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Multiplexed cytokine detection in microliter microdialysis samples obtained from activated cultured macrophages

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Abstract

Microdialysis sampling probes were used to collect cytokine samples from lipopolysaccharide (LPS)-stimulated macrophages. The probes were immersed into cell culture wells containing either RAW 264.7 or isolated peritoneal macrophages. Dialysates (15 μL) from these wells were subjected to a multiplexed cytokine sandwich immunoassay platform analyzed by flow cytometry that measures up to six separate cytokines, interleukin-6 (IL-6), interleukin-10 (IL-10), interleukin-12p70 (IL-12p70), interferon-γ (IFN-γ), macrophage chemoattractant protein-1 (MCP-1), and tumor necrosis factor-α (TNF-α) in a single 15-μL sample. In vitro microdialysis sampling relative recovery experiments showed that only IFN-γ, IL-6, MCP-1, and TNF-α could be recovered across a commercially-available 100-kDa MWCO microdialysis membrane. Eleven hours after LPS addition (1 μg/mL), RAW 264.7 macrophages secreted greater than 8000 pg/mL of TNF-α and greater than 1000 pg/mL MCP-1. With the peritoneal macrophages, greater than 6000 pg/mL of IL-6, MCP-1, and TNF-α were obtained. The maximum dialysate concentrations obtained from the RAW macrophages were 1300 pg/mL for TNF-α and 55 pg/mL for MCP-1. Maximum cytokine concentrations from peritoneal macrophage dialysates reached approximately 2000 pg/mL, 1100 pg/mL and 500 pg/mL for TNF-α, MCP-1 and IL-6, respectively. Microdialysis sampling allowed for 20-min samples to be collected during the cytokine release from the activated macrophages. These results demonstrate that microdialysis sampling can be used for collection of selected cytokines with improved temporal resolution.

Keywords: Cytokines; Interleukin-6 (IL-6); Interleukin-10 (IL-10); Interleukin-12p70 (IL-12p70); Interferon-γ (IFN-γ); Macrophage chemoattractant protein-1 (MCP-1); Tumor necrosis factor-α (TNF-α); Macrophage; Microdialysis; Flow cytometry

Cytokines are a diverse group of bioactive proteins with molecular weights between 8 and 80 kDa. Cytokines are produced by various cell types including lymphocytes, neutrophils and monocytes/macrophages. At femtomolar concentrations that are transient and tightly regulated, cytokines affect cells via paracrine, autocrine, or endocrine actions to modify and modulate immunological and inflammatory reactions [1]. Cytokines form the basis of a sophisticated cellular communication network for normal immune function that is often dysregulated in disease states [2,3]. To fully elucidate the exact nature of an immune response requires detection of multiple cytokines rather than detection of one single cytokine.

Microdialysis sampling is a diffusion-based membrane separation technique that has principally been used to obtain representative samples from the extracellular fluid of living tissue [4,5]. During microdialysis sampling, a perfusion fluid is passed through the device at low microliter per minute flow rates. Analyte recovery or extraction efficiency (EE) is dependent upon a variety of physical and chemical parameters including the analyte diffusivity through the membrane and sample medium as well as the membrane length, and flow rate (Q_d). The EE is mathematically defined in Eq. (1), where C_{outlet} is the analyte outlet

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Table 1
Physicochemical properties of the cytokines studied[a]

Cytokine	MW (kDa)	Conformation	Active protein (kDa)	p*I*	R_G (Å)[b]	Amino acids
IL-12p70	35 and 40	Heterodimer	75	7.1	28.1	506
TNF-α	17.3	Homotrimer	51.9	5.6	21.0	468
IL-10	18.8	Homodimer	37.6	8.1	22.6	320
IFN-γ	15.9	Homodimer	31.8	5.7	24.5	266
MCP-1	13.1	Homodimer	26.2	10.5	13.5	250
IL-6	21.7	Monomer	21.7	6.5	16.0	187

[a] Data from [21].
[b] R_G, gyration radius was calculated using QuaSAR-Descriptor, MOE computer program (Chemical Computing Group Inc.) based on cytokine 3-D structure information from protein database bank (http://www.rcsb.org/pdb/).

concentration in the dialysate, C_{inlet} is the analyte inlet concentration (typically zero), $C_{sample,\infty}$ is the analyte concentration far

$$EE = \frac{C_{outlet} - C_{inlet}}{C_{sample,\infty} - C_{inlet}} = 1 - \exp\left(\frac{-1}{Q_d(R_d + R_m + R_e)}\right) \quad (1)$$

from the probe. Analyte mass transport is a coupled process that involves resistance terms associated with the three regions of microdialysis sampling, the dialysate (R_d), membrane (R_m) and external sample medium (R_e) [6]. The combination of the above-mentioned parameters coupled with the perfusion fluid passing through the membrane inner fiber lumen only once causes the EE to rarely reach 100%.

Recently, highly sensitive multiplexed immunoassay platforms have become commercially available allowing cytokine measurements in 50-μL sample volumes [7–10]. The detection limits for these assays fall into the low pg/mL range and have been compared and validated against standard enzyme-linked immunosorbent assay (ELISA) methods [11].

Commercially available microdialysis probes with 100-kDa MWCO membranes have been reported for in vivo microdialysis of a few select cytokines [12–14]. The use of particle-based immunoassays has potential to be highly advantageous to the sample-limited microdialysis process since several analytes can be analyzed in a single low volume sample. For example, if six separate cytokines were to be quantified in microdialysis samples using standard ELISA techniques more than 600 μL of sample would be needed. Thus, a flow rate of 1 μL/min would require 10 h of microdialysis sampling. By using the bead-based immunoassay only 15–20 min of sampling is needed at the same flow rate to achieve sufficient sample volume for the analysis. Here, we report the use of microdialysis sampling in macrophage cell culture as a model system to demonstrate the use of cytometric bead array systems for multiplexed cytokine analysis in as little as 15 μL of dialysate.

1. Materials and methods

1.1. Materials

BD™ Mouse Inflammation Cytokine Cytometric Bead Array (CBA) (BD Biosciences Pharmingen, San Diego, CA) kits were used for all the experiments. These kits contain a batch of six sets of polymeric beads (7 μm) each with its own distinct fluorescence intensity. Beads with the same fluorescence intensity are coated with a specific antibody for each cytokine (Interleukin-12p70 (IL-12p70), tumor necrosis factor-α (TNF-α), interferon-γ (INF-γ), macrophage chemoattractant protein-1 (MCP-1), IL-10, and IL-6) (Table 1). Phycoerythrin (PE) conjugated detection antibodies are also included in the kit. The kit contains proprietary mixtures of assay diluent and wash buffer. The wash buffer contains a proprietary concentration of phosphate-buffered saline (PBS), serum proteins, and detergent. The cytokines and cytokine antibody-coated bead related samples were stored at 4 °C prior to use.

Phosphate-buffered saline was prepared using dried Difco™ FA Buffer solid (Becton Dickinson and Company, Sparks, MD, USA) according to the manufacturer (10 g/L water). This PBS solution contains approximately 8.5 g sodium chloride, 1.1 g disodium phosphate, and 0.32 g monosodium phosphate in 1 L. Bovine serum albumin (BSA) and Tween®-20 were purchased from Sigma (Sigma Chemical Co., St. Louis, MO, USA).

1.2. Microdialysis system

A 3-syringe bracket microdialysis pump with Bee Syringe Pump Controller (Bioanalytical System Inc., West Lafayette, IN, USA) was used. Commercially available CMA/20 10-mm 100 kDa molecular weight cutoff (MWCO) polyethersulfone (PES) microdialysis probes were used for all experiments (CMA Microdialysis, North Chelmsford, MA, USA). The membrane external diameter is 500 μm.

1.3. Cytokine in vitro microdialysis

In vitro microdialysis samples were collected in a sand bath at 37 °C. The BD wash buffer was used as the microdialysis perfusion fluid. The probe was perfused at flow rates between 1.0 and 2.0 μL/min, and 20-μL samples were collected and stored at 4 °C prior to sample preparation for detection. Dialysate samples (15 μL) were incubated with 15 μL mixed cytokine antibody-coated beads and 15 μL PE detection reagent for 2 h in 12 mm × 75 mm polystyrene round-bottom tubes (Becton Dickson and Company, Franklin Lakes, NJ, USA) as per the BD instructions. After incubation, 0.70 mL of wash buffer was added into the tubes and vortexed. The samples were centrifuged at 200 × *g* for 5 min and carefully aspirated to discard the super-

natant from each test tube. Wash buffer (200 μL) was added to each assay tube to resuspend the beads prior to flow cytometric analysis.

Solutions containing 1250 pg/mL of each cytokine were prepared in the assay diluent solution provided by the manufacturer. One milliliter of the cytokine sample was placed into a 1.5-mL microcentrifuge tube. The microdialysis probe was immersed into this quiescent solution. Cytokine concentrations in the sample medium were determined in duplicate via splitting of a 40-μL aliquot taken before and after in vitro microdialysis sampling. The average cytokine concentration between these duplicate samples was used as the actual cytokine concentration in the sample medium for microdialysis EE calculations. All the microdialysis experiments were performed in triplicate at each flow rate using one probe.

The ultrafiltration through the PES microdialysis membrane was determined using five separate perfusion fluids. These solutions contained phosphate-buffered saline, PBS with 0.1% (w/v) BSA, PBS with 0.1% (w/v) BSA and 0.05% (w/v) Tween®-20, the BD assay diluent, and the BD wash buffer. Dialysates were collected every 20 min at 2.0 μL/min. Dialysates were weighed on a tared Ohaus Analytical Plus Electronic Balance sensitive to 0.00001 g (Ohaus Corporation, Florham Park, NY, USA).

1.4. Flow cytometry

A FACScan flow cytometer (Becton Dickinson, San Jose, CA) equipped with a 488-nm laser and the capability to detect and distinguish fluorescence emissions at 576 and 670 nm was used for multiplexed cytokine analysis. The software was BD CellQuest that included the BD CBA software. Instrument setup was carried out according to BD Pharmingen CBA instructions. Cytokine standards included one negative control (0 pg/mL cytokines) and additional standards between 20 and 5000 pg/mL. Standard curves were plotted using a four-parameter logistic curve-fitting model inherent to the BD software.

1.5. Cell culture

RAW 264.7 cells (American Type Culture Collection, TIB-71, Manassas VA), a murine macrophage cell line, were used for this study. The cells were cultured in Dulbecco's modification of Eagle's medium (DMEM) (Fisher Scientific, Pittsburgh, PA, USA) with 10% (v/v) filtered fetal bovine serum (Biowhittaker, Walkersville, MD, USA) supplemented with penicillin (100 U/mL) and streptomycin (100 μg/mL) (Fisher Scientific, Pittsburgh, PA, USA). For all experiments the cells were incubated at 37 °C with 5% CO_2. Cells were added to 24-well plates at 1×10^6 per well and were incubated for 1 h. The cells were then stimulated to release cytokines with bacterial lipopolysaccharide (LPS) (*E. coli*, K-235; Sigma) by replacing the media with DMEM containing LPS at 1 μg/mL. Controls did not contain LPS.

The microdialysis probes were placed into the wells through a specially machined cover for the tissue culture plate as previously described [15]. Cell culture media from control and LPS stimulated wells were collected over a 10-h period. Three dialysate samples (20 min collection/sample) were collected consecutively every other hour using a 1.0 μL/min perfusion flow rate. Cytokine concentrations in the media were quantified in duplicate from 40 μL of sample collected at various time points using the CBA kit from BD PharmMingen.

1.6. Preparation of peritoneal macrophages

Male Swiss–Webster mice weighing 20–25 g were used to obtain peritoneal macrophages. These mice received food and water ad libitum and were maintained on a 12 h on/off lighting cycle. All procedures were approved by the local IACUC committee at Albany Medical College. Each mouse was injected with 2 mL of 3% thioglycolate (i.p.) 2 days before macrophage isolation [16]. Prior to macrophage harvesting, the mice were euthanized using a halothane overdose. Peritoneal macrophages were isolated by two separate 8-mL lavages of the peritoneal cavity with cold phosphate-buffered saline. The cells were washed, suspended in DMEM macrophage culture medium and added to 24-well plate at 1.0×10^6 /mL. Fresh medium was added 2 h after plating. The following day the culture medium was again replaced with fresh medium and 1 μg/mL LPS was added. The collection of dialysates and culture medium was the same as described for the RAW 264.7 cells.

2. Results and discussion

2.1. Microdialysis perfusion fluid composition

Microdialysis EE is a complex function of physical components of the microdialysis system including membrane chemistry, pore size, probe geometry, length, analyte diffusive properties and perfusion fluid flow rate. For peptides and proteins, the microdialysis EE is also affected by the perfusion fluid composition. Serum proteins and detergents are commonly added to microdialysis perfusion fluids to improve protein recovery [17,18]. Five different perfusion fluids with varying serum protein and detergent compositions were studied to determine their effect on the microdialysis EE for these cytokines. A 100-kDa MWCO polyethersulfone (PES) membrane was used in these studies to allow collection of the high molecular weight cytokines. This particular type of microdialysis membrane has a propensity to exhibit ultrafiltration due to its larger pores. The extent of this ultrafiltration is shown in Table 2.

Table 2
Microdialysis membrane ultrafiltration

	Dialysate mass (g)[a]
PBS	0.02985 ± 0.00349
PBS + 0.1 wt.% BSA	0.03897 ± 0.00077
PBS + 0.1 wt.% BSA + 0.05 wt.% Tween®-20	0.03898 ± 0.00012
BD assay diluent	0.03800 ± 0.00051
BD wash buffer	0.03842 ± 0.00038

[a] Dialysate masses were mean ± standard deviation (S.D.), $n = 4$. Dialysates were collected every 20 min at 2.0 μL/min. The expected mass is approximately 0.04 g.

When phosphate-buffered saline was used as the perfusion fluid, an approximate 20% volume loss was observed across the membrane due to membrane ultrafiltration. Osmotic agents such as large molecular weight dextrans or albumin are typically included in the microdialysis perfusion fluid to compensate for fluid loss across these 100-kDa MWCO membranes [19]. With 0.1% (w/v) bovine serum albumin added to the PBS, the perfusion fluid loss was reduced to less than 5% volume loss. This result is similar to what others have reported for BSA concentrations as high as 4% (w/v) [18]. Thus, BSA can be used as an osmotic agent to reduce the ultrafiltration. When 0.05 wt.% Tween®-20 was added into the 0.1 wt.% BSA solution in phosphate buffer, the microdialysis perfusion fluid loss was not statistically different than the 0.1 wt.% BSA phosphate-buffered saline solution. The in-house prepared solutions containing protein and detergent exhibited similar ultrafiltration loss data when compared to the BD wash buffer and assay diluent. The BD solutions are proprietary mixtures containing serum proteins and detergents. Thus, it is not unexpected that similar results would be obtained between the BD solutions and the in-house prepared solutions.

In addition to preventing fluid loss, the inclusion of different agents in the perfusion fluid serves to improve the microdialysis EE for the cytokines. Fig. 1 shows the cytokine concentrations collected from a standard concentration of 1250 pg/mL for different perfusion fluid compositions. The microdialysis EE for the cytokines was lowest when PBS was used as a perfusion fluid. Inclusion of BSA and Tween®-20 in the perfusion fluid improved the microdialysis EE of these cytokines as previously reported [17,18]. Addition of Tween®-20 to the BSA solution did not create a statistically significant improvement in the microdialysis EE as compared to BSA alone. Surfactants reduce the adsorption of cytokine molecules on the microdialysis membrane and the outlet dialysis tubing. Serum proteins are commonly used as blocking buffers in ELISA. Thus, BSA can block the nonspecific adsorption sites on the membrane and outlet tubing for cytokine molecules during microdialysis sampling. Since both the BD wash buffer and the BD assay diluent contain serum proteins and detergent, inclusion of these solutions gave similar cytokine microdialysis EE values as compared to the in-house prepared BSA and BSA/Tween®-20 solutions. In the remaining experiments, the BD wash buffer was chosen as the perfusion fluid.

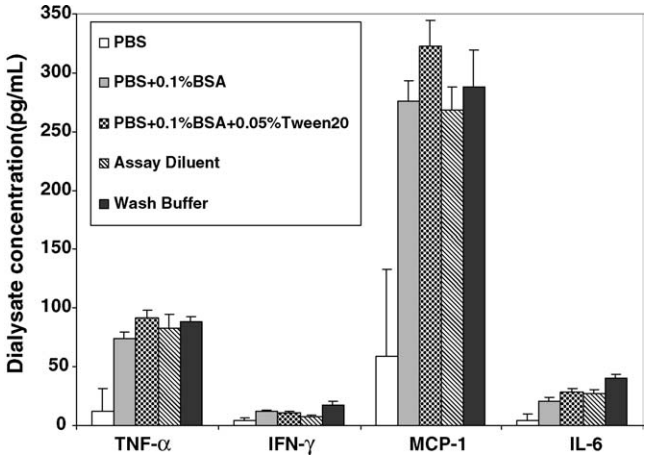

Fig. 1. Perfusion fluid effect on cytokine microdialysis EE (1.0 μL/min, $n = 3$). The microdialysis probe was immersed into a quiescent spiked (1250 pg/mL) cytokine standard solution. Error bars denote mean ± S.D.

2.2. In vitro microdialysis extraction efficiency

An important quantitative aspect of microdialysis sampling is that it provides a relative measure of the analyte concentrations external to the probe. Table 3 shows the in vitro microdialysis EE for different flow rates for cytokines spiked into culture media and the BD assay diluent at 37 °C in a sand bath. MCP-1 shows the highest EE reaching greater than 15% using the 1.0 μL/min flow rate. Among the six different cytokines, MCP-1 has the second lowest molecular weight (26.2 kDa). IL-6 has the lowest molecular weight (21.7 kDa) among this set of cytokines and exhibits an EE of approximately 5% which is much less than MCP-1 and less than TNF-α (51.9 kDa), a larger MW cytokine. IFN-γ (31.8 kDa) exhibited low microdialysis EE (1–2%) at all the perfusion flow rates. IL-12p70 and IL-10 were not detected in the dialysates in the 1250 pg/mL standard.

To determine if the microdialysis EE was less than 1% for IL-12p70 and IL-10, a 10,000 pg/mL cytokine standard was used. Three perfusion flow rates (0.5, 1.0, and 0.2 μL/min) were used to achieve the highest possible EE in an effort to recover measurable amounts of these cytokines. IL-12p70 was not detected with these conditions due to its molecular weight (75 kDa) and size approaching that of the PES membrane MWCO.

MWCO values for dialysis membranes are typically determined using equilibrium conditions. It is important to note that during microdialysis sampling, equilibrium conditions are not achieved. The membrane surface area and perfusion fluid flow rate combined with only one pass of the perfusion fluid through the inner fiber lumen generally prevent high recoveries. Thus, it is not surprising that IL-12p70 cannot easily pass through the 100-kDa MWCO dialysis membrane used for these studies. The microdialysis extraction efficiency of IL-10 was found to be less than 0.3% although IL-10 has a much lower molecular weight (27.6 kDa) than IL-12p70.

A possible explanation for the wide variance of the cytokine microdialysis EE values may be due to differences among the

Table 3
Cytokine in vitro microdialysis EE

	Cell culture media (μL/min)		BD assay diluent (μL/min)	
	1.0	2.0	1.0	2.0
IL-12p70	n.d.	n.d.	n.d.	n.d.
TNF-α	9.0 ± 0.7	3.8 ± 0.4	6.6 ± 0.8	4.7 ± 0.6
IFN-γ	2.1 ± 0.3	1.3 ± 0.3	1.2 ± 0.2	0.8 ± 0.1
MCP-1	17.9 ± 0.3	9.6 ± 0.6	13.2 ± 1.4	9.0 ± 1.5
IL-10	n.d.	n.d.	n.d.	n.d.
IL-6	5.7 ± 0.5	3.4 ± 0.4	4.3 ± 0.6	3.1 ± 0.4

A 100-kDa MWCO PES probe with a 10-mm length was used (mean ± S.D., $n = 4$). Cytokine standards (2500 pg/mL) were spiked into BD assay diluent and macrophage cell culture media. Microdialysis sampling was performed using a sand bath at 37 °C. n.d., Not detected.

cytokine 3-D structures. While molecular weight will affect size, the 3-D structure and the cytokine radius will also affect analyte diffusion through the membrane pores. Table 1 shows the radius of gyration (R_G) for each of the cytokines studied. The R_G is a characteristic length of the molecule [20]. IFN-γ, which exhibits a low microdialysis EE when compared with the other cytokines including the larger molecular weight TNF-α has a significantly larger R_G compared to other cytokines. The IL-10 structure is similar to that of IFN-γ and it has a similar R_G (Table 1) [21]. For IL-10, it appears the low EE may be due to a diffusion restriction caused by a hindered diffusion process through the pore caused by analyte shape rather than the molecular weight. Furthermore, the interactions between the membrane polymeric microdomains and the proteins are presently poorly understood. It is possible that additional interactions between the protein and the membrane material may hinder the diffusion process as well.

2.3. Control RAW 264.7 macrophage cytokine concentrations

The cytokine concentrations in the resting (i.e., unstimulated) macrophage cell culture wells were measured by taking 40-μL aliquots directly from the well at different time points. Six cytokines (IL-12p70, TNF-α, IFN-γ, MCP-1, IL-10, and IL-6) were quantified in duplicate from these samples. No significant difference in the cytokine concentrations was observed between cytokines spiked into either BD assay diluent or the fresh cell culture media (data not shown). This suggests that the culture media does not interfere with the immunoassay. Macrophages without stimulation produced TNF-α (about 800 pg/mL) and MCP-1 (about 90 pg/mL) in the media 11 h after plating as shown in Fig. 2. The TNF-α concentration increased gradually during the period. Dialysates were collected in these wells and the mean maximum concentration of TNF-α was 82 pg/mL. MCP-1 was not detected in the dialysates. IL-6, IL-10, IL-12p70, and IFN-γ were not detected in culture media aliquots obtained from these incubations at any time point using the CBA kit assay.

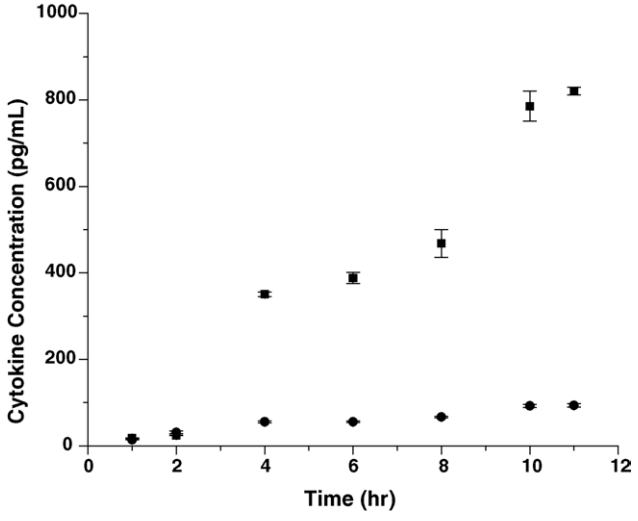

Fig. 2. Cytokine concentrations in the control macrophage media as a function of time. Data are plotted as a range of the sample aliquot measured in duplicate. Symbols denote TNF-α (■) and MCP-1 (●).

2.4. Cytokines released from LPS stimulated RAW 264.7 macrophages

LPS is commonly used to stimulate the production of some inflammatory cytokines in vivo and in vitro. Microdialysis sampling was used to collect samples so as to follow the changes in cytokine release after using a physiological stimulus. Cytokine production following LPS addition was detected in the culture media and dialysates. TNF-α and MCP-1 concentrations in the macrophage media increased to the range of approximately 8200 and 1230 pg/mL, respectively, 11 h after LPS addition (Fig. 3). IL-6, IL-10, IL-12p70, and IFN-γ were not detected in the macrophage culture media following LPS addition.

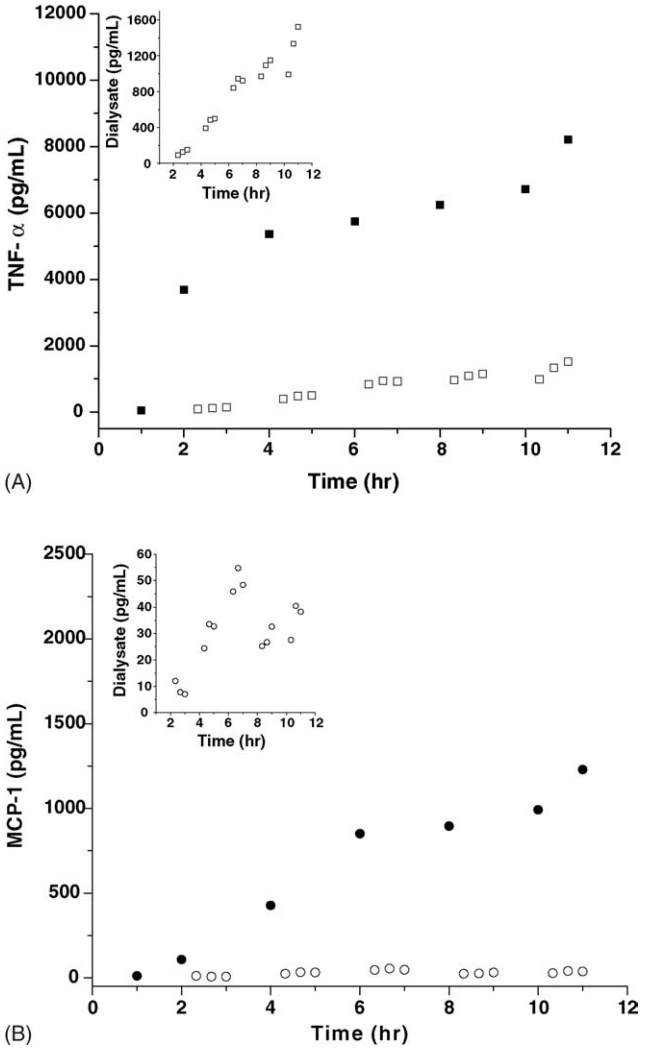

Fig. 3. (A) Cytokine concentrations in the culture media and dialysate for TNF-α. (B) Cytokine concentrations in the culture media and dialysate for MCP-1. The microdialysis flow rate was 1.0 μL/min. Symbols denote TNF-α ((■) culture well, (□) dialysate) and MCP-1 ((●)-culture well, (○) dialysate). LPS (1.0 μg/mL) stimulated macrophage cell media were measured in duplicate and the symbol denotes an average. Each dialysate collection is denoted with its own symbol. The insets show the dialysate concentrations for these cytokines.

For TNF-α, the concentration detected in the culture media increased rapidly from 50 to 3680 pg/mL in the second hour, but slowed considerably by 6 h (Fig. 3A). The detected dialysate TNF-α concentrations were representative of the concentrations detected in the culture media. In the dialysates, the TNF-α concentration increased gradually from non-detectable to a range of 1283 ± 270 pg/mL averaged over the one hour collection period in the 11th hour (Fig. 3A, $n = 3$).

The microdialysis EE for TNF-α increased initially and then approached a steady value (from 3.3% to about 15%) during the 11-h period. Note that these EE values are approximate as the concentrations were not in a steady-state which is generally the underlying assumption used for EE calculations [6]. Furthermore, there may be a gradient between the bottom of the culture well and the top. The solution surrounding the microdialysis probe may not be entirely homogeneous immediately following LPS addition. Over time the concentrations throughout the culture media may become more homogenous as the cytokine release kinetics decrease.

The MCP-1 concentrations detected in the macrophage culture media increased at a slower rate than those for TNF-α (Fig. 3B). The detected MCP-1 concentrations increased slowly during the first 2-h and then increased rapidly from about 100 pg/mL to more than 800 pg/mL 6 h after LPS addition. After 6 h, the macrophage media MCP-1 concentrations approached a plateau concentration of 1200 pg/mL.

The dialysate MCP-1 concentration initially increased and then approached a steady state which was similar to the concentrations in the macrophage cell media surrounding the microdialysis probe. However, the MCP-1 dialysate concentrations were low (reaching 55 pg/mL). The microdialysis EE of MCP-1 from activated macrophage culture media was about 5% (between 3.5 and 8%), which was less than the in vitro microdialysis EE (∼18%). This reduction in EE may be due to a variety of reasons including the lack of an absolute steady state of concentrations as well as experimental variability. Similar to activated macrophage media, the other four cytokines in the CBA assay kit (IL-6, IL-10, IL-12p70, and IFN-γ) were not detected in the microdialysis dialysates.

2.5. Cytokines released by peritoneal macrophages

Only two (TNF-α and MCP-1) of the six cytokines that can be quantified from the CBA kit were detected from the LPS-activated RAW 264.7 macrophage cells. To determine if freshly prepared macrophages may produce additional cytokines, the microdialysis experiments were also performed in isolated peritoneal macrophages from male Swiss–Webster mice. Fig. 4 shows the cytokine concentrations obtained from the LPS-activated peritoneal macrophage culture medium. Three cytokines (TNF-α, MCP-1 and IL-6) were readily detected in the peritoneal macrophage medium after LPS addition. All the three cytokine concentrations increased with time after the LPS addition and reached concentrations of 6000 pg/mL or greater within 6 h. IL-6 was not detected from LPS-stimulated RAW macrophage cells while significant IL-6 concentrations were obtained from the isolated peritoneal macrophages. Similarly, for MCP-1 only 1400 pg/mL was obtained from the RAW cells and greater than 6000 pg/mL MCP-1 obtained from the LPS-stimulated peritoneal macrophages. Similar TNF-α release kinetics and concentrations were observed between both LPS-stimulated RAW macrophages and peritoneal macrophages. IL-10, IL-12p70, and IFN-γ were not detected in the peritoneal macrophage culture media following LPS addition.

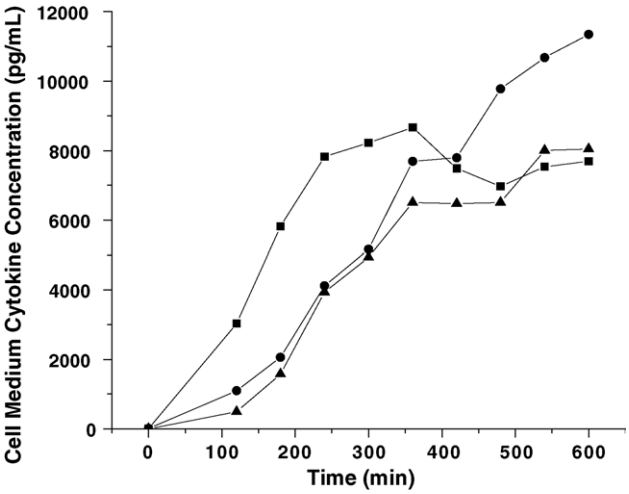

Fig. 4. Detected medium cytokine concentrations released by isolated mouse peritoneal macrophages at 1.0×10^6/mL density after addition of LPS (1 μg/mL): TNF-α (■), MCP-1 (●), and IL-6 (▲). Data were average value of duplicate measurements. Two hours after LPS addition, medium samples were taken every hour until 10 h.

Fig. 5 showed the detected absolute dialysate cytokine concentrations from LPS-stimulated peritoneal macrophages. The cytokine releasing patterns shown in the culture medium in Fig. 4 from the peritoneal macrophages were reflected in the dialysates. All three cytokine concentrations in the dialysates continuously increased and reached 1500 pg/mL for TNF-α, 1000 pg/mL for MCP-1, and 500 pg/mL for IL-6, respectively. The microdialysis extraction efficiency (EE) of these three

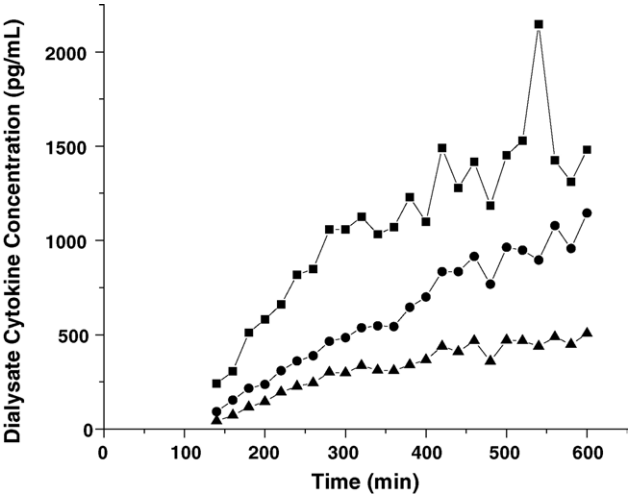

Fig. 5. Dialysate (absolute, not corrected for extraction efficiency) cytokine concentrations after LPS addition (1 μg/mL) to the peritoneal macrophage culture medium TNF-α (■), MCP-1 (●), and IL-6 (▲). Dialysate collection began 2 h after LPS addition.

cytokines were calculated to be as follows ($n = 24$): $15.7 \pm 5.0\%$ for TNF-α, $8.4 \pm 1.2\%$ for MCP-1, and $5.6 \pm 0.8\%$ for IL-6, respectively.

The culture medium samples were obtained directly from the well surface and the macrophages attach to the well bottom. Cytokines were released from macrophages attached to the bottom of the well that has a depth of approximately 1.4 cm. The diffusion of cytokines is slow especially for larger proteins such as TNF-α (MW 51.9 kDa), leading to potential concentration gradients from well bottom to the surface. The microdialysis membrane was immersed vertically below the medium surface. This may be the reason for the higher TNF-α microdialysis EE (15%) obtained from the macrophages as compared to the in vitro microdialysis EE (9%) using only spiked cell culture medium. IL-6 (21.7 kDa) is smaller than TNF-α and exhibited similar microdialysis EE from the macrophages as well as from spiked culture medium. However, MCP-1 (26.2 kDa) showed an unexpected lower microdialysis EE (8%) in the cell culture than its in vitro value (18%) obtained using the sand bath.

3. Summary

Protein collection from complex biological matrices via microdialysis sampling is still in its infancy [22]. Protein and peptide collection across microdialysis sampling membranes causes analytical challenges due to their larger size and thus smaller aqueous diffusion coefficients that result in low EE. In addition to the EE challenges, most proteins are typically quantified using immunoassays which may require 100 μL or more of sample. These constraints have limited the temporal resolution during microdialysis sampling in the past.

Cytokines produced from LPS stimulated macrophages were collected using microdialysis sampling with a 100-kDa MWCO PES membrane. Using the right conditions, different macrophage cells produce combinations of IL-6, MCP-1, and TNF-α. Some of the cytokines (IFN-γ, IL-10, and IL-12p70) that are included in the BD inflammation kit were not detected. All six of these cytokines have important roles during inflammatory responses; however, not all of these cytokines are made by macrophages. For example, IFN-γ is produced by T-cells. Macrophages produce very low concentrations of IL-12p70 and IL-10. Therefore, non detection of these cytokines was expected. The failure of these cells to make IL-6 was unexpected and was verified by standard ELISA. Experiments with spiked samples show the problems with detection of such large molecules, but also show that it is possible to collect several cytokines which was the purpose of this study.

The BD cytokine bead-based assay kit allows for measurement of these cytokines from 15-μL samples of dialysates collected from the macrophage cultures. As more researchers use microdialysis sampling for protein collection, the range of cytokines that can be reliably measured in dialysates will be determined. These results show that microdialysis sampling using 100-kDa MWCO membranes coupled with particle-based flow cytometric immunoassay is a viable multiplexed detection method for some, but not all cytokines.

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References

[1] F.R. Balkwill, F. Burke, Immunol. Today 10 (1989) 299–304.
[2] M.G. Cavallo, A. Toto, A.J.H. Gearing, T. Forsey, D. Andreani, R. Thorpe, P. Pozzilli, Immunology 75 (1992) 664–668.
[3] S. Keshav, L.-P. Chung, S. Gordon, Diagn. Microbiol. Infect. Dis. 13 (1990) 439–447.
[4] T.E. Robinson, J.B. Justice Jr., Microdialysis in the Neurosciences, Elsevier, Amesterdam, 1991.
[5] M. Muller, BMJ 324 (2000) 588–591.
[6] P.M. Bungay, P.F. Morrison, R.L. Dedrick, Life Sci. 46 (1990) 105–119.
[7] U. Prabhakar, E. Eirikis, H.M. Davis, J. Immunol. Methods 260 (2002) 207–218.
[8] K.L. Kellar, R.R. Kalwar, K.A. Dubois, D. Crouse, W.D. Chafin, B.E. Kane, Cytometry 45 (2001) 27–36.
[9] T.B. Martins, B.M. Pasi, J.W. Pickering, T.D. Jaskowski, C.M. Litwin, H.R. Hill, Clin. Chem. 118 (2002) 346–353.
[10] K.L. Kellar, J.P. Douglass, J. Immunol. Methods 279 (2003) 277–285.
[11] K.L. Kellar, M.A. Iannone, Exp. Hematol. 30 (2002) 1227–1237.
[12] A.J. Mayberry, B. Klitzman, J.A. Mathy, L.S. Levin, T.M. Phillips, S.A. Brown, Surg. Forum 49 (1998) 616–617.
[13] F. Sjögren, C. Svensson, C. Anderson, Br. J. Dermatol. 146 (2002) 375–382.
[14] J. Riese, S. Boecker, W. Hohenberger, P. Klein, W. Haupt, Surg. Infect. 4 (2003) 11–15.
[15] L. Sun, J.A. Stenken, J. Chromatogr. B 796 (2003) 327–338.
[16] D.J. Loegering, M.R. Lennartz, Inflammation 26 (2002) 305–310.
[17] T.M. Phillips, L.M. Kennedy, E.C. De Fabo, J. Chromatogr. B 697 (1997) 101–109.
[18] W.J. Trickler, D.W. Miller, J. Pharm. Sci. 92 (2003) 1419–1427.
[19] H. Rosdahl, U. Ungerstedt, J. Henriksson, Acta Physiol. Scand. 159 (1997) 261–262.
[20] M. Daune, Molecular Biophysics: Structures in Motion, Oxford University Press, Oxford, 1999, pp. 10–11.
[21] K.A. Fitzgerald, L.A.J. O'Neill, A.J.H. Gearing, R.E. Callard, The Cytokine Facts Book, second ed., Academic Press, San Diego, 2001.
[22] R.J. Schutte, S.A. Oshodi, W.M. Reichert, Anal. Chem. 76 (2004) 6058–6063.