RESEARCH ARTICLE

Effects of artemisinin and hydroxychloroquine on cytokines in experimental sepsis

Büyükcavlak, M.[1*], Duman, I.[2], Eryavuz, O.D.[3], Ünlü, A.[3], Duman, A.[4]

[1]University of Health Sciences, Konya City Hospital, Department of Anesthesiology and Reanimation, 42020, Karatay, Konya, Turkey
[2]Necmettin Erbakan University, Meram Faculty of Medicine, Department of Medical Pharmacology, 42080, Meram, Konya, Turkey
[3]Selcuk University, Faculty of Medicine, Department of Medical Biochemistry, 42130, Selçuklu, Konya, Turkey
[4]Selcuk University, Faculty of Medicine, Department of Anesthesiology and Intensive Care, 42130, Selçuklu, Konya, Turkey
*Corresponding author: mustafabcavlak@gmail.com

ABSTRACT

Pro-and anti-inflammatory cytokines mediate the inflammatory response in sepsis. Therefore, regulation of cytokines with medications in risky situations may protect the patients from sepsis. Hydroxychloroquine and artemisinin are antimalarial drugs with immunomodulatory properties. In this study, we intended to investigate the effects of artemisinin and hydroxychloroquine on the cytokines released during sepsis in the rat model. Twenty-four rats were randomized into four groups. The control group received oral saline, the sepsis group received oral saline and intraperitoneal *lipopolysaccharide toxin (LPS)*, the artemisinin-treated sepsis group received oral 33.33 mg/kg of artemisinin, and the hydroxychloroquine-treated sepsis group received oral 33.33 mg/kg of hydroxychloroquine before *LPS* injection. Three hours later, serum cytokines were measured. An increase was detected in TNF-α, IL-1, and IL-6 levels in the sepsis group compared to the control (p<0.01). Oral pretreatment with artemisinin resulted in significant downregulation only of IL-1 levels (p<0.01). Cytokines IL-1 and IL-6 were significantly downregulated in the serum of LPS-induced rats pretreated with oral hydroxychloroquine than rats with sepsis (p<0.01). Decreases observed in TNF-α and IL-10 levels were insignificant. These results demonstrated that both artemisinin and hydroxychloroquine attenuate the release of pro-inflammatory cytokines three hours after LPS-induced sepsis in rats. A significant decrease was observed in serum IL-1 and IL-6 levels with hydroxychloroquine and IL-1 levels with artemisinin. Based on our findings, we suggest that the therapeutic potential of artemisinin and hydroxychloroquine may be beneficial in preventing cytokine storm during sepsis, and further research is needed to determine the optimal timing of administration.

Keywords: Artemisinin; cytokine; hydroxychloroquine; interleukin; sepsis.

INTRODUCTION

Sepsis is a diverse syndrome induced by the dysregulated host response to an infection. Recent "Sepsis-3 consensus" defines sepsis as life-threatening organ dysfunction resulting from an abnormal host response to infection (Singer *et al*., 2016). The host response involves sustained excessive inflammation, immune suppression, and, a failure to return to normal homeostasis (van der Poll *et al*., 2017). Sepsis is one of the most important causes of mortality in intensive care units. It has seen a significant increase in its incidence in recent years and is expected to increase further.

During sepsis, the host response is complex and involves releasing inflammatory cytokines from leukocytes and endothelial cells. These cytokines induce new cytokine production during the cytokine storm that will eventually cause cell and organ damage (van der Poll *et al*., 2017). When the innate immune system recognizes the pathogen-associated molecular patterns (PAMPs) of the invading pathogen through pattern recognition receptors (PRRs), a complex array of intersecting pathways of the immune system is strongly activated, leading to sepsis. This inflammatory response mediated by pro-inflammatory cytokines, which includes tumor necrosis factor α (TNF-α), interleukin-1 (IL-1), IL-2, IL-6, IL-8, and IL-17, is typically balanced by a counter-response with the release of anti-inflammatory cytokines such as IL-4, IL-10, and IL-13 (Jacobi, 2002). Therefore, TNF-α, IL-1, IL-6, and IL-10 have been widely studied cytokines to evaluate the level of immune response and to diagnose sepsis (Chaudhry *et al*., 2013; Chousterman *et al*., 2017; Raveendran *et al*., 2019). Due to the vital role of cytokines in the pathophysiology of sepsis, numerous anti-inflammatory treatments have been investigated. However, none has been shown to be effective in sepsis (Singer *et al*., 2016).

Hydroxychloroquine (HCQ) and artemisinin (ART) are first-line antimalarial drugs that have saved millions of lives in the malaria-endemic areas of the world. In addition to antimalarial activity, experimental and clinical studies have demonstrated immunomodulatory and anti-inflammatory properties for both drugs and derivatives (Ben-Zvi *et al*., 2012; Hou & Huang, 2016; An *et al*., 2017). Therefore, both antimalarial agents have been proposed as

potential therapeutic agents during the cytokine storm of COVID-19 sepsis (Krishna *et al.*, 2020). However, research on their effectiveness in sepsis is limited (Long *et al.*, 2016; Lu *et al.*, 2019; Zhang *et al.*, 2020). Although both ART artemisinin and HCQ hydroxychloroquine have been studied separately in sepsis, there has yet to be a study investigating both drugs together as a pretreatment. In the present study, we investigated the effects of ART and HCQ on cytokines in an *in vivo* rat model in the early phase of *lipopolysaccharide (LPS)* toxin-induced sepsis.

MATERIAL AND METHODS

Chemicals and reagents
Artemisinin (ART, 361593) and *LPS* (*LPS*, 055:B5, sigma) was purchased from Sigma-Aldrich (St. Louis, MO, USA); Hydroxychloroquine (HCQ, A14208) was obtained from Adooq Bioscience (Irvin, CA, USA). Elisa kits for rat TNF-α (E0764), rat IL-1 (E0107), rat IL-6 (E0135), and rat IL-10 (E0108) were provided by BT LAB (Shanghai, China).

Animals
Twenty-four 15-18 weeks old *Wistar Albino* rats (6 per each experiment) weighing 400-550 g were provided by the Experimental Medicine Application and Research Center of Selcuk University. The study was approved by our Institutional Animal Care and Use Committee (27/2020). Experimental procedures were performed following the Guide for the Care and Use of Laboratory Animals (National Research Council Committee for the Update of the Guide for the and Use of Laboratory, 2011). Rats were housed 3 per cage at 23±2°C under a 12 h/12 h light/dark cycle, with water and food available *ad libitum.*

Study groups
After one week of acclimatization, the animals were randomly divided into four groups of six rats. Control (group 1) received saline by oral gavage one day before and on the day of the experiment. *Intraperitoneal* saline was administered 2 hours after the oral saline on the experiment day. The sepsis group (group 2) received oral saline one day before and on the day of the experiment. *Intraperitoneal* 5 mg/kg of LPS was administered 2 hours after oral saline on the day of the experiment. ART-treated sepsis group (group 3) received oral 33.33 mg/kg of ART (in soybean oil) one day before and on the day of the experiment. *Intraperitoneal* 5 mg/kg of LPS was administered half an hour after oral ART on the day of the experiment. HCQ-treated sepsis group (group 4) received oral 33.33 mg/kg of HCQ (in saline) one day before and on the day of the experiment. *Intraperitoneal* 5 mg/kg of *LPS* was administered 2 hours after oral HCQ on the experiment day. Oral gavages were administered in a 5 ml/kg volume, and the *intraperitoneal* injections were 2 ml/kg.

Blood collection
Three hours after the LPS injection, under *intraperitoneal* 10 mg/kg xylazine hydrochloride and 50mg/kg ketamine anesthesia, blood samples were obtained from the heart, and the rats were sacrificed. The serum was separated by centrifugation for 10 min at 3000 rpm for biochemical analysis and stored at -80°C until assay.

Biochemical analysis
ELISA kits were used to measure cytokines, IL-1 (E0107Ra), IL-6 (E0135Ra), IL-10 (E0108Ra), and TNF-α (E0764Ra). The assays were detected according to the manufacturers' recommended protocol.

Statistical analysis
Statistical analysis was carried out with SPSS 21.0 software (Chicago, IL, USA). The normality of the data was checked with the Shapiro-Wilk normality test, Q-Q graphics, and Box-Plot graphics, and the homogeneity of the group variances was checked with the Levene variance homogeneity test. Data were presented as mean ± standard deviation. In the intergroup comparisons of the markers IL-1 and TNF-α used in the study, Tukey HSD multiple comparisons followed One-Way Analysis of Variance (ANOVA) was The Welch. The F test and the subsequent Games-Howell multiple comparison tests were used in the intergroup comparison of IL-6 and IL-10 (due to the violation of the homogeneity assumption of group variances). Values of $p < 0.05$ were considered significant.

RESULTS

We detected a significant increase in serum cytokine TNF-α, IL-1, and IL-6 levels in the sepsis group three hours after *intraperitoneal* administration (Figure 1A, 1B, 1C). The increase of IL-10 in the sepsis group was nonsignificant (Figure 1D). The increase in pro-inflammatory cytokines (TNF-α, IL-1, and IL-6) was statistically significant compared to the control, confirming the onset of sepsis in *LPS*-treated rats ($p<0.01$).

Artemisinin: Oral pretreatment with ART resulted in significant downregulation of serum IL-1 levels ($p<0.01$) (Figure 1B). The decrease in TNF-α (Figure 1A), IL-6 (Figure 1C), and IL-10 (Figure 1D) levels were statistically insignificant.

Hydroxychloroquine: The results of ELISA illustrated that inflammatory cytokine IL-1 (Figure 1B) and IL-6 (Figure 1C) were significantly downregulated in serum of *LPS*-induced rats pretreated with oral HCQ compared to rats with sepsis ($p<0.01$). Decreases observed in TNF-α (Figure 1A) and IL-10 (Figure 1D) levels were insignificant.
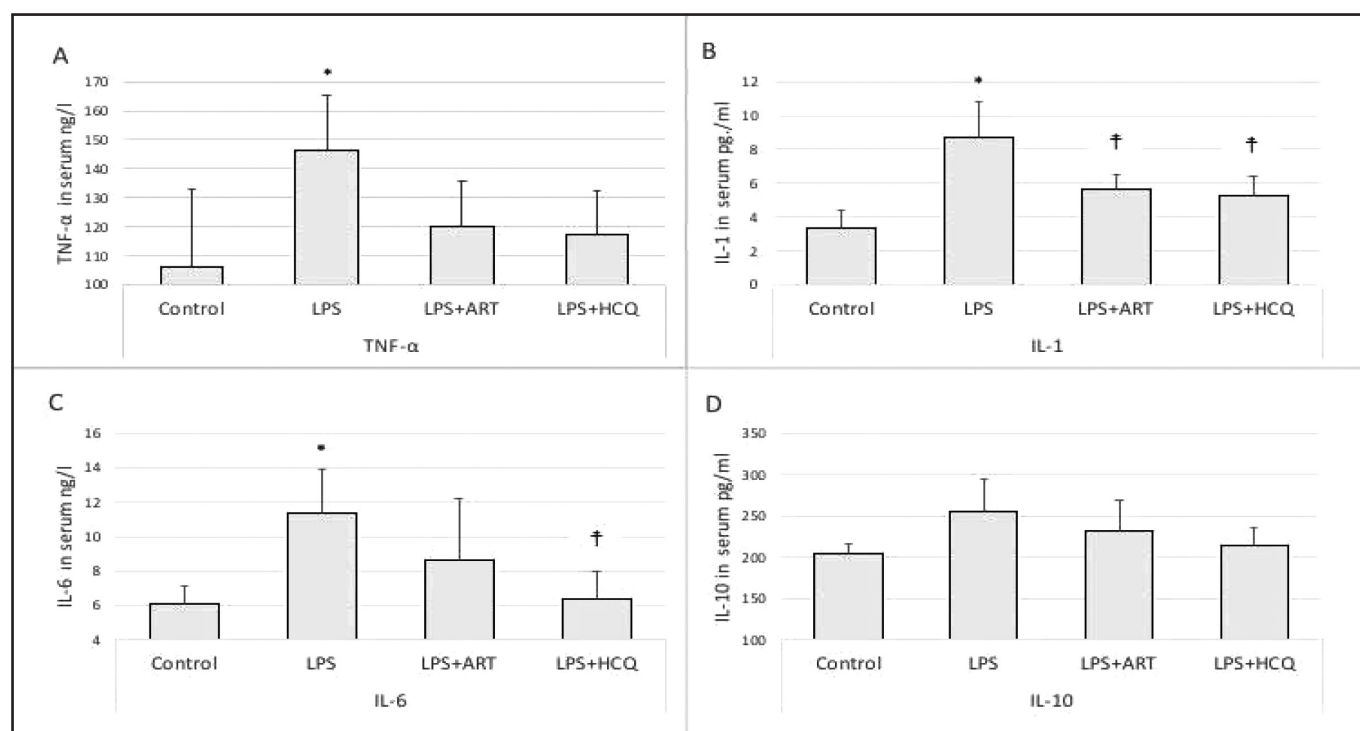
Figure 1. Effects of artemisinin and hydroxychloroquine on LPS induced cytokine production; 1A: TNF-α, 1B: IL-1, 1C: IL-6, and 1D: IL-10. ART: artemisinin, LPS: lipopolysaccharide toxin, Values are mean ± SD (n=6). *p<0.01 vs control, †p<0.01 vs LPS.

DISCUSSION

Our results demonstrate that HCQ and ART inhibit cytokine release in the early stage of sepsis model rats challenged with *intraperitoneal LPS*.

Cytokines are small proteins, including interleukins, tumor necrosis factors, chemokines, interferons, and growth factors. Pro- and anti-inflammatory cytokines are released during infection. IL-1, IL-6, and TNF-α are pro-inflammatory cytokines (Dinarello, 2000; Opal & DePalo, 2000). IL-1 subgroup IL-1 and IL-6 have been shown to correlate with higher mortality in patients with sepsis (Terregino *et al.*, 2000). Recently, IL-6 receptor- neutralizing monoclonal antibody has been approved for the treatment of autoimmune diseases and has also been used to manage severe COVID-19 pneumonia (Toniati *et al.*, 2020). TNF-α is the primary mediator of natural immunity and is the first cytokine to increase during sepsis. However, it rapidly returns to its basal level within hours due to its short half-life (Dinarello, 2000). IL-10 is a cytokine that limits the inflammatory response to infection by reducing the production of pro-inflammatory cytokines. IL-10, the most known anti-inflammatory cytokines, is released in the late stages of infection (Opal & DePalo, 2000).

Antimalarial drugs HCQ and ART may be promising candidates for sepsis treatment. In rodents, *LPS* induces the activation of *NF-κB* and the release of inflammatory cytokines, such as TNF-α, IL-1β, and IL-6 production (Zhao *et al.*, 2017; Zhang *et al.*, 2020). Previous trials have shown that ART family drugs could inhibit inflammatory cytokines and induce anti-inflammatory cytokine production, such as IL-10 (Hou & Huang, 2016). In a previous sepsis model in mice, ART derivative artesunate inhibited *LPS*-induced IL-6 and TNF-α by inhibiting autophagic activation (Kuang *et al.*, 2018). In two mice studies on artesunate not involving sepsis models, artesunate reduced IL-6, IL-1β, and TNF-α (Wan & Li, 2017; Zhang *et al.*, 2020). Previously similar to our study Wang *et al.* (2006) administered ART orally before mice were challenged with LPS. They reported that pretreatment with ART resulted in a higher survival rate. The survival of mice pretreated with 50 mg/kg of ART was similar to higher doses of ART in the first 24 hours after the challenge with *LPS*. This protection was correlated with the ability of ART to inhibit the release of pro-inflammatory cytokines, TNF-α and IL-6. In the present study, different degrees of inhibition were observed in serum levels of IL-1, IL-6, IL-10, and TNF-α, three hours after the LPS challenge. ART resulted in statistically significant downregulation of IL-1 levels, while the decreases in TNF-α, IL-6, and IL-10 were not significant. There are differences in our methodology, which may explain the dissimilarity of our cytokine results. We used different species of animal and the parent drug ART. Unlike previous works with longer sampling times, our blood samples were taken 3 hours after the *intraperitoneal LPS* injection. In rodent models, serum TNF-α and IL-1 levels show significant increases in 2 to 4 hours (Villa *et al.*, 1995). Three hours may not have been enough time to observe a significant change in IL-6 level, which is expected to peak at 4-6 hours after infection and stay in circulation for up to 10 days (Van Snick, 1990).

HCQ has been used in humans for a long time for therapeutic purposes, including malaria and autoimmune diseases. Despite limited *in vitro* and *in vivo* data about the effect of HCQ on cytokines, the safety profile, low cost, and easy availability encouraged HCQ to be used to treat COVID-19 (Wozniacka *et al.*, 2006). In healthy human volunteers, HCQ treatment lowers the level of IL-6 (Van den Borne *et al.*, 1997). Sperber *et al.* (1993) studied the effect of HCQ on cytokine production by human monocytes and T cells. Similar to our results, HCQ inhibited the production of IL-1α and IL-6, but TNF-α production was not affected. They concluded that preferential inhibition of IL-1α production by monocytes and IL-6 production by T cells and monocytes might contribute to its anti-inflammatory effect in autoimmune diseases. Van den Borne *et al.* (1997) compared the *in vitro* effects of HCQ on cytokine production in peripheral blood mononuclear cells. They observed that HCQ inhibited phytohemagglutinin-induced TNF-α and *LPS* induced TNF-α and IL-6 production, while phytohemagglutinin-induced IL-6 production was not affected.

Our study found that pretreatment with HCQ significantly inhibited the serum levels of IL-1 and IL-6 after *intraperitoneal LPS* initiated the inflammatory process. Therefore, it was thought that the lack of significance in the inhibition of TNF-α might be due to a rapid decrease following the rapid increase in TNF-α serum levels.

In the present study, the increase in IL-10 levels was not significant three hours after *intraperitoneal LPS*. However, the selected sample time may need to be longer for IL-10 levels to increase because IL-10 is an anti-inflammatory cytokine secreted in the late period of sepsis. Eventually, we could not show any effect of ART or HCQ on IL-10.

There are limitations to the present study. First, immune responses between species vary considerably. Therefore, it is difficult to extrapolate our *LPS*-induced rat sepsis model to humans, but experimental studies form a base for human research. Second, the production and elimination of cytokines may differ at other time points in the course of sepsis. Third, no agreed time interval defines the early stage of sepsis. Fourth, our design does not include molecular pathways of their anti-inflammatory effects. Finally, our study assesses the effects of a single dose delivered at two points before the challenge, with measurements taken only at the third hour of *LPS*-induced sepsis, which we considered to represent the early phase of experimental sepsis. The reason for assessing a single time point was to avoid sacrificing more animals. Additionally, drugs were administered before the *LPS* injection as a pretreatment in our study. Since medications are generally administered after the onset of symptoms in clinical practice, a future study investigating the ability of the drugs to reduce cytokines after *LPS* injection might provide valuable information.

In conclusion, our results demonstrated that both ART and HCQ attenuate the release of pro-inflammatory cytokines three hours after *LPS*-induced sepsis in rats. Furthermore, the downregulation in serum cytokines is prominent for IL-1 and IL-6 with HCQ and IL-1 with ART. Based on our results, we suggest that the therapeutic potential and optimal time of application of ART and HCQ in preventing cytokine storm during sepsis needs further research.

Conflicts of interest
The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this study.

REFERENCES

An, J., Minie, M., Sasaki, T., Woodward, J.J. & Elkon, K.B. (2017). Antimalarial drugs as immune modulators: new mechanisms for old drugs. *Annual Review of Medicine* **68**: 317-330. https://doi.org/10.1146/annurev-med-043015-123453

Ben-Zvi, I., Kivity, S., Langevitz, P. & Shoenfeld, Y. (2012). Hydroxychloroquine: from malaria to autoimmunity. *Clinical Reviews in Allergy & Immunology* **42**: 145-153. https://doi.org/10.1007/s12016-010-8243-x

Chaudhry, H., Zhou, J., Zhong, Y., Ali, M.M., McGuire, F., Nagarkatti, P.S. & Nagarkatti, M. (2013). Role of cytokines as a double-edged sword in sepsis. *In Vivo* **27**: 669-684.

Chousterman, B.G., Swirski, F.K. & Weber, G.F. (2017). Cytokine storm and sepsis disease pathogenesis. *Seminars in Immunopathology* **39**: 517-528. https://doi.org/10.1007/s00281-017-0639-8

National Research Council (US) Committee for the Update of the Guide for the Care and Use of Laboratory Animals. (2011). Guide for the Care and Use of Laboratory Animals. 8th edition. Washington (DC): National Academies Press (US), pp. 1-220. https://doi.org/10.17226/12910

Dinarello, C.A. (2000). Proinflammatory cytokines. *Chest* **118**: 503-508. https://doi.org/10.1378/chest.118.2.503

Hou, L. & Huang, H. (2016). Immune suppressive properties of artemisinin family drugs. *Pharmacology & Therapeutics* **166**: 123-127. https://doi.org/10.1016/j.pharmthera.2016.07.002

Jacobi, J. (2002). Pathophysiology of sepsis. *American Journal of Health-System Pharmacy* **59**: S3-S8. https://doi.org/10.1093/ajhp/59.suppl_1.S3

Krishna, S., Augustin, Y., Wang, J., Xu, C., Staines, H.M., Platteeuw, H., Kamarulzaman, A., Sall, A. & Kremsner, P. (2020). Repurposing antimalarials to tackle the COVID-19 pandemic. *Trends in Parasitology* **37**: 8-11. https://doi.org/10.1016/j.pt.2020.10.003

Kuang, M., Cen, Y., Qin, R., Shang, S., Zhai, Z., Liu, C., Pan, X. & Zhou, H. (2018). Artesunate attenuates pro-inflammatory cytokine release from macrophages by inhibiting TLR4-mediated autophagic activation via the TRAF6-Beclin1-PI3KC3 pathway. *Cellular Physiology and Biochemistry* **47**: 475-488. https://doi.org/10.1159/000489982

Long, H., Xu, B., Luo, Y. & Luo, K. (2016). Artemisinin protects mice against burn sepsis through inhibiting NLRP3 inflammasome activation. *The American Journal of Emergency Medicine* **34**: 772-777. https://doi.org/10.1016/j.ajem.2015.12.075

Lu, L-H., Chao, C-H. & Yeh, T-M. (2019). Inhibition of autophagy protects against sepsis by concurrently attenuating the cytokine storm and vascular leakage. *Journal of Infection* **78**: 178-186. https://doi.org/10.1016/j.jinf.2018.12.003

Opal, S.M. & DePalo, V.A. (2000). Anti-inflammatory cytokines. *Chest* **117**: 1162-1172. https://doi.org/10.1378/chest.117.4.1162

Raveendran, A.V., Kumar, A. & Gangadharan, S. (2019). Biomarkers and newer laboratory investigations in the diagnosis of sepsis. *Journal of the Royal College of Physicians of Edinburgh* **49**: 207-216. https://doi.org/10.4997/jrcpe.2019.308

Singer, M., Deutschman, C.S., Seymour, C.W., Shankar-Hari, M., Annane, D., Bauer, M., Bellomo, R., Bernard, G.R., Chiche, J.D., Coopersmith, C.M. *et al*. (2016). The third international consensus definitions for sepsis and septic shock (Sepsis-3). *JAMA* **315**: 801-810. https://doi.org/10.1001/jama.2016.0287

Sperber, K., Quraishi, H., Kalb, T.H., Panja, A., Stecher, V. & Mayer, L. (1993). Selective regulation of cytokine secretion by hydroxychloroquine: inhibition of interleukin 1 alpha (IL-1-alpha) and IL-6 in human monocytes and T cells. *The Journal of Rheumatology* **20**: 803-808.

Terregino, C.A., Lopez, B.L., Karras, D.J., Killian, A.J. & Arnold, G.K. (2000). Endogenous mediators in emergency department patients with presumed sepsis: are levels associated with progression to severe sepsis and death? *Annals of Emergency Medicine* **35**: 26-34. https://doi.org/10.1016/S0196-0644(00)70101-6

Toniati, P., Piva, S., Cattalini, M., Garrafa, E., Regola, F., Castelli, F., Franceschini, F., Airo P., Bazzani, C., Beindorf, E.A. *et al*. (2020). Tocilizumab for the treatment of severe COVID-19 pneumonia with hyperinflammatory syndrome and acute respiratory failure: A single center study of 100 patients in brescia, Italy. *Autoimmunity Reviews* **19**: 102568. https://doi.org/10.1016/j.autrev.2020.102568

Van den Borne, B.E., Dijkmans, B.A., De Rooij, H.H., Le Cessie, S. & Verweij, C.L. (1997). Chloroquine and hydroxychloroquine equally affect tumor necrosis factor-alpha, interleukin 6, and interferon-gamma production by peripheral blood mononuclear cells. *The Journal of Rheumatology* **24**: 55-60.

van der Poll, T., van de Veerdonk, F.L., Scicluna, B.P. & Netea, M.G. (2017). The immunopathology of sepsis and potential therapeutic targets. *Nature Reviews Immunology* **17**: 407-420. https://doi.org/10.1038/nri.2017.36

Van Snick, J. (1990). Interleukin-6: an overview. *Annual Review of Immunology* **8**: 253-278.

Villa, P., Sartor, G., Angelini, M., Sironi, M., Conni, M., Gnocchi, P., Isetta, A.M., Grau, G., Buurman, W., Van Tits, L.J.H. *et al*. (1995). Pattern of cytokines and pharmacomodulation in sepsis induced by cecal ligation and puncture compared with that induced by endotoxin. *Clinical and Diagnostic Laboratory Immunology* **2**: 549-553. https://doi.org/10.1128/cdli.2.5.549-553.1995

Wan, R.J. & Li, Y.H. (2017). Effects of Artesunate prevent nephritis via the Toll like receptor 4/nuclear factor κB signaling pathway in rats. *Molecular Medicine Reports* **16**: 6389-6395.
https://doi.org/10.3892/mmr.2017.7362

Wang, J., Zhou, H., Zheng, J., Cheng, J., Liu, W., Ding, G., Wang, L., Luo, P., Lu, Y., Cao, H. *et al*. (2006). The antimalarial artemisinin synergizes with antibiotics to protect against lethal live Escherichia coli challenge by decreasing proinflammatory cytokine release. *Antimicrobial Agents and Chemotherapy* **50**: 2420-2427. https://doi.org/10.1128/AAC.01066-05

Wozniacka, A., Lesiak, A., Narbutt, J., McCauliffe, D.P. & Sysa-Jedrzejowska, A. (2006). Chloroquine treatment influences proinflammatory cytokine levels in systemic lupus erythematosus patients. *Lupus* **15**: 268-275.
https://doi.org/10.1191/0961203306lu2299oa

Zhang, E., Wang, J., Chen, Q., Wang, Z., Li, D., Jiang, N. & Ju, X. (2020). Artesunate ameliorates sepsis-induced acute lung injury by activating the mTOR/AKT/PI3K axis. *Gene* **759**: 144969.
https://doi.org/10.1016/j.gene.2020.144969

Zhao, D., Zhang, J., Xu, G. & Wang, Q. (2017). Artesunate protects LPS-induced acute lung injury by inhibiting TLR4 expression and inducing Nrf2 activation. *Inflammation* **40**: 798-805.
https://doi.org/10.1007/s10753-017-0524-6