PHOTOIMMUNOTHERAPY OF MELANOMA VIA COMBINATION OF HYPERICIN-PHOTODYNAMIC THERAPY AND *IN VIVO* STIMULATION OF DENDRITIC CELLS BY PNGVL3-HFLEX PLASMID DNA

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SUMMARY

Recently, combination treatment of photodynamic therapy (PDT) with dendritic cell (DC)-based immunotherapy also termed photoimmunotherapy has been shown to be an effective anti-tumor treatment. In these studies, the DCs were expanded *in vitro* and primed *in vivo* via intratumoral injection of DCs. In the present study, the anti-tumor effectiveness of a novel anti-cancer treatment via photoimmunotherapy uitilizing the combination of hypericin (HY)-PDT and *in vivo* stimulation of DCs via pNGVL3-hFlex plasmid DNA was investigated in murine B16 melanoma. The anti-tumor effectiveness of PDT alone, photoimmunotherapy and control were compared *in vivo* in various murine models including a primary tumor model, distant established tumor (metastatic) model and when exposed to a second tumor challenge (tumor vaccine model). Photoimmunotherapy was superior to both control and PDT alone in suppressing tumor growth on a small established contralateral tumor and when exposed to a second tumor challenge. However, it was not effective in suppressing the growth of a large established contralateral tumor. Photoimmunotherapy was also not superior to PDT alone in controlling the primary tumor.

In conclusion, photoimmunotherapy using HY-based PDT and *in vivo* DC expansion by pNGVL3-Flex plasmid DNA is a novel anti-cancer modality which results in an effective systemic tumor specific anti-tumor immune response which suppresses tumor growth at distant sites.

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LIST OF ABBREVIATIONS

AJCC	American Joint Committee on Cancer
APC	antigen presenting cell
BCG	bacillus Calmette-Guerin
DC	dendritic cell
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
FBS	fetal bovine serum
GMCSF	granulocyte-monocyte colony stimulating factor
HBSS	Hanks buffered salt solution
HSP	heat shock protein
IFN	interferon
IL	interleukin
IR	infrared
MHC	major histocompatibility
MIP	macrophage inflammatory protein
mRNA	messenger ribonucleic acid
NF	nuclear factor
NK	natural killer
PBS	phosphate buffer solution
PDT	photodynamic therapy
ROS	reactive oxygen species
RPMI	Roswell Park Memorial Institute

SPG	schizophyllin
TAA	tumor associated antigen
TLR	toll-like receptor
TNF	tumor necrosis factor
UV	ultraviolet

PUBLICATION/PRESENTATION

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CHAPTER 1

INTRODUCTION

1.1 Photodynamic Therapy (PDT)

Photodynamic therapy (PDT) is a clinically established physicochemical modality for the local treatment of cancer (Korbelik and Sun, 2006). It is also presently utilized for the treatment of various non-malignant diseases (Dougherty et al., 1998). Although light has been used for the treatment of various diseases for over thousands of years, the development of PDT as a therapeutic modality for human diseases has occurred only over 100 years ago (Daniell and Hill, 1991; Ackroyd et al., 2001; Moan and Peng, 2003). At present, PDT has been advocated as a promising therapeutic modality for many human cancers, and clinical trials testing its use are being performed for malignancies afflicting almost any organ in the human body. Some of these include cancers involving the head and neck region, brain, breast, lung, skin, liver, bile ducts, bladder and gastrointestinal tract (Dougherty, 2002, Dolmans 2003). Currently, PDT is approved for use as curative treatment for early-stage cancers and for palliation in advanced malignancies.

1.1.1 PDT-induced cell death

PDT induces both apoptotic and necrotic cell death. The balance between apoptosis and necrosis after PDT *in vitro* depends on several factors including photosensitizer concentration, light fluence rate, oxygen concentration and type of tumor (Castano et al., 2005). Numerous *in vivo* and *in vitro* studies have been reported examining the pathways of apoptosis induced after PDT. These studies have described various signaling pathways, mitochondrial events and apoptotic mediators (Castano et al., 2006; Agostinis et al., 2004; Moor, 2000). The mechanism of PDT's tumoricidal effects is a complex interplay of various biochemical processes *in vivo*. The 3 key components

considered essential for effective PDT are the presence of the photosensitizer, light and oxygen. Briefly, PDT involves the systemic administration of a photosensitizer that demonstrates preferential accumulation in tumor cells, followed by illumination of the tumor with a laser beam. This generates a complex photochemical reaction which produces cytotoxic intermediates such as reactive oxygen species (ROS) that can destroy the tumor cells (Dougherty et al., 1998). Tumor destruction results not only from these direct cytotoxic effects but also from the induction of a local inflammatory response (Dougherty et al., 1998). The preferential accumulation of the photosensitizer in tumors is a critical step in PDT. This process allows targeting of tumor tissue and reduces damage to normal tissue (collateral damage). Although the mechanism of photosensitizer retention in tumor compared to normal tissue has not been fully elucidated, a multitude of factors have been proposed which contribute to this preferential distribution of photosensitizers to tumor tissue. Changes in properties of the tumor tissues itself such as decrease in pH, elevation of low density protein receptors, and presence of macrophages may contribute to this preferential distribution. Other factors such as presence of a large interstitial space, leaky vasculature, compromised lymphatic drainage, and high lipid content have also been postulated to favor preferential distribution of photosensitizers to tumor tissues (Dougherty et al., 1998).

Presently, it is believed that several biochemical processes contribute to the anti-tumor effects of PDT. Some of these key processes include: 1) direct tumor cell killing induced by photooxidative damage, (Penning and Dubbelman, 1994) 2) vascular damage and occlusion causing tumor cell damage via deprivation of oxygen and nutrients (Fingar, 1996) and 3) immune-mediated anti-tumor effects (de Vree et al., 1996; Korbelik et al.,

1996; Korbelik et al., 1997). The relative contribution of each of these mechanisms of tumor destruction is difficult to determine but it is highly likely that all of these are necessary for successful outcome after treatment (Jalili et al, 2004).

Direct tumor cell damage by oxygen free radicals is the main mechanism of tumor cell killing via PDT. When the photosensitizer absorbs light, it is activated to an excited singlet state. The activated photosensitizer is then rapidly converted to the longer-lived triplet state due to intersystem crossing (Ryter and Tyrell, 1998). Eventually, the latter can undergo two types of reactions. In type I mechanisms, a photosensitizer radical is produced which in the presence of oxygen can generate superoxide radical anions, peroxides and hydroxyl radicals (Ali and Olivo, 2003). Alternatively, in type II mechanisms, singlet oxygen is produced by reaction of the triplet state of the photosensitizer with oxygen.

Studies have demonstrated that vascular damage occurs after PDT which leads to severe and persistent post-PDT tumor hypoxia and nutrient depletion which may contribute to long-term tumor control. PDT has been shown to cause vessel constriction, vessel leakage, thrombus formation and leukocyte adhesion leading to platelet activation and thromboxane release which results in vessel damage and blood flow stasis (Fingar et al., 1993; Fingar, 1996). Inhibition of nitric oxide production and release by PDT also results in vasoconstriction (Gilissen et al., 1993). These PDT-induced changes in the tumor results in microvascular damage and collapse leading to tumor cell destruction.

1.1.2 PDT-induced immune response

Besides the direct anti-tumor effects via ROS and ischemic tumor death via vascular damage, there is accumulating evidence that PDT results in a strong anti-tumor immune response. The anti-tumor immune response after PDT is composed of both the non-tumor specific response secondary to the acute inflammatory reaction and tumor-specific immune reaction. After PDT, a wide range of photooxidative lesions produced in the cytoplasm and membrane of tumor cells, tumor vasculature and surrounding stromal elements results in the rapidly induced massive damage that threatens local homeostasis (Korbelik and Sun, 2006). These result in a strong host response which aims to contain the altered homeostasis, remove dead tissue and promote tissue healing of the affected region (Korbelik and Cecic, 2003). This host reaction to PDT manifests as the inflammatory reaction, immune response and acute phase response (Dougherty et al., 1998). Various inflammatory mediators are released and expressed at the PDT treatment site including heat shock proteins (HSP), cytokines, archidonic acid metabolites and proteins from the complement system (Cecic and Korbelik, 2002; Gollnick et al., 2003; Korbelik et al. 2005). Key components of the innate immune system such as Toll-like receptors (TLR) and the complement system are activated and play a critical role in PDT-mediated tumor destruction. Practically every component of the innate immune system participate in tumor destruction including neutrophils, mast cells, macrophages, natural killer cells and elements of the complement system such as opsonins and membrane attack complex (Korbelik and Sun, 2006; Dougherty et al., 1998). Subsequently, the activation of the innate immune system culminates in the development of the acquired tumor specific immune response (Dougherty et al, 1998). Innate immune cell presence and activation is essential for the development of acquired immunity and innate cell

infiltration into the treated tumor bed is a hallmark of PDT (Kousis et al., 2007; Dougherty et al., 1998). The acute inflammation caused by PDT-induced tumor cell necrosis attracts leucocytes to the tumor and increases antigen presentation. Heat shock protein (HSP) 70 which is thought to be one of the most important cellular factors involved in PDT-induced immune response is released and is involved in various interactions with antigen presenting cells (APCs) including dendritic cells (DCs).

Tumor-specific immune response has been shown to be an important mechanism in PDT-induced tumor destruction. There are numerous preclinical studies that suggest that PDT enhances the systemic anti-tumor immune response although the mechanism behind this enhancement remains unclear (Castano et al., 2006). Dougherty et al pointed out that the tumor specific immune response may not be important in initial tumor cell damage but its effect may be essential in maintaining long-term tumor control (Dougherty et al., 1998). APCs such as DCs, macrophages and B lymphocytes are important mediators in the initial step of tumor-specific immune response. Cancer cells damaged or destroyed by PDT are processed by APCs and the antigens are presented on the cell membranes via major histocompatibility (MHC) class molecules. These tumor antigens are recognized by T helper lymphocytes which are than activated and subsequently sensitize cytotoxic T lymphocytes to the tumor antigens. The activation, expansion and differentiation of T lymphocytes lead to the development of tumor-specific immunity. These tumor sensitized lymphocytes have the potential to eliminate disseminated tumor cells. Thus, PDT may be associated with a systemic immune reaction and anti-tumor effect although PDT by itself is by definition a local therapeutic modality.

The findings of several studies that lymphoid cells are essential for preventing the recurrence of PDT-treated tumors provide further support for the role of the tumor-specific anti-tumor immune reaction in PDT. Korbelik et al documented that PDT-mediated curability of mouse cancers was reduced or non-existent in severe combined immune deficient mice (Korbelik and Dougherty, 1999; Korbelik et al., 1999). This could be restored after bone marrow transplant or T-cell transfer from immunocompetent mice. Furthermore, immune memory cells could be recovered from distant lymphoid sites suggesting that long-lasting systemic immunity was raised against even poorly immunogenic tumors (Korbelik and Dougherty, 1999; Korbelik et al., 1999). Hendrzak-Henion et al., 1999 also demonstrated that after PDT treatment, immune-deficient mice could not demonstrate complete tumor remission as opposed to immune-competent mice which were permanently cured. The results of these studies suggest that PDT can generate immune memory cells and thus has the potential to be combined with immunotherapy protocols in the treatment of malignant tumors. This potential has since been confirmed by several studies which have demonstrated that immune-stimulating cytokines, immunomodulators and adoptive transfer of immune cells have the ability to enhance the anti-tumor effectiveness of PDT (Golab et al., 2000; Krosl et al., 1996; Korbelik et al., 2001). Further evidence of the anti-tumor immune effects of PDT were the observations in some studies that localized therapy with PDT was capable of controlling distant disease (Gomer et al., 1987). In the recent study by Kabingu et al., 2007, the investigators found that PDT-treatment of subcutaneous tumors resulted in inhibition of the growth of distant lung metastases. This study was the first to demonstrate that $CD8^+$ T cell was responsible for the control of tumors growing outside

the treatment field following PDT. Earlier studies showing inhibition of distant tumor growth away from the treatment field did not attempt to determine the specific effector cell-type responsible for tumor control (Gomer et al., 1987; Blank et al, 2001). $CD8^+$ T lymphocyte mediated control of the distant lung tumors was found to be independent of $CD4^+$ T lymphocytes but dependent on natural killer (NK) cells (Kabingu et al., 2007). These results were consistent with the earlier findings of Korbelik and Dougherty, 1999 whereby depletion of $CD8^+$ T cells substantially impaired the ability of PDT to suppress the long-term growth of EMT6 as opposed to the depletion of $CD4^+$ T cells. Anecdotal clinical cases of regression of distant tumors after local PDT have also been reported in the literature (Thong et al., 2007; Naylor et al., 2006). Thong et al. reported an interesting case of a histologically-proven multifocal cutaneous angiosarcoma of the head and neck region. The patient underwent localized Fotolon-PDT of several selected lesions (Thong et al., 2007). Spontaneous regression was subsequently observed in several of the cutaneous lesions at distant sites. Biopsies demonstrated that these distant lesions were infiltrated by $CD8^+$ T-cell clones which suggest that PDT resulted in a systemic acquired immune response which resulted in the systemic anti-tumor activity.

1.1.3 PDT-generated anti-tumor vaccines

In 2002, Gollnick et al., 2002 performed the first study to directly demonstrate the ability of PDT to enhance tumor immunogenicity and to generate an effective anti-tumor vaccine. They demonstrated that PDT-generated cell lysates were immunogenic and PDT-generated vaccines were more effective than ultraviolet (UV) or ionizing radiation-generated vaccines. These vaccines were tumor-specific, induced a cytotoxic T-cell

response and did not require the co-administration of an adjuvant to be effective. PDT-generated lysates were shown to activate DCs to express interleukin (IL)-12 which is critical in inducing a cytotoxic T-cell response. This capacity of PDT to stimulate both phenotypic and functional maturation of DCs was postulated to be the key reason behind the effectiveness of PDT in generating an effective anti-tumor vaccine. Subsequently, their findings were confirmed more recently by Korbelik and Sun, 2006. In a similar study, Korbelik and Sun, 2006 demonstrated that PDT-generated vaccines were significantly superior to vaccines generated by lysed cells or X-ray treated cells in producing tumor growth retardation, tumor regression and even complete tumor cures. This study further confirmed the unique advantage of PDT for the generation of anti-tumor vaccines. Moreover, the PDT-generated vaccines were tumor-specific as documented by its lack of efficacy against mismatched tumors. This finding was a firm indication that the observed anti-tumor effects were due to a PDT-induced tumor-specific immune response. It also further demonstrated that vital components of the tumor-specific immune response such as DCs, memory T- and memory B-cells were dramatically increased at the tumor site and its draining nodes. Korbelik and Sun, 2006 also demonstrated that vaccine cells retrieved from the treatment site 1 hour after injection were intermixed with DCs, expressed HSP70 on their surface and were opsonized by complement C3 verifying the findings of several earlier studies (Castano et al., 2006). More recently, Kousis et al., 2007 demonstrated that the induction of the anti-tumor immune response after PDT is dependent on neutrophil infiltration into the treated tumor bed. They further suggested that neutrophils may be responsible for directly

stimulating T-cell proliferation and/or survival. However, these did not seem to affect DC maturation or T-cell migration.

Unlike PDT, most of the routinely used anti-cancer therapies cause immunosuppression. Radiotherapy and chemotherapy delivered at doses sufficient to produce tumor destruction are well-known to be toxic to the bone marrow which results in myelosuppression and hence, immunosuppression (Castano, et al., 2006). Nonetheless, it is important to note that low doses of radiotherapy and chemotherapy may enhance the immune system including induction of HSPs (Sierra-Rivera et al., 1993). Major surgery has also been reported to produce immunosuppression, leading to diminished lymphocyte and natural (NK) cell function (Ng et al., 2005). Hence, unlike these traditionally available therapeutic modalities, PDT has the properties of an ideal cancer therapy which not only results in primary tumor destruction but also triggers the immune system to recognize and destroy remaining tumor cells which may be local or distant (Castano et al., 2006).

1.2 Hypericin (HY)-mediated PDT.

The ideal photosensitizer for PDT should have the following properties including: chemical purity, minimal dark toxicity, significant light absorption at wavelengths that penetrate tissue deeply, high tumor selectivity and rapid clearance from normal tissue (Ali and Olivo, 2003; Pass, 1993; Fisher et al., 1995). Various photosensitizers have been approved and are currently used for the clinical treatment of cancer. These include Metvix (5-aminiolevulinic acid- methylesther), Foscan (meta-tetrahydroxyphenylchlorin) and Photofrin (Hematoporphyrin derivative). The most commonly used photosensitizer

presently is probably Photofrin. However, this first generation photosenstizer has several notable limitations including the low light absorption, low tumor tissue selectivity and long duration of cutaneous photosensitivity (Dolmans et al., 2003). This has led researchers search for newer improved drugs with properties closer to that of an ideal photosensitizer.

HY is a chemical found in the *Hypericum* species of herb of which hypericum perforatum or St John's Wort is the most common genum. It is a herb with golden yellow flowers (Lavie et al, 1995). The proto-forms of HY and its congener pseudo-hypericin exist as dark-coloured granules in minute glands of St John's Wort (Southwell and Bourke, 2001). These structures of partially cyclic precursors are transformed into naphthodianthrone analogues; HY and pseudoHY on light irradiation. The chemical structure of HY is demonstrated in Figure 1.

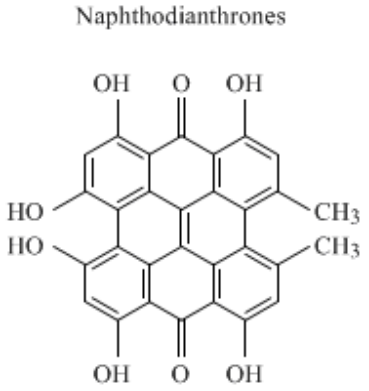


Figure 1. Chemical structure of HY

Under physiological conditions, HY is present as a monobasic salt. It can be taken up by cellular lipid membrane structures and behave as lipophilic ion pairs (Lavie, et al, 1995). HY exhibits bright fluorescence detection in organic solvent, which makes it an ideal diagnostic tool for fluorescence detection (Olivo et al, 2003). The utility of intra-vesical instillation of HY for the detection of flat bladder neoplasms have been demonstrated in a clinical trial (D'Hallewin et al, 2002). HY is a powerful photosensitizer as it demonstrates a high yield of singlet oxygen and its minimal dark toxicity makes it a very promising and useful clinical tool (Agostinis, 2002). It is metabolized rapidly *in vivo* and has minimal toxic properties when administered sytemically (Meruelo et al., 1988). *In vivo* studies have demonstrated that HY binds well to tumor cells and are retained for longer periods than normal tissues (Chung et al., 1984). HY has been studied in several clinical trials for the treatment of skin cancers, brain tumors and cutaneous lymphoma (Alecu et al., 1998, Lavie et al., 2000). However, its use has never been evaluated in malignant melanoma.

HY has an extremely broad absorption spectra making it readily excitable by a variety of light sources (Miller et al., 1995; Schempp et al., 1999) It is maximally activated at a wavelength of light of approximately 470 nm (Ali and Olivo, 2003). The photodynamic effects of HY have been well-investigated by numerous investigators. It has been shown that high PDT doses induce rapid cell necrosis whereas lower intermediate doses induce apoptosis (Agostinis et al., 2002, Ali et al., 2001). The apoptotic pathway of cell death after HY-PDT has been well-elucidated. This has been shown to be mediated by the mitochondria followed by activation of the caspase cascade (Ali et al., 2001) possibly via hydrogen peroxide production (Ali et al., 2002). Assefa et

al. demonstrated that activation of c-Jun N-terminal kinase (also known as stress activated protein kinase) and p38 mitogen-activated protein kinase increases the resistance to HY-induced apoptosis (Assefa et al., 1999; Hendrickx et al., 2003). Other effects of HY-PDT reported include activation of lipid peroxidation (Chaloupka et al., 1999; Miccoli et al., 1998), inhibition of protein kinase C, inhibition of growth factor stimulated protein kinase (Agostinis et al., 1995; de Witte et al., 1993) and increase in matrix metalloproteinase-1 (Du, et al., 2004). In NPC/HK1 nasopharyngeal carcinoma cells, it has been shown that HY-PDT produced maximal tumor regression in mice when the incubation period was 1 hour and 6 hours after instillation of HY whereas HY-PDT was less effective when incubation periods were between this time interval (Du et al., 2003). Du et al., 2003 further demonstrated that at an incubation of 1 hour the HY concentration was maximal in the mouse plasma whereas at an incubation of 6 hours, HY concentration was maximal in the tumor tissue and low in plasma. Hence it was postulated that HY-PDT could induce tumor necrosis and shrinkage via 2 mechanisms ie. via vascular damage and direct tumor cell killing.

It is essential to note that different cell types may demonstrate a different response to HY-PDT (Kyriakis, 1999; Lavie et al., 1999). It is well-known that the mechanism of tumor destruction by HY-PDT hinges on several important factors including type of tumor cell, tumor microvasculature, host inflammatory response and host immune response (Dougherty et al., 1998).

1.3 Immunotherapy with DCs

Protective immunity results from the combined action of the innate and adaptive immune system (O'Neill and Bhardwaj, 2007). The innate arm of the immune system is composed of phagocytic cells, NK cells and complement which provide an early and rapid non-specific immune response. Both B and T cells make up the adaptive immune system which is critical for the generation of immunologic memory. Proper functioning of both the innate and adaptive immunity is critical against the development of malignant tumors (Smyth et al, 2006). APCs provide an important link between the two arms of the immune system. They process intra- and extra-cellular proteins into antigenic peptides which are then presented to cells of the adaptive immune system. Although, B cells, macrophages, monocytes and DCs can all function as APCs, DCs are thought to be the most potent APC of them all (Banchereau, et al, 2000). This has been demonstrated by both *in vitro* and *in vivo* experiments (Steinman and Pope, 2002).

As with other APCs, DCs play an important role in activating both the innate and adaptive components of the immune system via interaction with naïve T-cells (Steinman, 1997). DCs drive both the cell-mediated and humoral arms of the adaptive immune response. They express high levels of major histocompatibility (MHC) molecules and immune co-stimulatory accessory molecules and are responsible for the secretion of many potent T-cell-activating cytokines which are critical for an effective immune response (Fong and Hui, 2002). DCs specialize in acquiring, processing and presenting antigens to naive, resting T-cells activating them to induce an antigen-specific immune response (Banchereau et al., 1998). The process of efficiently capturing antigens is restricted to the immature stage of development when DCs express low levels of MHC and co-stimulatory molecules. During this immature stage, DCs are inefficient APCs.

Additional signals frequently referred to as danger signals are essential for inducing the maturation of DCs and to transform them into effective APCs. Although this danger signals necessary for the activation of DCs has not been unequivocally resolved, there is mounting evidence that some of the HSPs play a critical part (Flohe et al., 2003, Chen et al., 1999). DCs are capable of processing both endogenous (synthesized within the DC cytosol) and exogenous (from the extra-cellular environment) antigens (Aloysius et al., 2006). Examples of exogenous antigens include viruses, bacteria, cell products from necrotic or apoptotic cells, immune complexes and HSPs. These antigens are captured through various receptors via various mechamisms like endocytosis, pinocytosis or phagocytosis. These captured antigens are processed into immunogenic fragments which bind to MHC class I and II molecules which are transported to the cell surface for recognition by and activation of antigen-specific T-cells. Endogenous antigens on the other hand are broken down in the cytosol. These are then transported into the endoplasmic reticulum via special transporters (transporters of antigen presentation). The peptides are loaded to MHC class I molecules within the endoplasmic reticulum and are transferred to the cell surface via the golgi-body network for presentation to $CD8^+$ T cells.

DCs are derived from bone marrow progenitors and circulate in the blood as immature precursors. They migrate to various tissues such as the subepithelial compartment of the respiratory tract, basal layer of the epidermis, in the lamina propria of gut wall and in organized lymph follicles such as Peyer's patches (Aloysius et al., 2006). Here, they constantly sample the micro-environment for foreign antigens. These are then captured, processed and than presented on the cell surface linked to MHC molecules.

After stimulation, DCs undergo further maturation and subsequently migrate to secondary lymphoid tissues where they present antigens to T-cells and induce an antigen specific immune response (Austyn et al., 1988). When matured, DCs lose their ability to take up antigen. The homing of DCs into nearby regional lymph nodes has been shown to be dependent on the expression of chemokine receptor 7. DCs are also responsible for inducing the humoral arm of the acquired immune system (Aloysius et al., 2006). They induce memory B cell differentiation into effector plasma cells and regulate B cell priming. More recent evidence also suggests that DCs play a crucial role in regulating the hosts innate immunity (Degli-Espost and Smyth, 2005). The complex interaction and cross-talk between DCs and NK cells play a vital role in this process.

Cell surface phenotyping has shown that are as many as 5 distinct subtypes of DCs at least in mice (O'Neill and Bhardwaj, 2007; Shortman and Liu, 2002; Clark et al., 2005). In humans, the three best characterized DCs include cells resembling epidermal Langerhans cells, cells resembling dermal or interstitial DCs and plasmacytoid DCs (O'Neill and Bhardwaj, 2007). The precise origin of the different DC subtypes is unclear although it has previously been thought that most DCs are of myeloid origin. In mice, it has been shown that DC can be derived from common myeloid and common lymphoid progenitors as well as a third progenitor cell without either myeloid or lymphoid potential (del Hoyo et al., 2002)

There is presently vast amount of data in the literature which support the concept that cancer patients can spontaneously develop specific adaptive immune responses to tumor associated antigens (TAAs) (Aloysius et al., 2006). Various tumor antigens have been discovered in different malignancies which are potential immunological targets for

T-cells. Effective T-cell response to these antigens forms the basis for immune elimination of tumor cells. DCs are professional APCs which are responsible for presenting TAAs to immature T-cells in regional tumor draining lymph nodes leading to the expression of tumor specific $CD8^+$ T-cells. However, there is evidence to suggest that there is a decrease in the absolute numbers of peripheral circulating DCs and tumor infiltrating DCs in various malignancies. Moreover, there also appears to be abnormal differentiation and maturation of DCs in cancer patients. As a consequence of this impaired tumor recognition and antigen presentation mechanisms by DCs, immune evasion occurs and the tumor progresses (Aloysius et al., 2006). These findings have prompted some investigators to utilize regimes involving *ex vivo* differentiation and maturation of DCs in an optimal milieu before using them for anti-cancer DC immunotherapy.

Cancer immunotherapy has a very long history (although unrecognized) (Du, 2004). It was noted by the Egyptians that surgical opening of the tumor site could induce tumor regression, presumably through the generation of infection and activation of the immune system (Hoption Cann et al., 2003; Castano et al., 2006). More then a hundred years ago, William Coley who was a surgeon found that certain infections could induce tumor regression and he created a 'vaccine' based initially on bacteria (Castano et al., 2006). The legacy of his findings continues until today. For example, the bacillus Calmette-Guerin (BCG) vaccine derived from Mycobacterium bovis which is used for the prevention of tuberculosis is still presently utilized for the treatment of superficial bladder cancer (Bassi, 2002). Since these initial studies, groundbreaking discoveries in immunology have identified the key roles of various mediators in propogating the anti-

tumor immune response and numerous immunotherapy modalities using ILs, DCs and lymphocytes have been generated.

DCs have generated great interest as a vaccine adjuvant because of their potent immuno-stimulatory capacity and ability to prime immature T-cells (Banchereau and Palucka, 2005). As DCs are present in most tissues especially tumors at a very low frequency (which is therefore the most likely rate-limiting step), the addition of autologous DCs should theoretically result in a stronger and more durable tumor-specific immunity (Saji et al., 2006). DC-based immunotherapy can be broadly classified into *in vivo* mobilization and *in vitro* manipulation techniques. Presently, the most common approach for DC immunotherapy is to isolate large numbers of DCs by culturing bone marrow progenitors *ex vivo* in the presence of cytokines, loading the DCs with antigens and reinjecting them back to the host (O'Neill and Bhardwaj, 2007). This approach has been extremely successful in murine models whereby numerous studies have demonstrated that these DC-based vaccines can protect mice against a second tumor challenge and can even cure mice harboring established tumors (Celluzzi et al., 1998; Gilboa et al., 2007). In humans, DC-based immunotherapy have also demonstrated promising results although these have not been as dramatic as those seen in mice. Clinical and immune responses have been reported for various malignancies in patients including B cell lymphoma, metastatic melanoma and metastatic renal cell carcinoma (Banchereau et al., 2001; Tuenttenberg et al., 2006; Wierecky et al., 2006). A significant and notable drawback of *ex vivo* DC-based vaccines is that the *ex vivo* production of individually tailored cellular therapies is laborious and costly. Hence, the use of *in situ* approaches which take advantage of the biological properties of DCs *in vivo* has generated a

tremendous amount of interest. Approaches that can mobilize DCs to accessible sites where they can be matured and primed with antigens *in vivo* are being developed in the hope that this may lead to effective therapies without the need for expensive and laborious processes (Steinman and Pope, 2002). Some of these approaches include systemic mobilization of DCs using Flt3 ligand, local injection of chemokines such as macrophage inflammatory protein (MIP)-3β, use of DNA vaccines containing bacterial CpG motifs and the use of topical compounds such as Imiquimod (a TLR 7 agonist) (O'Neill and Bhardwaj, 2007; Homey et al., 2002)

Administration of DCs loaded e*x vivo* with tumor antigens have been shown to elicit both potent anti-tumor and anti-viral immune response *in vitro* and *in vivo* (Pagilla et al., 1996, Celluzzi et al., 1996). DCs, regardless of the route of administration has been shown to induce antigen specific T-cell immunity in cancer patients (Fong et al., 2001). DCs pulsed with tumor derived peptides, genes or lysates, as well as DCs fused with tumor cells, have all been shown to be effective as therapeutic cancer vaccines (Saji et al., 2006). DC-based vaccination has demonstrated promising results in clinical trials involving patients with various advanced malignancies. These are well-tolerated and are capable of inducing specific anti-tumor T-cell responses resulting in tumor regression. However, on the whole the therapeutic efficacy of DC-based vaccination has been limited and various investigators have suggested combination therapy with other therapeutic modalities to enhance its potency. Anti-tumor treatment modalities such as systemic anti-tumor drugs, radiation and radiofrequency ablation have all been combined effectively with DC immunotherapy (Saji et al., 2006). PDT is another modality which has been shown to demonstrate great potential when used in combination with immunotherapy.

This combination modality which is still under investigation is termed photoimmunotherapy (Jalili et al., 2004). This modality of treatment is the main focus of the present study and will be discussed in detail later.

Intra-tumoral injection of DCs offers the theoretical advantage of *in vivo* loading and activation of DCs which should be superior to *in vitro* loading of DCs with tumor antigens. The inflammatory milieu *in vivo* with its abundance of cytokines, various immune mediators and cells allows a broad and complex range of immune interactions which may result in an effective anti-tumor immune response. However, this technique is still associated with the problems associated with the culturing of DCs from bone marrow (BM) progenitors *in vitro.* This *in vitro* technique is associated with many practical problems including the required usage of many expensive cytokines in the growth medium, contamination of cultures and inducing possible changes in the physiological properties of DCs (Fong and Hui, 2002). Hence, some investigators have proposed that the expansion of DCs *in vivo* may be advantageous as the generation of potentially multiple DC subsets might be of great importance in eliciting optimal antigen-specific responses (Liu et al., 2001). Recently, the administration of the novel human hematopoietic growth factor, FLT-3 ligand (hFlt-3L) had been shown to have a profound effect on the generation of functional mature DCs in various organs (Maraskovsky et al., 1996). Subsequently, several studies have also shown that Flt-3L also results in recruitment of DCs to the tumor site (Lynch et al., 1997; Esche et al., 1998). Presently, despite the immense potential of DC-based anti-tumor vaccines being frequently demonstrated in pre-clinical studies; the clinical efficacy of DC-based vaccines remains limited (Saji et al., 2006; Fong and Hui., 2002). One of the many possible reasons

proposed for its limited clinical applicability and the variable results obtained in inducing strong anti-tumor immunity, particularly cytotoxic T-cell responses is that DCs are activated *in vitro* by antigen loading (Saji et al., 2006). The main problem with *in vitro* activation is that DCs are loaded with only 1 or a few tumor antigens whereas *in vivo* tumors potentially contain a few thousand antigens (Saji et al., 2006). Hence, *in vivo* activation of DCs may overcome this limitation and several studies combining DC- based vaccines and chemotherapy or radiotherapy have demonstrated this potential advantage (Yu et al., 2003; Teitz-Tennenbaum et al., 2003; Song and Levy, 2005). The *in vivo* expansion and generation of mature DCs offers many other potential theoretical advantages over the traditional *in vitro* culturing of DCs. *In vivo* expansion of DCs has the potential to generate various distinct DC subsets with different immune functions in order to elicit an optimal immune response (Fong and Hui, 2002). Although the exact lineage from which DCs are derived remain controversial, there is growing evidence that DCs can be sub-classified into myeloid and lymphoid subsets (Ardavin et al., 1993; Wu et al., 1996; Sauders et al., 1996) and these may have synergistic roles in generating an effective immune response. In addition, DC expansion *in vivo* in lymphoid and non-lymphoid organs could also greatly increase the chance of interaction with precursor T cells (Fong and Hui, 2002). It has also been reported that granulocyte monocyte- colony stimulating factor (GM-CSF)-treated mice generate a significant increase in DCs in the lymphoid and non-lymphoid compartments (Braun et al., 1999). This provides indirect evidence that DCs cultured *in vitro* are different from that *in vivo* and other presently unknown factors are critical for the generation of DC *in vivo*. Nonetheless, it is important to take note that concerns had been raised previously about the *in vivo* mobilization

method of DC-based immunotherapy as it has been shown that DCs from cancer patients are not only quantitatively defective but also qualitatively impaired due to tumor-induced inhibition of DC differentiation and maturation (Gabrilovich et al., 2004). Hence, *in vivo* generation of unprimed DCs may just result in an increase of immature non-functional DCs in cancer patients. Worse still, it has also been shown that increased mobilization and hence, numbers of immature DC may result in immune tolerance rather than produce an immunostimulatory effect. Thus, the effects of *in vivo* mobilization of DC may be counter-productive (Lutz and Schuler, 2002).

It has recently been demonstrated by Wu et al., 2001 that the administration of the recombinant gene encoding hFlt-3L gene into mice could also result in the *in vivo* expansion of DCs. Subsequently, a follow-up study demonstrated that the use of pNGVL3-hFlex plasmid DNA to expand DCs *in vivo* could induce a potent tumor-specific immune response when primed with a tumor peptide (Fong and Hui, 2002). In this study, mice primed with hFlt-3L gene and a tumor specific peptide were able to elicit an antigen-specific cytotoxic T-cell response which retarded tumor growth. A single injection of the plasmid DNA resulting in a peak elevation of DCs in various lymphoid and non-lymphoid organs 7 to 10 days post-immunization suggesting that this was the optimal time for antigen presentation. Hence, this study showed that fears that *in vivo* mobilization of immature DCs may be detrimental in malignancy is unfounded. This is with the caveat that an appropriate stimulus is present to prime naive DCs to mature.

1.4 Photoimmunotherapy

PDT used in combination with other immunostimulatory agents or strategies also termed photoimmunotherapy have been recently reported in several studies (Jalili et al., 2004; Castano et al., 2006). This combination approach can be broadly divided into 3 categories ie. 1) PDT with microbial adjuvants 2) PDT and cytokine therapy and 3) PDT with regulatory T-cells and adoptive cellular therapies (Castano et al., 2006).

PDT and microbial adjuvants. Agents derived from microbial stimulators of the innate immune system can be injected intra or peri-tumorally before, during or after PDT. These agents function as activators of TLRs or similar pattern-recognition molecules found on macrophages and DCs (Castano et al., 2006; Takeda et al., 2003). TLRs function as detectors of danger signals. Activation of TLR pathways induce nuclear transcription factor (NF)κβ which consequently results in the expression of several genes involved in immune system activation (Seya et al., 2003). Based on these observations, several studies were performed to test the effectiveness of combination therapy involving the administration of immunoadjuvants (potential TLR ligands) and PDT (Castano et al., 2006). Korbelik et al., 2001 demonstrated that PDT used in combination with a single dose of BCG was superior to PDT alone in treating subcutaneous EMT6 tumors in mice irregardless of the photosensitiser utilized. Photoimmunotherapy significantly increased the number of cured tumors and the number of memory T-cells in tumor draining lymph nodes as compared to PDT alone. In another study, schizophyllan (SPG) used in combination with Photofrin-mediated PDT of mice harboring SCCVII increased the tumor cure rate threefold as compared to PDT alone (Krosl and Korbelik, 1994). SPG is a fungal polysaccharide which is a potent inducer of humoral and cell-mediated immunity via macrophage dectin 1 receptor as well as TLR (Castano et al., 2006). After observing

that the complement system was activated during PDT, Korbelik et al demonstrated that tumor-localized treatment with zymosan, an alternative complement pathway activator could reduce the number of recurrent tumors after PDT (Korbelik et al., 2004).

PDT and cytokine therapy. Another class of photoimmunotherapy involves the administration of cytokines in combination with PDT. A single dose of intravenously administered recombinant tumor necrosis factor (TNF)α was shown by Bellnier to potentiate Photofrin-mediated PDT of murine SMT-F adenocarcinoma (Bellnier, 1991). Others also demonstrated that localized tumor treatment with GCSF or GMCSF with PDT resulted in a significant reduction of tumor growth, increased survival of mice and even complete cure of mouse tumors (Golab et al., 2000; Krosl et al., 1996).

PDT with regulatory T cells and adoptive cellular therapies. The 3rd category of photoimmunotherapy includes interventions designed to modify and augment the cellular arm of the adaptive immune system (Castano et al., 2006). This includes PDT combined with DC-based immunotherapy which is the subject of the present study. Recently, combination treatment of PDT with DC-based immunotherapy as a form of photoimmunotherapy has been shown to be an effective anti-tumor treatment for colorectal cancer and melanoma in murine models (Saji et al., 2006; Jalili et al., 2004). Theoretically, the unique mechanism of PDT-induced tumor destruction which not only mediates apoptotic and necrotic tumor cell death but also alters the tumor microenvironment through the release of proinflammatory cytokines such as TNF α, IL-1 and IL-6 (Saji et al., 2006; Dougherty et al.,1998; Gollnick, 1997) creates an environment that favours DC maturation and antigen-loading (Engleman, 2004). One of the common reasons attributed to the limited clinical efficacy of DC-based immunotherapy is their

variable ability to induce a strong anti-tumor immune particularly cytotoxic T lymphocyte response which may be due to problems associated with tumor antigen selection and activation (Saji et al, 2006). Most of these clinical trials have included single or only a few tumor antigens to activate and load DCs *in vitro* whereas *in vivo* tumors potentially contain thousands of antigens. Hence, photoimmunotherapy with intra-tumoral DC injection theoretically overcomes this limitation as the activation and loading of DCs after photoimmunotherapy with intra-tumoral DC injection is in *vivo*. Two recent studies (Saji et al, 2006; Jalili et al, 2004) reporting on the outcome of photoimmunotherapy using PDT and DCs for malignancies were based on this hypothesis. In both these studies, DCs were harvested *ex vivo* and injected intra-tumorally. Both studies found that combination therapy (photoimmunotherapy) induced a strong anti-tumor immunity which resulted in destruction of both the targeted tumor and tumors at distant sites (Saji et al., 2006; Jalili et al., 2004).

1.5 Melanoma.

Although malignant melanoma is extremely rare in Black and Asian populations, it is relatively more common in Caucasians. This is presumably due to the sensitivity of white skin to sun exposure (Markovic et al., 2007). Its incidence is reported to be increasing at a faster rate than any other cancer in the United States and Western European countries (Pilla et al., 2006). In the United States, it is presently the fifth most common cancer affecting men and the sixth most common cancer in women (Markovic et al., 2007). Malignant melanoma is a highly lethal disease, accounting for only 4% of all skin cancers but causing almost 80% of skin cancer deaths. A well-know feature of

malignant melanoma is that the tumor cells can spread haematogenously and lead to distant metastases. At the time of diagnosis, 80-85% of patients have stage I or II disease (local), 10-13% have stage III disease (regional) and 2-5% have stage IV disease (distant disease) (Balch et al., 2001). Several factors have been identified as important prognostic factors in melanoma including depth of invasion, ulceration, presence of microsattelites, satellites and in-transit metastases, lymph node involvement and distant metastases (Markovic et al., 2007).

The treatment of choice for stage I to III melanoma is surgical excision. The choice of surgical margin is dependent on the depth of the tumor which has been shown to be an important prognostic factor in melanoma (Balch et al., 2001). Although, surgical resection is effective for early stage tumors, advanced stage melanoma ie. American Joint Committee on Cancer (AJCC) stage III and IV cancers are associated with a poor prognosis. Adjuvant systemic therapy such as levamisole, vaccines, interferon (IFN) and chemotherapy have been administered after surgical resection for high risk primary melanoma to reduce the risk of systemic disease recurrence and death (Verma, et al., 2005). However, systemic review of numerous randomized trials do not demonstrate any significant overall survival benefit with any of these adjuvant therapies (Verma, et al., 2005).

Melanoma with distant metastases is associated with a median survival of 6 to 9 months and the 5-year survival rates are reported to be in the range of 1 – 5% (Balch et al., 2001). This is especially so with regards to patients with advanced disease associated with cutaneous or subcutaneous metastases whom have an extremely poor prognosis. Presently, many of these patients are offered palliative treatment with intravenous

chemotherapy, isolated limb perfusion, interferon or IL-2 therapy (Naylor et al, 2006). However, palliative control of widespread cutaneous metastases is extremely difficult with currently available treatment modalities. Presently, the most widely used chemotherapeutic agent for melanoma is dacarbazine alone or in combination with other drugs (Pilla et al., 2006). These regimens have been reported to produce response rates ranging from 30 to 50% in Phase II trials. However in large Phase III randomized studies, chemotherapy has had limited impact on overall patient survival (Chapman et al., 1999). Similarly, while chemotherapy regimens in combination with systemic administration of cytokines such as IL-2 and IFN-α have produced promising results in terms of response-rate and progression-free survival in early Phase II trials, subsequent Phase III studies have failed to demonstrate improvement in overall survival (Ridolfi et al., 2002; Keiholz et al., 2005). Due to the poor outcome of metastatic malignant melanoma to traditional chemotherapy, numerous systemic options such as immunotherapy have been investigated as possible alternative treatment options (Pilla et al., 2006).

1.5.1 PDT for melanoma

Although, PDT has been established as a therapeutic option for various primary and secondary skin malignancies, the use of PDT has been traditionally found to be of limited benefit in melanoma (Biel, 1996; Nowak-Sliwinska et al., 2006). This has been attributed to the presence of large amounts of light-absorbing melanin pigment that prevents light penetration into the tumor tissue. Hence, it was previously believed that only amelanotic melanoma such as melanoma of the iris respond satisfactorily to PDT (Favilla et al., 1991). Melanin are natural pigments found in many organisms and tissues

(Lim et al., 2004). The formation of melanin in human skin offers protection against UV light via 2 mechanisms. Firstly, it absorbs and scatters incident light. Secondly, melanin is also responsible for scavenging ROS such as superoxide anions, hydroxyl radicals and singlet oxygen and for inhibiting lipid peroxidation. Studies have demonstrated that there is good correlation between the degree of pigmentation and response to PDT (Nelson et al., 1988). Presently, it is also believed that the microevironment of melanoma tumors which may be hypoxic *in vivo* contributes to the ineffectiveness of PDT (Brurberg et al., 2004). This is attributed to the fact that melanoma cells exhibit a high oxygen consumption due to respiration, melanogenesis and physicochemical interaction between oxygen and melanin (Pajak et al., 1980; Hopwood et al., 1985; Nowak-Sliwinska et al., 2006).

Most of the earlier studies studying the effect of PDT on melanoma utilized first generation photosensitizers such as Photofrin (Peeva et al, 1999; Pass, 1993) which activated light at a wavelength of 630 nm. The competition between the light absorption regions of melanin and these photosensitizers resulted in the poor yield of PDT-mediated melanoma destruction. However, in 1998, Haddad et al., 1998 conducted a study which demonstrated that PDT could be effective in the treatment of melanoma. They demonstrated that PDT using aluminium phthalocyanine decreased B16 melanoma cell viability *in vitro*. More importantly, PDT retarded the growth of B16 tumors and prolonged survival of mice inoculated with B16 melanoma. The authors further hypothesized that as melanin converts a large fraction of light into heat (Polla et al., 1982), PDT could also cause tumor death via hyperthermia. This additive, synergistic effect of PDT and tumor hyperthermia was consistent with the findings demonstrated by

Leunig et al., 1994 in their study evaluating the effect of PDT-induced heating of melanoma *in vivo*. Subsequently, several others have confirmed that PDT using various photosensitizers can result in efficient tumor destruction of melanoma (Busseti et al., 1999; Lim et al., 2004, Kolarova et al. 2007).

The efficiency of PDT on melanoma has been shown to be dependent on the type of photosensitizer used (Peeva et al., 1999). Melanin absorbs light over broad spectrum with a peak absorption at about 335 nm. Its absorption of light decreases with longer wavelengths until its absorption of light is almost completely attenuated at wavelengths over 700 nm. Lim et al demonstrated efficient tumor destruction *in vivo* and *in vitro* of B16F10 melanoma cells with silkworm-pheophorbide *a* (Lim et al., 2004) This was attributed to its long wave-length of light absoprtion at 665nm. It is well-documented that the longer the light absorption wavelength of photosensitizers used the deeper the skin penetration (Marcus, 1990). In another interesting study, Nowak-Sliwinska et al., 2006 compared the efficiency of PDT utilizing various photosensitisers against melanoma. They found that Verteporfin was superior to merocyanine C540 and photofrin II in achieving tumor control *in vitro.* They attributed this finding to the mechanism of tumor destruction of Verteporfin which strongly depended on the high yield of singlet molecular oxygen.

Initial studies on the clinical use of PDT for skin metastases from melanoma yielded poor results with a clinical effect produced in only 20-30% of patients (Biel, 1996; Feyh, 1996). However, subsequent clinical studies utilizing different photosensitizers produced more promising results. Sheleg et al., 2004 studied the effect of chlorin e_6-mediated PDT on 14 patients with skin metastases. They found complete

regression in all 14 patients with 8 patients requiring only 1 session of PDT. There was also minimal toxicity observed with PDT treatment. Two patients developed pyrexia and rigors and 8 developed pain several days after PDT controlled with oral morphine. There were no major toxicities observed such as photodermatitis, renal or hepatic injury.

1.5.2 Immunotherapy for melanoma

Interest in the use of immunotherapy for melanoma arose when observations from clinical and epidemiological studies revealed anecdotal cases of spontaneous regression of primary melanoma and that an increased incidence of melanoma occurs in the immune-suppressed (Euvrard et al., 2003). This was further confirmed by histopathological studies which demonstrated improved prognosis of malignant melanoma when the primary lesions were associated with T lymphocyte infiltrate (Clemente et al., 1996). The seminal work by van der Brugge et al in 1991, whereby the first human melanoma antigen recognized by CD^+ T cells was cloned opened the door to numerous other studies whereby many other melanoma antigenic peptides were identified (Novellino et al., 2005). Subsequently, numerous immunotherapy regimes were formulated for advanced AJCC stage IV melanoma and evaluated in clinical trials. Broadly, the various vaccines for malignant melanoma can be classified into cell-based vaccines, peptide-based vaccines, DC-based vaccines, recombinant virus vaccines, plasmid or naked deoxyribonucleic acid (DNA) vaccines and vaccination with HSPs.

Thus far, despite considerable investment of time and money in immunotherapy for melanoma, both first and second generation vaccines have failed to demonstrate superior clinical efficacy in large Phase III randomized trials over chemotherapy or even

best supportive care (Schadendorf et al., 2006; Rosenberg et al., 2004; Pilla et al., 2006). However, despite the wide gap between the results of highly promising preclinical studies and the poor efficacy of vaccines shown by clinical trials thus far, considerable effort to produce a clinically-useful vaccine continues. This is because the potential clinical applicability of vaccines in melanoma has been fully demonstrated (Pilla et al., 2006). The high immunological response rates demonstrated in clinical trials coupled with highly successful results in a limited number of patients have corroborated the possibility of successful immunotherapy in the treatment of melanoma. The challenge of successful immunotherapy in melanoma is to induce a clinically relevant immune response. Presently, it is believed that the inability of vaccines to induce a sufficient quantity and quality of anti-tumor cell response is responsible for its initial failures (Pilla et al., 2006). The challenge to generate an adequate qualitative T-cell response consists of the identification of a strong stimulus to induce an adequate anti-tumor T-cell response. PDT may be the answer to this challenge as it has been shown in numerous studies as discussed previously to result in strong anti-tumor T- cell immune response by activating both the innate and adaptive arms of the immune system. Hence, interest has arisen on the combined use of PDT with immunotherapy (photoimmunotherapy) for the treatment of melanoma.

DC-based vaccination has been studied extensively in stage IV malignant melanoma. As melanoma-associated peptide antigens have been well-characterized, these have been utilized for pulsing DCs. Other cancer associated antigens including acid-eluted tumor peptides, tumor lysates, messenger ribonucleic acid (mRNA) and tumor-DC hybrids have also been used (Proudfoot et al., 2007). An important Phase I trial

demonstrated that 13 of 18 patients generated an INF-$_\gamma$ T-cell immune response to at least 2 melanoma peptides after four vaccinations with subcutaneous DC loaded with four melanoma peptides (Fay et al., 2006). In that study, the overall median survival of patients was 20 months (range, 2-83 months) and this was significantly higher for patients that mounted an immune response. This was especially so for those responding to more than 1 melanoma antigen. However, despite the success demonstrated with Phase II trials, Phase III trials have failed to demonstrate good melanoma tumor response to DC-based vaccines. A notable Phase III trial was conducted to compare the efficacy of autologous peptide-pulsed DCs with dacarbazine in metastatic melanoma utilizing a cocktail of class I and II-restricted peptides representing melanoma antigens. After interim analysis of 108 patients, the study was closed early as there was no significant advantage of the DC arm versus the dacarbazine arm in terms of response rate (3.8% vs 5.5%) and overall survival (Schadendorf et al., 2006).

The main reason for the disappointed results of immunotherapy for melanoma has been attributed to the presence of immune barriers at the level of the melanoma tumor microenvironment (Gajewski, et al., 2007). Two of the key evasive mechanisms which have been identified to confer tumor resistance to the effector phase of the anti-tumor T-cell response are poor chemokine-mediated trafficking of effector cells and the action of negative regulatory pathways that inhibit T-cell function (Gajewski et al., 2007). Some of these negative regulatory pathways include T-cell anergy, suppression by regulatory T-cells, action of inhibitory ligands and metabolic dysregulation (Gajewski et al., 2007).

1.5.3 Photoimmunotherapy for melanoma

The first study conducted using DC-based photoimmunotherapy for melanoma was performed recently by Saji et al., 2006. The investigators found that PDT alone could not induce any tumor suppression in even very small 3mm diameter B16 melanoma cells (Saji et al., 2006). However, they subsequently demonstrated the superior potential of photoimmunotherapy using ATX-S10 Na(II)-PDT and intra-tumoral DC over PDT alone to treat local and systemic B16 melanoma cells in mice (Saji et al., 2006). This study was important as it not only demonstrated the ability of combination immunotherapy (intra-tumoral DC injection) to enhance the anti-tumor effects of PDT but also that this synergistic effect of immunotherapy could be demonstrated in poorly immunogenic tumors (B16 tumors).

More recently, a clinical study reported promising results on the palliative use of *in situ* photoimmunotherapy in 2 patients with advanced stage melanoma with cutaneous metastases. In that the study, PDT was performed using a near infrared (IR) 805 nm laser with intralesional injection of indocyanine green. Immunotherapy was administered via topical imiquimod a TLR agonist which has been reported to be effective against melanoma (Steinmann et al., 2000; Hesling et al., 2004; Vereecken et al., 2003). The study demonstrated that photoimmunotherapy was well-tolerated, could effectively clear local cutaneous and subcutaneous deposits and most importantly demonstrated beneficial systemic anti-tumor effects such as regression of lung metastases.

1.6 Scope of study

In this study, we hypothesize that PDT combined with the *in vivo* expansion of DCs may induce anti-tumor immunity. As such this study was designed to determine if

photoimmunotherapy via the combination of HY-PDT with the *in vivo* expansion of DCs using pNGVL3-hFlex plasmid DNA was effective in inducing antitumor immunity.

The objectives of this study are to:

1. Evaluate if HY-PDT was effective in inducing tumor destruction of murine B16 melanoma
2. Determine the appropriate HY and light dose for effective PDT of B16 melanoma
3. Establish the predominant mode of cell death after PDT of B16 tumor and if incubation period had an effect on this
4. Compare the anti-tumor effect of photoimmunotherapy versus PDT alone versus control on B16 melanoma (primary tumor model)
5. Determine if photoimmunotherapy generated an effective anti-tumor vaccine compared to PDT alone and control
6. Evaluate if photoimmunotherapy retarded the growth of a large and small established contralateral tumor compared to PDT alone and control (metastatic model)
7. Ascertain if the effect of photoimmunotherapy was tumor specific
8. Determine if the splenocytes of photoimmunotherapy treated mice harbored immune cells which could be used to generate an effective tumor vaccine (adoptive transfer)

CHAPTER 2

MATERIALS AND METHODS

2.1 Cell culture

Murine B16 melanoma cell line (original source - American Type Culture Collection) and RMA lymphoma parental cell line (originally from Dr Acres, Transgene, Strasbourg, France) were used in the study. Both B16 melanoma and RMA cell lines were gifts from Prof. K.M. Hui, Division of Cellular and Molecular Research, National Cancer Centre, Singapore (Fong and Hui, 2002). Both cell lines were cultured in Roswell Park Memorial Institute (RPMI) 1640 supplemented with 10% fetal bovine serum (FBS; Hyclone, Logan, UT, USA), 100 IU/ml penicillin, 100 μg/ml streptomycin, 292 μg/ml L-glutamine (GIBCOBRL, Life Sciences, NY, USA) and 1 mM sodium pyruvate (JRH Biosciences, KS, USA). These were maintained in an atmosphere of 5 % CO2 at 37ºC.

2.2 Mice

Female C57BL/6 mice, 8-12 weeks of age were used in the experiments. These were purchased from the Animal Laboratory Unit of the National University of Singapore. The animals were housed in the National Cancer Centre Animal Holding Unit and were fed with pellets (Glen Forrest stockfeeder, Australia). The animals were sacrificed via delivery of 100% carbon dioxide. They were euthanized when the tumor diameter exceeded 30 mm or when they were in distress, which ever event occurred first.

All experiments were performed in compliance with the National Advisory Committee for Laboratory Animal Research Guidelines in Singapore and all procedures were approved by the Institutional Animal Care and Use Committee, Singhealth Singapore in accordance with international standards.

2.3 Tumor model

The caudal half of the mice was shaved and 0.5 x 10^6 cells (B16 cells or RMA cells) suspended in 100 μL Hanks Buffered Salt Solution (HBSS) were injected subcutaneously into the lower right or left flank of the mice. The cells were counted using a hemocytometer. Tumor growth was subsequently monitored. The tumors were measured in 3 dimensions: length, breath and thickness using an electronic Vernier caliper. The tumor volumes were subsequently estimated according to the formula: tumor volume (mm^3) = $\pi/6$ x length x breath x thickness.

2.4 Photosensitizer

HY, a photosensitizer with a maximum absorption of 590 nm (Du et al., 2003) was obtained from Molecular Probes (Eugene, OR, USA). This was prepared as a stock solution of 1mg/ml in dimethyl sulfoxide (DMSO) and stored at -20°C in the dark. The photosensitizer was diluted in phosphate buffer saline (PBS) before injection into mice.

2.5 Light source

A broadband halogen light source (Zeiss KL1500), fitted with a customized 560-640 nm band-pass filter (46152, Edmund Scientific Inc.) was used as the light source. A power meter (Laser check, Coherent, USA) was used to quantitate light intensities.

2.6 Photodynamic treatment of tumors

The mice were restrained in a self-made restrainer and 5 mg/kg of hypericin in 0.1ml PBS were administered parenterally via the tail veins of the tumor-bearing mice.

The mice were subsequently housed in cages covered with aluminium foil and protected from surrounding light. Subsequently, prior to photodynamic therapy; the mice were anesthetized with 1:1 cocktail of diazepam (5 mg/ml) and ketamine (50 mg/ml) at a dose of 1 ml/kg given intraperitoneally. They were then placed in a specially designed holder and only the tumor-bearing region to be treated with light was exposed.

2.7 Plasmid DNA

The PNGVL3-hFlex plasmid containing the extracellular domain (secreted form) of the human Flt-3L gene was originally obtained from the Vector Core Laboratory at the University of Michigan (Ann Arbor, MI, USA) and has been described previously (Lyman et al., 1994). These were also kindly provided by Professor K. M. Hui. Plasmid purification was performed with the QIAGEN® plasmid kit (QIAGEN N.V., Netherlands) as described in the manufacturer's handbook (QIAGEN® Plasmid Purification Handbook, 2003). These were delivered *in vivo* via hydrodynamic-based tail vein injection (Liu et al., 1999). Briefly 10 μg of DNA was diluted in 1.5-2 ml of sterilized 0.9% NaCl solution and injected into the tail vein over 10 s using a 271/2 g needle (Wu et al., 2001).

2.8 Transmission Electron Microscopy (TEM)

i) Fixation of tissues

The tissues were immersed in cold 2% paraformaldehyde and 3 % glutaraldehyde in 0.1 M sodium phosphate buffer (pH 7.4) and fixed for a duration of 4 hours. The specimens

were subsequently rinsed 3 times with 5% sucrose solution in 0.1 M phosphate buffer (pH 7.4).

ii) Osmication

The specimens were cut into small pieces under a dissecting microscope (Nikon SMZ-1, Japan). These were fixed in 1% osmium tetroxide in phosphate buffer containing 1.5% potassium ferrocyanide for 2 hours in a fume cupboard. Specimens, were then washed twice with deionized water.

iii) Dehydration and embedding

Dehydration was performed with an ascending series of ethanol. Specimens were immersed in 25% ethanol for 5 minutes, and in 50%, 75%, 95% and 100% ethanol for 10 minutes each, before processing. They were subsequently processed in absolute acetone for 10 minutes with 2 changes. A 100% acetone and araldite mixture (1:6) was used to infiltrate the specimens for 2 hours and before 3 changes of fresh resin. The specimens were incubated in an oven at 40 °C, 45 °C and 50 °C for 1 hour during each change. Finally, the tissues were embedded in dried araldite capsules and allowed to polymerize in an oven at 60 °C for 24 hours.

iv) Trimming and ultra-thin sectioning

The capsule blocks were trimmed with an electrical trimmer to remove excess portions of araldite. Subsequently, the capsule blocks were cut with an ultramicrotome (Ultracut E, Reichert-Jung 200M) to obtain sections of 1 μm. The sections were mounted on glass slides stained with 1% toluidine blue and examined under the light microscope (Leitz Aristoplan). Subsequently, ultra-thin sections (0.1 μm) were cut and a fine hair brush was than used to place the ultra-thin sections on G100 formvar-coated copper grids.

v) Staining of sections

All sections were stained with 2% uranyl acetate and lead citrate. The grids bearing sections were put on drops of saturated uranyl acetate for 10 minutes. These were than washed with deionized water. After drying, they were allowed to float on drops of lead citrate for 8 minutes. The grids were than rinsed again with deionized water and dried.

vi) Examination under TEM

A TEM (Philips CM120 Biotwin, Oregon, USA) was operated at an accelerating voltage of 60-80 KV and was used to examine the tissue sections.

2.9 *In vivo* experiments

2.9.1 Effective PDT of B16 melanoma in C57BL/6 mice

Aim: To determine the light dose for effective PDT without causing mouse toxicity

As no previous study has been published on PDT with HY on B16 melanoma tumors, preliminary experiments had to be conducted to identify the effective drug dose and light setting for the destruction of B16 tumors *in vivo* without major toxicity to the mouse. [based on a previously published protocol (Du et al., 2003)]. B16 cells (0.5×10^6 cells per mouse) in 100 μL HBSS were injected subcutaneously into the lower right flank of pre-shaven C57BL/6 mice. Between days 8 to 10 when the tumors were established, the mice were administered with 5 mg/kg of HY via tail vein injection followed by light irradiation of the tumor after an incubation period of 1 or 6 hours later. The 2 incubation periods of the drug (1 and 6 hours) were selected as it has been shown previously that cell death occurred predominantly via apoptosis at 1 hour and via necrosis at 6 hours (Du, 2004) after HY-PDT. These 2 modes of cell death may have an effect or the immune response

generated by PDT. PDT was than administered with a HY dose of 5 mg/kg and the light dose was increased step-wise from 30, 60, 90 to 120 J/cm^2 for each incubation period (1 or 6 hours) respectively. Three mice were assigned to each of the 8 groups. The tumors on the mice were subsequently observed and the treatment was considered effective when the tumor was completely destroyed with no gross tumor visible (usually after 3 days).

2.9.2 Effect of the mode of cell death after HY-PDT and if incubation period (1 vs 6 hours) influenced the mode of cell death

Aim: To determine if the mode of cell death and if incubation period has an effect on the mode of cell death after PDT of B16 tumor

Three mice were each assigned to 2 groups (incubation period 1 hour and 6 hours). B16 tumor cells were than injected into both flanks of all 6 mice. Between days 8 to 10, when the tumors were established, PDT was than administered to the tumors in the right flank. The control group consisted of the tumors in the left flank. At 1 hour or 6 hours after PDT the mice were sacrificed and the tumors were harvested and prepared for electron microscopy. The specimen slices were studied to determine if the mode of cell death was predominantly via apoptosis or necrosis for the 2 incubation periods.

2.9.3 Growth curve of B16 and RMA tumor model

Aim: To determine the growth curve of B16 and RMA tumor model

B16 and RMA tumors cells were inoculated into the right flank of mice and the growth of these tumors were measured. Three mice were used for each tumor model.

2.9.4 Effect of photoimmunotherapy and PDT in a B16 primary tumor model

Aim: To determine and compare the effect of photoimmunotherapy versus PDT alone versus control on a B16 primary tumor model.

PDT was administered as previously described (2.9.1). With regards to photoimmunotherapy; on day 0, B16 tumor cells were injected into the lower right flank of the mice. The plasmid DNA was injected into the tail vein on day 2 and on day 9, PDT was administered. An interval of 7 days after injection of the plasmid DNA was chosen before PDT as it has been demonstrated previously that this was the time interval whereby the maximum cytotoxic T-cell response could be observed (Fong and Hui, 2002). The mice were divided into 7 groups (5 to 8 mice per group) for comparing the effects on tumor growth. The two control groups comprised of Group 1 which had tumor cells inoculated but no administration of plasmid or PDT and Group 2 where plasmid alone was injected without photodynamic therapy. There were 2 PDT groups; Group 3 and Group 4 whereby tumor cells were inoculated and PDT was administered at incubation periods of 1 hour and 6 hours but no plasmid was injected. Group 5 and 6 were the photoimmunotherapy groups, both intravenous plasmid injection and PDT were performed at incubation periods of 1 hour and 6 hours respectively. The tumor growths were subsequently monitored and compared.

2.9.5 Effectiveness of photoimmunotherapy in generating an anti-tumor vaccine

Aim: To determine if photoimmunotherapy generated an effective anti-tumor vaccine.

PDT and photoimmunotherapy were administered as previously described. However, this time the mice were challenged with a second tumor in the left flank at day 15 after the

initial tumor inoculation (5 days after PDT). The time interval of 5 days was arbitrarly selected to presumably allow sufficient time for an adequate immune memory response. The mice were divided into 5 groups and compared (minimum of 5 mice per group): 1 control group with no treatment (Group 1), 2 PDT groups with incubation of 1 and 6 hours (Group 2 and 3) and 2 photoimmunotherapy groups with incubation of 1 hour and 6 hours (Group 4 and 5). The tumors in the left flank were then measured serially and compared. As no significant difference in terms of anti-tumor effect was observed between the 2 incubation periods, the 1 hour incubation period was used in subsequent experiments for convenience.

2.9.6 Effect of photoimmunotherapy on a contralateral tumor (metastasis model)

Aim: To determine if photoimmunotherapy retarded the growth of an established contralateral tumor (Small and large metastasis model)

Treatment protocols were performed as previously described.

For the small metastasis model, mice were inoculated with a second tumor (metastasis) in the left flank on day 4 after the primary tumor was inoculated to simulate tumor metastasis. The mice were divided into 3 groups (minimum of 5 mice per group): Group 1 (control group) with no treatment, Group 2 (PDT alone) and Group 3 (photoimmunotherapy). The volumes of tumors in the left flank were then monitored.

For the large metastasis model, the second tumor (left) was inoculated on the same day as the primary tumor (right). PDT was administered at day 14. The mice were divided into 3 groups (minimum of 5 mice per group): Group 4 (control group) with no treatment, Group 5 (PDT alone) and Group 6 (photoimmunotherapy group). We selected the tumor

implantation metastatic model instead of a lung metastasis model as this model would allow tumor metastases to be reproduced more consistently as compared to the unpredictable number and size of metastases in the lung metastasis model. Furthermore, the response of tumor metastases to treatment in the implantation model would be easier to quantify (by measuring tumor size) as compared to the lung metastatis model.

2.9.7 Tumor specificity

Aim: To determine if the effect of photoimmunotherapy was tumor specific.

PDT and photoimmunotherapy were administered as previously described 2.9.5. However, this time the mice were challenged with a different second tumor (RMA) in the left flank at day 15 after the initial tumor inoculation (5 days after PDT). The mice were divided into 3 groups and compared (minimum of 5 mice per group): 1 control group with no treatment (Group 1), 1 PDT groups with incubation of 1 hour (Group 2) and 1 photoimmunotherapy groups with incubation of 1 hour (Group 3). The RMA tumors in the left flank were then measured serially and compared.

2.9.8 Adoptive immune transfer

Aim: To determine if the splenocytes of photoimmunotherapy treated mice harbored immune cells that could be used to generate an effective tumor vaccine.

Mice with B16 tumors were treated with photoimmunotherapy as before and euthanized at 5-7 days after PDT. The spleens were harvested and mashed in a plate. Splenocytes were obtained after the mashed spleen products were passed through a cell strainer and centrifuged. Before injection into mice, the red blood cell component of the splenocytes

were lysed using tris-buffered ammonium chloride. The cells were counted and 5 x 10^7 splenocytes were infused i. v. into 2 groups of mice. The control group was composed of mice inoculated with B16 in the right flank and RMA on the left flank. The treatment group (adoptive transfer) was also composed of B16 in the right flank and RMA in the left flank but with splenocytes injected i.v. at day 2.

2.10 Statistical analyses

All statistical analyses were conducted using the computer programme Statistical Package for Social Sciences for Windows, version 10.0 (SPSS Inc, Chicago, IL, USA) and Microsoft Excel 2003. Graphs were generated using Microsoft Excel. Results were expressed as mean ± SEM and unpaired 2 tailed t-tests were used to compare means. All tests were 2-sided and a P-value of less than 0.05 was considered statistically significant.

CHAPTER 3

RESULTS

3.1 Effective PDT of B16 tumor

PDT treatment was administered to the mice in escalating doses from 30, 60, 90 to 120 J/cm^2 with incubation periods of 1 and 6 hours. Effective tumor destruction without toxicity to the mice was grossly found only when PDT was performed with a HY dose of 5 mg/kg and 120 J/cm^2 light irradiation (100mW/cm2 x 25 minutes) after both 1 and 6 hours incubation (Figure 2). There was minimal or no visible effect when doses of 30, 60, 90 J/cm^2 were used.

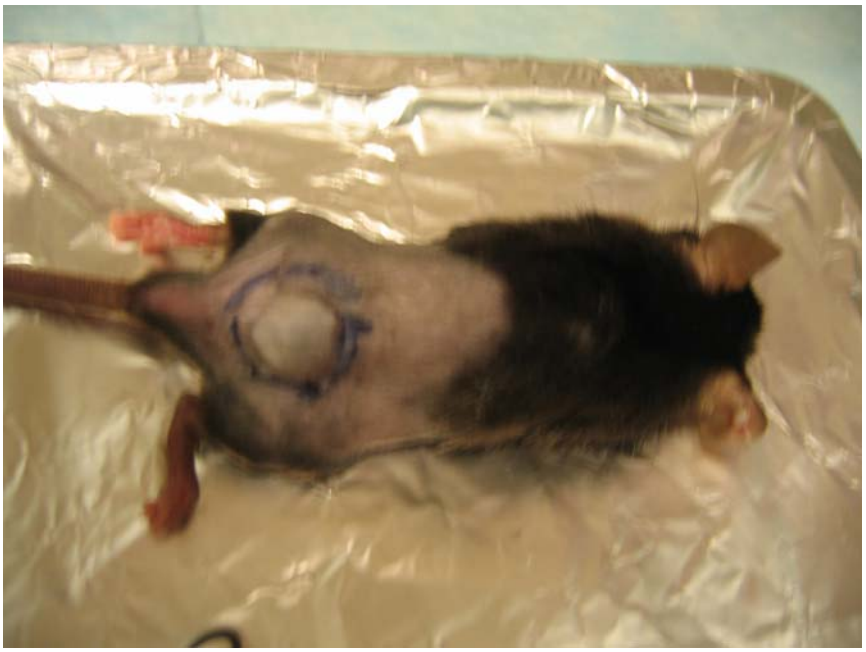


Figure 2A. Photograph of female C57BL/6 mouse with established B16 tumor 9 days after subcutaneous inoculation in a shaved mouse.

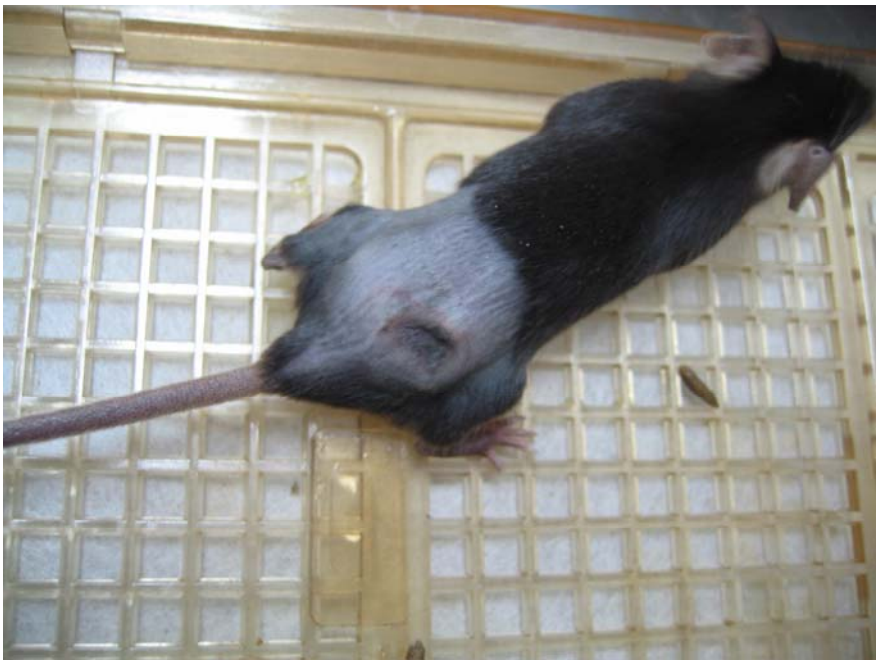
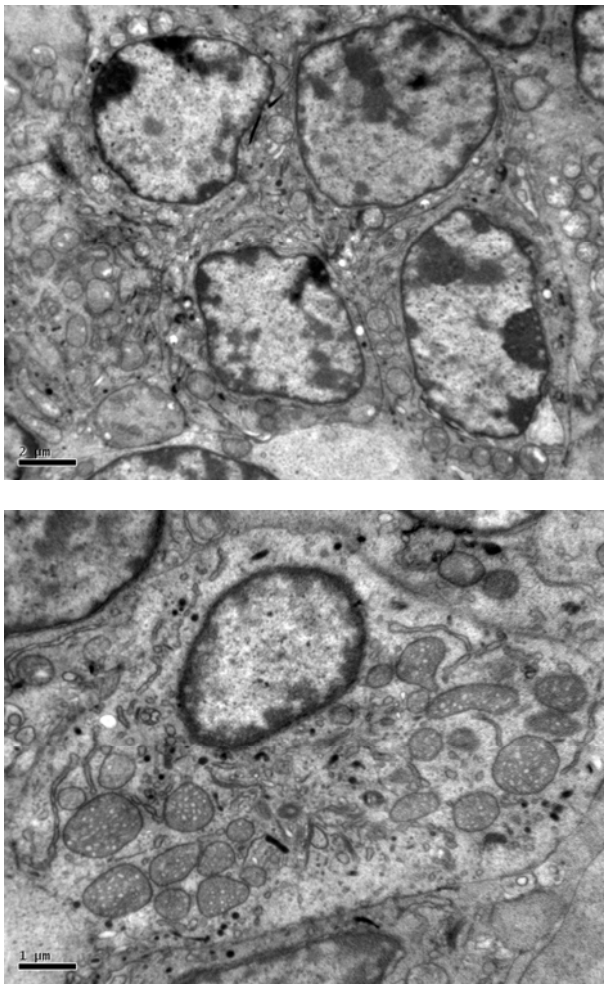


Figure 2B. Photograph of female C57BL/6 mouse demonstrating the destroyed B16 tumor seen 3 days after HY-PDT with a light dose of 120 J/cm^2

3.2 Mode of tumor cell death after HY-PDT

The EM appearance of an untreated B16 tumor cell from the control group is demonstrated in Figure 3. HY-PDT of B16 tumors was performed with a HY concentration of 5 mg/kg and light dose 120 J/cm^2 at an incubation period of 1 and 6 hours. The predominant mode of tumor cell death was necrosis and this was regardless of the incubation period (Figure 4). Only a small proportion of cells demonstrated some degree of apoptosis (Figure 5).



Figures 3A and B. Electron photomicrogram. Control: tumor cells were intact with normal nucleus and cell membrane

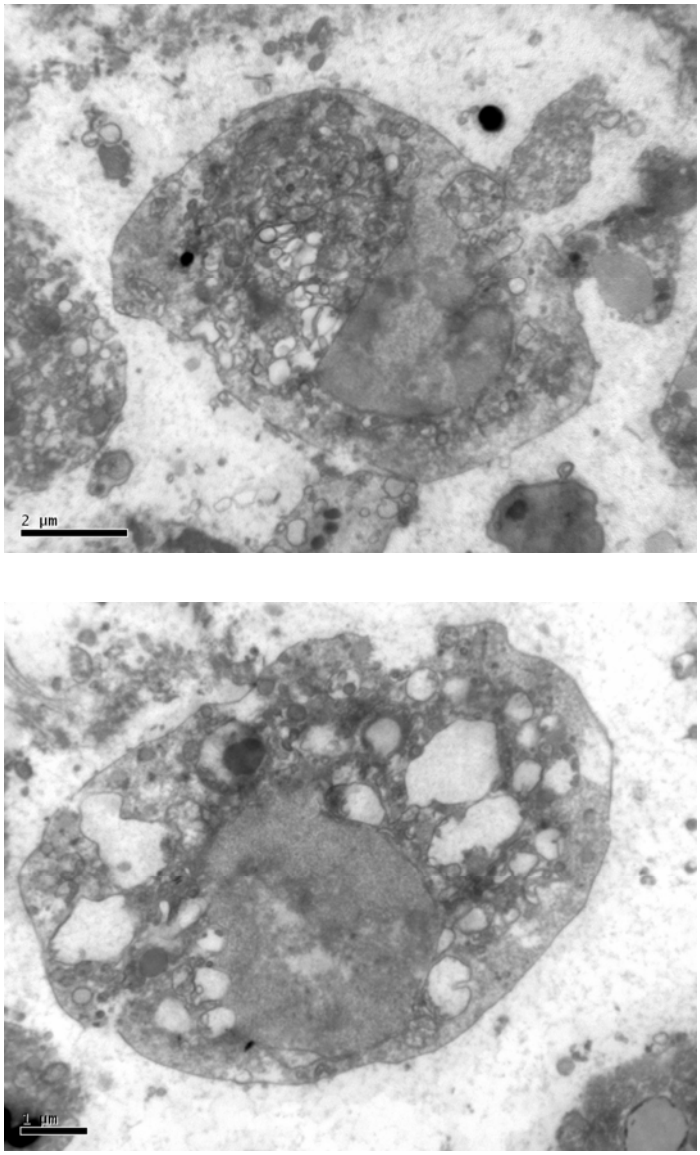


Figure 4. Electron photomicrogram of the tumor cells after PDT. The cells have mainly undergone necrosis displaying loss of integrity of the cell membrane, vacuolation, disintegration of cytoplasm and absence of apoptotic nuclear changes. **A.** B16 cells demonstrating features of necrosis after HY-PDT at incubation of 1 hour. **B.** B16 cells also demonstrating features of necrosis after PDT at incubation of 6 hours

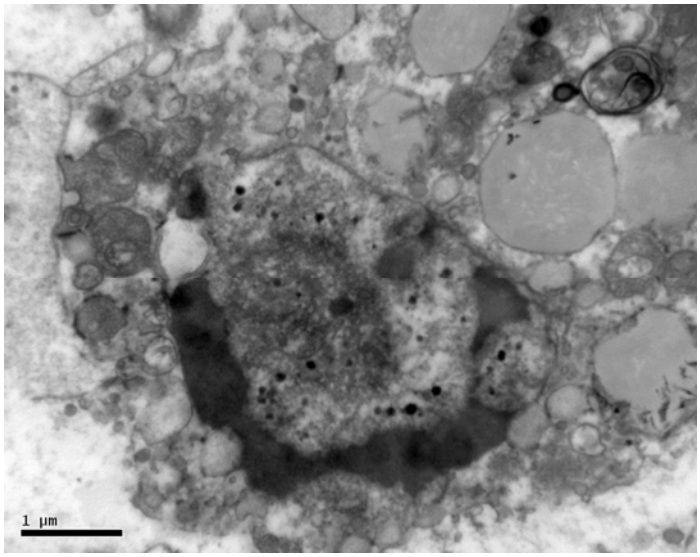


Figure 5. Electron photomicrogram: Some cells demonstrating secondary nucleus with peripheral margination of the chromatin in the nucleus suggestive of apoptosis.

3.3 Growth curve of B16 and RMA tumor model

The growth curves of B16 murine melanoma and RMA murine lymphoma tumors in mice following inoculation of 0.5 x 10^6 cells subcutaneously. Both tumors demonstrate a exponential increase in tumor volume with rapid tumor growth at approximately 12 to 15 days after tumor inoculation (Figures 6 and 7). The tumors were grossly visible at 7 to 10 days with mean tumor volumes of about 40 mm^3 and PDT treatment in subsequent experiments was administered during this time interval. The tumor volumes were estimated by measuring the tumor length, breath and thickness and calculated according to the formula: tumor volume (mm^3) = π/6 x length (mm) x breath (mm) x thickness (mm).

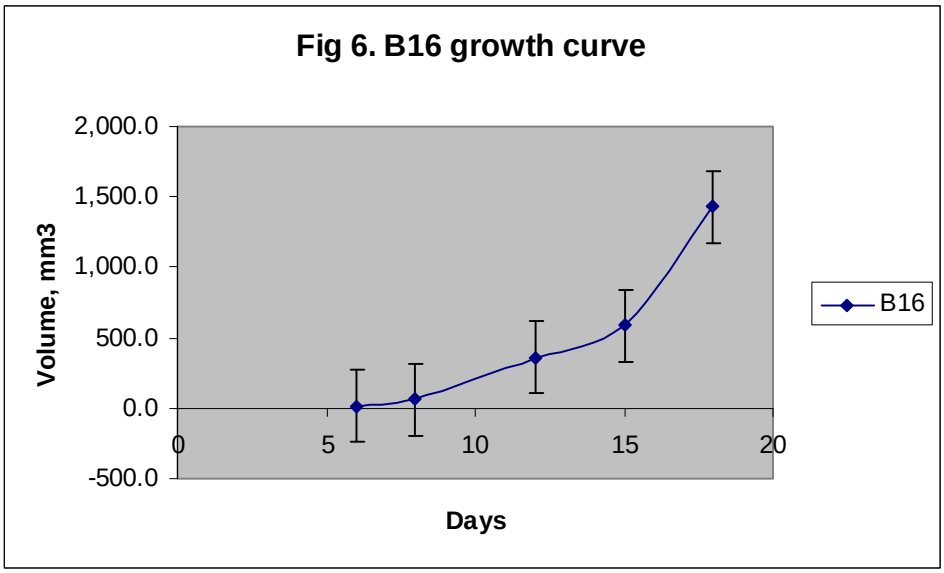


Figure 6. Growth curve of B16 murine melanoma tumors after subcutaneous injection of approximately 0.5 x 10^6 cells. Values were represented as mean ± SE of 3 animals.

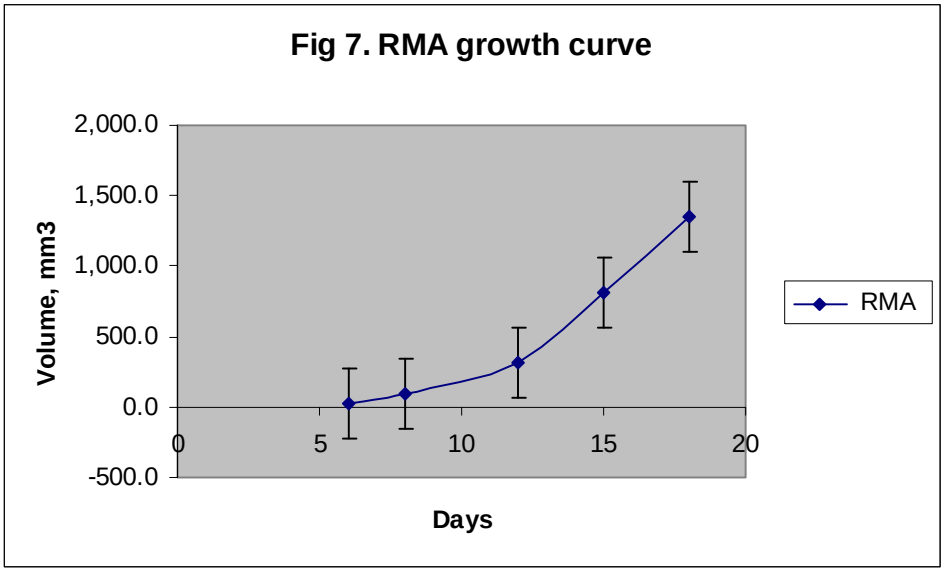


Figure 7. Growth curve of RMA murine lymphoma tumors after subcutaneous injection of approximately 0.5 x 10^6 cells. Values were represented as mean ± SE of 3 animals.

3.4 Effect of photoimmunotherapy and PDT in a B16 primary tumor model

There was no difference in the tumor growth curves when both control groups (Groups 1, no PDT or plasmid administered and 2, no PDT but plasmid administered) were compared. In fact, the tumor growth curves were almost mirror images of each other (Figure 8). Similarly, the growth curves of the 2 PDT groups (Groups 3, incubation period 1 hour and 4, incubation period 6 hours) (Figure 9) and the 2 photoimmunotherapy groups (Groups 5, incubation period 1 hour and 6, incubation period 6 hours) (Figure 10) were similar with no statistical difference. The growth curves of the control, PDT and photoimmunotherapy groups were then plotted together in the same graph and compared. This demonstrated that both the PDT group and photoimmunotherapy groups was effective in inhibiting primary tumor growth (Figure 11). Effective gross tumor destruction was demonstrated with both methods. Both the PDT and photoimmunotherapy groups were superior to the control group in retarding B16 tumor growth. However, photoimmunotherapy was not superior when compared with PDT alone in retarding primary tumor growth.

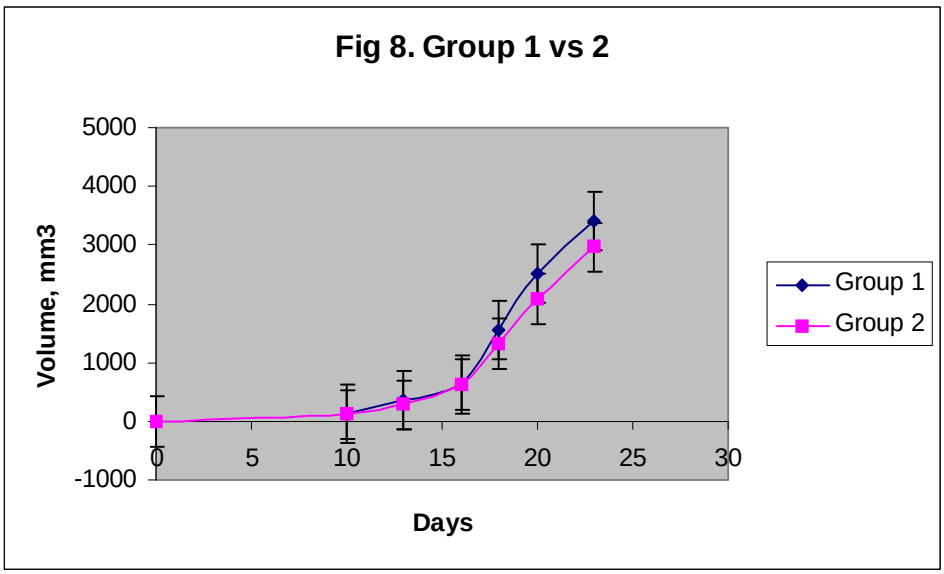


Figure 8. Comparison between the growth curves of B16 tumor in the 2 control groups (Group 1, no PDT or plasmid administered and Group 2, no PDT but plasmid was administered). There was no statistical difference between the 2 curves. The P-value at day 10, 13, 16, 18, 20 and 23 were .908, .353, .998, .590, .461, .165 respectively. Values are represented by mean ± SE of at least 5 animals.

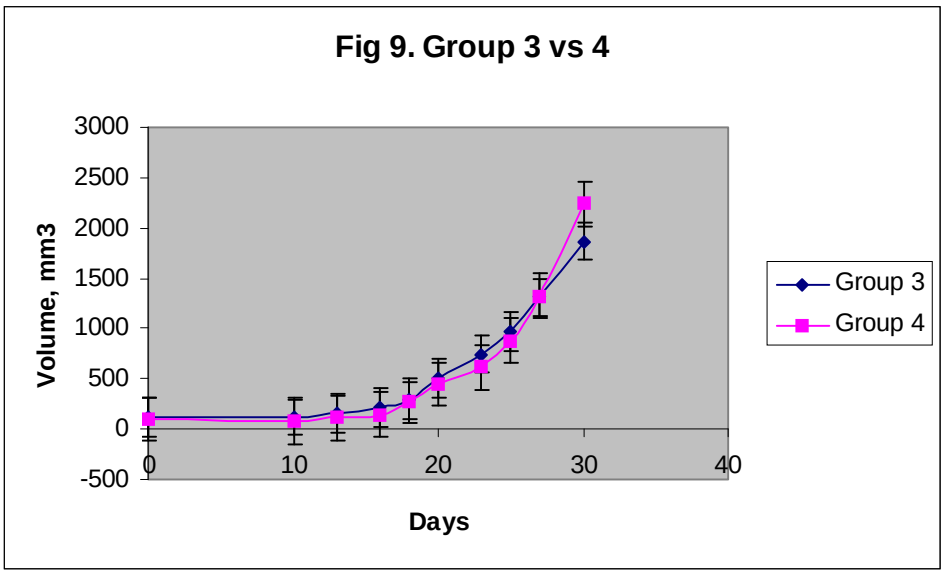


Figure 9. Comparison between the growth curves of B16 tumor in the 2 PDT groups (Group 3, incubation period 1 hour and Group 4, incubation period 6 hours). There was no statistical difference between the 2 curves. The P-values were .736, .431, .519, .398, .379, .782, .701, .836, .979, .637 at day 10, 13, 16, 18, 20, 23, 25, 27, 30 and 33 respectively. Values are represented by mean ± SE of at least 5 animals.

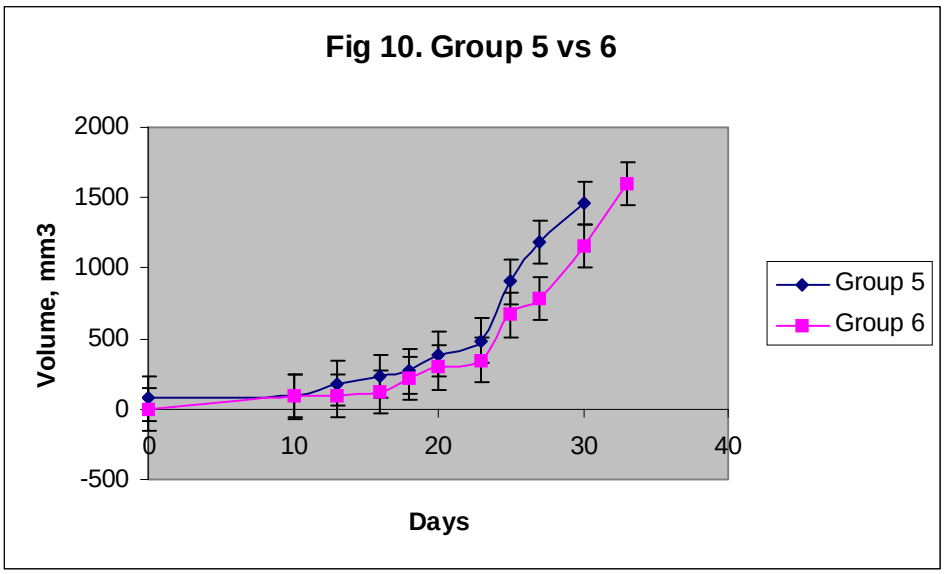


Figure 10. Comparison between the growth curves of B16 tumor in the 2 photoimmunotherapy groups (Group 5, incubation period 1 hour and Group 6, incubation period 6 hours). There was no statistical difference between the 2 curves. The P-values were .100, .877, .703, .938, .862, .526, .578, .598, .910 and .682 at day 10, 13, 16, 18, 20, 23, 25, 27, 30 and 33 respectively.Values are represented by mean ± SE of at least 5 animals.

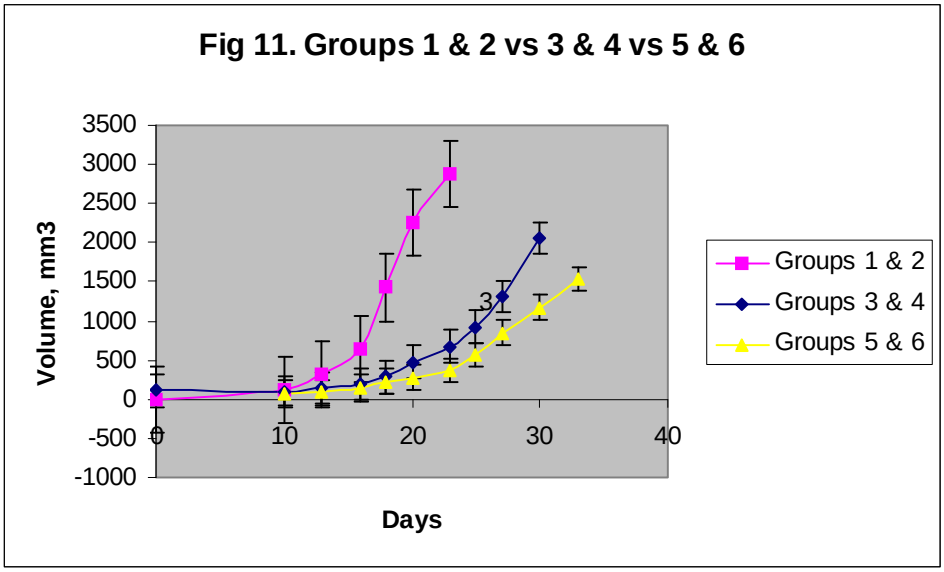


Figure 11. Comparison between the growth curves of B16 tumor in the control (Groups 1 and 2) versus PDT (Groups 3 and 4) versus photoimmunotherapy group (Group 5 and 6). Both the PDT and photoimmunotherapy groups were significantly superior to the control group in retarding tumor growth. The P-values were .914, .007, .003, <.001, <.001, <.001 and .242, .004, .003, <.001, <.001, <.001 at day 10 to 25 respectively. However, there was no statistical difference between the growth curves of the PDT versus the photoimmunotherapy group. P-values were .395, .836, .737, .595, .972, .368, .580, .771, .633 and .301 at days 10 to 33 respectively. Values are represented by mean ± SE of at least 10 animals.

3.5 Effectiveness of photoimmunotherapy in generating an anti-tumor vaccine

The mice were challenged with inoculation of a second B16 tumor in the opposite flank (left side).There was no difference in the second tumor growth curves between the 2 PDT groups (Group 2, 1 hour incubation period and Group 3, 6 hours incubation period) (Figure 12) and between the 2 photoimmunotherapy groups (Figure 13). However, when the second tumor growth curves of the control (Group 1), PDT alone group (Groups 2 and 3) and photoimmunotherapy group were compared (Groups 4 and 5), mice with tumors treated with photoimmunotherapy were more resistant to a second tumor challenge as compared to PDT treated mice and mice with no treatment respectively (Figure 14). Mice in the PDT alone treated group seemed to be more resistant than the control to the second tumor challenge although this was not statistically significant. Overall, these results suggest that photoimmunotherapy resulted in immunologic memory.

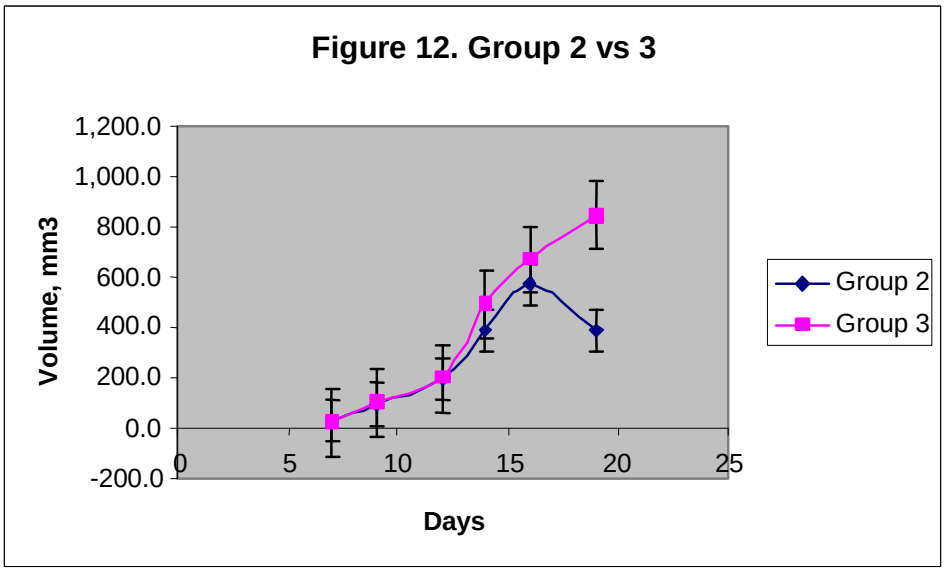


Figure 12. Comparison between the growth curves of the second B16 tumor (left side) in the 2 PDT groups (Groups 2, 1 hour incubation period and 3, 6 hours incubation). There was no statistical difference between both curves. On the 7^{th}, 9^{th}, 12^{th}, 14^{th}, 16^{th} and 19^{th} day, the P-value was .664, .953, .920, .608, .690 and .065 respectively. Values are represented by mean ± SEM of at least 5 animals.

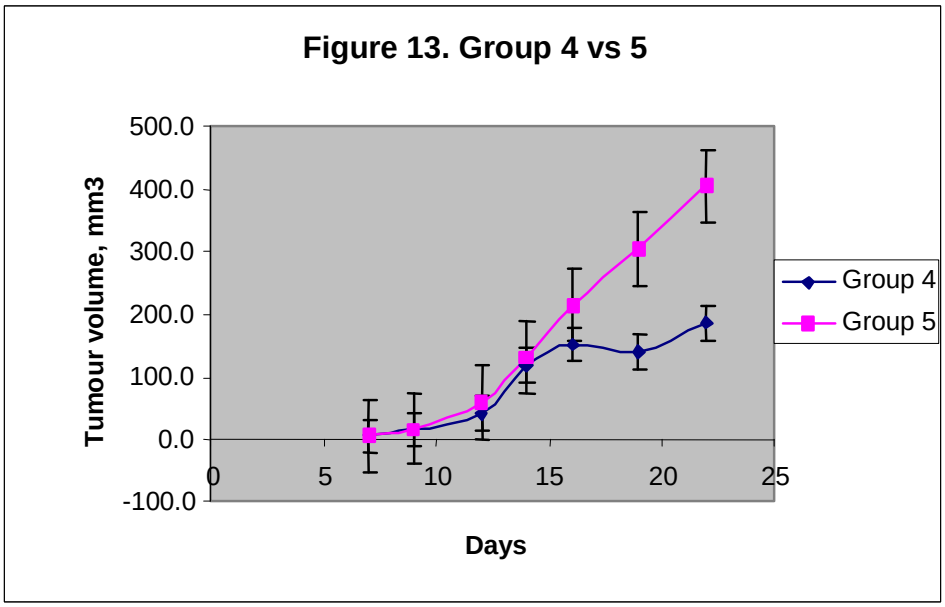


Figure 13. Comparison between the growth curves of the second B16 tumor (left side) in the 2 photoimmunotherapy groups (Groups 4, 1 hour incubation period and 5, 6 hours incubation). There was no statistical difference between both curves. On the 7th, 9th, 12th, 14th, 16th, 19th and 22nd day, the P-value was .731, .949, .261, .736, .436, .026 and .098 respectively. Values are represented by mean ± SEM of at least 5 animals.

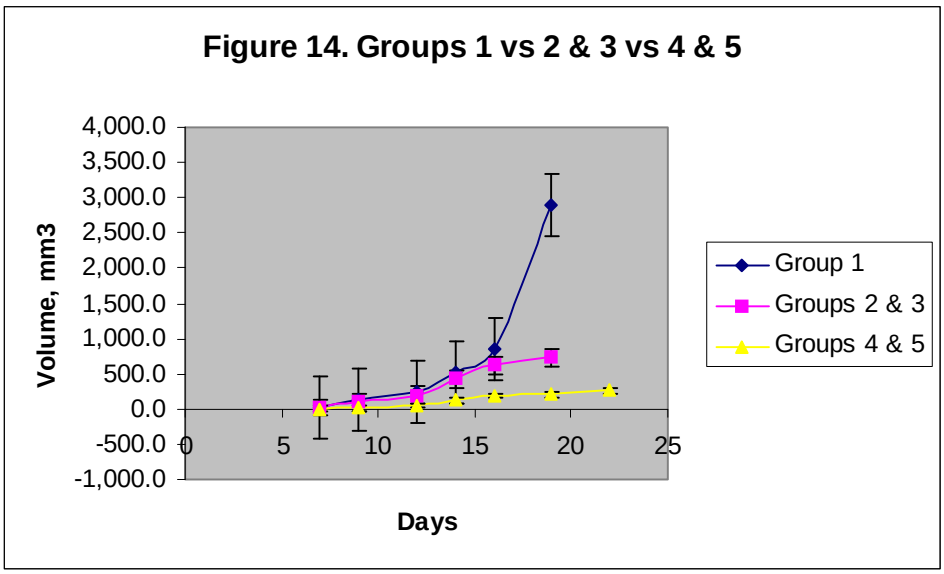


Figure 14. Comparison between the growth curves of the second B16 tumor (left side) in the control (Groups 1) versus PDT (Groups 2 and 3) versus photoimmunotherapy group (Groups 4 and 5). The photoimmunotherapy group was significantly superior to both the control group, P-value on 7^{th}, 9^{th}, 12^{th}, 14^{th}, 16^{th} and 19^{th} day were <.001 and PDT group, P-value on 7^{th}, 9^{th}, 12^{th}, 14^{th}, 16^{th} and 19^{th} day were <.001, .001, <.001, <.001, <.001 and <.001 respectively in retarding tumor growth. The PDT group appeared to be superior to the control group but this was not statistically significant, P-values were .370, .614, .509, .512, .253 and .004 on the 7^{th} to 19^{th} day, respectively. Values are represented by mean ± SEM of at least 5 animals.

3.6 Effect of photoimmunotherapy on a preexisting contralateral tumor (metastatic model)

The results showed that photoimmunotherapy conferred systemic antitumor effects against B16 melanoma cells. As demonstrated in Figure 15, the growth of the contralateral tumor was significantly suppressed compared to the 2 control groups (PDT and no treatment). However, when the pre-established tumor was large (Figure 16), there was no significant difference between the photoimmunotherapy group and control groups.

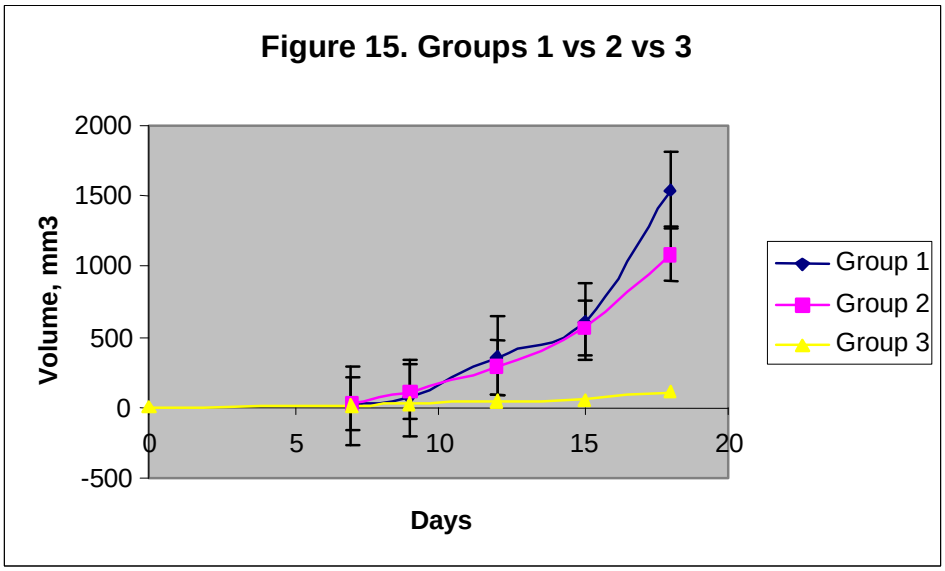


Figure 15. Small metastasis model. Comparison between the growth curves of the second B16 tumor (left side) in the control (Groups 1) versus PDT (Groups 2) versus photoimmunotherapy group (Group 3). The photoimmunotherapy group was significantly superior to both the control group, P = .53, <.001, <.001, <.001, <.001 and PDT group, P = .29, <.001, <.001, <.001, <.001 at day 7, 9, 12, 15 and 18 respectively in retarding tumor growth. There was no difference between the tumor growth curve of the PDT group compared to the control group. Values are represented by mean ± SE of at least 5 animals.

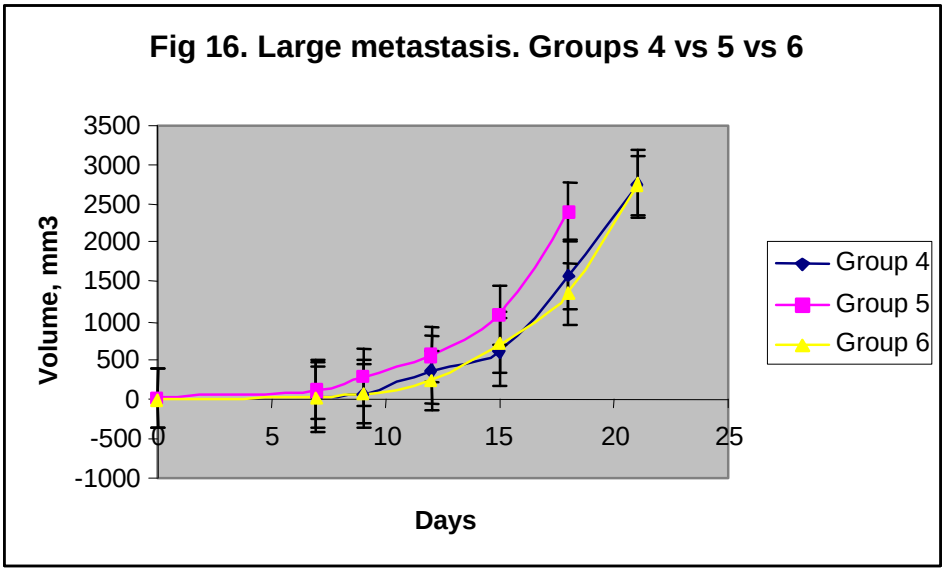


Figure 16. Large metastasis model. Comparison between the growth curves of the second B16 tumor (left side) in the control (Groups 4) versus PDT (Groups 5) versus photoimmunotherapy group (Group 6). The photoimmunotherapy group was not superior to both the control group and PDT group in retarding tumor growth $P > .05$. Values are represented by mean ± SE of at least 5 animals.

3.7 Tumor specificity.

This experiment confirmed the tumor-specific nature of photoimmuntherapy. Although previously, photoimmunotherapy was effective in retarding the growth of the second B16 tumors, it did not demonstrate any effect on the second RMA tumor. There was no significant difference in the growth curves of RMA tumors in the control, PDT or photoimmunotherapy group.

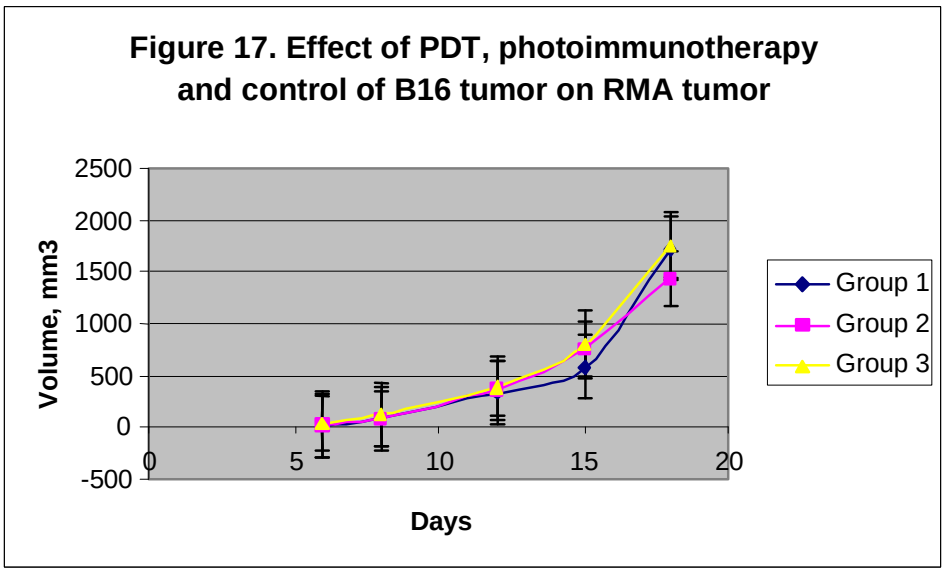


Figure 17. Tumor specificity. There was no significant difference in tumor growth between RMA tumors in the control group (Group1), PDT group (Group 2) and photoimmunotherapy group (Group 3).

3.8 Adoptive immune transfer

The results showed that adoptive transfer of primed splenocytes failed to result in growth retardation of B16 tumors. The B16 tumor growth curves were similar with or without the injection of primed splenocytes (Figure 18). As expected, there was no significant difference between the growth curves in the control group (RMA) (Figure 19).

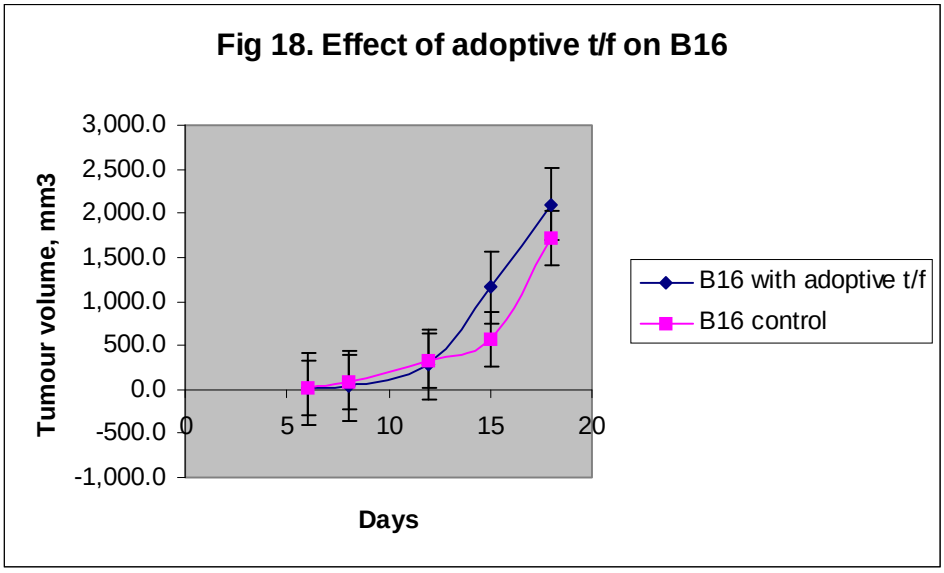


Figure 18. There was no significant difference between the growth curves of B16 tumor cells with and without adoptive transfer. The P-values on the 6^{th}, 8^{th}, 12^{th}, 15^{th} and 18^{th} days post inoculation were .198, .223, .609, .004, .517 respectively. Values are represented by mean ± SEM of at least 5 animals.

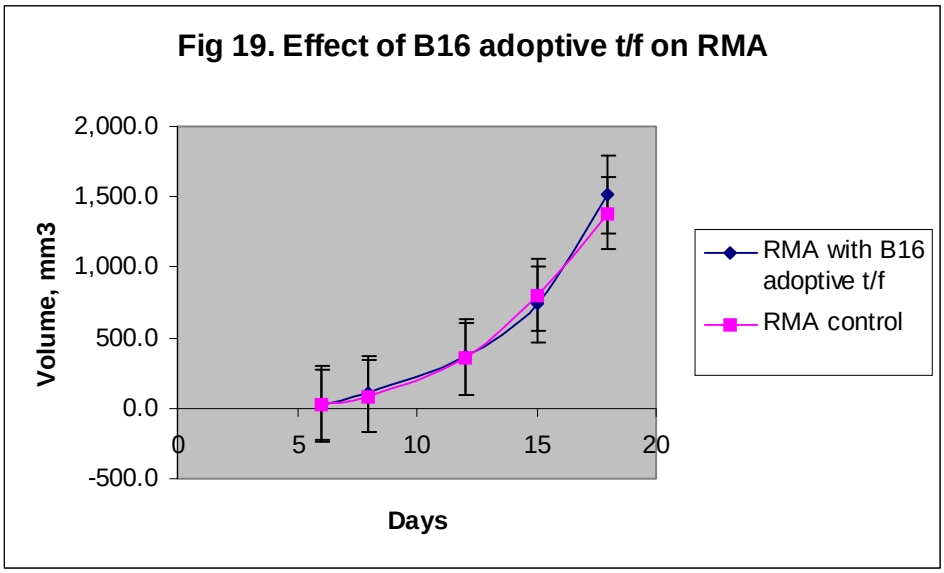


Figure 19. Not unexpectedly, there was also no difference between the growth curves of the RMA tumor before and after the adoptive transfers of B16-primed T lymphocytes. The P-values were .318, .280, .875, .487 and .458 on the 6th to 18th post-inoculation day respectively.

CHAPTER 4

DISCUSSION

4.1 PDT in melanoma

Presently, most researchers regard the role of PDT in the treatment of melanoma as limited (Biel, 1996) as traditionally, it has been well-documented that PDT alone is ineffective against melanoma (Biel, 1996; Schoenfeld et al., 1994; Saji et al., 2006). The large amounts of light-absorbing melanin pigment that prevents light penetration into the tumor tissue has been proposed as the reason behind this phenomenon. Previous studies on PDT using ATX-S10 Na (II) and 5-ALA with light of wavelengths more then 600 nm demonstrated the resistance of these tumors against PDT (Saji et al., 2006; Schoenfeld et al, 1994). However, others have confirmed that PDT using various photosensitizers can result in efficient tumor destruction of melanoma (Busseti et al., 1999; Lim et al., 2004, Kolarova et al. 2007). The present study confirms the findings that PDT can be effective against melanoma if higher light doses than usual are utilized. To the best of our knowledge this is the first study to date to demonstrate the ability of HY-PDT to produce tumor destruction in murine melanoma.

In this study by using a higher light dose of 120 J/cm^2, gross B16 tumor destruction as shown in Figure 2B could be produced even in tumors of 10 mm in diameter. These light doses were twice of that (60 J/cm^2) used in a previous study on the effect of HY-PDT on HK1 and CNE-2 nasopharyngeal cancer cells (Du et al., 2003). Importantly, these high light doses used were not toxic to the mice which survived weeks after PDT treatment. Interestingly, the wavelength of light used in our study was 580 nm which was shorter than that used in previous studies on PDT for melanoma. Melanin absorbs light over a broad-sprectum of wavelengths demonstrating maximal absorption at a wave-length of 335 nm. Its ability to absorb light decreases logarithmically as the

wavelength increases till almost negligible levels for light with a wavelength longer than 700 nm. Hence, theoretically, PDT utilizing light with a longer wavelength should demonstrate better penetration through melanin pigment. The present observation suggests that wavelength alone (and hence, light penetration alone) may not be the sole determinant of effective PDT for melanoma and that other biochemical products which may be activated during PDT may be important for inhibiting or activating tumor destruction. Very recently, investigators from Germany have demonstrated that the expression of heme oxygenase 1 (HO-1) is at least partly responsible for the resistance of melanoma to ALA-PDT (Frank et al., 2007). The inactivation of HO-1 by gene silencing resulted in an approximately 50% increase in the tumor cell death rate after ALA-PDT in melanoma. Further studies, are needed to determine the model of tumor destruction of melanoma after HY-PDT.

4.2 HY-PDT induced cell death

The mode of HY-PDT mediated cell death (and PDT in general) may vary from both necrosis to apoptosis or both (Ali et al., 2002; Thong et al., 2006). The mode of cell death after PDT is influenced by several factors including photosensitiser dose, tumor type, light dose, light-fluence rate, drug-light interval, oxygen concentration and intracellular localization of photosensitiser (Thong et al, 2007). In general, most studies demonstrate that the mode of cell death shifts from apoptosis to necrosis when HY concentration and light dose is increased (Vantieghem et al., 2001; Kamuhabwa et al., 2001). Cell type also has an important influence on the mode of cell death (Wyld et al., 2001). For example, in a study analyzing the effect of HY-PDT in HK1 NPC cell lines,

PDT only induced necrosis irrespective of dose (Du, 2004). Agostinis et al., 2002 found that malignant cells often had an impaired ability to undergo apoptosis. Some investigators believe that the extent of oxidative injury determines the mode of cell death (Girotti et al., 2001; Fiers et al., 1999). Less extensive oxidative damage that exceeds the repair capacity of the cell is likely to trigger apoptosis whereas more extensive damage which leads to membrane lysis would induce necrosis. Hence it is likely that cells more susceptible to oxidative stress such as HK1 are more likely to undergo necrosis rather than apoptosis (Du, 2004).

This is the first study investigating the use of HY-PDT in B16 melanoma cell lines *in vivo*. The results demonstrated that at a HY dose of 5 mg/kg and light dose of 120 J/cm^2, the mode of cell death was predominantly via necrosis with only a minor degree of apoptosis. The results in this study was similar to that of the study on nasopharngeal carcinoma cells conducted by Du et al. that the anti-tumor HY-PDT effects were similar after drug incubations of 1 hour and 6 hours (Du, 2004). In that study, it was demonstrated that at 1 hour incubation, HY concentration was maximal in plasma and the mode of tumor destruction was vascular-mediated resulting in cell death mainly apoptosis. At 6 hours incubation HY concentration was maximal in the tumor and the mode of cell death was mainly via necrosis. However, these observations were only applicable to CNE-2 cells whereas for HK-1, the mode of cell death was predominantly via cell necrosis irrespective of PDT dose or incubation period. The results from the present study also demonstrated that regardless of the incubation period tumor cell death of B16 melanoma occurred mainly via necrosis. These findings emphasize the important influence of tumor cell type on the mode of cell death (Wyld et al., 2001). The findings

were also consistent with those demonstrated by other investigators that the mode of cell death of melanoma cells after PDT were predominantly that of necrosis (Lim, et al., 2004). However, it is important to note that the localization of HY after administration was not analyzed in this study and it is possible that the pharmacokinetics and dynamics of the drug maybe different in the present model from the NPC model. Also, as the mode of cell death was not specifically studied with various drug and light dosages, it may be entirely possible that apoptotic cell death of B16 melanoma can still be induced with HY-PDT.

Presently, it is controversial whether necrosis or apoptosis serves as a superior source of tumor-associated antigens for the activation of DCs (Albert et al., 1998; Sauter B et al., 2000; Rovere et al., 1998). However, evidence from the study by Gollnick et al suggested that cell death via both necrosis and apoptosis induces the most effective anti-tumor response compared to either alone hence the superiority of PDT-generated vaccine over UV and ionizing radiation generated vaccines (Gollnick et al., 2002). Nonetheless, the relationship between mode of tumor cell death and efficiency of induction of the immune response remains unclear. Many studies examining this relationship by non-PDT treatment modalities both *in vivo* and *in vitro* have produced conflicting results (Magner and Tomasi, 2005; Bartholomae et al., 2004). Some reports have demonstrated that apoptotic cells are superior to necrotic tumor cells in inducing an immune response (Scheffer et al., 2003; Shaif-Muthana et al, 2000). On the other hand, others have shown that modalities inducing predominantly tumor cell necrosis are actually superior at activating the immune system as compared to methods causing predominantly apoptosis (Zitvogel et al., 2004; Melcher et al., 1999). After tumor cell necrosis, cytosolic contents

spill out to the extracellular space through the damaged plasma membranes and induce an acute inflammatory response. Host leukocytes are attracted to the tumor and potentiate the immune response possibly by increased antigen presentation. The present study suggests that tumor cell death occurring predominantly via necrosis can induce an effective immune response after photoimmunotherapy but not PDT alone. Further studies are needed to determine if apoptosis alone or a combination of apoptosis and necrosis will generate a more effective immune response.

4.3 Photoimmunotherapy with DC-based vaccines

The combination of PDT with DC-based vaccines or photoimmunotherapy has not been well-studied (Gollnick et al., 2002; Jalili et al., 2004; Saji et al., 2006). The first study to demonstrate the ability of PDT to enhance tumor cell immunogenicity and to generate an effective tumor vaccine was conducted by Gollnick et al., 2002 on murine EMT6 and P815 tumor models. They successfully demonstrated that PDT-treated tumors were highly immunogenic and PDT-generated lysates resulted in highly effective vaccines in the absence of an adjuvant (Gollnick et al., 2002). In that study, PDT-generated tumor lysates formed potent vaccines which were superior to tumor vaccines generated by UV or ionizing irradiation. The authors postulated that the unique mechanism of PDT-mediated cell death which resulted in both apoptosis and necrosis as opposed to the predominantly apoptotic cell death after UV treatment and predominantly necrotic cell death after ionizing radiation could be the reason why PDT-treated tumors were more immunogenic. As, it has been previously shown that uptake of apoptotic cells alone are insufficient to activate and induce maturation of DC and a second danger signal

is required (Nestle et al., 2001), the authors hypothesized that it is possible that UV and IR-generated tumor vaccines were only able to partially activate DCs and hence resulted in a less optimal T-cell immune response. This was supported by their findings that UV-generated lysates were only able to partially activate DCs as evidenced by the increase in MHC class II and CD86 expression but no increase in IL-12 production, and that both UV and ionizing radiation-generated vaccines were not as effective as PDT vaccines in stimulating tumor specific IFN-γ-secreting cells and increasing splenic cytolytic activity.

Subsequently, Korbelik and Sun, 2006 conducted a similar study examining the effects of PDT-generated tumor vaccine on poorly immunogenic murine squamous cell carcinoma (SCCVII). They too discovered that the efficacy of PDT-generated vaccines was superior to lysed cell and x-ray-treated cell generated vaccines. They identified two elements important for the unique capacity of PDT to generate an effective cancer vaccine ie. surface expression of HSP 70 and binding of complement proteins on PDT-treated cells.

Thus far, only 2 studies on DC-based photoimmunotherapy have been reported and both have shown the benefit of this combined therapy (Saji et al, 2006; Jalili et al., 2004). Both these studies utilized intra-tumoral injection of DCs harvested *ex vivo*. Intra-tumor injection of DCs overcomes the problems associated with *ex vivo* antigen-loading of DCs. *In vivo* loading of DCs is thought to be superior to *ex vivo* loading as this exposes and primes DCs to the potentially thousands of antigens present in tumors as opposed to the 1 or 2 antigens used in *ex vivo* techniques. However, it is essential to remember that their methods were still not entirely *in vivo* as the expansion of DCs were performed *ex*

vivo which has been shown to be associated with potential limitations and practical problems especially cost and manpower.

The method utilized in the present study of DC-based immunotherapy which is entirely *in vivo* will theoretically overcome all the potential problems associated with both *ex vivo* DC expansion and *in vitro* antigen loading and activation of DCs. The potential advantages of the entirely *in vivo* method is not only that DCs are potentially primed by numerous tumor antigens but that DCs of different lineages are expanded which may be required to produce an optimal T-cell response. However, it is important to highlight important potential limitations associated with the *in vivo* expansion of DCs. It has been reported that DCs in patients with malignancy are functionally impaired DCs and theoretically, DC expansion may result in increased in numbers of DCs which are not useful. Furthermore, it has also been demonstrated that an increase in the numbers of immature DC may result in immune tolerance rather than immune stimulation (Lutz and Schuler, 2002).

The results in the present study demonstrate that photoimmunotherapy utilizing the *in vivo* expansion of DC has a similar systemic anti-tumor immunity effect as that of the *in vitro* expansion and intratumoral injection of DCs method utilized by Jalili et al and Saji et al (Jalili et al., 2004; Saji et al., 2006). Photoimmunotherapy not only retarded contralateral tumor growth when exposed to a second tumor challenge but it also suppressed the growth of an established contralateral tumor. These findings suggest that PDT destruction of melanoma results in an optimal milleu for the priming and maturation of DCs resulting in an effective tumor specific immune response. These findings also suggest that photoimmunotherapy may play an important role in the clinical setting in the

delay or prevention of the development of metastases as well as for the palliation of advanced metastatic disease. The effectiveness of photoimmunotherapy demonstrated in this study on B16 melanoma cells which is considered poorly immunogenic (Saji et al., 2006) also suggests that it need not be restricted to only highly immunogenic tumors.

The pathophysiology of combination therapy with PDT and DC expansion has been previously described (Saji et al., 2006; Jalili et al., 2004). PDT induces cell death via both necrosis and apoptosis following oxidative stress. It also induces an intense local inflammatory reaction via the rapid release of proinflammatory mediators released from tumor cells and endothelial cells (Agarwal, et al., 1993). Cytokines that are involved in the recruitment of neutrophils and myeloid cells such as TNF, IL-1β and IL-6 are also secreted by PDT-treated cells (Jalili et al., 2004). These complex reactions induce an anti-tumor immune response and are able to activate DCs. The induction of HSPs by PDT-treated cells have been proposed as a key step in the activation of DCs (Jalili et al., 2004). HSPs have been identified as key activators of DCs giving rise to potent immunoregulatory activities (Feng, et al., 2001; Flohe et al., 2003) and several studies have demonstrated that HSPs are expressed after PDT (Gomer et al., 1996; Wang et al., 2002). Finally, cytotoxic T cells have been shown to be responsible for the regression of the tumor and $CD8^+$ T cells have been further identified as the major effector cells.

4.4 Effectiveness of photoimmunotherapy on primary tumor.

The present study demonstrated that both PDT and photoimmunotherapy were effective in retarding primary tumor growth as compared to control. However, the effect of photoimmunotherapy was not superior to PDT alone in the treatment of primary B16

tumors when a therapeutic dose of PDT was used. This is in contrast to the findings of both Jalili et al and Saji et al that photoimmunotherapy with intra-tumoral injection of DCs was superior to PDT alone in controlling primary tumor growth of CT26 colorectal carcinoma cells and both CT26 colorectal carcinoma and B16 melanoma cells respectively (Jalili et al, 2004., Saji et al., 2006). It may be possible to demonstrate the superiority of photoimmunotherapy over PDT if a sub-therapeutic dose of PDT with minimal tumor destruction is administered as in the study by Saji et al., 2006. Hence, although Saji et al., 2006 demonstrated the superiority of photoimmunotherapy over PDT alone in the treatment of primary B16 tumors, it is likely that this is probably true only when a sub-therapeutic dose of PDT is administered. In the clinical setting, the benefit of photoimmunotherapy over PDT alone for the treatment of primary tumors will likely be limited to situations whereby one is unable to administer a therapeutic dose of PDT to the entire tumor due to toxicity.

4.5 Effectiveness of photoimmunotherapy in generating an tumor specific anti-tumor vaccine

The findings in this study were consistent with that of Saji et al., 2006 that photoimmunotherapy treated mice demonstrated anti-tumor immunity and were more resistant to a second tumor challenge. The findings also demonstrated that the anti-tumor immunity was specific as photoimmunotherapy of B16 cells had no effect on a second tumor challenge with RMA (Figure 17). This finding provides evidence that the anti-tumor response induced by photoimmunotherapy is tumor-specific and hence is most likely mediated by the tumor-specific acquired immune response. This finding is

consistent with the findings from previous studies on photoimmunotherapy and PDT-generated vaccines that the acquired immune response which is tumor specific plays a key role in anti-tumor activity after PDT (Korbelik and Sun, 2006). Clinically, these effects may be useful in preventing the development of metachronous tumors especially in malignancies associated with field change such as head and neck cancers and hepatocellular carcinoma whereby the development of second primary cancers are not uncommon. However, further experiments need to be performed to determine if the tumor-specific immune effects of photoimmunotherapy are durable.

4.6 Effect of photoimmunotherapy on a pre-established contralateral tumor (metastatic model)

In this study, photoimmunotherapy was effective and superior to PDT and control in retarding the growth of the contralateral tumor. This too was consistent with the findings of Jalili et al., 2004 and Saji et al., 2006. However, photoimmunotherapy was ineffective when the established contralateral tumor was large (Figure 16) suggesting that when tumors were large, the immune system was overwhelmed and photoimmunotherapy was ineffective. This finding has important implications in the clinical setting suggesting that photoimmunotherapy should be administered early when distant metastases are small and an efficient anti-tumor response can be mounted.

4.7 Adoptive transfer

The development of effective adoptive transfer of T-cells as a strategy for immunotherapy in human malignancies has met with many obstacles (Riddel, 2007). One

of the major limitations of adoptive therapy is to be able to isolate antigen-specific T lymphocytes consistently and successfully (Stauss et al., 2007). Efficient methods for selective isolation of high avidity T cells are still in the process of being developed. Moreover, it is not easy to isolate T cells with high avidity for tumor-associated antigens because of acquisition of tolerance to tumor antigens (Drake et al., 2006). Low avidity cytotoxic T-lymphocytes have been shown to be less effective in tumor protection *in vivo* (Zeh et al., 1999). There is also the problem of ensuring that transferred T cells are still tumor-reactive in the *in vivo* state following adoptive transfer (Blattman and Greenberg, 2004; Gattinoni et al., 2006). Failure of clinical response to adoptive transfer of laboratory expanded T cells have been attributed to evasion of tumor-specific immune responses by immunosuppressive factors (Lizee et al., 2007). Another possible reason for the failure of adoptive transfer in this study is the timing at which the spleen of photoimmunotherapy treated mice were harvest. In this study, splenocytes were harvested 5 to 7 days after treatment as opposed to the study by Saji et al. (2006) whereby the splenocytes were harvested 4 weeks after treatment. It is possible that the shorter time period in this study was insufficient for the development of adequate numbers of tumor-specific memory T-cells in the spleen for effective adoptive transfer. It was not possible to wait 4 weeks for the harvesting of splenocytes in this study as before this time period the tumors had already reached the size threshold whereby the mice were required to be euthanized.

The lack of efficacy seen in adoptive transfer in this study may be due to the above reasons. The efficacy of PDT-generated vaccine has also been shown to be dependent on the number of cells used for vaccination. In a recent study by Korbelik and Sun, the

efficacy of PDT-generated vaccine was compared using various concentrations of 5, 10, 20 and 50 million cells. It was shown that a vaccine using 20 million cells produced the greatest tumor retardation (Korbelik and Sun, 2006). It is possible that the number of lymphocytes used in this study was insufficient. Furthermore the presence of regulatory cells that has the potential to inhibit T-cell responses cannot be excluded (Ward and Kaufman, 2007). In addition there is also the possibility of hitherto unknown tumor escape mechanisms. Interestingly, a very recent finding by Matter et al. (2007) showed that contrary to expectation, adoptive T-cell therapy could reduce tumor surveillance and enhance tumor growth rather than regression.

4.8 Conclusion

The main findings in this study are listed below:

1. HY-PDT can induce tumor destruction in melanoma in mice without toxic effects
2. Higher light doses are required for effective tumor destruction
3. The mode of cell death of B16 melanoma after HY-PDT after both 1 hour and 6 hours incubation was mainly via necrosis. Apoptosis occurred at a much lesser extent.
4. Both photoimmunotherapy and PDT retarded the growth of primary B16 melanoma cells.
5. The anti-tumor effect of photoimmunotherapy against the primary tumor was not superior to PDT.
6. Photoimmunotherapy was effective in generating an anti-tumor vaccine. Tumors treated with photoimmunotherapy were resistant against a second tumor challenge.
7. The anti-tumor immunity generated by photoimmunotherapy was tumor-specific.

8. Photoimmunotherapy was effective in conferring systemic anti-tumor effects against small distant untreated tumors.

9. The systemic anti-tumor effect of photoimmunotherapy was ineffective when distant untreated tumors were large in size.

10. Adoptive transfer of splenocytes of photoimmunotherapy-treated mice failed to produce an effective anti-tumor response.

In conclusion, the data in our present study demonstrates that high doses of HY-PDT is effective against melanoma and is the first study to demonstrate the anti-tumor effects of HY-PDT on melanoma. It also demonstrates that photoimmunotherapy using HY-PDT and *in vivo* DC expansion by pNGVL3-Flex plasmid DNA results in an effective systemic anti-tumor immune response which is tumor-specific and suppresses tumor growth at distant sites. This is the first study to demonstrate the effective use of photoimmunotherapy via *in vivo* DC expansion. These results suggest that the addition of DC-based immune therapy to PDT results in systemic effects which may be useful in the clinical setting for delaying and preventing the development of a second primary and for treating distant metastases. The results of our study are summarized in Figure 20.

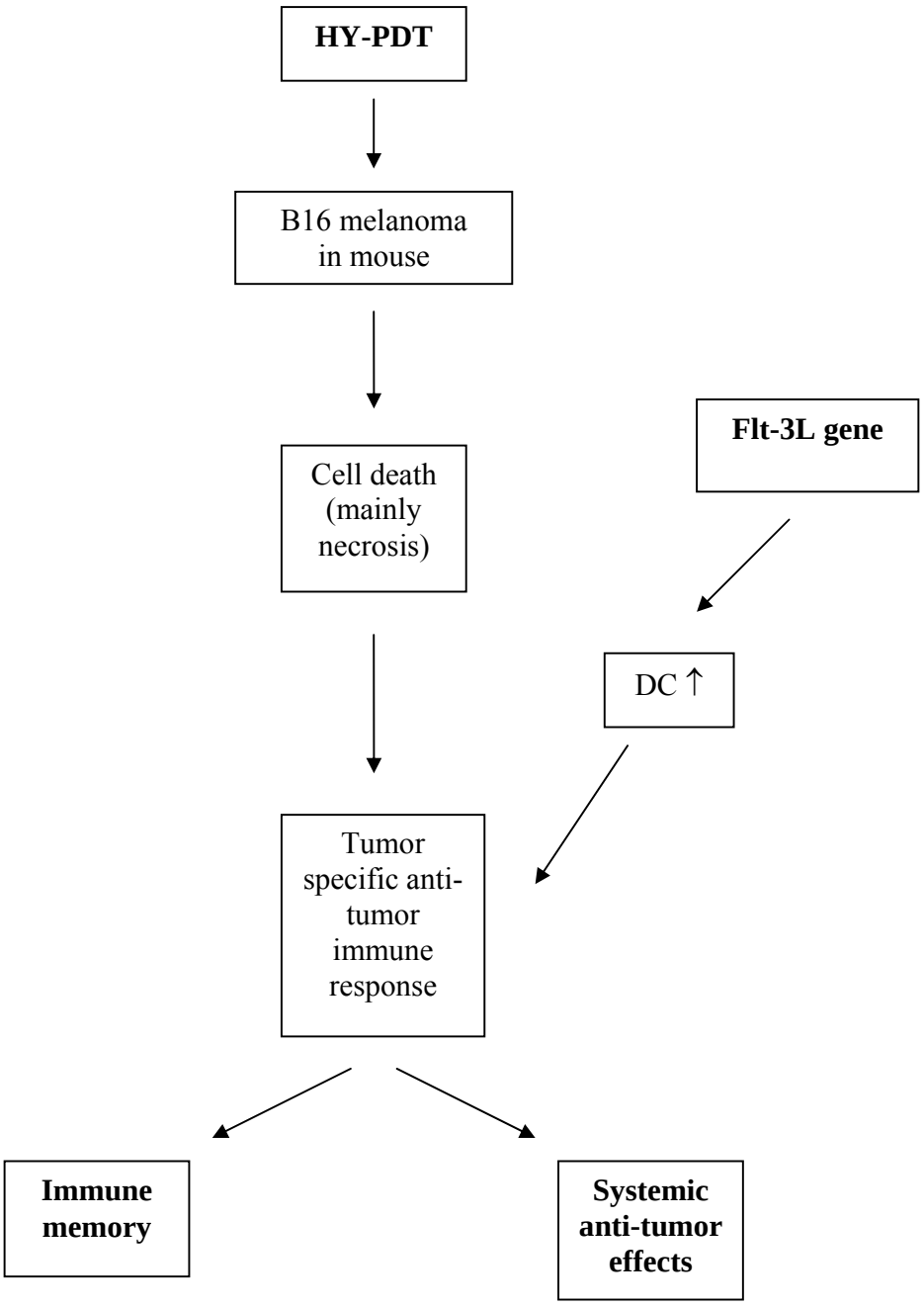


Figure 20. Effect of photoimmunotherapy on B16 melanoma

4.9 Further studies

Preclinical studies

Further studies should be performed to determine the effect of different light and drug dose of HY-PDT on mode of cell death ie. apoptosis and necrosis. Once this is determined, it would be interesting to investigate how the relative proportions of apoptosis and necrosis affect the immune response triggered and more importantly the outcome of photoimmunotherapy. Studies should be also conducted to determine the pathophysiology of HY-mediated tumor destruction in melanoma. It is intriguing how HY-PDT despite its short wavelength is able to mediate the destruction of melanoma.

Further experiments should be performed to obtain further insight and to compare the host response and pathophysiology of PDT and photoimmunotherapy induced tumor-destruction. Flow cytometry of can be used to examine the cells of regional lymph nodes (tumor-draining lymph nodes) to determine the percentage increase of T-lymphocyte, B-lymphocyte and DC population. Furthermore, the proportion of various subtypes of T-cells especially cells bearing the $CD44^{+}$ $CD45RB^{-}$ memory phenotype should be determined (Korbelik and Sun, 2006). Flow cytometry should also be used to analyze the cells retrieved from the tumor site comparing cells from tumors in the control, PDT and photoimmunotherapy treated mice. Specifically, in addition to proportion of DCs and lymphocytes, the cells should be studied to determine if complement proteins and HSPs are expressed as these have been fingered as possible key players for effective photoimmunotherapy. The role of neutrophils in photoimmunotherapy should also be studied as it has been recently shown that they play a key role in PDT-induced systemic anti-tumor immunity (Kousis et al., 2007). Experiments should be performed to

determine the efficacy of photoimmunotherapy using light doses which stimulate different levels of neutrophil infiltration (Henderson et al., 2004) to determine the role of neutrophils on DC-based photoimmunotherapy. ELISPOT assays can also be used for the *in vitro* characterization of the antitumor immune response induced by PDT and photoimmunotherapy (Saji et al., 2006). These essays can be used to determine if splenocytes of photoimmunotherapy treated mice contained significantly more tumor-specific IFN-λ-secreting cells than splenocytes from the other treatment groups.

Cytotoxic activity of lymph node cells and splenocytes after PDT and photoimmunotherapy should be tested in a standard (^{51}Cr) release assay, as described previously (Golab et al., 2003). After photoimmunotherapy and PDT, the cytotoxic assays are used to measure spontaneous activity (NK cells) and specific cytotoxicity ($CD8^+$ T cells) (Jalil et al., 2004). Confocal laser microscopy studies can be performed to determine the increased expression of various HSPs.

Clinical studies

Considering the large numbers of patients whom have been treated via PDT over the past 30 years (Castano et al., 2006), there have been few studies examining the effects of PDT on the human immune system much less the effects of photoimmunotherapy. Presently, only a few case reports examining the effects of PDT on the immune system and the effects of photoimmunotherapy in humans have been reported in the literature (Abel-Hady et al., 2001; Shikowitz et al., 2005; Thong et al., 2007). Systematic studies examining the effects of PDT and photoimmunotherapy on cancers and the resultant immune responses in humans are long overdue. Phase I and II clinical trials should also

be performed to study the effects of *in vivo* DC-based photoimmunotherapy in human cancers especially melanoma as there is presently no effective treatment for this.

Currently, although the use of PDT in combination with immune therapy ie. photoimmunotherapy is still in its infancy, its future looks bright as this modality seems to be based on extremely sound scientific principles. However, it is difficult to determine if PDT-induced anti-tumor immunity and photoimmunotherapy would someday become a standard treatment modality for human cancers in the future or that reports of its use would be relegated from scientific journals to history books. Only time will provide us with this answer.

CHAPTER 5

REFERENCES

References

Abdel-Hady ES, Martin-Hirsch P, Duggan-Keen M, Stern PL, Moore JV, Corbitt G, Kitchener HC, Hampson IN. Immunological and viral factors associated with the response of vulval intraepithelial neoplasia to photodynamic therapy. Cancer Res 2001;61:192-6.

Ackroyd R, Kelty C, Brown N, Reed M. The history of photodetection and photodynamic therapy. Photochem Photobiol 2001;74:656-69.

Agarwal ML, Larkin HE, Zaidi SI, Mukhtar H, Oleinicjk NL. Phospholipase activation triggers apoptosis in photosensitized mouse lymphoma cells. Cancer Res 1993;53:5897-902.

Agostinis P, Buytaert E, Breyssens H, Hendricks N. Regulatory pathways in photodynamic therapy induced apoptosis. Photochem Photobiol Sci 2004;3:721-9.

Agostinis P, Vandenbogaerde A, Donella-Deana A, Pinna LA, Lee KT, Goris J, Merlevede W, Vandenheede JR, de Witte PA. Photosensitized inhibition of growth factor-regulated protein kinases by hypericin. Biochem Pharmacol 1995;49:1615-22.

Agostinis P, Vantieghem A, Merlevede W, de Witte PA. Hypericin in cancer treatment: more light on the way Int J Biochem Cell Biol 2992;34:221-41.

Albert ML, Sauter B, Bhardwaj N. Dendritic cells acquire antigen and apoptotic cells and induce class I-restricted CTLs. Nature 1998;392:86-9.

Alecu M, Ursaciuc C, Halalau F, Corma G, Merlevede W, Waelkens E, de Witte PM. Photodynamic treatment of basal cell carcinoma and squamous cell carcinoma with hypericin. Anticancer Res 1998;4651-4.

Ali SM, Chee SK, Yuen GY, Olivo M. Hypericin and hypocrellin induced apoptosis in human mucosal carcinma cells. J Photochem Photobiol B 2001;65:59-73.

Ali SM, Chee SK, Yuen GY, Olivo M. Photodynamic therapy induced Fas-mediated apoptosis in human carcinoma cells. Int J Mol Med 2002;9:257-70.

Ali SM, Soo KC, Gan YY, Olivo M. Hypocrellins and hypericin induced apoptosis in human tumor cells: a possible role of hydrogen peroxide. Int J Oncol 2002;9:461-72.

Ali SM, Olivo M. Mechanisms of action of phenanthroperylenequinones in photodynamic therapy (review). Int J Oncol 2003;22:1181-91.

Aloysius MM, Takhar A, Robins A, Eremin O. Dendritic cell biology, dysfunction and immunotherapy in gastrointestinal cancers. Surgeon 2006;4:195-210.

Ardavin C, Wu L, Li CL, Shortman K. Thymic dendritic cells and T Cells develop simultaneously in the thymus from a common precursor population. Nature 1993;362:761-3.

Assefa Z, Vantieghem A, Declercq W, Vandenabeele P, Vandenheede JR, Merlevede W, de witte P, Agostinis P. The activation of c-Jun N-terminal kinase and p38 mithen-activated protein kinase signalling pathways protects HeLa cells from apoptosis following photodynamic therapy with hypericin. J Biol Chem 1999;274:8788-96.

Austyn JM, Kupiec-Weglinski JW, Hankins DF, Morris PJ. Migration patterns of dendritic cells in mouse. Homing to T cell-dependent areas of spleen, and binding within marginal zone. J Exp Med 1988;167:646-51.

Balch CM, Buzaid AC, Soong SJ. Final version of the American Joint Committee on Cancer staging system for cutaneous melanoma. J Clin Oncol 2001;19:3635-48.

Banchereau J, Briere F, Caux C, Davoust J, Lebecque S, Liu YJ, Pulendran B, Palucka K. Immunobiology of dendritic cells. Annual Review of Immunology 2000;18:767-811.

Banchereau J, Palucka AK, Dhodapkar M, Burkeholder S, Tauqet N, Rolland A, Taquet S, Coquery S, Wittkowski KM, Bhardwaj N. Immune and clinical responses in patients with metastatic melanoma to CD34+ progenitor-derive dendritic cell vaccine. Cancer Res 2001;61:6451-8.

Banchereau J, Steinman RM. Dendritic cells and the control of immunity. Nature 1998;392(6673):245-52.

Bartholomae WC. T cell immunity induced by live, necrotic, and apoptotic tumor cells. J Immunol 2004;173:1012-22.

Bassi P. BCG (Bacillus of Calmette Guerin) therapy of high-risk superificial bladder cancer. Surg Oncol 2002;11:77-83.

Biel MA. Photodynamic therapy and the treatment of head and neck cancers . J Clin Laser Med Surg 1996;14:239-44.

Bellnier DA. Potentiation of photodynamic therapy in mice with recombinant human tumor necrosis factor-α.. J Photochem Photobiol B 1991;8:203-10.

Blank M, Lavie G, Mandel M, Keisari Y. Effects of photodynamic therapy with hypericin in mice bearing highly invasive solid tumors. Oncol Res 2001;12:409-18.

Blattman JN, Greenberg PD. Cancer immunotherapy: a treatment for the masses. Science. 2004;305:200-5.

Braun SE, Chen K, Blazar BR, Orchard PJ, Sledge G, Robertson MJ, Broxmeyer HE, Cornetta K. Flt3 ligand antitumor activity in a murine breast cancer model: a comparison with granulocyte-monocyte colony-stimulating factor and a potential mechanism of action. Hum Gene Ther 1999;10:2141-51.

Brurberg KG, Graff BA, Olsen DR, Rofstad EK. Tumor-line specific O_2 fluctuations in human melanoma xenografts. Int J Radiat Oncol Biol Phys 2004;58:403-9.

Busetti A, Soncin M, Mori G, Rodgers MA. High efficiency if benzoporphyrin derivative in the photodynamic therapy of pigmented malignant melanoma. Br J Cancer 1999;79:821-4.

Castano A, Demidova TN, Hamblin MR. Mechanisms in photodynamic therapy: part two- cellular signalling, cell metabolism and modes of cell death. Photodiagn Photodyn Ther 2005;1-23.

Castano AP, Mroz P, Hamblin MR. Photodynamic therapy and anti-tumor immunity. Nat Rev 2006;6:535-45.

Chapman PB, Einhorn LH, Meyers ML, Saxman S, Destro AN, Panageas KS, Begg CB, Agarwala SS, Schuchter LM, Ernstoff MS, Houghton AN, Kirkwood JM. Phase III multicenter randomized trial of the Dartmouth regimen versus dacarbazine in patients with metastatic melanoma. J Clin Oncol 1999;17:2745-51.

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

Celluzzi CM, Mayordomo JI, Storkus WJ, Lotze MT, Falo LD Jr. Peptide-pulsed dendritic cells induce antigen-specific CTL-mediated protective immunity. J Exp Med 1996;183:283-7.

Chaloupka R, Obsil T, Plasek J, Suereau F. The effect of hypericin and hypocrellin-A on lipid membranes and membrane potential of 3T3 fibroblasts. Biochem Biophys Acta 1999;1418:39-47.

Chen W, Syldath U. Belmann K, Burkart V, Kolb H. Human 60k-Da heat-shock protein: a danger signal to the innate immune system. J Immunol 1999;162:3212-9.

Chung PS, Saxton RE, Paiva MB, Rhee CK, Soudant J, Mathey A, Foote C, Castro DJ. Hypericin uptake in rabbits and nude mice transplanted with human squamous cell carcinomas: study of a new sensitizer for laser phototherapy. Laryngoscope 1984;104:1471-6.

Clark G, Munster D, Yusuf S, Hart DN. Eighth leucocyte differentiation antigen workshop DC section summary. Cell Immunol 2005;236:21-8.

Clemente CG, Mihm MC Jr, Bufalino R, Zurrida S, Collini P, Cascinelli N. Prognostic value of tumor infiltrating lymphocytes in the vertical growth phase of primary cutaneous melanoma. Cancer 1996;77(7):1303-10.

Daniell MD, Hill JS. A history of photodynamic therapy. Aust N Z J Surg 1991;61:340-8,
De Vree WJ, Essers MC, de Bruijn HS, Star WM, Koster JF, Sluiter W. Evidence for an important role of neutrophils in the efficacy of photodynamic therapy *in vivo*. Cancer Res 1996;56:2908-11.

Degli-Espostil MA, Smyth MJ. Close encounters of different kinds: dendritic cells and NK cells take centre stage. Nat Rev Immunol 2005;5(2):112-24.

Del Hoyo GM, Martin P, Vargas HH, Ruiz S, Arias CF, Ardavin C. Charaterization of a common precursor population for dendritic cells. Nature 2002;415:1043-7.

D'Hallewin MA, Kamuhabwa AR, Roskams T, de Witte PA, Baert L. Hypericin-based fluorescence diagnosis of bladder carcinoma. BJU Int 2002;89:760-3.

Dolmans DE, Fukumura D, Jain RK. Photodynamic therapy for cancer. Nat Rev Cancer 2003;338:607-13.

Dougherty TJ. An update on photodynamic therapy applications. J Clin Laser Med Surg 2002;20:3-7.

Dougherty TJ, Gomer CJ, Henderson BW, Jori G, Kessel D, Korbelik M. Moan J, Peng Q. Photodynamic therapy. J Natl Cancer Inst 1998;90:889-5.

Drake CG, Jaffee E, Pardoll DM. Mechanisms of immune evasion by tumors. Adv Immunol. 2006;90:51–81.

Du HY. Hypericin-mediated photodynamic therapy in nasopharyngeal cancer. PhD thesis 2004, Department of Anatomy, Faculty of Medicine, National University of Singapore.

Du HY, Bay BH, Olivo M. Biodistribution and photodynamic therapy with hypericin in a human NPC murine tumor model. Int J Oncol 2003;22:1019-24.

Du HY, Olivo M, Mahendran R, Bay BH. Modulation of matrix metalloproteinase-1 in nasopharyngeal cancer cells by photoactivation of hypericin. Int J Oncol 2004;24:657-62.

Engleman EG. Dendritic cell-based cancer immunotherapy. Semin Oncol 2003;30:23-9.

Esche C, Subbotin VM, Maliszewski C, Lotze MT, Shurin MR. Flt-3 ligand administration inhibits tumor growth in murine melanoma and lymphoma. Cancer Res 1998;58:380-3.

Euvrard S, Kaniakis J, Claudy A. Medical progress: skin cancers after organ transplantation. N Engl J Med 2003;348:1681-91.

Favilla I, Barry WR, Gosbell A, Ellims P, Burgess F. Photo therapy of posterior uveal melanoma. Br J Ophthalmol 1991;75:719-21.

Fay JW, Palucka AK, Paczesny S, Dhodapkar M, Johnston DA, Burkeholder S, Ueno H, Banchereau J. Long-term outcomes in patients with metastatic melanoma vaccinated with melanoma peptide-pulsed $CD34^{+}$ progenitor-derived dendritic cells. Cancer Immunol Immunother 2006;55:1209-18.

Feng H, Zeng Y, Whitsell L, Katsanis E. Stressed apoptotic tumor cells express heat shock proteins and elicit tumor-specific immunity. Blood 2001;97:3505-12.

Feyh J. Photodynamic treatment for cancers of the head and neck. J Photochem Photobiol B: Biol 1996;36:175-7.

Fiers W, Beyaert R, Declercq W, Vandenabeele P. More than one way to die: apoptosis, necrosis and reactive oxygen damage. Oncogene 1999;18:7719-30.

Fingar VH. Vascular effects of photodynamic therapy. J Clin Lased Med Surg 1996;14:323-8.

Fingar VH, Wieman TJ, Karavolos PS, Doak KW, Quellet R, van Lier JE. The effects of photodynamic therapy usiing different substituted zinc phthalocyanines on vessel constriction, vessel leakage and tumor response. Photochem Photobiol 1993;58:251-8.

Fisher AM, Murphee AL, Gomer CJ. Clinical and preclinical photodynamic therapy. Lasers Surg Med 1995;17:2-31.

Flohe SB, Bruggemann J, Lendemans S, Nikulina M, Meierhoff G, Flohé S, Kolb H. Heat shock protein 60 induces maturation of dendritic cells versus Th1-promoting phenotype. J Immunol 2003;162:3212-9.

Fong CL, Hui KM. Generation of potent and specific cellular immune responses via in vivo stimulation of dendritic cells by pNGVL3-hFlex plasmid DNA and immunogenic peptides. Gene Therapy 2002;9:1127-38.

Fong L, Brockstedt D, Benike C, Wu L, Engleman EG. Dendritic cells injected via different routes induce immunity in cancer patients. J Immunol 2001;166:4254-9.

Frank J, Lornejad-Schafer MR, Schoffl H, Flaccus A, Lambert C, Biesalski HK. Inhibition of heme oxygenase-1 increases responsiveness of melanoma cells to ALA-based photodynamic therapy. Int J Oncol 2007;31:1539-45.

Gabrilovich D. Mechanisms and functional significance of tumour-induced dendritic cell defects. Nat Rev Immunol 2004;4:941-52.

Gajewski TF. Failure at the effector phase: immune barriers at the level of the melanoma tumor microenvironment. Clin Cancer Res 2007;13:5256-61.

Gilboa E. DC-based cancer vaccines. J Clin Invest 2007;117:1195-203.

Gilissen MJ, van de Merbel-de Wit LE, Star WM, Koster JF, Sluiter W. Effect of photodynamic therapy on the endothelium-dependant relaxation of isolated rat aortas. Cancer Res 1993;53:2548-52.

Girotti AW. Photosensitized oxidation of membrane lipids: reaction pathways, cytotoxic effects, and cytoprotective mechanisms. Photochem Photobiol B 2001;63:103-13.

Gattinoni L, Powell DJ Jr, Rosenberg SA, Restifo NP. Adoptive immunotherapy for cancer: building on success. Nat Rev Immunol 2006;6:383-93.

Golab J, Wilczyński G, Zagozdzon R, Stokłosa T, Dabrowska A, Rybczyńska J, Wasik M, Machaj E, Ołda T, Kozar K, Kamiński R, Giermasz A, Czajka A, Lasek W, Feleszko W, Jakóbisiak M. Potentiation of the anti-tumor effects of Photofrin-based photodynamic therapy by localized treatment with G-SCF. Br J Cancer 2000;82:1484-91.

Gollnick SO, Evans SS, Baumann H, Owczarczak B, Maier P, Wang WC, Ungar E, Henderson BW. Role of cytokines in photodynamic therapy-induced local and systemic inflammation. Br J Cancer 2003;88:1772-9.

Gollnick SO, Vaughan L, Henderson BW. Generation of effective antitumor vaccines using photodynamic therapy. Cancer Res 2002;62:1604-8.

Gomer CJ, Ferrairo A, Murphree AL. The effect of localized porphyrin photodynamic therapy on the induction of tumor metastasis. Br J Cancer 1987;56:27-32.

Gomer CJ, Ryter SW, Ferrario A, Rucker N, Wong S, Fisher AM. Photodynamic therapy-mediated oxidative stress can induce expression of heat shock proteins. Cancer Res 1996;56:2355-60.

Haddad R, Blumen feld A, Siegal A, Kaplan O, Cohen M, Skornick Y, Kashtan H. In vitro and in vivo effects of photodynamic therapy on murine malignant melanoma Ann Surg Oncol 1997;5:241-247.

Hendrickx N, Volanti C, Mens U, Seternes OM, de Witte P, Vandenheede JR, Piette J, Agostinis P. Up-regulation of cylcooygenase-2 and apoptosis resistance by p38 MAPK in hypericin=mediated photodynamic therapy of human cancer cells. J Biol Chem 2003;278:52231-9.

Hedrzak-Henion JA, Knisely TL, Cincotta L, Cincotta E, Cincotta AH. Role of the immune system in mediating the antitumor effect of benzophenothiazine photodynamic therapy. Photochem Photobiol 1999;69:111-9.

Hesling C, D'Incan M, Mansard S, Franck F, Corbin-Duval A, Chèvenet C, Déchelotte P, Madelmont JC, Veyre A, Souteyrand P, Bignon YJ. In vivo and in situ modulation of the expression of genes involved in metastasis and angiogenesis in a patient treated with topical imiquimod for melanoma skin metastases. Br J Dermatol 2004;150:761-7.

Homey B, Muller A, Zlotnik A. Chemokines: agents for the immunotherapy of cancer? Nat Rev Immunol 2002;2:175-84.

Hoption Cann SA, van Netten JP, van Netten C. Dr William Coley and tumor regression: a place in history or in the future. Postgrad Med J 2003, 79:672-80.

Hopwood LE, Swartz HM, Pajak S. Effect of melanin on radiation response of CHO cells. Int J Radiat Oncol Biol Phys 2004;58:403-9.

Jalili A, Malowski M, Wilczynski GM, Wilczek E, Chorazy-Massalska M, Radzikowska A, Maslinski W, Bialy L, Sienko J, Sieron A, Adamek M, Basak G, Mroz P, Krasnodebski IW, Jakobisiak M, Golab J. Effective photoimmunotherapy of murine colon carcinom induced by the combination of photodynamic therapy and dendritice cells. Clin Cancer Res 2004;10:4498-508.

Kabingu E, Vaughan L, Owczarczak B, Ramsey KD, Gollnick SO. CD8 T cell-mediated control of distant tumors following local photodynamic therapy is independent of CD4 T cells and dependent on natural killer cells. Br J Cancer 2007;96:1839-48.

Kamuhabwa AR, Agostinis PM, D'Hallewin MA, Baert L, de Witte PA. Cellular photodestruction induced by hypericin in AY-27 rat bladder carcinoma cells. Photochem Photobiol 2001;74:126-32.

Keilholz U, Punt CJ, Gore M, Kruit W, Patel P, Lienard D, Thomas J, Proebstle TM, Schmittel A, Schadendorf D, Velu T, Negrier S, Kleeberg U, Lehman F, Suciu S, Eggermont AM. Dacarbazine, cisplatin, and interferon α-2b with or without interleukin-2 in metastatic melanoma: a randomized Phase III trial (18951) of the European Organisation for Research and Treatment of Cancer Melanoma Group. J Clin Oncol 2005;23:6747-55.

Kolarova H, Hevrelova P, Bajgar R, Jirova D, Kejlova K, Strnad M. In vitro photodynamic therapy melanoma cell lines with phthalocyanine. Toxicol In Vitro 2007;21:249-53.

Korbelik M, Cecic I. Mechanism of tumor destruction by photodynamic therapy. In: Nalwa HS (ed) Handbook of photochemistry and photobiology, vol 4. American scientific publishers, Stevenson Ranch, pp39-77.

Korbelik M, Dougherty GJ. Photodynamic therapy-mediated immune response against subcutaneous tumors. Cancer Res 1999;59:1941-6.

Korbelik M, Krosl G, Krosl J, Dougherty GJ. The role of host lymphoid populations against in the response of mouse EMT6 tumor to photodynamic therapy. Cancer Res 1996;5647-52.

Korbelik M, Narapaju VR, Yamamoto N. Macrophage-directed immunotherapy as adjuvant to photodynamic therapy of cancer. Br J Cancer 1997;75:202-7.

Korbelik M, Sun J. Cancer treatment by photodynamic therapy combined with adoptive immunotherapy using genetically altered natural killer cell line. Int J Cancer 2001;93:269-74.

Korbelik M, Sun J. Photodynamic therapy-generated vaccine for cancer therapy. Cancer Immunol Immunother 2006;55:900-9.

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;65:1018-26.

Korbelik M, Sun J, Cecic I, Serrano K. Adjuvant treatment for complement activation increases the effectiveness of photodynamic therapy of solid tumors. Photochem Photobiol Sci 2004;3:812-6.

Korbelik M, Sun J, Posakony JJ. Interaction between photodynamic therapy and BCG immunotherapy responsible for the reduced recurrence of treated mouse tumors. Photochem Photobiol 2001;73:403-9.

Kousis PC, Henderson BW, Maier PG, Gollnick SO. Photodynamic therapy enhancement of antitumor immunity is regulated by neutrophils. Cancer Res 2007;67:10501-10.

Krosl G, Korbelik M. Potentiation of photodynamic therapy by immunotherapy: the effect of schizophyllan (SPG). Cancer Lett 1994;84:43-9.

Krosl G, Korbelik M, Krosl J, Dougherty GJ. Potentiation of photodynamic therapy-elicited antitumor response by localized treatment with granulocyte-macrophage colony-stimulating factor. Cancer Res 1996;56:3281-6.

Kyriakis JM. Making the connection coupling of stress-activated ERK/MAPK (extracellular-signal-ragulated kinase/mitogen-activated protein kinase) core signalling modules to extracellular stimuli and biological responses. Biochem Soc Symp 1999;64:29-48.

Lavie G, Kaplinsky C, Toren A, Aizman I, Meruelo D, Mazur Y, Mandel M. A photodynamic pathway to apoptosis and necrosis induced by dimethyl tetrahydroxyhelianthrone and hypericin in leukaemic cells: possible relevance to photodynamic therapy. Br J Cancer 1999;79:423-32.

Lavie G, Mazur Y, Lavie D, Meruelo D. The chemical and biological properties of hypericin-a compond with broad spectrum of biological activities. Med Res Rev 1995;15:111-9.

Lavie G, Meruelo D, Aroyo K, Mandel M. Inhibition of the CD8+ T-cell mediated cytotoxic reaction by hypericin: potential for treatment of T-cell mediated diseases. Int Immunol 2000;12:479-86.

Leunig M, Leunig A, Lankes P, Goetz AE. Evaluation of photodynamic therapy-induced heating of hamster melanoma and its effect on local tumour eradication. Int J Hyperthermia 1994;10:297-306.

Lim DC, Ko SH, Lee WY. Silkworm-pheophorbide *a* mediated photodynamic therapy against B16F10 pigmented melanoma. J Photochem Photobiol B 2004;74:1-6.

Lizée G, Cantu MA, Hwu P. Less yin, more yang: confronting the barriers to cancer immunotherapy. Clin Cancer Res 2007;13:5250-5.

Liu F, Song YK, Liu D. Hydrodynamics-based transfection in animals by systemic administration of plasmid DNA. Gene Therapy 1999;6:1258-66.

Liu YJ. Dendritic cells subsets and lineages, and their functions in innate and adaptive immunity. Cell 2001;106:259-62.

Lutz MB, Schuler G. Immature, semi=mature and fully mature denritic cells: which signals reduce tolerance or immunity? Trends Immunol 2002;23:445-9.

Lyman SD, James L, Johnson L, Brasel K, de Vries P, Escobar SS, Downey H, Splett RR, Beckmann MP, McKenna HJ. Cloning of the human homologue of the murine flt-3 ligant: a growth factor for early hematopoietic progenitor cells. Blood 1994;83:2795-801.

Lynch DH Andreasen A, Maraskovsky E, Whitmore J, Miller RE, Schuh JC. Flt-3 ligand induces tumor regression and anti-tumor immune responses *in vivo*. Nat Med 1997;3:625-31.

Magner WJ, Tomasi TB. Apoptotic and necrotic cells induced by different agents vary in their expression of MHC and costimulatory genes. Mol Immunol 2005;42:1033-42.

Maraskovsky E Brasel K, Teepe M, Roux ER, Lyman SD, Shortman K, McKenna HJ. Dramatic increases in the numbers of functionally mature dendritic cells in Flt-3 ligand-

treated mice: mulitple dendritic cell subpopulations identified. J Exp Med 1996;184:1953-62.

Markovic SN, Erickson LA, Rao RD, Weenig RH, Pockaj BA, Bardia A, Vachon CM, Schild SE, McWilliams RR, Hand JL, Laman SD, Kottschade LA, Maples WJ, Pittelkow MR, Pulido JS, Cameron JD, Creagan ET. Malignant melanoma in the 21st century, part 1: epidemiology, risk factors, screening, prevention, and diagnosis. Mayo Clin Proc 2007;82:364-80.

Markovic SN, Erickson LA, Rao RD, Weenig RH, Pockaj BA, Bardia A, Vachon CM, Schild SE, McWilliams RR, Hand JL, Laman SD, Kottschade LA, Maples WJ, Pittelkow MR, Pulido JS, Cameron JD, Creagan ET. Malignant melanoma in the 21st century, part 2: staging, prognosis, and treatment. Mayo Clin Proc 2007;82:490-513.

Marcus SL. Clinical applications: a review, in future directions and applications of photodynamic therapy, in: CJ Gomer (Ed). Photodynamic therapy, SPIE, Bellingham, WA, 1990. pp 1-23.

Matter M, Pavelic V, Pinschewer DD, Mumprecht S, Eschli B, Giroglou T, von Laer D, Ochsenbein AF. Decreased tumor surveillance after adoptive T-cell therapy. Cancer Res 2007;67:7467-76.

Meruelo D, Lavie G, Lavie D. Therapeutic agents with dramatic antiretroviral activity and little toxicity at effective doses: aromatic polycyclic diones hypericin and pseudohypericin. Proc Natl Acad Sci USA 1988;85:5230-4.

Melcher A, Gough M, Todryk S, Ville R. Apoptosis or necrosis for tumor immunotherapy: what's in a name? J Mol Med 1999;77:824-33.

Miccoli L, Beurdeley-Thomas A, de Pinieux G, Sureau F, Oudard S, Dutrillaux B, Poupon MF. Light-induced photoactivation of hypericin affects the energy metabolism of human glioma cells by inhibiting hexokinase bound to mitochondria. Cancer Res 1998;58:5777-86.

Miller GG. Brown K, Moore RB, Diwu ZJ, Liu J, Huang L, Lown JW, Begg DA, Chlumecky V, Tulip J, McPhee MS. Uptake kinetics and intracellular localization of hypocrellin photosensitizers for photodynamic therapy: a confocal microscopy study. Photochem Photobiol 1995;61:632-8.

Moan J, Peng Q. An outline of the hundred-year history of PDT. Anticancer Res 2003;23:3591-600.

Moor AC. Signalling-pathways in cell death and survival after photodynamic therapy. J Photochem Photobiol B 2000;57:1-13.

Naylor MF, Chen WR, Teague TK, Perry LA, Nordquist RE. In situ photoimmunotherapy: a tumour-directed treatment for melanoma. Brit J Dermatol 2006;155:1287-92.

Nelson JS, McCullough JL, Berns MW. Photodynamic therapy of human malignant melanoma xenografts in athymic nude mice. J Natl Cancer Inst 1998;80:56-60.

Nestle FO, Banchereau J, Hart D. Dendritic cells: on the move from bench to bedside. Nat Med 2001;7:761-5.

Ng CS, Lee TW, Wan S, Wan IY, Sihoe AD, Arifi AA, Yim AP. Thoracotomy is associated with significantly more profound suppression in lymphocytes and natural killer cells than video-assisted thoracic surgery following major lung resections for cancer. J Invest Surg 2005;18:81-8.

Novellino L, Castellin C, Parmiani G. A listing of human tumor antigens recognized byb T cells: March 2004 update. Cancer Immunol Immunother 2005;4:187-207.

Nowak-Sliwinska, Karocki A, Elas M, Pawlak A, Stocjel G, Urbanska K. Verteporfin, photofrin II and merocyanine 540 as PDT photosensitizers against melanoma cells. J Biochem Biophy Res Comm 2006;349:549-5.

O'Neill DW, Bhardwaj N. Exploiting dendritic cells for active immunotherapy of cancer and chronic infections. Mol Biotechnol 2007;36:131-41.

Pagilla 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;183:283-7.

Pajak S, Subczynski W, Panz T, Lukiewicz S. Rate of oxygen consumption of hamster melanoma cells as a factor influencing their radioresistance. Folia Histochem Cytochem 1980;18:33-9.

Pass HI. Photodynamic therapy in oncology: mechanisms and clinical use. J Natl Cancer Inst 1993;85:443-56.

Peeva M, Shopova M, Stoichkova N, Michailov N, Wohrle D, Muler S. Comparative photodynamic therapy of B16 pigmented melanoma with different generations of senstizers. J Porphyr Phthalocya 1999;3:380-7.

Penning LC, Dubbelman TM. Fundamentals of photodynamic therapy: cellular and biochemical aspects. Anticancer Drugs 1994;5:139-46.

Pilla L, Valenti R, Marrari A, Patuzzo R, Santinami M, Parmiani G, Rivoltini. Vaccination: role in metastatic melanoma. Expert Rev Anticancer Ther 2006;6:1305-18.

Polla LL, Margalis RJ, Dover JS Melanosomes are a primary target of Q-switched ruby laser irradiation in guinea pig skin. J Invest Dermatol 1982;89:281-6.

Proudfoot O, Pouniotis D, Sheng KC, Loveland BE, Pietersz GA. Dendritic cell vaccination. Expert Rev Vaccines 2007;6:617-33.

QIAGEN® plasmid purification handbook, 2nd edition, August 2003.

Riddel SR. Engineering antitumor immunity by T-cell adoptive immunotherapy. Hematology Am Soc Hematol Educ Program. 2007;:250-6.

Ridolfi R, Chiarion-Sileni V, Guida M, Romanini A, Labianca R, Freschi A, Lo Re G, Nortilli R, Brugnara S, Vitali P, Nanni O; Italian Melanoma Intergroup. Cisplatin, dacarbazine with or without subcutanoeus interleukin-2, and interferon α-2b in advanced melanoma outpatients: results from an Italian multicenter Phase III randomized clinical trial. J Clin Oncol 2002;20:1600-7.

Rosenberg SA, Yang JC, Restifo NP. Cancer immunotherapy: moving beyond current vaccines. Nat Med 2004;10:909-15.

Rovere P, Vallinoto C, Bondanza A, Crosti MC, Rescigno M, Ricciardi-Castagnoli P, Rugarli C, Manfredi AA. Bystander apoptosis triggers dendritic cell maturation and antigen-presenting function. J Immunol 1998;161:4467-71.

Ryter SW, Tyrell RM. Singlet molecular oxygen ($^1O_2$): a possible effector of eukaryortic gene expression. Free Radic Biol Med 1998;24:1520-34.

Saji H, Song W, Furumoto K, Kato H, Engleman EG. Systemic antitumor effect of intratumoral injection of dendritic cells in combination with local photodynamic therapy. Clin Cancer Res 2006;12:2568-74.

Saunders D, Lucas K, Ismaili J, Wu L, Maraskovsky E, Dunn A, Shortman K. Dendritic cell development in culture from thymic precursor cells in the absence of granulocyte/macrophage colony-stimulating factor. J Exp Med 1996;184:2185-96.

Sauter B, Albert ML, Francisco L, Larsson M, Somersan S, Bhardwaj N. 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;191:423-34.

Schadendorf D, Ugurel S, Schuler-Thurner B Nestle FO, Enk A, Bröcker EB, Grabbe S, Rittgen W, Edler L, Sucker A, Zimpfer-Rechner C, Berger T, Kamarashev J, Burg G, Jonuleit H, Tüttenberg A, Becker JC, Keikavoussi P, Kämpgen E, Schuler G; DC study

group of the DeCOG. Dacarbazine (DTIC) versus vaccination with autologous peptide-pulsed dendritic cells (DC) in first-line treatment of patients with metastatic melanoma: a randomized Phase III trial of the DC study group of the DeCOG. Ann Oncol 2006;17:563-70.

Schempp CM, Simon-Haarhaus B, Heine A, Schopf E, Simon JC. In vitro and in vivo activation of hypericin with incoherent light source PDT 1200 SOA (520-750 nm) and with solar simulated radiation (290-1500 nm). Photodermatol Photoimmunol Photomed 1999;15:13-7.

Scheffer SR Nave H, Korangy F, Schlote K, Pabst R, Jaffee EM, Manns MP, Greten TF. Apoptotic, but not necrotic, tumor cell vaccines induce a potent immune response in vivo. Int J Cancer 2003;103:205-11.

Schoenfeld N, Mamet R, Nordenberg Y, Shafran M, Babushkin T, Malik Z. Protophorphyrin biosynthesis in melanoma B16 cells stimulated by 5-aminolevulinic acid and chemical inducers: characterization of photodynamic activation. Int J Cancer 1994;56:106-12.

Schuler-Thurner B, Schultz ES, Berger TG, Weinlich G, Ebner S, Woerl P, Bender A, Feuerstein B, Fritsch PO, Romani N. Rapid induction of tumor-specific type 1 T helper cells in metastatic melanoma patients by vaccination with mature, cryopreserved, peptide-loaded monocyte-derived dendritic cells. J Exp Med 2002;195:1279-88.

Seya T Akazawa T, Uehori J, Matsumoto M, Azuma I, Toyoshima K. Role of toll-like receptors and their adaptors in adjuvant immunotherapy for cancer. Anticancer Res 2003;23:4369-76.

Shaif-Muthana M, McIntyre C, Sisley K, Rennie I, Murray A. Dead or alive: immunogenicity of human melanoma cells when presented by dendritc cells. Cancer Res 2000;60:6441-7.

Sheleg SV, Zharvid EA, Khodina TV, Kochubeev GA, Istomin YP, Chalov VN, Zhuravkin IN. Photodynamic therapy with chlorin e_6 for skin metastases of melanoma. Photodermatol Photoimmunol Photomed 2004;20: 21-6.

Shikowitz MJ Abramson AL, Steinberg BM, DeVoti J, Bonagura VR, Mullooly V, Nouri M, Ronn AM, Inglis A, McClay J, Freeman K. Clinical trial of photodynamic therapy with meso-tetra (hydroxyphenyl) chlorine for respiratory papillomatosis. Arch Otolaryngol Head Neck Surg 2005;131:99-105.

Shortman K, Liu Y. Mouse and human dendritic cell subtypes. Nat Rev Immunol 2002;2:151-61.

Sierra-Rivera E, Voorhess GJ, Freeman ML. γ irradiation increases HSP-70 in Chinese hamster ovary cells. Radiat Res 1993;135:40-5.

Slingluff Jr CL, Chianese-Bullock KA, Bullock TN, Grosh WW, Mullins DW, Nichols L, Olson W, Petroni G, Smolkin M, Engelhard VH. Immunity to melanoma antigens: from self-tolerance to immunotherapy. Adv Immunol 2006;90:243-95.

Smyth MJ, Dunn GP, Schreiber RD. Cancer immunosurveillance and immunoediting: the roles of immunity in suppressing tumor development and shaping tumor immunogenicity. Advances in Immunology 2006;90:1-50.

Song W, Levy R. Therapeutic vaccination against murine lymphoma by intratumoral injection of naïve dendritic cells. Cancer Res 2005;65:5958-64.

Southwell IA, Bourke CA. Seasonal variation in hypericin content of Hypericum perforatum L. (St. John's Wort). Phytochemistry 2001;56:437-441.

Stauss HJ, Cesco-Gaspere M, Thomas S, Hart DP, Xue SA, Holler A, Wright G, Perro M, Little AM, Pospori C, King J, Morris EC. Monoclonal T-cell receptors: new reagents for cancer therapy. Mol Ther. 2007 15:1744-50.

Steinmann A, Funk JO, Schuler G, von den Driesch P. Topical imiquinod treatment of cutaneous melanoma metastasis. J Am Acad Dermatol 2000;43:555-6.

Steinman RM, Pope M. Exploiting dendritice cells to improve vaccine efficacy. J Clin Invest 2002;109:1519-26.

Steinman RM, Pack M, Inaba K. Dendritic cells in the T-cell areas of lymphoid organs. Immunol rev 1997;156:25-37.

Tacken PJ, de Vries JM, Torensma R, Figdor CG. Dendritic-cell immunotherapy: from *ex vivo* loading to *in vivo* targeting. Nat Rev Immunol 2007;7:790:802.

Takeda K, Kaisho T, Akira S. Toll-like receptors. Annu Rev Immunol 2003;21:335-76.

Teitz-Tennenbaum S, Li Q, Rynkiewicz S, et al. Radiotherapy potentiates the therapeutic efficacy of intratumoral dendritic cell administration. Cancer Res 2003;63:8466-75.

Terando AM, Faries MB, Morton DL. Vaccine therapy for melanoma: current status and future directions. Vaccine 2007;25S:B4-B16.

Tuettenberg A, Becker C, Huter E, Knop J, Enk AH, Jonuleit H. Induction of strong and persistent MelanA/MART-1-specific immune responses by adjuvant dendritic cell-based vaccination of stage II melanoma patients. Int J Cancer 2006;118:2617-27.

Thong PS, Ong KW, Goh NS, Kho KW, Manivasager V, Bhuvaneswari R, Olivo M, Soo KC. Photodynamic-therapy-activated immune response against distant untreated tumours in recurrent angiosarcoma. Lancet Oncol 2007;8:950-2.

Thong PS, Watt F, Ren MQ, Tan PH, Soo KC, Olivo M. Hypericin-photodynamic therapy (PDT) using an alternative regime suitable for multi-fraction PDT. J Photochem Photobiol B 2006;82:1-8.

Wang HP, Hanlon JG, Rainbow AJ, Espiritu M, Singh G. Upregulating Hsp27 plays a role in the resistance of human colon carcinoma HT29 cells to photooxidative stress. Photochem Photobiol 2002;76:98-104.

Wierecky J, Muller MR, Wirths S, Halder-Oehler E, Dorfel D, Schmidt SM, Hantschesl M, Brugger W, Schroder S, Horger, SM, Hantschel M, Brugger W, Schroder S, Horger MS. Immunologic and clinical responses after vaccinations with peptide-pulsed dendritic cells in metastatic renal cancer patients. Cancer Res 2006;66:5910-8.

Van der Bruggen P, Traversari C, Chomez P Lurquin C, De Plaen E, Van den Eynde BJ, Knuth A, Boon T. A gene encoding an antigen recognized by cytolytic T lymphocyts on a human melanoma. Science 1991;254:1643-7.

Vantieghem A, Xu Y, Declercq W, Vandenabeele P, Denecker G, Vandenheede JR, Merlevede W, de Witte PA, Agostinis P. Different pathways mediate cytochrome c

release after photodynamic therapy with hypericin. Photochem Photobiol 2001;74:133-42.

Vereecken P, Mathieu A, Laporte M, Petein M, Velu T, Awada A, Heenen M. Management of cutaneous locoregional recurrences of melanoma: a new therapeutic perspective with imiquimod. Dermatology 2003;206:279-80.

Verma S, Quirt I, McCready D, Bak K, Charette M, Iscoe N. Systemic review of systemic adjucant therapy for patients at high risk for recurrent melanoma. Cancer 2006;106:1431-42.

Ward RC, Kaufman HL. Targeting costimulatory pathways for tumor immunotherapy. Int Rev Immunol. 2007 26:161-96.

Wu L, Li CL, Shortman K. Thymic dendritic cell precursors: relationship to the T lymphocyte lineage and phenotype of the dendritic cell progeny. J Exp Med 1996;184:903-11.

Wyld L, Reed MW, Brown NJ. Differential cell death response to photodynamic therapy is dependent on dose and cell type. Br J Cancer 2001;84:1384-6.

Wu X, He Y, Falo LD Jr, Hui KM, Huang L. Regression of human mammary adenocarcinoma by systemic administration of recombinant gene encoding the fFlex-trail fusion protein. Mol Ther 2001;3:368-74.

Yu B, Kusmartsev S, Cheng F, Paolini M, Nefedova Y, Sotomayor E, Gabrilovich D. Effective combination of chemotherapy and dendritic cell administration for the treatment of advanced-stage experimental breast cancer. Clin Cancer Res 2003;9:285-94.

Zeh HJ 3rd, Perry-Lalley D, Dudley ME, Rosenberg SA, Yang JC. High avidity CTLs for two self-antigens demonstrate superior in vitro and in vivo antitumor efficacy. J Immunol 1999;162:989-94.

Zitvogel L Casares N, Péquignot MO, Chaput N, Albert ML, Kroemer G. Immune response against dying tumor cells. Adv Immunol 2004;84:131-79.