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Flow cytometry analysis of transcription factors in T lymphocytes

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Abstract

Detection of transcription factors expressed in immune cell populations, particularly in subpopulations that are represented at low frequencies in lymphoid and nonlymphoid organs, presents a particular challenge when using traditional methods such as western blot analysis. Therefore, development of flow cytometry-based methods which allow identification of transcription factors in specific immune cell populations is of main interest. Here we developed and optimized a protocol for rapid and convenient detection of the transcription factor BCL11B (CTIP2) in T lymphocyte subpopulations using flow cytometry. The method employs saponin and Tween 20 both during the fixation and permeabilization steps, and we demonstrate that it is efficient for three independent antibody clones against distinctive BCL11B epitopes. In addition, we prove that the method preserves the staining of surface markers.

Keywords

transcription factors; BCL11B/CTIP2; T lymphocytes; CD4⁺ T lymphocytes; flow cytometry

1. Introduction

Western blot analysis of total cell lysates or nuclear fraction is most commonly employed for the detection of the transcription factors presence in different types of cells. Different from most cell lines, primary cells are composed of heterogeneous populations of cells. Therefore, analysis of transcription factors by western blot analysis has major limitations because it requires prior purification of subpopulations of cells, and a large number of cells (at least 0.5×10^6). For cells of the immune system, such separation of subpopulations can be achieved by fluorescent-activated cell sorting (FACS) based on surface markers. However, many subpopulations contain limited numbers of cells, which makes western blot analysis difficult. On the other hand, immunocytochemistry offers the advantage of allowing the identification of the transcription factors in small populations of cells and also permits to determine whether the transcription factor is present in the cytoplasmic or nuclear fraction. This technique allows surface staining as well and identification of subpopulations, however with limited number of fluorochromes at the same time. In addition, there are limitations in terms of evaluation of significant numbers of cells using this technique.

The method of choice that permits rapid and simultaneous identification of subpopulations expressing the transcription factors of interest in small but significant number of cells without prior purification is flow cytometry. The identification of subpopulations which

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expresses the transcription factor can be achieved by knocking in reporter genes, such as Green Fluorescence Protein (GFP) downstream of the transcription factor promoter, such as it has been achieved for Foxp3¹ and RORgammat². In the absence of such models, the populations expressing the transcription factor can be identified by cell surface staining for subpopulation specific markers and intranuclear staining with antibodies for the specific transcription factor. This second goal requires permeabilization of the cellular and nuclear membranes to allow antibodies to reach the nuclear epitopes. Specifically, following surface staining, fluorochrome-labeled antibodies bound to surface markers will be fixed. Cells can be then permeabilized, which allows exposure of nuclear epitopes, followed by staining with transcription factor-specific antibodies either directly coupled with a fluorochrome or with fluorochrome-labeled secondary antibodies. At the end, samples are run on a flow cytometer and specific subpopulations are identified based on the surface markers together with the transcription factor of interest. This technique offers the advantage of single cell analysis, which enables the determination of both the presence of the transcription factor of interest in subpopulations of primary cells, and the frequencies of the primary cell subpopulations which express the transcription factor without the need of the prior purification of the subpopulations. The technique was proven to render significant data after acquisition and analysis of a small number of cells, as little as 3×10^4 . In addition, the technique allows quantification of the transcription factor in subpopulations of cells by evaluation of mean fluorescence intensity (MFI).

BCL11B, known also as CTIP2, is a sequence-specific DNA binding transcription factor³ expressed in immune system⁴⁻⁷, brain⁸⁻¹² and skin^{13, 14}. It has been demonstrated that its expression in immune system is confined to T lymphocytes⁴⁻⁷. It is therefore critical to establish a methodology based on flow cytometry analysis that allows determination of various T cell subpopulations which express BCL11B.

2. Materials

Mice

5–10 weeks C57BL/6 female or male mice. Mice were housed in the Albany Medical Center animal research facility and all the animal procedures were approved by the Institutional Animal Care and Use Committee.

Instruments and Disposables

- Sterile surgical instruments: forceps and scissors (Roboz Surgical Instrument Co., Gaithersburg, MD).
- 40 μ m nylon cell strainers (BD Falcon, Franklin Lakes, NJ)
- 50 ml conical tube (BD Biosciences, Franklin Lakes, NJ)
- 5 ml syringes
- sterile transfer pipettes
- MACS multistand (Miltenyi Biotec, Bergisch Gladbach, Germany)
- Minimacs separation unit (Miltenyi Biotec)
- MS columns (Miltenyi Biotec)
- Sorval Legend RT with cytospin system and plate holders (Thermo Scientific, Waltham, MA)
- Olympus BX61 Microscope (Olympus, Center Valley, PA)

- FACS Calibur Flow Cytometer (Beckton Dickinson, San Jose, CA)

Media and Buffers

- Complete Medium (CM): RPMI 1640 media (Gibco/BRL, Bethesda, MD) supplemented with 10% heat inactivated FCS (Hyclone, Logan, UT), 100 U/ml Penicillin, 100 U/ml Streptomycin solution (Gibco), 2 mM L-glutamine (Gibco), 0.05 mM beta mercaptoethanol (Sigma, St. Louis, MO) and 25 mM HEPES
- Red Blood Cell Lysis Buffer (RBCLB): 0.83% NH_4Cl , 0.1% KHCO_3 , and 0.0037 % EDTA (all from Sigma)
- Staining buffer (SB): 1% BSA and 0.01% sodium azide in PBS
- MACS buffer: PBS supplemented with 0.5% BSA and 2 mM EDTA
- BN Buffer: 15 mM Tris pH 7.5, 60 mM KCl, 5 mM MgCl_2 , 15 mM NaCl, 250 mM sucrose, 0.3% NP40, protease inhibitor cocktail (Sigma)
- NEB Buffer: 25 mM Tris pH 8.0, 250 mM NaCl, 10% glycerol, 0.2% NP40, protease inhibitor cocktail (Sigma)
- Blocking buffer: 4% rabbit serum in PBS
- Immunocytochemistry wash buffer: PBS and 0.1% Tween.
- Hoechst 33258 (Molecular Probes, Eugene, OR) (1 $\mu\text{g/ml}$) in PBS

Antibodies and Reagents

- supernatants of HB-197 hybridoma (ATCC, Rockville, MD) containing 2.4G2
- anti-CD4 antibody, clone GK1.5, (FITC) (eBioscience, San Diego, CA)
- anti-CD8 antibody, clone 53–6.7, (PE-Cy7) (eBioscience)
- anti-BCL11B antibodies clones BL1801, BL1800, BL1798 and BL1799 (Bethyl laboratories, Montgomery, TX) (Note 1).
- anti-HDAC2 antibody, clone H54 (Santa Cruz Biotechnology, Santa Cruz, CA)
- anti- α -actin mouse monoclonal antibody (Sigma)
- Alexa 647-conjugated goat anti-rabbit secondary antibodies (Invitrogen Corp., Carlsbad CA).
- Fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit secondary antibodies (Southern Biotechnology Associates, Inc., Birmingham, AL)
- CD4 L3T4 Microbeads (mouse) ((Miltenyi Biotec)
- Formaldehyde 37% (VWR, West Chester, PA)
- saponin (Sigma)
- Tween 20 (Fisher Scientific, Pittsburgh, PA)
- RNase (Sigma) 10 mg/ml
- Fluoromount (Southern Biotechnology Associates, Inc.)

¹The epitopes recognized by BL1800, BL1801, BL1798 and BL1799 map to the following regions of the human BCL11B using the numbering given in Gene ID 64919: residues 500–550 (BL1800), residues 881–894 (BL1801), residues 11–26 (BL1798), and residues 245–259 (BL1799), respectively. The epitopes recognized by BL1800, BL1801, BL1798 and BL1799 are 90%, 100%, 100% and 94%, respectively, identical between mouse and human proteins.

- V-bottomed 96 well plates (Corning Costar Corp, Cambridge, MA)
- FACS tubes (Krackeler Scientific, Albany, NY)

3. Methods

Here we developed and optimized a protocol for rapid and convenient detection of the transcription factor BCL11B in T lymphocyte subpopulations using flow cytometry. As previously demonstrated in the case of ectopical expression of BCL11B homologue, BCL11A/CTIP1, in HEK293 cells ⁸, the presence of endogenous BCL11B was restricted to the nuclei of CD4⁺ T lymphocytes, as demonstrated both by immunocytochemistry and western blot analysis of cytoplasmic and nuclear fractions (Fig. 1B and C). Detection of BCL11B by western blot analysis of the nuclear fraction requires at least 0.5×10^6 purified CD4⁺ T lymphocytes (Fig. 1C). The use of flow cytometry allowed us to detect simultaneously the subpopulations of T lymphocytes, CD4⁺ and CD8⁺ in this specific case (Fig. 4) and, BCL11B (Fig. 2 and 3) without prior purification. In addition, flow cytometry enabled the quantification of BCL11B levels through MFI (Fig. 3). In brief, in the protocol we developed cells were first stained for the surface markers CD4 and CD8, followed by fixation in the presence of non-ionic detergents, further followed by a permeabilization step, preceding the nuclear staining. We found, as shown bellow in detail, that the addition of the non-ionic detergents during both the fixation and subsequent permeabilization steps allowed for the optimal detection of the transcription factor BCL11B (Fig. 2). We used two non-ionic detergents, saponin, known to permeabilize cellular membranes ¹⁵, and Tween 20, a polysorbate surfactant, known also to permeabilize membranes and stabilize nuclear antigen-antibody binding ¹⁶. Neither saponin nor Tween 20 alone, or saponin and Tween 20 together added only during the permeabilization step (Fig. 2, Methods 3 through 5) allowed optimal detection of BCL11B. Addition of saponin during both fixation and permeabilization steps improved the detection (Fig. 2, Method 1), however the protocol which yielded the best results employed both saponin and Tween 20 during the fixation and subsequent permeabilization steps (Fig. 2, Method 2). Due to the high abundance of RNA in the cells, we rationalized that the addition of RNase may further reduce the nonspecific background. Indeed, further addition of RNase in the permeabilization buffer improved the staining and reduced the background (Fig. 3) (Note 2). The optimized method allows the preservation of the surface markers CD4 and CD8 (Fig. 4) (Note 3).

Four antibodies developed against specific peptide epitopes of BCL11B (Note 1) were tested with the optimized protocol, out of which three allowed the detection of BCL11B (Fig. 3), while the forth one, BL1799, though previously tested to work both in western blots and immunoprecipitations (data not shown) could not detect BCL11B by flow cytometry (Fig. 3). Antibodies BL1800, BL1801 and BL1798 allowed detection of BCL11B both in gated CD4⁺ and CD8⁺ T lymphocytes, while the CD4⁺CD8⁻ fraction, known to lack expression of BCL11B, was, as expected, negative, in agreement with the previously published data ⁴⁻⁷.

Tissue and cells suspension preparation

Spleen or lymph nodes were removed from the mouse using sterile surgical forceps. Spleens were cut into pieces using surgical scissors and placed on a PBS pre-wet 40 μ m cell strainer on top of a 50 ml conical-bottom tube. Spleen pieces were gently pressed against the strainer with a 5 ml syringe plunger using an up and down motion (Note 4). The strainer was washed

²This might not be appropriate for transcription factors known to be associated with RNA, as their three dimensional organization and epitope exposure might be disturbed by elimination of RNA.

³The optimized protocol can be employed for detection of BCL11B further in other T cell subpopulations. In addition, it might be of interest to test this protocol for other immune-specific transcription factors using monoclonal or polyclonal antibodies developed against specific peptides. Titrating of the antibodies and further optimization of the protocol might be necessary.

with 10 ml CM. Total splenocytes were centrifuged at 300 x g for 10 min at 4°C, resuspended in 1 ml pre-warmed RBCLB and incubated at 37°C for 5 min. CM was immediately added and the cells were centrifuged at 300 x g for 10 min. Cells were resuspended in 10 ml CM, mixed, and a small aliquot was removed for counting. The same procedure was also used for lymph nodes without the red blood cell lysis step.

Purification of CD4⁺ T lymphocytes

Single cell suspensions isolated from spleen and lymph nodes by the protocol presented above were washed two more times in PBS and counted. The cells were then washed once in MACS buffer. Cells were resuspended in MACS buffer at 1×10^7 cells/30 μ l MACS buffer. 5 μ l CD4 MACS beads were added to each 1×10^7 cells. The cells were incubated in the dark at 4°C for 15 minutes to allow for the beads to bind. Post-incubation the cells were washed with 1–2 ml MACS buffer to remove any unbound CD4 MACS beads. The cells were then resuspended in 500 μ l MACS buffer and applied to the MS column. The columns were hung on the magnetic column holder and the cells were passed through the columns. The columns were washed 3 times with 500 μ l MACS buffer. After the final wash, the column was removed from the magnetic column holder and placed into a fresh 15ml conical tube. 1 ml MACS buffer was then placed in the column and the CD4⁺ T cells were flushed out by firmly pushing the plunger into the column. With one MS column we usually purify approximately 1×10^7 CD4⁺ T lymphocytes.

Immunocytochemistry

CD4⁺ T lymphocytes were cytospun on 0.1% gelatin-coated coverslips using a Cytospin system adapted for Sorval Legend RT. Following this, cells were fixed in 4% formaldehyde in PBS at 4°C for 20 min, followed by treatment with 0.1 % Tween 20 in PBS for 30 minutes for permeabilization. The cells were then incubated with blocking buffer for 1 hr, and then with anti-BCL11B, clone BL1801 primary antibodies (1 μ g in 200 μ l) or prebleed rabbit serum in blocking buffer, plus 0.1% Tween 20, overnight at 4°C. After four washes the cells were incubated with the FITC-conjugated secondary antibodies, 1 μ g in 500 μ l blocking buffer, plus 0.1% Tween 20, for 1hr at room temperature, followed by three washes and 10 minutes staining with Hoechst 33258 (1 μ g/ml) in PBS. Cells were then mounted in Fluoromount and visualized under Olympus BX61 Microscope.

Cytoplasmic/Nuclear Fractionation

CD4⁺ T lymphocytes purified as described above were spun down and the cell pellet was washed once with 5 ml cold PBS and then resuspended in 1ml cold PBS and transferred to a 1.5 ml microfuge tube and spun down again. The supernatant was removed and the cells were resuspended in 100 μ l BN buffer plus a protease inhibitor cocktail. The cells were incubated for 5 minutes on ice and then spun down for 10 minutes at 200 x g in a microfuge at 4°C. The supernatant (cytosolic fraction) was transferred to a new tube. The pellet was then resuspended in 50 μ l NEB buffer plus a protease inhibitor cocktail, and incubated for 30 minutes on a rotator at 4°C. After incubation, the samples were sonicated 3X for 5 seconds and then spun down at 13,000 RPM for 15 minutes. The supernatant (nuclear fraction) was transferred to a fresh tube. A volume corresponding to at least 0.5×10^6 CD4⁺ T lymphocytes was used to run on a 7.5% SDS-polyacrylamide gel.

Staining of lymph node or spleen cellular suspensions for flow cytometry analysis

Cell staining was performed in V-bottomed 96 well plates.

⁴Grinding motion, which can damage the cells, should be avoided.

Surface staining for CD4 and CD8 was first performed. For this purpose 1×10^6 cells/well were incubated in 100 μ l HB-197 hybridoma supernatant on ice for 20 min to block Fc receptors. 0.4 μ g anti-CD4-FITC and 0.08 μ g anti-CD8-PE-Cy7 antibodies were then added and suspensions were then incubated for 30 min on ice in the dark followed by 2 washes with 150 μ l SB.

Cells were then fixed in the following buffers for 30 min at 4°C (Notes 5 and 6)

- For method # 1, 3% formaldehyde in PBS plus 0.1% saponin (Note 7) (Fig. 2)
- For method # 2, 3% formaldehyde in PBS plus 0.1% saponin, plus 0.5% Tween 20 (Fig. 2)
- For methods # 3, 4, and 5, 3% formaldehyde in PBS (Fig. 2)

Fixed cells were spun at 500 x g for 10 min, and washed twice with 150 μ l permeabilization buffer, then resuspended in 75 μ l of permeabilization buffer and incubated for 30 min at 4°C.

The permeabilization buffers contains the following

- For methods # 1 and 4, SB plus 0.5% saponin (Fig. 2)
- For methods # 2 and 3, SB plus 0.5% saponin, plus 0.5% Tween 20 (Fig. 2)
- For method # 5, SB plus 0.5% Tween 20 (Fig. 2)

To decrease the nonspecific binding, the permeabilization buffer was further optimized by addition of 10 μ g/ml RNase (Fig. 3).

Addition of anti-BCL11B and secondary antibodies

- 0.1 μ g of anti-BCL11B antibodies (clones BL1801, BL1800, BL1798 and BL1799) were added in 25 μ l permeabilization buffers (see above) for another 30 min on ice.
- Unbound primary antibodies were washed out with permeabilization buffer for a total of three washes
- 0.16 μ g anti-rabbit IgG- Alexa 647 was added to the 100 μ l cell suspension in permeabilization buffers. Cells were incubated for another 20 min at 4°C in the dark, and washed three times, each time with 150 μ l of permeabilization buffers (Note 8). Cells were then resuspended in SB buffer and transferred into flow tubes right before reading on the flow cytometer.

Flow cytometry analysis: data acquisition and analysis

- 3×10^4 cells were acquired on FACS Calibur flow cytometer upgraded to 5 colors (Cytek, Fremont, CA), and data was analyzed using Flowjo software (Tree Star, Inc, Ashland, OR).

⁵Fixation was conducted 150 μ l of the listed buffers.

⁶Solutions for fixation and permeabilization were prepared fresh. Due to formaldehyde toxicity, fixation/permeabilization solution was prepared in the fume hood.

⁷For both fixation and permeabilization, saponin was added from a 10% stock solution in 1x PBS. Saponin is light-sensitive, thus, the stock solution should be kept in the dark.

⁸As permeabilization with saponin is a reversible process it is important to continue to work in permeabilization buffer once the cells are permeabilized. However, cells were resuspended in SB after the last wash to allow for the pores of the membranes to close.

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Abbreviations

BCL11B	B-cell leukemia/lymphoma
CTIP2	Chicken ovalbumin upstream promoter transcription factor (COUP-TF)-interacting protein 2
HDAC2	histone deacetylase 2

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Figure 1.

BCL11B transcription factor is localized in the nucleus of CD4⁺ T lymphocytes. **(A)** Prior to immunocytochemistry and western blot analysis CD4⁺ T lymphocytes were isolated from spleen and lymph nodes by MACS technology, using anti-CD4 antibody-coated magnetic microbeads and magnetic column separation, as described in Material and Methods. The degree of purification was evaluated by staining an aliquot of the purified CD4⁺ T lymphocytes with anti-CD4-FITC-labeled antibodies and analyzed by flow cytometry. Purity of the separated CD4⁺ T lymphocytes was 92.4%. **(B)** Left: immunocytochemistry staining of cells cytopun on coverslips with anti-BCL11B antibodies (Clone BL1801) or prebleed rabbit serum, followed by FITC-conjugated secondary antibodies, as described in Material and Methods. Middle: Hoechst counterstained of the cells shown in the left; right: Overlay. Images were obtained on an Olympus BX61 Microscope with 100 X objectives. **(C)** Left: Western blot analysis of cytoplasmic and nuclear extracts from CD4⁺ T lymphocytes purified as described in A. Right: Western blot analysis of nuclear extracts from 0.5 × 10⁶ (left lane) or 1.5 × 10⁶ CD4⁺ T lymphocytes (right lane). Actin and HDAC2 were used as loading controls.

Figure 2.

Detection of BCL11B by Flow Cytometry. Total splenocytes were surface stained with anti-CD4-FITC and anti-CD8-PE-Cy7 antibodies as described in detail in Material and Methods. This staining was followed by several combinations of fixation/permeabilization and permeabilization treatments, as follows:

Method #1:	<ul style="list-style-type: none"> Fix/Perm: 3% formaldehyde + 0.1% saponin; Perm: SB + 0.5% saponin.
Method #2:	<ul style="list-style-type: none"> Fix/Perm: 3% formaldehyde + 0.1% saponin + 0.5% Tween 20; Perm: SB + 0.5% saponin + 0.5% Tween 20.
Method #3:	<ul style="list-style-type: none"> Fix/Perm: 3% formaldehyde; Perm: SB + 0.5% saponin + 0.5% Tween 20.
Method #4:	<ul style="list-style-type: none"> Fix/Perm: 3% PFA; Perm: SB + 0.5% saponin.
Method #5:	<ul style="list-style-type: none"> Fix/Perm: 3% formaldehyde; Perm: SB + 0.5% Tween 20.

Anti-mouse (clone BL 1801) was used followed by secondary Alexa 647-conjugated goat anti-rabbit secondary antibodies. Histograms depict BCL11B expression in gated CD4⁺ (solid black line), CD8⁺ (dotted black line), and CD4⁻CD8⁻ (solid gray line) cells. Secondary antibody (gray shaded). Comparison of the five methods used indicates that method # 2 allowed BCL11B detection with low background staining in CD4⁻CD8⁻ cells.

Figure 3.

Three out of the four anti-BCL11B antibody tested clones recognize nuclear epitopes exposed through the optimized method of staining. Total splenocytes were surface stained with anti-CD4-FITC and anti-CD8-PE-Cy7 antibodies, fixed and permeabilized using Method #2 (Fig. 2) further optimized by the addition of 10 µg/ml RNase in the permeabilization buffer. Anti-BCL11B antibodies, clones BL1801, BL1800, BL1799, and BL1798 were used for BCL11B detection followed by Alexa-647-conjugated goat anti-rabbit secondary antibodies. The histograms show that the staining with BL1800 and BL1801 anti-BCL11B antibodies on gated CD4⁺ (solid black lines), and CD8⁺ (dotted black lines), T lymphocytes resulted in optimal detection of BCL11B with minimal staining on CD4⁻CD8⁻ cells (solid gray line). BL1798 clone was less optimal in detecting BCL11B expression in CD8⁺, compared to CD4⁺ T lymphocytes, and also gave a higher background on CD4⁻CD8⁻ cells. This clone requires further optimization. Clone BL1799 did not detect BCL11B in any of the T lymphocytes subpopulations tested. Therefore, BL1799 is not recommended for detection of BCL11B by flow cytometry. Secondary antibody (gray shaded).

Figure 4. Staining for the surface markers CD4 and CD8 is preserved following fixation and permeabilization with optimized Method #2. Total splenocytes were surface stained with anti-CD4-FITC and anti-CD8-PE-Cy7 antibodies as described in detail in Material and Methods, followed by Method #2 of fixation and permeabilization and staining with anti-BCL11B antibodies. The dot plot graph depicts the percentages of CD4⁺ and CD8⁺ T lymphocytes in the spleen.