Original Research Article

Kinetics of Intracellular, Extracellular and Production of Pro-Inflammatory Cytokines in Lipopolysaccharide-Stimulated Human Peripheral Blood Mononuclear Cells

Endang Kumolosasi*, Emil Salim, Ibrahim Jantan and Waqas Ahmad
Drug and Herbal Research Center, Faculty of Pharmacy, Universiti Kebangsaan Malaysia, Jalan Raja Muda Abdul Aziz, 50300, Kuala Lumpur, Malaysia

For correspondence: **Email: e_kumolosasi@yahoo.co.id; **Tel:** +60149208598; **Fax:** +603-26983271*

Abstract

Purpose: To determine the detailed time course of intracellular, extracellular and production of pro-inflammatory cytokines (TNF-α, IL-1β, IL-1α, IL-6 and IL-8) in normal human peripheral blood mononuclear cells (PBMC).
Methods: PBMC were isolated from human whole blood, stimulated by 0.1 µg/ml lipopolysaccharide (LPS) and incubated at 37ºC, 5% CO_2. Samples were harvested at different time intervals (4, 8, 12, 16, 20 and 24 h) after stimulation. ELISA was employed for the measurement of the extracellular and intracellular cytokine levels of the samples.
Results: The release of TNF-α, IL-6 and IL-8 on LPS-stimulated PBMC were significantly higher with concentrations in the range of 3161 ± 162.5 to 4027 ± 361.5 pg/ml ($p < 0.001$), 3921.5 ± 879.3 to 11628.3 ± 2647.3 pg/ml ($p \leq 0.030$), and 4122.0 ± 382.9 to 5898.6±115.8 pg/ml ($p \leq 0.049$), respectively compared to intracellular levels that were very low (TNF-α, 23.5 ± 5.0 to 69.5 ± 13.8 pg/ml; IL-6, 22.5 ± 16.5 to 96.5 ± 9.6 pg/ml; and IL-8, 501.1 ± 221.0 to 1452.5 ± 415.7 pg/ml) and remained unchanged during 24 h. In contrast, both IL-1α and IL-1β were secreted gradually. Secretion and production of IL-6 was significantly higher at 8 h (9394.4 ± 846.3 pg/ml; $p = 0.002$) and at 20 h (11628.3 ± 2647.3 pg/ml; $p = 0.014$) than other cytokines.
Conclusion: The differences in the characteristic kinetics of cytokines may be caused by different mechanisms of secretion and function. IL-1, TNF-α and IL-8 play a role as proinflammatory cytokines, whereas IL-6 consecutively plays a dual role as pro-inflammatory cytokine and anti-inflammatory.

Keywords: Pro-inflammatory cytokine, Tumor necrosis factor-α, Interleukin-1α, Interleukin-1β, Interleukin-6, Interleukin-8.

INTRODUCTION

Cytokines are low molecular weight proteins produced by cells in response to a variety of stimuli. After genesis, they influence the behavior of target cells [1,2]. These molecules are essentially involved in a number of biological processes, including cell proliferation, inflammation, immunity, migration, fibrosis, repair and angiogenesis [3]. Cytokines that promote inflammation are known as pro-inflammatory cytokines [4]. Tumor necrosis factor-α (TNF-α), Interleukin-1β (IL-1β), Interleukin-1α (IL-1α), Interleukin-6 (IL-6) and Interleukin-8 (IL-8) are pro-inflammatory cytokines which even though are dissimilar proteins transcribed from different genes, share important roles in acute inflammation [5].

TNF-α is involved in phagocyte activation, endotoxic shock, tumor cytotoxicity and IL-1 plays important roles in cell proliferation and differentiation and pyrexia. Meanwhile, IL-6 has a role in cell differentiation into plasma cells, immunoglobulin production and IL-8 recruits and retains neutrophils at sites of inflammation [6].

Many studies on the kinetics of cytokine production have been carried out in experimental animals, whole blood models and isolated cells [7-12]. Kwak *et al* reported intracellular and extracellular cytokine production by human mixed mononuclear cells in response to group B *Streptococci* and *E. coli* LPS [13]. However the effect of *Salmonella enteritica* LPS on the kinetics of intracellular, extracellular and production of these pro-inflammatory cytokines in normal human PBMC not been reported. TNF-α is one of the prominent pro-inflammatory cytokines in cancer and rheumatoid arthritis and some studies have reported that TNF has a key role in the regulation of inflammatory processes [7, 8, 14, 15].

However, the roles of other pro-inflammatory cytokines are not well studied. It is interesting to evaluate the involvement of other pro-inflammatory cytokines in inflammation induced by lipopolysaccharide (LPS), especially IL-6 which has been reported to possess both pro- and anti-inflammatory properties. In the present study, the detailed time course of intracellular, extracellular and production of these pro-inflammatory cytokines was determined in normal human peripheral blood mononuclear cells (PBMC) induced by *Salmonella enteritica* LPS.

EXPERIMENTAL

Isolation of PBMC

Human whole blood from healthy volunteers (≥ 18 years old) was collected for the isolation of PBMC. The inclusion criteria were considered: non-smoker, fasted overnight and did not take any medicine or supplements. Venous blood was collected in heparinized tubes and processed immediately. PBMC were isolated by centrifugation on Lymphoprep (Axis-Shield PoC AS, Oslo, Norway) at 600 g for 20 min at 18 – 20°C. Next, cells were washed twice in RPMI-1640 at 300g for 10 min at 4°C. PBMC were resuspended in RPMI-1640 media with L-glutamin (Sigma, Steinheim, Germany) supplement with 10% heat inactivated newborn calf serum (Sigma, Steinheim, Germany), 10 mM

HEPES (Sigma, Steinheim, Germany), and 100 U/ml penicillin and 100 μg/ml streptomycin (PAA, Pasching, Austria) in culture tubes. The cells were adjusted to 5×10^5 cells/ml and counted using a hemocytometer (Hausser Scientific, Horsham, USA) and light microscope (Olympus Optical, Tokyo, Japan). The use of human blood in this study was approved by the Human Ethical Committee of Universiti Kebangsaan Malaysia (UKM) (approval No. UKM 1.5.3.5/244/NF-040-2011) and conformed to the principles outlined in the declaration of Helsinki [16].

Kinetic study

PBMC were stimulated with 0.1 μg/ml of LPS from *Salmonella enteritica* (Sigma, Steinheim, Germany) and incubated at 37ºC, 5% CO_2. Samples were harvested at different time intervals (4, 8, 12, 16, 20 and 24 h) after stimulation with LPS. Supernatants were collected for measuring extracellular cytokines. Pelleted cells were washed with RPMI-1640 and lysed based on the procedure described by Hazuda *et al* [10] which used lysis buffer (40mM; 50 mM Tris-HCl, pH 8.0, 0.1 mM EDTA, 1 mM dithiothreitol) plus 2 mM PMSF followed by sonication for 30 s, with modification by changing lysis buffer with water followed by gentle shaking for 10 s and left at room temperature for 1 h for measuring intracellular cytokines. This modification was performed to avoid the use of chemicals which may influence the stability of the cytokines. A control group was also performed to examine cytokine release but in the absence of added LPS. The cells used as a control group and stimulated group using LPS were taken from the same subjects. Cytokine production was determined by measuring the intracellular and extracellular levels of cytokines.

Immunoassay for TNF-α, IL-1α, IL-1β, IL-6 and IL-8

Enzyme-Linked immunosorbent assay (ELISA) method was used for measuring TNF-α, IL-1α, IL-1β, IL-6 (Cayman Chemicals,Ann. Arbor, MI) and IL-8 (Abnova, Taipei, Taiwan) according to manufacturer's instructions.

Statistical analysis

All statistical analyses were performed using SPSS® Statistic 20. One-way ANOVA, followed by a post-test (Tukey's multiple comparison) were used when significant differences at $p < 0.05$ were present. Cytokine assays by ELISA were determined in duplicate, and data were obtained from three different donors. The results are

expressed as mean ± standard error of the mean (n = 3).

RESULTS

The results show that the levels of TNF-α secreted in response to LPS stimulation were significantly higher (3161 ± 162.5 to 4027 ±

361.5 pg/ml; $p<0.001$) than those of intracellular TNF-α and the control group throughout the 24 h incubation period. Moreover, the results showed no significant difference between intracellular and extracellular TNF-α levels in control group. Additionally, there was no significant difference between LPS-stimulated and non-stimulated intracellular TNF-α levels (Figure 1A).
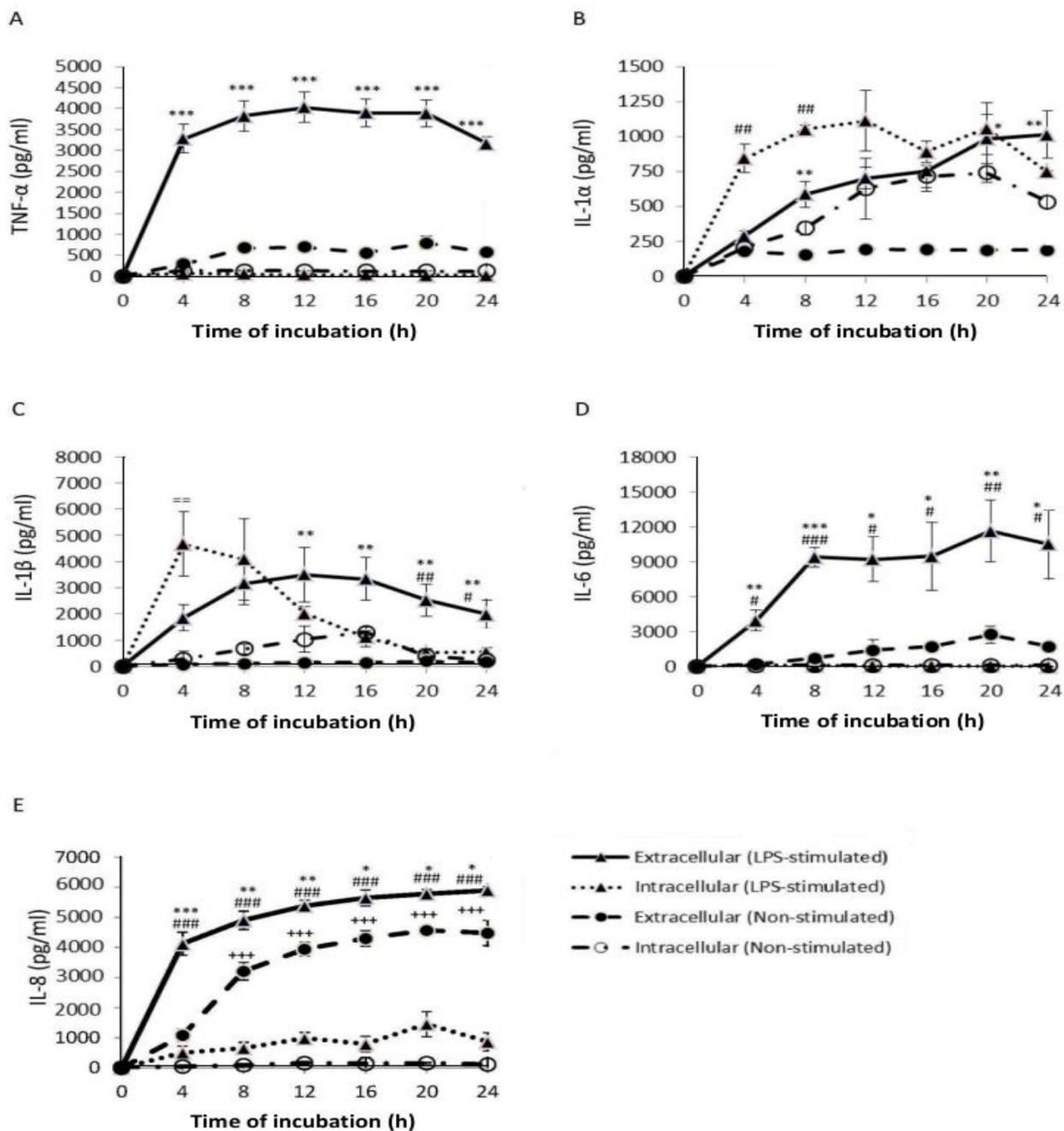
Fig 1: Time course of TNF-α (A), IL-1α (B), IL-1β (C), IL-6 (D) and IL-8 (E) extracellular and intracellular on LPS-stimulated and non-stimulated human PBMC. [#]$p < 0.05$, [##] $p < 0.01$, [###]$p < 0.001$ for the LPS-stimulated extracellular cytokines versus the intracellular. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ for the LPS-stimulated extracellular cytokines versus the non-stimulated. [+]$p < 0.05$, [++]$p < 0.01$, [+++]$p < 0.001$ for the non-stimulated extracellular cytokines versus the intracellular. =$p < 0.05$, ==$p < 0.01$ for the LPS-stimulated intracellular cytokines versus the non-stimulated

In response to LPS stimulation, the intracellular IL-1α levels were significantly higher at 4 - 8 h (843.0 ± 101.1 – 1051 ± 29.0 pg/ml; $p \leq 0.002$) than the extracellular and control groups but no significant difference was observed at 12 - 24 h. Additionally, extracellular IL-1α concentrations in response to LPS-stimulation were significantly higher compared to non-stimulated extracellular at 8 h (586.2 ± 91.8 pg/ml; $p = 0.003$) and 20 - 24 h (982.5 ± 176.2 to 1013.2 ± 171.7 pg/ml; $p \leq 0.013$). The results also showed that there was no significant difference between extracellular and intracellular levels of IL-1α in non-stimulated cells (Figure 1B).

Intracellular IL-1β levels on LPS-stimulated PBMC were significantly higher than non-stimulated PBMC at 4 h (4674.3 ± 1236.7 pg/ml; $p = 0.007$). However, the levels decreased gradually and reached the lowest level at 20 h (539.8 ± 124.4 pg/ml). The extracellular level of IL-1β in response to LPS stimulation was significantly higher than its intracellular level at 20 h (2531.4 ± 616.1 pg/ml; $p = 0.009$) and at 24 h (1986.2 ± 541.3 pg/ml; $p = 0.029$). Also, the IL-1β secreted on LPS-stimulated PBMC was significantly higher than non-stimulated PBMC at 12 - 24 h (1986.2 ± 541.3 to 3506.0 ± 1033.7 pg/ml; $p \leq 0.01$) (Figure 1C).

The levels of IL-6 released in response to LPS stimulation were significantly higher than its intracellular levels and the control group throughout 4-24 h (3921.5 ± 879.3 to 11628.3 ± 2647.3 pg/ml; $p \leq 0.030$). Moreover, the results showed that there was no significant difference between non-stimulated IL-6 extracellular and intracellular and between stimulated and non-

stimulated IL-6 intracellular (Figure 1D). Figure 1E shows that the levels of IL-8 secreted increased gradually in response to LPS and was significantly higher than its intracellular levels and the control group throughout 4-24 h (4122.0 ± 382.9 to 5898.6 ± 115.8 pg/ml; $p \leq 0.049$). The results also showed that the concentrations of non-stimulated IL-8 extracellular increased gradually and were significantly higher than its intracellular levels throughout 8 - 24 h (3209.3 ± 291.4 to 4481.6 ± 420.9 pg/ml; $p = 0.001$).

Figure 2A shows that in the non-stimulated PBMC, IL-8 was the most concentrated cytokine as it was released gradually and reached its maximum level at 4481 ± 420.9 pg/ml at 24 h. This was followed by IL-6 which reached its maximum level of 2732.4±340.3 pg/ml at 20 h. Whereas the levels of TNF-α, IL-1α and IL-1β were very low and remained unchanged during 24 h time course. Figure 2B shows that in LPS-stimulated cells, the extracellular levels of IL-6 were significantly higher at 8 h (9394.4 ± 846.3; p=0.002) and 20 h (11628.3 ± 2647.3pg/ml, p=0.014) as compared to other cytokines, followed by IL-8, TNF-α, IL-1β and IL-1α in order of decreasing concentrations. In addition, there was no significant difference between IL-1α and IL-1β secretion during the 24 h period.

In the intracellular non-stimulated PBMC, the levels of IL-1β gradually increased until it reached maximum value at 16 h (1306.5 ± 491.0 pg/ml) and then decreased onwards. While the levels of IL-1α increased gradually until it reached a maximum value at 20 h (741.6 ± 79.1 pg/ml) and then decreased.
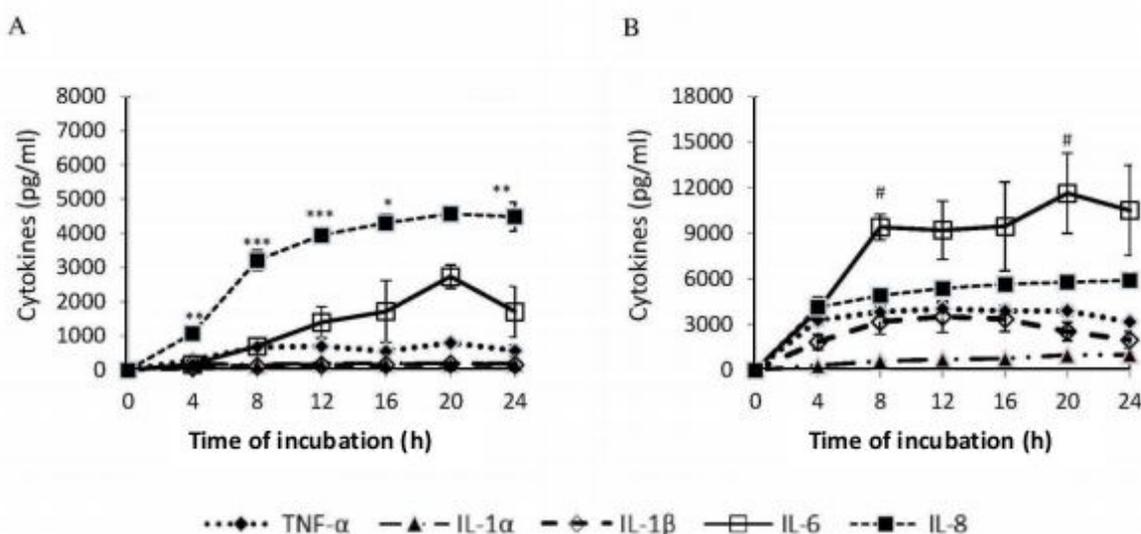
Fig 2: Time course of TNF-α, IL-1α, IL-1β, IL-6 and IL-8 extracellular of non-stimulated (A) and LPS-stimulated (B) human PBMC. *p<0.05, **p<0.01 and ***p<0.001 are significantly different for the IL-8 compared to other cytokines. #p < 0.05 is significantly different for IL-6 compared to other cytokines
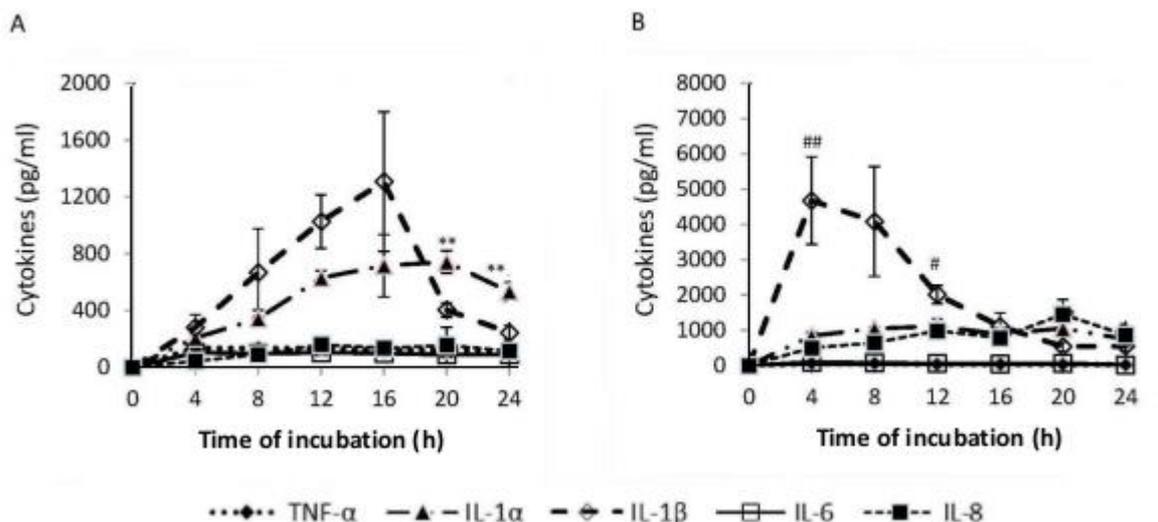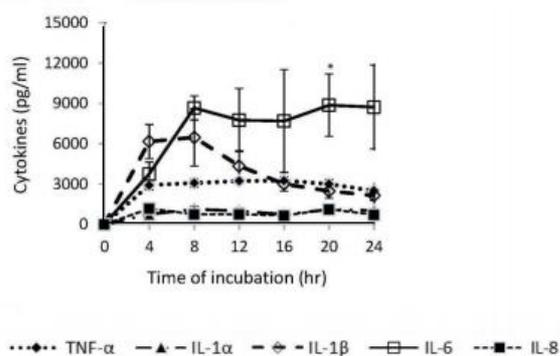
Fig 3: Time course of TNF-α, IL-1α, IL-1β, IL-6 and IL-8 intracellular of non-stimulated (A) and LPS-stimulated (B) human PBMC. *$p < 0.05$, **$p < 0.01$ are significantly different for the IL-1α compared to other cytokines. #$p <$ 0.05, ## $p < 0.01$ are significantly different for IL-1β compared to other cytokines

Whereas TNF-α, IL-6 and IL-8 were in low levels (≤159.6 ± 17.0 pg/ml) and remained unchanged during the 24 h period (Figure 3A).

Figure 3B also shows that the level of IL-1β intracellular in response to LPS was significantly higher at 4 h (4674.3 ± 1236.7 pg/ml; p≤0.05) and at 12 h (2013.6 ± 258.8; p≤0.27) as compared to other cytokines. Whereas the levels of TNF-α, IL1-α, IL-6 and IL-8 were very low (≤ 1452.5 ± 415.7 pg/ml) during the 24 h time course.

Figure 4 shows the time course of pro-inflammatory cytokine production in that IL-6 was the highest cytokine produced at 8 to 24 h.

Fig 4: Time course of TNF-α, IL-1α, IL-1β, IL-6 and IL-8 production on human PBMC stimulated by LPS. *p<0.05 is significantly different for the IL-6 compared to other cytokines

All the data is represented as mean ± SEM. Net production of cytokines calculated by deducting the extra and intracellular cytokine extent of non-stimulated cells from stimulated cells.

DISCUSSION

The results indicated that there were differences in the kinetics of intracellular, extracellular and production of cytokines in human PBMC stimulated by LPS. TNF-α, IL-6 and IL-8, were secreted directly in high quantities, but the intracellular level of these cytokines were very low and remained unchanged during 24 h of incubation. On the contrary, both IL-1α and IL-β were secreted gradually. The difference in their characteristics was due to the different functions and different pathways of cytokine secretion. In the macrophages, once LPS stimulates via toll-like receptor 4 (TLR4), the type II trans-membrane precursor pro-TNF is quickly synthesized and builds up in the Golgi and delivered to the cell surface [17-18]. Then it is cleaved by TNF alpha converting enzyme (TACE) and A disintegrin and a metalloproteinase 17 (ADAM17) [19].

Likewise, LPS stimulation leads to the accumulation of newly synthesized IL-6 in the Golgi complex in macrophages, which is subsequently loaded into tubo-vesicular carriers that bud off the Trans-Golgi network (TGN) either as the sole labeled content or jointly with TNF [20]. Since IL-6 is a soluble cytokine rather than a trans-membrane cytokine like TNF, it uses

multiple carriers (including p230 and Golgin-97) for its export from the Golgi [21]. After export from the Golgi, both TNF and IL-6 colocalize with recycling endosomes (REs) in macrophages during their transport to the cell surface following LPS stimulation [18]. Furthermore, it was shown that IL-6 and TNF were localized in different sub-compartments of REs [20]. It was suggested that REs may in addition function to sort these two cytokines for release at different points on the cell surface. Additionally, TNF-α and IL-6 are mainly secreted in the supernatant because they have a hydrophobic group in their precursor amino acid sequences that are most likely signal peptides [7].

In contrast, IL-1 is released by a non-classical secretory route in which a number of cytokines synthesized in the cytoplasm are released through the plasma membrane without passing through the traditional organelles such as REs and Golgi complex [22]. IL-1β lacks a signal peptide and is produced on free ribosomes in the cytoplasm and cleaved by caspase-1 of the inflammasome preceding its release [23-24]. How these cytokines break out from cells is still mainly a mystery. Numerous pathways and mediators have been called upon, including ATP-binding cassette (ABC) transporters, secretory lysosomes, endosomes, membrane blebbing [25-27] and most newly, transport via multi-vesicular body-derived exosomes [28]. Although there are functional similarities between IL-1α and IL-1β [29], yet they are different in terms of secretion. The phosphorylation of the intracellular precursor of IL-1α may facilitate the processing and release of IL-1α, but this phosphorylation does not take place on IL-1β [11]. The mechanism of how IL-8 secreted is still poorly understood.

The presence of extracellular and intracellular cytokines, respectively, even without stimulation by LPS may be due to the highly sensitive PBMC in response to media or culture conditions [30-34].

These differences in the kinetics of cytokine secretion and production undoubtedly provide a reflection of the functional differences of these mediators. Although TNF and IL-1 share numerous biological activities, the major distinction being that TNF has no direct effect on lymphocyte proliferation. Although IL-6 shares several activities with IL-1, including induction of pyrexia and the production of acute phase proteins, IL-6 is considered the most important hepatocyte-inducer for synthesis of acute phase proteins. In contrast to these pro-inflammatory effects, IL-6 mediates several anti-inflammatory effects by terminating up-regulatory inflammatory cascade and inhibits IL-1 and TNF synthesis [35]. On the other hand, IL-8 mediates the recruitment and activation of neutrophils in inflamed tissue [4-5].

On extracellular LPS-nonstimulated, the level of IL-8 was significantly higher compared to other cytokines. The level of IL-8 gradually increased until it reached a maximum value at 20 hours, while other cytokines remained unchanged during 24 h. This finding can be explained by the fact that IL-8 is an autocrine regulator of its self production in monocytes [36]. However, extracellular LPS-stimulated PBMC showed that the level of IL-6 was higher than those of other cytokines. This profile may be explained by the fact that many cytokines such as TNF-α, IL-1 induce the release of IL-6 [5]. It was also showed that at 20 h the level of IL-6 increased but at the same time the level of IL-1β and TNF-α decreased, which may be caused by the anti-inflammatory effects of IL-6 by suppressing IL-1β and TNF-α at the level of 11628 pg/ml. This finding is in agreement with those reported by Schindler *et al.* [7]. IL-6, although mostly regarded as a pro-inflammatory cytokine, also has many regenerative and anti-inflammatory activities. Pro-inflammatory responses of IL-6 are mediated by trans-signaling. IL-6 will bind to soluble interleukin-6 receptor (sIL-6R), afterwards this IL-6/sIL-6R complex binds to glycoprotein 130 (gp130) receptors to produce pro-inflammatory signal. Whereas the anti-inflammatory response of IL-6 is mediated by classic signaling. In the classic signaling IL-6 first binds to the membrane-bound non signaling α-receptors IL-6R (mbIL-6R) then this complex binds to two molecules of gp130 and leads to IL-6-signal transduction [37].

However there has been no explanation of how these two opposing activities can occur. That interesting phenomenon can be explained by our findings. The results show that when LPS triggers the production of pro-inflammatory cytokines, IL-6 led excretion of other cytokines, this was shown by its highest extracellular concentration compared with other cytokines at 24 h incubation. IL-6 level was gradually increased to maximum level during 20h and at that period pro-inflammatory effect was produced. When the maximum level (11628.3 ± 2647.3 pg/ml) was reached, sIL-6R were saturated (at this time IL-6 release rate was higher than Fas ligation, resulting in ADAM17 mediated shedding of membrane IL-6R (mIL-6R)

from the surface), that is when IL-6 start to bind mIL-6R, classic signaling will occur and produces anti-inflammatory activity. This process may have been caused by the affinity of IL-6 to the sIL-6R being higher than mbIL-6R.

Anti-inflammatory activity also occurs when IL-6 level reached the maximum level and some of IL-6 start binding to mbIL-6R, this binding causes a decline of extracellular IL-6 after 20 h incubation. It may be that at the same time, IL-6 starts to inhibit secretion of TNF-α and IL-1β. This explanation is also supported by Figure 4 which shows that IL-6 production was highest from 8 h until 24 h incubation. The fact that IL-6 suppresses IL-1β and TNF production was reported by Schindler *et al* [7] and also stated by Kwak *et al* [13]. Furthermore, IL-6 also stimulates synthesis of IL-1 receptor antagonist (IL-1ra) [35].

CONCLUSION

The differences in the characteristic kinetics of cytokines may be caused by different mechanisms of secretion and function. IL-1, TNF-α and IL-8 are proinflammatory cytokines, whereas IL-6 acts as a pro-inflammatory cytokine or an anti-inflammatory cytokine. The change in its activity may be affected by its level.

ACKNOWLEDGEMENT

REFERENCES

1. Clemens MJ. Cytokines. UK: BIOS Scientific Publishers Limited; 1998; p 121.
2. Feldman M. Many cytokines are very useful therapeutic targets in disease. J Clin Invest 2008; 118: 3533-3536.
3. Oppenheim JJ. Cytokines: past, present, and future. Int J Hematol 2001; 74: 3-8.
4. Dinarello CA. Proinflammatory cytokines. CHEST 2000; 118: 503-508.
5. Feghali CA, Wright TM. Cytokines in acute and chronic inflammation. Front Biosci 1997; 2: 12-26.
6. Tayal V, Kalra BS. Cytokines and anti-cytokines as therapeutics-An update. Eur J Pharmacol 2008; 579: 1–12.
7. Schindler R, Mancila J, Endres S, Ghorbani R, Clark SC, Dinarello CA. Correlations and interactions in the production of interleukin-6 (IL-6), IL-1, and tumor necrosis factor (TNF) in human blood mononuclear cells: IL-6 suppresses IL-1 and TNF. Blood 1990; 75(1): 40-47.
8. Jansky L, Reymanova P, Kopecky J. Dynamics of cytokine production in human peripheral blood mononuclear cells stimulated by LPS or infected Borelia. Physiol Res 2003; 52: 593-598.
9. Kaplanski G, Farnarier C, Kaplanski S, Porat R, Shapiro L, Bongrand P, Dinarello CA. Interleukin-1 induces interleukin-8 secretion from endothelial cells by a juxtacrine mechanism. Blood 1994; 84: 4242-4248.
10. Hazuda DJ, Lee JC, Young PR. The kinetics of interleukin-1 secretion from activated monocyte. J Biol Chem 1988; 263: 8473-8479.
11. Lonnemann G, Endres S, Van JWM, Cannon JG, Koch KM, Dinarello, CA. Differences in the synthesis and kinetics of release of interleukin-1α, interleukin-1β and tumor necrosis factor from human mononuclear cells. Eur J Immunol 1989; 19: 1531-1536.
12. De Forge LE and Remick DG. Kinetic of TNF, IL-6, and IL-8 gene expression in LPS-stimulated human whole blood. Biochem Biophys Res Commun 1991; 174: 18-24.
13. Kwak DJ, Augustine NH, Borges WG, Joyner JL, Green WF, Hill HR. Intracellular and extracellular cytokine production by human mixed mononuclear cells in response to group B Streptococci. Infect Immun 2000; 68(1): 320-327.
14. Brown KK, Heitmeyer SA, Hookfin EB, Hsieh L, Buchalova M, Taiwo YO, Janusz MJ. P38 MAP kinase inhibitors as potential therapeutics for the treatment of joint degeneration and pain associated with osteoarthritis. J Inflamm 2008; 5(22): 1-8.
15. Ma J, Zhang YG, Xia Y, Sun J. The inflammatory cytokine tumor necrosis factor modulates the expression of Salmonella typhi murium efector proteins. J Inflamm 2010; 7(42): 1-12.
16. World Medical Association. Declaration of Helsinki - Ethical Principles for Medical Research Involving Human Subjects, 2008 [cited 2013 Apr 9]. Available from:
http://www.wma.net/en/30publications/10policies/b3/index.html
17. Lieu ZZ, Lock JG, Hammond LA, La Gruta NL, Stow JL, Gleeson PA. A trans-golgi network golgi is required for the regulated secretion of TNF in activated macrophages in vivo. Proc Natl Acad Sci USA 2008; 105: 3351-3356.
18. Murray RZ, Kay JG, Sangermani DG, Stow JL. A role for the phagosome in cytokine secretion. Science 2005; 310: 1492-1495.
19. Black RA, Rauch CT, Kozlosky CJ, Peschon JJ, Slack JL, Wolfson MF, Castner BJ, Stocking KL, Reddy P, Srinivasan S, et al. A metalloproteinase disintegrin that releases tumor necrosis factor alpha from cells. Nature 1997; 385: 729–733.
20. Manderson AP, Kay JG, Hammond LA, Brown DL, Stow JL. Subcompartments of the macrophage recycling

endosome direct the differential secretion of IL-6 and TNF alpha. J Cell Biol 2007; 178: 57–69.

21. Stanley AC, Lacy P. Pathways for cytokine secretion. Physiol 2010; 25: 218-229.

22. Nickel W. The mystery of nonclassical protein secretion. A current view on cargoproteins and potential export routes. Eur J Biochem 2003; 270: 2109-2119.

23. Dinarello CA. Interleukin-1. Cytokine Growth Factor Rev 1997; 8: 253-265.

24. Ogura Y, Sutterwala FS, Flavell RA. The inflammasome: first line of the immune response to cell stress. Cell 2006; 126: 659-662.

25. Andrei C, Dazzi C, Lotti L, Torrisi MR, Chimini G, Rubartelli A. The secretory route of the leaderless protein interleukin-1beta involves exocytosis of endolysosome related vesicles. Mol Biol Cell 1999; 10: 1463–1475.

26. Carta S, Tassi S, Semino C, Fossati G, Mascagni P, Dinarello CA. Histone deacetylase inhibitors prevent exocytosis of interleukin-1beta-containing secretory lysosomes: role of microtubules. Blood 2006; 108: 1618–1626.

27. Hamon Y, Luciani MF, Becq F, Verrier B, Rubartelli A, Chimini G. Interleukin-1beta secretion is impaired by inhibitors of the ATP binding cassette transporter, ABC1. Blood 1997; 90: 2911–2915.

28. Qu Y, Franchi L, Nunez G, Dubyak GR. Nonclassical IL-1 beta secretion stimulated by P2X7 receptors is dependent on inflammasome activation and correlated with exosome release in murine macrophages. J Immunol 2007; 179: 913–925.

29. Hunter CA, Timans J, Pisacane P, Menon S, Cai G, Walker W, Aste-Amezaga M, Chizzonite R, Bazan JF, Kastelein RA. Comparison of the effects of interleukin-1alpha, interleukin-1 beta and interferon-gamma-inducing factor on the production of interferon-gamma by natural killer. Eur J Immunol 1997; 27(11): 2787-2792.

30. Lijnen P, Saavedra A, Petrov P. In vitro proliferative response of human peripheral blood mononuclear cells to concanavalin A. Clin Chim Acta 1997; 264: 91-101.

31. Hugin AW, Rudikoff E, Morse HC. Variability in growth sustaining capacity of medium batches. J Immunol Methods 1995; 186: 151-154.

32. Causey AL, Westen RM, Clem LW, Bly JE. A serum – free medium for human primary T-Lymphocyte culture. J Immunol Methods 1994; 175: 15-21.

33. Weichert H, Lehmann J, Ambrosius H. Culture and stimulation of human T- and B-Lymphocyte. Biomed Biochim Acta 1990; 49: 999-1004.

34. Blaehr H, Ladefoged J. Mitogen-induced lymphocyte transformation in four different serum-free media. J Immunol Methods 1988; 111: 125-129.

35. Borish LC, Steinke JW. Cytokines and chemokines. J Allergy Clin Immunol 2003; 111: S460-475.

36. Browning DD, Diehl WC, Hsu MH, Schraufstatter IU, Ye RD. Autocrine regulation of Interleukin-8 production in human monocytes. Am J Physiol Lung Cell Mol Physiol 2000; 279: 1129-1136.

37. Scheller J, Chalaris A, Arras DS, John SR. The pro- and anti-inflammatory properties of the cytokine interleukin-6. Bio Chim Biophys Acta 2011; 1-11.