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A Study of the Activity Control of T-cells by Irradiation with Atmospheric Oxygen Plasma

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A Study of the Activity Control of T-cells by Irradiation with Atmospheric Oxygen Plasma

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A thesis submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy (Ph.D.)

in the

Department of Advanced Energy Engineering Sciences

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KYUSHU UNIVERSITY

Abstract

Interdisciplinary Graduate School of Engineering Sciences
Department of Advanced Energy Engineering Sciences

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A Study of the Activity Control of T-cells by Irradiation with Atmospheric Oxygen Plasma

by Sitti Subaedah

Plasma at atmospheric pressure has been intensively studied for potential medical applications, including bacterial sterilization, dermatological interventions, improvement of adaptive immunity, and cancer immunotherapy. Plasma at atmospheric pressure is in a discharge mode in which a broad plasma region can be formed between the electrodes. Thus, it is the most efficient and capable of generating a large number of active particles. Plasma irradiation in medical applications directs the active particles generated from streamers to cells or tissues. Plasma sterilization proceeds in a highly regulated manner by very short time reactions within cells. Several studies have shown that plasma irradiation in cancer therapy is one of the most promising medical treatments that can trigger a cancer-specific immune response.

On the other hand, malaria is a severe infectious disease of humans caused by the lasmodium parasite and is a primary global health problem responsible for the death of over a million people every year. Cerebral malaria is a life-threatening complication of the disease by the parasite plasmodium falciparum; this complication is characterized by parasite-infected red blood cells

accumulating in the brain that induce an immune response during inflammation, such as T lymphocytes (antibody-helpers). Several previous studies have reported that immune responses to malaria are linked to antibody-helper CD4+ T-cells that appear sequentially during the primary infection as a humoral and cellular immune response. Antibody-helper CD4+ T-cells reactive to plasmodium infection in red blood cells indirectly stimulate immune cell proliferation and possibly increase proteins (IFN-γ) that may orchestrate secreted antibody responses or induce the engulfing and absorbing of parasites.

However, many studies show it will take about three weeks to obtain the desired cell number in Plasmodium infection in red blood cells. In addition, at this proliferation rate, the immune cells can be defeated by the rapidly increasing parasites when using currently available techniques. Excessive immune cells damage the blood cell in brain inducing the coagulation of red blood cells. Therefore, new approaches are required to rapidly regulate T-cells for an immune response, especially when Plasmodium parasites invade the body. The success of any novel immune system treatment for infection in red blood cells depends on its ability to rapidly and selectively target T-cells while minimizing damage to healthy cells. Therefore, it is necessary to examine the activation of antibody-helper CD4+ T lymphocytes, i.e., T helper 1 (Th1) and T helper 2 (Th2) cells, that appear sequentially during primary infection. Th1/Th2 cells produce interferon-gamma (IFN-γ) that plays a role in controlling acute infection, and also produce IL-4 as a humoral immune response, which assists in the maturation of B cells into plasma cells. In this study, we experimentally investigated the activation of two types of T lymphocytes, i.e., EL4 T-cells and BW5147 T-cells sensitized with activator-CD3/CD28 and irradiated with atmospheric oxygen plasma to evaluate the respective and combined effects of sensitization and plasma irradiation on cell activation.

Results obtained, the total amount of cells for two subsets of T-cells, i.e., EL4 T-cells and BW5147 T-cells, combined with sensitization by an anti-CD3/CD28 increased after plasma irradiation under most conditions due to the active oxygen species generated in the oxygen plasma that influenced the cell to grow. In this study, to find the active oxygen species from plasma has difficulty interacting with BW5147 T-cells, causing the IFN-γ levels not to increase significantly for any irradiation conditions. Meanwhile, for EL4 T-cells, the IFN-γ levels significantly increased after plasma irradiation for all of the irradiation conditions. Likewise, oxygen plasma irradiation in EL4 T-cells can be used to control IFN-γ production, and gene expression analysis indicated that MAPK related ERK genes and IFNG genes were upregulated when oxygen plasma was used to irradiate the T-cells.

Overall, these results imply that the IFN-γ production and cytokine levels in T-cells that increased due to oxygen plasma irradiation can be balanced out by means of altered gene expression. Therefore, it can be concluded that plasma irradiation will be helpful for infectious diseases of malaria treatment. The summarizes the results obtained in this research as follows:

- Chapter 1 describes background information concerning the potential of atmospheric oxygen plasma in medical treatments and the proliferation of the immune in the cells, including problem formulation, uniqueness of this research, the framework, and the objectives of this research.

- Chapter 2 presents a literature review regarding atmospheric oxygen plasma irradiation. A variety of models having been developed in the immune system treatment using plasma irradiation are introduced.

- Chapter 3 explains the experimental apparatus in this research, describes the irradiation schematic and preparation of the cells for the experiment, and the methods used in analyzing data. It also introduces, what the cells can be utilized, what kind of analytic results are obtained,

why the data should be calculated, and what kind of challenges are involved when handling the data from experimental results.

- Chapter 4 discusses some of the highlighted analytic results generated from the experiment of activating EL4 T-cells irradiated with atmospheric oxygen plasma. This approach using varied discharge voltage (in the range of 4.2-6.0 kV), and the plasma irradiation period is changed from 0 s (without irradiation) to 40 s. Then at first, the active species in DBD Plasma, i.e., gas temperature, ozone concentration, and light emission intensity in each discharge voltage, are observed. Next, the fundamental characteristics of the plasma and T-cells in each discharge voltage of plasma irradiation, enhancement of T-cell proliferation, IFN-γ production, and gene expression of T-cells are reported.

- Chapter 5 proposes an approach with conducting an advanced experiment to compare the growth characteristic of T-cells between two types of T Lymphocyte, i.e., BW5147 T-cells and EL4 T-cells using atmospheric oxygen plasma irradiation. It also explained the growth and differentiation characteristics of each two types of T-cells, comparing IFN-γ production and gene expression of each two kinds of T-cells irradiated with oxygen plasma.

- Finally, concludes the thesis in Chapter 6. It summarizes the experiment results and the findings of this research and discusses some topics for future scientific research to investigate the activation of T-cells irradiated with atmospheric oxygen plasma.

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List of Abbreviations

ATC	**A**rtemisinin-based **C**ombination **T**herapy
ASK1	**A**poptosis **S**ignal-regulating **K**inase 1
APCs	**A**ntigen **P**resenting **C**ells
DAVID	**D**atabase for **A**nnotation, **V**isualization and **I**ntegrated **D**iscovery
DBMEM	**D**ulbecco's **M**odified **E**agles **M**edium
DBD	**D**ielectric **B**arrier **D**ischarge
HV	**H**igh **V**oltage
HSC	**H**uman oral **S**quamous **C**arcinoma
IRF	**I**nterferon **R**egulatory **F**actor
JNK	c-**J**un **N**-terminal **K**inase
kV	**k**ilo**V**olt
LHRI	**H**uman **R**etrovirology and **I**mmunoinformatics
MHC	**M**ajor **H**istocompatibility **C**omplex
MAPK	**M**itogen **A**ctivated **P**rotein **K**inase
NKs	**N**atural **K**iller cell**s**
NHBE	**N**ormal **H**uman **B**ronchial **E**pithelial
ppm	**p**arts **p**er **m**illion
TNF	**T**umor **N**ecrosis **F**actor
TCR	**T**-cells **R**eceptor
RNA	**R**ibonucleic **A**cid
RPMI	**R**oswell **P**ark **M**emorial **I**nstitute
ROS	**R**eactive **O**xygen **S**pecies
RNS	**R**eactive **N**itrogen **S**pecies

List of Symbols

BW5147	T lymphocyte (naïve T-cell T-cells)
EL4	T lymphocyte (T-cells)
ECT	Electrochemotherapy
ERK	Extracellular clues from cell surface receptors
p38	MAPK Antibody
B16/F1	Cancer cell line
CD3/CD28	Dynabeads® Mouse T-Activator
H_2O_2	Hydrogen peroxide
IFNγ	IFN-gamma
IFI213	Mouse(genes)
IL	Interleukin
mL	Milliliter
mm	Millimeter
min	Minute
nm	Nanometre/nanometer
NO	Nitric oxide
OH	Hydroxyl radical
O_2	Oxygen
Th	T-cells helper
$^\circ$	Temperature
C	Celsius
s	second
O(^5P) and O(^1D)	oxygen atoms

Chapter 1

Introduction

This chapter introduces the background of this thesis. It discusses background, problem formulation, the uniqueness of this research, research objectives, and the framework of the research.

1.1 Background

The research field of oxygen plasma in medical treatments is innovatively combining plasma physics, life science, and clinical medicine. It is principally focused on the application of atmospheric oxygen plasma in therapeutic settings. Based on its capability to inactivate microorganisms and stimulate cell regeneration, current atmospheric oxygen plasma in medical applications are focused on the sterilization of medical devices and treatment of skin diseases [1].

Since atmospheric oxygen plasma can also be used as a human immune therapies system, it is use in cancer treatment has become a promising field of clinical plasma application. It was shown the plasma application in cancer treatment are exposed the active particles generated from streamers and to direct cells or tissue [2]. Plasma irradiation processes are highly regulated by very short reactions within cells and are very potential as a stimulus to enhance the growth of the cells [1] [3]. Thus, the treatment of cells succeeds in triggering a cancer-specific immune response [2][3].

On the other hand, malaria of the most severe infectious disease of humans caused by plasmodium parasites is a primary global health problem responsible for the death of over a million people every year [4] [5]. Cerebral malaria is a life-threatening complication of disease with the parasite Plasmodium falciparum; this complication is characterized by the parasite-infected red blood cells accumulating in the brain [6]. It will induce the immune response during inflammation, such as T-Lymphocyte (antibody-helper) [5] [6].

Along with recent advances in our understanding of the human immune system, immune therapies for cancer have been increasingly utilized. The immune cells, such as macrophages, dendritic cells, T-cells, B-cells, and natural killer (NK) cells, that are used in such therapies must be proliferated outside of the body (i.e., in vitro) to obtain thousands of times the initial number of cells [2] [3] [7]. In the case of the T-cells, a type of lymphocyte which develops in the thymus gland and plays a central role in the immune response, it takes about 3 weeks to obtain the desired cell number.

Likewise, for Malaria disease, various therapies to enhances the body's immune response to an antigen such as T Lymphocyte have been evaluated to reduce high mortality in patients with severe malaria, but none have proven to be effective [8]. Exchange transfusion has been proposed to remove infected red blood cells from the circulation, thereby lowering the parasite burden and replacing with unparasitized cells. But no evidence exists to support its efficacy as adjunctive therapy in severe malaria [9]. Cytokines such as tumor necrotic factor (TNF-α) and interleukin-1 (IL-1) probably contribute to the pathogenesis of severe malaria, and several case reports have suggested that plasma application is an effective adjunctive therapy [8] [10]. In addition, several studies also reported a patient with Plasmodium falciparum malaria is treated successfully with intravenous artesunate and adjunctive therapy with plasma application.

The immune response during inflammation tends to be exaggerated; for example, the immune response of T-cells to malaria parasite infections tends to be excessive, especially in the brain [5]. In immune responses (such as the response to the malaria parasite), T-cells are sensitized by macrophages (a type of phagocyte) and, in turn, produce interferon gamma (IFN-γ), which promotes further immune functions to inactivate the foreign body. However, IFN-γ also induces an inflammatory response in red blood cells, causing them to coagulate and occlude smaller blood vessels; this effect damages brain tissue. Moreover, mutations are more likely to occur in rapidly proliferating T-cells. Therefore, it is important to appropriately regulate the immune response when antigens invade the body [6].

Numerous techniques for controlling cells have been developed, but the protocol can vary significantly between models [2] [4] [11]. In addition, several study noticed new methods are required to proliferate immune cells rapidly (by factors of thousands in less than 3 weeks) for practical application in immune therapies medicine [12] [13].

Therefore, in this study, experimentally investigated the activation of T Lymphocyte i.e., EL4 and BW5147 T-cells and sensitized with antibodies against CD3/CD28 (activator-CD3/CD28) then irradiated with atmospheric oxygen plasma to evaluate the respective and combined effects of sensitization and plasma irradiation on cell activation.

1.2 Uniqueness of this Research

Recently, discharge plasmas have been recognized as one of immune therapy stimulation, especially for enhancement growth of the cells. They include various kinds of ions, electrons, radicals, UV light, electric fields, and metastables [14] [15] [16]. On the other hand, several types of active species are examined to contribute to the antimicrobial (cells) effects of gas plasma.

However, it is essential to note that these active species may differ depending on the types of gas used for plasma production and the plasma production methods. Also, the effects of plasma irradiation on the immune cells have not been clarified. The protein, the interferon-gamma (IFN-γ), and genetic mechanisms behind the plasma effects are not well understood. Moreover, the growth characteristics of T-cells of immunity to malaria have not been examined directly using plasma irradiation [4]. Genotoxic stress resulting from using atmospheric oxygen plasma and chemical agents, including the malaria parasite, severely impacts the cells.

In this study examine the active species produced in dielectric barrier discharge (DBD) plasma. Next, investigate the growth of cells irradiated directly by the plasma by focused on changes in the T Lymphocyte (T-cells), i.e., EL4 and BW5147 T-cells sensitized by the anti CD3/CD28 reagent in advance.

The uniqueness of this research are outlined as follows: 1. Cells are irradiated by plasma directly; 2. Reduce the influence of chemical species produced in culture cell; 3. Comparison of effects of plasma treatment on normal cells and irradiated cells and 4. Examined intracellular signal change by plasma treatment.

1.3 Research Question

The primary research questions of this work concern revealing the factors involved in and ways to achieve the goal of this study, i.e., to clarify the effect of active oxygen species generated by the torch-type dielectric barrier discharge (DBD) plasma on the growth enhancement of T-cells.

In this study, many results will confirm the potential to use the atmospheric oxygen plasma to enhance the immunotherapy cell. The followings are the research questions addressed in this research.

- Question 1: Finding of the appropriate condition of the active species in DBD plasma varying the discharge voltage, material gas flow rate, discharge electrode structure, and distance from the discharge electrode.
- Question 2: Clarification of any correlation between the gaseous ozone concentration around the cells and the various discharge voltage increased from 4.2 to 5.1 to 6.0 kV.
- Question 3: Variation of the enhancement characteristics of the cell number with varying time period irradiation (10 s interval).
- Question 4: Clarification of the production characteristics of IFN-γ (immunity against) for both types of T lymphocytes (musculus, mouse), EL4 and BW5147 T-cells in the effect of oxygen plasma irradiation.
- Question 5: Significant impacts on gene expression of T-cells irradiated with oxygen plasma.
- Question 6: Does this study be concluded that the atmospheric oxygen plasma will promise the treatment of malaria disease?

1.4 Objective and Scope

The primary objective of this study is the activity control of T-cells by irradiation with atmospheric oxygen plasma. This study uses atmospheric-pressure plasma was generated by cylindrical-type dielectric barrier discharge (DBD).

Therefore, the study mainly examines the significant factors, i.e., clarification of the effect of active oxygen species generated by torch-type DBD plasma on cells. Then, the growth and differentiation characteristics of cells are measured.

The objectives and scope of this research are outlined as follows:

1. Active species generated by plasma irradiations, the concentrations of gaseous ozone around T-cells in the microplate well were measured as an index for T-cells growth and activation.

2. Adjusting the amount of active oxygen produced by changing the discharge voltage and the irradiation time, it is possible to selectively control only T-cells in which many active oxygen species are accumulated in the cells.

3. Evaluate T-cells activation in terms of the increase in the cell number due to atmospheric oxygen plasma irradiation; two types of T-cells (EL 4 T-cells and BW5147 T-cells) sensitized by the anti CD3/CD28 were irradiated with the plasma for a period from 0 (i.e., no irradiation) to 50 s.

4. Clarify the plasma treatment effect on the immunity intracellular pathogens of T-cells by means of the production of IFN-γ for both types of T-cells.

5. Investigate the characteristics of cell growth due to oxygen plasma irradiation as the result of the immune function enhancement of EL4 T-cells using the gene expression analysis.

1.5 Research Framework

The framework of this research is to study the activity control of T-cells by irradiation with atmospheric oxygen plasma. Figures. 1.1 and 1.2 shows the conceptual framework and purpose of this research, respectively.

The framework of this research are outlined as follows:

1. Material Preparation and Preliminary Experiment: The process begins with the preparation of the experimental apparatus and cell culture. The atmospheric plasma was generated by cylindrical-type dielectric

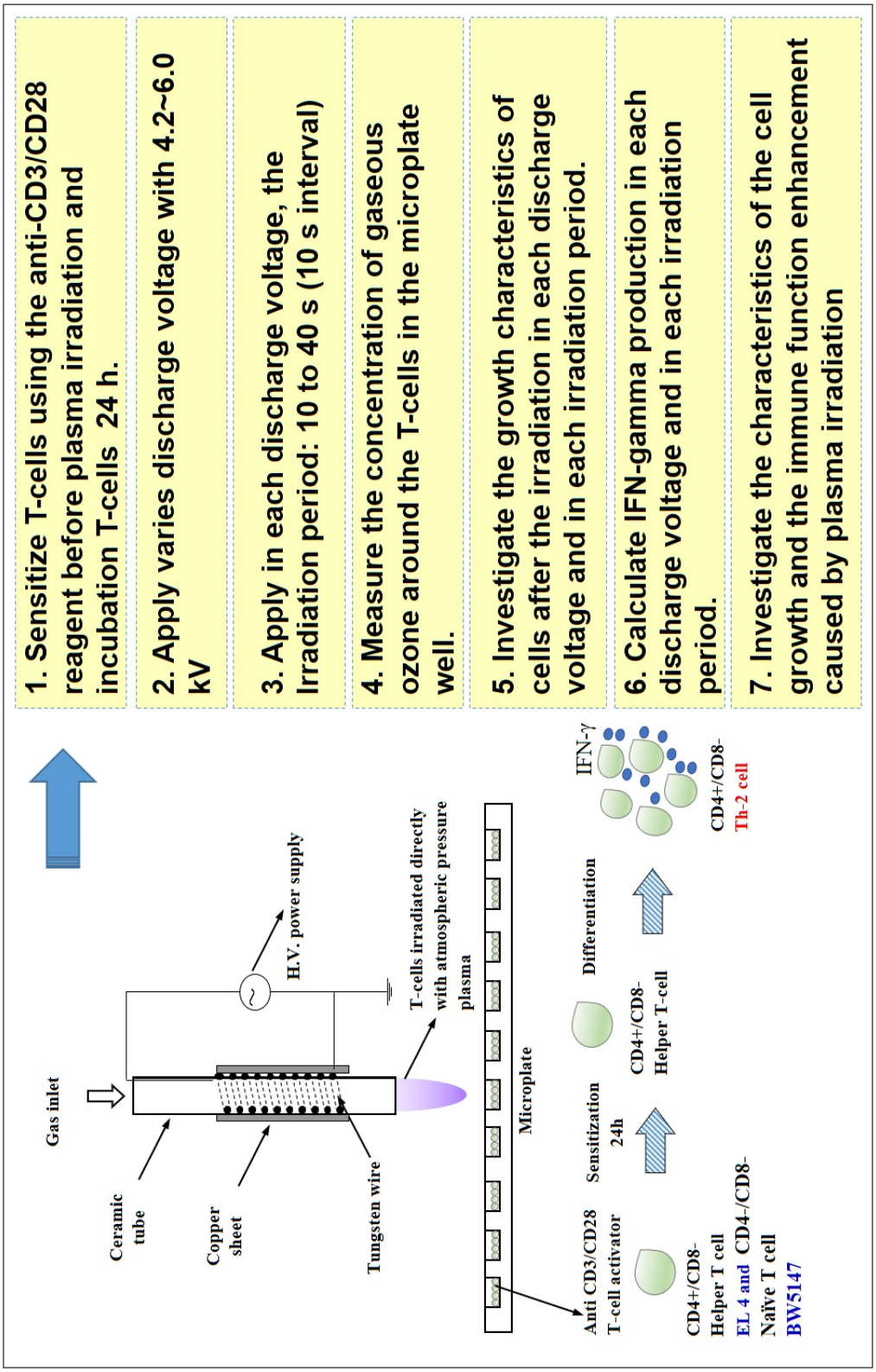

FIGURE 1.1: Framework of this research

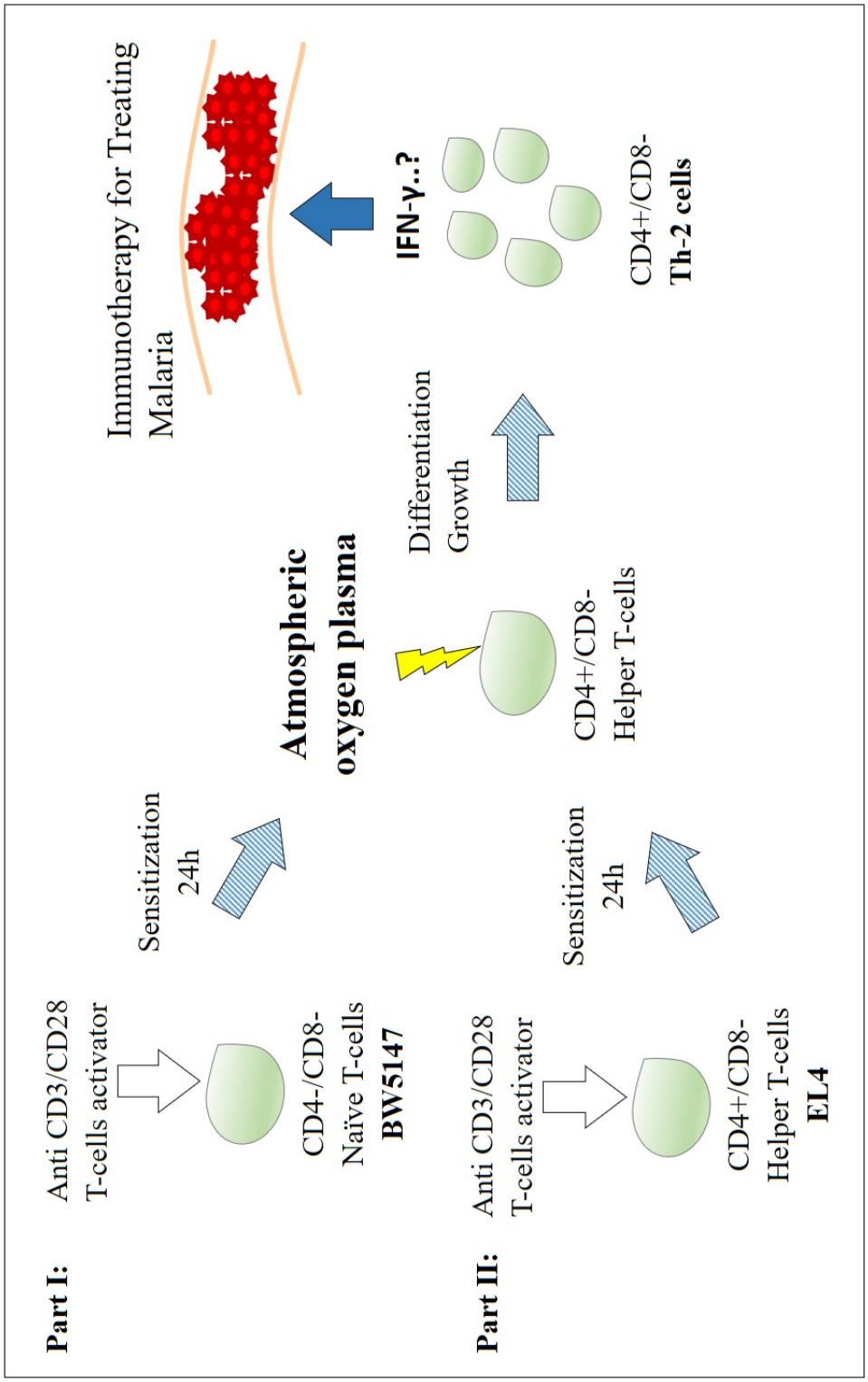

FIGURE 1.2: Goal of this research

barrier discharge (DBD). Pure oxygen gas was supplied as the material gas at a fixed flow rate of 0.6 L/min, and a high voltage (in the range of 4.2-6.0 kV). The cells were kept at approximately 24°C during the plasma irradiation by controlling the plasma gas temperature. The gas temperature can be controlled by varying the discharge voltage, material gas flow rate, discharge electrode structure, and distance from the discharge electrode. The cells were cultured in Roswell Park Memorial Institute medium with L-glutamine and sodium. Before plasma irradiation, the cell population was kept constant at 5.3×10^5 cells/ml throughout the experiment. The T-cells used in this experiment were sensitized by applying a mixture of microbeads conjugated with antibodies against T-cells surface glycoproteins CD3/CD28; this sensitization technique has been shown to produce a spontaneous increase in the number of T-cells without an external stimulus. The details are described in Chapters 3 and 4.

2. Advanced Experiment: This stage covers the advanced experiment to investigate the ability, rapidly, and selectivity of the target T-cells to minimize damage to healthy cells. This work focused on growth enhance of two subsets of T lymphocytes (T-cells) naïve T-cells(BW5147) and EL4 T-cells sensitized with anti CD3/CD28 (activator-CD3/CD28) then irradiated with atmospheric oxygen plasma. The irradiation period varies from 0 to 50 s (interval 10 s) and evaluate the respective and combined effects of sensitization and plasma irradiation on cell activation. Further, to be expected is the utilization for immune therapy in malaria disease. The details are described in Chapter 5

1.6 Thesis Outline

The thesis is organized as follows:

Chapter 1 describes background information concerning the potential of atmospheric oxygen plasma in medical treatments and the proliferation of immune cells, including problem formulation, uniqueness of this research, the framework, and the objectives of this research.

Chapter 2 presents a literature review regarding atmospheric oxygen plasma irradiation. A variety of models have been developed in the immune system treatment using plasma irradiation.

Chapter 3 explains the experimental apparatus in this research, describes the irradiation schematic and preparation of the cells for the experiment, and the methods used in analyzing obtained data. In addition, explains what the cells can be utilized, what kind of analytic results are obtained, what the data should be calculated, and what kind of challenges are involved when handling the data from experimental results.

Chapter 4 discusses some of the highlighted analytic results generated from the experiment of activating EL4 T-cells irradiated with atmospheric oxygen plasma. This approach using different discharge voltage (in the range of 4.2-6.0 kV), and the plasma irradiation period is changed from 0 s (without irradiation) to 40 s. Then, the active species in DBD Plasma, i.e., gas temperature, ozone concentration, and light emission intensity in each discharge voltage, are observed. Next, the fundamental characteristics of the plasma and T-cells in each discharge voltage of plasma irradiation, enhancement of T-cell proliferation, IFN-γ production, and gene expression of T-cells were reported.

Chapter 5 proposes an approach with conducting the growth comparison between two types of T Lymphocyte, i.e., BW5147 T-cells and EL4 T-cells using atmospheric oxygen plasma irradiation. In this chapter, also explained the growth and differentiation characteristics of each two types of T-cells, comparing IFN-γ production of each two kinds of T-cells irradiated with oxygen plasma.

In Chapter 6, the thesis concludes with a summary of the experimental results and the findings of this research, and discusses some topics for future scientific research to investigate the further activation of T-cells irradiated with atmospheric oxygen plasma.

Chapter 2

Related Work

2.1 Introduction

This chapter presents a literature review of conceptual, theoretical and methodological topics related to the atmospheric oxygen plasma irradiation in medical application. Through the literature review, it was summarized the fundamentals of the methods of plasma irradiation and their potential application, as well as the efficiency of these disinfection/sterilization methods against various microorganisms and for immune system treatment.

2.2 Atmospheric Pressure Plasma

Plasma is defined as an ionized (or energized) gas with an equal number of positively and negatively charged particles. plasma is often regarded as the fourth state of matter (the other three being solids, liquids and gases) because, while plasma is neither a normal gas or a liquid, its properties are similar to those of both gases and liquids. Plasma can be categorized as either high-temperature and low-temperature (low-pressure plasma) based [17]. A good example of a naturally occurring high-temperature plasma is lightning. High-temperature plasma can be artificially generated using a high-voltage high-temperature arc, which is the basis for the corona discharge process and for plasma torches used to vaporize and redeposit metals.

Low-pressure plasma can be carried out at low-temperatures without residual remaining toxic gases. It is still a relevant technique today, as it gives good control over various plasma operating parameters [18] [19]. On the other hand, the mode of operation of the atmospheric pressure plasma is changed by varying the process parameters such as pressure, power, process time, gas flow and gas composition. Thus, several effects can be achieved in a single process step [20]. Therefore, low-pressure plasma have gained interest over atmospheric pressure plasma as it possesses various advantages used in surface modification including sterilization, surface activation, chemical vapor deposition, biotreatment of microorganisms, and material processing, are ionized gases generated under deep vacuum conditions [7] [20].

There are many discharge techniques in the atmospheric pressure plasma method that produced stable gas discharge. One of them is Dielectric Barrier Discharge (DBD) was gained interest over atmospheric pressure plasma as it possesses various advantages [21] [22]. Dielectric barrier discharge, also known as silent discharge, is a particular type of AC (High Voltage: HV) discharge that distributes intense thermodynamic, nonequilibrium plasma at gas pressure and moderate atmospheric temperatures. DBD atmospheric pressure plasma has an approximate electron frequency of 1010 density/cm^3 and a power density of about 0.1 w/cm^3 [19] [20] [21].

In operation, DBD are generated when an alternating (1–100 kHz) high voltage (0.5–20 kV) is applied between two electrodes, in which at least one is covered with a dielectric material. In discharge, excited, ionized, active oxygen species and radicals are generated via the impact of energetic electrons and reactant particles. The appearance of a dielectric barrier is one of the most obvious ways to produce nonequilibrium atmospheric pressure discharge as it prevents short-circuiting and arcing among the two electrodes [1] [19].

Furthermore, the dielectric layer prevents electrode corrosion and etching. DBD discharges at atmospheric pressure are characterized by forming a large number of short-lived independent current filaments or micro discharges [1] [23]. For certain conditions, a homogeneous treatment can be achieved similar to the distinctive glow discharge obtained at lower pressures by evading the streamers [23]. Compared with corona dischargers, DBD systems are capable of more homogeneous treatment of large areas. It is geometrically more versatile than a corona discharge, where the distance between the two electrodes is usually much smaller, confining the volume [19] [24].

Another advantage over other plasma systems is its capacity to manage without the use of large-scale vacuum systems, reducing the acquisition and maintenance cost. Finally, it is characterized by relatively high deposition rates and allows for a straight-forward implementation of direct processing on the object [23] [24]. Therefore, the DBD Atmospheric pressure plasma has an advantage of medical applications, including bacterial sterilization, dermatology, promotion of adaptive immunity, and cancer immunotherapy [17] [25] [26].

2.3 Potential in Medical Applications

Atmospheric pressure plasma in medical applications has developed in the last few years into an innovative and growing field of research and development. It combines several areas: physics, chemistry, engineering, biology, microbiology and medicine into an extensive multidisciplinary research effort. Current activities relate to the design of specialized plasma sources and delivery techniques, understanding the processes that lead to active particles and microorganism effects without harming cells, maintaining prescribed

safety standards, and investigating the relevant processes in the treatment [27] [28].

Several studies currently explore the potential applications of atmospheric pressure plasma in cancer therapy. It benefits from various of reactive oxygen species (ROS) and reactive nitrogen species (RNS), which can promote oxidative stress and trigger different signaling pathways in cells [29] [30]. The opportunity of a novelty therapy in cancer treatment and the selectivity to ablate cancer disease (e.g., melanoma cell) while the corresponding normal cells remain unaffected have already been discussed in [30], the detachment of SW900 ($CVCL_1731$) cancer cell line from the culture vessel after plasma irradiation has been examined and the results are quite promising. In contrast, no detachment was observed when normal human bronchial epithelial (NHBE) cells were treated.

Furthermore, Hayashi *et al.* (2018) [2] conducted a study in vitro that clarifies the inactivation effect of active oxygen species generated by torch-type DBD plasma on oral cancer cells. The Human oral squamous carcinoma cell line (HSC-3) and Human keratinocyte cell line (HaCaT) after plasma treatment has been observed. This study reported, by adjusting the amount of active oxygen produced by changing the discharge voltage or the irradiation time, it was possible to inactivate only cancer cells without affecting normal cells selectively. HSC3 has the characteristic that phosphorylation of Ser46 of p53 does not occur. However, oxygen plasma treatment causes phosphorylation of Ser46 and induces cell death [2].

Next, in vivo study was examined cold plasma selectivity and the possibility of a paradigm shift in cancer therapy [27]. The ability of atmospheric pressure plasma in a murine tumor cell line has been investigated. Plasma application selectively eradicates cancer cells in vitro without damaging normal cells, and they found crucially reduces tumor size in vivo. It is shown that reactive oxygen species metabolism and oxidative stress-responsive genes

are deregulated [27]. Another field investigated the efficacy of atmospheric pressure plasma as a potential therapy for cancer treatment. Georg *et al.* (2013) [31], compared the antitumor efficacy of atmospheric pressure plasma with electrochemotherapy (ECT) in a melanoma mouse model. A single atmospheric pressure plasma treatment is directed to a significant obstacle of tumor growth acceleration. However, this was less effective compared to ECT, whereas the combination of atmospheric pressure plasma and ECT had the most substantial effect. In light of these findings, the authors discussed that cold plasma presents a potential option to ECT and may serve as a new possibility for palliative skin melanoma treatment, either alone or in combination with ECT [31]. A murine cell line (B16/F10) has been practiced to elucidate the effect of atmospheric pressure plasma on melanoma cells in vitro, showing a loss of viability of almost 100% at 48 hours after atmospheric pressure plasma treatment for 3 minutes [32].

Another on going challenge in the field of conception and development, the possible use of atmospheric pressure plasma for non-cancer treatment has also been examined in vitro and vivo studies for several other diseases, including actinic keratosis, scar treatment, skin tumors, wound healing, and immunogenicity like malaria diseases [30] [33] [34] [35] [36].

Overall in these studies above, increased repression of different cell lines in culture and decreased parasite growth or pathogen volume reduction have been observed in most various of the mouse cell line models after atmospheric pressure plasma treatment. Although the molecular mechanisms for the efficacy of plasma irradiation on cells are not fully understood, reactive oxygen species (ROS) and charged particles have been determined to be significant contributors to plasma-induced cell death [27] [37]. In addition, atmospheric pressure plasma treatment the protective effects of the immunogenicity are exploited to specifically target the cancerous cells while sparing normal counterparts. These a more innovative approach in which the focus

is on overcoming the cell failure in the processes that prevent cancers in the body. In the normal course of events, cells of the immune system identify and destroy mutated cells before they can establish diseases like tumors in the body. Identified key players of the anti-tumor immune response are antigen-presenting cells (APCs), T-cells, and natural killer cells (NK-cells) [38].

However, the complexity of both plasma generation, charged particles, electric current, and reactive gas species on the one hand, and specific application conditions demands in medicine on the other, have to be determined from the beginning when plasma devices for medical applications will be developed [11] [39]. The fact that most of the critical features of atmospheric pressure plasma applications have to be identified and optimized at best before conduct an experiment makes the atmospheric pressure plasma research and development a novel and deeply important field in plasma medicine [1] [31] [40].

2.4 Cerebral Malaria

Malaria is a severe infectious disease of humans caused by the Plasmodium parasite. It is a primary global health problem responsible for the death of over a million people every year [4] [5]. Cerebral malaria is a life-threatening complication of the disease by the parasite Plasmodium falciparum; this complication is characterized by parasite-infected red blood cells accumulating in the brain [6] that induce an immune response during inflammation [3] [6].

As shows in Figures 2.1 and 2.2 malaria is transmitted when Plasmodium parasite forms are deposited into the dermis during parasite-infected. Parasites exit the dermis and transit through the circulation to infect hepatocytes in the liver. Over the next several days of asymptomatic liver-stage infection, parasites undergo amplification and differentiation into merozoites.

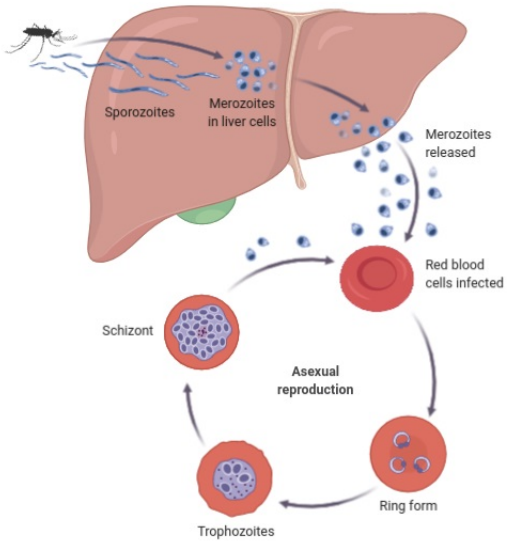

FIGURE 2.1: Malaria causing Plasmodium parasite. Reprinted from "Malaria Asexual Reproduction Cycle", by BioRender, May 2019, retrieved from https://app.biorender.com/biorender-templates/t-5e62a0ac9501410088a02097-malaria-asexual-reproduction-cycle, 22/06/2021. Copyright 2021 by BioRender.

Merozoites emerge from infected hepatocytes either individually or as part of a merosome and represent an antigenically distinct form of the parasite that targets host erythrocytes to establish blood-stage infection, which is the phase responsible for all clinical signs and disease symptoms [41]. Therefore, cellular and humoral adaptive immune responses are essential for limiting Plasmodium parasite replication and the severity of malaria this phase. In immune response, parasite-specific cytotoxic CD8+ T cells are thought to eliminate infected hepatocytes following recognition of parasite antigens presented on MHC class I molecules; by contrast, CD4+ T cell-dependent antibody responses will prevent sporozoites malaria invasion of hepatocytes [42] [43]. Both of these immune mechanisms effectively prevent the progression from asymptomatic to acute infection [43]. Moreover, during the blood stage

of Plasmodium infection in naive or partially resistant hosts, parasitized erythrocytes (which lack functional MHC expression) are indirectly targeted by CD4+ T helper (Th) cells and possibly T lymphocytes that may orchestrate secreted antibody responses or the anti-parasitic activity of phagocytes [43] [44].

On the other hand, there is a variety of different traditional and novel treatment options for malaria diseases, antimalarial therapy, antimalarial drugs, artemisinin-based combination therapy (ACT) and immunotherapy. However, it takes about three weeks to obtain the desired cell number; at this proliferation rate, the immune cells can be outnumbered by the rapidly increasing pathogen when using currently available treatment [21].

Furthermore, the success of any novel immune system treatment for malaria disease depends on its ability to rapidly and selectively target T-cells while minimizing damage to healthy cells. It is necessary to examine the activation of antibody-helper CD4+ T lymphocytes, i.e., Th1 and Th2 cells, that appear sequentially during primary infection. Th1/Th2 cells produce IFN-γ that plays a role in controlling acute infection, and also produce IL-4 as a humoral immune response, which assists in the maturation of B cells into plasma cells [4] [45] [46]. Therefore, new approaches are required to rapidly modulate T-cells for an immune response, especially when Plasmodium parasites invade the body [47] [48].

2.5 Summary

In summary, the principally new elements in plasma irradiation in medicine are the molecular delivery of oxygen active species and the corresponding ability to access even micron-sized regions, the possible use of ions as active agents [46] [49]. The combination of charging by electrons and the induced

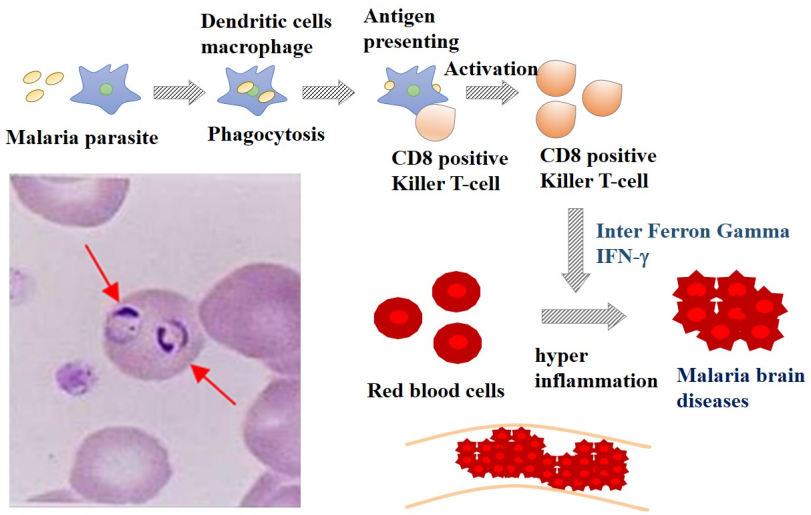

FIGURE 2.2: Sequence of malaria brain disease

electric fields, leading to increased permeability of cell walls for drug delivery immediately [2] [29] [50].

In general, from the literature review above, plasma irradiation for medicine is relatively mature and has found a niche application for coagulation to avoid excessive bleeding. An exciting development is recognizing gas plasma instruments in precision cutting to minimize tissue damage, improve recovery and reduce complications. An even more exciting development is the use of atmospheric pressure plasma for cancer treatment, with emerging evidence that cancer cells respond differently to normal cells when exposed to gas plasma, either directly or indirectly.

The underpinning science is still in active development with important questions remaining. Synergies appear to exist between reactive species, but mechanisms and the most effective synergy combinations remain to be fully understood. Especially in malaria Plasmodium infection, the immune cells, such as macrophages, dendritic cells, T-cells, B-cells, and natural killer (NK) cells that are used in such therapies must be proliferated outside of the body (i.e., in vitro) to obtain thousands of times the initial number of cells [21] [49]

[51].

Therefore, by the following above the related work, the present study will be an attempt in the direction to experimentally investigated the activation of the sensitized T lymphocytes, i.e., EL4 and BW5147 T-cells sensitized with antibodies against CD3/CD28 (activator-CD3/CD28), which are irradiated with atmospheric oxygen plasma and then the respective and combined effects of sensitization and plasma irradiation on cell activation are evaluated.

Chapter 3

Experimental Apparatus, Materials and Methodologies

3.1 Introduction

This chapter explains the experimental apparatus and materials used in this research and describes the experiment apparatus and cell culture preparation. The present study shows what atmospheric oxygen plasma irradiation can be utilized, what the experimental should be handled, what it is to culture cells, and what to analyze the data obtained from the experiment.

3.2 Experimental Apparatus

As described early in Chapter 2 in Section 2.2, in this study, the atmospheric plasma was used generated by cylindrical-type dielectric barrier discharge (DBD). Here, briefly introduce a DBD device that works. In general, the DBD device consists of two dielectrical structures, and at least one of these electrodes is covered by a cylindrical layer, as shown in Figure 3.1.

Figure 3.1 and Table 3.1 shows a schematic diagram and conditions of the experimental apparatus. The atmospheric pressure plasma was generated by cylindrical-type dielectric barrier discharge (DBD). The discharge electrode, a stainless-steel mesh, was set inside a ceramic tube with an inner diameter of

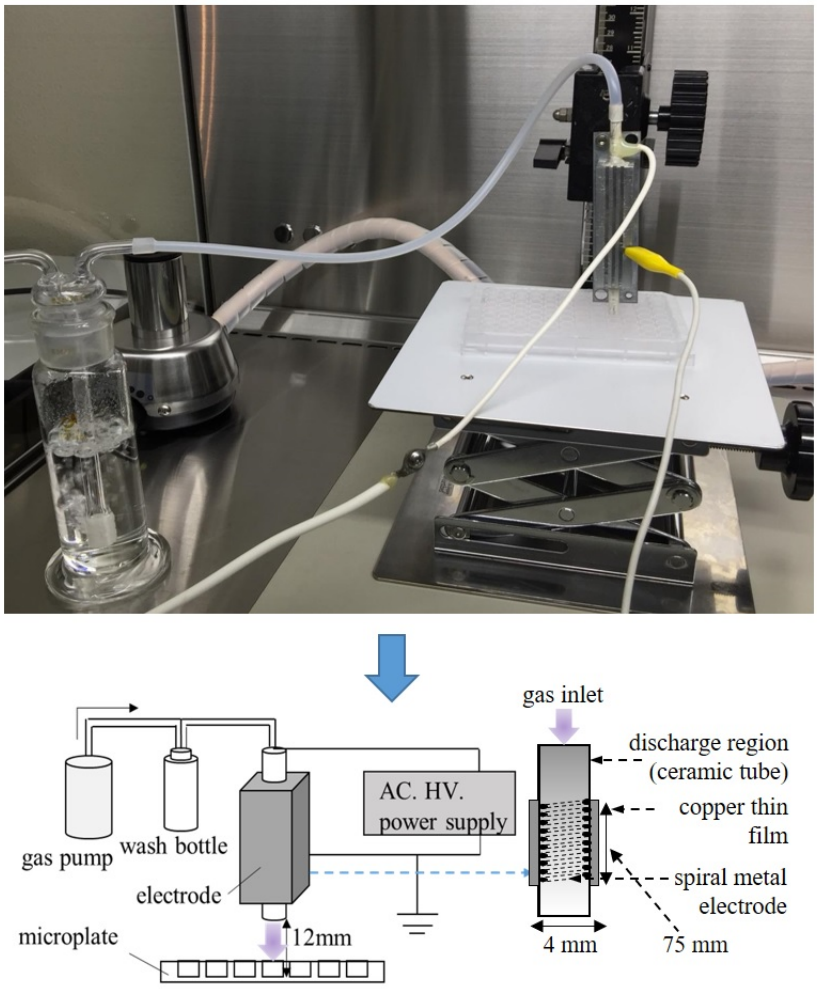

FIGURE 3.1: Schematic diagram of the experimental apparatus

4 mm, and a cupper thin film as the grounded electrode was wound around the outer wall of the ceramic tube. Pure oxygen gas was supplied through the ceramics tube as the material gas at a fixed flow rate of 0.6 L/min, and a high variance voltages was applied to the mesh electrode to generate DBD around the discharge electrode on the inner surface of the ceramic tube.

The ozone and active oxygen species generated by this process were blown out of the ceramic tube with a distance between the discharge electrode and cells on the microplate well bottom of 12 mm. A gas detection tube was specification used to measure the ozone concentration as an indicator of oxygen plasma production [52].

TABLE 3.1: Experimental conditions

Types of cells	: BW5147 T-cells & EL4 T-cells
Material gas	: Oxygen (O^2)
Gas flow rate	: 0.6 L/min
Irradiation period	: 10∼50 s (10 s interval)
Discharge voltage	: 4.2∼6.0 kV
Input power	: 60 W
Frequency	: 13.56 MHz

Active species ejected from the tube were applied to the cells in a 96 well microplate with a culture medium volume of 0.2 ml. During the plasma irradiation, cells were kept at less than or equal to 37°C, as cells require this temperature, and the discharge voltage was maintained at less than 6.3 kV in all experiments. Figure 3.2 shows the irradiation process of atmospheric pressure plasma.

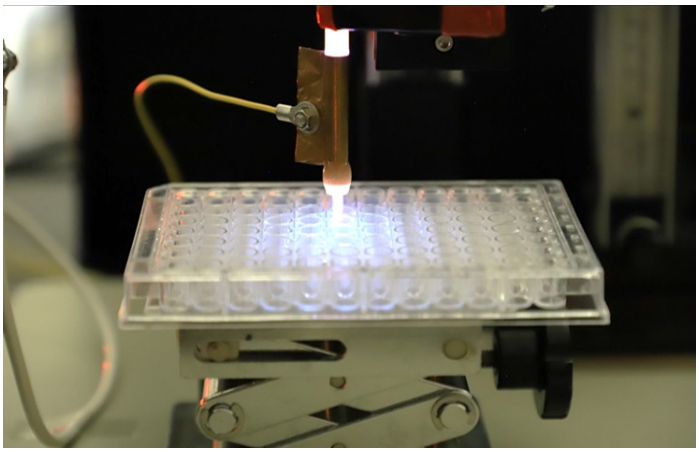

FIGURE 3.2: Irradiation process of atmospheric pressure plasma

3.3 T Lymphocytes (T-cells)

T lymphocytes originate from hematopoietic stem cells, which are developed in the bone marrow. Some of these multipotent cells will become lymphoid progenitor cells that leave the bone marrow and travel to the thymus via the blood as shown in Figure 3.3.

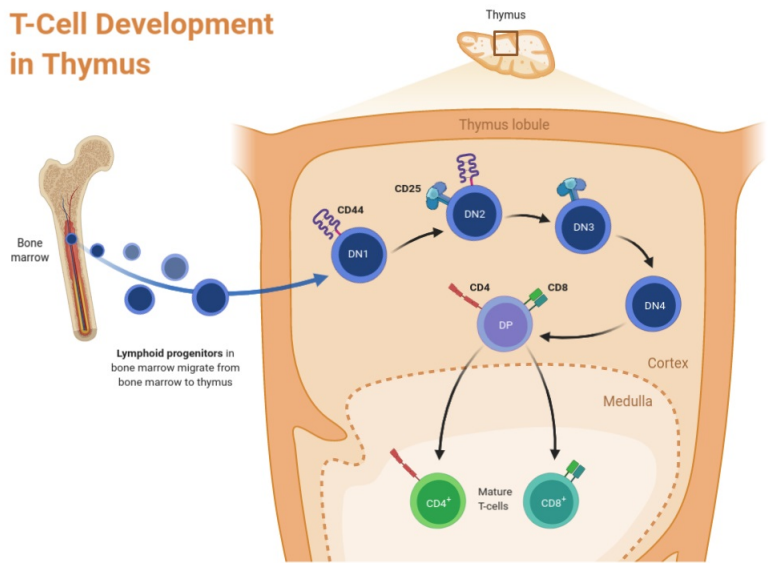

FIGURE 3.3: Development of T lymphocytes (T-Cells). Reprinted from "T-Cell Development in Thymus",by BioRender, June 2020, retrieved from https://app.biorender.com/biorender-templates/t-5ece831af7ae9e00b3bdce3e-t-cell-development-in-thymus, 22/06/2021. Copyright 2021 by BioRender.

T lymphocytes encounter a selection process in the thymus, which the majority of developing T cells (called thymocytes) will not survive. Thymocytes that have receptors to self-antigen molecules receive negative signals and are removed from the repertoire [53]. While T lymphocyte that survives will develop its own T-cells receptor (TCR) that is specific for a particular antigen. T lymphocytes that sustain thymic selection will mature and leave the thymus. They will circulate through the peripheral lymphoid organs and ready to encounter a specific antigen and become activated. Once activated, the T-cells will reproduce and differentiate into an effector T-cells. Moreover, the thymus atrophies also produce naïve T lymphocytes over time [53] [54].

Next, naïve T lymphocytes (Naïve T-cells) are cells that have not yet encountered their specific antigen. In peripheral lymphoid organs, naïve T-cells can interact with antigen-presenting cells (APCs), which use an MHC molecule to present antigen. If the Naïve T-cells recognize a specific antigen,

it will increase and differentiate into effector T lymphocytes of a particular type. Effector T lymphocytes will associate with host cells (rather than the pathogen) to carry out their immune function [55]. The cell either uses a co-receptor called CD8/CD4 to bind to the MHC molecule. These proteins help to differentiate significant effector groups of T lymphocytes. Naïve T-cells will become cytotoxic T lymphocytes (CD8+ T-cells), and those with CD4 will become T helper lymphocytes, each of which are specialized in particular tasks [53] [56].

Therefore, T-cells are the central mediators of both humoral and cellular adaptive immune responses. Particular receptor-mediated clonal selection and expansion of T-cells assure antigen-specific immunity. In addition, encounters with cognate antigens generate immune memory, the capacity for long-term, antigen-specific immunity against previously encountered pathogens. Activated T-cells can respond rapidly and secrete effector cytokines even in the absence of antigen stimulation [55] [57]. Recent studies have pay attention to the importance of activation of CD4+ T cells in infection clearance and autoimmune pathogenesis and the existence of a distinct innate-like immunological function [58].

In order to investigate the optimum conditions to obtain growth enhancement and activation effects, two subsets of T lymphocytes (T-cells), i.e., naïve BW5147 T-cells and EL4 T-cells, were used. BW5147 T-cells is an organism from mus musculus (mouse) established from a spontaneous AKR/J thymoma [59]. While EL4 T-cells is also an organism from mus musculus (mouse) was established from a lymphoma induced in a C57BL mouse 9,10-dimethyl-1,2-benzanthracene [59].

3.4 Cell Culture

In this study, two subsets of T lymphocytes (T-cells) are used, i.e., BW5147 T-cells are a kind of naïve cell differentiated to helper T-cells, and EL4 T-cells obtained from the lymph are also determined helper T-cells. Before plasma irradiation, these two cells were sensitized in advance by anti-CD3/CD28 antibodies (Dynabeads™ Mouse T-Activator [60]).

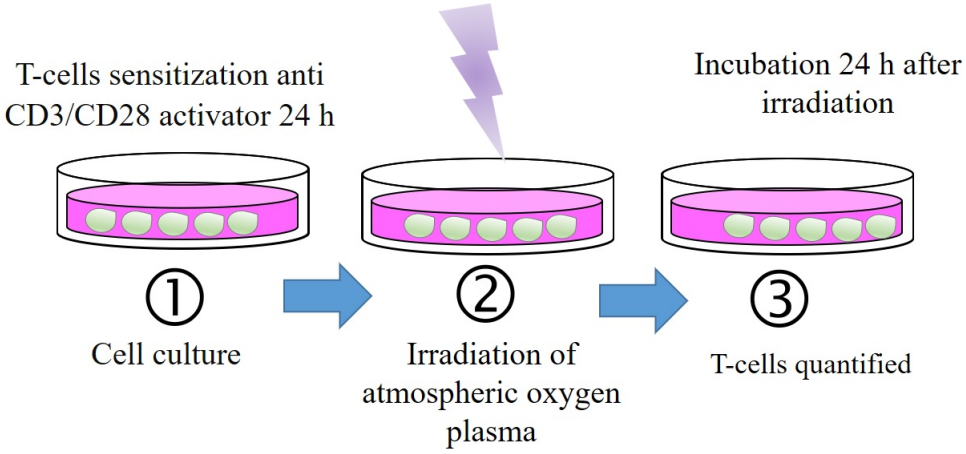

FIGURE 3.4: Schematic diagram of the experiment process

To induce precise responses in cells in suspension, EL4 T-cells were cultured in Roswell Park Memorial Institute (RPMI) and BW5147 T-cells were cultured in Dulbecco's Modified Eagles Medium (DMEM). For each T-cell subculture, the population was kept constant at 2.0×10^5 cells per well at the beginning of the experiment. After the irradiation process, the cells were cultured in an incubator at 37°C for 24 hours, and then the cell number was quantified using a cell counting kit (WST-8) [61] [62].

For every measurement, four wells of the micro-well plate were used to obtain average values and the same parameters to confirm the repeatability of each experiment. The amount of IFN-γ produced by the two types of T-cells was measured using a sandwich enzyme-linked immunosorbent assay (ELISA) assay. Next, variations in gene expressions in T-cells were analyzed

using a microarray method [3] [63]. Ribonucleic acid (RNA) was extracted from cells at 24 hours after plasma irradiation, and the microarray is fabricated. The microarray with the RNA of T-cells after plasma irradiation is scanned using microarray scanner, and then the expressed gene list is obtained. A gene annotation table of expressed genes was obtained from the list of expressed genes with $p < 0.05$ using the database for annotation, visualization, and integrated discovery (DAVID) [64] [65]. Next, introduces briefly regarding Cell Counting method was used in this study.

3.5 Cell Counting

An essential stage in this work is to involve the counting of cells number. Knowing the number of input cells is vital for standardizing experiments and for measuring assay atmospheric plasma impact on the cells. This phase is required accurate and consistent numbers of input cells and cell proliferation. Therefore, counting the number of cells grown in tissue culture is essential to determine the confluence level before diluting the cells into smaller aliquots for optimal cell growth [62] [66].

Figure 3.5 shows the cell counting device used in this study. For the cell counting device using the hemocytometer and a plate reader (450 nm filter) with 96-well plate, and CO_2. Inoculating cell suspension $100\mu l$ in a 96-well plate, adding 10 μl of the CCK-8 solution to each well of the plate, incubating the plate for 1 hour in the incubator, and then measuring the absorbance at 450 nm using a microplate reader. Next, count the number of cells as follows. Number of cells (cells / ml) = (measured total number of cells) / (measured number of compartments) x dilution ratio x 10^4, and total number of cells (cells) = 1 ml number of cells per ml x volume of cell suspension.

The cell number was quantified using a cell counting kit (WST-8). Then, the enzyme-linked immunosorbent assay (ELISA) to assay the amount of

IFN-γ produced by T-cells after plasma irradiation and the database for annotation, visualization, and integrated discovery (DAVID) was used to obtain gene expression of cells.

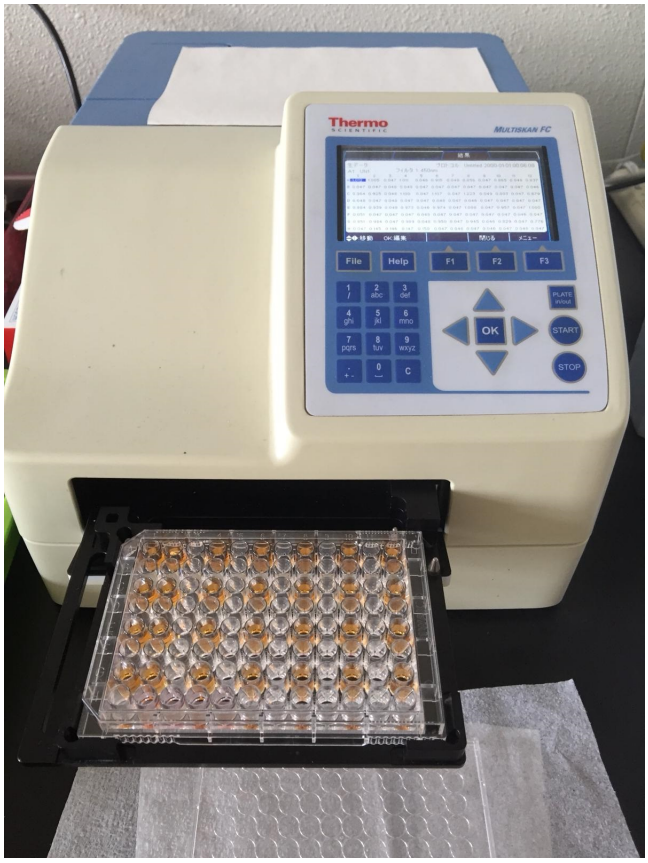

FIGURE 3.5: Cell counting device

3.5.1 Cell Counting Kit 8 (WST-8)

Cell Counting Kit 8 (WST-8) provides a convenient and robust way of performing a cell viability assay. The kit uses a water-soluble tetrazolium salt to quantify the number of live cells by producing an orange formazan dye upon bio-reduction in the presence of an electron carrier. WST-8/CCK8 solution is added directly to the test cells with no premixing of components required [61] [62].

Cellular dehydrogenases reduce WST-8/CCK8 tetrazolium salt to an orange formazan product soluble in tissue culture medium. The amount of

formazan produced is measured by absorbance at 450 nm and directly proportional to the number of living cells. The excellent stability and little cytotoxicity of the WST-8 / CCK8 solution make the kit useful for assays that require prolonged incubation (such as 24 to 48 hour) [62] [67].

3.5.2 The Database for Annotation, Visualization and Integrated Discovery (DAVID)

Practical annotation of differentially expressed genes is an essential and critical step in the analysis of microarray data. Therefore, DAVID (the database for annotation, visualization, and integrated discovery) is a free online bioinformatics resource developed by the Laboratory of Immunopathogenesis and Bioinformatics [65] was used. All instruments in the DAVID Bioinformatics Resources aim to provide a practical examination of large lists of genes obtained from genomic libraries, e.g., microarray and proteomics studies. For more detail, DAVID can be found at Laboratory of Human Retrovirology, and Immunoinformatics (LHRI) [64].

The DAVID Bioinformatics Resources consists of the DAVID Knowledgebase and five integrated, web-based functional annotation tool suites: the DAVID Gene Functional Classification Tool, the DAVID Functional Annotation Tool, the DAVID Gene ID Conversion Tool, the DAVID Gene Name Viewer, and the DAVID NIAID Pathogen Genome Browser [64] [65].

The expanded DAVID Knowledgebase now integrates almost all prominent and well-known public bioinformatics resources centralized by the DAVID Gene Concept, a single-linkage method to agglomerate tens of millions of diverse gene/protein identifiers and annotation terms from a variety of public bioinformatics databases. For any uploaded gene arrangement, the DAVID Resources now presents not only the typical gene-term enrichment analysis but also new mechanisms and functions that enable users to consolidate

large gene lists into gene functional groups, convert between gene/protein identifiers, imagine many-genes-to-many-terms relationships, cluster irrelevant and heterogeneous terms into groups, search for interesting and relevant genes or terms, dynamically view genes from their lists on bio-pathways and more [64] [68].

Chapter 4

Activation of EL4 T-cells Using Atmospheric Oxygen Plasma

4.1 Introduction

Recent years have seen a renaissance in the research linking cancer with the immune cells playing a central role in the human immune system. The immune cells, such as macrophages, dendritic cells, T-cells, B-cells, and natural killer (NK) cells that are used in such therapies must be proliferated outside of the body (i.e., in vitro) to obtain thousands of times the initial number of cells [2] [3] [7].

The adaptive immune system consists of T-cells and B-cells, which express specific antigen recognition receptors and develop highly specialized effector functions with the ability to form long-term immunological memory. In addition, T-cells a type of lymphocyte which grows in the thymus gland and plays a central role in the immune response; it takes about three weeks to obtain the desired cell number. However, at this rate of proliferation, the immune cells can be defeated by the rapidly increasing cancer cells when using currently available techniques [2] [69] [70]. Therefore, new methods are required to increase immune cells rapidly (by factors of thousands in less than three weeks) for practical application in immune therapies.

The activation of T cells in response to antigen, and their subsequent proliferation and differentiation, constitutes a primary immune response. At the same time as providing armed effector T cells, this response generates immunological memory, which gives protection from subsequent challenge by the same pathogen [45]. In immune responses (such as the response to the malaria parasite), T-cells are sensitized by macrophages (a type of phagocyte) and, in turn, produce IFN-γ, which promotes further immune functions to inactivate the pathogen. However, IFN-γ also induces an inflammatory response in red blood cells, causing them to coagulate and occlude smaller blood vessels; this effect damages brain tissue. Moreover, mutations are more likely to occur in rapidly proliferating T-cells. Therefore, it is important to appropriately modulate the immune response when antigens invade the body [6].

Several techniques for controlling cells have been developed, but the protocol can vary significantly between models [2] [4] [11]. Plasma at atmospheric pressure has drawn considerable attention for its potential medical applications including bacterial sterilization, dermatology, promotion of adaptive immunity, and cancer immunotherapy [2] [21]. Plasmas have very short lifetimes and do not remain for a long time; therefore, they do not induce side effects in the human body. In addition, atmospheric oxygen plasma in medical treatments have been shown to promote cell proliferation, which is highly regulated by a series of reactions within a cell [70]. Here, it was used this technique to activate T-cells, that is, enhancements of cell proliferation and cytokine production. It is known that certain signaling factors, such as the anti-CD28 emitted by macrophages and dendritic cells, can sensitize T-cells, resulting in increased cell proliferation [51].

Therefore, the hypothesize that T lymphocytes in the sensitized state will be more strongly activated by oxygen plasma irradiation and exhibit enhanced cell proliferation compared with cells that are not sensitized (i.e.,

naïve T-cells) [3]. In this study, experimentally investigate the growth enhancement of T lymphocyte (EL4 T-cells) sensitized with antibodies against CD3/CD28 (anti-CD3/CD28) irradiated with atmospheric oxygen plasma to evaluate the respective and combined effects of sensitization and plasma irradiation on cell activation.

4.2 Experimental Setup

Figure 4.1 shows the experimental apparatus used in this experiment. The atmospheric pressure plasma was generated by cylindrical-type dielectric barrier discharge (DBD)device. The discharge electrode, a spiral metal electrode, was set inside a dielectric (ceramic) tube, and a grounded electrode was wound around the outer wall of the tube. Pure oxygen gas was supplied as the material gas at a fixed flow rate of 0.6 L/min, and a high voltage (in the range of 4.2-6.0 kV) was applied to the mesh electrode to produce DBD on the inner surface of the ceramic tube [3] [49] [69].

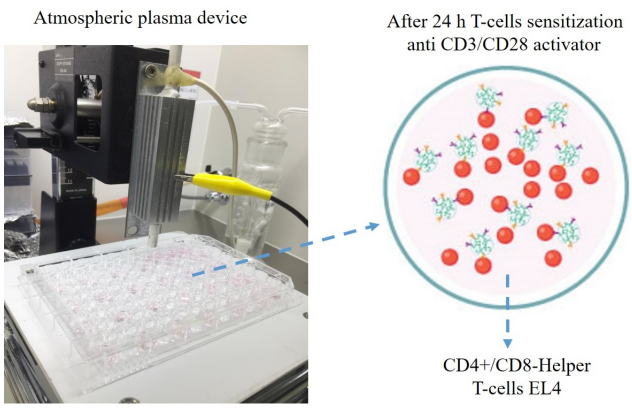

FIGURE 4.1: Plasma torch device and experimental setup

The material gas for plasma production is pure oxygen gas to obtain active oxygen species. The produced ozone and active oxygen species are blown off from the ceramic tube, then ozone concentrations as the indicator of the oxygen plasma production are measured using the gas detection

tube [2]. The plasma from the tube was applied to the cells in the 96-well microplate. The cells were kept at approximately 37°C during the plasma irradiation by controlling the material gas temperature.

4.3 Fundamental Characteristics of the Plasma and T-cells

4.3.1 Gas Temperature

Figure 4.2 shows the temperature of ambient air near the surface of the culture medium (volume: 0.2 ml) covering the T-cells in the microplate well. The temperature of the medium was approximately the same as that of the surrounding gas. The gas temperature can be controlled by varying the discharge voltage, material gas flow rate, discharge electrode structure, and distance from the discharge electrode. In this study, the discharge voltage was varied to achieve approximately proportionate changes in the gas temperature. As animal cells require temperatures approximately 37°C, the discharge voltage was maintained at less than 6.3 kV in all experiments .

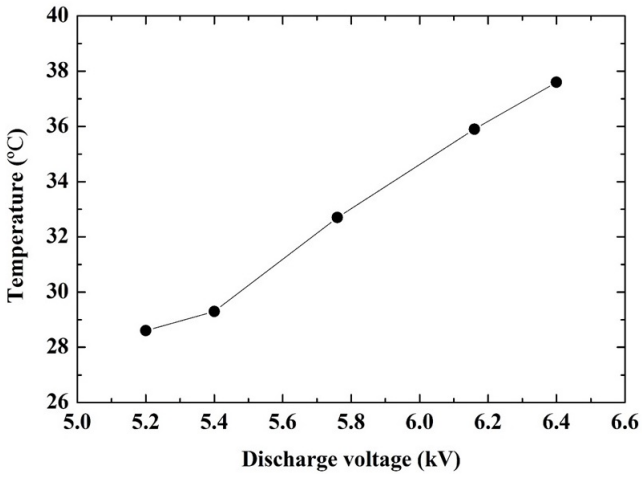

FIGURE 4.2: Temperature of ambient air as a function of the discharge voltage

4.3.2 Active Species in DBD Plasma

The concentration of gaseous ozone around the T-cells in the microplate well was measured as an index for the concentration of singlet atomic oxygen, $O(^1D)$, which dissociates from ozone and is considered the major particle responsible for T-cells activation. The ozone concentration in the vicinity of the T-cells was found to increase with the discharge voltage, as shown in Figure 4.3. As the discharge voltage was increased from 4.2 to 6.0 kV, the ozone concentration on the microplate well as a function of the discharge voltage increased from approximately 200 to 600 ppm, respectively.

The plasma irradiation period is changed from 0 (without irradiation) to 40 s. When the cell number decreases with the irradiation period longer than 40 s, the deviation of each cell number data point increased, and the significance of each data decreased. Although the reason or mechanism for the observed data deviation has not been specified, this data deviation could be due to the variation of responses of cells to the excess active oxygen in the culture medium.

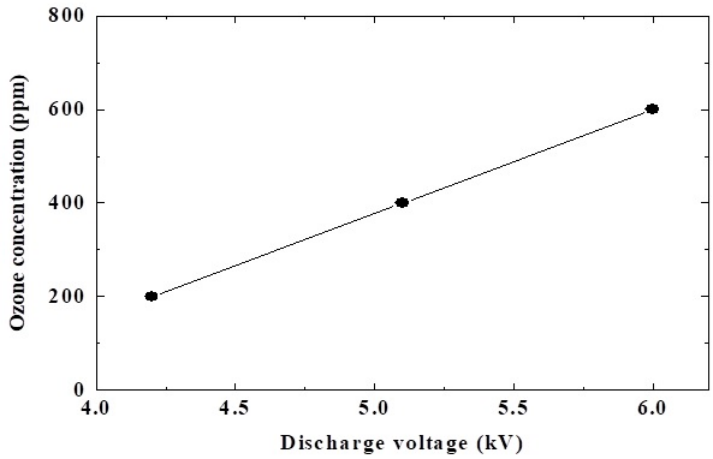

FIGURE 4.3: Ozone concentration on the microplate well as a function of the discharge voltage

In order to confirm the reactive oxygen species reacting with the cells, the typical emission spectrum of the oxygen plasma was obtained between the

ceramic torch tube edge and the culture medium surface in which ambient air is dissociated into atomic oxygen and nitrogen by high energy electrons from the discharge. In this region, ambient air is dissociated into atomic oxygen and nitrogen by high energy electrons from the discharge.

Figure 4.4 shows the typical light emission intensity of the oxygen plasma with the wavelength at 777.25 nm in different discharge voltages. The emission intensity from $O(^5P)$ was measured vertically to the axis of the ceramic tube just below the opening edge as shown in Figure 4.5. Also, active nitrogen species that do not appear on the spectrum are also generated in the gaseous region. Active oxygen $O(^5P)$ has enough energy for the dissociation of water at the surface of the culture medium, which generates OH radicals. The OH radicals then combine to form hydrogen peroxide (H_2O_2). Also, dissociated atomic oxygen and nitrogen are combined to NOx in the gas phase. OH radicals and NOx have enough energy to modify proteins such as enzymes and hormones in cells.

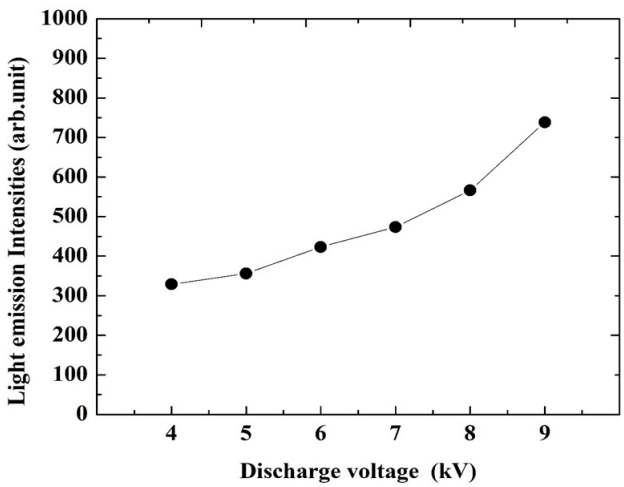

FIGURE 4.4: Light emission intensity of atomic oxygen $O(^5P)$ of each T-cells in different discharge voltages

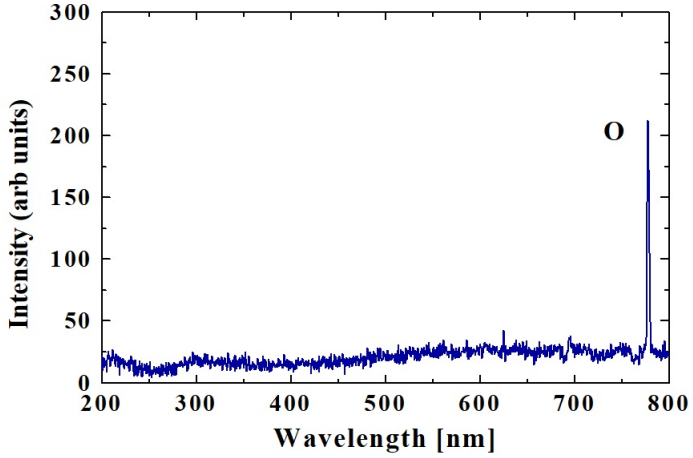

FIGURE 4.5: Typical spectrum of atomic oxygen O(^{5}P) of each T-cells in different discharge voltages

4.3.3 Enhancement of T-cells Proliferation

The effects of atmospheric oxygen plasma irradiation on the enhancement of T-cell proliferation were investigated as a function of the irradiation period from no irradiation (i.e., 0 second, T-cells without sensitization) to 40 s (i.e., T-cells sensitized by the anti-CD3/CD28). The activation of T-cells was evaluated in terms of the increase in the cell number. Figure 4.6 illustrates the cell number after irradiation for different periods from 0 to 40 s, followed by 24 h incubation.

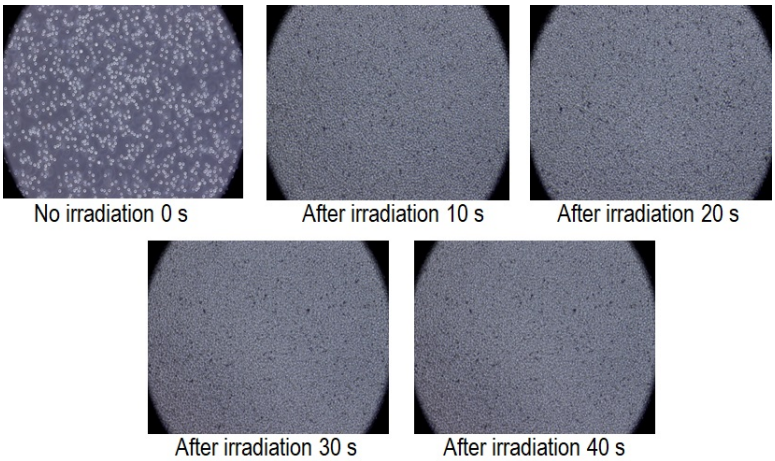

FIGURE 4.6: Enhancement of T-cells proliferation as a function of the irradiation period

Next, to examine the detailed enhancement of T-cell proliferation due to the effects of atmospheric oxygen plasma irradiation on the activation of T-cells were investigated as a function of the irradiation period from 0 (i.e., no irradiation) to 40 s. T-cells without antigen contact were irradiated with atmospheric oxygen plasma [71] [72] [73]. The activation of T-cells was evaluated in terms of the increase in the cell number.

Figure 4.7 shows the cell number after irradiation for different periods from 0 to 40 s, followed by 24 h incubation. The number of T-cells slightly increased only slightly (by less than 10%) due to the oxygen plasma irradiation. In a previous study, active oxygen species were shown to activate apoptosis-signal-regulating kinase 1 (ASK1), thus promoting cell proliferation [48] [74].

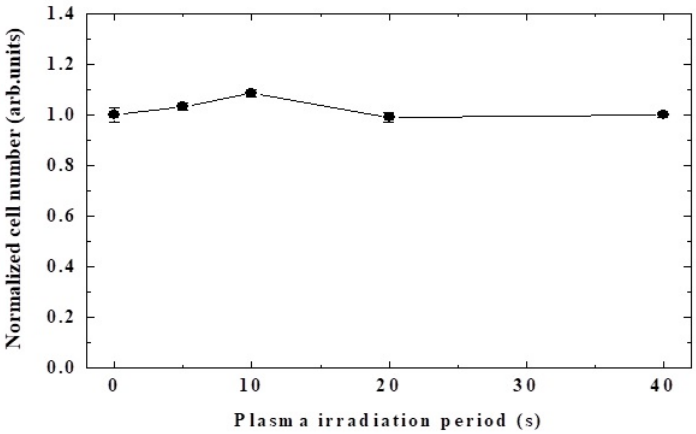

FIGURE 4.7: Number of T-cells after plasma irradiation and 24 hours of incubation as a function of the plasma irradiation period; the cell numbers were normalized to the cell number in control wells that were not exposed to plasma irradiation

In general, T-cells sensitization by cytokines emitted by other immune cells (e.g., macrophages and dendritic cells) causes the cells to enter a state that is appropriate for growth and division; thus, cell sensitization results in enhanced cell growth and division in response to a stimulus [75] [76]. Therefore, the sensitization was expected to enhance the T-cells proliferation due to oxygen plasma irradiation. The T-cells were sensitized by applying anti-CD3/CD28 before the plasma irradiation [77]. To confirm the enhancement

of T-cells proliferation by sensitization, the number of T-cells after 24 h incubation following sensitization by anti-CD3/CD28 was compared with that in the absence of sensitization.

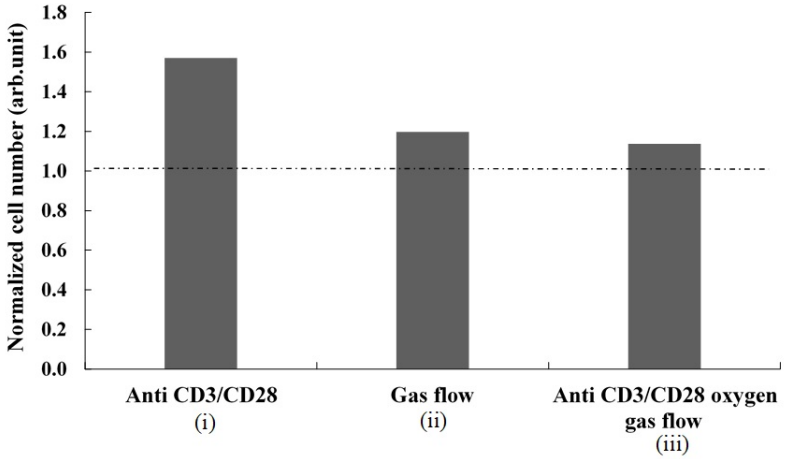

FIGURE 4.8: Number of T-cells after (i) sensitization with an anti-CD3/CD28 mixture, (ii) application of oxygen gas, and (iii) anti-CD3/CD28 sensitization in combination with oxygen gas; the cell numbers were normalized to the cell number in control wells that were not exposed to sensitization or oxygen gas flow

The result shown in Figure 4.8(i) indicates that anti-CD3/CD28 sensitization caused the number of T-cells to increase to approximately 1.57 times that without sensitization. To investigate the effect of the gas flow on the cells, raw oxygen gas without plasma was blown over the T-cells prior to the plasma irradiation. Following the exposure to oxygen gas, the cell number was 1.19 times that of the untreated cell population, as shown in Figure 4.8(ii). Exposure to oxygen gas without discharge was used as a control in this experiment. The oxygen gas flow was found to suppress the sensitization effect of anti-CD3/CD28 on the T-cells. The number of T-cells following anti-CD3/CD28 combined with oxygen gas flow was only 1.1 times that the untreated cell population [78] [79], as shown in Figure 4.8(iii)).

In addition, the sensitization of cells anti CD3/CD28 irradiation with plasma oxygen increased compared to irradiation with gas flow. In general, when the cell is significantly increased, the difference effect using plasma

oxygen plasma not always larger than gas flow, due to growth by excess active oxygen the above difference is deduced from the maximum growth. the effect of increasing by air plasma, this shows the above difference by gas flow which may be related to the content in gas stream. these results suggest that the growth-enhancing effect is due to the active species in the plasma. In this experiment the cells anti CD3/CD28 irradiation with oxygen plasma increase than those irradiation with gas flow.

It is confirmed experimentally used bubbly that the high-energy active oxygen species can be penetrated into the very thin liquid layer, which is covered by the very thin liquid layer, is discolored by the oxygen plasma irradiation [18]. Also, the removal of the culture media around the cells does not provide any significant influences on the cells. Because these cells are attributed to skin cells, they are durable in drying.

Thus, it was concluded that oxygen gas flow counteracts T-cells activation. The results shown in Figures 4.7 and 4.8 indicate that the T-cells activation effect of anti-CD3/CD28 exceeds that with the oxygen gas flow. Next, the number of T-cells after anti-CD3/CD28 sensitization and oxygen plasma irradiation are investigated with a discharge in the range of 4.2 to 6.0 kV

a. Number of T-cells with A Discharge Voltage of 4.2 kV

Figure 4.9 shows the number of T-cells after anti-CD3/CD28 sensitization and oxygen plasma irradiated with a discharge voltage of 4.2 kV for different periods in the range of 0–40 s after 24 h incubation; the cell numbers were normalized by that of the control population (T-cells without plasma irradiation). Plasma irradiation for 10 s was found to increase the cell number by approximately 1.4 times compared with that in the control population. The T-cells emitted active oxygen species, which inactivate microorganisms and abnormal cells, and provide the oxygen supply that T-cells require as they

proliferate. There was an excess of active oxygen when the irradiation period was 20 and 40 s.

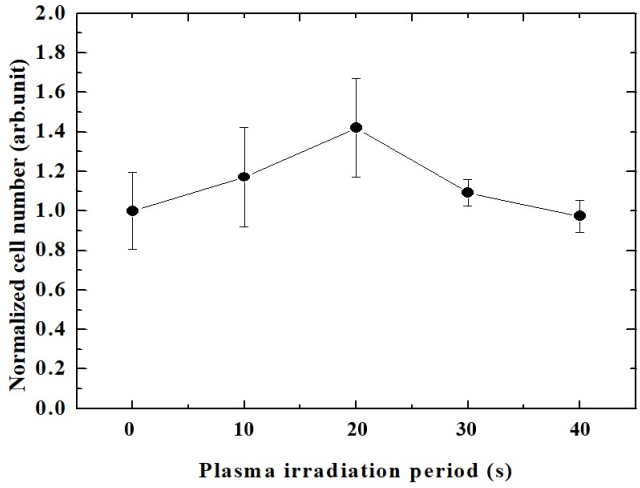

FIGURE 4.9: Normalized number of pre-activated T-cells as a function of the plasma irradiation period with a discharge voltage of 4.2 kV

b. Number of T-cells with A Discharge Voltage of 5.1 kV

Figure 4.10 shows the normalized number of T-cells when the discharge voltage was 5.1 kV and the irradiation period was varied from 0 to 40 s. The number of T-cells increased with the plasma irradiation period, indicating that the irradiation supplementation increased significantly and that T-cells growth depends strongly on the plasma irradiation period.

Furthermore, there is an optimum irradiation condition at which the level of active oxygen is ideal. The results show that the cell number reached approximately 5 times that of the control (untreated) T-cells when the irradiation period was 40 s. This effect was significantly higher than the effects of oxygen gas, oxygen plasma, or anti-CD3/CD28 alone. Student's t-test indicated that the difference between the cell numbers in the sample exposed to plasma irradiation and the control was statistically significant with ($p < 0.05$). However, there were no statistically significant differences in the cell

numbers following plasma irradiation for 10, 20 and 30 s compared with the control. These results suggest that the number of T-cells can be increased more effectively by combining the sensitization treatment and the plasma irradiation.

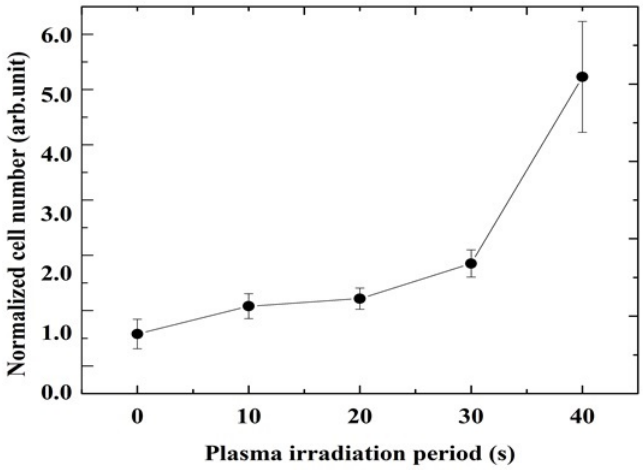

FIGURE 4.10: Normalized number of pre-activated T-cells as a function of the plasma irradiation period with a discharge voltage of 5.1 kV

c. Number of T-cells with A Discharge Voltage of 6.0 kV

Figure 4.11 shows the number of T-cells after 24 h incubation following plasma irradiation with a discharge voltage of 6.0 kV. The cell number decreased compared with the control without plasma irradiation for all irradiation periods. This result contrasts with the finding that the growth rate increased in response to plasma irradiation with discharge voltages of 4.2 and 5.1 kV compared with the control. When the discharge voltage was 6.0 kV, the ozone concentration was 600 ppm, which would indicate to an excess amount of oxygen species, even with a short irradiation period of 10 s. Therefore, cell proliferation was restricted under these conditions due to increasing levels of active oxygen density associated with higher discharge voltages and longer irradiation period.

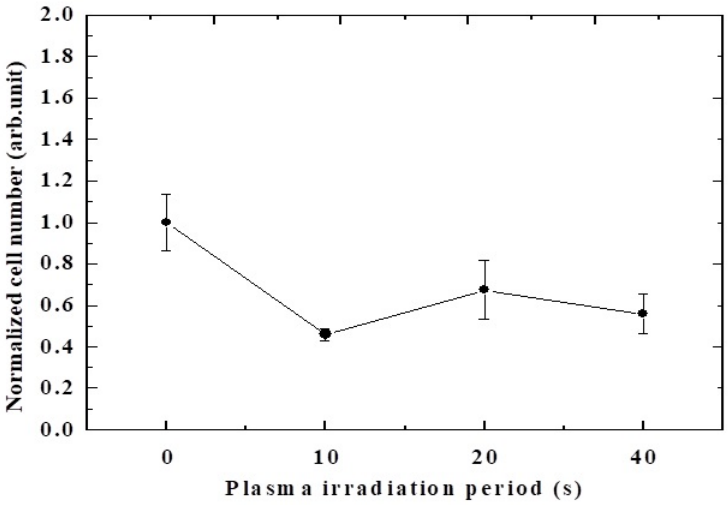

FIGURE 4.11: Normalized number of pre-activated T-cells as a function of the plasma irradiation period with a discharge voltage of 6.0 kV

The ozone and active oxygen species (ROS) cause unintended interactions with the components of the culture medium, such as lipid peroxidation, releasing by-products that negatively impact cell proliferation. Although there is some scatter in the T-cells number when varying the discharge period, the cell number after the plasma irradiation with 6.0 kV decreases from the control value. In this case, the active oxygen species phosphorylate the JNK and p38 involved in the MAPK cascade, leading to cell apoptosis [78] [79] [80] [81].

In addition, the plasma irradiation effects were further evaluated in terms of the CT (concentration over time) value of the gaseous ozone around the surface of the culture medium. The CT value of the ozone is considered to be proportional to the ozone dose amount injected into the culture medium. Although the CT value associated with plasma irradiation at 5.1 kV for 40 s is approximately equal to that associated with irradiation at 6.0 kV for 20 s (about 270), the proliferation rates associated with these two irradiation conditions differed significantly (the cell numbers were 5.28 and 0.67 times that of the control, respectively). While there was a clear relationship between

the cell number and the treatment period when the discharge voltage was 5.1 kV, there was no such correlation with discharge voltages of 4.2 and 6.0 kV. These facts indicate that the cell number cannot be controlled by the ozone CT value.

Overall, there are other factors influencing the T-cells activation aside from the density of atomic oxygen dissociated from ozone, which depends on the duration of the ozone generation associated with the plasma discharge. For example, different discharge voltages may produce different active oxygen species, and chemically active species such as OH radical tend to cause damage to the cells.

4.3.4 IFN-gamma (IFN-γ) Production

IFN-γ is a cytokine produced by activated T-cells and plays a role in pathways that inactivate pathogens; thus, in this study, it was used as a measure of T-cells activation due to the plasma irradiation with anti-CD3/CD28 sensitization [21] [69]. The IFN-γ amount was measured using ELISA method after 24 h incubation following the plasma irradiation. The total amount of IFN-γ in each microplate well was normalized to the cell number under each respective condition. Figure 4.12 shows the amount of IFN-γ in one cell when varying the plasma irradiation period and the discharge voltage. The active oxygen species generated in the oxygen plasma influence the production of IFN-γ. The total amount of IFN-γ increased with the plasma irradiation with the discharge voltages of 4.2 and 6.0 kV compared with the control. However, the IFN-γ amount in Figure 4.12 indicates negative correlation with the T-cells proliferation in Figures 4.9 to 4.11. For example, with a discharge voltage of 6.0 kV, the IFN-γ level increased with the plasma irradiation period but the cell proliferation did not change significantly.

In addition, with a discharge voltage of 5.1 kV, the amount of IFN-γ was

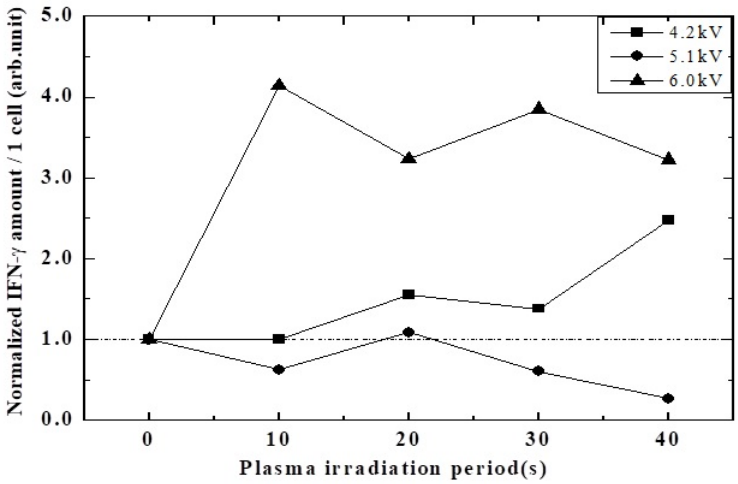

FIGURE 4.12: Normalized IFN-γ amount/one cell as a function of the plasma irradiation period with discharge voltages of 4.2, 5.1, and 6.0 kV

lower than that in the control. The IFN-γ amount in Figure 4.12 was measured after 14 h from the plasma irradiation, which is according to the protocol of the INF-γ measurement reagent used in this experiment. With the 5-fold increase in T-cells, it may take several days for T cells to produce IFN-γ. These results suggest that T-cells proliferation and IFN-γ production would occur via different pathways. Therefore, sensitized T-cells are activated by oxygen plasma irradiation, but the effects on T-cells vary significantly depending on the discharge voltage.

4.3.5 Gene Expression of T-cells

When an appropriate amount of the oxygen plasma is applied to T-cells, the thioredoxin on the ASK1 in the T-cells is considered to be oxidized [82] [83], initiating a cascade leading to the activation of MAPKs such as ERK, JNK, and p38. When ERK, a cell growth signaling factor, is phosphorylated, the TCA circuit is promoted and CDC2 cyclin is activated, which accelerates the cell cycle. Active oxygen species have the capability to upregulate the transcription of genes coding for enzymes that catalyze rate-controlling reactions

in the TCA circuit or the cell cycle. The influence of plasma irradiation on the cell cycle would require several tens of hours.

In order to investigate the characteristics of the cell growth and the immune function enhancement caused by the plasma irradiation, comprehensive variations of the gene expressions in T-cells were analyzed using the microarray method after 24 hour from the plasma irradiation. A gene annotation table of expressed genes obtained from the list of expressed genes with $p < 0.05$ shows that genes associated with the cell growth factor, MAPK that is the protein kinases specific to the amino acids serine and threonine family, ERK, and cyclin enzyme for the cell cycle were differentially expressed by the plasma irradiation to the sensitized T-cells with a discharge voltage of 5.1 kV and irradiation period of 40 s. At the same time, the JNK is one of the major signaling cassettes of the MAPK signaling pathway, p38 is a class of MAPK that is responsive to stress stimuli, such as cytokines, ultraviolet irradiation, heat shock, and osmotic shock. Also, JNK is involved in genes that code for proteins in cells that underwent, apoptosis in response to oxygen plasma irradiation. However, the expression levels are lower than that of the ERK.

To clarify the production characteristics of the IFN-γ produced by the plasma irradiated on T-cells, expressions of genes concerning the interferon are observed. Table 4.1 shows indexes of gene expression amount, Z-score and gene expression ratio, which relate to the interferon productions with different discharge voltages.

TABLE 4.1: Gene expressions of T-cells in the case of the discharge voltages of 4.2 kV(a), 5.1 kV(b), and 6.0 kV(c).

(a)	Gene expression	Z-score	Ratio
	IRF4	2.31	2.21
	IRF8	-2.06	0.62
(b)	Gene expression	Z-score	Ratio
	IRF4	3.38	5.59
	IFNG	2.76	9.93
	IRF4	2.36	7.13
(c)	Gene expression	Z-score	Ratio
	IRF4	2.66	4.96
	IFI213	2.11	3.55

IRF4 is a kind of gene that encodes a transcription factor protein with functions as a regulator of interferons and interferon-inducible genes, and IRF8 (Interferon regulatory factor 8). This is specifically binds to the upstream regulatory region of type interferon regulatory factor l (IRF l) and IFN-γ inducible MHC class I genes, and play a negative regulatory role in cells of the immune system. IFI213 gene codes enzymes catalyzing the Interferon regulatory factors (IRFs), which play important roles in the immune response, including immune cell development, differentiation and responses to pathogens [76] [77].

Table 4.1(a) shows that, in the case of the discharge voltage 4.2 kV, the annotation table of expressed genes shows significant expressions of IRF4 and IRF8 genes. Increase of the gene expression amount in IRF4 and decrease of the amount in IRF8 contribute to the increase of the IFN-γ production, as shown in Figure 4.12, where the cell number gradually increase by the oxygen plasma irradiation. Table 4.1(b) shows that the expressions of the IFNG gene and two genes with same IRF4 annotation are upregulated in

cells those suffer the plasma irradiation with 5.1 kV. Both IFNG and IRF4 genes enhance the Interferon productions. Each Z-score is enough high for the expressions. At 5.1 kV, the cell number increases significantly with the plasma irradiation period, and then the cell doubling time becomes short. Then, the preparation period of the IFN-γ in cells decreases with increase of the plasma irradiation period.

Therefore, the production amount of the IFN-γ in one cell would decrease from the control level, as shown in Figure 4.12. The gene expression amounts of IFNG and IRF4 would increase to compensate the decreased IFN-γ amount to the control level. Gene expression variations of IFN-γ have not been observed on the table at the discharge voltage of 4.2 and 6.0 kV. In the case of the discharge voltage 6.0 kV, as shown in Table 4.1(c), the gene expression shows there is IRF4 gene coding enzyme catalyzing the Interferon production. Also, the gene expression IFI213 gene that increase the interferon production. The expression of above genes would be increased by the oxygen plasma irradiation.

These results imply that the IFN-γ production in T-cells with increasing the number due to oxygen plasma irradiation can be balanced out by means of altered gene expression [52] [84].

4.4 Summary

In this study, the effects of atmospheric oxygen plasma irradiation on the increase in the number of T-cells were investigated with different periods of the irradiation. The total amount of IFN-γ in cells increased after plasma irradiation under most conditions due to the active oxygen species generated in the oxygen plasma. However, while the IFN-γ levels were uncorrelated with T-cells proliferation, the effects on T-cells vary significantly depending on the discharge voltage.

Also, the moderate amount of active oxygen species in the plasma in combination with sensitization by an anti-CD3/CD28 was shown to significantly enhance T-cells proliferation. The oxygen plasma irradiation can be used to control IFN-γ production. Gene expression analysis indicated that MAPK related ERK genes and IFNG genes are upregulated when oxygen plasma irradiates the T-cells.

Chapter 5

Growth Comparison of Two Subset of T Lymphocytes

5.1 Introduction

Most morbidity and mortality from cerebral malaria are caused by infection with Plasmodium falciparum [4] [5]. Cerebral malaria is a life-threatening complication of the disease by the parasite Plasmodium falciparum; this complication is characterized by parasite-infected red blood cells accumulating in the brain [6] that induces an immune response during the inflammation, such as T lymphocytes (antibody-helpers) [3] [6].

Several previous studies reported, there is substantial evidence that cytotoxic lymphocytes can recognize and destroy intrahepatic parasites. More importantly, the secretion of IFN-(γ) by CD4 and CD8+ T cells has been associated with protective immune responses. In addition, antibody-helper CD4/CD8+ T-cells reactive to Plasmodium infection in red blood cells indirectly stimulate immune cell proliferation and possibly increase IFN-(γ) those may orchestrate secreted antibody responses or induce the engulfing and absorbing of pathogen[4] [45] [46]. Thus, raising the production of the immune system is necessary to control malaria infection.

On the other hand, the success of any novel immune system treatment for

malaria disease depends on its ability to rapidly and selectively target T-cells while minimizing damage to healthy cells. Therefore, it is necessary to examine the activation of antibody-helper CD4+ T lymphocytes, i.e., T helper 1 (Th1) and T helper 2 (Th2) cells, that appear sequentially during primary infection. Th1 cell produces IFN-γ that plays a role in controlling acute infection, and while Th2 cell produces IL-4 as a humoral immune response, which assists in the maturation of B cells into blood cells. However, it takes about three weeks to obtain the desired cell number at this proliferation rate; the immune cells can be defeated by the rapidly increasing parasites when using currently available techniques [52].

Therefore, this study conducted an advanced experiment to investigate the activation of two subsets of T lymphocytes (T-cells), i.e., EL4 T-cells and BW5147. T-cells sensitized with antibodies against CD3/CD28 (activator-CD3/CD28) are irradiated with atmospheric oxygen plasma, and then the respective and combined effects of sensitization and plasma irradiation on cell activation are evaluated.

5.2 Experimental Setup

As describes early in Chapter 3 in Section 3.2, the atmospheric plasma was also generated by cylindrical-type dielectric barrier discharge (DBD). The discharge electrode, a stainless-steel mesh, was set inside a ceramic tube with an inner diameter of 4 mm, and a copper thin film as the grounded electrode was wound around the outer wall of the ceramic tube. Pure oxygen gas was supplied through the ceramics tube as the material gas at a fixed flow rate of 0.6 L/m, and a high voltage 4.2 kV was applied to the mesh electrode to cause DBD around the discharge electrode on the inner surface of the ceramic tube. The ozone and active oxygen species generated by this process were blown out of the ceramic tube with a distance between the discharge

electrode and cells on the microplate well bottom of 12 mm. A gas detection tube was used to measure the ozone concentration as an indicator of oxygen plasma production [3] [2]. Active species ejected from the tube were applied to the cells in a microplate with a culture medium volume of 0.2 ml. During the plasma irradiation, temperature of cells were kept at less than or equal to 37°C. Cells require this range of the temperature, and the discharge voltage was maintained at less than 6.3 kV in all experiments. Active species in DBD Plasma, i.e., gas temperature, ozone concentration, and light emission intensity of atomic oxygen as a function of the discharge voltage, have been described early in Chapter 4 in Section 4.3.2.

In order to examine the optimum conditions in the differentiation, growth enhancement, and activation effects, two subsets of T lymphocytes (T-cells), i.e., naïve T-cells (BW5147) and EL4 T-cells, were used. BW5147 T-cells are a kind of naïve cell, and EL4 T-cells obtained from the lymph are differentiated helper T-cells. Before plasma irradiation, these two cell cultures were sensitized in advance by anti-CD3/CD28 antibodies (Dynabeads™ Mouse T-Activator anti CD3/CD28) [60].

For the practical use of this method, T-cells were extracted from the the thymus and lymph and had already been sensitized spontaneously. To induce precise responses in cells in suspension, EL4 T-cells were cultured in Roswell Park Memorial Institute (RPMI) and BW5147 T-cells in Dulbecco's Modified Eagles Medium (DMEM). For each T-cells type subcultured, the population was kept constant at 2.0×10^5 cells per well at the beginning of the experiment. After the irradiation process, the cells were cultured in a CO_2 incubator at 37°C for 24 hours, and then the cell number was quantified using a cell counting kit (WST-8). For every measurement, 4 wells of the micro-well plate to obtain average values were used for the same parameters to confirm the repeatability of each experiment.

Next, the amount of IFN-γ produced by the two types of T-cells was measured using a sandwich enzyme-linked immunosorbent assay (ELISA) assay.

5.3 Enhancement of T-cells Proliferation

The role of atmospheric oxygen plasma in maintaining and enhancing antibody production was examined using two subsets of T lymphocytes (T-cells), i.e., naïve T-cells (BW5147) and EL4 T-cells. Before these T-cells were irradiated with the plasma, the T-cells were sensitized using anti-CD3 and anti-CD28 antibodies during cultivation for 24 hours. This technique activates the T-cells and increases the number of CD3/CD28 receptors on the T-cells surface, and has been proven to cause the spontaneous increase in the number of T-cells without external inducement [2] [3] [7] [71].

Therefore, first, it was evaluated each of two subsets T-cells' growth and differentiation characteristics and next compare the IFN-gamma (IFN-γ) production of each of two subsets T-cells.

5.3.1 Growth and Differentiation Characteristics of T-cells

To evaluate T-cells activation in terms of the increase in the cell number due to atmospheric oxygen plasma irradiation described above, two types of T-cells were irradiated with plasma for a period from 0 (i.e., no irradiation) to 50 s. Figure 5.1 shows the enhancement characteristics of the cell number after irradiation for each time period.

First, the number of EL4 T-cells sensitized with anti-CD3/CD28 increased with the irradiation period rapidly from 0 (control/no irradiation) to 50 s. This increase can be seen in the upward movement for irradiation times of 10 and 20 s, and then the cell number slightly decreased (by less than 10%) with irradiation times of 30 and 40 s. After 20 s, the cell number was almost saturated. Meanwhile, BW5147 T-cells sensitized with anti-CD3/CD28 also

showed an increase from 0 (control/no irradiation) to 50 s of irradiation, i.e., for irradiation times of 10 s, 20 s, 30 s, and 50 s, then also slightly decreased at an irradiation time of 40 s. BW5147 T-cells increased almost constantly with the irradiation period even though there was some fluctuation.

This result indicates that active oxygen species activated apoptosis-signal-regulating kinase 1 (ASK1) and then promoted cell proliferation, as in our previous study [3]. Also, this results indicated that the irradiation supplementation increase depends strongly on the plasma irradiation period, where there is an optimum irradiation time at which the level of active oxygen is ideal.

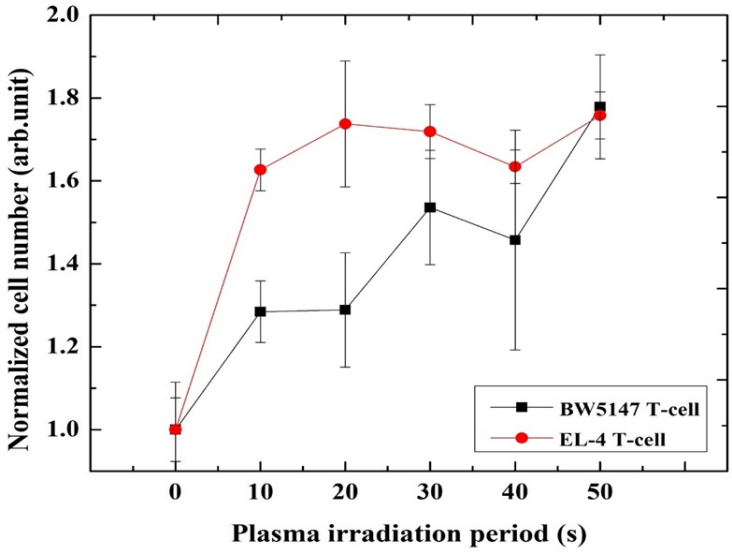

FIGURE 5.1: Normalized number of pre-activated each T-cells as a function of the plasma irradiation period 4.2 kV

As shown in Figure 5.1 for both T-cells (EL4 and BW5147), the cell number reached approximately 1.8 times that of control (0 irradiation) T-cells when the irradiation period was 50 s. This clarified that the effect of oxygen plasma irradiation with cell sensitization is significantly higher than the impact of individual treatments of oxygen gas, oxygen plasma, or anti-CD3/CD28 alone. According to a comparison of the cell numbers using a t-test, the difference in numbers between plasma-irradiated cells and control cells was statistically

significant ($p < 0.05$) for both types of T-cells. Overall, these results proved that the number of T-cells could be increased more effectively using plasma irradiation and the sensitization treatments.

5.3.2 Comparison of IFN-gamma (IFN-γ) Production

CD4+ T-cells are a crucial mediator for protection and immunity against intracellular pathogens. CD4+ T-cells have distinctive cytokine production patterns and functional capacity; Th1 cells produce IFN-γ as an essential mediator of host immunity against intracellular pathogens, while Th2 cells produce the IL-4 cytokine, which mediates immunity toward extracellular pathogens and collaboration with B cells for antibody production [85] [86]. IFN-γ activates macrophages to enhance their phagocytic function and stimulate the production of reactive oxygen species that are toxic to Plasmodium [47]. The characteristics of IFN-γ production by EL4 and BW5147 T-cells were compared in those irradiated with the atmospheric oxygen plasma.

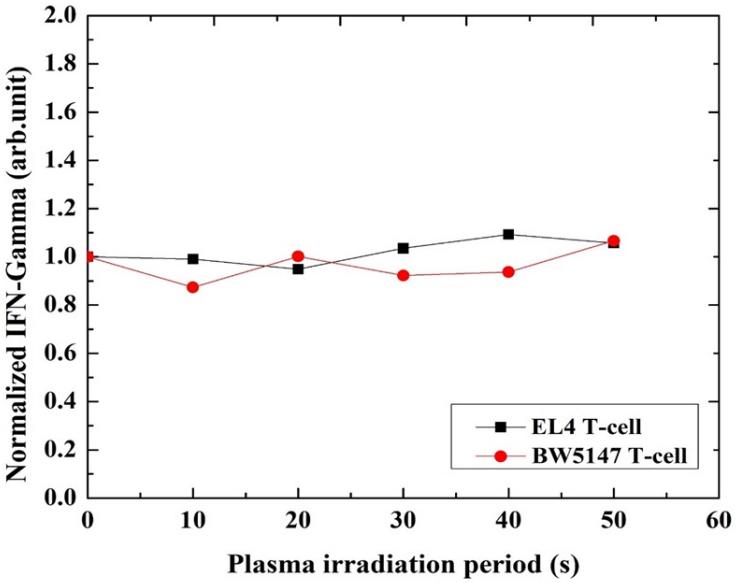

FIGURE 5.2: Normalized IFN-γ of each T-cells as a function of the plasma irradiation period

As discussed early in Chapter 3 in Section 3.5, the production of IFN-γ for both types of T-cells was measured using ELISA after a 24 hours incubation after plasma irradiation. The total amount of IFN-γ in each microplate was normalized to the cell number under each respective condition.

Figure 5.2 shows the amounts of IFN-γ of the two types of T-cells, and that varying the plasma irradiation period influenced the production of IFN-γ due to the impact the active oxygen species generated in the oxygen plasma. The production of IFN-γ by BW5147 T-cells after oxygen plasma irradiation was almost at the same level as that without plasma irradiation (control).

Although IFN-γ showed small increases after 20 s and 50 s of irradiation, overall, IFN-γ did not significantly increase conditions compared with the control. BW5147 T-cells are extracted from the thymus and are either undifferentiated naïve cells or only differentiated into CD4+/CD8- single-positive naïve cells. Each receptor that appears on the surface of the cell is underdeveloped, so active oxygen species from plasma have difficulty interacting with BW5147 T-cells. Therefore, IFN-γ in the cells did not increase for all irradiation periods of oxygen plasma compared with the control.

EL4 T-cells are a kind of helper T-cells obtained from lymph nodes and have already been differentiated into single-positive CD4/CD8+ T-cells from double-negative cells. Receptors on the surface of the T-cells have been developed and so active oxygen species from the oxygen plasma can react with the receptors, as shown in Figure 5.3. However, EL4 T-cells did not show enhancement of the IFN-γ production when the plasma irradiation period changed with the ozone concentration of approximately 200 ppm. IFN-γ production in T-cells was not affected by the irradiation period of active oxygen species, even though the T-cells were sensitized and differentiated into helper T-cells. Therefore, oxygen plasma does not enhance the differentiation of EL4 T-cells into Th1 cells. There is a possibility of differentiation into Th2 cells after irradiation with the oxygen plasma for several tens of seconds. Due

to the oxygen plasma irradiation increased the EL4 T-cells number without influencing the IFN-γ production.

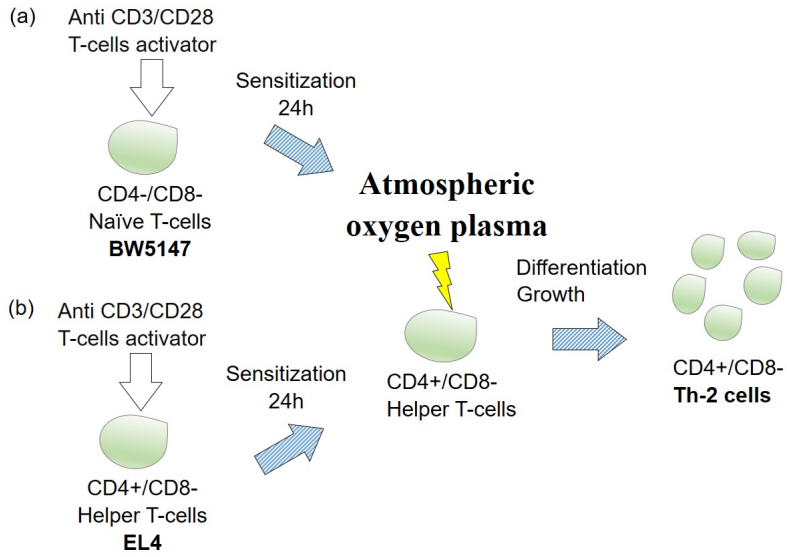

FIGURE 5.3: Sequences of the plasma irradiation and sensitization for the (a) BW5147 T-cells and (b) EL4 T-cells

From the above results, the differentiation sequences of different types of T-cells exposed to oxygen plasma irradiation are estimated, as shown in Figures 5.3(a) and (b). Figure 5.3(a) shows the plasma irradiation and sensitization sequences for BW5147 T-cells with antibodies against CD3/CD28 (anti-CD3/CD28) that would have been differentiated into single-positive CD4+/CD8- cells. When the oxygen plasma irradiates the cells, the cell number changes, and BW5147 T-cells would differentiate into Th2 cells. The EL4 T-cells those are extracted from the thymus have received some antigens and have already been differentiated into CD4+/CD8- helper T cell in the thymus. Figure 5.3(b) shows that EL4 T-cells are sensitized using antibodies against CD3/CD28 (anti-CD3/CD28). When the EL4 T-cells are irradiated with the oxygen plasma with the same parameter as the irradiation for BW5147, the T cell would differentiate into the Th-2 cell, because the significant increase of the IFN-γ has not been observed.

Overall, our results have proven that both the BW5147 and the EL4 T-cells differentiated into the Th-2 category, which produces IL-4 cytokine for the B cell activation, when the cells are irradiated with the oxygen plasma with the discharge voltage of 4.2 kV and the oxygen gas flow rate of 0.6 L/min.

5.4 Summary

The effects of atmospheric oxygen plasma irradiation on the two types of T-cells were investigated with the same irradiation parameter. The two types of T-cells, the BW5147 and the EL4, which are sensitized by the anti CD3/CD28, are irradiated with the oxygen plasma with the condition where the both T cell grow effectively due to active oxygen species in the plasma. The active oxygen species from plasma have difficulty interacting with BW5147 T-cells, thus causing the IFN-γ levels not to increase significantly for any of the irradiation conditions.

Meanwhile, for EL4 T-cells, the IFN-γ levels significantly increased after plasma irradiation for all of the irradiation conditions. Likewise, oxygen plasma irradiation in EL4 T-cells can be used to control IFN-γ production, and gene expression analysis indicated that MAPK related ERK genes and IFNG genes were upregulated when oxygen plasma was used to irradiate the T-cells.

Chapter 6

Conclusion and Future Work

6.1 Conclusion

First, the activation of T-cells (EL4 T-cells) was examined when they were sensitized with monoclonal antibodies against CD3/CD28, followed by irradiation with atmospheric oxygen plasma. Then, the proliferation of EL4 T-cells was evaluated by varying the discharge voltage and varying time period irradiation (10 s interval) as a function of the duration of oxygen plasma irradiation. Results obtained that plasma irradiation significantly promoted cell proliferation; when plasma irradiation was applied to sensitized T-cells, the cell number increased compared with cells that were not exposed to irradiation. Likewise, oxygen plasma irradiation in EL4 T-cells can be used to control IFN-γ production, and gene expression analysis indicated that MAPK related ERK genes and IFNG genes were upregulated when oxygen plasma was used to irradiate the T-cells.

Next, an advanced experiment by comparing the growth enhancement of two subsets of T lymphocytes (T-cells) naïve T-cells(BW5147) and EL4 T-cells sensitized with anti-CD3/CD28 (activator-CD3/CD28) then irradiated with atmospheric oxygen plasma. The effects of atmospheric oxygen plasma irradiation on the two types of T-cells grow effectively due to active oxygen species in the plasma. In addition, the IFN-γ levels significantly increased on the EL4 T-cells after plasma irradiation for all of the irradiation conditions.

Meanwhile, for the naïve T-cells(BW5147), The active oxygen species from plasma have difficulty interacting with BW5147 T-cells, thus causing the IFN-γ levels not to increase significantly for any of the irradiation conditions.

It was concluded that two subsets of T lymphocytes, i.e., EL4 and BW5147 T-cells differentiate into the Th2 category, which produces IL-4 as the major cytokine irradiated with atmospheric oxygen plasma significantly increased. It will be promoted B-cell activation and modulate macrophage responses to Plasmodium infection like malaria diseases.

6.2 Recommendations for Future Research

One of the principally potential of the atmospheric pressure plasma on the cell growth enhancement is the particle delivery of active oxygen species (ROS) and the corresponding ability to access even micron-sized regions. The beneficial use of oxygen as active agents is the combination of charging by electrons and varying time period irradiation, leading to increased permeability of cell growth for immune therapy.

Therefore, further building on this study, and future research should focus on the specific production of ROS into cells leading to the various abundances and combinations of particles generated by atmospheric pressure plasma. In addition, the possibility of conducting the multiple mechanisms and parameters for the investigation of efficacy the atmospheric pressure plasma to various types of T-cell.

Appendix A

Publish Work

A.1 Domestic and International Conference Proceedings

- Activation and growth of EL-4 T-cell Using Atmospheric Oxygen Plasma. Sitti Subaedah, Haruka Uematsu, and Nobuya Hayashi. The 12th International Workshop on Plasma Application and Hybrid Functionally Materials (IAPS 2019).

- Growth Characteristics of EL-4 T-cell with the activator CD3/CD28 Using Atmospheric Oxygen Plasma for Malaria Disease. Sitti Subaedah, Haruka Uematsu, and Nobuya Hayashi. The 41st International Symposium on Dry Process (DPS2019).

- Impact of Reactive Oxygen Species (ROS) in the Cell Using the Plasma Irradiation Sitti Subaedah, Haruka Uematsu, and Nobuya Hayashi. The Japan Society of Applied Physics (JSAP), The 81st Autumn Meeting 2020

- Enhanchement of Growth and Cytokine Production of EL-4 T-cell by Irradiation the Athmosheric Oxygen Plasma. Sitti Subaedah, Haruka Uematsu, and Nobuya Hayashi. 22nd International Conference of Cross

Straits Symposium on Energy and Environmental Science and Technology (CSS-EEST2020)

- Activation of T Lymphocyte (BW5147) by Using Athmospheric Oxygen Plasma. Sitti Subaedah, Haruka Uematsu, and Nobuya Hayashi. The 30th Materials Research Society of Japan (MRS-J) Annual Meeting.

- EL-4 and T-lymphocyte (BW5147) Regulatory T-cells are Critical in Immune Response of Cerebral Malaria. 13th International Symposium on Advanced Plasma Science and Its Application for Nitrides and Nanomaterial, 14th International Conference on Plasma-Nano Technology and Science (ISPlasma2021/IC-Plants2021). Sitti Subaedah, Haruka Uematsu, and Nobuya Hayashi.

A.2 International Journal

- Activation of EL-4 T-cells By Irradiation with Atmospheric Oxygen Plasma. Sitti Subaedah, Haruka Uematsu, and Nobuya Hayashi. Japanese Journal of Applied Physics 59.SJ (2020): SJJF03.

- Growth Comparison Between Lymphocyte BW5147 T-cells and EL4 T-cells Using Atmospheric Oxygen Plasma Irradiation. Sitti Subaedah, Haruka Uematsu, and Nobuya Hayashi. Plasma Medicine Journal.

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