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Western Analysis of Intracellular Interleukin-8 in Human Mononuclear Leukocytes

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Abstract

Most cytokines are stored in the cytoplasm until their release into the extracellular environment; however, some cytokines have been reported to localize in the nucleus. Traditional whole cell extract preparation does not provide information about the intracellular localization of cytokines. Here, we describe how to prepare cytoplasmic and nuclear extracts that can be analyzed by immunoblotting. While in this chapter we use this method to analyze intracellular localization of interleukin-8 (IL-8) in human mononuclear leukocytes, this protocol is adaptable to any cell type or protein of interest.

Keywords

Cytokines; Interleukin-8; Intracellular localization; Cytoplasmic extract; Nuclear extract; Immunoblotting

1 Introduction

Interleukin-8 (IL-8) is a potent pro-inflammatory chemokine. Based on its structure it belongs to the CXC chemokine class and is also denoted as CXCL8. The expression of IL-8 is mainly regulated by NFκB transcription factor and it is synthesized as 99-amino acid precursor that is subsequently processed to a 72-amino acid mature form. While it is produced by many cell types, leukocytes are the major source of secreted IL-8 [1, 2]. Secretion of IL-8 can be stimulated by many different signals, including bacterial lipopolysaccharide (LPS) or other pro-inflammatory signals such as Tumor Necrosis Factor (TNFα). Extracellular IL-8 binds G-protein-coupled receptors CXCR1 and CXCR2 leading to the activation of multiple downstream signaling pathways including pathways that regulate cell proliferation and survival [2]. IL-8 plays a critical role in the immune response of the host by mediating the recruitment of neutrophils and monocytes to sites of inflammation. However, the unresolved secretion of IL-8 can lead to the development of chronic inflammatory diseases such as acute respiratory distress syndrome [3, 4]. IL-8 expression has also been shown to be associated with the progression of cancer [2], therefore the regulation of IL-8 is an important area of research.

There are a variety of methods to study and measure secreted cytokines as described in other chapters of this book. This chapter describes the detection of intracellular cytokines by

immunoblotting. Unlike immunofluorescence microscopy techniques that detect intracellular cytokines in their native forms, immunoblotting uses denaturing conditions. Most cytokines are localized in the cytoplasm before secretion, however some exhibit nuclear localization, such as HMGB1, IL-1α, and TNFα [5–7]. Whole cell extract preparations represent total protein expression levels, while cytoplasmic and nuclear extracts can provide information about localization in these cellular compartments. We have previously shown cytoplasmic localization of IL-8 by subcellular fractionation [7]. Here we describe how to prepare cytoplasmic and nuclear extracts, and analyze IL-8 expression in LPS-stimulated human peripheral blood mononuclear cells (PBMCs) by immunoblotting. The preparation of cytoplasmic and nuclear extract is outlined in Fig. 1; it involves the sequential lysing of plasma and nuclear membranes in order to separate the two cellular compartments. First, cells are incubated in hypotonic lysis buffer without detergent to swell up the cells. The plasma membrane of the swollen cells is then lysed by adding nonionic, nondenaturing detergent and the nuclei are pelleted. Cytoplasmic fraction (supernatant) is collected, and both the cytoplasmic fraction and the nuclei are denatured in sample buffer. The denatured proteins can be resolved on denaturing polyacrylamide gel, transferred to nitrocellulose or PVDF membrane, and protein expression analyzed by immunoblotting. This chapter focuses on the analysis of intracellular IL-8 in human PBMCs (Fig. 2); however, this protocol can be adapted to any cell type to analyze any intracellular protein.

2 Materials

2.1 Stock Solutions

All solutions are prepared in double-distilled water and stored at room temperature unless noted otherwise.

1. 1 M Hepes, pH 7.5, store at 4 °C.
2. 1 M KCl.
3. 1 M NaCl.
4. 1 M $MgCl_2$.
5. 0.5 M EGTA.
6. 0.5 M EDTA, pH 8.0: To prepare 100 mL, add 20.81 g of endotoxin-tested EDTA (MW 416.2) to ~50 mL of cell-culture grade water. Add NaOH pellets as necessary to start dissolving EDTA. EDTA will not dissolve until the pH is close to 8.0. Adjust pH to 8.0, bring to final volume. Filter. Store at room temperature.
7. 0.5 M Tris–HCl, pH 6.8.
8. 50 % glycerol.
9. 10 % SDS.
10. 1 % (w/v) Bromophenol blue.
11. 10 % NP-40 (Nonidet P-40, can be substituted by IGEPAL CA-630).

2.2 Isolation of Peripheral Blood Mononuclear Cells (PBMCs)

1. 15 mL polypropylene centrifuge tubes, sterile and pyrogen-free.
2. Transfer pipette, sterile and pyrogen-free.
3. Ficoll-Paque PLUS (endotoxin-tested). Store at 4 °C.
4. Phosphate-buffered saline without Ca^{2+} and Mg^{2+} (PBS), pH 7.4, sterile.
5. 10× red blood cell (RBC) lysis buffer: 1.55 M NH_4Cl, 100 mM $NaHCO_3$, 1 mM EDTA. To prepare 100 mL of 10× stock, dissolve 8.291 g NH_4Cl and 0.8401 g of $NaHCO_3$ (endotoxin-tested) in cell-culture grade water. Add 1 mL of 0.5 M EDTA, pH 8.0. Adjust pH to 7.4. Bring to 100 mL and filter sterilize. Store at 4 °C. Use at 1× to lyse red blood cells.
6. Culture medium: RPMI 1640 supplemented with heat-inactivated fetal bovine serum (FBS) at 10 %. Penicillin-streptomycin at 1 % is optional.
7. Wright/Giemsa stain.

2.3 Stimulation of PBMCs

1. 1.5 mL microcentrifuge tubes, sterile and pyrogen-free.
2. Lipopolysaccharide reconstituted at 1 mg/mL with PBS and stored at −20 °C.

2.4 Preparation of Cytoplasmic and Nuclear Extracts

1. Hypotonic lysis buffer (detergent-free): 10 mM Hepes, pH 7.5, 10 mM KCl, 3 mM NaCl, 3 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA. Filter. Store at 4 °C. Add inhibitors just before use.
2. Protease inhibitors stock concentration: 1 M dithiothreitol (DTT; 500×), 100 mM phenylmethylsulfonyl fluoride (PMSF) prepared in absolute ethanol (50×), 20 mM sodium orthovanadata (20×), protease inhibitor cocktail for mammalian cell extract (10×).
3. 5× sample buffer: 62.5 mM Tris–HCl, pH 6.8, 10 % (v/v) glycerol, 2 % (w/v) SDS, 0.01 % (w/v) bromophenol blue, 5 % (v/v) β-mercaptoethanol. β-mercaptoethanol is added right before use. Stock without β-mercaptoethanol can be stored at room temperature.

3 Methods

3.1 Isolation of PBMCs

This section describes the procedure to isolate PBMCs from freshly collected, heparinized whole blood (*see* **Note 1**). PBMCs are mixture of lymphocytes and monocytes (*see* **Note 2**). It is not necessary to perform this procedure in cell culture hood unless cells will be cultured

[1]The procedure to collect whole blood must be reviewed and approved by the respective Institutional Review Board. Start procedure to isolate PBMCs immediately after blood was collected. Keep whole blood at room temperature until isolation.
[2]Monocytes and lymphocytes can be separated using additional methods [8].

for longer than 24 h; however, it is critical to minimize pre-activation of cells prior to stimulation. To prevent pre-activation, use all materials that come into contact with cells, including solutions and plastic ware, certified endotoxin-tested/pyrone-free; check to use cell-culture grade (endotoxin-tested) powdered chemicals in preparing stock solutions. In addition, proceed in a timely fashion and keep isolated PBMCs cold until stimulation.

3.1.1 Ficoll Gradient Sedimentation

1. Add 5 mL of Ficoll to 15 mL tube.
2. Carefully layer 8 mL of undiluted heparinized blood over 5 mL of Ficoll using a pyrogen-free transfer pipette (*see* **Note 3**).
3. Centrifuge at 400 × *g* for 40 min. For the remaining of the procedure, keep cells and all solutions cold on ice. This is to minimize pre-activation of cells.
4. Collect the interphase layer (between serum and Ficoll layers) using a transfer pipette into a new 15 mL tube (*see* **Note 4**).
5. Bring up to 10 mL with cold PBS.
6. Invert few times to rinse.
7. Spin cells at 300 × *g* for 10 min.
8. Discard supernatant.

3.1.2 RBC Lysis (Optional, See Note 5)

1. Add 10 mL of cold 1× RBC lysis buffer to cell pellet.
2. Resuspend cell pellet using a transfer pipette.
3. Gently mix by inverting tube 10–20 times (*see* **Note 6**).
4. Centrifuge at 300 × *g* 10 min.
5. Discard supernatant.

3.1.3 Washing

1. Add 10 mL of cold PBS to cell pellet.
2. Resuspend cell pellet using a transfer pipette.
3. Invert few times to rinse cells.
4. Centrifuge at 200 × *g* for 10 min (*see* **Note 7**).

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[3]When layering whole blood over Ficoll, make sure not to break the interface between Ficoll and blood; mixing Ficoll and whole blood will destroy the gradient and prevent successful PBMC isolation.
[4]Ficoll gradient creates four distinct layers: the top yellow layer contains serum, followed by the clear Ficoll layer in the middle, and red layer at the bottom containing RBCs and granulocytes. There is a thin, cloudy layer between the serum and Ficoll layers. It is possible to directly collect the interphase using a transfer pipette. Alternatively, serum layer may be removed first. Take care to minimize collecting the Ficoll layer as this layer can be a source of non-PBMC cells.
[5]RBC lysis is optional. There are usually little contaminating RBCs in the interphase layer collected from adult blood; however, the interphase is usually heavily contaminated from a cord blood source.
[6]This step is time sensitive. Time in RBC lysis buffer should be minimized to prevent lysis of non-RBCs.
[7]The slower speed allows removal of contaminating platelets.

5. Discard supernatant.

6. Repeat washing twice more (*see* **Note 8**).

3.1.4 Resuspending Cells in Culture Medium

1. After the last wash, discard supernatant, resuspend cell pellet in residual fluid and pool cells if there are multiple 15 mL tubes.

2. Bring to 5 mL with cold culture medium.

3. Count cells; use trypan blue to determine viability. Adjust volume for final cell concentration of 5×10^6 cells/mL (or as optimal for cell type used).

4. To determine PBMC purity by differential morphology, smear ~100 μL of cell suspension onto a glass slide and air dry. Stain with Wright/Giemsa stain according to manufacturer's instructions. Count at least 100 cells. Expected purity is ~95 %.

3.2 Stimulation of PBMCs

1. Add 1 mL of cell suspension to 1.5 mL pyrogen-free test tubes (*see* **Note 9**).

2. Transfer tubes to 37 °C water bath. Equilibrate tubes for 15 min to bring to 37 °C.

3. Add LPS to tubes at a final concentration of 100 ng/mL. Invert tubes to mix and start timing stimulation and place back into water bath.

4. Incubate until desired times.

5. Place tubes on ice to cool (*see* **Note 10**).

3.3 Preparation of Cytoplasmic (CE) and Nuclear Extracts (NE)

Keep cells and solutions cold on ice at all times. All centrifugation steps are performed at 4 °C. Volumes can be scaled to number of cells (e.g., for 2.5×10^6 cells use half the volume at each step).

1. Centrifuge cells for 3 min at 300 × *g* at 4 °C in a microcentrifuge.

2. Aspirate supernatant. Wash cells by adding 1 mL of cold PBS and resuspend cell pellet by pipetting up and down. Centrifuge as in **step 1** (*see* **Note 11**)

3. While the cells are centrifuging add protease inhibitors to hypotonic lysis buffer (*see* **Note 12**).

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[8] At this point the cell suspension can be used separate monocytes or lymphocytes [8].

[9] Alternatively, cells can be stimulated in cell culture dishes. If preparing whole cell extracts, cells may be directly lysed in the cell culture dish after stimulation. However, for preparing cytoplasmic and nuclear extracts, cells have to be collected into 1.5 mL centrifuge tubes first.

[10] Alternatively, sample can be quick-spun for 10 s and lysed immediately. The time to quick-spin and aspirate supernatant is part of the incubation time, and the hypotonic lysis buffer is added exactly at the end of the incubation time point.

[11] This step is optional.

[12] Inhibitors should be added right before use as some protease inhibitors have short half-lives once in aqueous solution; e.g., PMSF has a half-life of ~30 min. If the time points are more than 30 min apart we recommend adding inhibitors freshly at each time point. Each cell type has its own unique combination of proteases. The inhibitors listed here are minimal required; hypotonic lysis buffer can be supplemented with additional protease inhibitors and optimized for working concentrations as necessary depending on the cell type used.

4. Aspirate supernatant and add 150 μL of hypotonic lysis buffer (with protease inhibitors). Resuspend cell pellet gently by pipetting up and down. Keep on ice for 15 min (*see* **Note 13**).
5. Add 8 μL of 10 % NP-40, at a final concentration of 0.5 %. Vortex vigorously for 10 s. Keep on ice for 10 s (*see* **Note 14**).
6. Centrifuge for 5 min at 845 × *g* at 4 °C in a microcentrifuge (*see* **Note 15**). Place tubes back on ice. Keep both the supernatant and the pellet.
7. Transfer supernatant to new 1.5 mL microcentrifuge tube. This is the cytoplasmic extract (CE) fraction.
8. The pellet is the nuclear fraction. Wash nuclear pellet by adding 300 μL of hypotonic lysis buffer (with protease inhibitors) to the nuclear pellet and resuspend gently by pipetting up and down. Centrifuge 5 min at 2400 × *g* in a microcentrifuge.
9. Save 5 μL of the cytoplasmic fraction to measure protein concentration.
10. Add 36 μL of 5× sample buffer to the remaining cytoplasmic fraction and heat immediately for 5 min at 95 °C (*see* **Note 16**). Spin and store CE at −20 °C.
11. Aspirate supernatant from the washed nuclear pellet. Resuspend the nuclear pellets in 50 μL of 2× sample buffer. Vortex well. Heat immediately for 5 min at 95 °C. Centrifuge 5 min at 9400 × *g* in a microcentrifuge. Transfer supernatant (NE) to a new tube and store at −20 °C.
12. Determine protein concentration of cytoplasmic fraction. If your laboratory does not have a routine method already to measure protein concentration, there are commercially available kits such as Coomassie Plus.

3.4 Immunoblotting

1. Due to the small size of cytokines (IL-8 is ~8 kDa), use 14 or 15 % polyacrylamide gel.
2. Load equal protein amounts per lane; volumes are calculated based on protein concentrations. The recommended volumes for loading are 20 μL of CE and 5 μL of NE, equivalent of ~5×10^5 cells.
3. Proteins can be transferred onto nitrocellulose or PVDF membrane.
4. Monitor purity of cytoplasmic and nuclear fractions by analyzing lactate dehydrogenase (LDH; cytoplasmic-specific) and lamin B (nuclear-specific) expression, as shown in Fig. 2.

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[13]During the incubation in hypotonic lysis buffer cells will swell up; do not exceed more than 30 min at this step as it may result in partial lysis of cells and loss of cytoplasmic material.
[14]This step is time sensitive. Do not exceed the indicated times as NP-40 may start lysing the nucleus as well, resulting in the cross-contamination of the cytoplasmic fraction.
[15]The speed used to pellet the nuclei may have to be optimized for other cell types, e.g., 200 × *g* in a microcentrifuge is sufficient for human neutrophils.
[16]If a heat block is not available, tubes may be suspended in boiling water.

5. Detection can be done by preferred method; chemiluminescence was used in Fig. 2.

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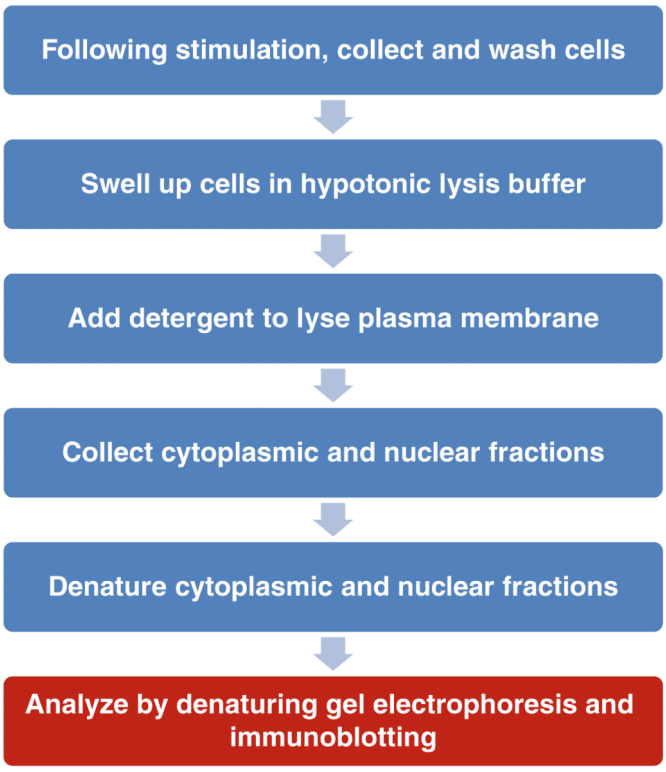

Fig. 1.
Schematic outline of preparing cytoplasmic and nuclear extracts

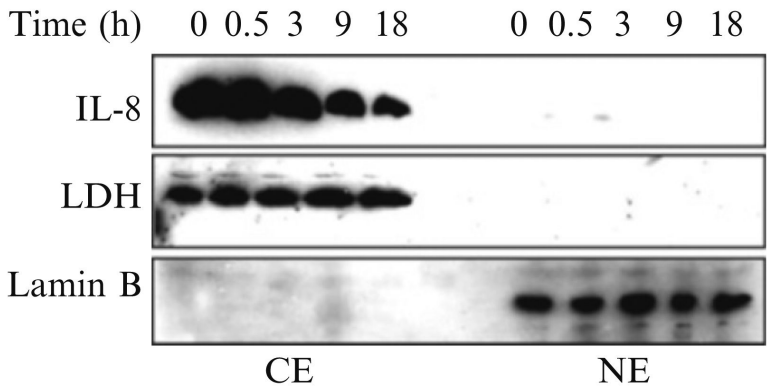

Fig. 2.

Intracellular localization of IL-8 in LPS-stimulated human PBMC. Western blot analysis of cytoplasmic (CE) and nuclear extracts (NE) prepared from human PBMC (5×10^6) stimulated with LPS (100 ng/mL) over time as indicated. CE and NE were resolved on 14 % denaturing polyacrylamide gels and transferred to nitrocellulose membrane. IL-8 expression was analyzed by using polyclonal IL-8 antibody (Santa Cruz, sc-7922). The purity of CE and NE was monitored by LDH and lamin B antibodies, respectively. Each lane contains approximately 5×10^5 cells