**RESEARCH ARTICLE**

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# Inflammation protein quantification by multiple reaction monitoring mass spectrometry in lipopolysaccharide-stimulated THP-1 cells

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**Rationale:** Inflammation is a cascade of events mediated by a cytokine network triggering the cellular response. In order to monitor the modulation of the crucial inflammatory proteins, e.g., Tumour Necrosis Factor-α (TNF-α), Interferon-γ (INF-γ), Interleukin-8 (IL-8) and Interleukin-10 (IL-10), upon stimulation with endotoxins, differentiated and undifferentiated THP-1 cells were treated with lipopolysaccharides (LPSs) from *E. coli*, key cell wall components of Gram-negative bacteria.

**Methods:** The multiple reaction monitoring mass spectrometry (MRM-MS) method was optimized by using the standard proteins to be quantified, in order to construct external calibration curves and define the analytical parameters. The developed method was used to quantify the above-mentioned inflammatory proteins in THP-1 differentiated cells upon stimulation with LPSs with high accuracy, sensitivity, and robustness.

**Results:** The analysis of such proteins in MRM mode allowed the kinetics of stimulation along the time up to 24 h to be followed and the MS results were found to be comparable with those obtained by Western-blotting. A significant increase in TNF-α release triggered a cascade mechanism leading to the production of INF-γ and IL-8. IL-10, instead, was found to be constant throughout the process.

**Conclusions:** The developed MRM-MS method allowed the quantification of TNF-α, INF-γ, IL-8 and IL-10 along a time-course from 2 to 24 h. Hence, a trace of the kinetics of the inflammatory response in THP-1 cells upon stimulation with *E. coli* LPSs was obtained. Finally, the extensibility of the developed MRM method to serum samples and other matrices demonstrated the versatility of the approach and the possibility to quantify multiple target proteins in different biological samples by using a few microliters in a single analysis.

# 1 | INTRODUCTION

Inflammation is typically associated with secretion of specific cytokines and chemokines, which coordinate cell and tissue activities.[1] Toll-like receptors (TLRs) play a key role in the inflammatory pathway, having the prominent biological function to promote synthesis and release of cytokines to trigger inflammatory response.[2] TLR4 was the first identified mammalian Toll-like receptor, belonging to the cytomembrane type I transmembrane glycoprotein receptor, that can be activated by the lipopolysaccharides (LPSs) of pathogenic microorganisms, lipoproteins and peptidoglycans, to stimulate cells to produce cytokines, chemokines, adhesion molecules and acute phase proteins, in order to regulate the inflammatory responses.[3] A variety of acute inflammatory responses is induced by administering small doses of intravenous endotoxins to humans.[4] The response induced by endotoxins is qualitatively similar to those that occur during the early stages of septic shock. LPSs, the key cell wall components of Gram-negative bacteria, are among the most powerful bacterial virulence factors in terms of pro-inflammatory properties,[4,5] playing a central role in the pathogenesis of septic shock.

In the context of inflammatory response, the best cellular model to study cell response to pathogens such as *Streptococcus pneumoniae* and *Mycobacterium marinum* is the THP-1 cell line, a human monocytic leukaemia cell line.[6,7] Indeed, THP-1 cells have been widely used to study immune responses as it is possible to easily differentiate THP-1 monocytes into macrophages by incubation with phorbol 12-myristate 13-acetate.[8] In order to analyse inflammatory processes in *in vitro* experiments, differentiated and undifferentiated THP-1 cells can be stimulated with inflammatory activators, such as LPSs, or with pro-inflammatory cytokines. It has been extensively reported that exposure of THP-1 cells to LPSs is responsible for significant variations in the expression levels of a number of inflammation-related genes (IL-1β, IL-6, IL-8, IL-10 and TNF-α).[9,10] These variations can be detected within 1 h from cell stimulation, and, in some cases, variations in expression levels persist even after 6 h. In the present study, mass spectrometry (MS) analyses in Multiple Reaction Monitoring (MRM) ionization mode have been used to develop a method able to quantify the production of relevant protein markers, such as Tumour Necrosis Factor-α (TNF-α), Interferon-γ (INF-γ), Interleukin-8 (IL-8) and Interleukin-10 (IL-10), upon the stimulation of THP-1-differentiated cells with *E. coli* LPSs. The key advantages of MRM methodology are the ability to target specific peptide sequences, including protein variants and modified forms, and the possibility of multiplexing hundreds of peptides upon the injection of a single complex sample. The targeted approach of MRM experiments guarantees very high sensitivity, selectivity and specificity,[11] concomitantly avoiding the problems related to antibody availability for all the target molecules to be analyzed, and the cross-reactivity typical of immune-enzymatic assays. The quantification of the mentioned inflammatory proteins along a time-course from 2 to 24 h has allowed us to trace the kinetics of the inflammatory response induced by cell stimulation with *E. coli* LPSs. The

observation that the MS results are in line with those obtained by Western-blotting gives more robustness to the developed method.

Preliminary data also indicated the applicability of the optimized MRM-MS method to serum samples, thus indicating the great potentiality of the used technique. Actually, the main advantage of the MRM-MS assay is the inestimable possibility to quantify multiple target proteins in a single analysis by using a few microliters of samples and by reaching sensitivities comparable with those of conventional immune-enzymatic methods as well as the broad extensibility to any biological matrix.

# 2 | RESULTS AND DISCUSSION

## 2.1 | Differentiation of THP-1 cells and stimulation with bacterial LPSs

A preliminary study on THP-1 cells stimulated with bacterial LPSs was carried out as described below. To differentiate THP-1 monocytes, cells were plated into six-well plates ($5 \times 10^6$ cells/well), and then 2 nM of phorbol 12-myristate 13-acetate (PMA) was added to the culture medium for 96 h at 37°C. To induce the inflammatory process, differentiated macrophages were stimulated with 1 μg/mL of LPS from *E. coli* for 40 min at 37°C. Afterwards, we analyzed the activation of NF-kB (nuclear factor kappa-light-chain-enhancer of activated B cells), a key factor in regulating the immune response to infections, by following the phosphorylation of its inhibitor IκB. Upon phosphorylation and consequent degradation of IκB, the NF-κB complex is released and enters into the nucleus where it can 'turn on' the expression of specific genes endowed with DNA-binding sites for NF-κB. The activation of these genes by NF-κB leads to the inflammatory or immune response. NF-κB turns on the expression of its own repressor, IκB. Thus, the analysis of IκB levels has to be performed in a specific time interval, in particular prior to its re-synthetization (40 min at most).[12] THP-1 cells were stimulated with LPS and, subsequently, Western-blotting analyses were performed by using antibodies specifically recognizing IκB. A significant decrease (4.4-fold) in intracellular levels of IκB upon treatment of THP-1 cells with *E. coli* LPS was found, strongly suggesting an activation of the NF-κB pathway (Figure 1A). Upon induction of the inflammation process, a further marker of inflammation was followed, such as TNF-α, in order to evaluate the release of cytokines in the conditioned medium. TNF-α is a cytokine playing a key role in systemic inflammation and is one of the cytokines involved in the acute phase reaction. It is mainly produced by activated macrophages,[13] but it is also produced by a broad variety of cell types including lymphoid cells, mast cells, endothelial cells, cardiac myocytes, adipose tissue, fibroblasts, and neurons. Large amounts of TNF-α are released in response to inflammation induced by cell exposure to lipopolysaccharides, bacterial products, and IL-1.[14]

To evaluate the release of this protein, cells were incubated with 1 μg/mL of LPS from *E. coli* for 6 h and, subsequently, the conditioned medium was analyzed by Western-blotting by using antibodies

## (A)



## (B)
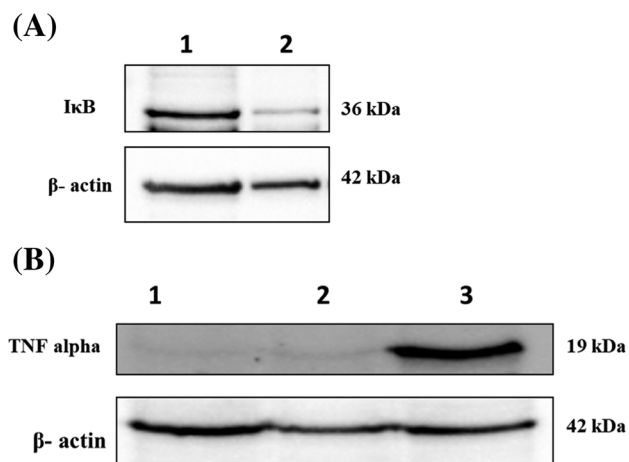


**FIGURE 1** A, Western-blotting analyses of intracellular levels of IκB activated by the treatment of differentiated THP-1 cells with LPS. Lane 1, total proteins extracted from untreated cells; lane 2, total proteins extracted from cells treated with 100 ng/mL of LPS. Western-blotting analyses were performed by using antibodies specifically recognizing IκB. Endogenous β-actin was used as an internal standard. B, Western-blotting analysis of TNF-α intracellular levels upon inflammation induction by treatment of differentiated THP-1 cells with LPS. Lane 1, total proteins extracted from untreated cells; lane 2, total proteins extracted from cells treated with 300 ng/mL of brefeldin A (3 h); lane 3, total proteins extracted from cells co-treated for 3 h with 300 ng/mL of brefeldin A and 1 μg/mL of LPS. Western-blotting analyses were performed by using antibodies specifically directed towards TNF-α. Endogenous β-actin was used as an internal standard

specifically recognizing TNF-α, selected as a marker of inflammation. It has to be noted that, because of the low sensitivity of Western-blotting methodology, no signal associated with TNF-α was observed (data not shown). For this reason, upon stimulation of cells with LPS (1 μg/mL) for 3 h at 37°C, protein secretion was inhibited by co-treating cells with LPS (1 μg/mL) and brefeldin A (300 ng/mL) for a further 3 h at 37°C. In this way, TNF-α intracellular levels are expected to increase because of inflammation induction and concomitant block of protein secretion. Under these experimental conditions, a significant increase in TNF-α intracellular level (8.6-fold) was observed upon inflammation induction (Figure 1B).

## 2.2 | Setup of the MRM-MS method

Although other authors quantified the cytokines by benefiting from the selectivity and sensitivity of the MRM approach, some differences distinguished the current work from others.[15,16] The majority of them quantified the cytokines secreted into the conditioned medium, whereas, in the present work, the intracellular content of selected cytokines was quantified after blocking their release from THP-1 cells by brefeldin. Additionally, the present work can be considered a further progress of the previous ones addressed at the point-time quantification of inflammatory proteins released in secretome[16] and in response to interleukin-1beta stimulation.[15] The main goal of the

present study is to analyze the kinetics of the production of some cytokines, in order to clarify the cellular response to the external stimuli. The immunological response to external viruses or bacteria is a topic of great interest nowadays. Although different methodologies of detection, e.g., ELISA and MS, were used to quantify cytokines in THP-1 cells, similar concentration values were recorded.[17] Actually, within MS strategies, large-scale approaches were comprehensively used for the exploration of numerous cytokines in both lysate cells and secretome samples.[18]

Finally, the current work provided the development of an MRM method for the detection and quantification of some cytokines by a TQ-S mass spectrometer, in order to optimize the instrumental parameters to be tested on clinical samples. Indeed, sera from female patients were preliminarily analyzed providing good instrumental responses for all monitored proteins. Such results encouraged us to carry out further investigations, in order to provide all the necessary analytical parameters in serum samples.[16]

A kinetic study was performed by stimulating macrophage cells ($1 \times 10^6$ cells/well) with 1 μg/mL of LPS from *E. coli* for different time points (2, 4, 6, 9 and 24 h). This allowed the exploration of both "primary markers of inflammation", activated through the immediate response of the immune system to the action of LPS, and of "late markers of inflammation", that are generally activated 10 h after the stimulation. Cell lysates collected at the different time points were subjected to MRM analyses and, for each time point, a volume corresponding to the same amount of protein content (100 μg) was used to carry out the digestion and subsequently the liquid chromatography (LC)/MRM-MS analysis. This allowed us to normalize the obtained data to protein content.

In order to set up the MRM-MS method, the best ionizing peptides *per* protein and the best precursor ion-product ion transitions were obtained by Skyline software. The selection of the peptides to be monitored was figured out by matching the Skyline software data and those collected into the online repositories, e.g., the SRM Atlas.[19] This strategy allowed the selection of proteotypic peptides for each target protein. Stringent criteria of selection were used in Skyline to increase the selectivity of the method, such as the exclusion of peptides containing methionine residues within the sequence, consensus sequences for N-glycosylation or other amino acidic modifications compromising the optimal ionization efficiency.

The choice to monitor two or three peptides as satisfactory for the identification and subsequent quantification of a large number of target proteins by MRM-MS analyses is shared from other authors.[20] Matrix interference was assessed by spiking samples with hydrolyzed protein standards, thus performing a quantitative analysis by the external standard method that did not require labelled peptides as references.[21]

For each target protein, the peptide showing the highest Total Ion Current (TIC) for all the monitored transitions was assigned as Quantifier, while the other monitored peptides were used as Qualifiers to enhance the specificity in protein recognition in a real sample.

Solutions containing a known concentration (fmol/μL) of each standard were analyzed to study the linearity of instrumental response. The calibration curves were obtained by plotting the sum of the peak areas for all the monitored transitions for the quantifier peptide versus the concentration of standard protein (fmol/μL). Analyses were performed in triplicate for each point of the calibration curves and linear functions were then applied to the calibration curves.

## 2.3 | MRM-MS method validation

Analytical parameters were determined and the limits of detection (LOD), limits of quantification (LOQ), working ranges, linearity ranges, the calculated y-intercepts and the angular coefficients obtained for the calibration curves are reported in Table 2. Values of $R^2$ tending to 1 indicated a strong correlation between concentration and instrumental response for each calibration curve.

Repeatability and accuracy for each standard are summarized in Table 1. These values were obtained by considering n = 10 replicate analyses on a 100 fmol/μL solution of the mixture of standards. The matrix effect was evaluated by spiking THP-1-unstimulated cells with a known amount of the mixture of standards (500 fmol/μL each). This spiked sample was treated as reported in section 3 and then analyzed n = 10 times under the same conditions as the standard samples and on the same day to evaluate the effects of interfering substances in standard quantification (Table 2).

## 2.4 | MRM-MS methodology applied to THP-1-differentiated cells stimulated with *E. coli* LPSs

The optimized MRM-MS procedure was applied to THP-1-differentiated cells ($1 \times 10^6$) stimulated with LPS molecules from *E. coli*, in order to induce the inflammation process. To perform kinetic analyses, samples were analyzed at different time points upon cell

**TABLE 1** List of selected proteins with corresponding selected peptide sequences, precursor ions *m/z*, product ions *m/z* and collision energies (CE)

| Protein | Peptide | Precursor ion *m/z* | Product ions *m/z* | CE (V) |
|---|---|---|---|---|
| TNF-α | R.ANALLANGVELR.D [108–119] | 620.85++ | A [y10] - 1055.62+ | 20 |
| | | | L [y9] - 984.58+ | |
| | | | L [y8] - 871.49+ | |
| | | | A [y7] - 758.41+ | |
| | | | N [y6] - 687.37+ | |
| | R.IAVSYQTK.V [158–165] | 455.25++ | A [y7] - 796.41+ | 15 |
| | | | V [y6] - 725.38+ | |
| | | | S [y5] - 626.31+ | |
| | | | Y [y4] - 539.28+ | |
| INF-γ | K.SVETIK.E [91–96] | 338.69++ | V [y5] -589.35+ | 12 |
| | | | E [y4] -490.21+ | |
| | | | T [y3] - 361.24+ | |
| | K.FFNSNK.K [103–108] | 378.68++ | F [y5] -609.29+ | 11 |
| | | | N [y4] - 462.23+ | |
| | R.DDFEK.L [112–116] | 327.14++ | D [y4] - 538.25+ | 11 |
| | | | F [y3] - 423.22+ | |
| IL-8 | R.ELCLDPK.E [74–80] | 437.72++ | L [y6] - 745.39+ | 14 |
| | | | C [y5] - 632.30+ | |
| | | | L[y4] - 472.27+ | |
| | K.ENWVQR.V [81–86] | 416.20++ | N [y5] - 702.36+ | 11 |
| | | | W [y4] - 588.32+ | |
| IL-10 | K.DQLDNLLLK.E [58–66] | 536.30++ | Q [y8] - 956.57+ | 20 |
| | | | L [y7] - 828.51+ | |
| | | | D [y6] - 715.43+ | |
| | | | N [y5] - 600.40+ | |
| | K.ESLLEDFK.G [67–74] | 490.75++ | S [y7] - 851.45+ | 18 |
| | | | L [y6] - 764.41+ | |
| | | | L [y5] - 651.33+ | |
| | | | E [y4] - 538.25+ | |

stimulation with LPS, i.e., 2 h, 4 h, 6 h, 9 h, and 24 h. MRM-MS analyses were performed in triplicate for each time point, to evaluate the reproducibility of the method in complex matrices.

On the basis of the MRM TIC chromatograms, reported in Figure 2, the developed method allows the identification of the ELCLDPK peptide from the IL-8 protein with high specificity and selectivity, without any other interfering peak.

All the results obtained by MRM-MS analysis of THP-1-differentiated cells stimulated with *E. coli* LPSs are summarized in the histograms of Figure 3, reporting the intracellular expression trend of each target protein, as an average of three replicates, at different time points upon cell stimulation with bacterial LPSs. Each point of the time-course analysis was compared with a cell sample not stimulated with *E. coli* LPS (CTRL), in order to determine the content of each target protein at the baseline level.

TNF-α production was found to increase 2 h and 4 h after stimulation of differentiated cells with bacterial LPSs (Figure 3), thus confirming the pro-inflammatory role exerted by TNF-α in the recruitment of macrophages, and in the stimulation of complex signalling cascades that control several intracellular functions. Indeed, this cytokine acts in synergism with IFN-γ, and stimulates the migration of immune cells to the infection site, thus contributing to the granuloma formation, capable of controlling the disease progression.[22]

In agreement with TNF-α production, the amount of INF-γ was found to progressively increase in a time interval of 4 h following cell stimulation with LPS (Figure 3). Afterwards, the amount of protein was found to decrease and to increase again after 24 h from cell stimulation with bacterial LPS (Figure 3), in line with the role of INF-γ in the acute and late inflammation processes. Indeed, INF-γ up-

**TABLE 2** Analytical parameters for TNF-α, INF-γ, IL-8 and IL-10 quantifier peptides

| Quantifier peptide | m | q | $R^2$ | LOD | LOQ | Linearity range [fmol/μL] | %recovery | %accuracy | %RSD |
|---|---|---|---|---|---|---|---|---|---|
| TNF-α R.ANALLANGVELR.D [108–119] | 117.6 | 106.1 | 0.998 | 4.8 | 14.8 | 1–250 | 92.3 | 85–115 | 3.5 |
| INF-γ K.SVETIK.E [91–96] | 2.07 | 34.6 | 0.995 | 2.5 | 7.5 | 1–1000 | 94 | 92–99 | 8.7 |
| IL-8 R.ELCLDPK.E [74–80] | 306.6 | −4.355 | 0.998 | 1.2 | 3.6 | 1–2000 | 90 | 87–108 | 9.2 |
| IL-10 K.DQLDNLLLK.E [58–66] | 2.06 | 28.10 | 0.997 | 3.2 | 9.6 | 1–1000 | 91 | 89–100 | 5.5 |

LOD, LOQ, repeatability (%RSD), accuracy (%), matrix effect (%recovery), linearity range (fmol/μL), y-intercept, angular coefficient for the calculated calibration curves obtained for quantifier peptides of standard proteins are reported
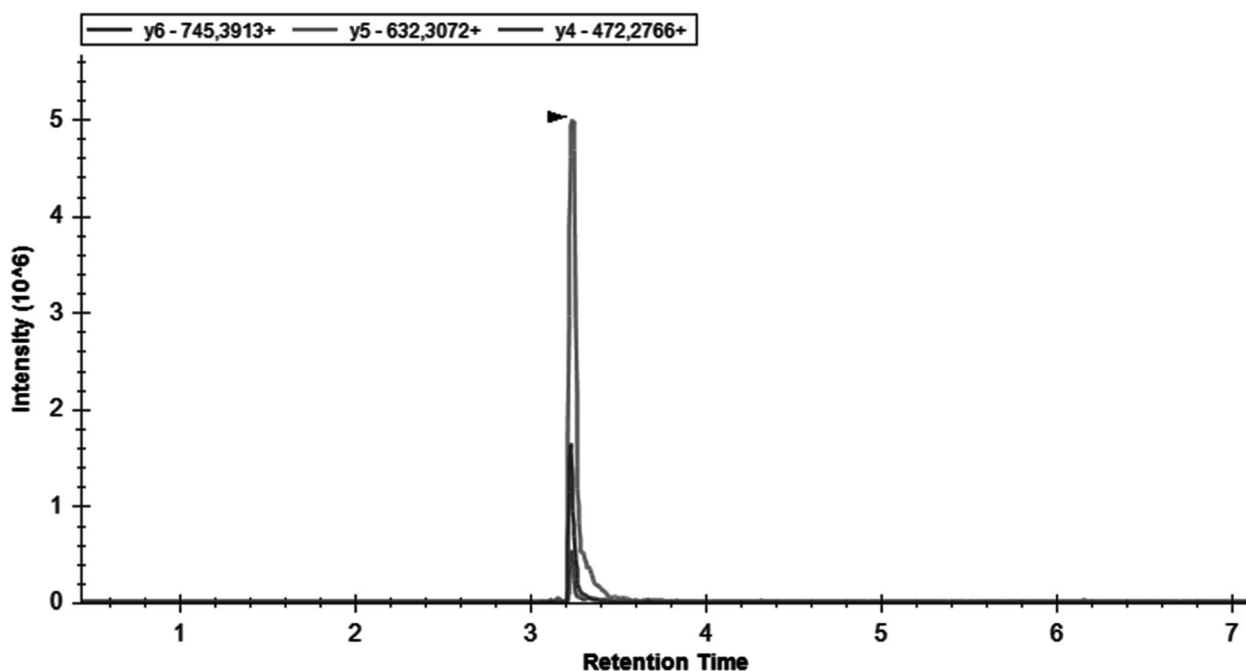


**FIGURE 2** MRM TIC chromatograms for ELCLDPK peptide of IL-8 from THP-1 cell sample after 6 h of LPS stimulation. Monitored transitions are perfectly co-eluted at 3.2 min
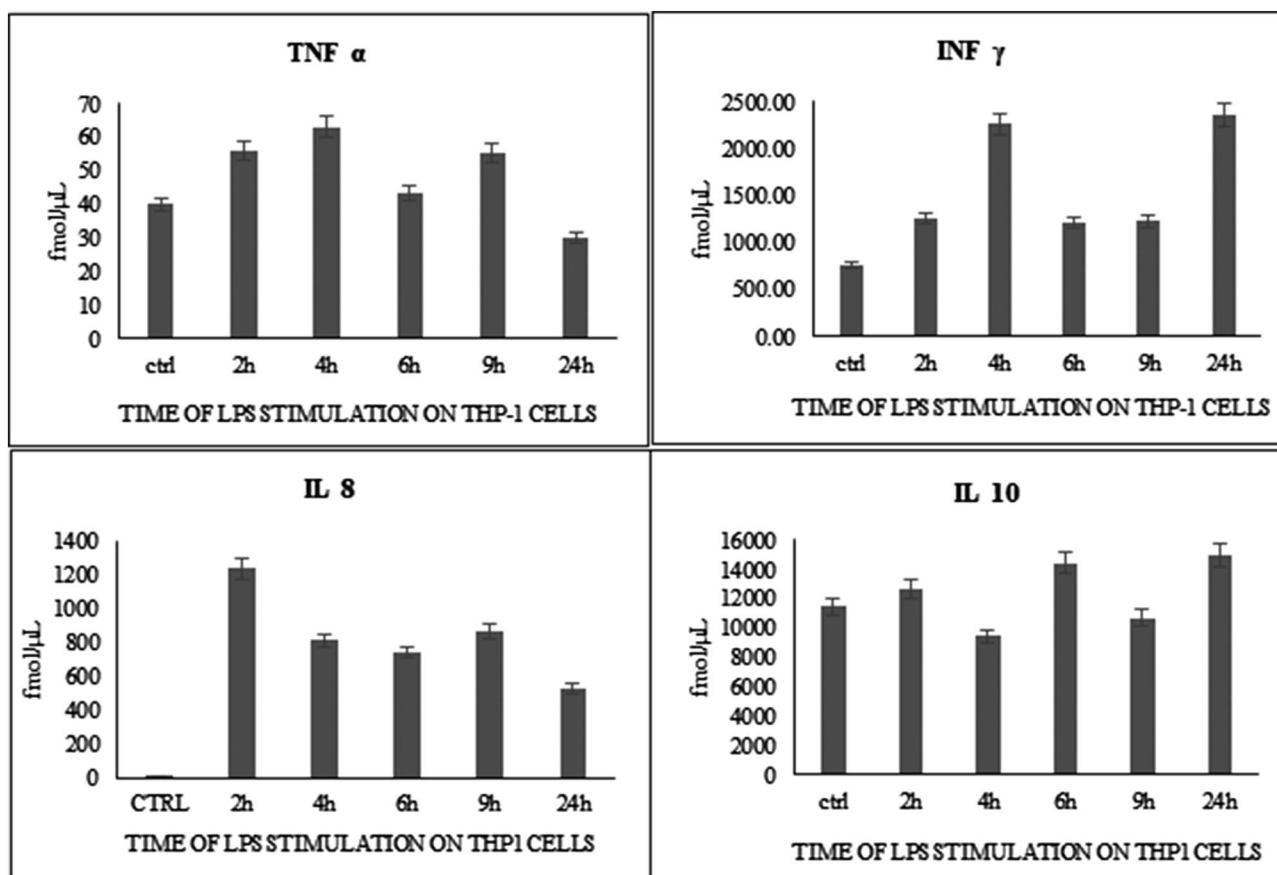
**FIGURE 3** Graphical representation of kinetics of target protein content. Protein content was expressed as fmol/μL for each sample of THP-1 cells stimulated with *E. coli* LPS. Each sample was analyzed in triplicate (n = 3) and the calculated standard deviations were found to be comprised in the range 8–11%

regulates expression of inflammatory mediators, especially primary mediators, such as TNF-α and interleukin (IL)-1, which induce alveolar macrophages to produce a large number of secondary inflammatory cytokines, such as IL-6 and IL-8.[21] IL-8 production was found to significantly increase after 2 h from cell stimulation with bacterial LPS, and then to progressively decrease (Figure 3). This behaviour is in line with the role of IL-8 in acute inflammation processes, as it is involved in the accumulation and activation of inflammatory cells, the release of inflammatory mediators, and in neutrophil chemotaxis to sites of inflammation to regulate the inflammatory response.[16,21,23] In the case of IL-10, a slight increase in production is observed only at 6 and 24 h after cell stimulation with LPS; otherwise its concentration remains almost constant and similar to that recorded for non-stimulated cell samples.

Indeed, IL-10 is considered to be an inhibitory cytokine crucial for preserving the adequate balance between inflammatory and immune-pathological responses.[6,24] Due to its ability to inhibit production of cytokines from T lymphocytes, IL-10 is considered a cytokine synthesis inhibitory factor (CSIF), that acts by inhibiting the production of pro-inflammatory cytokines (IFN-γ, TNF-α and IL-12), and by blocking the activation of T lymphocytes through the inhibition of MHC class II molecule expression.

## 2.5 | Application of the MRM-MS assay to the sera samples

A preliminary attempt to apply the set-up method (MRM-MS) to real samples was carried out by using four pooled serum samples belonging to women of childbearing age. Thus, four sera samples were prepared by pooling different samples (10 sera each). Samples were treated as previously described and quantification results are summarized in Table 3. The Western-blotting analyses performed on the same sera samples pointed out that no positive signal was detected by using antibodies specifically recognizing TNF-α (Figure 4, lane 4). To define the detection limits of the Western-blotting methodology, increasing amounts of standard protein were analyzed, and 6.25 ng (Figure 4, lane 10), corresponding to about 35 fmol//μL, was defined as the lowest protein amount detectable by this technique. Considering that the LOD for TNF-α by the MRM method was set up at 4.8 fmol/μL, this method seems to be promising for the detection and quantification of these inflammatory proteins in sera.

Obtained data showed protein concentrations (Table 3) significantly higher than the physiological values measured by immunological assays and reported by others.[25] Actually, these

**TABLE 3** Concentrations of target proteins determined by the MRM-MS approach in serum samples

| Serum sample | TNF-α (fmol/μL) | IL-10 (fmol/μL) | INF-γ (fmol/μL) | IL-8 (fmol/μL) |
|---|---|---|---|---|
| S | 4.52 | 589.03 | 252.00 | 4.57 |
| Pool 2 | 4.06 | 856.34 | 229.83 | 3.80 |
| Pool 3 | 33.71 | 903.11 | 287.55 | 4.11 |
| Pool 4 | 22.10 | 746.43 | 221.32 | 5.50 |
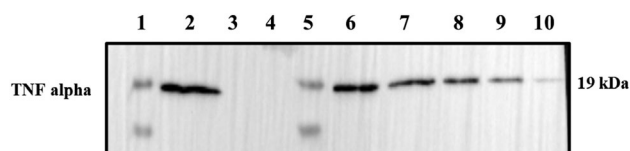


**FIGURE 4** Western-blotting analysis of TNF-α levels in the serum of patients with inflammatory diseases. Lane 1, pre-stained protein markers; lane 2, pure TNF-α protein (200 ng); lane 3, control serum; lane 4, serum from patients with inflammatory disease; lane 5, pre-stained protein markers; lane 6, pure TNF-α protein (100 ng); lane 7, pure TNF-α protein (50 ng); lane 8, pure TNF-α protein (25 ng); lane 9, pure TNF-α protein (12.5 ng); lane 10, pure TNF-α protein (6.25 ng). Western-blotting analyses were performed by using antibodies directed towards TNF-α protein

findings could be due to the different specificity of the two compared techniques, considering that, to the best of our knowledge, few reference methods have been reported to assess serum levels of these proteins by using targeted mass spectrometry in clinical investigations. However, the obtained data seems to be very promising, paving the way to the applicability of mass spectrometry in MRM mode also to the clinical setting, ensuring high reproducibility, sensitivity and savings in terms of time and costs.

## 3 | EXPERIMENTAL

### 3.1 | Materials and methods

#### 3.1.1 | Chemicals and reagents

Interleukin 8, interleukin 10, tumor necrosis factor-α and interferon-γ standard proteins, guanidine, tris(hydroxymethyl) aminomethane hydrochloride (TrisHCl), dithiothreitol (DTT), ethylenediaminetetraacetate (EDTA), trypsin, iodoacetamide (IAM), ammonium bicarbonate (AMBIC), and trichloroacetic acid (TCA) were purchased from Sigma-Merck (Milan, Italy). Formic acid (HCOOH), methanol, chloroform, and acetonitrile (ACN) were from J.T. Baker (Phillipsburg, NJ, USA). Pipette tips C18 (Zip tips) and centrifugal filter units were purchased from Merck Millipore (Schaffhausen, Switzerland).

Serum samples were obtained from consenting women. All experimental protocols were approved by the Ethic Committees of the Local Health Authority of the three areas and accepted by the Italian National Institute of Health in December 2017.

#### 3.1.2 | Cell culture

THP-1 monocyte cells were from the American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were cultured in RPMI-1640 (Sigma-Merck, Milan, Italy), supplemented with 10% fetal bovine serum (HyClone, GE Healthcare Lifescience, Chicago, IL, USA), L-glutamine and antibiotics, in a 5% $CO_2$ humidified atmosphere at 37°C. THP-1 cells were differentiated by adding 2 nM PMA (Sigma-Merck, Milan, Italy) to macrophages for 96 h.

#### 3.1.3 | Lipopolysaccharides (LPSs) and antibodies

Lipopolysaccharides from *E. coli* were purchased from Sigma-Merck (Milan, Italy). The primary antibodies anti-IκB, anti-TNF-α and anti-β-actin were purchased from Santa Cruz Biotechnology (Dallas, TX, USA).

#### 3.1.4 | Western-blotting analyses

THP-1 monocyte cells were plated into six-well plates ($5 \times 10^5$ cells/well), and then 2 nM of PMA was added to the culture medium for 96 h at 37°C to induce the cell differentiation. Afterwards, to induce the inflammatory process, differentiated macrophages were stimulated with 1 μg/mL of LPS from *E. coli*. To prepare cell lysates, both untreated and treated cells were scraped off in phosphate-buffered saline (PBS), centrifuged at 1000 *g* for 10 min, and then suspended in lysis buffer (1% NP-40 in PBS, pH 7.4) containing protease inhibitors. After 30 min of incubation on ice, lysates were centrifuged at 14000 *g* for 30 min at 4°C. Upon determination of total protein concentration in the supernatant by the Bradford assay, samples were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) followed by Western-blotting using specific antibodies directed towards IkB (Cell Signalling Technology) or TNF-α (ProteinTech). For normalization to internal standard signals, antibodies against β-actin (Sigma-Merck, Milan, Italy) were used.

#### 3.1.5 | Preparation of standard solutions and analysis of peptide mixtures by in-solution digestion

Stock solutions were prepared by following the procedures recommended by Sigma-Merck and stored at −20°C until the analysis was performed.

Each standard protein (2 μg) was lyophilized and subsequently used to prepare peptide mixtures useful to obtain the calibration

curves by the external standard method. To this purpose, standard proteins were submitted to reduction, alkylation and tryptic digestion. Samples were dissolved in denaturing buffer (6 M urea, 300 mM TrisHCl pH 8.0, 10 mM EDTA) containing DTT in a 10-fold molar excess with respect to Cys residues at 37°C for 2 h. Following incubation, IAM was added to perform carboamidomethylation by using an excess of alkylating agent (5-fold molar excess with respect to thiol residues). The mixture was then incubated in the dark at room temperature for 30 min. The proteins were purified by a standard protocol of precipitation by using chloroform/methanol/water. Supernatants were removed, and the pellets were dried under vacuum. Digestion of protein mixtures was carried out in 10 mM AMBIC using trypsin at 50:1 (protein/enzyme) mass ratio. The samples were incubated at 37°C for 16 h and, following acidification with 10% HCOOH, they were dried under vacuum.

To eliminate any impurities, the samples were suspended in 200 μL of 100 mM AMBIC, filtered by centrifugal filter units (0.22 μm), and dried in a speed-vacuum concentrator. Peptide mixtures were then suspended in 0.1% HCOOH to a final concentration of 10 pmol/μL.

By mixing 10 μL of each tryptic peptide standard mixture and 10 μL of 0,1% HCOOH, a solution at a final concentration of 2 pmol/μL for each standard was obtained. Serial dilutions were prepared in the concentration range 1–2000 fmol/μL. Six concentration points were analyzed in triplicate to construct the calibration curves.

All standards were kept at −20°C before LC/MRM-MS analyses.

## 3.1.6 | In-solution digestion of THP-1 cellular extracts following LPS stimulation and serum samples

Total protein lysates were obtained from $1 \times 10^6$ cells, for both control and LPS-stimulated samples, and quantified by Bradford assay. An aliquot of the serum (50 μL) was used for in-solution digestion. Both protein suspensions (cellular and serum ones) were subjected to reduction, carboamidomethylation, and tryptic hydrolysis of proteins as previously described for standard proteins. Purified peptide mixtures were then suspended in 50 μL of 0.1% HCOOH, and stored at −20°C until LC/MRM-MS analysis.

## 3.1.7 | LC/MS/MS instrumentation and conditions: MRM targeted proteomic approach

Peptide mixtures, once purified, were analyzed by LC/MS/MS using a Xevo TQ-S instrument (Waters, Milford, MA, USA) equipped with an IonKey UPLC Microflow source coupled to an Acquity ultra-performance liquid chromatography (UPLC) system (Waters). The peptide mixtures (1 μL) were injected and separated on a TS3 analytical reversed-phase (RP) column (1.0 mm × 150 mm; Waters) at 45°C with a flow rate of 3 μL/min using 0.1% HCOOH in water (LC–MS grade) as eluent A and 0.1% HCOOH in ACN as eluent

B. Peptides were eluted (starting from 1 min after injection) with a linear gradient of eluent B in A from 7% to 95% in 55 min. The column was re-equilibrated at initial conditions for 4 min. The MRM-MS analyses were performed in positive ion mode using an MRM detection window of 0.5–1.6 min per peptide; the duty cycle was set to automatic and dwell times were minimal 5 ms. Cone voltage was set to 35 V.

In order to set up a targeted MRM method, Skyline software (3.7, 64-bit version; MacCoss Lab Software, University of Washington, USA)[26–28] was used for the *in silico* selection of peptides with unique sequences for each selected protein. For each peptide, the *m/z* value of the precursor ion, the *m/z* values of the product ions and the relative collision energy were provided by Skyline. Peptides with zero missed cleavages were considered and the best two to five transitions per peptide were selected from the top ranked y-fragments. As a result, nine peptides for the four selected proteins were selected and 29 transitions were monitored during a single analysis. Table 1 summarizes the amino acid sequence for all the selected peptides for each protein. Moreover, Table 1 reports the best transitions, *m/z* precursor ion – *m/z* product ions, with the corresponding collision energy (V) values to optimize the MRM-MS method.

## 3.1.8 | Method validation: Determination of LOD and LOQ

Quantitative analysis on THP-1 cells stimulated with *E. coli* LPS samples was conducted using the external standard method by using standard proteins hydrolyzed with trypsin as standard. Standard solutions ranging from 2000 fmol/μL to 1 fmol/μL were used to construct the calibration curves. Each point of the calibration curve was analyzed in triplicate by using the LC/MRM-MS method. The upper limit of the working range has been defined for each selected standard compound as the concentration where the instrumental response becomes non-linear.

The limit of detection (LOD) and the limit of quantification (LOQ) were estimated by using the following equations:

$$LOD = \frac{3.3s}{b}; LOQ = \frac{10s}{b}$$

where s is the standard deviation of the y-intercept and b is the angular coefficient of the calibration curves calculated for each single standard.

To evaluate repeatability and accuracy, a 100 fmol/μL standards' mixture was analyzed 10 times (n = 10) on the same day and under the same conditions. Therefore, repeatability was calculated as relative standard deviation (%RSD) according to:

$$\%RSD = \frac{\sigma}{media_{n=10}} \times 100$$

Accuracy was defined as: $\%Accuracy = \frac{c_{exp}}{c_{std}} \times 100$ where $c_{exp}$ is the quantification result and $c_{std}$ the known concentration of the standard analyte.

The matrix effect was evaluated by spiking a mixture of standards into 50 μL of THP-1-unstimulated cells to reach a final concentration of each standard molecule of 500 fmol/μL. This mixture was treated as reported for THP-1-stimulated cells and then analyzed 10 times on the same day and under the same conditions. Matrix effect of quantification of standards was evaluated as the recovery value of standards as reported in the following equation:

$$\%\text{Recovery} = \frac{c_{exp} - c_{not\ spiked}}{c_{std}} \times 100$$

where $c_{exp}$ is the analyte concentration measured in the spiked THP-1 cells, $c_{not\ spiked}$ is the analyte concentration measured in the non-spiked THP-1-unstimulated cells and $c_{std}$ refers to the 500 fmol/μL standard analyte added to the THP-1 cells.

## 4 | CONCLUSIONS

Cells respond to an inflammatory stimulus primarily by activating signalling pathways that lead to the release of proteins, such as cytokines and chemokines, that play a crucial role. The quantification of such proteins responsive to cell stimuli is a fascinating research field contributing to the study of inflammatory cascade of events. The development of MRM-MS methodology, as described herein, has the main advantage of allowing the identification and quantification of a wide panel of target proteins in a single analysis step, overcoming cross-reactivity problems associated with the use of antibodies. The proposed experimental approach, allowing the simultaneous analysis of several target proteins, also offers the possibility to easily perform complex kinetic analyses of inflammatory processes. Indeed, in the present study, we quantified several protein markers, such as TNF-α, INF-γ, IL-8 and IL-10, in the conditioned medium of stimulated cells with high accuracy, selectivity, sensitivity, robustness, and with good reproducibility. Hence, the MRM-MS methodology described here appears to be a powerful tool to determine the inflammation state of cells. Indeed, the methodology not only allows quantitative analyses of low-abundance proteins, but also delivers reliable quantitative data when proteins are analyzed across multiple samples.[22] In the present work, differentiated THP-1 cells were treated with bacterial LPSs, in order to induce the inflammatory process, and to study cell system reactions to applied stimulus. Kinetic analyses were performed by collecting samples at 2 h, 4 h, 6 h, 9 h, and 24 h after stimulation with LPS. A mass spectrometry method in MRM mode for the simultaneous monitoring of four proteins, such as TNF-α, INF-γ, IL8, and IL10, has been developed to investigate the production trend of these protein markers. We demonstrated that, upon cell stimulation with LPSs, an increase in TNF-α production occurred, triggering a cascade mechanism that led to the production of INF-γ and IL-8. IL-10, instead, remained almost constant throughout the process, thus confirming and supporting its action as an anti-inflammatory cytokine.

In order to demonstrate the extensibility of the method to applications in the clinical field, the developed MRM method was applied to pooled serum samples from women of childbearing age. These preliminary experiments were carried out in an attempt to compare the MRM-MS method with enzyme-immunoassay sensitivity. The satisfactory detection of monitored cytokines by MRM-MS will encourage researchers to use this approach as an alternative tool to the immunologic assay.

## DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article, further inquiries can be directed to the corresponding author.

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