

Original Research Article

Effect of vitamin D and *Tinospora cordifolia* on SDF-1 expression during bacterial infection

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Abstract

Purpose: To evaluate the activity of vitamin D (VD), *Tinospora cordifolia* (TC) ethanol extract, and a combination of the two (VD + TC) on Stromal Cell-Derived Factor-1 (SDF) 1 expression in a subset of GR-1⁺ myeloid cells and B220⁺ B lymphocytes cells using flow cytometry analysis.

Methods: Female mice of the *Mus musculus* strain BALB/c were divided into groups of 5 mice each, comprising: normal group (N), infection group (I), vitamin D group (VD; 100 µg/kg body weight), TC group (TC; 100 mg/kg BB), and combination group at the same doses (VD + TC). Treatment of VD, TC and their combination was given to mice for 28 days. On day 29, all mice except the normal group were infected with *Escherichia coli* (10⁶ CFU/mL) by intraperitoneal (IP) injection. Six hours post-infection, immune responses were evaluated by measuring SDF-1 expression in GR-1⁺ myeloid cells and B220⁺ B lymphocytes.

Results: The results of the analysis on Gr-1 cells showed that the expression of SDF-1 in the infection group (I; 7.62 %) was significantly higher than that of all other groups, including normal group (4.54 %), TC (4.44 %), VD (4.99 %), and VD+TC (5.22 %) groups, ($p < 0.05$). Similar results were observed in the subset of B220⁺ cells, where SDF-1 expression in group I (12.35 %) was significantly higher compared to the normal (5.92 %), TC (6.22 %), VD (7.47 %), and the combination of VD + TC (6.06 %; $p < 0.05$).

Conclusion: *Tinospora cordifolia* and vitamin D modulate the expression of SDF-1 in Gr-1⁺ and B220⁺ cells both singly and in combination.

Keywords: SDF-1, Gr-1, B220⁺, vitamin D, *Tinospora cordifolia*

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INTRODUCTION

Stromal Cell-Derived Factor-1 (SDF-1) is one of the chemokines that plays a role in the chemotactic activity of immunocompetent cells. SDF-1, also known as CXCL12, has a role in the migration, homing and activation process of various types of immune cells [1]. The SDF-1 molecule mediates the migration of immune cells

to inflammatory sites or lymphoid tissues through their interaction with CXCR4 receptors, which are broadly expressed in various subsets of immune cells. In addition, SDF-1 also contributes to other important physiological processes such as hematopoiesis, angiogenesis, and tissue regeneration [2].

The regulation of SDF-1 expression in a subset

of immune cells, such as granulocytes and B lymphocytes, is an important aspect that deserves close monitoring. Granulocytes, characterized by the expression of the Gr-1 marker, are the frontline component in the innate immune response [3], while B lymphocytes, recognized through the expression of the B220 marker, play a central role in humoral immunity [4]. Based on the results of literature search, there is little information related to the expression of SDF-1 in a subset of granulocyte cells, Gr-1⁺ and B220⁺. SDF-1 molecules are more often said to be produced by bone marrow cells [5]. That attracts the movement of these two types of immunocompetent cells [6]. Therefore, monitoring the expression of SDF-1 in these cells is one of the things that provide additional important information in the field of immunobiology.

The expression of SDF-1 in infected conditions is increased in infected tissues, which helps the tissue in recruiting more immune effector cells [7]. The abundance of SDF-1 molecules affects the ability of granulocyte cells to migrate to the site of infection. Neutrophils produce CXCL4, which is a receptor of the SDF-1 molecule, so that they will respond to the SDF-1 concentration gradient in the target tissue [8]. This immune cell response mechanism is important in the early stages of infection because neutrophils (including granulocyte cells) are frontline defense cells that are important in the infectious response through phagocytosis, nitric oxide (NO) production and proteolytic enzymes [9]. Meanwhile, in B220⁺ cells, SDF-1 expression plays an important role in the differentiation programming process and the movement of naïve B cells towards lymphoid organs. In addition, SDF-1 expression is important in the context of antibody formation by mature B cells [10]. Based on these things, SDF-1 expression is an important factor in the functionality of granulocyte cells and B cells as important components of the cellular immune system. Although SDF-1 is generally associated with a protective immune response, some studies suggest that excessive expression of SDF-1 may correlate with poor prognosis in various pathological conditions such as chronic inflammation, cancer, and systemic infections [1]. Therefore, it is important to keep SDF-1 regulation under control so that it remains within the production limit that is beneficial and does not give rise to destructive activities in the body.

Regarding aspects of controlling or modulating the body's immune system, there are several natural substances that have the potential to be immunomodulators, including vitamin D. Vitamin D, in addition to being an essential nutrient in

maintaining bone health, also has a function in maintaining the homeostasis of the immune system. This essential natural ingredient is known to have immunomodulatory activity that has a homeostasis effect on T cells and B cells, including the production of pro-inflammatory and anti-inflammatory cytokines [11]. Vitamin D receptors (VDRs) are expressed in various immune cells such as monocytes, macrophages, dendritic cells, T cells, B cells and other immunocompetent cells [12]. Activation of VDR after exposure to vitamin D in its active form (1,25-dihydroxyvitamin D3) activates the production of various immunomodulatory substances, including antimicrobial peptides such as cathelicidin and defensin [13]. The results of in vitro and in vivo studies have shown that vitamin D has the ability to increase phagocytosis activity and antibody production [14]. However, it is not known directly how vitamin D affects the expression of SDF-1 in granulocyte and lymphocyte cells.

In addition to vitamin D, *Tinospora cordifolia* (TC), the menispermaceae family, locally known as Bratawali. It is widely distributed in the Mediterranean region, South Asia, and Southeast Asia, including Indonesia. Its ethanolic stem extract is known to have immunomodulatory activity [15]. Plants of the Menispermaceae family are known to contain bioactive compounds of the alkaloid group, glycosides, isoquinolin, terpenoids and various carboxylic acids that have immunomodulatory as well as anti-inflammatory activity [16]. One of the active compounds detected in TC, namely magnoflorin, is known to increase phagocytosis activity, increase nitric oxide (NO) production, increase CD4⁺ and CD8⁺ cell populations, and proliferation of B and T lymphocyte cells and antibodies [17]. In addition, TC is also known to lower several inflammatory mediators, such as TNF- α , through inhibition of the NF- κ B pathway [18]. Based on the data from the results of these studies, it is projected that these two ingredients, namely VD and TC, affect the expression of SDF-1 in immune cells, especially Gr-1 and B220 cells.

The purpose of this study is to explore the potential effects of VD, TC and the combination of the two on SDF-1 expression on two subsets of immunocompetent cells representing the innate and adaptive immune systems (granulocytes and lymphocytes) through flow cytometry analysis. The results of this study are expected to provide new insights related to the synergistic activity of the two natural immunomodulatory agent ingredients. Therefore, this study will provide useful information in the

development of immunomodulatory nutraceutical supplement formulas or immunotherapy and the development of more targeted immunological intervention strategies in dealing with infections and hyperinflammation.

EXPERIMENTAL

Materials

The vitamin D was obtained from Blackmores Vitamin D3 1000 IU in liquid form. Meanwhile, the stem simplicia of *Tinospora cordifolia* (TC) was obtained on August 2, 2023, from UPT Laboratorium Herbal Materia Medica, Batu (Jl. Lahor No.87, Pesanggrahan, Batu City, East Java; GPS: -7.86749391539108, 112.51929985598086). Plant material was officially identified at the same institution, with identification number 074/540/102.20-A/2023. The antibodies used include: Fluorescein Isothiocyanate-Conjugated (FITC) rat anti-mouse Ly-6G/Ly-6C (Gr-1; clone RB6-8C5, BD Biosciences), Peridinin-Chlorophyll-Protein Complex (PerCP) anti-Human/Mouse CXCL12/SDF-1 (clone #79018, R&D Systems), and Phycoerythrin (PE) anti-mouse B220 (clone RA3-6B2, BD Pharmingen).

Extraction and preparation of ingredients

The TC powder was extracted through the modified Zahirudin method of maceration [19]. A total of 500 g of powdered stem simplicia of TC was macerated with 2500 mL of 96 % ethanol (ratio of 1:5 w/v) for 24 hours. After that, the residue was filtered and this process was repeated once daily for three days. On the second and third days, the residue of the re-filtration was dissolved with new solvent (2500 mL of 96 % ethanol), filtered, and all the filtrates from the first to the third day were collected. The collected filtrate was then evaporated using a rotary evaporator until it reached a stable weight. The concentrated extract that had been stabilized was then weighed to determine the final result (yield) of the extraction, and then stored in the refrigerator at a temperature of 4 °C until use.

Acclimatization of test animals

This study used female mice of the *Mus musculus* strain BALB/c, obtained from Malang Wistar Farm, Dau District, Malang Regency, East Java. A total of 25 female BALB/c mice in healthy condition, with a body weight ranging from 20 – 25 g and aged 6 – 8 weeks, were kept in cages under controlled conditions of temperature (25 ± 2 °C), relative humidity (70 – 80 %), and a 12-

hour light/dark cycle. During the acclimatization period of 7 days, the animals were given standard feed in the form of pellets and drinking water *ad libitum* (free access) every day.

Ethical approval

This study received ethical approval from the Health Research Ethics Committee of the University of Muhammadiyah Malang (approval no. E.5a/254/KEPK-UMM/XII/2024).

Test animal treatment

During the 28-day treatment period, the mice were fed standard feed and drinking water (Aquadest) *ad libitum*. Mice are divided into five groups of oral treatments as follows:

N (Normal): untreated control group, received only Aquadest orally,

I (Infection): infection control group, received only Aquadest orally,

VD: the group given VD at a dose of 100 µg/kg body weight, [20].

TC: group given TC extract at a dose of 100 mg/kg body weight (BW), [21,22].

VD+TC: the group received a combination of vitamin D (100 µg/kg BW) and TC extract (100 mg/kg BW) [23].

All treatments were administered once daily orally using a probe for 28 days. On the 29th day, after fasting, all groups except the normal control were injected intraperitoneally with an inactivated *Escherichia coli* suspension at a concentration of 10⁶ CFU/mL.

Data collection from test animals

On the 29th day after treatment, six hours post-infection with *E. coli*, the mice were euthanized via cervical dislocation. Next, surgery was performed to remove the spleen. The spleen was then cleaned using *Phosphate Buffer Solution* (PBS). Each organ was processed separately according to different observation objectives and methods [24].

The cleaned spleen was placed in a petri dish, then 1 mL of PBS was added and gently rubbed clockwise until it became a suspension. This suspension was then centrifuged at 4 °C for 5 minutes at a speed of 2500 rpm. After the supernatant was removed, the homogenate was

re-added with 1 mL of PBS and re-centrifuged under the same conditions.

Immunostaining and flow cytometry

Pellets obtained from the spleen were divided into microtubes using micropipettes, then stained according to the target immunocompetent cells using extracellular staining techniques. The dyes used include FITC anti-mouse rat Ly-6G/Ly-6C (Gr-1; RB6-8C5 clone, BD Biosciences) to mark Gr-1 cells, and PE anti-mouse B220 (RA3-6B2 clone, BD Pharmingen) to mark B220 cells. The dyes obtained from the pellets were incubated for 20 minutes at 4 °C under dark conditions [24].

Next, sample fractionation was carried out using Cytofix/Cytoperm, followed by intracellular staining using anti-Human/Mouse PerCP antibody CXCL12/SDF-1 (clone #79018, R&D Systems), which reacts to all human, rat, and mouse species. This intracellular staining was also incubated at 4 °C in a dark room. Afterwards, the sample was analyzed using the Flow Cytometer (BD Biosciences FACS Cantoll), and the data were further analyzed with the FlowJo v10 for Windows (FlowJo LLC, Ashland) software to determine the relative proportions of each cell type based on the observed parameters.

Data analysis

Data from the Flow Cytometry analysis are presented in the form of mean values $\pm$ standard deviations. To determine the differences between groups, a one-way ANOVA statistical test was used, which was then followed by a follow-up test of Tukey HSD as a post hoc test, with a significance level set at $p < 0.05$. Each experimental group consisted of 5 mice, with a total of five groups included in the study.

RESULTS

The results of the study obtained for the VD, TC and the combination of VD+TC groups on the expression of SDF-1 in both Gr-1⁺ and B220⁺ cells are presented in Figures 1-2. The data showed that bacterial infection with *E. coli* significantly increased SDF-1 expression ($p < 0.05$) in GR-1⁺ cells compared to the normal control. Six hours post-infection, the infection group (I) exhibited an average of 7.62 % GR-1⁺ cells expressing SDF-1, which was significantly higher ($p < 0.05$) than the VD (5.64 %), TC (5.31 %), and VD+TC (6.40 %) treatment groups. These results indicate that administration of VD, TC, or their combination effectively reduced SDF-1 expression in GR-1⁺ cells relative to the infection group.

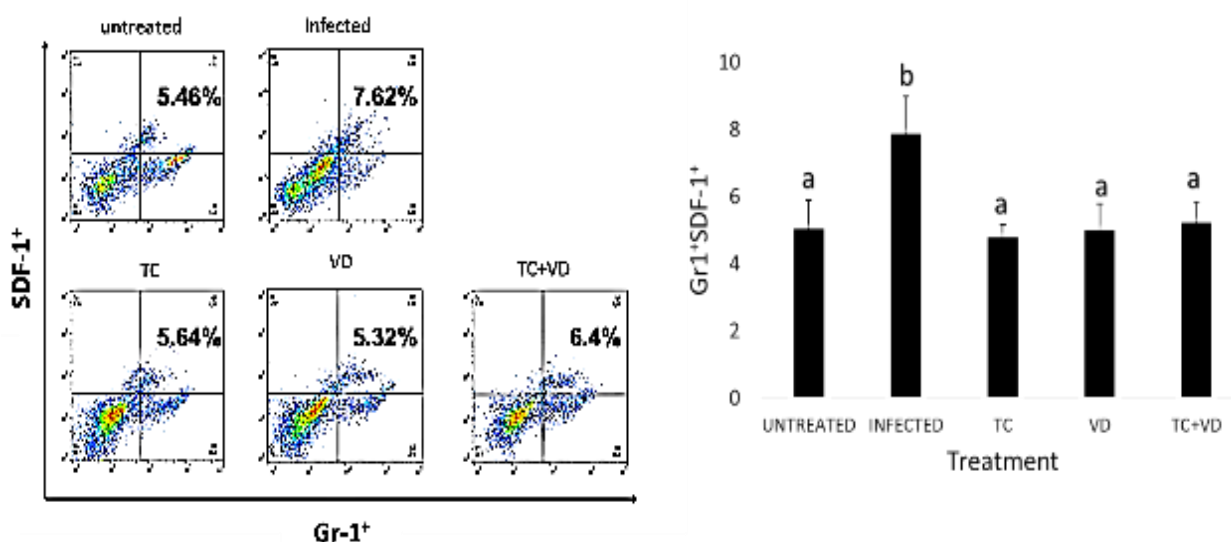


Figure 1: The results of the *Flow Cytometry* analysis of SDF-1 expression in Gr-1⁺ cells. The results of dot plot analysis show the realistic number of population distributions of Gr-1⁺ cells expressing SDF-1 (left). Histogram of comparison of SDF-1 expression in Gr-1⁺ cells after treatment with *T. cordifolia* (TC), vitamin D (VD), and combination (VD+TC) for 28 days, then given *E. coli* infection by IP on day 29 (right). Samples were taken and analyzed using flow cytometry after 6 hours of infecting mice. **Key:** Data representing the mean SDF-1 expression in Gr-1⁺ cells were statistically analyzed using the one-way ANOVA method followed by the Tukey HSD test with a significance value of $p < 0.05$. Different letters above the histogram bars indicate significant differences ($p < 0.05$)

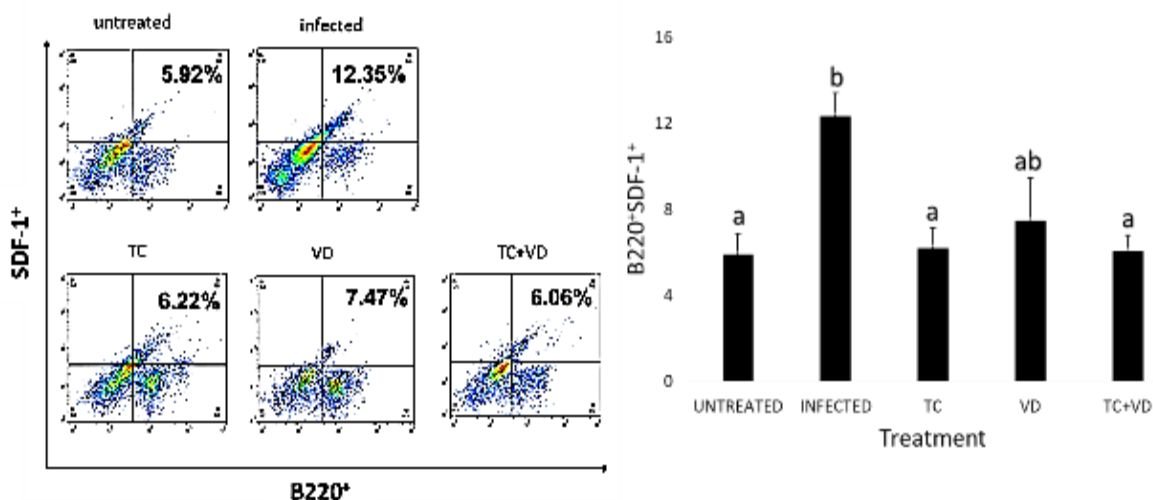


Figure 2: Results of *Flow Cytometry* analysis of SDF-1 expression in B220+ cells. The results of dot plot analysis show the realistic number of B220+ cell populations expressing SDF-1 (left). Comparative histogram of SDF-1 expression in B220+ cells after treatment with *T. cordifolia* (TC), vitamin D (VD), and combination (VD+TC) for 28 days, then given *E. coli* by IP on day 29. Samples were taken and analyzed via flow cytometry after 6 hours of infecting mice. **Key:** Data in the form of the average value of SDF-1 expression in B220+ cells were statistically analyzed using the one-way ANOVA method followed by the Tukey HSD test with a significance value of $p < 0.05$. Different letters above the histogram bars indicate significant differences ($p < 0.05$)

Harmonized results were also observed in the B220+ cell subset. Infection increased the population of B220+ cells expressing SDF-1 to 12.35 %, which was significantly higher ($p < 0.05$) than the normal control (5.92 %). Administration of VD, TC, and the VD+TC combination reduced SDF-1 expression to 7.47, 6.22 and 6.06 %, respectively. These reductions were statistically significant compared with the infection group for TC and VD+TC ($p < 0.05$), whereas the decrease observed in the VD group was not statistically significant ($p > 0.05$).

DISCUSSION

These results show that infection is a triggering factor for increased SDF-1 expression in both Gr-1+ and B220+ cells. It is known that bacterial infections cause the activation of several immune cell receptors, including Toll-Like Receptor (TLR)3/4 and Nucleotide-Binding Oligomerization Domain-Containing Protein 2 (NOD2) receptors. Peptidoglycan on the bacterial cell wall activates the TLR3/4 and NOD2 pathways, which in turn activate the MAPK pathway, resulting in the production of various proinflammatory cytokines that direct effector immune cells to the site of infection [25]. Although there is no specific information that SDF-1 is activated through this mechanism, with its function as a chemokine that recruits immune cells so that they come to the site of infection, this result correlates with the activation of the MAPK pathway, which has the same function as a response to infection.

SDF-1 has a receptor called CXCR4; the interaction of both (SDF-1 and CXCR4) results in a response of immune cells that move them to the site of infection. CXCR4 is expressed in B lymphocyte cells and granulocyte cells; its activation makes these cells respond with migratory activity to the environment that requires it, including the site of infection [26]. A side effect that occurs when too many CXCR4 pathways are activated is the occurrence of hyperinflammation, which is harmful to the body if not controlled. Therefore, inhibition of CXCR4 activity is one of the crucial steps in controlling the negative effects of infection in the form of hyperinflammation or acute inflammation [27]. In addition to systemic infectious conditions, increased expression of SDF-1 may be a compensatory signal to accelerate the recruitment and activation of effector immune cells in an effort to produce antibodies and regulate cellular immune responses. Nevertheless, excessively high expression of SDF-1 has the potential to lead to tissue homeostasis dysregulation and excessive inflammation, which worsens the clinical prognosis [28].

Vitamin D (VD) is known to lower inflammatory chemokines. However, there is no previous research data that is directly related to decreased SDF-1 expression due to VD and TC treatment. Therefore, the decrease in SDF-1 expression in Gr-1+ and B220+ cells suggests that both control SDF-1 expression. These

findings demonstrate the role of vitamin D as an immunomodulatory agent that regulates the activity of T and B cells and influences the expression of various chemokine and cytokine molecules. Several studies have shown that vitamin D decreases the expression of proinflammatory chemokines in different cell types in the inflammatory context, through a mechanism that involves inhibition of the NF- κ B signaling pathway [29].

The results in this study were obtained by observing the expression of SDF-1 with TC treatment. And it is well known that TC is a plant with various bioactive compounds that have anti-inflammatory and immunomodulatory activities. TC bioactive compounds have the potential to inhibit the production of proinflammatory chemokines and cytokines through their effects on the MAPK and JAK-STAT pathways [30]. In this regard, it is reasonable to suspect that the difference in the population of Gr-1⁺ and B220⁺ cells that express SDF-1, which was less than that of the infection group, indicates the influence of controlling the expression of SDF-1 by TC bioactive compounds.

This study as a whole has been able to provide an overview that both VD and TC, in individual or combination form, have a role in controlling the expression of SDF-1 in innate and adaptive immune cells, in this case Gr-1⁺ and B220⁺. These results illustrate the opportunity to utilize both ingredients as a formula for immunomodulatory supplement preparations, especially immunosuppression, in the context of immune system homeostasis and prevention of negative effects of acute infections.

This brief study has limitations related to dose variation and sampling time to comprehensively describe fluctuations in chemokines such as SDF-1 in some post-infection and treatment periods. Therefore, it is recommended to continue this study with the addition of treatment variations in dosage and sampling time in a similar testing scheme. The study also confirms the need for further investigation of molecular mechanisms to better understand the individual and combined effects of these natural compounds on the immune system. These findings may serve as a basis for the development of additional therapeutic strategies in the management of infectious diseases using natural compounds.

CONCLUSION

This study uncovered the potential role of vitamin D, *Tinospora cordifolia*, and their combination as

immunomodulators in regulating the expression of SDF-1 in a subset of immune cells, specifically granulocytes GR-1⁺ and B220⁺ cells, during bacterial infection. The results showed that bacterial infection multiplied the population of cells expressing SDF-1 in GR-1⁺ and B220⁺ cells, while the administration of vitamin D or a combination of vitamin D and *Tinospora cordifolia* appeared to result in fewer populations of cells expressing SDF-1. These findings suggest the supportive role of vitamin D in controlling chemokine-mediated immune responses under infectious stress conditions.

DECLARATIONS

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Conflict of interest

No conflict of interest is associated with this work.

Contribution of authors

We declare that this work was done by the author(s) named in this article, and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Poncojari Wahyono served as the corresponding author and played a central role in the conceptualization of the study, the development of the research methodology, and the preparation of the original draft. K. Sya'dyah and Husamah contributed significantly to data curation and formal analysis, ensuring the reliability and integrity of the data used. M. A. Muchlisin was responsible for conducting the investigation and developing visual representations of the findings. A.S. Jamil contributed to the formal analysis and provided critical input through reviewing and editing the manuscript. All authors have read and approved the final version of the manuscript.

Ethical approval

This research has received ethical feasibility approval from the Health Research Ethics

Committee of the University of Muhammadiyah Malang (KEPK-UMM), with an ethical feasibility certificate (no. E.5a/254/KEPK-UMM/XII/2024).

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Use of Artificial Intelligence/large language models

Artificial Intelligence (AI) tools, including large language models, were used solely to enhance the language and clarity of the manuscript. No AI tools were employed to generate, analyze, or interpret any research data or to create scientific content related to the study.

Use of a research reporting tool

No specific research reporting tools or checklists (such as CONSORT, PRISMA, STROBE, etc.) were used in the preparation of this manuscript.

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