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Immunomodulatory Properties of *Citrus limon* Extracts on BALB/c Mouse Lymphoid and Myeloid Lineage Cells

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Abstract

To maintain or restore immunological homeostasis, life forms frequently rely on immunomodulators, also known by many terms such as biological response modifiers, immunostimulants, and immune restoratives. Lemon (*Citrus limon* (L.) Burm) is another well-known source of micronutrients and bioactive phytochemicals that serve as antioxidant against oxidative stress. In this present study, we aimed to examine the immunomodulatory properties of *Citrus limon* extracts (CLE) on lymphoid and myeloid lineage cells from BALB/c mouse. Six-weeks-old mice were treated by four different doses of CLE, 0, 200, 400, and 800 mg/kg BW for 14 days. Several markers of immune cells were investigated including CD8, CD62L, CD4, B220, VLA-4, TER119, CD55, Gr1, and CD11b antibodies which cover the population of lymphoid and myeloid lineage cells. Flow cytometry analysis was used to identify the specific subset of studied cell population. The results showed that CLE significantly increased the number of $Gr1^+$ granulocyte cells but did not affect other cell types. As a result, we surmised that the components of CLE might have a particular impact on granulocyte cells. Importantly, more investigation is needed to learn how CLE boosts granulocyte cell production.

Keywords: *Citrus limon*, granulocyte, immunomodulator, lymphoid, myeloid

1. Introduction

The immune system is undoubtedly one of the human body's most complex and dynamic systems (Huntington and Graym, 2018). Immune system comprises a network of innate and adaptive immune cells that continuously monitor their respective microenvironment by constantly recognizing and distinguishing between self and non-self-antigens while maintaining communication (Cao *et al*. 2019; Horwitz *et al*. 2019; Netea *et al*. 2020). Those responses are carried out by specific immune cell types, which are generally further categorized into two main groups, the effector and regulatory cells (Mezheyeuski *et al*. 2018; Zemmour *et al*. 2018). The alteration of these delicate balances could lead to an autoimmune problem when the effector cells become aberrantly reactive to self-antigens or their responses become exaggerated. Also, the amplitude of particular immune responses is highly influenced by the amount of pro-inflammatory and anti-inflammatory cells activated during the event. Maintaining immune homeostasis is critical to maintaining a normal and sufficient immune response (Cicchese *et al*. 2018; Sozzani *et al*. 2017).

On the other hand, the immune responses could also be compromised due to primary or secondary immune deficiency. Genetic factors often cause the first one, while the latter is caused by various environmental stress and diseases, such as actively taking cancer therapy, including radiation and chemotherapy, malnutrition, alcohol consumption, and smoking. These conditions significantly reduce the ability of the immune cells to fight any potential threat, causing longer and more severe infections and worsening the disease's prognosis. They interfere with MAPK and NF-κB signaling pathways, reducing pro-inflammatory cytokines synthesis such as IFN-γ, TNF-α, and IL-1β, hampering MHC II expression in APCs, increasing TLRs expression, decreasing anti-inflammatory cell counts such as Th2 and Tregs, reducing the number of $CD4^+$ and $CD8^+$ T cells, and also lowering IgG expression and avidity (Bourke *et al*. 2016; Qiu *et al*. 2016; Romeo *et al*. 2007).

To maintain or recover immunological homeostasis, we often depend on various immunomodulators, which are referred to by various names, including biological response modifiers, immunostimulants, and immune restoratives (Ogbue *et al*. 2022; Sapkota *et al*. 2022; Shamliyan and Dospinescu 2017). Its mechanism of action might involve the augmentation of anti-infective immunity of immune system cells such as lymphocytes, macrophages, dendritic cells, and natural killer cells. Other processes may occur, including activating or restoring immunological effector activity (Ferrari *et al*. 2020; Machado *et al*. 2020; Riaz *et al*. 2019). Some of the most often used immunomodulators include drugs produced from natural or synthetic components and microbial compounds. For instance, one of the most intensively studied herbal extracts, the extract of ginseng significantly reduces the ROS-induced IL-6 and

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IL-8 expression preventing pro-inflammatory hypercytokinemia while also increasing the ratio of $CD4^+/CD8^+$ T cells, NK cell cytolytic activity, as well as increasing serum level of IgA, IgG and IgM (Hong *et al*. 2012; Lee *et al*. 2014; Predy *et al*. 2005; Riaz *et al*. 2019; Zhou *et al*. 2014).

Another highly capitalized source for its micronutrients and bioactive phytochemicals is lemon. It contains a wide variety of natural antioxidants, including vitamin C. It also contains bioflavonoids, a group of antioxidants that help protect the body from oxidative stress. In our previous study, we found that *C. limon* extract have ameliorative effect on breast cancer mouse model (Putra *et al.* 2023). Some studies suggested that it could lower the concentration of C-reactive protein in the blood plasma, soluble vascular cell adhesion molecule-1 (sVCAM-1), and the soluble endothelial leukocyte adhesion molecule-1 (sE-selectin), both in normal individuals and persons with metabolic-disorder (Alhabeeb *et al*. 2022; Asgary *et al.* 2014; Buscemi *et al.* 2012). The highly dominant bioflavonoids identified on it are hesperidin and its aglycone variant, hesperetin (Miles and Calder 2021; Pyrzynska 2022; Zanwar *et al*. 2014). Interestingly, both hesperidin and hesperetin showed immunomodulatory effects through reducing the expression of TNF-α, IL-1β, and ICAM-1. Furthermore, these compounds also increasing the phosphorylation rate of the p38 MAPK and activating c-Jun-N-terminal kinase pathway (Choi and Lee 2010; Karthikeyan *et al*. 2021; Miles and Calder 2021).

On the other hand, vitamin C could optimize the phagocytosis capability of various phagocytic cells in the innate immune system (Gombart *et al*. 2020; Leal *et al*. 2017), as well as increase T cell proliferation and the concentration of various Ig in the blood serum (Miles and Calder 2021). Besides, the high content of flavonoids, terpenoids, fibers, and minerals in lemon has an essential function in preventing several severe diseases like obesity, diabetes, hypertension, cardiovascular disease, and certain malignancies (Asgary *et al*. 2018; Mahmoud *et al*. 2019; Saini *et al*. 2022), making it one of the best sources of various bioactive compounds which could be studied further particularly as immunomodulator compounds. As a result, the lemon extract is hypothesized to have immunomodulatory properties that affect both lymphoid and myeloid lineage cells.

2. Materials and Methods

2.1. Research Design

We applied a completely randomized design approach in this study by altering the treatment to examine the effect of the intervention by varying the variables used. This study was done in vivo on healthy BALB/c mice weighing about 20-25g, using four treatment groups: negative control (no lemon extract), lemon extract dosage 1 (200 mg/kg BW), dose 2 (400 mg/kg BW), and dose 3 (800 mg/kg BW). Each treatment had five replicates. The spleen was then examined to see how every treatment affected it.

2.2. The Lemon Extraction

The extraction mechanism we applied during this study was the crude extraction mechanism. About 100 g of lemons was blended until homogenous and then filtered. The filtrate of the crude lemon extract was then freeze-dried until it became a paste that was kept at 4°C until usage. Before the oral admission, we dissolved the crude extract paste that had been weighed before with distilled water into several working dosages. They are dose 1 (200 mg/kg BW), dose 2 (400 mg/kg BW), and dose 3 (800 mg/kg BW).

2.3. Oral Administration of CLE to BALB/C Mice Model

Mice about six-weeks-old were kept in cages in the Animal Physiology Laboratory of the Department of Biology, Faculty of Mathematics and Natural Sciences, Brawijaya University, Malang, for ten days to allow the mice to adjust to their new environment. Five mice were placed in each cage. They were kept at room temperature with food and water daily, *ad libitum*. The cages were cleaned every two days. The acclimatized mice were then orally administered lemon extract in three doses. The following were the treatment doses: dose 1 (200 mg/kg BW), dose 2 (400 mg/kg BW), and dose 3 (800 mg/kg BW). The oral administration of lemon extract was carried out for 14 days.

2.4. Sacrificing and Isolation of the BALB/c Mice Model

All mice models were sacrificed by neck-dislocating method, then sprayed with 70% alcohol before surgery to retrieve the mouse's spleen conducted from the left dorsal to ventral. The spleen of each treated mouse was washed with PBS solution and homogenized with 10-ml PBS. The homogenized spleen were placed into a 15-ml propylene tube. The acquired cell suspension was placed in a propylene tube. The cell suspension in the propylene tube was then centrifuged for 5 minutes at a speed of 2500 rpm and a temperature of 10°C. After discarding the supernatant, the pellet was resuspended in 1 ml of PBS. All animal treatments in this study have been approved by Ethical Board Commission of Brawijaya University with ethical approval number 779-KEP-UB.

2.5. Antibody Staining and Flow Cytometry Analysis

The isolated cells were resuspended, and 80 µl was transferred to a microtube. Then, 400 µl of PBS was added to the sample microtube and centrifuged for 5 minutes at a speed of 2500 rpm and a temperature of 10°C. The combination staining used for the flow cytometry analysis including PE-CD8, PE-Cy5-CD62L, PE-CD4, PE-Cy5-B220, PE-VLA-4, PE-Cy5-TER119, PE-CD55, FITC-Gr1, and FITC-CD11b antibodies (Figure 1) is as described below: The pellets were removed from the supernatant and stained with 50 µl of extracellular antibody (1 µl stock solution of antibody diluted with 50 µl of PBS and 10% FBS) before being incubated on ice for 20 minutes. The cell suspension was washed with 500 µl of washperm and resuspended to clean the fixative solution. The suspension was then centrifuged for 5 minutes at a speed of 2500 rpm at a temperature of 10°C. The pellets were then separated from the supernatant. After extracellular antibody staining, the cells were incubated in 300-500 µl of PBS before being transferred to a cuvette for flow cytometry analysis as our previous protocols (Putra *et al.* 2016; Putra *et al.* 2015).

2.6. Data Analysis

The BD Cellquest Pro^{TM} software was used to analyze the flow cytometry results. Then, we analyzed the data using a One-way ANOVA parametric analysis ($p \leq 0.05$), followed by Tukey's posthoc test to determine the

significance between treatment groups. We used SPSS version 16 for Windows for all statistical analyses mentioned.

3. Results and Discussion

3.1. The Effect of CLE Administration on Memory T Cells

Flow cytometry analysis revealed that the relative number of effector memory T cells ($CD8^+CD62L^-$) in the first dosage treatment, was 68.14%. Meanwhile, the relative number in the negative control, dose 2, and dose 3 treatments was 58.72%, 55.39%, and 66.07%, respectively (Figure 2). Compared to other treatment groups, dose 1 was the most efficacious in increasing the relative number of $CD8^+CD62L^-$. Previously, we also found that chloramphenicol induce the $CD8^+CD62L^-$ T cells (Putra *et al.* 2020; Putra and Rifa'i 2019). However, there were no significant statistical differences among all treatment groups in this present study.

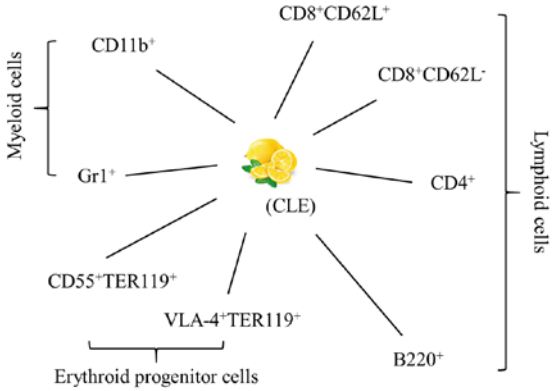

Figure 1. Different subsets of major immune cells evaluated after CLE administration.

The T cells that have matured and been released from the thymus have not yet encountered their appropriate antigen, expressing the CD62 protein (L-selectin) and C-C Chemokine receptor type 7 (CCR7) on their surface and are known as naïve T cells (Horna *et al*. 2019; Hunter *et al*. 2016). To transform into activated T cells, they migrate to secondary lymphoid organs such as the spleen, lymph nodes, tonsils, Peyer's patches, and other mucosal tissues, interacting with antigen, antigen-presenting cells, other lymphocytes through their respective cell receptors (Farber *et al*. 2014; Krummel *et al*. 2016). Because effector memory T cells circulate in the periphery and have direct effector activities when they encounter antigens, they do not express L-selectin (Watson *et al*. 2019). This subtype of memory T cells has a high cytotoxicity level yet lower proliferation ability (Martin and Badovinac 2018; Meryk *et al*. 2020). Without previous challenge from antigens, it is suggested that the improved number of memory T cells in lemon extract-treated groups is because vitamin C in the extract indirectly promotes memory T cells proliferation by enhancing phosphorylation of MAPK and ERK1/2, as well as increasing the activation of NF-κB in the dendritic cells. This phenomenon increases the synthesis of IL-15, promoting survival and proliferation of memory T cells through activating MAPK/PI3K-AKT pathway (Hong *et al*. 2016; Van Gorkom *et al*. 2018; Watkinson *et al*. 2021).

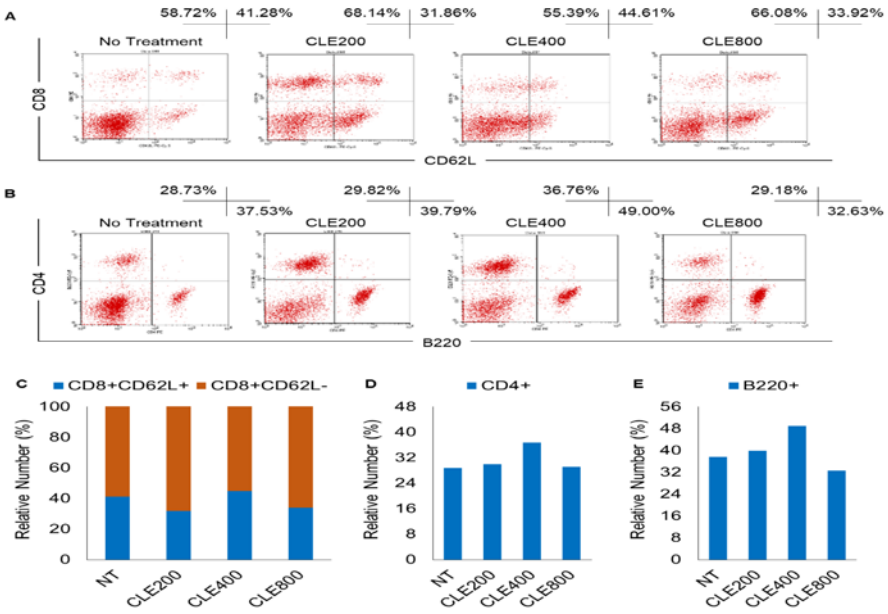

Figure 2. Immunomodulatory evaluation of CLE on $CD8^+CD62L^+$, $CD8^+CD62L^-$, $CD4^+$, and $B220^+$ subsets. (A). Flow cytometry graph of $CD8^+CD62L^+$ and $CD8^+CD62L^-$; (B). Flow cytometry graph of $CD4^+$ and $B220^+$; (C). Bar graph of $CD8^+CD62L^+$ and $CD8^+CD62L^-$; (D). Bar graph of $CD4^+$; and (D). Bar graph of $B220^+$.

The direct mechanism of vitamin C in the lemon extract that could stimulate memory T cell proliferation is still unknown. However, it is suggested that the memory T cells' immediate effect of vitamin C intake through sodium-dependent vitamin C transporters (SVCT) reduces apoptosis and induces more proliferation in a limited dose, known as the physiological dose (Van Gorkom *et al*. 2018). Folate in the extract could also have a similar effect in the T cells population, but with a far less known mechanism; it is predicted to influence the transcription process needed for proliferation and protein synthesis (Duthie *et al*. 2010; Miles and Calder 2021). Based on the results, dose 1 administration is the most effective dose to stimulate memory T cell proliferation without prior antigen exposure.

The ROS, which is involved in triggering cell death and terminating the immunological response, modulates naïve T cell activation because it is accumulated immediately upon activation (Hong *et al*. 2016). T cell differentiation is also influenced by ROS, but not their activation or proliferation. A proper T-cell response occurs under physiological conditions when there is an equilibrium between ROS and antioxidants in the tissue microenvironment and intracellular compartment (Belikov *et al*. 2015). Compared to memory and effector $CD8^+$ T cells, activated $CD8^+$ T cells have a distinct genetic signature. Chronically infected mice models and chronically infected people with HIV, EBV, or CMV do not express or re-express CD62L or CCR7, rendering them dysfunctional in their route and lymph node localization (Nolz *et al*., 2011).

3.2. The Effect of CLE Administration on B cells

According to the flow cytometry data, the relative number of B cells, also known as $B220^+$ cells, in the dose 2 treatment was 49.00%, while the negative control, dose 1, and dose 3 had a relative number of B cells of 37.53%, 39.79%, and 32.63%, respectively (Figure 2). We suggest that the dose 2 treatment was the most effective in increasing the relative number of B lymphocytes (B220) among other dosages. However, in this present study we found there were no statistically significant differences between these treatment dosages.

A particular compound in the lemon extract, naringenin, stimulates the proliferation of B cells in the spleen. (Maatouk *et al*. 2016). Vitamin C is also thought to increase the percentage of B cells and retain their viability. However, the result is still inconsistent among studies, and the molecular mechanism for both compounds remains unclear (Carr and Maggini 2017; Maatouk *et al*. 2016; Van Gorkom *et al*. 2018). For instance, a human-based study suggests that vitamin C intake positively correlates to the level of antibodies in the blood plasma, particularly IgM, IgA, and IgG, while others suggest the contrary results (Carr and Maggini 2017; Van Gorkom *et al*. 2018). A study that may explain these phenomena suggests that vitamin C is an important cofactor to ten-eleven translocation enzyme (TET) type 2 and 3 and promotes cytosine demethylation at Blimp1, which later triggers the activation mechanism of B cells towards plasma cells lineage, later increasing the concentration of antibodies (Qi *et al*. 2020).

The increasing number of mature B cells in the spleen without prior antigen challenge could be due to three main factors: increasing proliferation of B cell progenitor in the bone marrow, increasing proliferation capacity of B cells in the spleen, and increasing viability of mature or naïve B cells in the spleen during the selection mechanism (Ruiz-Iglesias *et al*. 2020; Shahaf *et al*. 2016; Van Gorkom *et al*. 2018). The second one is suggested as the main action mechanism of hesperidin, a major flavonoid in the extract, to increase the number of B cells in the spleen (Ruiz-Iglesias *et al*. 2020; Sassi *et al*. 2017).

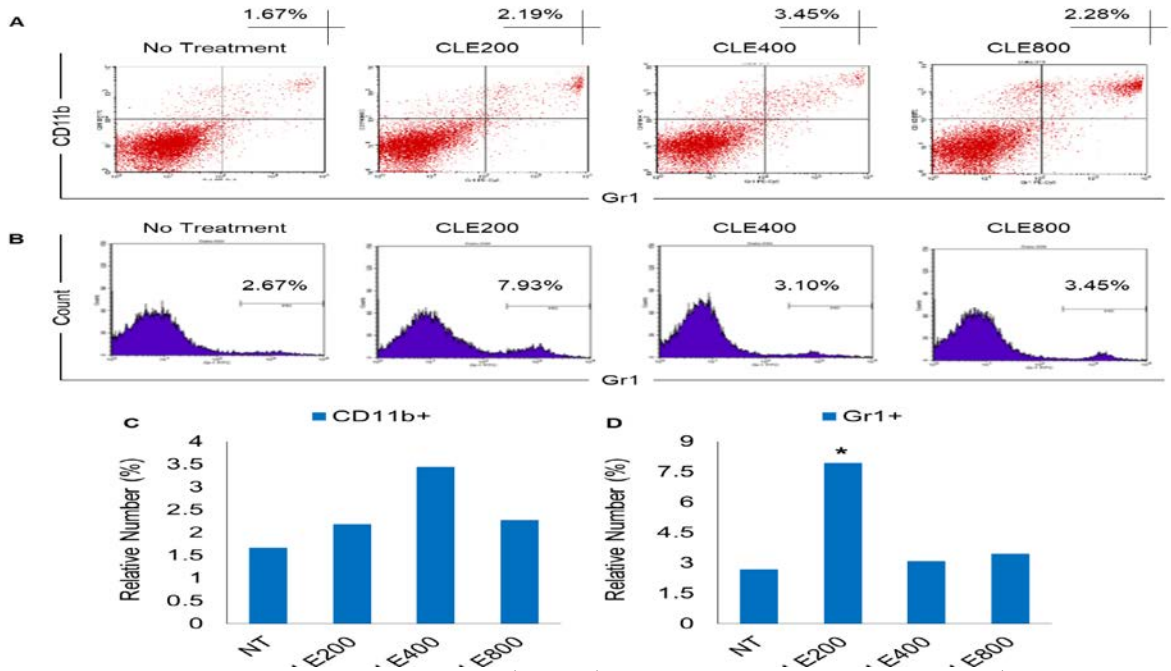

Figure 3. Immunomodulatory evaluation of CLE on $CD11b^+$ and $Gr1^+$ subsets. (A). Flow cytometry graph of $CD11b^+$; (B). Flow cytometry graph of $Gr1^+$; (C). Bar graph of $CD11b^+$; and (D). Bar graph of $Gr1^+$. The asterisk indicates the statistically significance compared to the other groups with p-values < 0.05.

On the other hand, research suggests that polysaccharide content in the extract could increase the viability of mature B cells in the spleen. The polysaccharide increases the viability of B cells by interacting with Toll-like receptor 4 (TLR4) and activating the MAPK signaling pathway (Xie *et al.* 2020). Pectin, furthermore could also increase the number of mature B cells in the spleen by increasing the production of IL-4 in the spleen, subsequently stimulating the B cells' differentiation and proliferation, as well as inhibit BCR-mediated apoptosis towards the mature B cells (Granato *et al*. 2014; Merheb *et al*. 2019; Zhou *et al*. 2020).

3.3. The Effect of CLE Administration on Helper T cells

According to the flow cytometry results, the relative number of helper T cells ($CD4^+$) in the dosage 2 treatment was 36.76%, whereas the relative number of the helper T cells in the negative control, dose 1, and dose 3 treatments was 28.73%, 29.82%, and 29.18%, respectively (Figure 2). Among the different treatment doses, it was clear that dose 2 was the most efficient in boosting the relative proportion of helper T cells. But the results of these treatments did not differ in a way that was statistically important.

Although there is no significant difference between groups, we suggest that lymphocytes, particularly T cells, have been known to accumulate a certain amount of vitamin C using sodium-dependent vitamin C transporter protein type 2 (SVCT2) (Hong *et al*. 2016; Oyarce *et al*. 2018). However, the results involving vitamin C and T cells primarily resulted in conflicting and inconsistent results. All of the previous research agrees that the cells tend to accumulate intracellular ROS after activation as a byproduct of mitochondrial activity as well as the vitamin C mentioned before, but at some point, it did not behave as an antioxidant as most predicted. Primarily, vitamin C is suggested to act as a cofactor for epigenetic regulation through interaction with Jumonji C histone–lysine demethylase, which acts as primary hydroxylate agent for methylcytosine residues specifically in Cd4 and Cd8 genes, as well as histone demethylation (Manning *et al*. 2013). It is also suggested that it triggers the continuation of the developmental stage of T cells from double-negative to double-positive stage, which is highly needed to begin the functional TCR$\alpha\beta$ selection process (Manning *et al*. 2013). These exact mechanisms also drive the polarization of naïve helper T cells into subsequent subsets (Song *et al*. 2017; Van Gorkom *et al*. 2018).

The higher number of $CD4^+$ T helper cells in the spleen could be attributed to their higher viability caused by vitamin C intake. It significantly decreases the T cell apoptosis rate and increases their proliferation (Carr and Maggini 2017; Miles and Calder 2021; Van Gorkom *et al*. 2018). As an antioxidant, it denies the promotion of activation-induced cell death by ROS through activation of the NF-κB pathway by lowering the concentration of the ROS (Carr and Maggini 2017; Hong *et al*. 2016; Kawashima *et al*. 2015). However, a study's results proved the opposite conclusion by stating that it exerts a toxic effect after some point. Unfortunately, the exact mechanism remains unclear, but they predict that it nullified the ROS amount and role as an important control mechanism during differentiation and functional stages (Belikov *et al*. 2015; Hong *et al*. 2016; Yarosz and Chang 2018).

Apart from vitamin C, other sources mentioned that the effect of *Citrus limon*'s flavonoids on the population of helper T cells is much more limited. Hesperidin in the citrus extract could also influence the increasing number of $CD4^+$ T helper cells by influencing the lymphoid tissue. However, the exact mechanism still needs to be figured out (Ruiz-Iglesias *et al.* 2020). A study suggests that hesperidin intake in healthy people had no notable effect (Perche *et al*. 2014). Its aglycone counterpart, hesperetin, is thought to have immunostimulatory characteristics by promoting T cell proliferation. Unfortunately, its exact mechanisms also remain unclear (Sassi *et al*. 2017). Polysaccharides such as pectin could also increase the number of splenic $CD4^+$ cells, particularly in IL-10 deficient mice, by downregulating pro-inflammatory cytokine expression (Beukema *et al*. 2022; Ye and Lim 2010). We still need further investigation to clearly picture why both lymphocytes' numbers peaked at the intermediate dose rather than the highest one.

3.4. The Effect of CLE Administration on Macrophages

The relative number of macrophage cells ($CD11b^+$) in the dosage 2 treatment was 3.45%, whereas the lowest relative number of macrophage cells was in the negative control group with 1.67%. The rest of the treatment doses had a relative number of macrophages around 2%, with dose 1 and dose 3 numbers being 2.19% and 2.28%, respectively (Figure 3). Among the several treatment dosages, dose 2 has the highest ability to increase the relative number of macrophage cells. Those results showed no statistically significant changes.

Other studies suggest that administering flavonoids or other bioactive compounds lowers the cell count and inhibits the maturation process of macrophages by inhibiting LPS-induced inflammatory response (Carr and Maggini 2017; Han *et al*. 2022; Zhang *et al*. 2014). Instead, we found that the number of macrophages increased without statistical significance. We cannot find any sufficient explanation for why this phenomenon occurs. A study in traumatic ulcer rats showed that the number of macrophages increased after the administration of *Citrus limon* peel oil and suggested that fumarate acid and d-limonene could act as a free radical scavenger which suppresses the production of ROS and iNOS, protecting macrophages from oxidative damage and prevent them from undergoing apoptosis, hence promoting their proliferation (Surboyo *et al*. 2019).

Another study using *Citrus limon* peel essential oil on guinea pig suggests that the induction of it causes type IV hypersensitivity, which triggers the antigen recognition mechanism by APCs, introduced to T-helper cells and initiating the synthesis of IFN-γ, promoting macrophage activation and proliferation. These inflammatory responses, however, did not show any sign of inflammation. They suggest that the increasing number of macrophages may not be fully driven towards pro-inflammatory response but also contribute to anti-inflammatory responses because some flavonoids like d-limonene and linalool drive the macrophage polarization towards M2 phenotype and induce the synthesis of IL-10, inhibiting the inflammatory responses and minimizing the

effect (Sulaiman et al., 2022; Mahdani *et al*. 2020). Rutin, another flavonoid, also stimulates the $CD11b^+$ cells toward the M2 phenotype (Ferraz *et al*. 2020).

3.5. The Effect of CLE Administration on Myeloid Cells Quantity

Flow cytometry results show that all treatment groups have a higher relative number of Gr-1 cells than the negative control at 2.67%. It was significantly higher in the dose 1 group, with 7.93%, while the other dosages were consistent at around 3%, with dose 2 at 3.1% and dose 3 at 3.45% (Figure 3). The dose 1 treatment was the most effective in inducing Gr-1 myeloid cell differentiation. The relative number of Gr-1 neutrophils in the dose 1 group increased by 5.26% but remained within acceptable limits. If the relative number exceeds the acceptable limit, it is considered neutrophilia, defined as an increased number of neutrophils beyond the average threshold caused by inflammation, stress, the corticosteroid reaction, excessive exercise, and the epinephrine response (Goldman and Schafer 2020). It also developed due to physiological events such as corticosteroid induction, inflammation, and neoplasia.

Granulocytes are white blood cell subgroups distinguished by granules' presence in their cytoplasm. Because of the different forms of the nucleus, which generally contains a three-segment gap, granulocytes, also known as polymorphonuclear leukocytes (PMNs), consist of three types of cells: neutrophils, basophils, and eosinophils (Breedveld *et al*. 2017). Neutrophils are white blood cells with the highest population among PMNs cells, accounting for over 70% of the total number of cells. It has an extremely crucial role in innate immune responses because of its phagocytic nature in circulation (Rosales 2018). It is one of the first cells to migrate toward the site of inflammation during the early acute inflammatory phase, generally caused by bacterial infection, environmental exposure, or malignancy by following chemical signals such as IL-8, C5a, fMLP, leukotriene B4, and H_2O_2 (Kraus and Gruber 2021; Selders *et al*. 2017).

One of the few compounds in the extract thought to have a critical effect on myeloid cells is coumarin. It has a vital function in the immune system by regulating the activity of white blood cells, particularly the granulocytes, making them, particularly the neutrophils, work more efficiently because it prevents extensive and potentially dangerous activation of neutrophils has been proposed as a critical injury-limiting way by acting as scavengers for ROS generated by the neutrophils (Chen *et al*. 2015). Conversely, vitamin C acts as an antioxidant by scavenging free radicals in activated leukocytes due to lipid oxidation, preserving normal membrane fluidity and motility (Miles and Calder 2021). It also influences immune function by regulating redox-sensitive cell communication pathways. Another distinctive bioactive compound in lemon is auraptene, which has immunomodulatory properties by increasing the activities of β-glucuronidase and acid phosphatase in macrophages (Genovese and Epifano 2011), as well as stimulating the production of IL-1β and TNF-α (Hsia *et al*. 2021).

As mentioned previously, vitamin C is highly critical for various immune cells, including myeloid cell lineage, so normally they maintain it at an adequate intracellular level (Ang *et al*. 2018; Liugan and Carr 2019). A study in septic patients suggests that vitamin C could prevent the peripheral neutrophils from undergoing apoptosis by increasing the level of Bcl-2 while simultaneously lowering apoptosis-promoting factors such as caspase-3 and poly-ADP-ribose polymerase in the downstream (Liugan and Carr 2019), as well as the upstream by inhibiting caspase-8 activation induced by Fas-ligand interaction, and Fas-induced apoptosis as general primarily as a consequence of its properties as antioxidant (Ang *et al*. 2018). On the other hand, hesperidin also had a similar effect on inhibiting granulocyte apoptosis by acting as an antioxidant in the ROS-induced apoptosis pathway (Adefegha *et al*. 2017). These mechanisms could be the factor behind the higher number of granulocytes we found in this study.

3.6. The Effect of CLE Administration on Erythroid Cells Quantity

Flow cytometry analysis revealed that the relative proportion of $TER119^+VLA\text{-}4^+$ cells in dose 2 was 73.31% and 65.65% in dose 3. The relative number of erythroid cells in both dosages dropped from the negative control of 75.27%. In contrast, the relative number of $TER119^+VLA\text{-}4^+$ cells in the dose 1 group grows by 9.68% compared to the normal group, to 84.95%, although the increase is not statistically significant (Figure 4). These findings suggest that the dose 1 treatment is the most efficient in stimulating the differentiation process in erythrocytes. The results also showed that the relative number of $TER119^+CD55^+$ cells dropped to 23.78%, 18.49%, and 24.19%, respectively, for those three dosages, compared to the normal control group, 28.96%. However, the declining number in these three treatments was not statistically significant compared to each other. It is suggested that the dose 1 treatment is the most effective in preventing cell lysis.

Iron ion is a necessary precursor in the synthesis of hemoglobin in erythrocytes. In contrast, vitamin C is an exogenous antioxidant that plays a vital role in erythrocyte synthesis because it acts as an enzyme cofactor and promotes the mobilization of the ferrous form of iron to transferrin, increasing its bioavailability (Cimmino *et al*. 2018; Imam *et al*. 2017). It can reduce oxidative stress and prevent free radical damage to erythrocyte cells (Milošević *et al*. 2018; Suleman *et al*. 2019). Consequently, the vitamin C concentration of lemon extract, particularly in dose 1, may have a substantial role in erythrocyte precursor synthesis, although there is no statistical significance compared to other doses. Nevertheless, the high concentration of vitamin C in the other two dosages did not increase the number of red blood cell precursors because it may trap superoxide anions and have the ability to interrupt the cycle of radical reactions caused by the lipid peroxidation process exclusively in low concentration, but not in high concentration (Juan *et al*. 2021; Kaźmierczak-Barańska *et al*. 2020). In this study, the administration of lemon juice from the three doses did not significantly differ, presumably caused by the lipid peroxidation process. The lipid peroxidation on the erythrocyte membrane could reduce the membrane fluidity and enhance the fragility of the erythrocyte membrane, leading to a higher rate of hemolysis (Duchnowicz *et al*. 2021; Maćczak *et al*. 2017). Suppose the body does not have an adequate number of antioxidants. In that case, it is

conceivable that the number of erythrocytes and hemoglobin levels may drop, resulting in anemia because the erythrocyte membrane is the most vulnerable part of erythrocyte to lipid peroxidation due to direct and continual exposure to high oxygen partial pressure and saturated fatty acids (Pisoschi *et al*. 2021).

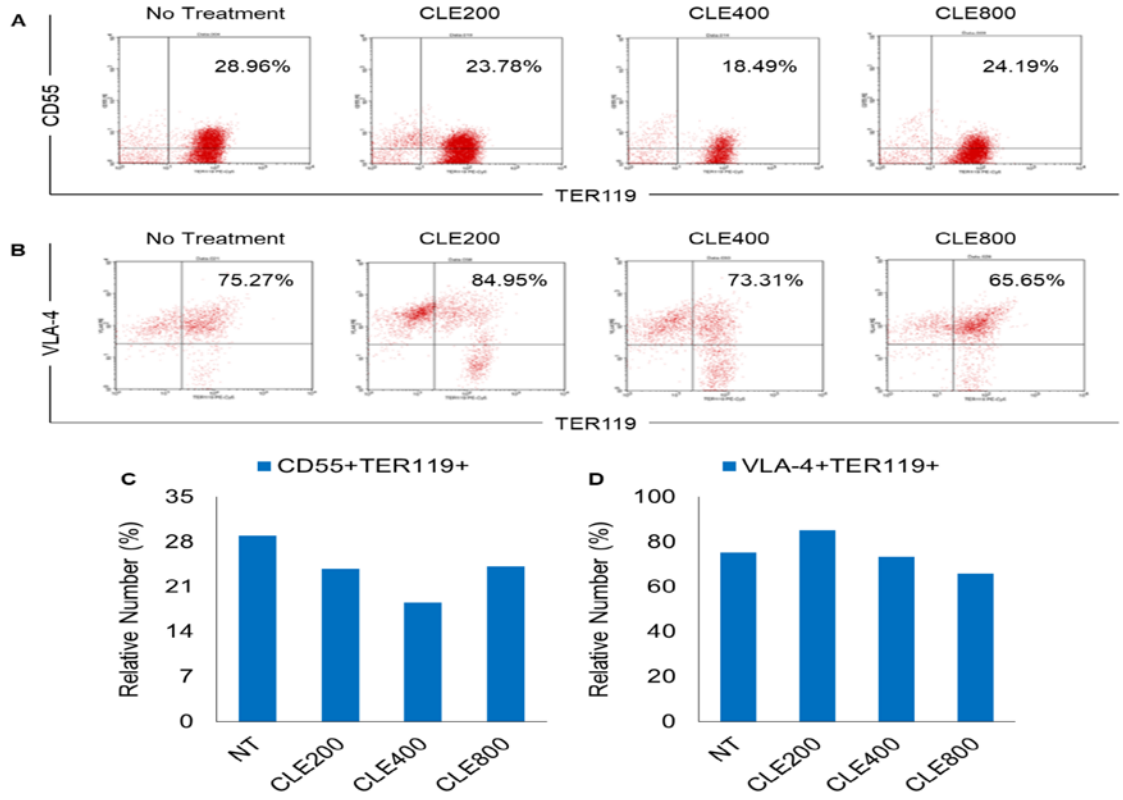

Figure 4. Immunomodulatory evaluation of CLE on $CD55^{+}TER119^{+}$ and $VLA\text{-}4^{+}TER119^{+}$ subsets. (A). Flow cytometry graph of $CD55^{+}TER119^{+}$; (B). Flow cytometry graph of $VLA\text{-}4^{+}TER119^{+}$; (C). Bar graph of $CD55^{+}TER119^{+}$; and (D). Bar graph of $VLA\text{-}4^{+}TER119^{+}$.

On the other hand, erythrocytes use both enzymatic and non-enzymatic antioxidants to combat free radicals generated by lipid peroxidation. Those two ideas stated that the quantity of adult red blood cells should be at least equivalent to or even higher than the normal group of the presence of vitamin C, which reduces hemolysis, supported by CD55, which further protects the cells from lysis. However, the statistical analysis suggests that all treatment groups had a lower relative number of erythroid cells, probably due to the high degree of lipid peroxidation in mice models.

4. Conclusion

In the present investigation, numerous subsets of lymphoid and myeloid immune cells were examined. According to our findings, CLE has a considerable effect in increasing the number of $Gr1^{+}$ granulocyte cells, but not other cell types. Thus, we hypothesized that the chemicals in CLE could have a specific effect on granulocyte cells. Importantly, additional research must be conducted to determine how CLE increases the number of granulocyte cells.

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Conflict of Interest

No conflict of interest

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