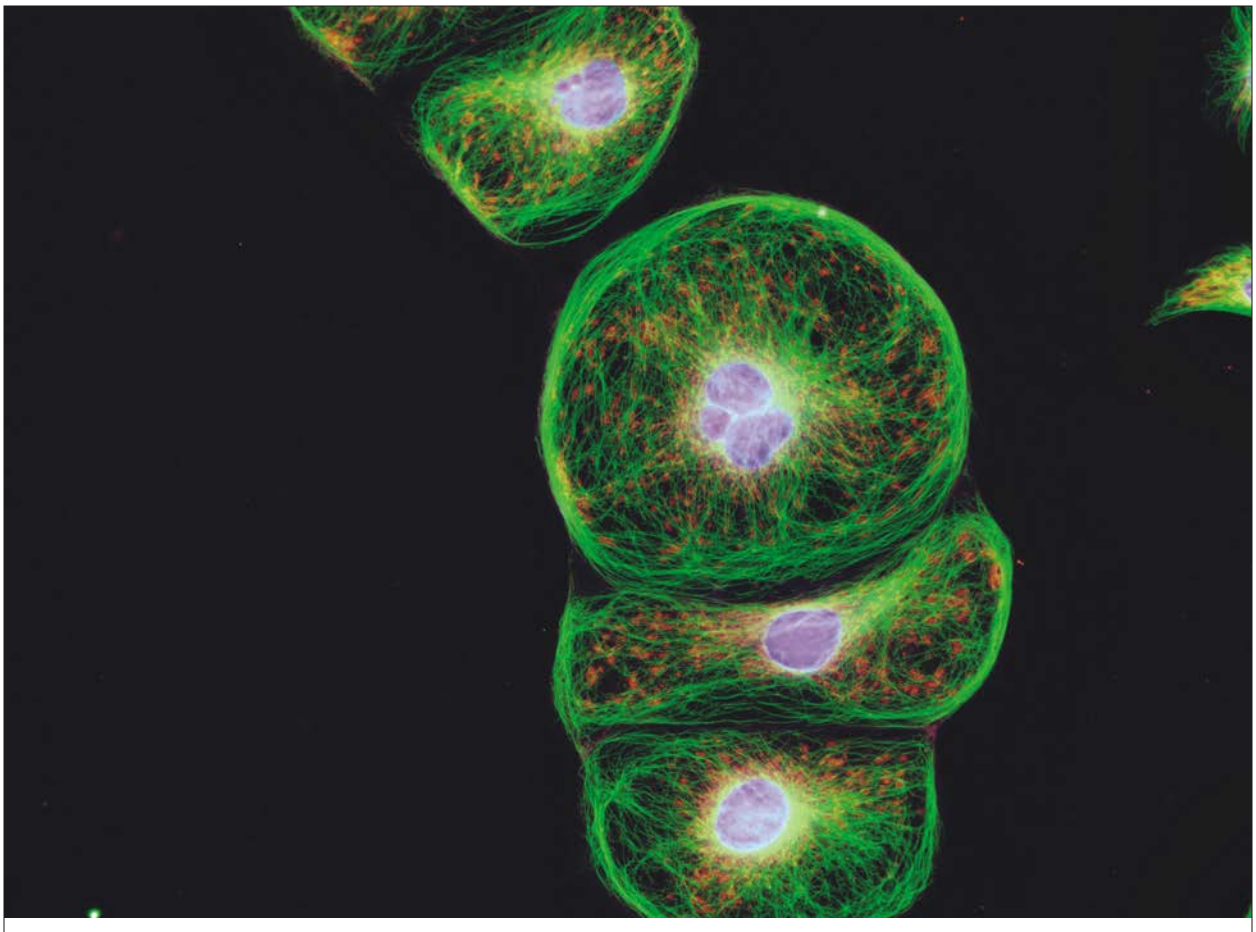

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Decreasing the Expression Level of Macrophage Cell, Pro-Inflammatory Cytokines and NF-κB by Using VipAlbumin® *in vitro*

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ABSTRACT

Inflammation is a physiological response to an injury and disruption by the external factor. VipAlbumin® is a one of the supplement that has anti-inflammatory activity. This study wants to know the effect of VipAlbumin® to macrophage, pro-inflammatory cytokines and transcriptional factor NF-κB. The experiment was done by cultured cells from healthy mice spleen in RPMI with 10% FBS, stimulant anti-CD3 and LPS, 2-Mercaptoethanol and VipAlbumin®'s concentration were 0, 0.33, 33.3 and 3333.3 μg mL^{-1}. This study showed that VipAlbumin® did not only decrease the number of macrophage cells, TNF-α and IFN-γ cytokines produced by $CD4^+$ T cells and IL-6 cytokines produced by macrophage cells, but also suppress NF-κB activation in $CD4^+$ and $CD8^+$T cells and macrophage cells significantly ($p<0.05$) compared to control. Those results proved that this supplement can be used to cure many kinds of disease which is caused by inflammation.

Key words: Cytokines, inflammation, NF-κB, *Ophiocephalus striatus*, VipAlbumin®

INTRODUCTION

Inflammation is a part of the body response to either internal or external environmental stimuli. Inflammation can become chronic inflammation and it may lead to various diseases such as cardiovascular diseases, pulmonary diseases, autoimmune diseases, cancer and diabetes mellitus (Kelly *et al*., 2001; Aggarwal, 2004; Yen *et al*., 2006). Chronic inflammation has also been linked to various steps including cellular transformation, promotion, survival, proliferation, invasion, angiogenesis and metastasis involved in tumorigenesis (Coussens and Werb, 2002; Mantovani, 2005).

Inflammation, the response of tissue to injury, is characterized in the acute phase by increased blood flow and vascular permeability along with the accumulation of fluid, leukocytes and inflammatory mediators such as a group of secreted polypeptides known as cytokines. Several cytokines play key roles in mediating acute inflammatory reactions, namely IL-1, TNF-α, IL-6, IL-11 and IL-8. The inflammatory response is characterized by coordinate activation of various signaling pathways that regulate expression of both pro- and anti-inflammatory mediators in resident tissue cells and leukocytes recruited from the blood (Arican *et al*., 2005; Lawrence, 2009; Qu *et al*., 2013). Aggarwal *et al*. (2006) also mentions that chronic inflammation has been linked with the role of pro-inflammatory cytokines, chemokines, adhesion molecules and inflammatory enzymes. Pro-inflammatory cytokines are produced predominantly by activated macrophages ($CD68^+$) and are involved in the up-regulation of inflammatory reactions. There is abundant evidence that certain pro-inflammatory cytokines such as TNF-α, IFN-γ and IL-6 are participating in the process of pathological pain (Zhang and An, 2007).

Inflammation caused by immunocompetent cells, mediated by NF-κB (Bonizzi and Karin, 2004). Tak and Firestein (2001) also explain that activation of the NF-κB plays a central role in inflammation through its ability to induce transcription of pro-inflammatory genes. This pathway is activated on precise cellular stimulation, usually by signals related to stress or pathogen. Park *et al*. (2000) and Wu *et al*. (2003) explains the role of NF-κB in macrophage cells that coordinate expression of the gene encoding iNOS, COX-2, TNF-α and another protein. NF-κB is required for the ability and reaction of TNF-α to stimulation of TNF-α and IFN-γ synergistically from Bf gene (Huang *et al*., 2002; Eckmann *et al*., 2008).

VipAlbumin® is a supplement from snakehead fish (*Ophiocephalus striatus*) with high content of albumin compare to the other kinds of fish. One of the albumin's benefits is as anti-inflammation. Thus, the aim of this study was to proof this anti-inflammatory activity through the change of macrophage cell number, pro-inflammatory cytokines production and NF-κB on lymphocyte T and macrophage cell *in vitro*.

MATERIALS AND METHODS

Medium preparation: Medium that used was RPMI 1640 supplemented with 10% Fetal Bovine Serum (FBS), 1% antibiotic Penicillin and Streptomycin 10x and 2-Mercaptoethanol (2-ME) 1 μL per 10 mL medium. Medium added by stimulant anti-CD3 as much as 10 μL mL^{-1} medium and 2 μL mL^{-1} medium Lipopolysaccharide (LPS). Medium filtered by millipore membrane 0.20 μm. All of that procedure has done with aseptic method in Laminar Air Flow (LAF). VipAlbumin® added into medium with a concentration 0 μg mL^{-1} (K); 0.33 μg mL^{-1} (D1); 33.3 μg mL^{-1} (D2) and 3333.3 μg mL^{-1} (D3).

Cell isolation: Mice were dislocated and dissected. Spleen was isolated and washed with PBS in petri dish. Cells were isolated from spleen by crushed it in PBS. Homogenates were centrifuged at a speed of 2500 rpm, at a temperature of 10°C, for 5 min. Supernatant was discarded, while the pellet resuspended in 1 mL of medium.

Counting the number of cells: The cell suspension was taken 5 μL, added by evans blue 10x as much as 95 μL (20×dilution) and homogenized with a pipette. Cells were counted using a hemocytometer with a microscope. The number of cells counted in the formula to determine the actual number of cells. The formula for computing the number of cells is: Σ Cells = Σ the cell count×5×dilution×10^4 cells mL^{-1}.

Cell culture and harvesting: Medium control, dose 1, 2 and 3 were added with cell as much as 3 million mL^{-1} and then mixed gently. Cells were grown in 48 well culture plate as much as 1 mL $well^{-1}$. The cells were incubated in a 5% CO_2 incubator at 37°C for 5 days. After the incubation period, the cells were harvested by pipetting medium of each treatment to made cell homogene and then moved it into 15 mL polypropylene tube and then centrifuged at 2500 rpm at a temperature of 10°C for 5 min. Pellet were resuspended in 1 mL of PBS and continued to immunocytochemistry procedure.

Immunocytochemistry: Spleen cell suspensions were divided into 4 microtubes (A, B, C, D). Microtubes A, B, C and D were centrifuged at a speed of 2500 rpm for 5 min at a temperature of 10°C. Supernatant was discarded and the pellets were stained with antibodies. The combinations of dye that used were 4 types, the antibody composition as follows: dye A: FITC-conjugated rat

anti-mouse CD4, PE-conjugated rat anti-mouse CD8 and PE/Cy5-conjugated rat anti-mouse NF-κB, dye B: FITC-conjugated rat anti-mouse CD4, PE-conjugated rat anti-mouse TNF-α and PE/Cy5-conjugated rat anti-mouse IFNg, dye C: FITC-conjugated rat anti-mouse CD68 and PE/Cy5-conjugated rat anti-mouse NF-κB and dye D: FITC-conjugated rat anti-mouse CD68 and PE/Cy5-conjugated rat anti-mouse IL-6.

Cells were stained with extracellular antibodies then incubated for 20 min in the ice box at 4°C. Subsequently, the cells added with a fixative solution cytofix/cytoperm as much as 50 μL and incubated for 20 min in the ice box. Residual of fixative solution removed by washing solution washperm as much as 500 μL and then centrifuged at a speed of 2500 rpm at a temperature of 10°C for 5 min. Supernatant was discarded, while the pellets were stained with intracellular antibodies then incubated for 20 min in the ice box.

Flowcytometry: Cells that have been incubated either in the extracellular and intracellular staining procedure added with 500 μL of PBS. Each sample was transferred into a flowcytometry cuvet and then was analyzed by flowcytometer.

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

RESULTS

VipAlbumin® can decrease the relative number of macrophage cells ($CD68^+$): It was known that macrophage plays a crucial role in the inflammation process as the largest contributor of pro-inflammatory cytokines. VipAlbumin® proven can reduce the relative number of macrophages ($CD68^+$) (Fig. 1). Relative number of macrophages *in vitro* test in the control group was 44.53% and decreased significantly ($p<0.05$) to 40.8% (D1), 39.72% (D2) and 40.12% (D3). All of three VipAlbumin® treatment D1, D2 and D3) did not significantly different from each other ($p>0.05$).

VipAlbumin® can decrease production of pro-inflammatory cytokines TNF-α, IFN-γ and IL-6: Pro-inflammatory cytokine that plays a role in chronic inflammation include TNF-α, IFN-γ and IL-6. *In vitro* assays performed in this study showed that VipAlbumin® in cell culture for 5 days can reduce the relative number of these cytokines significantly compared to the control group. Relative number of TNF-α that produced by $CD4^+$ T cells in the control was 5.72% and decreased significantly ($p<0.05$) in VipAlbumin® D1 and D3 to 4.77 and 4.27%. The D2 group was not significantly different with the control, but the numbers were still relatively lower at 5.40% (Fig. 2).

VipAlbumin® could also decrease the relative number of IFN-γ cytokine that produced by $CD4^+$ T cells. Relative number of IFN-γ that produced by $CD4^+$ T cells in the control was 7.47%. Groups D1 and D2 could decrease relative number of IFN-γ that produced by produced by $CD4^+$ T cells was significantly different ($p<0.05$) than the control became 4.41 and 4.58%, while D3 was not significantly different from the control but relatively lower than the control, it was 6.41% (Fig. 3). IL-6 cytokine that was detected in this study was IL-6 that produced by macrophages. *In vitro* test showed that IL-6 that produced by macrophages in the control group was 6.91% and significantly ($p<0.05$) decreased in group D1 VipAlbumin® to 3.99%, while in group D2 and D3 did not significantly different with control but relatively lower, 5 and 5.24% (Fig. 4).

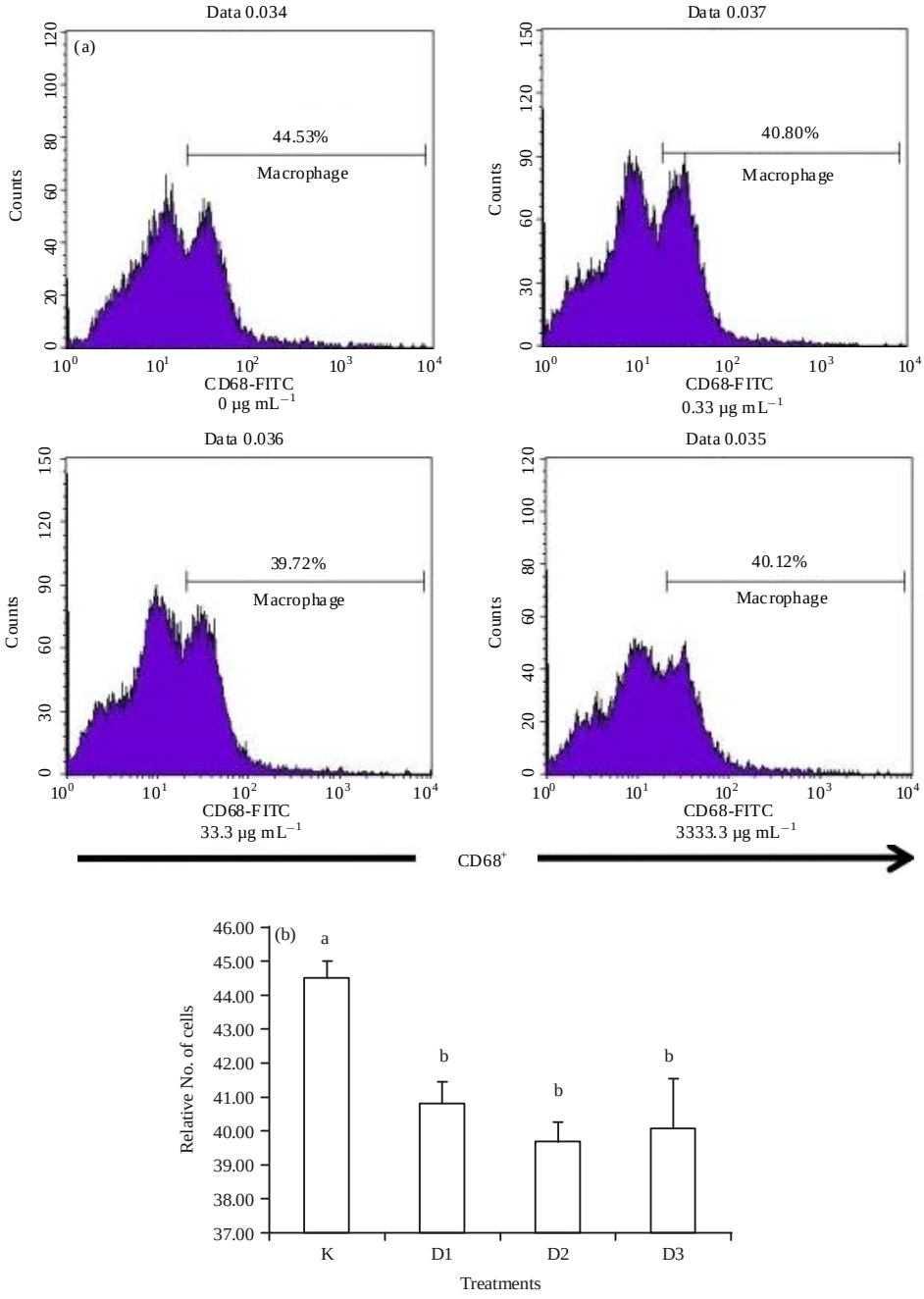

Fig. 1(a-b): Stimulation of cells using VipAlbumin® showed the decreasing of macrophage ($CD68^+$) cells. Spleen cells were cultured in RPMI medium with 10% FBS, anti-CD3 and LPS for five days. The K is control group. In D1 treatment culture was added with 0.33 µg mL^{-1} VipAlbumin®, D2 treatment was added with 33.3 µg mL^{-1} VipAlbumin® and then D3 was added with 3333.3 µg mL^{-1} VipAlbumin®. On day 5, cell cultures were harvested and analyzed using flowcytometry (left) and tabulated into Microsoft Excel (right). Macrophage ($CD68^+$) cells were presented in relative number. Data are Mean±SD in each group with p-value<0.05

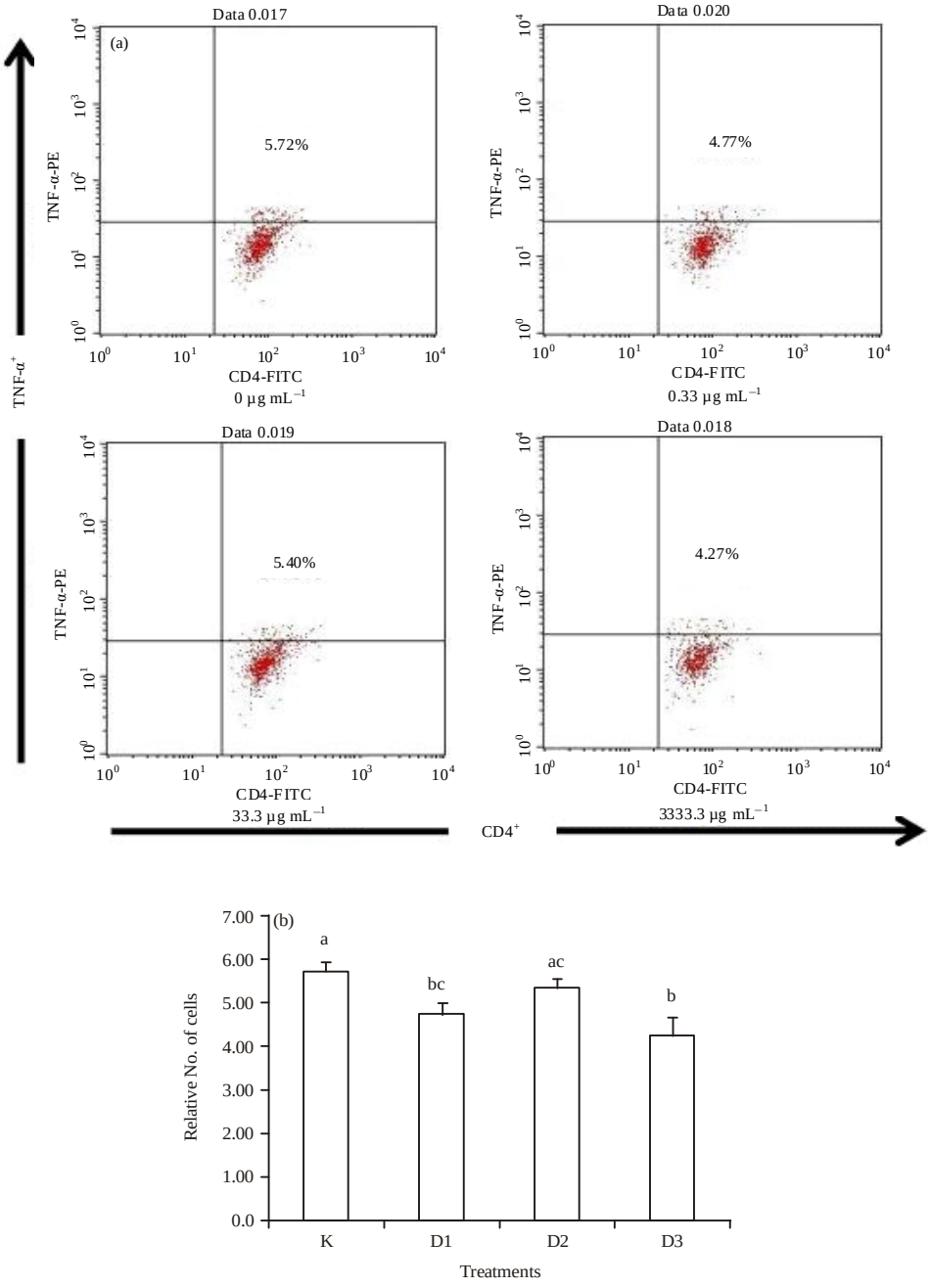

Fig. 2(a-b): VipAlbumin® was able to decrease TNF-α that produced by $CD4^+$ T cells. Spleen cells were cultured in RPMI medium with 10% FBS, anti-CD3 and LPS for five days. The K is control treatment. In D1 treatment culture was added with 0.33 µg mL^{-1} VipAlbumin®, D2 treatment was added with 33.3 µg mL^{-1} VipAlbumin® and then D3 was added with 3333.3 µg mL^{-1} VipAlbumin®. On day 5, cell cultures were harvested and analyzed using flowcytometry (left) and tabulated into Microsoft Excel (right). The TNF-α that produced by $CD4^+$ T cells was presented in relative number. Data are Mean±SD in each group with p-value<0.05

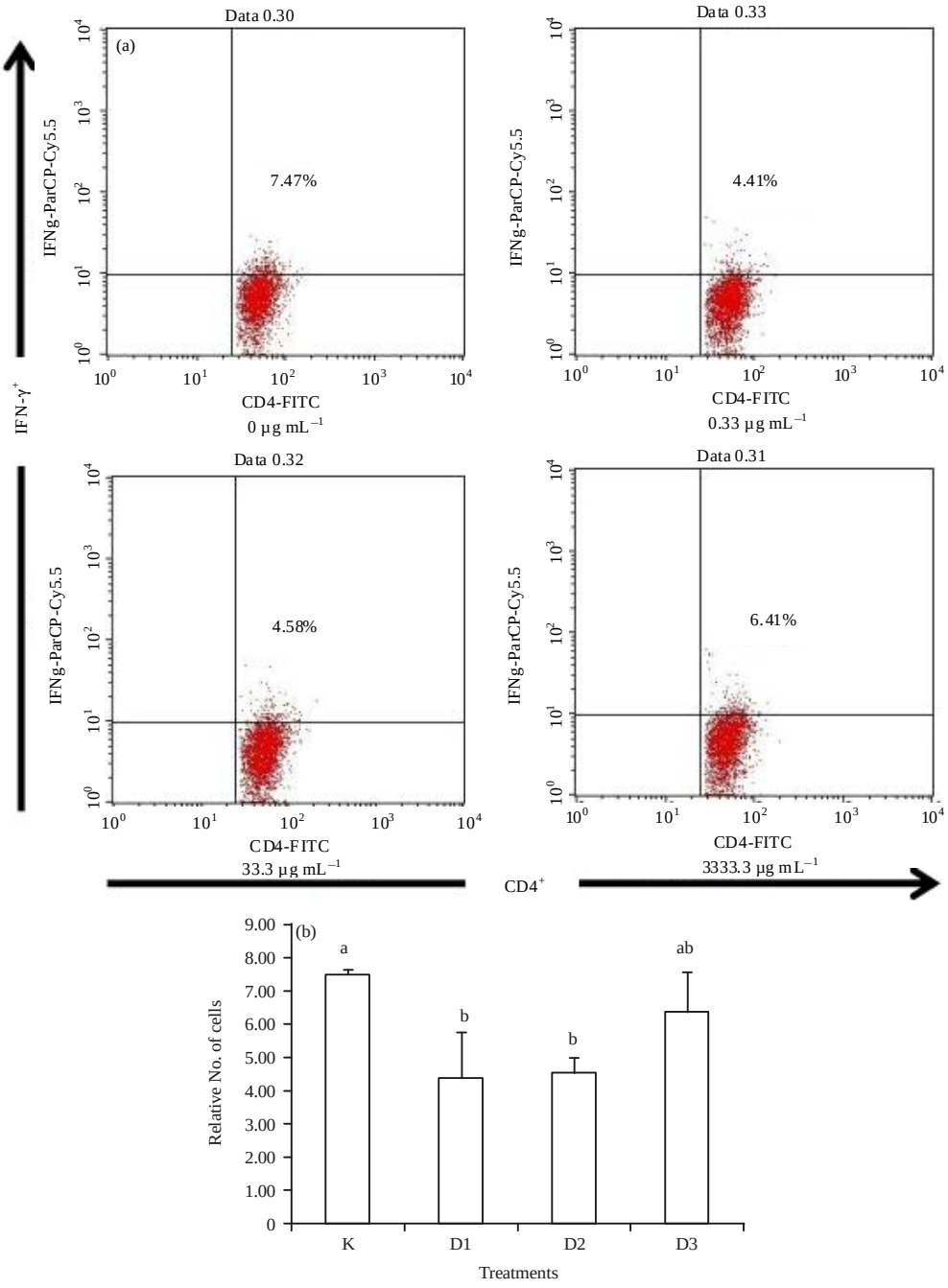

Fig. 3(a-b): Stimulation of lymphocyte cells using VipAlbumin® was decrease the IFN-γ that produced by $CD4^+$ T cells. Spleen cells were cultured in RPMI medium with 10% FBS, anti-CD3 and LPS for five days. The K is control treatment. In D1 treatment culture was added with 0.33 µg mL^{-1} VipAlbumin®, D2 treatment was added with 33.3 µg mL^{-1} VipAlbumin® and then D3 was added with 3333.3 µg mL^{-1} VipAlbumin®. On day 5, cell cultures were harvested and analyzed using flowcytometry (left) and tabulated into Microsoft Excel (right). The IFN-γ that produced by $CD4^+$ T cells was presented in relative number. Data are Mean±SD in each group with p-value<0.05

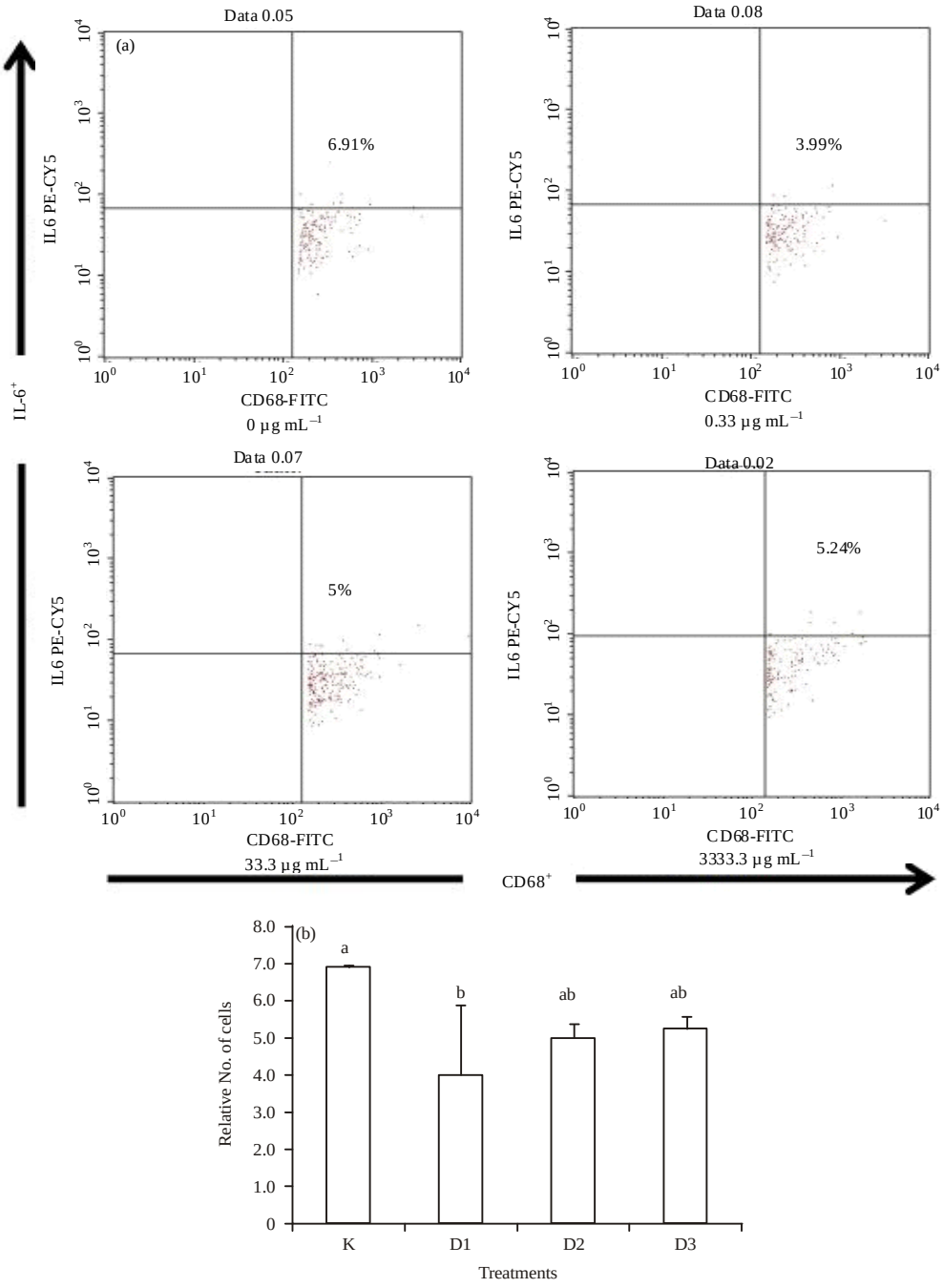

Fig. 4(a-b): VipAlbumin® had an efficacy to decrease IL-6 that produced by macrophage cells. Spleen cells were cultured in RPMI medium with 10% FBS, anti-CD3 and LPS for five days. The K is control treatment. In D1 treatment culture was added with 0.33 µg mL^{-1} VipAlbumin®, D2 treatment was added with 33.3 µg mL^{-1} VipAlbumin® and then D3 was added with 3333.3 µg mL^{-1} VipAlbumin®. On day 5, cell cultures were harvested and analyzed using flowcytometry (left) and tabulated into Microsoft Excel (right). The IL-6 that produced by macrophage cells was presented in relative number. Data are Mean±SD in each group with p-value<0.05

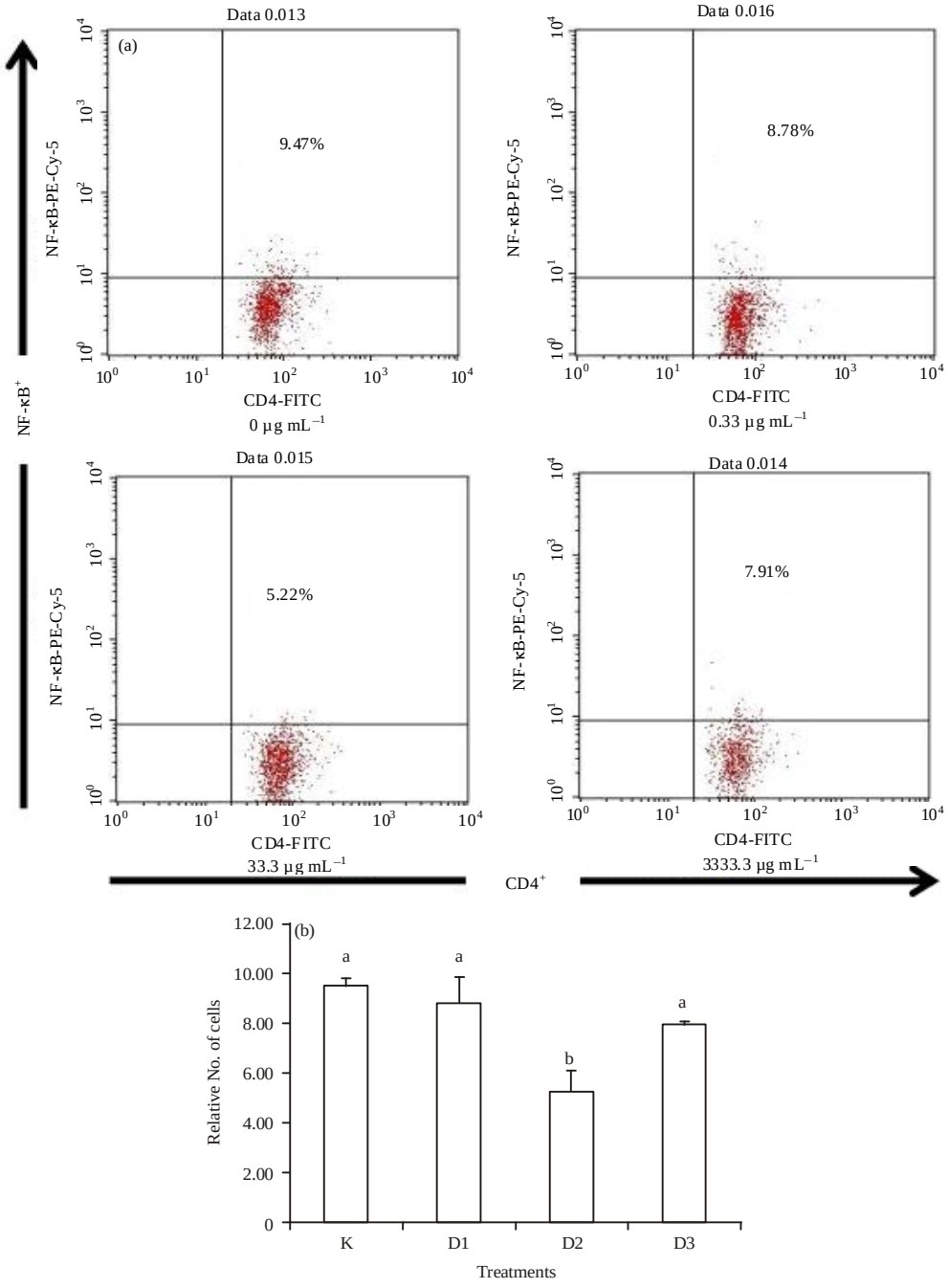

Fig. 5(a-b): Stimulation of lymphocyte cells using VipAlbumin® proved the decreasing of NF-κB on $CD4^+$ T cells. Spleen cells were cultured in RPMI medium with 10% FBS, anti-CD3 and LPS for five days. The K is control treatment. In D1 treatment culture was added with 0.33 µg mL^{-1} VipAlbumin®, D2 treatment was added with 33.3 µg mL^{-1} VipAlbumin® and then D3 was added with 3333.3 µg mL^{-1} VipAlbumin®. On day 5, cell cultures were harvested and analyzed using flowcytometry (left) and tabulated into Microsoft Excel (right). The NF-κB on $CD4^+$ T cells were presented in relative number. Data are Mean±SD in each group with p-value<0.05

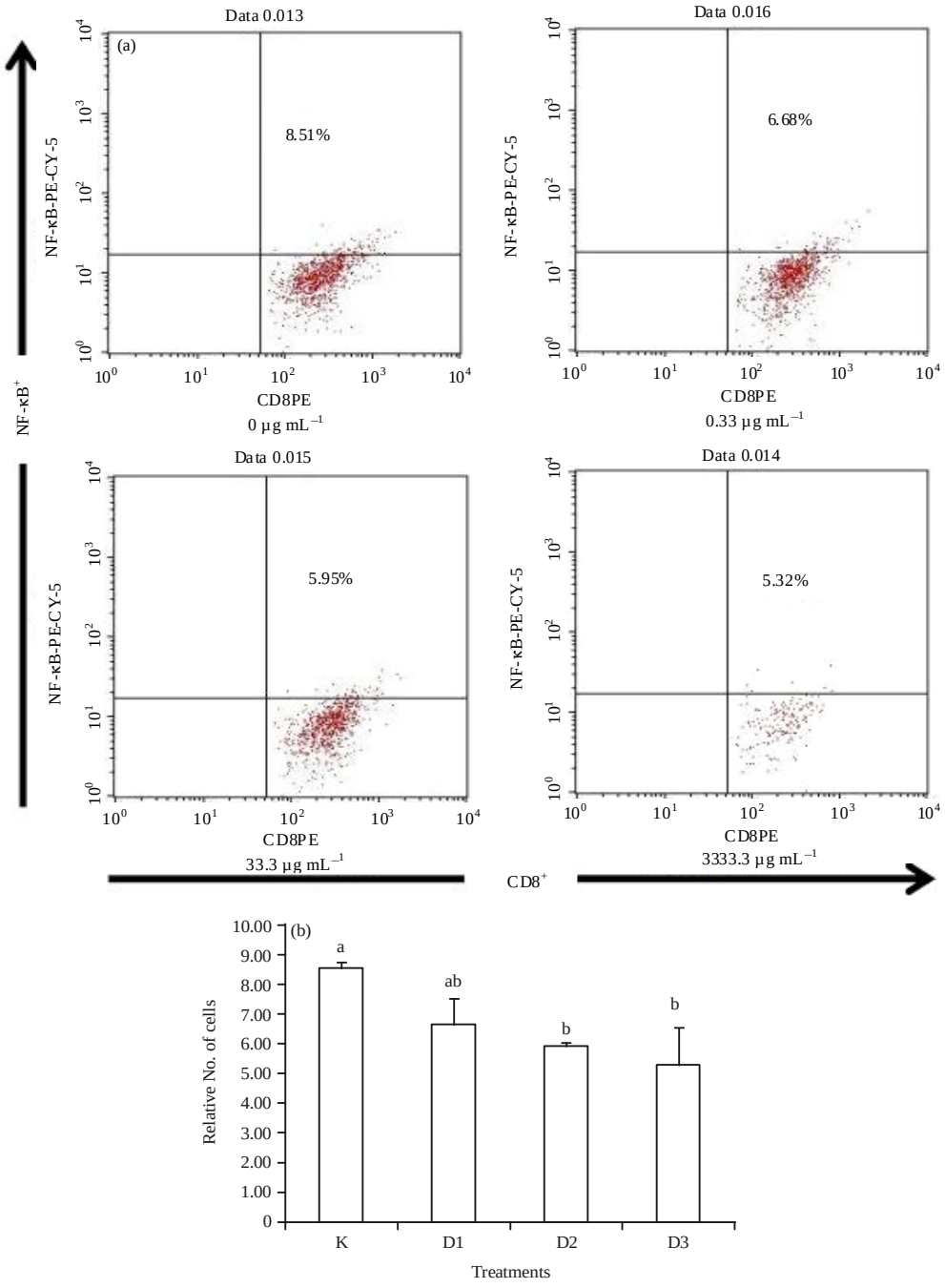

Fig. 6(a-b): Cultured of lymphocyte cells using VipAlbumin® stimulation showed the decreasing of NF-κB on $CD8^+$T cells. Spleen cells were cultured in RPMI medium with 10% FBS, anti-CD3 and LPS for five days. The K is control treatment. In D1 treatment culture was added with 0.33 μg mL^{-1} VipAlbumin®, D2 treatment was added with 33.3 μg mL^{-1} VipAlbumin® and then D3 was added with 3333.3 μg mL^{-1} VipAlbumin®. On day 5, cell cultures were harvested and analyzed using flowcytometry (left) and tabulated into Microsoft Excel (right). The NF-κB on $CD8^+$ T cells were presented in relative number. Data are Mean±SD in each group with p-value<0.05

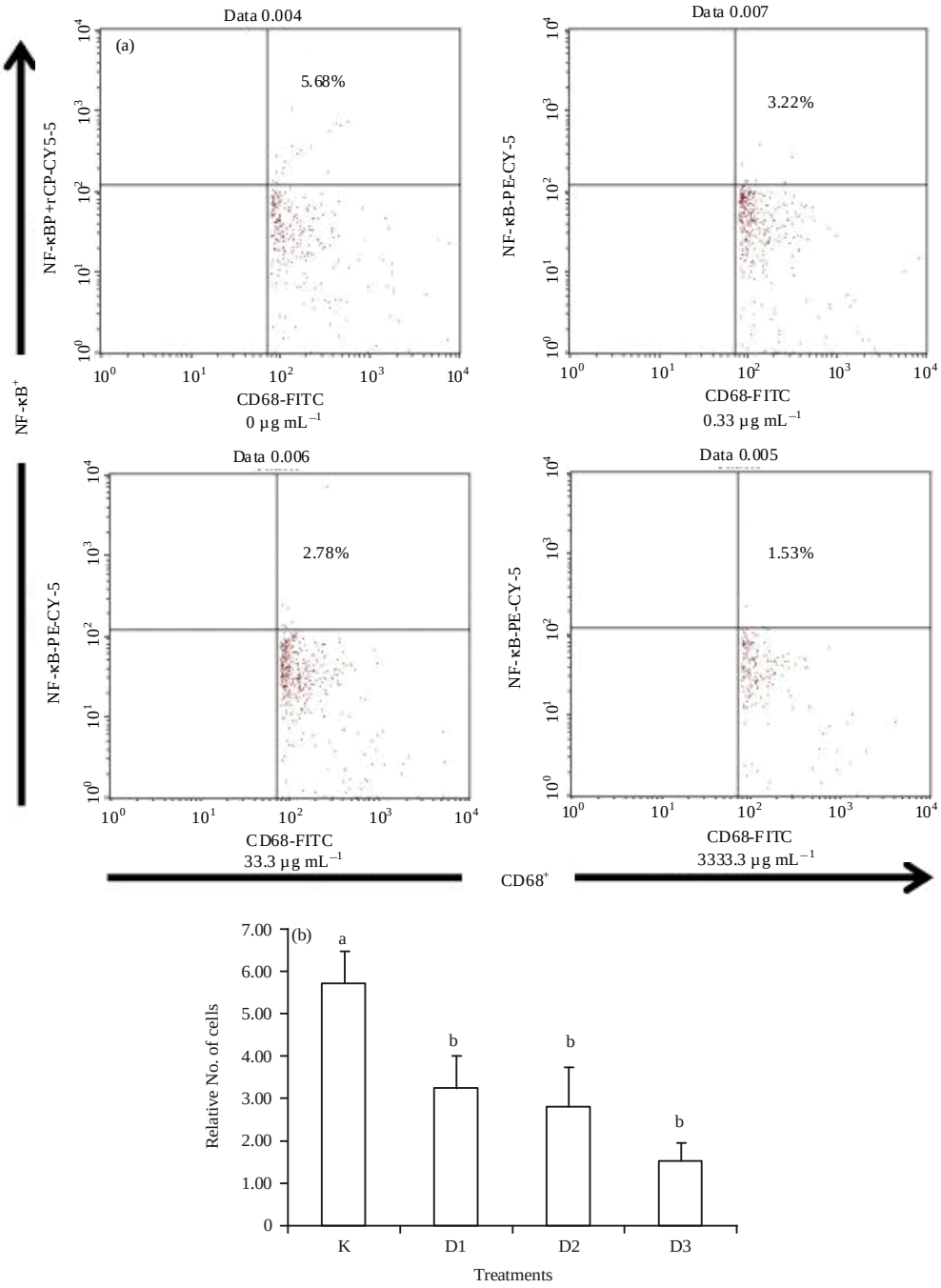

Fig. 7(a-b): Stimulation of VipAlbumin® was able to decrease the activation of NF-κB on macrophage cells. Spleen cells were cultured in RPMI medium with 10% FBS, anti-CD3 and LPS for five days. The K is control treatment. In D1 treatment culture was added with 0.33 μg mL^{-1} VipAlbumin®, D2 treatment was added with 33.3 μg mL^{-1} VipAlbumin® and then D3 was added with 3333.3 μg mL^{-1} VipAlbumin®. On day 5, cell cultures were harvested and analyzed using flowcytometry (left) and tabulated into Microsoft Excel (right). The NF-κB on macrophage cells were presented in relative number. Data are Mean±SD in each group with p-value<0.05

VipAlbumin® can suppress transcription factor NF-κB on T lymphocyte and macrophage cells: The NF-κB in immune cells plays a role as a transcription factor for pro-inflammatory cytokines. VipAlbumin® was proved to suppress NF-κB among in $CD4^+$ and $CD8^+$ T cells and macrophages ($CD68^+$). NF-κB in $CD4^+$ T cells *in vitro* assays can reduced significantly by D2 group compared with control 9.47% became 5.22% (Fig. 5).

VipAlbumin® also showed suppressant activity to NF-κB on $CD8^+$ T cells. Groups D2 and D3 *in vitro* treatment showed the significant decrease in the relative number of $CD8^+NF\text{-}\kappa B^+T$ cells compared to control 8.51% became 5.95 and 5.31% (Fig. 6). The last, activity of NF-κB suppression by VipAlbumin® also observed in macrophages ($CD68^+$). Therefore, the result proved that VipAlbumin® D1, D2 and D3 was able to suppress the relative number of $CD68^+NF\text{-}\kappa B^+$ cells significantly ($p<0.05$) compared to controls 5.68% became 3.22, 2.78 and 1.53% (Fig. 7).

DISCUSSION

The result of this study proved that VipAlbumin® have anti-inflammatory activity. Besides containing albumin, which has long been known to have benefit as an anti-inflammatory (Mustafa *et al*., 2012), VipAlbumin® taken from a crude extract from snakehead fish so it also contains other complex protein, omega-3 fatty acids, amino acids such as glycine, histidine, cysteine, glutamine and tryptophan, vitamin A, D_3 and E and mineral magnesium which also has a function as an anti-inflammatory. According to Simopoulos (2002) and Wall *et al*. (2010), Omega-3 fatty acids from fish act as anti-inflammatory because they can suppress a number of pro-inflammatory cytokines such as IL-1, IL-6 and TNF-α. Amino acids such as glycine, histidine, cysteine, glutamine and tryptophan have anti-inflammatory effects by suppressing the production of cytokines TNF-α, IFN-γ, IL-6, IL-1, preventing activation of macrophages and effector T cells and also inhibit NF-κB activation (Wheeler and Thurman, 1999; Stachlewitz *et al*., 2000; Liboni *et al*., 2005; Son *et al*., 2005; Hasegawa *et al*., 2012). Moreover, vit. A can inhibit cytokine IL-12, TNF-α and IFN-γ that produced by Th1 lymphocytes (Maggini *et al*., 2007). The $1,25(OH)_2D_3$, the active form of vit. D_3, also serves as an anti-inflammatory. $1,25(OH)_2D_3$ can inhibit maturation of Dendritic Cells (DC) that much secrete proinflammatory cytokines (DeLuca and Cantorna, 2001). The vit. E can prevent the production of pro-inflammatory cytokines including IL-1, IL-6, TNF and chemokines IL-8 that produced by monocytes and macrophages (Devaraj and Jialal, 1999; Munteanu and Zingg, 2007) and IFN-γ that produced by T cells (Winkler *et al*., 2007). Magnesium plays an important role in inflammatory disease because lack of magnesium in the body can cause several inflammatory disorders such as type 2 DM (Schulze *et al*., 2007; Villegas *et al*., 2009).

Macrophages are the dominant phagocyte at sites inflammation and the cellular and acellular debris encountered by macrophages can have profound effects on their inflammatory profile (Scull *et al*., 2010). This study proved that VipAlbumin® significantly decreased the relative number of macrophage cells compared to control. Fujiwara and Kobayashi (2005) also mentioned that macrophages participate in the auto-regulatory loop in the inflammatory process because macrophages produce a wide range of biologically active molecules involved in both beneficial and detrimental outcomes in inflammation. Activated macrophages are deactivated by anti-inflammatory cytokines and cytokine antagonists that are mainly produced by macrophages and also by anti-inflammatory substance.

The anti-inflammatory activity of VipAlbumin® could also decrease the relative number of pro-inflammatory cytokine namely TNF-α and IFN-γ that produced by $CD4^+$ T cells and also IL-6 that produced by macrophage ($CD68^+$) cells. TNF-α, also known as cachectin, acts on several

different signaling pathways through two cell surface receptors, TNFR1 and TNFR2 regulate apoptotic pathways, NF-κB activation in inflammation and activate stress-activated protein kinases (SAPKs). TNF-α has been shown to play important roles in both inflammatory and neuropathic hyperalgesia (Schafers *et al*., 2003). Induces of class I MHC on all somatic cells, induces class II MHC on APCs and somatic cells and activates macrophages, neutrophils and NK cells (Yoshimura *et al*., 2008). The IL-6 has been shown to play a central role in the neuronal reaction to nerve injury. The IL-6 is also involved in microglial and astrocytic activation as well as in regulation of neuronal neuropeptides expression (Ozaktay *et al*., 2006). There is an evidence that IL-6 contributes to the development of neuropathic pain behavior following a peripheral nerve injury (Zhang and An, 2007).

The decrease of cytokines number may relate with deactivation of NF-κB because the result showed the reduction in the number of NF-κB on $CD4^+$ and $CD8^+$ T lymphocytes and macrophages cells. This statement is in line with (Aupperle *et al*., 2001; Lawrence, 2009), who explains that the nuclear factor NF-κB pathway has long been considered a prototypical proinflammatory signaling pathway, mostly based on the role of NF-κB in the expression of proinflammatory genes including cytokines, chemokines and adhesion molecules. Thus, it means that the decreasing of that pro-inflammatory cytokines by VipAlbumin® could decrease both acute and chronic inflammation.

CONCLUSION

VipAlbumin® could decrease the number of macrophage cells, pro-inflammatory cytokines TNF-α, IFN-γ and IL-6 and also NF-κB on $CD4^+$ and $CD8^+$ T cells and macrophage cells. Hence, it proved that VipAlbumin® can be used to cure inflammatory disease because it has anti-inflammatory activity.

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