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The role of VipAlbumin® as an immunostimulatory agent for controlling homeostasis and proliferation of lymphoid cells

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Abstract

VipAlbumin® is a supplement from snakehead fish (Ophiocephalus striatus) which has high content of albumin that is very important to develop new cells. The aims of this study were to know the effect of VipAlbumin® to cell proliferation, expression level of $CD4^+CD62L^+$ T cell, regulatory T cell, and B220+ cell, and immunocompetent cell cycle. Cell isolated from spleen of pathogen free mice were cultured in RPMI 1640 with 10% FBS, 1% Pen/Strep 10×, 2-Mercaptoetanol, anti-CD3 and LPS. The concentrations of VipAlbumin® used were 0 µg/ml; 0.33 µg/ml; 33.3 µg/ml; and 3333.3 µg/ml. The cell was incubated in CO_2 5% incubator 37°C for 3 days for cell cycle and 5 days for proliferation analysis and cell expression. FACS analysis was done to know cell proliferation profile, status of cell, and cell cycle. Concentration 33.3 µg/ml and 3333.3 µg/ml significantly can increase cell proliferation and induce cell enter G2/M phase ($p < 0.05$) compared to control. VipAlbumin can significantly increase the relative number of $CD4^+CD62L^+$ T cell, regulatory T cell, and B220+ cell ($p < 0.05$) compared to control. This study gives scientific evidence that VipAlbumin can be used as an immunostimulant which accelerates immunocompetent cells growth.

Key words: *proliferation, cell cycle, immunostimulatory, Ophiocephalus striatus, VipAlbumin®.*

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Introduction

VipAlbumin® is supplement from snakehead fish (*Ophiocephalus striatus*) extract. In the recent years, some researches reveal the nutritional content of snakehead fish. Zuraini *et al.* [1] had conducted an analysis of amino acid composition and fatty acids, and it its known that this fish contain amino acids: glutamic acid, aspartame acid, and lysine, and fatty acids: palmitic acid, stearic acid, arachidonic acid (omega-6) and docosahexanoic acid (omega-3). Mustafa *et al.* [2] explained that protein content of snakehead fish is 25.5%, higher than milkfish 20.0%, goldfish 16.05%, snapper (20.0%), and sardines (21.1%). Majority of snakehead fish protein extract is albumin which is 64.61% from protein total.

Albumin has a function to regulate blood osmotic pressure and maintain water balance in the blood plasma to maintain blood volume. It also has a function to transport elements which are less soluble in water, such as free fatty acids, calcium, iron and some drug elements to pass through the blood plasma and fluid cells. It can be synergized with mineral Zn which is very important to cell development and the formation of new tissue for wound healing and wound healing due to surgery [2]. Based on that, it is assumed that albumin also play role in proliferation of immunocompetent cells which influence the cell cycle.

Cell cycle is regulated by cyclin levels. Cyclin D initiates cells enter from G0 phase to G1 phase. Cyclin D can bind to Cdk4 and Cdk6. Cyclin D and Cdk4 complex cause hypophosphorylation Retinoblastoma protein (pRB) and facilitate the expression of cyclin E. Cyclin E and cyclin A are able to bind Cdk2 and induce cell transition from G1 phase to S phase. Cyclin B1 and B2 can bind Cdk1, Cdc2, and P34 kinase and initiate cell to enter M phase (mitosis) to perform division [3-5].

The cells of immunostimulatory compound target are T and B lymphocytes cells. Therefore, activities of VipAlbumin® expected to increase the number of $CD4^+CD62L^+$ T cells, Regulatory T cells (Treg) and B220+ cells. T cells play a role in cellular immune system (T cell mediated immunity) whereas B cells play a role in humoral immune system. Treg are $CD4^+$ T cells that express many CD25 molecules, so they are usually called $CD4^+CD25^+$ T cell. The function of Treg in the body is to maintain homeostasis immunocompetent cells by regulating reactive cells [6]. Analyzing the presence of Treg cells requires specific markers such as Foxp3 [7, 8] or by triple staining with

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anti-CD62L which is a marker for naive cells so Treg cells will be labeled with $CD4^+CD25^+CD62L^+$.

Increasing the number of immunocompetent cells will have an impact on improving the immune system. Increasing of immune system activity is caused by immunostimulatory compounds. Immunostimulatory as the immune system enhancer works by increasing proliferation of immunocompetent cells. Immunostimulatory compound induces these cells enter to S phase or G2/M phase so that the cells proliferate actively. The aims of this study are to determine the ability VipAlbumin® in improving the immune system through an increase in immunocompetent cell proliferation including an increase in the number of $CD4^+CD62L^+$ T cells, $CD4^+CD25^+CD62L^+$ T cells, and $B220^+$ cells.

Material and methods

Medium preparation

The medium was RPMI 1640 supplemented by 10% fetal bovine serum (FBS), 1% antibiotic Penicillin/Streptomycin, and 50 µM 2-Mercaptoetanol (2-ME). It was added with 1 µg/ml of soluble anti-CD3 mAb and 10 ng/ml of lipopolysaccharide (LPS). It was filtered by miliophore membrane 0.20 µm. All of that procedure has done with aseptic method in Laminar Air Flow (LAF). VipAlbumin® was added into medium with concentration 0 µg/ml (K); 0.33 µg/ml (D_1); 33.3 µg/ml (D_2); and 3333.3 µg/ml (D_3). Those doses were obtained from the conversion of the human dose 33.3 mg/kg BW, we assumed that 1 mg of BW is equal to 1 ml medium. Because of VipAlbumin® is a powder, it should be diluted with RPMI medium first.

Cell isolation

Cells were collected from Balb/C mice pathogen free. Cells were isolated from spleen. The homogenates of cells were centrifuged with a speed of 2500 rpm, at a temperature of 10°C, for 5 minutes. Supernatant was discarded while the pellet was resuspended in 1 ml of medium.

Counting the number of cells

The required cell suspension was 5 µl, added by evans blue 10× as much as 95 µl (20× dilution) and homogenized with a pipette. Cells were counted by a haemocytometer counting chamber. The number of cells was counted in the formula to determine the actual number of cells. The formula for compute the number of cells is: Σ cells = Σ the cell count × 5 × dilution × 10^4 cells/ml.

Proliferation assay

Proliferation was measured by flow cytometry using 5,6-carboxyfluorescein diacetate succinimidyl ester (CFSE). The suspension of spleen cells were incubated with 2 µM CFSE in room temperature. After 10 minutes, the suspension was added with 500 µl FBS and then centrifuged. Supernatant was discarded while pellet resuspended in 1 ml of PBS, and then centrifuged. Pellet was resuspended in 1 ml of medium and then centrifuged. Pellet then was resuspended with medium. Control, dose 1, 2, and 3 medium were added with cell which had already been resuspended with medium, as much as 3 million of cells/ml and then mix gently. Cells were grown on 48 well culture plates of 1 ml/well. Cells were incubated in 5% CO_2 incubator at 37°C for 3 days.

Cell cycle and cell expression analysis

Control, dose 1, 2, and 3 medium added with 3 million of cells/ml and then mixed gently. Cells were grown in 48 well culture plates of 1 ml/well. Cells were incubated in 5% CO_2 incubator at 37°C for 5 days.

FACS analysis

Proliferation

Spleen cell suspension was centrifuged in 2500 rpm for 5 minutes at 10°C. Supernatant was discarded and the pellet was ready to analyze.

Cell cycle and cell expression

Spleen cell suspension was divided into 3 tubes A, B and C then centrifuged with a speed of 2500 rpm for 5 minutes at a temperature of 10°C. Cell in micro tube A was used for cell cycle analysis. Pellets were added with 1 ml of 70% ethanol and incubated for 30 minutes in ice box. After incubation, the cell was centrifuged with a speed of 2500 rpm at a temperature of 10°C for 5 minutes. Pellets were washed in 1 ml PBS, centrifuged with a speed of 2500 rpm at a temperature of 10°C for 5 minutes. Pellets were then stained with Propidium iodide and incubated for 30 minutes in box with room temperature.

Cells in micro tube B and C were used for cell expression analysis. Cells in tube B were stained with FITC-conjugated rat anti-mouse CD4, PE-conjugated rat anti-mouse CD25, and PE/Cy5-conjugated rat anti-mouse CD62L. While in tube C were stained with PE-conjugated rat anti-mouse B220. All of sample then incubated for 20 minutes in the 4°C ice box. After conducting all procedure, all samples were added with 500 µl of PBS. Each sample was transferred into a flow cytometry cuvette and then analyzed by flow cytometer.

Statistical analysis

Data were analyzed by BD cellquest PRO™ software then tabulated and analyzed statistically. The statistical analysis used a parametric one-way ANOVA analysis with significance of 0.05% and was followed by Tukey test. The application for statistical analysis was SPSS version 16 for Windows.

Results

VipAlbumin® was able to increase immunocompetent cell proliferation

Immunocompetent cell labeled with CFSE showed higher fission activity when stimulated with VipAlbumin® *in vitro* for 5 days. Cells that divide were on the left peak on flow cytometry analysis because it showed a decrease in CFSE luminescence. As shown in Fig. 1, control treatment did not show two different peaks which meant cells in control treatment did not proliferate maximally. Whereas, VipAlbumin® treatment especially D_2 and D_3 could make two different peaks in FACS result proving that VipAlbumin® was able to increasing cell immunocompetent cell proliferation. Figure 1 also showed that D_2 and D_3 of VipAlbumin® treatment were able to increase the cell proliferation became 36.67% and 42.28% significantly higher ($p \leq 0.05$) than control (28.64%) whereas D_1 (33.61%) did not significant ($p > 0.05$).

VipAlbumin® was able to increase the relative number of naïve T cells ($CD4^+CD62L^+$), regulatory T cells ($CD4^+CD25^+CD62L^+$), and B lymphocyte cells ($B220^+$)

Because of VipAlbumin® ability in increasing immunocompetent cell proliferation, we investigated its immunostimulant activity through expression level of naïve T cells ($CD4^+CD62L^+$), regulatory T cells ($CD4^+CD25^+CD62L^+$), and B lymphocyte cells (B220+). Figure 2 proved that D_2 and D_3 of VipAlbumin® specifically increased the number of $CD4^+CD62L^+$ T cells became 81.85% and 78.71% ($p \leq 0.05$) compared to control (68.37%) treatment. Among D_1, D_2, and D_3 of VipAlbumin® could increase the relative number of regulatory T cells (Treg) (Fig. 3) from 64.5% in control treatment became 71.15% (D_1), 68.67% (D_2), and 67.58% (D_3) significantly different ($p \leq 0.05$). VipAlbumin® not only could increase the expression level of T lymphocyte cells but also

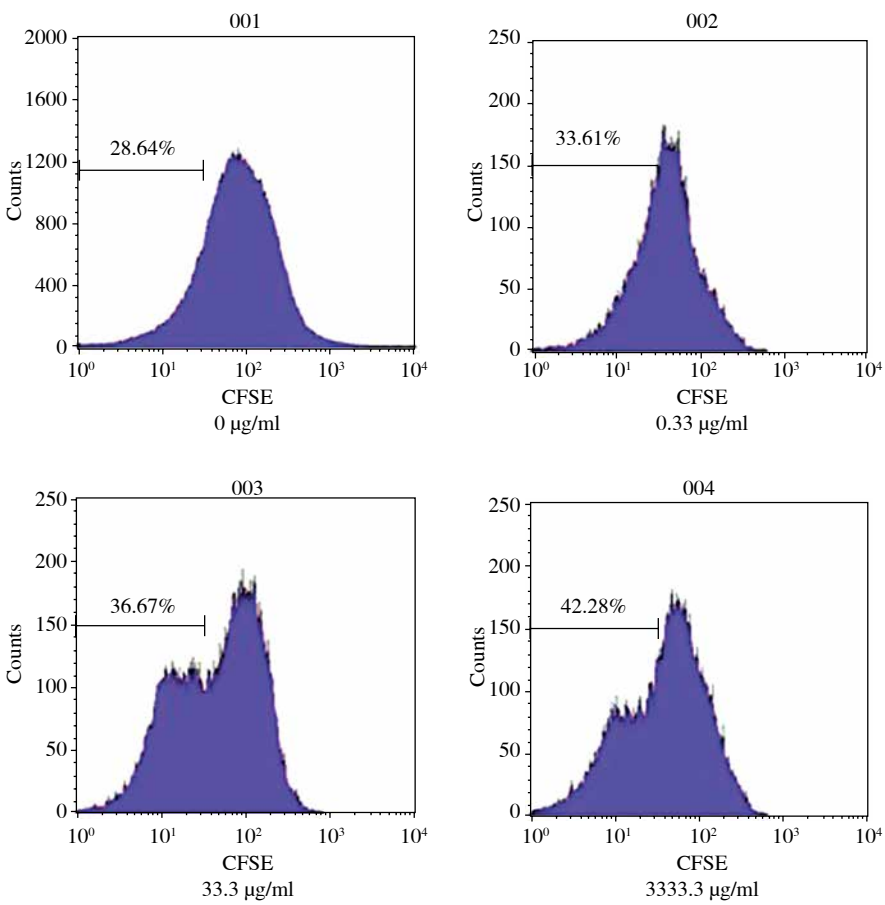

Fig. 1. Stimulation cells from spleen by using VipAlbumin® for 5 days showed the increase of cell proliferation relative number. Spleen cells were cultured in RPMI medium with 10% FBS, anti-CD3 and LPS for five days. On day 5, cell cultures were harvested and analyzed by flow cytometry and tabulated into Microsoft Excel. The cell proliferation was presented in relative number and obtained from all living cells. Data were mean ± SD in each group with p value ≤ 0.05

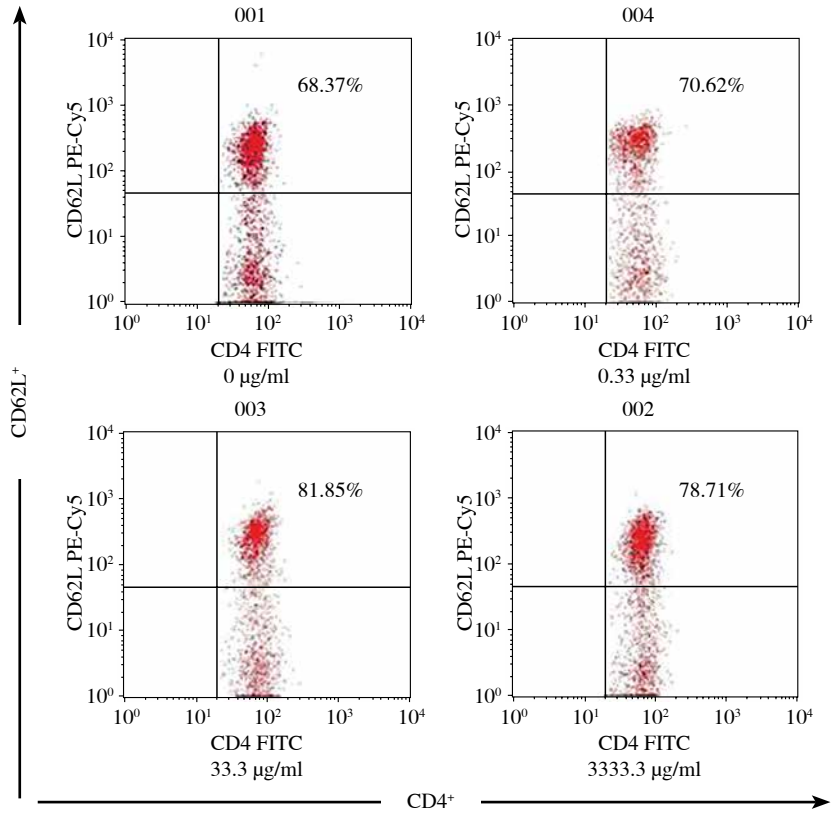

Fig. 2. Stimulation cells from spleen by using VipAlbumin® for 5 days showed the increase of $CD4^+CD62L^+$ relative number. Spleen cells were cultured in RPMI medium with 10% FBS, anti-CD3 and LPS for three days. On day 5, cell cultures were harvested and analyzed by flow cytometry and tabulated into Microsoft Excel. $CD4^+CD62L^+$ T cell were presented in relative number and obtained from all $CD4^+$ T cells population. Data were mean ± SD in each group with p value ≤ 0.05

B lymphocyte cells ($B220^+$). This statement was showed in Fig. 4. The expression levels of $B220^+$ cells in control treatment was 51.44% and increased became 54.09% in D_1 treatment, 57.10% in D_2 treatment, and 64.57% in D_3 treatment. T lymphocyte cells play a role in cellular immune response whereas B lymphocyte cells played a role in humoral immune response which functioned as extracellular protection. This result proved that VipAlbumin® might have a function to activate both cellular and humoral immune system.

VipAlbumin® can induce cells enter to G2/M phase in cell cycle

The increase of cell proliferation activity as a result of VipAlbumin® stimulus was reinforced with cell cycle data. VipAlbumin® did not affect G0/G1 phase due to the relative number of cells both in the control treatments and VipAlbumin® treatment did not show any difference ($p > 0.05$). As shown in Fig. 5, the decrease in S phase occurred because cells stimulated to enter G2/M phase. Like Fig. 1, Fig. 5 also showed that D_2 and D_3 of VipAlbumin® treatment were able to stimulate cell to enter G2/M phase which was significantly higher ($p \leq 0.05$) than control whereas D_1 was not significant ($p > 0.05$). The relative number of cell that enter to G2/M phase in control treatment was 7.75% and increased became 12.54% in D_2 and 13.69% in D_3 of VipAlbumin® treatment. This result absolutely proved the efficacy of VipAlbumin® in increasing cell proliferation due to its ability in stimulating the transition of cells from S phase to G2/M phase.

Discussion

The increase in relative number of cells proliferation in VipAlbumin® treatment indicates that VipAlbumin® has the ability as an immunostimulant which enhance the immune system. Immunostimulant as immune system enhancer works by increasing proliferation of immunocompetent cells. Albumin contained in VipAlbumin® can be synergized with mineral zinc which is play a role in cell development and the formation of new tissue. Besides,

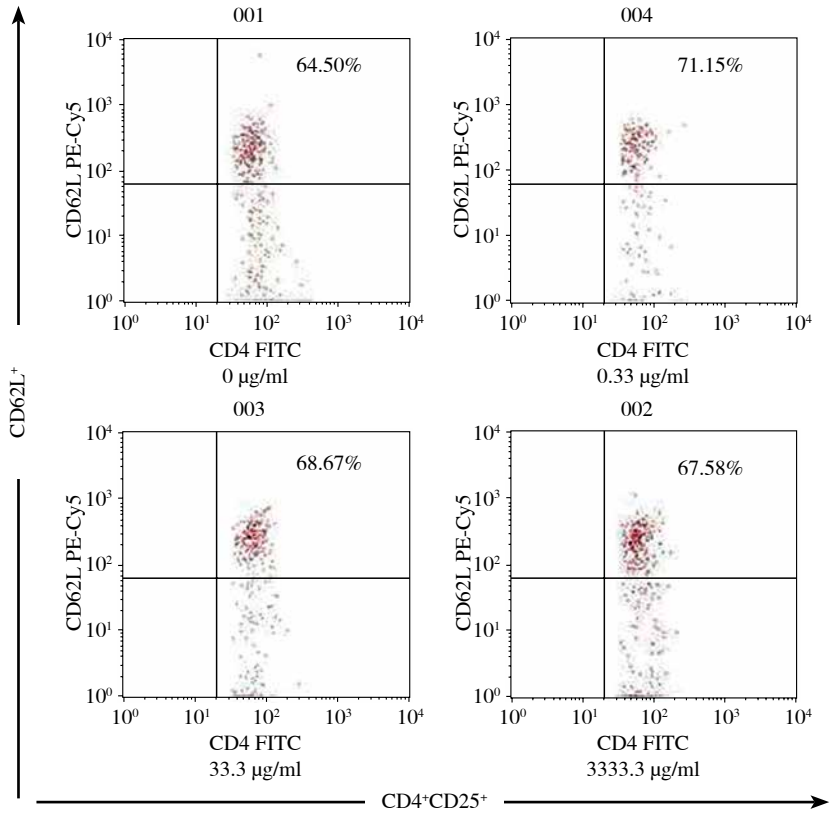

Fig. 3. Stimulation cells from spleen by using VipAlbumin® for 5 days showed the increase of regulatory T cell relative number. Spleen cells were cultured in RPMI medium with 10% FBS, anti-CD3 and LPS for five days. On day 5, cell cultures were harvested and analyzed by flow cytometry and tabulated into Microsoft Excel. $CD4^+CD25^+CD62L^+$ T cell were presented in relative number and obtained from all $CD4^+CD25^+$ T cells population. Data were mean ± SD in each group with p value ≤ 0.05

Baie and Sheikh [9] described that some of snakehead fish extracts compound such as amino acids and fatty acids play an important role in the cell formation and wound healing process.

The targets of immunostimulatory compounds are macrophages, granulocytes, and lymphocytes T and B. The compounds contained in VipAlbumin® which acts as an immunostimulant including zinc, vitamin A, B_6, B_{12}, and D_3. Zinc is required for high cell proliferation, especially in the immune system and affects the function of the innate and acquired immune systems. It also plays a role in increasing antibody responses produced by B lymphocytes [10]. Vitamin B_6 is needed in the biosynthesis of proteins and nucleic acids so that vitamin B_6 impact on immune system function as antibodies and cytokines formed from amino acids and requires vitamin B_6 as coenzyme for metabolism [11]. Vitamin B_6 deficiency can affect the change of T helper cells ($CD4^+$ T cells) percentage [10]. According to Tamura *et al*. [12], vitamin B_{12} acts as an immunomodulator in cellular immune system. Vitamin B_{12} can enhance T cell proliferation and immunoglobulin synthesis by B cells. Vitamin B_{12} deficiency can cause suppression for the immune response toward viruses and bacteria in experimental animals.

ATRA, the main metabolite of vitamin A, can change the naďve $CD4^+FoxP_3^-$ T cells into Treg cells $FoxP_3^+$. The bound of ATRA with the nuclear retinoic acid receptor α can induce histone acetylation in the $FoxP_3$ gene promoter, resulting in expression of $FoxP_3$ protein in $CD4^+$ T cells and becoming Treg cells. Gregori *et al*. [13] and Issazadeh-Navikas *et al*. [14] explained that 1,25-$(OH)_2D_3$, the active form of the vitamin D_3, can induce an increase in Treg cells.

The results of this study proves that the crude extract of snakehead fish found in VipAlbumin® contains very complex compounds and is useful as an immunostimulant by increasing immunocompetent cell proliferation, especially $CD4^+CD62L^+$ T cells which plays a role in cellular immune response, $B220^+$ cell which acts in humoral immune response and antibody formation, and Treg cells which are indispensable for maintaining homeostasis immunocompetent cells. According to Rifa'i [15], the presence of Treg

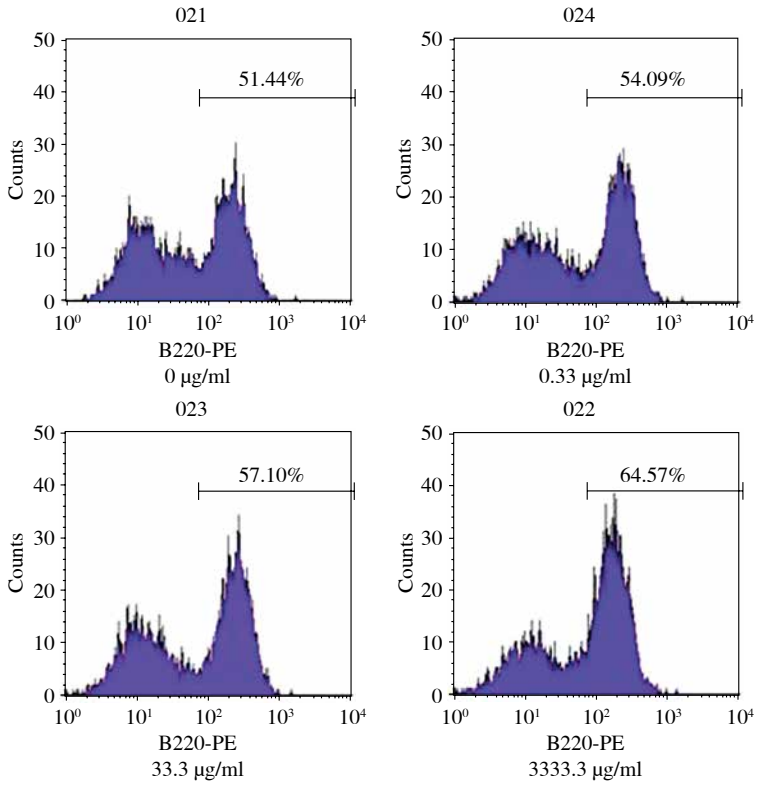

Fig. 4. Stimulation cells from spleen by using VipAlbumin® for 5 days showed the increase of B220+ cell relative number. Spleen cells were cultured in RPMI medium with 10% FBS, anti-CD3 and LPS for five days. On day 5, cell cultures were harvested and analyzed by flow cytometry and tabulated into Microsoft Excel. B220+ cell were presented in relative number and obtained from all lymphocyte cells population. Data were mean ± SD in each group with p value ≤ 0.05

cells absolutely necessary in order to an individual lives normally. Rifa'i [16] and Josefowicz *et al*. [17] explained that Treg cells serves as a suppressant which play a role in an important mechanism of negative regulation of the inflammatory response caused by the immune system which usually occurs in autoimmune, autoinflamasi, allergies, acute and chronic infections, cancer, and metabolic inflammation.

Cell proliferation is closely related to the progression of the cell cycle. An increased cell proliferation can occur if cells are induced to enter S phase or G2/M phase. In this study, compounds contained in VipAlbumin® proved can induce the cells enter to G2/M phase in cell cycle. The compound contained in VipAlbumin®, especially vitamin E, is assumed to be able to increase the level of cyclin A by increase cytokine IL-2. Adolfsson *et al*. [18] explained that vitamin E can increase cytokine IL-2. Interleukin 2 is a growth factor for immunocompetent cell which is able to increase the level of cyclin D_2, E, and A.

In cell cycle, after passing through S phase, cyclin A will take off Cdk2 and bind to Cdk1 to regulate cell transition from S phase to G2 phase [19]. Cyclin A-Cdk1 complex cause chromatin condensation required for cell division [20]. Entering the M phase, cyclin A will be degraded and expression of cyclin B will be increased and bind to Cdk1.

The complex of cyclin B_1 and B_2 with Cdk1 is maturing phase component factor (MPF), which regulates the process spindle formation and sister chromatid pairs. Pines and Hunter [21] explained that the cyclin B increased during the mitotic cell phase. The complex of cyclin B1/Cdk1 will stimulate mitosis and play an important role in the control of microtubule rearrangement during mitosis [19, 20]. Martinvalet *et al*. [22] and Wang *et al*. [23] explained that the complex of cyclin B1/Cdk1 is the matrix of mitochondria and mitochondrial proteins phosphorylate groups including complex I (CI) in the respiratory chain. Cyclin B1/Cdk1 can increase mitochondrial respiration by increasing the utilizing of oxygen and ATP so bioenergy for cell adequate during G2/M transition and cells proliferate actively. All of these statements answer clearly how VipAlbumin® can stimulate the proliferation of immunocompetent cell.

In summary, this study demonstrated that VipAlbumin® plays a role in increasing the proliferation of immunocompetent especially subset naďve T cells, regulatory T cells, and B lymphocyte cells by inducing the cells entering to G2/M phase in cell cycle. VipAlbumin® may play as an immunostimulant which can accelerate immunocompetent cells growth. VipAlbumin® supplement as immunostimulatory agents is more effective than consum-

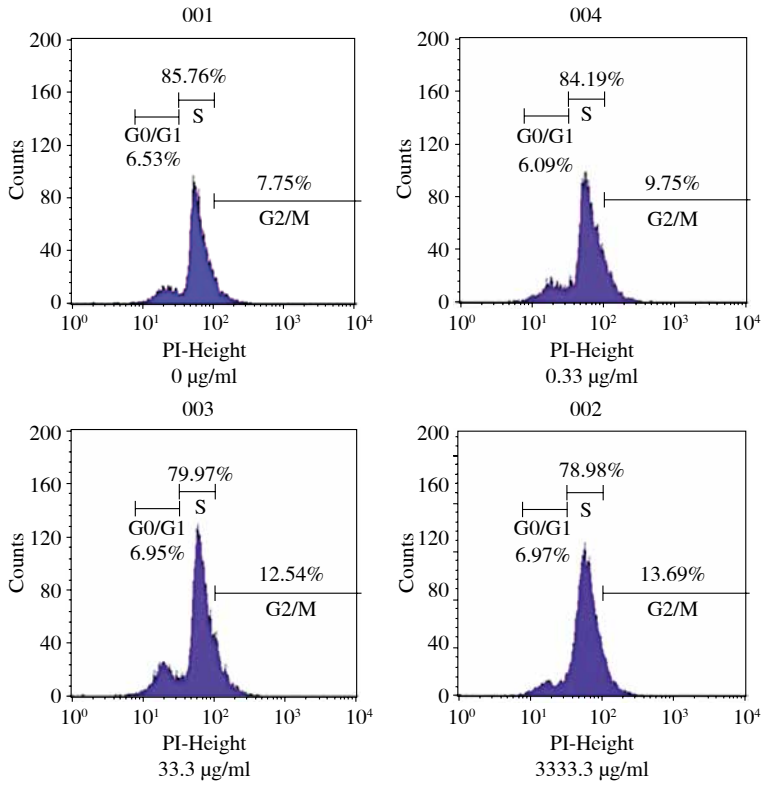

Fig. 5. Stimulation cells from spleen by using VipAlbumin® for 3 days showed the increase of the relative number cells in G2/M Phase Cell Cycle. Spleen cells were cultured in RPMI medium with 10% FBS, anti-CD3 and LPS for three days. On day 3, cell cultures were harvested and analyzed by flow cytometry and tabulated into Microsoft Excel. The cell cycle phase was presented in relative number and obtained from all living cells. Data were mean ± SD in each group with p value ≤ 0.05

ing snakehead fish directly because the nutrient content of snakehead fish extract in one capsule of VipAlbumin® higher than one snakehead fish. Although our investigations provide information about the possible use of VipAlbumin® as an immunostimulant, further studies especially in vivo studies are necessary to determine the mechanism of its immunostimulant action.

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The authors declare no conflict of interest.

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