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The Influence of Ethanol Extract *Dioscorea alata* L. on $CD4^+CD62L^+$ and $CD8^+CD62L^+$ Profile of BALB/c Mice Model Digestive Tract Allergy

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Abstract. Naive T cells and activated T cells express different adhesion molecules and patterns of migration. Naive T cells express CD62L. The research aims to assess the $CD4^+CD62L^+$ and $CD8^+CD62L^+$ profile of BALB/c mice model allergy on the sensitivity, the challenge and the gastrointestinal tract allergy phase after treatment with ethanol extract of *Dioscorea alata* L. (EEDA). An experimental study with post test only control group design using 63 BALB/c mice was divided into seven groups: control group (I) and treatment groups with the ethanol extract of *D. alata* L. tubers dose (0.00, 0.17, 2.01, 10.04) $g \cdot kg^{-1}$ bw (II-V); an antihistamine drug treatment (VI) and diosgenin treatment of 200 $mg \cdot kg^{-1}$ bw (VII). Treatment groups induced with ovalbumin i.p. on 15 d and 22 d, orally on 23 d to 30 d subsequently. Mice were sacrificed on (18, 25 and 31) d, the spleen was taken and isolated to measure $CD4^+CD62L^+$ and $CD8^+CD62L^+$ profile with flowcytometry. The results showed that EEDA dose (0.17, 10.04) $g \cdot kg^{-1}$ and drug on sensitivity phase, EEDA dose 2.01, 10.04 $g \cdot kg^{-1}$; and drug on challenge phase; EEDA dose (0.17, 2.01, 10.04) $g \cdot kg^{-1}$, antihistamine drug and diosgenin on gastrointestinal tract allergy phase increased the percentage of $CD4^+$ expressing $CD62L^+$, but not diosgenin on sensitivity and challenge phase, EEDA dose (0.17, 2.01, 10.04) $g \cdot kg^{-1}$, and antihistamine drug increased the percentage of $CD8^+$ T cells expressing $CD62L^+$ on the sensitivity, challenge, and gastrointestinal tract allergy phase. The conclusion is EEDA and antihistamine drug increase the percentage of $CD4^+$ T cells and $CD8^+$ T cells expressing $CD62L^+$ on the sensitivity, challenge, and gastrointestinal tract allergy phase and diosgenin increase the percentage of $CD4^+$ T cells and $CD8^+$ T cells expressing $CD62L^+$ on the gastrointestinal tract allergy phase of BALB/c mice digestive tract allergy model.

Keywords: BALB/c mice model digestive tract allergy, $CD4^+CD62L^+$, $CD8^+CD62L^+$, ethanol extract *Dioscorea alata* L.

INTRODUCTION

Purple yam (*Dioscorea alata* L.) is a source of biological tubers that has not been used optimally. Some nutrients contained in Dioscorea species are carbohydrates, essential amino acids, minerals, polyphenols, mucilage (glycoprotein), a purine derivative (e.g. allantoin) and steroidal saponin [1–10]. *Dioscorea alata* L. also contains diosgenin [11] which is the main aglycone steroidal saponin which acts as steroid intermediates in the pharmaceutical industry. Steroidal saponin is the most important bioactive compounds because it has multiple biological functions, including the activity of anticarcinogenic, antithrombotic, antiviral, haemolytic, hypocholesterolemic, hypoglycemic, immunostimulatory, antitumorigenic, antimutagenic, immunomodulatory and

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anti-inflammatory depending on its structure [5–8]. Some researcher also suggest that steroidal saponins have hypoallergenic activity [12].

Allergens that enter in the body will be processed by APC (antigen presenting cells). Peptides of allergen are presented by APC induce lymphocyte activation T naive cells, T naive cells further activates lymphocytes to produce Th2, its cytokines [13]. Each T lymphocytes express the TCR (T cell receptor) which is unique to the cell surface as a result of clonal selection in the process of maturation in the thymus. Mature T lymphocytes known as naïve T cells, circulating through the blood and lymphatic systems and are naïve T lymphocytes in secondary lymphoid organs. Naive T cells are T cells that have not been exposed to foreign antigens and not activated [14].

CD62L (L-selectin) is a homing receptor of T cells that play an important role as well as a marker for the development of naive T cells. T cells expressing CD62L and CD62L interaction with its ligands are essential for T cells to enter lymph nodes through high endothelial venules [15]. CD62L expression quickly disappears as soon as the T cell receptor binds to T cells and expression of T cells into $CD62L^-$ deemed to have been exposed to antigen and activated [16, 17].

Activated T cells rapidly proliferate (clonal expansion), migrate through the tissue to the antigen exists and perform effector functions such as a cell-mediated cytotoxicity and production of various cytokines (soluble mediators of the immune response). Cytotoxic T cells $CD8^+$ very effectively can directly lyses infected cells or malignant cells that face antigens, while the T helper cells $CD4^+$ produce cytokines that can be directly toxic to the target cells or to stimulate the function another of effectors T cells and antibody production of B cells, and to mobilize strongly inflammatory mechanisms [14].

CD4 or CD8 glycoprotein expression has attracted the attention of immunologist because its play an important role in the immune response. Besides, the exclusive expression of CD4 or CD8 is characterized by antigen specificity and different functions. For instance, CD4 T cells are restricted MHC class II and programmed for helper function, whereas CD8 T cells restricted by MHC class I and programmed for cytotoxic function. Subsets CD4 and CD8 are the biggest part of the T cell antibody and major component of the immune response mediated by T cells in the thymus. They differentiate from precursors double positive $CD4^+CD8^+$ [18, 19] and play important aspect of this process, matching lineage differentiation of CD4 or CD8 (cytotoxic vs helper functions) against the specificity of MHC (Major Histocompatibility Complex) class II or MHC class I respectively [20–23].

The aims of the research are to examine the $CD4^+CD62L^+$ and $CD8^+CD62L^+$ profile of BALB/c mice model digestive tract allergy on the sensitivity phase, the challenge phase, and gastrointestinal tract allergy phase after treatment with ethanol extract of *Dioscorea alata* L. (EEDA).

MATERIALS AND METHODS

The research was conducted at the Laboratory of Animal Physiology, Laboratory of Molecular Biology Department of Biology, Faculty of Mathematics and Natural Sciences and Biomedical Laboratory, Faculty of Medicine, University of Brawijaya, Malang, East Java, Indonesia.

Experimental animals are BALB/c mice (*Mus musculus*), 6 wk to 8 wk, with a healthy condition. This research has taken the certificate of eligibility of ethics (Ethical Clearance) of the Research Ethics Committee (Animal Care and Use Committee) University of Brawijaya number KEP-144-UB.

Ethanol extract tubers of *Dioscorea alata* L. was made according to a previous study by Lee *et al*. [24]. The tuber of *Dioscorea alata* L. peeled, washed, cut thin, then dried in the sun and covered with a black cloth to dry further pulverized in a blender into tiny particles known as simplicia. Simplicia is made an extract with a solution of 70 % ethanol for seven days with maceration method.

Dose of ethanol extract of the tubers of *Dioscorea alata* L. converted from human dose with 70 kg body weight into 20 g of mice, multiplied by the conversion value 0.0026, so we get the first dose of 0.17 $g \cdot kg^{-1}$, second doses of 2.01 $g \cdot kg^{-1}$ and third doses of 10.04 $g \cdot kg^{-1}$.

Mice Model Allergic Digestive Tract

BALB/c mice intraperitoneally sensitized and challenged with OVA (ovalbumin). Mice immunized with intraperitoneal injections on 15 d with 0.15 mL OVA in $Al(OH)_3$ which is made of 2.5 mg OVA dissolved in 7.75 mL of aluminium hydroxide. On 22 d, there was intraperitoneal injection with OVA in 0.15 mL of distilled water per 2.5 mg OVA mice was dissolved in 10 mL of distilled water. On 23 d until 30 d, mice received oral booster with OVA in 0.15 mL of distilled water which is made by mixing 2.5 mg OVA in 10 mL of distilled water [25, 26].

On 18 d, 25 d and 31 d, three mice from each group were sacrificed by cervical dislocation. Mice were dissected and the spleen is taken to isolate its lymphocytes.

Isolation of Lymphocytes

The spleen is isolated from the body of mice. Spleen washed used PBS (phosphate-buffered saline) twice and cleaned of its fat. Once the organ is pressed clockwise by using the base of the syringe, it was filtered with a wire. Homogenates were mixed with propylene tube containing PBS 15 mL. PBS was added until the volume reached 10 mL. It was centrifuged at 2 500 rpm (1 rpm = 1/60 Hz), 4 °C for 5 min afterwards. Supernatant was discarded while the pellet was resuspended with 1 mL PBS and homogenized.

Preparation and Analysis of Flow Cytometry

Suspension pellets 50 µL of each sample was added to a sterile microtube containing 500 mL of PBS. The suspension is subsequently centrifuged at 2 500 rpm, 4 °C for 5 min. Pellets then added with 500 mL of PBS and were taken each 50 µL into the tube. For extracellular staining, 50 µL homogenates which was added with monoclonal antibody that is PE (phycoerythrin)-conjugated anti-mouse CD8 (clone 53-6.7), FITC (fluorescein isothiocyanate) conjugated anti-mouse CD4 (clone GK 1.5) and PE-conjugated anti-mouse CD62L then stored in the ice box. Then made the connection between computers by means of flowcytometry in a state of "acquiring" and setting the BD Cell Quest software PRO ™. Do set the plot in acquiring mode, labeled $CD4^+$ on the X axis and $CD8^+$ on the Y axis, labeled $CD4^+$ on the X axis and $CD62L^+$ on the Y axis, labeled $CD8^+$ on the X axis and $CD62L^+$ on the Y axis and conducted gating area (G1 = R1). Flowcytometry certainly in a state of Low-Run. After flowcytometry ready, included in the sample cuvette then pipetting. Cuvette mounted at the nozzle flow cytometry FACS Calibur TM BD Biosciences. Data analysis was performed by flow cytometry using FACS Calibur TM with cell Quest software.

Statistical Analysis of the Data

Data are presented as means ± SD. Comparison between groups was performed by the one-way analysis of variance continued with Tukey test. Considered a value of $p < 0.05$ was statistically significant.

RESULT AND DISCUSSION

$CD4^+$ T cells play an important role in the pathogenesis of allergic diseases. $CD4^+$ T cells include T helper (Th)1, Th2, Th17 and regulatory T cells. Th2 cells are the major cell types contributing to pathological changes of the allergic disease [27]. In some other cases, Th1 cells also contribute to the pathogenesis of allergy, such as allergic asthma [28]. Th2 cell polarization shift is one of the main pathological features of allergy. After activation of TCR (T Cell Receptor) by a particular antigen, the antigen specific Th2 cells proliferate and produce pro-inflammatory cytokines, such as IL-4, IL-5 and IL-13 to induce allergic inflammation in the local network. In general, after the activation, T cells can undergo programmed cell death (apoptosis) to be eliminated. This condition is designed to AICD (Activation Induced Cell Death) [29].

Analysis of Relative Number of $CD4^+CD62L^+$ T cells

In the sensitivity phase, after intraperitoneal injection of OVA on 15 d, the ethanol extract of *Dioscorea alata* L. (EEDA) with dose 0.00 $g \cdot kg^{-1}$ group showed insignificant decreased ($p > 0.05$) percentage of $CD4^+$ T cells expressing $CD62L^+$ compared to the control group. The percentage of $CD4^+$ T cells that express $CD62L^+$ increased in mice which are given EEDA dose 0.17 $g \cdot kg^{-1}$; EEDA dose 10.04 $g \cdot kg^{-1}$, mice which is given the antihistamine drug compared with EEDA dose 0.00 $g \cdot kg^{-1}$ group, but the percentage of $CD4^+$ T cells that express $CD62L^+$ on mice which is given the EEDA dose of 2.01 $g \cdot kg^{-1}$ and the Diosgenin treatment group did not increase as seen in Fig 1 (a). In the challenge phase, after intraperitoneal injection of OVA on 15 d and 22 d, EEDA with dose 0.00 $g \cdot kg^{-1}$ group showed significant decreased ($p < 0.05$) percentage of $CD4^+$ T cells expressing $CD62L^+$ compared to the control group. The percentage of $CD4^+$ T cells expressing $CD62L^+$ increased in mice which are given EEDA dose 2.01 $g \cdot kg^{-1}$; EEDA 10.04 $g \cdot kg^{-1}$ and the antihistamine drug treated compared to the EEDA dose 0.00 $g \cdot kg^{-1}$

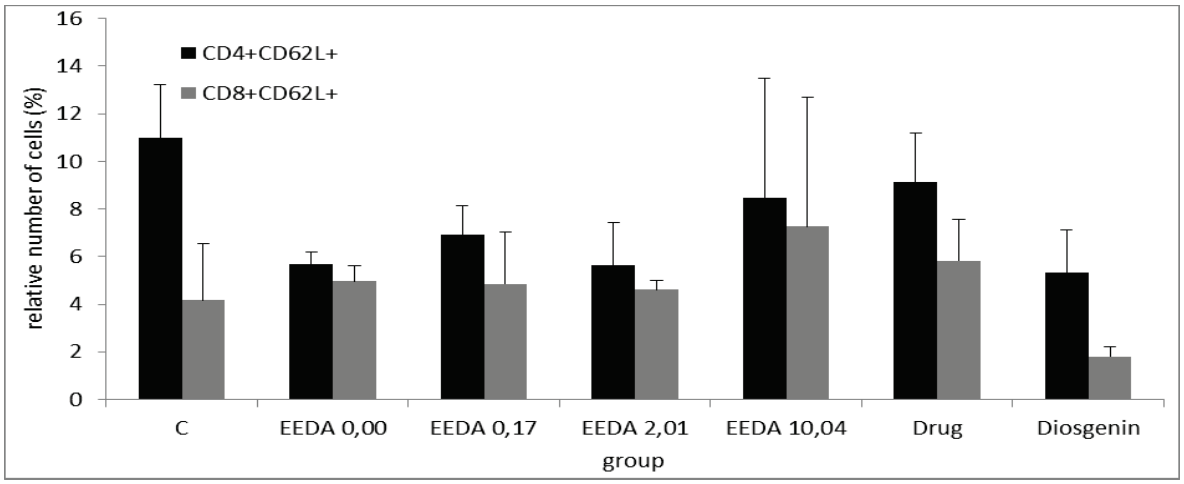
group. However, the percentage of $CD4^+$ T cells expressing $CD62L^+$ did not increase in EEDA dose 0.17 g · kg^{-1} group and diosgenin treatment group as seen in Figure 1(b).

In the digestive tract allergy phase, after intraperitoneal injection of OVA on 15 d and 22 d continued with orally on 23 d until 30 d, EEDA dose 0.00 g · kg^{-1} showed significant decreased ($p < 0.05$) percentage of $CD4^+$ T cells expressing $CD62L^+$ compared to the control group. The percentage of $CD4^+$ T cells expressing $CD62L^+$ increased in the EEDA dose 0.17 g · kg^{-1}, 2.01 g · kg^{-1}, 10.04 g · kg^{-1}, treatment group with antihistamine drug, and diosgenin treated group compared to the EEDA dose 0.00 g · kg^{-1} group as shown in Fig. 1(c).

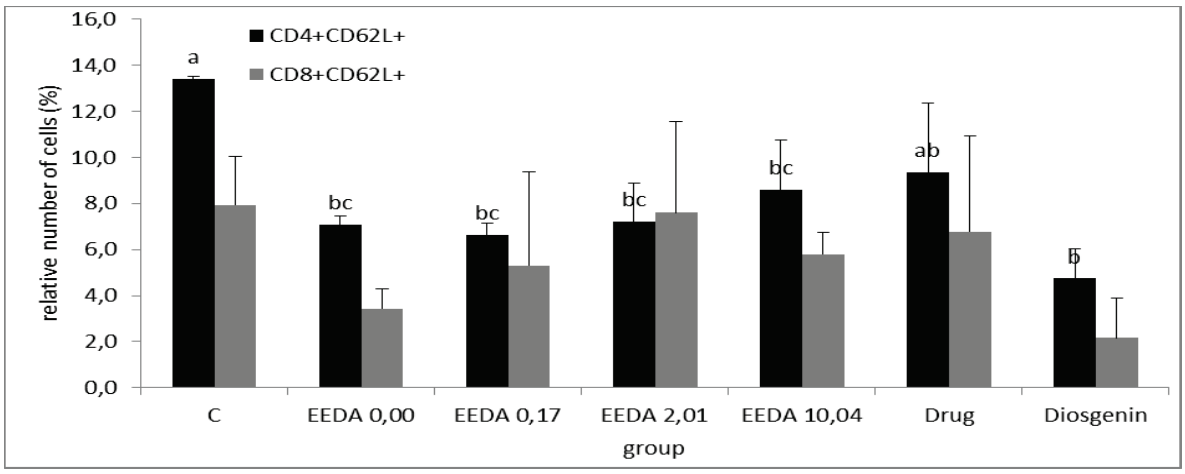
This result indicates that the active compound in the ethanol extract of *Dioscorea alata* L. (EEDA) can increase the number of naïve CD4 T cells in the sensitivity phase, challenge phase and digestive tract allergy phase of BALB/c mice model digestive tract allergy. EEDA at low doses (0.17 g · kg^{-1}) can increase insignificantly higher the number of naïve CD4 T cells than EEDA dose of 2.01 g · kg^{-1}. The percentage of $CD4^+$ T cells expressing $CD62L^+$ on challenge phase showed an increase in EEDA dose 2.01 g · kg^{-1} and EEDA 10.04 g · kg^{-1} significantly. The percentage of $CD4^+$ T cells expressing $CD62L^+$ on digestive tract allergy phase showed an increase in EEDA dose 0.17 g · kg^{-1}, EEDA dose 2.01 g · kg^{-1} and EEDA 10.04 g · kg^{-1} significantly. This is consistent with the results of research Knight *et al*. [30] that the OVA sensitization causes a decrease in L-selectin (CD62L). This naïve T cell decline is caused by increased expression of cytokines IL-4 at the beginning of the OVA and increased significantly after oral administration OVA.

The results showed that the activation of T cells in the spleen caused by oral administration of OVA allergen. Naïve T cells have a variety of cell surface molecules, one of which is the adhesion molecule CD62L, as a marker of the presence of naive T cells. A decrease in T cells expressing CD62L, caused by exposure to OVA that trigger differentiation of naïve T cells into effector cells, such as $CD4^+$ T cells, expressing $CD8^+$, $CD69^+$, $CD25^{+,}$ and $CD44^+$. CD62L is a marker of cell activation, thus decreasing number of T cells $CD4^+CD62L^+$ indicates activity naïve cells that turn into $CD4^+$ T cell subsets, such as regulatory T cells caused by exposure to allergens into the body [31].

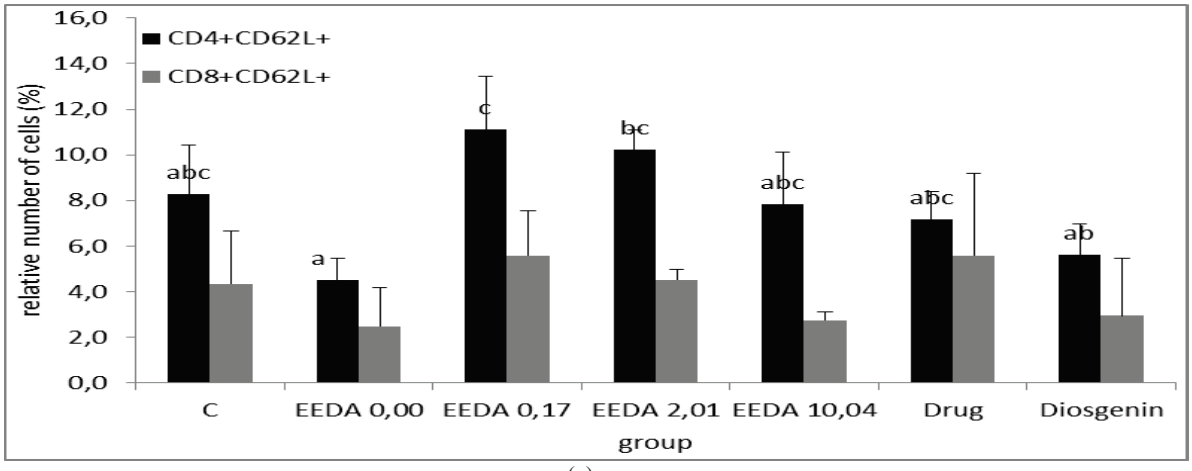
In the digestive tract allergy phase, giving diosgenin dose of 200 mg/mouse increase the percentage of $CD4^+$ T cells expressing $CD62L^+$ but not on the sensitivity phase and the challenge phase. In the sensitivity phase, EEDA dose of 0.17 g · kg^{-1} and 10.04 g · kg^{-1} can also increase the percentage of $CD4^+$ T cells expressing $CD62L^+$ compared to the EEDA dose 2.01 g · kg^{-1}. However, there was no significant difference among treatment groups of EEDA, drugs, and diosgenin. According to Huang *et al*. [32], the content of diosgenin on *Dioscorea alata* L. tubers can reduce the expression of IL-4 and GATA-3 intestinal in BALB/c mice which were sensitized to ovalbumin. According to the research by Huang *et al*. [32], the reduced expression of IL-4 (Th2 cytokine) causes increased $CD4^+$ T cells expressing $CD62L^+$. On the challenge phase, there was also no significant difference among the treatment groups. There were significant differences between the control group and the diosgenin treatment group. On the digestive tract allergies phase, percentage of $CD4^+$ T cells expressing $CD62L^+$ was also significant different among EEDA dose 0.00 g · kg^{-1} group, EEDA dose 0.17 g · kg^{-1} group, and the diosgenin treatment group.



(a)



(b)



(c)

FIGURE 1. Percentage relative number of $CD4^+$ T cells and $CD8^+$ T cells expressing $CD62L^+$ in the sensitivity phase (a), the challenge phase (b) and digestive tract allergy phase (c) after the ethanol extract of *Dioscorea alata* L. (EEDA) on control group (c), EEDA 0.00 g · kg^{-1} (T0), EEDA 0.17 g · kg^{-1} (T1); EEDA 2.01 g · kg^{-1} (T2); EEDA 10.04 g · kg^{-1} (T3), antihistamine drugs (T4) and diosgenin (T5). Description: The bar chart followed by the same letters means no significant difference among the groups ($p > 0.05$)

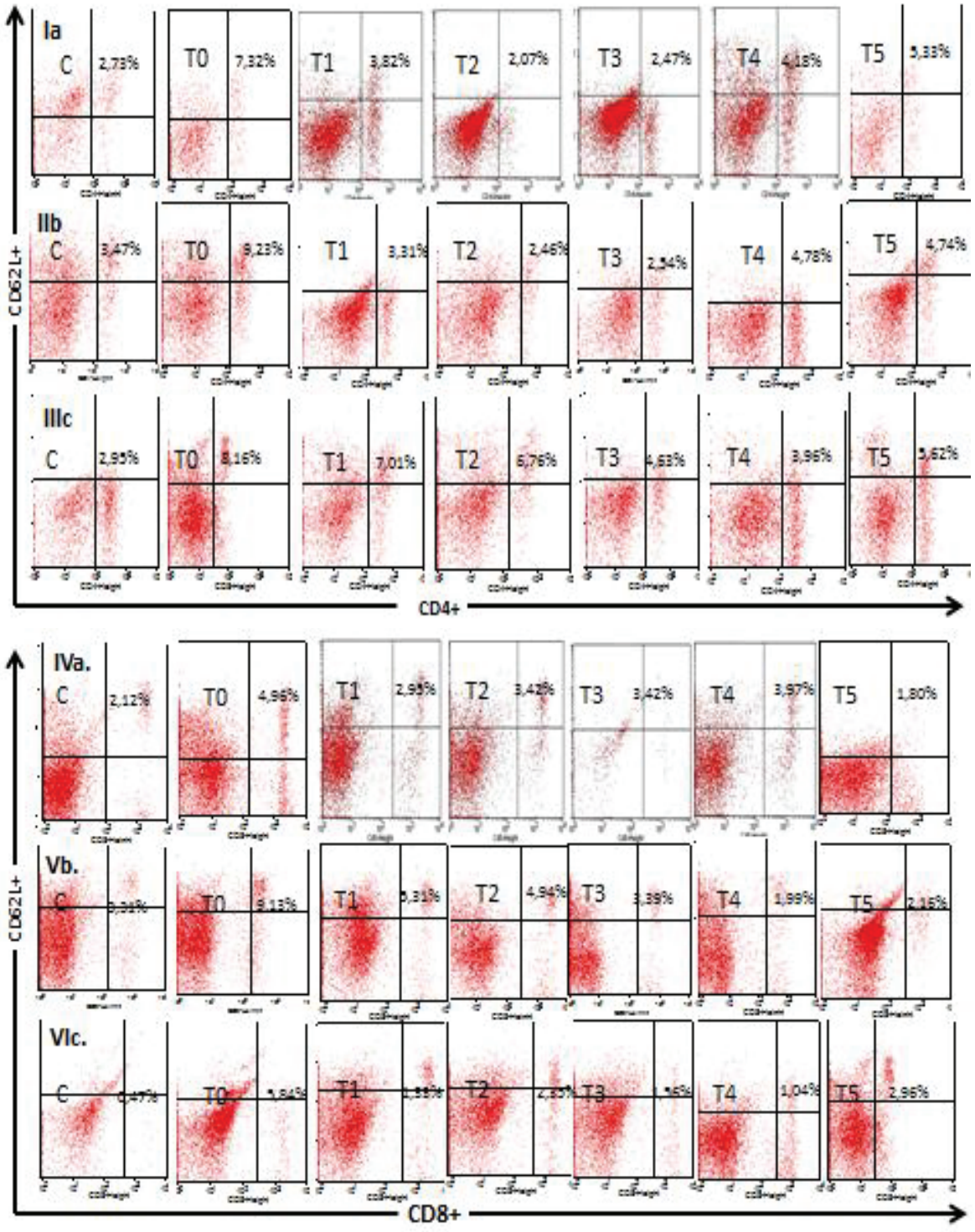


FIGURE 2. Profile dot plot $CD4^+$ T cells and $CD8^+$ expressing $CD62L^+$ of spleen of mice model of allergic gastrointestinal tract after given ethanol extract *Dioscorea alata* L. tubers on sensitization phase (Ia, IVa), challenge phase (IIb, Vb) and digestive tract allergy phase (IIIa, VIc) in the control group (C), EEDA 0.00 g · kg^{-1} (T0), EEDA 0.17 g · kg^{-1} (T1), EEDA 2.01 g · kg^{-1} (T2), EEDA 10.04 g · kg^{-1} (T3), antihistamine drugs (T4) and diosgenin (T5).

Treatment of ethanol extract of *Dioscorea alata* L. with dose 0.17 g · kg^{-1}, 2.01 g · kg^{-1} and 10.04 g · kg^{-1} was not significantly different with the percentage of $CD4^+$ T cells expressing $CD62L^+$ BALB/c mice model of digestive tract allergy. This indicated that *Dioscorea alata* L. with high doses of ethanol extract could not increase the number

of $CD4^+$ T cells expressing $CD62L^+$ BALB/c mice model of digestive tract allergy. The variation within the dose of ethanol extract of *Dioscorea alata* L. was insignificantly different, thereby indicating that the influence of variations of low dose, medium dose, or high dose of ethanol extract of *Dioscorea alata* L. might improve naïve $CD4^+$ T cells. The active compound in the ethanol extract of *Dioscorea alata* L. showed significant gain to suppress the proliferation of $CD4^+$ T cells in the BALB/c mice model digestive tract allergy, but the ethanol extract of *Dioscorea alata* L. at the high dose was not able to suppress the proliferation and activation of $CD4^+$ T cells.

Figure 2 shows the dot plots profile of the analysis of flow cytometry FACS CaliburTM with software cellQuest percentage of $CD4^+$ T cells that express $CD62L^+$ on the sensitization phase, challenge phase and digestive tract allergy phase of BALB/c mice model digestive tract allergy after ethanol extract of *Dioscorea alata* L. was given.

Analysis of Relative Number of $CD8^+CD62L^+$ T cells

The sensitivity phase, ethanol extract of *Dioscorea alata* L. (EEDA) with dose 0.00 g · kg^{-1} group showed the percentage of $CD8^+$ T cells expressing insignificant increase $CD62L^+$ ($p > 0.05$) compared to the control group. Compared to EEDA dose 0.00 g · kg^{-1} group, the percentage of $CD8^+$ T cells expressing $CD62L^+$ increased at EEDA dose 0.17 g · kg^{-1}, 2.01 g · kg^{-1}, 10.04 g · kg^{-1} and the antihistamine drug treated. However, the percentage of $CD8^+$ T cells expressing $CD62L^+$ in mice with diosgenin treatment did not increase as shown in Fig. 1 (a).

In the challenge phase, EEDA 0.00 g · kg^{-1} group showed insignificant decrease ($p > 0.05$) in the percentage of $CD8^+$ T cells that express $CD62L^+$ compared to the control group. Compared to EEDA dose 0.00 g · kg^{-1} group, the percentage of $CD8^+$ T cells expressing $CD62L^+$ increased at EEDA dose 0.17 g · kg^{-1}, 2.01 g · kg^{-1}, 10.04 g · kg^{-1} and antihistamine drug treated. On the contrary, percentage of $CD8^+$ T cells expressing $CD62L^+$ decreased in mice with diosgenin treatment as seen in Fig. 1 (b).

The digestive tract allergy phase, EEDA 0.00 g · kg^{-1} group showed insignificant decrease ($p > 0.05$) in the percentage of $CD8^+$ T cells expressing $CD62L^+$ in mice compared to a control group. Compared to EEDA dose 0.00 g · kg^{-1} group, the percentage of $CD8^+$ T cells expressing $CD62L^+$ increased at the EEDA dose 0.17 g · kg^{-1}, 2.01 g · kg^{-1}, 10.04 g · kg^{-1}, antihistamine drug treated and diosgenin treated group as seen in Fig. 1 (c).

The results showed that the digestive tract allergy phase EEDA 0.00 g/kg group after intraperitoneal injection of OVA on 15 d and 22 d continued with orally on 23 d until 30 d showed percentage $CD8^+CD62L^+$ T cells an increased significantly ($p = 0.037$) compared to the control group mice. The percentage of $CD8^+CD62L^+$ T cells increased in mice which is given the EEDA dose 0.17 g · kg^{-1}, 2.01 g · kg^{-1}, 10.04 g · kg^{-1}, mice which is given the antihistamine drug and mice which is given diosgenin compared to EEDA dose 0.00 g · kg^{-1} group as shown in Fig. 1(c).

In general, T cell responses can be divided into four distinct phases: activation, expansion, contraction and memory. After antigen stimulation, naïve $CD8^+$ T cells specific antigen become activated and undergo rapid expansion and differentiation of effector cells, where they increase the amount of up to 50 000-fold [33–36]. After the peak of the expansion, ~ 90 % to 95 % of the effector cells to undergo apoptosis, leaving the memory of long lived population that develop over the next few weeks [37]. This renewable memory cell population will increase protection in part because rapid expression and localization of the effector function non lymphoid network against secondary antigenic challenge [38, 39].

$CD8^+$ T cell activation would reveal many adhesion molecules on the cell membrane and cytokine profile different from its rest state. Adhesion molecules and cytokines working as an indispensable mediator involved in cross-talk immune cells in the immune response. Therefore, in the same way, the activation of $CD8^+$ T cells may produce specific immunity and shows the potential function of their regulations on the dendritic cells and then eventually to the activation and proliferation of $CD4^+$ T cells. But, until now, the exact role of $CD8^+$ T cell activation in immunoregulation can not be shown. Results of the study Chen *et al*., exploring the function of the activation of $CD8^+$ T cells in the regulation of immunity using T-cell/DC cocultured *in vitro* and the $CD8^+$ T cells adoptive transfer and activation *in vivo* found that activation of $CD8^+$ T cells negatively regulates the proliferation of $CD4^+$ T cells through changes in phenotype and dendritic cells function [40].

CONCLUSION

Ethanol extract of *Dioscorea alata* L. and antihistamine drug increase the percentage of $CD4^+$ T cells and $CD8^+$ T cells that express $CD62L^+$ on the sensitivity, challenge and digestive tract allergy phase. Diosgenin increased the percentage of $CD4^+$ T cells and $CD8^+$ T cells that express $CD62L^+$ on the gastrointestinal tract allergy phase of BALB/c mice digestive tract allergy model.

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