The Effects of Liposomale Methylprednisolone Palmitate on the Production of TNFα in Mice

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Abstract: Introduction & Aim: Liposomes are used in this study as the carrier of an immunosuppressive drug, namely methylprednisolone palmitate (MPLP), to reduce the drug side effects on various organ systems, such as musculoskeletal, gastrointestinal, and cardiovascular. This study aimed to investigate the effects of low-dose liposomal methylprednisolone palmitate (L-MPLP) on the in vitro and in vivo production of tumor necrosis factor alpha (TNFα) derived from the culture of C3H mice spleen. Methods: For the in vitro culture, the TNFα levels were identified using the splenic lymphocyte cultures. Meanwhile, for the in vivo culture, the mice were divided into eight groups of five (5) mice randomly. Forty-eight hours after the drug administration, these mice were sacrificed. The spleen was removed and used for lymphocyte culture. The TNFα levels were measured with ELISA at a wavelength of 450 nm. Results: The in vitro and in vivo assays showed that, when administered at the same dose, L-MPLP produced lower TNFα than methylprednisolone (MPL). Conclusion: At small doses, L-MPLP can significantly inhibit the in vitro and in vivo production of TNFα, as opposed to the control group MPL.

1 INTRODUCTION

Liposomes are phospholipid vesicles containing polar and non-polar groups with a phospholipid membrane structure similar to the cell membrane. Liposomal vesicles are formed spontaneously when lipids or phospholipids exposed to water. Liposomes carry drug molecules in various ways, namely the encapsulation of hydrophobic drug substances by interacting with the lipophilic substance membrane and the entrapment of hydrophilic substance inside the vesicle (Jone, 2013).

As a drug carrier, liposomes have been tested in animals and humans either by oral or parenteral route. The oral administration of liposomes is ineffective because they are enzymatically hydrolyzed before entering the blood circulation. They are also unstable in the intestines, and they can interact with bile salts. Meanwhile, their parenteral administration, especially intravenous, is more easily monitored in terms of extravasation and uptake by tissues. Several studies have used liposomes as a drug carrier with the following reasons: liposomes extend the half-life of drugs and increase the distribution of drugs to the targeted organs, which consequently reduce drug dose and minimize the drug side effects (Anwekar *et al.*, 2011; Ait-Oudhia *et al.*, 2014).

MPL is a lipophilic glucocorticoid containing a hydroxyl (-OH) group at C_{21} which makes it amphoteric and able to form micelles. The formation of micelles makes MPL easily detached from the liposome membrane, resulting in unstable liposomes. Benameur *et al.* (1993) incorporate dexamethasone palmitate (DMP) into liposomes by replacing the -OH group at C_{21} with a palmitate group, forming a more lipophilic drug and creating a better DMP interaction with liposome membrane than dexamethasone alone. Shaw *et al.* (1976) also manage to incorporate cortisone in the form of cortisone palmitate into liposomes. Following the success of Benaumer *et al.* (1993) and Shaw *et al.* (1976) and relying on the similarity of the basic structures of dexamethasone- and cortisone-incorporated methylprednisolone, this research hypothesized that the addition of a palmitate group to the C_{21} of methylprednisolone would give similar results. The initial experiment in our research proved

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that the preparation of liposomal methylprednisolone palmitate (L-MPLP) using phosphatidylcholine from egg yolk (Egg-Yolk Phosphatidyl Choline/EPC) resulted in an incorporation of about 70% and that the combination of EPC and 2.5 mol% tetraether lipid (TEL) increased the incorporation of MPLP into the liposome membrane to 95%.

In spite of having strong anti-inflammatory effects, dexamethasone has a long half-life, stimulates activity in bone demineralization, and suppresses the growth factor better than methylprednisolone; therefore, it is not suitable for long-term therapy, for example in post-organ transplantation (Becker, 2013). This condition is the reason behind the exclusion of dexamethasone in our study. Immunosuppressants play an important role in preventing the recipient's body from rejecting transplanted organ or tissue and in treating several autoimmune diseases. Immunosuppressive drugs promote the success of organ transplants, such as kidney, bone marrow, liver, heart, pancreas, and lungs. Some autoimmune diseases and disorders, such as hemolytic anemia, idiopathic thrombocytopenia purpura, Hashimoto's thyroiditis, systemic lupus erythematosus, acute glomerulonephritis, and acquired hemophilia, have improved with the use of this drug (Luisa and Piedras, 2013).

Liposomes are used as drug carriers, which in this case is methylprednisolone palmitate (MPLP), to reduce the drug side effects and retain the therapeutic function of the resultant liposomal drug even when administered at a small dose. MPLP is a novel compound created using the same synthesis method as that of dexamethasone palmitate developed by Benameur *et al.* (1993), and it has been successfully incorporated into the liposome membrane by Purwaningsih *et al.* (2007), forming methylprednisolone palmitate (L-MPLP). However, this new compound has never been evaluated for its immunosuppressive effect. The initial step of the biological activity test of L-MPLP is assessing its immunosuppressive effects on lymphocyte proliferation. This new compound is expected to inhibit the proliferation of lymphocytes, which is indicated by the decreased production of TNFα, as the mitogen 'concanavalin A' stimulates the other glucocorticoids in cultured lymphocytes.

TNFα is a cytokine playing a major role in the activation of immune reactions, including the specific and non-specific immune system. The other roles of TNFα are to stimulate endothelial cells, express adhesion molecules, activate inflammatory cells, and stimulate other cells in expressing major histocompatibility (MHC) class I molecules on the cytotoxic T cells (Keystone and Ware, 2010). Lymphocyte proliferation test mainly aims to determine the proliferation ability of lymphocyte cells after mitogen stimulation with or without drugs. Some mitogens stimulate a specific subpopulation of lymphocytes. For instance, concanavalin A (con-A) stimulates T lymphocyte cells (Abbas *et al.*, 2014).

This study aimed to evaluate the biological effects of L-MPLP by measuring the TNFα levels in the lymphocyte cultures of C3H mice spleen after the administration of L-MPLP with different concentrations, namely 0.005 mM, 0.05 mM, and 0.5 mM, and 48 hours after the intravenous administration of L-MPLP at different doses, i.e., 2, 8, and 16 mg/kg BW.

2 MATERIALS AND METHODS

2.1 Materials

The liposomes and liposomal methylprednisolone palmitate (L-MPLP) were made fresh from Egg-Yolk Phosphatidylcholine (EPC), the Tetraether Lipid (TEL) was obtained from Purwaningsih *et al.* (2007), and the MPLP was donated by Bernina Biosystems GmBH. The methylprednisolone-Na succinate (Solu-Medrol) was purchased from Upjohn. This research also used ethyl acetate, methanol, chloroform PA, NaOH, HCl, Tris buffer solution (pH 7.4) from Merck (filtered through a Millipore membrane before use), and Aquabidest (sterilized water) from IKA Farma. The other materials were RPMI 1640 (pH 7.2-7.4, Gibco), fetal bovine serum/FBS (ICN Flow), gentamicin (Gibco), fungizone (Gibco), sodium bicarbonate (ICN Flow), concanavalin A (Sigma) as a mitogen, nylon wool (Biotest), nylon mesh, 2M HCl, aqua Millipore, aquabidest, 70% alcohol, sterile cottons, candles, CO_2 gas from Perum Aneka Gas, and TNFα ELISA kits (Quantikine). The male C3H mice (12-16 weeks old, 20-22 gr in weight) were obtained from the Department of Anatomic Pathology, Faculty of Medicine, Universitas Indonesia. Before the experiment, the test animals were acclimatized in captivity in the Laboratory of Pharmacology, Faculty of Medicine. They were given ad libitum access to food and drink. The study approval (No 556/PT02.FK/ETIK/2012) was obtained from the Ethics Committee, Faculty of Medicine, Universitas Indonesia before the study began.

2.2 Methods

2.2.1 In Vitro Measurement of TNFα Levels

The TNFα assay was performed in the splenic lymphocyte cultures using the Tris buffer solution and liposomes as controls. RPMI solution containing 1×10^{-6} cells/ml and 2.5 g/ml concanavalin A were added to each of the following groups: the liposome solution (20 ml, 0.5 mM), MPL, and the three L-MPLP concentrations (0.5 mM, 0.05 mM, and 0.005 mM). After 48-hour incubation, the cultures were centrifuged at 2,000g for 10 min. The supernatants were discharged and stored at -20^0C before being used for the measurement of TNFα levels using ELISA at a wavelength of 450 nm.

2.2.2 In Vivo Measurement of TNFα Levels

Drug Administration
The drug was administered intravenously to each mouse based on the group division, i.e., eight (8) groups of 5 mice, via vena lateralis in the tail. The group division was as follows: Group I was used as a control (Tris buffer, 5ml/kg); Groups II, III, and IV were administered with methylprednisolone sodium succinate/MPL at different concentrations, i.e., 8, 16, and 32 mg/kg BW, respectively; Groups V, VI, and VII were given liposomal methylprednisolone (L-MPLP) at different concentrations, i.e., 2, 8, and 16 mg/kg BW, respectively; and Group VIII was treated with 0.5 mM liposomes. Forty-eight hours after drug administration, these mice were sacrificed by exposing them to the aether. Their spleens were removed using scissors, and the lymphocyte culture was collected with tweezers.

The Lymphocyte Culture
The spleens were placed in a sterile 60mm Petri dish containing 5 ml of RPMI medium. To get the lymphocyte cell suspension, the cell was filtered through a sterile nylon mesh, and the erythrocytes were lysed with ammonium chloride buffer solution. The amount of T lymphocytes was enriched by flowing the cell suspension through a nylon wool column, which had been soaked in 2M HCl overnight and then washed with distilled water, for 5-6 times and aerating it to allow drying.

Nylon wool fibers were separated with tweezers and inserted into a polyethylene plastic syringe. As much as 0.3 gram of nylon wool was inserted into a 5ml syringe to form the column for the spleen cell suspension. The insertion was conducted with moderate pressure and followed by the slow release

of the piston. The column, piston, and needle syringe were sterilized in an autoclave for 15 minutes at a temperature of $110\text{-}115^0$ C. The column was preincubated with RPMI 1640 medium containing 5% fetal bovine serum (FBS). The cell suspension was resuspended to achieve a population of 5×10^6 cells/ml. It was loaded onto the column and, then, incubated at 37^0 C for 60 minutes. The unretained lymphocytes were eluted with a medium (eluent) as much as one volume of the syringe. The eluate (i.e., T lymphocytes) was washed two (2) times and centrifuged at 2,000g for 5 minutes. Afterward, a population of 1×10^6 cells/ml was prepared from the cell suspension. Lymphocytes with a concentration of 1×10^6 cells/ml were maintained in RPMI 1640 medium containing 5% FBS, fungizone, gentamicin, and concanavalin A (10 μg/ml). The in vitro cultures were created by adding MPL and L-MPLP with four different concentrations (i.e., 0.005 mM, 0.05 mM, 0.5 mM, and 0.5 mM) to the mice spleen. After a 48-hour incubation, the cultures were centrifuged at 2,000g for 10 min. The supernatants were discharged and stored at -20^0C before being used for the measurement of TNFα levels using ELISA at a wavelength of 450 nm (Wohler & Barnum, 2010; Bhattacharjee & Das, 2008)

2.2.3 Data Analysis

The data obtained from in vitro and in vivo cultures were analyzed in SPSS 20.0. The mean values of the measurement results of the groups were compared using One-way ANOVA and expressed as mean±SD. The p-value of <0.05 meant that the difference between the mean values was statistically significant.

3 RESULTS AND DISCUSSION

The results of the TNFα level measurement from the in-vitro culture are shown in Figure 1A, while the percentage of TNFα level in each group to the TNFα level in the control group (represented as % levels of TNFα) is depicted in Figure 1B. The TNFα levels and the % levels of TNFα from the in-vivo culture can be seen in Figure 2A and Figure 2B, respectively.
The statistical test results showed that percentages of the TNFα levels in Groups MPL1, MPL2, MPL3, L-MPLP1, and liposomes were not significantly different from the control group (p>0.05). Meanwhile, the percentages of the TNFα levels in Groups L-MPLP2 and L-MPLP3 were significantly
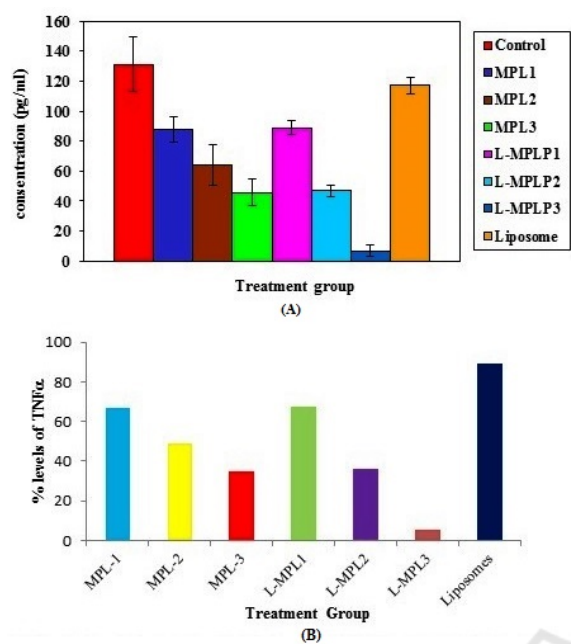
Figure 1: (A) TNFα levels (mean ± SD) and (B) the percentages of TNFα levels in each group compared to the TNFα level in the control group (in vitro culture). (Control group) Tris Buffer 5 ml/kg BW; (MPL1) 0.5 mM methylprednisolone sodium succinate; (MPL2) 0.05 mM methylprednisolone sodium succinate; (MPL3) 0.005 mM methylprednisolone sodium succinate; (L-MPLP1) 0.5 mM liposomes methylprednisolone palmitate; (L-MPLP2) 0.05 mM liposome methylprednisolone palmitate; (L-MPLP3) 0.005 mM liposome methylprednisolone palmitate

different (p <0.05) from the control group (without the administration of drugs or MPL). The administration of L-MPLP at a dose of 2 mg/kg BW resulted in an equal TNFα level to MPL given at a dose of 16 mg/kg BW, i.e., 94.85% (see Figure 2B). Meanwhile, at the same dose of 8 mg/kg BW, the treatment using L-MPLP produced lower TNFα than MPL by 1.5 times. At a dose of 16 mg/kg BW, L-MPLP yielded TNFα level 3 times lower than MPL at the same dose.

Several theories suggest that liposomes as drug carriers can extend the half-life of drugs and increase the distribution of drugs into the organ selectively so that the drug dose can be minimized (Sercombe *et al.*, 2015; Mishina *et al.*, 1994; Binder *et al.*, 1994). Mishina *et al.* (1994) state that, when compared to methylprednisolone (MPL) at a dose of 2 mg/kg BW IV), the same dose of liposomal methylprednisolone (L-MPL) in male Sprague-Dawley rats can prolong the half-life from 0.48 hours to 30.13 hours and increase the distribution
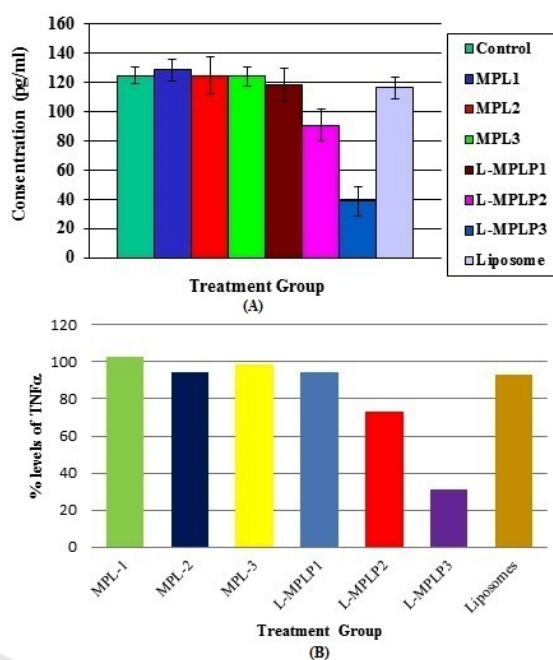
Figure 2: (A) TNFα Level (mean ± SD) and (B) the percentages of TNFα levels in each group compared to the TNFα level in the control group (in vivo culture). (Control group) Tris Buffer 5 ml/kg BW; (MPL1) 0.5 mM methylprednisolone sodium succinate; (MPL2) 0.05 mM methylprednisolone sodium succinate; (MPL3) 0.005 mM methylprednisolone sodium succinate; (L-MPLP1) 0.5 mM liposomes methylprednisolone palmitate; (L-MPLP2) 0.05 mM liposome methylprednisolone palmitate; (L-MPLP3) 0.005 mM liposome methylprednisolone palmitate

volume from 2.1 L/kg to 21.87 L/kg. However, the use of L-MPLP at a dose of 2 mg/kg BW in our study did not exhibit any biological effects on mice. This finding contradicts the research conducted by Mishina *et al.* (1994) and Binder *et al.* (1994) where the administration of L-MPL at this dose exhibits biological effects. These two studies successfully examine the immunosuppressive effects of the same dose of L-MPL in male Sprague-Dawley rats, as evident in the increase of survival to 30 days after a heart transplant (instead of only 10-day survival in the control group).

Although Mishina *et al.* (1994) and Binder *et al.* (1994) identify the immunosuppressive effects of MPL without measuring the TNFα level, these two studies provide a conclusion that at a dose of 2 mg/kg BW, L-MPL gives a good immunosuppressive effect. Meanwhile, in our study, the use of L-MPLP at the same dose has no biological effects on male C3H mice because of several reasons. The first is the different strains of

the test animals. This study uses mice with C3H strain, whereas Mishina *et al.* (1994) and Binder *et al.* (1994) use the Sprague-Dawley strain. Such difference in the test animal species leads to dissimilar sensitivity to glucocorticoids. The second reason is the type of the liposome used in the experiments. These two studies use liposome made from a combination of EPC and phosphatidylglycerol. Meanwhile, the liposome in our research is small-sized with a diameter of 73 nm, and it is made from a combination of EPC and TEL. The different types of phospholipids determine the size and diameter of the liposome, which, in turn, affect the speed of the drug uptake (Sercombe *et al.,* 2015; Shashi *et al.*, 2012).

The small unilamellar liposome vesicles (SUVs) are absorbed at a slower pace compared to the large-sized ones (LUVs). Therefore, the circulation time of liposomal SUVs is longer than the liposomal LUVs. Liposomes made from the combination of EPC and phosphatidylglycerol are medium-sized (MUVs), i.e., about 100 nm (Sercombe *et al.*, 2015; Kumar *et al.*, 2012), whereas the liposomes in our research are small-sized (SUVs), i.e., about 73 nm (Purwaningsih *et al.*, 2007). The third reason is that both Mishina *et al.* (1994) and Binder *et al.* (1994 use MPL, which is already widely used and known for its effects, whereas our study chooses a novel compound (MPLP) that is expected to be a pro-drug. Therefore, even though administered during the same period, MPLP has not shown its effect yet, as opposed to MPL.

Methylprednisolone (MPL) sodium succinate is a pro-drug (an MPL derivative) that is rapidly hydrolyzed to methylprednisolone with a half-life of 2.5 hours in humans when administered at a dose of 1 mg/kg BW. At higher doses than 10 mg/kg BW, the half-life becomes longer, i.e., up to 3.6 hours. The half-life of MPL sodium succinate after the intravenous administration to the C3H mice was 10-30 minutes. MPL sodium succinate did not decrease the TNFα levels due to its short half-life. Accordingly, after 48 hours of administration, the drug levels in the blood were very low. This finding is similar to several references that mention that the administration of a single dose of glucocorticoids reduces lymphocytes, monocytes, basophils, and eosinophils for 4-6 hours and returns them to their normal levels after 48 hours.

Figure 2A shows that L-MPLP is still exhibiting an effect on T lymphocytes and reducing the TNFα levels after 48 hours of treatment. This condition shows that the use of liposomes as drug carriers can

extend the half-life of MPLP, but the length of the half-life of MPLP remains unclear. Using different concentrations of dexamethasone (DEX), namely at a concentration of 0.3, 0.8, and 10 μg/ml, against the mitogen-induced proliferation of lymphocytes in rats, Miller *et al.* (1991) explain that DEX influences T cell proliferation and the in vitro mitogen-induced proliferation of lymphocyte in cell cultures.

As seen in Figure 1A, the different concentrations of MPL and L-MPLP (i.e., 0.5, 0.05, and 0.005 mM) added to the cultures before being incubated for 48 hours inhibit lymphocyte proliferation, which is significantly different from the control group (without drug administration, p<0.05). The figure also shows that at a concentration of 0.5 mM, L-MPLP inhibits the lymphocyte proliferation by decreasing the TNFμ levels, which are significantly different from the control MPL at the same concentration (p<0.05). Furthermore, the application of liposome without any immunosuppressive drugs at a concentration of 0.5 mM does not decrease the TNFα levels (p>0.05). This finding shows that liposomes do not affect lymphocyte proliferation and TNFα level. It also indicates that 0.5 mM liposomes comprised of a combination of EPC and TEL are not toxic to C3H mice. From the results of a toxicity study of 6 μg/ml TEL in L5178Y murine lymphoma cells (EMAT cells) and mutagenicity or antimutagenicity tests of *Salmonella typhimurium* strain TA 100, Freisleben *et al.* (1993) affirm that TEL is nontoxic and nonmutagenic.

The additions of MPL and L-MPLP to the cultures before 48 hours of incubation resulted in the proliferation of lymphocyte that reduced the TNFα levels significantly, as opposed to the controls (p <0.05). This condition is explainable by the ability of glucocorticoids to suppress lymphocyte proliferation. According to Cidlowski (2013), the sensitivity of glucocorticoid receptor varies depending on the antigen or mitogen used.

Benameur *et al.* (1995) state that liposomes as drug carriers are useful for reducing the administration dose of dexamethasone palmitate while retaining its therapeutic effects. As an initial test to assess the biological activity of liposomal-dexamethasone (DMP-SUVs), they employ lymphocyte proliferation test and interferon gamma level (IFNγ) measurement and compare the results to the use of dexamethasone (DEX) without liposomes at 1/6 times of the dose of DEX. Based on the proliferation test and measurement results, they conclude that DMP-SUVs inhibit lymphocyte proliferation and reduce the levels of IFNγ six (6)

times greater than dexamethasone alone. In other words, the use of DMP-SUVs in therapy can be reduced without altering their pharmacological effects because liposomes carry the drug to the targeted organs, especially the ones that are rich in reticulum-endothelial systems like liver and spleen (Anwekar *et al.*, 2011). The sustained drug release offered by liposomes prolongs the drug exposure in the cells, allowing more drug cellular uptake (Jone, 2013).

In our study, the administration of L-MPLP at a dose of 2 mg/kg to the C3H mice inhibited the formation of TNFα. This result was equal to the administration of MPL at a dose of 16 mg/kg. Meanwhile, the administrations of L-MPLP at the doses of 8 mg/kg and 16 mg/kg inhibited the formation of TNFα, respectively, by 1.4 times and 3 times greater than MPL at the same doses.

The in vitro administrations of 0.005 mM L-MPLP and MPL to the cell cultures inhibited the formation of TNFα by nearly equal numbers. Meanwhile, the applications of 0.05 mM and 0.5 mM L-MPLP inhibited the formation of TNFα, respectively, by 1.4 and 6.6 times greater than MPL at the same concentrations.

Tumor Necrosis Factor (TNF) plays an important role in a broad range of immune and inflammatory processes, including cellular activation, survival, and proliferation, as well as cell death by necrosis and apoptosis (Keystone, 2010). Glucocorticoids are used as immunosuppressant drugs that inhibit or prevent cellular and humoral immunity. They suppress cellular immune responses that suppress hinder T cell proliferation by inhibiting enzymes with the following cytokines: IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-8, and TNF. Glucocorticoids suppress humoral immunity's response and lead to the expression of IL-2 and IL-2 receptors on B cells (Rathee *et al.*, 2012). Applying 10 μg/ml lipopolysaccharide to male CBA/J mice in an in vivo study, Nguyen *et al.* (1990) affirm that the 2-week administration of cyclosporine at a dose of 75 mg/kg decreases the levels of TNFα, which are significantly different from the TNFα level in the control group. They also affirm the inhibition of in vitro TNFα formation by using murine macrophages that are stimulated with lipopolysaccharide and administered with cyclosporine at a dose of 0.001 to 1 g/ml. This inhibition is significantly different from the one exhibited in the control groups (without cyclosporine) (Nguyen *et al.*, 1990). Our research used a small dose of liposomal methylprednisolone palmitate, which also inhibited the formation of TNFα. The resulting TNFα level was significantly

different from the one in the control groups. In this case, such administration is expected to reduce the side effects and toxicity of methylprednisolone palmitate.

4 CONCLUSIONS

A small dose of liposomal methylprednisolone palmitate (L-MPLP) can significantly inhibit both in vivo and in vitro productions of TNFα, as opposed to the control group, i.e., methylprednisolone (MPL).

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