Vitamin D and *Tinospora cordifolia* modulate TLR3 and TLR4 pathways, reduce inflammation, and maintain antimicrobial peptide levels in infected mice

Ahmad Shobrun Jamil[1,2], *Sri* Widyarti[3], *Meddy* Setiawan[4], *Muhaimin* Rifa'i[3*],

[1]Student of the Doctoral Program, Department of Biology, Faculty of Mathematics and Natural Sciences, Brawijaya University, Indonesia
[2]Department of Pharmacy, Faculty of Health Sciences, University of Muhammadiyah Malang, Indonesia
[3] Department of Biology, Faculty of Mathematics and Natural Sciences, Brawijaya University, Indonesia
[4]Department of Medicine Education, Faculty of Medicine, University of Muhammadiyah Malang, Indonesia

Abstract. The activation of Toll-Like Receptor-3 (TLR3) and Toll-Like Receptor-4 (TLR4) signalling pathways is a regular pathway for immune system activation during infection. This study aimed to investigate the effects of vitamin D (VD) and *Tinospora cordifolia* ethanol extract (TC) on TLR3 and TLR4 receptor protein expression, proinflammatory cytokine (IL-1 and IL-6) production, and antimicrobial peptide cathelicidin (CAP) production in CD11b+ cells of mice infected with *Escherichia coli*. The treatments consisted of administration of VD (0.325 µg/kg bw), TC (100 mg/kg bw), and a combination of both in the same dose for 28 days, followed by induction of *E. coli* infection on day 29. The flow cytometry method was analyzed of TLR3, TLR4, IL-1, IL-6, and CAP expression in CD11b+ cells of experimental animals. The following measurement results were compared with healthy controls and infected animals with the significance of differences between treatments analyzed by One-way ANOVA with $p < 0.05$. The results showed that administering VD, TC, and a combination of both reduced the expression of TLR3, TLR4, and IL-1 compared to treating infected animals. The combination treatment of VD + TC increased CAP production more than all other treatments. This significant finding suggests that the combination of VD + TC has the potential to control inflammation without disrupting the body's defence mechanisms against infection, providing valuable insights for the field of immunology.

* Corresponding author: immunobiology@ub.ac.id

1 Introduction

Infectious diseases continue to be a global threat due to their significant impact on human health, economy, and societal stability. The COVID-19 pandemic shows how infectious diseases have devastated global society if adequate prevention and treatment strategies are unavailable [1]. The human body has a natural defence system to deal with infectious diseases. The inflammation at the infection site is the body's generic response to overcome infection. When inflammation occurs, immunocompetent cells can rush to the site of infection to eradicate pathogenic microorganisms. However, excessive inflammation can be a new problem in infectious diseases, causing a cytokine storm that damages cells and tissues as a whole [2].

Inflammation can be triggered by the activation of the TLR-3 and TLR-4 pathways due to the entry of particles or microorganism cells into the site of infection in the human body. So, the TLR-3 and TLR-4 pathways are part of the pathways that determine the inflammation process as an initial response to infection. TLR3 is a transmembrane receptor on immunocompetent cells that identifies the presence of viruses at the site of infection, resulting in a robust antiviral response [3]. Meanwhile, TLR4 is activated to identify lipopolysaccharide (LPS), a common component of bacterial cell walls [4]. When TLR4 is triggered, events produce proinflammatory cytokines, which help recruit immune cells to fight and destroy bacteria [5].

However, the inflammatory response triggered by TLR pathway activation needs to be controlled so that hyperinflammation involves significant increases in Interleukine-1 (IL-1) and IL-6, as in the case of Corona Viruses Disease-19 (COVID-19) [6] while maintaining the effectiveness of immune system function in controlling infection. Therefore, efforts are needed to explore and formulate various potential natural ingredients to obtain ideal therapeutic conditions in the case of infections that have the potential to cause hyperinflammation with a combination of natural ingredients that effectively control inflammation while still maintaining its function as an anti-infection. Naturally, the human body has antimicrobial peptides such as cathelicidin (CAP) that can increase the immune system's effectiveness in controlling infections [7]. Cathelicidin has pleiotropic properties [8], which, on the one hand, can modulate the immune system so that immunocompetent cells immediately react to ward off infection. Still, in certain circumstances, it also has anti-inflammatory properties, so its existence can be used to prevent cases of hyperinflammation [8].

Extracts of several plant species are known to have activities that can interfere with TLR signaling pathways, giving new hope in developing hyperinflammatory case management strategies. Plant extracts *Castanea sativa*, *Cinchona pubescens*, and *Cinnamomum verum* can decrease proinflammatory cytokines in THP-1 monocyte cells and TLR4-transfected HeLa cells via affecting TLR2 and TLR4. [9]. Additionally, extracts of *Mentha pulegium* L. It lowers the levels of proinflammatory cytokines, proteins, and transcription factors in LPS-stimulated PBMCs. The extract dramatically lowered mRNA levels of Tumor Necrosis Factor alpha (TNF-α), IL-1β, IL-6, TLR4, inducible nitric oxide synthase (iNOS), and Nuclear Factor Kappa Beta (NFKB), as well as protein levels of TNFα, IL-1β, and TLR4. [10]. Research on *Hyssopus officinalis* L. extract activates endosomal TLRs (TLR3, TLR7, TLR8, and TLR9) and increases MyD88 and NFKB expression in PBMCs, leading to antiviral innate immune responses [11].

Tinospora is a genus of plants widely used in Southeast Asia; in Indonesia, this plant is traditionally used as an anti-diabetes, malaria fever, and heart disease [12]. Related to anti-inflammatory activities, *Tinospora cordifolia* reduces TNF-α, TGF-β proinflammatory cytokines, and TLR4 receptor expression levels in *in-vivo* testing [13]. It also reduces the proinflammatory cytokines IL-1 and IL-6 [13]. This plant, extensively distributed throughout

Southeast Asia, particularly in Indonesia, can be an anti-inflammatory medication ingredient. In addition to controlling inflammation of the TLR3 and TLR4 pathways by utilizing plant extracts, efforts to optimize the immune system through the administration of drug formulas need to be supplemented with efforts to maximize the levels of antimicrobial peptides in the body, which play an essential role as a first line barrier against infection. Vitamin D is known today as a substance that effectively increases the expression of cathelicidin through the vitamin D receptor pathway that activates transcription factors that trigger an increase in cathelicidin expression/production in its producing cells [14–16]. However, there is still little information to show how combining vitamin D and plant extracts can control the expression of TLR3, TLR4, IL-1, and IL-6 and may increase cathelicidin production. This research was conducted to fill the information gap related to the effectiveness of combinations of *T. cordifolia* and vitamin D in controlling inflammation via the reduction of the expression of TLR3, TLR4 receptor proteins, the proinflammatory cytokines IL-1 and IL-6 and its effect in increasing cathelicidin production, especially in one of the subsets of immunocompetent cells with CD11b markers commonly possessed by macrophages.

The modulation of TLR3 and TLR4 pathways by vitamin D and *Tinospora cordifolia* (TC) has important clinical implications for controlling inflammation and enhancing immune response in infections [17, 18]. These two ingredients demonstrated their potential to regulate proinflammatory cytokines and maintain antimicrobial peptide levels, which are critical for practical immune function. The results of this research are relevant in developing new therapeutic strategies that can improve the treatment of infections and autoimmune diseases, exploiting the synergistic benefits between nutrition and herbs to support the immune system. Further research is needed to understand the mechanisms underlying the interactions between vitamin D, *Tinospora cordifolia*, and the TLR pathway and how this combination can be optimized in clinical practice.

2 Material and Method

2.1 Antibodies

PE- Anti-CAP-18 Antibody (G-1) (Santa Cruz; sc-166055 PE), FITC anti-mouse/human CD11b (BioLegend; clone: M1/70,), PerCP anti-mouse/human IL-1(LSBio; clone: 11n92,), PerCP anti mouse IL-6 (AP-MAB0847), PE anti-mouse CD283(TLR3) (BioLegend; clone: 11F8,), PE/Cy7 anti-mouse CD284 (TLR4) (BioLegend; clone SA15-21).

2.2 Plant identification

Tinospora cordifolia (stem and leaf parts) was obtained from and identified by UPT Balai Materia Medika, Batu City, East Java, Indonesia, identification letter number 074/540/1-2.20-A/2022.

2.3 Ethical clearance

The Health Research Ethics Committee of the University of Muhammadiyah Malang granted ethical approval for the experimental animal treatment process under approval number E.5a/254/KEPKUMM/XII/2022.

2.4 Preparation of Vitamin D and *Tinospora cordifolia* extract

The liquid dose form of Blackmores Vitamin D3 1000 IU contains vitamin D (cholecalciferol). The herb Simplicia of Tinospora cordifolia (TC) was acquired and examined at UPT Balai Materia Medika in Batu City, East Java, Indonesia. The simplicia was crushed into a powder and macerated in a 1:3 (w/v) solution of 96% ethanol for three days. After the macerate was filtered, a rotary evaporator concentrated the filtrate until it reached a steady weight. After that, TC was kept in a refrigerator at 4^{o}C until it needed to be utilized.

2.5 Experimental animals

The study employed female BALB/c mice obtained from the Malang Wistar Farm in the Dau District of Malang, East Java, Indonesia. In cages with controlled conditions, 25 healthy female BALB/c mice weighing between 20 and 25 g and between 6 and 8 weeks of age were housed. Throughout the experiment, they had unrestricted access to water and a daily pellet meal. The experimental animal was divided into five groups:

Untreated (UT)	: healthy mice without *E. coli* injection
E. coli group	: *E. coli* injection on the 29th without additional treatment
Vitamin D (VD) group	: vitamin D 0.325 µg/kg body weight every day for 28 days + *E. coli* injection on the day 29th
Tinospora cordifolia (TC)	: TC extract 100 mg/kg body weight every day for 28 days + *E. coli* injection on the day 29th
VD + TC group	: vitamin D 0.325 µg/kg bw + TC extract 100 mg/kg bw daily for 28 days + *E. coli* injection on the day 29th

The rationalization of TC dosage is based on Tiwari's research [19], which gave TC extract to mice at a dose of 100 mg/kg body weight. Waqas Ahmad's research [20] gave *T. crispa* extract (one genus with TC) at a concentration of 100, 200, and 300 mg/kg body weight, and it appears that the 100 mg/kg body weight treatment has affected several parameters of the innate immune response test in Wistar Kyoto rats. The dose of vitamin D with 0.325 µg/kg body weight of mice results from calculating the effective dose conversion for humans of 1000 IU/day [21].

2.6 Immunofluorescent staining and flow cytometry

Mice treated for 28 days were fasted on the 29th day but were still provided with water to drink, injected intraperitoneally with *E. coli* suspension 0.1 ml concentration of cells was 10^6 CFU/ml and then sacrificed with neck dislocation after six hours injection. After collecting spleen samples, they were cleaned with sterile PBS and crushed in a cold mortar until a single-cell solution was thought to have been obtained. This prepared single-cell suspension conforms to standard protocols [22]. At 10°C, the single-cell suspension was centrifuged for five minutes at 2500 rpm. After removing the supernatant, FITC anti-mouse/human CD11b was used to stain the pellet (BioLegend; clone: M1/70) for extra cell dye and CD11b cell subset markers. Cells were then fixated with cytofix/cytoperm buffer (BD-Biosciences, Pharmingen) and washed with buffer. To observe the expression of cytokines (IL-1, IL-6, TLR3, TLR4) and cathelicidin (CAP), cells were then stained with anti-mouse/human PerCP antibodies IL-1 (clone: 11n92, LSBio), anti-mouse PerCP IL-6 (AP-MAB0847), anti-mouse PE, anti-mouse PE CD283(TLR3) (clone: 11F8, BioLegend), PE/Cy7 anti-mouse CD284 (TLR4) (clone SA15-21, BioLegend), and PE-Anti-CAP-18 Antibody (G-1) (SANTA CRUZ

sc-166055 PE). Following that, the cells are incubated for 30 minutes at 4oC. The FACS CantoIITM instrument (BD-Biosciences, San Jose, CA) was then used to perform flow cytometry analysis on the stained cell samples. The FlowJo v10 for Windows program (FlowJo LLC, Ashland, OR) was then used to evaluate the flow cytometry data.

2.7 Statistical analysis

The average results ± standard deviation (SD) are the results that are reported. The demonstrated data is percentage data, statistically analyzed using SPSS for Windows (Version 23.0) using a one-way analysis of variance (ANOVA). The significant data was later analyzed using the Tukey HSD post hoc test. A statistically significant outcome is one where P is less than 0.05.

3 Result and discussion

3.1 The administration of Vitamin D and *T. cordifolia* extract decreased the expression of $CD11b^+TLR3^+$ and $CD11b^+TLR4^+$ in the infected group

TLR3 and TLR4 are two immunocompetent cell receptors, including a subset of CD11b cells that recognize pathogens through LPS material and double-stranded RNA. The treatment of infection in mice made the expression of TLR3 in CD11b cells and TLR4 in the CD11 cells higher than that of the standard group. Interestingly, the administration of vitamin D (VD) treatment and extracts of *T. cordifolia* (TC) in mice for 28 days made the expression of TLR3 and TLR4 higher than that of the regular group but not as high as the treatment of infection without the administration of VD and TC (Figures 1A and 1B). Another interesting result was that the combination of VD and TC administration did not make $CD11b^+TLR3^+$ and $CD11b^+TLR4^+$ expression higher or lower than that of VD and TC administration alone.

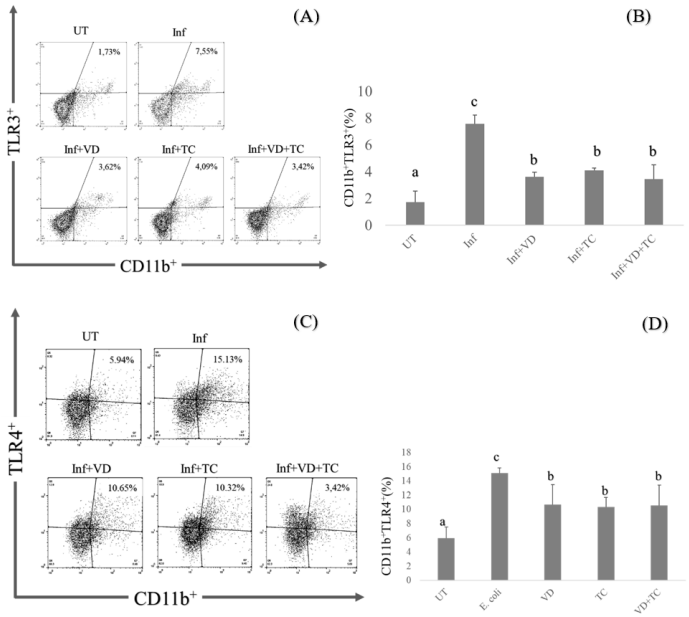

Fig 1. The TLR3 and TLR4 expression profile in CD11b cells following treatment. (A) Analysis of dot plots of $CD11b^+TLR3^+$ cells using flow cytometry. (B) Comparative graph of TLR3 expression

in CD11b cells (C) Dot plot analysis of TLR4 expression in CD11b cells by flow cytometry. (D) TLR4 comparison graph in CD11b cells. Statistical analysis was performed using one-way ANOVA, followed by Post Hoc, and Tukey's HSD tests with $p < 0.05$ are significantly different results. The real difference is expressed with other letters.

3.2 Administration of VD and TC results in lower production of IL1 and IL6 in LPS-induced CD11b$^+$ cells

Both IL-1 and IL-6 are proinflammatory cytokines, yet IL-6 can also be anti-inflammatory in certain conditions. The infection treatment increased the production of IL-1 and IL-6 compared to the standard group. IL-1 in the treatment of VD, TC, and VD+TC was lower than that of the infection group and had the same level as the standard group. Interestingly, in IL-6, it is known that VD treatment lowers its expression than that of the infection group, and this is not the case in the TC group and the VD+TC combination. IL-6 expression in the TC and VD+TC groups did not differ statistically significantly from the infection group and was higher than that of the standard group (Figures 2A and 2B).

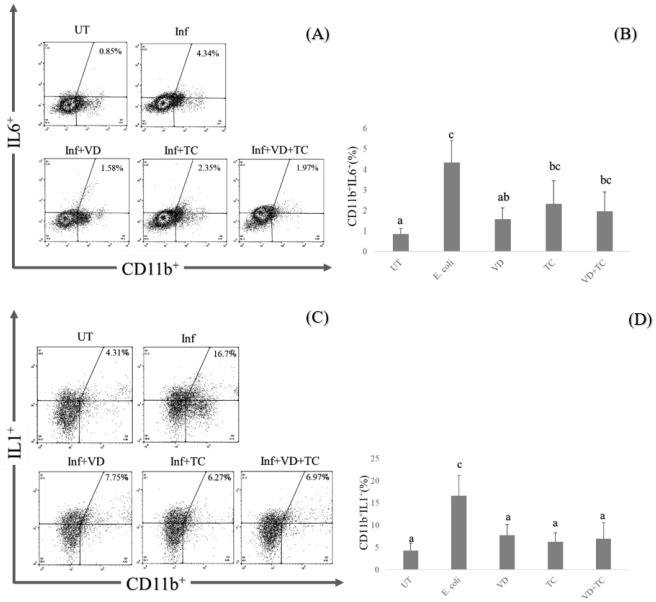

Fig 2. Expression of IL6 and IL1 in CD11b cells. (A) Analysis of dot plots of CD11b$^+$IL6$^+$ cells using flow cytometry. (B) Comparative graph of IL6 expression in CD11b cells (C) Analysis of dot plots of CD11b+IL1+ cells using flow cytometry. (D) IL1 comparison graph in CD11b cells±. Statistical analysis included one-way ANOVA, Post Hoc, and Tukey's HSD tests, with p-values < 0.05 indicating significant differences. The significant difference is stated using different letters.

3.3 Combination of VD and TC administration makes CAP expression higher

Cathelicidin (CAP) is an antimicrobial peptide produced by several cells that acts as a first-line barrier in the immune system. Our results showed that the infection treatment did not appear to make CAP expression higher than the standard group and the administration of VD and TC (Figure 3). Interestingly, the combination of VD+TC was statistically significant, making CAP expression higher than that of the standard group.

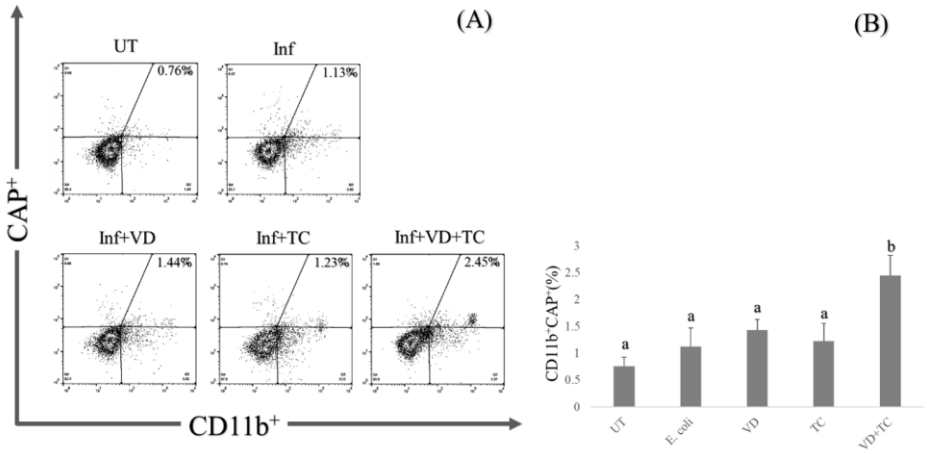

Fig 3. CAP expression in CD11b cells. (A) Analysis of dot plots of CD11b+CAP$^+$ cells using flow cytometry. (B) CAP comparison graph in CD11b cells±. Statistical analysis was done based on one-way ANOVA followed by the Post Hoc and Tukey's HSD tests. $p < 0.05$ is a significantly different result. The real difference is expressed with other letters.

4 Discussion

TLR pathway activation is a key phase that activates immunocompetent cells' immunological response pathways to activate NFKB and create proinflammatory cytokines, including IL-1 and IL-6 [23]. This process will then proceed with translocating other immunocompetent cells at the location of infection and generating antimicrobial peptides such as CAP in these cells [24]. There is a process of elimination of antigens and pathogenic microbes that infect until the body returns to normal. TLR3 and TLR4 are two critical receptors in CD11b macrophage cells that play a role in recognizing viral and bacterial infections. TLR3 recognizes double-stranded RNA, common to viruses, while TLR4 recognizes the presence of LPS, a component of bacterial cell walls [3]. CD11b cells, generally a subset of macrophage cells, are one of the subsets of cells expressing both receptors [4].

Decreased expression of TLR3 and TLR4 can significantly impact the regulation of inflammatory responses in the body, as shown in Figure 1 and Figure 2. TLR3 and TLR4 are essential parts of the innate immune system that detect infections and release proinflammatory cytokines, including IL-1 and IL-6 [25]. According to the findings of this study, when TLR3 and TLR4 expression declines, it also affects the activation of signaling pathways related to the inflammatory response. This is because TLR activation will initiate a signaling pathway that promotes the secretion of proinflammatory cytokines such as TNF-α, IL-6, and IL-1b [26]. So, inhibition of TLR expression causes a reduced inflammatory response [27]. TLR4 activation by LPS will trigger the upregulation of proinflammatory cytokines by activating transcription factors NFKB and AP-1 [28]. The results of this study indicate that VD, TC, and their combination treatments can reduce the expression of TLR3 and TLR4 (Figure 1), thereby reducing the level of proinflammatory cytokine expression (Figure 2).

Based on the present study's findings, TC and VD can lower TLR3 and TLR4 expression compared to the infection group. The treatment of administering VD, TC, and a combination of the two for 28 days was followed by infection induction *of E. coli* on the 29th day. The results indicated that TRL3 and TLR4 expression was lower than in the infection group that did not receive VD, TC, or VD+TC. As previously studied, vitamin D reduces TLR3

expression [21]. Other results also show that TC can decrease TLR4 expression [29]. The results of these studies were confirmed in this study where with a dose of vitamin D of 0.325 micrograms/ml and TC dose of 100 mg/ml, it was possible to change the expression of TLR3 and TLR4 to a lower level either in the administration of VD or TC alone or when both were given together (Figure 1A-D).

According to our research, TC and VD can change IL-1 and IL-6 production to decrease to that of the infection group. This is demonstrated by the dot plot flow cytometry analysis results, which showed that the expression of IL-1 and IL-6 by $CD11b^+$ cells in the VD, TC, and VD+ETC treatment groups was statistically significantly smaller than that of group E. coli. Meanwhile, the VD, ETC treatment, and the combination of the two statistically had the same IL-1 expression level as the control (healthy animals) (Figure 2C-D) and IL-6, which was closer to the expression level in healthy animals (Figure 2A-B). TC is known by Philip et al. [24], and it can decrease the expression of these inflammatory genes. The same thing was obtained from the results of other studies that vitamin D also has an anti-inflammatory effect by reducing the expression of proinflammatory proteins such as IL-1 and IL-6. The decrease in inflammatory cytokines occurs because Vitamin D activity inhibits the differentiation of M1 cells that produce proinflammatory cytokines and stimulates the multiplication of differentiation of M2 cells that produce anti-inflammatory cytokines such as IL-10 [30].

TC is known as a plant with anti-inflammatory activity, as several other identified plant extracts also play an important role in the immune response, especially anti-inflammatory. One of them is known that Glycine max extract elicited by Saccharomyces cerevisiae reduces the expression of TLR3 and TLR4 in CD11b cells [22]. Pristiawan's [31] research shows that propolis ethanol extract also reduces the expression of TLR3 and TLR4 in B cells. Apart from the two studies above, not many researchers have revealed the effect of plant extracts on the expression of TLR3 and TLR4 in immunocompetent cells. However, various research results link TLR pathway activity with anti-inflammatory effects. Among them, it is stated that methanol extract from *Caragana rosea* is known to inhibit TLR4 signaling significantly. Inhibition of TLR4 signaling is known to be influenced by suppressing the NFKB and IRF3 pathways, which causes a decrease in proinflammatory cytokines such as TNF-α and Il-6 [32]. Cannabinoids contained in plant extracts, especially Δ9-tetrahydrocannabinol (THC) and cannabidiol (CBD), have been shown to modulate TLR3 and TLR4 signaling in immunocompetent cells. Combining the two compounds can effectively reduce inflammation induced by TLR3 activation [33]. The xanthone-rich fraction of *Securidaca appendiculate* has been identified as a selective TLR4 inhibitor, showing significant anti-inflammatory effects by inhibiting TLR4 dimerization and reducing NF-κB activity [34].

This study also showed that although IL-1 and IL-6 were lower in production, they did not affect the production of CAP antimicrobial peptides (Figure 3A-B). Administration of VD and TC to animals acutely infected with *E. coli* It was found that it could make $CD11b^+TLR3^+$ and $CD11b^+TLR4^+$ expression lower than that of the infection group (Figure 1A-D). The lower expression of TLR affects the production of IL-1 and IL-6. Our results showed lower expression of TLR3 and TLR4 in the VD, TC, and VD+TC groups, followed by lower expression in IL-1 and IL-6 (Figure 2A-D). This result is reinforced by several related studies that state that decreased TLR expression and activity leads to reduced expression of proinflammatory cytokines such as IL-1 and IL-6 [35, 36]. However, the low expression of TLR3, TLR4, IL-1, and IL-6 in this study did not directly impact the production of CAP antimicrobial peptide in CD11b cells. This is because, in addition to the CAP biosynthesis pathway, there is another pathway, namely the direct pathway, that activates the Vitamin D receptor (VDR) [30]. However, further research on the molecular mechanism of combination activity between VD and TC needs to be carried out to determine the factors that make the two substances able to synergistically increase CAP production in CD11b cells,

as shown in Figure 3. The CAP biosynthesis pathway and proinflammatory cytokines such as TNF-α, IL-1, and IL-6 are predicted to partially converge on NFKB and AP-1 as transcription factors [37]. This makes sense because CAP biosynthesis is often associated with increased proinflammatory cytokines. However, it is also known that the CAP biosynthesis pathway also goes through the activation of the vitamin D receptor (VDR) [15] and the endoplasmic reticulum stress induction pathway [38]. So, there is a non-inflammatory pathway in CAP biosynthesis. This allows for synergistic action for the combination of VD + TC treatment in CAP production and in controlling inflammation.

5 Conclusion

This study concludes that the administration of vitamin D (VD) and Tinospora cordifolia extract (TC) effectively lowers the expression of Toll-like receptor-3 (TLR3) and Toll-like receptor-4 (TLR4) compared to infected animals in their $CD11b^{+}$ cells. Decreased expression of TLR3 and TLR4 causes lower production of proinflammatory cytokines, Interleukin-1 (IL-1), and the same trend in IL-6, although not yet statistically significant. These results indicate the potential of combining VD and TC ingredients in controlling excessive inflammation. Another interesting result is that the combination of VD and TC does not interfere with the production of the antimicrobial peptide Cathelicidin (CAP), indicating that the combination treatment of VD+TC can modulate the inflammatory response without reducing the function of the immune system to fight infection, especially in the aspect of the production of the antimicrobial peptide cathelicidin. These findings indicate the potential of VD and TC as candidate therapeutic agents that can be formulated as pharmaceutical preparations to manage inflammation and improve immune function in infectious diseases.

We extend our gratitude to the Education Fund Management Agency (Lembaga Pengelola Dana Pendidikan/LPDP) of the Ministry of Finance, Republic of Indonesia, for generously funding this research under grant number 0001004/IPA/D/PDD-2020.

References

[1] W.J. Metsemakers, H.C. van der Mei, R.G. Richards, T.F. Moriarty, Editorial – infectious-disease research during a pandemic: The importance of global unity. Eur. Cells Mater. **42**, 154–155 (2021). doi: 10.22203/eCM.v042a11.

[2] S. Chen, W.Ye, W. Zheng, Z. Xu, Y. Chen, L. Jin, Increased serum antimicrobial peptide LL-37 and HBD-2 combined with 25-hydroxyvitamin D3 deficiency in infants with pertussis. J. Infect. Dev. Ctries. **14**, 1164–1169 (2020). doi: 10.3855/jidc.12317.

[3] T. Karrasch, A. Höpfinger, A. Schäffler, A. Schmid, The adipokine C1q/TNF-related protein-3 (CTRP-3) inhibits Toll-like receptor (TLR)-induced expression of Cathelicidin antimicrobial peptide (CAMP) in adipocytes. Cytokine. **148**, 155663 (2021). doi: 10.1016/j.cyto.2021.155663.

[4] M.R.E. Wuryandari, M.F. Atho'illah, R.D. Laili et al., *Lactobacillus plantarum* FNCC 0137 fermented red *Moringa oleifera* exhibits protective effects in mice challenged with *Salmonella typhi* via TLR3/TLR4 inhibition and down-regulation of proinflammatory cytokines. J. Ayurveda Integr. Med. **13**, 100531 (2022). doi: 10.1016/j.jaim.2021.10.003.

[5] J.F. Burgueño, A. Barba, E. Eyre, C. Romero, M. Neunlist, E. Fernández, TLR2 and TLR9 modulate enteric nervous system inflammatory responses to lipopolysaccharide. J. Neuroinflammation. **13**, 187 (2016). doi: 10.1186/s12974-016-0653-0.

[6] E. Purwanti, F.E. Hermanto, J.W. Souhaly, W. Prihanta, T.I. Permana, Exploring public health benefits of Dolichos lablab as a dietary supplement during the COVID-19 outbreak: A computational study. J. Appl. Pharm. Sci. **11**, 135–140 (2021). doi: 10.7324/JAPS.2021.110217.

[7] R.M. van Harten, E. van Woudenbergh, A. van Dijk, H.P. Haagsman, Cathelicidins: Immunomodulatory antimicrobials. Vaccines. **6**, (2018). doi: 10.3390/vaccines6030063.

[8] D. Xhindoli, S. Pacor, M. Benincasa, M. Scocchi, R. Gennaro, A. Tossi, The human cathelicidin LL-37 - A pore-forming antibacterial peptide and host-cell modulator. Biochim. Biophys. Acta - Biomembr. **1858**, 546–566 (2016). doi: 10.1016/j.bbamem.2015.11.003.

[9] A. Schink, J. Neumann, A.L. Leifke et al., Screening of herbal extracts for TLR2-and TLR4-dependent anti-inflammatory effects. PLoS One. **13**, 1–27 (2018). doi: 10.1371/journal.pone.0203907.

[10] F. Mohammadi, K. Rahimi, A. Ahmadi, Z. Hooshmandi, S. Amini, A. Mohammadi, Anti-inflammatory effects of *Mentha pulegium* L. extract on human peripheral blood mononuclear cells are mediated by TLR-4 and NF-κB suppression. Heliyon. **10**, e24040 (2024). doi: 10.1016/j.heliyon.2024.e24040.

[11] M. Ghasempour, M. Hosseini, M.S. Soltani-Zangbar et al., The impact of Hyssop (*Hyssopus officinalis*) extract on activation of endosomal toll like receptors and their downstream signaling pathways. BMC Res. Notes. **15**, 1–7 (2022). doi: 10.1186/s13104-022-06253-3.

[12] D. Pandiangan, M. Silalahi, F. Dapas, F. Kandou, Diversity of medicinal plants and their uses by the Sanger tribe of Sangihe Islands, North Sulawesi, Indonesia. Biodiversitas. **20**, 621–631 (2019). doi: 10.13057/biodiv/d200301.

[13] A. Parveen, S. Zahiruddin, N. Agarwal, M.A. Siddiqui, S.H. Ansari, S. Ahmad, Modulating effects of the synergistic combination of extracts of herbal drugs on cyclophosphamide-induced immunosuppressed mice. Saudi J. Biol. Sci. **28**, 6178–6190 (2021). doi: 10.1016/j.sjbs.2021.06.076.

[14] W.J. Lee, H.W. Cha, M.Y. Sohn, S.J. Lee, D.W. Kim, Vitamin D increases expression of cathelicidin in cultured sebocytes. Arch. Dermatol. Res. **304**, 627–632 (2012). doi: 10.1007/s00403-012-1255-z.

[15] M.B. Lowry, C. Guo, Y. Zhang et al., A mouse model for vitamin D-induced human cathelicidin antimicrobial peptide gene expression. J. Steroid Biochem. Mol. Biol. **198**, 105552 (2020). doi: 10.1016/j.jsbmb.2019.105552.

[16] M. S. Gönen, M. Alaylıoğlu, E. Durcan et al., Rapid and effective vitamin D supplementation may present better clinical outcomes in COVID-19 (SARS-CoV-2) patients by altering serum INOS1, IL1B, IFNg, Cathelicidin-LL37, and ICAM1. Nutrients. **13**, 1–19 (2021). doi: 10.3390/nu13114047.

[17] F.N. Hamza, S. Daher, H.M.A. Fakhoury, W.B. Grant, P.R. Kvietys, K. Al-Kattan, Immunomodulatory properties of vitamin D in the intestinal and respiratory systems. Nutrients. **15** (2023). doi: 10.3390/nu15071696.

[18] J. Jacob, B.M. Babu, M.C. Mohan, A.P. Abhimannue, B.P. Kumar, Inhibition of proinflammatory pathways by bioactive fraction of *Tinospora cordifolia*. Inflammopharmacology. **26**, 531–538 (2018). doi: 10.1007/s10787-017-0319-2.

[19] P. Tiwari, S. A. Ali, B. Puri, A. Kumar, A.K. Datusalia, *Tinospora cordifolia* Miers enhances the immune response in mice immunized with JEV-vaccine: A network pharmacology and experimental approach. Phytomedicine. **119**, 1–11 (2023). doi:

10.1016/j.phymed.2023.154976.

[20] W. Ahmad, I. Jantan, E. Kumolosasi, S.N.A. Bukhari, Immunostimulatory effects of the standardized extract of *Tinospora crispa* on innate immune responses in Wistar Kyoto rats. Drug Des. Devel. Ther. **9**, 2961–2973 (2015). doi: 10.2147/DDDT.S85405.

[21] J. Martínez-Moreno, J.C. Hernandez, S. Urcuqui-Inchima, Effect of high doses of vitamin D supplementation on dengue virus replication, Toll-like receptor expression, and cytokine profiles on dendritic cells. Mol. Cell. Biochem. **464**, 169–180 (2020). doi: 10.1007/s11010-019-03658-w.

[22] M.F. Atho'illah, Y.D. Safitri, F. . Nur'aini, S. Widyarti, H. Tsuboi, M. Rifa'i, Elicited soybean extract attenuates proinflammatory cytokines expression by modulating TLR3/TLR4 activation in high−fat, high−fructose diet mice. J. Ayurveda Integr. Med. **12**, 43–51 (2021). doi: 10.1016/j.jaim.2021.01.003.

[23] S.R. Pakadang, S. Ratnah, A.M. Salasa, Jumain, M. Hatta, Toll Like Receptor 4 expression profile in mice infected *Mycobacterium tuberculosis* given with miana leaves extract (*Coleus scutellarioides* (L.) Benth) (tuberculosis preventive and curative mechanisms). Pharmacogn. J. **14**, 497–505 (2022). doi: 10.5530/pj.2022.14.63.

[24] A. Padhi, K. Pattnaik, M. Biswas, M. Jagadeb, A. Behera, A. Sonawane, *Mycobacterium tuberculosis* LprE suppresses TLR2-dependent cathelicidin and autophagy expression to enhance bacterial survival in macrophages. J. Immunol. **203**, 2665–2678 (2019). doi: 10.4049/jimmunol.1801301.

[25] M.S. Diamond, T.D. Kanneganti, Innate immunity: the first line of defense against SARS-CoV-2. Nat. Immunol. **23**, 165–176 (2022). doi: 10.1038/s41590-021-01091-0.

[26] A. Stierschneider, C. Wiesner, Shedding light on the molecular and regulatory mechanisms of TLR4 signaling in endothelial cells under physiological and inflamed conditions. Front. Immunol. **14**, 1–15 (2023). doi: 10.3389/fimmu.2023.1264889.

[27] J. Zi, F. Wang, Z. Liu et al., Impact of Toll-like Receptor 4 expression on inflammatory responses related to premature membrane rupture induced by lipopolysaccharide. Discov. Med. **35**, 429 (2023). doi: 10.24976/discov.med.202335176.43.

[28] H.J. Kim, H. Kim, J.H. Lee, C. Hwangbo, Toll-Like Receptor 4 (TLR4): new insight immune and aging. Immun. Ageing. **20**, 1–11 (2023). doi: 10.1186/s12979-023-00383-3.

[29] N.S. Ghatpande, A.V. Misar, R.J. Waghole, S.H. Jadhav, P.P. Kulkarni, *Tinospora cordifolia* protects against inflammation associated anemia by modulating inflammatory cytokines and hepcidin expression in male Wistar rats. Sci. Rep. **9**, 1–11 (2019). doi: 10.1038/s41598-019-47458-0.

[30] L. Malaguarnera, Vitamin D3 as potential treatment adjuncts for COVID-19. Nutrients. **12**, 1–19 (2020). doi: doi:10.3390/nu12113512.

[31] B. Pristiwanto, A. Soewondho, S.B. Sumitro, M. Rifa'i, Inhibition of toll-like receptor 3-4 with ethanolic extract of propolis on innate immunity in diabetes mellitus mice. AIP Conf. Proceeding. **1844**, 020008 (2017). doi: 10.1063/1.4983419.

[32] A. Mitra *et al.*, *Caragana rosea* Turcz methanol extract inhibits lipopolysaccharide-induced inflammatory responses by suppressing the TLR4/NF-κB/IRF3 Signaling pathways. Molecules. **26**, 1–23 (2021).

[33] J. Fitzpatrick, B. Hackett, L. Costelloe, W. Hind, E.J. Downer, Botanically-derived Δ9-tetrahydrocannabinol and cannabidiol, and their 1:1 combination, modulate toll-like receptor 3 and 4 signalling in immune cells from people with multiple sclerosis. Molecules. **27**, 1–22 (2022).

[34] M. Tao, C. Ji, Y. Wu, J. Dong, Y. Li, O.J. Olatunji, 1,7-dihydroxy-3,4-dimethoxyxanthone inhibits lipopolysaccharide-induced inflammation in RAW264.7 macrophages by suppressing TLR4 / NF- κ B signaling cascades. Inflammation. **43**, 1821–1831 (2020). https://doi.org/10.1007/s10753-020-01256-3.

[35] M. Cutolo, R. Campitiello, E. Gotelli, S. Soldano, The Role of M1/M2 macrophage polarization in rheumatoid arthritis synovitis. Front. Immunol. **13**, 1–14 (2022). doi: 10.3389/fimmu.2022.867260.

[36] E. Verweyen, D. Holzinger, T. Weinhage, et al., Synergistic signaling of TLR and IFNα/β facilitates escape of IL-18 expression from endotoxin tolerance. Am. J. Respir. Crit. Care Med. **201**, 526–539 (2020). doi: 10.1164/rccm.201903-0659OC.

[37] G. Li, J. Domenico, Y. Jia, J.J. Lucas, E.W. Gelfand, NF-κB-dependent induction of cathelicidin-related antimicrobial peptide in murine mast cells by lipopolysaccharide. Int. Arch. Allergy Immunol. **150**, 122–132 (2009). doi: 10.1159/000218115.

[38] K. Park, P.M. Elias, Y. Oda et al., Regulation of cathelicidin antimicrobial peptide expression by an Endoplasmic Reticulum (ER) stress signaling, vitamin D receptor-independent pathway. J. Biol. Chem. **286**, 34121–34130 (2011). doi: 10.1074/jbc.M111.250431.