Journal of Immunological Methods 464 (2019) 131–137

Contents lists available at ScienceDirect

Journal of Immunological Methods

journal homepage: www.elsevier.com/locate/jim

Technical Note

Secreted and intracellular cytokines are complementary measures for human monocytes treated with Toll-like receptor agonists

Yang Song[a,b], Julie Nigro[a,c], Lijia Yu[a,c], Mario Congiu[a,c], Narelle Skinner[a,c], Alexander Thompson[a,d], Kumar Visvanathan[a,c,*]

[a] Department of Medicine, University of Melbourne, Melbourne, Australia
[b] School of Medicine, Tsinghua University, Beijing, China
[c] Immunology Research Centre, St. Vincent's Hospital, Melbourne, VIC, Australia
[d] Department of Gastroenterology, St. Vincent's Hospital, Melbourne, VIC, Australia

A B S T R A C T

Cytokine production by human peripheral blood mononuclear cells including monocytes, is frequently assessed by measuring secreted cytokines using **e**nzyme **l**inked **i**mmuno**s**orbent **a**ssay (ELISA), whereby the total concentration of one cytokine of interest is obtained without information regarding the cell type responsible for making the cytokine. Cytokines can be retained inside the cell using protein transport inhibitors. Subsequent analysis by flow cytometry not only identifies the cell type producing the cytokine but can semi-quantitate the amount of cytokine produced by measuring the **g**eometric **m**ean **f**luorescence **i**ntensity (gMFI) and is amenable to analyzing more than one protein associated with the same cell (multiplexing). We hypothesized that a more comprehensive and biologically meaningful cytokine profile could be acquired by measuring both secreted and the retained intracellular cytokines in parallel cultures of magnetic-sorted CD14+ monocytes. Peripheral monocytes were isolated from 18 healthy donors and treated with standardized molecules that stimulate cytokine production; **T**oll-**l**ike **r**eceptor (TLR)4 agonist (**l**ipo**p**oly**s**accharide, LPS) or TLR7/8 agonist (R848). Pro-inflammatory cytokines (**i**nter**l**eukin (IL)-6, IL-8 and **t**umour **n**ecrosis **f**actor (TNF)) secreted into the culture medium were measured by ELISA. Parallel cultures were treated with LPS and R848 in the presence of brefeldin A (protein transport inhibitor) and the accumulated intracellular cytokines measured by flow cytometry. Each cytokine (IL-6/IL-8/TNF) gave a unique general pattern when secreted versus intracellular cytokine measurements (frequency and gMFI) were plotted to determine correlation. For monocytes treated with the TLR4 agonist, secreted IL-8 correlated with the frequency of IL-8 positive cells ($R = 0.559$, $p = .016$) and not with the amount (gMFI) of IL-8 per cell. In contrast, monocytes treated with the TLR7/8 agonist showed no correlation of secreted IL-8 with the frequency of IL-8 positive cells, but with this treatment secreted IL-6 was correlated with an increase in the frequency of IL-6 positive cells ($R = 0.501$, $p = .034$). TNF secretion from monocytes treated with either the TLR4 or TLR7/8 agonist did not correlate with the frequency or gMFI of TNF positive cells. However, there were significant correlations between the TLR4 and TLR7/8 induced TNF response (secreted and gMFI). We conclude that there are fundamental differences in secreted and intracellular IL-6/IL-8/TNF production after monocytes are treated with TLR agonists. Furthermore, secreted and intracellular cytokine analyses are complementary measures that should be used in parallel to explore inflammatory response and cytokine biology.

1. Introduction

The measurement of cytokine production is commonly used to determine the activity of the immune system (Choy et al., 2017). The innate immune system is the body's first line of defense against invading pathogens. Pathogens are recognized by the binding of pathogen-associated molecular pattern (PAMPs) to pattern recognition receptors such as Toll-like receptors (TLRs) (Takeuchi and Akira, 2010). Binding of TLRs with their respective ligands, triggers a pro-inflammatory signaling cascade that has antimicrobial and antiviral activity as well as stimulating the adaptive immune response through cytokine production and the activation of antigen presenting cells (APCs). There are two main techniques used to assess cytokine production in clinical samples and cell culture, the **e**nzyme-**l**inked **i**mmuno**s**orbent **a**ssay (ELISA) and flow cytometry.

ELISA is the 'gold standard' to quantitatively measure cytokines within a liquid medium with high specificity and sensitivity however, it does not provide information on the cell type producing the cytokine (Schuerwegh et al., 2003). In contrast, protein transport inhibitors can be used to retain cytokines in the cell and flow cytometry used to identify cytokine production by individual cells or cell populations. The latter allows the determination of the frequency (%) of cytokine producing cells within a population and an arbitrary measure of the amount of cytokine per cell determined by the **g**eometric **m**ean **f**luorescence **i**ntensity (gMFI) of the positive population (Amel Kashipaz et al., 2003).

Cytokine release may not be uniform across a homogeneous cell population, as each cell is in a distinct point in their cell cycle, and

* Corresponding author at: The University of Melbourne, Department of Medicine - St. Vincent's Hospital, PO Box 2900, Fitzroy, VIC 3065, Australia.
E-mail address: kv@unimelb.edu.au (K. Visvanathan).

https://doi.org/10.1016/j.jim.2018.11.003
Received 19 July 2018; Received in revised form 23 October 2018; Accepted 1 November 2018
Available online 03 November 2018
0022-1759/ © 2018 Published by Elsevier B.V.

varies between multiple different cells in a heterogeneous population. The average cytokine production by a cell population can be driven by a few individual groups of single cells showing significant heterogeneity (Shalek et al., 2014). The measurement of bulk cytokine release by ELISA cannot differentiate increased cytokine response due to increased cell number or from increased cytokine per cell. Moreover, incomplete and even conflicting cytokine data suggest there is a need to present results from different assays side by side (Chao et al., 1991). In this study we hypothesized that the analysis of isolated CD14+ cells should simplify the comparison between secreted and intracellular cytokines.

Only one study has compared extracellular cytokines with retained intracellular cytokines (IL-1β/IL-6/IL-10/IL-12) of CD14+ monocytes treated with a **T**oll-**l**ike **r**eceptor (TLR)4 agonist (**l**ipo**p**oly**s**accharide, LPS), showing good correlation between the two (Schuerwegh et al., 2003). However in that study, a flow cytometry bead array method was used to measure extracellular cytokines, not ELISA. In our study we have used ELISA to measure cytokines secreted by monocytes treated with either a TLR4 or TLR7/8 agonist in a first head-to-head comparison with retained intracellular cytokines measured by flow-cytometry. We were particularly interested in the degree of correlation between the two methodologies, and two TLR agonists and whether flow cytometry provides biological insight into the process of cytokine production over and above the information provided by ELISA.

2. Methods

Eighteen healthy volunteers (aged 22–61 years) gave a blood donation to the study.

2.1. Isolation of peripheral monocytes

Peripheral blood was collected into lithium-heparin tubes. **P**eripheral **b**lood **m**ononuclear **c**ells (PBMC) were isolated by density gradient centrifugation using SepMate™ tubes (STEMCELL Technologies) containing Ficoll-Paque™ Plus (GE Healthcare). To isolate monocytes, PBMCs were filtered through a 40 μm cell strainer, centrifuged (300 ×*g*, 10 min), resuspended in **p**hosphate-**b**uffered **s**aline (PBS)/0.5% ($^{w}/_{v}$) **b**ovine **s**erum **a**lbumin (BSA)/2 mM **e**thylene-**d**iamine**t**etra**a**cetic acid (EDTA, 80 μL/10^7 cells) and incubated with CD14 magnetic MicroBeads (Miltenyi Biotec, Catalogue Number 130-050-201, 20 μL beads/10^7 cells) for 15 min at 4 °C. The cell-MicroBead suspension was washed with PBS/0.5%BSA/2 mM EDTA (2 mL) and centrifuged (300 ×*g*, 10 min). The cell pellet was resuspended in PBS/0.5%BSA/2 mM EDTA (500 μL) and applied to an autoMACS™ Separator (Miltenyi Biotec). The positive selection program was used to collect the CD14+ fraction. For each donor, the purity of CD14+ monocytes was determined by flow cytometry and ranged from 95% to 100% (mean = 98%).

2.2. Stimulation of cytokine production by monocytes

After CD14+ monocytes were isolated, viable cells were counted using trypan blue (Gibco) staining. Viable monocytes (1×10^6) were transferred to a sterile round bottom polystyrene tube (5 mL Falcon) with culture medium (1 mL) comprising: RPMI-1640 (Gibco), 2 mM GlutaMAX, 100 units/mL penicillin, 100 μg/mL streptomycin and 5% ($^{v}/_{v}$) **f**etal **b**ovine **s**erum (FBS). Monocytes were incubated in the presence and absence of the TLR4 ligand LPS (0.01–100 ng/mL or 100 ng/mL, LPS-EK Ultrapure from *Escherichia coli* strain K12, InvivoGen, San Diego, CA) or the TLR7/8 ligand R848 (5 μg/mL = 14.25 μM, imidazoquinoline compound InvivoGen, San Diego, CA) for 6 h in a CO_2 incubator (Sanyo, Osaka, Japan) at 37 °C, with 95% humidity and 5% CO_2. After treatment, the monocytes were centrifuged (524 ×*g*, 5 min, 20 °C), the supernatants transferred to new tubes and stored at −80 °C for subsequent ELISA.

To measure intracellular cytokines, parallel monocyte cultures were treated with 1 mL culture medium (no stimulant), LPS (0.01–100 ng/mL or 100 ng/mL) or R848 (14.25 μM) for 2 h in a CO_2 incubator at 37 °C, with 95% humidity and 5% CO_2. After this period, the cultures were spiked with brefeldin A (final concentration was 1.0 μg/mL or 3.57 μM, Catalogue No. 555029, BD Biosciences, San Jose, CA) and incubated for a further 4 h in a CO_2 incubator at 37 °C, with 95% humidity and 5% CO_2. After incubation, monocyte cultures were centrifuged (524 ×*g*, 5 min, 20 °C) and prepared for flow cytometry analysis.

2.3. Intracellular cytokine measurements by flow cytometry

Monocytes (1×10^6/100 μL) were incubated with 3 μL anti-human CD14-APC-Cy7 (clone MφP9, Catalogue No. 557831, BD Biosciences, San Jose, CA) for 20 min on ice, protected from light, prior to the addition of PBS/2% FBS (1 mL). Cells were centrifuged (524 ×*g*, 5 min, 4 °C), the supernatant was discarded and the cell pellet fixed and permeabilized with Cytofix/Cytoperm™ Fixation/Permeabilization solution (100 μL, BD Biosciences) for 16 h at 4 °C. Cells were centrifuged (1455 ×*g*, 5 min, 20 °C), supernatant discarded and washed twice with BD Perm/Wash™ buffer (1 mL) prior to staining with a cocktail (100 μL) of anti-human IL-6-FITC (clone MQ2-6A3, Catalogue Number 554696, 5 μL), IL-8-PE (clone G265–8, Catalogue Number 554720, 5 μL) and TNF-APC (clone MAb11, Catalogue Number 554514, 3 μL) all from BD Biosciences (San Jose, CA). Cells were incubated for 30 min on ice, protected from light before adding PBS (1 mL) and centrifuged (1455 ×*g*, 5 min, 20 °C). Cells were analyzed on the BD FACSCanto II flow cytometer (BD Biosciences, San Jose, CA) using FACS Diva software Version 6.0 (BD Biosciences, San Jose, CA). Computer analysis software (FLOWJO® Version 9, Tree Star Inc., Ashland, OR) was used to analyze the data. The gating strategy included selection of single cells on forward scatter/side scatter plots (Fig. Error! Reference source not found.A), followed by selection of the CD14 positive population versus side scatter (Fig. 1B) and further analysis of this population for IL-6/IL-8/TNF (Fig. 1C). The software was used to calculate the frequency (%) and **g**eometric **m**ean **f**luorescence **i**ntensity (gMFI, as a measure of production) of cytokine producing cells.

2.4. Secreted cytokines measured by ELISA

The cell culture supernatants from treated monocytes were tested with separate BD Biosciences OptEIA™ kits for IL-6 (Catalogue No. 555220), IL-8 (Catalogue No. 555244) and TNF (Catalogue No. 555212), according to the manufacturer's instructions. Briefly, 96-well, flat-bottom, high-protein binding ELISA plates (Nunc MaxiSorp™, Thermo Scientific) were coated with biotinylated anti-human IL-6, IL-8 or TNF antibodies in 0.1 M $NaHCO_3$/0.1 M $Na_2CO_3^-$ (pH 10.0) for 16 h at 4 °C. The unbound antibody was aspirated and non-specific binding of the bound antibody was blocked with PBS/10% FBS (200 μL/well, 1 h, 20 °C). The blocking reagent was aspirated and undiluted cell culture supernatant or recombinant protein standards (100 μL/well) were added to duplicate wells and incubated for 2 h at 20 °C. After washing three times (PBS/0.05% Tween-20, 400 μL/well), biotinylated detection antibody plus horse radish peroxidase-streptavidin were added for 60 min at 20 °C (100 μL/well). After washing 3 times (PBS/0.05% Tween-20, 400 μL/well), 3,3′, 5,5;**t**etra**m**ethyl**b**enzidine (TMB) chromogenic solution (100 μL/well, SeraCare, Catalogue No. 5120-0076) was added for 30 min before stopping the reaction with 1 M H_2SO_4 (50 μL/well). The absorbance was measured at 450 nm on a FLUOstar Omega (BMG Labtech, Cary, NC) and readings were determined from standards (in pg/mL) prepared in different wells on the same plate.

2.5. Statistical analyses

The flow cytometry parameters (frequency (%) and gMFI) of cytokine producing cells were correlated with the ELISA results on semi-log

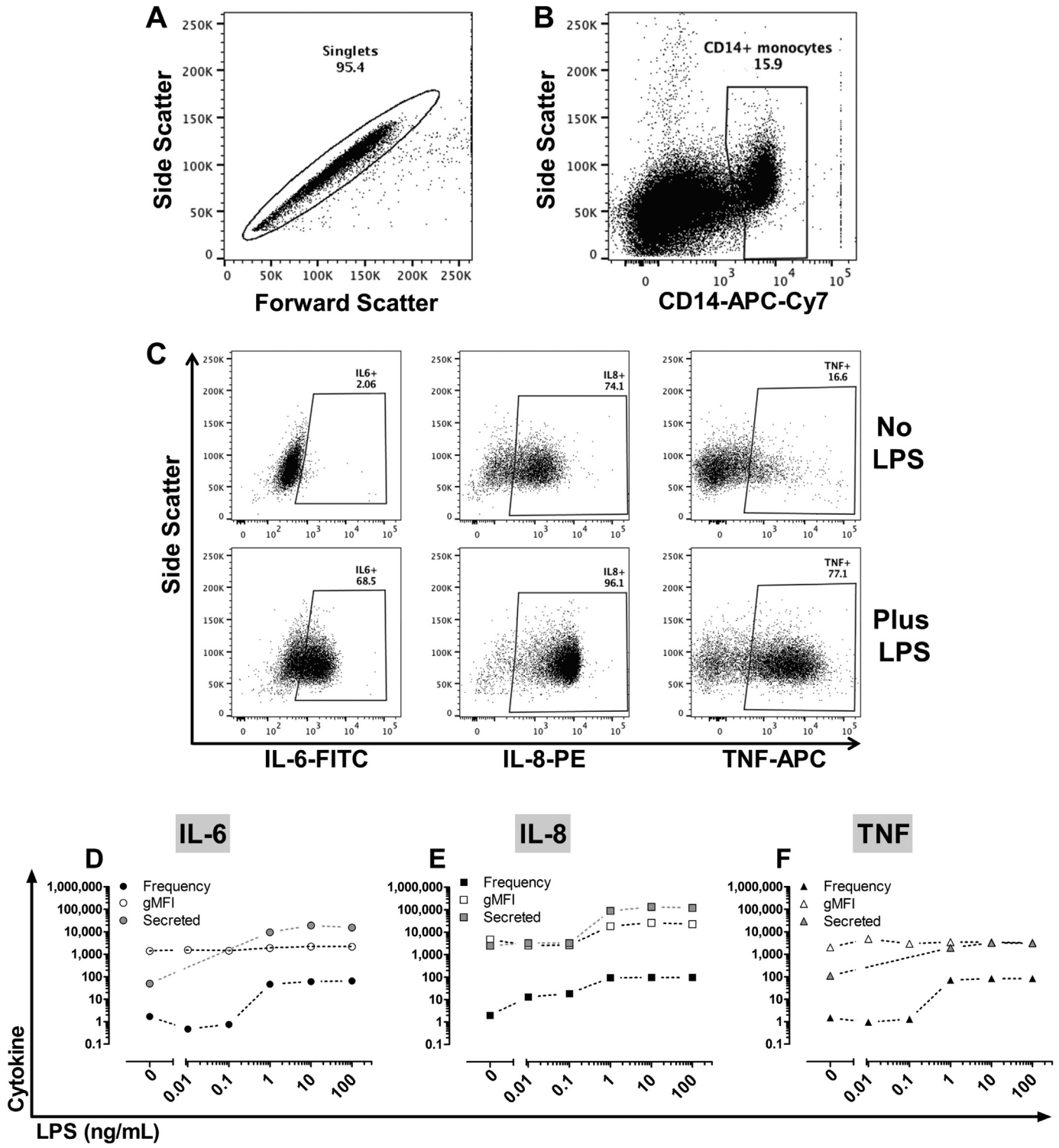


Fig. 1. Representative gating strategy and concentration-response to LPS. Monocytes from one donor were analyzed by flow cytometry. The gating strategy was based on forward scatter/side scatter and selection of single cells (A), followed by selection of CD14 positive cells on the CD14-APC-Cy7 versus side scatter plot (B). The CD14+ population was analyzed for cytokines IL-6, IL-8 and TNF, showing the difference between monocytes incubated in the absence and presence of LPS (100 ng/mL, C). The CD14+ monocytes from 1 donor were treated with LPS (0.01–100 ng/mL) and analyzed for intracellular (frequency and gMFI) and secreted IL-6 (D), IL-8 (E) and TNF (F). Data points are connected by a dotted line for visualization only.

and log x-y plots. Statistical analyses were performed using Prism 6. The Spearman's test was used for describing the correlation between the continuous variables.

3. Results

An in vitro model of the stimulated innate immune system was established by treating magnetic-sorted CD14+ cells with ligands for TLR4 (LPS) and TLR7/8 (R848). Secreted cytokines (IL-6/IL-8/TNF) were measured by ELISA and intracellular cytokines by measuring the

frequency/gMFI of cytokine producing cells by flow cytometry (Fig. 1A–C). The 6 h incubation of MAC-sorted CD14+ monocytes reduced the proportion of CD14+ cells independent of the treatment as represented in Fig. 1B. Between donors, the CD14+ population for unstimulated, LPS and R848 ranged from 12 to 39%, 7–56% and 17–56%, respectively.

3.1. Correlation between the frequency and gMFI of cytokine positive cells

To demonstrate that measuring intracellular cytokines can provide a deeper understanding of the underpinning biology, we tested whether or not there was a correlation between the frequency of cytokine positive cells and the gMFI of the cytokine positive cells. The treatment of magnetic-sorted CD14+ cells with LPS (0.01–100 ng/mL) resulted in a concentration-response with regards to the frequency of IL-6, IL-8 and TNF positive cells and the amount of secreted cytokine (Fig. 1D–F). In contrast, a concentration-response to LPS treatment was observed for the gMFI of IL-8 positive cells (Fig. 1E) and not for IL-6 and TNF positive cells (Fig. 1D, F). Subsequent experiments were done with 100 ng/mL LPS or 14.25 nM R848 as previously described for PBMCs by our laboratory (Howell et al., 2013).

Magnetic-sorted CD14+ cells were treated with LPS (100 ng/mL) or R848 (14.25 μM) for 6 h before analysis. Compared with untreated monocytes, cultures treated with LPS and R848 showed a higher frequency and gMFI of IL-6, IL-8 and TNF (Fig. 2, closed symbols). After monocyte treatment with LPS, the frequency of TNF positive cells correlated with the gMFI of TNF positive cells (Fig. 2C, $R = 0.485$, $p = .041$). Similarly, when monocytes were treated with R848, the frequency of TNF positive cells correlated with the gMFI of TNF positive cells (Fig. 2F, $R = 0.783$, $p = .0001$). The frequency and gMFI of IL-6/IL-8 positive cells did not correlate with each other, after treatment with either LPS or R848 (Fig. 2A, B, D, E). For IL-8 positive cells, this may be due to a relatively high basal frequency in unstimulated monocytes (Fig. 2B, E). These data suggest that for some cytokines, an increase in the number of cytokine producing cells does not always correlate with an increase in the production of that cytokine.

3.2. Correlation between secreted and intracellular cytokines

The complex cytokine biology can be analyzed with x-y plots of secreted and intracellular cytokine data (Fig. 3). For unstimulated monocytes, secreted IL-8 and TNF correlated with the frequency of IL-8 positive cells and TNF positive cells (Fig. 3B and C, respectively). In the absence of stimulant, secreted TNF correlated with the gMFI of TNF positive cells (Fig. 3I, $R = 0.477$, $p = .045$).

When treated with LPS, the amount of IL-6 secreted by monocytes correlated with intracellular gMFI of IL-6 positive cells (Fig. 3G, $R = 0.476$, $p = .046$). Secreted IL-8 correlated with the frequency of IL-8 positive cells after monocyte treatment with LPS (Fig. 3B, $R = 0.559$, $p = .016$).

When treated with R848, the amount of IL-6 secreted by monocytes correlated with the frequency of IL-6 producing cells (Fig. 3D, $R = 0.501$, $p = .034$). Secreted TNF correlated with the intracellular gMFI of TNF positive cells after treatment with R848 (Fig. 3 L, $R = 0.463$, $p = .053$).

By comparing with basal levels, general patterns show that for IL-6 where the basal gMFI is comparable with that of stimulated cultures (Fig. 3G, J), the increased IL-6 secretion following TLR ligand treatment is likely the result of increased frequency of IL-6 positive cells (Fig. 3A, D). In contrast, increased basal secretion of IL-8 following TLR ligand treatment is likely the result of increased gMFI (Fig. 3H, K) because the frequency of IL-8 positive cells is similar to untreated cells (Fig. 3B, E). In contrast to both IL-6 and IL-8, the increased TNF secretion from monocytes treated with either TLR4 or TLR7/8 ligands may be the

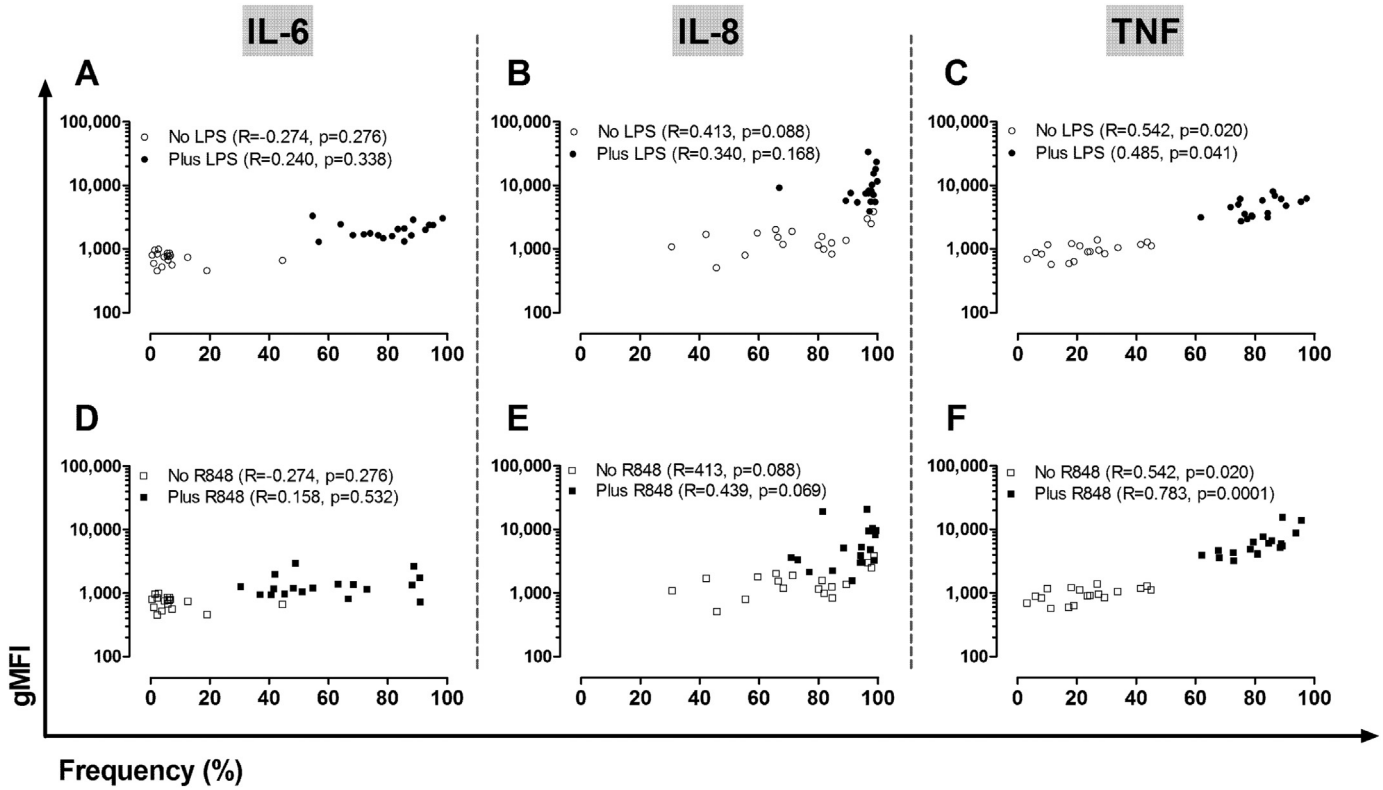
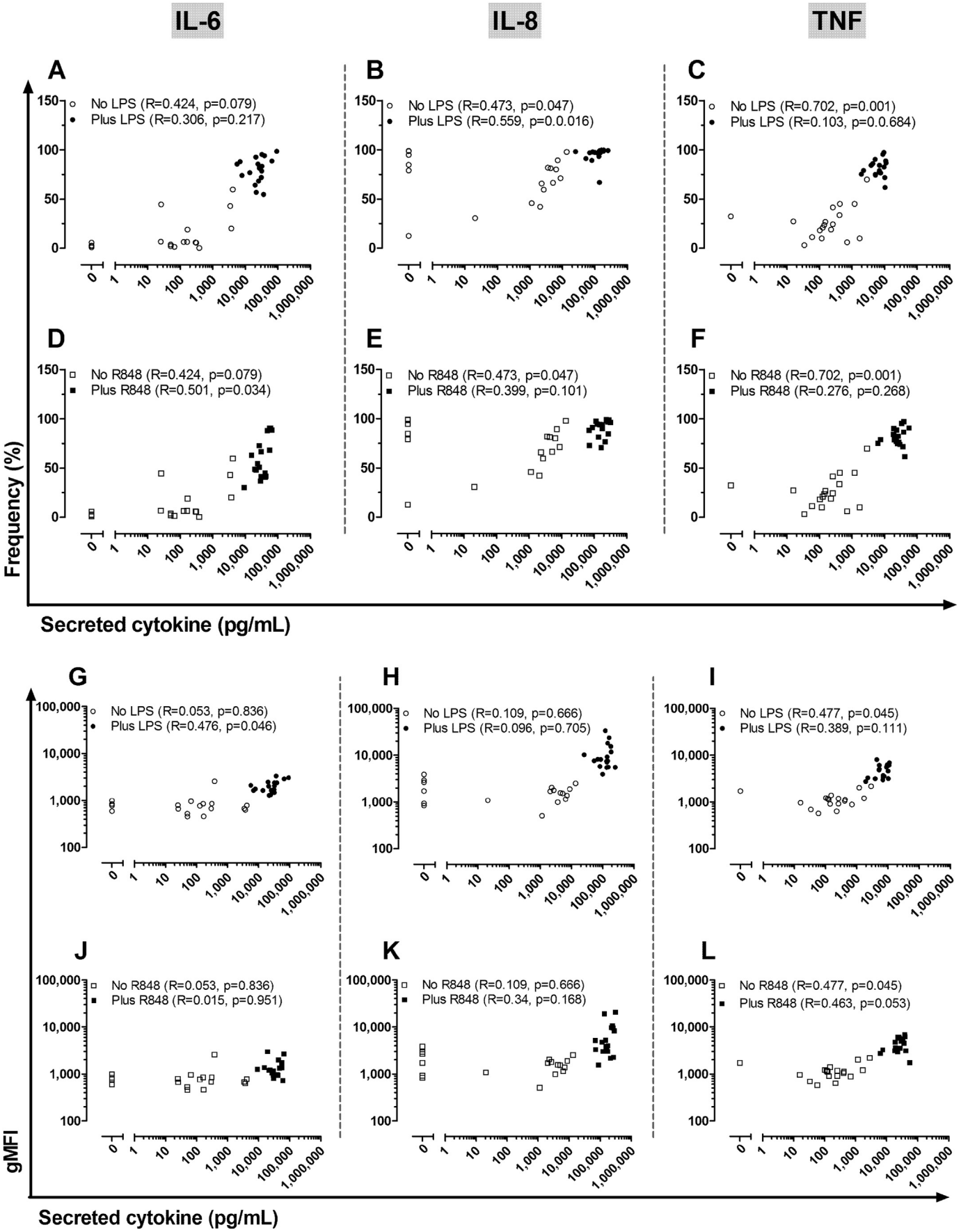


Fig. 2. Correlation between the frequency and gMFI of cytokine positive monocytes. Peripheral monocytes from 18 donors were positively selected with CD14 magnetic beads and treated with no stimulant (open symbols), a TLR4 agonist (LPS, closed circles) or TLR7/8 agonist (R848, closed squares) for 6 h. Intracellular IL-6 (A, D)/IL-8 (B, E)/TNF (C, F) were measured by flow cytometry. The frequency of cytokine positive cells and the gMFI of the same cytokine were graphed together as x-y plots on a semi-log scale. The Spearman's test was used to calculate the P value and correlation co-efficient stated on each graph.



(caption on next page)

Fig. 3. Correlation between secreted and intracellular cytokines. Peripheral monocytes from 18 donors were treated with a TLR4 agonist (LPS, A-C and G-I, closed circles) or TLR7/8 agonist (R848, D-F and J-L, closed squares) for 6 h. Secreted IL-6/IL-8/TNF measured by ELISA, was graphed on the x-axis (log scale) with intracellular cytokine parameters; either frequency (A-F, linear scale) of the cytokine or the gMFI (G-L, log scale) of the cytokine. The corresponding measurements from unstimulated cells are presented as open symbols in each graph. The Spearman's test was used to calculate the P value and correlation co-efficient stated on each graph.

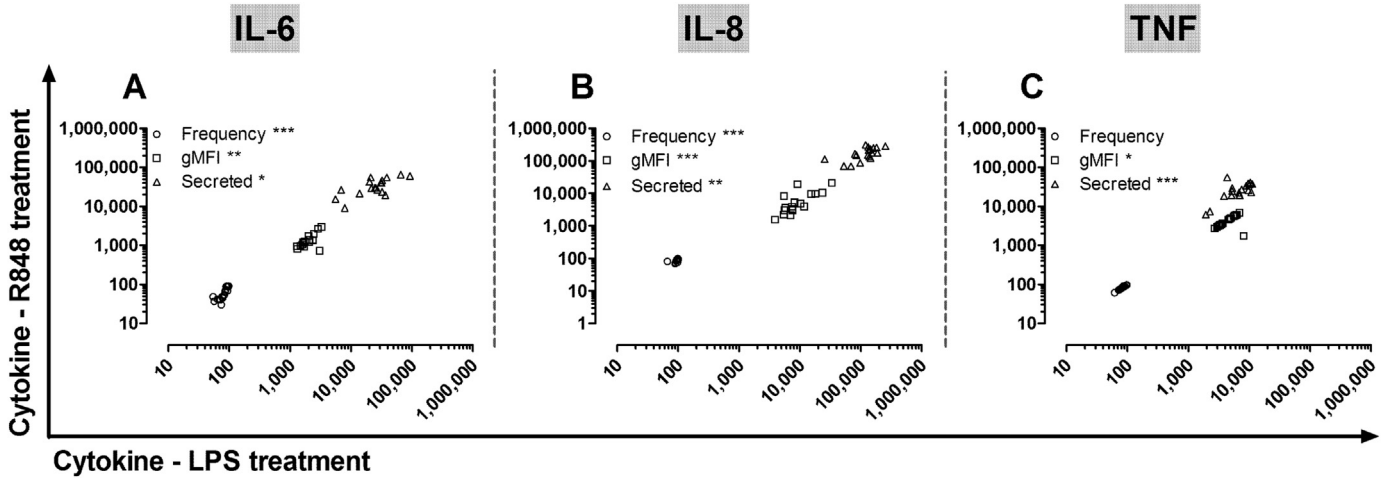


Fig. 4. Correlation of TLR4 and TLR7/8 induced cytokine stimulation. The data from monocytes treated with either TLR agonist were graphed together (LPS vs R848) to evaluate frequency (open circles), gMFI (open squares) and secreted cytokines (open triangles). The Spearman's test was used to calculate the P value and correlation co-efficient. (A) IL-6, Frequency R = 0.888, p < 0.001 (***), gMFI R = 0.639, p = 0.004 (**), secreted R = 0.573, p = 0.013 (*). (B) IL-8, Frequency R = 0.779, p < 0.001 (***), gMFI R = 0.744, p = 0.0004 (***), secreted R = 0.618, p = 0.0063 (**). (C) TNF, Frequency R = 0.263, p = 0.2911, gMFI R = 0.548, p = 0.0186 (*), secreted R = 0.761, p = 0.0002 (***).

result of both increased frequency (Fig. 3C, F) and increased gMFI of TNF positive cells (Fig. 3I, L).

3.3. Correlation of TLR4 and TLR7/8 induced cytokine stimulation

The intracellular and secreted cytokine data were used to compare the monocytes capacity to respond to two TLR agonists (LPS and R848), plotted on the same graph (Fig. 4). There was a significant correlation between the responses to LPS and R848 related to the frequency ($R = 0.888$, $p < .0001$) and gMFI ($R = 0.639$, $p = .004$) of IL-6 positive cells and secreted IL-6 ($R = 0.573$, $p = .013$, Fig. 4A). Similarly, a significant correlation between the responses to LPS and R848 related to the frequency ($R = 0.779$, $p = .0001$) and gMFI ($R = 0.744$, $p = .004$) of IL-8 positive cells and secreted IL-8 ($R = 0.618$, $p = .0063$, Fig. 4B). In contrast, only the gMFI of TNF positive cells ($R = 0.548$, $p = .0186$) and secreted TNF ($R = 0.761$, $p = .0002$) were associated with a significant correlation between LPS and R848 treatment (Fig. 4C). Overall, these data suggest that a cytokine response to a TLR4 ligand corresponds with a response to a TLR7/8 ligand.

4. Discussion

Monocytes predominantly express the principal family of pattern recognition receptors, up to thousands of TLRs per cell (Visintin et al., 2001). Moreover, monocytes produce a large amount of cytokines after stimulation of TLR4 and TLR7/8 (Bjork et al., 1992), and are an important model for studying the cytokine profile (Schildberger et al., 2013). By focusing on a specific cell type (human primary monocytes) we have extended on previous reports (Schuerwegh et al., 2003) by showing significant improvement in the validation for measuring secreted and intracellular cytokines using two assays. Purified monocytes from 18 healthy donors were stimulated and multidimensional data generated for intracellular IL-6/IL-8/TNF. Secreted cytokines from magnetic-sorted monocytes were measured by ELISA whereas the frequency of cytokine positive cells and amount of intracellular cytokine per cell was measured by flow cytometry. This is the first report to demonstrate that monocytes treated with TLR agonists have different secreted and intracellular cytokine response profiles.

Treatment of monocytes with either a TLR4 or TLR7/8 agonist, increased IL-8 production to levels generally one order of magnitude greater than IL-6 and TNF. Known for recruiting granulocytes, **n**atural **k**iller (NK) cells and T cells (Taub et al., 1996), IL-8 synergizes with interferon-alpha in NK cells (Dunn et al., 2007). In clinical conditions where the immune system is compromised, such as chronic hepatitis B patients undergoing a hepatic flare (acute increase in liver inflammation), peripheral IL-8 is the only cytokine that is consistently elevated and a biomarker for activation of the innate immune system (Tan et al., 2010). Moreover, the onset of hepatic flares is preceded by higher IL-8 production (Dunn et al., 2007; Tan et al., 2010). In this study, monocytes produced more IL-8 compared with IL-6 and TNF, which supports the use of this cell model for the study of cytokines in clinical conditions and their biological role for recruiting and synergizing with other immune cells.

Measuring the amount of secreted cytokines by ELISA is informative but does not give cell-specific cytokine information. Flow cytometry is complementary to ELISA because it provides advantageous semi-quantitative data for cell-specific cytokines. In our study, each stimulated cytokine (IL-6/IL-8/TNF) gave a unique pattern when measurements for secreted vs intracellular were compared (Fig. 3); supporting the use of both techniques to provide a deeper understanding of cytokine biology.

Using gMFI we were only able to detect a small amount of IL-6 production following LPS stimulation and IL-6/IL-8 production following R848 stimulation. In contrast, significant production was demonstrated by measuring secreted cytokines by ELISA. However, the ELISA data are misleading and cannot discriminate cytokines from monocytes that have retained/lost CD14 after treatment which occurred in unstimulated, LPS and R848 treated cells. The loss of CD14 from monocytes occurs during culture and has been reported by others (Ruppert et al., 1993).

The LPS treated monocytes secreted IL-8 and this correlated with the frequency of IL-8 producing cells (Fig. 3B) but not with their gMFI

(Fig. 3H). Under these conditions, the frequency was more sensitive than gMFI. The possible biological mechanism for this discrepancy may be due to the heterogeneity of CD14+ monocytes which exist as two populations; CD14 + CD16- (classical, 85% of all monocytes) and CD14 + CD16+ (intermediate, 10% of all monocytes) (Ancuta et al., 2009; Belge et al., 2002). These two monocyte populations can respond differently to TLR ligands. For example, CD14+/CD16+ monocytes treated with LPS produced 3-fold more TNF and IL-10 compared with CD14 + CD16- (Belge et al., 2002; Skrzeczynska-Moncznik et al., 2008). This could mean that in our study after LPS treatment, whilst more CD14+ cells were positive for IL-8 (i.e. frequency), only a small percentage of the population may have been producing the majority of IL-8, and this increased gMFI signal was likely lost amongst the majority of CD14+ cells that had little increase in IL-8 production. To test this, future studies should include an antibody to CD16 to further subgroup the CD14+ monocytes.

Only one other study has compared intracellular production with extracellular cytokines (IL-2/IL-4/IL-6/TNF/IL-10/IL-12) of CD14+ monocytes treated with LPS (TLR4 agonist) showing good correlation between the two (Schuerwegh et al., 2003). However in that study, a flow cytometry bead array method was used to measure extracellular cytokines after stimulation of whole blood with LPS. To extend and clarify the previous report, we used the 'gold standard' ELISA to measure cytokines secreted by monocytes and directly compared this with retained intracellular cytokines measured by flow-cytometry. Additionally, rather than stimulating whole blood with only one TLR4 agonist (LPS), we used purified monocytes and treated these with either a TLR4 or TLR7/8 agonist.

To semi-quantitate the amount of intracellular cytokines in the CD14+ population, cells were stimulated for 6 h and Brefeldin A was added in the last 4 h. There is a possibility that CD14+ cells secrete cytokines within the first 2 h with TLR agonists and this may cause a difference in the secreted vs intracellular cytokines observed. However, this is unlikely based on our own unpublished findings and previous cytokine kinetic studies that show little to no difference of IL-8 and TNF cytokines secretion from monocytes in the first 2 h of LPS treatment compared with untreated cells (Kaufmann et al., 1999).

We note that the concentration of brefeldin A recommended by the manufacturer and used in this study (3.57 μM), is 2.5 times the concentration used in the other study (1.4 μM) (Schuerwegh et al., 2003). With our experimental conditions, the viability of the monocytes at the end of the treatment period was > 85% (data not shown). Despite the difference in concentration of brefeldin A between our study and the previous report, both studies found a correlation between intracellular and secreted IL-6 after monocytes were treated with LPS (Fig. 2.2D).

In conclusion, different cytokine profiles between IL-6/IL-8/TNF were evidenced by flow cytometry and ELISA. Flow cytometry provided a complementary and detailed insight beyond ELISA in revealing cell-specific intracellular cytokines. Our study shows that when assessing immune cell culture models, analysis of both secreted and intracellular cytokines gives a more comprehensive understanding of the biology surrounding cytokine production.

Author contributions

KV and AT conceived and designed the experiments. YS, LY and NS performed the experiments. YS and LY analyzed the data. MC performed experiments and drafted the manuscript. YS and KV wrote the paper. JN redrafted significant parts of the work and critically revised it so as to contribute to the interpretation.

Acknowledgements

This work was supported by a project grant to KV from the Australian National Health and Medical Research Council (ID: 1066536). YS was supported by the China Scholarship Council.

References

Amel Kashipaz, M.R., et al., 2003. Normal production of inflammatory cytokines in chronic fatigue and fibromyalgia syndromes determined by intracellular cytokine staining in short-term cultured blood mononuclear cells. Clin. Exp. Immunol. 132 (2), 360–365.
Ancuta, P., et al., 2009. Transcriptional profiling reveals developmental relationship and distinct biological functions of CD16+ and CD16- monocyte subsets. BMC Genomics 10, 403.
Belge, K.U., et al., 2002. The proinflammatory CD14+CD16+DR++ monocytes are a major source of TNF. J. Immunol. 168 (7), 3536–3542.
Bjork, L., et al., 1992. Endotoxin and Staphylococcus aureus enterotoxin A induce different patterns of cytokines. Cytokine 4 (6), 513–519.
Chao, C.C., et al., 1991. Altered cytokine release in peripheral blood mononuclear cell cultures from patients with the chronic fatigue syndrome. Cytokine 3 (4), 292–298.
Choy, M.C., Visvanathan, K., De Cruz, P., 2017. An overview of the innate and adaptive immune system in inflammatory bowel disease. Inflamm. Bowel Dis. 23 (1), 2–13.
Dunn, C., et al., 2007. Cytokines induced during chronic hepatitis B virus infection promote a pathway for NK cell-mediated liver damage. J. Exp. Med. 204 (3), 667–680.
Howell, J., et al., 2013. Toll-like receptor 3 and 7/8 function is impaired in hepatitis C rapid fibrosis progression post-liver transplantation. Am. J. Transplant. 13 (4), 943–953.
Kaufmann, A., et al., 1999. Induction of cytokines and chemokines in human monocytes by Mycoplasma fermentans-derived lipoprotein MALP-2. Infect. Immun. 67 (12), 6303–6308.
Ruppert, J., et al., 1993. Down-regulation and release of CD14 on human monocytes by IL-4 depends on the presence of serum or GM-CSF. Adv. Exp. Med. Biol. 329, 281–286.
Schildberger, A., et al., 2013. Monocytes, peripheral blood mononuclear cells, and THP-1 cells exhibit different cytokine expression patterns following stimulation with lipopolysaccharide. Mediat. Inflamm. 2013, 697972.
Schuerwegh, A.J., et al., 2003. Comparison of intracellular cytokine production with extracellular cytokine levels using two flow cytometric techniques. Cytometry B Clin. Cytom. 55 (1), 52–58.
Shalek, A.K., et al., 2014. Single-cell RNA-seq reveals dynamic paracrine control of cellular variation. Nature 510 (7505), 363–369.
Skrzeczynska-Moncznik, J., et al., 2008. Peripheral blood CD14high CD16+ monocytes are main producers of IL-10. Scand. J. Immunol. 67 (2), 152–159.
Takeuchi, O., Akira, S., 2010. Pattern recognition receptors and inflammation. Cell 140 (6), 805–820.
Tan, A.T., et al., 2010. A longitudinal analysis of innate and adaptive immune profile during hepatic flares in chronic hepatitis B. J. Hepatol. 52 (3), 330–339.
Taub, D.D., et al., 1996. T lymphocyte recruitment by interleukin-8 (IL-8). IL-8-induced degranulation of neutrophils releases potent chemoattractants for human T lymphocytes both in vitro and in vivo. J. Clin. Invest. 97 (8), 1931–1941.
Visintin, A., et al., 2001. Regulation of Toll-like receptors in human monocytes and dendritic cells. J. Immunol. 166 (1), 249–255.