Video Article

Proteomic Profiling of Macrophages by 2D Electrophoresis

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Abstract

The goal of the two-dimensional (2D) electrophoresis protocol described here is to show how to analyse the phenotype of human cultured macrophages. The key role of macrophages has been shown in various pathological disorders such as inflammatory, immunological, and infectious diseases. In this protocol, we use primary cultures of human monocyte-derived macrophages that can be differentiated into the M1 (pro-inflammatory) or the M2 (anti-inflammatory) phenotype. This *in vitro* model is reliable for studying the biological activities of M1 and M2 macrophages and also for a proteomic approach. Proteomic techniques are useful for comparing the phenotype and behaviour of M1 and M2 macrophages during host pathogenicity. 2D gel electrophoresis is a powerful proteomic technique for mapping large numbers of proteins or polypeptides simultaneously. We describe the protocol of 2D electrophoresis using fluorescent dyes, named 2D Differential Gel Electrophoresis (DIGE). The M1 and M2 macrophages proteins are labelled with cyanine dyes before separation by isoelectric focusing, according to their isoelectric point in the first dimension, and their molecular mass, in the second dimension. Separated protein or polypeptidic spots are then used to detect differences in protein or polypeptide expression levels. The proteomic approaches described here allows the investigation of the macrophage protein changes associated with various disorders like host pathogenicity or microbial toxins.

Video Link

The video component of this article can be found at http://www.jove.com/video/52219/

Introduction

Macrophages are heterogeneous and plastic cells that are able to acquire distinct functional phenotypes. *In vivo,* these cells respond to a large variety of micro environmental signalssuch as microbial products, cytokines, *etc*.[1]. *In vitro*, the pro-inflammatory phenotype (M1) of macrophage can be induced by lipopolysaccharide (LPS) and the anti-inflammatory phenotype (M2) by some cytokines such as interleukin-4 (IL-4). Moreover, macrophages can switch from an activated M1 to M2 phenotype, and conversely, upon specific signals[2].

Depending on the phenotype, macrophages will have different functions. M1 macrophages are cells that produce pro-inflammatory cytokines, such as tumor necrosis factor α (TNF-α), to kill microorganisms or tumor cells[3]. In contrast, M2 macrophages prevent these inflammatory response like in wound healing and fibrosis by producing anti-inflammatory factors such as TGF-β[3,4].

Human peripheral blood mononuclear cells from healthy donors were isolated by Ficoll density gradient centrifugation as previously described[5] using a technique adapted from Boyum[6]. Macrophages in culture can be differentiated into M1 or M2 phenotype after 6 days of primary culture[7].

Analysis of protein expression or protein changes between the two subtypes of macrophages under various controlled stimuli, such as host pathogenicity or microbial toxins, will be helpful to decipher the functionality of the pro- and anti-inflammatory macrophages.

Proteomics are unique tools for direct monitoring of proteins that are specifically up- or down-regulated in human cultured macrophages under various stimuli. Fluorescent dyes have resolved some of the limitations of 2D gel electrophoresis, such as low sensitivity and image analysis[8]. The dyes reacting with cysteine residues have increased the detection sensitivity compared to those reacting with lysine residues[9]. In a previous study, we demonstrated the usefulness of DIGE saturation labelling for the analysis of scarce samples[10] compared to the classical silver-stained 2D electrophoresis[11]. This technology is helpful in rapidly analysing protein modifications between the two subtypes of macrophages or between untreated and treated macrophages from the same subtype.

The advantages of this proteomic technique are having access to the information of protein size and post-translational modifications by analysing the 2D gel[12]. It should be taken into account that this is not a high throughput technique, limiting the number of samples that can be analysed. The development of high throughput assays based on mass spectrometry as reviewed recently[13] can improve this.

Here we present how to perform 2D DIGE analysis from the protein extraction of cultured macrophages through the processes of electrophoresis, isoelectrofocusing, and SDS-PAGE as well as information on the usefulness of adequate 2D software.

Protocol

The protocol follows the guidelines of our institutions human research ethics committee. Buffy coat from healthy human donors were obtained from the Regional Blood Transfusion Center (Lille, France). Samples obtained from the buffy coats are declared as an Inserm collection (n °DC2010-1209).

1. Material and Culture Media Preparation

1. Dilute 10x Phosphate Buffer Saline (PBS) in sterile distilled water to obtain 1x PBS.
2. Make RPMI 1640 medium supplemented with gentamicin (40 µg/ml) and L-glutamine (2 mM), with and without 10% pooled human serum.

2. Primary Cultures of Monocyte-derived Macrophages (MDM)

1. Dilute buffycoat (25 ml) with 1x PBS. Carefully load on a Ficoll/leucosep tube and centrifuge at 1,600 x g for 20 min, at room temperature.
2. Collect monocytes at the interface into a new tube. Wash 3x with 10 ml 1x PBS containing 0.1% ethylenediaminetetraacetic acid (EDTA) by successive centrifugations at 1,000 x g, 370 x g and 160 x g for 10 min each, and then once in 1x PBS alone at 160 x g for 10 min.
3. Resuspend the cell pellet in 5 ml RPMI-1640 medium without serum and seed the cells in 35 mm dishes at a density of 1×10^6 cells per dish.
4. After sedimentation for 90 min in the incubator, discard the supernatant containing the non-adherent cells. Wash the adherent cells, consisting of monocytes, 3x with 1 ml PBS; then add 1 ml of fresh medium containing 10% (v/v) human serum to the previously serum free cells.
5. After 6 days of culture, to yield alternative differentiated macrophages (M2), add recombinant human IL-4 (15 ng/ml) and maintain for 6 days. Then, treat differentiated macrophages with lipopolysaccharide (100 ng/ml) at day 12 for 4 hr to obtain M1 macrophages.

3. Extraction of M1 and M2 Macrophage Proteins for 2D Electrophoresis

1. Wash macrophages three times with 25 mM Tris, pH 7.4, and scrap in buffer containing 30 mM Tris pH 8, 4% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), 2 M thiourea and 7 M urea.
2. Lyse cells using a mixer suitable for 1.5 ml microcentrifuge tubes for 5 min in ice and store at -20 °C.
3. Determine protein concentration using a commercial Bradford reagent. Store 100 µl aliquots of the proteins at -20 °C until use.

4. Isoelectrofocusing

 NOTE: Perform all labelling procedures in the dark.

1. Reduce 5 µg of each sample (M1 and M2 macrophages) adjusted to 9 µl with lysis buffer with 2 mM Tris(2-carboxyethyl)phosphine (TCEP) for 1 hr at 37 °C.
2. Add cyanine 3 (Cy3) to M1 extract and M2 sulfhydryl-reactive dye extract at a concentration of 0.8 nM/µg protein. Add cyanine 5 (Cy5) to M1 and M2 extracts at a concentration of 0.8 nM/µg protein and incubate for 30 min at 37 °C.
3. Stop the reaction with the addition of an equal volume of sample buffer containing 7 M urea, 2 M thiourea, 4% CHAPS, 130 mM dithiothreitol (DTT) and 2% pharmalytes.
4. Mix Cy3-labeled M1 samples with Cy5-labeled M2 samples in one hand and Cy3-labeled M2 samples with Cy5-labeled M1 samples in another hand.
5. Rehydrate an Immobilized pH gradient (IPG) strip (240 mm, pH 3-10 linear gradient) with 450 µl of labelled mixed samples in buffer containing 7 M urea, 2 M thiourea and 4% CHAPS on a isoelectric focusing (IEF) cell system for 24 hr without applying any current.
6. Perform focusing at 300 volts (V) for 3 hr, and then at a gradient to 1,000 V for 6 hr, at a gradient to 8,000 V for 3 hr and finally at 8,000 V for 3 hr.

5. Second Dimension

 NOTE: Perform all electrophoresis procedures in the dark.

1. Incubate the IPG strips in equilibration buffer containing 0.1 mM Tris-HCl (pH 8), 6 M urea, 2% (w/v) sodium dodecyl sulfate (SDS) and 30% (v/v) glycerol for 10 min.
2. Transfer the equilibrated IPG strips for the second dimension (SDS-polyacrylamide gel electrophoresis (PAGE)) onto 12.5% PAGE gels and seal with low-melting agarose.
3. Carry out electrophoresis at 20 °C using an Ettan-Daltsix system at a constant voltage of 70 V overnight followed by 300 V until the bromophenol blue front reaches the bottom of the gel.

6. Image Acquisition and Bioinformatic Analysis

1. Scan gels cast between the two low-fluorescence glass plates with a DIGE Imager scanner at excitation/emission wavelengths of 532/580 nm for Cy3 and 633/670 nm for Cy5 to yield images with a pixel size of 100 µm.
2. Perform image analysis with commercial software as previously detailed[12].
3. Calculate and normalize spot volumes in each image. Assign a normalized spot volume as a proportion of the total value of each spot detected in the gel.

4. Analyse the differences in protein spot volumes for each type of macrophages by comparing the normalized spot volume value between the two groups (M1 and M2). Consider the difference between the spot volumes to be significant if the change is 1.5 fold (p <0.05, one-way ANOVA analysis).

Representative Results

To perform appropriate differential proteomic analysis, the processing of samples to be analysed should be verified.

In the example presented, the cell culture quality of macrophages is required for morphological and molecular aspects as previously published[5]. The differentiation of monocytes into macrophages and the homogeneity of the culture was followed by phase-microscopy. **Figure 1** showed an example of primary cultures of M1 and M2 macrophages. As shown, we verified the homogeneity of M1 (**Figure 1A, B**) and M2 (**Figure 1C, D**) subtypes of macrophages at day 12 of culture by phase-microscopy at low (**Figure 1A, C**) and high (**Figure 1B, D**) magnification. Clearly, we observed a distinct morphology of macrophages M1 and M2 after 12 days of primary culture. As previously published, we verified by RT-PCR the presence of mRNA coding for TNF and IL1B in M1 macrophages and for *MRC1* and *CCL18* in M2 macrophages[14] (not shown).

Only cultures combining those criteria were used for proteomic analysis.

We determined the 2D protein patterns of the two subtypes of macrophages, M1 (pro-inflammatory) and M2 (anti-inflammatory). For that purpose, we extracted proteins from M1 and M2 cultured macrophages as detailed in the protocol and M1 and M2 macrophages were either labelled with Cy3 or Cy5 saturation dyes to analyse the proteins by 2D DIGE electrophoresis. **Figure 2** presents representative 2D gels of M1 (**Figure 2A, B**) and M2 (**Figure 2C, D**) macrophages of sufficient quality for bioinformatic analysis. The same pattern of proteins was observed for either M1 or M2 independently of the cyanine dyes used Cy3 (**Figure 2A, C**) or Cy5 (**Figure 2B, D**). In this example, a large linear pH gradient was used (3-10) and for detailing proteins with an acidic or basic pI, narrow pH gradient can be used.

Interestingly, the bioinformatic analysis of the 2D gel revealed 20 areas containing spots that can be analysed and compared using the 2D software. **Figure 3** is an example of numerised 2D gel in which the 20 areas of spots are located. The 2D software is able to quantify and compare the volume of each spot between several 2D gels from different samples. The software can quantify the increase or decrease of spot volume and this can be visualized by colour as indicated in **Figure 3**. Spots are considered to have significant differential expression if the fold-change of normalized spot volumes as indicated in paragraph 6 of protocol section was greater than 1.5 with a p-value <0.05. The presence or absence of a protein or polypeptide spot between the two groups of macrophages can be detected by the software.

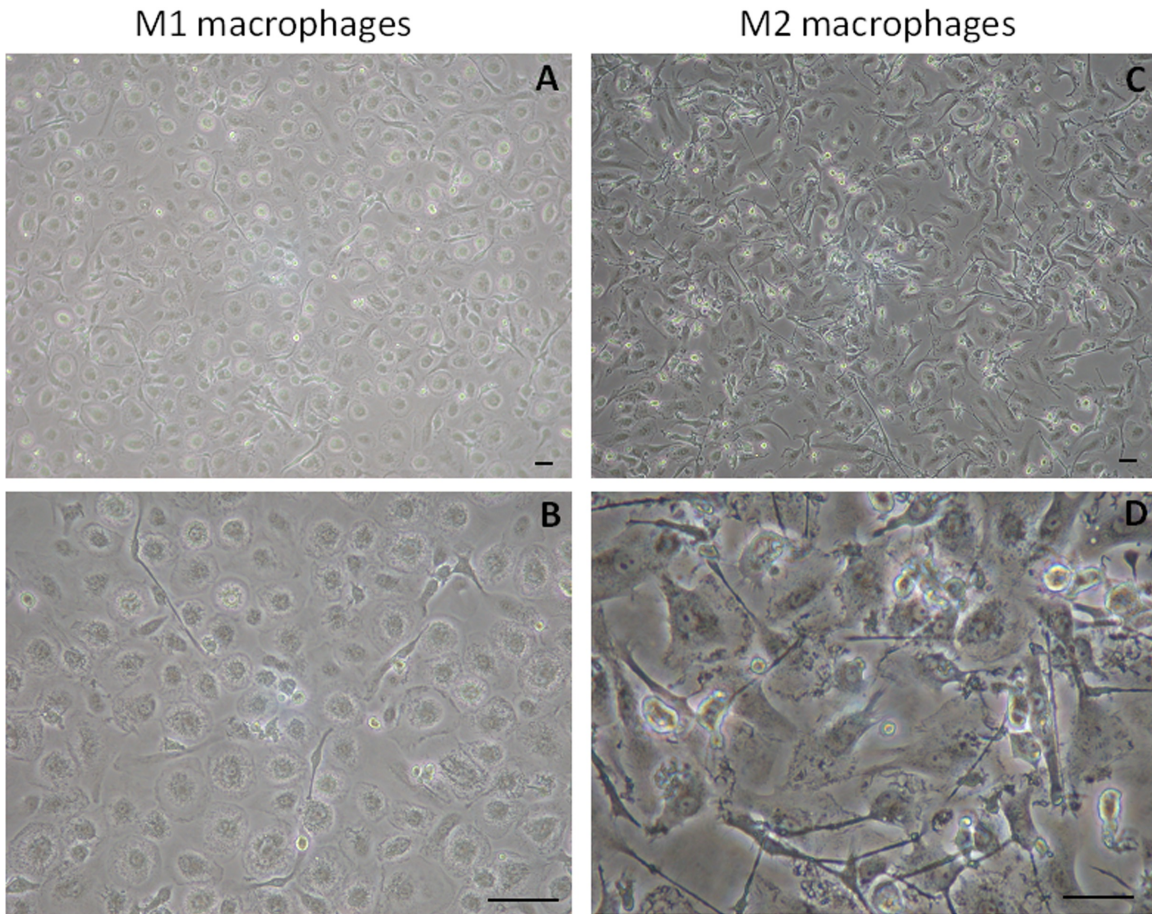**Figure 1: Photographs of phase-contrast microscopy of primary culture of macrophages.** Example of morphology of M1 **(A, B)** and M2 **(C, D)** macrophages. M1 macrophages were obtained by lipopolysaccharide treatment for 4 hr at day 12. M2 macrophages were obtained by IL-4 treatment starting from day 6 until day 12. Phase-contrast microscopy allows clear identification of both subtypes of macrophages. Photographs at low (10X) (**A, C**) and high (40X) (**B, D**) magnification were performed at day 12 of culture. Scale bar: 50 µm. Please click here to view a larger version of this figure.
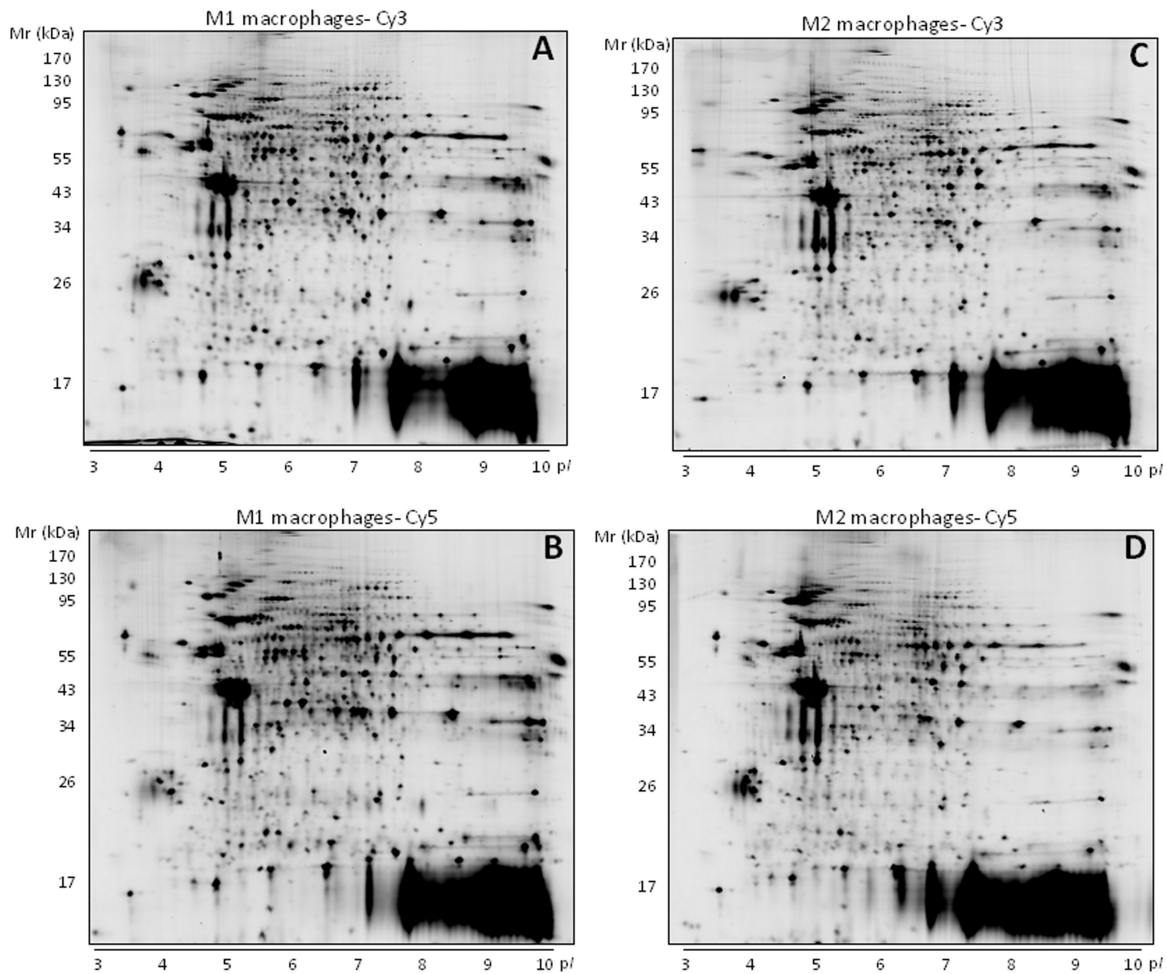
Figure 2: Representative 2D DIGE gel of M1 and M2 cultured macrophages. Proteins (5 µg) were labelled with either Cy3 **(A, B)** or Cy5 **(C, D)** from M1 **(A, B)** and M2 **(C, D)** macrophages cultured for 12 days. The same pattern of 2D gel for M1 or M2 macrophages was obtained independently of the labelled cyanine used. The positions of molecular weight (Mr) standards are indicated on the left, and the pI is indicated on the bottom of the gel. Please click here to view a larger version of this figure.
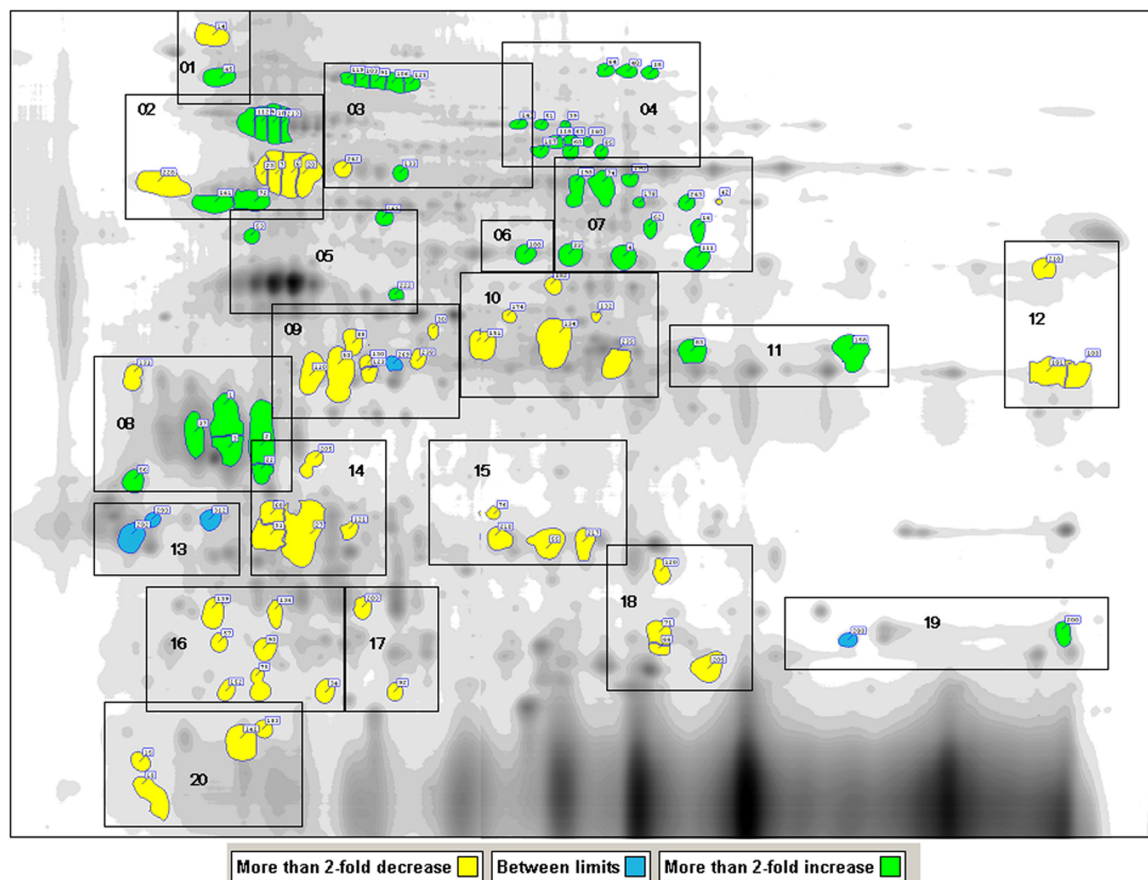
Figure 3: Bioinformatic analysis of a representative 2D DIGE gel from macrophage proteins with Progenesis SameSpots software. Twenty areas were detected to perform the differential analysis between M1 and M2 macrophages. The bottom of the figure indicates the color code for the fold-change of volume spots. Please click here to view a larger version of this figure.

Discussion

The protocol described herein details a method to analyse the impact of various stimuli of the two subtypes of macrophages, M1 (pro-inflammatory) and M2 (anti-inflammatory). Primary cultures of M1 and M2 macrophages were obtained from the differentiation of monocytes as previously published[7].

The procedure of 2D DIGE gel electrophoresis requires specialized materials and equipment, such as IEF cell for isoelectrofocusing, low-fluorescence plates for the SDS-PAGE in order to scan twice the gel at excitation/emission wavelength for Cy3 and Cy5, a scanner for fluorescence and 2D software. This method requires some practice and manual dexterity.

There are several critical steps for successful 2D gel electrophoresis: 1) the extraction of proteins is critical in an environment without any contaminants such as albumin or keratin, and 2) performing the labelling and electrophoresis in the dark. Avoid the use of DTT for protein reduction in the lysis buffer as the technique requires the use of TCEP for reducing peptide sulfide bonds in proteins before labelling with cyanine reactive dyes.

This sensitive 2D DIGE technique allows the assessment of the differential expression of protein spots by macrophages under various environmental conditions, in the presence or absence of proteins depending on the subtype.

One of the limitations of this 2D DIGE technique is the need to label the proteins on the cysteine residue, which is absent in 14% of the protein. The other limitation is the relatively small number of samples that can be analysed at the same type in comparison to high throughput technology like quantitative mass spectrometry, though 2D DIGE is less costly.

Microbial stimuli, such as LPS as well as Th1 cytokines, activate macrophages into an M1 inflammatory state, while Th2 cytokines, such as IL-4, polarize cells to an M2 phenotype with immunoregulatory, repairing, and anti-inflammatory functions[3]. Phenotyping M1 and M2 cultured macrophages with or without host pathogenicity through proteomics approaches will provide a more detailed picture of the complex functional role of individual subtypes. The 2D DIGE electrophoresis may be helpful for the identification of polypeptides/proteins or post-translational modifications of these proteins differentially modified in one subtype or between the two subtypes of macrophages under specific stimuli or environment.

Disclosures

There are no declared conflict of interest.

Acknowledgements

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