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Vitamin D and Tinospora cordifolia Modulate TLR3 and TLR4 Pathways Reduce Inflammation and Maintain Antimicrobial Peptide Levels in Infected Mice

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Abstract. The activation of Toll-Like Receptor-3 (TLR3) and Toll-Like Receptor-4 (TLR4) signaling 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 (100 mcg/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 suggests that the combination of VD + TC has the potential to control inflammation without disrupting the body's defense mechanisms against infection.

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

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diseases have devastated global society if adequate prevention and treatment strategies are unavailable [1]. The human body has a natural defense system to deal with infectious diseases. The mechanism of inflammation at the site of infection 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 pro-inflammatory 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 IL-1 and IL-6, as in the case of 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 inhibit the production of pro-inflammatory cytokines by influencing the TLR2 and TLR4 pathways in THP-1 monocyte cells and TLR4-transfected HeLa cells [9]. In addition, extracts of *Mentha pulegium L*. It reduces the expression of pro-inflammatory mediators, proteins, and transcription factors in LPS-stimulated PBMCs. This extract significantly reduced the levels of mRNA TNF-α, IL-1β, IL-6, TLR-4, iNOS, and NF-κB p65, as well as decreased the levels of TNF-α, IL-1β, and TLR-4 proteins [10]. Research on extracts *Hyssopus officinalis* L. demonstrated that this extract was able to induce antiviral innate immune responses by activating endosomal TLRs such as TLR3, TLR7, TLR8, and TLR9, as well as increasing the expression of MyD88 and NF-κB in PBMCs [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* is known to reduce TNFa, TGFb pro-inflammatory cytokines, and TLR4 receptor expression levels in in vivo testing [13] and also decreases the proinflammatory cytokines IL-1 and IL-6 [14]. As a plant widely spread in Southeast Asia and especially in Indonesia, this plant can be developed as an anti-inflammatory drug 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 to this day as a substance that effectively increases the expression of cathelicidin (Austria et al., 2021) through the vitamin D receptor pathway that activates transcription factors that trigger an increase in cathelicidin expression/production in its producing cells (Lee et al., 2012; Lowry et al., 2020; Gönen et al., 2021). 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 pro-inflammatory 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.

2 Material and Method

2.1 Antibodies

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

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 4ºC 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. Twenty-five normal female BALB/c mice, weighing approximately 20–25 g and aged between 6 and 8 weeks, were housed in cages

with controlled environments. 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 dose of 100 micrograms/kg body weight every day for 28 days + *E. coli* injection on the day 29th
Tinospora cordifolia (TC) : TC extract dose of 100 mg/kg body weight every day for 28 days + *E. coli* injection on the day 29th
VD + TC group : vitamin D 100 micrograms/kg bw + TC extract 100 milligrams/kg bw daily for 28 days + *E. coli* injection on the day 29th

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. Spleen samples were taken and then washed with sterile PBS and crushed using a cold mortar until it was estimated that a single-cell suspension was obtained. This prepared single-cell suspension conforms to standard protocols (Atho'illah et al., 2021). The single-cell suspension was centrifuged at 2500 rpm for 5 minutes at 10°C. The supernatant was removed, and the pellet was stained with FITC anti-mouse/human CD11b (clone: M1/70, BioLegend) for extra cell dye and CD11b cell subset markers. Cells were then fixated with cytofix/cytoperm buffer (BD-Biosciences, Pharmingen) and washed with buffer. Cells were then stained with intracellular dye using 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) to see cathelicidin expression. The cells are then incubated at 4°C for 30 minutes. The stained cell samples were then analyzed by flow cyometry using the FACS CantoIITM device (BD-Biosciences, San Jose, CA). The flow cytometry readings were then analyzed using FlowJo v10 for Windows software (FlowJo LLC, Ashland, OR).

2.7 Statistical analysis

The reported results are in the form of average results ± standard deviation (SD). The data displayed is percentage data, which is then statistically analyzed by analysis of variance using a one-way analysis of variance (ANOVA) with Tukey HSD post hoc test. A $P < 0.05$ is a statistically significant result.

3 Result and Discussion

3.1 Administration of Vitamin D and T. cordifolia extract made the expression of CD11b+TLR3+ and CD11b+TLR4+ lower in the infection 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 CD11b+TLR3+ and CD11b+TLR4+ 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.

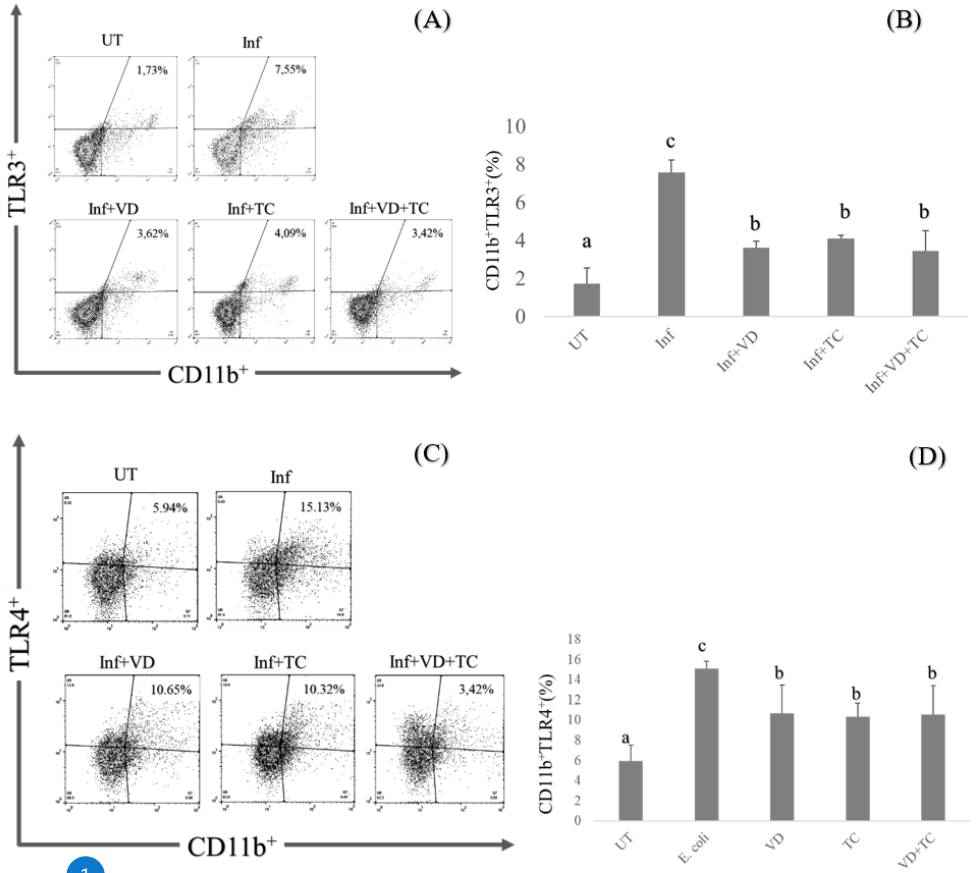

Figure 1. Expression of TLR3 and TLR4 in CD11b cells. (A) Analysis of dot plots of CD11b+TLR3+ cells using flow cytometry. (B) Comparative graph of TLR3 expression in CD11b cells (C) Dot plot analysis of CD11b+TLR4+ cells using flow cytometry. (D) TLR4 comparison graph in CD11b cells±. Statistical analysis was carried out 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.

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

IL-1 and IL-6 are pro-inflammatory cytokines, although IL-6 can also be anti-inflammatory cytokines under certain conditions. Infection treatment made the production of IL-1 and IL-6 higher than 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).

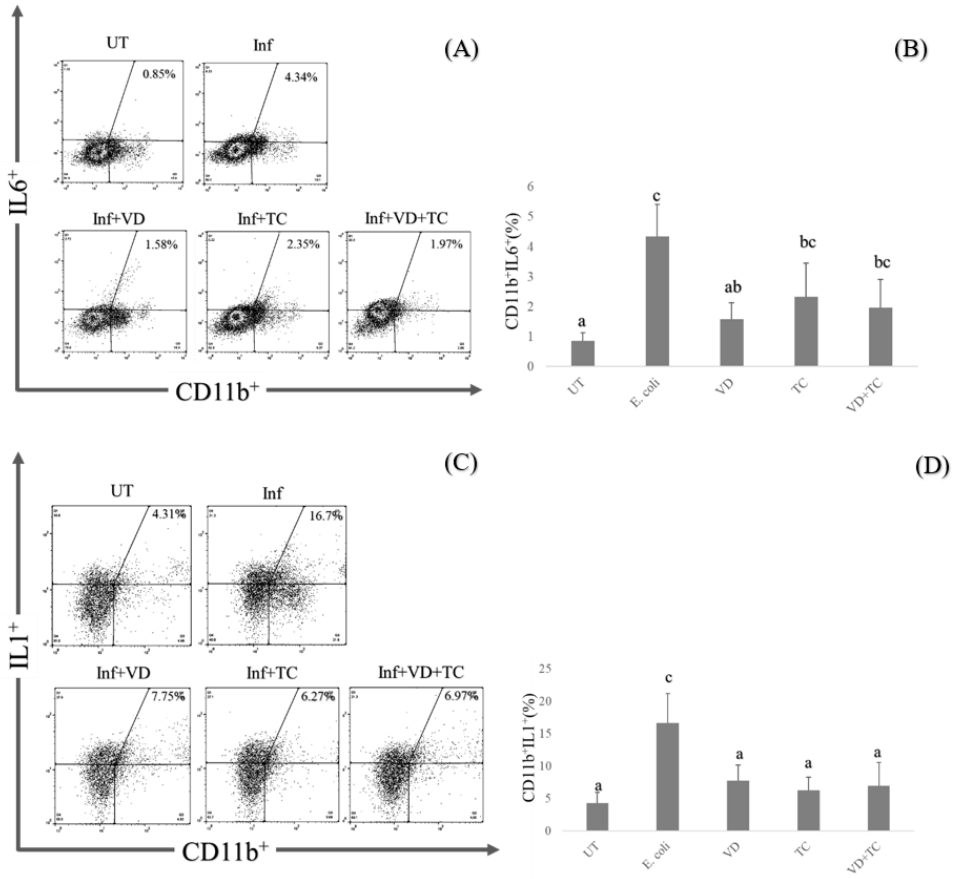

Figure 2. Expression of IL6 and IL1 in CD11b cells. (A) Analysis of dot plots of $CD11b^{+}IL6^{+}$ cells using flow cytometry. (B) Comparative graph of IL6 expression in CD11b cells (C) Analysis of dot plots of CD11b+IL1+ cells using flow cytometry. (D) IL1 comparison graph in CD11b cells±. Statistical analysis was carried out 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.

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.

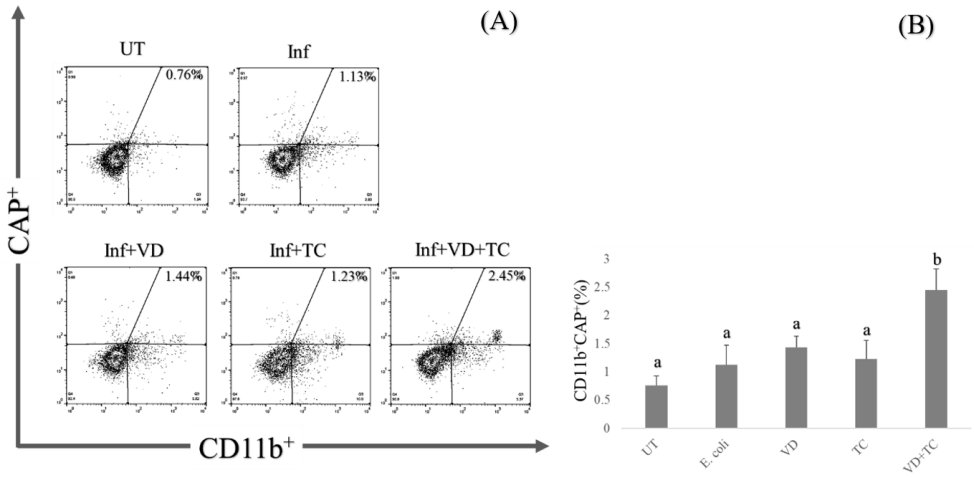

Figure 3. CAP expression in CD11b cells. (A) Analysis of dot plots of CD11b+CAP⁺ cells using flow cytometry. (B) CAP comparison graph in CD11b cells±. Statistical analysis was carried out based on one-way ANOVA followed by the Post Hoc and Tukey's HSD tests. $p < 0.05$ is a significantly different result.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

4 Discussion

TLR pathway activation is a critical phase that activates immunocompetent cell immunological response pathways that will activate NFKB so that pro-inflammatory cytokines such as IL-1 and IL-6 are produced [20]. This process will then continue with the movement of other immunocompetent cells at the site of infection and the production of antimicrobial peptides such as CAP in these cells [21]. 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 produce proinflammatory cytokines, including IL-1 and IL-6 [22]. Based on the results of this study, it is known that the activation of signaling pathways related to the inflammatory response also decreases along with the decrease in TLR3 and TLR4 expression. This causes less IL-1 and IL-6, expected to help reduce inflammation. This is useful when excessive inflammation, including autoimmune and chronic inflammatory diseases.

Based on the results of this study, 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 showed that the expression of TRL3 and TLR4 was lower than that of the infection group that was not given VD, TC, and VD+TC. As previously studied, vitamin D reduces TLR3 expression [23]. Other results also show that TC can decrease TLR4 expression [13]. The results of these studies were confirmed in this study where with a dose of vitamin D of 100 micrograms/ml and an extract 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).

Our study shows that VD and TC and their combination can alter the production of IL-1 and IL-6 to be lower than that of the infection group. This can be seen from the results of dot plot flow cytometry analysis where CD11b+ cells in the VD, TC, and VD+ETC treatment groups were statistically significantly lower in expression of IL-1 and IL-6 compared to 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). The study's results also show that the induction of *E. coli* intraperitoneally increased the expression of IL-1 and IL-6 than that of controls (healthy animals). *E. coli*, as commonly known, is a Gram-negative bacterium with a cell wall arrangement in the form of lipopolysaccharides (LPS). LPS is a unique material that makes up the bacteria's cell wall and is a ligand on the TLR receptor. Activating this TLR will impact the production of IL-1, IL-6, and various inflammatory cytokines such as TNFa, IFNg, and other inflammatory proteins such as iNOS and COX-2 [24]. TC is known by Philip Research 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 pro-inflammatory 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 pro-inflammatory cytokines and stimulates the multiplication of differentiation of M2 cells that produce anti-inflammatory cytokines such as IL-10 [25].

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 pro-inflammatory cytokines such as IL-1 and IL-6 [26], [27]. 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) [25]. 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.

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 pro-inflammatory 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.

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