Study of the PD-1 expression in lung carcinoma cells and T lymphoblasts by *Euglena gracilis* water extract

by

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Abstract

Lung cancer is the leading cause of cancer related deaths in the United States. Natural products are a diverse source of anticancer compounds. *Euglena gracilis (E. gracilis)* is a unicellular freshwater green alga rich in nutrients that is used as a dietary supplement. It has been studied for its many properties, including anti-inflammatory, antiviral, and antitumoral. We have been investigating the preventative effects of *E. gracilis* against lung cancer development. Our previous studies demonstrate water extract from *E. gracilis* (EWE) possesses anti-cancer activity against lung carcinomas and stimulates T-cell cytotoxicity against lung cancer cells. However, it is unknown how EWE directly attenuates the growth of lung carcinoma cells and directly stimulates T cells, and what component(s) within EWE are responsible for these biological effects.

In the current study, we investigated the relationship between EWE and the mRNA expression of PD-1 in lung carcinoma cells and T cells. Orally administered EWE in an orthotopic lung cancer syngeneic mouse model was revealed to increase the expression of PD-1 and PD-L1 in tumor-bearing mouse lungs, which was correlated with a decrease in tumor weights in the mice. *In vitro,* EWE was found to increase the mRNA expression of PD-1 and PD-L1 in both murine Lewis lung carcinoma (LLC) cells and murine splenocytes, and EWE also increased the mRNA expression of PD-1 and granzyme B in human Jurkat T lymphoblasts. Using the EWE-induced stimulation of these proteins as assay tools, the bioactive compound in EWE was purified by FPLC with a size exclusion column. Three specific fractions were found to increase the mRNA expression of PD-1 and PD-L1 in LLC cells and the mRNA expression of PD-1 and granzyme B in Jurkat cells. The FPLC fraction which showed highest bioactivity was further fractionated by C18 reverse phase HPLC. The resulting HPLC fraction that increased the expression of PD-1 in LLC cells was subjected to the structural analysis by liquid chromatography mass spectroscopy and nuclear magnetic resonance, which is currently underway.

These results suggest that there is a relationship between the EWE-induced attenuation of lung tumor growth and the EWE-induced increased PD-1 expression, and that EWE treatment may enhance its therapeutic efficacy by stimulating antitumor immunity in lung cancer. The PD-1 stimulator present in EWE was preserved throughout fractionation with FPLC and HPLC in

tandem, and, once identified, could provide clear insights into the mechanisms by which EWE achieves these biological effects.

Table of Contents

List of Figures

List of Tables

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Dedication

I would like to dedicate my thesis to my mom, dad, brother, and family.

Chapter 1 - Literature-based study of lung cancer & natural product-based lung cancer prevention

Lung cancer in humans:

Lung cancer is the leading cause of cancer-related deaths in the United States. Although the lung cancer death rate in both men and women has decreased from the respective peaks in 1990 and 2002, lung cancer still causes more deaths each year than colorectal, breast, and prostate cancers combined [1]. In 2024, it is estimated that 20% of cancer deaths in males and 21% of cancer deaths in females will be due to lung cancer [1].

Lung cancer has two subsets. 80-85% of all lung cancers are non-small cell lung cancer (NSCLC) and the remaining 15% are small cell lung cancer [2]. NSCLC can be divided into squamous cell carcinomas, which account for 30% of all lung cancers [3], adenocarcinomas, which account for 40% of all lung cancers [4], and large cell carcinomas, which account for the remaining 15% of all lung cancers [5]. Squamous cell carcinomas evolve from the squamous epithelial cells that line the airways [3], adenocarcinomas usually evolve from the glandular cells of the lungs [4], and large cell carcinomas lack the features of small cell lung cancer and show neither glandular nor squamous differentiation [6]. According to the World Health Organization, early-stage lung cancer refers to lung tumors that have not metastasized to other organs or lymph nodes, and advanced disease refers to lung cancers where the cancer has spread to other organs or lymph nodes [7].

Although small cell lung cancer is difficult to treat, many treatments have been developed for NSCLC [2]. Despite different subsets, general treatment for NSCLC includes surgical removal, chemotherapy, radiation therapy, and immune checkpoint inhibitor (ICI) therapy. Chemotherapy is often utilized in early-stage lung cancer or metastasized tumors. A combination

of two chemotherapeutics, typically cisplatin or carboplatin with another drug, are injected intravenously over a few minutes or as an infusion [8]. Radiation therapy can be used with multiple stages of NSCLC but is typically associated with early stages. It uses high-energy x-rays or particles to kill cancer cells [9] and is considered a noninvasive alternative to patients unable or unwilling to undergo surgical removal [10].

Another treatment for NSCLC is immune checkpoint inhibitor (ICI) therapy. ICI therapy uses monoclonal antibodies to block the negative regulatory checkpoints of the immune system and lead to activation of the immune system. The immune system can recognize cancer cells due to tumor antigens, which differ enough to be recognizable as non-self [11]. The immune system has these inhibitory checkpoints in place because the cytotoxic T cells designed to eliminate threats to the body could also attack normal, healthy cells and organs if left unrestrained, causing autoimmune diseases such as multiple sclerosis, rheumatoid arthritis, inflammatory bowel disease, and Type 1 diabetes [12]. Thus, the immune system implements several of these checkpoint proteins such as Programmed Death-1 (PD-1), Programmed Death Ligand-1 (PD-L1), and Cytotoxic T-lymphocyte associated protein 4 (CTLA-4) to maintain self-tolerance. Mice genetically modified to be deficient in PD-1, PD-L1, or CTLA-4 frequently developed autoimmune diseases, with some of them being fatal [11] [12]. PD-1 can be found on the surface of T-cells, and interacts with PD-L1, which is expressed in tumor cells, epithelial cells, and immune cells [11]. CTLA-4 is found in the intracellular vesicles in regulatory T cells or on the surface of activated T cells, and ‘competes’ with CD28 for their ligands CD80 and CD86 [13]. CTLA-4 produces a strong inhibitory signal when bound to CD80 or CD86, while CD28 produces a stimulatory signal when bound to CD80 or CD86 that activates the T cell [13]. CD80 and CD86 are expressed on the surface of antigen presenting cells of the immune system and

have a higher affinity for CTLA-4 over CD28 [13]. Unfortunately, cancer cells are able to take advantage of these immune checkpoint inhibitor systems and produce the ligands in abnormally high amounts to inactivate and escape the immune system from destroying them [14]. The goal of ICI therapy is to block these inhibitory interactions to stimulate apoptosis in the cancer cells.

ICI therapy has revolutionized lung cancer treatment with its high efficacy [15] [16], and some such as ipilimumab (monoclonal anti-CTLA-4 antibody), atezolizumab (monoclonal anti-PD-L1 antibody), pembrolizumab (monoclonal anti-PD-1 antibody), and nivolumab (monoclonal anti-PD-1 antibody) are FDA approved [17]. A 5-year survival rate of greater than 10% was not achieved previously using cytocidal anticancer drugs [15], while it has been reported that ICI therapy can achieve a greater than 10% 5 year survival rate [15 [18]. Studies have found that it increased both long-term overall survival and progression-free survival compared to docetaxel, an anticancer drug which has been commonly used [18], and increased the response rate in patients when combined with chemotherapy compared to patients receiving chemotherapy alone [19]. In a study comparing the immune related adverse events of anti-PD-1 ICI therapy compared to docetaxel, they found no potential new adverse events, and the anti-PD-1 ICI therapy had a favorable safety profile compared with docetaxel [18]. Despite the revolutionary success of ICI therapy, more research is still needed to improve its efficacy, as not all lung cancers respond to ICI therapy. In general, tumor response can be determined by their tumor microenvironment being inflamed by T cells and other immune cells, or the lack of inflammation by the immune system. Because ICI therapy relies on the interaction between T cells and cancer cells, it is less effective in tumors lacking this immune cell infiltration [20].

Common preclinical *in vitro* assays for studying the anticancer effects of novel compounds on cancers

Many *in vitro* assays are used to assess the anticancer effects of novel compounds on cancers. These assays test the effectiveness of the novel compounds by analyzing the cancer cell viability or induced apoptosis of cancer cells, cancer cell cycle arrest, or cancer cell migration and invasion. Some cell viability or apoptosis studies *in vitro* also assess immortalized noncancerous cell lines to determine the specificity of the effects. Alternatively, some studies assess the effects of these compounds on the immune system's ability to target and eliminate cancer through coculture studies or by analyzing T cell activation.

Cancer cell viability tests *in vitro* assess the direct effect of a compound on the health of the cells, some of which assess the metabolic activity of the cells or if the cell membrane has become impaired. These tests do have disadvantages, as there are many factors that can impact the metabolic activity of the cells without being an indicator of cell health, and the cell membrane is assessed by dyes that will eventually also permeate healthy cells, thus requiring strict timing [21]. Apoptosis tests *in vitro* analyze the activity of the enzymes involved or signs seen in apoptosis at various different steps. These can range from detecting DNA fragmentation or nucleus changes to analyzing the mitochondrial and/ or cell membrane integrity [22]. Cell cycle arrest refers to the process of the cancer cells entering the G_0 stage of the cell cycle, where the cells are metabolically active but no longer proliferate unless prompted by extracellular signals [23]. These tests typically look for markers associated with cell cycle arrest or evidence of modifications in the DNA's histones [24]. Cancer cell migration analyzes the ability of the cancer cells to migrate from one area to another, while invasion assays determine if the cells can penetrate a barrier in order to move from one area to another. A commonly used assay for cell

migration involves having two chambers on top of each other separated by a porous membrane to determine if the cells can move from the upper compartment to the lower one. A commonly used cell invasion assay uses the same principle, but there is an added layer to the porous membrane [25]. These assays are endpoint assays, and removing the cells in the upper compartment without disturbing the lower compartment can be difficult [25]. Cell migration can also be assessed in real time by scraping off an area of cells in a dish then microscopically monitoring any cells that move into the new area, which has its disadvantages in being unable to assess a long period of time such as greater than twenty-four hours and the scrape can be uneven or result in artifacts by the mechanical cell damage [25]. Alternatively, some tests assess the ability of the compound to activate the immune system. Coculture studies can range from involving the direct interaction of the two cell types in the same dish, the transfer of the conditioned media from one dish of cultured cells to another, or the indirect interaction of the two cells where a barrier prevents direct interaction, but the media is shared [26]. These assays allow for the study of the interactions between the immune cells and cancer cells but are limited in their inability to fully replicate the tumor environment [26]. Some *in vitro* studies instead focus on culturing and analyzing cytotoxic T cells. These tests look for the production of cytokines associated with activated cytotoxic T cells such as tumor necrosis factor alpha (TNFα), which induces cell death [27], perforins that cause pores in the target cell membrane, or granzymes such as granzyme B that initiate apoptosis in the target cell [28].

These *in vitro* assays are useful for screening novel compounds for anticancer activity can be easily conducted, and cost less resources as preliminary studies.

Murine models for studying NSCLC:

In addition to *in vitro* tests, many mouse models have been developed for studying NSCLC *in vivo* or for preclinical trials. *In vivo* models are able to more accurately represent the microenvironment or development of tumors and assess the toxicity of a drug or novel compound in other organs or systems outside of the tumor location.

The xenograft mouse model involves injecting human lung cancer cells into immunocompromised mice. Another type of xenograft model is the *ex vivo* model, where a patient's surgically removed tumor tissue is grafted into the immunocompromised mouse. These models utilize human tumor tissue, allowing for human-specific complexities to be studied or, in the case of *ex vivo* models, provide testing and insights for an individual patient. Despite these advantages, there are also limitations in xenograft models. These models use immunocompromised mice, since a competent murine immune system would reject the human tissue as foreign. Thus, the effects of a competent immune system are unable to be evaluated [29].

Another *in vivo* mouse model is the syngeneic murine model, where immunologically compatible cancer cells are injected into immunocompetent mice. This allows for the natural immune response and toxicity of the therapeutic to be evaluated. For studying NSCLC, the only widely available syngeneic model is the Lewis lung carcinoma (LLC) model [29]. LLC cells have a high number of mutations, which, in addition to the presence of immunocompetent cells, make the LLC model ideal for studying immunotherapy, as cancers with a high number of mutations often respond positively to immunotherapy [30]. However, being a syngeneic murine model, the conclusions or results often are not easily transferable to humans [29].

Genetically modified mouse models are also used to study lung cancer. These can be utilized for spontaneous tumorigenesis *via* gene mutations including those that knock out tumor suppressor genes. Gene mutation-induced lung cancer mouse models closely resemble the human cancer phenotype [31], which often possess several mutations in tumor suppressor and/ or promotor genes [32]. The tumorigenesis in these genetically modified models can also be induced by regulating the gene through the addition of a compound, typically a chemical, which is known as a conditional model. These models allow for a more accurate representation of lung cancer tumorigenesis by spatially and temporally regulating the genes that have the potential to cause tumorigenesis [29]. Although conditional models reduce the embryonic lethality and off-target metastasis of mutation-induced models [33], conditional models have limitations in invasive metastasis resulting in difficulties studying early cancer events, or limited metastasis depending on the mutation [29].

Another model used for studying NSCLC is the carcinogen-induced model, which utilize carcinogens to induce mutations in mice susceptible to spontaneous tumorigenesis. Depending on the carcinogen, these models are able to more accurately predict the clinical efficiency of therapeutic agents and allow for all stages of lung cancer to be observed but come with the disadvantages of low or inconsistent metastatic potential due to the variability in administering the carcinogen and long maturation times for the tumors to grow to notable sizes [29].

Of the many mouse models used to evaluate therapeutics for treating NSCLC, the most commonly used model for preclinical trials is the xenograft model due to its ability to represent the human tumor microenvironment for testing drug therapies, and it was used for testing chemotherapies such as gemcitabine, cisplatin, and carboplatin in preclinical trials [29].

However, as mentioned, the LLC syngeneic mouse model is the most frequently used when studying immunotherapy in mice with an intact immune system.

Lung cancer prevention by natural products derived from algae

According to the National Cancer Institute, more than half of today's chemotherapeutics are derived from natural sources such as plants or microbes [34] [35] [36]. Microalgae are a source of a variety of small molecules with anticancer properties [37] [38] [39]. Table 1.1 lists a summary of various components and extracts derived from green algae demonstrating anticancer properties against NSCLC.

Table 1.1 Summary of extracts and components derived from green algae demonstrating inhibitory effects against lung cancers

Alga Species	Component	Model	Effect
Auxenochlorella pyrenoidosa	β-cryptoxanthin	Carcinogen-induced mouse model, cigarette smoke ferret model, *in vitro* cell migration assay	Inhibited lung tumor growth, prevented lung tumor development, prevents cigarette smoke-induced squamous cell transformation and lung inflammation, [44] [45] [46]
Bryopsis pennata	Kahalaide F	HOP6 & A549 cells *in vitro*, xenograft *in vivo* model, Human trial (NCSLC),	*In vitro* lung cancer cell inhibition, *in vivo* lung tumor inhibition [53] Patients (human trial) experienced clinical benefits [54]

Chlorella sorokiniana	Filtered extract	Human lung cancer xenograft *in vivo* model, A549 *in vitro*	Inhibits cell growth, decreased tumor growth, increased expression of apoptosis factors [57]
Chlorella vulgaris	Carbon dioxide extract	H1299, A549, and H1437 cells *in vitro*	Inhibits lung cancer cell growth, inhibits cancer cell migration ability, [56]
Codium decorticatum	Glycoprotein	A549 cells *in vitro*	Inhibits lung cancer cell growth, induces membrane damage in lung cancer cells [51]
Dunaliella salina	β-carotene	A549 cells *in vitro*	Enhances the growth inhibition of trichostatin A against lung cancer cells [48]
Dunaliella salina	Ethanol extract	A549 cells *in vitro*	Induces apoptosis, induces cell cycle arrest [55]
Haematococcus pluvialis	Astaxanthin	A549 and H1703 cells *in vitro*	Inhibits lung cancer cell growth, synergistically inhibited cell growth when combined with an antitumoral antibiotic [42]
Ulva Lactuca	Ulvan	A549 cells *in vitro*	Inhibits lung cancer cell viability [50]
Ulva prolifera	Polysaccharides	A549 and H1650 cells *in vitro*	Blocks tumor metastasis, inhibits cancer cell growth [49]

Carotenoids

Carotenoids are pigments exhibiting yellow, orange, red, and purple colors that become precursors of vitaimin A, antioxidants, and immune enhancers in animals [40]. High levels of serum carotenoids have been linked to a lower risk of lung cancer death in US adults [41]. Astataxnthin is a carotenoid with a red pigment from the green alga *Haematococcus pluvialis* found to induce cytotoxic effects in human lung cancer cell lines and decreased the expression of Rad51, a molecule that is linked to chemoresistance [42]. Rad51 is a DNA-binding protein normally associated with DNA repair mechanisms but, in tumors, repairs the chemotherapy-induced damaged DNA, leading to chemoresistant carcinomas [43]. Astataxnthin also was found to increase the anticancer effects of mitomycin C, an anticancer antibiotic [42]. β-cryptoxanthin is a carotenoid pigment produced by the green alga *Auxenochlorella pyrenoidosa* found to inhibit lung cancer cell migration [44] in an *in vitro* cell migration assay. In a nicotine-induced cancer mouse model, β-cryptoxanthin was also found to inhibit lung tumorigenesis and emphysema, or lung inflammation, [45] and reduced cigarette smoke-induced emphysema, oxidative DNA damage and lung squamous metaplasia in ferrets [46]. β-carotene is a carotenoid produced by the green alga *Dunaliella salina* [47] found to enhance the cytotoxic effects of the anti-cancer drug trichostatin A against human lung carcinoma cells in a cell viability study [48].

Polysaccharides and Polypeptides

In addition to carotenoids, many organic compounds isolated from green algae have been found to have anticancer effects. Polysaccharides derived from *Ulva prolifera* have been found to inhibit cell proliferation in human lung carcinoma cells [49]. Similarly, Ulvan, a sulfated hetero-polysaccharide extracted from *Ulva Lactuca* was found to exhibit cytotoxicity against human lung carcinoma cells in a cell proliferation study [50]. One glycoprotein isolated from

Codium decorticatum was found to exhibit cytotoxicity and membrane damaging effects against human lung carcinoma cells in *in vitro* studies [51]. A depsipeptide is a cyclic peptide where at least one of its amine groups is instead replaced with an analogous ester group [52]. Kahalaide F is a cyclic depsipeptide produced by the green alga *Bryopsis pennata* found to exhibit cytotoxicity against NSCLC [53] and completed Phase I clinical trials in treating patients with advanced solid tumors including NSCLC [54].

Unidentified Extracts

Some extracts obtained from green algae have demonstrated preventative effects against lung cancer. The ethanol extract of *Dunaliella salina* induced apoptosis and cell cycle arrest in human lung cancer cells [55]. A carbon dioxide extract from *Chlorella vulgaris* inhibited both lung cancer cell growth and migration *in vitro* [56]. In NSCLC cells, a filtered extract from *Chlorella sorokiniana* was found to induce apoptosis and inhibited xenograft tumors *in vivo* when admistered orally in mice [57]. Similarly, another green alga, *Euglena gracilis*, has demonstrated anticancer activity and benefits with its extracts.

Euglena gracilis extracts

Euglena gracilis is a unicellular alga that is rich in nutrients and used as a dietary supplement [58] [59]. Studies have reported the *E. gracilis* contains 29.4% carbohydrates, 42.3% protein, 19% lipid, 6.1% ash, and 3.2% water [60][61], and many have focused on paramylon, a carbohydrate produced by *E. gracilis* as its energy storage, for cancer-related studies. Paramylon can account for up to 95% of its cell mass when grown under certain conditions [62]. Studies focused on paramylon have found that it has immune-enhancing effects [63] [60] and anticancer effects in cancers other than lung cancer [64] [65].

Other extracts from *E. gracilis* have also been studied for various biological effects. One study found that their *E. gracilis* extract could inhibit adipocyte-differentiation without cytotoxicity [66]. The water-soluble fraction of *E. gracilis* has also been studied for its anticancer effects. Our lab studied the effects of orally administered *E. gracilis* extract on lung cancer and discovered it attenuates lung cancer growth and stimulates cytotoxic immune cells [67] and alters the gut-lung axis [68]. The gut-lung axis refers to the complex communication and interaction between the intestinal environment including microbiota and gut immunity, and the lungs, which ultimately impacts the functions of the lung. The gut micorbiota can activate immune cells that infiltrate the lungs and activate immune responses in the lungs [69]. Some studies have found that responders to anti-PD-1 ICI therapy have different microbiota profiles compared with nonresponders to anti-PD-1 ICI therapy [70], and anitbiotics targeting gram positive bacteria can reduce the effectiveness of cyclophosphamide, an anticancer chemotherapeutic drug, in lung cancer patients [71]. The *E. gracilis* studies conducted by our lab have not identified the compound within these extracts responsible for these various biological effects. Although paramylon is studied for anticancer effects, the water-soluble fraction, which does not contain paramylon [67], was found to be more effective than paramylon at attenuating lung cancer growth when orally administered in mice [68]. Thus, the goal of this research is to study the characteristics of this *Euglena gracilis*-derived water extract and further characterize their anticancer effects.

Chapter 2 - Experiment-based study of Euglena gracilis extract-based lung cancer control

This chapter is part of a full manuscript currently under review at International immunopharmacology as: A combination treatment with a water extract from *Euglena gracilis* and anti-PD-1 antibody strongly inhibits growth of lung cancer in mice through stimulating tumor-infiltrating lymphocytes.

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Introduction

Lung cancer remains the highest cause of cancer related death, despite the lung cancer death rate dropping 59% and 36% from the peaks for men and women, respectively [1]. It causes more deaths than that by the next two deadliest cancers, colorectal and prostate, combined [1]. Lung cancer remains a prevalent form of cancer even in populations that have never smoked, and the lung is an organ commonly metastasized by other types of cancer [72] [73] [74]. Therefore, novel preventative and therapeutic strategies for both primary and metastatic lung cancers are urgently required. Currently, immune checkpoint inhibitor (ICI) therapy is one of the major cancer therapies used to treat various cancers including lung cancer [75]. ICI therapy targets molecules such as Cytotoxic T-lymphocyte associated protein 4 (CTLA-4), Programmed Death-

1 (PD-1) and Programmed Death-Ligand 1 (PD-L1). PD-1 is found on activated T cells, including cancer antigen-specific cytotoxic T lymphocytes (CTLs) [76] [77], and suppresses CTL-dependent oncolysis [78] when bound to its ligand, PD-L1. PD-L1, is expressed on cancer cells so they can escape the immune system and avoid cell death [15]. In NSCLC, PD-L1 is a biomarker for ICI therapy, and the FDA indicates ICI therapy use in patients with tumor PD-L1 ≥1% for CTLA-4 and PD-1 ICI therapy, and tumor PD-L1 ≥50% or the PD-L1 expression in tumor-infiltrating immune cells covering ≥10% of the tumor area for PD-L1 ICI therapy [79]. ICI therapy targeting PD-1/PD-L1 is designed to interrupt the binding between these molecules, resulting in enhanced anti-tumor immunity, while the efficacy of the therapy is dependent on the expression of PD-L1 in cancer cells [80] as well as the activity of the cancer antigen-specific CTLs.

Microalgae are a diverse source of natural product-derived anticancer compounds [37] [81]. *Euglena gracilis (E. gracilis)* is a single-celled green microalga rich in nutrients [58] [59] as a dietary supplement [82]. It has been extensively studied and found to have ant-inflammatory [83] [84], anticancer [85][64][65] and antiviral [86] [87] properties. Our previous studies found that water extracted from *E. gracilis* attenuates lung cancer growth [67] [68] [85] and stimulates T lymphoblasts in a spheroid coculture [67]. The aims of the present study are to elucidate the mechanisms by which *E. gracilis* water extract (EWE) stimulates anticancer immunity in T lymphoblast cells and determine EWE's potential as an adjuvant to ICI therapy. Here, the present study reports new insights of the effect of EWE on the modulation of anti-tumor immunity through the modulation of PD-1 and PD-L1 expression in tumor tissues. Oral administration of EWE, which started 3 weeks before cancer cell inoculation, inhibited tumor growth and increased both PD-1 and PD-L1 expression.

Materials and Methods

Animals

Female C57BL/6 mice were obtained from Charles River Laboratories International, Inc. and were housed humanely according to university, state, and federal guidelines (AAALAC) in the AAALAC-accredited animal resource facilities of the Kansas State University College of Veterinary Medicine. All animal experiments adhered strictly to protocols approved by the Kansas State University Institutional Animal Care and Use Committee (Protocol # 4867) and Institutional Biosafety Committee (Protocol # 1609). The mice were housed in a clean facility under controlled conditions of temperature (20–26 °C), with 30%–70% relative humidity and light (12:12 h light-dark cycles). All mice were held for a week to acclimatize before the treatment. The mice were observed daily, and body weights were obtained on alternate days.

EWE preparation:

Dried powder of *E. gracilis* was obtained from Euglena Co., Ltd. (Tokyo, Japan). It is composed of 29.4% carbohydrates, 42.3% protein, and 19.0% lipids. Approximately 70–80% of the carbohydrate content is paramylon [87]. The *Euglena* water extract (EWE) was prepared using a protocol described previously [68]. Dried powder of *E. gracilis* was suspended in sterile PBS (40 ml) and the suspension was incubated at 37 °C for 30 min with periodic sonication for 30 sec. The suspension was centrifuged at 11,405×g for 20 min at room temperature, removing Euglena cell debris and mature paramylon as a pellet. The supernatant was filtered through one layer of water-soaked Whatman #1 filter paper (Cytiva, Marlborough, MA, USA) to remove floating materials. The filtrate was incubated in boiling water for 10 min as a heat treatment, followed by centrifugation at 11,405×g for 10 min at room temperature. The supernatant was

filtered by a sterile 0.22 μm disk syringe filter (Midwest Scientific, Valley Park, MO, USA) and used as partially purified water extracts from *E. gracilis* (EWE). All mature paramylon granules (granule size > 0.2–0.3 μm diameter) were removed by these two steps of centrifugation and membrane filtration [67]. The dry weight was calculated after drying a 300 μl of aliquot at 55 °C for 48 hours from which the dry weight of PBS was subtracted (8.69 mg dry powder (salt) per 0.3 ml PBS). All the extracts were stored at −20 °C until further use. An analysis on the basal components in the EWE was conducted previously [68].

Cell culture

The murine Lewis lung carcinoma (LLC) cell line (CRL-1642) and human lymphoblast cell line Jurkat (TIB-152) were purchased from American Type Culture Collection (ATCC, Manassas, VA). Dulbecco's Modified Eagle's Medium (DMEM) and RPMI 1640 was obtained from Mediatech, Inc (Manassas, VA). Fetal bovine serum (FBS) was obtained from Biowest (Riverside, MO, USA). The penicillin-streptomycin stock was obtained from Lonza Rockland, Inc. (Allendale, NJ, USA). The LLC murine Lewis lung carcinoma cells were cultured in DMEM supplemented with 10% v/v FBS and 1% v/v penicillin-streptomycin, and the Jurkats were cultured in RPMI 1640 supplemented with 10% v/v FBS and 1% v/v penicillin-streptomycin.

The cells were cultured at 37°C in a humidified atmosphere containing 5% CO_2. The cell lines were authenticated by short tandem repeat (STR) DNA profiling. Both cells were maintained in low passages (<15) for this study.

Effect of orally administrated EWE on lung carcinoma tumor growth in a syngeneic mouse lung

The anti-tumor effect of EWE was evaluated in female C57BL/6 mice using an LLC murine lung carcinoma syngeneic mouse model (*n*=5). The mice were pre-treated with 200 mg/kg EWE *via* drinking water *ad lib* starting 3 weeks prior to cancer cell inoculation (Fig. 2.1A). Mice were injected with 1.5×10^6 LLC cells in200 µl PBS *via* tail vein. The PBS was treated as control *via* drinking water with the same schedule. All mice were sacrificed 21 days after LLC injection by cervical dislocation after exposure to saturated CO_2. The lungs and spleens were weighed and fixed in 10% formalin for histological analysis. The tumor weights in the lungs were calculated by subtracting the average weight of an age-matched normal mouse lung (142.3±11.9 mg, $n = 5$).

PD-1 and PD-L1 expression in EWE-treated tumor-bearing mouse lungs, LLC cells, and murine splenocytes

To evaluate the PD-1 and PD-L1 expression in EWE-treated tumor-bearing mouse lungs, the RNA was extracted from formalin-fixed paraffin-embedded (FFPE) lungs using Quick-RNA FFPE MiniPrep (Zymo Research Corporation, Irvine, CA, USA). The expression of these genes was also evaluated in EWE-treated LLC cells, human T lymphoblast or Jurkat cells, and murine splenocytes prepared from normal C57BL/6 mouse spleens. Briefly, LLC cells (5×10^4 cells/well) were seeded into a 6-well plate and Jurkat cells (1×10^4 cells/ well) were seeded in a 12-well plate. After twenty-four hours, the cells were treated with 0.1, 1, and 10 µg/ml EWE. Forty-eight hours after treatment, the RNA was extracted using TRIzol following the manufacturer's instruction (Thermo Fisher Scientific, Waltham, MA, USA). One step reverse transcription

quantitative real-time PCR (RT-qPCR) was performed using the iTaq Universal SYBR Green One-Step Kit (Bio-Rad, Hercules, CA, USA). The RT-qPCR was performed as follows: 45 cycles of 15 s at 95 °C, and 60 s at 60°C. The results were quantified by the comparative Ct method [89]. Table 2.1 displays the sequences of the primers.

Table 2.1 Primers used for RT-qPCR analysis in splenocytes and LLC cells.

Primer		Sequence	Size
Murine PD-1	Forward (5'-3')	CCGCCTTCTGTAATGGTTTGA	65 bp
	Reverse (5'-3')	GGGCAGCTGTATGATCTGGAA	
Murine PD-L1	Forward (5'-3')	TGGACAAACAGTGACCACCAA	66 bp
	Reverse (5'-3')	CCCCTCTGTCCGGGAAGT	
Human PD-1	Forward (5'-3')	CGTGGCCTATCCACTCCTCA	61 bp
	Reverse (5'-3')	ATCCCTTGTCCCAGCCACTC	
Human PD-L1	Forward (5'-3')	AAATGGAACCTGGCGAAAGC	60 bp
	Reverse (5'-3')	GATGAGCCCCTCAGGCATTT	
Human Jurkat	Forward (5'-3')	TGCAGGAAGATCGAAAGTGCG	180 bp
	Reverse (5'-3')	GAGGCATGCCATTGTTTCGTC	
18S	Forward (5'-3')	GAGGTTCGAAGACGATCAGA	315 bp
	Reverse (5'-3')	TCGCTCCACCAACTAAGAAC	

Immunohistochemical analyses on PD-1 and PD-L1 expression in EWE-treated mouse lungs and spleens

To analyze the PD-1 and PD-L1 expression in EWE-treated mouse lungs and spleens, immunohistochemical staining was conducted as described previously [90]. Antibodies for PD-1 (D7D5W) and PD-L1 (D5V3B) were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). Sections were incubated with primary antibodies for mouse PD-1 (1:100 dilution) or PD-L1 (1:100 dilution) at 4 °C overnight, then incubated with a biotin-conjugated anti-rabbit IgG antibody (Vector Laboratories, Burlingame, CA, USA) at a 1:100 dilution for 1 hour at 37 °C.

Statistical analysis

All values are expressed as the mean ± standard division of mean. Statistical significance was assessed by an unpaired t-test or ANOVA followed by Tukey's test. Statistical significance was set at $p<0.05$.

Results

To examine the relation between oral treatment of EWE and the expression of PD-1 and PD-L1 in the tumors, mice were orally administrated with EWE (200 mg/kg/day) for 6 weeks, which include 3 weeks pre-treatment before cancer cell inoculation (Fig. 2.1A). As shown in Figs. 2.1B and 2.1C, the tumor growth in the mouse lungs was significantly attenuated by EWE treatment (289.9±87.7 mg, $p<0.05$) compared with the PBS control group (591.2±173.6 mg) (line graph in both Figs. 2.1B and 2.1C). To evaluate the effect of EWE on PD-1 and PD-L1 expression in the lung tumors, the total RNA was extracted from formalin-fixed paraffin-embedded lung tissues and PD-1 and PD-L1 mRNA levels were determined using real-time

PCR. Oral administration of EWE increased the mRNA expression of PD-1 (bar graph in Fig. 2.1B) and PD-L1 (bar graph in Fig. 2.1C) in the lung tissues compared to the PBS control group. Notably, the mRNA expression levels of PD-1 or PD-L1 in the tumor-bearing mouse lung and the tumor weights of the same mice in the PBS control and EWE treated group are negatively correlated (Figs. 2.1B and 2.1C).

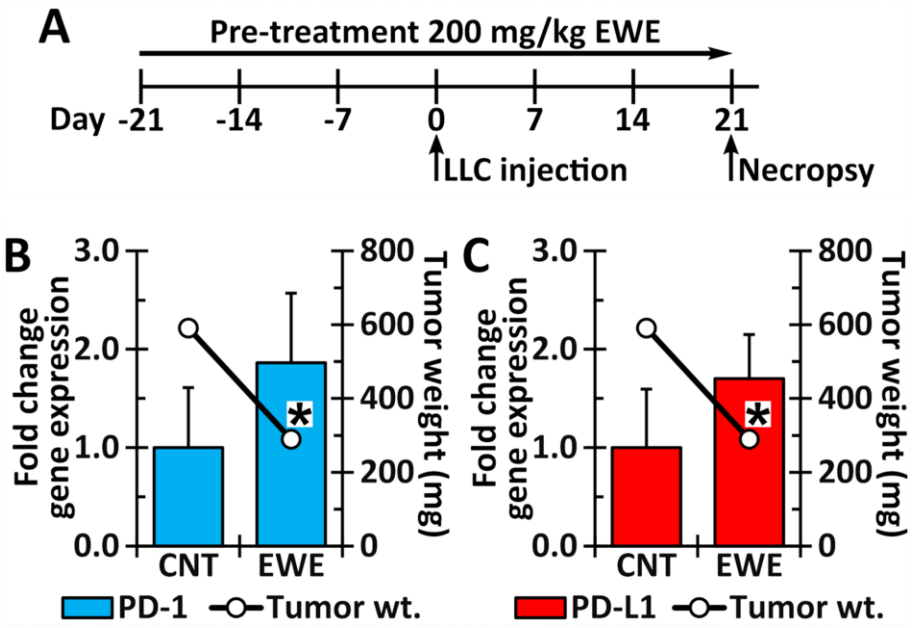

Figure 2.1 The effect of oral EWE treatment on tumor growth and the expression of both PD-1 and PD-L1 in mouse lungs.

(A), Schematic illustration of a mouse study. (B and C), Correlation of average tumor weight (line graph) and (B) PD-1 and (C) PD-L1 mRNA expression in lung tumor-bearing mouse lung (*n*=5). *: $p<0.05$.

To clarify the localization of the PD-1 and PD-L1 expressing cells, an immunohistochemical analysis with anti-PD-1 and PD-L1 antibodies was conducted in PBS- or EWE-treated mouse tumors and spleens. As shown in Fig. 2.2A, both PD-1^+ and PD-$L1^+$ cells were observed in the intratumoral microenvironment. EWE treatment significantly increased the expression levels of PD-1 and PD-L1 proteins in both the tumor and spleen; notably the PD-1 expression level in the

tumors of the EWE treated group was significantly higher than that in the PBS control group (p<0.05), whereas the PD-L1 expression levels in the lung did not show a statistical significance (Fig. 2.2A). The expression levels of PD-1 and PD-L1 proteins in the spleens were much lower than those in the lungs and no statistical differences were observed in the EWE treated and PBS control groups (Fig. 2.2B).

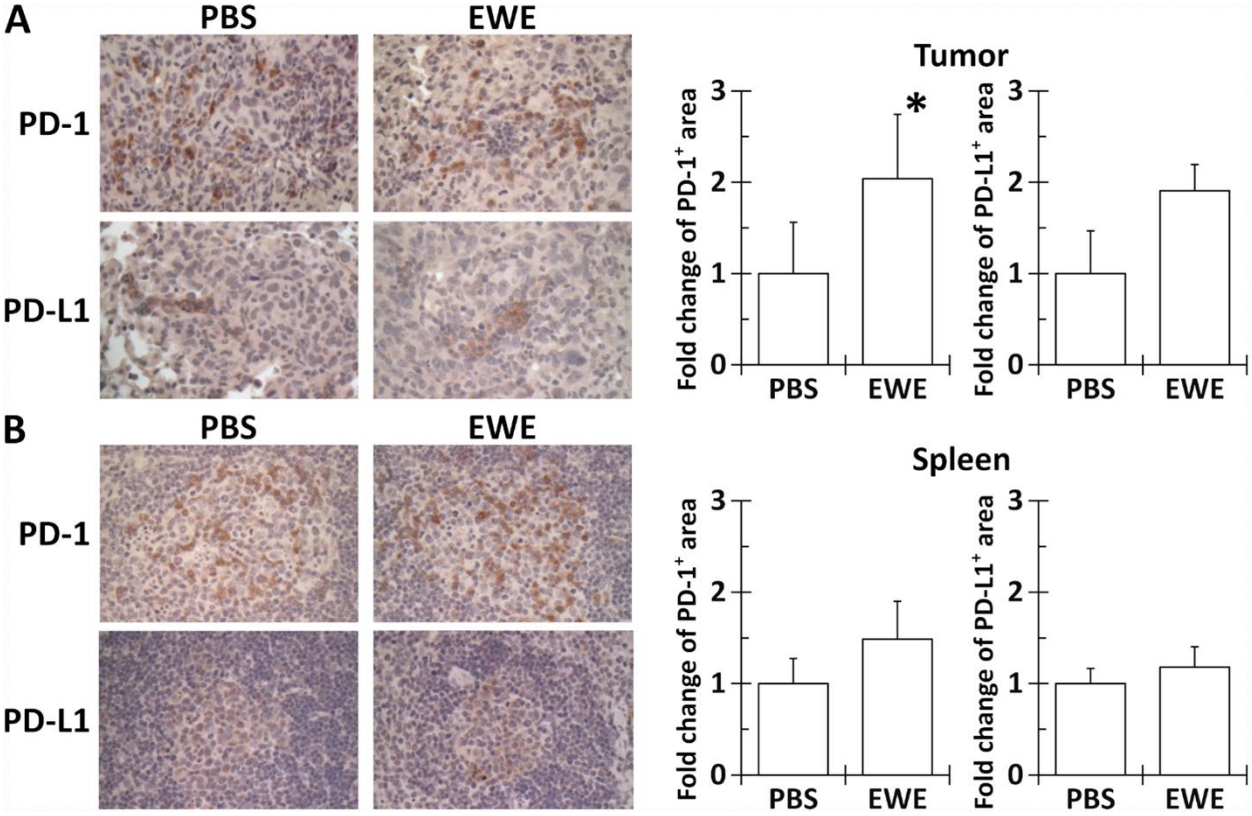

Figure 2.2 Immunohistochemical analysis of PD-1 and PD-L1 expression in the PBS/EWE-treated mouse lung tumor (A) and spleen (B).

Typical images (Left, dark brown cells indicate the antibody-stained cells) and quantitative analyses of the positive area of each protein (Right, n=3–5). *: p<0.05.

To clarify if EWE directly stimulates PD-1 and PD-L1 expression in lung tumor cells and normal lymphocytes, LLC cells, and splenocytes from normal C57BL/6 mice were treated with low concentrations of EWE and the mRNA expression of PD-1 and PD-L1 were examined using

real-time PCR. The EWE-induced increase in both PD-1 and PD-L1 expression was observed in EWE-treated LLC cells in cell culture (Fig. 2.3A). The EWE-dependent increase of PD-1 mRNA was approximately two times higher than that of PD-L1. The EWE-induced increase in the expression of both PD-1 and PD-L1 was also observed in EWE-treated splenocytes in a cell culture (Fig. 2.3B) although the expression was lower than that in LLC cells (Fig. 2.3A).

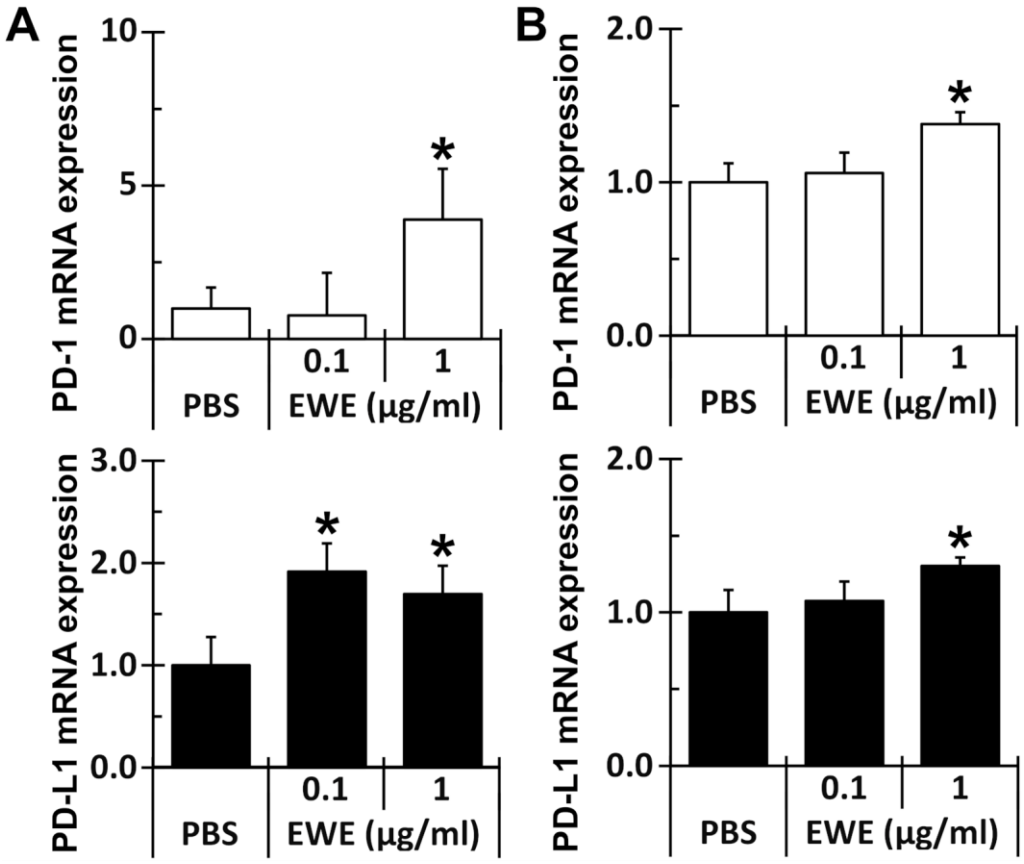

Figure 2.3 PD-1 and PD-L1 mRNA expression in EWE-treated LLC cells and in EWE-treated splenocytes.

The mRNA expression of PD-1 and PD-L1 in LLC cells (*n*=4, panel A) and splenocytes (*n*=3, panel B) at 48 hours after treatment was measured by real-time PCR. *: $p<0.05$.

Discussion

PD-1 and PD-L1 are extensively studied regulators of the immune system and are successful targets for ICI therapy in lung cancer [15]. A lack of PD-1 causes a breakdown of

peripheral immune tolerance, which causes multiple autoimmune features in mice; therefore PD-1 plays an important role in maintaining immune homeostasis [91]. PD-L1 is also important to maintain immune homeostasis by regulating immune responses [92]. PD-L1 is expressed on immunosuppressive immune cell populations such as myeloid-derived suppressor cells (MDSC), dendritic cells, and tumor-associated macrophages (TAM) [93]. These cells regulate T cell immune function.

In this study, we report orally administered *Euglena* water extract (EWE) increased the expression of PD-1 and PD-L1 in murine lung tumors (Fig. 2.1B and 2.1C), which was correlated with a decrease in tumor weights (Fig 2.1B and 2.1C). Although non-small cell lung cancer (NSCLC) is a good-responder type cancer to ICI therapy, only a subset of NSCLCs is sensitive to this therapy, namely, cancers with a high expression of the PD-L1, a high tumor infiltration of lymphocytes, and/or a high tumor mutation burden [79] [94] [95]. Further investigation by IHC found that PD-1 but not PD-L1 was significantly increased with oral EWE administration in the murine tumors (Fig. 2.2). Additionally, EWE dose-dependently increased the expression of PD-1 and PD-L1 in murine splenocytes and lung carcinoma cells (Fig. 2.3) *in vitro*. Recent human studies demonstrated that NSCLCs expressing either PD-1 or PD-L1 have a significantly better prognosis when they are treated with ICI therapy [96] [97] [98] [99] [100]. Therefore, controlling PD-1/PD-L1 expression in NSCLC tumors may provide new insights for cancer therapies. Natural product-based PD-L1 expression modulation in two different murine cancer cells has been reported. An extract from *Caesalpinia spinosa* (P2Et) induced PD-L1 expression in B16-F10 melanoma cells [101] and 4T1 breast cancer cells [102]. It is also reported that P2Et treatment combined with αPD-L1 antibody synergistically attenuated growth of a B16-F10 tumor, but not that of a 4T1 tumor [102]. Another natural product, egg

polysaccharide from *Strongylocentrotus nudus* induced PD-L1 expression in B16-F10 melanoma cells and showed a synergistical tumor attenuating effect with αPD-L1 antibody [103]. These previous studies indicate that the findings of the Euglena extract-induced potentiation of ICI against lung cancer in mice is not an isolated incident and a potential human application should be considered. To the best of our knowledge, this is the first study to describe the increases of PD-1 and PD-L1 expression by EWE, which does not contain paramylon, in both lung carcinoma cells and splenocytes *in vitro*.

Chapter 3 - Purification and Characterization of the PD-1 expression stimulator in *Euglena gracilis* extract

Introduction

Natural products are the source of many anticancer drugs. In addition to disrupting the cancer cell-cycle, several are also able to affect the transcription and expression of molecules [35]. PD-1 and PD-L1 are regulatory molecules that are also targets for cancer immune checkpoint inhibitor therapy (ICI therapy) [80]. An extract from *Caesalpinia spinosa* and an egg polysaccharide from *Strongylocentrotus nudus* were reported to induce PD-L1 expression in melanoma and breast cancer cells [101] [102] [103]. Several studies have found that lung cancer patients with increased PD-L1 or PD-1 expression led to better overall survival compared to those with lower PD-L1 or PD-1 [99] [104].

Microalgae have been the source for many natural product-derived anticancer therapeutics [34] [38]. *Euglena gracilis* (*E. gracilis*) is a microalga rich in nutrients [58] used as a dietary supplement [82]. Studies have reported the *E. gracilis* contains 29.4% carbohydrates, 42.3% protein, 19% lipid, 6.1% ash, and 3.2% water [60] [61] and many have focused on its carbohydrate storage, paramylon [62]. Paramylon can account for up to 95% of its cell mass when grown under certain conditions. Studies focused on paramylon have found it has immune-activation effects [63] [60], anticancer properties [64] [65] [85] and can alleviate acute liver injury in a mouse model [105].

The water-soluble fraction of *E. gracilis* has also been shown to exhibit similar biological effects, including attenuating lung cancer [67] [68] [88], stimulate T lymphoblasts *in vitro* [67] and inhibit adipocyte-differentiation [66]. In fact, the water-soluble fraction seemed to be more effective compared to paramylon when both were administered orally to mice [68]. However, it is still unknown what within this water-soluble fraction, which is devoid of paramylon [67] exhibits these effects, and by what mechanisms it achieves them. Here, we report the purification of the PD-1 expression stimulator in the water-soluble extract from *E. gracilis.*

Materials and Methods:

EWE preparation:

Dried powder of *E. gracilis* was obtained from Euglena Co., Ltd. (Tokyo, Japan). The *Euglena* water extract (EWE) was prepared using a protocol described previously. [68]. Dried powder of *E. gracilis* was suspended in sterile PBS (40 ml) and the suspension was incubated at 37 °C for 30 min with periodic sonication for 30 sec. The suspension was centrifuged at 11,405×g for 20 min at room temperature, removing Euglena cell debris and mature paramylon. The supernatant was filtered through one layer of water-soaked Whatman #1 filter paper (Cytiva, Marlborough, MA, USA) to remove floating materials. The filtrate was incubated in boiling water for 10 min, followed by centrifugation at 11,405×g for 10 min at room temperature. The supernatant was filtered by a sterile 0.22 μm disk syringe filter (Midwest Scientific, Valley Park, MO, USA) and used as partially purified water extracts from *E. gracilis* (EWE). All mature paramylon granules (granule size > 0.2–0.3 μm diameter) were removed by these two steps of centrifugation and membrane filtration [67]. The dry weight was calculated after drying a 300 μl of aliquot at 55 °C for forty-eight hours from which the dry weight of PBS was subtracted (8.69 mg dry powder (salt) per 0.3 ml PBS). All the extracts were stored at −20 °C until further use. An analysis on the basal components in the EWE was conducted previously [88].

Cell lines:

The murine Lewis lung carcinoma (LLC) cell line (CRL-1642) and human lymphoblast cell line Jurkat (TIB-152) were purchased from American Type Culture Collection (ATCC, Manassas, VA). Dulbecco's Modified Eagle's Medium (DMEM) and RPMI 1640 was obtained from Mediatech, Inc (Manassas, VA). Fetal bovine serum (FBS) was obtained from Biowest (Riverside, MO, USA). The penicillin-streptomycin stock was obtained from Lonza Rockland, Inc. (Allendale, NJ, USA). The cells were cultured at 37°C in a humidified atmosphere containing 5% CO_2. The cell lines were authenticated by short tandem repeat (STR) DNA profiling. Both the cells were maintained in low passages (<15) for this study. The LLC murine Lewis lung carcinoma cells were cultured in DMEM supplemented with 10% v/v FBS and 1% v/v penicillin-streptomycin, and the Jurkats were cultured in RPMI 1640 supplemented with 10% v/v FBS and 1% v/v penicillin-streptomycin.

Effect of whole EWE on Jurkats and LLC cells

To evaluate the PD-1, PD-L1, and granzyme B (GZMB) expression in EWE-treated LLC cells and Jurkat cells, LLC cells (5×10^4 cells/well) were seeded into a 6-well plate and Jurkat cells ($1x10^4$ cell/ well) were seeded into a 12-well plate. After twenty-four hours, the cells were treated with 0.1, 1, and 10 µg/ml EWE. Twenty-four and forty-eight hours after treatment, the RNA was extracted using TRIzol following the manufacturer's instruction (Thermo Fisher Scientific, Waltham, MA, USA). One step reverse transcription quantitative real-time PCR (RT-qPCR) was performed using the iTaq Universal SYBR Green One-Step Kit (Bio-Rad, Hercules, CA, USA). The RT-qPCR was performed as follows: 45 cycles of 15 s at 95 °C, and 60 s at 60°C. The results were quantified by the comparative Ct method [89]. Table 3.1 displays the sequences of the primers.

Table 3.1 Primers used for RT-qPCR analysis in Jurkat cells and LLC cells.

Primer		Sequence	Size
Murine PD-1	Forward (5'-3')	CCGCCTTCTGTAATGGTTTGA	65 bp
	Reverse (5'-3')	GGGCAGCTGTATGATCTGGAA	
Murine PD-L1	Forward (5'-3')	TGGACAAACAGTGACCACCAA	66 bp
	Reverse (5'-3')	CCCCTCTGTCCGGGAAGT	
Human PD-1	Forward (5'-3')	CGTGGCCTATCCACTCCTCA	61 bp
	Reverse (5'-3')	ATCCCTTGTCCCAGCCACTC	
Human PD-L1	Forward (5'-3')	AAATGGAACCTGGCGAAAGC	60 bp
	Reverse (5'-3')	GATGAGCCCCTCAGGCATTT	
Human Jurkat	Forward (5'-3')	TGCAGGAAGATCGAAAGTGCG	180 bp
	Reverse (5'-3')	GAGGCATGCCATTGTTTCGTC	

18S	Forward (5’-3’)	GAGGTTCGAAGACGATCAGA	315 bp
	Reverse (5’-3’)	TCGCTCCACCAACTAAGAAC	

Fast Protein Liquid Chromatography (FPLC) Fractionation of EWE:

Whole EWE (6mg/ml) was fractionated by FPLC using Superdex 75 column chromatography with 0.1M ammonium bicarbonate (pH 8.0) as the running buffer. The elution profile is shown in Figure 3.3A. The resulting fractions were stored at -80°C. These fractions were evaluated for their biological activity in stimulating the expression of PD-1, PD-L1, or Granzyme B in LLC cells or Jurkat cells. LLC cells (1×10^4) or Jurkat cells (2×10^4) were seeded in 12-well plates. After twenty-four hours, 3 µl of each FPLC fraction (equivalent of 0.018 mg) was added to the cells at 4 fractions/ well (two duplicate determinations, *n*=4) . After incubation for forty-eight hours, the mRNA of the LLC or Jurkat cells was extracted and analyzed by RT-PCR for the expression of PD-1 and PD-L1 (LLC cells, two duplicate determinations, *n*=4) or PD-1, PD-L1, and granzyme B (Jurkats, two duplicate determinations, *n*=4) . To confirm our findings, the same column chromatography was repeated using the same starting amount.

High Performance Liquid Chromatography (HPLC) Fractionation of EWE:

Superdex 75 fractions 35 and 36 (100µl each), obtained from the Superdex FPLC injection, were combined and completely dried by a Speed Vac. They were then dissolved with 50 µl initial mobile phase buffer (5% MeOH in 0.1% TFA), then filtered through a 0.2 µm pore filter centrifugation. The filtrate was injected into a C18 reverse phase column (4.6 x 150 mm, XbridgeTM Shield RP18, 3.5 µm (Part No. 186003045)) equilibrated with 5% MeOH in 0.1% TFA acidified filtered water. The flowrate was 1 ml/min with a gradient elution of 5% - 90% MeOH in 0 - 30 min and maintaining 90% MeOH for a final 10 min. This HPLC fractionation was conducted using Waters HPLC (model# 1525 Binary HPLC Pump) with Photodiode allay detector (Model # 299). The elution profile is shown in Fig. 3.5A. The resulting fractions were collected and 100 µl of each fraction was combined at 3 fractions/ group, dried completely using a Speed Vac and stored at -80°C. These fractions were evaluated for their biological activity in

stimulating the expression of PD-1 and/or granzyme B in LLC cells or Jurkat cells. 1×10^4 LLC cells or 2×10^4 Jurkat cells were seeded in 12-well plates. After twenty-four hours, the combined fraction precipitates (?) were reconstituted in 450 µl of media (DMEM), then heated at 37°C for 15 minutes to dissolve the fractions and check for any pH changes. 200 µl of media was removed from the incubating cells, then 200 µl of the HPLC fraction-media was added to the cells. After incubation for forty-eight hours, the mRNA was collected and analyzed for the expression of PD-1 and/or granzyme B (two duplicate determinations, n=4). Table 1 displays the sequences of the primers.

Statistical analysis:

All values are expressed as the mean ± standard deviation of the mean. For all the experiments, statistical significance was assessed by an unpaired t-test and was conducted with multiple sample determinations. A p-value of <0.05 was considered statistically significant.

Results:

Whole EWE

To further characterize EWE's ability to stimulate T cells, we investigate its effect on the expression of PD-1 and PD-L1 in murine Lewis lung cancer (LLC) cells and Jurkat (human T lymphoblast) cells. These cells were treated with 0.1, 1, and 10 µg/ml EWE and incubated for twenty-four or forty-eight hours. Then, the mRNA was extracted, and a RT-qPCR was done to analyze the mRNA expression of PD-1 and PD-L1 in LLC cells or PD-1, PD-L1, and granzyme B (GZMB) in Jurkat cells. EWE dose-dependently increased the expression of PD-1 and PD-L1 at forty-eight hours, which was statistically significant in the 1 µg/ml and 10 µg/ml treatments (Fig 3.1). In Jurkat cells, EWE dose-dependently and transiently increased the expression of PD-1 at twenty-four hours, which was statistically significant (Fig 3.2B), but did not increase the expression of PD-L1 at either time point (Fig 3.2A).EWE also dose-dependently increased the expression of GZMB at forty-eight hours, which was statistically significant in the 1 µg/ml and 10 µg/ml treatments (Fig 3.2C).

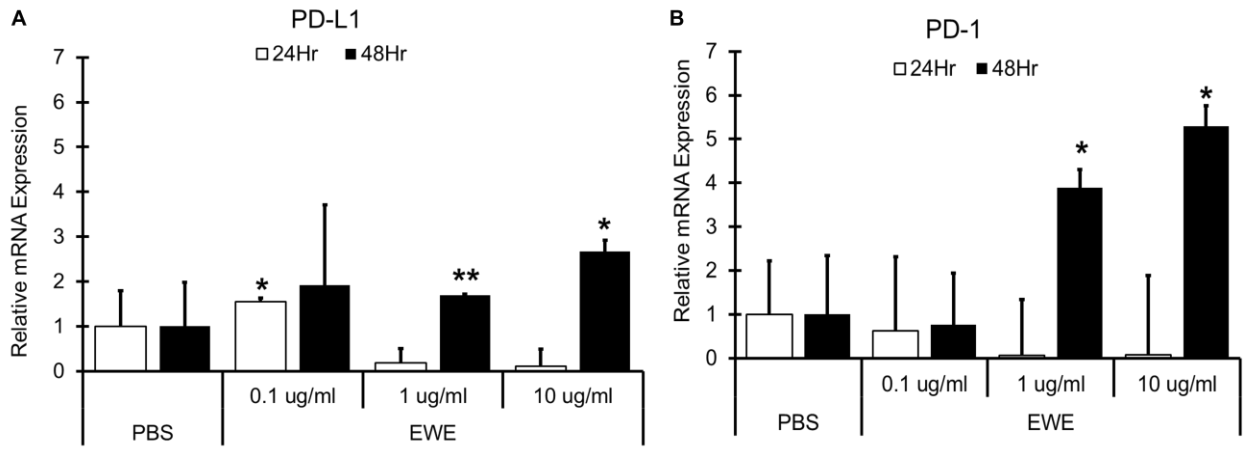

Figure 3.1 The mRNA expression of PD-1 and PD-L1 mRNA in EWE-treated LLC cells. The mRNA expression of PD-1 (A) and PD-L1 (B) in LLC cells (two duplicate determinations, *n*=4) , at 24 and 48 hours after treatment measured by real-time PCR. *: p<0.05, **: p<0.01.

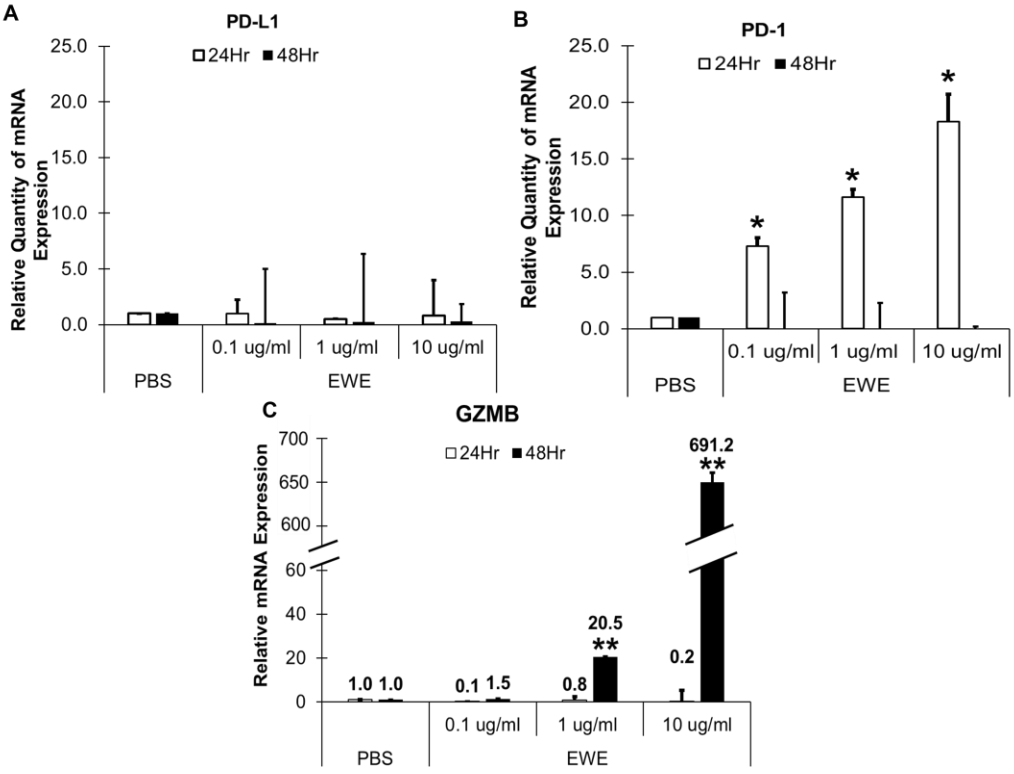

Figure 3.2 The expression of PD-1 and PD-L1 mRNA in EWE-treated Jurkat cells.

The mRNA expression of PD-1 (A) and PD-L1 (B) in Jurkat cells (two duplicate determinations, $n = 4$), at 24 and 48 hours after treatment measured by real-time PCR. *: $p<0.05$, **: $p<0.01$.

FPLC

Whole EWE (6mg/ml) was fractionated by FPLC using a Superdex 75 column with 0.1 mol/L ammonium bicarbonate (pH 8.0) as the running buffer. The FPLC fractions were grouped together, with 3 µl of each added to LLC cells. After forty-eight hours, the mRNA of the cells was extracted and analyzed by RT-PCR for the expression of PD-1 and PD-L1. As shown in Figure 3.3B, fractions 1-4, 5-8, 17-20, 21-24, 29-32, and 33-36 significantly increased the expression of PD-1 in LLC cells. Similarly, all fractions excluding 25-28 significantly increased the expression of PD-L1 in LLC cells (Fig. 3.3C). However, the increase in PD-1 expression was more remarkable compared to that of PD-L1.

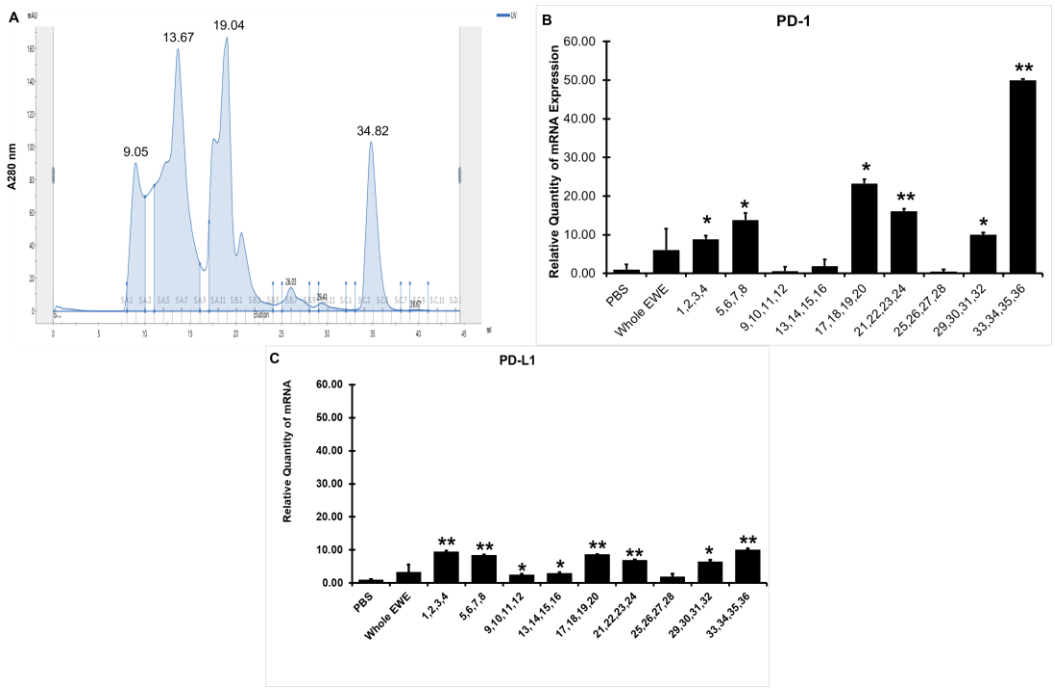

Figure 3.3 The expression both of PD-1 and PD-L1 in LLC cells treated with FPLC Superdex75 EWE fractions.

(A), Elution profile of Superdex 75 FPLC of EWE. The mRNA expression of PD-1 (B) and PD-L1 (C) in LLC cells was measured 48 hours after treatment with grouped fractions from Superdex 75 FPLC by real-time PCR (two duplicate determinations, *n*=4). *: $p<0.05$, **: $p<0.01$.

To evaluate the fractions' ability to stimulate PD-1 or PD-L1 in Jurkat cells, the FPLC fractions were grouped together, with 3 µl of each added to Jurkat cells. After forty-eight hours, the mRNA of the cells was extracted and analyzed by RT-PCR for the expression of PD-1 and GZMB. As shown in Figure 3.4A, fractions 1-4, 9-12, 13-16, 17-20, and 33-36 significantly

increased the mRNA expression of PD-1. Fractions 1-4, 9-12, 13-16, 17-20, 25-28, 29-32, and 33-36 all significantly increased the expression of GZMB in Jurkat cells (Fig. 3.4B).

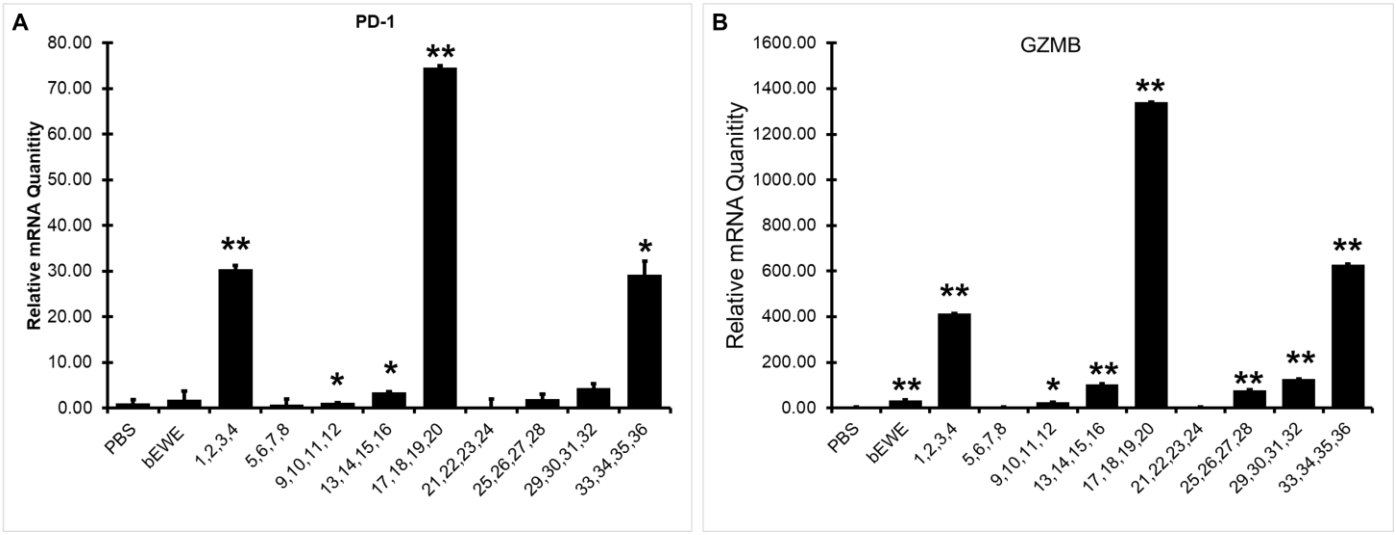

Figure 3.4 The mRNA expression of PD-1 and Granzyme B in Jurkat cells treated with Superdex75 FPLC fractions of EWE.

The mRNA expression of PD-1 (A) and Granzyme B (B) in Jurkat cells was measured 48 hours after treatment with Superdex 75 FPLC fractions of EWE by real-time PCR (two duplicate determinations, *n*=4). *: $p<0.05$, **: $p<0.01$.

HPLC

Based on these results, FPLC fractions 35-36 were combined and completely dried by Speed Vac for HPLC analysis. These combined fractions were kept at -80°C until analysis, where they were reconstituted by 5% methanol and filtered through a 0.2 µm pore filter before being injected into the HPLC column. The elution profiles at an absorbance of 220 nm (Fig. 3.5A) and 254 nm (Fig. 3.5B) were monitored. The resulting fractions were kept at 4°C until the bioassay. These HPLC fractions were combined at 3 fractions/ treatment group, then dried completely by Speed Vac. The resulting dried precipitate was kept at -80 C. To compare the effect of these HPLC fractions on the PD-1 mRNA expression in LLC with that of whole EWE,

450 µl of media was added to the precipitate, then heated at 37°C for 15 minutes to dissolve the fractions and check for any pH changes. Then, 200 µl of media was removed from the wells, and 200 µl of media containing the HPLC eluate was added to each well. After forty-eight hours, the mRNA was extracted and analyzed by RT-PCR. As shown in Figure 3.5C, fractions 1-3, 4-6, 10-12, 13-15, 16-18,25-27, 28-30, 31-33, 34-36, and 37-39 significantly increased the expression of PD-1 in LLC cells. Due to the noticeable increase in PD-1 expression stimulated by fractions 25-27, these 3 fractions were chosen for mass spectrometry and proton ^{1}HNMR spectroscopy analysis to further characterize the PD-1 stimulator in EWE.

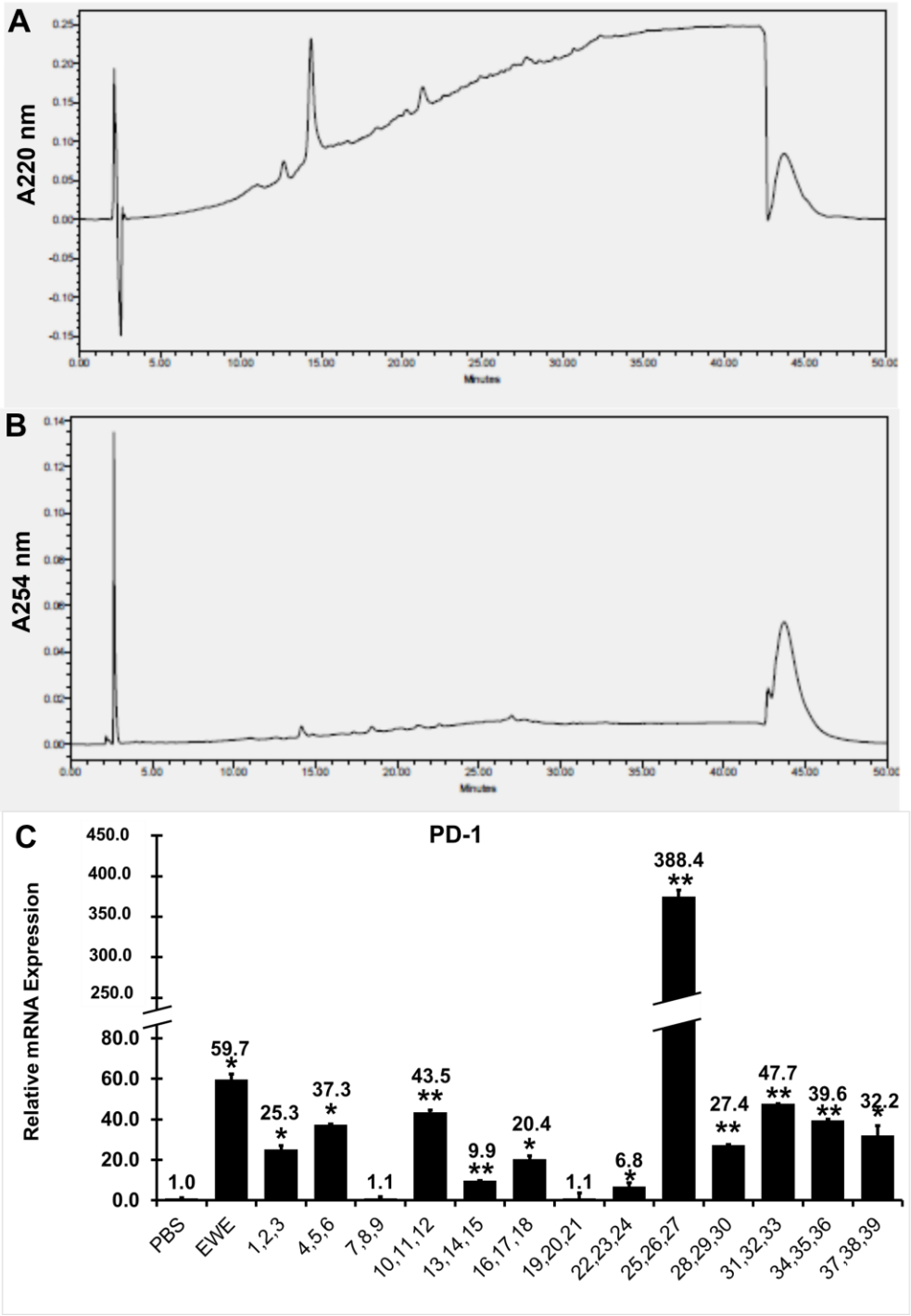

Figure 3.5 The expression of PD-1 in LLC cells treated with HPLC fractions of EWE.
The elution profile of the fractionation of FPLC35-36 by HPLC at 220nm (A) and 255 nm (B). (C), the mRNA expression of PD-1 in LLC cells, which were treated with grouped HPLC fractions for 48 hours, was measured by real-time PCR (two duplicate determinations, *n*=4).*: $p<0.05$, **: $p<0.01$.

Discussion

This study presents the characterization and purification of a PD-1 stimulator in the water-soluble extract from *Euglena gracilis*. EWE dose-dependently increased the expression of PD-1 and PD-L1 in Lewis lung carcinoma (LLC) cells at forty-eight hours, which was statistically significant in the 1 µg/ml and 10 µg/ml treatments (Fig. 3.1). In Jurkat cells, EWE dose-dependently increased the expression of PD-1 at twenty-four hours, which was statistically significant but did not increase the expression of PD-1 at forty-eight hours (Fig 3.2B) or PD-L1 at either time point (Figure 3.2A). EWE also dose-dependently increased the expression of GZMB at forty-eight hours, which was statistically significant in the 1 µg/ml and 10 µg/ml treatments (Fig 3.2C). Although Jurkat cells are human T lymphoblasts and LLC cells are murine lung carcinoma cells, Jurkat cell-derived microparticles have been shown to inhibit cancer cell proliferation regardless of this species difference [106]. Thus, we decided to focus on these two cell types for studying the effects of EWE on anti-lung cancer immunity for *in vitro* studies.

Next, we fractionated the *Euglena* extract by FPLC and analyzed the resulting fractions for their ability to stimulate PD-1, PD-L1, or granzyme B expression in LLC or Jurkat cells. We found that fractions 1-4, 5-8, 17-20, 21-24, 29-32, and 33-36 increased the expression of PD-1 in LLC cells, which was statistically significant (Fig. 3.3B). Similarly, all fractions excluding 25-28 increased the expression of PD-L1 in LLC cells (Fig. 3.3C), which was statistically significant. In Jurkats, fractions 1-4, 9-12, 13-16, 17-20, and 33-36 increased the mRNA expression of PD-1, which was statistically significant (Fig. 3.4A), and fractions 1-4, 9-12, 13-16, 17-20, 25-28, 29-32, and 33-36 all increased the expression of GZMB in Jurkat cells with statistical significance (Fig. 3.4B). Fractions 35-36 exhibited a statistically significant increase in PD-1 expression in LLC cells (Fig. 3.3B) and Jurkat cells (Fig. 3.4A) and showed a statistically significant increase

in the expression of granzyme B in Jurkat cells (Fig. 3.4B). These fractions were matched with a strong isolated absorption peak late in the elution profile (Fig. 3.3A). Due to these factors, we decided to further fractionate FPLC fractions 35-36 by HPLC.

When treated with the HPLC fractions, the LLC cells showed a statistically significant increase in the expression of PD-1 expression in fractions 1-3, 4-6, 10-12, 13-15, 16-18, 25-27, 28-30, 31-33, 34-36, and 37-39, which showed statistical significance. Due to how significant the stimulation in PD-1 expression from fractions 25-27 was, these have been chosen for identification by LC-MS and proton NMR spectroscopy. To the best of our knowledge, this is the first study to report on the fractionation of the PD-1 stimulator within the *Euglena gracilis* water extract.

PD-1 and its ligand, PD-L1, are regulatory proteins that play an immunosuppressive role in the immune system for both PD-1 and PD-L1. They have also become very successful targets for cancer immunotherapy (ICI therapy) [15]. ICI therapy relies on the presence of these regulatory proteins such as PD-1 or PD-L1, and interrupts their interaction with each other, resulting in T cell activation and cancer cell apoptosis [15]. Granzyme B is an important cytokine secreted by cytotoxic T cells that results in apoptosis in cancer cells [107] [108].

While PD-1 is often expressed on immune cells and PD-L1 is expressed on both immune and cancer cells, some recent studies have discovered the existence of PD-1 in cancers such as lung cancer [109] [110]. Studies have shown that an up-regulation in PD-1 or PD-L1 can improve the efficacy in patients' response to ICI therapy [94] [96] [111]. Thus, further studies are needed to identify and characterize the PD-1 stimulator in *Euglena gracilis* water extract and its effect when combined with ICI therapy.

Chapter 4 - Conclusion

This research aimed to investigate the relationship between the *Euglena gracilis* water extract (EWE)-induced inhibition of lung tumor growth and the expression of PD-1 in both lung cancer cells and T cells *in vitro* and *in vivo*. EWE significantly attenuated Lewis lung carcinoma (LLC) tumor growth in mice and increased the expression of PD-1 and PD-L1 in the tumor-bearing mouse lung. Detailed analysis revealed that EWE-induced expression of these proteins is restricted primarily in the tumor-infiltrating lymphocytes. In cell culture, EWE was found to increase the mRNA expression of PD-1 and PD-L1 in LLC cells and the mRNA expression of PD-1 and granzyme B in Jurkat cells. These bioactivities that stimulated the expression of PD-1 and PD-L1 mRNA in LLC cells and the expression of granzyme B and PD-1 mRNA in Jurkat cells were well preserved during Superdex 75 FPLC and C18 reverse phase HPLC. The FPLC purified EWE stimulated the mRNA expression of PD-1 in LLC cells and both PD-1 and granzyme B in Jurkat cells significantly more than whole EWE. Likewise, further purifying EWE by HPLC significantly increased the mRNA expression of PD-1 in LLC cells, which was a stronger stimulation than that by whole EWE or the FPLC purified EWE bioactive fractions. These increases may be due to the removal of impurities that inhibit the bioactivities of EWE, which would require additional experiments to confirm. Nevertheless, these observations may suggest that the purified bioactive substances may possess a stronger bioactivity than those of the unpurified bioactive substances in EWE. Therefore, these bioactivities, which increase the expression of PD-1 in LLC cells and both PD-1 and granzyme B in Jurkat cells, may play an important role in EWE-induced antitumor immunity.

Future Directions

Further investigation of the bioactive compound(s) responsible for stimulating the mRNA expression of PD-1 by LC-MS and 1H-NMR is currently ongoing. Once the chemical structure has been identified, we would like to determine if this component is related to EWE's ability to attenuate the growth of lung carcinoma.

This study found that EWE stimulates the mRNA of PD-1 in lung cancer cells and T lymphoblasts, and granzyme B in T lymphoblasts. To the best of our knowledge, this study is the first to report on these findings and suggest the potential for EWE as an enhancer for immune checkpoint inhibitor therapy targeting PD-1.

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