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IN VITRO BOOSTING AND EXPANSION OF TUMOR INFILTRATING KILLER T CELLS

A thesis
Presented to
the Graduate School of
Clemson University

In Partial Fulfillment of the
Requirements for the Degree
Master of Science
Microbiology

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by
Chunlei Mei
November 2010

Accepted by
Dr. Yanzhang Wei, Committee Chair
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Dr. T. R. Jeremy Tzeng

ABSTRACT

Immune cell tumor infiltration represents one of the mechanisms that the immune system responds to tumor cells. The tumor infiltrated immune cells include lymphocytes (TIL), dendritic cells (DCs), macrophages, and natural killer (NK) cells. Although TILs have been extensively studied in order to develop adoptive transfer based immunotherapies, how to effectively isolate, culture, and *in vitro* boosting the killer cells from TILs remains a challenge. Meanwhile, OK-432, a heat and penicillin-treated lyophilized preparation of the low-virulence strain of *Streptococcus pyogenes*, has been shown to exhibit immunomodulatory activities and potential antitumor therapeutic function, including the activation of DCs, neutrophils, macrophages, lymphocytes, and NK cells as well as the induction of multiple cytokines production, such as IL-1, IL-2, IL-6, TNF-a, IFN-y, and IL-12. This research aims to establish an *in vitro* culture system, in which highly cytotoxic and tumor cells specific CTLs can be expanded, using OK-432, IL-2 and other cells and/or cytokines. TILs were extracted from experimental mouse melanoma B16F0 tumors grown on mice using two different methods. The isolated TILs were then cultured *in vitro* in four different culture conditions: 1) low dose IL-2, 2) low dose IL-2 + OK-432, 3) low dose IL-2 + DCs, and 4) low dose IL-2 + OK-432 + DCs. The cell count, phenotype, and cytotoxicity of these cultured TILs were evaluated using techniques including fluorescent activated cell sorting (FACS) and cytotoxicity assay. The results demonstrated that the additions of OK-432 and DCs into the culture system increased the

cell growth rate and the percentages of CD8+ cells in some culture conditions. More importantly, the addition of OK-432 and DCs into the culture system significantly increased the cytotoxicity of 14-day cultured TILs from 18% (low dose IL-2 alone), to 44% (low dose IL-2 + OK-432), 40% (low dose IL-2 + DC), or 73% (low dose IL-2 + OK-432 + DC). Therefore, this research provides an improved *in vitro* TIL culture system that effectively enhances the cytotoxicity of the killer cells.

Key words: Melanoma, TIL, Immunotherapy, IL-2, OK-432, CTL, DC

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1. INTRODUCTION

1.1 Overview of melanoma

Malignant melanoma mainly occurs in skin but may also arise in the bowel and the eyes (Figure 1). Although it is not the most common type of skin cancer, it causes the majority of skin cancer-related deaths. Melanoma incidence and related death have been steadily increasing in the U. S. in recent years. The lifetime risk estimate has reached 1 in 75 in the U. S.[1, 2] While surgical excision is effective when primary tumors are thin, at least one third of patients with early stage disease will develop metastases. Patients with metastatic melanoma have a mean survival rate of six to eight months, with only 5% surviving more than year five.[3] Therefore, great efforts have been made to improve treatments for metastatic melanoma patients. Many agents have been investigated for antitumor activity in melanoma, but few have shown the response rate that is more than 10%. Among these new developments, immunotherapy has occupied a prominent place. In some studies significant tumor-specific immune responses were measured. [1, 3, 4]

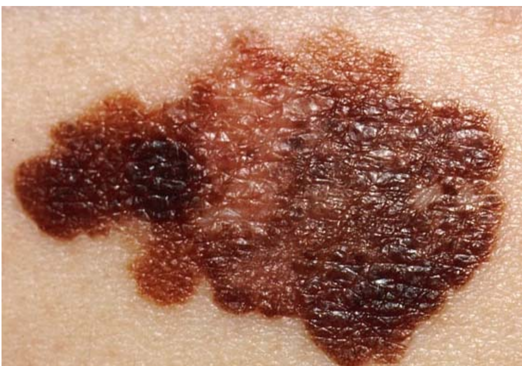

Figure 1. Melanoma develops in different cities, but mainly on the skin [5]

1.2 Tumor Infiltrating Lymphocytes (TILs)

Many researchers believe that TILs represent the immune response to melanoma cells and other tumor cells. This response is usually measured by the level of lymphocytic infiltratation present at the base of the vertical growth phase of the tumor and is sometimes categorized as brisk, nonbrisk, or absent. The most brisk tumor-infiltrating lymphocytic response is always found in thin tumors. [6, 7] TILs are mainly composed of T lymphocytes as well as other subsets including: dendritic cells, macrophages, natural killer (NK) cells, and B lymphocytes. T lymphocytes of TILs are mainly comprised of varying numbers of CD4+ helper and/or CD8+ cytotoxic T cells (CTL). [8, 9] TILs can play a key role in antitumor immune responses in the host by enhancing T lymphocyte cytotoxicity and inducing apoptosis in tumor cells. CD8+ TILs function in a tumor-specific manner, while CD8+ T cells recognize antigens including tumor antigens and tumor associated antigens presented by MHC class I molecules. After recognition and activation, CTLs directly kill tumor cells by injecting lytic granules containing preforin and granzymes into target cells. They also deliver Fas ligand mediated death signals into tumor cells (Figure 2). Besides the direct killing, CTLs also release cytokinges that will destroy or help to destroy target cells.[6, 10, 11] CD4+ TILs are also essential in order to establish and maintain the function of tumor-specific CD8+ TILs in tumor tissue. CD4+ T cells (T_H) recognize antigens, including tumor antigens and tumor associated antigens presented by MHC class II molecules. Upon activation, CD4+ T helper cells release cytokines, which orchestrate immune responses (Figure 3). [7, 11] There is a third group of tumor infiltrated lymphocytes, which are CD4+ and CD25+. The function of this group of cells is immune suppression. [12] It is possible that the ratio of CD4+/CD8+ T

lymphocytes is one of the key factors for appropriate function of TILs. TILs also produce soluble cytokines such as interferon-γ for the cellular immune response. Some studies on IL-2-cultured TILs showed that the composition of TILs varies ranging from an infiltration with 90% CD4+ T cells to an infiltrate with 90% CD8+ T cells in individual patients.[9]

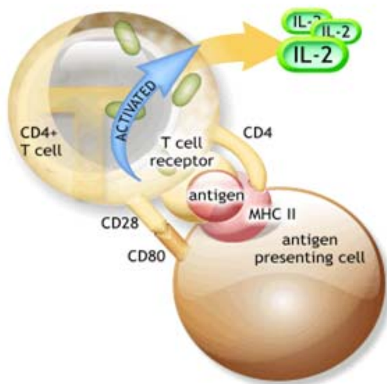

Figure 2. CD8+ T cell pathway [13]

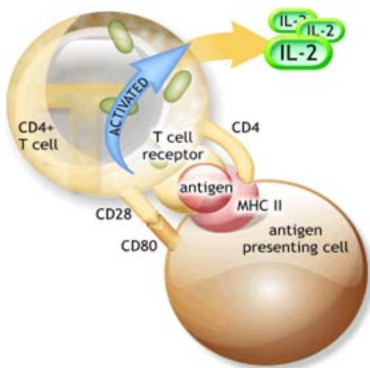

Figure 3. CD4+ T cell pathway [13]

The work of Gershon and Kondo 1971 first identified the so-called "suppressor" cells. Sakaguchi et al. stirred interest in T regulatory cells (Tregs) by identifying CD4+ T cells highly expressing CD25, which are able to prevent autoimmunity in a murine model. [12, 14, 15] Tregs represent 5-10% of human CD4+ T cells. Tregs have several subpopulations including naturally occurring CD4+CD25+ high Treg cells, induced Treg cells(Tr1 and TH3 cells), as well as CD4+CD25+ high Treg cells developed in the periphery by conversion of CD4+CD25– T cells. A subpopulation of CD8+ cells is also classified as Tregs, but little is known about that subpopulation.[14, 16, 17] All these Treg subpopulations coexist and contribute to immune suppression. Among specific Treg markers, the transcription factor FOXP3 has been reported as uniquely expressed in Treg cells in mice. Therefore, CD4+/CD25+high/Foxp3+ can be the marker to identify Tregs.[14, 17] CD4+CD25+high/FOXP3+ Tregs are characteristic in their anergic state (Figure 4.). They are able to actively inhibit CD4+CD25– T cells, CD8+ T cells, dendritic cells (DCs), natural killer (NK) cells, natural killer T (NKT) cells, and B cells in a cell-to-cell contact and dose-dependent manner. Although Tregs contribute significantly to the maintenance of immune system homeostasis and tolerance to self-antigens, they may represent the main obstacle to immunotherapy for cancers because of their suppressive behavior to the other immune cells. [16-18]

Figure 4. Model for the suppression of conventional T cells by regulatory T cells [19]

1.3 The function of IL-2

One of the most important growth factors of TIL are interleukin (IL)-2, which have been studied for more than 20 years in the therapy of melanoma. High dose IL-2 therapy has been developed to treat metastatic melanoma patients. [20-22] Some patients displayed significantly positive immune responses to the treatment and tumor regression was observed in these patients, although the relative response rate among patients is still very low. [20-22] Another advantage of this treatment is that once the initial treatments are complete, remission can be gained and further maintenance therapies are not needed. However, in some cases, the surgical removal of residual disease is required. [20, 22] This treatment also has some very severe side effects such as vomiting, nausea, diarrhea, fatigue and flu like symptoms such as fever, chills, and muscle aches. [22, 23] Despite the miserable side effects, the treatment typically does no permanent damage. Some researchers have developed high or low dose IL-2 plus other reagents or immune peptides treatment such as gp100 analogue peptide.[22, 24] Compared to the IL-2 only treatment, response rate and progression-free survival rate were significantly higher. Also, the

reduction of administrated dose of IL-2 can significantly reduce the side effects. All of the aforementioned studies suggest that the treatments combining IL-2 with other reagents or immune peptides are the right track for the immunotherapy of melanoma. [21, 25, 26]

Smith et al. indicated that IL-2 can enhance the proliferation rate of antigen specific TILs and/or their cytotoxicity. [27] To expand the effective T cells *in vitro*, IL-2 must be added during culture as a growth factor. T cells produce IL-2 following antigen presentation, and also express IL-2 receptor on their surface. The growth, differentiation, and survival of antigen selected T cells can be stimulated by IL-2 and IL-2 receptor interaction. T cell immunologic memory and induction of regulatory T cell activity also require IL-2. [21, 28, 29] Additionally, IL-2 can induce the differentiation and proliferation of natural killer (NK) cells and facilitate the production of antibodies by B cells. The Food and Drug Administration (FDA) approved recombinant IL-2 for the treatment of metastatic renal cell carcinoma and melanoma in 1992. [21, 28, 29]

Although IL-2 is widely used in the cultivation and expansion of T cells, the concentrations of IL-2 vary greatly among different studies from 10 IU/mL to 6000 IU/mL. Several studies have compared the effect of different IL-2 concentrations on the proliferation rate of T cells but not on cytotoxicity function. A recent paper reported that T cell cultures grown in very high IL-2 concentrations (600-6000 IU/mL) had the highest proliferation rate and secreted the most interferon (IFN)-r in response to antigenic stimulation but exhibited lower direct cytotoxicity. [27] On the contrary, TIL cultures

grown in low concentrations of IL-2 (10-600 IU) had a lower proliferation rate and rarely secreted IFN-r but displayed higher cytotoxic activity. [27] The author also used a combined approach, starting with low concentration of IL-2 (10-120 IU) the first week, then increasing the IL-2 concentration to 6000 IU/mL during the second week. In this way, T cells expanded well, maximally produced IFN-r and were highly cytotoxic against tumor cells. [27]

1.4 The function of DCs

Dendritic cells (DC), originating from bone marrow, are one kind of antigen presenting cells (APC's) and one of the best equipped APCs to prime the response of T cells because they can constitutively express MHC II antigens and numerous T cell co-stimulatory molecules. [30] Immature DCs are phagocytic cells that can sample antigens at the site of injury or infection and migrate to secondary lymphoid organs, where they can present antigens to T cells. However, immature DCs are weak immunogens and can even induce the antigen-specific Tregs. Mature DCs on the other hand have surface stimulatory molecules such as CD40, CD80, CD86 and some adhesion molecules. Upon activation, DCs can migrate to the lymphoid tissues where they interact with T cells and B cells to initiate and shape the adaptive immune response. [30-32]

1.5 Coley' toxin and OK-432

It was reported that acute bacterial infection can induce regression in some malignant tumor long time ago. Based on this work, Busch infected a patient with soft tissue sarcoma with erysipelas in 1868, and rapid tumor shrinkage was observed after infection

[33]. However, this response was only partial and tumor recurrence subsequently occurred. *Streptococcus* was identified as the causative agent of erysipelas 13 years later. [33, 34]

Dr. William B. Coley, a New York surgeon found a substantial number of this kind of publications in 1890s and started his own research. He infected *Streptococcus* to an inoperable patient who had an extensive lymphoma of the neck. He observed that the tumor underwent extensive necrosis, although with sever erysipelas after infection. The patient remained disease-free for 8 years. Encouraged by this outcome, he injected *Streptococcus* to treat patients with a variety of malignant tumors. [34] The clinical results were variable. He observed that different tumor types had different outcomes and the severity of infection also highly affect the result. He also noticed that the presence of *Serratia marcescens* can improve the outcome. He incorporated *Serratia marcescens* into the *Streptococcal* vaccine. The combined injection of these two heat-killed bacteria was called "Coley's toxin" since then. [35]

Coley concluded that the infection induced a systemic response, which led to tumor destruction. Administration of the toxin was very complicated and may result in significant morbidity and even mortality. High fever is essential for a good outcome. [34, 35] After treating various kinds of tumors, he noticed that the best response was from patients with inoperable soft tissue sarcomas. The five years disease-free survival rate was approximately 50%. [34, 35]

Coley's observations illustrated that acute infection may lead to stimulation of immune response of the host. This results in recognition of the tumor by the immune system, initiation of immune response to tumors, and tumor destruction. [34] However, Coley's toxin was abandoned long ago, mainly to avoid life-threatening infection. Contemporary science still cannot define the active elements in Coley's toxin, and attempts to improve safety such as heat treatment, resulted in diminished clinical efficacy. Some researchers suggested that plasminogen might be activated by administration of fresh *Streptococcus* which leads to the regression of tumors. [36]

One of the plasminogen activators is streptokinase (SK) whose activity may be lost after purification and heat treatment, due to elimination of live bacteria. SK is produced by virulent and when combined stoichiometrically with host plasminogen leads to the formation of enzymically active plasmin. The function of plasmin, which has relatively broad substrate specificity, is to initiate some protease cascades and degrades various plasma and extracellular matrix proteins. At the same time, SK could function as a streptococcal spreading factor which facilitates the dissection of organisms through tissue planes. [37] Some studies show that a host factor, which may be plasminogen, is needed for the antitumor effect. [38, 39] Some researchers suggest that administrating of SK or other plasminogen activators, or plasmin may reproduce the systemic streptococcal infections. There are several possible mechanisms of plasminogen on antitumor effects such as a direct cytoxicity to tumor cells, disruption of the tumor extracellular matrix, alteration of tumor growth factor activity, and inhibition of metastasis. Besides SK, other

plasminogen activators are being tested in the lab, including LPS, which is widely used to activate DCs in the lab, and OK-432. [37-39]

OK-432, developed by Okamoto et al., is a heat and penicillin-treated lyophilized preparation of the low-virulence strain Su of *Streptococcus pyogenes*. OK-432 has immunomodulatory and potential antitumor therapeutic function by activating DCs, neutrophils, macrophages, lymphocytes, and NK cells, as well as inducing multiple cytokines including IL-1, IL-2, IL-6, TNF-a, IFN-y, and IL-12. It has been reported that OK-432 can improve the antitumor function induced by a granulocyte-macrophage (GM)-CSF gene-modified tumor vaccine. [40-46] OK-432 has also been used clinically in Japan as an adjuvant treatment for patients with lung cancer, esophageal cancer, gastrointestinal cancer, bladder cancer, and peritoneal carcinomatosis. A great improvement has been made in cancer survival rate when immunotherapy and OK-432 are concomitantly used with chemotherapy. However, the precise mechanism of OK-432's anti-cancer activity remains unknown. OK-432 has not been approved by FDA to be used in the U. S. at the time of this publication. [47, 48]

1.6 Cellular immunotherapy

Cellular immunotherapy (CI) started with the use of lymphokine-activated killer cells (LAK) in the early 1980s and advanced to the use of immunologically specific, tumor-infiltrating lymphocytes (TIL) today. One of the recent immunotherapy strategies consists of expansion of large numbers of autologous tumor reactive lymphocytes (TILs from tumors or peripheral blood) *ex vivo* and subsequently reinfused those cells into the

patients' circulatory system. [6, 7, 49, 50] Melanoma is an ideal tumor model for studying CI because of the relatively easy melanoma-TIL culture conditions and the higher frequency of clinical responses. [51] Tumor-specific CD4+ and/or CD8+ T cells can be harvested from the culture of enzymatically digested tumor biopsies with high concentration IL-2. Stable long-term tumor cell lines also can be derived from harvested cells which make detailed immunologic analysis easier. Antitumor TIL can be derived from different sites such as cutaneous and visceral lesions. [7, 51, 52]

At the beginning of CI therapy, people hoped that more durable clinical successes in a wider range of tumor types could be achieved. TIL transfer therapy was considered by many researches to be the most promising immunotherapeutic strategy. Successful tumor eradication in murine models has been achieved by this strategy. [6] Unfortunately, this is not the case in clinical practice. TILs extracted and expanded from tumor tissues are sometimes composed of quiescent and/or functionally anergic T cells. They are unable to persist or perform their immunosurveillance function after adoptive transfer and consequently, they fail to co-deliver effective cytoreductive and/or immunosuppressive chemotherapy, especially when non-specific T cell populations are used. The function of TILs is always weakened by the accumulation of Tregs, which are able to actively inhibit the function of TILs. Tumor burden contributes to a significant tumor and/or host derived suppressive environment which is also a reason to dampen the initial hope. [8, 9, 49, 50]

TIL treatment showed little effect in colon cancer, esophageal cancer, breast cancer, follicular lymphomas, ovarian cancer, renal cell cancer, and non-small cell lung cancer.

This is likely due to non-specific TIL or TIL with no functional reactivity being the only products obtained, despite using standard TIL culture conditions. This is due to the experimental inability to remove fresh tumor cells from the mixture. [7, 9, 51]

2. MATERIALS AND METHODS

2.1 Mice, cell line, and reagents

C57BL/6J mice (male or female) at 6–8 weeks of age were purchased from Jackson Laboratories (Bar Harbor, ME) and housed in our pathogen-free animal facilities. The animal experiments were carried out in accordance with the Guidelines for the Care and Use of Laboratory Animals (NIH Publication Number 85-23), and institutional guidelines. B16F0 mouse melanoma cells (ATCC #CRL 6322, Rockville, MD) were cultured in DMEM (GIBCO BRL, Grand Island, NY) containing 10% fetal bovine serum (FBS, Hyclone, Logan, Utah) and 50 μg/ml of gentamicin (GIBCO BRL). Recombinant mouse IL-2 (mIL-2, herein refered to as IL-2) (Cat. No. 402-ML) was purchased from R&D (Minneapolis MN). β_2-microglobulin (Cat. No.058K0814) was purchased from Sigma (St. Louis, MO). RPMI 1640 (Cat. No.10 -040-CU) was purchased from Mediateeh (Manassas, VA). Dynalbeads FlowComp^{TM} Mouse Pan T (CD90.2) (Cat. No. 114.65D) was purchased from Invitrogen (Oslo, Norway). Sodium pyruvate (Cat. No. 25-000-C1) was purchased from Mediateeh (Manassas, VA). Hepes Hyclone (Cat. No. SH30851.01) was purchased from Hyclone Laboratories, Inc (Logan, Utah). Amino acids (Cat. No. SH30238.01) was purchased from Hyclone Laboratories, Inc (Logan, Utah). GiBco 12605 TrypLE Red (Lot. No. 564859) was purchased from Invitrogen (Oslo, Norway). All of the antibodies of CD3, CD4, CD8, CD25 Foxp3, CD19, CD80 and CD86 were purchased from BD Pharmingen ^{TM} (San Jose, California).

2.2 Extraction of TILs from tumor

Two different procedures were employed in the study to isolate TILs, i.e., ficol gradient centrifugation method and Dynabeads® FlowComp™ Mouse Pan T method.

2.2.1 Ficol gradient centrifugation method

To extract TILs from mice melanoma tumor tissues, 1×10^6 B16F0 tumor cells were subcutaneously injected to each of 4 female C57BL/6J mice two weeks before harvesting the tumor tissue. The mice were euthanized and the tumor tissues were aseptically dissected and cut into small pieces in a petri dish. 20 ml of digestion solution (collagenase, 1.4 mg/ml and DNase, 0.392 mg/ml) were added to the tumor tissues. The digestion suspension was transferred into a T-75 flask and incubated at 37°C for 2 hrs with slow shaking. The cell suspension was strained using a 70 µm cell strainer to remove un-digested tissues. The cells were then washed twice with all free DMEM by spinning at 900xg for 10 min each time. The cells, resuspended in 20 ml all free DMEM were gently added over 15 ml of ficol in a 50 ml centrifuge tube without disturbing the interface. The tubes were centrifuged at 900xg for 30 min with brake off. Cells at the interface after centrifugation were carefully collected, washed twice with all free DMEM, and resuspended in culture medium containing 10% FBS. In order to isolate T cells, the cell suspension was added onto a nylon wool column and incubated at 37°C for 1h. The cells that passed through (mainly T lymphocytes) were collected into a new tube, washed twice with all free DMEM and resuspended in 10ml cell culture medium with 10% FBS.

2.2.2 Dynabeads® FlowComp™ Mouse Pan T method

To extract TILs from mice melanoma tumor tissues, 1×10^6 B16 cells were subcutaneously injected into each of 4 female C57BL/6J mice two weeks before

harvesting the tumor tissue. The mice were euthanized and the tumor tissues were aseptically dissected and cut into small pieces in a petri dish. The tumor tissues were then ground by using the coarse part of 2 glass slides, the cell suspension was passed through a 70 μm cell strainer. After two times of washing with all free DMEM, the cells were resuspended in 15 ml ammonia-chloride-potassium (ACK) solution (ACK lysis buffer: 0.15 M NH_4Cl; 1.0 mM $KHCO_3$; 0.1 mM Na_2EDTA; pH 7.2) to remove red blood cells and then resuspended in isolation buffer from the dynabeads FlowComp Mouse Pan T (CD90.2) kit at a concentration of $1x10^8$ cells/ml.

FlowComp™ Mouse CD90.2 Antibody from the kit was added into the cell suspension at a ratio of 25 µl antibodies per 500 µl cell suspension ($5×10^7$ cells), mixed well and incubated for 10 minutes at 2–8°C. After incubation, cells were washed by adding 2 ml of isolation buffer and centrifuging at 900xg for 8 min and resuspended in 1 ml isolation buffer. 75 µl of resuspended FlowComp™ Dynabeads were added into the tube, mixed well, and incubated for 15 min at room temperature with rolling and tilting. The tube was then placed on a magnet for a minimum of 1 min. With the tube still on the magnet, the supernatant was carefully removed and discarded. The washing step was repeated once. The tube was removed from the magnet and the cell pellet was carefully resuspended with 1 ml FlowComp™ Release Buffer and incubated for 10 min at room temperature under rolling and tilting. The tube was placed on the magnet for 1 min, and the supernatant containing the bead-free cells (TILs) was transferred to a new tube. The cells were then washed with 2 ml of isolation buffer by centrifuging at 900xg for 8 min and

responded in cell culture medium with 10% FBS. The cell suspension was kept on 2–8°C until further use in downstream applications.

2.3 Culture of dendritic cells from bone marrow

To improve cytotoxicity, DCs were added into the culture medium. To extract DCs from mice, the mice were euthanized in CO_2 chamber. Femur and tibia bones were dissected and placed in a small petri dish. 5-10 ml (for bones of 1 mouse) of all free RPMI was put in a petri dish. The bone marrow cells were passed through a 40 µm cell strainer into a 50 ml tube. After washing twice with 10 ml all free RPMI medium by spinning at ca. 700xg for 5 min, the cells were resuspended with 10 ml of ACK lysis buffer for cell from the bones of one mouse and incubated at room temperature for 5 min. After another two wash cycles, the cells were resuspended in 10 ml of DC medium and cultured at 37°C for 6 days with addition of 10 ml DC medium into the culture dish on day 4. On day 6, 10 ml of medium were gently removed from the dish and 10 ml DC medium containing LPS (200 ng/ml), which activates DCs, was added into the dish. The cells were then further cultured for 24 to 48 hrs.

2.4 Culture of TIL cells

In order to expand the TILs isolated from tumors *in vitro* using different methods as described above, the cells were cultured in four different culture conditions.

2.4.1 Culture condition A: IL-2 alone

Cells were cultured in RPMI with 10% FBS, 10 mM MOPS, 0.05 mM 2-ME, 100 U/ml penicillin, 100 μg/ml streptomycin, 216 mg/l L-glutamine, 116 mg/l L-arginine, 36 mg/l L-asparagine, 6 mg/l colic acid, 110 mg/l sodium pyruvate, 2 g/l $NaHCO_3$. IL-2 at a dose of 400 IU/ml was also added into the medium. Cultures were maintained in 5% CO_2 at 37°C for up to 4 weeks for most of the studies. When necessary, the cells were cultured longer.

2.4.2 Culture condition B: IL-2 + OK-432

Culture condition B was the same as Culture condition A except OK-432 at a concentration of 1 μg/ml was added. Cultures were maintained in 5% CO_2 at 37°C for up to 4 weeks for most of the studies. When necessary, the cells were cultured longer.

2.4.3 Culture conditions C: IL-2 + DC

In culture condition C, activated DCs were added into the culture system as Culture Condition A at a TIL to DC ratio of 10 to 1. Cultures were maintained in 5% CO_2 at 37°C for up to 4 weeks for most of the studies. When necessary, the cells were cultured longer.

2.4.4 Culture condition D: IL-2 + OK-432 + DC

Culture condition D was the same as Culture condition C except OK-432 at a concentration of 1 μg/ml was added into the culture medium. Cultures were maintained in 5% CO_2 at 37°C for up to 4 weeks for most of the studies. When necessary, the cells were cultured longer.

2.5 Fluorescent Activated Cell Sorting (FACS) analysis

To phenotypically characterize TILs, freshly isolated cells and cells cultured for various length of time in different culture conditions were subjected to the FACS analysis. 2×10^5 cells from each sample were collected and washed twice with FACS staining buffer. Different antibodies (1 μg/$1x10^6$ cells) were added to the cells in 50 μl staining buffer and incubated for 30 min on ice. After staining, the cells were washed twice with staining buffer and resuspended in 500 μl of staining buffer and analyzed with FACS Calibur using CellQuest software. The following antibodies or their combinations were used: Isotype controls, H-$2D^b$-FITC, H-$2D^b$-PE, CD3-PE, CD4-PE/CD3-Cy5, CD8-PE/CD3-Cy5, CD25-PE/CD3-Cy5, Faxp3/CD3-Cy5.

In order to stain the intracellular antigen, Foxp3, after the CD3-Cy5 staining, the cells were washed twice with the staining buffer, treated with BD's CYTOFIX CYTOPEM on ice for 30 min, and stained with Foxp3- PE overnight on ice.

Before putting into culture, DCs were also analyzed with FACS using antibodies of CD33, CD80 and CD86.

2.6 Cytotoxicity analysis

The cytotoxicity of TIL cells, fresh or cultured in various culture conditions, was measured by a standard ^{51}Cr release assay using B16F0 tumor cells as target cells. $2x10^6$ B16F0 cells were collected, washed twice with PBS, and resuspended in 0.1 ml culture medium containing 20% FBS. 100 μCi of ^{51}Cr were added into the cell suspension. The

tube was gently mixed and incubated at 37°C for 1h with gentle mixing every 20 min to allow for cellular uptake of ^{51}Cr. The cells were washed twice with PBS and resuspended in RPMI medium at a concentration of 5×10^{4} cells/ml. 100 µl of the cells ($5x10^{3}$ cells) were transferred to each well of 96-well round bottom plate. While labeling the target cells, the effector cells were prepared at concentrations of $5.5x10^{5}$ /ml, $1.5x10^{5}$ /ml, and $5x10^{4}$ /ml, respectively. 100 µl of each effector cells were added into corresponding wells which contain 100 µl of the labeled target cells so that the effector to target ratio was 11:1, 3:1, and 1:1. After spinning for 30 sec at 228xg to settle the cells, the plate was incubated at 37°C for 4 hrs. 30 min before finishing the incubation, 100 µl of 2% Triton-100 was added into some of the wells containing only the target cells as maximum release control. After spinning at 228xg for 2 min, 50 µl of the supernatant from each well were transferred into a new plate correspondingly, and 150 µl of the scintillation liquid were added into each well. The radioactivity of the samples was measured with the Trilux scintillation counter. The cytotoxicity of the killer cells was calculated as:

$$\text{Cytotoxicity} = \frac{(\text{average CPM of group} - \text{background CPM})}{\text{maximal CPM} - \text{background CPM}}$$

CPM denotes for count per minute.

3. RESULTS

3.1 Yield and phenotype of TILs isolated with different methods

Several experiments using the ficol gradient centrifugation method were performed. The isolated TIL cells were stained with different antibodies and analyzed by FACS. Since there were many tumor cell contaminants present in the TIL cell preparation, anti-CD3 antibody was used to gate T cells in the FACS analysis. As shown in Table 1, 42% of the CD3+ cells are CD4+ and 35% of the CD3+ cells are CD8+. CD25+ cells accounts for 17% and Foxp3+ cells accounts for 7%.

Since the ficol gradient centrifugation method leaves many tumor cells in the TIL preparation, we employed the Dynabeads® FlowComp™ Mouse Pan T method. This method positively selects for all T cells using a pan T antibody, anti-CD90.2. As expected, this method generated highly pure T cells with no tumor cell contaminants present in the preparation. As shown in Table 2, 37.07% of the CD3+ cells are CD4+ and 41.22% of the CD3+ cells are CD8+. CD25+ cells accounts for 2.07% and Foxp3+ cells accounts for 0.07%. The problem of this method is the limited yield of TILs.

Table 1: Phenotype of fresh TILs isolated using the ficol gradient centrifugation method

CD4+	CD8+	CD25+	Foxp3+
42.00%	35.23%	17.58%	7.31%

Table 2: Phenotype of fresh TILs isolated using the Dynabeads® FlowComp™ Mouse Pan T protocol.

CD4+	CD8+	CD25+	Foxp3+
37.07%	41.22%	2.07%	0.07%

In conclusion, the Dynabeads® FlowComp™ Mouse Pan T method seems to be the better choice to isolate TILs from mouse tumor tissues. As a result, most of the following experiments were performed with TILs isolated using this method. However, since only 90.2+ cells are selected, some functional TILs in the tumor tissue may be lost.

3.2 IL-2's role on expansion, phenotype, and cytotoxicity of cultured TILs

3.2.1 Cell count and surface marker of cultured TIL with low dose IL-2

In this culture condition, TILs grew fast in the first week (290%) and second week (130%) and then slowed down. (Table 3) The growth rate reported for each week was calculated based upon the amount of cells in the week prior to amount of cells of the week. The reason is that certain amounts of cells were taken out of the cell culture to perform experiments. The percentages of CD4+ cells and CD8+ cells in the culture decreased significantly (from 37% to 5% and from 41% to 12%, respectively), while CD25+ cells increased significantly from 2% to 35% (Table 4), which means the cells that expressed CD25+ were activated.

Table 3. Cell count of cultured TILs in low dose IL-2 (400 IU/ml)

Time	**Cell Count** (cells/ml)	**Growth Rate**
Week 0	0.4×10^6	---
Week 1	1.4×10^6	290%
Week 2	2.9×10^6	130%
Week 3	1.7×10^6	-24%
Week 4	1.1×10^6(from 1.3×10^6)	-24%

Table 4. Phenotype of cultured TILs in low dose IL-2 (400 IU/ml)

Time	CD4+	CD8+	CD25+	Foxp3+
Week 0	37.04%	41.22%	2.07%	0.07%
Week 2	1.54%	12.77%	6.62%	0.04%
Week 4	5.15%	12.64	35.05%	ND

ND, not detectable.

3.2.2 Cytotoxicity of cultured TIL with low dose IL-2

TILs cultured for 14 days and 28 days in low dose IL-2 were harvested and used to perform a cytotoxicity assay using B16F0 tumor cells as target cells. Due to the limitation of cell numbers, only three effector to target ratios were evaluated: 11:1, 3:1 and 1:1. As shown in Figure 5, the cytotoxicity of TILs cultured in this condition is relatively low (less than 20%), although the cytotoxicity of 14-day cultured TILs (18%) is higher than that of 28-day cultured TILs 12% at the effector to target ratio of 11:1.

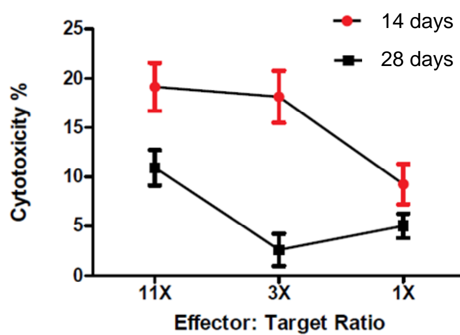

Figure 5. Cytotoxicity of TILs cultured in low dose IL-2 (400 IU/ml) for 14 and 28 days.

3.3 OK-432's role on expansion, phenotype, and cytotoxicity of cultured TILs

3.3.1 Cell count and surface marker of cultured TIL with OK-432

As illustrated on Table 5, when OK-432, at a concentration of 1μg/ml, was added into the TIL culture together with low dose IL-2 (400 IU/ml), cell growth was promoted. More importantly, under this culturing condition we were able to culture the TILs for up to 14 weeks and maintain cell growth. (Table 5) Under this culture condition CD4+ cells decreased significantly, similar to the IL-2 alone culture condition. CD8+ cells increased significantly in the first two weeks of culture, in contrast to the IL-2 alone culture condition. (Table 6)

Table 5. Cell count of cultured TILs in low dose IL-2 (400 IU/ml) plus OK-432

Time	**Cell Count** (cells/ml)	**Growth Rate**
Week 0	$0.6x10^6$	0
Week 1	$1.9x10^6$	217%
Week 2	$2.93x10^6$	54%
Week 3	$4.62x10^6$	58%
Week 4	$3.24x10^6$ (from $1x10^6$)	224%
Week 5	$2.85x10^6$ (from $1x10^6$)	185%
Week 6	$4.85x10^6$	70%
Week 7	$4.62x10^6$	-5%
Week 8	$3.57x10^6$	-23%
Week 9	$2.5x10^6$ (from $1x10^6$)	150%
Week 10	$1.13x10^6$	-55%
Week 11	$2.35x10^6$	52%
Week 12	$2.8x10^6$	19%
Week 13	$1.71x10^6$	-39%
Week 14	$1.13x10^6$(from $0.55x10^6$)	105%

Table 6. Percentages of different cells in cultured TILs in low dose IL-2 (400 IU/ml) plus OK-432

Time	CD4+	CD8+	CD25+	Foxp3+
Week 0	37.04%	41.22%	2.07%	0.07%
Week 1	1.62%	73.62%	41.01%	1.06%
Week 2	0.35%	63.31%	27.53%	0.72%
Week 3	0.24%	48.97%	32.96%	0.25%
Week 4	0.79%	36.49%	35.79%	1.84%

3.3.2 Cytotoxicity of cultured TIL with OK-432

TILs cultured for 14 days and 28 days in low dose IL-2 plus OK-432 were harvested and used to perform a cytotoxicity assay using B16F0 tumor cells as target cells. Due to the limitation of cell numbers, only three effector to target rations were performed: 11:1, 3:1 and 1:1. As Figure 6 shows, both of the effector cells collected from day 14 culture and day 28 culture had significant killing of the target tumor cells. When the effector to target ratio is 11:1, the cytotoxicity reached 44.2% and 42%, respectively. This cytotoxicity is significant higher that of TILs cultured in IL-2 alone culture condition (19% and 12%, respectively).

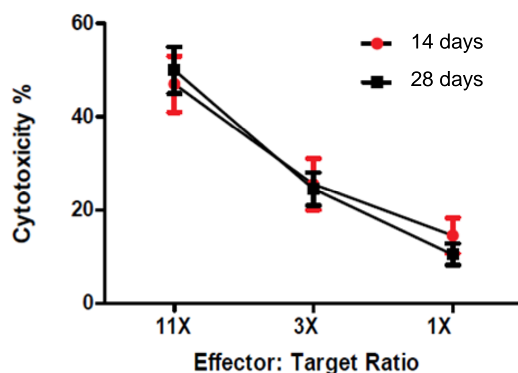

Figure 6. Cytotoxicity of TILs cultured in 400 IU/ml IL-2 and 1 µg/ml OK-432 for 14 and 28 days.

3.4 Dendritic cell's role on cytotoxicity of cultured TILs

In order to further improve the cytotoxicity of TILs cultured in low dose IL-2 and OK-432, bone marrow derived dendritic cells were added into the culture system. The cultured cells were harvested at various time points and used to perform the cytotoxicity assay using B16F0 tumor cells as target cells. The cytotoxicity of TILs cultured in low dose IL-2 with DCs for 14 and 28 days is shown on Figure 7. The cytotoxicities of TILs cultured under this condition for 14 and 28 days are 40% and 60%, respectively. Figure 8 shows the cytotoxicity of TILs cultured in low dose IL-2 with DCs plus OK-432. When the ratio of effector to target was 11:1, the cytotoxicities of TILs cultured in this condition reached 73% and 57%, respectively.

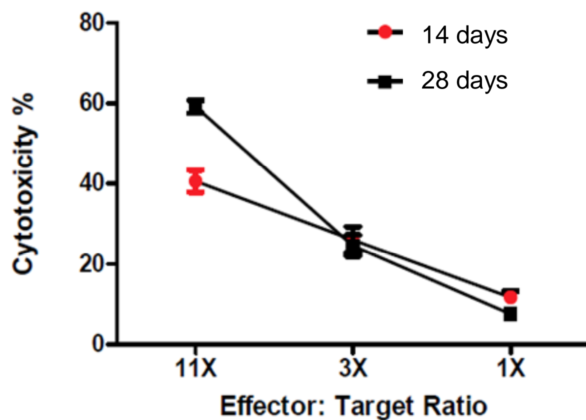

Figure 7. Cytotoxicity of TILs cultured in low dose IL-2 (400 IU/ml) and DCs (TIL:DC=10:1) for 14 and 28 days.

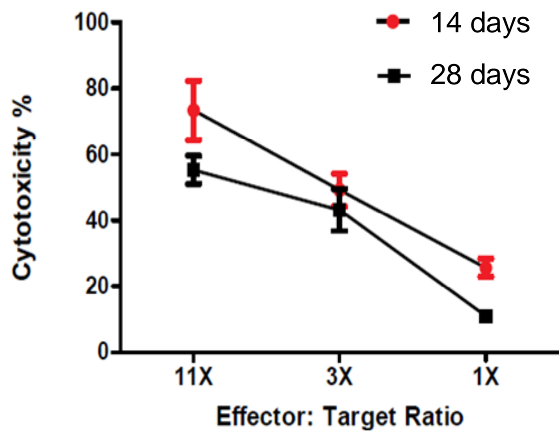

Figure 8. Cytotoxicity of TILs cultured in low dose IL-2 (400 U/ml), 1 μg/ml OK-432 and DCs for 14 and 28 days.

4. DISCUSSION

Although TIL therapy has achieved notable progress in treatment of melanoma in preclinical animal models, the clinical outcomes are not always promising. [6] Researchers have developed several new ideas to enhance the proliferation and cytotoxicity of the cultured TILs by adding different concentrations of IL-2 or GM-CFS and/or other cytokines to the culture. [7, 9] This project was designed to establish an *in vitro* culture system in which highly cytotoxic and tumor cell specific CTLs can be expanded, while inhibiting Tregs by using OK-432, IL-2 and other cells and/or cytokines. Experimental subcutaneous tumors of mouse B16F0 melanoma were developed in mice and T lymphocytes of TILs were extracted from tumors directly. The isolated cells were then cultivated in four different culturing conditions. The first challenge was how to effectively isolate highly pure TILs with sufficient amount from tumor tissues. Many studies have used ficol gradient to separate TILs from tumors. However, many TILs are lost in the ficol centrifugation step. Another challenge is that the cell preparations from ficol gradient contain many tumor cells, and it is difficult to separate TILs from tumor cells. In this work, we first used the ficol gradient approach to isolate TILs from tumor tissues. Although a significant number of TILs was obtained, the cell preparation contained many tumor cells. Furthermore, TILs contain T cells, B cells and NKs etc.[8, 9] Our main goal is to separate T cells. It is also challenging to eliminate other lymphocytes from TILs as well. We then employed another method to isolate T lymphocytes directly from tumor tissues, i.e., the Dynabeads® FlowComp™ Mouse Pan T method. Our data support that the Dynabeads® FlowComp™ Mouse Pan T protocol represents a better method, although it risks losing some functional T cells.

Many researchers believe that TILs represent the immune response to tumor cells. TILs are mainly composed of T lymphocytes and other subsets. T lymphocytes of TILs are mainly comprised of varying numbers of CD4+ helper and CD8+ cytotoxic T cells (CTL). [6-9] In order to expand T cells from TILs *in vitro*, many researchers used IL-2 in their culture systems. The concentration of IL-2 used in culture becomes a major issue. It is debated whether high dose IL-2 (over 1000 IU/ml) or low dose IL-2 (lower than 600 IU/ml) is better in terms of enhancing the proliferation rate and cytotoxicity of T cells.[27] A recent paper reported that T-cell cultures grown in very high IL-2 concentrations (6000 IU/ml) had the highest proliferation rate but exhibited lower direct cytotoxicity. On the contrary, T cells grown in low concentrations of IL-2 (600 IU/ml) had a lower proliferation rate but displayed higher cytotoxicity. [27] Based upon these results, we used low dose IL-2 (400 IU/ml) in our approach. The results demonstrate that T cells cultured in this dose of IL-2 proliferate well and showed low cytotoxicity against B16F0 tumor cells. (Table 3 and Figure 5)

Meanwhile, many studies have reported that OK-432, a heat and penicillin-treated lyophilized preparation of the low-virulence strain of *Streptococcus pyogenes*, exhibits immunomodulatory activities and has potential antitumor therapeutic function, including the activation of DCs, neutrophils, macrophages, lymphocytes, and NK cells, and the induction of multiple cytokines production, such as IL-1, IL-2, IL-6, TNF-a, IFN-y, and IL-12. [40-46] We hypothesized that OK-432, when combined with low dose IL-2, will enhance the proliferation and cytotoxicity of TILs in culture. As shown in Table 5 and Figure 6, TILs grew well in culture condition containing low dose IL-2 and OK-432 at a

concentration of 1 μg/ml. More importantly, cytotoxicity of T cells cultured in low dose IL-2 and OK-432 increased significantly (44%), when compared to that of T cells culture in low dose IL-2 alone (18%).

DCs are antigen presenting cells and one of the best equipped APCs to prime the response of T cells because they can constitutively express MHC II antigens and numerous T cell co-stimulatory molecules. [30, 31] In order to further improve the tumor cell specific cytotoxicity of the cultured TILs, bone marrow derived DCs were first loaded with B16F0 tumor antigens and added into the *in vitro* culture system. When TILs are cultured in low dose IL-2 plus DCs, the cytotoxicity of the TILs improved significantly from 18% for low dose IL-2 alone to nearly 60% for low dose IL-2 plus DCs. (Figure 7) The cytotoxicity of culture TILs increased to up to 73%, (Figure 8) when all the three reagents were added into the culture system, i.e., low dose IL-2 (400 IU/ml), OK-432 (1 μg/ml), and DCs (TIL: DC=10:1).

According to our results, proper concentration of TILs in culture is very important. TILs cannot expand if the concentration is too low. On the other hand, nutritive elements become limiting if the concentration is too high. Another issue encountered in this research is that mice have to be euthanized when tumors are still small. The animal protocol requires that mice be euthanized when tumors start to become necrotic. Unfortunately, B16 melanoma tumors become necrotic when tumors are still small. This affects the collections of significant numbers of TILs from the tumors. The number of

TILs in tumors is affected by tumor size. The number of TILs may be small when the size of tumors is small. This may also be a consequence of the immune system not being activated when tumors are small. This directly means that only a small amount of TILs can be harvested, which negatively affects culture and phenotype analysis.

In conclusion, an improved *in vitro* TIL culture system was established by adding OK-432 into the culture system with low dose IL-2. Furthermore, when dendritic cells are added into the culture system, the cytotoxicity of the cultured TILs is significantly enhanced.

5. REFERENCES

1. Demierre, M.F., et al., *State of the science 60th anniversary review: 60 Years of advances in cutaneous melanoma epidemiology, diagnosis, and treatment, as reported in the journal Cancer.* Cancer, 2008. **113**(7 Suppl): p. 1728-43.
2. Markovic, S.N., et al., *Malignant melanoma in the 21st century, part 1: epidemiology, risk factors, screening, prevention, and diagnosis.* Mayo Clin Proc, 2007. **82**(3): p. 364-80.
3. Roudier-Pujol, C. and P. Saiag, *[Malignant melanoma. Epidemiology, screening, diagnosis, evolution, prognostic clinical and histopathologic criteria].* Rev Prat, 1998. **48**(15): p. 1729-35.
4. Vosmik, F., *[Malignant melanoma of the skin. Epidemiology, risk factors, clinical diagnosis].* Cas Lek Cesk, 1996. **135**(13): p. 405-8.
5. Mukherjee, K., *Melanoma Observation, Detection Health Centers Helpful in Early Diagnose*, in *Top news*. 2010. [cited 2010 Nov. 3] Available from: http://topnews.net.nz/content/23726-melanoma-observation-detection-health-centers-helpful-early-diagnose
6. Oble, D.A., et al., *Focus on TILs: prognostic significance of tumor infiltrating lymphocytes in human melanoma.* Cancer Immun, 2009. **9**: p. 3.
7. Yannelli, J.R. and J.M. Wroblewski, *On the road to a tumor cell vaccine: 20 years of cellular immunotherapy.* Vaccine, 2004. **23**(1): p. 97-113.
8. Boon, T., et al., *Human T cell responses against melanoma.* Annu Rev Immunol, 2006. **24**: p. 175-208.
9. Chiou, S.H., et al., *Current concepts of tumor-infiltrating lymphocytes in human malignancies.* J Reprod Immunol, 2005. **67**(1-2): p. 35-50.
10. Harlin, H., et al., *Tumor progression despite massive influx of activated CD8(+) T cells in a patient with malignant melanoma ascites.* Cancer Immunol Immunother, 2006. **55**(10): p. 1185-97.
11. Roszkowski, J.J., et al., *Simultaneous generation of CD8+ and CD4+ melanoma-reactive T cells by retroviral-mediated transfer of a single T-cell receptor.* Cancer Res, 2005. **65**(4): p. 1570-6.
12. Antony, P.A. and N.P. Restifo, *CD4+CD25+ T regulatory cells, immunotherapy of cancer, and interleukin-2.* J Immunother, 2005. **28**(2): p. 120-8.
13. Sheppard, T., *Acquired Immunity*, in *blobs.org*. 2009. [cited 2010 Nov. 3] Available from: http://www.blobs.org/science/article.php?article=12
14. Beyer, M. and J.L. Schultze, *CD4+CD25highFOXP3+ regulatory T cells in peripheral blood are primarily of effector memory phenotype.* J Clin Oncol, 2007. **25**(18): p. 2628-30; author reply 2630-2.
15. Oved, K., et al., *Predicting and controlling the reactivity of immune cell populations against cancer.* Mol Syst Biol, 2009. **5**: p. 265.
16. Liyanage, U.K., et al., *Prevalence of regulatory T cells is increased in peripheral blood and tumor microenvironment of patients with pancreas or breast adenocarcinoma.* J Immunol, 2002. **169**(5): p. 2756-61.
17. Viguier, M., et al., *Foxp3 expressing CD4+CD25(high) regulatory T cells are overrepresented in human metastatic melanoma lymph nodes and inhibit the function of infiltrating T cells.* J Immunol, 2004. **173**(2): p. 1444-53.
18. Wolf, A.M., et al., *Increase of regulatory T cells in the peripheral blood of cancer patients.* Clin Cancer Res, 2003. **9**(2): p. 606-12.

19. Oberle, D.r.n.N., *Regulatory T cells*, in *Dkfz. German Cancer Research Center*. 2010. [cited 2010 Nov. 3] Available from: http://www.dkfz.de/en/immungenetik/Elisabeth_group/Elisabeth_englisch.html
20. Albertini, M.R., et al., *Systemic interleukin-2 modulates the anti-idiotypic response to chimeric anti-GD2 antibody in patients with melanoma.* J Immunother Emphasis Tumor Immunol, 1996. **19**(4): p. 278-95.
21. Atkins, M.B., *Interleukin-2 in metastatic melanoma: establishing a role.* Cancer J Sci Am, 1997. **3 Suppl 1**: p. S7-8.
22. Dutcher, J., *High-dose interleukin-2 in metastatic disease: renal cell carcinoma and melanoma.* Oncology (Williston Park), 2002. **16**(11 Suppl 13): p. 3.
23. Quan, W.D., Jr., et al., *High-dose intensity pulse interleukin-2 with famotidine has activity in metastatic melanoma.* Cancer Biother Radiopharm, 2008. **23**(5): p. 641-6.
24. Cragun, W.C., et al., *Low-dose IL-2 induces cytokine cascade, eosinophilia, and a transient Th2 shift in melanoma patients.* Cancer Immunol Immunother, 2005. **54**(11): p. 1095-105.
25. Atkins, M.B., *Interleukin-2: clinical applications.* Semin Oncol, 2002. **29**(3 Suppl 7): p. 12-7.
26. Eisner, R.M., A. Husain, and J.I. Clark, *Case report and brief review: IL-2-induced myocarditis.* Cancer Invest, 2004. **22**(3): p. 401-4.
27. Besser, M.J., et al., *Modifying interleukin-2 concentrations during culture improves function of T cells for adoptive immunotherapy.* Cytotherapy, 2009. **11**(2): p. 206-17.
28. O'Brien, G.C., et al., *Co-immunotherapy with interleukin-2 and taurolidine for progressive metastatic melanoma.* Ir J Med Sci, 2006. **175**(1): p. 10-4.
29. Misawa, E., et al., *Booster effect of interleukin-2 on natural killer 1.1+ cells stimulated by administration of macrophage colony-stimulating factor in mice.* J Immunother, 2003. **26**(1): p. 21-30.
30. Schuler, G., *Dendritic cells in cancer immunotherapy.* Eur J Immunol. **40**(8): p. 2123-30.
31. Segovia, M., M.C. Cuturi, and M. Hill, *Preparation of mouse bone marrow-derived dendritic cells with immunoregulatory properties.* Methods Mol Biol. **677**: p. 161-8.
32. Shinozaki, Y., et al., *Tumor-specific cytotoxic T cell generation and dendritic cell function are differentially regulated by interleukin 27 during development of antitumor immunity.* Int J Cancer, 2009. **124**(6): p. 1372-8.
33. McCarthy, E.F., *The toxins of William B. Coley and the treatment of bone and soft-tissue sarcomas.* Iowa Orthop J, 2006. **26**: p. 154-8.
34. Bickels, J., et al., *Coley's toxin: historical perspective.* Isr Med Assoc J, 2002. **4**(6): p. 471-2.
35. Hoption Cann, S.A., J.P. van Netten, and C. van Netten, *Dr William Coley and tumour regression: a place in history or in the future.* Postgrad Med J, 2003. **79**(938): p. 672-80.
36. Zacharski, L.R. and V.P. Sukhatme, *Coley's toxin revisited: immunotherapy or plasminogen activator therapy of cancer?* J Thromb Haemost, 2005. **3**(3): p. 424-7.
37. Huang, T.T., H. Malke, and J.J. Ferretti, *Heterogeneity of the streptokinase gene in group A streptococci.* Infect Immun, 1989. **57**(2): p. 502-6.
38. Christensen, E.A., *Infection and malignant tumours. 1. Growth of Brown-Pearce carcinoma in rabbits treated with living or killed haemolytic streptococci.* Acta Pathol Microbiol Scand, 1959. **46**: p. 285-95.
39. Christensen, E.A. and E. Kjems, *Infection and malignant tumours. 2. Inhibition of growth of Brown-Pearce carcinoma in young rabbits treated with phage lysates of haemolytic streptococci.* Acta Pathol Microbiol Scand, 1959. **46**: p. 296-304.
40. Ojima, T., et al., *Streptococcal preparation OK-432 promotes the capacity of dendritic cells (DCs) to prime carcinoembryonic antigen (CEA)-specific cytotoxic T lymphocyte*

responses induced with genetically modified DCs that express CEA. Int J Oncol, 2008. **32**(2): p. 459-66.
41. Fujimoto, T., et al., *Streptococcal preparation OK-432 is a potent inducer of IL-12 and a T helper cell 1 dominant state.* J Immunol, 1997. **158**(12): p. 5619-26.
42. Wang, X., et al., *Streptococcal preparation OK-432 enhances the antitumor activity of CPT-11 by increasing Th1-cytokine production in mice.* Anticancer Res, 2001. **21**(4A): p. 2505-10.
43. Fujino, A., et al., *A role of cytokines in OK-432 injection therapy for cystic lymphangioma: an approach to the mechanism.* J Pediatr Surg, 2003. **38**(12): p. 1806-9.
44. Tatsugami, K., et al., *Local injections of OK432 can help the infiltration of adoptively transferred CD8+ T cells into the tumor sites and synergistically induce the local production of Th1-type cytokines and CXC3 chemokines.* Cancer Immunol Immunother, 2000. **49**(7): p. 361-8.
45. Okamoto, T., et al., *Local injection of OK-432 can augment the TH1-type T-cell response in tumor-draining lymph node cells and increase their immunotherapeutical potential.* Int J Cancer, 1997. **70**(5): p. 598-605.
46. Yamagiwa, S., et al., *Liposome-encapsulated OK-432 specifically and sustainedly induces hepatic natural killer cells and intermediate T cell receptor cells.* J Gastroenterol Hepatol, 2000. **15**(5): p. 542-9.
47. Poldervaart, M.T., et al., *Treatment of lymphatic malformations with OK-432 (Picibanil): review of the literature.* J Craniofac Surg, 2009. **20**(4): p. 1159-62.
48. Takahashi, K., et al., *OK-432 develops CTL and LAK activity in mononuclear cells from regional lymph nodes of lung cancer patients.* Int J Immunopharmacol, 1998. **20**(8): p. 375-88.
49. de la Cruz-Merino, L., et al., *Cancer and immune response: old and new evidence for future challenges.* Oncologist, 2008. **13**(12): p. 1246-54.
50. Janeway CA, T.P., Walport M, Shlomchik MJ., ed. *Immunobiology: The Immune System in Health and Disease.* . 2005, Garland Science: New York.
51. Kawakami, Y., *New cancer therapy by immunomanipulation: development of immunotherapy for human melanoma as a model system.* Cornea, 2000. **19**(3 Suppl): p. S2-6.
52. Dudley, M.E., et al., *Cancer regression and autoimmunity in patients after clonal repopulation with antitumor lymphocytes.* Science, 2002. **298**(5594): p. 850-4.