TH9402 Mediated Photodynamic Therapy: Implications for Ex Vivo Purging and Tumor Vaccination.

by

Qin Yong Dai

Faculty of Medicine, Division of Experimental Medicine,
McGill University, Québec, Canada

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This thesis is dedicated to my parents. Most of the truly important things in life I learned from them.

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Abstract

Photodynamic therapy (PDT) is a powerful strategy to eliminate a variety of tumor and alloreactive T cells. While the cytotoxic effect of the novel rhodamine-derived TH9402 photosensitizer has been attributed to the generation of radical oxygen species, the cell death pathways involved have not been extensively investigated. In this study, we evaluated the ability of PDT to induce apoptosis and necrosis in EL4 cells, and assessed its underlying molecular mechanisms. We found that the dominant mode of cell death after PDT was influenced by the conditions of treatment. PDT using TH9402 triggers a caspase-dependent intrinsic apoptotic pathway. However, the incapacity of caspase inhibition to block the induction of apoptosis and to limit cell death indicates that apoptosis induced by PDT also involves a caspase-independent pathway. The observation that the TH9402 photosensitizer mediates cytotoxicity through activation of a number of death pathways suggests that it could effectively limit the capacity of tumor cells to become resistant to TH9402.

Secondly, PDT using TH9402 induces a time- and dose-dependent decrease in target cell viability resulting in high levels of elimination of multiple myeloma cells. In an attempt to evaluate the clinical relevance of these findings and simulate minimal residual disease in an apheresis product, the combination of RPMI-8226 myeloma cells admixed with human apheresis cells from healthy donors was PDT treated. Again high levels of elimination of myeloma cells were observed while sparing normal cells. This important cytotoxic activity was observed in conditions which preserve more that 50% of hematopoietic progenitor cells. These results strongly suggest that TH9402 PDT represents an appealing strategy in *ex vivo* purging of autologous stem cell transplants in patients with multiple myeloma.

Finally, we have found that immunization with PDT-treated whole tumor cells was able to delay tumor cell growth. This finding encouraged us to investigate whether a DC-based PDT vaccine could protect from tumor cell progression. In this study, purified DCs were generated from murine bone marrow. We also identified a cytokine combination capable of promoting DC activation. Under these conditions, PDT-treated tumor cells did not increase IL-12 production by DCs, but they provoked partial maturation of DCs. Once these activated DCs were loaded with whole tumor cells treated by PDT, a tumor protection effect was observed in two different murine models in vivo . Such a vaccine may be particularly useful when tumor cells do not represent a pure cell population and are rather dispersed among normal cells. The present studies therefore uncovered unique apoptotic pathways of TH9402 PDT, and identified two appealing approaches to take advantage clinically of those peculiar photochemical properties in an attempt to improve the survival of patients with cancer.

Résumé

La thérapie photodynamique (PDT) est une stratégie très efficace pour éliminer une grande variété de cellules tumorales ainsi que les cellules T alloréactives. Alors que l'effet d'un nouvel agent photodynamique dérivé de la rhodamine, le TH9402, a été attribué à la génération de radicaux libres, les mécanismes de la mort cellulaire en cause n'avaient pas encore été étudiés extensivement. Dans la présente étude, nous avons évalué la capacité de la PDT à induire l'apoptose et la nécrose dans des cellules EL4 ainsi que les mécanismes sous-jacents à son activité. Nous avons observé que le mode de mort cellulaire prédominant après la PDT était influencé par les conditions de traitement. La PDT avec le TH9402 entraîne l'apopotose par une voie dépendante des caspases. Par contre, l'incapacité de bloquer l'induction de l'apoptose induite par la PDT peut également impliquer une voie indépendante des caspases. Notre observation révélant que l'agent photosensible TH9402 entraîne la cytotoxicité via l'activation de plusieurs voies de mort cellulaire suggère, de façon intéressante, que ceci pourrait limiter la capacité des cellules tumorales à devenir résistantes au TH9402.

En second lieu, nous avons démontré que la PDT utilisant le TH9402 induit une diminution de la viabilité cellulaire qui dépend de la durée d'incubation et de la dose de TH9402, et résulte en de hauts niveaux d'élimination des cellules de myélome multiple. Dans l'optique d'évaluer l'utilité clinique que pourraient avoir ces résultats, nous avons mimé la maladie minimale résiduelle dans des produits d'aphérèse humains. Pour ce faire, les cellules de myélome (RPMI-8226) ont été mélangées avec des cellules d'aphérèse de donneurs sains. Encore une fois, de hauts niveaux d'élimination des cellules de myélome ont été observés ainsi que la préservation des cellules normales. Cette activité cytotoxique importante a été observée dans des conditions préservant plus de 50% des progéniteurs

hématopoïétiques. Ces résultats suggèrent fortement que la PDT avec le TH9402 représente une stratégie intéressante pour la purge cellulaire de greffons de cellules souches autologues chez les patients atteints de myélome multiple.

Finalement, nous avons démontré que l'immunisation avec des cellules tumorales traitées avec le TH9402 retarde la croissance des cellules tumorales. Cette découverte nous a encouragé à déterminer si un vaccin à base de cellules dendritiques et de PDT pourrait protéger contre la progression des cellules tumorales. Dans cette partie du projet, nous avons généré des cellules dendritiques à partir de la moelle osseuse de souris donneuses. Nous avons aussi identifié une combinaison de cytokines permettant l'activation des cellules dendritiques. Dans ces conditions, les cellules tumorales traitées avec la PDT n'ont pas entraîné d'augmentation de la production d'IL-12 par les cellules dendritiques mais ont provoqué une maturation partielle de celles-ci. Une fois que ces cellules dendritiques ont été en contact avec les cellules tumorales traitées avec la PDT, une protection contre la croissance des tumeurs a été observée dans 2 différents modèles murins de cancer in vivo. Un tel vaccin pourrait être particulièrement utile lorsque les cellules tumorales ne représentent pas une population pure de cellules mais sont plutôt dispersées parmi des cellules normales. Les présentes études ont ainsi permis de découvrir des voies d'apoptose uniques de la PDT au TH9402, et d'identifier deux approches innovatrices pour prendre avantage cliniquement de ces propriétés photochimiques particulières dans le but d'améliorer la survie des patients atteints de cancer.

Acknowledgements

First and foremost, I would like to express my eternal gratitude to my PhD supervisor Dr. Denis Claude Roy. He is truly a devoted hematologist and scientist who excels in both fundamental and clinical research. He will remain a role model for me. His enthusiasm, persistence and tirelessness deeply impress me. His infectious dedication and commitment to scientific excellence played a big part in driving me to overcome and complete this often controversial, at times difficult, but always challenging project.

I would like to take this opportunity to thank all the members of my thesis committee: Dr.Andrew Muland, Dr. Fernando Congote, Dr.Lambert Busque and Dr. Marika Sarfati for useful comments and guidance during the course of my studies. I would also like to emphasize the special attention and commitment of Dr. Congote who helped significantly in making this thesis a reality.

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I reserve a very special gratitude to Dr. Andrew Bateman, who allowed me to begin my PhD program in his lab. Special memory goes to Dr. Gerald B. Price, one of the most influential teachers I met.

Finally, I want to extend my gratitude to all my family and friends for their encouragement and support during the time I spent in Montreal.

Preface

In accordance with the "guidelines for thesis preparation" stipulated by the Faculty of Graduate Studies and Research of McGill University, I have elected to present my research in a manuscript-based format. According to these guidelines, I have written a general literature review as chapter 1. The next three chapters include results presented at the 34th Annual Scientific Meeting of International Society for Experimental Hematology (chapter 2), a second manuscript that will be summitted for publication later this year and will contain additional data using primary cell cultures (chapter 3), and a third manuscript (chapter 4) that will be presented at the 35th Annual Meeting of the International Society for Experimental Hematology. All three papers will be submitted once we get the permission from Celmed Biosciences Inc. The manuscripts are:

1. **Q. Y. Dai**, J. Filep, D. El Kebir, P. Dubé, C. Scotto, D.C. Roy. Both apoptotic and necrotic cell death pathways are induced differentially using photodynamic therapy in EL4 cells. Experimental Hematology. 2005;33 (7)Supplement; 100

2. **Q. Y. Dai**, L. Busque, C. Maldonado, A. Balassy, D. C. Roy. Elimination of multiple myeloma cells in an ex vivo purging model using photodynamic therapy with the rhodamine-derived TH9402 photosensitizer.

3. **Q. Y. Dai**, M Guérin, C. Maldonado, C., D. C. Roy. Specific antitumor effect of a dendritic cell-based vaccine loaded with photodynamically treated tumor cells.

Chapters 2, 3, 4 are followed by a general discussion summarizing the data presented herein, but also primarily focusing on future directions.

Contributions of the authors

I am responsible for all the data presented in chapters 2, 3 and 4. A list of the work contributed by the coauthors of each manuscript is as follows:

Charpter 2: Dr El Kebir repeated Western blots for AIF and EndoG in treated cells at various time points. Pascale Dubé helped to measure the caspase activity using a colorimetric assay. Cecilia Maldonado participated in the preparation of PDT treated cells. C. Scotto participated to the evaluation of the results.

Charpter 3: Cecilia Maldonado performed the extract of DNA in myeloma cells. Antonia Balassy performed the progenitor cultures with semi-solid and LTC-IC assays. This data was used in figure3.3.

Charpter 4: Mireille Guerin repeated some flow cytometry analyses for the phenotype of DCs and ELISA tests for IL-12 and IFNγ. Cecilia Maldonado and Mireille Guerin also cultured DCs and helped with the measurements of tumor size and to monitor the mice after vaccination. Technicians at the animal facility collected blood samples from mice.

List of abbreviations:

AAD	Aminoactinomycin
ABA	Aaminobenzamide
AIF	Apoptosis inducing factor
ALA	Aminolevulinic acid
ATA	Aurintricarboxylic acid
ATCC	American Type Culture collection
BFU-E	Burst forming units-erythroid
BPD	Benzo-porphyrin derivatives
CFU	Colony forming units
CFU-GEMM	CFU-granulocyte/erythroid/monocyte/macrophages
CFU-GM	CFU-granulocyte/macrophage
CML	Chronic myeloid leukemia
CTL	Cytotoxic T lymphocytes
DC	Dendritic cells
Endo G	Endonuclease G
FBS	Fetal bovine serum
FSC	Forward Scatter
G-CSF	Granulocyte colony-stimulating factor
GM-CSF	Granulocyte macrophage colony stimulating factor
GvHD	Graft versus host disease
HPD	Hematoporphyrin derivative
HSC	Hematopoietic stem cell

HSP	Heat shock protein
IFN	Interferon
IL	Interleukin
LDA	Limitting Dilution Assay
LTC-ICs	Long-term culture–initiating cells
MBD	Methylene blue derivative
MDR	Multidrug resistance
MHC	Major histocompatibility complex
MM	Multiple myeloma
MPT	Mitochondrial permeability transition
NHL	Non-Hodgkin's lymphoma
PARP	Poly(ADP-ribose) polymerase
PBS	Phosphate buffered saline
PBSC	Peripheral blood stem cells
PDT	Photodynamic therapy
Pgp	P-glycoprotein
ROS	Reactive oxygen species
SSC	Side Scatter
TAA	Tumor associated antigen
TNF	Tumor necrosis factor

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Contributions to original knowledge

Chapter 2 shows that both caspase-dependent and–independent pathways are involved in EL4 cell apoptosis. TH9402-mediated PDT evoked disruption of mitochondrial membrane potential, resulted in cytochrome c release, and induced a caspase-dependent apoptosis. However, TH9402 evoked the release of AIF and Endo G from the mitochondria, suggesting the involvement of a caspase-independent pathway. This is the first demonstration of the participation of Endo G in PDT-induced apoptosis.

Chapter 3 presents the results concerning the use of TH9402 for ex vivo purging. The high selectivity of action of the photosensizer contributed to the elimination of myeloma cells, while preserving normal progenitor cells. Since there are very few reports of successful cancer cell purging with PDT in a clinical setting, these results indicate that TH9402 represents a very promising candidate for PDT-mediated tumor cell elimination in future clinical trials.

In chapter 4, we are providing the first demonstration that DC maturation can be induced by a combination of TNFα, IFNγ, IL-1β, and CD40L. A few reports show PDT can mediate an increase of immune responses. Two studies have described the use of vaccines based on PDT-treated cells for both preventive and therapeutic purposes. The data shown in chapter 4 indicate that PDT-treated cells can induce DC maturation and provide support to the hypothesis that DC-based PDT vaccines can successfully prevent tumor growth. This strategy could be beneficial to prevent disease recurrence in cancer patients. In addition, it may explain favorable results observed in patients receiving PDT-treated autologous stem cell grafts.

CHAPTER 1

Literature Review

1.1 Introduction to photodynamic therapy.

Photodynamic therapy (PDT) is a promising new modality for the treatment of cancers and some benign diseases. As currently practiced, it requires a sensitizer that is selectively activated by light in an appropriate wavelength. This treatment results in damage of the target cells/tissue through the release of molecular oxygen.

Treatments using light and light-activated compounds can be traced back in ancient times and were used to treat a wide variety of disorders.[1] Finsen discovered that light treatment could control skin manifestations of tuberculosis, a very common disease at that time. Similarly light could successfully treat other significant medical conditions such as rickets and neonatal hyperbilirubinemia. The use of an added chemical photosensitizer, rather than a natural chromophore was developed progressively. In Raab's initial work, when the dyes were added to Petri dishes of paramecia, an unexplained death during daylight experiments occurred. This was not observed during evening experiments. Rather than ignoring these findings, Raab systemically proved the connection between light activation of these dyes and therapeutic outcome. Continued work revealed the basis for the oxygen- and light-dependent photodynamic reaction and resulted in the important term of PDT.[2,3]

The current era of PDT research was initiated by Lipson and Schwartz at the Mayo Clinic, who reported in 1960 that injection of crude preparations of hematoporphyrin led to fluorescence of neoplastic lesions, visualized during surgery. Schwartz treated hematoporphyrin with acetic acid and sulfuric acid and obtained a porphyrin mixture that he termed "hematoporphyrin derivative" (HPD). Interestingly, HPD was employed first to try to render human tumors fluorescent for diagnostic purposes,[4,5] but some difficulty arose due to the fact that illumination of the tissues sometimes caused destruction of the

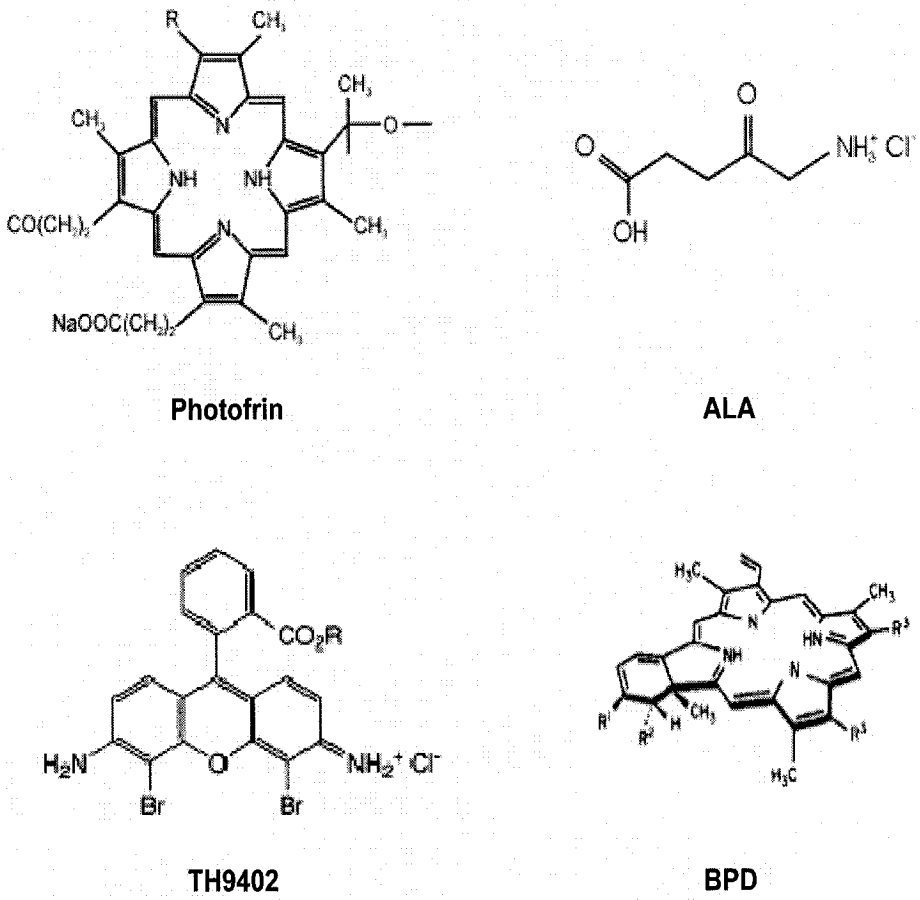


Fig.1.1 Molecular structures of photosensitizers

tumors as well as substantial clinical skin photosensitization. This finding gave rise to the first intentional PDT on a patient by Lipson, who treated a recurrent breast cancer with HPD, achieving partial improvement. Successful PDT was reported in glioma cells.[6] Further studies indicated that HPD consisted of a complex of many products, representing a mixture of some inert and active components. It contains several porphyrin, monomers as well as dimers and oligomers.[7] Dougherty analyzed and improved the HPD synthetic process to create a sensitizer which was more active and easy to produce. The less-active porphyrin monomers were removed and HPD was partially purified to form Photofrin.[8] Clinical research is directed toward usage of PDT for the treatment of neoplastic diseases which are otherwise difficult to control, including tumors of the skin, brain, head and neck, bladder and anywhere where light can be delivered either by surface irradiation or by fiber optics. Treatment of macular degeneration appears to be the major current clinical application.

Recently, extracorporeal PDT has been developed in a purging procedure, which uses safe photosensitizers and innovative irradiation devices for the effective eradication of neoplastic cells from grafts in autologous stem cell transplantation or for the deletion of alloreactive T cells in the treatment of graft versus host disease (GvHD).

1.2 Photosensitizer families.

Only three photosensitizers have received approval by the Food and Drug Administration (FDA) to date: Photofrin (Porfimer sodium), ALA (5-aminolevulinic acid) and Verteporfin (BPD, benzo-porphyrin derivative). (Structures shown in Fig.1.1)

Although Porfimer sodium is a complex mixture, it is now used widely and remains the most common photosensitizer for the treatment of non-dermatological tumors. The drug

has been approved for use in advanced and early stage lung cancers, superficial gastric cancer, oesophageal adenocarcinoma, cervical cancer, and bladder cancer.[9-12] It destroys tumors effectively and is non-toxic in the absence of light. No long-term safety issues have been reported and it was used systemically by intravenous administration in thousands of patients for more than 20 years. Photofrin can be used repeatedly without limit, even combined with other radiotherapy or chemotherapy. The major adverse reaction is a persistent photosensitization of the skin,[13] so that a patient has to remain away from bright light for several weeks after therapy. The initial selectivity between tumor tissue and healthy tissue can be low.[14] Moreover, the absorbance at 630nm, a wavelength to which tissues are moderately transparent, is very weak, therefore the depth of the effect is limited. This led to the development of other sensitizers.

Another porphyrin, which has had clinical and commercial success, is Verteporfin. Verteporfin is a benzoporphyrin derivative, which is clinically active when formulated with liposomes.[15] It activates at a higher wavelength for increased depth of light penetration and eliminates quickly from the body so that skin photosensitization is minimal.[16] Most of the clinical responses from Verteporfin sensitization are based on vascular disruption and shutdown; therefore, it would seem ideal for lesions depending on neovasculature. This drug is one of the most useful ophthalmology drugs ever developed and might have implication for cancer treatment.[17-19]

A notable element of 5- aminolevulinic acid (ALA) is a metabolic precursor of the photosensitizer protoporphyrin . ALA is the third sensitizer to be commercially available. It is a pro-drug that affects the biosynthesis of heme to create an excess of the natural porphyrin, protoporphyrin IX. While protoporphyrin eventually leaks into the circulation, it is sufficiently concentrated initially so that irradiation has a phototoxic effect.[20,21] ALA-

Table 1. Photosensitizer families.

Porphyrin platform
HPD (Photofrin)
BPD (Verteporfin)
ALA (Levulan)
Texaphyrins (Lutex)
Chlorophyll platform
Chlorins (Temoporfrin, NPe6, LS11)
Purpurins (Purlytin)
Bacteriochlorins (SQN400)
Dyes
Phtalocyanine (Photosens)
Napthalocyanine

based PDT is highly successful against basal cell and squamous cell cancers of the skin.[22,23] Caution is to be observed as lesions approaching 1cm will not usually be successfully treated by surface illumination and pain remains a common morbidity during therapy.

In clinical tests, photosensitizers arise from three families: porphyrins, chlorophylls, and dyes. (Shown in Table1) Chlorophyll like substances termed chlorines have excellent photosensitizing properties, as expected. Multiple drugs have been created including modifications of chlorophyll and chemically synthesized compounds. The synthetic chlorine temoporfin is a very potent sensitizer with a quantum yield of singlet oxygen of about 0.87. It is activated at 652nm, with a residual photosensitivity of only 2 weeks. Phase IIb studies in head and neck cancers have been completed, and this photosensitizer has also been studied in bronchial and esophageal tumors and in some gynecological applications. A developmental bacteriochlorin product SQN400 (mTHPBC, metatetrahydroxyphenyl bacteriochlorin), which absorbs light at 740nm, has entered phase I studies for the treatment of colorectal liver metastasis. Purins, degradation products of chlorophyll, also are clinically relevant. Dyes remain a fertile ground for the development of successful photosensitizers. Several other drugs are under investigation, such as tinetiopurpurin and phtalocyanine.[24]

1.3 Rhodamine derivative TH9402.

Extensive chemical and biological research has been done over the past 20 years to identify new photosensitizers with improved properties. There are four properties that would make a sensitizer optimal for use in photochemical cytotoxicity. a) selective retention or uptake by tumor, b) high quantum yields of singlet oxygen, c) sufficient

tissue penetration, and d) photolability.[25] Lipophilic cationic dyes, such as rhodamine 123, have been used as probes for membrane potentials in isolated mitochondria. These compounds penetrate plasma and mitochondrial membranes easily and can be concentrated up to 1000 fold in mitochondria. There is increased uptake or retention in malignant cells compared with normal epithelial cells.[26] Rhodamine dyes, because of their low toxicity and rapid elimination, are potentially useful reagents for PDT. However, the high quantum yields of fluorescence and consequently, low quantum yields of triplet formation limit their application. The specific objective was to design, synthesize and purify photoactive rhodamine derivatives, to enhance the formation of the triplet excited state from the singlet excited state. The addition of heavy-atoms could generate highly potent photosensitizers. Indeed, 4,5-dibromorhodamine 123 (TH9402) was selected because of its photophysical properties, low toxicity and stability. Singlet oxygen quantum yield is observed in brominated rhodamine 123. Calculated triplet quantum yield and singlet oxygen quantum yield values indicate that the energy transfer from the triplet state to singlet oxygen of dyes TH9402 and TH9402 butyl ester is almost 100%. Because of high quantum yields of singlet oxygen formation, this new photosensitizing molecule can be easily activated by light emission to affect tumor-cell killing.[27] Observations with fluorescence spectra and fluorescence microscopy indicate that TH9402 is localized only in mitochondria. Two binding sites are present at the lipid membrane of mitochondria.[28]

1.4 Selective tumor uptake and retention of photosensitizer.

Photosensitizers are selectively retained in tumor cells. The mechanisms involved in the preferential distribution of sensitizers in tumors are not fully understood.

In vivo, some photosensitizing drugs can reach higher concentrations in tumor tissue than in surrounding healthy tissue. Properties of tumor tissue may contribute to this selective distribution. These include elevated numbers of low-density lipoprotein receptors, the presence of macrophages, and a decreased pH value.

The association of the photosensitizer to low-density lipoprotein could result in selective or preferential release to neoplastic cells.[29-31] It is thought that tumors may catabolize low-density lipoproteins at higher rates than normal cells via endocytosis, suggesting that there could be selectivity in the uptake of photosensitizer bound by low-density lipoproteins. Many types of tumor cells express a high number of membrane receptors for low-density protein, which promote the internalization of the low-density protein-bound photosensitizer. Moreover, tumor-associated macrophages play a role in the tumor-selective uptake of aggregated sensitizers. In animal tumors, macrophages take up large amounts of HPD and Photofrin.[32-34] Selective retention may also be related to the decreased pH in tumors. Sensitizers become more water soluble as pH is decreased and would therefore be selectively retained. The interstitial fluid is the fluid surrounding the cells and localized between their plasma membranes and vascular walls. The pH value of interstitial fluid is lower and the content of lactic acid is higher in tumors than in most normal tissues. Although porphyrins is a complex with different ionic species, generally the lipophilicity as well as the cell uptake increases with decreasing pH.[35-37] In tumor-bearing animals, selective uptake of Photofrin takes place in some tumors.[38,39] Other theories regarding the selective retention of sensitizer include the abnormal structure of tumor stroma, a leaky vasculature and poor lymphatic drainage.[40]

Moreover, some, if not all, cationic drugs are recognized by the multidrug transporter, which excludes many cationic agents from cells that express multidrug resistance (MDR).

The mitochondrial localizing drug rhodamine-123 has been used to identify tumor cells expressing this phenotype by their low fluorescence. P-glycoprotein (Pgp), gene product of MDR, is associated with the efflux of cationic lipophilic compounds.[41] In previous studies, we found that, *in vitro,* $CD34^+$ cells accumulated lower amounts of TH9402 than B or T cells, and their corresponding myeloid and erythroid clonogenic progenitors were less sensitive to the PDT process. In addition, the lowest dye retention was observed in the more immature $CD34^+CD38^-$ cells, and reflected preservation of LTC-IC progenitors, which were least affected by the PDT process. Interestingly, immature progenitors are also known to retain less rhodamine and feature higher expression of P-glycoprotein (Pgp) compared to mature cells.[42,43] The addition of Cyclosporine A and verapamil, which are known for their ability to inhibit Pgp, will increase the intracellular concentration of TH9402 and drastically increase Pgp-expressing cell death.[44] Therefore, differential Pgp expression profile in various cells could explain why long-term hematopoietic progenitors are less sensitive to PDT than tumor cells. TH9402 phototherapy eliminates various cell populations according to a hierarchy that primarily reflects the profile of expression of Pgp. However, Pgp is not the sole determinant of dye retention in leukemias.[45-48] Indeed, PDT was found to be active against a large panel of tumor cell lines expressing high levels of Pgp, while doxorubicin, another known Pgp substrate, was effective only against a fraction of those lines.[49] The existence of rhodamine efflux+/Pgp- cells or rhodamine efflux-/Pgp+ subtypes in acute myelocytic leukemic cells also indicate that other mechanisms are also involved in rhodamine transport and retention.[50,51]

1.5 Intracellular photosensitizer localization and targets.

The subcellular localization of the photosensitizer has been shown to be a key factor in the outcome of PDT and has a strong influence on whether and to what extent cells undergo apoptosis or necrosis in response to photoactivation. Because of a short-life and a limited migration of singlet oxygen ($^1O_2$) from the site of its formation, initial cell damage of PDT is closely related to the localization of the sensitizer.[52] Many hydrophobic photosensitizers localize in the mitochondrial membranes and exert their primary action there, such as ALA and BPD.[53] Usually these sensitizers are very rapid inducers of apoptosis, in contrast to photosensitizers localized in lysosomes or at the plasma membrane. Cationic dyes usually preferentially target the mitochondria, which are important sites of action.[54] For TH9402, over 98% of intracellular fluorescence could be visualized around the mitochondrial membrane by fluorescence microscopy.

The most striking and immediate cellular in vitro response observed following HPD-based PDT is damage to the plasma membrane. Membranes are good targets by virtue of the water-lipid partition coefficient. Porphyrin uptake starts with a clearly defined initial binding within the plasma membrane, followed by migration to internal cellular regions.[55] Sensitizers localized at the plasma membrane are likely to cause necrosis during light exposure. Several photosensitizers are localized in lysosomes and cause cell death via two different routes: the release of lysosomal enzymes in the cytosol, or relocalization of the photosensitizer after illumination to other non-lysosomal targets.[56] Apoptosis induction is a much slower process than that induced by mitochondrial based photosensitizers.[57] Moreover, since most PDT sensitizers do not accumulate in cell nuclei, PDT has generally a low potential of causing DNA damage, mutations, and carcinogenesis.[58]

1.6 Mechanism of PDT effect on tumor destruction.

PDT requires exposure of cells to a photosensitizer followed by irradiation with visible light of an appropriate wavelength. It works only in the presence of oxygen. When oxygen is not present in the system or is present at levels of less than 2%, cells are resistant to PDT.[59,60] PDT cytotoxicity most likely occurs through photooxidative reactions. Upon absorption of a photon, the photosensitizer undergoes one or more energy transitions and usually emerges in its excited triplet state. There are two major reaction pathways open to the excited sensitizer. The triplet can participate in a one-electron oxido-reduction (Type I photochemistry) with a neighboring molecule, producing free radical intermediates that can react with oxygen to generate various reactive oxygen species (ROS). Alternatively, the triplet-state photosensitizer can transfer energy to ground state oxygen (Type II photochemistry), generating singlet molecular oxygen, a highly reactive form of oxygen that reacts with many biological molecules, including lipids, proteins, and nucleic acids.[61-64] The dominant mechanism for PDT in most circumstances in cells and tissues is type II photochemistry, since most photosensitizers for PDT are efficient producers of singlet oxygen in simple chemical systems. The interaction between the excited photosensitizer and molecular oxygen produces singlet oxygen as well as other ROS to induce cell death.

While PDT can result in direct killing of malignant cells, it was realized that there were other vital elements of the process. This included the partial or complete shut-down of the tumor vasculature.[65] Vascular damage contributes to long-term tumor control. Microvascular collapse can be readily observed following PDT and can lead to severe and persistent post-PDT tumor hypoxia.[66,67] The mechanism underlying the vascular effect of PDT is involved in vessel obstruction.

1.7 Immune mechanism and PDT.

The positive results obtained with PDT in a clinical setting have stimulated much interest in the mechanisms responsible for determining the efficacy of this treatment modality. In particular, important advances have recently been made in the understanding of PDT-elicited antitumor immune responses and their relevance to the therapeutic benefit of this approach.[68] Briefly, at least three major factors appear to be involved in the induction of a strong immune response against PDT-treated cancers. PDT-mediated oxidative stress triggers a variety of cellular signal transduction pathways,[69] that lead to increased expression of stress proteins and the induction of downstream early response genes, the products of which are transcription factors regulating the expression of various genes. Of particular importance, PDT has been shown to activate nuclear factor-κB and AP-1, which in turn control the expression of various cytokines and other immunologically important genes. Among the cytokines whose expression has been reported to be modulated by PDT are IL-6, IL-10, and tumor necrosis factor-α,[70,71] whereas several others, including IL-1ß, IL-2, and granulocyte-colony stimulating factor, may also be affected.[72,73] PDT is also known to increase the expression of various genes involved in cell adhesion or antigen presentation and these may further contribute to the development of the inflammatory/immune response elicited by this therapy.

Another important factor that contributes to the induction of PDT-mediated immune responses is the proinflammatory damage generated in cellular membranes and the vasculature of treated tumor and normal tissues.[74] These photooxidative lesions are responsible for the extensive release of various potent inflammatory mediators that provoke a prompt and strong inflammatory reaction at the PDT-treated site. A dominant event in such PDT-induced inflammation is a rapid and massive invasion of activated

inflammatory cells, including granulocytes, mast cells, and monocytes/macrophages, from the circulation to the PDT-treated site. These cells appear to be the main contributors to the inflammation-primed immune development process associated with PDT.

The nature, rate, and extent of tumor cell death induced by PDT may also play a crucial role in determining the generation of effective antitumor immune response. Large amounts of cellular debris are generated at a tumor site within a short time interval of PDT treatment. The particular nature of such material facilitates the uptake and presentation of putative tumor antigens by macrophages and dendritic cells recruited to the tumor site in response to PDT-induced inflammatory signals, ensuring the recognition of tumor-specific epitopes by T lymphocytes and their subsequent activation.[75]

The initial photooxidative injury (inflicted during exposure of solid cancers to photodynamic light) triggers a variety of responses, some of which indirectly lead to tumor destruction. Hence, in addition to the direct killing of tumor cells, secondary events including ischemia (subsequent to vascular damage), ischemia-reperfusion injury, the antitumor activity of activated inflammatory cells and tumor-specific T lymphocytes may contribute to the eradication of PDT-treated lesions.[76] Although the immune reaction may be less important than the other antitumor effects in the early stages of tumor ablation after PDT, its role can be decisive in attaining long-term tumor control.

1.8 Regulation of cell death in PDT.

Apoptosis has been found to be a prominent form of cell death in response to PDT. There are numerous examples in which the ability of PDT to initiate the apoptotic process differs depending on the cell line, the photosensitizer and its subcellular location, the overall dose and other conditions.

In all cases of death after PDT, outcome may depend on cellular mutations. Cells lacking critical components of the apoptotic /necrotic pathway may not display the full morphological characteristic of apoptosis. For example, the death response to ALA-PDT may depend on the particular cell type.[77] In some cases, more than one mechanism may be demonstrated, or a shift from apoptosis to necrosis may occur, probably due to multiple targets.

The subcellular location of a photosensitizer has a strong influence on whether and to what extent cells undergo apoptosis in response to photoactivation. Sensitizers that localize in mitochondria are likely to induce apoptosis, while sensitizers localized in the plasma membrane are likely to cause necrosis during light exposure. Dellinger[78] found apoptosis in CV-1 cells if they were photoirradiated 24 hours after introduction of Photofrin when the photosensitizer was internalized, whereas after only 1 hour, when Photofrin was primarily in the plasma membrane, necrosis was the predominant form of cell death. Kessel and Luo[79]studied a series of photosensitizers in L1210 leukemia and other cells and demonstrated that photosensitizers that bind to mitochondria induce apoptosis upon photoirradiation, whereas those that bind to the plasma membrane or lysosomes, but not to mitochondria, kill cells less efficiently and by a non-apoptotic mechanism.

The cellular response to PDT varies with its overall dosage. The photosensitizer methylene blue derivative (MBD) localizes in mitochondria of V79 cells. When MBD was present at concentrations above 0.05 μg/ml, photoactivation of the cells caused early (within 3 hours) apoptosis as the dominant mechanism of cell death. At 0.05 μg/ml, the process was much slower, and apoptotic cells appeared only after 1 day.[80] When different pathways of energy production are inhibited by high (glycolysis) or low (oxidative

phosphorylation) concentrations of Methylene Blue derivative, immediate or delayed apoptosis, respectively, are induced, whereas inhibition of both leads to necrosis. It appears that high photodynamic doses can cause a switch from apoptosis to necrosis when energy sources are highly depleted, because the former has a requirement for ATP.

To further evaluate the role of the subcellular localization of photosensitizers in the killing of V79 cells by PDT, Noodt *et al.*[81] compared two lipophilic porphyrins (3THPP and Photofrin) that localize to intracellular membranes, including mitochondria, and two relatively hydrophilic sulfonated porphines ($TPPS_{2a}$ and $TPPS_4$), that are taken up into lysosomes by endocytosis. PDT with either of the membrane-localizing photosensitizers resulted in increasing numbers of cells becoming apoptotic (TUNEL positive) during the first 12 hours, but apoptotic bodies were not observed. In contrast, after photoactivation of the lysosome-localized photosensitizers, apoptotic cells were not detected until after 12 hours but extensive fragmentation of the cells into apoptotic bodies was found. These data provide evidence for at least two distinct pathways by which PDT can induce apoptosis.

Although PDT can produce apoptosis or necrosis, or evoke a combination of the two outcomes, in many cases PDT is highly efficient in inducing apoptosis.[82] Thus, lower doses than those needed to produce necrosis may be very effective in eliciting cell killing. Furthermore, the efficient induction of apoptosis by PDT implies that PDT may be able to bypass mechanisms that make cells resistant to apoptosis in response to chemotherapeutic drugs and ionizing radiation.

1.9 Measurement of apoptosis.

Quantification of the precise extent of apoptosis *vs.* necrosis and total cell death in cultured cells is difficult and several issues need to be considered. Apoptosis is a

biochemical process that passes through several stages each with its own hallmarks and timing. The kinetics of the PDT-mediated apoptotic process depends upon a variety of factors, including the dose of the inducing agent, post irradiation time, light intensity and the ability of different cell types to carry out steps in the process. Thus, the percentage of cells in apoptosis will vary with time after initiation. Cells treated with doses higher than those needed to cause apoptosis may die without exhibiting the usual characteristics of apoptosis, and are generally regarded as undergoing necrosis. Late apoptotic cells or bodies can eventually lose the ability to maintain their membrane pumps and as a result may be unable to exclude vital dyes, such as trypan blue. Such apoptotic cells or bodies would then appear as necrotic cells. Therefore, one cannot conclude that a particular cell type or treatment does not produce an apoptotic response without studying a range of doses of the initiating agent and assaying for apoptotic markers over a wide range of times.

There are numerous assays for apoptosis. The two most common endpoints of apoptosis are morphological changes (cell shrinkage, condensation of nuclear chromatin, formation of apoptotic bodies) and DNA fragmentation into large fragments (300 bp and 50 kbp) and then to oligonucleosome-sized fragments (multiples of ~200 bp) that appear as a " ladder" of DNA bands upon agarose gel electrophoresis. Although observation of these endpoints is an indicator of apoptosis, quantification of the percentage of cells in a population that are in apoptosis is difficult or impossible. For this purpose, many investigators use the TUNEL assay during which fluorescent-labeled biotinylated nucleotides are added to the ends of DNA fragments within fixed cells. The cells are then stained in their DNA with propidium iodide and can be viewed on a microscope stage, or they can be quantified by flow cytometry to indicate the presence of viable, apoptotic,

necrotic, and late apoptotic cells. Apoptotic cells can also be detected with the protein Annexin V, which binds in a highly selective manner to phosphatidyl serine; this phospholipid flips from the inner to the outer leaflet of the plasma membrane during apoptosis.[83] This assay is also adaptable to quantification by flow cytometry or to imaging. Since many of the enzymatic steps in apoptosis are known, it is often desirable to follow the process by monitoring those steps. Antibodies are available commercially for all of the common caspases, so that procaspase levels and their proteolytic processing can be monitored by western blot analysis. In addition, model peptide substrates can also be purchased for many of the caspases in fluorogenic or chromogenic forms, permitting assay of cell extracts for the presence and level of individual active caspases. Inhibitors based on active site peptide motifs can be used to test the participation of suspected caspases. However, because caspases are not completely specific for the amino acid motifs, the model substrates and inhibitors cannot provide absolute identification of the involved caspase. Caspase action can also be monitored by assessing the cleavage of intracellular protein substrates; *e.g.*, cleavage of PARP or lamin B is a common indicator of the action of caspase-3 or -7 in apoptosis.

With any of these or other assays, it is advisable to study sufficient doses and post-treatment times so that the progress of cell death may be observed. In addition, it is necessary to use more than one assay to confirm results. Finally, if some or all of the cells do not undergo apoptosis, one cannot conclude that the cells remain alive, without conducting an assay for total cell death. The best assay for this purpose is a clonogenic assay, which measures the ability of each cell in a population to grow, replicate, and form a colony. Unlike assays for apoptosis or for viability, such as dye exclusion or tetrazolium reduction, clonogenic assays register all cell death events integrated over time.[84]

1.10 The role of mitochondria in apoptosis.

As numerous candidate photosensitizers have been developed and evaluated, one of the most prominent features of successful agents is targeting of mitochondria. Mitochondrial damage is a major cause of phototoxicity. Observations of mitochondrial damage after photosensitization have been made since early days of experimentation with PDT. This has been demonstrated by enzyme assays, electron microscopy of treated cells and tumors and fluorescence microscopy showing depolarization of mitochondria. That mitochondrial damage is responsible for cell death has been postulated for many years,[85] but the cause and effect relationship of mitochondrial damage and cell death was difficult to resolve. Many of the early experiments were performed at one to several hours after irradiation, and thus provided evidence of damage a long time after triggering events. Information showing that mitochondrial depolarization was an early step leading to cell death,[86] and that release of cytochrome c from mitochondria activated cytotoxic caspases gave indications that mitochondria were central in effecting death signals from upstream events.[87,88] Correlations were found between phototoxic cell death and very rapid mitochondrial depolarization, cytochrome c-release and activation of caspases within minutes after PDT.[89,90] Additionally, photosensitizers thought to be localized to non-mitochondrial organelles also caused depolarization of mitochondria,[91,92] suggesting a common pathway or low levels of highly active photosensitizer in mitochondria. It should be noted that although many of these events resulted in apoptosis, mitochondrial depolarization may also be an early event associated with necrosis.[93]

1.11 Apoptotic pathways.

The mitochondrial apoptotic pathway is triggered by diverse extracellular and intracellular stresses and developmental cues and requires the permeabilization of the outer mitochondrial membrane leading to the release of apoptogenic molecules, including cytochrome c, into the cytosol. Cytosolic cytochrome c binds to Apaf-1 which, in the presence of dATP or ATP, leads to pro-caspase-9 activation in the oligomeric complex called the apoptosome. Caspase-9 cleaves and activates downstream caspases including pro-caspase-3, precipitating apoptosis.[94] Concurrent with cytochrome c, other mitochondrial intermembrane proteins have been shown to be released as well into the cytosol. Apoptosis inducing factor (AIF) and endonuclease G (Endo G), once released into the cytosol, rapidly translocate to the nucleus where they promote nuclear DNA breakdown in a caspase-independent fashion.[95]

Chiefly two models have been proposed to explain how mitochondrial intermembrane proteins, including cytochrome c, are released from the mitochondria during apoptosis. A first model evokes the participation of the mitochondrial permeability transition (MPT), which has been defined as a sudden increase of the mitochondrial inner membrane permeability.[96] The MPT is thought to be due to opening of a regulated proteinaceous pore, which is a dynamic multimeric complex located at the contact site between the inner and the outer mitochondrial membranes. MPT pore openers cause permeabilization of the inner membrane thus dissipating the H^- gradient and causing a membrane potential loss which uncouples the respiratory chain. This model predicts osmotic matrix swelling and outer membrane rupture allowing cytochrome c and other intermembrane proteins to escape from mitochondria.

A second model predicts that the structural integrity of mitochondrial membrane would be maintained and that the formation of a specific outer membrane pore of the appropriate size would allow the passage of cytochrome c from the intermembrane space to the cytosol. Although the molecular nature of such pore is still elusive, a good candidate is the pro-apoptotic Bcl-2 family member Bax. Upon a death signal, cytosolic pro-apoptotic members relocalize to membranes, especially the mitochondrial outer membrane and exert their pro-apoptotic function by promoting the efflux of cytochrome c to the cytosol.[97,98]

1.12 Models of PDT in the clinic.

Regularly, PDT is used in a light directed manner. Photosensitizer is injected into patients intravenously or topically. The sensitizer will be accumulated at tumor sites. When the light is delivered through a fiber-optic device, the activated photosensitizer will release the singlet oxygen and reactive oxygen species (ROS) to induce cell death. Meanwhile, numerous preclinical studies have shown PDT *in vivo* enhances the host antitumor immune response. This immunologic effect could involve PDT-induced alterations in tumor microenvironment via indirect stimulation of proinflammatory cytokines, including tumor necrosis factor α (TNFα), IL-6 and IL-1.[99,100] The primary role of light directed PDT is to kill the tumor cells and this occurs through two major pathways: a direct killing, either by apoptosis and/or necrosis,[101] and an indirect pathway that will cause the recruitment of inflammatory cells (neutrophils, macrophages, etc) and the nonspecific activation of the immune system.[102,103]

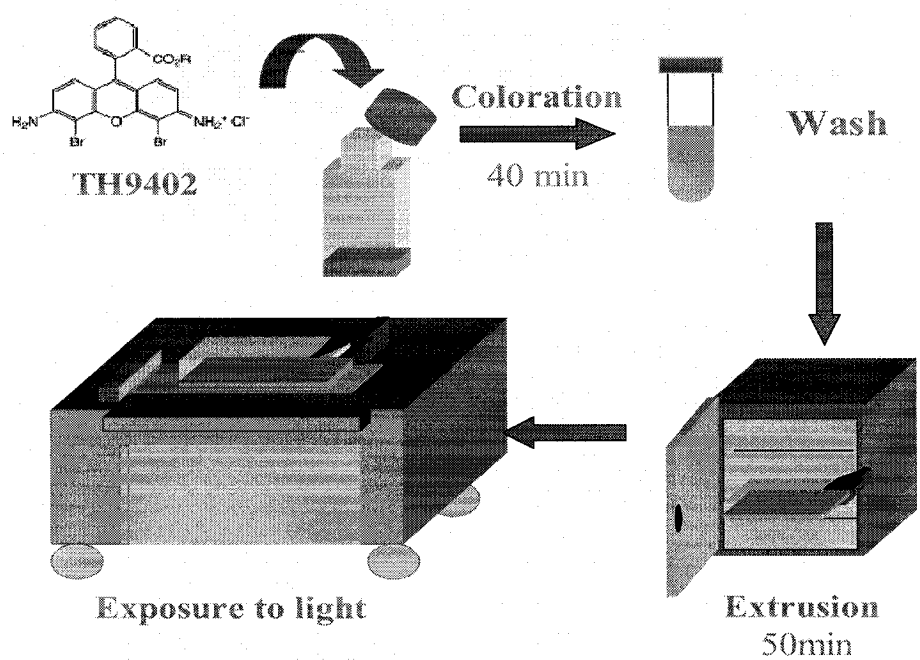


Fig.1.2 Purging procedure ex vivo

In addition to the above oncology trials, *in vitro* PDT has been tested for the purification of malignant hematologic components for autologous transplantation or elimination of alloreactive T cells in GvHD.[104,105] The whole procedure includes the incubation of cells with photosensitizer, extrusion of dye from the cells and exposure under the light (Fig 1.2). In many aspects, extracorporeal PDT harbors a greater flexibility than *in vivo* PDT. There is less stringent requirement for photosensitizer absorption spectra, as the target cells can be treated in a controlled environment where they can be photosensitized homogeneously using appropriate media, container, cell concentration and thickness of target area. The light source can easily be positioned directly adjacent to the target cells, allowing for the utilization of fairly simple set ups of irradiation devices.

1.13 Tumor cell purging.

Autologous bone marrow and stem cells are able to reconstitute hematopoiesis after myeloablative therapy for malignant diseases, resulting in cure in a substantial proportion of patients.[106] Disease relapse, however, is a common occurrence, and it has been hypothesized that, in some cases, this is due to reinfusion of small numbers of viable clonogenic tumor cells contaminating the stem cell inoculum.[107] Effective techniques for removal, or purging, of viable tumor cells from autologous harvests would theoretically circumvent this problem.[108]

For this strategy to be successful in practice, several problems must be overcome. As with all forms of autologous hematopoietic stem cell (HSC) transplantation, effective conditioning regimens must be available to eradicate residual disease before the transplant. Second, it is necessary to have access to relatively normal autologous HSC

with the least possible contamination with malignant cells. Finally, a highly selective purging process must be devised, capable of removing clonogenic tumor cells while sparing normal hematopoietic cells required for marrow engraftment. Because the cellular properties of the tumor cells in bone-marrow-derived diseases closely resemble those of normal stem cells, the selectivity of the purging process is a difficult and critical issue.

A variety of ingenious techniques have been devised for this purpose. First, the utilization of immunologic antibodies which bind preferentially to antigens expressed on the tumor cells but not on normal marrow stem cells. The removal of antibody-coated cells can be achieved by direct killing, using complement-mediated cytotoxicity or specifically constructed immunotoxins, or, alternatively, by coupling monoclonal antibodies to magnetic micropheres. Antibody-coated cells can be physically removed from marrow suspensions by placement in a strong magnetic field.[109]

The other major purging approach has been the use of pharmacological agents to treat marrow ex vivo. Pharmacologic purging is less selective than immunologic methods and relies upon the relative insensibility of resting marrow stem cells to those cytotoxic drugs that are capable of eliminating clonogenic tumor cells.[110]

Purging has also been attempted by positive selection of $CD34^+$ stem and progenitor cells, as opposed to negative selection of tumor cells. Advantages and disadvantages can be identified for either positive or negative selection strategies, but it has been difficult, if not impossible, to clearly compare these approaches, primarily because of limited accessibility and the emphasis given to optimize individual methodologies.

1.14 PDT bone marrow purging.

Despite all previously described research efforts, methods for bone marrow purging require significant improvements. An ideal agent for bone marrow purging would be characterized by high selectivity for malignant cells, with minimal toxicity to committed progenitor cells and stem cells. The procedure should be practical for clinical use and cost effective. Finally, it should be effective against drug-resistant tumor cells. Several other interesting approaches to marrow purging have been investigated at the preclinical level, with autografts also being performed in pilot studies in some instances. The sensitivity of some tumor cells, including leukemia, to light exposure after photosensitization with agents such as merocyanine 540 has been exploited for marrow purging, in view of the resistance of normal marrow cells to photolysis.[111,112] Indeed, photodynamic therapy has been successful in the selective elimination of alloreactive T cells[113]and treatment of graft rejection and graft-versus-host disease (GVHD). These results have revealed the interest in this approach.[114,115] Photosensitizers can be accumulated not only in immunoreactive T cells but also in malignant cells. There is now considerable evidence to suggest that it will be useful as a bone marrow purging technique in autologous bone marrow transplantation.

1.15 TH9402 in purging strategy

In a previous study from our laboratory, a successful purging of chronic myelocytic leukemia was achieved. Mixtures of apheresis product from healthy donors and leukemia cells K562 were treated by extracorporeal PDT using TH9402. This process resulted in the significant reduction or complete elimination of leukemic cells and sparing of normal progenitors. Four samples from patients were purged with conditions known to eradicate the cell line and yielded negative RT-PCR for *bcr/abl*. Normal progenitors were detected

with an average recovery of more than 80% of colony forming unit-granulocyte macrophages (CFU-GM).[116] These results demonstrated the efficacy of the purging procedure and encouraged us to develop this approach in various hematopoietic malignancies, such as non-Hodgkin's lymphoma (NHL) or multiple myeloma (MM). The whole procedure includes:1) cells suspension at certain concentration and incubation with TH9402 for 40 minutes. 2) dye efflux of cells in serum free medium for 50 minutes. 3) exposure under the light at 540 nm wavelength.

1.16 Cellular immunotherapy for cancer.

The importance of the interaction between the immune system and cancer cells was recognized in the 1890s when William Coley used streptococcal cultures to treat patients with advanced sarcoma. Numerous subsequent clinical trials have been conducted, particularly using irradiated whole tumor cells mixed with bacterial adjuvants. These attempts to activate general immunity led to clinical responses. More recently, antibodies and T cells that identify tumor antigens have been isolated from patients. Tumor and dendritic cell vaccines stimulate antitumor activity in the patient. The aim is to harness potent immunological weapons to destroy cancer cells.[117]

A potential target for cancer immunotherapy is the tumor associated antigen (TAA). It is immunogenic and expressed on tumor cells, but not on normal cells. Identification of tumor-specific antigens has been an important goal in the field of cancer therapy. Unfortunately, most tumor antigens are not sufficiently immunogenic to induce an effective immune response, and tumor specific antigens may vary between individuals with the same tumor type. Despite recent advances in the identification of an array of tumor antigens, it is still unclear which antigens are clinically relevant for the majority of

tumors. For this reason, interest developed in the use of whole tumor cells as vaccines, based on the concept that tumor antigens will be more immunogenic than more ubiquitously expressed antigens. Whole tumor cells carry all the potential TAA, that will be processed and presented on the DC surface MHC class I and class II molecules resulting in a polyclonal expansion of both $CD4^+$ and $CD8^+$ T cells.

1.17 Whole cell vaccines.

One of the earliest forms of cellular therapy consisted of whole tumor cells rendered safe by irradiation and mixed with an immunological adjuvant in some mouse strains. Mice could be partially protected from subsequent tumor challenge by a vaccination of tumour cells mixed with adjuvants.[118] Initial clinical studies showed the safety of this approach. An autologous irradiated vaccine with BCG as an adjuvant was used to treat 40 melanoma patients. There were five positive responses: four complete and one partial remission.[119] In patients with colorectal cancer vaccinated with autologous tumor cells vaccines mixed with BCG, although there was no difference in overall survival, significant improvements were seen in recurrence free survival, with the most benefit seen in patients with the lowest tumor burden.

With the increasing realization that immune responses against tumors are relevant, administration of cytokines is a non-specific way to activate the immune system on tumor regression. Peritumoral injection of low doses of cytokines could enhance the anti-tumor immune response without the side effects associated with systemic treatment.[120] A more recent approach is the use of vaccines containing genetically modified cells-gene modified vaccines, in which genes encoding key components of the immune response can be introduced into the tumor cells in vitro to increase the immunogenecity of the vaccine.

The most common gene modified vaccines use cytokines. The cytokine is produced in high concentration in the vicinity of the tumor cells, where it alters the local immunological environment and enhances the activities of antigen presenting cells and the activation of tumor specific T cells. This approach avoids the side effects associated with systemic treatment with cytokines. A number of cytokines have been used, with varying degrees of success. One murine study compared a number of different cytokines, using a number of poorly and moderately immunogenic tumor models, and demonstrated that of the cytokines used, tumors transduced with granulocyte macrophage colony stimulating factor (GM-CSF) produced the greatest degree of systemic immunity. Importantly, tumors genetically modified to express GM-CSF were able to cure pre-established tumors.[121,122]

1.18 Role of T cells in antitumor activity.

Several preclinical studies have demonstrated that activation of both $CD4^+$ and $CD8^+$ T cells is critical for generating the most potent antitumor immune responses.[123,124] These antigen-specific T-cell responses are initiated by professional antigen-presenting cells (APCs) (Fig.4). Any protein in the tumor cell is a potential tumor antigen. These antigens are released by secretion, shedding, or tumor lysis and captured by APCs. Uptaken antigens are processed and presented by major histocompatibility complex (MHC) class I and MHC class II molecules for priming and activation of $CD8^+$ and $CD4^+$ T cells, respectively. Eight– to 10–amino acid peptide fragments are presented on MHC class I molecules, whereas peptides presented on class II molecules are between 12 and 20 amino acids long.[125] Activated $CD4^+$ T cells provide important costimulation via cytokine secretion, which can initiate and also amplify the $CD8^+$ T-cell response. In addition,

memory $CD4^+$ T cells play a critical role in maintaining the protective immunity.[126,127] Ultimately, activated antigen-specific $CD8^+$ T cells become cytotoxic and lyse tumor cells.[128]

1.19 DC and tumor vaccines.

To date, tumor immunotherapy has only limited success. This can be explained by the limited availability of tumor-associated antigens and the inability to deliver such antigens in a manner that renders them immunogenic in patients with cancer. However, recent insights into the role of dendritic cells (DC) as the pivotal APC that initiate immune responses may provide the basis for generating more effective antitumor immune responses.[129]

The ability of DCs to generate antitumor immune responses *in vivo* has been documented in many animal models. Most of these experiments have involved *in vitro* isolation of DCs, followed by loading of the DCs with tumor antigen and injection of the antigen-bearing DCs into syngeneic animals as a cancer vaccine. Immunity produced by vaccines depends largely on the efficiency of antigen presentation. DCs are probably the means by which most vaccines work. They possess an extraordinary capacity to capture and process antigen and contain all that is needed to stimulate T cell immunity, including high levels of major histocompatibility complex (MHC), costimulatory molecules and adhesion molecules. These properties, coupled with the fact that it is now possible to generate, *ex vivo*, large numbers of functional DCs, have led to considerable interest in the use of DC vaccines as a mean to induce antitumor effect.

1.20 Defining DCs.

DCs have a distinct morphology characterized by irregular shape with the presence of numerous membrane processes that can extend for up to hundreds of micrometers. These processes can take the form of dendrites, pseudopods, or veils. Additional morphologic features of DCs include high concentrations of intracellular structures related to antigen processing such as endosomes, lysosomes, and the Birbeck granules of Langerhans cells. DCs are also characterized by the presence on their surface of large amounts of MHC class II molecules and the absence of lineage-specific markers including CD14 (monocyte), CD3 (T cell), CD19,20,24 (B cell), CD56 (natural killer cell), and CD66b (granulocyte).[130] Not surprisingly, in light of their antigen-presenting functions, DCs also express various adhesion and costimulatory molecules. Examples of the former include CD11a (LFA-1), CD11c, CD50 (ICAM-2), CD54 (ICAM-1), CD58 (LFA-3), and CD102 (ICAM-3), although all of these markers can be found on monocytes and macrophages.[131] Costimulatory molecules such as CD80 (B7.1) and CD86 (B7.2), and molecules regulating costimulation such as CD40 are also expressed on mature myeloid DCs.[132,133] DC phenotypes vary with different stages of maturation and activation. Human DC precursors circulating in the blood initially can express CD2, 4, 13, 16, 32, and 33, but they gradually lose their expression of these antigens with maturation. In contrast, adhesion molecules, costimulatory molecules, and MHC antigens increase with maturation. CD80 and 86 are upregulated with activation, particularly with CD40 ligation. CD86 tends to appear earlier in maturation, while CD80, which is almost unmeasurable in blood precursors, appears later.[134]

Several antibodies have been described that preferentially but not exclusively stain mature DCs. Antibodies reactive against human DCs include anti-CD83 and CMRF-44.

Antibodies to CD83 stain mature activated DCs, but not DC precursors, and also cross-react with activated B cells.[135] CMRF-44 can stain circulating blood DCs as well as activated DCs, but it also cross-reacts with macrophages and monocytes.[136] Antibodies directed at mouse DC include 33D1, N418 (antiCD11c), and DEC-205, although these antibodies also stain monocytes to varying degrees.[137-139]

With DC activation and migration from the tissues, antigen uptake activity and the associated antigen receptors are downregulated, resulting in a switch in APC function from antigen uptake to antigen presentation.[140] DCs are capable of processing antigen via classical pathways: endogenous antigens via the proteasome into the MHC class I compartment, and exogenous antigens via endocytic lysosomes into the MHC class II compartment.[141] DCs also possess alternative pathways of antigen processing and can route exogenous antigen into the MHC class I pathway through a mechanism known as cross-priming.[142] DC may also utilize molecular chaperones such as heat shock protein HSP96 to deliver antigens via the class I pathway.[143]

Sensitizing the immune system to specific antigens is certainly the most pertinent function for DCs, and this has been examined both in vitro and in vivo. Early studies in mice demonstrated that DCs exposed to infectious influenza virus or influenza nucleoprotein peptide, in vitro, induce a primary proliferative and antiviral CTL response.[144] In vivo priming of $CD4^+$ helper and $CD8^+$ cytotoxic T cells with antigen-loaded DCs has been demonstrated in several animal models. DCs can also prime immune responses to antigens in vivo in humans.[145,146] Moreover, administration of DCs loaded with tumor-associated peptides or proteins can lead not only to specific proliferative responses and CTL, but also to tumor protection in animal models.[147-150]

These observations provide a compelling rationale for pursuing DC-based immunotherapy for cancer.

1.21 Isolation of Dendritic cells.

Dendritic cells (DCs) are a trace but highly specialized subset of antigen-presenting cells (APC). Because no dendritic cell line exists, these cells must be freshly isolated from tissue before each experiment. Two methods are established for preparing dendritic cells. The first method involves the isolation of DCs from mouse spleen, resulting in a cell population that is highly enriched in accessory cell and APC function.[151] The isolation relies upon a transient exposure of the cells to tissue culture plates, which allows for the depletion of adherent monocytes. Lymphocytes are then depleted by passage over a bovine serum albumin (BSA) gradient to isolate low-density DCs. Collagenase digestion of splenocyte suspensions can be used to increase the yield of dendritic cells. But, in general, these techniques yield low numbers of DCs, making experiments difficult to perform. The second method involves generating large numbers of DCs from mouse bone marrow progenitor cells.[152] Bone marrow progenitor cells are cultured in the presence of granulocyte/macrophage colony-stimulating factor (GM-CSF) to stimulate proliferation and maturation of DCs. Once generated, the large numbers of DCs can be used for cell biology studies, genetic modification, and *in vivo* immunization. In our study, we used a novel bulk-culture method for generating mature dendritic cells from mouse bone marrow cells, as described before.[153] There are several advantages: First, it is easy and less time-consuming to establish the culture, since unseparated bulk BM cells were taken as DC source. Second, yield and purity of bulk-DC are higher than those obtained by the conventional method when compared on the same day of culture. Third, bulk-DCs are

more potent in stimulating DCs. Fourth, bulk-DCs are more potent in stimulating allogeneic T cells and presenting peptide antigens to T cells, compatible with their more matured phenotypes. Finally, the bulk-culture method potentially costs less, since no Abs and complement are needed for the depletion of T-and B-lineage cells.

As with the animal models, more recent approaches for generating DC from human bone marrow precursors utilize $CD34^+$ cells cultured in the presence of exogenous GM-CSF, usually in combination with IL-4 and/or TNF-α.[154-157] Stem cell factor (SCF) and/or Flt3-ligand (FL) are often added to increase DC yields by inducing the proliferation of DC progenitors.[158,159] Sources for human $CD34^+$ precursors include bone marrow, cord blood, and G-CSF-mobilized peripheral blood.

Many groups have generated DC-like cells by culturing $CD14^+$ monocyte enriched PBMC in vitro. When cultured for 1–2 weeks with media supplemented with GM-CSF and IL-4, monocytes give rise to large numbers of cells that are morphologically and phenotypically similar to the "classical" density purified DCs. These cytokine-generated DCs require additional maturation in vitro with TNF-α or monocyte-conditioned media in order to fully stimulate in an allogeneic MLR or prime antigen-specific T cell responses in vitro and in vivo. Moreover, without this additional maturation step, the DC phenotype can revert to that of a monocyte. Nonetheless, these "monocyte-derived DCs" are capable of inducing strong antitumor responses in vivo, in mice.[160,161] Recently, clinical trials using these cells in patients with melanoma and renal cell carcinoma have demonstrated induction of specific immune responses as well as tumor regression.[162,163] One advantage of this approach includes easy access to $CD14^+$ monocytes, but it requires ar least one week of culture *in vitro*.

It is now possible to generate large numbers of DCs from mobilized $CD34^+$ haematopoeitic stem cells or from peripheral blood monocytes, and to define them phenotypically by a panel of surface markers. There is still no single marker that is specific for DCs.

1.22 Interaction of T cells and Dendritic Cells.

For an effective T-cell mediated immune response, T-cells require antigens to be presented to them to sensitize naive T-cells and to re-stimulate primed T-cells. Antigen presentation is therefore a crucial step in the initiation of an effective immune response. To initiate T-cell immunity, peptides from infected cells located anywhere in the body must be recognized by circulating T-cells. Tumors have few MHC molecules on their surface and usually lack co-stimulatory molecules. DCs enable the immune system to tackle these difficulties and this property has led to an interest in the development of DC vaccines. The unique capacity of these "mature" DCs to activate T-cells is probably related to the presence of an exceptionally high number of MHC, co-stimulatory and adhesion molecules. DCs generated *ex vivo* and loaded with tumour antigen prior to re-infusion are now entering clinical trials.

In order to effectively exploit DC function, the different stages of DC maturation must be understood. DC precursors migrate from bone marrow and circulate in the blood to specific sites in the body where they mature and act as sentinels for the immune system.[164] Tissue-resident DCs process and present antigens in the context of MHC class I and II molecules. Sources of antigen can include viral proteins, bacterial proteins, or apoptotic bodies.[165,166]

In addition to encountering antigen, DCs are thought to require an antigen-independent danger signal in order to become activated. These signals can include LPS, interferon (IFN) α and γ, IL-1β, and potentially direct signals from viruses and bacteria. With activation, DCs downregulate their antigen uptake and processing functions, shift to antigen presentation and upregulate their expression of MHC, costimulatory, and adhesion molecules as described earlier. With maturation during in vitro culture, MHC class I and II densities on the cell surface increases 10–100-fold, while MHC synthesis is reduced. Class II peptide-MHC complex turnover falls dramatically, as reflected by the change in surface halflife of these complexes from a few hours on immature DCs to 2 days on mature DCs, providing a stable source of processed antigen when danger signals are received.[167]

Once activated, DCs leave the tissues and migrate via the afferent lymphatics to the T cell–rich paracortex of the draining lymph nodes. Within the secondary lymphoid organs, activated DC attracts naive and memory T cells for priming. Activated DCs also secrete cytokines including IL-7 and IL-12, which contribute to their unique functions. IL-7 can induce CD4 and CD8 T cell proliferation and B cell differentiation.[168,169] IL-12 biases T helper responses toward a Th1 pattern.[170,171] This combination of cytokines will typically bias T helper responses to Th2 or antibody generation. This stimulatory milieu produced by activated DCs, combined with the presentation of epitopes in MHC class I and class II and the expression of costimulatory molecules, contributes to the generation of potent antigen-specific immune responses.[172] Within the secondary lymphoid tissues, activated $CD4^+$ T cells can further activate DCs through CD40/ 40L interactions, and this also provides a survival signal to DCs.[173-175] Furthermore, CD40 ligation allows DCs to prime CTL, even in the absence of immediate $CD4^+$ T cells help.

1.23 PDT-generated vaccines.

The PDT generated vaccines belong to the category of target tumor-derived whole cancer cell-based vaccines. Antigenic repertoire contained in whole tumor cell derived vaccines allows for circumventing the problems associated with shedding or downregulation of specific antigens as well as with MHC-restricted epitope identification for individual patients. Moreover, targeting tumor antigens unique to individual patients generated by random mutation in cancer cells may be important for securing an effective therapeutic response.[176,177] In PDT, cells can be induced through apoptosis and necrosis. Secondary necrosis is readily formed over time. Such a dynamic and viable process may be used as a source of TAAs for a vaccine strategy. Two elements seem relevant for the unique capacity of PDT for cancer vaccine generation: surface expression of HSP70 and binding of complement proteins on PDT-treated cells. There are reports on the induction by PDT of HSP70 expression on the surface of treated cells,[178] as well as on the fixation of complement proteins on these cells.[179] Both HSP70 and complement proteins are expressed in high levels on the surface of PDT-vaccine cells retrieved from the vaccination site after their tumor-localized administration. The surface exposure of HSP70 facilitates the opsonization of C3 or its fragments on PDT-treated cells. This statement is supported by results showing that binding of C3/C3 fragments on vaccine cells was inhibited by the co-administration of HSP70-blocking antibodies at the time of vaccination. Complement proteins may be opsonizing peptides associated with surface-expressed HSP70, recognized as altered self-targets by the complement system. Alternatively, they could directly bind HSP70 directly.[180] Relevance of complement in the efficacy of PDT-vaccine is demonstrated by the lack of therapeutic benefit against tumors growing in C3-deficient mice.

Generation of antitumor vaccines has be exploited in both preventive and therapeutic treaments. Gollnick et al[181] were successful in providing protection against EMT6 tumor challenge by vaccinating naïve mice with supernatants of PDT-treated EMT6 cells. They showed that PDT-generated tumor cell lysates have the capacity to stimulate both the phenotypic and functional maturation of DCs, and to induce a cytotoxic T cell response. Korbelik et al[182] developed a PDT-generated therapeutic cancer vaccine. They found that the growth of established subcutaneous, poorly immunogenic mouse tumors could be retarded by lesion-localized injection of tumor cells of the same origin, which were previously treated in vitro by PDT. In the present study, we describe the development of tumor vaccine using photosensitizer TH9402.

Specific aims and preface to chapter 2.

Photodynamic therapy (PDT) is a cancer treatment showing dramatic antitumor activity. The novel photosensitizer TH9402 is a rhodamine derivative with favorable photophysical properties, low intrinsic toxicity and high stability. While the cytotoxic effect of TH9402 has been described previously, the induction of cell death pathways has not been investigated.

PDT may generate either apoptosis, necrosis, or both. The goal at the start of this project was to modulate the different conditions of PDT to induce EL4 cell death. Investigation of the potential mechanism in EL4 cell death pathways will be helpful to test the advantage of PDT to overcome tumor cell resistance during the treatment. The establishment of the best conditions inducing EL4 cell death is a prerequisite for subsequent evaluation of the different whole tumor cell vaccines in vitro and to identify optimal sources for antigenic stimulation.

CHAPTER 2

Both apoptotic and necrotic cell death pathways are induced differentially using photodynamic therapy.

2.1 Abstract

Purpose: Photodynamic therapy (PDT) is a powerful strategy to eliminate a variety of tumor cells and alloreactive T lymphocytes. While the cytotoxic effect of the novel rhodamine-derived TH9402 photosensitizer has been attributed to the generation of reactive oxygen species and singlet oxygen, the cell death pathways involved have not been fully elucidated. In this study, we evaluated the ability of PDT to induce apoptosis and necrosis, and assessed underlying molecular mechanisms.

Results: We found a direct correlation between TH9402 concentration (0-10μM) and intensity of light exposure (0-10J/cm^2) with the extent and rapidity of elimination of mouse lymphoma EL-4 cells. Increasing the intensity of PDT shifted the ratio from predominantly apoptotic to necrotic cell death as measured by Annexin-V/7AAD at 2 to 4h post-PDT. PDT (10μM and 5J/cm^2) evoked marked decreases in mitochondrial transmembrane potential (assessed with $DiOC_6(3)$) as early as 2h after PDT. These were associated with decreased presence of Bax in the cytosol. PDT also resulted in activation of caspase-9 and caspase-3 as early as 15 minutes after PDT through the release of cytochrome C from the mitochondria. However, pan-caspase blockade with ZVAD-FMK or a specific caspase-3 inhibitor DEVD-CHO failed to alter the percentage of PDT-induced AnnV+/7AAD- cells and did not produce detectable changes in DNA fragmentation, indicating involvement of a caspase-independent death pathway. Indeed, TH9402 also evoked the release of AIF and endonuclease G from the mitochondria, suggesting involvement of a caspase-independent pathway.

Conclusion: Under conditions that induce apoptosis, PDT with the TH9402 photosensitizer activates both caspase-dependent and -independent death pathways.

TH4902 activation of multiple death pathways suggests that TH9402 could effectively limit the capacity of tumor cells to become resistant to this compound.

2.2 Introduction:

Photodynamic therapy (PDT) has the potential to become a useful anti-cancer strategy.[1] Compared with other irradiation techniques and chemotherapy, PDT is associated with low toxicity that allows its repeated usage.[2,3] It has been shown active against locally advanced malignant disease in a context of palliation, and for long-term control of early stage cancer.[4-7] Interestingly, other agents with chemotherapeutic effects or oxygen carrier properties can be combined with PDT to enhance its effect and accelerate tumor cell death.[8,9]

The procedure requires exposure of cells or tissues to a photosensitizing drug followed by irradiation with visible light of the appropriate wavelength, usually in the red or infrared region, as compatible with the absorption spectrum of the dye. Once activated by the light, the photosensitizer absorbs energy directly and interacts with molecular oxygen to yield an excited form called singlet oxygen. This highly reactive form with direct cytotoxic activity contributes to the destruction of tumor cells.[10] A few products with photosensitizing ability have been used in clinical trials for a wide variety of cancer treatment indications.[11-15] However, limitations often include persistent photosensitization of the skin and poor tissue penetration, associated with red light usage. Such limitations have motivated the development of molecules with improved pharmacokinetics and photochemical characteristics.

Rhodamine-123 is a potentially appealing molecule because of its high penetration in tissue membranes and preferential retention in malignant cells.[16] Among a large number of rhodamine derivatives, 4,5-dibromorhodamine 123 (TH9402) was selected because of its favorable photophysical properties, low toxicity and stability. Because of high

quantum yields of singlet oxygen formation, this photosensitizing molecule can be easily activated by light emission to induce cell death.[17]

The biological effect of PDT depends on either apoptosis or necrosis, or a combination of the two outcomes by reactive oxygen species and singlet molecular oxygen.[18-20] Recent studies have explained the mechanism of apoptosis after PDT in relation to mitochondrial photodamage. Photosensitizers are known to induce apoptosis rapidly through activation of the mitochondrial pathway. This includes cytochrome c and other apoptotic factors release, caspase activation, PARP cleavage, chromatin condensation and DNA fragmentation.[21-25]

In this study, we investigated the ability of TH9402 PDT to evoke apoptosis and the impact of treatment conditions on modulation of cell death pathways. We also studied the apoptotic pathways involved.

2.3 Materials and methods:

Cell culture. Mouse T lymphoma cell line EL4 was obtained from the American Type Culture Collection (ATCC). Cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% horse serum, 100 U/mL penicillin, 100 μg/mL streptomycin (all from Invitrogen) in a humidified atmosphere at 37^0C with 5% CO_2.

Kinetic of PDT-mediated cytotoxicity. Cells were incubated at a concentration of 10^6/ml for up to 90 min in 10ml DMEM phenol red-free medium (Invitrogen, Grand Island, NY) containing 2.5% serum or without serum in different concentrations of TH9402 (2.5, 5 or 10μM). After 40 min incubation, cells were centrifuged and resuspended in

photosensitizer-free medium with 10% serum. The cells were taken at different time points during the incorporation and clearance periods and TH9402 content was determined immediately using flow cytometry.

Photodynamic therapy. Cells were incubated with 2.5, 5 or 10μM TH9402 (Celmed BioSciences, Montreal, Canada) for 40 minutes in medium containing 2.5% serum, centrifuged and resuspended in a dye-free medium with 10% serum for 50 minutes extrusion (dye efflux), and subsequently exposed to 5 or 10 J/cm^2 light at 540nm wavelength using a light scanning device (Theralux, Celmed Biosciences). Cells in medium without dye during incubation were used as controls. In apoptosis studies, cells were preincubated with Z-VAD-FMK (BD-Pharmingen, San Diego, CA), a pan-caspase inhibitor, DEVD-CHO (Calbiochem, San Diego, CA), a specific caspase-3 inhibitor; aurintricarboxylic acid (ATA), a general DNase inhibitor, (Sigma, Canada) or 3-aminobenzamide (3ABA) (Sigma), a Poly(ADP-ribose) polymerase (PARP) inhibitor for 1 hour before PDT treatment.

Assessment of cell death. To assess apoptosis and necrosis in the same cell population, cells in exponential growth phase were seeded at a final concentration of 10^6 cells/ml either in a six-well plates, sample thickness 3mm, or in a flask, sample thickness 10mm. After PDT, cells were stained with Annexin V-CY5 and 7AAD (BD-Pharmingen) according to manufacturer's protocol and fluorescence evaluated by flow cytometry (Becton Dickinson).

Measurement of mitochondrial membrane potential ($\Delta\Psi_m$). Changes in $\Delta\Psi_m$ were monitored by the uptake of $DiOC_6(3)$. $DiOC_6(3)$ (Molecular Probes, Eugene, OR) was dissolved in ethanol at 0.5mM. After PDT, cells were centrifuged and resuspended in culture medium containing 25nM $DiOC_6(3)$, and then incubated at 37°C. Cell aliquots were obtained at 2, 4, and 6 hours and fluorescence determined by flow cytometry.

Caspase activity assay. After PDT, cells were centrifuged and resuspended in culture medium. Caspase-9 and caspase-3 activities were measured at 15, 30 and 45 minutes post-PDT using colorimetric assay kits (Chemicon International, Temecula, CA) following manufacturer's instructions.

Western Blot analysis. Total cellular protein extracts were prepared in lysis buffer (20 mM Tris, 1 mM EGTA, 2 mM Na_3VO_4, 25 mM NaF, 0.5% (vol/vol) Triton X-100, 2 mM PMSF, 40 μg/ml aprotinin, and 10 μg/ml each of chymostatin, leupeptin, and pepstatin A). The extraction of proteins in mitochondria and nuclei were performed using corresponding kits (Pierce, Rockford, IL) according to the manufacturer's instructions. The protein content was measured using Bio-Rad (Hercules, CA) protein assay reagents and aliquots were analyzed on 12.5% SDS-PAGE gels. After transferring protein onto PVDF membrane, the proteins were probed with monoclonal anti-Bax, anti-cytochrome c, anti-AIF (Santa Cruz), anti-endonuclease G (EndoG, Chemicon), anti-actin (NeoMakers, Fremont, CA) and anti-histone H1 antibodies (Upstate, Lake Placid, NY). The immune complexes were detected by a chemiluminescence system (Amersham).

Analysis of DNA fragmentation. DNA fragmentation was assayed by gel electrophoresis as described.[26] Briefly, following cell lysis, low molecular weight DNA was precipitated, resuspended in Tris-EDTA buffer, treated with 25 μg/ml RNase A (Roche, Switzerland) for 15 min, denaturated for 5 minutes at 65°C, and then subjected to electrophoresis on 1.5% agarose. After staining with ethidium bromide, DNA was visualized by UV examination for image analysis.

Statistical analysis. Data are expressed as mean ± S.E from a minimum of three experiments, unless otherwise indicated. The significance of differences between experimental conditions was examined using Student's *t*-test.

2.4 Results:

Kinetics of TH9402 retention in EL4 cells.

As a rhodamine derivative, fluorescent emission of TH9402 can easily be detected using flow cytometry, and fluorescence intensity corresponds to the actual intracellular concentration of the dye.[27] Cell samples were taken at different time points during the incorporation and clearance periods. Initial experiments showed that maximum dye incorporation occurred within 40 minutes for all groups exposed to 2.5, 5, 10 μM TH9402 and that intracellular clearance reached a stable level in 50 minutes (Fig.2.1). Therefore, 40 minutes incubation, 50 minutes extrusion was chosen for the dye accumulation and retention during the PDT treatment.

Uptake of TH9402 in EL4 cells in the medium with or without serum.

Cells incubated without serum were induced more rapidly into death with a lower concentration of TH9402 (1 and 2.5μM) than cells exposed to TH9402 in presence of serum (data not shown). In order to determine if this difference was attributable to different levels of TH9402 intracellular content, TH9402 retention was measured using flow cytometry (Fig.2.2). Incorporation of TH9402 was greater in samples without serum than in those with serum. In serum-free conditions, even the lowest concentration (2.5 μM) led to intracellular saturation of TH9402, while much higher dye concentrations were required to achieve similar levels in presence of serum.

Cell killing induced by PDT is dose dependent.

A direct correlation was detected between TH9402 concentration (0-10μM) or intensity of light exposure (0-10J/cm^2) and the extent of elimination of EL-4 cells. TH9402 at 5μM and 10 μM in the presence of serum evoked cell death. Increases in light intensity enhanced the cell death process. These results indicate that both TH9402 concentration and light intensity have an impact on the induction of EL4 cell death (Fig.2.3). Similar results were obtained at 24h post-PDT (data not shown).

PDT induces both apoptosis and necrosis.

Cells treated under different conditions of PDT revealed different profiles of cell death. At 2h post-PDT using 10μM TH9402 and 10J/cm^2 illumination, the majority of cells were 7AAD positive (Fig.2.4A). Necrosis was evident soon after PDT. However, decreasing light intensity by decreasing energy delivery (5J/cm^2) and increasing sample

thickness shifted the ratio from predominantly necrotic to apoptotic cell death (Fig.2.4B). Apoptotic cells progressed to a 7AAD+ status and necrosis at 4h and 24h post-PDT.

Disruption of mitochondrial membrane potential in PDT.

To study the induction of apoptosis, we measured the impact of PDT on mitochondrial transmembrane potential using the fluorochrome $DiOC_6(3)$ and flow cytometry[28]. PDT induced a rapid loss of $\Delta\Psi_m$. At 2h post-PDT, cells retained less DiOC6 because of the collapse of membrane potential. At 6h post- PDT, a marked decrease as represented by the dramatic shift to left of fluorescence intensity was detected (Fig.2.5). Most cells had lost their membrane integrity. In contrast, no change in $\Delta\Psi_m$ was observed in untreated cells. Thus, these results indicate that TH9402 is involved in mitochondrial damage.

Involvement of a caspase-dependent pathway in apoptosis.

Since PDT-mediated disruption of the mitochondrial membrane would be expected to induce sequential activation of caspase-9 and caspase-3, their enzymatic activity was measured after PDT. Caspase-9 and caspase-3 activity was barely detectable in non-treated EL4 cells. PDT using TH9402 at 10μM, $5J/cm^2$ resulted in marked increases in caspase activity as early as 15 minutes after PDT (Fig.2.6). The maximum level of enzyme activity for caspase-3 was maintained until 2h post-PDT (data not shown). These results indicate that TH9402 induced cell apoptosis via a caspase-dependent mechanism.

To investigate whether TH9402 activates the intrinsic or mitochondrial pathway of apoptosis, Western blot analysis was performed to examine the presence of AIF and cytochrome c in mitochondria. The level of AIF in the mitochondrial fraction

significantly decreased within 30 minutes of PDT. Cytochrome c also decreased 60 min post-PDT. While Bax levels in whole cell extracts remained stable throughout the 60 min time period after PDT, cytosolic Bax level decreased with time and was barely detectable at 60 min post-PDT (Fig.2.7A). AIF was not detectable in the cytoplasm of untreated cells. However, steadily increasing amounts of AIF were detected in the cytosolic fraction up to 1h post-PDT (Fig.2.7B).

TH9402-induced apoptosis in EL4 cells is also caspase-independent.

To assess the role of caspases in TH9402-induced cell death, EL4 cells were preincubated with the pan-caspase inhibitor Z-VAD-FMK at 20μM or with the caspase-3 specific inhibitor DEVD-CHO at 10μM before PDT. DEVD-CHO completely inhibited caspase-3 activity (Fig.2.8A). Surprisingly, Z-VAD-FMK had little effect on caspase-3 activity in EL4 cells compared with that observed in human neutrophil granulocytes (data not shown). Caspase-3 inhibition did not result in changes in the percentage of apoptotic cells, indicating involvement of pathway(s) other than caspases in TH9402-induced apoptosis (Fig.2.8B).

DNA fragmentation was also examined after PDT. In the absence of PDT, there was no DNA fragmentation in a control sample. However, marked DNA fragmentation was detected in cells at 2h post-PDT, and the intensity of the bands seems increased at 4h post-PDT (Fig.2.9B). The endonuclease G inhibitor, ATA, with DEVD-CHO partially inhibited DNA fragmentation, whereas blockade of caspase-3 or PARP alone produced no detectable effects (Fig.2.9A). Nuclear content of EndoG in TH9402 treated cells was consistently higher than that observed in nuclei of untreated cells (Fig.2.7C).

2.5 Discussion:

PDT using a single reagent TH9402, a novel rhodamine derivative photosensitizer, combined with a photoilluminator, offers a platform which could be easily applied in extracorporeal purging for the deletion of immunoreactive T cells in GvHD or neoplastic cells in autologous bone marrow transplantation.[29-32] The cytotoxic effect of TH9402 has been attributed to the generation of reactive oxygen species, in particular to the formation of singlet oxygen. In this study, we evaluated the ability of PDT to induce apoptosis and necrosis in EL4 cells, and assessed the underlying molecular mechanisms.

We found complete dye saturation in cells treated with 10μM TH9402 in serum-supplemented medium. Similar results were obtained with a lower concentration (2.5μM) in serum free conditions. The most likely explanation for this phenomenon is that the presence of serum facilitates dye efflux. The extent of photodynamic activity depends not only on the efficacy of singlet oxygen production or free radical generation, but also on the kinetics of the dye-binding sites, cellular retention and extrusion. Thus, our results indicate that the augmented intracellular retention of lower concentrations of TH9402 in serum-free conditions could result in effects similar to those detected with higher concentrations of TH9402 in the presence of serum. This phenomenon will need to be investigated further since it could promote enhanced tumor cell elimination and facilitate the purging procedure in clinical applications.

Although PDT can induce apoptosis, necrosis, or both, in most cases PDT with agents other than TH9402 was found to be highly efficient in inducing apoptosis.[33-36] Quantification of the precise extent of apoptosis versus necrosis was done by flow cytometry monitoring.[37] The ratio between necrosis and apoptosis could depend on the

concentration of photosensitizer and /or the light dose.[38,39] PDT with TH9402 was found to induce high levels of cell death. Decreased light intensity and/or increased sample thickness, which also lowers energy delivery, resulted in a switch from necrosis to apoptosis. Since TH9402 is a photosensitizer localized in mitochondria, induction of necrotic and apoptotic pathways might be attributed to photodynamic damage of different mitochondrial targets. High photodynamic doses can cause a shift from apoptosis to necrosis when energy sources are depleted, because the former has a requirement for ATP.[40-42] Photodynamic modulation of target cell death pathways could be used for whole cell vaccine preparation, since apoptotic or necrotic mortality may induce different effects on the presentation of tumor antigens to dendritic cells.

The mitochondrial apoptotic pathway is triggered by oxidative stress and requires the permeabilization of the outer mitochondrial membrane. This leads to the release of pro-apoptotic molecules, including cytochrome c and AIF, into the cytosol. Cytosolic cytochrome c together with Apaf-1 and pro-caspase-9 form the apoptosome, leading to activation of caspase-9. Caspase-9 cleaves and activates downstream caspase including pro-caspase-3, precipitating apoptosis.[43] In our study, PDT resulted in immediate decreases in mitochondrial content in both cytochrome c and AIF and marked increases in the activity of caspase-9 and caspase-3.

It has been proposed that permeabilization of the inner mitochondrial membrane and subsequent loss of $\Delta\Psi_m$ triggers the release of intermembrane proteins.[44] Our results showed that $DiOC_6(3)$ fluorescence was decreased by 40% at 2h after PDT, suggesting that TH9402 induced the collapse of $\Delta\Psi_m$ through the induction of mitochondrial inner membrane permeabilization.

Furthermore, another model involving the mitochondrial pathway of apoptosis is related to Bax, a cytosolic pro-apoptotic Bcl-2 family member. Upon a death signal, it relocalizes to the mitochondrial outer membrane and exerts its pro-apoptotic function by promoting the efflux of cytochrome c to the cytosol. [45,46] In the current study, we found cellular redistribution of Bax from the cytosolic to a membrane-bound form taking place within 1h after PDT. Although it is not clear whether Bax is the primary stimulus for initiating cytochrome c release, this finding is consistent with earlier reports on Bax involvement in mitochondria-mediated apoptosis.[47-50]

However, 35% of EL4 cells incubated with the caspase-3 inhibitor DEVD-CHO before PDT underwent apoptosis after treatment with TH9402. Furthermore, caspase-3 inhibition did not block DNA fragmentation, a characteristic feature of apoptosis. Likewise, in ALA-based PDT of HL60 leukemia cells, blockade of caspase-3 with DEVD-FMK did not lead to a concomitant inhibition of apoptosis.[51] Other studies have also proposed involvement of caspase-independent mechanisms in cell demise following PDT.[52]

The present results reveal that PDT evokes AIF release from mitochondria to cytosol. AIF then can translocate, presumable by a poly(ADP-ribose) polymerase-dependent mechanism, to nuclei and stimulate chromatin condensation and fragmentation. However, the poly(ADP-ribose) polymerase inhibitor 3ABA failed to affect DNA fragmentation induced by PDT. Interestingly, DNA fragmentation was partially reduced by treatment with ATA, a putative inhibitor of endonuclease G. In addition, we detected enhanced nuclear accumulation of endonuclease G 1 h post-PDT. These observations are consistent with those of Ishihara et al, who reported the involvement of endonuclease G in nucleosomal DNA fragmentation under sustained oxidative stress.[53] Mitochondria are the

target of TH9402, where it produces singlet oxygen and ROS. This, in turn, may mediate release of pro-apoptotic proteins through changes in mitochondrial permeability transition. Our results provide further evidence for caspase-independent induction of apoptosis by PDT. The present results imply for the first time a role for EndoG in PDT-induced apoptosis, although we can not exclude the possibility of the involvement of other DNases.

In conclusion, TH9402 PDT can be modulated in order to predominantly induce apoptotic cell death. Our results indicate that PDT evokes mitochondrial permeability transition and Bax translocation to the mitochondria, leading to the release of pro-apoptotic proteins, cytochrome c, AIF and EndoG. Using conditions that induce apoptosis, PDT causes activation of both caspase-dependent and -independent death pathways. Furthermore, the observations that the TH9402 photosensitizer mediates cytotoxicity through activation of a number of death pathways suggest that it may effectively limit the capacity of tumor cells to become resistant to PDT.

2.6 Figures:

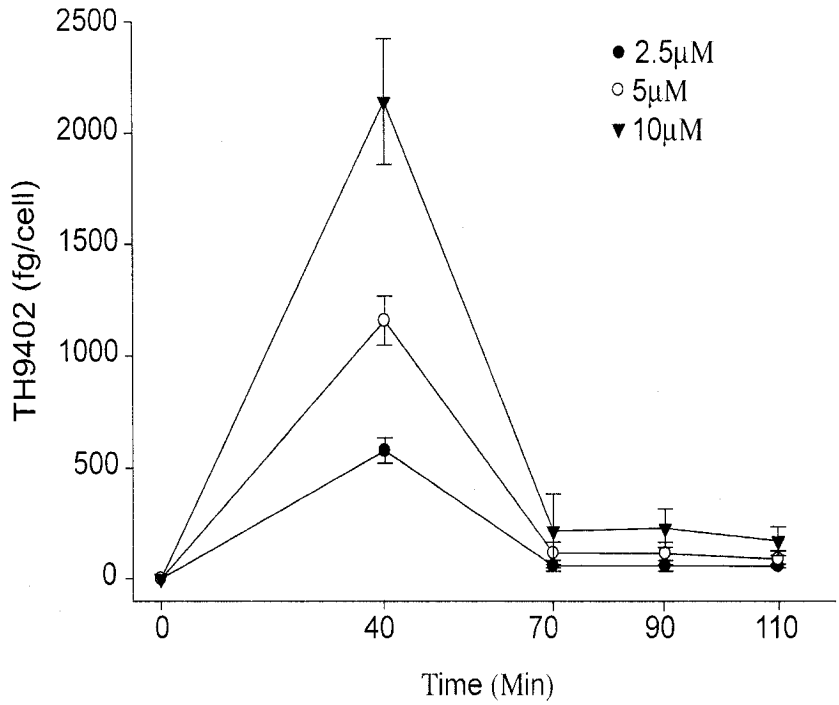


Figure 2.1: Uptake of TH9402 by EL4 cells. Cells (10^6/ml) were incubated for 40 minutes in a medium containing 2.5% serum and various concentrations of TH9402 followed by a 70 minutes washout period. Values are the mean ± SE of 3 experiments in duplicate.

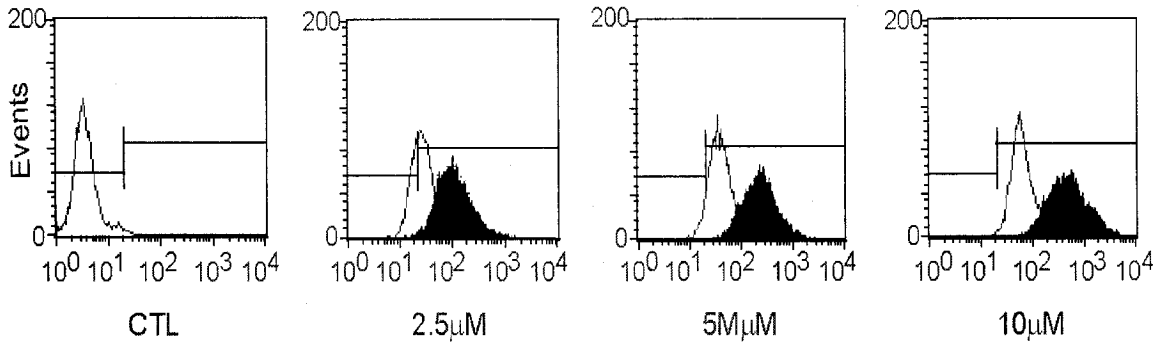


Figure 2.2: Cellular retention of TH9402 after 50 minutes of dye efflux in medium supplemented or not with serum. After 40 minutes incubation with 2.5, 5 and 10 μM TH9402, dye efflux was favored by resuspending in medium with or without serum. TH9402 fluorescent intensity was measured in intact cells using flow cytometry (FL1). (□ serum+, ■ serum-). CTL=Untreated cells. These results are representative for 3 independent experiments.

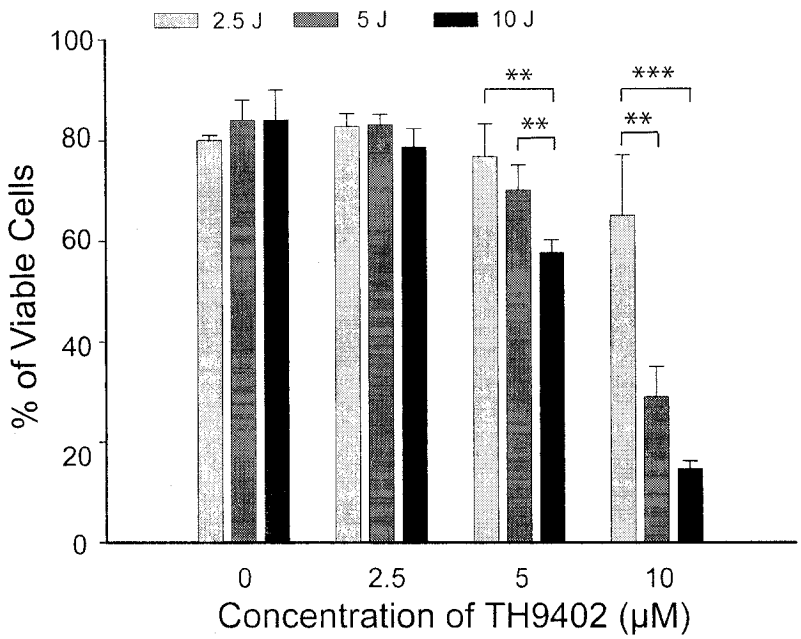


Figure 2.3: Dose-dependent death of PDT-treated EL4 cells. The cells were treated with TH9402 at 0, 2.5, 5, or 10μM and exposed to light at 2.5, 5, 10 J/cm^2. Cell viability was assessed at 2h post-PDT following staining with Annexin V and 7AAD. Viable cells were defined as both Annexin V and 7 AAD negative cells. Values are the mean±SE of 3 experiments. ** $P<0.01$, *** $P<0.001$.

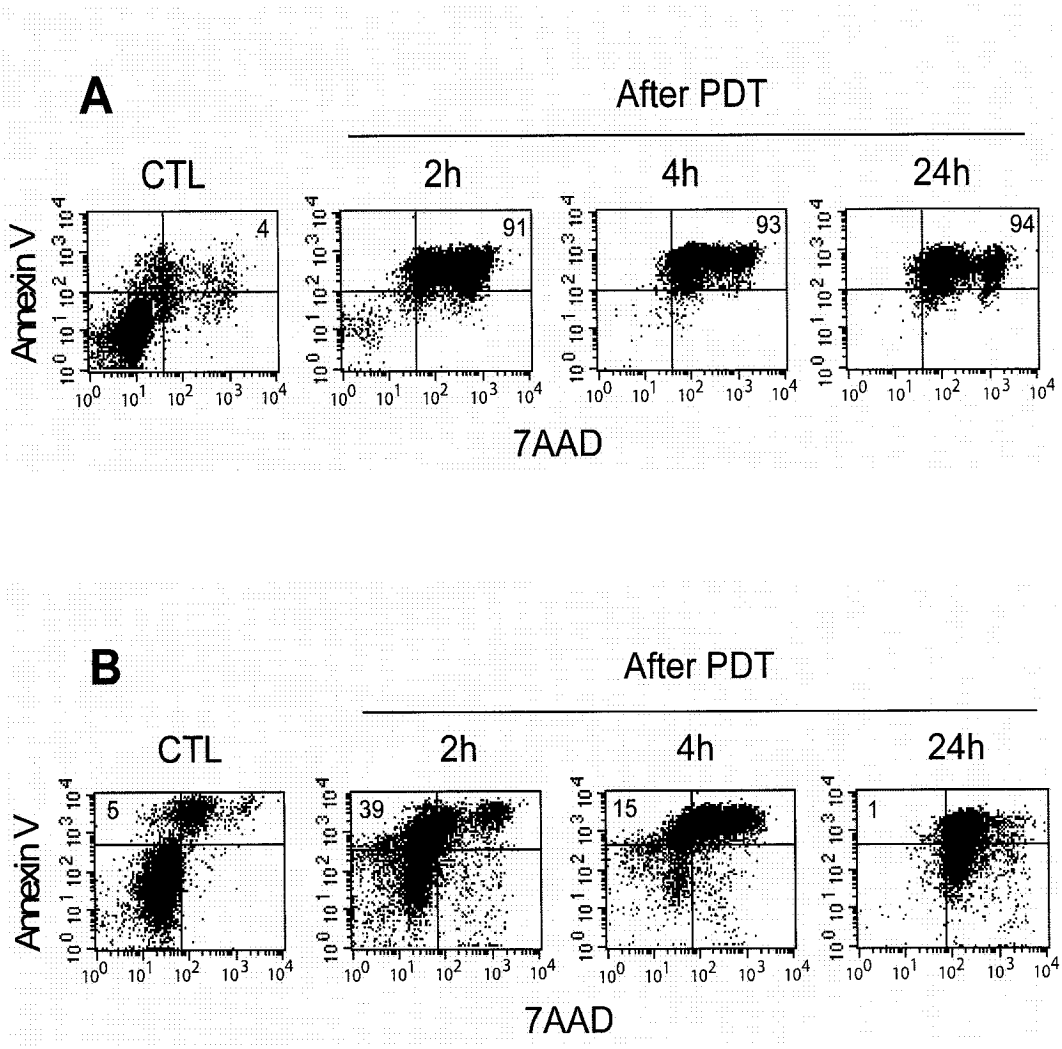


Figure 2.4: Cell necrosis or apoptosis after PDT. **A)** Cells were treated with TH9402 at 10μM and exposed to light at 10 J/cm^2 under sample thickness of 1.7 mm. Cell viability (staining with 7AAD) was assessed at 2, 4, and 24h post-PDT. Numbers show the percentage of dead cells. **B)** Cells were treated with TH9402 at 10μM and exposed to light at 5 J/cm^2 under sample thickness of 3 mm. The development of apoptosis (Annexin V binding) was assessed at 2, 4, and 24h post-PDT. Numbers show the percentage of apoptotic cells. These results are representative for 3 independent experiments.

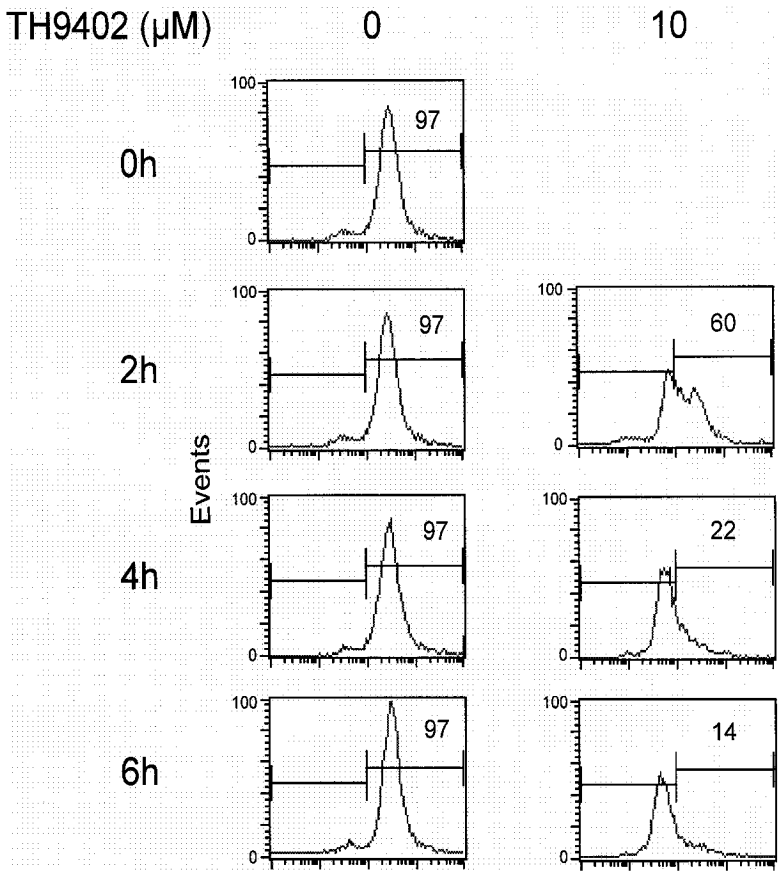


Figure 2.5: Changes in mitochondrial transmembrane potential following PDT. Apoptosis was induced in EL4 cells using TH9402 at 10μM, 5J/cm^2. After PDT, cells were loaded with $DiOC_6(3)$ and fluorescence intensity measured by flow cytometry at 2, 4 and 6 hours post-PDT. Numbers show the percentage of intact cells. Results are representative for 3 independent experiments.

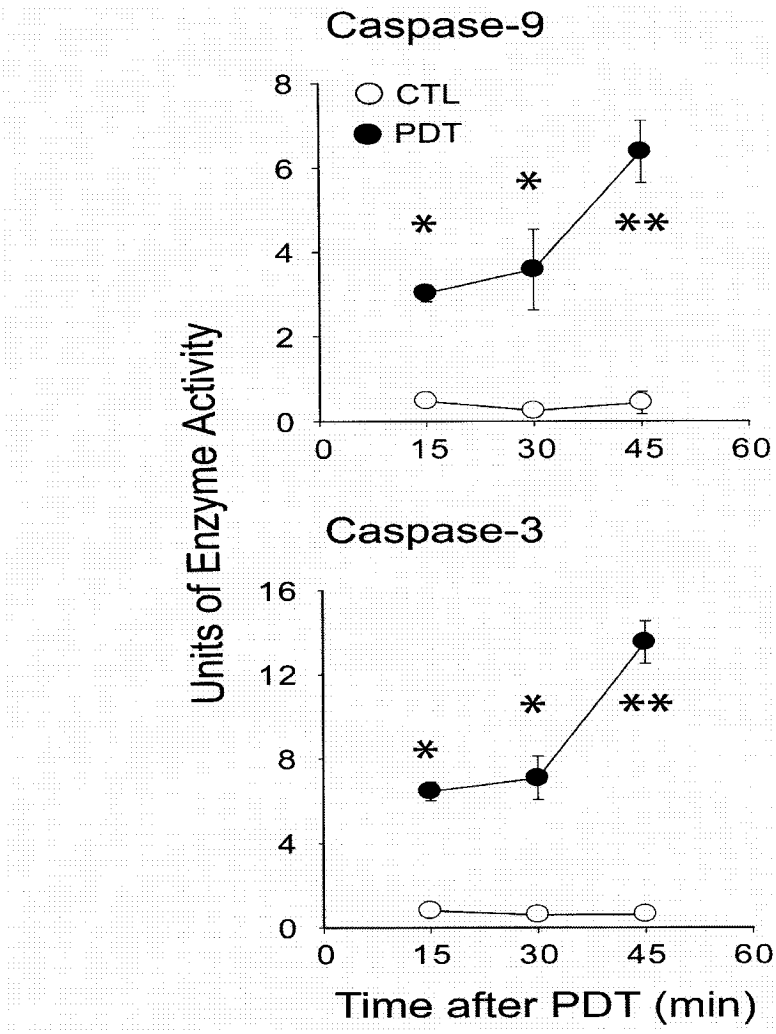
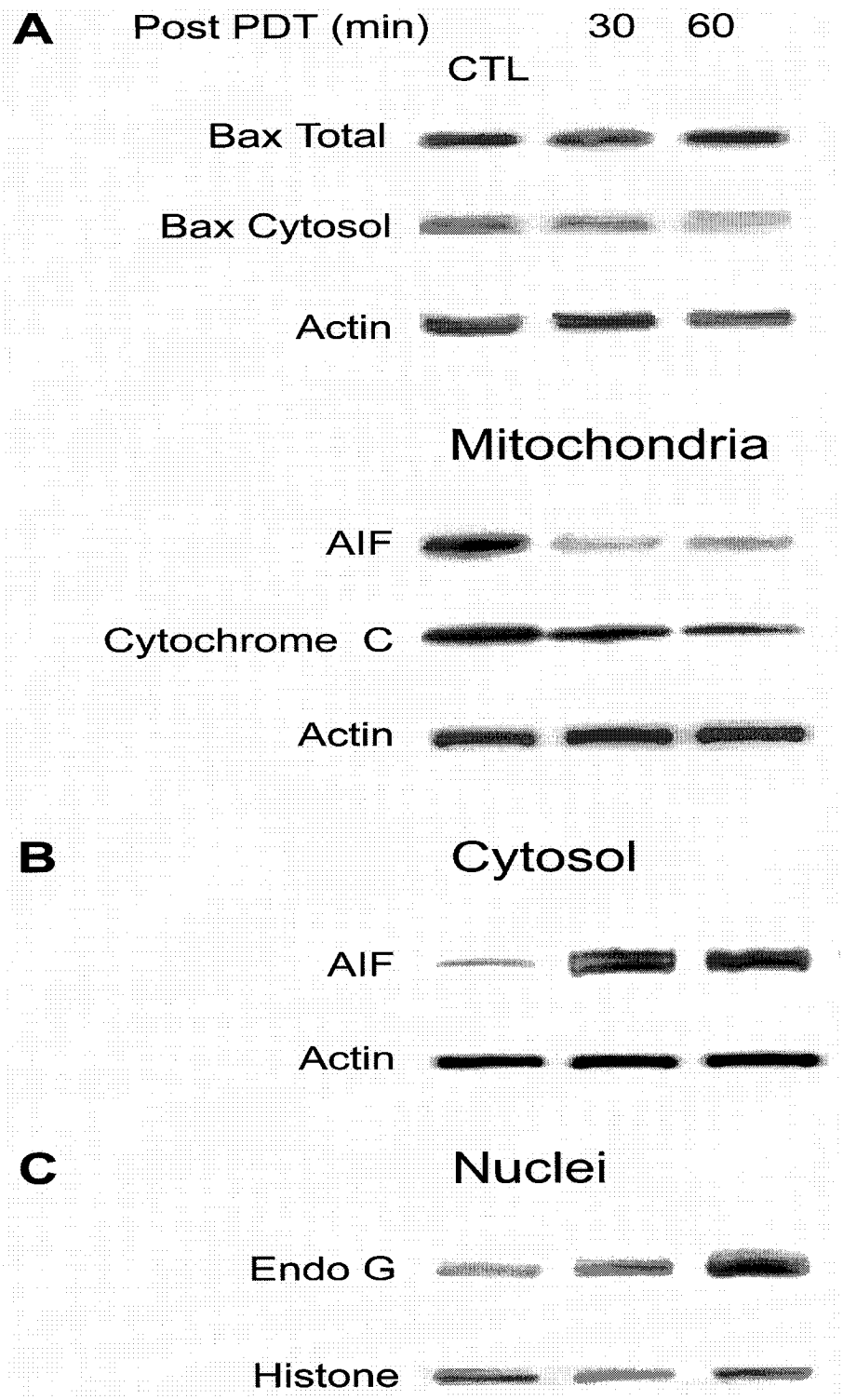


Figure 2.6: Activation of caspase-9 and caspase-3 in PDT-treated EL4 cells. Cells were exposed to PDT at 10μM TH9402, 5J/cm^2. Cells were collected and lysed at the indicated times after PDT. Caspase activity was determined using a colorimetric assay. Values represent mean ± SE of 3 experiments in duplicate. * $P<0.05$, ** $P<0.01$. (vs untreated cells in same time periods)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Actin
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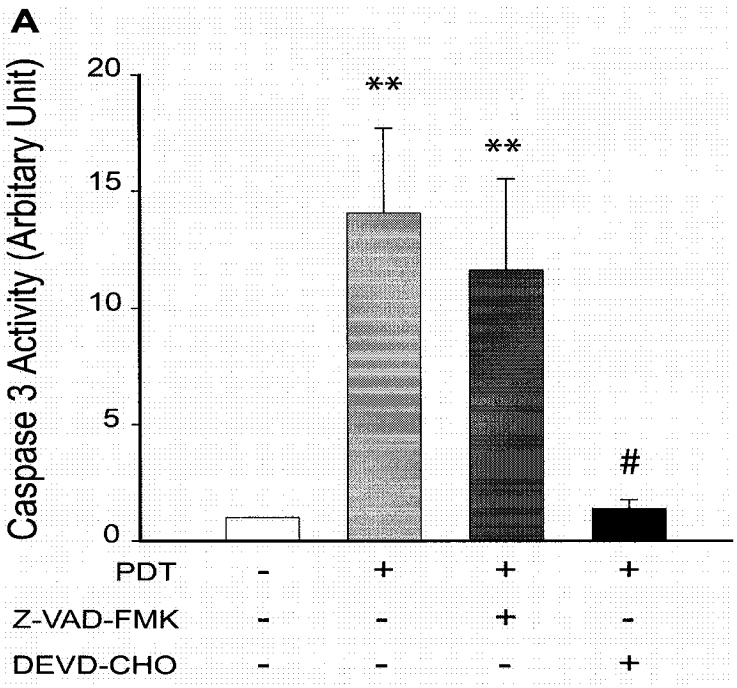
Nuclei
Endo G
Histone

Figure 2.7: Expression and localization of apoptosis-related proteins. Western blot analysis was used to determine Bax, Cytochrome C, AIF and EndoG in different organelles. Cells were induced to apoptosis with PDT at 10μM TH9402, 5J/cm^2. Cells were lyzed and fractionated at 30, 60 minutes post-PDT. **A)** Total cell and cytosolic protein extracts were analysed for Bax. β-actin served as control for equal protein loading. **B)** Mitochondrial protein extracts were immunoblotted to determine the expression of cytochrome C and AIF. Actin served as an internal control. **C)** Expression of EndoG in the nuclear protein fraction. Histone H1 served as control for equal protein loading. Similar results were observed in at least two independent experiments.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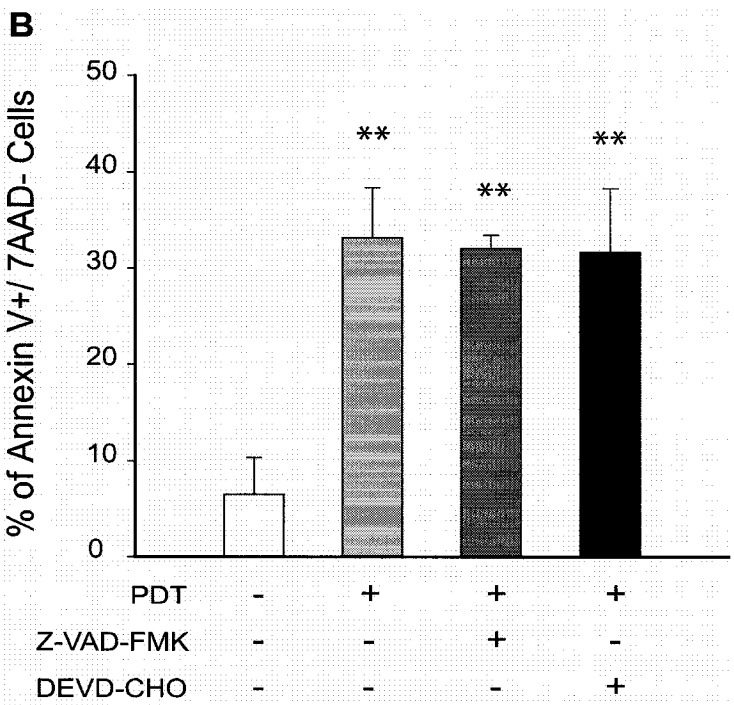
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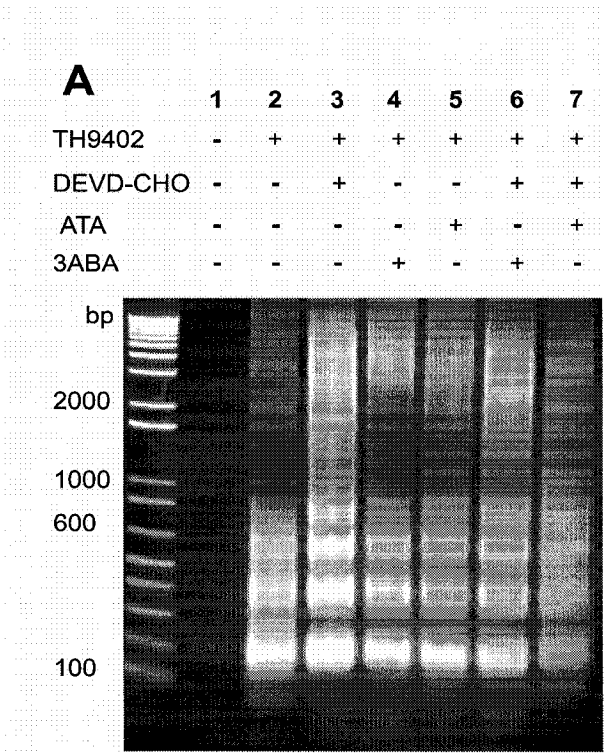
Z-VAD-FMK - - + -
DEVD-CHO - - - +

Figure 2.8: Effect of caspase inhibition on EL4 cell apoptosis. Cells were preincubated with Z-VAD-FMK or DEVD-CHO at 10μM for 1 hour, followed by PDT at 10μM, 5J/cm^2. Aliquots of EL4 cells were stained with Annexin V/7AAD and caspase 3 activity was measured. Results are the mean ± SE of 3 experiments. ** $P<0.01$, # $P>0.05$


A
1 2 3 4 5 6 7
TH9402 - + + + + + +
DEVD-CHO - - + - - + +
ATA - - - - + - +
3ABA - - - + - + -
bp
2000
1000
600
100

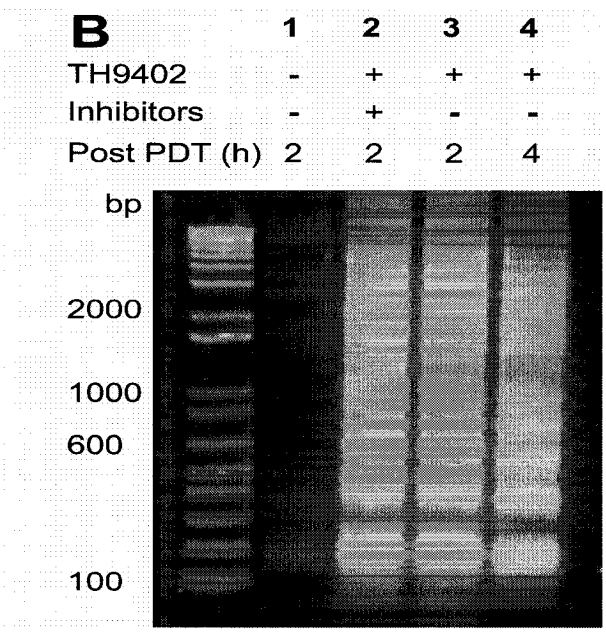

B
1 2 3 4
TH9402 - + + +
Inhibitors - + - -
Post PDT (h) 2 2 2 4
bp
2000
1000
600
100

Figure 2.9: DNA fragmentation induced by TH9402 at 10μM, 5J/cm^2. After PDT, cells were lysed, and low molecular weight DNA was isolated and subjected to agarose gel electrophoresis. **A)** Effects of caspase and endonuclease G inhibition. Cells were preincubated with DEVD-CHO (10μM), 3ABA (2mM), ATA (100μM) alone or in combination as indicated for 1 hour before PDT. Cells were processed at 2h post-PDT. **B)** inhibitors include DEVD-CHO, 3ABA, and ATA together.

2.7 Reference List:

(1) Dougherty TJ, Gomer CJ, Henderson BW et al. Photodynamic therapy. J Natl Cancer Inst. 1998;90:889-905.

(2) Hopper C. Photodynamic therapy: a clinical reality in the treatment of cancer. Lancet Oncol. 2000;1:212-219.

(3) Cortese DA, Edell ES, Kinsey JH. Photodynamic therapy for early stage squamous cell carcinoma of the lung. Mayo Clin Proc. 1997;72:595-602.

(4) Moghissi K, Dixon K, Stringer M et al. The place of bronchoscopic photodynamic therapy in advanced unresectable lung cancer: experience of 100 cases. Eur J Cardiothorac Surg. 1999;15:1-6.

(5) Moghissi K, Dixon K, Thorpe JA, Stringer M, Moore PJ. The role of photodynamic therapy (PDT) in inoperable oesophageal cancer. Eur J Cardiothorac Surg. 2000;17:95-100.

(6) Lightdale CJ, Heier SK, Marcon NE et al. Photodynamic therapy with porfimer sodium versus thermal ablation therapy with Nd:YAG laser for palliation of esophageal cancer: a multicenter randomized trial. Gastrointest Endosc. 1995;42:507-512.

(7) Savary JF, Grosjean P, Monnier P et al. Photodynamic therapy of early squamous cell carcinomas of the esophagus: a review of 31 cases. Endoscopy. 1998;30:258-265.

(8) Kamuhabwa AR, Huygens A, Roskams T, De Witte PA. Enhancing the photodynamic effect of hypericin in human bladder transitional cell carcinoma spheroids by the use of the oxygen carrier, perfluorodecalin. Int J Oncol. 2006;28:775-780.

(9) Nonaka M, Ikeda H, Inokuchi T. Effect of combined photodynamic and chemotherapeutic treatment on lymphoma cells in vitro. Cancer Lett. 2002;184:171-178.

(10) Oleinick NL, Morris RL, Belichenko I. The role of apoptosis in response to photodynamic therapy: what, where, why, and how. Photochem Photobiol Sci. 2002;1:1-21.

(11) Nseyo UO, DeHaven J, Dougherty TJ et al. Photodynamic therapy (PDT) in the treatment of patients with resistant superficial bladder cancer: a long-term experience. J Clin Laser Med Surg. 1998;16:61-68.

(12) Nseyo UO, Shumaker B, Klein EA, Sutherland K. Photodynamic therapy using porfimer sodium as an alternative to cystectomy in patients with refractory

transitional cell carcinoma in situ of the bladder. Bladder Photofrin Study Group. J Urol. 1998;160:39-44.

(13) Allison R, Mang T, Hewson G, Snider W, Dougherty D. Photodynamic therapy for chest wall progression from breast carcinoma is an underutilized treatment modality. Cancer. 2001;91:1-8.

(14) Bernstein ZP, Wilson BD, Oseroff AR et al. Photofrin photodynamic therapy for treatment of AIDS-related cutaneous Kaposi's sarcoma. AIDS. 1999;13:1697-1704.

(15) Taber SW, Fingar VH, Wieman TJ. Photodynamic therapy for palliation of chest wall recurrence in patients with breast cancer. J Surg Oncol. 1998;68:209-214.

(16) Modica-Napolitano JS, Aprille JR. Basis for the selective cytotoxicity of rhodamine 123. Cancer Res. 1987;47:4361-4365.

(17) Boumedine RS, Roy DC. Elimination of alloreactive T cells using photodynamic therapy. Cytotherapy. 2005;7:134-143.

(18) Kennedy JC, Pottier RH, Pross DC. Photodynamic therapy with endogenous protoporphyrin IX: basic principles and present clinical experience. J Photochem Photobiol B. 1990;6:143-148.

(19) Noodt BB, Berg K, Stokke T, Peng Q, Nesland JM. Different apoptotic pathways are induced from various intracellular sites by tetraphenylporphyrins and light. Br J Cancer. 1999;79:72-81.

(20) Wyld L, Reed MW, Brown NJ. Differential cell death response to photodynamic therapy is dependent on dose and cell type. Br J Cancer. 2001;84:1384-1386.

(21) Chiu SM, Oleinick NL. Dissociation of mitochondrial depolarization from cytochrome c release during apoptosis induced by photodynamic therapy. Br J Cancer. 2001;84:1099-1106.

(22) Granville DJ, Carthy CM, Jiang H et al. Rapid cytochrome c release, activation of caspases 3, 6, 7 and 8 followed by Bap31 cleavage in HeLa cells treated with photodynamic therapy. FEBS Lett. 1998;437:5-10.

(23) Kessel D, Luo Y. Photodynamic therapy: a mitochondrial inducer of apoptosis. Cell Death Differ. 1999;6:28-35.

(24) Varnes ME, Chiu SM, Xue LY, Oleinick NL. Photodynamic therapy-induced apoptosis in lymphoma cells: translocation of cytochrome c causes inhibition of respiration as well as caspase activation. Biochem Biophys Res Commun. 1999;255:673-679.

(25) He J, Whitacre CM, Xue LY, Berger NA, Oleinick NL. Protease activation and cleavage of poly(ADP-ribose) polymerase: an integral part of apoptosis in response to photodynamic treatment. Cancer Res. 1998;58:940-946.

(26) Filep JG, Baron C, Lachance S, Perreault C, Chan JS. Involvement of nitric oxide in target-cell lysis and DNA fragmentation induced by murine natural killer cells. Blood. 1996;87:5136-5143.

(27) Brasseur N, Menard I, Forget A et al. Eradication of multiple myeloma and breast cancer cells by TH9402-mediated photodynamic therapy: implication for clinical ex vivo purging of autologous stem cell transplants. Photochem Photobiol. 2000;72:780-787.

(28) Rottenberg H, Wu S. Quantitative assay by flow cytometry of the mitochondrial membrane potential in intact cells. Biochim Biophys Acta. 1998;1404:393-404.

(29) Chen BJ, Cui X, Liu C, Chao NJ. Prevention of graft-versus-host disease while preserving graft-versus-leukemia effect after selective depletion of host-reactive T cells by photodynamic cell purging process. Blood. 2002;99:3083-3088.

(30) Guimond M, Balassy A, Barrette M et al. P-glycoprotein targeting: a unique strategy to selectively eliminate immunoreactive T cells. Blood. 2002;100:375-382.

(31) Villeneuve L. Ex vivo photodynamic purging in chronic myelogenous leukaemia and other neoplasias with rhodamine derivatives. Biotechnol Appl Biochem. 1999;30 (Pt 1):1-17.

(32) Brasseur N, Menard I, Forget A et al. Eradication of multiple myeloma and breast cancer cells by TH9402-mediated photodynamic therapy: implication for clinical ex vivo purging of autologous stem cell transplants. Photochem Photobiol. 2000;72:780-787.

(33) Fabris C, Valduga G, Miotto G et al. Photosensitization with zinc (II) phthalocyanine as a switch in the decision between apoptosis and necrosis. Cancer Res. 2001;61:7495-7500.

(34) Jalili A, Makowski M, Switaj T et al. Effective photoimmunotherapy of murine colon carcinoma induced by the combination of photodynamic therapy and dendritic cells. Clin Cancer Res. 2004;10:4498-4508.

(35) Morgan J, Oseroff AR. Mitochondria-based photodynamic anti-cancer therapy. Adv Drug Deliv Rev. 2001;49:71-86.

(36) Oleinick NL, Evans HH. The photobiology of photodynamic therapy: cellular targets and mechanisms. Radiat Res. 1998;150:S146-S156.

(37) Vermes I, Haanen C, Steffens-Nakken H, Reutelingsperger C. A novel assay for apoptosis. Flow cytometric detection of phosphatidylserine expression on early apoptotic cells using fluorescein labelled Annexin V. J Immunol Methods. 1995;184:39-51.

(38) Piette J, Volanti C, Vantieghem A et al. Cell death and growth arrest in response to photodynamic therapy with membrane-bound photosensitizers. Biochem Pharmacol. 2003;66:1651-1659.

(39) Takahashi H, Itoh Y, Miyauchi Y et al. Activation of two caspase cascades, caspase 8/3/6 and caspase 9/3/6, during photodynamic therapy using a novel photosensitizer, ATX-S10(Na), in normal human keratinocytes. Arch Dermatol Res. 2003;295:242-248.

(40) Dellinger M. Apoptosis or necrosis following Photofrin photosensitization: influence of the incubation protocol. Photochem Photobiol. 1996;64:182-187.

(41) Noodt BB, Rodal GH, Wainwright M et al. Apoptosis induction by different pathways with methylene blue derivative and light from mitochondrial sites in V79 cells. Int J Cancer. 1998;75:941-948.

(42) Noodt BB, Berg K, Stokke T, Peng Q, Nesland JM. Different apoptotic pathways are induced from various intracellular sites by tetraphenylporphyrins and light. Br J Cancer. 1999;79:72-81.

(43) Hengartner MO. The biochemistry of apoptosis. Nature. 2000;407:770-776.

(44) Martinou JC, Green DR. Breaking the mitochondrial barrier. Nat Rev Mol Cell Biol. 2001;2:63-67.

(45) Martinou JC, Green DR. Breaking the mitochondrial barrier. Nat Rev Mol Cell Biol. 2001;2:63-67.

(46) Puthalakath H, Strasser A. Keeping killers on a tight leash: transcriptional and post-translational control of the pro-apoptotic activity of BH3-only proteins. Cell Death Differ. 2002;9:505-512.

(47) Chiu S, Evans HH, Lam M, Nieminen A, Oleinick NL. Phthalocyanine 4 photodynamic therapy-induced apoptosis of mouse L5178Y-R cells results from a delayed but extensive release of cytochrome c from mitochondria. Cancer Lett. 2001;165:51-58.

(48) Chiu SM, Oleinick NL. Dissociation of mitochondrial depolarization from cytochrome c release during apoptosis induced by photodynamic therapy. Br J Cancer. 2001;84:1099-1106.

(49) Chiu SM, Xue LY, Usuda J, Azizuddin K, Oleinick NL. Bax is essential for mitochondrion-mediated apoptosis but not for cell death caused by photodynamic therapy. Br J Cancer. 2003;89:1590-1597.

(50) Zong WX, Lindsten T, Ross AJ, MacGregor GR, Thompson CB. BH3-only proteins that bind pro-survival Bcl-2 family members fail to induce apoptosis in the absence of Bax and Bak. Genes Dev. 2001;15:1481-1486.

(51) Grebenova D, Kuzelova K, Smetana K et al. Mitochondrial and endoplasmic reticulum stress-induced apoptotic pathways are activated by 5-aminolevulinic acid-based photodynamic therapy in HL60 leukemia cells. J Photochem Photobiol B. 2003;69:71-85.

(52) Granville DJ, Cassidy BA, Ruehlmann DO et al. Mitochondrial release of apoptosis-inducing factor and cytochrome c during smooth muscle cell apoptosis. Am J Pathol. 2001;159:305-311.

(53) Ishihara Y, Shimamoto N. Involvement of endonuclease G in nucleosomal DNA fragmentation under sustained endogenous oxidative stress. J Biol Chem. 2006;281:6726-6733.

Preface to chapter 3

High-dose chemotherapy followed by autologous transplantation using peripheral blood stem cells (PBSC) represents a very potent cancer treatment. However, malignant cells can persist in patients undergoing autologous transplantation and are also mobilized during peripheral stem cell collection. A purging strategy would be most useful to eliminate tumor cells contaminating autografts. In this chapter, the selective cytotoxicity of PDT towards cancer cells will be evaluated.

In chapter 2, it was found that PDT induced cell death in a time- and dose- dependent manner. We have also found that there is highly selective ablation of B cells in PDT. This prompted us to investigate the purging effect of PDT toward human multiple myeloma cells. To mimic the clinical situation in myeloma patients, RPMI8226 myeloma cells were mixed with cells from healthy apheresis products. The purpose of this chapter is to prove the efficacy of TH9402 mediated cell death by PDT in clinical conditions where tumor cells are admixed with normal progenitors and evaluate the possible application of PDT for the purging of myeloma cells from stem cell grafts.

CHAPTER 3

Elimination of multiple myeloma cells in an ex vivo purging model using photodynamic therapy with a novel photosensitizer TH9402

3.1 Abstract:

Purpose: High-dose chemotherapy followed by autologous transplantation using peripheral blood stem cells (PBSC) is considered as the standard treatment for multiple myeloma patients. However, most patients will eventually relapse after transplantation. This is attributable either to malignant cells in the patients that escape eradication by the preparative regimen, or to the reinfusion of viable clonogenic tumor cells within the autograft. Cell purging strategies are being developed to address the later possibility. Photodynamic purging with TH9402 has been considered as a potential approach to eliminate tumor cells in autologous stem cell transplantation, particularly because of its selectivity for malignant cells, with sparing of normal immune cells such as resting T cells. In addition, the rhodamine derivative TH9402 has demonstrated most favorable induction of cell death pathways and the ability to cause rapid and potent phototoxicity.

Results: TH9402-mediated photodynamic therapy (PDT) induces human multiple myeloma RPMI8226 cell death in a time- and dose-dependent manner. RPMI8226 cells treated by photodynamic therapy (PDT) using 5μM TH9402 and 10J/cm^2 light exposure were efficiently destroyed as measured by flow cytometry. Moreover, a tumor clonogenic assay showed that PDT can eradicate up to 5 logs (99.999%) of myeloma cells compared with non-treated cells. To mimic clinical conditions, human aphereses products from healthy donors were mixed with 10% RPMI8226 cells and PDT treated under conditions which have been shown to preserve more than 50% of hematopoietic progenitor cells, including the more primitive immature progenitors. At 4 h post-PDT, RPMI8226 cells were eliminated from the apheresis product mixture, as shown by flow cytometry. Furthermore, the purging process eliminated residual myeloma cells below detectable thresholds of a sensitive PCR methology.

Conclusion: These results strongly suggest that TH9402 represents an appealing reagent to optimize graft composition in multiple myeloma patients undergoing autologous PBSC transplantation.

3.2 Introduction:

Multiple myeloma (MM) is a neoplastic disorder of plasma cells which accounts for approximately 10% of malignant hematologic diseases.[1] Autologous transplantation is considered as the standard treatment for multiple myeloma patients, particularly for those individuals younger than age 55.[2,3] The effectiveness of this treatment modality is superior to conventional chemotherapy in terms of both event-free and overall survival.[4,5] However, most patients eventually relapse after treatment either because (i) these cells have developed resistance mechanism to circumvent high-dose chemotherapeutic damage, or (ii) small numbers of viable clonogenic tumor cells are reinfused along with normal progenitor cells in the autograft.[6]

To eradicate the contaminating tumor cells, effective techniques for removal of viable tumor cells from autologous harvests have been developed. Methods to reduce tumor cell contamination include exposure to chemical agents, tumor targeting monoclonal antibodies, long-term marrow culture or hyperthermia.[7-11] Only $CD34^+$ selection, a strategy able to significantly reduce myeloma cell contamination in PBSC collections, has been evaluated clinically in a Phase III study with patients receiving an unmanipulated graft in the control arm. Although malignant cell levels decreased with the CD34 selection, no improvement in disease-free or overall survival was achieved.[12] Interestingly, patients infused with CD34 purified progenitor cells also developed greater numbers of infectious complications. This may reflect the simultaneous elimination of immune cell populations, such as T cells, with their anti-infection and anti-tumor potential, that occurs during the selection process. More recently, photodynamic purging processes have been reported as cytotoxic against a variety of cancer cell lines.[13,14] In addition, PDT has been shown to spare resting T cells that could react toward infectious organisms and malignant

cells.[15,16] This peculiar ability to exert selective and extensive anti-tumor effects while preserving hematopoietic stem and T cells prompted us to evaluate its use as a purging strategy.

The phototosensitizer 4,5-dibromorhodamine methyl ester (TH9402) is a rhodamine derivative with favorable photophysical properties, low toxicity and stability. Because of high quantrum yields of singlet-oxygen formation, this photosensitizing molecule can be easily activated by light emission to induce tumor-cell death. Previous reports have shown that rhodamine123 (R123) is specially retained by mitochondria of various carcinoma cells.[17-19] The observation that early hematopoietic progenitors fail to accumulate native rhodamine-123 in significant amounts[20-22] suggested that this rhodamine derivative would induce limited hematologic toxicity. Indeed, the preferential accumulation of TH9402 resulted in the eradication of 3 to 6 logs of non-Hodgkin's lymphoma (NHL) and chronic myeloid leukemia (CML) cells with preservation of normal hematopoietic progenitors for engraftment of autologous purged stem cell.[23,24] In this study we investigated the tumoricidal action of TH9402 as an *ex vivo* photodynamic agent against the human multiple myeloma.

3.3 Materials and methods:

Photosensitizer. TH9402 was supplied by Celmed Biosciences Inc. (Montreal, Canada). The stock solution (858μM) was freshly prepared by dissolving 2 mg of the lyophilized dye in 4 ml of sterile water.

Human cell line. The myeloma cell line RPMI8226, established from a male myeloma patient, was obtained from the American Type Culture collection (ATCC, VA) and

maintained in complete medium consisting of RPMI1640, 2mML-glutamine, 50 units/ml penicillin, 50μg/ml streptomycin, and 10% fetal bovine serum (FBS) (all from Invitrogen, Grand Island, NY).

Apheresis product (AP). All aphereses products originated from healthy female donors after obtaining informed consent. Before collection, the donors were given 10 μg/kg granulocyte colony-stimulating factor (G-CSF) for 5 consecutive days to mobilize PBSCs. AP cells were washed in phosphate buffered saline (PBS) twice and then mixed with myeloma cells.

Effect of PDT on myeloma cells. Cells in exponential growth phase were seeded in six-well plates at 1×10^6 cells/ml and a liquid thickness of 3mm. After 40 minutes of incubation in X-Vivo15 (Bio-Whittaker, Walkersville, MD) medium of containing 2,5% FBS and 5 or 10μM TH9402, cells were centrifuged and resuspended in dye-free medium with 10% FBS. After 50 minutes of dye efflux in a humidified atmosphere at 37°C and 5% CO_2, cells were exposed to 10 Joules/cm^2 using a light scanning device. Control cells (incubated in medium without dye) were exposed to the light as well. After PDT, the treated cells were evaluated by flow cytometry staining with Annexin V and 7AAD.

Limiting Dilution Assay (LDA). After treatment with PDT, control and treated cells were washed twice and plated in a limiting dilution assay, as described previously.[25] Briefly, each treatment sample was serially diluted from 2×10^5 to 0.25 cells per 125μl in RPMI1640 complete culture medium. Then, 24 aliquots of each dilution were plated in

flat-bottom 96-multiwell plates (Nunclon, Nunc, Roskilde, Denmark). Cells were incubated at 37°C for 10 days and fed every 3 days. Growth at each serial dilution was assessed in an "all-or-nothing" (positive or negative) fashion under an inverted phase microscope. The frequency of clonogenic cells within the test population was estimated using chi-square minimization.[26]

Cell culture for hematopoietic progenitors. Hematopoietic progenitors were evaluated by plating in semi-solid methylcellulose medium (MethoCult H4434; StemCell Technologies) in 35-mm plastic suspension culture dishes, according to the manufacturer's instructions. Colonies were enumerated after 13 to 16 days of culture at 37°C in a fully humidified 5% CO_2 atmosphere.[27] Assays were at least in duplicate. Non-treated cells were plated as controls.

The number of long-term culture–initiating cells (LTC-ICs) was determined after plating on irradiated M2-10B4 (ATCC) stromal feeder cells established in collagen-coated 96-well flat-bottom plates (Becton Dickinson Labware, Bedford, MA). Cells were seeded in limiting dilution conditions and maintained for 5 weeks with weekly half-medium changes using Myelocult medium (StemCell Technologies Inc.) containing 10^{-6}M of hydrocortisone sodium succinate (Abbot Laboratories, Montreal, Canada). At the end of this period, individual wells were evaluated for the presence or absence of cobblestone area forming cells (CAFCs). This score was confirmed by overlaying wells with 100 µl MethoCult (StemCell Technologies Inc.). Wells were assessed for the presence or absence of secondary colony forming cells after 14 days of incubation at 37°C in humidified 5% CO_2 atmosphere.[28]

PDT purging of myeloma cells contaminating apheresis product. AP and RPMI8226 cells were immunophenotyped by flow cytometry with monoclonal antibodies anti-CD38 and anti-CD138 (BD Biosciences, Mississauga, ON), conjugated with APC or PerCP, to identify markers for detection of efficacy before and after purging procedure. AP cells were mixed with myeloma cells to simulate 10% contamination. Cell mixtures were incubated in medium containing 5μM TH9402 for 40 minutes, followed by extrusion for 50 minutes and finally exposed to 10 Joules/cm^2 light. To mimic clinical applications, cell mixtures were seeded at a concentration of 20×10^6 cells/ml and thickness of 10 mm, and treated under the above PDT conditions.

Molecular detection of residual myeloma cells.

Genomic DNA was extracted by DNAzol (Invitrogen) according to the instructions of the manufacturer. Genomic DNA (50ng) was amplified using a consensus primer (ATGCCTTCATCAGGATGCTA) and an antisense primer (GCTAGTATTTTGTTATGGACTTT) derived from human Y chromosome. The reaction was carried out for 35 cycles (denaturation 94 °C for 30s, annealing 53 °C for 30s, extension 72 °C for 60s) with a final extension of 7 min to generate a 734pb fragment. PCR products were visualized on a 1.5% agarose gel stained with ethidium bromide.

3.4 Results:

Induction of RPMI8226 cell death after PDT treatment.

Multiple myeloma RPMI8226 cells were exposed to 5 and 10 μM TH9402 based on preliminary experiments indicating that 5μM was the dose required to eliminate myeloma

cells (data not shown). Most cells exposed to light alone without dye were viable even after 24h of culture (Fig.3.1). In contrast, treatment of cells with TH9402 resulted in a time-dependent decrease in cell viability. Cell death was accelerated by increasing the concentration of TH9402. Nevertheless, the condition using 5μM TH9402 and 10J/cm^2 ultimately resulted in a degree of cell death similar to 10μM and 10J/cm^2. In both cases, cells were almost completely killed 24h post-PDT.

Depletion of RPMI8226 cells after PDT as assessed in a LDA.

To determine the extent of the effectiveness of PDT, RPMI8226 cells were exposed to 5 and 10μM TH9402 PDT and plated in a limiting dilution fashion in 96-well plates and cultured for 10 days. TH9402 decreased the number of positive clones to below the detectable threshold of the LDA, which corresponds to the elimination of more than 5 logs of myeloma cells (Fig.3.2). TH9402 at 5 or 10μM appeared to be equally potent in eliminating RPMI8226 cells.

Preservation of hematopoietic progenitors.

To assess the capacity of TH9402 to maintain sufficient numbers of hematopoietic progenitors for engraftment, mobilized peripheral blood cells obtained from healthy donors underwent TH9402 PDT, followed by progenitor evaluation with colony-forming and LTC-IC assays. Mean survival rates of 59% for CFU-GM, 58% for BFU-E and 80% for CFU-GEMM were observed in cells treated with 5μM, 10 J/cm^2 at 10 mm thickness (Fig 3A). The effect of PDT on the more primitive LTC-IC progenitors was measured in 3 samples. A mean of 80% of LTC-IC survived PDT (Fig.3.3B).

Purging of RPMI8226 cells from apheresis product after PDT.

Apheresis cells were found to be $CD38^+$ and $CD138^-$, whereas RPMI8226 cells expressed both CD38 and CD138 (Fig.3.4). This differential expression of CD138 could be used to further study the purging effect of myeloma cells, when admixed with apheresis product. Indeed, before PDT, we detected two predominant cell populations: $CD38^+/CD138^-$ and $CD38^+/CD138^+$ (Fig.3.5). In contrast, at 4h and 24h post-PDT, positive staining for CD138 was disappeared. Double positive cells were reduced from 13% to 1%, which indicates the selective removal of double positive malignant cells.

Elimination of molecularly detectable residual myeloma cells.

To confirm PDT mediated elimination of residual neoplastic cells, we treated a mixture of RPMI8226 cells (established cell line from a male patient) and apheresis product obtained from a healthy female donor. A PCR assay was then used to amplify a 734 bp fragment of the Y chromosome as a marker of tumor cells. As expected, PCR amplified this fragment from RPMI8226 cells as well as from the cell mixture not exposed to PDT, and not from the apheresis product without myeloma cells (Fig.3.6). However, this fragment disappeared after PDT.

3.5 Discussion:

The combination of high dose chemotherapy and autologous stem cell transplantation is effective for treating a number of malignant disorders. Nevertheless, it is still limited by high relapse rates due in part to tumor cell contamination of autologous grafts. Despite

considerable research efforts, methods for stem cell graft purging require significant improvement.[29]

Multiple myeloma is a malignant disorder affecting primarily late stage B cells (plasma cells). We found that PDT using TH9402 induced RPMI8226 cell death in a time- and dose-dependent manner. Cell viability was decreased rapidly (within hours). PDT using TH9402 at 5μM and 10J/cm^2 almost completely killed these cells 24h post-PDT. This result is in line with our previous study showing that TH9402 eliminates B cells at pre-plasma cell levels extremely rapidly and efficiently.[30] In addition, RPMI8226 cells were reduced by up to 5 logs in a clonogenic assay, which suggests that TH9402 is an effective reagent to eliminate myeloma cells at levels commensurate with the large number of cells present in stem cell grafts. We have previously showed that PDT using TH9402 causes activation of both caspase-dependent and independent pathways. The mechanism of myeloma cell death involved apoptosis, as assessed by Annexin V staining in flow cytometry, probably in addition to the radical oxygen species-mediated necrosis (Fig.3.1) Our observation that cell cytotoxicity is conducted through activation of a number of death pathways (manuscript in preparation), implies that TH9402 may effectively limit the capacity of tumor cells to become resistant. Encouraged by these favorable preclinical studies, we developed a purging procedure using TH9402 in multiple myeloma.

To simulate the clinical situation of minimal residual disease in an apheresis product, human apheresis product from healthy donors was mixed with RPMI8226 myeloma cells and treated by PDT under conditions shown to preserve more that 50% of hematopoietic progenitor cells. Because RPMI8226 cells are both CD38 and CD138 positive, the

detection of tumor cells remaining after purging was conducted by flow cytometry. Myeloma cells were destroyed by rapid phototoxicity, as expected with a combination of high quantum of singlet oxygen production and apoptosis. Further PCR testing proved that PDT eliminated residual tumor cells detectable in apheresis. These results indicate that a dramatic purging effect can be achieved within a 10% tumor burden, which is much higher than amounts encountered frequently in clinical situations (0.01-1%).[31]

An ideal agent for stem cell purging would be characterized by high selectivity against malignant cells in order to achieve effective tumor cell eradication, with minimal toxicity toward progenitor and stem cells. Currently, there is a lack of antineoplastic agents which induce selective destruction of cancer cells while leaving intact the normal cellular population. Therefore the use of photosensitizers for hematopoietic stem cell graft purging has been explored in patients with leukemia and lymphoma.[32-36] Because of their low toxicity and rapid elimination, rhodamine dyes are potentially useful reagents for PDT. They preferentially target the mitochondrial structure and function, an ideal site for PDT action. When chronic myelocytic leukemia cells were incubated with photosensitizers, including rhodamine123, they exhibited preferential uptake and retention as compared with normal cells.[37-40] Previous studies using TH9402 mediated PDT resulted in a significant reduction or complete elimination of leukemia cells. Samples yielded negative RT-PCR for bcr/abl after PDT in four chronic myelogenous leukemia (CML) patients. Importantly, these treatment conditions had no significant impact on progenitor cells. Similar results were obtained when K562 leukemia cells were mixed with normal PBSC.[24,41] Here, TH9402 clearly demonstrated limited toxicity toward normal hematopoietic progenitors. Greater than 50% of BFU-E, CFU-GM, and CFU-Mix

were preserved under conditions that eradicated more than 5 logs of myeloma cells (Fig.3.3A). Even lower toxicity was observed with more immature progenitors (LTC-IC, Fig.3.3B). Therefore, TH9402 represents a safe and specific agent for tumor cell elimination.

The differences in the extent of PDT-mediated cytotoxicity depended on TH9402 accumulation and clearance kinetics in target cell subsets. The selectivity of TH9402 is related to the functional transporter, P-glycoprotein (Pgp).[16,41] Pgp has been shown to play an important role in rhodamine retention and transport.[42] Interestingly, immature progenitors feature higher expression of Pgp compared to mature cells.[21,43] The $CD34^{+}CD38^{-}$ subset, believed to represent immature hematopoietic progenitors, retained the smallest amount of TH9402 among all cell types analyzed in PBSC collections (data not shown). This may explain the observed resistance of clonogenic progenitors to PDT.

In summary, this study demonstrates the efficacy of TH9402 to induce RPMI8226 cells death and eradicate myeloma cells in a clonogenic assay after PDT. Moreover, the potent and selective elimination of malignant cells and preservation of normal progenitor cells suggests that TH9402 represents a potentially useful reagent for *ex vivo* purging of autologous PBSC transplants in patients with multiple myeloma.

3.6 Figures:

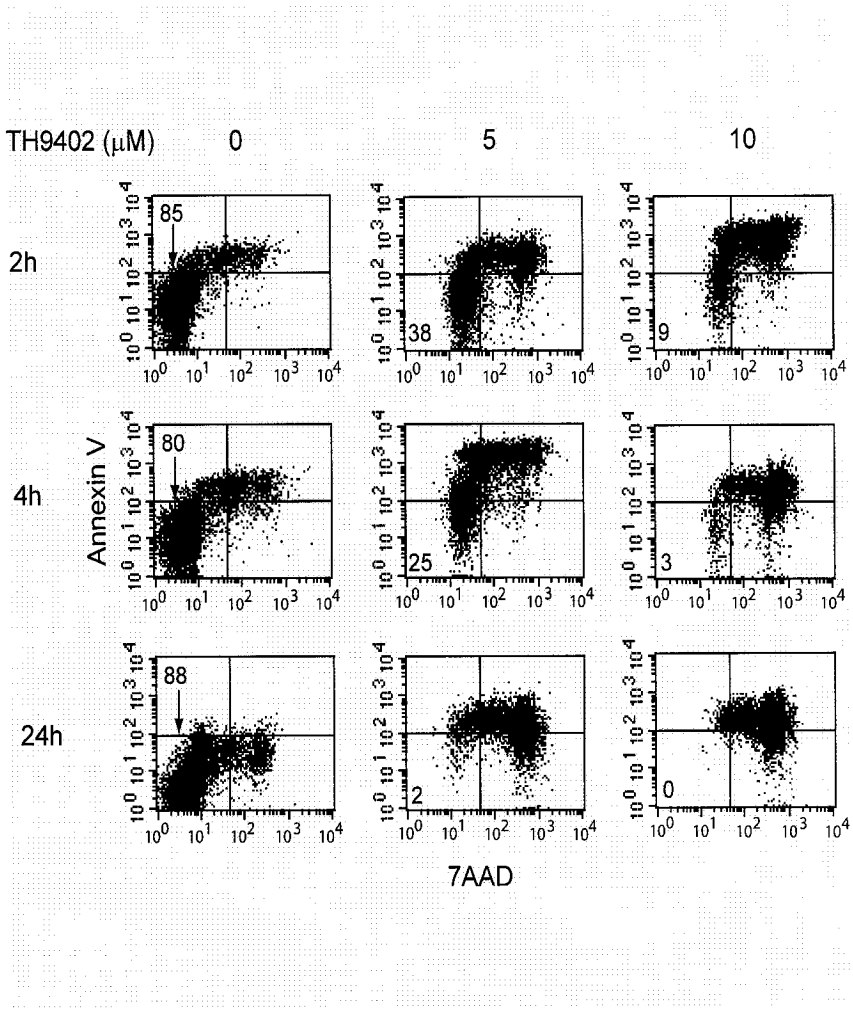


Figure 3.1: Kinetics of cell death following PDT with TH9402. RPMI8226 cells were treated with light alone (0μM) or treated with TH9402 (5 or 10μM). Cell viability (staining with 7AAD) and development of apoptosis (Annexin V binding) were assessed at 2, 4, and 24h post-PDT. Numbers show the percentage of viable cells. The results are representative of 3 independent experiments.

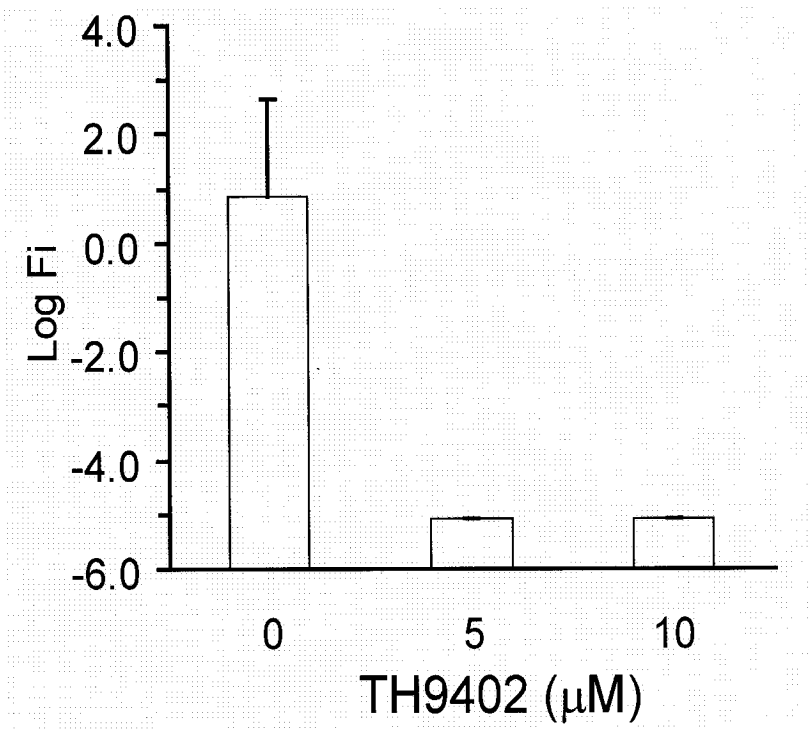
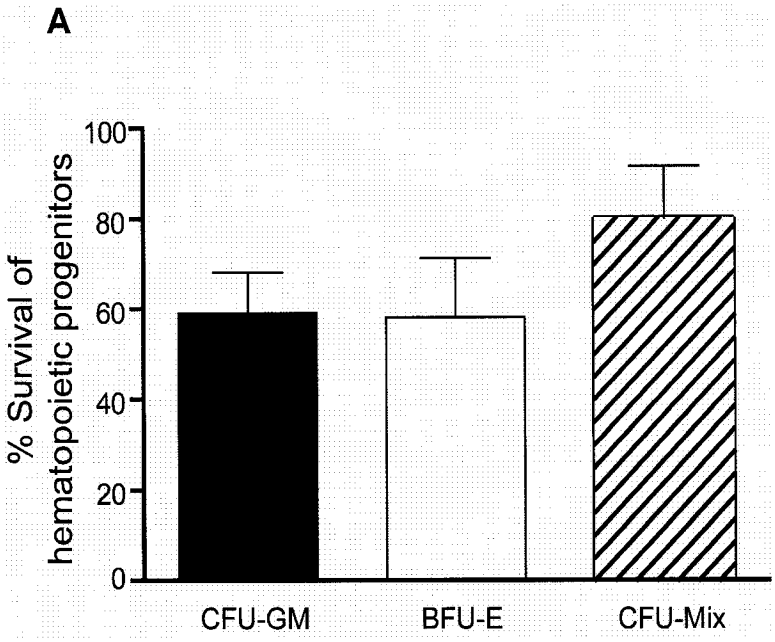


Figure 3.2: Elimination of RPMI8226 cells after TH9402 mediated PDT. RPMI8226 admixed with PBSC in a 1:10 ratio were PDT treated using 5 and 10μM TH9402 and cultured for 10 days in a limiting dilution assay. For both PDT conditions, the frequency of clonogenic cells was below the threshold level indicated for the 5 and 10μM doses. Values are the mean ± SE of 3 independent experiments.


A
% Survival of hematopoietic progenitors
100
80
60
40
20
0
CFU-GM
BFU-E
CFU-Mix

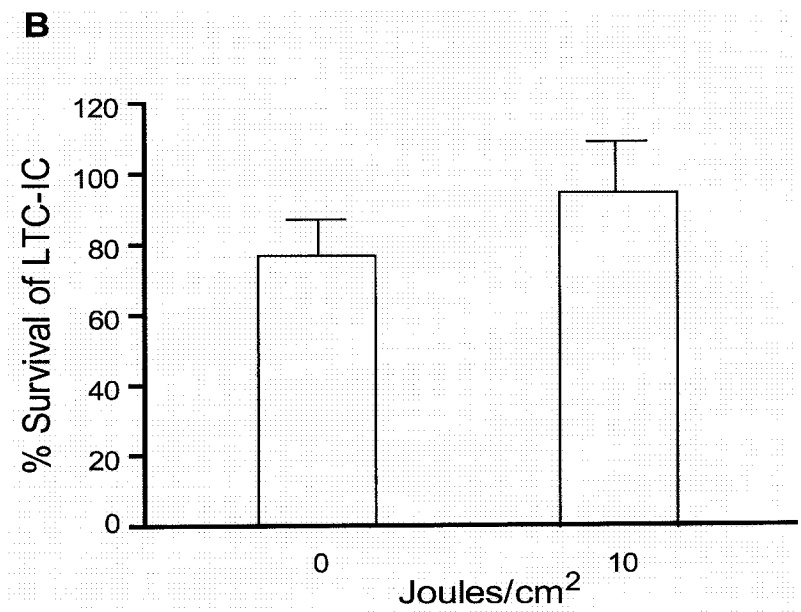

B
% Survival of LTC-IC
120
100
80
60
40
20
0
0
10
Joules/cm2

Figure 3.3: Effect of TH9402 mediated PDT on normal progenitor cells. **(A)** Apheresis cells from healthy donors were exposed to 5μM TH9402 for 40 minutes followed by a 50 minutes clearance period and light illumination at $10J/cm^2$ and 10 mm thickness. Hematopoietic progenitors were measured using a methylcellulose-based clonogenic assay. Results are expressed as mean ± SE of 4 experiments. **(B)** Survival of LTC-IC progenitors was measured after PDT under the above conditions. Mean ± SE of 3 experiments.

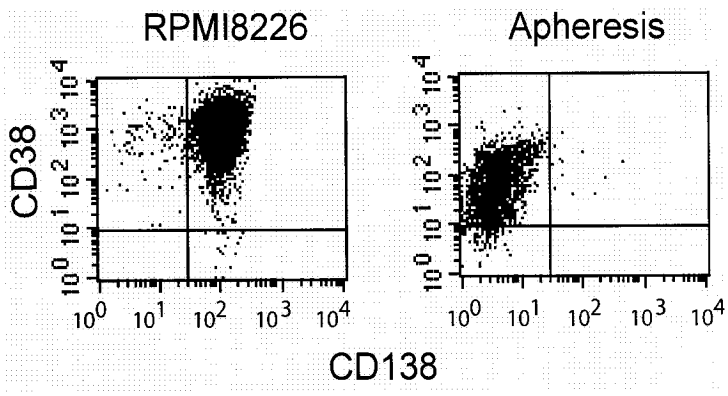


Figure 3.4: Phenotype of RPMI8226 cells and cells obtained from apheresis. The cells were stained with an APC-labeled anti-CD38 and a PerCP-labeled anti-CD138 mAbs. Immunostaining was assessed by flow cytometry. Results are representative of 3 independent measurements.

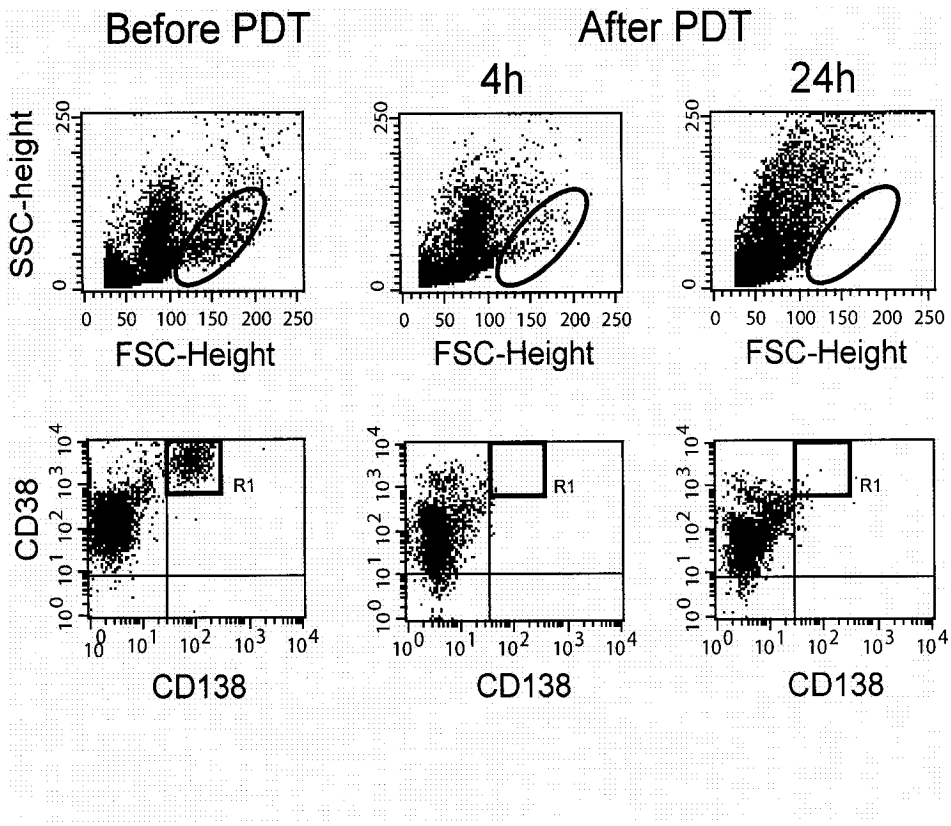


Figure 3.5: Purging of RPMI8226 cells. RPMI8226 cells (10%) were mixed with apheresis product before PDT and exposed to TH9402 under the same PDT condition as described in Figure3.3. Cells were stained with anti-CD38 and anti-CD138 mAbs. Double-color immunostaining was analyzed by flow cytometry at 0h, 4h and 24h post-PDT. The R1 region corresponds to double positive cells. Results are representative of 3 independent experiments.

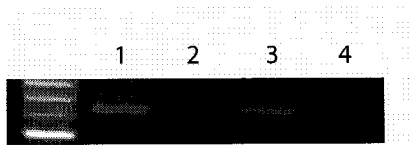


Figure 3.6. Expression of a fragment of the Y chromosome. PDT-treated mixture of RPMI8226 cells at 10% in apheresis product were cultured for 8 days. Genomic DNA was extracted and a 734pb fragment of the Y chromosome was amplified by PCR. PCR products were visualized on a 1.5% agarose gel. Lane1: RPMI8226 cell line established from a male patient. Lane2: Apheresis product obtained from a female donor. Lane3: Mixture of RPMI8226 cells at 10% in apheresis product before PDT. Lane4: Mixture of RPMI8226 cells at 10% in apheresis product after PDT. Left lane shows markers of 600, 700, 800, 900 bp.

3.7 Reference List

(1) Rajkumar SV, Gertz MA, Kyle RA, Greipp PR. Current therapy for multiple myeloma. Mayo Clin Proc. 2002;77:813-822.

(2) Blade J, Vesole DH, Gertz M. High-dose therapy in multiple myeloma. Blood. 2003;102:3469-3470.

(3) Kumar A, Loughran T, Alsina M, Durie BG, Djulbegovic B. Management of multiple myeloma: a systematic review and critical appraisal of published studies. Lancet Oncol. 2003;4:293-304.

(4) Attal M, Harousseau JL, Stoppa AM et al. A prospective, randomized trial of autologous bone marrow transplantation and chemotherapy in multiple myeloma. Intergroupe Francais du Myelome. N Engl J Med. 1996;335:91-97.

(5) Gertz MA, Lacy MQ, Dispenzieri A, Hayman SR, Kumar SK. High-dose chemotherapy with autologous hematopoietic stem cell transplantation in patients with multiple myeloma. Expert Rev Anticancer Ther. 2006;6:343-360.

(6) Jansen J, Falkenburg JH, Stepan DE, LeBien TW. Removal of neoplastic cells from autologous bone marrow grafts with monoclonal antibodies. Semin Hematol. 1984;21:164-181.

(7) Ciobanu N, Paietta E, Andreeff M, Papenhausen P, Wiernik PH. Etoposide as an in vitro purging agent for the treatment of acute leukemias and lymphomas in conjunction with autologous bone marrow transplantation. Exp Hematol. 1986;14:626-635.

(8) Gribben JG, Freedman AS, Neuberg D et al. Immunologic purging of marrow assessed by PCR before autologous bone marrow transplantation for B-cell lymphoma. N Engl J Med. 1991;325:1525-1533.

(9) Gribben JG, Saporito L, Barber M et al. Bone marrows of non-Hodgkin's lymphoma patients with a bcl-2 translocation can be purged of polymerase chain reaction-detectable lymphoma cells using monoclonal antibodies and immunomagnetic bead depletion. Blood. 1992;80:1083-1089.

(10) Okamoto S, Olson AC, Berdel WE, Vogler WR. Purging of acute myeloid leukemic cells by ether lipids and hyperthermia. Blood. 1988;72:1777-1783.

(11) Turhan AG, Humphries RK, Eaves CJ et al. Detection of breakpoint cluster region- negative and nonclonal hematopoiesis in vitro and in vivo after transplantation of cells selected in cultures of chronic myeloid leukemia marrow. Blood. 1990;76:2404-2410.

(12) Stewart AK, Vescio R, Schiller G et al. Purging of autologous peripheral-blood stem cells using CD34 selection does not improve overall or progression-free

survival after high-dose chemotherapy for multiple myeloma: results of a multicenter randomized controlled trial. J Clin Oncol. 2001;19:3771-3779.

(13) Brasseur N, Menard I, Forget A et al. Eradication of multiple myeloma and breast cancer cells by TH9402-mediated photodynamic therapy: implication for clinical ex vivo purging of autologous stem cell transplants. Photochem Photobiol. 2000;72:780-787.

(14) Tsujino I, Anderson GS, Sieber F. Postirradiation hyperthermia selectively potentiates the merocyanine 540-sensitized photoinactivation of small cell lung cancer cells. Photochem Photobiol. 2001;73:191-198.

(15) Chen BJ, Cui X, Liu C, Chao NJ. Prevention of graft-versus-host disease while preserving graft-versus-leukemia effect after selective depletion of host-reactive T cells by photodynamic cell purging process. Blood. 2002;99:3083-3088.

(16) Guimond M, Balassy A, Barrette M et al. P-glycoprotein targeting: a unique strategy to selectively eliminate immunoreactive T cells. Blood. 2002;100:375-382.

(17) Davis S, Weiss MJ, Wong JR, Lampidis TJ, Chen LB. Mitochondrial and plasma membrane potentials cause unusual accumulation and retention of rhodamine 123 by human breast adenocarcinoma-derived MCF-7 cells. J Biol Chem. 1985;260:13844-13850.

(18) Lampidis TJ, Bernal SD, Summerhayes IC, Chen LB. Selective toxicity of rhodamine 123 in carcinoma cells in vitro. Cancer Res. 1983;43:716-720.

(19) Oseroff AR, Ohuoha D, Ara G et al. Intramitochondrial dyes allow selective in vitro photolysis of carcinoma cells. Proc Natl Acad Sci U S A. 1986;83:9729-9733.

(20) Udomsakdi C, Eaves CJ, Sutherland HJ, Lansdorp PM. Separation of functionally distinct subpopulations of primitive human hematopoietic cells using rhodamine-123. Exp Hematol. 1991;19:338-342.

(21) Zijlmans JM, Visser JW, Kleiverda K et al. Modification of rhodamine staining allows identification of hematopoietic stem cells with preferential short-term or long-term bone marrow-repopulating ability. Proc Natl Acad Sci U S A. 1995;92:8901-8905.

(22) Smeets M, Raymakers R, Vierwinden G et al. A low but functionally significant MDR1 expression protects primitive haemopoietic progenitor cells from anthracycline toxicity. Br J Haematol. 1997;96:346-355.

(23) Dallaire N, Leonard B Moreau B et al. Preferential eradication of B-lineage lymphoma cells over T cells using TH9402 photodynamic cell therapy and

hierarchical preservation of myeloid and erythroid progenitors. Blood. 2001;98:391a

(24) Roy DC, Boileau J, Laplante T et al. Phase I study of autologous progenitor cell transplantation purged with a photodynamic approach for patients with chronic myeloid leukemia. Blood. 2000;96:583a.

(25) Roy DC, Felix M, Cannady WG, Cannistra S, Ritz J. Comparative activities of rabbit complements of different ages using an in-vitro marrow purging model. Leuk Res. 1990;14:407-416.

(26) Taswell C. Limiting dilution assays for the determination of immunocompetent cell frequencies. I. Data analysis. J Immunol. 1981;126:1614-1619.

(27) Roy DC, Ouellet S, Le Houillier C et al. Elimination of neuroblastoma and small-cell lung cancer cells with an anti-neural cell adhesion molecule immunotoxin. J Natl Cancer Inst. 1996;88:1136-1145.

(28) Sutherland HJ, Lansdorp PM, Henkelman DH, Eaves AC, Eaves CJ. Functional characterization of individual human hematopoietic stem cells cultured at limiting dilution on supportive marrow stromal layers. Proc Natl Acad Sci U S A. 1990;87:3584-3588.

(29) O'Reilly RJ. New promise for autologous marrow transplants in leukemia. N Engl J Med. 1986;315:186-188.

(30) Roy DC, Dallaire N, Krosl G et al. Normal and neoplastic B cells are unduly susceptible to photodynamic cell therapy. Biol Blood Marrow Transplant. 2003;9:115.

(31) Cooper BW, Moss TJ, Ross AA, Ybanez J, Lazarus HM. Occult tumor contamination of hematopoietic stem-cell products does not affect clinical outcome of autologous transplantation in patients with metastatic breast cancer. J Clin Oncol. 1998;16:3509-3517.

(32) Kessel D, Luo Y. Mitochondrial photodamage and PDT-induced apoptosis. J Photochem Photobiol B. 1998;42:89-95.

(33) Levy JG. Photosensitizers in photodynamic therapy. Semin Oncol. 1994;21:4-10.

(34) Levy JG. Photodynamic therapy. Trends Biotechnol. 1995;13:14-18.

(35) Mulroney CM, Gluck S, Ho AD. The use of photodynamic therapy in bone marrow purging. Semin Oncol. 1994;21:24-27.

(36) Reszka KJ, Bilski P, Chignell CF et al. Photosensitization by anticancer agents. 11. Mechanisms of photosensitization of human leukemic cells by

diaminoanthraquinones: singlet oxygen and radical reactions. J Photochem Photobiol B. 1992;15:317-335.

(37) Jamieson C, Hornby A, Richter A, Mitchell D, Levy J. Relative sensitivity of leukemic (CML) and normal progenitor cells to treatment with the photosensitizer benzoporphyrin derivative and light. J Hematother. 1993;2:383-386.

(38) Jamieson C, Richter A, Levy JG. Efficacy of benzoporphyrin derivative, a photosensitizer, in selective destruction of leukemia cells using a murine tumor model. Exp Hematol. 1993;21:629-634.

(39) Jamieson CH, McDonald WN, Levy JG. Preferential uptake of benzoporphyrin derivative by leukemic versus normal cells. Leuk Res. 1990;14:209-219.

(40) Keating A, Jamieson C, Hornby A, Wang XH, Levy J. Photodynamic elimination of clonogenic Ph+ chronic myeloid leukemia cells. Leuk Lymphoma. 1993;11 Suppl 1:265-269.

(41) Roy DC, Paqutte Y, Balassy A et al. Elimination of chronic myeloid leukemia cells with a novel photodynamic treatment. Blood. 1999;94:144a.

(42) Weaver JL, Pine PS, Aszalos A et al. Laser scanning and confocal microscopy of daunorubicin, doxorubicin, and rhodamine 123 in multidrug-resistant cells. Exp Cell Res. 1991;196:323-329.

(43) Chaudhary PM, Roninson IB. Expression and activity of P-glycoprotein, a multidrug efflux pump, in human hematopoietic stem cells. Cell. 1991;66:85-94.

Preface to chapter 4.

The effect of PDT on antitumor immune responses indicates that the role of PDT is not restricted to the eradication of malignant cells and that PDT-generated vaccines may have clinical potential in cancer treatment. Because of the extraordinary capacity to capture and process tumor antigens, DCs most likely represent crucial determinants of cancer vaccines. In this study, we investigated the vaccine effect of DCs, previously exposed in vitro to whole tumor cell antigens generated by PDT. This approach is based on the observation that PDT treated cells may provide a strong danger signal, and promote the immunization process. As we have shown before, PDT treatment yields a population containing a mixture of viable, apoptotic, and necrotic cells. Thus, PDT using TH9402 represents an interesting approach to provide a broad and varied source of tumor antigens and has the potential to trigger the activation of DCs for enhanced vaccination efficiency.

CHAPTER 4

Specific antitumor effect of a dendritic cell-based vaccine loaded with photodynamically treated tumor cells

4.1 Abstract

Purpose: DCs have been a major focus of basic and clinical research within the last years. With their putative therapeutic value as potent antigen presenting cells, they are often used as a target cell for immunotherapy. Despite the identification of increasing numbers of tumor specific antigens, whole tumor cell vaccines remain a viable option. Since TH9402 mediated phototoxicity generated apoptotic and necrotic cell death, the ability of PDT to induce an anti-tumor response associated with efficient recognition of tumor antigens has prompted us to investigate whether PDT can be exploited to generate a DC-based antitumor vaccine.

Results: We found the combination of TNFα, IL-1β, IFNγ and CD40L induces DC maturation *in vitro* both phenotypically and functionally. A synergistic effect of IL-12 secretion was observed, in addition to high expression of cell surface markers. PDT-treated tumor cells partially induced DC maturation with increased expression of CD86 and MHC class II. DCs pulsed with antigens generated from syngeneic PDT-treated tumor cells inhibited tumor growth in both DBA and B6SJL murine models. The survival curves demonstrated protective effects of PDT vaccination with tumor-free animals. Serum levels of IFNγ in vaccinated group were significantly higher than those in the control group. The specific anti-tumor activity was confirmed by demonstrating targeted cytotoxicity. Indeed, antigen primed DCs significantly increased cytolytic activity present in the spleens of vaccinated mice.

Conclusion: In this study, we designed and optimized a protocol of DC-based cancer vaccine loaded by PDT-treated whole tumor antigens. This vaccine strategy, which uses a broad display of antigens through PDT targeting rather than single antigen immunization,

could stimulate tumor specific cytotoxic T lymphocyte (CTL) activity and generate an anti-tumor immune response. Such a vaccine may be particularly useful when tumor cells do not represent a pure cell population and are rather dispersed among normal cells.

4.2 Introduction

Dendritic cells (DCs) are the best know professional antigen presenting cells (APC) and the most effective inducers of adaptive immunity.[1,2] These cells acquire antigens from apoptotic and necrotic tumor cells, which represent a source of tumor associated antigens (TAA) for processing and presentation. Controversy exists with respect to the optimal form of killed tumor cells for stimulating effective immune priming and antitumor activity in DC-based vaccine strategies. Several authors have postulated that cells dying in apoptosis might not elicit significant cellular immune responses. In contrast, because necrosis does not occur in the absence of external stimuli, cells dying of necrosis may prove more effective in stimulating a cellular immune response.[3-7] Immature DCs present antigens quite inefficiently. Additional signals, often referred to as danger signals are needed to induce maturation, which transforms DCs into effective APCs that migrate to regional lymph nodes for the activation of T lymphocytes.[8]

Whole tumor cell vaccines are one of the earliest forms of cellular therapy. This approach avoids the need for specific identification and selection of tumor antigens before treatment and favors presentation of a wide array of putative tumor antigens in the context of appropriate costimulation.[9] Usually whole tumor antigens are generated by irradiation, boiling, or freeze-thaw lysis. From a clinical prospective, the ability of the latter to stimulate an antitumor immune response remains uncertain. Moreover, when tumor cells are dispersed among normal cells, the use of non-specific approaches may enhance the risk of immunizing against normal antigenic structures.

Photodynamic therapy is a promising treatment for various malignant disorders and can be incorporated easily into clinical practice. Both necrotic and apoptotic cells are generated after PDT, accompanying oxidative stress leading to the release of heat shock

proteins (HSP).[10,11] Of particular importance, PDT modulates the expression of several inflammatory mediators including tumor necrosis factor α (TNFα), IL-6 and IL-1,[12-14] and increases the expression of various genes involved in cell adhesion or antigen presentation.[15-18] These underlying mechanisms were found to play an important role in the therapeutic outcome. We have found that PDT can be implemented easily and yields apoptotic or necrotic cells in varying proportions under different treatment conditions. Moreover, secondary necrotic cells are readily formed from apoptotic cells over time. Therefore, PDT creates a unique condition to provide tumor antigens with danger signals for the effective stimulation of immature DCs.[19]

Clinical trials with DC-based tumor vaccines using tumor cell lysates have had some but limited success.[20,21] The peculiar biophysical effects of PDT and its potential to induce antitumor immune responses prompted us to investigate whether PDT can be exploited to generate a DC-based antitumor vaccine.

The purpose of this work was to optimize the production of antigen-loaded DC for vaccination. Various stimuli were selected to induce DCs maturation *in vitro*. Then, PDT-treated tumor cells were immediately exposed to DCs for antigen priming. The DCs-based vaccines generated a cellular mediated cytotoxic immune response that protected mice against tumor growth.

4.3 Materials and methods

Animals and tumor cells. Two strains of mice B6SJL and DBA/2J were obtained from the Jackson laboratory. All breeding and experimental procedures with mice were approved by the Animal Care Committee and followed the guidelines of the Canadian Council on Animal Care. Murine P815 mastocytoma cells, EL4 thymoma cells, A20 B lymphoma

cells and B16.F0 melanoma cell lines were obtained from the American Type Culture Collection (ATCC). P815 and B16.F0 cells were grown in DMEM and A20 cells in RPMI1640 supplemented with 10% FBS, 2mM L-glutamine, 100U/mL penicillin, and 100μg/mL streptomycin. EL4 cells were cultured in DMEM supplemented with 10% horse serum, 100U/mL penicillin, and 100μg/mL streptomycin (all from Invitrogen, Grand Island, NY) in a humidified atmosphere at 37^0C with 5% CO_2.

Photodynamic therapy. The 4,5 dibromorhodamine methyl ester (TH9402) photosensitizer was provided by Celmed BioSciences (Montreal, Canada). Exponentially growing P185 or EL4 cells were exposed to 2.5μM or 10μM TH9402, respectively. Cells were plated in a six-well plate (Sarstedt, Newton, NC), at a final concentration of 2×10^6 cells and sample thickness of 3mm. After 40 minutes of incubation in medium containing 2.5% serum, cells were resuspended in dye-free medium with 10% serum for 50 minutes extrusion, and subsequently exposed to visible light (540nm wavelength) at 5 J/cm^2 using a light scanning device.

Isolation and culture of bone marrow DCs. DCs were prepared from DBA/2J or B6SJL mice as described previously.[22] Briefly, DCs were obtained from bone marrow precursors by flushing of long bones, and RBCs lysed in a hypotonic buffer. Cells were then cultured in six-well plates in RPMI1640 medium supplemented with 10% FBS, 2mM-glutamine, 1mM 2-mercaptoethanol, 100U/mL penicillin, and 100μg/mL streptomycin in the presence of 10ng/ml GM-CSF and 20ng/ml IL-4 (BioSource, Camarillo CA). On day 6, nonadherent cells were collected and isolated by gradient centrifugation in 14.2%

metrizamide. DC purity was assessed by flow cytometry using APC-conjugated anti-CD11c.

Activation of DCs. Murine TNFα (20ng/ml), IL-1β (0.1μg/ml), IFNγ (0.1μg/ml) (all from BioSource) and CD40L (1μg/ml) (Alexis Biochemicals, San Diego, CA) were added in culture medium to stimulate DC maturation. DCs were cocultured with PDT-treated tumor cells at a ratio of 1:3 without any inflammatory mediators. After 48h-incubations, DCs underwent flow cytometry evaluation. Culture supernatants were harvested for the analysis of IL-12. As a positive control, DCs were incubated with 1μg/ml lipopolysaccharide (LPS, Sigma Chemical, St Louis, MO). DCs incubated with medium alone were used as negative controls.

DCs phenotypic analysis. DCs were subjected to Fc blocking using anti-CD16/CD32 monoclonal antibodies (mAbs), then stained with PE and FITC-conjugated mAbs specific for CD11c, CD86, I-A^b or I-A^d, and CD40 (all from BD Biosciences, Mississauga, ON). Only CD11c positive cells were analyzed for CD86, MHC class II and CD40 expression. Immunofluorescence reactivity was evaluated by automated multi-parameter flow cytometry (FACSSort; Becton Dickinson, Mountain View, CA) At least 1 X 10^4 cells were analyzed in each sample.

Vaccine preparation and immunization. PDT treated cells were pipetted onto the DCs at a ratio of 3:1 and cultured for 48 hours in presence of TNFα, IL-1β, IFNγ and CD40L as described above. DCs were isolated and washed twice in PBS prior to vaccination. Six to

10-week-old mice were immunized 3 times weekly. DCs were injected intradermally on both sides of the shoulder with 2×10^5 tumor antigens priming DCs or non-priming DCs. Control mice received PBS alone. One week after the last vaccine, mice were challenged on the flank with 2×10^4 tumor cells harvested from exponentially growing cultures. Tumor size was measured three times weekly in three dimensions with a caliper. Mice were sacrificed when the largest tumor diameter reached 17 mm. Tumor growth was monitored for 90 days. At least five mice were used in each group.

Monitoring of cytokine secretion. IL12-p40 concentration (BioSource, Camarillo CA) in culture supernatant and IFNγ levels (Bender MedSystems, Vienna, Austria) in serum collected from mice one week after tumor cell challenge were determined using commercially available ELISA kits according to the instructions of the manufacturer.

Chromium (^{51}Cr)-release assay. One week after the last vaccine, splenocytes were isolated from vaccinated and control mice. Cytolytic activity was measured as described previously.[23] Splenocytes were restimulated with irradiated (5000cGy) P815 cells, and IL-2 (10 units/ml, BioSource) added to the culture. At day 6, viable cells were harvested and assessed in a standard 4-h ^{51}Cr release assay for their ability to lyse P185 cells. Specificity was determined by measuring lysis of the different H-2 matched tumor cell line A20. Briefly, ^{51}Cr-labeled P815 or A20 cells were incubated with effector cells at different E:T ratios in triplicate wells, and ^{51}Cr-release was determined by analyzing the supernatants in a gamma counter. Spontaneous release was less than 15%. Results for individual wells were expressed as a percentage of specific lysis calculated as follows: %

specific lysis= 100 × [(experimental release-spontaneous release)/(maximum release-spontaneous)]. Spontaneous release and maximum release were obtained from wells containing target cells incubated in medium alone or in 1% Triton X, respectively.

Statistical analysis. Statistical differences in survival were calculated using the log-rank test. Other comparisons were determined using a two-tailed *t* test. Analyses were considered significant when reaching the 0.05 alpha level.

4.4 Results

The combination of TNFα, IL1β, IFNγ and CD40L induces DCs maturation.

In order to investigate the optimal condition to activate DCs maturation, the impact of TNFα, IL1β, IFNγ and CD40L on DC phenotypic maturation was evaluated. After 6 days culture in presence of GM-CSF and IL4, highly purified DCs (CD11c marker > 90%) were collected (data not shown). DCs were then incubated with TNFα, IL1β, IFNγ or CD40L separately for 48h. Expression of CD86, MHC II and CD40 was determined by flow cytometry. Fig.4.1 shows that TNFα and IFNγ were particularly efficient at inducing DCs maturation with increased expression levels of cell surface markers ($P<0.05$). There was a slight trend with the other mediators in inducing DC maturation.

The functional state of DCs was evaluated by measuring IL-12 level in the supernatants collected from the DC culture medium. We did not find enhanced secretion of IL12 when DCs were exposured to TNFα, IL1β, IFNγ and CD40L either alone or when combined as two. However, when DCs were grown in contact with a mixture comprising all mediators (TNFα, IL1β, IFNγ and CD40L), the level of IL12-P40 was increased significantly

($P<0.05$), indicating a synergistic effect of cytokines. LPS was used as a positive control (Fig.4.2).

PDT-treated tumor cells partially induce DCs maturation.

The capacity of PDT-treated tumor cells to promote the maturation of DCs in vitro was evaluated by measuring expression of CD86 and MHC class II molecules. DCs were cultured for 6 days in presence of GM-CSF and IL-4. Two populations of the easily dislodged cells were distinguished according to their expression level of CD86, and MHC II molecules. These populations were 54% and 57% CD86 positive, 25% and 41% MHC II positive, respectively in both DBA and B6SJL cells. After 48h incubation with PDT-treated P815 or EL4 cells, DCs expressed more surface antigens. As shown in Fig.4.3, the percentage of DCs expressing CD86 reached to 72% and 75%, and to 36% and 55% for MHC II, respectively. The increase for CD86 and MHC II was significant ($P<0.05$). The mean intensity of these molecules was also increased.

DCs antigen primed with PDT-treated tumor cells effectively prevent tumor growth.

To compare the immune responses in normal or vaccinated hosts, we investigated the effect of vaccine prepared from DCs with PDT-treated tumor cells. The survival curves demonstrated that protective effects in two tumor model systems (Fig.4.4). DCs loaded with PDT-treated tumor cells antigen significantly protect against tumor growth when compared with DCs alone or controls ($P<0.05$ and $P<0.01$ respectively).

To test the specificity of tumor vaccines, vaccinated B6SJL mice were inoculated with H-2 matched unrelated melanoma cell line B16.F10. Vaccination with DCs loaded on EL4

cells antigen provided no protection against growth of B16.F10 melanoma cells (data not shown), which demonstrates that this vaccine effect was tumor specific. Similar results were observed in the DBA model. The specificity of the vaccine effect was confirmed in tests of cytotoxicity.

Moreover, monitoring of tumor growth after the vaccination revealed significant differences in the growth rate between the control and the vaccinated group. The mean volume at day 23 was significantly lower in the vaccinated group than in controls in both DBA and B6SJL models ($P<0.05$ and $P<0.01$ respectively) (Fig.4.5).

DCs-based vaccines induce IFNγ secretion and produce cytotoxic activity.

Serum collected from mice vaccinated with antigen priming DCs contained significant levels of IFNγ (Fig.4.6), as compared with those from mice vaccinated with DCs without antigen priming or vaccinated with PBS ($P<0.05$).

To examine the generation of CTL response against parental tumor cells, splenocytes from DBA/2 mice vaccinated with antigen priming DCs were cultured with irradiated parental P815 tumor cells for 6 days. As shown in Fig4.7, effector cells from mice vaccinated were able to lyse parental P815 cells. Antigen priming DCs significantly increased cytolytic activity present in the spleens of vaccinated mice when compared with the activity in mice vaccinated with PBS alone ($P<0.001$ at E:T ratio of 100:1). This lysis was tumor specific, because a significant difference existed between the lysis of parental P185 cells and syngeneic non-related target cells A20 ($P<0.01$ at E:T ratio of 100:1).

4.5 Discussion

Some clinical studies indicate that vaccination with DC loaded TAA could be therapeutically relevant in cancer.[21,24,25] The aim of this strategy is to generate $CD8^+$ cytotoxic T lymphocytes (CTL) that would specifically recognize and destroy residual tumor cells. To activate a TAA specific T cell response, antigens have to presented by "professional" APC such as DC, to prime naïve T cells.[26] Activated $CD4^+$ T cells provide an important costimulation via cytokine secretion, which can initiate and also amplify the $CD8^+$ T-cell response. Ultimately, activated antigen-specific $CD8^+$ T cells become cytotoxic and lyse tumor cells.[27] However, in many cases, tumor antigens have not been clearly identified. Therefore, the use of whole tumor cells as TAA for loading of DCs is a popular approach. Whole tumor cells express an array of target antigens that will be processed and presented to both MHC class I and class II molecules. This results in a polyclonal expansion of both $CD4^+$ and $CD8^+$ T cells.[28-32] In this study, we use PDT-treated whole tumor cells as the source of TAA to be loaded onto DCs for the prevention of tumor growth in mice model.

The process by which tumor cells die during therapy is highlighted as being important in the generation of immune responses against tumor antigens.[33,34] Controversy exists with respect to the optimal form of killed tumor cells (apoptotic vs. necrotic) for stimulating effective immune anti-tumor activity in DC-based vaccine strategies. Vaccines will typically contain cell numbers in excess of what can be disposed of by scavenging macrophages. Cells undergoing secondary necrosis would be particularly suitable in providing a danger signal.[35]

We have previously shown that PDT using TH9402 yields a population containing a mixture of viable, apoptotic and necrotic cells in varying proportions. Secondary necrotic

cells are readily formed from apoptotic cells over time (manuscript in preparation). The protocol of PDT treatment used in this study resulted in the appearance of both apoptotic and necrotic cells. Such a dynamic and variable process may be used as a source of TAAs for DC-based vaccines.

The nature of activating factor in PDT-generated tumor cell is unknown. PDT-mediated oxidative stress triggers a variety of cellular signal transduction pathways leading to increased expression of stress proteins and the induction of TNFα, IL-1, and IL-6. Although the nature of the danger signals necessary for the effective stimulation of DCs interacting with dying tumor cells is unknown, there is increasing evidence suggesting that some heat shock proteins (HSP) might play this role.[36-38] This hypothesis may explain our findings that PDT-treated tumor cells appear to partially activate DCs, as supported by the increased CD86 and MHC II expression. This partial DC activation explains the weak vaccine effect of tumor growth retardation in our preliminary experiments.

To improve the antitumor activity, bone marrow-derived DCs were chosen for the vaccine preparation. Although the methodology and the criteria for an efficient DC-based vaccine have not yet been standardized, a major criterion for an effective DC vaccine is to generate cells which are both phenotypically and functionally maturation, produce IL-12 and elicit a $CD8^+$ response. Gollnick et al reported that PDT with Photofrin generated tumor cell lysates have the capacity to stimulate both the phenotypic and functional maturation of DCs *in vitro*, and to induce a cytotoxic T cell response *in vivo*.[39] Korbelik et al developed PDT-derived therapeutic cancer cell vaccine and found a large number of DCs colocalized with the vaccine cells as revealed in the lesion injection site.[40] These

findings indicate that DCs play an important role in PDT generated vaccines, by initializing an antitumor immune response.

The cross-presentation of antigens from dying cells requires antigen uptake by immature DCs, followed by DC maturation.[41] *In vitro*, DC maturation usually needs some additional maturation signals. In this study, we compared the effects of TNFα, IL-1β, IFNγ and CD40L on the maturation of bone marrow derived DCs. TNFα does not necessarily increase IL-12 secretion even though it induces DC phenotypic maturation. IFNγ is a major factor in the development of Th1 priming.[42] IFNγ-producing $CD4^+$ helper cells are necessary for the generation and maintenance of CTL. IL-1β was tested as a possible candidate molecule that could induce IL-12 due to potential analogy of signaling pathways between members of the IL1β receptor family and the toll-like receptor superfamily.[43] CD40/ CD154 (CD40L) interactions are critical for the priming and expansion of $CD4^+$ Th cells and $CD8^+$ cytotoxic T cells in response to protein antigens.[44] We demonstrated that either individual or dual treatment failed to induce a full maturation of DCs (data not shown). These results are not consistent with published observations using human DC. In human DCs, combination of CD40L and IFNγ induces the production of high levels of IL-12. Furthermore, IFN γ+ IL-1β induces a significant phenotypic and functional maturation.[45,46] The detailed mechanism is unclear, but it might be due to the human/murine species difference. However, a mixture of all these mediators induced a functional maturation, as evidenced by increased IL-12 secretion. These optimal conditions to activate DCs, together with PDT induced partial maturation by itself, prompted us to investigate whether this combined treatment could generate an effective vaccine to be used *in vivo*. Both prevention and retardation of tumor growth

were evident.(Fig.4.4 and Fig.4.5) The specificity of the vaccine was documented by the negative results obtained with mismatched tumors cells and non related tumor cell lysis in CTL.

Since PDT has been approved for successful removal of the residual cancer cells in autologous BM purging, these treated tumor cells could be reinfused to the patient. Our study implies that the patient may benefit in tumor clearance and reduce recurrence through a vaccine effect. Future studies should define the optimal conditions required for the human DC preparation to provide the most efficient antitumor activity.

4.6 Figures:

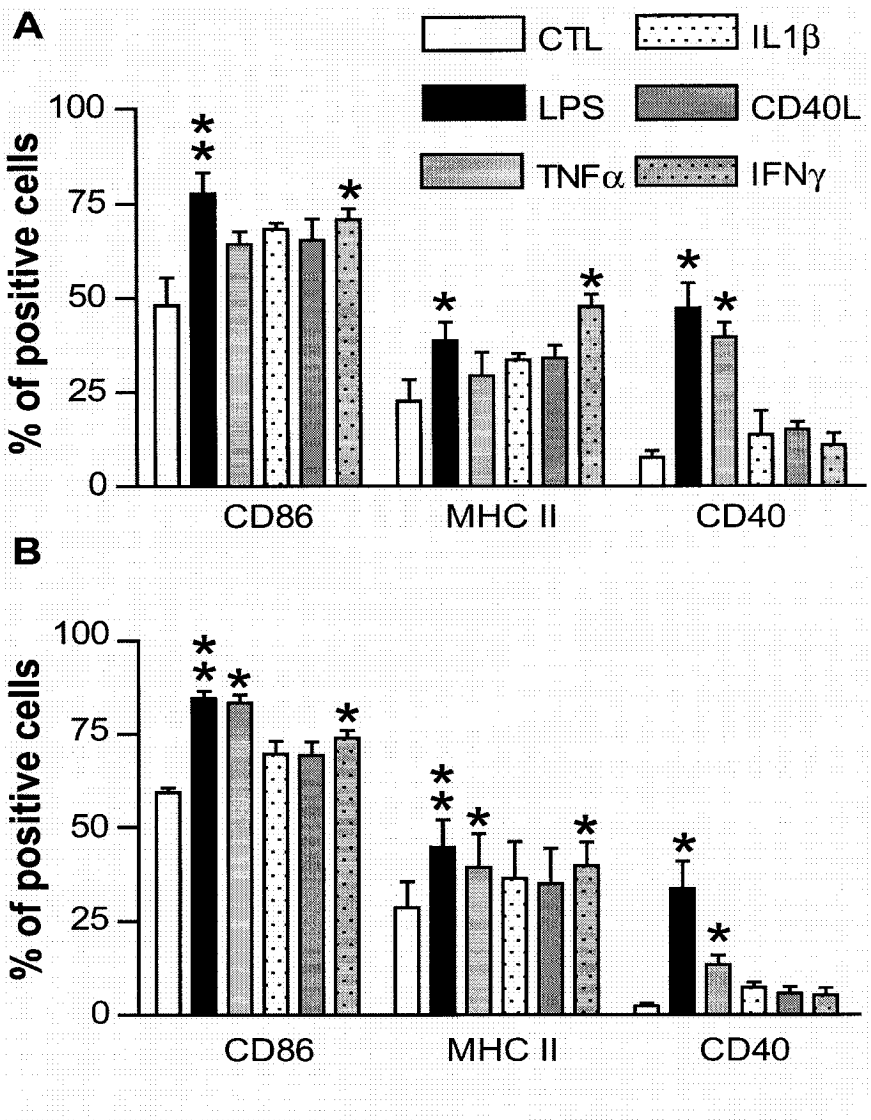

A
CTL
IL1β
LPS
CD40L
TNFα
IFNγ
% of positive cells
100
75
50
25
0
CD86
MHC II
CD40
B
% of positive cells
100
75
50
25
0
CD86
MHC II
CD40

Figure 4.1: Phenotypic maturation of DCs activated by different inflammatory mediators. Bone marrow cells from mice were differentiated into DCs in presence of GM-CSF (10ng/ml) and IL-4 (20ng/ml). At day 6, isolated immature DCs were cultured with TNFα (20ng/ml), IL-1β(0.1μg/ml), IFNγ(0.1μg/ml) or CD40L (1μg/ml) for 48h. Cells were stained with mAbs for CD86, MHC II and CD40 markers. The surface expression was analyzed by flow cytometry. DCs stimulated with LPS were considered as internal control. Results are shown as mean percentage of positive cells ± SE for three to five experiments. * $P<0.05$, ** $P<0.01$ compared with DCs without activation. **A**: DBA/2, **B**: B6SJL

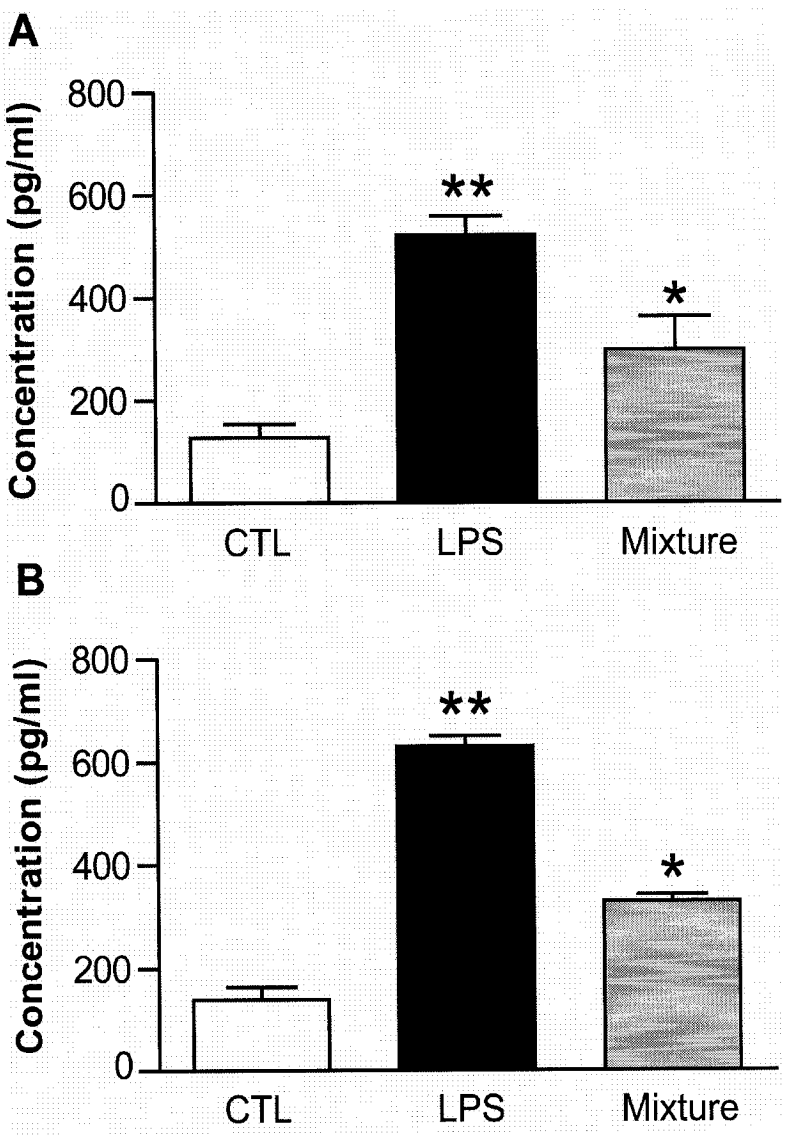

A
Concentration (pg/ml)
800
600
400
200
0
**
*
CTL
LPS
Mixture
B
Concentration (pg/ml)
800
600
400
200
0
**
*
CTL
LPS
Mixture

Figure 4.2: Secretion of IL-12 in DCs. IL-12 p40 secreted by DCs activated with a combination of TNFα, IL-1β, IFNγ and CD40L for 48h was measured directly from culture supernatants using ELISA assay. LPS was used as internal control. Values in triplicates are the concentration with a mean ± SE. This is one representative of three experiments. * $P<0.05$ ** $P<0.01$ compared with DCs without activation. **A**: DBA/2, **B**: B6SJL

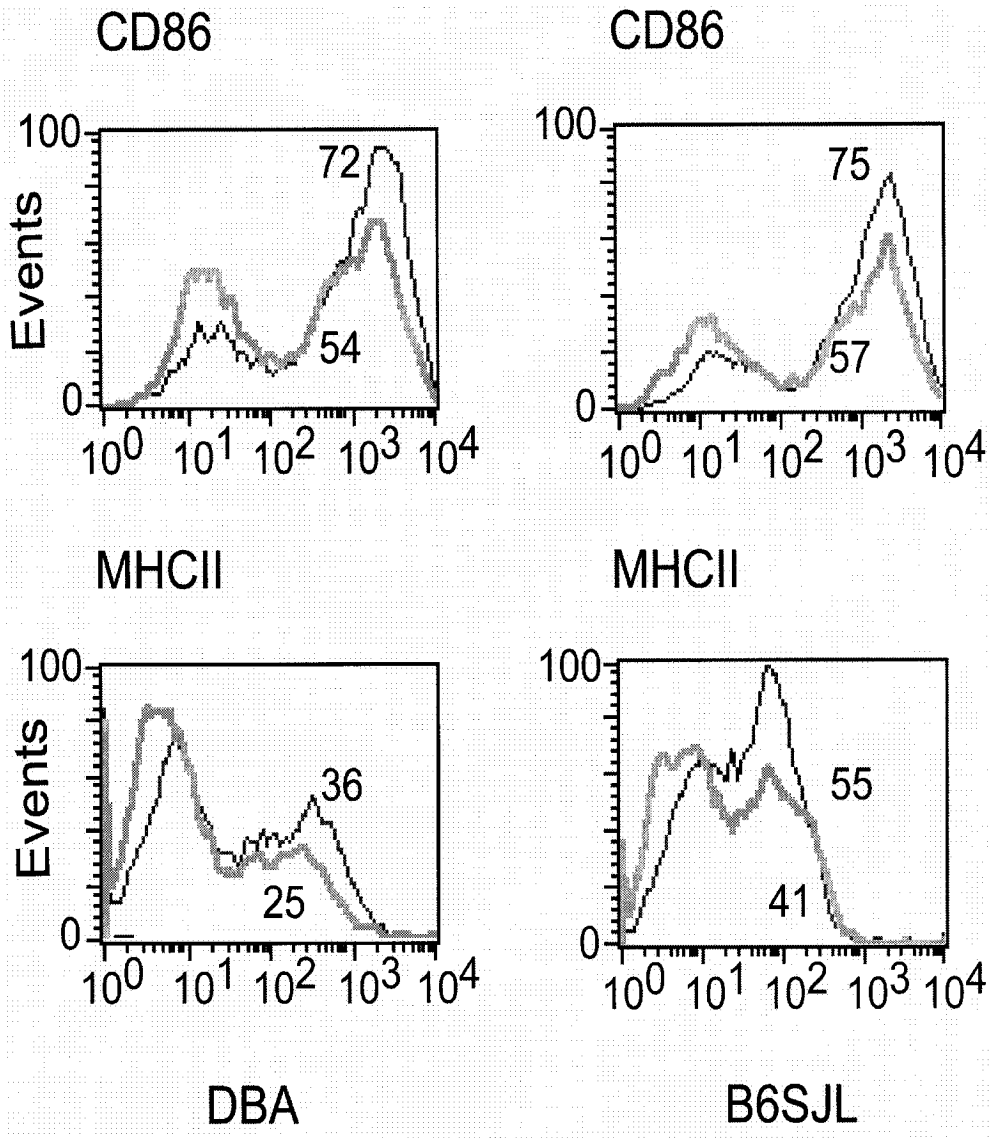

CD86
CD86
100
Events
0
72
54
75
57
10^0 10^1 10^2 10^3 10^4
MHCII
MHCII
100
Events
0
36
25
55
41
10^0 10^1 10^2 10^3 10^4
DBA
B6SJL

Figure 4.3: Increased expression of CD86 and MHC class II on DCs surface. Bone marrow derived DCs were cocultured with PDT-treated tumor cells for 48h. Surface expression of CD86 and MHC class II were analyzed by flow cytometry. Light line represents DCs without antigen priming and dark line represents DCs with antigen priming from PDT-treated tumor cells. The values are the percentage of positive cells. This is one representative of three experiments.

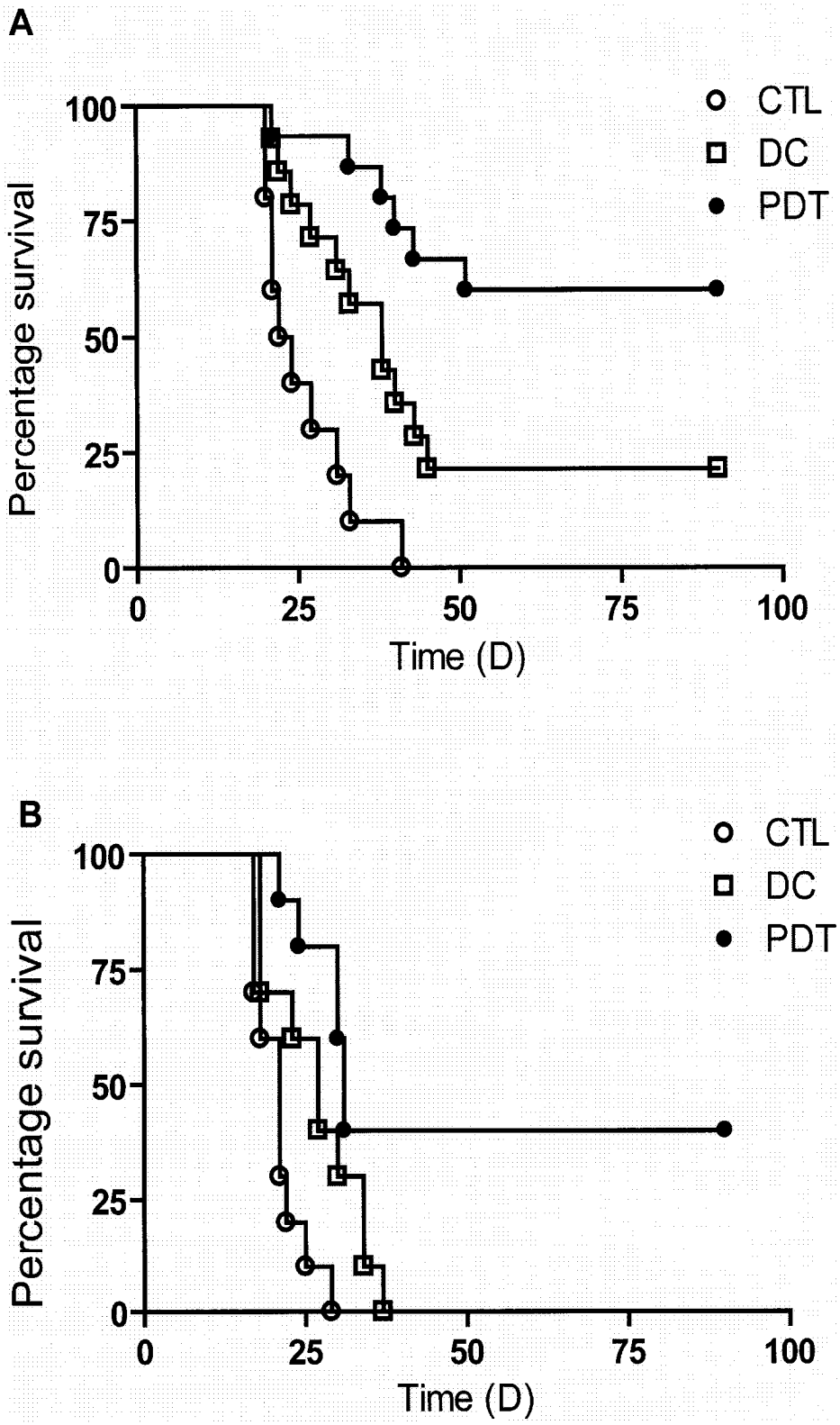

A
100
75
50
25
0
Percentage survival
0
25
50
75
100
Time (D)
CTL
DC
PDT
B
100
75
50
25
0
Percentage survival
0
25
50
75
100
Time (D)
CTL
DC
PDT

Figure 4.4: Tumor free survival of mice vaccinated with antigen priming DCs . **A,** naïve DBA mice were vaccinated with DCs antigens loaded with PDT-treated P815 cells (PDT group, n=15), or unloaded DCs (DC group, n=14) or PBS alone (Control group, n=10) once a week for 3 weeks. One week after last vaccine, mice were challenged with 2×10^4 parental P815 cells and tumor growth was monitored for 90 days. **B,** naïve B6SJL mice were vaccinated with DCs antigens loaded with PDT-treated EL4 cells (PDT group, n=10), or unloaded DCs (DC group, n=10) or PBS alone (control group, n=10) once a week for 3 weeks. One week after last vaccine, mice were challenged with 2×10^4 parental EL4 cells and tumor growth was monitored for 90 days. Results are presented as the percentage of mice which were tumor-free.

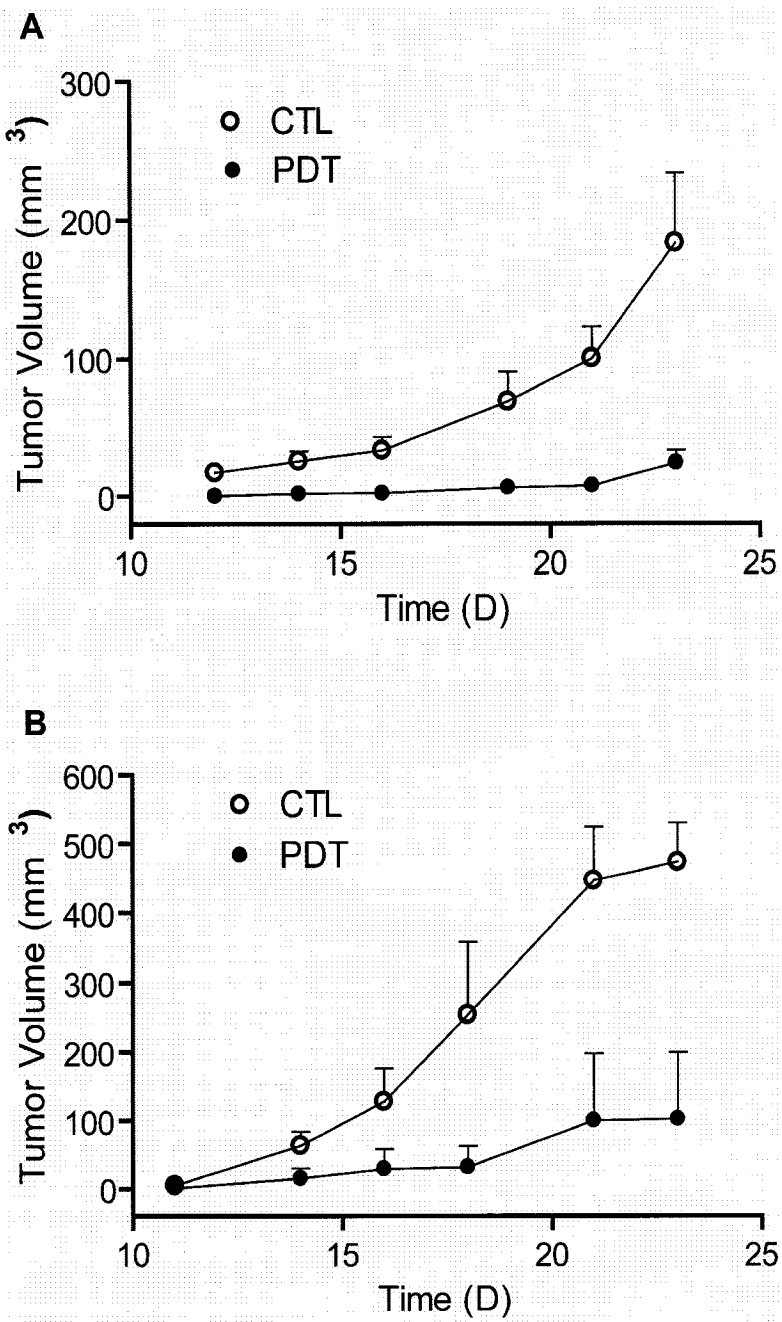

A
300
200
100
0
Tumor Volume (mm 3)
CTL
PDT
10
15
20
25
Time (D)
B
600
500
400
300
200
100
0
Tumor Volume (mm 3)
CTL
PDT
10
15
20
25
Time (D)

Figure 4.5: The effect of vaccine on tumor growth rate. Response to the vaccine treatment in two mice models (**A**: DBA, **B**: B6SJL) was determinated by subsequent tumor size measurement. Results are shown as mean ± SE. Each group consisted of at least five mice. Data are representative of at least two experiments.

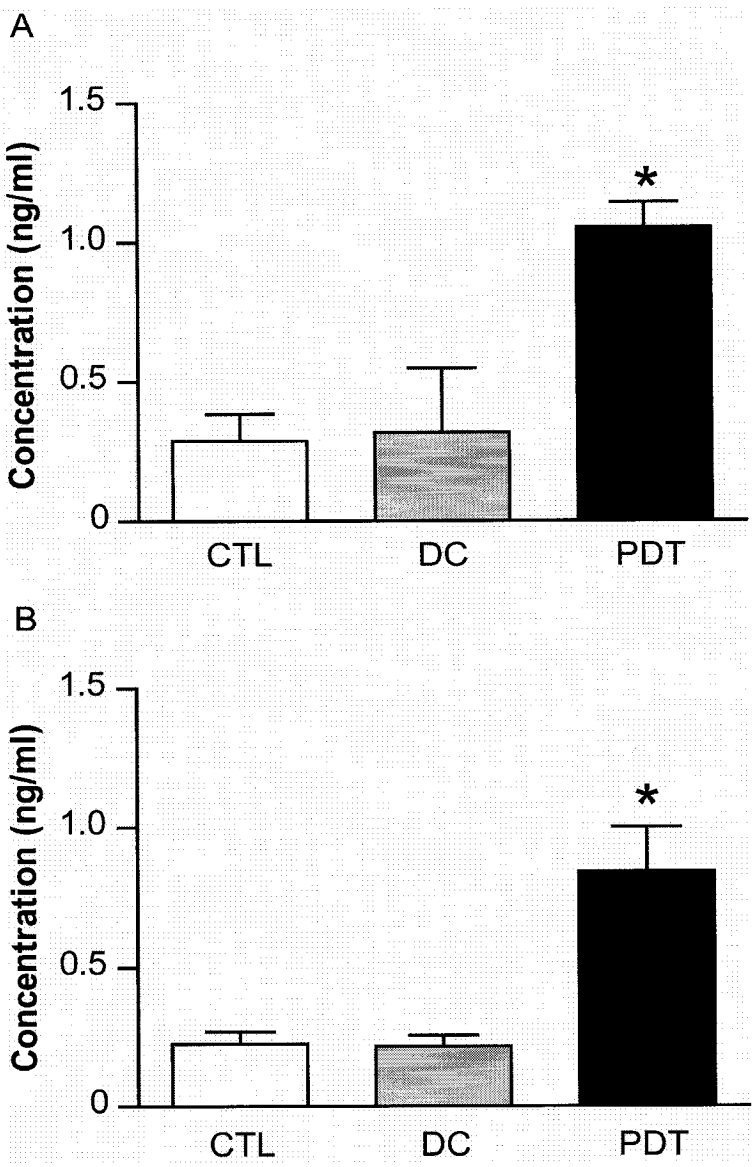

A
1.5
1.0
0.5
0
Concentration (ng/ml)
*
CTL
DC
PDT
B
1.5
1.0
0.5
0
Concentration (ng/ml)
*
CTL
DC
PDT

Figure 4.6: Serum level of IFNγ in mice. After tumor challenge in mice (**A**: DBA/2, **B**: B6SJL), serum was collected from each group. IFNγ levels were determined with an ELISA kit. Values are presented as mean ± SE. At least three mice were included in each group. The experiments were repeated twice. * $P<0.05$ compared with group vaccinated with PBS.

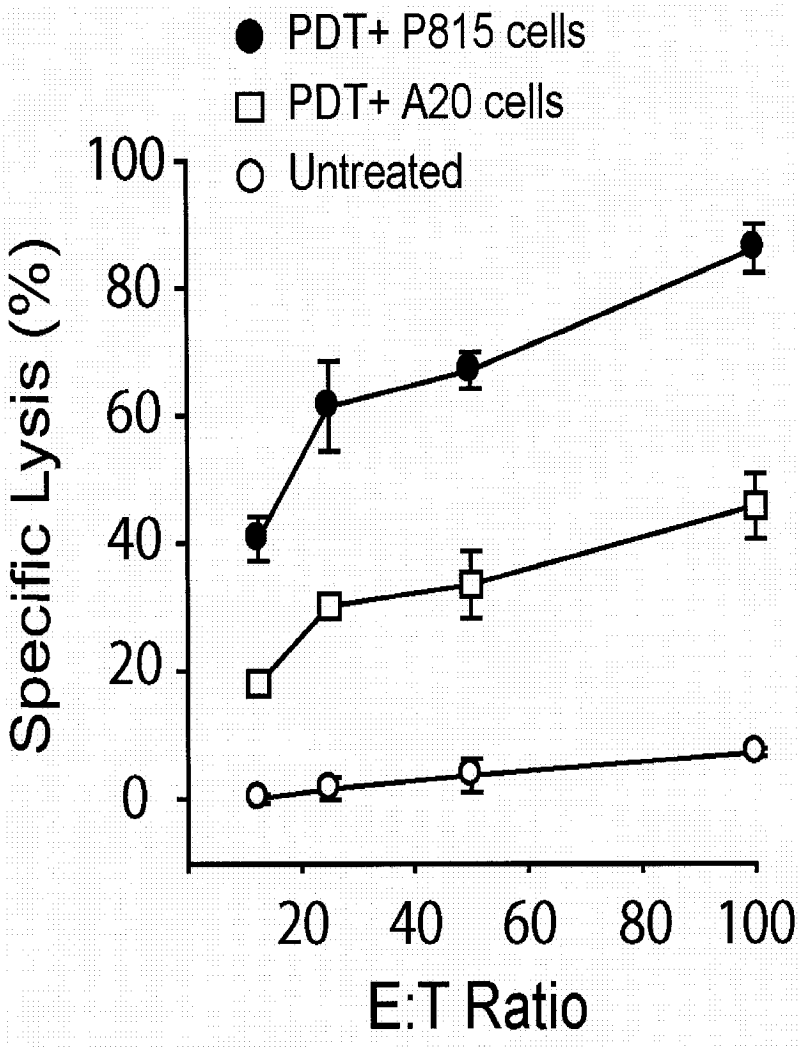

PDT+ P815 cells
PDT+ A20 cells
Untreated
Specific Lysis (%)
100
80
60
40
20
0
20
40
60
80
100
E:T Ratio

Figure 4.7: CTL activity induced by antigen priming DCs vaccination. Spleen cells from DBA/2 mice vaccinated with antigen priming DCS were restimulated *ex vivo* for 6 days with irradiated (5000cGy) parental P815 tumor cells. Cytotoxic activity against P815 cells was measured in a standard 4-h ^{51}Cr release assay. Non related target cells (A20) were chosen to evaluate specificity. Data are represented as mean specific lysis of triplicate values (%) ± SE at different E:T ratios. Data from one representative experiment of three independent analyses.

4.7 Reference List

(1) Banchereau J, Steinman RM. Dendritic cells and the control of immunity. Nature. 1998;392:245-252.

(2) Guermonprez P, Valladeau J, Zitvogel L, Thery C, Amigorena S. Antigen presentation and T cell stimulation by dendritic cells. Annu Rev Immunol. 2002;20:621-667.

(3) Albert ML, Sauter B, Bhardwaj N. Dendritic cells acquire antigen from apoptotic cells and induce class I-restricted CTLs. Nature. 1998;392:86-89.

(4) Basu S, Binder RJ, Suto R, Anderson KM, Srivastava PK. Necrotic but not apoptotic cell death releases heat shock proteins, which deliver a partial maturation signal to dendritic cells and activate the NF-kappa B pathway. Int Immunol. 2000;12:1539-1546.

(5) Gallucci S, Lolkema M, Matzinger P. Natural adjuvants: endogenous activators of dendritic cells. Nat Med. 1999;5:1249-1255.

(6) Rovere P, Vallinoto C, Bondanza A et al. Bystander apoptosis triggers dendritic cell maturation and antigen-presenting function. J Immunol. 1998;161:4467-4471.

(7) Sauter B, Albert ML, Francisco L et al. Consequences of cell death: exposure to necrotic tumor cells, but not primary tissue cells or apoptotic cells, induces the maturation of immunostimulatory dendritic cells. J Exp Med. 2000;191:423-434.

(8) Gallucci S, Matzinger P. Danger signals: SOS to the immune system. Curr Opin Immunol. 2001;13:114-119.

(9) Ward S, Casey D, Labarthe MC et al. Immunotherapeutic potential of whole tumour cells. Cancer Immunol Immunother. 2002;51:351-357.

(10) Fabris C, Valduga G, Miotto G et al. Photosensitization with zinc (II) phthalocyanine as a switch in the decision between apoptosis and necrosis. Cancer Res. 2001;61:7495-7500.

(11) Korbelik M, Sun J, Cecic I. Photodynamic therapy-induced cell surface expression and release of heat shock proteins: relevance for tumor response. Cancer Res. 2005;65:1018-1026.

(12) de Vree WJ, Essers MC, Koster JF, Sluiter W. Role of interleukin 1 and granulocyte colony-stimulating factor in photofrin-based photodynamic therapy of rat rhabdomyosarcoma tumors. Cancer Res. 1997;57:2555-2558.

(13) Gollnick SO, Liu X, Owczarczak B, Musser DA, Henderson BW. Altered expression of interleukin 6 and interleukin 10 as a result of photodynamic therapy in vivo. Cancer Res. 1997;57:3904-3909.

(14) Kick G, Messer G, Goetz A, Plewig G, Kind P. Photodynamic therapy induces expression of interleukin 6 by activation of AP-1 but not NF-kappa B DNA binding. Cancer Res. 1995;55:2373-2379.

(15) Blom DJ, Schuitmaker HJ, Waard-Siebinga I, Dubbelman TM, Jager MJ. Decreased expression of HLA class I on ocular melanoma cells following in vitro photodynamic therapy. Cancer Lett. 1997;112:239-243.

(16) King DE, Jiang H, Simkin GO et al. Photodynamic alteration of the surface receptor expression pattern of murine splenic dendritic cells. Scand J Immunol. 1999;49:184-192.

(17) Margaron P, Sorrenti R, Levy JG. Photodynamic therapy inhibits cell adhesion without altering integrin expression. Biochim Biophys Acta. 1997;1359:200-210.

(18) Runnels JM, Chen N, Ortel B, Kato D, Hasan T. BPD-MA-mediated photosensitization in vitro and in vivo: cellular adhesion and beta1 integrin expression in ovarian cancer cells. Br J Cancer. 1999;80:946-953.

(19) Jalili A, Makowski M, Switaj T et al. Effective photoimmunotherapy of murine colon carcinoma induced by the combination of photodynamic therapy and dendritic cells. Clin Cancer Res. 2004;10:4498-4508.

(20) Kugler A, Stuhler G, Walden P et al. Regression of human metastatic renal cell carcinoma after vaccination with tumor cell-dendritic cell hybrids. Nat Med. 2000;6:332-336.

(21) Nestle FO, Alijagic S, Gilliet M et al. Vaccination of melanoma patients with peptide- or tumor lysate-pulsed dendritic cells. Nat Med. 1998;4:328-332.

(22) Son YI, Egawa S, Tatsumi T et al. A novel bulk-culture method for generating mature dendritic cells from mouse bone marrow cells. J Immunol Methods. 2002;262:145-157.

(23) Brichard VG, Warnier G, Van Pel A et al. Individual differences in the orientation of the cytolytic T cell response against mouse tumor P815. Eur J Immunol. 1995;25:664-671.

(24) Gong J, Chen D, Kashiwaba M, Kufe D. Induction of antitumor activity by immunization with fusions of dendritic and carcinoma cells. Nat Med. 1997;3:558-561.

(25) Hsu FJ, Benike C, Fagnoni F et al. Vaccination of patients with B-cell lymphoma using autologous antigen-pulsed dendritic cells. Nat Med. 1996;2:52-58.

(26) Germain RN. Immunology. The ins and outs of antigen processing and presentation. Nature. 1986;322:687-689.

(27) Boon T, Cerottini JC, Van den EB, van der BP, Van Pel A. Tumor antigens recognized by T lymphocytes. Annu Rev Immunol. 1994;12:337-365.

(28) Fields RC, Shimizu K, Mule JJ. Murine dendritic cells pulsed with whole tumor lysates mediate potent antitumor immune responses in vitro and in vivo. Proc Natl Acad Sci U S A. 1998;95:9482-9487.

(29) Celluzzi CM, Falo LD, Jr. Physical interaction between dendritic cells and tumor cells results in an immunogen that induces protective and therapeutic tumor rejection. J Immunol. 1998;160:3081-3085.

(30) Inaba K, Metlay JP, Crowley MT, Steinman RM. Dendritic cells pulsed with protein antigens in vitro can prime antigen-specific, MHC-restricted T cells in situ. J Exp Med. 1990;172:631-640.

(31) Inaba K, Turley S, Yamaide F et al. Efficient presentation of phagocytosed cellular fragments on the major histocompatibility complex class II products of dendritic cells. J Exp Med. 1998;188:2163-2173.

(32) Geiger JD, Hutchinson RJ, Hohenkirk LF et al. Vaccination of pediatric solid tumor patients with tumor lysate-pulsed dendritic cells can expand specific T cells and mediate tumor regression. Cancer Res. 2001;61:8513-8519.

(33) Larsson M, Fonteneau JF, Bhardwaj N. Dendritic cells resurrect antigens from dead cells. Trends Immunol. 2001;22:141-148.

(34) Melcher A, Gough M, Todryk S, Vile R. Apoptosis or necrosis for tumor immunotherapy: what's in a name? J Mol Med. 1999;77:824-833.

(35) Ronchetti A, Rovere P, Iezzi G et al. Immunogenicity of apoptotic cells in vivo: role of antigen load, antigen-presenting cells, and cytokines. J Immunol. 1999;163:130-136.

(36) Chen W, Syldath U, Bellmann K, Burkart V, Kolb H. Human 60-kDa heat-shock protein: a danger signal to the innate immune system. J Immunol. 1999;162:3212-3219.

(37) Feng H, Zeng Y, Whitesell L, Katsanis E. Stressed apoptotic tumor cells express heat shock proteins and elicit tumor-specific immunity. Blood. 2001;97:3505-3512.

(38) Flohe SB, Bruggemann J, Lendemans S et al. Human heat shock protein 60 induces maturation of dendritic cells versus a Th1-promoting phenotype. J Immunol. 2003;170:2340-2348.

(39) Gollnick SO, Vaughan L, Henderson BW. Generation of effective antitumor vaccines using photodynamic therapy. Cancer Res. 2002;62:1604-1608.

(40) Korbelik M, Sun J. Photodynamic therapy-generated vaccine for cancer therapy. Cancer Immunol Immunother. 2006;55:900-909.

(41) Labeur MS, Roters B, Pers B et al. Generation of tumor immunity by bone marrow-derived dendritic cells correlates with dendritic cell maturation stage. J Immunol. 1999;162:168-175.

(42) Das G, Sheridan S, Janeway CA, Jr. The source of early IFN-gamma that plays a role in Th1 priming. J Immunol. 2001;167:2004-2010.

(43) Hilkens CM, Kalinski P, de Boer M, Kapsenberg ML. Human dendritic cells require exogenous interleukin-12-inducing factors to direct the development of naive T-helper cells toward the Th1 phenotype. Blood. 1997;90:1920-1926.

(44) van Kooten C, Banchereau J. Functions of CD40 on B cells, dendritic cells and other cells. Curr Opin Immunol. 1997;9:330-337.

(45) Nakahara T, Urabe K, Fukagawa S et al. Engagement of human monocyte-derived dendritic cells into interleukin (IL)-12 producers by IL-1beta + interferon (IFN)-gamma. Clin Exp Immunol. 2005;139:476-482.

(46) Snijders A, Kalinski P, Hilkens CM, Kapsenberg ML. High-level IL-12 production by human dendritic cells requires two signals. Int Immunol. 1998;10:1593-1598.

CHAPTER 5

General discussion

General discussion:

In chapter 2 to 4, the results were extensively discussed. Therefore, the present chapter is a brief compilation of those discussions. Furthermore, I will put forward some ideas about future directions of research on this topic.

PDT has become an approved clinical treatment for a variety of cancers. In addition to the direct killing of tumor cells, attained when vital cell targets are inactivated by photo-oxidative damage, multiple secondary antitumor events are elicited by the PDT-induced oxidative stress and are of a critical importance for the successful outcome of this therapy.[67,169]

Currently, a chart showing a general overview of PDT (such one shown in chapter 1) shows the history of the development in clinical reality for the cancer treatment. More than 25 years have past since PDT was proposed for the first time as a useful tool in oncology. Nevertheless, this approach is only now being used more widely in the clinic. Compared with surgery and radiotherapy, it is non-invasive and can be targeted and repeated without dose limitations. Moreover, it has been developed for other clinical applications. One of the most promising applications is the purification of malignant hematological components for transplantation. Furthermore, PDT has immunological consequences inducing a systemic antitumor response.

Despite its complexity, the biology of PDT is becoming increasingly well understood. While the cytotoxic effect of the novel rhodamine-derived TH9402 photosensitizer has been attributed to the generation of radical oxygen species, the cell death pathways involved have not been investigated. In chapter 2, we evaluated the ability of PDT to induce apoptosis and necrosis in EL4 cells, and assessed the underlying molecular mechanisms. We found that EL4 cells respond to TH9402 predominantly in

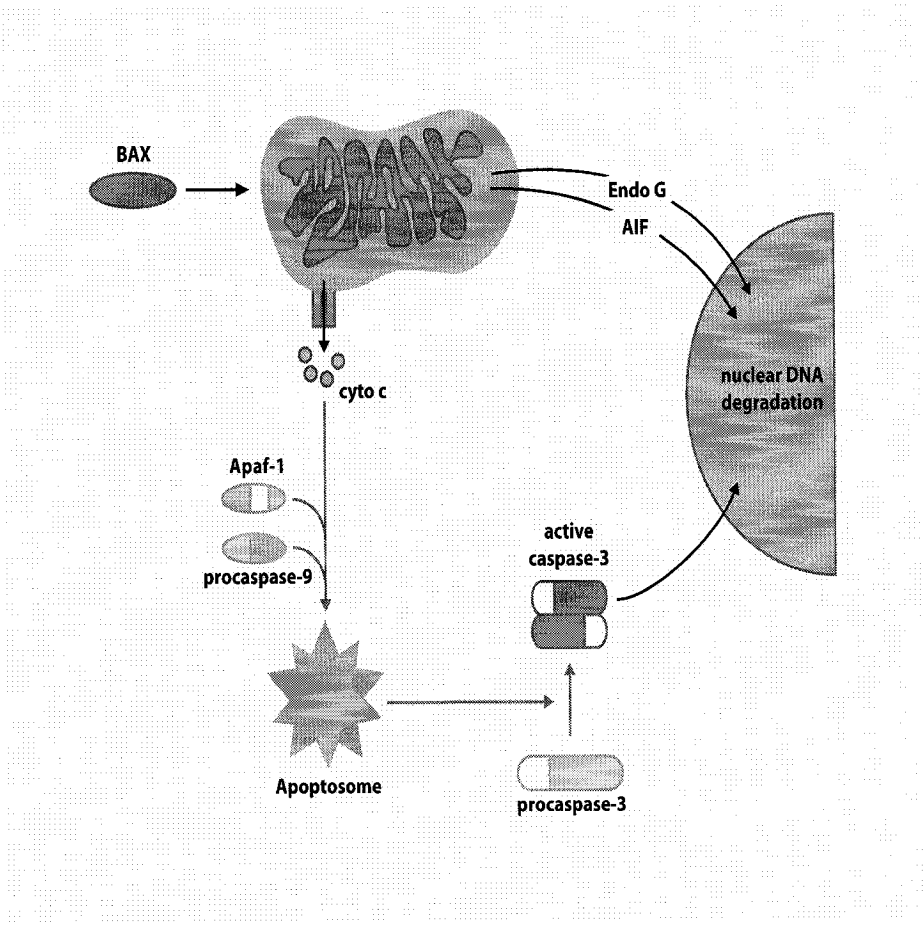


Figure 5. Scheme of apoptotic cell death pathway induced by TH9402

apoptosis. A secondary necrosis was formed evidently over time. The dominant mode of cell death after PDT was influenced by the conditions of treatment. Some adjustments in dose and light intensity could cause a switch from necrosis to apoptosis. These results show very clearly the complexity of cell death signals induced by photosensitizers.

In fact, TH9402 evoked mitochondrial permeability transition and Bax translocation to mitochondria, which resulted in cytosolic release of cytochrome C, with an increase in caspase 3 and 9 activity. This implies a role of the intrinsic caspase-dependent apoptotic pathway. However, the ability of caspase inhibition to block the induction of apoptosis and to limit cell death indicates that apoptosis induced by PDT can also involve a caspase-independent pathway, associated with AIF and Endo G release from mitochondria.(Figure 5) The observation that the TH9402 photosensitizer mediates cytotoxicity through activation of a number of death pathways suggests that it could effectively limit the capacity of tumor cells to become resistant to TH9402.

Purging cancer cells from autologous stem cell grafts is currently used to reduce the possibility of cancer relapse, but new methodologies need to be developed to improve the efficacy of treatment. TH9402-mediated PDT is a promising technique for this application. Based on the results in chapter 3, PDT using TH9402 eliminated myeloma cells efficiently among normal hematopoietic progenitors and achieved high levels of tumor cell eradication with minimal toxicity toward normal stem cells. These interim results suggest that TH9402 is able to target and eliminate cancer cells without compromising engraftment. Future experiments will include studies using primary myeloma cells coming from individual patients. This will require first to set up the methodology to identify the clone marker for each patient.

Our preliminary results also show that immunization with whole tumor cells treated by PDT was able to delay tumor cell growth. This finding encouraged us to investigate whether a DC-based vaccine could protect the mouse from tumor cell progression. The ability of DCs to generate an anti-tumor immune response in vivo has been documented in some animal models. In chapter 4, purified DCs were generated from mouse bone marrow cultured in the presence of GM-CSF and IL-4. DCs were activated to produce IL12 in the presence of a mixture of TNF, IL-1β, IFNγ and CD40L *in vitro*. This cytokine combination was also capable of promoting DC activation and it is the combination that was retained for our studies. PDT-generated tumor cells did not increase IL-12 production by DCs, but they provoked partial maturation of DCs, as evidenced by the increase in MHC II and CD86 expression. Once these activated DCs were loaded with whole tumor cells treated by PDT, a tumor protection effect was observed in two mouse models. Thus, our tumor vaccine strategy, that uses a broad display of antigens through PDT targeting rather than single antigen immunization, could stimulate tumor specific CTL activity and generate an anti-tumor immune response. Such a vaccine may be particularly useful when tumor cells do not represent a pure cell population and are rather dispersed among normal cells.

Controversy exists with respect to the optimal pathway of cell death that is able to stimulate effective immune priming and antitumor activity in DC-based vaccine strategy. The function of DCs can be dramatically affected by both apoptotic and necrotic cells. However, the response of the DCs to these two stimuli has been postulated to be very different, and this may be one important basis for the response of the immune system. The uptake of necrotic cells by DC results not only in presentation of peptides on the cell surface, but also in activation of the DC to express

co-stimulatory molecules, which are necessary for T cell activation and induction of an inflammatory response. In contrast, apoptotic cells do not seem to trigger co-stimulatory molecules, and the MHC peptide complexes have been hypothesized to selectively inactivate T cell recognition.[170,171] In our experiments, we found EL4 cells were quite sensitive to be induced on the way of cell death by necrosis or apoptosis, the latter would proceed rapidly into necrosis. In our preliminary experiments, the vaccine with cell lysates generated by PDT (induced mostly in necrosis) or whole-treated cells (induced predominantly in apoptosis) delayed the incidence of tumor after challenge with an equivalent effect.

Recently, uric acid released from dying cells has been shown to act as a danger signal for the immune system, stimulating DC maturation and enhancing T-cell responses to foreign antigens.[172] Adjuvants are thought to work, at least in part, by stimulating DCs maturation and by increasing expression of costimulatory molecules.[173,174] Shi et al[175] reported that endogenous adjuvant activity markedly increases in cells when they are injured in ways that cause them to undergo apoptosis. Consistent with this observation, uric acid in EL4 cells increased markedly after treatment with heat shock, cyclohemiximide and emetine, the factors which were shown to increase adjuvant activity.[176] When uric acid was added to cultures of primary bone-marrow-derived DCs (>95% $CD11c^+$), it stimulated DCs to increase rapidly their expression of CD86. Such a role is consistent with our observations and may partially explain the PDT effect on DCs maturation in chapter 4. This provides an insight into the mechanism underlying the increase in endogenous adjuvant in PDT-treated tumor cells. Future studies could consider the signal transduction pathways involve in DCs maturation induced by TH9402-mediated PDT, in particularly concerning the development of danger signals such as uric acid synthesis.

Since PDT using TH9402 induces cell death efficiently in either apoptosis or necrosis through different signal transduction pathway, it represents a promising preclinical method of effect on myeloma cell purging which could be potentially useful in clinical practice. Patients may benefit from the reinfusion of PDT treated autografts to prevent disease recurrence, because these tumor antigens generated from PDT may increase host immune responses through a vaccine effect with DCs.

In conclusion, TH9402-mediated PDT can be beneficial at different levels, by purging of tumor cells in autografts and eliciting host immune responses when used in combination with DCs.

Reference List for chapter 1 and Chapter 5

(1) Allison RR, Mang TS, Wilson BD. Photodynamic therapy for the treatment of nonmelanomatous cutaneous malignancies. Semin Cutan Med Surg. 1998;17:153-163.

(2) Von Tappenier H, Jodlbauer A. On the effect of photodynamic (fluorescent) substances on protozoa and enzmes. Arch Klin Med. 1904;80:427-87.

(3) Jodlbauer A, Von Tappenier H. On the participation of oxygen in the photodynamic effect of fluorescent substances. Munch Med Wochenschr. 1904;52:1139-41.

(4) LIPSON RL, BALDES EJ, OLSEN AM. Hematoporphyrin derivative: a new aid for endoscopic detection of malignant disease. J Thorac Cardiovasc Surg. 1961;42:623-629.

(5) LIPSON RL, BALDES EJ, OLSEN AM. The use of a derivative of hematoporhyrin in tumor detection. J Natl Cancer Inst. 1961;26:1-11.

(6) Diamond I, Granelli SG, McDonagh AF et al. Photodynamic therapy of malignant tumours. Lancet. 1972;2:1175-1177.

(7) Bonnett R, Berenbaum MC. HPD-a study of its components and their properties. Adv Exp Med Biol. 1983;160:241-250.

(8) Dougherty TJ. A brief history of clinical photodynamic therapy development at Roswell Park Cancer Institute. J Clin Laser Med Surg. 1996;14:219-221.

(9) Lightdale CJ, Heier SK, Marcon NE et al. Photodynamic therapy with porfimer sodium versus thermal ablation therapy with Nd:YAG laser for palliation of esophageal cancer: a multicenter randomized trial. Gastrointest Endosc. 1995;42:507-512.

(10) Biel MA. Photodynamic therapy and the treatment of head and neck neoplasia. Laryngoscope. 1998;108:1259-1268.

(11) Nseyo UO, Shumaker B, Klein EA, Sutherland K. Photodynamic therapy using porfimer sodium as an alternative to cystectomy in patients with refractory transitional cell carcinoma in situ of the bladder. Bladder Photofrin Study Group. J Urol. 1998;160:39-44.

(12) Moghissi K, Dixon K, Stringer M et al. The place of bronchoscopic photodynamic therapy in advanced unresectable lung cancer: experience of 100 cases. Eur J Cardiothorac Surg. 1999;15:1-6.

(13) Moriwaki SI, Misawa J, Yoshinari Y et al. Analysis of photosensitivity in Japanese cancer-bearing patients receiving photodynamic therapy with porfimer sodium (Photofrin). Photodermatol Photoimmunol Photomed. 2001;17:241-243.

(14) Gilson D, Ash D, Driver I, Feather JW, Brown S. Therapeutic ratio of photodynamic therapy in the treatment of superficial tumours of skin and subcutaneous tissues in man. Br J Cancer. 1988;58:665-667.

(15) Houle JM, Strong A. Clinical pharmacokinetics of verteporfin. J Clin Pharmacol. 2002;42:547-557.

(16) Houle JM, Strong HA. Duration of skin photosensitivity and incidence of photosensitivity reactions after administration of verteporfin. Retina. 2002;22:691-697.

(17) Brown SB, Mellish KJ. Verteporfin: a milestone in opthalmology and photodynamic therapy. Expert Opin Pharmacother. 2001;2:351-361.

(18) Messmer KJ, Abel SR. Verteporfin for age-related macular degeneration. Ann Pharmacother. 2001;35:1593-1598.

(19) Richter AM, Kelly B, Chow J et al. Preliminary studies on a more effective phototoxic agent than hematoporphyrin. J Natl Cancer Inst. 1987;79:1327-1332.

(20) Malik Z, Djaldetti M. 5-Aminolevulinic acid stimulation of porphyrin and hemoglobin synthesis by uninduced Friend erythroleukemic cells. Cell Differ. 1979;8:223-233.

(21) Kennedy JC, Pottier RH. Endogenous protoporphyrin IX, a clinically useful photosensitizer for photodynamic therapy. J Photochem Photobiol B. 1992;14:275-292.

(22) Wang I, Bendsoe N, Klinteberg CA et al. Photodynamic therapy vs. cryosurgery of basal cell carcinomas: results of a phase III clinical trial. Br J Dermatol. 2001;144:832-840.

(23) Morton CA, Whitehurst C, McColl JH, Moore JV, MacKie RM. Photodynamic therapy for large or multiple patches of Bowen disease and basal cell carcinoma. Arch Dermatol. 2001;137:319-324.

(24) Hopper C. Photodynamic therapy: a clinical reality in the treatment of cancer. Lancet Oncol. 2000;1:212-219.

(25) Moan J. Properties for optimal PDT sensitizers. J Photochem Photobiol B. 1990;5:521-524.

(26) Modica-Napolitano JS, Aprille JR. Basis for the selective cytotoxicity of rhodamine 123. Cancer Res. 1987;47:4361-4365.

(27) Pal P, Zeng H, Durocher G et al. Phototoxicity of some bromine-substituted rhodamine dyes: synthesis, photophysical properties and application as photosensitizers. Photochem Photobiol. 1996;63:161-168.

(28) Villeneuve L, Pal P, Durocher G et al. Spectroscopic and photophysical investigations on the nature of localization of rhodamine-123 and its dibromo derivative in different cell lines. J Fluores. 1996;6:209-219.

(29) Allison BA, Pritchard PH, Richter AM, Levy JG. The plasma distribution of benzoporphyrin derivative and the effects of plasma lipoproteins on its biodistribution. Photochem Photobiol. 1990;52:501-507.

(30) Allison BA, Waterfield E, Richter AM, Levy JG. The effects of plasma lipoproteins on in vitro tumor cell killing and in vivo tumor photosensitization with benzoporphyrin derivative. Photochem Photobiol. 1991;54:709-715.

(31) Allison BA, Pritchard PH, Levy JG. Evidence for low-density lipoprotein receptor-mediated uptake of benzoporphyrin derivative. Br J Cancer. 1994;69:833-839.

(32) Bugelski PJ, Porter CW, Dougherty TJ. Autoradiographic distribution of hematoporphyrin derivative in normal and tumor tissue of the mouse. Cancer Res. 1981;41:4606-4612.

(33) Korbelik M, Krosl G, Olive PL, Chaplin DJ. Distribution of Photofrin between tumour cells and tumour associated macrophages. Br J Cancer. 1991;64:508-512.

(34) Korbelik M, Krosl G, Chaplin DJ. Photofrin uptake by murine macrophages. Cancer Res. 1991;51:2251-2255.

(35) Bohmer RM, Morstyn G. Uptake of hematoporphyrin derivative by normal and malignant cells: effect of serum, pH, temperature, and cell size. Cancer Res. 1985;45:5328-5334.

(36) Moan J, Smedshammer L, Christensen T. Photodynamic effects on human cells exposed to light in the presence of hematoporphyrin. pH effects. Cancer Lett. 1980;9:327-332.

(37) Pottier R, Kennedy JC. The possible role of ionic species in selective biodistribution of photochemotherapeutic agents toward neoplastic tissue. J Photochem Photobiol B. 1990;8:1-16.

(38) Peng Q, Moan J, Cheng LS. The effect of glucose administration on the uptake of photofrin II in a human tumor xenograft. Cancer Lett. 1991;58:29-35.

(39) Thomas JP, Girotti AW. Glucose administration augments in vivo uptake and phototoxicity of the tumor-localizing fraction of hematoporphyrin derivative. Photochem Photobiol. 1989;49:241-247.

(40) Brown SB, Brown EA, Walker I. The present and future role of photodynamic therapy in cancer treatment. Lancet Oncol. 2004;5:497-508.

(41) Weaver JL, Pine PS, Aszalos A et al. Laser scanning and confocal microscopy of daunorubicin, doxorubicin, and rhodamine 123 in multidrug-resistant cells. Exp Cell Res. 1991;196:323-329.

(42) Chaudhary PM, Roninson IB. Expression and activity of P-glycoprotein, a multidrug efflux pump, in human hematopoietic stem cells. Cell. 1991;66:85-94.

(43) Zijlmans JM, Visser JW, Kleiverda K et al. Modification of rhodamine staining allows identification of hematopoietic stem cells with preferential short-term or long-term bone marrow-repopulating ability. Proc Natl Acad Sci U S A. 1995;92:8901-8905.

(44) Guimond M, Balassy A, Barrette M et al. P-glycoprotein targeting: a unique strategy to selectively eliminate immunoreactive T cells. Blood. 2002;100:375-382.

(45) Bailly JD, Muller C, Jaffrezou JP et al. Lack of correlation between expression and function of P-glycoprotein in acute myeloid leukemia cell lines. Leukemia. 1995;9:799-807.

(46) Denis-Gay M, Petit JM, Ratinaud MH. Rhodamine 123: is it an appropriate dye to study P-glycoprotein activity in adriamycin-resistant K562 cells? Anticancer Res. 1995;15:121-126.

(47) Marie JP, Helou C, Thevenin D, Delmer A, Zittoun R. In vitro effect of P-glycoprotein (P-gp) modulators on drug sensitivity of leukemic progenitors (CFU-L) in acute myelogenous leukemia (AML). Exp Hematol. 1992;20:565-568.

(48) Marie JP, Faussat-Suberville AM, Zhou D, Zittoun R. Daunorubicin uptake by leukemic cells: correlations with treatment outcome and mdr1 expression. Leukemia. 1993;7:825-831.

(49) Canti G, Lattuada D, Morelli S et al. Efficacy of photodynamic therapy against doxorubicin-resistant murine tumors. Cancer Lett. 1995;93:255-259.

(50) Lamy T, Drenou B, Grulois I et al. Multi-drug resistance (MDR) activity in acute leukemia determined by rhodamine 123 efflux assay. Leukemia. 1995;9:1549-1555.

(51) Peng Q, Moan J, Nesland JM. Correlation of subcellular and intratumoral photosensitizer localization with ultrastructural features after photodynamic therapy. Ultrastruct Pathol. 1996;20:109-129.

(52) Ratcliffe SL, Matthews EK. Modification of the photodynamic action of delta-aminolaevulinic acid (ALA) on rat pancreatoma cells by mitochondrial benzodiazepine receptor ligands. Br J Cancer. 1995;71:300-305.

(53) Kessel D, Luo Y. Mitochondrial photodamage and PDT-induced apoptosis. J Photochem Photobiol B. 1998;42:89-95.

(54) Kessel D. Sites of photosensitization by derivatives of hematoporphyrin. Photochem Photobiol. 1986;44:489-493.

(55) Berg K, Moan J. Lysosomes and microtubules as targets for photochemotherapy of cancer. Photochem Photobiol. 1997;65:403-409.

(56) Noodt BB, Berg K, Stokke T, Peng Q, Nesland JM. Different apoptotic pathways are induced from various intracellular sites by tetraphenylporphyrins and light. Br J Cancer. 1999;79:72-81.

(57) Moan J. Porphyrin photosensitization and phototherapy. Photochem Photobiol. 1986;43:681-690.

(58) Lee SK, Forbes IJ, Betts WH. Oxygen dependency of photocytotoxicity with haematoporphyrin derivative. Photochem Photobiol. 1984;39:631-634.

(59) Mitchell JB, McPherson S, DeGraff W et al. Oxygen dependence of hematoporphyrin derivative-induced photoinactivation of Chinese hamster cells. Cancer Res. 1985;45:2008-2011.

(60) Niedre M, Patterson MS, Wilson BC. Direct near-infrared luminescence detection of singlet oxygen generated by photodynamic therapy in cells in vitro and tissues in vivo. Photochem Photobiol. 2002;75:382-391.

(61) Ravanat JL, Di Mascio P, Martinez GR, Medeiros MH, Cadet J. Singlet oxygen induces oxidation of cellular DNA. J Biol Chem. 2000;275:40601-40604.

(62) Weishaupt KR, Gomer CJ, Dougherty TJ. Identification of singlet oxygen as the cytotoxic agent in photoinactivation of a murine tumor. Cancer Res. 1976;36:2326-2329.

(63) Wright A, Bubb WA, Hawkins CL, Davies MJ. Singlet oxygen-mediated protein oxidation: evidence for the formation of reactive side chain peroxides on tyrosine residues. Photochem Photobiol. 2002;76:35-46.

(64) Wieman TJ, Mang TS, Fingar VH et al. Effect of photodynamic therapy on blood flow in normal and tumor vessels. Surgery. 1988;104:512-517.

(65) Chen Q, Chen H, Hetzel FW. Tumor oxygenation changes post-photodynamic therapy. Photochem Photobiol. 1996;63:128-131.

(66) Henderson BW, Fingar VH. Relationship of tumor hypoxia and response to photodynamic treatment in an experimental mouse tumor. Cancer Res. 1987;47:3110-3114.

(67) Korbelik M. Induction of tumor immunity by photodynamic therapy. J Clin Laser Med Surg. 1996;14:329-334.

(68) Gomer CJ, Luna M, Ferrario A et al. Cellular targets and molecular responses associated with photodynamic therapy. J Clin Laser Med Surg. 1996;14:315-321.

(69) Anderson C, Hrabovsky S, McKinley Y et al. Phthalocyanine photodynamic therapy: disparate effects of pharmacologic inhibitors on cutaneous

photosensitivity and on tumor regression. Photochem Photobiol. 1997;65:895-901.

(70) Gollnick SO, Liu X, Owczarczak B, Musser DA, Henderson BW. Altered expression of interleukin 6 and interleukin 10 as a result of photodynamic therapy in vivo. Cancer Res. 1997;57:3904-3909.

(71) de Vree WJ, Essers MC, Koster JF, Sluiter W. Role of interleukin 1 and granulocyte colony-stimulating factor in photofrin-based photodynamic therapy of rat rhabdomyosarcoma tumors. Cancer Res. 1997;57:2555-2558.

(72) Nseyo UO, Whalen RK, Duncan MR, Berman B, Lundahl SL. Urinary cytokines following photodynamic therapy for bladder cancer. A preliminary report. Urology. 1990;36:167-171.

(73) Dougherty TJ, Gomer CJ, Henderson BW et al. Photodynamic therapy. J Natl Cancer Inst. 1998;90:889-905.

(74) Noodt BB, Berg K, Stokke T, Peng Q, Nesland JM. Apoptosis and necrosis induced with light and 5-aminolaevulinic acid-derived protoporphyrin IX. Br J Cancer. 1996;74:22-29.

(75) Dellinger M. Apoptosis or necrosis following Photofrin photosensitization: influence of the incubation protocol. Photochem Photobiol. 1996;64:182-187.

(76) Noodt BB, Rodal GH, Wainwright M et al. Apoptosis induction by different pathways with methylene blue derivative and light from mitochondrial sites in V79 cells. Int J Cancer. 1998;75:941-948.

(77) Oleinick NL, Evans HH. The photobiology of photodynamic therapy: cellular targets and mechanisms. Radiat Res. 1998;150:S146-S156.

(78) Vermes I, Haanen C, Steffens-Nakken H, Reutelingsperger C. A novel assay for apoptosis. Flow cytometric detection of phosphatidylserine expression on early apoptotic cells using fluorescein labelled Annexin V. J Immunol Methods. 1995;184:39-51.

(79) Brown JM, Wouters BG. Apoptosis, p53, and tumor cell sensitivity to anticancer agents. Cancer Res. 1999;59:1391-1399.

(80) Singh G, Jeeves WP, Wilson BC, Jang D. Mitochondrial photosensitization by Photofrin II. Photochem Photobiol. 1987;46:645-649.

(81) Zamzami N, Marchetti P, Castedo M et al. Reduction in mitochondrial potential constitutes an early irreversible step of programmed lymphocyte death in vivo. J Exp Med. 1995;181:1661-1672.

(82) Yang J, Liu X, Bhalla K et al. Prevention of apoptosis by Bcl-2: release of cytochrome c from mitochondria blocked. Science. 1997;275:1129-1132.

(83) Kluck RM, Bossy-Wetzel E, Green DR, Newmeyer DD. The release of cytochrome c from mitochondria: a primary site for Bcl-2 regulation of apoptosis. Science. 1997;275:1132-1136.

(84) Granville DJ, Carthy CM, Jiang H et al. Rapid cytochrome c release, activation of caspases 3, 6, 7 and 8 followed by Bap31 cleavage in HeLa cells treated with photodynamic therapy. FEBS Lett. 1998;437:5-10.

(85) Kessel D, Luo Y. Photodynamic therapy: a mitochondrial inducer of apoptosis. Cell Death Differ. 1999;6:28-35.

(86) Kessel D, Antolovich M, Smith KM. The role of the peripheral benzodiazepine receptor in the apoptotic response to photodynamic therapy. Photochem Photobiol. 2001;74:346-349.

(87) Kessel D, Luo Y, Mathieu P, Reiners JJ, Jr. Determinants of the apoptotic response to lysosomal photodamage. Photochem Photobiol. 2000;71:196-200.

(88) Hengartner MO. The biochemistry of apoptosis. Nature. 2000;407:770-776.

(89) van Loo G, Saelens X, van Gurp M et al. The role of mitochondrial factors in apoptosis: a Russian roulette with more than one bullet. Cell Death Differ. 2002;9:1031-1042.

(90) Martinou JC, Green DR. Breaking the mitochondrial barrier. Nat Rev Mol Cell Biol. 2001;2:63-67.

(91) Puthalakath H, Strasser A. Keeping killers on a tight leash: transcriptional and post-translational control of the pro-apoptotic activity of BH3-only proteins. Cell Death Differ. 2002;9:505-512.

(92) Korbelik M, Dougherty GJ. Photodynamic therapy-mediated immune response against subcutaneous mouse tumors. Cancer Res. 1999;59:1941-1946.

(93) Oleinick NL, Morris RL, Belichenko I. The role of apoptosis in response to photodynamic therapy: what, where, why, and how. Photochem Photobiol Sci. 2002;1:1-21.

(94) Cecic I, Korbelik M. Mediators of peripheral blood neutrophilia induced by photodynamic therapy of solid tumors. Cancer Lett. 2002;183:43-51.

(95) Granville DJ, McManus BM, Hunt DW. Photodynamic therapy: shedding light on the biochemical pathways regulating porphyrin-mediated cell death. Histol Histopathol. 2001;16:309-317.

(96) Goggins TF, Chao N. Depletion of host reactive T cells by photodynamic cell purging and prevention of graft versus host disease. Leuk Lymphoma. 2003;44:1871-1879.

(97) Mulroney CM, Gluck S, Ho AD. The use of photodynamic therapy in bone marrow purging. Semin Oncol. 1994;21:24-27.

(98) Dicke KA, Jagannath S, Spitzer G et al. The role of autologous bone marrow transplantation in various malignancies. Semin Hematol. 1984;21:109-122.

(99) Jansen J, Falkenburg JH, Stepan DE, LeBien TW. Removal of neoplastic cells from autologous bone marrow grafts with monoclonal antibodies. Semin Hematol. 1984;21:164-181.

(100) O'Reilly RJ. New promise for autologous marrow transplants in leukemia. N Engl J Med. 1986;315:186-188.

(101) Gordon MY, Goldman JM, Gordon-Smith EC. 4-Hydroperoxycyclophosphamide inhibits proliferation by human granulocyte-macrophage colony-forming cells (GM-CFC) but spares more primitive progenitor cells. Leuk Res. 1985;9:1017-1021.

(102) Atzpodien J, Gulati SC, Strife A, Clarkson BD. Photoradiation models for the clinical ex vivo treatment of autologous bone marrow grafts. Blood. 1987;70:484-489.

(103) Sieber F, Krueger GJ. Photodynamic therapy and bone marrow transplantation. Semin Hematol. 1989;26:35-39.

(104) Barr ML, Meiser BM, Eisen HJ et al. Photopheresis for the prevention of rejection in cardiac transplantation. Photopheresis Transplantation Study Group. N Engl J Med. 1998;339:1744-1751.

(105) Greinix HT, Volc-Platzer B, Kalhs P et al. Extracorporeal photochemotherapy in the treatment of severe steroid-refractory acute graft-versus-host disease: a pilot study. Blood. 2000;96:2426-2431.

(106) Roy DCPYBAeal. Elimination of chronic myeloid leukemia cells with a novel photodynamic treatment. Blood. 1999;94:144a.

(107) Armstrong AC, Eaton D, Ewing JC. Science, medicine, and the future: Cellular immunotherapy for cancer. BMJ. 2001;323:1289-1293.

(108) Shu S, Rosenberg SA. Adoptive immunotherapy of a newly induced sarcoma: immunologic characteristics of effector cells. J Immunol. 1985;135:2895-2903.

(109) Berd D, Maguire HC, Jr., McCue P, Mastrangelo MJ. Treatment of metastatic melanoma with an autologous tumor-cell vaccine: clinical and immunologic results in 64 patients. J Clin Oncol. 1990;8:1858-1867.

(110) Forni G, Fujiwara H, Martino F et al. Helper strategy in tumor immunology: expansion of helper lymphocytes and utilization of helper lymphokines for experimental and clinical immunotherapy. Cancer Metastasis Rev. 1988;7:289-309.

(111) Borrello I, Pardoll D. GM-CSF-based cellular vaccines: a review of the clinical experience. Cytokine Growth Factor Rev. 2002;13:185-193.

(112) Dranoff G, Jaffee E, Lazenby A et al. Vaccination with irradiated tumor cells engineered to secrete murine granulocyte-macrophage colony-stimulating factor stimulates potent, specific, and long-lasting anti-tumor immunity. Proc Natl Acad Sci U S A. 1993;90:3539-3543.

(113) Golumbek PT, Lazenby AJ, Levitsky HI et al. Treatment of established renal cancer by tumor cells engineered to secrete interleukin-4. Science. 1991;254:713-716.

(114) Germain RN. Immunology. The ins and outs of antigen processing and presentation. Nature. 1986;322:687-689.

(115) Swain SL, Bradley LM, Croft M et al. Helper T-cell subsets: phenotype, function and the role of lymphokines in regulating their development. Immunol Rev. 1991;123:115-144.

(116) Mackay CR. Immunological memory. Adv Immunol. 1993;53:217-265.

(117) Boon T, Cerottini JC, Van den EB, van der BP, Van Pel A. Tumor antigens recognized by T lymphocytes. Annu Rev Immunol. 1994;12:337-365.

(118) Neves AR, Ensina LF, Anselmo LB et al. Dendritic cells derived from metastatic cancer patients vaccinated with allogeneic dendritic cell-autologous tumor cell hybrids express more CD86 and induce higher levels of interferon-gamma in mixed lymphocyte reactions. Cancer Immunol Immunother. 2005;54:61-66.

(119) Hart DN. Dendritic cells: unique leukocyte populations which control the primary immune response. Blood. 1997;90:3245-3287.

(120) Hart DN, Prickett TC. Adhesion molecules in tonsil DC-T cell interactions. Adv Exp Med Biol. 1993;329:65-69.

(121) Banchereau J, Bazan F, Blanchard D et al. The CD40 antigen and its ligand. Annu Rev Immunol. 1994;12:881-922.

(122) Fagnoni FF, Takamizawa M, Godfrey WR et al. Role of B70/B7-2 in CD4+ T-cell immune responses induced by dendritic cells. Immunology. 1995;85:467-474.

(123) McLellan AD, Starling GC, Williams LA, Hock BD, Hart DN. Activation of human peripheral blood dendritic cells induces the CD86 co-stimulatory molecule. Eur J Immunol. 1995;25:2064-2068.

(124) Zhou LJ, Schwarting R, Smith HM, Tedder TF. A novel cell-surface molecule expressed by human interdigitating reticulum cells, Langerhans cells, and activated lymphocytes is a new member of the Ig superfamily. J Immunol. 1992;149:735-742.

(125) Hock BD, Starling GC, Daniel PB, Hart DN. Characterization of CMRF-44, a novel monoclonal antibody to an activation antigen expressed by the allostimulatory cells within peripheral blood, including dendritic cells. Immunology. 1994;83:573-581.

(126) Nussenzweig MC, Steinman RM, Witmer MD, Gutchinov B. A monoclonal antibody specific for mouse dendritic cells. Proc Natl Acad Sci U S A. 1982;79:161-165.

(127) Metlay JP, Witmer-Pack MD, Agger R et al. The distinct leukocyte integrins of mouse spleen dendritic cells as identified with new hamster monoclonal antibodies. J Exp Med. 1990;171:1753-1771.

(128) Kraal G, Breel M, Janse M, Bruin G. Langerhans' cells, veiled cells, and interdigitating cells in the mouse recognized by a monoclonal antibody. J Exp Med. 1986;163:981-997.

(129) Hart DN, McKenzie JL. Isolation and characterization of human tonsil dendritic cells. J Exp Med. 1988;168:157-170.

(130) Lanzavecchia A. Mechanisms of antigen uptake for presentation. Curr Opin Immunol. 1996;8:348-354.

(131) Norbury CC, Chambers BJ, Prescott AR, Ljunggren HG, Watts C. Constitutive macropinocytosis allows TAP-dependent major histocompatibility complex class I presentation of exogenous soluble antigen by bone marrow-derived dendritic cells. Eur J Immunol. 1997;27:280-288.

(132) Arnold-Schild D, Hanau D, Spehner D et al. Cutting edge: receptor-mediated endocytosis of heat shock proteins by professional antigen-presenting cells. J Immunol. 1999;162:3757-3760.

(133) Macatonia SE, Taylor PM, Knight SC, Askonas BA. Primary stimulation by dendritic cells induces antiviral proliferative and cytotoxic T cell responses in vitro. J Exp Med. 1989;169:1255-1264.

(134) Hsu FJ, Benike C, Fagnoni F et al. Vaccination of patients with B-cell lymphoma using autologous antigen-pulsed dendritic cells. Nat Med. 1996;2:52-58.

(135) Nestle FO, Alijagic S, Gilliet M et al. Vaccination of melanoma patients with peptide- or tumor lysate-pulsed dendritic cells. Nat Med. 1998;4:328-332.

(136) Flamand V, Sornasse T, Thielemans K et al. Murine dendritic cells pulsed in vitro with tumor antigen induce tumor resistance in vivo. Eur J Immunol. 1994;24:605-610.

(137) Mayordomo JI, Zorina T, Storkus WJ et al. Bone marrow-derived dendritic cells pulsed with synthetic tumour peptides elicit protective and therapeutic antitumour immunity. Nat Med. 1995;1:1297-1302.

(138) Paglia P, Chiodoni C, Rodolfo M, Colombo MP. Murine dendritic cells loaded in vitro with soluble protein prime cytotoxic T lymphocytes against tumor antigen in vivo. J Exp Med. 1996;183:317-322.

(139) Zitvogel L, Mayordomo JI, Tjandrawan T et al. Therapy of murine tumors with tumor peptide-pulsed dendritic cells: dependence on T cells, B7 costimulation, and T helper cell 1-associated cytokines. J Exp Med. 1996;183:87-97.

(140) Steinman RM, Kaplan G, Witmer MD, Cohn ZA. Identification of a novel cell type in peripheral lymphoid organs of mice. V. Purification of spleen dendritic cells, new surface markers, and maintenance in vitro. J Exp Med. 1979;149:1-16.

(141) Inaba K, Inaba M, Romani N et al. Generation of large numbers of dendritic cells from mouse bone marrow cultures supplemented with granulocyte/macrophage colony-stimulating factor. J Exp Med. 1992;176:1693-1702.

(142) Son YI, Egawa S, Tatsumi T et al. A novel bulk-culture method for generating mature dendritic cells from mouse bone marrow cells. J Immunol Methods. 2002;262:145-157.

(143) Romani N, Gruner S, Brang D et al. Proliferating dendritic cell progenitors in human blood. J Exp Med. 1994;180:83-93.

(144) Bernhard H, Disis ML, Heimfeld S et al. Generation of immunostimulatory dendritic cells from human CD34+ hematopoietic progenitor cells of the bone marrow and peripheral blood. Cancer Res. 1995;55:1099-1104.

(145) Sallusto F, Lanzavecchia A. Efficient presentation of soluble antigen by cultured human dendritic cells is maintained by granulocyte/macrophage colony-stimulating factor plus interleukin 4 and downregulated by tumor necrosis factor alpha. J Exp Med. 1994;179:1109-1118.

(146) Caux C, Dezutter-Dambuyant C, Schmitt D, Banchereau J. GM-CSF and TNF-alpha cooperate in the generation of dendritic Langerhans cells. Nature. 1992;360:258-261.

(147) Maraskovsky E, Brasel K, Teepe M et al. Dramatic increase in the numbers of functionally mature dendritic cells in Flt3 ligand-treated mice: multiple dendritic cell subpopulations identified. J Exp Med. 1996;184:1953-1962.

(148) Rosenzwajg M, Canque B, Gluckman JC. Human dendritic cell differentiation pathway from CD34+ hematopoietic precursor cells. Blood. 1996;87:535-544.

(149) Lotze MT, Hellerstedt B, Stolinski L et al. The role of interleukin-2, interleukin-12, and dendritic cells in cancer therapy. Cancer J Sci Am. 1997;3 Suppl 1:S109-S114.

(150) Austyn JM, Larsen CP. Migration patterns of dendritic leukocytes. Implications for transplantation. Transplantation. 1990;49:1-7.

(151) Svensson M, Stockinger B, Wick MJ. Bone marrow-derived dendritic cells can process bacteria for MHC-I and MHC-II presentation to T cells. J Immunol. 1997;158:4229-4236.

(152) Albert ML, Sauter B, Bhardwaj N. Dendritic cells acquire antigen from apoptotic cells and induce class I-restricted CTLs. Nature. 1998;392:86-89.

(153) Pure E, Inaba K, Crowley MT et al. Antigen processing by epidermal Langerhans cells correlates with the level of biosynthesis of major histocompatibility complex class II molecules and expression of invariant chain. J Exp Med. 1990;172:1459-1469.

(154) Zhou LJ, Tedder TF. A distinct pattern of cytokine gene expression by human CD83+ blood dendritic cells. Blood. 1995;86:3295-3301.

(155) Armitage RJ, Namen AE, Sassenfeld HM, Grabstein KH. Regulation of human T cell proliferation by IL-7. J Immunol. 1990;144:938-941.

(156) Heufler C, Koch F, Stanzl U et al. Interleukin-12 is produced by dendritic cells and mediates T helper 1 development as well as interferon-gamma production by T helper 1 cells. Eur J Immunol. 1996;26:659-668.

(157) Yawalkar N, Brand CU, Braathen LR. IL-12 gene expression in human skin-derived CD1a+ dendritic lymph cells. Arch Dermatol Res. 1996;288:79-84.

(158) Steinman RM. The dendritic cell system and its role in immunogenicity. Annu Rev Immunol. 1991;9:271-296.

(159) Ridge JP, Di Rosa F, Matzinger P. A conditioned dendritic cell can be a temporal bridge between a CD4+ T-helper and a T-killer cell. Nature. 1998;393:474-478.

(160) Bennett SR, Carbone FR, Karamalis F et al. Help for cytotoxic-T-cell responses is mediated by CD40 signalling. Nature. 1998;393:478-480.

(161) Schoenberger SP, Toes RE, van der Voort EI, Offringa R, Melief CJ. T-cell help for cytotoxic T lymphocytes is mediated by CD40-CD40L interactions. Nature. 1998;393:480-483.

(162) Hoos A, Levey DL, Lewis JJ. Autologous heat shock protein-peptide complexes for vaccination against cancer: from bench to bedside. Dev Biol (Basel). 2004;116:109-115.

(163) Lewis JJ. Therapeutic cancer vaccines: using unique antigens. Proc Natl Acad Sci U S A. 2004;101 Suppl 2:14653-14656.

(164) Korbelik M, Sun J, Cecic I. Photodynamic therapy-induced cell surface expression and release of heat shock proteins: relevance for tumor response. Cancer Res. 2005;65:1018-1026.

(165) Cecic I, Serrano K, Gyongyossy-Issa M, Korbelik M. Characteristics of complement activation in mice bearing Lewis lung carcinomas treated by photodynamic therapy. Cancer Lett. 2005;225:215-223.

(166) Prohaszka Z, Singh M, Nagy K et al. Heat shock protein 70 is a potent activator of the human complement system. Cell Stress Chaperones. 2002;7:17-22.

(167) Gollnick SO, Vaughan L, Henderson BW. Generation of effective antitumor vaccines using photodynamic therapy. Cancer Res. 2002;62:1604-1608.

(168) Korbelik M, Sun J. Photodynamic therapy-generated vaccine for cancer therapy. Cancer Immunol Immunother. 2005;1-10. *****

(169) Henderson BW, Dougherty TJ. How does photodynamic therapy work? Photochem Photobiol. 1992;55:145-157.

(170) Green DR, Beere HM. Apoptosis. Gone but not forgotten. Nature. 2000;405:28-29.

(171) Sauter B, Albert ML, Francisco L et al. Consequences of cell death: exposure to necrotic tumor cells, but not primary tissue cells or apoptotic cells, induces the maturation of immunostimulatory dendritic cells. J Exp Med. 2000;191:423-434.

(172) Hu DE, Moore AM, Thomsen LL, Brindle KM. Uric acid promotes tumor immune rejection. Cancer Res. 2004;64:5059-5062.

(173) Cox JC, Coulter AR. Adjuvants--a classification and review of their modes of action. Vaccine. 1997;15:248-256.

(174) Schijns VE. Immunological concepts of vaccine adjuvant activity. Curr Opin Immunol. 2000;12:456-463.

(175) Shi Y, Zheng W, Rock KL. Cell injury releases endogenous adjuvants that stimulate cytotoxic T cell responses. Proc Natl Acad Sci U S A. 2000;97:14590-14595.

(176) Shi Y, Evans JE, Rock KL. Molecular identification of a danger signal that alerts the immune system to dying cells. Nature. 2003;425:516-521.

Appendix