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The Potential of VipAlbumin® to Chronic Inflammation in Type 2 Diabetes Mellitus Balb/C Mice Model

Dinia Rizqi Dwijayanti, M. Sasmito Djati, Mansur Ibrahim and Muhaimin Rifa'i

Department of Biology, Brawijaya University, Malang, Indonesia

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Corresponding Author:
Muhaimin Rifa'i
Department of Biology, Brawijaya University, Malang, Indonesia
Email: rifa123@ub.ac.id

Abstract: Diabetes Mellitus (DM) is one of diseases which have increasing number of sufferers every year. Almost all DM patients are type 2 DM. One of the causes of type 2 DM is a chronic inflammation due to an increase of circulating proinflammatory cytokines such as TNF-α, IFN-γ and IL-6. VipAlbumin® from snakehead fish extract is expected to be useful as an alternative treatment of type 2 DM because of its proinflammatory activity. The aim of this study was to determine the effect of VipAlbumin® on regulatory T cell activation, macrophage cells, proinflammatory cytokines and NF-κB. The experiments were done by inducing mice model of type 2 DM with STZ 100 mg/kg BW and then gave them oral administration of VipAlbumin® 0 mg/g BW, 0.01664 mg/g BW, 0.0416 mg/g BW and 10.4 mg/g BW for 14 days. The data were statistically analyzed with one way ANOVA with significance value 0.05% and Tukey test using SPSS version 16 for Windows. The results showed the decreasing number of regulatory T cells in type 2 DM mice, increasing number of macrophage cells and proinflammatory cytokines TNF-α, IFN-γ and IL-6 as well as the increasing number of NF-κB as a transcription factor of inflammatory mediators in $CD4^+$ T cells, $CD8^+$ T cells and macrophages ($CD68^+$) compared to healthy mice ($p \leq 0.05$). Oral administration of VipAlbumin® for 14 days was proven to cure type 2 DM mice (K^+) by increasing the number of regulatory T cells, decreasing the number of macrophage cells and proinflammatory cytokines TNF-α, IFN-γ and IL-6 and inhibiting NF-κB in T lymphocytes $CD4^+$, $CD8^+$ and macrophages at the level equivalent to healthy mice and significantly different ($p \leq 0.05$) compared with K+ group.

Keywords: Inflammation, *Ophiocephalus Stiatus*, Regulatory T Cells, Type 2 DM, VipAlbumin®

Introduction

Diabetes Mellitus (DM) is one of pathophysiology condition that causes disturbance of glucose homeostatic mechanisms in blood. DM currently affects 170 million people worldwide and will increase to 365 million in 2030 (Wild *et al*., 2004). More than 90% of DM patients are type 2 DM (Yumi *et al*., 2013). In type 2 DM, a patient experiences a decrease in the ability of pancreatic β cells to and thus the insulin resists in peripheral tissue such as liver and kidney. One of the main causes of secrete insulin type 2 diabetes mellitus is a chronic inflammation that involves the role of immunocompetent cells and proinflammatory cytokines.

The chronic inflammatory phase in β cells is caused by an increase of Free Fatty Acid (FFA) which circulates proinflammatory cytokines, produces leptin and also decreases adiponectin (Yumi *et al*., 2013). Those factors will induce lipotoxicity, oxidative stress, ER stress and mitochondrial dysfunction in β cells so that the β cells can not secrete insulin normally (Hotamisligil, 2010; Dunmore and Brown, 2013).

Dula *et al*. (2010) explained that the circulation of inflammatory cytokines is associated with fatty tissue and β cell dysfunction. Pancreas of mice that was exposed to the cytokine TNF-α, IL-1β and IFN-γ overnight showed the damage of calcium regulation and when there was an was extended exposure combined with stress conditions, it will lead to β cell dysfunction.

Production of cytokines in β cells blocks the function of β cell due to the activation of NF-κB, transcription factor to proinflammatory cytokine (Weaver, 2012). According to Donath and Shoelson (2011), CXCL8 and CXCL10 and other chemokine produced by β cells are chemotactic for monocytes and macrophages. Brooks-Worrell *et al*. (2011) mentions that in the peripheral blood of patients with type 2 DM, it was also found 42% increase in T cells that react against β cells. These indicate that either innate or adaptive immune response is considered as one of the factors associated with type 2 diabetes.

Basically, the treatment of type 2 DM can be done directly by inhibiting proinflammatory cytokines and activation of regulatory T cells (Treg). Treg cell functions to regulate the performance of effector T helper cells (Th) and T cell cytotoxic (Tc). That regulation causes effector cells do not work reactively and do not secrete cytokines (Corthay, 2009; Sakaguchi *et al*., 2009; Rifa'i, 2014). However, a synthetic drug used to treat type 2 DM only focuses on increasing the level of insulin and its long-term use can cause side effects such as liver and kidney damage. Hence, it needs alternative treatments from natural substance to reduce these side effects.

Snakehead fish (*Ophiocephalus stiatus*) is one of Indonesia's natural resources that contain proteins, including albumin, which is higher compared to the other kind of fish. Several recent studies reveal many efficacies of albumin such as increasing Hb level, accelerating wound healing, increasing the number of serum albumin, anti-cancer, anti-inflammatory and so on (Mustafa *et al*., 2012). One of the supplements gotten from albumin snakehead fish is VipAlbumin® which is believed to be useful in the process of therapy of type 2 DM. Therefore, the aim of this study was to determine the effect of VipAlbumin® to regulatory T cells, macrophage cells, proinflammatory cytokines produced by immunocompetent cells and NF-κB on effector cells.

Materials and Methods

Induction of Type 2 DM Mice Model

Mice model of type 2 DM were obtained by injecting STZ at neonatal mice 5 day old at a dose of 100 mg/kg BW intraperitoneal. The mice were allowed to grow, fed and watered ad libitum. Blood glucose levels were measured with a glucometer after 4 weeks old. Mice were declared to suffer from type 2 diabetes when their blood glucose levels exceed 200 mg dL^{-1}.

Oral Administration of VipAlbumin® to Type 2 DM Mice Model

Positive diabetic type 2 mice were given VipAlbumin® orally at a dose of 0 mg/g BW (K+); 0.01664 mg/g BW (D1); 0.416 mg/g BW (D2); and 10.4 mg/g BW (D3). The dose is obtained from the conversion of the human dose that is 33.3 mg/kg BW. The administration of VipAlbumin® was conducted for 14 days. Blood glucose levels were measured every 3 days during the administration of VipAlbumin® to determine the change of blood glucose level.

Cell Isolation from Spleen

Mice were dislocated and dissected. Spleen was isolated and washed in petri dish containing PBS. Cells from mice spleen were isolated by crushing spleen in PBS. Homogenates of cell were centrifuged with a speed 2500 rpm, 10°C, for 5 min. Pellet was resuspended in 1 mL of PBS.

Immunocytochemistry and FACS Analysis

Spleen cell suspensions were divided into micro tubes A, B, C, D and E and then centrifuged with a speed of 2500 rpm, 10°C, for 5 min. Pellets were stained with antibodies. The combinations of antibodies used were A: FITC-conjugated rat anti-mouse CD4, PE-conjugated rat anti-mouse CD25 and PE/Cy5-conjugated rat anti-mouse CD62L; B: FITC-conjugated rat anti-mouse CD4, PE-conjugated rat anti-mouse CD8 and PE/Cy5-conjugated rat anti-mouse NF-κB; C: FITC-conjugated rat anti-mouse CD4, PE-conjugated rat anti-mouse TNF-α and PE/Cy5-conjugated rat anti-mouse IFN-γ; D: FITC-conjugated rat anti-mouse CD68 and PE/Cy5-conjugated rat anti-mouse NF-κB; E: FITC-conjugated rat anti-mouse CD68 and PE/Cy5-conjugated rat anti-mouse IL-6.

Extracellular staining was done to cell in micro tube A. Cells were stained with 1 µL of antibody that had been diluted with 50 µL of PBS 10% FBS and then incubated for 20 min in ice box at 4°C. Intracellular staining was done to cell in micro tube B, C, D and E. These cells were added with extracellular antibody for 20 min in ice box. After that, cells were added with 50 µL fixative solution cytofix/cytoperm and incubated for 20 min in ice box. Fixative solution was removed by 500 µL washing solution washperm and then centrifuged with a speed of 2500 rpm, 10°C, for 5 min. Pellets were stained with intracellular antibodies then incubated for 20 min in the ice box. After extracellular and intracellular staining procedure, cells were added with 500 µL of PBS and transferred into a flow cytometry cuvet. Each sample was analyzed with flow cytometer.

Experimental Design

This study is an explorative experiment and consists of five treatments. They are negative control (healthy mice and without VipAlbumin® administration), positive control (type 2 DM mice model and without VipAlbumin® administration), D1, D2 and D3 (type 2 DM mice model and with different concentration of VipAlbumin® administration). Each treatment was repeated 5 times.

Data Analysis

The data were analyzed by using BD cellquest PRO™ software then tabulated using Microsoft Excel and analyzed statistically. Statistical analysis used was a parametric one-way ANOVA with significance of 0.05% and followed with Tukey test.

Results

VipAlbumin® Increased the Number of Regulatory T Cells

The administration of VipAlbumin® can increase significantly ($p \leq 0.05$) the relative number of regulatory T (Treg) cells in type 2 DM mice models compared to control (Fig. 1). The number of Treg cells in control group of type 2 DM mice models (K+) significantly decreased ($p \leq 0.05$) compared to healthy mice (K-) from 53.24% became 43.79%. Relatively, the number of type 2 DM mice models which were given orally VipAlbumin® was increasing and it became 63.54% in D1, 64.40% in D2 and 65.55% in D3 group.

VipAlbumin® Decreased the Relative Number of Macrophage Cells ($CD68^+$)

It is found out that the number of macrophage cells increases in type 2 DM patients, especially in pancreas tissue. It happens because macrophage plays an important role in chronic inflammation as a proinflammatory cytokine producer. In this study, the number of macrophages cells increased significantly in mice models of type 2 DM (K+) compared to healthy mice (K-), 4.09% doubling to 8.23%. The administration of VipAlbumin® in mice model of type 2 DM, among D1, D2 and D3, significantly decreased the number of macrophages cells compared to controls (K+) became 1.64, 1.57 and 1.44% (Fig. 2).

VipAlbumin® Decreased the Number of Proinflammatory Cytokine TNF-α, IFN-γ and IL-6

Proinflammatory cytokines produced by immune cells is one of chronic inflammation mediators in pancreas that causes pancreatic β cells damage. These proinflammatory cytokines include TNF-α, IFN-γ and IL-6. In vivo test, it showed a decrease in the relative number of TNF-α produced by $CD4^+$ T cells in mice models of type 2 DM after oral administration of VipAlbumin® for 14 days (Fig. 3). Relative number of TNF-α produced by $CD4^+$ T cells in healthy mice (K-) was 1.49% and increased significantly ($p \leq 0.05$) became 6.93% in mice models of type 2 DM (K+). The administration of VipAlbumin® in group D1, D2 and D3 significantly decreased the relative number of TNF-α produced by $CD4^+$ T cells ($p \leq 0.05$) than K+ group became 1.11%, 1.89% and 0.39%.

VipAlbumin® reduced the relative number of cytokine IFN-γ t produced by CD4+ T cells. This study showed the increase of relative number of IFN-γ secreted by $CD4^+$ T cells in type 2 DM mice model (K+) compared to healthy mice (K-), 1.12% became 4.63% or 4 times higher than normal conditions (Fig. 4). Mice model of type 2 DM which were given oral administration of VipAlbumin® showed that it was like a control (K-) relative number; they were 0.84% (D1), 2.18% (D2) and 1.64% (D3). These numbers were significantly different ($p \leq 0.05$) with K+ group.

Cytokines IL-6 detected in this study was IL-6 produced by macrophages ($CD68^+$). Relative number of cytokine IL-6 produced by macrophages in normal conditions (K-) was 1%. In mice models of type 2 DM (K+) this number increased significantly ($p \leq 0.05$) became 4.6%. On the other hand, oral administration of VipAlbumin® D1, D2 and D3 in type 2 DM were proven significantly reduced that number ($p \leq 0.05$) became 2.26, 1.47 and 1.55%. The numbers of IL-6 produced by macrophages were not significantly different from normal conditions (Fig. 5).

VipAlbumin® Suppressed NF-κB in T Lymphocyte and Macrophage Cells

Proinflammatory cytokine produced by immune cells is played by NF-κB, the transcription factor for proinflammatory cytokines. In this study, NF-κB in T cells and macrophages in type 2 DM mice model (K+) were significantly increased compared with healthy mice (K-). VipAlbumin® suppressed NF-κB among in lymphocytes $CD4^+$, $CD8^+$ T cells and macrophages ($CD68^+$). Oral administration of VipAlbumin® in type 2 DM had suppression activity to relative number of $CD4^+NF\text{-}\kappa B^+$ in VipAlbumin® treatment group (D1, D2, D3) significantly ($p \leq 0.05$) compared with control (K+), 9.42% became 1.51% in D1, 2.32% in D2 and 1.94% in D3, close to normal conditions (K-) was 1.18% (Fig. 6).

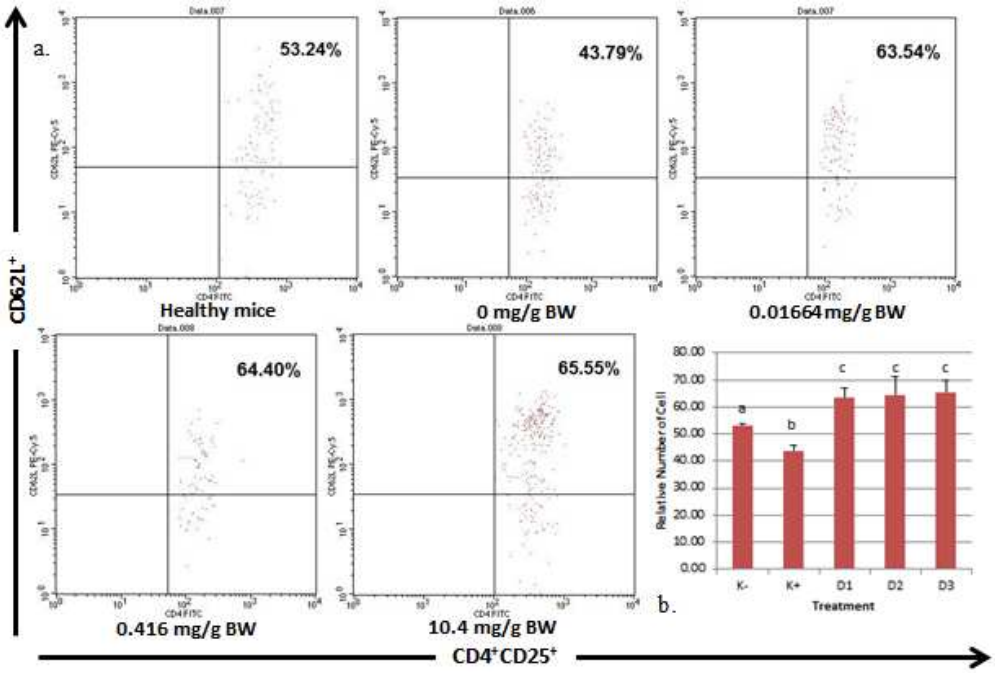

Fig. 1. The administration of VipAlbumin® in type 2 DM mice model can increase relatively good number of T regulatory cells. K- is healthy mice group. Mice model of type 2 DM were treated with VipAlbumin® 0 mg/gr BW (K+); 0.01664 mg/gr BW (D1); 0.416 mg/gr BW (D2); and 10.4 mg/gr BW (D3) for 14 days. After 14 days, mice were dissected, cells were isolated from spleen and then analyzed using flow cytometry (a) and tabulated using Microsoft Excel (b). T regulatory cell ($CD4^+CD25^+CD62L^+$) were presented in relative number. The data are mean±SD in each group with p value ≤0.05.

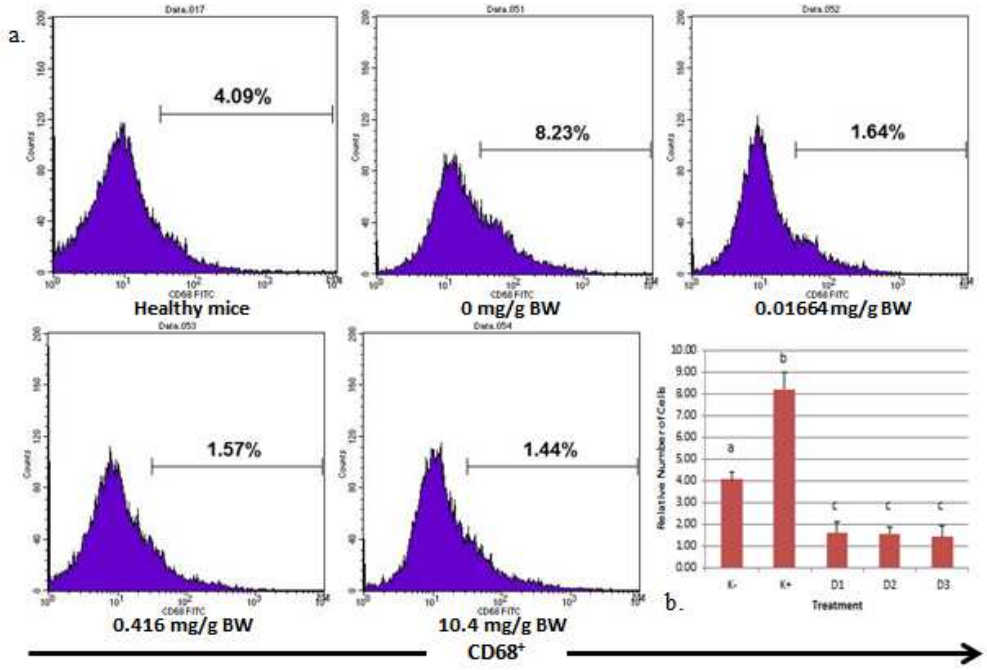

Fig. 2. The administration of VipAlbumin® in type 2 DM mice model can decrease relative number of macrophage cells. K- is healthy mice group. Mice model of type 2 DM were treated with VipAlbumin® 0 mg/gr BW (K+); 0.01664 mg/gr BW (D1); 0.416 mg/gr BW (D2); and 10.4 mg/gr BW (D3) for 14 days. After 14 days, mice were dissected, cells were isolated from spleen and then analyzed using flow cytometry (a) and tabulated using Microsoft Excel (b). Macrophage ($CD68^+$) cells were presented in relative number. The data are mean±SD in each group with p value ≤0.05

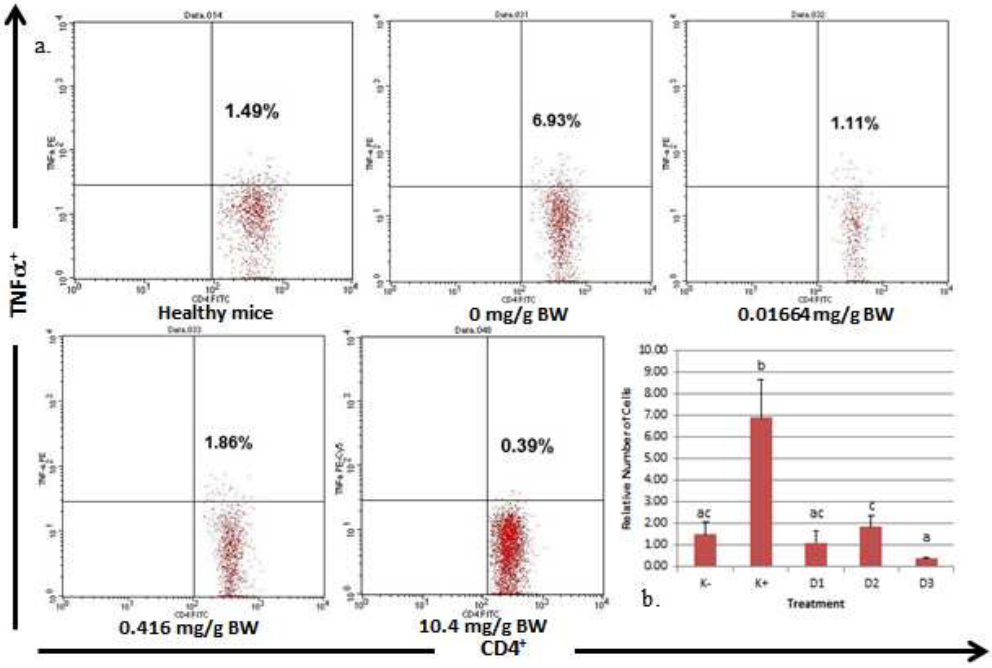

Fig. 3. The administration of VipAlbumin® in type 2 DM mice model can decrease relative number of TNF-α produced by $CD4^+$ T cells. K- is healthy mice group. Mice model of type 2 DM were treated with VipAlbumin® 0 mg/gr BW (K+); 0.01664 mg/gr BW (D1); 0.416 mg/gr BW (D2); and 10.4 mg/gr BW (D3) for 14 days. After 14 days, mice were dissected, cells were isolated from spleen and then analyzed using flow cytometry (a) and tabulated using Microsoft Excel (b). TNF-α produced by $CD4^+$ T cells was presented in relative number. The data are mean±SD in each group with p value ≤0.05

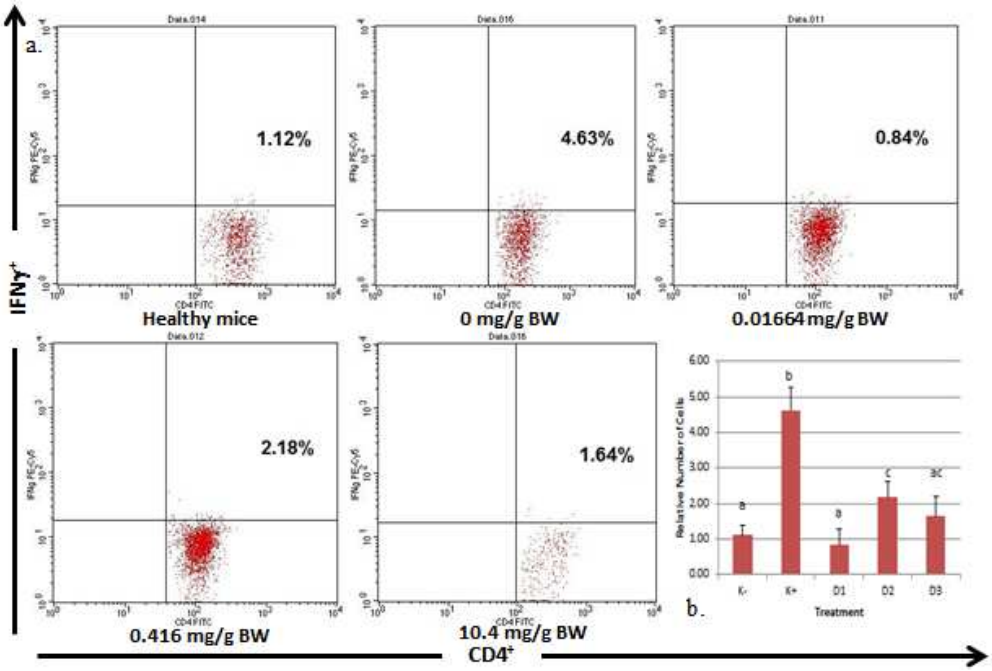

Fig. 4. The administration of VipAlbumin® in type 2 DM mice model can decrease relative number of IFN-γ produced by $CD4^+$ T cells. K- is healthy mice group. Mice model of type 2 DM were treated with VipAlbumin® 0 mg/gr BW (K+); 0.01664 mg/gr BW (D1); 0.416 mg/gr BW (D2); and 10.4 mg/gr BW (D3) for 14 days. After 14 days, mice were dissected, cells were isolated from spleen and then analyzed using flow cytometry (a) and tabulated using Microsoft Excel (b). IFN-γ produced by $CD4^+$ T cells was presented in relative number. The data are mean±SD in each group with p value ≤0.05

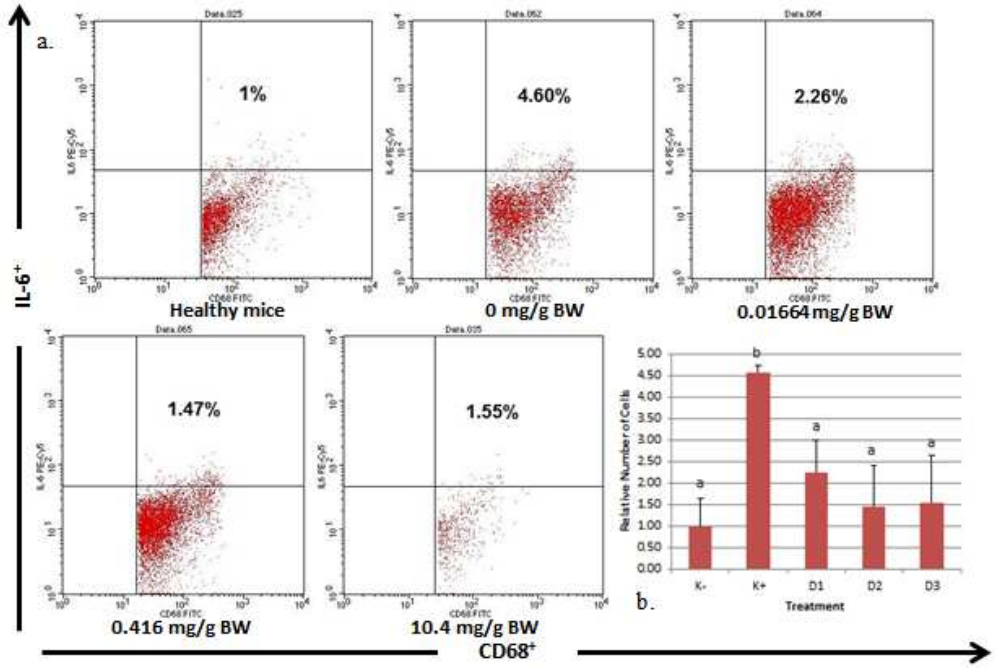

Fig. 5. The administration of VipAlbumin® in type 2 DM mice model can decrease relative number of IL-6 produced by $CD68^+$ cells. K- is healthy mice group. Mice model of type 2 DM were treated with VipAlbumin® 0 mg/gr BW (K+); 0.01664 mg/gr BW (D1); 0.416 mg/gr BW (D2); and 10.4 mg/gr BW (D3) for 14 days. After 14 days, mice were dissected, cells were isolated from spleen and then analyzed using flow cytometry (a) and tabulated using Microsoft Excel (b). IL-6 produced by macrophage cells were presented in relative number. The data are mean±SD in each group with p value ≤0.05

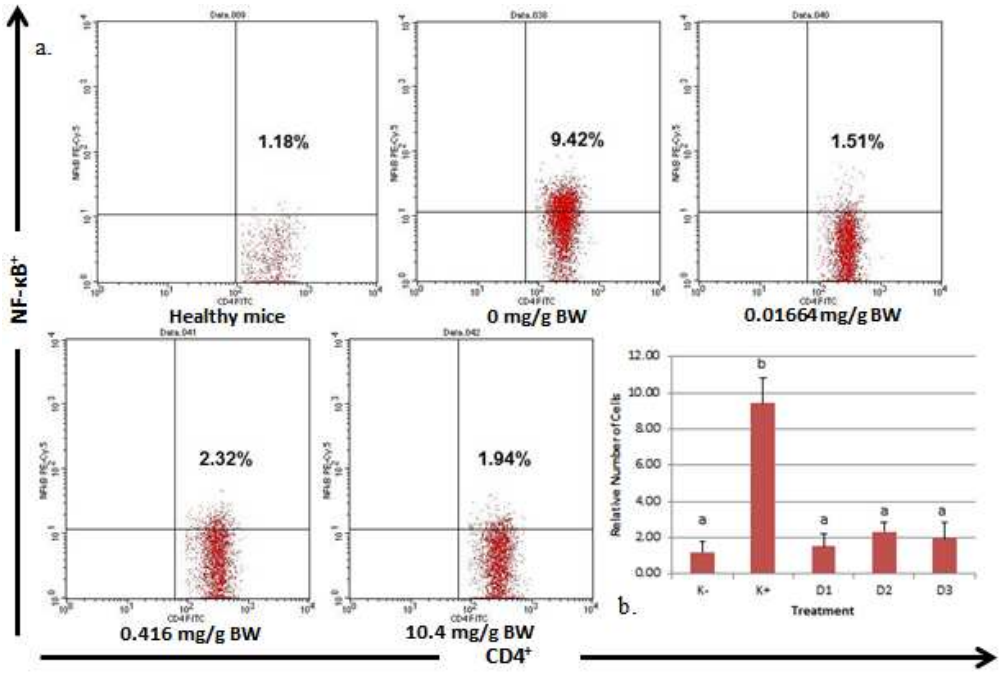

Fig. 6. The administration of VipAlbumin® in type 2 DM mice model can decrease relative number of NF-κB on $CD4^+$ T cells. K- is healthy mice group. Mice model of type 2 DM were treated with VipAlbumin® 0 mg/gr BW (K+); 0.01664 mg/gr BW (D1); 0.416 mg/gr BW (D2); and 10.4 mg/gr BW (D3) for 14 days. After 14 days, mice were dissected, cells were isolated from spleen and then analyzed using flow cytometry (a) and tabulated using Microsoft Excel (b). NF-κB on $CD4^+$ T cells were presented in relative number. The data are mean±SD in each group with p value ≤0.05

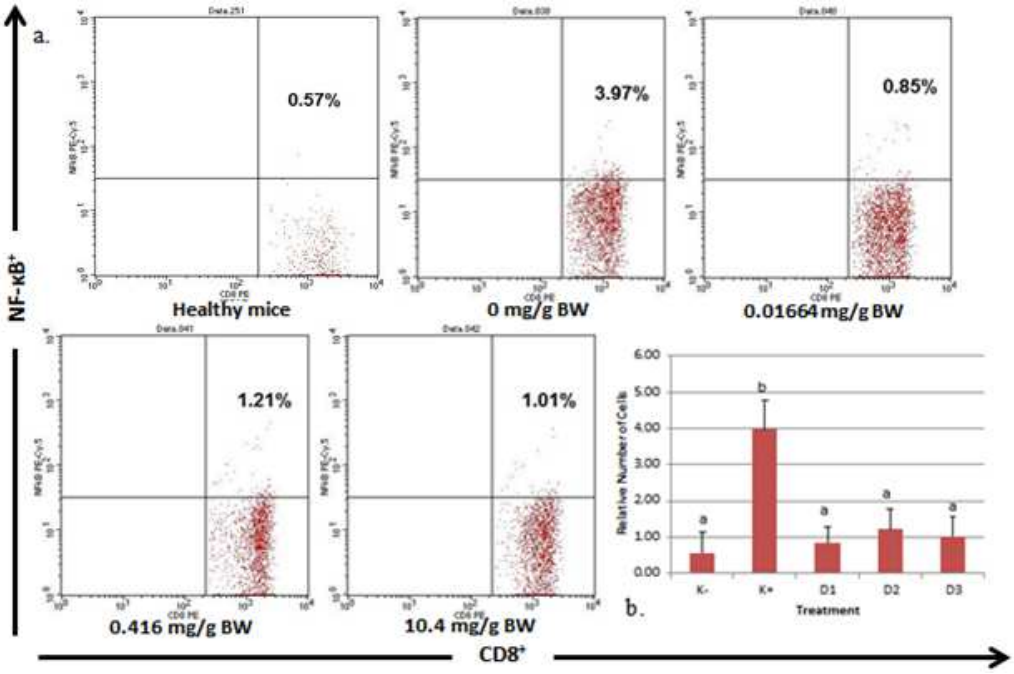

Fig. 7. The administration of VipAlbumin® in type 2 DM mice model can decrease relative number of NF-κB on $CD8^+$ T cells. K- is healthy mice group. Mice model of type 2 DM were treated with VipAlbumin® 0 mg/gr BW (K+); 0.01664 mg/gr BW (D1); 0.416 mg/gr BW (D2); and 10.4 mg/gr BW (D3) for 14 days. After 14 days, mice were dissected, cells were isolated from spleen and then analyzed using flow cytometry (a) and tabulated using Microsoft Excel (b). NF-κB on $CD8^+$ T cells were presented in relative number. The data are mean±SD in each group with p value ≤ 0.05

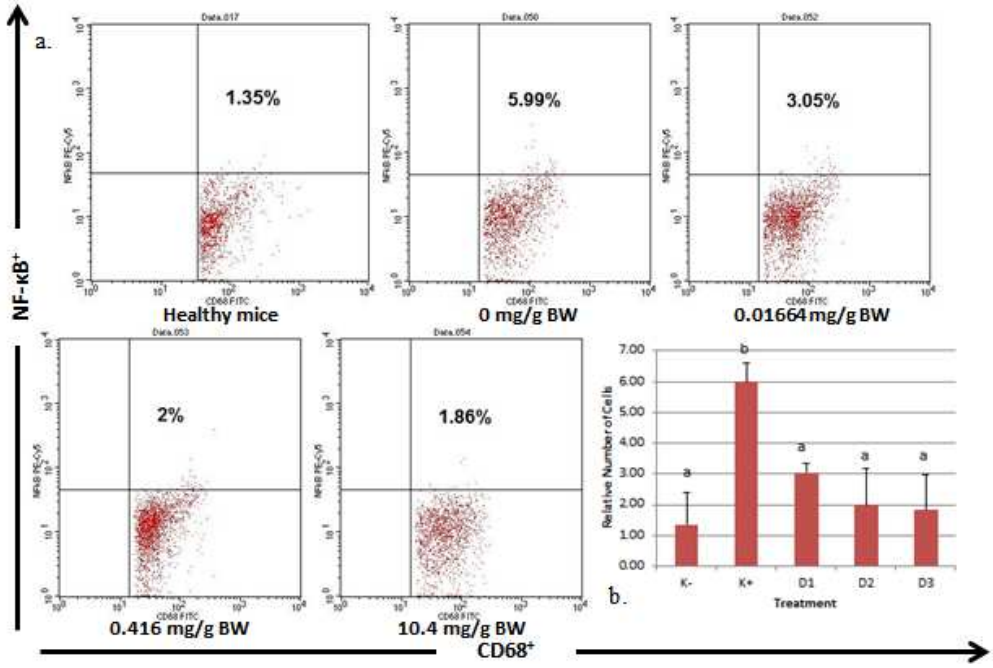

Fig. 8. The administration of VipAlbumin® in type 2 DM mice model can decrease relative number of NF-κB on macrophage cells. K- is healthy mice group. Mice model of type 2 DM were treated with VipAlbumin® 0 mg/gr BW (K+); 0.01664 mg/gr BW (D1); 0.416 mg/gr BW (D2); and 10.4 mg/gr BW (D3) for 14 days. After 14 days, mice were dissected, cells were isolated from spleen and then analyzed using flow cytometry (a) and tabulated using Microsoft Excel (b). NF-κB on macrophage cells were presented in relative number. The data are mean±SD in each group with p value ≤ 0.05

VipAlbumin® also showed suppression activity to NF-κB in $CD8^+$ T cells. Relative number of $CD8^+$NF-$κB^+$ T cells in treatment group was lower than control group (K+) and significantly different ($p \leq 0.05$) from 3.97% became 0.85, 1.21 and 1.01%. These numbers were not significantly different from healthy mice (K-), which was 0.57 (Fig. 7).

Finally, the suppression activity of NF-κB by VipAlbumin® was also observed in macrophages ($CD68^+$). In treatment group, the number of $CD68^+$NF-$κB^+$ cells was significantly lower ($p \leq 0.05$) than control (K+) group which was 5.99% became 3.05% in D1, 2% in D2 and 1.86% in D3 (Fig. 8). The results of treatment groups were not significantly different ($p > 0.05$) from healthy mice group (K-) which was 1.35%. Overall, the number of NF-$κB^+$ cells in treatment group did not show a significant difference with healthy mice group. It means that the treatment group of mice had returned to normal condition or became healthy.

Discussion

Treg cell has a function as a suppressant which mediated suppression as a vital mechanism of negative regulation of immune-mediated inflammation that usually occurs in autoimmune, autoinflammation, allergies, acute and chronic infections, cancer and chronic inflammation including cases of diabetes mellitus (Rifa'i, 2010; Josefowicz *et al*., 2012). This study proved that mice model of type 2 DM have decreased the number of Treg cells significantly compared to the control. It showed that homeostasis of Treg cell number greatly affected the chronic inflammation that occurs in type 2 DM.

The efficacy of VipAlbumin® in increasing the number of Treg cells in type 2 DM can help the healing process of type 2 DM by slowing down the chronic inflammation that occurs in pancreas. Components contained in VipAlbumin® may bind to receptors on the surface of T cells that have an impact on the activation of PKC-θ, Bcl-10, CARMA1 or IKK2. Four of these molecules are signaling pathway from TCR to NF-κB activation that leads the role in the process of differentiation of naïve T cells into Treg cells (Feuerer *et al*., 2009).

VipAlbumin® components that affect the activation of Treg cells are vit.A and Vit.D. Main metabolite of Vit.A, ATRA can change the naïve $CD4^+FoxP_3^-$ T cells into Treg cells $FoxP3^+$. Histone acetylation in the $FoxP_3$ gene promoter is induced when ATRA binds to the nuclear retinoic acid receptor α, resulting in the expression of $FoxP_3$ protein in $CD4^+$ T cells and becomes Treg cells. ATRA also can prevent the conversion of Treg cells into Th17 proinflammatory cells induced by IL-6 (Issazadeh-Navikas *et al*., 2011). Besides vit.A, Vit.D especially D_3 also affects the activation of Treg cells. Gregori *et al*. (2002) and Issazadeh-Navikas *et al*. (2011) explains that the active form of Vit.D_3, 1,25-$(OH)_2D_3$, can induce an increase in Treg cells.

Alpan *et al*. (2004) and Rifa'i and Widodo (2011) explained that the role of Treg cells is to create the tolerance conditions and homeostasis in immune system because Treg cells is able to regulate and control the effector cells that have been activated to avoid autoreactivity of effector cells, including the ability of effector cells to secrete proinflammatory cytokine (Yamazaki *et al*., 2003). Suppressive activity of Treg cells on effector cells can be through four main mechanisms. First, Treg cells have capability to secrete cytokines that can inhibit the function of effector cells directly, such as IL-10, TGF-β and IL-35. These cytokines are classified as inhibitors cytokine that contribute to the inhibition of effector cell function directly (Collison *et al*., 2007). Second, it acts as competitors for effector cells in utilizing IL-2 because Treg cells express three molecules which have a high affinity for IL-2, namely CD25 (receptor α chain IL-2), CD122 and CD132 (Yu *et al*., 2009). Third, activated Treg cell has a function as cytotoxic cells that secrete granzyme A and granzyme B and some other types of perforin that cause apoptosis of effector cells directly (Grossman *et al*., 2004; Gondek *et al*., 2005). The last, Treg cells can express molecules such as galectin-1 which is one of a family of β-galactosidase binding protein that will cause cell cycle arrest and apoptosis in effector cells (Garin *et al*., 2007). These mechanisms can suppress the activity of effector cells especially in production of proinflammatory cytokines.

Macrophages are phagocytic cells that are most responsible for chronic inflammatory process, especially in type 2 DM because macrophages can produce proinflammatory cytokines (Scull *et al*., 2010). This study proved that the number of macrophages ($CD68^+$) in type 2 DM mice model is higher than normal condition and VipAlbumin® can reduce this number almost equivalent to healthy mice. This decreasing number can occur because activated Treg can suppress macrophage cells through four main mechanisms which have been described previously. On the other hand, the content of VipAlbumin® also acts directly as an anti-inflammatory and thus inhibits the activation of macrophage cells. Fujiwara and Kobayashi (2005) also explain that macrophages can be deactivated by an anti-inflammatory agent.

As mentioned before, one of the causes of type 2 DM is a systemic inflammation that occurs in β cells, adipose tissue, liver and muscle (Morino *et al*., 2006). The main trigger in β cells inflammation is the same as fatty tissue, liver and muscle as they are the excessive of saturated Free Fatty Acids (FFA), lipid mediators such

as 12 (S)-hydroxyeicosatetraenoic acid [12(S)-HETE], an increase of glucose level and proinflammatory cytokines and chemokines such as TNF-α, IFN-γ and IL-6 (Yumi *et al*., 2013). The results of this study are in line with the theory stating that proinflammatory cytokine TNF-α, IFN-γ and IL-6 significantly increase in mice model of type 2 DM compared to the control. In the case of type 2 DM, proinflammatory cytokines secreted by immunocompetent cells is one of the inflammatory mediators that cause β cell dysfunction. VipAlbumin® was proven to reduce the number of proinflammatory cytokines TNF-α, IFN-γ and IL-6 in mice models of type 2 DM at an equal level to the number of cytokine in healthy mice.

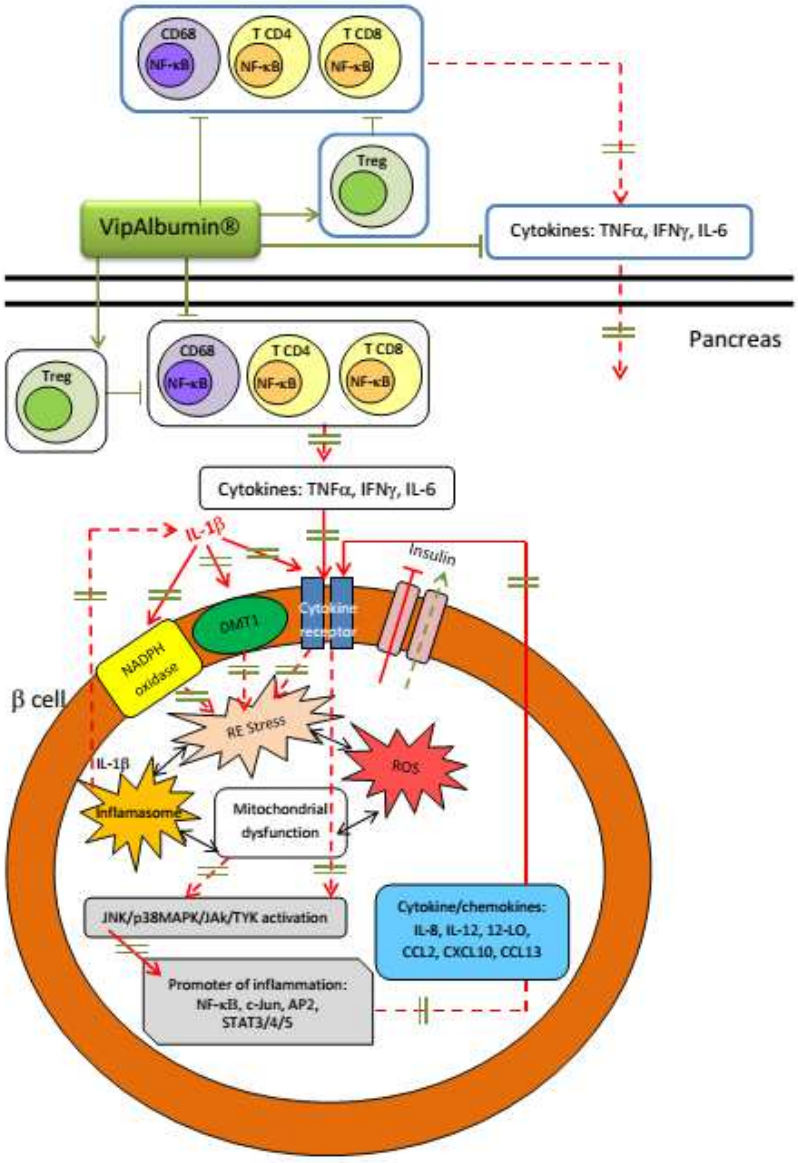

Fig. 9. The mechanism of VipAlbumin® in curing type 2 DM. Interleukin-1β is a pro-inflammatory cytokines that can activate NADPH oxidase and Divalent Metal Transporter 1 (DMT1); both lead to an increase in Reactive Oxygen Species (ROS) and oxidative stress. ROS activates JNK, p 38 MAPK or nuclear factor NF-κB. Inflammation in pancreatic β cells will lead to the recruitment and activation of immune cells in pancreas tissues such as $CD4^+$ T cells, $CD8^+$ T cells and macrophages ($CD68^+$). These immune cells will secrete pro-inflammatory cytokines. Cytokines produced locally or from circulation such as TNF-α, IFN-γ and IL-6 also activate inflammatory pathways via specific cytokine receptors in β cells. Insulin secretion is further reduced by this chronic inflammation. VipAlbumin is expected to activate regulatory T cell, inhibit proinflammatory cytokines and suppress activation of NF-κB in effector cells. The reduction of proinflammatory cytokines will reduce chronic inflammation on pancreatic β cells so pancreatic β cells can secrete insulin normally

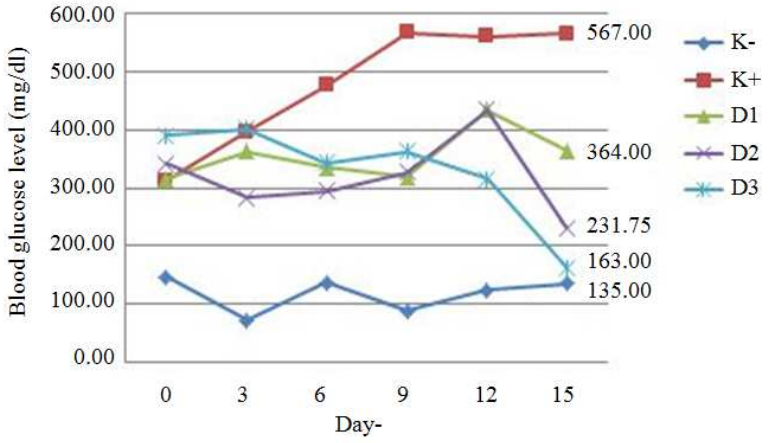

Fig. 10. Blood glucose level of mice in each treatment

In effector cells, NF-κB is a transcription factor that binds to the promoter gene of inflammatory mediators that affect the production and secretion of proinflammatory cytokines (Silverman and Maniatis, 2001; Caamano and Hunter, 2002; Liang *et al*., 2004; Yumi *et al*., 2013). The decrease of NF-κB level will decrease proinflammatory cytokine such as TNF-α, IFN-γ and IL-6 (Huang *et al*., 2002; Lawrence, 2009). This study also showed that NF-κB played an important role in the inflammatory process in type 2 DM due to an increase in NF-κB significantly in mice models of type 2 DM compared to controls. VipAlbumin® in this study can decrease the number of NF-κB in $CD4^+$ and $CD8^+$ T cells and macrophages cells at levels which are not significantly different from healthy mice.

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 also contains other complex protein, omega-3 fatty acids, amino acids glycine, histidine, cysteine, glutamine and tryptophan and the mineral magnesium which also has a function as an anti-inflammatory. According to Simopoulos (2002) and Wall *et al*. (2010), Omega-3 fatty acids, especially those derived from fish, act as anti-inflammatory and they are proven to suppress a number of pro-inflammatory cytokines such as IL-1, IL-6 and TNF-α. Several types of amino acids such as glycine, histidine, cysteine, glutamine and tryptophan have anti-inflammatory effects by preventing the production of cytokines TNF-α, IFN-γ, IL-6, IL-1, preventing activation of effector T cells and macrophages, as well as inhibiting NF-κB activation (Wheeler and Thurman, 1999; Stachlewitz *et al*., 2000; Son *et al*., 2005; Liboni *et al*., 2005; Hasegawa *et al*., 2011). Magnesium also plays a role as an anti-inflammatory due to lack of magnesium in the body can cause several metabolic disorders such as type 2 DM (Schulze *et al*., 2007; Villegas *et al*., 2009).

The efficacies of VipAlbumin® in increasing the number of Treg cells, decreasing the number of macrophage cells and proinflammatory cytokine and inhibiting NF-κB in immunocompetent cells proved that it had a direct impact on reducing inflammation and further will impact on the reparation of pancreatic β cells, so they can secrete insulin normally (Fig. 9). Furthermore, the inflammation that occurs in other tissues such as the liver and muscle will slow down, so that insulin receptors on this tissue will work normally. The healing process of mice model of type 2 DM after oral administration of VipAlbumin® was in line with a decrease of blood glucose levels closer to normal condition < 200 mg dL^{-1} (Fig. 10).

Conclusion

Potential of VipAlbumin® as an anti-inflammatory can be used as a supplement to cure the chronic inflammation, especially type 2 DM. Healing activity that occurs through the activation of Treg cells decreases the relative number of macrophage cells and proinflammatory cytokines TNF-α, IFN-γ and IL-6 and inhibits NF-κB in $CD4^+$, $CD8^+$ T lymphocytes and macrophage cells.

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Author's Contributions

Dinia Rizqi Dwijayanti: Wrote the manuscript and participated in the study design.

M. Sasmito Djati: Participated in statistical analysis.

Mansur Ibrahim: Participated in the study design.

Muhaimin Rifa'i: Revised the manuscript and the study design.

Ethics

This article is original research paper and contains unpublished material. The corresponding author confirms that all of the other authors have read and approved this manuscript and there are no ethical issues involved.

References

Alpan, O., E. Bachelder, E. Isil, H. Arnheiter and P. Matzinger, 2004. 'Educated' dendritic cells act as messengers from memory to naive T helper cells. Nat. Immunol., 5: 615-225. DOI: 10.1038/ni1077

Brooks-Worrell, B.M., J.L. Reichow, A. Goel, H. Ismail and J.P. Palmer, 2011. Identification of autoantibody negative autoimmune type 2 diabetic patients. Diabetes Care, 34: 168-173. DOI: 10.2337/dc10-0579

Caamano, J. and C.A. Hunter, 2002. NF-κB family of transcription factors: Central regulators of innate and adaptive immune functions. Clin. Microbiol. Rev., 15: 414-429. DOI: 10.1128/CMR.15.3.414-429.2002

Collison, L.W., C.J. Workman, T.T Kuo, K. Boyd and K.M. Vignali *et al*., 2007. The inhibitory cytokine IL-35 contributes to regulatory T-cell function. Nature, 450: 566-571. DOI: 10.1038/nature06306

Corthay, A., 2009. How do regulatory T cells work? Scandinavian J. Immunol., 70: 326-336. DOI: 10.1111/j.1365-3083.2009.02308.x

Donath, M.Y. and S.E. Shoelson, 2011. Type 2 diabetes as an inflammatory disease. Nat. Rev. Immunol., 11: 98-107. DOI: 10.1038/nri2925

Dula, S.B., M. Jecmenica, R. Wu, P. Jahanshahi and G.M. Verrilli *et al*., 2010. Evidence that low-grade systemic inflammation can induce islet dysfunction as measured by impaired calcium handling. Cell Calcium, 48: 133-142. DOI: 10.1016/j.ceca.2010.07.007

Dunmore, S.J. and J.E. Brown, 2013. The role of adipokines in β-cell failure of type 2 diabetes. J. Endocrinol., 216: T37-T45. DOI: 10.1530/JOE-12-0278

Feuerer, M., J.A. Hill, D. Mathis and C. Benoist, 2009. $Foxp3^+$ regulatory T cells: Differentiation, specification, subphenotypes. Nat. Immunol., 10: 689-695. DOI: 10.1038/ni.1760

Fujiwara, N. and K. Kobayashi, 2005. Macrophages in inflammation. Curr. Drug Target-Inflammat. Allergy, 4: 281-286. DOI: 10.2174/1568010054022024

Garin, M.I., C.C. Chu, D. Golshayan, E. Cemuda-Morollon and R. Wait *et al*., 2007. Galectin-1: A key effector of regulation mediated by CD4+CD25+ T cells. Blood, 109: 2058-2065. DOI: 10.1182/blood-2006-04-016451

Gondek, D.C., L.F. Lu, S.A. Quezada, S. Sakaguchi and R.J. Noelle, 2005. Cutting edge: Contact-mediated suppression by $CD4^+CD25^+$ regulatory cells involves a granzyme B-dependent, perforin-independent mechanism. J. Immunol., 174: 1783-1786. DOI: 10.4049/jimmunol.174.4.1783

Gregori, S., N. Giarratana, S. Smiroldo, M. Uskokovic and L. Adorini, 2002. A 1α,25-dihydroxyvitamin D_3 analog enhances regulatory T-cells and arrests autoimmune diabetes in NOD mice. Diabetes, 51: 1367-1374. DOI: 10.2337/diabetes.51.5.1367

Grossman, W.J., J.W. Verbsky, W. Barchet, M. Colonna and J.P. Atkinson *et al*., 2004. Human T regulatory cells can use the perforin pathway to cause autologous target cell death. Immunity, 21: 589-601. DOI: 10.1016/j.immuni.2004.09.002

Hasegawa, S., T. Ichiyama, I. Sonaka, A. Ohsaki and S. Okada *et al*., 2011. Cysteine, histidine and glycine exhibit anti-inflammatory effects in human coronary arterial endothelial cells. Clin. Exp. Immunol., 167: 269-274. DOI: 10.1111/j.1365-2249.2011.04519.x

Hotamisligil, G.S., 2010. Endoplasmic reticulum stress and the inflammatory basis of metabolic disease. Cell, 140: 900-917. DOI: 10.1016/j.cell.2010.02.034

Huang, Y., P.M. Krein, D.A. Muruve and B.W. Winston, 2002. Complement factor B gene regulation: Synergistic effects of TNF-α and IFN-γ in macrophages. J. Immunol., 169: 2627-2635. DOI: 10.4049/jimmunol.169.5.2627

Issazadeh-Navikas, S., R. Teimer and R. Bockermann, 2011. Influence of dietary components on regulatory T cells. Molecular Med., 18: 95-110. DOI: 10.2119/molmed.2011.00311

Josefowicz, S.Z., L. Li-Fan and A.Y. Rudensky, 2012. Regulatory T cells: Mechanisms of differentiation and function. Ann. Rev. Immunol., 30: 31-64. DOI: 10.1146/annurev.immunol.25.022106.141623

Lawrence, T., 2009. The nuclear factor NF-κB pathway in inflammation. Inflamm. Biol. Group, 1: 1-10. DOI: 10.1101/cshperspect.a001651

Liang, Y., Y. Zhou and P. Shen, 2004. NF-κB and its regulation on the immune system. Cellular Molecular Immunol., 1: 343-350.

Liboni, K.C., N. Li and P.O. Scumpia, 2005. Glutamine modulates LPS-induced IL-8 production through IkB/NF-kB in human fetal and adult intestinal epithelium. J. Nutr., 135: 245-251.

Morino, K., K.F. Petersen and G.I. Shulman., 2006. Molecular mechanisms of insulin resistance in humans and their potential links with mitochondrial dysfunction. Diabetes, 55: S9-S13. DOI: 10.2337/db06-S002

Mustafa, A., M.A. Widodo and Y. Kristianto, 2012. Albumin and zinc content of snakehead fish (*Channa striata*) extract and its role in health. Int. J. Sci. Technol., 1: 1-5. DOI: 10.1.1.247.2648

Rifa'i, M., 2010. Andrographolide ameliorate rheumatoid arthritis by promoting the development of regulatory T cells. J. Tropical Life Sci., 1: 5-8.

Rifa'i, M., 2014. Aspek biologi sel T regulator CD_{4+} CD_{25+} pada transplantasi sumsum tulang. J. Exp. Life Sci., 4: 1-9.

Rifa'i, M. and Widodo, 2011. Sel T regulator $CD4^+$ $CD25^+$ mencegah terjadinya fenotip letal pada mencit defisiensi CD122. J. Veteriner, 12: 166-172.

Sakaguchi, S., K. Wing, Y. Onishi, P. Prieto-Martin and T. Yamaguchi, 2009. Regulatory T cells: How do they suppress immune responses? Int. Immunol., 21: 1105-1111. DOI: 10.1093/intimm/dxp095

Schulze, M.B., M. Schulz, C. Heidemann, A. Schienkiewitz and K. Hoffmann *et al*., 2007. Fiber and magnesium intake and incidence of type 2 diabetes: A prospective study and meta-analysis. Arch. Int. Med., 167: 956-965. PMID: 17502538

Scull, C.M., W.D. Hays and T.H. Fischer, 2010. Macrophage pro-inflammatory cytokine secretion is enhanced following interaction with autologous platelets. J. Inflammat., 53: 1-9. DOI: 10.1186/1476-9255-7-53

Silverman, N. and T. Maniatis, 2001. NF-κB signaling pathways in mammalian and insect innate immunity. Genes Dev., 15: 2321-2342. DOI: 10.1101/gad.909001

Simopoulos, A.P., 2002. Omega-3 fatty acids in inflammation and autoimmune diseases. J. Am. College Nutr., 21: 495-505. DOI: 10.1080/07315724.2002.10719248

Son, D.O., H. Satsu and M. Shimizu, 2005. Histidine inhibits oxidative stress- and TNF-α-induced interleukin-8 secretion in intestinal epithelial cells. FEBS Lett., 579: 4671-4677. DOI: 10.1016/j.febslet.2005.07.038

Stachlewitz, R.F., X. Li and S. Smith, 2000. Glycine inhibits growth of T lymphocytes by an IL-2-independent mechanism. J. Immunol., 164: 176-182. DOI: 10.4049/jimmunol.164.1.176

Villegas, R., Y.T. Gao, Q. Dai, G. Yang and H. Cai *et al*., 2009. Dietary calcium and magnesium intakes and the risk of type 2 diabetes: The shanghai women's health study. Am. J. Clin. Nutr., 89: 1059-1067. DOI: 10.3945/ajcn.2008.27182

Wall, R., R. Paul, F.F. Gerald and S. Catherine, 2010. Fatty acids from fish: The anti-inflammatory potential of long-chain omega-3 fatty acids. Nutr. Rev., 68: 280-289. DOI: 10.1111/j.1753-4887.2010.00287.x

Weaver, J.R., 2012. Integration of pro-inflammatory cytokines, 12-lipoxygenase and NOX-1 in pancreatic islet beta cell dysfunction. Molecular Cellular Endocrinol., 358: 88-95. DOI: 10.1016/j.mce.2012.03.004

Wheeler, M.D. and R. Thurman, 1999. Production of superoxide and TNF-alpha from alveolar macrophages is blunted by glycine. Am. J. Physiol., 277: L952- L959. PMID: 10564180

Wild, S., G. Roglic, A. Green, R. Sicree and H. King, 2004. Global prevalence of diabetes estimates for the year 2000 and projections for 2030. Diabetes Care, 27: 1047-1053. DOI: 10.2337/diacare.27.5.1047

Yamazaki, S., T. Iyoda, K. Tarbell, K. Olson and K. Velinzon *et al*., 2003. Direct expansion of functional $CD25^+$ $CD4^+$ regulatory T cells by antigen-processing dendritic cells. J. Exp. Med., 198: 35-47. DOI: 10.1084/jem.20030422

Yu, A.X., L.J. Zhu, N.H. Altman and T.R. Malek, 2009. A low interleukin-2 receptor signaling threshold supports the development and homeostasis of T regulatory cells. Immunity, 30: 204-217. DOI: 10.1016/j.immuni.2008.11.014

Yumi, I., A.D. Dobrian, M.A. Morris and J.L. Nadler, 2013. Islet inflammation: A unifying target for diabetes treatment? Trends Endocrinol. Metabolism, 24: 351-360. DOI: 10.1016/j.tem.2013.01.007