Journal of Ayurveda and Integrative Medicine 11 (2020) 414–420

Contents lists available at ScienceDirect

Journal of Ayurveda and Integrative Medicine

journal homepage: http://elsevier.com/locate/jaim

Original Research Article (Experimental)

Single garlic oil modulates T cells activation and proinflammatory cytokine in mice with high fat diet

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ARTICLE INFO

Article history:
Received 20 January 2019
Received in revised form
2 October 2019
Accepted 11 June 2020
Available online 12 August 2020

Keywords:
High-fat diet
Single garlic
T-cells

ABSTRACT

Background: Hyperlipidemia triggers atherosclerosis by involving immune cells, such as T-cells. T-cells plays a role in worsening conditions during a high-fat diet (HFD).
Objective: The research aimed to analyze the role of single garlic oil (SGO) on T-cells activation and its proinflammatory cytokine expression in HFD mice.
Methods: Mice were divided into six groups: ND (normal diet); HFD (high-fat diet without treatment); HFD + Simv (HFD + simvastatin 2.6 mg/kg body weight); and HFD + SGO 1–3 (high-fat diet + single garlic oil in a dose of 12.5, 25, and 50 mg/kg body weight), respectively. Treatments were orally given every day for 45 days. At the end of treatments, lymphocytes were isolated from mice spleen. The relative number of T-cells and proinflammatory cytokines were measured using flow-cytometry and analyzed using one-way ANOVA ($p < 0.05$).
Result: Our result indicated that HFD mice had lower naive T cells (CD4+CD62L+) than normal mice ($p < 0.05$). SGO treatment in HFD mice increased the relative number of naïve T cells. HFD treatment increased the expression of TNF-α and IFN-γ through NF-κB expression. Furthermore, SGO treatment improved the expression of TNF-α and IFN-γ.
Conclusion: Our study suggests that SGO could act as a promising prospect for therapy to improve chronic inflammation in a HFD.

1. Introduction

Hyperlipidemia is closely related to the increase of plasma lipids, especially cholesterol. A high-fat diet is realized as the primary factor causing an imbalance between cholesterol serum of low-density lipoproteins (LDL) and high-density lipoproteins (HDL) [1]. An increasing LDL level in serum is followed by a decrease in HDL level, which is the critical characteristic in atherosclerosis development [2]. Atherosclerosis is a complex disease that gradually develops in arterial blood vessels and the leading cause of cardiovascular disease [3].

Atherosclerosis is marked with the change in arterial size due to the formation of atherosclerosis plaques consisted of necrotic cells, calcified area, modified lipid accumulation, smooth muscle cells, endothelial cells, leukocyte, and foam cells [4]. Atherosclerosis is the leading risk factor of HFD and causing hyperlipidemia. The innate and adaptive immunity cells play a significant role in atherosclerosis progression [5,6].

T-lymphocyte is part of adaptive immunity that existed in the tunica adventitia of normal artery [7]. Dynamic change in blood lipid levels could trigger naïve $CD4^+$ T-cells to differentiate into effector cells and produce cytokine during atherogenesis [8]. T-helper 1 cell (Th1) is the subset of T lymphocyte mostly found in atherosclerotic lesions based on cytokine it produced. Th1 cells secrete IFN-γ and TNF-α proinflammatory cytokines to enhance immune response through macrophage activation, smooth muscle cells, and endothelial cells during atherogenesis [9].

Hyperlipidemia increase T-cells recruitment to the aorta in the initial and advanced stages [7]. Active T-cells will initiate proinflammatory mediator, thus cause an increase in immune response

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Peer review under responsibility of Transdisciplinary University, Bangalore.

https://doi.org/10.1016/j.jaim.2020.06.009

and worsen atherosclerosis development [10]. NFκB is the main transcription factor which plays a significant role in proinflammatory cytokines synthesis [11]. One of the therapeutic strategies to prevent and inhibit atherosclerosis lesion development is by using natural compounds to suppress T-cells activation and its proinflammatory cytokines production through NFκB.

Garlic (*Allium sativum*) is a cooking spice used globally. Also, it has a positive effect on health; thus, it commonly used as an ingredient for traditional medicine [12,13]. Organosulfur compounds in garlic, such as S-allyl-L-cysteine, diallyl sulfide, diallyl disulfide, diallyl trisulfide, ajoene, and allicin are the essential components [14,15]. The beneficial effects of garlic are among others, to protect from cardiovascular diseases [16] and as anti-inflammation [17].

Single garlic or in Indonesian called *Bawang lanang* is garlic with one bulb due to growth in an extreme environmental condition. Indonesian communities often use it as traditional medicine or supplement. However, the scientific information about single garlic, especially on atherosclerosis, is still limited. The present study focused on analyzing the effect of single garlic on T-cells activation along with its pro-inflammatory cytokines. The phytochemicals effect of single garlic and its derivate for health is expected to be one of the alternatives to prevent the formation of new or formed plaques in atherosclerosis through suppression of T-cells activation.

2. Materials & methods

2.1. Chemicals

FITC-conjugated anti-mouse CD4 (clone number: GK1.5), PE/Cy5-conjugated anti-mouse CD62L (clone number: MEL-14), PE-conjugated rat anti-mouse TNFα (clone number: MP6-XT22), and PerCP/Cy5.5-conjugated rat anti-mouse IFNγ (clone number: XMG1.2) antibodies were purchased from BioLegend (San Diego, CA). PE/Cy5-conjugated rabbit anti-mouse NFκB (Polyclonal) was purchased from Bioss Inc (Massachusetts, USA). Simvastatin (10 mg) was purchased from Dexa Medica, Tangerang, Indonesia.

2.2. SGO preparation

Single garlic was obtained from Ngadas Village, Malang Regency and Sarangan Village, Magetan Regency. Single garlic was identified

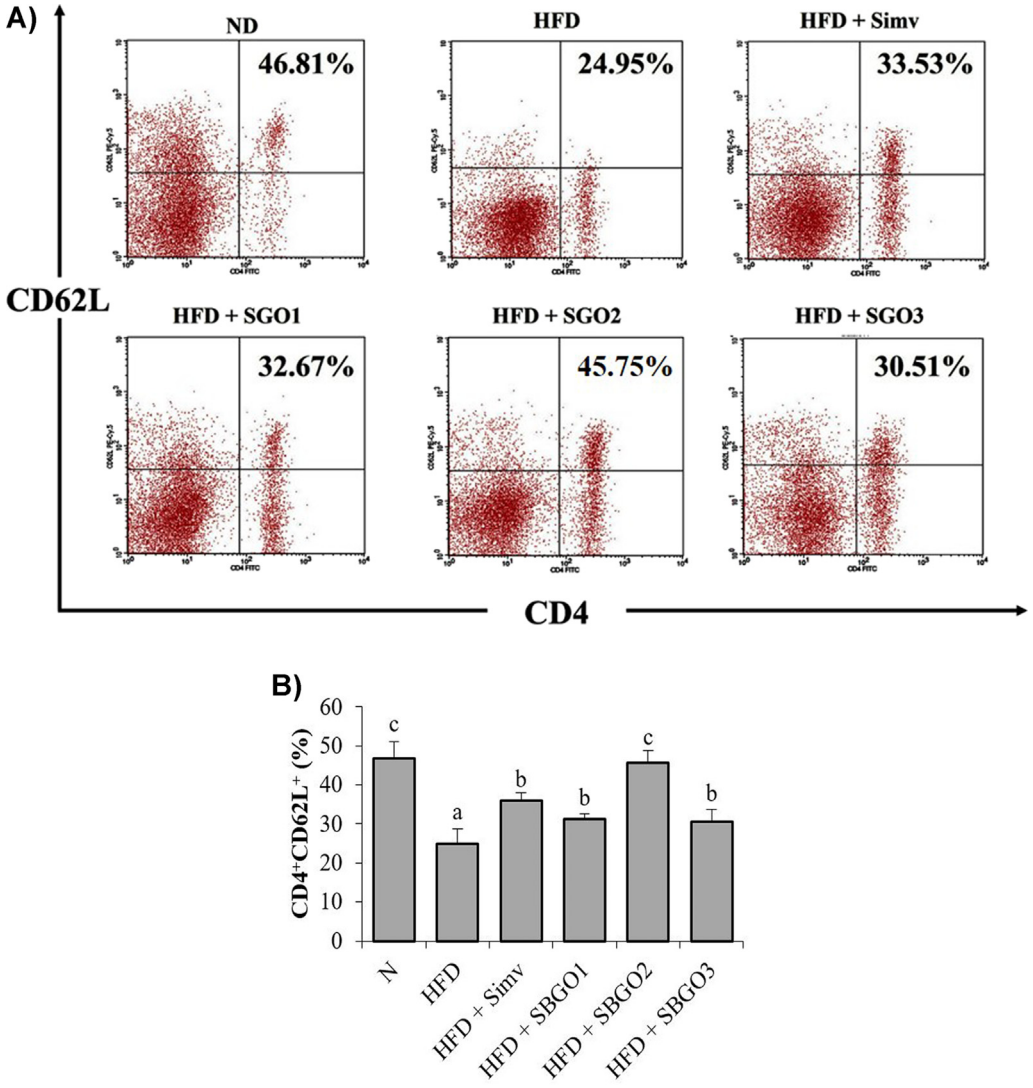

Fig. 1. SGO treatment increases $CD4^+CD62L^+$ T-cells in HFD mice. A) the relative number of $CD4^+CD62L^+$ T-cells resulted from flow-cytometry B) Bart chart of the relative number of $CD4^+CD62L^+$ T-cells represented in mean ± SD. Different letters indicate a significant difference ($p < 0.05$) based on the DMRT test. **SGO** = Single Garlic Oil; **ND** = Normal Diet; **HFD** = High-Fat Diet; **HFD + Simv** = HFD + Simvastatin 2.6 mg/kg; **HFD + SGO1** = HFD + SGO 12.5 mg/kg BW; **HFD + SGO2** = HFD + SGO 25 mg/kg BW; **HFD + SGO3** = HFD + SGO 50 mg/kg BW.

at UPT Materia Medica, Batu with no. specimen T0-T0975. The extraction process was conducted at UPT Materia Medika Batu, Indonesia. 250 g single garlic were dried at 50 °C. The garlic was then grounded into powder. The single garlic powder was extracted by soxhletation method with 1.5 L N-hexane. The extraction result was evaporated using a rotary evaporator and kept in a freezer at −4 °C until further use.

2.3. Experimental animals

Twenty-four male (*Mus musculus*) *Balb/C* mice, 10 ± 2 weeks old with 38 ± 5 g of body weight were used in this study. The mice obtained from The Integrated Research and Testing Laboratory-Unit IV, Gadjah Mada University, Yogyakarta. The mice acclimatized for one-week and given free access of food and ad libitum water.

2.4. Experimental design

After one-week acclimatized, the mice were divided into two groups, normal diet (ND) and HFD groups for 45 days. HFD contained with 37.33% carbohydrate, 35.85% fat, 11.17% protein, and mineral and fiber as remain. After 45 days, HFD mice groups were re-divided into five groups randomly : mice with high fat diet without treatment (HFD) + simv (HFD + simvastatin in a dose of 2.6 mg/kg body weight); HFD + SGO1 (12.5 mg/kg body weight); HFD + SGO2 (25 mg/kg body weight); and HFD + SGO3 (50 mg/kg

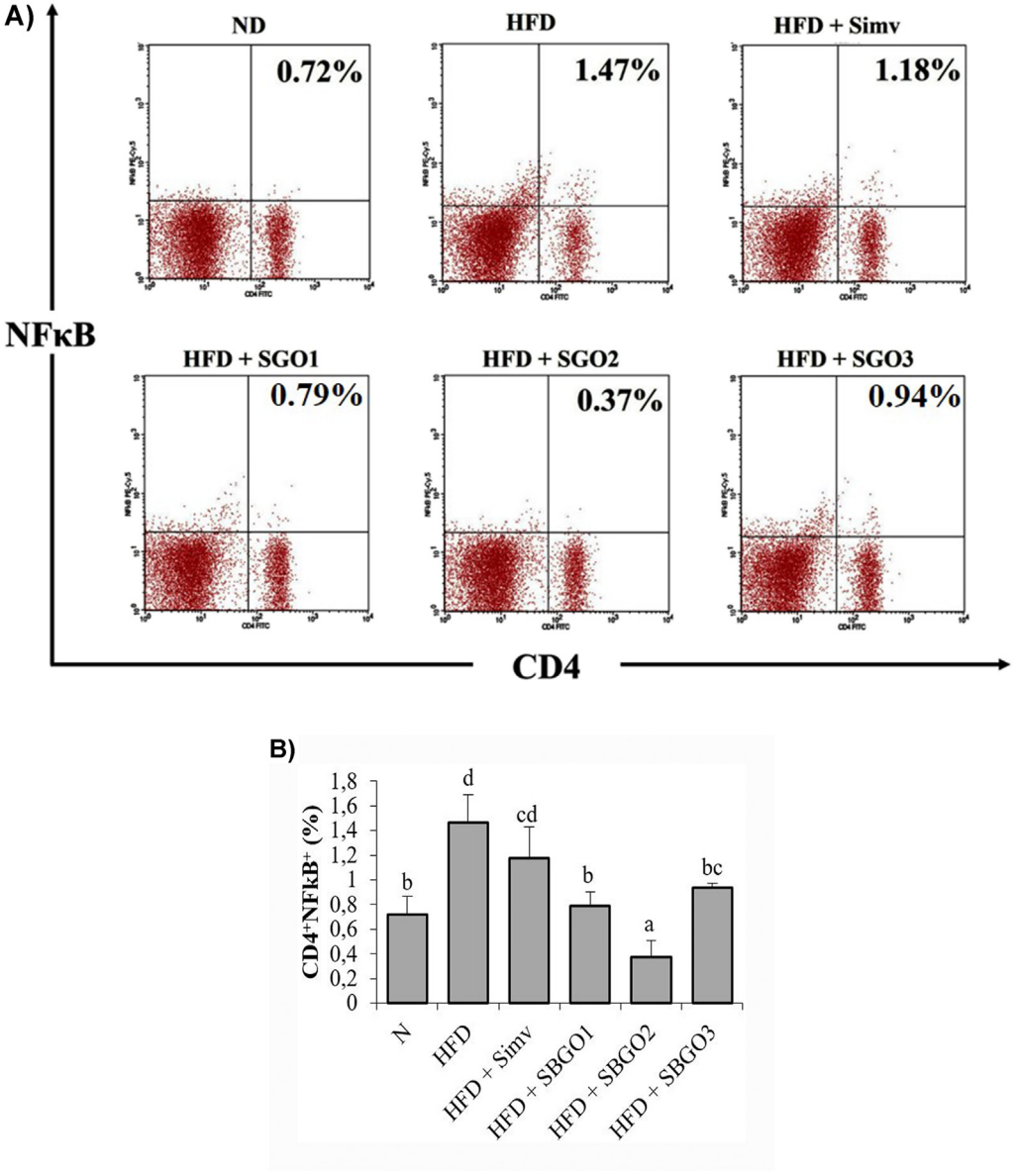

Fig. 2. SGO treatment suppresses $CD4^{+}NF\kappa B^{+}$ T-cells expression in HFD mice. A) The relative number of $CD4^{+}NF\kappa B^{+}$ T-cells resulted from flow-cytometry B) Bar chart of the relative number of $CD4^{+}NF\kappa B^{+}$ T-cells represented in mean ± SD. Different letters indicate a significant difference ($p < 0.05$) based on the DMRT test. **SGO** = Single Garlic Oil; **ND** = Normal Diet; **HFD** = High-Fat Diet; **HFD + Simv** = HFD + Simvastatin 2.6 mg/kg; **HFD + SGO1** = HFD + SGO 12.5 mg/kg BW; **HFD + SGO2** = HFD + SGO 25 mg/kg BW; **HFD + SGO3** = HFD + SGO 50 mg/kg BW.

body weight). Treatments were given orally every day for 4 weeks. The animal welfare and experimental procedures were approved by the Research Ethics Committee, Brawijaya University, Indonesia (Approval no. 880-KEP-UB).

2.5. Lymphocyte cells isolation, immunostaining, and FACS analysis

At the end of treatment, mice were fasted overnight and then dissected by using euthanasia. Spleen was collected and washed three times using PBS. The spleen then mashed in clockwise on a Petri dish containing 1 mL PBS. The suspension obtained was centrifuged at 2500 rpm for 5 min at 4 °C. Homogenate added with 1 mL PBS and re-centrifuged at 2500 rpm at a temperature of 4 °C for 5 min. Pellets were stained using FITC-conjugated anti-mouse CD4 (clone number: GK1.5) and PE/Cy5-conjugated anti-mouse CD62L (clone number: MEL-14) antibodies for extracellular staining.

Intracellular staining was performed by adding 100 µl Cytofix/Cytoperm (BD-Biosciences Pharmingen) cytofix-cytoperm into cells stained with FITC-conjugated anti-mouse CD4 antibody and incubated at 4 °C for 20 min. After incubation, 500 µL washsperm was added into cells suspension and then centrifuged at 2500 rpm,

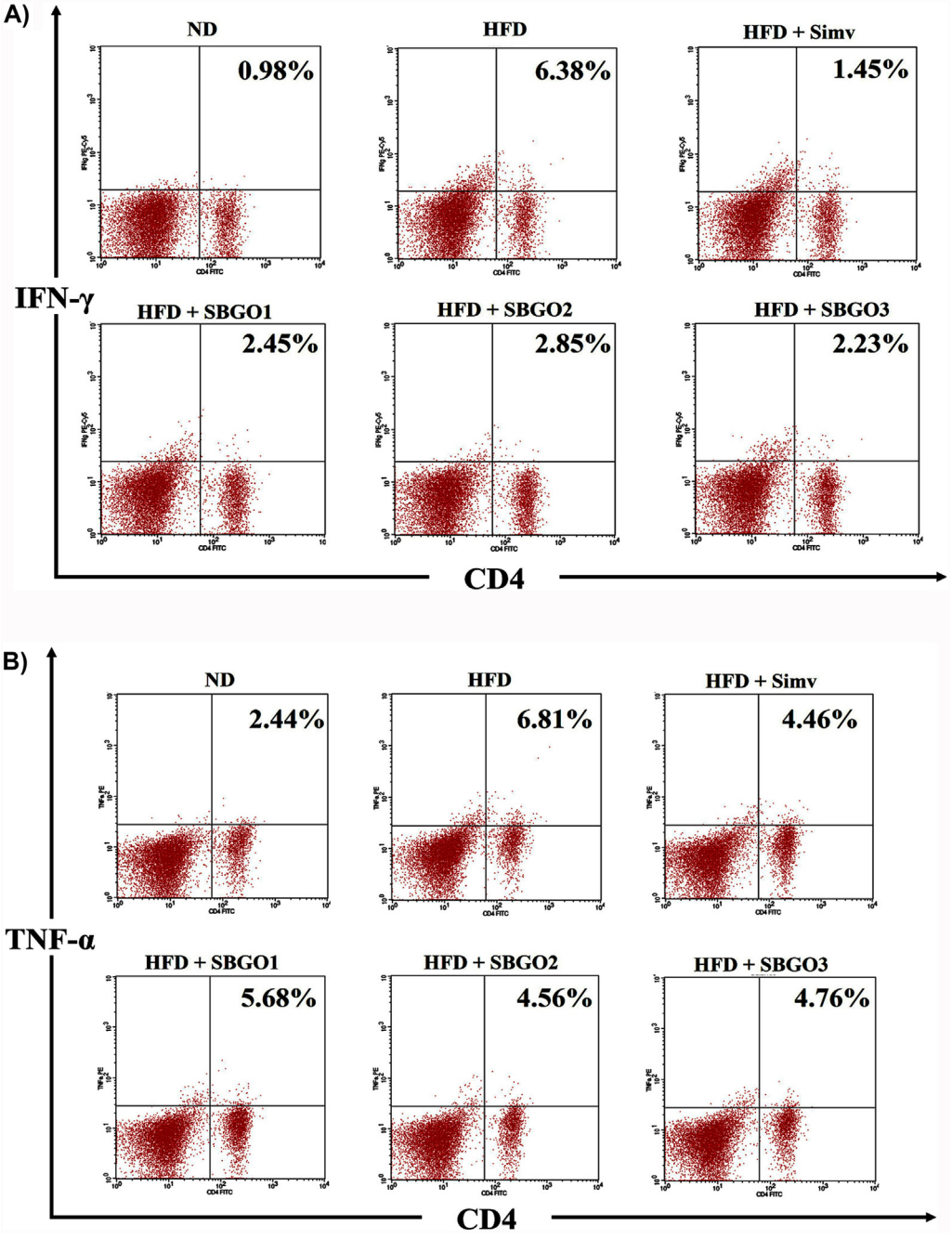

Fig. 3. SGO treatment decrease IFN-γ and TNF-α proinflammatory cytokines production by T-cells in HFD mice. A) The relative number of IFN-γ^+ synthesis $CD4^+$ T-cells resulted from flow-cytometry B) The relative number of $CD4^+$TNF-α^+T-cells, (C) Bar chart of the relative number of $CD4^+$IFN-γ^+ and $CD4^+$TNF-α^+ T-cells represented in mean ± SD. Different letters indicate a significant difference ($p < 0.05$) based on the DMRT test. **SGO** = Single Garlic Oil; **ND** = Normal Diet; **HFD** = High-Fat Diet; **HFD + Simv** = HFD + Simvastatin 2.6 mg/kg; **HFD + SGO1** = HFD + SGO 12.5 mg/kg BW; **HFD + SGO2** = HFD + SGO 25 mg/kg BW; **HFD + SGO3** = HFD + SGO 50 mg/kg BW.

4 °C for 5 min. The obtained supernatant was discarded, and pellets were stained using PE/Cy5-conjugated rat anti-mouse NFκB (Polyclonal), PE-conjugated rat anti-mouse TNFα (clone number: MP6-XT22), and PerCP/Cy5.5-conjugated rat anti-mouse IFNγ (clone number: XMG1.2) antibodies according to staining combination used. Stained pellets were incubated at 4 °C for 15 min in a dark room and analyzed using flow-cytometry (BD Biosciences FACS Calibur™). The relative number of each parameter were analyzed using BD CellQuest™ Pro sofware and then performed the statistical analysis.

2.6. Statistical analysis

The relative number of $CD4^+CD62L^+$, $CD4^+NF\kappa B^+$, $CD4^+IFN\text{-}\gamma^+$, and $CD4^+TNF\text{-}\alpha^+$ T-cells was analyzed using one-way ANOVA. If significance ($p < 0.05$), Duncan Multiple Range Test (DMRT) was performed as a post hoc test. Data presented as the mean ± standard deviation (SD).

3. Result

3.1. SGO suppress CD4 T-cells activation in high fat diet mice

Our result indicated that the $CD4^+CD62L^+$ has significantly difference between the normal group and the HFD group ($p < 0.05$) (Fig. 1A and B). HFD mice showed an increase in T-cells activation indicated by the decrease in the relative number of $CD4^+CD62L^+$ T-cells. SGO treatment restores the relative number of $CD4^+CD62L^+$ cells in HFD (Fig. 1A). Interestingly, simvastatin treatment did not significantly differ in the relative number of $CD4^+CD62L^+$ T-cells compared to SGO 12.5 mg/kg and 50 mg/kg (Fig. 1B). Furthermore, SGO 25 mg/kg did not have significantly differ in the relative number of $CD4^+CD62L^+$ T-cells compared to the normal group (Fig. 1B).

3.2. SGO suppress NFkB expression in high fat diet mice

Our result indicated that there was a significant difference in the $CD4^+NF\kappa B^+$ in the HFD group compared to the normal group ($p < 0.05$) (Fig. 2A and B). HFD mice showed an increase in NFκB activity in CD4 T-cells. SGO treatment decrease NFκB expression in T-cells in the HFD condition (Fig. 2A). Interestingly, SGO treatment had a better effect in suppressing NFκB activity on T-cells compared to simvastatin as drug control (Fig. 2B). In addition, the result indicates that SGO dose 25 mg/kg had lower NFκB activity in T-cells and even lower than the normal group (Fig. 2B).

3.3. SGO decrease proinflammatory cytokines production in high fat diet mice

Our result indicated that there was a significant difference $CD4^+IFN\text{-}\gamma^+$ and $CD4^+TNF\text{-}\alpha^+$ cells in the HFD group compared to the normal group ($p < 0.05$) (Fig. 3A–C). HFD mice have an increase in IFN-γ and TNF-α synthesis in CD4 T-cells. SGO treatment decreases the IFN-γ and TNF-α synthesis in CD4 T-cells in the HFD condition (Fig. 3A and B). Interestingly, simvastatin had a better effect in suppressing IFN-γ but had the same effect as SGO 25 mg/kg and 50 mg/kg in suppressing TNF-α synthesis (Fig. 3C).

4. Discussion

The role of nutrition as an agent for the prevention of degenerative disease receives rapid attention in the last few years. Phytochemicals diet can be used as a protective agent for the cardiovascular disease since it has beneficial effects, among others, as an antioxidant, and for fat homeostatic repair [13]. Rich fibre diet and antioxidant can be used as an option for cardiovascular disease management [18]. Garlic has known in the whole world for its medicinal and culinary values. Besides that, our previous study suggested that SGO were safe to consumes continuously [19]. Garlic contains sulfur compounds presumed to play a role in most of its biological activities [20,21].

Naïve T-cells are mature T-cells but not exposed to antigen and not yet differentiated [22]. The naïve T-cells express CD62L molecules functioned as an attachment and rolling in endothelium from blood to tissues. The number of $CD4^+$ T-cells effector/memory was significantly increased in HFD mice, whereas the number of naïve $CD4^+$ T-cells in HFD mice experienced a decrease compared to healthy mice [23]. A HFD triggers an increase in T-cells activation and imbalance between erythrocyte and lymphocyte [24] thus initiate inflammation [25].

Our result indicates that the HFD increased the activation of T-cells number, whereas SGO treatment suppress T-cells activation.

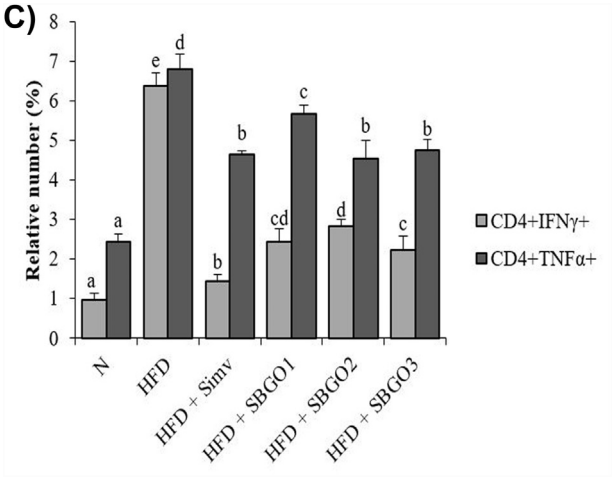

Fig. 3. (*continued*).

These result also in line with our previous study that lymphocyte concentration were decline in HFD mice after SGO treatment [26]. Allicin, one of the active compounds in garlic inhibits T-cell adhesion in endothelial cells by inhibiting ICAM-1 and VCAM-1 expression [27]. Other research results suggest that compound content in garlic could decrease cholesterol and LDL, prevent LDL oxidation, and decrease atherosclerosis progress [28,29]. Oxidized LDL could act as damage-associated molecular patterns (DAMP) in HFD conditions thus trigger the initiation of inflammation through TLR4 [30]. Allicin compound in garlic plays a vital role in preventing excessed LDL uptake into cells [24] thus it is assumed to participate in decreasing the number of oxidized LDL followed by the decrease in activated T-cell number.

NFκB is an inflammation mediator center that regulates more than 200 genes including those genes that involved in inflammation and innate immunity through proinflammatory cytokines production [11]. HFD triggers the activity of NFκB [31], which in turn promotes the excessive proinflammatory cytokine synthesis [32]. Our result indicated that SGO treatment decrease NFκB in CD4 T-cells significantly. Allicin compound plays a role in inhibiting NFκB activity. Thus it also inhibits pro-inflammatory cytokines production like TNF-α and IL-1β [27]. Organosulfur compounds in garlic can prevent IκB degradation. Thus NFκB activities reduced. Also, the sulfur content in garlic can inhibit p38 phosphorylation and ERK pathway [17].

Our next result indicated that a decrease in NFκB activity could decrease TNF-α and IFN-γ cytokines production. TNF-α has a strong proinflammatory activity, whereas IFN-γ induce local chemokine to recruit other immune cells, including increasing T-cells infiltration to tissues [33]. In line with our previous study, SGO treatment also reduce TNF- α concentration in serum [34]. Furthermore, SGO restores the regulatory T-cells (Tregs) and its anti-inflammatory cytokine IL-10 and TGF-β [35]. Thus resulting in the delay of T-cell activation and decline the proinflammatory cytokine. Our result indicates that phytochemical compounds in garlic oil had a robust anti-inflammation ability and can be used as a therapy in HFD caused inflammation.

5. Conclusion

SGO suppressed the CD4 T-cells activation through $CD4^{+}CD62L^{+}$ T-cells. The SGO decrease inflammation through the decrease in NFκB activities and its proinflammatory cytokines, such as TNF-α and IFN-γ. The result suggests that SGO played a role as an atheroprotective agent in the HFD condition. Phytochemicals content in garlic could be a promising therapeutic agent in the future for treatment of inflammatory-related diseases.

Source(s) of funding

The research was funded by Ministry of Research, Technology and Higher Education of the Republic of Indonesia no. grant 3.4.8/UN.32.14 LT/2018.

Conflict of interest

None

Acknowledgement

We would like to thank to Siti Nur Arifah and Alif Rofiqotun Nurul Alimah to assist this research.

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