Current Trends in Immunology
Vol. 20, 2019

Original Article

PRP's effect on the production of pro- and anti-inflammatory mediators from macrophage culture

Heidy[1,*], Andhika Rachman[2] and Heri Wibowo[3]

[1]Department of Physiology, Faculty of Medicine and Health Science, UNIKA Atma Jaya, Jakarta, Indonesia; [2]Division of Hematology & Medical Oncology, Department of Internal Medicine, Faculty of Medicine, Universitas Indonesia/Dr. Cipto Mangunkusomo Hospital, Jakarta, Indonesia; [3]Department of Parasitology and Integrated Laboratory, Faculty of Medicine, Universitas Indonesia, Jakarta, Indonesia.

ABSTRACT

Autologous platelet-rich plasma (PRP) (also called PRP) has been widely used in medicine, but the mechanism by which PRP heals the wound remains unknown. This study is aimed to analyze the effect of PRP on the production of pro- and anti-inflammatory mediators from macrophage culture because macrophages have an important role in wound healing process. This experimental research used macrophage culture and PRP which was taken from peripheral blood of healthy subjects. Macrophages were obtained from monocyte isolation which was cultured for 1 week, using Roswell Park Memorial Institute (RPMI) medium and granulocyte macrophage-colony stimulating factor (GM-CSF). The cultured macrophages were divided into 5 for different treatments to determine PRP's effect on pro- and anti-inflammatory mediator production. Pro- and anti-inflammatory mediators were measured using enzyme-linked immunosorbent assay (ELISA) technique. TNF-α levels on day 10 were high and decreased on day 14. Treatment groups receiving PRP experienced significant decrease in TNF-α production. IL-10 levels on day 10 were still low and began to rise on day 14. There was no significant difference between the treatment groups in terms of pro- and anti-inflammatory mediator level changes. PRP increased the production level of TNF-α when the macrophage was activated by LPS (Lipopolysaccharide) and after the macrophage activation began to decrease, the TNF-α production also decreased. PRP did not affect the production of IL-10 at the onset of macrophage activation, but may increase the production of IL-10 at the end of macrophage activation.

KEYWORDS: autologous, PRP, macrophage, TNF-α, IL-10.

INTRODUCTION

Platelet-rich plasma (PRP) is a plasma containing high platelet concentration. It has been widely applied since 1970 in regenerative medical therapies to enhance tissue regeneration, such as the wound healing of soft and bone tissues. The use of PRP is autologous, which means that the donor and recipient are the same subject. The advantages of using PRP are high concentration of natural growth factors, no risk of cross reactivity, no immune reaction or disease transmission [1, 2].

Each platelet contains 7 types of growth factors stored in every α-granule, including platelet-derived growth factor (PDGF α-β), transforming growth factor (TGF α-β), vascular endothelial growth factor (VEGF), epidermal growth factor (EGF), fibroblast growth factor (FGF), connective tissue growth factor (CTGF), and insulin-like

*Corresponding author: haihei_dee@yahoo.com

growth factor-1 (IGF-1). Every growth factor has its own role and function. These growth factors have a potential role in wound healing and regenerative tissue processes including chemotaxis, proliferation, differentiation, and angiogenesis [1, 2]. Cell studies in wound care have shown that multiple growth factors in PRP tend to be more effective than the use of a single recombinant human growth factor because healing tissue process needs a simultaneous cooperation between various growth factor [1, 3].

With increasing PRP levels, it is expected that the levels of growth factors and other substances that are important in wound healing will also increase [1, 4]. Although PRP is widely used, there is still little known about the mechanism by which PRP acts at the cellular level.

In addition to playing a role in hemostatic process and thrombus formation, platelets are also considered as immunomodulatory cells. Active platelets can bind with immune cells, which induces the activation of immune cells and release platelet's cytokines. Interaction between platelets and monocytes can increase the differentiation of monocyte into macrophage, control the cytokine release, and improve macrophage function. Platelets also induce phenotypic change from monocyte M1 into M2 [5].

In general, wound healing process consists of 3 phases: inflammation, proliferation, and remodeling. One of the inflammatory cells that plays an important role in the wound healing process is macrophage [6]. Macrophage is a phagocyte cell from innate immune system, located in various body tissues, and able to infiltrate the wound [2, 7].

When activated, macrophage will secrete pro-inflammatory mediators such as TNF-α, IL-6, IL-1β, IL-8, and IL-12 to begin inflammation, which aims to eliminate foreign objects. Besides being produced by activated macrophages, the inflammatory mediators are also produced by activated lymphocytes, endothelial cells, and fibroblasts. TNF-α is the first inflammatory mediator secreted by activated macrophages, having a strong effect as a response to any pathogen [8, 9].

Continuous TNF-α secretion can damage tissues, and therefore after pathogen has been eliminated, macrophage will secrete anti-inflammatory mediators such as IL-10, IL-4, and TGF-β. Anti-inflammatory mediators will suppress the inflammatory process, which simultaneously also suppresses the production of TNF-α and IL-6 [8, 10]. IL-10 is the strongest anti-inflammatory mediator, able to increase the regulation of endogenous anti-cytokine production and reduce the regulation of pro-inflammatory cytokine receptors [9].

In vivo inflammatory mediators are not only secreted by macrophage but also by others activated cells. Hence this research was carried out by using *in vitro* monocyte-derived macrophages. This study analyzes the effect of PRP on the production of pro- and anti-inflammatory mediator from macrophage culture.

MATERIALS AND METHODS

This is an experimental study that was conducted in the Integrated Laboratory of the Faculty of Medicine, Universitas Indonesia, from March to April 2017. Procedure of this study was approved by the Ethical Committee of the Faculty of Medicine, Universitas Indonesia (no. 543/UN2.F1/ETIK/2016). Written informed consent was given by participants with signature.

Six healthy 30-40 year-old males, who are the students of the biomedical master program in Universitas Indonesia, participated in this study. Subjects with tobacco-smoking habits, alcohol-drinking habits, using immunosuppressant drugs, or having autoimmune disease were excluded. On the first day of research, 15 mL peripheral blood was taken from all subjects, then PBMC was isolated, followed by monocyte isolation. Monocytes were cultured for 1 week to obtain macrophages, and then they were divided into 5 different treatments: Group I as a control received only 100 µL serum; Group II: received 100 µL serum + 100 $^{ng}/_{mL}$ LPS; Group III: 100 µL serum + 100 $^{ng}/_{mL}$ LPS + 5×10^6 platelets; Group IV: received 100 $^{ng}/_{mL}$ LPS + 5×10^6 platelets; Group V: received only 5×10^6 platelets.

The purpose of different treatments is to ensure PRP's effect on inflammatory mediators produced from macrophage cultures that have been activated by LPS. LPS is a wall component of gram-negative bacteria. When LPS is added into the macrophage cultures, it activates the macrophages

so that the macrophages can begin the inflammatory process and secrete pro-inflammatory mediators [10].

PRP and serum were obtained from peripheral blood on day 7. Inflammatory mediators' level was measured on day 10 and 14; we aimed to collect high levels of inflammatory mediators and measure them by ELISA. On day 10, it was expected that the level of pro-inflammatory mediators would be higher than the level of anti-inflammatory mediators; otherwise, on day 14 it was expected that the level of anti-inflammatory mediators would be higher than the level of pro-inflammatory mediators.

Monocyte-derived macrophage

Blood from healthy human donors was collected in heparin tubes and peripheral blood mononuclear cells (PBMCs) were isolated by using Medium Histopaque (Sigma-Aldrich) according to the manufacturer's instructions. Monocytes were further isolated by adherence method; 1×10^6 PBMCs per well were plated in a plate culture and incubated at 37 ºC for 30 minutes in an incubator. Non-adherent cells in the supernatant were removed and the adherent cells were washed carefully [11].

Monocytes isolated by adherence were cultured in 1 mL RPMI 1640 medium supplemented with 10% fetal bovine serum, 10 ng/ml recombinant human GM-CSF (Sigma-Aldrich), 1% antibiotic, and 1% antifungal. Media culture was changed on day 3 and 7. On day 7, monocytes differentiated into macrophages (Monocyte-Derived Macrophages) and were ready to be used for treatment. Five wells were used for 5 different treatments. Group I as a control received only 100 µL serum; Group II: received 100 µL serum + 100 $^{ng}/_{mL}$ LPS; Group III: received 100 µL serum + 100 $^{ng}/_{mL}$ LPS + 5×10^6 platelets; Group IV: received 100 $^{ng}/_{mL}$ LPS + 5×10^6 platelets; Group V: received only 5×10^6 platelets.

After treatments, macrophages were cultured without GM-CSF. On day 10 (day 3 after treatment), 500 µL supernatants from each well were collected and stored in refrigerator at -20 °C. On day 14 (day 7 after treatment), all supernatants were collected and stored in refrigerator at -20 °C. The production of mediators (TNF-α and IL-10) in the supernatant culture were measured using ELISA technique.

Autologous PRP isolation

Platelets were isolated from whole blood of healthy donors collected in citrate tubes. The whole blood was then centrifuged at 1.600 rpm for 5 minutes to separate the plasma, buffy coat and erythrocytes. The whole plasma containing platelets (hereafter called PRP) was isolated and then the platelet count was calculated using Sysmex. The platelets have to be activated before use; 5×10^6 platelets were incubated with 0.2 µL of 10 µM calcium ionophore A21387 (Sigma) for 10 minutes, then administered to Groups III, IV and V.

Serum isolation

Serum was isolated from whole blood of healthy donors collected in tubes without anti-coagulant and stored at room temperature for 20 minutes. Then it was centrifuged at 3.500 g for 10 minutes, and the supernatant was isolated using the pipette. 100 µL each serum was administered to Groups I, II, and III.

Analysis of mediators TNF-α and IL-10

Mediators TNF-α and IL-10 produced by macrophage culture were present in the supernatant. Supernatants were isolated into Eppendorf tube using pipette, then centrifuged at 14,000 g for 10 minutes. Supernatants from day 10 and day 14 were measured for TNF-α and IL-10 concentration using ELISA technique.

We used SPSS 24 program to analyse the collected data. The normality test used in this research was Shapiro-Wilk test; we used this method because our research samples were less than 50. To test our hypothesis, we used parametric T-dependent test for the normally-distributed data and non-parametric Wilcoxon test for the abnormally-distributed data.

RESULTS

In Group I: the median concentration of TNF-α decreased from 62.3 pg/mL on day 10 to 47.2 pg/mL on day 14, while the median concentration of IL-10 increased from 56 pg/mL on day 10 to 237.6 pg/mL on day 14. In Group II the median

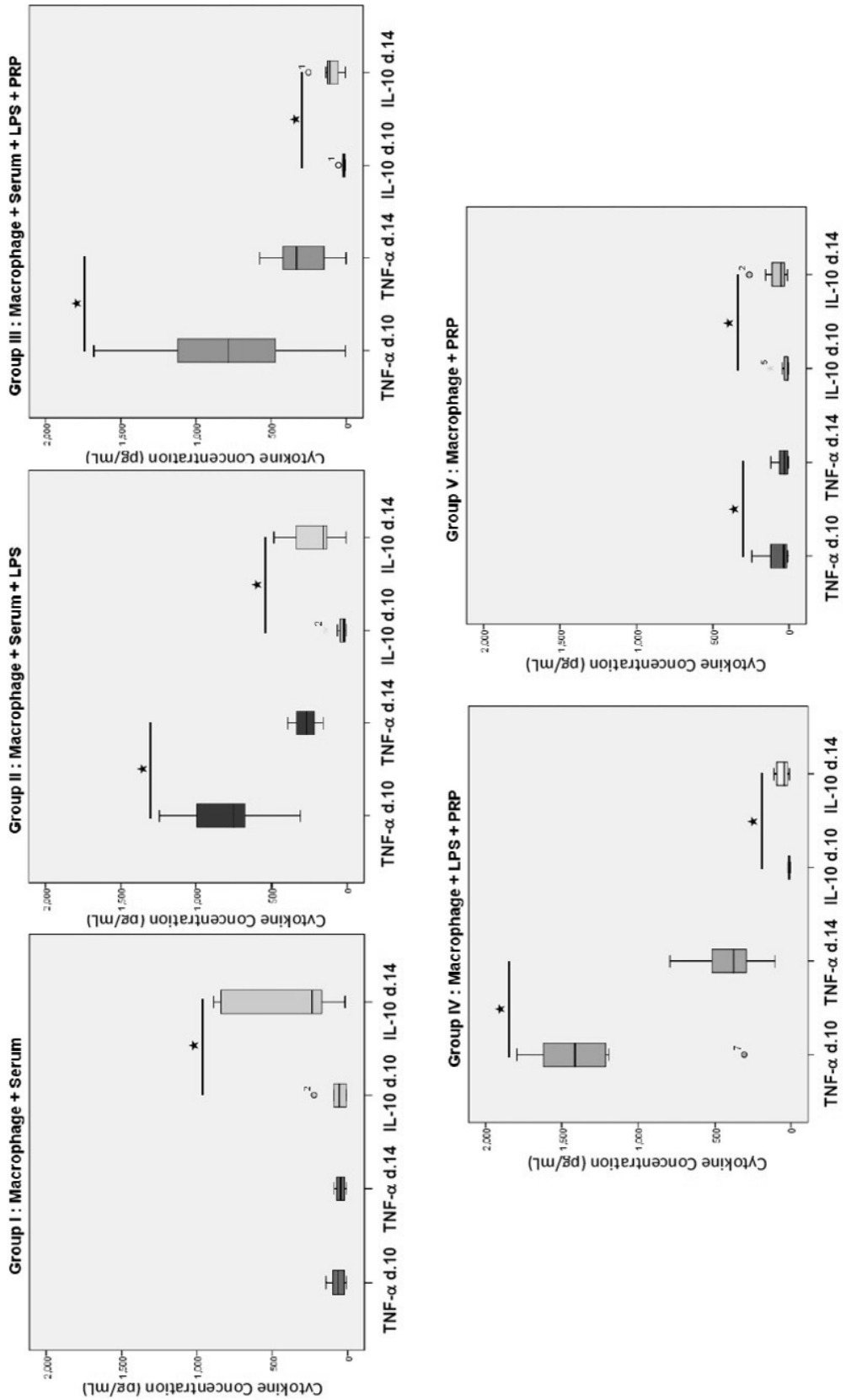

Figure 1. Comparison between TNF-α and IL-10 production in each group.
Box plots of cytokine concentration (pg/mL) of TNF-α and IL-10 from the supernatant on day 10 and day 14. *p < 0.05. Hypothesis was analyzed using T-dependent test for normal distribution data and Wilcoxon test for abnormal distribution data. TNF-α levels decreased on day 14 compared to day 10, while IL-10 levels increased on day 14 compared to day 10.

concentration of TNF-α decreased from 768.5 pg/mL on day 10 to 282.5 pg/mL on day 14, while the median concentration of IL-10 increased from 22.9 pg/mL on day 10 to 194.6 pg/mL on day 14. In Group III the median concentration of TNF-α decreased from 944.3 pg/mL on day 10 to 354.3 pg/mL on day 14, while the median concentration of IL-10 increased from 18.9 pg/mL on day 10 to 110.7 pg/mL on day 14. In Group IV the median concentration of TNF-α decreased from 1471.2 pg/mL on day 10 to 423 pg/mL on day 14, while the median concentration of IL-10 increased from 10.9 pg/mL on day 10 to 60.9 pg/mL on day 14. In Group IV the median concentration of TNF-α decreased from 61.5 pg/mL on day 10 to 25.9 pg/mL on day 14, while the median concentration of IL-10 increased from 12.7 pg/mL on day 10 to 59.9 pg/mL on day 14 (Figure 1).

The analysis of TNF-α and IL-10 level changes on day 14 and day 10 was done using the median differences of their concentrations in each group. Positive difference means that there's an increase in cytokine levels on day 14 compared to day 10. While negative difference means that there's an increase in cytokine levels on day 14 compared to day 10.

The results of these tests suggest that the median differences of TNF-α on day 14 and day 10 were negative. Negative difference means that TNF-α concentration decreased on day 14 compared to day 10. Significant decrease in median concentration of TNF-α occurred in Group II with $p = 0.001$, Group III with $p = 0.011$, and Group IV with $p = 0.000$ (Figure 2).

Meanwhile, the results of these tests suggest that the median differences of IL-10 on day 14 and

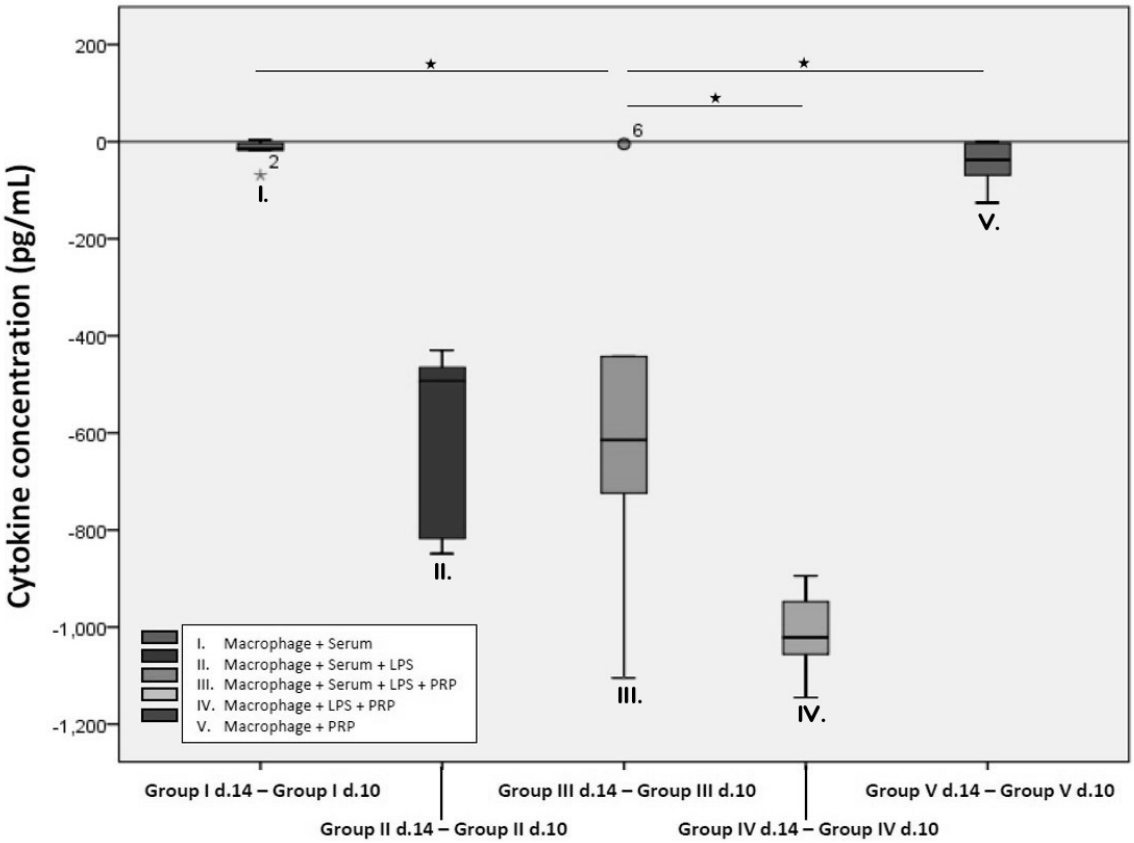

Figure 2. Difference in median concentration of TNF-α between day 14 and day 10.
Box plots of different average concentration of TNF-α on day 14 versus day 10. *$p < 0.05$. Hypothesis was analyzed using T-dependent test for normal distribution data and Wilcoxon test for abnormal distribution data. The highest decrease in TNF-α levels occurred in group IV, followed by groups III, II, V and I.

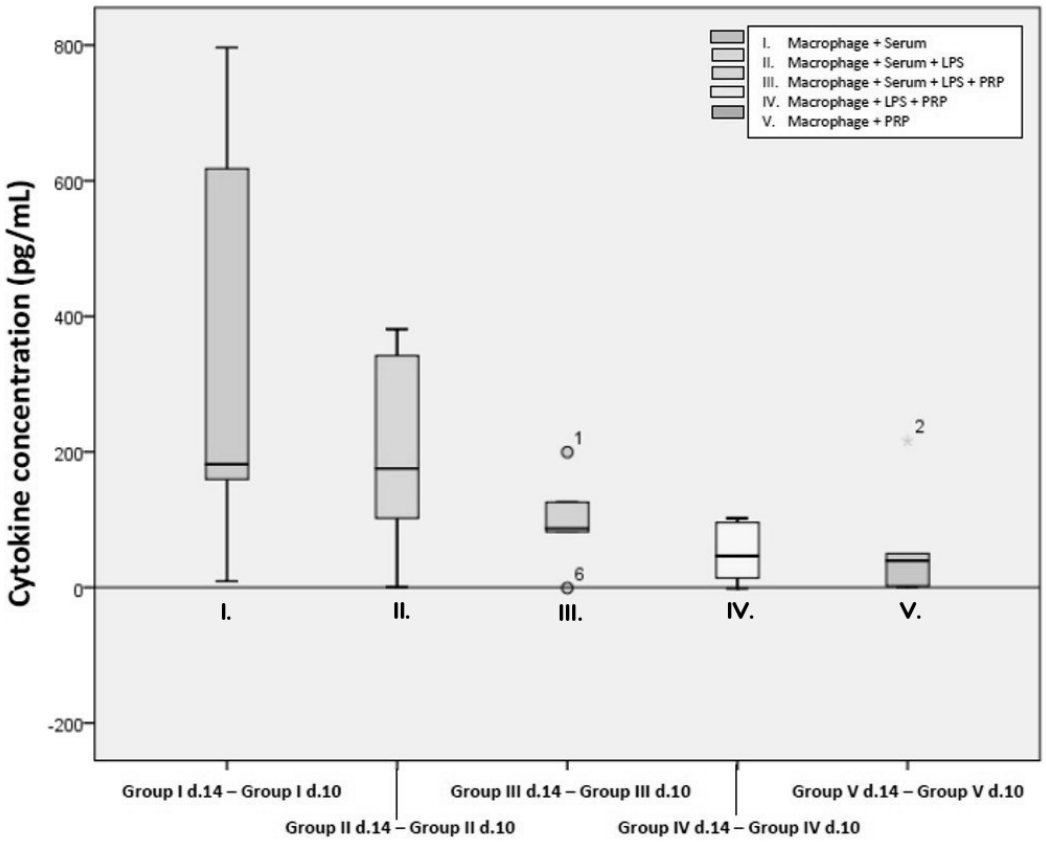

Figure 3. Difference in median concentration of IL-10 between day 14 and day 10.
Box plots of different average concentration of IL-10 on day 14 versus day 10. *p < 0.05. Hypothesis was analyzed using T-dependent test for normal distribution data and Wilcoxon test for abnormal distribution data. The elevation of IL-10 levels at day 14 was highest in group I, followed by groups II, III, IV, and V.

day 10 were positive. Positive difference means that IL-10 concentrations increased on day 14 compared to day 10. Significant increase in median concentration of IL-10 occurred in Group I with p = 0.049, Group II with p = 0.018, Group III with p = 0.017, Group IV with p = 0.030, and Group V with p = 0.018 (Figure 3).

DISCUSSION

This research used 5 different treatments to each macrophage culture from same subject, with the aim of finding out the most appropriate conditions so that the PRP can work optimally.

We used serum in this research with the aim of making the conditions of the macrophages resemble the condition in the human body. And we also used LPS (lipopolysaccharide) which is one of the wall components of gram-negative bacteria, with the aim of activating the macrophages which trigger inflammatory processes.

The results show that the macrophage group given LPS produces the pro-inflammatory mediator TNF-α more than those not given LPS. The decrease in TNF-α production on day 14 occurred most in Group IV, the treatment group given LPS and PRP. This shows that PRP can increase TNF-α production at the onset of inflammation and reduce TNF-α production when the inflammatory process is complete.

At the beginning of inflammation (on day 10) the level of anti-inflammatory mediator IL-10 is still low, and after inflammation begins to subside (on day 10) IL-10 levels begin to increase. The increase in IL-10 production occurred most in Group I, the treatment group given only serum. This shows that PRP does not affect IL-10 production.

The effect of platelets on cytokines produced by monocytes depends on the type of pathogen binding to the receptor of the platelet membrane. The platelet receptor that is able to recognize various components of the microbial wall is TLR. Platelets have TLR-2, TLR-4, TLR-7, and TLR-9 [12, 13]. In *in vitro* experiments, platelet receptors that play a role in the inflammatory process are TLR4 and TLR2. Pathogen components such as LPS (lipopolysaccharide) from gram-negative bacteria, that bind to TLR4 will increase the secretion of IL-1β, IL-6 and TNF-α from monocytes [5, 13].

Platelets also serve to suppress the inflammatory process that occurs in the cultured-cells, providing negative feedback.

CXCL4 on the platelet membrane not only acts as an inflammatory mediator, but also suppresses the CCR1, CCR2, and CCR5 chemotactic receptors in monocytes thereby inhibiting the monocyte migration process. The release of sCD40L from platelets also has an anti-inflammatory effect of monocytes with increased secretion of IL-10, which simultaneously also suppresses the production of TNF-α and IL-6 [5, 14, 15].

From this study it appears that platelets have a dual role as pro and anti-inflammatory. In the first process of macrophage activation by LPS, PRP induces the increase of pro-inflammatory cytokine production, characterized by an increase in the production of TNF-α on day 10. After a few days the production of anti-inflammatory cytokines increases suppressing the production of pro-inflammatory cytokines, characterized by an increase in production of IL-10 on day 14 and decrease in production of TNF-α on the same day.

TNF-α cytokine pro-inflammatory receptors were produced by activated macrophages using LPS through the classical pathway of M1 [16]. This process was accompanied with the morphological change of macrophages and release of various cytokines, such as TNF-α, IL-1 and IL-6 [5]. This is in accordance with the results of the study that the treatment group receiving LPS was able to produce high amount of TNF-α on day 10.

Macrophages' response to produce IL-10 cytokine anti-inflammatory receptors is slower than to produce cytokine pro-inflammatory receptors [12]. Therefore the IL-10 concentration was low on day 10, then began to increase on day 14. These IL-10 cytokine anti-inflammatory receptors were produced by activated macrophages through an alternative pathway, which was stimulated by IL-4 and IL-13 from the serum [5, 15].

On day 14, macrophage cell's ability to produce IL-10 was found to increase. Increased levels of IL-10 can also suppress the production of TNF-α produced by macrophages [17, 18].

TNF-α production was highest on day 10 and decreased levels of TNF-α were greatest on day 14 which occurred in group IV. However, in group IV there was no increase in the highest levels of IL-10 compared to the other groups. This can be due to the limited time and number of research subjects. Measurement of cytokine levels over a longer period is expected to show a trend of change in cytokine levels and would be more accurate.

These results suggest that PRP plays an important role in enhancing the macrophage cell's ability to produce TNF-α when macrophage cells are stimulated in the presence of LPS that can stimulate inflammation. This increase in TNF-α levels did not occur continuously. At the time when macrophage activation by LPS begins to decrease, TNF-α production also will decrease. This process was accompanied by the production of IL-10 by macrophages that suppress TNF-α production while acting as an anti-inflammatory agent.

The authors did not find any other researches that review the effect of PRP on inflammatory mediators from macrophage cultures. From this study, it is known that one of the mechanisms PRP can accelerate the wound healing process by increasing pro-inflammatory mediators produced by activated-macrophages, from the beginning of the inflammation.

CONCLUSION

From this research, it was found that the administration of PRP at the time of the inflammatory process can decrease the level of pro-inflammatory mediator TNF-α; however it has no effect on the level of anti-inflammatory mediator IL-10.

ACKNOWLEDGMENTS

This research was funded by Faculty of Medicine, UNIKA Atma Jaya. The authors want to thank all the staff of Department of Immunology and Integrated Laboratory, Faculty of Medicine, Universitas Indonesia for their assistance in this research. Thanks to all volunteers who kindly participated in this research and contributed to it, in particular the blood donors.

CONFLICT OF INTEREST STATEMENT

The authors declare that there is no conflict of interest regarding the publication of this paper.

REFERENCES

1. Cole, B. J., Seroyer, S. T., Filardo, G., Bajaj, S. and Fortier, L. A. 2010, Sports Health, 2(3), 203-210.
2. Kim, Y. H., Furuya, H. and Tabata, Y. 2014, Biomaterials, 35(1), 214-224.
3. Zollino, I., Candotto, V., Silvestre, F. X. and Lauritano, D. 2014, Annals of Oral & Maxillofacial Surgery, 2(1), 5.
4. Qian, Y., Han, Q., Chen, W., Song, J., Zhao, X., Ouyang, Y., Yuan, W. and Fan, C. 2017, Front. Chem., 5, 89.
5. Kral, J. B., Schrottmaier, W. C., Salzmann, M. and Assinger, A. 2016, Transfus Med. Hemotherapy, 43(2), 78-88.
6. Gonzalez, A. C., Costa, T. F., Andrade, Z. A. and Medrado, A. R. 2016, An. Bras. Dermatol., 91(5), 614-620.
7. Scull, C. M., Hays, W. D. and Fischer, T. H. 2010, J. Inflamm., 7, 53.
8. Guo, S. and DiPietro, L. A. 2010, J. Dent. Res., 89(3), 219-229.
9. Arango, G. and Descoteaux, A. 2014, Front. Immunol., 5, 491.
10. Stow, J. L., Ching, P., Offenhäuser, C. and Sangermani, D. 2009, Immunobiology, 214(7), 601-612.
11. Purnamasari, D. 2015, Efek vitamin d terhadap respons imun adaptif penyakit graves: telaah mengenai pematangan sel dendritik [dissertation], Jakarta: Universitas Indonesia, Indonesian.
12. Lam, F. W., Vijayan, K. V. and Rumbaut, R. E. 2015, Compr. Physiol., 5(3), 1265-1280.
13. Herter, J. M., Rossaint, J. and Zarbock, A. 2014, J. Thromb. Haemost., 12(11), 1764-1775.
14. Linke, B., Schreiber, Y., Picard-Willems, B., Slattery, P., Nusing, R. M., Harder, S., Geisslinger, G. and Scholich, K. 2017, Mediators Inflamm., 2017, 1463216.
15. Mantovani, A. and Garlanda, C. 2013, Nat. Immunol., 14(8), 768-770.
16. Parameswaran, N. and Patial, S. 2010, Crit. Rev. Eukaryot Gene Expr., 20(2), 87-103.
17. Kleiner, G., Marcuzzi, A., Zanin, V., Monasta, L. and Zauli, G. 2013, Mediators Inflamm., 2013, 434010.
18. Iyer, S. S. and Cheng, G. 2012, Crit. Rev. Immunol., 32(1), 23-63.