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Immunomodulatory Effect of Propolis Extract on Granzyme Expression in CD8$^+$ and CD4$^+$CD25$^+$ T Cells

Propolis Ekstresinin CD8$^+$ ve CD4$^+$CD25$^+$ T Hücrelerindeki Granzim İfadesi Üzerine Olan Bağışıklık Düzenleyici Etkileri

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Abstract

Objective: The purpose of this study was to explore the effect of propolis extract on CD8$^+$ and CD4$^+$CD25$^+$ regulatory T cell populations and granzyme expression in both cell populations, as propolis has been suggested as an immunomodulatory agent.

Materials and Methods: PBMC containing 1x10^6 cells/ml from cervical cancer stage 3 patients were isolated and cultured with and without propolis extract. The cells were plated in a 24-well plate with RPMI medium supplemented with 10% FBS, 10 U/ml penicillin, 100 U/ml streptomycin, 2 mM L-glutamine and 50 µM 2-ME. Propolis extract was added (5, 10, 25 and 50 µg/ml) to PBMC culture. Negative control was made from PBMC culture without propolis extract for comparison. The cells were incubated for 48 hours in a 5% CO_2 incubator at 37°C. The proportion of CD8$^+$ and CD4$^+$CD25$^+$ regulatory T cells that expressed granzyme were measured by flow cytometry.

Results: The results obtained that the level of CD4$^+$, CD4$^+$CD25$^+$, and CD8$^+$ T cells were not markedly depleted in propolis treatment in all doses compared to control group. The percentage of CD8$^+$Granzyme$^+$ and CD4$^+$CD25$^+$Granzyme$^+$ were significantly different in groups of propolis treatment compared to control group.

Conclusion: In summary, propolis extract may serve as an immunomodulatory agent in cervical cancer patients. Propolis extract could modulate the expression CD4$^+$CD25$^+$ T cells expressing granzyme and CD8$^+$ T cells expressing granzyme while it has no effect on CD4$^+$, CD4$^+$CD25$^+$, and CD8$^+$ T cells.

Keywords: CD8$^+$ T cells, CD4$^+$CD25$^+$ regulatory T cells, granzyme, propolis

Öz

Amaç: Çalışmanın amacı, propolis ekstresinin bağışıklık düzenleyici bir madde olarak CD8$^+$ ve CD4$^+$CD25$^+$ düzenleyici T hücrelerindeki granzim ifadesi üzerine olan etkisini incelemektir.

Gereçler ve Yöntemler: Evre 3 serviks kanserli hastalardan elde edilen 10(6) hücre/mL konsantrasyonundaki Periferik Mononükleer Kan Hücreleri(PMKH) izole edilerek propolis eklenen ve eklenmeyen kaplarda kültüre edildi. Hücreler, %10 FBS, 10 U/ml penisilin, 100 U/mL streptomisin, 2mM L-glutamin ve 50 mikroM 2-ME içeren 24 kuyulu kültür kaplarına paylaştırıldı. Bir PMKH popülasyonu Propolis uygulanmadan kontrol grubu olarak oluşturuldu. Hücreler %5 CO2 içeren kuluçka makinesinde(inkübatör) 48 saat kültüre edildi. Granzim ifade eden CD8+, CD4+CD25+ düzenleyici T lenfositleri akan hücre ölçer ile irdelendi.

Bulgular: Propolis uygulanan CD4$^+$, CD4$^+$CD25$^+$ ve CD8$^+$ T lenfositlerinin sayıları anlamlı ölçüde azalmadı. CD8$^+$Granzim$^+$ ve CD4$^+$CD25$^+$Granzim$^+$ hücrelerin sayıları, propolis uygulanan hastalarda farklı idi.

Sonuç: Sonuç olarak, propolis ekstresi serviks kanserli hastalarda bağışıklığı değiştiren bir madde olarak etki etmektedir. Ekstre, CD4$^+$CD25$^+$ T lenfositleri ile CD8$^+$ T lenfositlerinde granzim ifadesini değiştirmekte, ancak ekstrenin CD4$^+$, CD$^+$CD25$^+$ ve CD8$^+$ T lenfositlerinde granzim ifadesine etkisi görülmemektedir.

Anahtar Kelimeler: CD8$^+$ T Hücreleri, CD4$^+$CD25$^+$ düzenleyici T Hücreleri, granzim, propolis

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Introduction

It has been generally understood that the immune system has a major role in the formation of cancer cell and cancer cell growth. Physiologically, a human has a defense mechanism that works in an integrated manner to protect themselves from the invasion of a stranger and the development of cancer cells, this mechanism is known as immunosurveillance.[1] However, cancer cells make various efforts to escape

from immunosurveillance activity by manipulating the population and activity of various immune system components, affecting the population proportion of cells in the immune system, and stimulate the development of immune cells that are immunosuppressive so favorable for its development. One of the cells with the potential immunosuppression is regulatory T cells ($CD4^+CD25^+Foxp3^+$).[2,3]

Lymphocyte-mediated cytotoxicity is a major immune effector function required for normal defense against pathogens, control of neoplasms, and regulating immune responses. The role of cytotoxic T lymphocyte (CTL) in the immune response to a neoplasm has a very important position.[4] The most important way of CTL cytotoxic activity is via exocytosis of perforin and Granzyme. Granzyme is a subclass of serine proteases with diverse proteolytic specificities, while the main role of perforin is to permeabilize cell membrane to allow granzyme (the effector proteases), enter the target cells.[5] Granzyme is a major component of cytotoxic lymphocyte granules that trigger the apoptosis of target cells. The granzyme main role is to induce cell death to eliminate viruses and tumor cells.[6]

The immune response physiologically requires regulators to prevent an excessive immune response. One type of immune cell that has immunosuppressive activity is the regulatory T cell.[2,3] Regulatory T cells are a subset of T cells that express CD4, CD25, and the transcription factor Foxp3, and normally induce tolerance. An increase in these cells may reduce the immune response and suppress defense against tumors by inhibiting cytokines. Regulatory T cell activation is induced by TGF-β and IL-2 and is associated with a poor prognosis in cases of malignancy.[7] Regulatory T cells affect many components of the immune system, including $CD4^+$ T cells (Th1, Th2, and Th17), $CD8^+$ T cells, macrophage, dendritic cells (DCs), natural killer cells (NK), NKT cells, mast cells, osteoblasts and B cells[8] and have the capacity to reduce and inhibit an effective immune response to cancer cells. Activated regulatory T cells suppress the innate and adaptive immune responses through different mechanisms, including upregulating the production of immunosuppressive cytokines such as IL-10 and TGF-β[9], inhibiting the maturation of APC, in this case DCs by inhibiting the expression of the co-stimulatory molecules CD80 and CD86[10], inducing T cell cytolysis by releasing perforin and granzyme that can kill $CD4^+$ T cells, $CD8^+$ T cells, DCs and monocytes[2], and interfering with the metabolism of T cell responders, as regulatory T cells contain high concentrations of cAMP, which can inhibit proliferation, differentiation, and synthesis of IL-2 in T cells.[11]

Regulatory T cells are potent inhibitors of anti-tumor immunity and immunotherapy due to these immunosuppressive mechanisms.[12] Thus, regulatory T cells are an important target for cancer immunotherapy, as decreasing or inhibiting regulatory T cells is expected to improve the effectiveness of the immune response to cancer. Strategies inhibiting regulatory T cells target a variety of pathways, including the depletion of regulatory T cells in quantity and suppression/inhibition of their immunosuppressive function.

Both $CD8^+$ cytotoxic lymphocytes (CTL) and regulatory T cells express granzyme. For CTL, granzyme is an important effector molecule of cytotoxic activity against target cells, including cancer cells. Granzyme is expressed by regulatory T cells and triggers cytolysis of $CD4^+$ T cells, $CD8^+$ T cells, DCs and monocytes.[2,13]

Propolis is a substance produced by bees from plant resins collected and combined with the wax and secretions from the salivary glands of bees are rich in various enzymes.[14,15] Bee Propolis is used to coat the walls of the nest to protect the animals from the entry of the attacker from the outside, inhibit bacterial and fungal growth, strengthen cell walls also maintain an aseptic environment in a nest.[16] Propolis is a natural product with known immunomodulatory activity, but how it affects CTL and regulatory T cells is still unknown. This is a preliminary study to examine the effect of propolis extract on regulatory T cell ($CD4^+CD25^+$) and CTL ($CD8^+$) population and to assess the expression of granzyme in cultured PBMC obtained from of cervical cancer stage 3 patients.

Materials and Methods

Propolis extraction

The propolis extraction process was performed based on a modified method.[17] One hundred grams of propolis was cut into small pieces and frozen at -80 °C. After that, propolis was ground and extracted with ethanol (1:10). The solution was sonicated using an ultrasonic bath at 25 °C for 30 minutes in the dark. After sonication, the solution was filtered through Whatman No.1 filter paper. Sonication using 70% ethanol was repeated in order to

obtain the active compounds. The total solution then kept in the refrigerator for wax removal overnight. The solution was filtered through Whatman No.1 filter paper and evaporated to remove the organic solvent.

PBMC Isolation

The samples used in this study were obtained from the blood of cervical cancer stage 3 patients who previously diagnosed by oncologists of Saiful Anwar General Hospital, Malang Indonesia. The authors did not do the clinical characterization of the patients. All patients were new cases who had received no prior treatment, chemotherapy and/or radiation therapy before sample collection. Informed consent was obtained from all subjects before sample collection.

From each patient, 5 ml of peripheral blood was obtained using a 5-ml EDTA-containing syringe and kept in a Vacutainer with sodium heparin (BD, USA). The blood samples were mixed with the same volume of PBS. The diluted blood sample was layered on Ficoll-Paque Plus using a Pasteur pipette. The mixture was centrifuged at 4000x g for 40 minutes at 18-20 ºC. After centrifugation, the lymphocyte layer was transferred to a new tube and washed twice with PBS. The cell pellet was resuspended with Roswell Park Memorial Institute (RPMI)-1640 medium with 10% fetal bovine serum (FBS).

PBMC Culture

PBMC contained $1x10^6$ cells/ml were plated in a 24-well plate with RPMI medium supplemented with 10% FBS, 10 U/ml penicillin, 100 U/ml streptomycin, 2 mM L-glutamine and 50 µM 2-ME. Propolis extract was added (5, 10, 25 and 50 µg/ml) to the PBMC cultures. Negative control was made from PBMC culture without propolis extract for comparison. The cells were incubated for 48 hours in a 5% CO2 incubator at 37ºC.

Flow cytometry

Peripheral blood mononuclear cells were harvested for further flow cytometric analysis. To detect the percentage of $CD4^+CD25^+granzyme^+$ lymphocytes, cells were incubated with 5 µl of anti-human CD4 FITC and anti-human CD25 PE monoclonal antibodies (Biolegend, USA) for 30 minutes at room temperature in the dark. The appropriate isotype control antibodies (all from Biolegend) were simultaneously added to a separate tube containing cells from the same patient and incubated for 30 minutes in the dark. After centrifugation and washing with the washing solution, the samples were fixed using 300 µl of 1% paraformaldehyde and permeabilized using the same amount of 0.1% saponin (Sigma-Germany). Anti-human granzyme AlexaFluor 647 (Bios) was then added to the permeabilized cells and incubated for 30 minutes in the dark. The appropriate isotype control was simultaneously used in the control tube with permeabilized cells. To detect the percentage of $CD8^+granzyme^+$ cells that express granzyme, the same procedure was done as above using anti-human $CD8^+$ FITC (Biolegend) and anti-human granzyme AlexaFluor 647 (Bios).

After the final fixation, samples were analyzed immediately by using a BD FACS Calibur flow cytometer (BD, USA). Cellquest-pro software (BD-USA) was used for data acquisition and analysis. Briefly, the percentage of total CD4+ T cells was first calculated in the lymphocyte gate. In order to define the percentages of $CD25^+$ regulatory T cells among the lymphocyte population, the lymphocyte gate was applied on the quadrant of the CD4/FSC dot plot. The CD4 positive lymphocyte gate was finally applied on the $CD25^+/granzyme^+$ histogram and the percentage of $CD4^+CD25^+granzyme^+$ lymphocytes was considered as the percentage of regulatory T cells that expresses granzyme.

Statistical analysis

One-way ANOVA was used to analyze the data. The differences between groups were considered significant at $p<0.05$. All results were presented as the mean of ± SD values of 10 samples with three replicates in each group. Statistical analysis was performed by using SPSS software. This was followed by a posthoc Tukey's honestly significant difference (HSD) test to determine major changes and differences among $CD8^+$ and $CD4^+CD25^+$ regulatory T cells and also the proportion of $CD8^+$ and $CD4^+CD25^+$ T cells that expressed granzyme relative to the density mean values.

Results

The results show that the percentage of $CD4^+$, $CD4^+CD25^+$, and $CD8^+$ T cells were not markedly decreased in all doses of propolis extract compared to control group (Fig. 1 and Fig. 2).

In addition to observing changes in the $CD4^+CD25^+$and $CD8^+$ T cell populations, we observed the further study of their functional activity. In this case, we assessed

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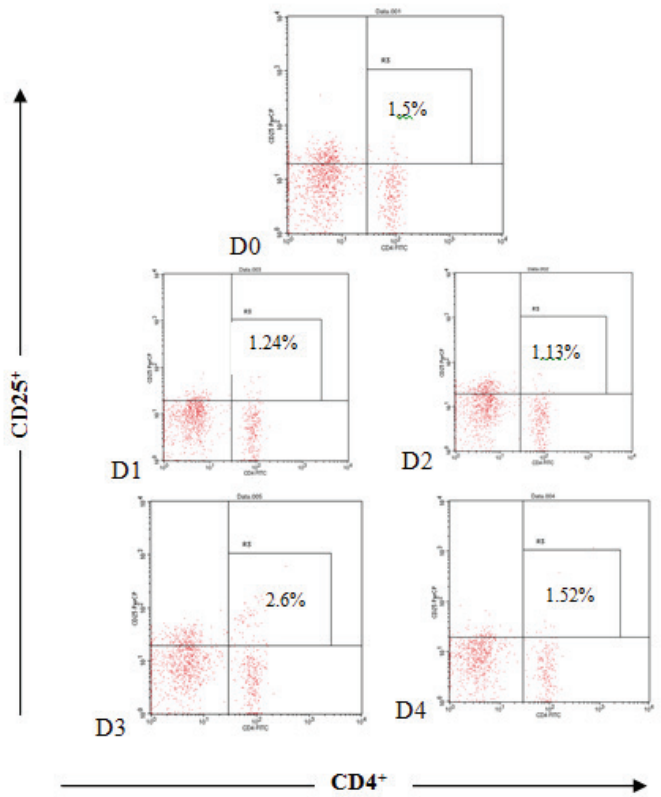

Figure 1. Propolis extract is not able to increase the level of $CD4^+CD25^+$ in all doses compared to control group. The relative number of $CD4^+CD25^+$ cells PBMC after 48 hours of propolis treatment and analyzed by flow cytometry (D0= PBMC culture without propolis extract/Control, D1 = PBMC culture with 5 µg/ml propolis extract, D2 = PBMC culture with 10 µg/ml propolis extract and D3 = PBMC culture with 25 µg/ml propolis extract, D4= PBMC culture with 50 µg/ml propolis extract). Data are mean of ± SD values 10 samples with three replications.

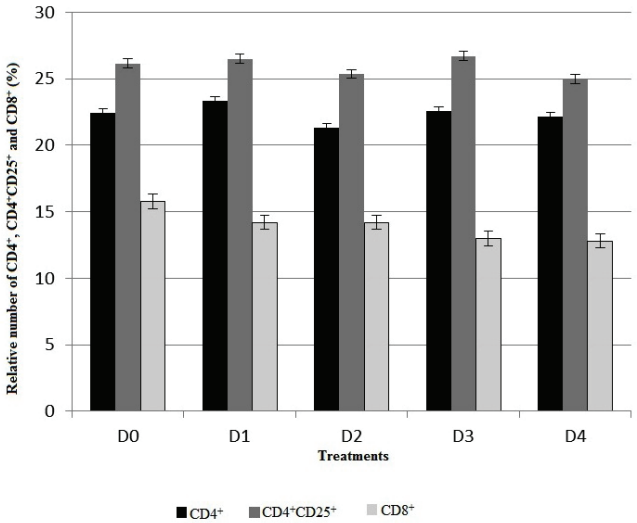

Figure 2. Propolis extract is not significantly changed the percentage of $CD4^+$, $CD4^+CD25^+$, and $CD8^+$ T cells population. The bars are the calculation of the number of $CD4^+$, $CD4^+CD25^+$ and $CD8^+$ T cells on PBMC from cervical cancer stage 3patients in vitro. PBMC from patients with stage 3 cervical cancer were treated as indicated for 48 hours and the expression of $CD4^+$, $CD4^+CD25^+$ and $CD8^+$ T cells were detected by flow cytometry. The differences between groups were considered significant at $p<0.05$. All results were presented as the mean ± SD values of 10 samples in each group with 3 replications.

the expression of granzyme by $CD8^+$ T cells and $CD4^+CD25^+$ regulatory T cells. In this study, D1 and D2 group of propolis extract did not significantly increase the percentage of $CD8^+$ T cells-expressing granzyme compared with the D0/control group. However, the level of $CD8^+$ T cells-expressing granzyme in D3 and D4 group of propolis extract were markedly depleted (Fig. 3). Otherwise, propolis extract was able to markedly increased the expression of granzyme between control and propolis-treated $CD4^+CD25^+$ regulatory T cells in D3 and D4 group (Fig. 4).

One-way ANOVA with LSD Post-Hoc test showed that D3 group (PBMC culture with 25 µg/ml propolis extract) and D4 (PBMC culture with 50 µg/ml propolis extract) have a significant difference of the relative number of $CD8^+$ expressing granzyme compared with control with p-value 0.044 and 0.019 respectively. The relative number of $CD4^+CD25^+$ expressing granzyme in D3 and D4 group also have a significant difference compared with control group with p-value 0.003 and 0.000 respectively.

Discussion

The effect of propolis extract to the population of $CD8^+$ and $CD4^+CD25^+$ regulatory T cells

In this study there was no significant change in the percentage of $CD4^+$ T-cell populations, $CD4^+$ $CD25^+$ and $CD8^+$, these results are different from a study by Rohmawati who reported that 200 mg/kgBW propolis extract given to normal mice increased the activation of $CD4^+$T cells ($CD4^+$ CD62L-) and decrease the level of $CD8^+$ 62L-T cells.[18] Different results were also obtained by Rifai (2014) who found that propolis extracts increased the number of $CD4^+$ $CD25^+$ T cells in normal mice but caused a decrease in diabetic model rats. These facts suggest that propolis extract can maintain immunological homeostasis so that propolis extract causes different effects when administered under different conditions.

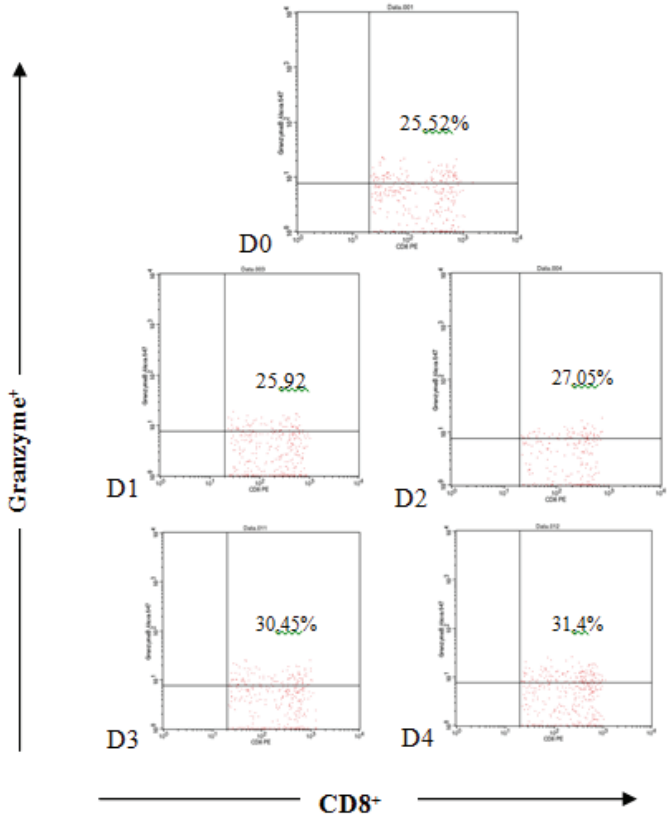

Figure 3. Propolis extract in D3 and D4 group is able to increase the level of $CD8^+$ T cells-expressing granzyme compared to control (D0) group. The relative number of $CD8^+$ cells-expressing granzyme after 48 hours of propolis treatment and analyzed by flow cytometry (D0 = PBMC culture without propolis extract/Control, D1 = PBMC culture with 5 µg/ml propolis extract, D2 = PBMC culture with 10 µg/ml propolis extract and D3 = PBMC culture with 25 µg/ml propolis extract, D4 = PBMC culture with 50 µg/ml propolis extract. Data are mean of ± SD values of 10 samples with 3 replications.

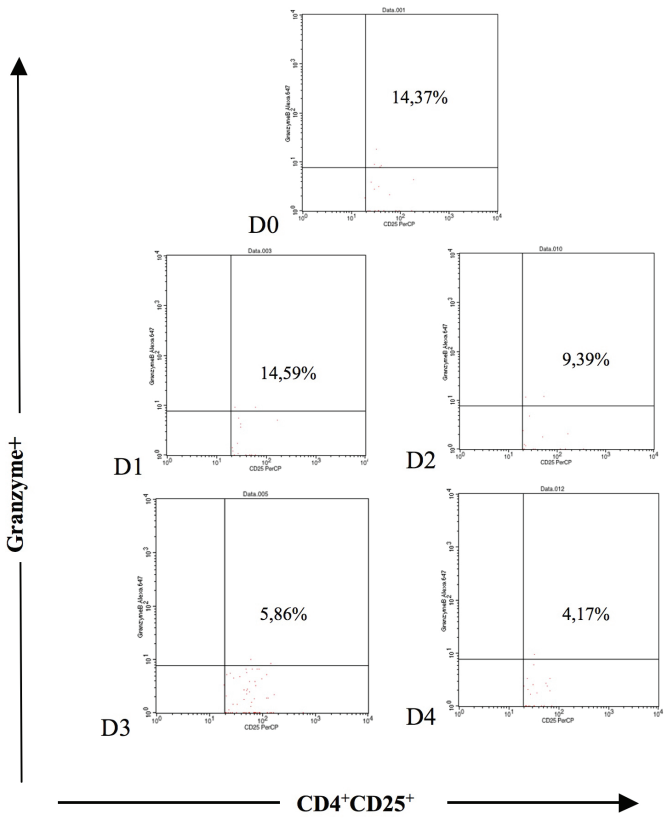

Figure 4. Propolis extract in D3 and D4 group is able to decrease the $CD4^+CD25^+$ T cells-expressing granzyme compared to control (D0) group. The relative number of $CD4^+CD25^+$ $Granzyme^+$ after 48 hours of propolis treatment and analyzed by flow cytometry (D0 = PBMC culture without propolis extract, D1 = PBMC culture with 5 µg/ml propolis extract, D2 = PBMC culture with 10 µg/ml propolis extract and D3 = PBMC culture with 25 µg/ml propolis extract, D4= PBMC culture with 50 µg/ml propolis extract). Data are mean ± SD values of 10 samples with 3 replications.

The therapeutic effect of propolis extract was probably derived from its active compounds, one of which is caffeic acid phenethyl ester (CAPE).[19] CAPE has been widely studied and has been shown to possess antioxidant,[20,21] anti-cancer[22–26], as well as immunomodulatory[27,28] capacity. As an immunomodulator, CAPE has been shown to inhibit the percentage of PBMC expressing CD25 following stimulation with staphylococcal enterotoxin B.[28] CAPE also lowered the expression of several cytokines, such as IL-12, IL-10 and IL-4, in cultured PBMC and T cells from a healthy donor after stimulation with pokeweed mitogen.[27]

In the context of tumor immunology, the cell population changes after the administration of propolis extract have a positive meaning and benefit. Regulatory T cells ($CD4^+CD25^+$ regulatory T cells) affect many components of the immune system, including $CD4^+$ T cells (Th1, Th2, and Th17), $CD8^+$ T cells, macrophages, DCs, NK cells, NKT cells, mast cells, osteoblasts, and B cells.[8] Regulatory T cells also have a functional activity to suppress the activity of $CD8^+$ T cells.[29] Via a variety of immunosuppressive mechanisms, regulatory T cells are potent inhibitors of anti-tumor immunity and immunotherapy. Decreasing or inhibiting effect of regulatory T cells is expected to improve the effectiveness of the immune response to destroy cancer cells.

Several studies had reported the effectiveness of using monoclonal antibodies to deplete regulatory T cells, including the anti-CD25 monoclonal antibody PC61, which was reported to cause the depletion of regulatory T

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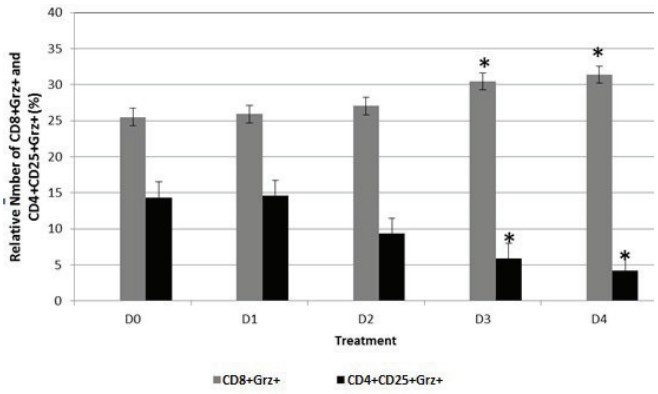

Figure 5. Effect of propolis extract to the percentage of the $CD8^+$ and $CD4CD25^+$ regulatory T cells-expressing granzyme. PBMC were treated as indicated for 48 hours and the expression of $CD8^+$granzyme$^+$ and $CD4^+CD25^+$granzyme$^+$ were detected by flow cytometry as described under Material and Methods. The differences between groups were considered significant at $p<0.05$. All results were presented as the mean ± SD values of 10 samples with three replicates in each group.

cells and inhibit the formation of tumors in experimental animals with mucin-like carcinoma associated antigen fibrosarcoma.[30] Moreover, the depletion of regulatory T cells in experimental animals increases the effectiveness of vaccination and HPV DNA to stimulate $CD8^+$ T cells specific for E7.[31] The anti-CD25 monoclonal antibody has also been used as a cancer immunotherapy, i.e. daclizumab. Provision of daclizumab in patients with metastatic breast cancer causes a decrease in $CD25^+FoxP3^+$ regulatory T cells in the peripheral blood. The result showed effective results in promoting the activity of cytotoxic T cells.[32]

In addition to a variety of beneficial effects, regulatory T cell depletion with anti-CD25 antibodies also has some disadvantages: the anti-CD25 monoclonal antibody is less effective against regulatory T-cells that do not express CD25 or when CD25 is expressed at low levels, and effector T cells that express CD25 can also be depleted. In addition, the depletion of regulatory T cells has also been reported to increase the concentration of circulating IgM autoantibodies.[30]

Effect of propolis extract on the expression of granzyme by $CD8^+$ T cells and $CD4^+CD25^+$ regulatory T cells The mechanism of propolis due to these effects on granzyme expression in $CD8^+$ and $CD4^+CD25^+$ regulatory T cells is still unclear, but the result of this study was very interesting and suggested the possibility of more favorable effects in cancer therapy. Granzyme is a cytolytic enzyme produced cytotoxic T cells that play a role in lysing target cells, including cancer cells. Granzymes in the past were thought to be expressed only by NK cells and cytotoxic T cells, which could be either $CD8^+$ T cells or cytolytic $CD4^+$ T cells, usually of the Th1 lineage.[6]

Granzyme is expressed in regulatory T cells and plays an important perforin-dependent role in regulatory T cell function in mice.[6] Granzyme which expressed by regulatory T cells could be used to perform immunosuppressive functions (i.e. to inhibit $CD8^+$ T cells). The ability of regulatory T cells to regulate antigen-specific $CD8^+$ T cell responses is mediated in part by granzyme, whether granzyme is required for regulatory T cells to suppress $CD8^+$ T cell proliferation in vitro. Wild-type regulatory T cells are able to efficiently suppress $CD8^+$ T proliferation in a dose-dependent manner, whereas granzyme negative regulatory T cells display less suppression of $CD8^+$ T cell proliferation.[2]

In conclusion, our results show that the increased level of $CD8^+$ T cells-expressing granzyme and a decreased level in $CD4^+CD25^+$ T cells-expressing granzyme by D3 and D4 propolis extract were both provide positive benefits in cancer therapy. Further research is needed to study whether propolis extract results in changes in cell populations and if the expression of granzyme can increase cancer cell death.

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DECLARATION OF CONFLICT INTEREST

The authors declared no conflict of interest with respect to the authorship and/or publication of this article.

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