Capture Kit (CTL, Cat # hCA3008F).

Human TNF-α/IgG/IgM Three-Color FluoroSpot Capture Kit (CTL, Cat # hCA3009F).

2.3 Media

1. CTL-Test Medium (CTL, Cat # CTLT-005).
2. CTL-Anti-Aggregate Wash Supplement (CTL, Cat # CTL-AA-005).
3. RPMI-1640 (Lonza, Cat # BW12167Q).
4. L-Glutamine (Gibco, Cat # 25030-081).

2.4 Cells, Cell Separation, and Cell Counting

1. Cryopreserved human PBMC, (CTL, ePBMC, http://www.immunospot.com/ImmunoSpot-ePBMC).
2. Human B cell Isolation Kit, by negative selection (Stemcell Technologies, Cat # 17954) *or*
   EasySep Release Human CD19 Positive Selection Kit (Stemcell Technologies, Cat # 17754).
3. EasySep Buffer (Stemcell Technologies, Cat # 20144).
4. Live-Dead Cell Counting kit with hemocytometers (CTL, Cat # CTL-LD-100).

2.5 Miscellaneous

1. B-Poly-S (CTL, Cat # CTL-hBPOLYS-200).
2. Lipopolysaccharide (LPS) (Sigma, Cat # L 4391).
3. Phosphate-buffered saline, PBS (Hyclone, Cat # SH30028-02).
4. Tween-20 (Sigma, Cat # P1379) to prepare 0.05% Tween-PBS.
5. Distilled water (Millipore, USA).
6. Ethyl alcohol, EtOH, 190 proof (Sigma, Cat # 493511).
7. Wide-orifice tips for plating cells (Rainin, Cat # 17007101).
8. 5 mL Round-Bottom Tube, 12 × 75 mm style, with cap (Falcon, Cat # 352003).
9. 25 cm^2 sterile culture flasks (Corning, Cat # 430639).
10. 50 mL conical centrifuge tubes (Falcon, Cat # 352070).
11. Sterile Gauze (Covidien, Cat # 8044).
12. Cell Reservoir (Denville, Cat # P8826-ST).
13. Parafilm "M" (Hach, Cat # PM-996).
14. Vacuum Manifold (Millipore, USA).
15. 10 mL syringe Luer Lok tip (BD, Cat # 309604).
16. 0.1 μm syringe filters, low protein binding (Millipore, Cat # SLVV033RS).

3 Methods

3.1 Preparation and Stimulation of PBMC

1. Thaw cryopreserved PBMC by placing them in a 37 °C metal bead bath for 15 min. Wipe vial with 70% EtOH to sterilize, and invert twice to mix.

2. Transfer contents of cryovial to a 50 mL conical polypropylene tube. Rinse cryovial with 1 mL warm (37 °C) Anti-Aggregate solution to recover remainder of cells, and add these to the conical tube. Slowly add dropwise, while swirling, an additional 9 mL warm Anti-Aggregate solution to minimize osmotic gradient as DMSO gets diluted.
3. Centrifuge cells at 300 × *g* for 10 min at room temperature (RT) with max brake.
4. Decant supernatant. Resuspend pellet by flicking the bottom of the tube. Add 10 mL Anti-Aggregate solution.
5. Take 20 μL of cell suspension for counting, and mix well with 20 μL Live/Dead Cell Counting dye. Load into hemocytometer and count live and dead cells on ImmunoSpot® Series 6 Ultimate Analyzer using ImmunoSpot® LDA 1.4 Software.
6. Centrifuge cells at 300 × *g* for 10 min at RT with maximal brake.
7. Decant supernatant and resuspend cells at five million live cells per milliliter in warm (37 °C) CTL-Test Medium.
8. Transfer cells to a sterile 25 cm^2 cell culture flask. Add B-Poly-S to the flask at 1 μL/mL final concentration. *See* also **Note 3**. Incubate at 37 °C with 9% CO_2 for 72 h.

3.2 Preparation of ELISPOT Plate

1. Remove underdrain of IPFL plate contained in ImmunoSpot® kit—this plate has a PVDF membrane that has been modified for low autofluorescence.
2. Pre-wet with 15 μL/well freshly prepared 70% EtOH and immediately wash with 150 μL PBS per well (*see* **Note 4**). Decant and repeat PBS wash two more times.
3. Blot plate on sterile gauze, replace the underdrain, and add 80 μL Capture Solution to each well. The Capture Solution contains the κ/λ capture antibodies as well as capture antibodies specific for each analyte to be detected in the multiplex assay, whereby each capture antibody is present at an optimized concentration. Cover plate and incubate overnight in a humidified chamber at 4 °C.

3.3 Isolation of CD19+ B Cells

1. Harvest the PBMC after 72 h polyclonal stimulation (continued from Subheading 3.1) transferring the cells from the culture flask to a sterile 50 mL conical tube. Wash with an excess of warm (37 °C) CTL-Test Medium by centrifugation at 300 × *g* for 10 min with max brake.
2. Decant supernatant. Resuspend pellet by flicking the bottom of the tube. Add 10 mL CTL-Test Medium.
3. Take 20 μL of cell suspension for counting, and mix well with 20 μL Live/Dead Cell Counting dye. Load into hemocytometer and count on CTL analyzer using Cell Counting software.

4. Centrifuge cells at 300 × *g* for 10 min at room temperature with max brake.
5. Decant supernatant and resuspend cells in EasySep Buffer at the concentration suggested in the manufacturer's protocol for the desired isolation kit. (In the following, we describe obtaining purified B cells by negative selection; *see* **Note 2** for positive selection). For negative selection, resuspend cells at a final concentration of 5×10^7 cells/mL.
6. Transfer cell suspension to a sterile 5 mL round-bottom tube. Follow manufacturer's protocol for isolation of CD19+ B cells from the pre-stimulated whole PBMC.
7. Add 50 μL/mL Cocktail Enhancer to sample.
8. Add 50 μL/mL Isolation Cocktail to sample. Mix and incubate at room temperature for 5 min.
9. Vortex RapidSpheres and add 50 μL/mL to sample. Mix well.
10. Add EasySep buffer to a final volume of 2.5 mL. Cap tube and invert gently to mix.
11. Place tube into EasySep magnet and remove lid. Incubate for 3 min. Do not agitate tube during incubation.
12. Pick up magnet and invert both magnet and tube to decant liquid into a new sterile 5 mL round-bottom tube. Do not tap or shake off any droplets that remain hanging from the tube.
13. Remove tube from the magnet and replace it with the new tube. Incubate a second time for 1 min within the magnet. Do not agitate tube during incubation.
14. Pick up magnet and invert both magnet and tube to decant liquid into a new sterile 15 mL conical tube. This is the isolated fraction.
15. Wash isolated fraction in CTL-Test Medium by centrifugation at 300 × *g* for 10 min at room temperature with max brake to remove traces of isolation buffer.
16. Decant supernatant. Resuspend pellet by flicking the bottom of the tube. Add 10 mL CTL-Test Medium per 10 million cells.
17. Take 20 μL of cell suspension for counting, and mix well with 20 μL Live/Dead Cell Counting dye. Load into hemocytometer and count on CTL analyzer using Cell Counting software.
18. Centrifuge cells at 300 × *g* for 10 min at room temperature with max brake.
19. Decant supernatant and flick tube to resuspend cell pellet. Adjust B cells to 1×10^6/mL in CTL-Test medium, and store in incubator at 37 °C until the cells are plated into the assay.

3.4 Plating of B Cells

1. Remove coated plate from humidified chamber (continued from Subheading 3.2). Decant coating solution and wash one time with 150 µL PBS/well. Decant PBS and blot.
2. If additional stimulation of B cells is required, plate stimulant in 100 µL CTL-Test at 2× final concentration (*see* **Note 3**). If no stimulant is required, add 100 µL CTL-Test to each well.
3. Using wide-orifice tips, add 100 µL per well isolated CD19+ B cells in serial titration (*see* **Note 5**).
4. Cover plate and tap gently on all sides to ensure even distribution of cells.
5. Incubate plate at 37 °C with 9% CO_2 for 24 h (*see* **Note 6**).

3.5 Detection of Antigen-Specific B Cells and Secretory Products

1. Prepare Detection Solution per manufacturer's protocol, containing all the detection antibodies specific for each analyte to be detected at an optimized concentration. If antigen-specific B cells are to be identified in addition, add labeled antigen as described in [5].
2. Filter Detection Solution through a low protein binding 0.1 µm syringe filter.
3. Remove plate from the incubator and decant. Wash 2× with 150 µL PBS, and 2× with 150 µL 0.05% Tween-PBS. Blot to remove residual liquid from the wells (*see* **Notes 7** and **8**).
4. Add 80 µL/well Detection Solution and cover plate. Incubate at room temperature for 2 h, protected from light.
5. Prepare Tertiary Detection solution per manufacturer's protocol. Filter through a low protein binding 0.1 µm syringe filter.
6. Decant plate and wash 3× with 150 µL/well 0.05% Tween-PBS. Blot to remove excess liquid from wells.
7. Add 80 µL/well Tertiary Detection solution and cover plate. Incubate at room temperature for 1 h, protected from light.
8. Decant plate and wash 3× with 150 µL/well distilled water. Remove underdrain and wash upside-down on a vacuum manifold 3× with distilled water to remove any excess fluorochrome.
9. Let plate dry completely, protected from light, before scanning and analysis.

3.6 Data Capture and Analysis

The plate is to be scanned and analyzed on a CTL Series 6 Ultimate Analyzer, or other compatible CTL fluorescent ELISPOT reader that is customized to avoid cross-bleeding of colors (Fig. 1) across detection channels (*see* **Note 1**). The theoretical and practical aspects of accurate multicolor ELISPOT analysis are described in dedicated chapters in this book [9, 18], respectively.

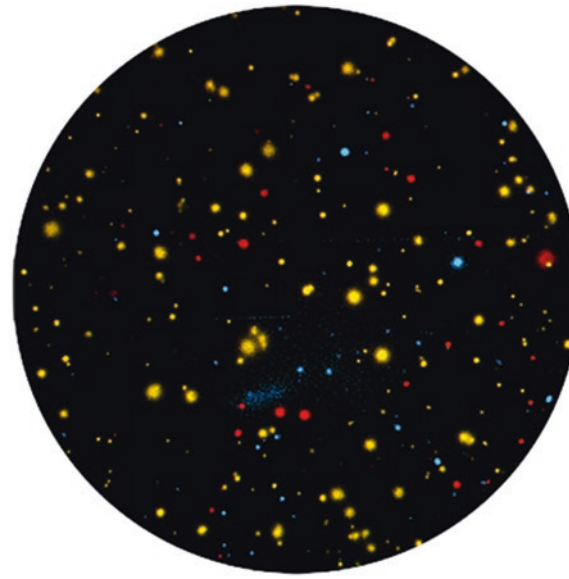

Fig. 1 Example of multicolor ImmunoSpot assay in which measurements of immunoglobulins are multiplexed with detection of cytokine. In this assay, IL-6 (in blue) was detected along with IgG (in yellow) and IgA (in red). The test procedure is detailed in the body of this chapter

4 Notes

1. For unambiguous detection of individual analytes using fluorochrome combinations, a setup is needed in which the dyes do not cross-bleed among the detection channels. Each channel is defined by a narrow excitation wavelength combined with a narrow emission filter and can be selected from an array of channels preinstalled on CTL fluorescent readers. Please consult CTL customer support for the selection of channels suitable for the fluorochrome combinations of choice.
2. To isolate CD19+ B cells by positive selection, the EasySep Release Human CD19 Positive Selection Kit can be used. Begin by resuspending whole PBMC at a final concentration of 1×10^8 cells/mL. Transfer cell suspension to a sterile 5 mL round-bottom tube and follow manufacturer's protocol for isolation of CD19+ B cells from the pre-stimulated whole PBMC.
3. For detection of several analytes, including Granzyme B, pre-stimulation may be necessary. The duration of pre-stimulation is dependent upon both the nature of the stimulatory agent and the donor's underlying state of immunobiology/pathology—it can last up to 1 week. With the need for pro-

longed activation, it is advised to add the stimulatory agent along with B-Poly-S at stage 3.1 of the protocol. Alternatively, add stimulant at Subheading 3.4, **step 3** of the protocol.

4. Activation of the membrane with ethanol is instantaneous and can be seen visually as a graying of the membrane. Ethanol should be washed off as quickly as possible following activation to avoid leakage of wells.
5. Numbers of analyte co-expressing B cell subpopulations vary largely and will depend upon the specific combination of analytes being detected, as well as the underlying immunobiology/immune pathology state of the donor. Therefore, titrations of cell concentration are very helpful in determining the optimal starting concentration for the assay.
6. Do not stack plates in the incubator, and do not allow incubator or plates to be disturbed during incubation. Doing so will cause cells within the wells to roll, and spots will become blurred and less distinct, resulting in increased difficulty detecting co-expression of analytes.
7. Do not allow membrane to dry at any point during the analyte detection process described in Subheading 3.5 until the plate is ready to be scanned in **step 9**.
8. Plate washes may be performed manually or with a suitable automated plate washer with adjusted pin length and flow rate, so that membranes and spots are not damaged (CTL recommends the CTL 405LSR).

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