Conference Paper

The Role Of IL-6 In TMPD-Treated Lupus Arthritis Mice

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Abstract

Lupus or systemic lupus erythematosus is a chronic autoimmune disease with systemic inflammation manifestations mainly in targeted organs. The appropriate animal model for lupus is necessary. The induction method by using 2,6,10,14 tetramethylpentadecane (TMPD) reveals more complex manifestations than other hydrocarbons. However, the autophagy of macrophages as an effect of TMPD makes differences to make the decision in lupus biomarker as a targeted therapy in lupus arthritis. Thus, this research focused on the role of CD68+IL-6 produced by macrophages and total IL-6 in lupus in correlation to the arthritis severity. The naïve and TMPD-treated groups (n=3) were induced by means of 0.5 ml TMPD i.p. After 6 months, the mice were sacrificed then the fresh spleens were prepared as isolated cells to be measured by using flow cytometry method. The knee joints were prepared for histology observation. The statistical analysis was performed by using T-test SPSS 22 version. The results showed the relative percentage of CD68+IL-6+ in the TMPD-treated group increased significantly ($P<0.05$) with the value of 62.38±9.97 %, compared to naïve group 49.70±2.34 %. Moreover, the total IL-6 did not increase significantly ($P>0.05$). Meanwhile, the arthritis severity score of the TMPD-treated group revealed severe erosion with the grade of 3.7±1.06, higher significantly ($P<0.05$) than the naïve group (0.5±0.71). The joint spaces in both groups were not significantly different. Finally, the observations gave the clear information that despite the autophagy potency, the CD68+IL-6 and the arthritis severity score were good markers in lupus preclinical study.

Keywords: CD68+IL-6; inflammation; lupus arthritis; TMPD.

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1. Introduction

The animal model for lupus arthritis is rarely studied. Hydrocarbons like complete Freund's adjuvant (CFA) and 2,6,10,14 tetramethylpenta-decane (TMPD) reveal arthritis manifestation although the similar baseline made by the hydrocarbons depends on

the immunity of experimental animal used [1, 2]. TMPD reveals more complex manifestations which the joint disorder occurs as a result of systemic immune imbalance. TMPD stimulates the production of IFN-α and IFN-β through immature monocytes (Ly6Chi). The production of IFN-I and pro-inflammatory cytokines could be stimulated by four main cell pathways by utilizing the differences between adaptor proteins or signaling intermediate of TRIF (TLR 3 and 4), MyD88 (TLR 7, 8, and 9), IPS-1 (Rig-I-like helicases, RLH)), and TBK1 or single-stranded RNA through TLR7 or TLR8 activates the gene expression of IFN-α and β. The process involves the adaptor proteins MyD88, some kinases and transcription factor interferon regulatory factor (IRF) 7 [3, 4]. All of the pathways come to nuclear factor kappa B (NFκB). Moreover, the overproduction of IFN-α and β by means of TMPD highly depends on TLR7-MyD88-IRF7 pathways. Finally, the organ disorders reveal the manifestation of glomerulonephritis, pulmonary bleeding, and arthritis [4, 5] after the sufficient lupus-specific antibodies produced, such as anti-Sm, anti-RNP, anti-ribosomal P, anti-Su, anti-dsDNA, and antichromatin [6–8].

Besides, according to Foncesa [9] and Janicahsvilli [10], the pro-inflammatory cytokines dominantly exist and then result in systemic inflammation. Cash [11] also says that IL-6 is a targeted therapy which is potential for lupus. In contrary, the lupus patients do not experience excessive inflammation except the local joint inflammation when the flaring period happens. Deretic [12] and Zhu [13] explain the phenomenon that TMPD could lead to autophagy of the macrophages in vitro and in vivo in the spleen. There is no clear information about the impact of the autophagy on the innate and adaptive immune system. Thus, in this research, we observed IL-6, a pro-inflammatory cytokine produced by macrophages, in TMPD-treated lupus mice. IL-6 is suggested as a cytokine that has a direct role in the tissue damage [14]. The IL-6 which is produced by macrophages is CD68+IL-6. It was compared to the total IL-6 and then be correlated to the arthritis severity score of the knee joint of the mice.

2. Materials and methods

2.1. Materials

Female Balb/c mice aged 4 weeks were received from LPPT UGM. These mice were species pathogen free with the certificate number of 352/LP3HP/29/VII/2015. TMPD (Pristane) was obtained from Sigma-Aldrich, Singapore. The anti-CD68 and anti-IL-6 were obtained from Biogenesis, USA. The PBS and aqua bidestillata Ikapharmindo were obtained from LDB Laboratory. The Verify reagent strips for urinalysis were obtained

from CV. Rachmandjaya Surabaya. The ethyl acetate (pro analysis grade) was obtained from Merck via PT Dianum Surabaya.

2.2. Methods

The experimental groups were a TMPD-treated group that was injected a volume of 0.5mL TMPD every 90 days (n=3) and a naïve group (n=3). The proteinuria measurement was done every 7 days. At the end of the experiment, mice were sacrificed. Then, the fresh spleen cells were immediately prepared to be 2measured by using flow cytometry method. This CD68+IL-6+ measurement would be analyzed by means of BD CellQuest program. Ethical clearance of this research was approved by ICUC of Veterinary Medicine Faculty Universitas Airlangga on January 12, 2016, with the number of 526-KE.

3. Results and discussion

Inflammation markers in lupus arthritis are potential biomarkers of lupus arthritis for drug development targets. Thus, it is a need to find markers that represent the disease severity. In this case, the pro-inflammatory marker IL-6 has a direct correlation to the tissue damage in spite of the macrophage autophagy in spleen which causes the inflammation decrease. This experiment discusses the phenomenon by means of the observation of the organoleptic data, the relative percentage of CD68+IL-6, the relative percentage of total IL-6, and the arthritis severity score (ASS) based on the Pritzker [15] scoring method.

In the organoleptic observation, the 6-month TMPD treated mice revealed a mild walking abnormality about 2-3 days and then the behavior normal the next day. There was no inflammation appeared in the joint, meanwhile the joint of the feet fingers seemed more red than normal. After the induction time over, the mice were sacrificed, and then the spleens were isolated. The spleen index was 227% than normal, reveals the functional disorder of the spleen as a secondary lymphoid organ. Figure 1 shows the organ appearance of TMPD-treated mice.

This white layer is predicted as a part of lipogranuloma as a result of TMPD induction in the peritoneal cavity. The thicker layer forms lipid spots inside the peritoneal cavity, but it does not interfere the liver and kidneys. This lipogranuloma is the trigger of the inflammation and immune imbalance processes caused by TMPD. Fresh spleens were collected in a closed tube which contained PBS, then the cells were prepared for flow

Figure 1: The spleen of TMPD-treated mouse reveals the darker color, membrane-like lipid layer, and the layer deposit in the middle of spleen surface.

Table 1: The relative percentage of CD68+IL-6 + in the spleen cells of naïve and TMPD-treated groups.

Groups	CD68+IL-6+ (%) ± SD
Naïve	49.70 ± 2.34
TMPD-treated	62.39 ± 9.97*

cytometry measurement. The processes were finished before 24 hours to keep the living cells above 70%, with periodic observation every 2 hours. After the cell counting finished, the data was analyzed by utilizing of BDCellQuest. The results are shown in Figure 3, Table 1 and Table 2.

The gate (R1) was decided in order to make a border to be applied to all samples in counting the IL-6 which is produced by macrophages. The chosen region was the monocytes and granulocytes region, so the counting was more specific.

Figure 3 shows the increase of cell number and the increase of the number of CD68+IL-6 in the up-right (UR) region of the sample. It was analyzed and then the result is the significant increase ($P<0.05$) of the relative percentage of CD68+IL-6+. Meanwhile, the total IL-6 is lower than the naïve mice. The decrease is not significant ($P>0.05$).

Both results were not in line, so we observed the outcome of the pathology processes to the joint of all mice. The results are shown in Figure 4 and Table 3. The TMPD-treated mouse histology observation shows the erosion of hyaline layer in both

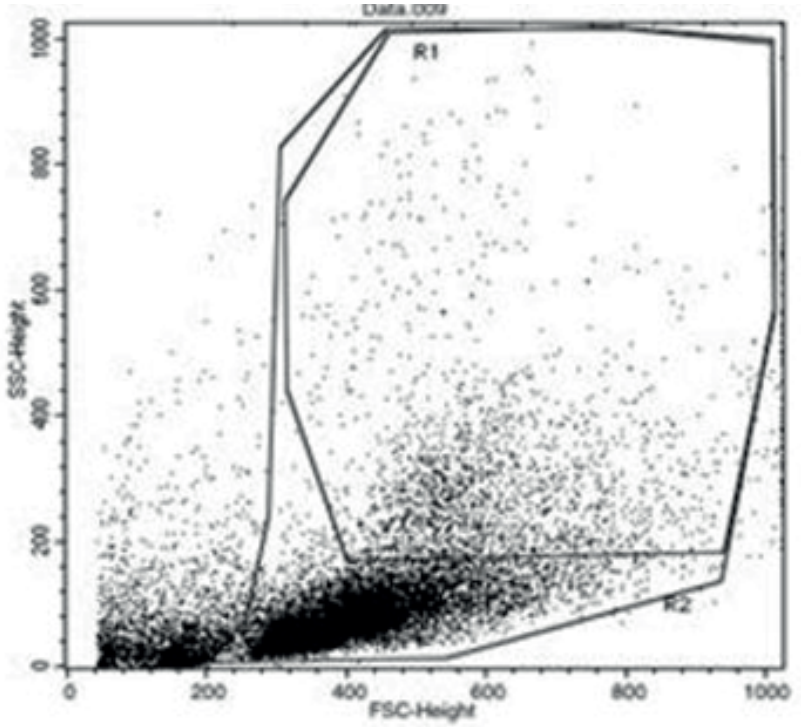


Figure 2: The gating of the macrophages in the BD CellQuest program which was connected to the flow cytometer.

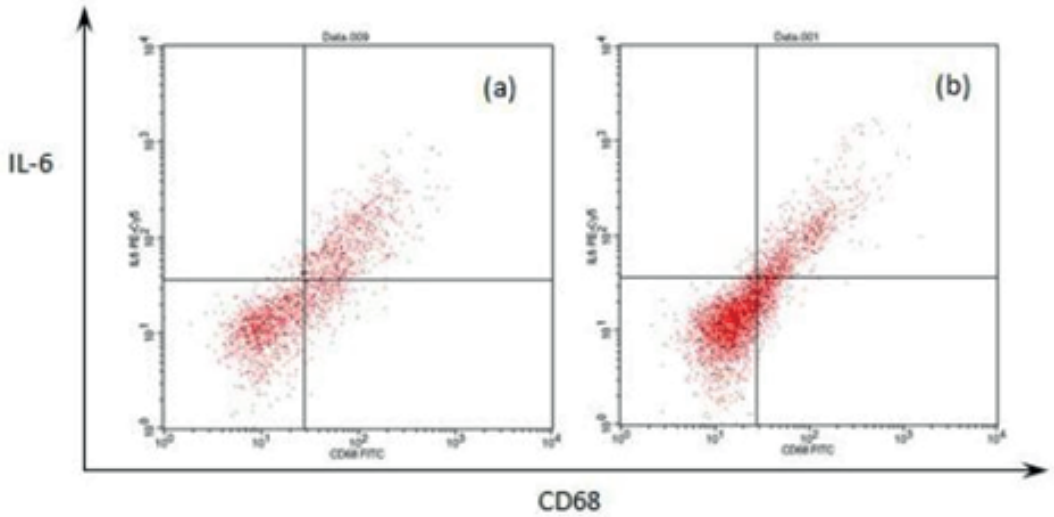


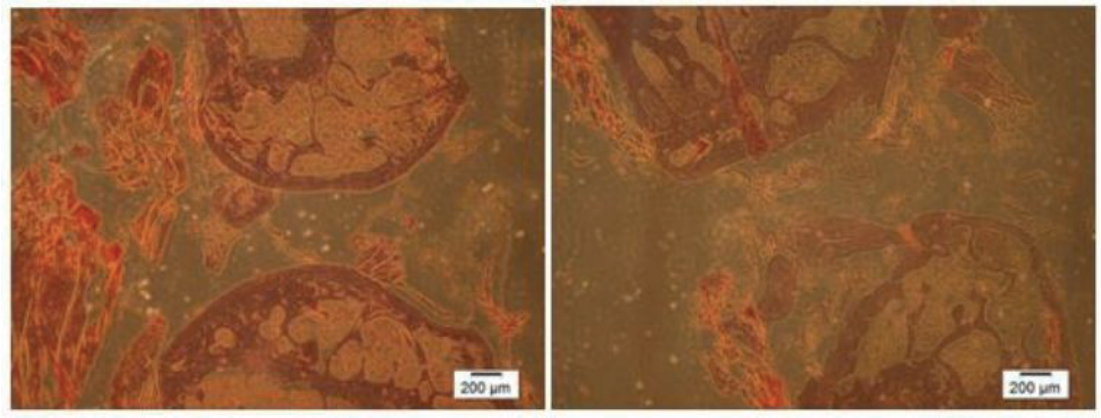
Figure 3: The relative percentage profiles of CD68+IL-6+ as the results of flow cytometry analysis of the fresh spleen cells of naïve group (a) and TMPD-treated group (b).

bones. The erosion seems to be involved in the compact bone destruction in a long term.

The results lead to a prediction that dominant IL-6 in TMPD-treated lupus mice is IL-6 which is produced by macrophages (innate immune system). There are no differences of total IL-6 in both groups. According to the high grade of the arthritis severity score

Table 2: The relative percentage of total IL-6 + in the spleen cells of naïve and TMPD-treated groups.

Groups	CD68+IL-6+ (%) ± SD
Naïve	5.53 ± 0.22
TMPD-treated	5.30 ± 0.46



(a) (b)

Figure 4: The histology observation of the joint of the naïve group (a) and TMPD-treated group (b) by facilitating of inverted microscope Olympus CXK41 at the magnitude of 40x.

results in the TMPD-treated group, the IL-6 results seem not relevant. The score is significantly higher ($P<0.05$) than the naïve one. Moreover, the joint space logically shows the severity of inflammation in the joint. In this case, the mean of the joint spaces of both groups are not significantly different ($P>0.05$). It is predicted that the autophagy mechanism which is stated by Zhu [13] occurs, so the joint disorder develops without the equal increase of total IL-6. It might be a result of upregulation of TLR 3 on macrophages [16] causes the control of marker cells in rheumatoid diseases [17]. The immune complex deposit could perform erosion of the hyaline layer of the joint [18, 19] and the imbalance of cytokines [20, 21].

4. Conclusion

In conclusion, CD68+IL6 and ASS are the appropriate markers to investigate new drugs for lupus by using TMPD-treated mice.

Table 3: The arthritis severity score (ASS) grade and the joint space of observed mice.

Groups	ASS Grade	Joint space (µm)
Naïve	0.5 ± 0.71	621.56 ± 334.52
TMPD-treated	3.7 ± 1.06*	672.34 ± 454.97

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