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Tumor abolition and antitumor immunostimulation by physico-chemical tumor ablation

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1. ABSTRACT

Tumor ablation by thermal, chemical and radiological sources has received substantial attention for the treatment of many localized malignancies. The primary goal of most ablation procedures is to eradicate all viable malignant cells within a designated target volume through the application of energy or chemicals. Methods such as radiotherapy, chemical and biological ablation, photodynamic therapy, cryoablation, high-temperature ablation (radiofrequency, microwave, laser, and ultrasound), and electric-based ablation have been developed for focal malignancies. In recent years a large volume of data emerged on the effect of *in situ* tumor destruction (ablation) on inflammatory and immune components resulting in systemic anti-tumor reactions. It is evident that *in situ* tumor ablation can involve tumor antigen release, cross presentation and the release of DAMPS and make the tumor its own cellular vaccine. Tumor tissue destruction by *in situ* ablation may stimulate antigen-specific cellular immunity engendered by an inflammatory milieu. Dendritic cells (DCs) attracted to this microenvironment, will undergo maturation after internalizing cellular debris containing tumor antigens and will be exposed to damage associated molecular pattern (DAMP). Mature DCs can mediate antigen-specific cellular immunity via presentation of processed antigens to T cells. The immunomodulatory properties, exhibited by *in situ* ablation could portend a future collaboration with immunotherapeutic measures. In this review are summarized and discuss the preclinical and clinical studies pertinent to the phenomena of stimulation of specific anti-tumor immunity by various ablation modalities and the immunology related measures used to boost this response.

2. INTRODUCTION

2.1. Immunotherapy of cancer

Globally, cancer claimed an estimated 7.6. million lives in 2008 and is on pace to double that number by 2030 (1). The role of the immune response in tumor development and treatment was discussed in a large volume of literature culminating in the understanding that immune surveillance and cancer immunoediting are part of the biology of tumors (2). The importance of the immune response in tumor progression and determent was re-emphasized by recent studies, which show that the immunological profile of the patient is a strong predicator for prognosis (3), which opened a global effort to analyze the “immunome” of the cancer patient and its correlation with the development of the disease.

Immunotherapy of cancer was a major target since 1891 when William Coley started an experimental treatment of cancer patients with bacterial derived products, actually introducing the first danger signal treatment, claiming that the beneficial effect is a result of activation of anti-tumor immunity (4). This effort is ongoing and seventeen immunotherapy products have received FDA approval in the past quarter century (5).

Several monoclonal antibodies targeting cancer-associated proteins (Her2/neu, EGFR, VEGF, CD20, CD52, CD22, CD30, CD38 and CD33) are approved for the treatment of solid and haematological malignancies (6). Other immune treatments that have received the FDA approval include recombinant cytokines, the most studied of these, IL-2, has demonstrated clinical responses in metastatic melanoma and metastatic renal cell carcinoma (7). Interferon-a is another agent that gained approval for ‘immunological cancers’ (that is, melanoma or renal cell cancer). Adoptive cell transfer (ACT) is another immunotherapeutic measure currently used to treat mainly melanoma patients. Immune cells from the patient are propagated *in vitro* and transferred back with the goal of transferring improved immune functionality and characteristics along with the cells back to the patient. Transferring autologous cells, or cells from the patient, minimizes graft-versus-host disease (GVHD) also described as tissue or organ rejection (8). Promising recent strategies include the use of lymphodepletion before T-cell infusion, and the engineering of new T-cell specificities with CARs (9, 10).

In recent years a major hype was formed around the ‘checkpoint blockade’ — referring to the use of antibodies that block immune-inhibitory pathways switched on by cancer cells. CTLA-4 and PD-1 are two key cell-surface receptors that, when bound by their ligands trigger such inhibitory pathways and inhibit T-cell activity. Antibodies that block CTLA-4 (ipilimumab), PD-1 (pembrolizumab and nivolumab) and its ligand PDL-1 have been approved to treat patients, and the clinical responses are often durable, with some patients remaining free from disease progression for many years. Until recently, the efficacy of these treatments has been noted mainly for melanoma and renal-cell carcinoma (11-13).

A major long time effort was devoted to developing anti cancer vaccines. Currently there are several vaccine types in clinical studies these include tumor cells, peptides and proteins, dendritic cell-Ag combinations, and recombinant vectors (For review see (14, 15)).

2.2. The tumor as its own vaccine

The difficulties to develop efficient cancer vaccines, due to problems with the identification of tumor-specific antigens, stimulated different efforts aiming to prime an anti-tumor immune response. The observation that irradiation of a tumor site can cause the decrease in size of distant tumor tissue (the abscopal effect) (16) raised the notion that destruction of the primary tumor can stimulate anti tumor immunity which will eradicate

residual tumor cells. It is becoming increasingly apparent that many standard cancer treatments may enhance the effectiveness of anti tumor immune reactions, possibly due to increased inflammation, release of antigen and danger signals, immunogenic cell death pathways and dampening the effects of regulatory cells.

This idea prompted a large volume of studies on the anti tumor immune response after *in situ* destruction (ablation) of solid tumors in preclinical and clinical settings. Tumor ablation is defined as the direct application of chemical, thermal or electrical energy to a specific focal tumor in an attempt to achieve eradication or cytoreduction inducing cellular necrosis. Image guided tumor ablation is widely used in the treatment of various solid tumors (17, 18). Apparently, any treatment modality, which destroys solid tumors *in situ* can be considered as an ablative treatment. The major types are radiation, thermal and electric based treatments, chemical and biological cytotoxic agents and photodynamic therapy.

A large volume of data is available stressing that destruction of the tumor by various therapeutic and ablative modalities can release tumor-associated antigens in the context of danger-associated molecular patterns (DAMPs) to the immune system resulting in the elimination of residual malignant cells in primary tumors and distant metastases. It was argued that this process could make the tumor its own cellular vaccine, and that it is differentially modulated by different treatments. Reports on this topic were summarized in a book (19) and two recent reviews (20, 21), stressing the point that any tumor ablation tried so far can stimulate anti-tumor immunity.

2.3. How to reinforce the anti-tumor immune reaction endorsed by ablation

The immune response triggered after ablation, which was mostly very weak, called for enforcement by immunomodulating agents. Indeed, many investigators are delving immunotherapy combinations with ablation modalities as promising strategies to improve cancer treatment (22-25).

Tumours escape immune attack by a variety of complementary mechanisms of immunosuppression loss of antigens, loss of MHC molecules, many of which operate in parallel. The presence of suppressive factors such as Treg cells or Myeloid-derived suppressor cells (MDSC) in the tumor microenvironment, and upregulation of surface ligands, which mediate T-cell anergy (or exhaustion) may explain the limited activity observed with previous immune-based therapies. This topic was dealt with in two reviews (26, 27).

The current review will bring evidence for ablation-mediated immune activation in preclinical models (section 3) and in patients (section 5). The published approaches for immunostimulation used in combination with various ablation modalities including the use of immunopotentiating agents such as adjuvants, dendritic cells, cytokines and growth factors, tumor vaccines and adoptive cell transfer will be outlined (sections 4 and 5). Measures to counteract suppressive mechanisms in combination with ablative procedures will be also discussed (sections 4.2. and 5). The relative potency of different ablation treatments to reinforce anti-tumor immunity will be also addressed (section 6).

3. STIMULATION OF ANTI-TUMOR IMMUNITY BY DIFFERENT ABLATION TREATMENTS

3.1. Radiation

Radiation oncology, which started in the discovery of X-rays in 1895 developed into a major tool in the treatment of cancer (28). Along with surgery and chemotherapy, radiation therapy is one of the most important methods of cancer treatment, and approximately 50-70% of cancer patients will receive radiation therapy. Radiation therapy involves photons (e.g. X-rays) or particles (e.g. protons, neutrons, alpha particles, heavy ions, and electrons). The most prevalent radiation treatment is the use of gamma or x-rays radiation (External Beam Radiation Therapy -EBRT). It is useful for treatment of local and regional disease sites, or where surgical excision of the tumor is not feasible due to the size and site of tumor, or patients` medical condition. The effectiveness EBRT is limited due to hypoxia in the tumor.

Several modes of EBRT were developed such as stereotactic radiotherapy (SRT), stereotactic body radiation therapy (SBRT), its extension stereotactic ablative radiotherapy (SABR), and lately, intensity modulated radiation therapy (IMRT) (29).

The report that irradiation of a tumor site can cause the decrease in size of distant tumor tissue (the abscopal effect) (16) triggered the concept that radiation and other aggressive *in situ* tumor destruction (ablation) modalities could stimulate anti-tumor immune reactivity, which is responsible for the systemic effects.

A considerable number of reports addressed this issue and experimental data could indicate that the radiation-induced tissue damage triggers production of generic “danger” signals that mobilize the innate and adaptive immune system. Danger microenvironment engenders a DC-mediated antigen-specific immune response (30-32). It was also suggested that the abscopal effect results from loss of growth stimulatory and/or immunosuppressive factors from the tumor (33). An extensive review article, published by leading investigators in this field, gathered information about the impact of RT on tumor immunity, including tumor-associated antigens, antigen-presenting cells,

and effector mechanisms. The review also discussed the experimental evidence supporting the contention that RT can be used as a tool to induce antitumor immunity (34).

Since gamma or X-ray radiation is the prevalent radiotherapeutic modality, most of the data about radiation-mediated immune response activation was obtained using this type of treatment. Yet, more recent studies indicate that heavy particles (neutrons, protons, alpha particles and heavy ions) can also destroy tumors and initiate anti-tumor immunity. Heavy particles defined as high-linear energy transfer (high-LET) radiation and deposit more energy along the path they take through tissue than do x-rays or gamma rays, interact directly with the critical target in the cell, and cause more damage to the cells they hit. The short range of alpha particles in tissue (less than 0.1. millimeter) has so far limited their medical applicability.

We developed a potent tumor ablation brachytherapy based on alpha irradiation. Our approach termed Diffusing Alpha emitters Radiation Therapy (DaRT) is based on the intra-tumoral insertion of radium-224 loaded wires, which release by recoil short-lived alpha-emitting atoms into the tumor. These atoms disperse in the tumor, and spray it with highly destructive alpha radiation particles. DaRT is the only modality currently available, which provides an efficient method for prolonged treatment of the entire volume of solid tumors by alpha radiation (35). DaRT achieved substantial tumor growth retardation, extended survival, reduced lung metastases and even complete cure of animals bearing murine squamous cell carcinoma (SCC), pancreatic, colon, prostate, and breast mouse derived tumors, and human derived tumors (summarized in (36)). Applied as a monotherapy, DaRT boosted the anti-tumor immune responses in both high and low immunogenic experimental tumor models (36). Moreover, DaRT in combination with CpG retarded the growth of DA3 derived tumors more effectively than each treatment alone (37).

Delivering alpha radiation (bismuth-213) using Radioimmunotherapy (RIT) to treat the murine adenocarcinoma MC-38 also induced a protective antitumor response that was mediated by tumor-specific T cells (38). Carbon ion beam (CIB) treatment at a clinically available dose to a poorly immunogenic squamous cell carcinoma cell line (SCCVII) primary tumors resulted not only in efficient elimination of the primary tumor but also in a dramatic reduction of tumor formation after secondary tumor challenge at a contralateral site. The antitumor effects were the result of tumor-specific, long-lasting antitumor immunity through CD8-positive T lymphocytes and was enhanced significantly by combining it with DC immunotherapy (39).

Radiation can affect anti-tumor immunity by either modulating inflammatory and adaptive immune components, or making the tumor cells more vulnerable to immune attack, or releasing tumor antigens and danger signals, or all of the above.

Tissue damage inflicted by radiation can release tumor antigens and danger signals into an inflammatory milieu, which will attract dendritic cells (DCs) among other cells. DCs undergoing maturation after internalizing apoptotic and necrotic cellular debris can promote a tumor specific immune response. Radiation can also promote anti-tumor immunity by increasing MHC class I expression on tumor cells and antigen presenting dendritic cells, along with tumor antigenic peptides and immune co-accessory molecules in tumor, stromal, and vascular endothelial cells. Radiation can induce production of pro-inflammatory and secretory molecules (cytokines, inflammatory mediators) like TNF and interleukin 1, and cell adhesion molecules by both cells and tissues (40-45).

Irradiation may have a direct effect on the immune response and produce potent immune adjuvant effects independent of its ability to induce tumor ablation. A non-myeloablative dose of total body irradiation followed by lymphocyte infusion results in a dramatic increase in responsiveness to tumor DNA vaccines against melanoma, with augmentation of T cell responses to tumor antigens and tumor eradication. Both a relative decrease in regulatory T cells and increase in activated dendritic cells were observed and corresponded with a brief window (24 hours) of augmented responsiveness to immunization. When immunizations were initiated within the period of augmented dendritic cell activation, mice develop anti-tumor responses that show increased durability as well as magnitude, and improved survival (46).

In spite of the positive data showing that RT can act to enhance anti-tumor immunity and aid in tumor cure, there are also examples of detrimental effects. Tumor-associated macrophages in the post-irradiated tumor microenvironment express higher levels of Arg-1, COX-2, and iNOS, and promote early tumor growth *in vivo* (47). The immune system is of course under considerable control by cells other than macrophages and the role for example of regulatory T cells has to be considered, and RT is also able to generate regulatory T cells as a response to damage just as it can generate immunity (48).

The studies of the last fifteen years intensified the understanding about the nature of the interrelationship between radiotherapy the immune response and the tumor microenvironment. Strategies aimed at interfering with the cross-talk between microenvironment tumor cells and their cellular partners were suggested, while taking into account that this new knowledge will probably translate into indication and objective of radiation therapy changes in the next future (49, 50).

3.2. Heat based tumor ablation

All thermal ablation techniques have in common the application of thermal energy to a tissue to produce tissue necrosis and tumor destruction. Thermal ablation, applied either in the whole body or locally, can be divided into the heat-based modalities, which include radiofrequency (RF), microwave (MW), high-intensity focused ultrasound (HIFU), and laser ablation, and the tissue-freezing technique referred to as cryoablation (51).

Delivering very high temperatures to tumors for short periods of time leads to significant temperature heterogeneity and targeted tumors likely contain cells exposed to the fever range of heating (37-41°C), the "hyperthermia range" (42°C-47°C) and the thermal ablation range (above 47°C). Each of these temperatures likely has different implications in terms of mechanisms of killing and interaction with the immune system. At lower temperatures, in the fever range (FRH) direct tumor cell killing is minimal and cell inactivation is due to profound immune stimulation of a wide range of immune cells. Cell death in the "hyperthermia range" (42-47°C) appears to be due to protein denaturation and is strongly enhanced by properties of the tumor microenvironment such as low glucose and reduced extracellular pH. Above 50 °C a different mode of tumor eradication is seen, characterized by cell necrosis and tissue coagulation (52). Thermal ablation of tumors such as hepatoma is carried out at temperatures exceeding 50 °C using radiofrequency (RF), microwaves and high intensity focused ultrasound (HIFU). At 48 °C - 55 °C the mechanisms of cell killing appear to differ from the hyperthermia range and involve much lower activation energies for cell inactivation. Interestingly, 50°C appears to be the threshold temperature needed to trigger tissue coagulation and necrosis in ablation therapy (53).

Over the past decades, thermo-ablative techniques for the therapy of localized tumors have gained importance in the treatment of patients not eligible for surgical resection. Anecdotal reports have described spontaneous distant tumor regression after thermal ablation, indicating a possible involvement of the immune system, hence an induction of antitumor immunity after thermal-induced therapy. In recent years, a growing body of evidence for modulation of both adaptive and innate immunity, as well as for the induction of danger signals through thermoablation, has emerged. Induced immune responses, however, are mostly weak and not sufficient for the complete eradication of established tumors or durable prevention of disease progression, and combination therapies with immunomodulating drugs are being evaluated with promising results (54).

Immune effects of thermal ablation may depend on the mode of cell death that is produced. In broad terms, apoptotic cell death is tolerogenic and absorption of apoptotic cell bodies by immune cells inhibits immunity. Hyperthermia range heating may lead to profound levels of apoptosis and its role in immunity is somewhat ambiguous. However, in the ablation range (above 47°C), cancer cell necrosis dominates and tumor specific immunity is observed, an effect that may play an important role in the outcome of treatment (52).

3.2.1. Hyperthermia range ablation

Local tumour hyperthermia for cancer treatment is currently used either for ablation purposes as an alternative to surgery or less frequently, in combination with chemotherapy and/or radiation therapy to enhance the effects of those traditional therapies. The "Hyperthermia Range" of heating is the one contemplated by exponents of this modality studied largely in the 1960s-1980s and comprises temperatures between 42°C and 47°C.

Data from animal models and human patients indicate that whole body and locoregional hyperthermia exerts many biological and therapeutic effects on immune competent cells and cytokines. Exposure of tumor bearing animals to temperatures in the hyperthermia range (below 50°C) may lead to apoptotic killing, conditions likely to induce immune tolerance to the tumor cells. It could be predicted that hyperthermia in this range might be immunogenic due to release of the abundant levels of HSPs that accumulate in heating. However, the effects of locally applied hyperthermia on tumor immunity are not consistent and both stimulation and inhibition of immunity are observed in this temperature range. The response of tumors to hyperthermia might thus involve competition between the immunogenic effects of Hsp70-peptide complexes and the tolerizing effects of apoptotic cells that occur in heated tumors *in vivo* (52). It should be mentioned that hyperthermia has been demonstrated to enhance the antigen presentation and consequently the activity of dendritic cells (55).

Further preclinical studies showed that locally heating tumours at 39–45° C can elicit anti-tumour immune responses by enabling tumour cells to stimulate the immune system through increased surface expression of MICA or MHC class I and release of HSPs and/or exosomes, by directly activating intra-tumoral immune cells such as NK cells, CD8+ T cells, and DCs, and by improving immune-cell trafficking between the tumour and lymphoid organs. Local tumour hyperthermia at 42–45°C in mice induces tumour-specific resistance against rechallenge in a CD8+ T cell-dependent manner. This efficacy is sensitive to small temperature differences, which means that there is a narrow optimal temperature range at least for a poorly immunogenic tumour. Thorough comparison of different temperatures/thermal doses is needed to fully understand what temperatures are most suitable for immune stimulation (56).

3.2.2. Radiofrequency ablation (RFA)

Radiofrequency ablation (RFA) is a minimally invasive therapy for the local destruction of primary

tumors and unresectable metastases, primarily in the liver. Electrode probes are placed within tumors percutaneously or during open or laparoscopic surgery. RFA delivers high-frequency electromagnetic waves (375–500 kHz) to the target tissue through a needle electrode. This causes local ionic oscillation and frictional heating that induces protein denaturation and leads to coagulation necrosis and irreversible cell death (57).

RFA is used when surgical resection of hepatocellular carcinoma HCC is not possible, or in patients suffering from unresectable liver metastasis from colorectal cancer. Beneficial responses of RFA have also been observed in other metastatic liver tumors, such as neuroendocrine tumors and primary intrahepatic cholangiocellular carcinoma, as well as in other cancers, such as lymphoma, head and neck cancer, prostate cancer, primary and metastatic tumors of the lung, breast cancer, bone metastases, and small renal cell carcinoma.

Tumor-destructing techniques, like radiofrequency ablation (RFA), may provide the immune system with an antigen source for the induction of antitumor immunity. After ablation tumor antigens become instantly available for antigen presenting cells (APCs), and the procedure itself creates an inflammatory environment that may further initiate anti-tumor immunity. The involvement of immunological phenomena after RFA suggests that combining this technique with immunotherapy may be promising to prevent local recurrences and induce long-term systemic protection against residual disease (58).

Early studies showed that RFA destroys tumoral tissue generating a local necrosis followed by marked inflammatory response with a dense T-cell infiltrate. Twenty-four hours after ablation, $CD3^+$ T cells infiltrated the hemorrhagic margin in the periphery of transplanted VX2 carcinomas in the liver of rabbits, and were present in the center of the tumor after 2 weeks. Increased levels of tumor-specific T cells were detected in peripheral (59). den Brok *et al*., (60) showed that antigen-presenting dendritic cells are crucial for the induction of potent immune responses. Adoptive transfer experiments further indicated that antitumor reactivity could be transferred to naïve mice by splenocytes (60). RFA also elevated DCs in the tumor-draining lymph nodes, which expressed higher levels of co-stimulatory molecules than DCs in untreated mice, and the cells that took up tumor antigens were the ones that matured (60). A significant increase in tumor-specific class I and II responses to minor histocompatibility (HY) antigens and tumor regression was observed in animals treated with subtotal RF ablation. RFA in combination with intratumoral dendritic cells (ITDC) resulted in tumor regression. However, combination therapy did not enhance tumor regression when compared with either treatment alone. Rechallenged mice in RF ablation, ITDC, and combination groups demonstrated significant tumor growth inhibition compared with controls (61).

3.2.3. Microwave ablation (MWA)

Microwave ablation is a special case of dielectric heating, where the dielectric material is tissue. Dielectric heating occurs when an alternating electromagnetic (EM) field is applied to an imperfect dielectric material. In tissue, heating occurs because the EM field forces water molecules in the tissue to oscillate. The bound water molecules tend to oscillate out of phase with the applied fields, so some of the EM energy is absorbed and converted to heat. The best EM absorbers contain a high percentage of water (e.g., most solid organs) while less heating occurs in tissues with low water content (e.g. fat). At microwave frequencies (typically 915 MHz or 2.4.5 GHz for ablative technologies), heating is more efficient in materials with a high conductivity. Microwaves are capable of propagating through materials with low or zero conductivity. This means that low-conductivity tissues inhibit RF current flow but allow better microwave propagation. This distinction between RF and microwave heating becomes more important as ablation of tissues outside of the liver becomes more common (62).

3.2.4. Laser induced ablation

Laser induced thermotherapy (LIT) uses optical fibers to deliver high-energy laser radiation to the target lesion. The mechanism of tumor destruction is temperature elevation within the tumor core (by the laser fiber) high enough to induce coagulation necrosis. Laser ablation was also designated as Focal laser ablation, photothermal therapy, laser interstitial therapy, and laser interstitial photocoagulation.

Selected tumors of liver CRC bearing mice and livers of mice without tumor induction were treated with laser ablation (LA). LA of the liver induced accumulation of CD3+ T-cells and Kupffer cells at the site of injury and systemic induction of immune responses as discerned by the presence of IFNγ secreting splenocytes. LA of liver tumors induced significant increase of CD3+ T-cells at site of injury, within normal liver parenchyma, and the tumor-host interface of both ablated and distant tumors. In contrast Kupffer cells only accumulated in ablated tumors and the liver parenchyma but not in distant tumors (63).

3.2.5. High Intensity focused ultrasound (HIFU)

Ultrasound is a form of vibrational energy, which propagates as a mechanical wave by the motion of particles in the medium. The wave propagation leads to compressions and rarefactions of the particles, so that a pressure wave is transmitted along with the mechanical movement of the particles. The ultrasound beam propagates through the body, it loses energy due to ultrasonic attenuation in tissue, and the absorption of energy causes a local temperature rise in tissue if

the rate of heating exceeds the rate of cooling. The ultrasound beams transmitted through living tissue can be focused into a small volume with concentrated high-energy distribution within the human body. This makes it possible to use an external source of focused ultrasound for therapeutic purposes. If ultrasonic energy is sufficiently concentrated in a focal volume, it can cause thermal ablation of a targeted tissue volume by generating temperatures of up to 60 °C causing coagulative necrosis. HIFU also causes acoustic cavitation, which occurs when acoustic pressure causes expansion and contraction of gaseous nuclei in cells, thereby leading to the collapse of the cell and nuclear membranes, the mitochondria and the endoplasmic reticulum. This technique is known as high intensity focused ultrasound (HIFU) or focused ultrasound surgery (FUS). It provides a noninvasive method of selective tissue ablation at depth without any damage to surrounding or overlying tissues (64).

Early studies indicated that HIFU treatment might stimulate host anti-tumor immunity and render mice resistant to the treated tumor (65). Studies in animal models showed that HIFU treatment could induce an enhanced CTLs activity *in vivo*, thus provides protection against subsequent tumor re-challenge (66). In addition, HIFU could upregulate *in vitro* and ex vitro molecule expression of HSP70 (67, 68), which are intracellular molecular chaperones that can enhance tumor cell immunogenicity, resulting in potent cellular immune responses. M-HIFU combined with surgery were found to significantly stimulate anti-tumor immunity against a transplanted prostate tumors, down-regulate intra-tumoral STAT3 activities, increase cytotoxic T cells in spleens and tumor draining lymph nodes (TDLNs), and improve the host survival. (69). HIFU ablation significantly increased the cytotoxicity of cytotoxic T lymphocytes, IFN-γ and TNF-α secretion, and the frequency of the MHC class I tetramer/CD8-positive cells. A stronger inhibition of tumor progression and higher survival rates were observed to be significant after adoptive immunotherapy in the HIFU group as compared to the sham-HIFU and control groups (70). Another way of achieving anti-cancer immune responses is by using ultrasound (US) in combination with microbubbles and nanobubbles to deliver genes and antigens into cells. US leads to bubble destruction and the forces released to direct delivery of the substances into the cytoplasm of the cells thus circumventing the natural barriers (for review see: 71).

Summary of pre-clinical data shows a link between HIFU- treatment and enhanced host antitumor immunity and the possibility that this is also the case in cancer treated patients is supported by clinical data (For review see: 64, 72).

3.2.6. Cryoablation

Cryoablation is increasingly being used as a primary treatment for localized cancers and as a salvage therapy for metastatic cancers. The cellular damage caused by cryoablation is the result of a combination of cellular events during cycles of tissue freezing and thawing. During freezing, decreased cellular metabolism causes tissue damage. Crystallization that occurs in the extracellular tissue and intracellular compartment disrupts organelle membranes and causes dehydration and further compromise in cellular function. During the thawing cycle the crystals coalesce into larger sizes, membranes are disrupted, and vessels occlude resulting in cell death (73).

Observations that distant untreated tumor foci began to regress after freezing a primary tumor lesion, pointed at the possibility that cryoablation can promote anti-tumor immunity. While most of the studies validated the ability of cryoablation to stimulate tumor recognition by the immune system, some studies demonstrated the opposite; tumor-bearing animals treated by cryoablation had diminished responses and increased tumor growth compared to controls. A comprehensive review of the history of cryosurgery for the treatment of cancer, the observations of distant tumor regression, the mechanisms by which cryoablation leads to cancer cell death, and how this can be altered by variations in cryosurgical technique, the pre-clinical data examining the relationship between cryoablation-induced cell death and both stimulatory and suppressive immune responses was published by M.S. Sabel (73). Another article reviews the preclinical and clinical evidence and discusses the mechanism of the antitumor immune response generated by cryoablation. The rationale and evidence behind several immunotherapy approaches that can be combined with cryoablation to devise a cryoimmunotherapeutic strategy with a potential to impact the progression of metastatic disease (74).

3.3. Electric based cancer ablation

Electric-based cancer ablation was developed for *in situ* ablation of solid tumors. Nordenstrom was the first to introduce tumor ablation by low-level direct electrical current as a palliative local treatment of solid tumors (75, 76). In later years the electric based treatments expanded and the electrical parameters used for treatment range from several volts per cm delivered for a long time period, to very high electric fields (up to 300 kV/cm). The treatment can be delivered as a continuous treatment or pulses. These treatments are either based on electro-stimulation alone or in conjunction with chemotherapeutic drugs. Low electric field or current treatments were designated as low-intensity electric fields (LIEF), electrochemical treatment (EChT), electrolytic ablation (EA) or Pulsed Electric current tumor ablation (LEFCT or PECTA). Additional electric-based tumor ablation treatments include the low-intensity alternating electric fields (TTFields), Electromagnetic radiation possessing extremely high frequency (EHF), the high voltage electroporation based treatments (Electroporation therapy (EPT), electrochemotherapy (ECT), or electrical

impulse chemotherapy (EIC), irreversible electroporation (IRE) and nanosecond pulsed electric fields (nsPEF) (for review see: 76, 77).

As for other ablation treatments the *in situ* destruction of the primary tumor by electric ablation may release antigenic material from the tumor and render it more accessible to the host's immune response. The involvement of antitumor immunity in the regression of mouse tumor nodules following low-voltage electrotherapy, was reported in Balb/c mice and Balb/c nu/nu athymic mice with colon 26 cell or Meth A cell tumor nodules (78). We performed extensive studies on the activation of anti-tumor immunity following ablation of solid metastatic tumors with pulsed low electric fields and currents without or with chemotherapeutic agents. The treatment was applied against mouse metastatic tumors such as: breast carcinoma (DA3), colon carcinoma (CT-26), squamous cell carcinoma (SQ2), prostate cancer (TRAMP-C1), and melanoma (B16F10) (79). As a result of ablation anti-tumoral immunity developed in the cured mice that destroyed residual cancer cells, both at the primary tumor site and in metastatic foci (for review see: 36). The anti tumoral activity was mediated by both CD8 and CD4 lymphocytes (80).

Electrochemotherapy (ECT) using high electric fields is a local drug delivery approach aimed at treatment with palliative intent of cutaneous and subcutaneous tumor nodules of different histotypes (for review see: 81). The first indication for a possible involvement of immune mechanisms in the eradication of the tumor by high voltage ECT came from studies, which showed that ECT successfully cured a higher ratio of tumors in immunocompetent mice than in immunodeficient nude mice, suggesting the involvement of the immune response in the effect of the treatment (82).

A similar ablation treatment, which is already in clinical use is Irreversible electroporation (IRE), IRE achieves cell death within the targeted tissue through a series of short duration pulsed high voltage electric fields that elevate the transmembrane potentials to an extent that permanently damages the lipid bilayers throughout the treated region (83, 84). Direct IRE completely ablated the tumor cells of a rat osteosarcoma. A significant increase in peripheral lymphocytes, especially CD3(+) and CD4(+) cells, as well as an increased ratio of CD4(+)/CD8(+) and increased percentage of IFN-γ-positive splenocytes was observed. Compared with the surgical resection group, the IRE group exhibited a stronger cellular immune response (85).

Over the last decade, nanosecond pulsed electric fields of (nsPEFs) have shown promise in pre-clinical studies. Non-thermal nanosecond pulsed electric field (nsPEF) therapy (30kV/cm) completely ablates UV-induced murine melanomas over a period of 12-29 days. In a melanoma allograft system, nsPEF treatment was superior to tumor excision at accelerating secondary tumor rejection in immune-competent mice, suggesting enhanced stimulation of a protective immune response by nsPEF-treated melanomas. This is also supported by the presence of CD4(+) -T cells within treated tumors (86). Similar results were reported when an orthotopic hepatocellular carcinoma (HCC) model in rats was treated with nsPEFs (50kV/cm). Tumours treated with nsPEFs expressed a significant number of cells with active caspase-3 and caspase-9, but not caspase-8, indicating an intrinsic apoptosis mechanism(s) as well as caspase-independent mechanisms. Rats with successfully ablated tumours failed to re-grow tumours when challenged with a second injection of N1-S1 cells. Infiltration of immune cells and the presence of granzyme B expressing cells within days of treatment suggest the possibility of an anti-tumour adaptive immune response (87).

3.4. Photodynamic therapy

Photodynamic therapy (PDT) is a clinically established modality for the treatment of cancerous and other diseased tissue by targeted activation of a photoreactive drug with light to generate cytotoxic reactive oxygen species in targeted lesions. Destruction of tumors or other targeted lesions by PDT is initiated by the administration of photosensitizing drugs such as Porfimer sodium (Photofrin) (HPD), 5-aminolevulinic acid (ALA), Temoporfin (Foscan) (mTHPC), compounds capable of capturing the energy of light at wavelengths of optimal tissue penetration (88). Absorbing the light energy transforms the photosensitizer molecule from its ground singlet state to an electronically excited singlet state and interacts with molecular oxygen that gets converted to highly reactive excited single state of oxygen. Singlet oxygen generated by PDT reacts rapidly and avidly with electron rich regions of lipids, proteins and other cell biomolecules producing oxidized species and cross-linking of polypeptides. The anti-tumor action of PDT is based on tumor cell killing by the direct phototoxic effect, and destruction of blood vessels, which causes death of tumor cells from hypoxia and starvation.

This therapeutic modality is now considered a treatment of choice for malignant and premalignant non-melanoma skin lesions, and an attractive option for a variety of other cancers including head and neck tumors, gastrointestinal malignancies, prostate and bladder cancers, early-stage lung cancer and malignant pleural mesothelioma, brain tumors, and intraperitoneal malignancies and can prolong survival in patients with inoperable cancers and significantly improve quality of life (89). Other types of therapeutic strategies using light to irradiate photosensible substances (PSs) include, in addition to photodynamic therapy (PDT), also photothermal therapy (PTT) and photoimmunotherapy (PIT). The main difference between PIT and PDT is that

in PIT, monoclonal antibodies (MABs) are associated to PSs to improve the selective binding of the PSs to the target tissues (90).

PDT produces damage at the treated site and rapid strong acute inflammatory reaction is provoked. This inflammatory process is integrated with acute phase response and other supporting host response processes. PDT is particularly effective in generating damage-associated molecular patterns (DAMPs) (89). Endogenous molecules such as heat shock proteins, calreticulin, phosphatidylserine, lysophosphatidylcholine, sphingosine-1-phosphate (S1P), extracellular matrix components, fibrinogen and high-mobility group box-1 protein may function as DAMPs. Binding of DAMPs on pattern recognition receptors (PRRs) triggers signal transduction pathways leading to leukocyte recruitment and activation of inflammatory and adaptive immune responses.

Changes in tumor microenvironment associated with the execution of these host-protecting responses trigger the development of adaptive immune response specific for the antigens of PDT-treated tumor (91). Early studies demonstrated that immune cells are essential for preventing the recurrence of tumors following the PDT treatment, and they include host lymphoid populations (92), neutrophils, cytolytic T cells, helper T cells and macrophages (93). In a later study, it was shown that a PDT regimen that induced a high level of neutrophilic infiltrate generated tumor-specific primary and memory CD8(+) T-cell responses. In contrast, immune cells isolated from mice treated with a PDT regimen that induced little or no neutrophilic infiltrate exhibited minimal antitumor immunity. These findings indicate that tumor-infiltrating neutrophils play an essential role in establishment of antitumor immunity following PDT (94).

The application of a novel photosensitizing methodology: vascular-targeted photodynamic therapy (VTP) also stimulated anti tumor immune response. This modality uses as a sensitizer Pd-bacteriochlorophyll and consequent spectral wavelength in the near infrared. The targets of VTP are the tumor-feeding arteries and draining veins whose almost instant occlusion (minutes) leads to tumor blood stasis and eradication (95).

Recently, photodynamic therapy (PDT) utilizing the photosensitizer, hypericin (Hyp), was characterized to stimulate anti-tumor immunity by inducing bona fide immunogenic cell death (ICD) (96). This led to the development of PDT vaccine protocols in which the patient is administered with a fragment of his tumor that was treated by PDT *ex vivo*. Such autologous whole-cell vaccines are optimally conditioned to target individualized, pertinent and even unique antigens in a patient-specific manner involving patient-matched MHC for recognition of tumor epitopes (97).

3.5. Chemical ablation and targeted therapy

3.5.1. Chemotherapy

Unlike other *in situ* ablation treatments chemotherapy is mostly given as a systemic treatment with a primary goal to kill cells, which spread out in the body. It is evident that chemotherapeutic drugs can either counteract the activity of immune response components or exhibit activities, which promote or complement anti-tumor immune reactivity. Chemotherapeutic drugs can:

a. Kill tumor cells and release tumor antigens and danger signals, which can activate anti-tumor immunity
b. Inhibit immunosuppressive cells such as regulatory T cells and myeloid derived suppressor cells (MDSC).
c. Stimulate CD8 lymphocytes and manipulate dendritic cells.
d. Increase the susceptibility of tumor cells to immune attack.

Early studies showed that DCs could collaborate with chemotherapy (mitomycin C) -induced apoptotic CT26 tumor cells and elicit improved antitumor immunity, probably through the acquisition of tumor-associated antigens from apoptotic tumor cells. Immunization of tumor bearing mice with DCs and apoptotic CT26 cells, but not with apoptotic CT26 alone, gave protection against tumor challenge. CT26 challenge was also rejected in 50% of the mice injected with mitomycin alone. A significantly higher level of cytotoxic T-cell activity and interferon-gamma production was seen in the protected mice (98).

Later studies revealed that chemotherapy could induce various tumor cell death modalities including ‘immunogenic cell death’, which leads to the delivery of a broad range of tumor-associated antigens and can trigger immune releasing tumor-derived antigen as well as danger signals. Cancer cells succumbing to anthracyclines (such as doxorubicin and mitoxantrone), oxaliplatin or ionizing irradiation can elicit vigorous anticancer immune responses when they are injected subcutaneously, in the absence of any adjuvant, into syngeneic immunocompetent mice (99, 100). The efficiency of anthracycline- and oxaliplatin-based chemotherapy against established tumors is lost when essential components of the immune system such as interferon-γ (IFN-γ), interleukin-1β (IL-1β) or their receptors are blocked by antibodies or eliminated by knockout technology (101, 102) ((For review see also: 24, 103-105). Chemotherapy–induced cell death deliver ‘danger signals’ that stimulate antigen-presenting cells such as dendritic cells to efficiently take up tumor antigens, process them, and cross-prime cytotoxic T lymphocytes, thus eliciting a tumor-specific cognate immune response. These include the pre-apoptotic exposure of calreticulin, release of HMGB1, ATP and HSPs, which can augment specific antitumor immune responses by enhancing antigen cross-presentation and

DC activation, and DNA-damage responses, which can activate innate immune responses (106, 107).

As stated previously, antitumor immunity driven by intratumoral dendritic cells contributes to the efficacy of anthracycline-based chemotherapy in cancer. It was recently reported that tumor-bearing mice deficient in the formyl peptide receptor 1 (FPR1), Fpr1(-/-), which is binding annexin-1, exhibited an impaired anti tumor immune response after treatment with anthracycline. The effect was attributed to the failure of Fpr1-deficient dendritic cells to process dying cancer cells. In this respect, Loss-of-function allele of the gene coding for formyl peptide receptor 1 (FPR1) was associated with poor metastasis-free and overall survival in breast and colorectal cancer patients receiving adjuvant chemotherapy (108).

Anti tumor cytotoxic drugs can also modulate the immune response by directly affecting the function of immune cells. Chemotherapeutic drugs such as cyclophosphamide can boost anti tumor immunity by inhibiting regulatory T cells (109) or MDSC (taxol, gemcitabine) (110). Chemotherapy such as docetaxel has the ability to modulate components of the immune system independent of antitumor activity. Studies in a preclinical model showed for the first time that docetaxel modulates CD4+, CD8+, CD19+, natural killer cell, and Treg populations in non-tumor-bearing mice. Docetaxel combined with recombinant viral vaccine was superior to either agent alone at reducing tumor burden, and docetaxel plus vaccine increases antigen-specific T-cell responses to antigen in the vaccine, as well as to antigens derived from the tumor (111). Another type of effect was exhibited by gemcitabine who reversed the defect in Ag cross-presentation of tumor infiltrating CD11b(+) DCs in a murine mesothelioma tumor AB1-HA model, rendering them able to induce tumor-specific T-cell activation or proliferation (112).

3.5.2. Targeted therapy

Targeted therapies such as anti-HER2 (trastuzumab), inhibitors of BCR-ABL translocation (imatinib), BRAFV600E mutation (Vemurafenib), EGFR mutation (erlotinib and gefitinib) and EML4-ALK fusion (crizotinib), which are now in clinical use in many solid tumors and haematological malignancies can produce tumor regression. It may be conceived that such agents can modulate the immune response by various mechanisms, such as release of antigens and danger signals from dead or dying cells and improved delivery of tumor antigen for presentation, affect immunosuppressive cells such as MDSCs or Tregs, as well effector T cells and DCs (107).

Imatinib mesylate (Gleevec), the tyrosine kinase inhibitor of KIT, PDGFR, ABL and BCR-ABL, exhibits improved survival benefit in patients with advanced gastrointestinal stromal tumor (GIST). In mice, a combination of imatinib and IL-2 resulted in the expansion of a population of effector cells that shared properties of both NK myeloid DCs and produce IFN-γ. These CD11c+ B220+ NK1.1.+ IKDCs, were able to lyse various target cells in the absence of NKG2D ligands or MHC class I molecules. Adoptive transfer of IKDCs but not B220− NK cells delayed tumor growth (113). Balachandran *et al*. (114) demonstrated the pre-existing role of CD8+ -mediated immune response, which is enhanced by imatinib therapy. In a model of transgenic GIST mice that develop spontaneous GIST, treatment with imatinib resulted in an increase in CD8+ T cell frequency, proliferation, activation as well as cytolytic capacity within the tumor and an increase in tumor-specific CD8+ T cells within the draining, but not the non-draining lymph nodes, and induced apoptosis of Tregs. A synergistic effect was seen in mouse GIST treated with imatinib and CTLA-4 blocking antibody compared to either drug alone. Finally, the authors demonstrated that in 36 patients undergoing surgery followed by either imatinib therapy or observation a greater frequency of CD8+, but lower of Tregs and of IDO mRNA was found in sensitive tumors compared to resistant tumors, therefore correlating with the preclinical data in mouse GIST.

Treatment of 16 patients with metastatic melanoma with either BRAF inhibitor alone (Vemurafenib) alone or BRAF + MEK inhibition (dabrafenib + trametinib) was associated with an increased expression of melanoma antigens and an increase in CD8+ T-cell infiltrate in tumor biopsies. This was also associated with a decrease in immunosuppressive cytokines (interleukin (IL)-6 and IL-8) and an increase in markers of T-cell cytotoxicity. Markers of T-cell exhaustion and the immunosuppressive ligand PDL1 are also increased with BRAF inhibition, further implying that immune checkpoint blockade may be critical in augmenting responses to BRAF-targeted therapy in patients with melanoma (115).

It has recently become apparent that upon oncogene inactivation, the immune response is critical in mediating the phenotypic consequences of oncogene addiction. In particular, CD4(+) T cells have been suggested to be essential to the remodeling of the tumor microenvironment, including the shutdown of host angiogenesis and the induction of cellular senescence in the tumor. Hence, oncogene inactivation may be an effective therapeutic approach because it both reverses the neoplastic state within a cancer cell and reactivates the host immune response (116).

3.6. Surgery

Results suggested that tumor resection not only led to the reversal of immune suppression, but also unmasked a population of primed T cells able to mediate protective immunity. To test this, mice were inoculated

s.c. with CMS5 cells and after 28 days the tumors were resected. A gradual normalization of the cellular phenotype of the spleen was observed. In particular, there was a decrease in the number of Mac1+/Gr1(high) cells and an increase in the number of CD3+ cells in the spleen within 24-48 h of tumor resection. By day 10, these values were normal. The functional implications of these changes were illustrated by the reduced growth rate or the complete rejection of a challenge of tumor cells in the resected mice. Both CD4+ and CD8+ cells were involved in the restoration of tumor immunity (117). In the breast carcinoma model, 4T1, cell-mediated and humoral adaptive immunity, as measured by rejection of allogeneic tumor, antigen-specific T-cell proliferation, and antigen-specific antibody responses, were suppressed in 4T1-bearing mice relative to tumor-free mice. Surgical removal of the primary tumor resulted in rebounding of antibody and cell-mediated responses, even in mice with metastatic disease. Macrophage activity and dendritic cell function were not suppressed in the tumor-bearing mice (118).

4. COMBINATION OF ABLATION AND IMMUNO-MANIPULATION TO REINFORCE THE IMMUNE RESPONSE

Tumor ablation by various methods can trigger an immune response against the tumor as outlined in the previous section. However ablation alone rarely induces effective anti-tumor immunity resulting in systemic tumor rejection. Immunotherapy can complement ablation to reinforce the anti-tumor immunity to better eradicate residual local and metastatic tumor cells. Various methods and agents were used to manipulate the immune response in combination with tumor ablation, and they can be divided into three major categories:

1. Agents, which stimulate immune response components. These include microbial or chemical immunoadjuvants, tumor vaccines, and cytokines. Such immunostimulators can promote the activity of dendritic cells and/or T lymphocytes.
2. Agents that inhibit cells and molecules, which suppress anti-tumor immune responses. These include agents, which inhibit the function or deplete immune suppressor cells such as myeloid derived suppressor cells (MDSC) or regulatory T cells (Tregs), or inhibitors of the suppressive function of immunological checkpoint molecules (CTLA-4, PD-1, PDL-1).
3. Adoptive transfer of anti-tumor T lymphocytes or antibodies.

Pre-clinical and emerging clinical evidence on combinations of ablation methods and immune modulating agents, capable of potentiating the immune response in the treatment of cancer in order to maximize cancer elimination and the prevention of escape mechanisms were previously published (21, 119).

4.1. Agents, which stimulate immune response components: Microbial or chemical immunoadjuvants, dendritic cells, cytokines and tumor vaccines

4.1.1. Immunoadjuvants

When a cell dies as the result of infection, the immune system responds rapidly and the system of Toll-like receptors (TLR) plays a key role in this process. Ligation of Toll-like receptors results in the induction of strong immune responses that may be directed against tumor-associated antigens. Unmethylated CpG-containing oligodeoxynucleotides are strong TLR agonists and activators of anti tumor immunity and of dendritic cell function (120).

CpG was used in many studies in combination with almost all ablation modalities and was found to significantly boost the anti-tumor immune response triggered by the destruction of the tumor by ablation. Unmethylated CpG-containing oligodeoxynucleotides (ODNs) enhanced the antitumor efficacy of chemotherapy (coramsine) (121). The intracellular signaling pathways that link TLR ligation with immune activation and where and how TLRs recognize their targets were addressed in the following article (122).

Further studies showed synergistic effects of gamma radiation and TLR-targeted immunotherapies in the treatment of cancer (123). Using our alpha-radiation-based ablation strategy, diffusing alpha-emitters radiation therapy (DaRT), to destroy local CT26 and DA3 tumors in combination with CpG resulted in a better control of the primary tumor and rendered the animals resistant to a re-inoculated tumor (37).

Electrochemotherapy (ECT) of tumors induced a massive recruitment of CD11c and CD11b positive cells in the tumors and a strong increase of TLR9 expression. ECT followed by the TLR-9 ligands, CpG oligodeoxynucleotides (CpG ODN), triggered both potent local synergistic antitumor effects, on the ipsi-lateral ECT-treated tumor, and a systemic antitumor response on the contra-lateral untreated tumor, in three tumor models. The systemic protection was T-cell dependent and was not observed in nude mice (124).

Treatment with photodynamic therapy (PDT) alone is often non-curative due to tumor-induced immune cell dysfunction and immune suppression. PDT mediated by verteporfin in combination with CpG oligodeoxynucleotides, for the treatment of 4T1 metastatic breast cancer in a BALB/c immunocompetent mouse model, gave improved local tumor control and a survival advantage compared to either treatment alone (125).

A combination treatment of cryoablation plus TLR9 stimulation via CpG-oligodeoxynucleotides was far more effective in the eradication of local and

systemic tumors than either treatment modality alone. Analysis of the underlying mechanism revealed that *in situ* tumor ablation synergizes with TLR9 stimulation to induce DC maturation and efficient cross-presentation in tumor-bearing mice, leading to superior DC function *in vivo* (126).

An innovative approach reported that Near-infrared light-responsive inorganic nanoparticles enhanced the efficacy of cancer photothermal ablation therapy. The design is based on chitosan-coated hollow copper sulfide (CuS) nanoparticles that assemble the immunoadjuvants oligodeoxynucleotides containing the cytosine-guanine (CpG) motifs. In this approach, photothermal ablation-induced tumor cell death reduced tumor growth and released tumor antigens into the surrounding milieu, while the immunoadjuvants potentiate host antitumor immunity. The results indicated that combined photothermal immunotherapy is more effective than either immunotherapy or photothermal therapy alone against primary treated and distant untreated tumors in a mouse breast cancer model (127).

The immunostimulant OK-432 was also tested in combination with ablation. Radiofrequency ablation of lung tumors in rabbits in combination with OK-432, prolongs survival, inhibited the growth of metastases and stimulated anti-tumor immunity (128, 129).

Non-microbial adjuvants were also effective in enhancing tumor ablation triggered anti tumor immunity. Saponin-based adjuvants and *in situ* tumor ablation, created a highly effective vaccine. Draining lymph node CD11c+ DCs acquired antigens more efficiently and become increasingly activated following ablation with saponin adjuvants relative to ablation alone (130). Laser induced photothermal ablation in combination with a local injection of an immunoadjuvant that consists of a semi-synthetic functionalized glucosamine polymer, N-dihydro-galacto-chitosan (GC), was also applied. This strategy proposed as an *in situ* autologous cancer vaccine (inCVAX) for the treatment of metastatic cancers was found to induce anti tumor immunity (131).

Immunostimulation not always boost the immune response after tumor ablation. Cryosurgical ablation of the normal rat ventral prostate and intra-prostatic Complete Freund's Adjuvant (CFA), does not protect against and can enhance the tumorigenicity of MatLyLu prostatic cancer cells at distant sites. This could be occurring through specific immunologic effects or non-specific mechanisms induced by cryosurgery and CFA (132). Toll like receptors may not always help to cure cancer. Gao *et al*. reported a novel Toll-like receptor 9 (TLR9) dependent mechanism that initiated tumor regrowth after local radiotherapy. Systemic inhibition of TLR9, but not TLR4, delayed tumor recurrence in mouse models of B16 melanoma, MB49 bladder cancer, and CT26 colon cancer after localized high-dose tumor irradiation. The tumorigenic effects of TLR9 depended on MyD88/NF-κB-mediated upregulation of interleukin (IL)-6 expression, which in turn resulted in downstream activation of Jak/STAT3 signaling in myeloid cells (133).

4.1.2. Dendritic cells

Dendritic cells (DC) are professional antigen-presenting cells that play a pivotal role in the induction of immunity. Dendritic cells control the initiation of stimulatory and regulatory immune responses. They are strategically located within tissues and continuously sample the microenvironment, displaying their internalized cargo on their cell surface. Activation of DC or facilitation of DC recruitment and function can promote anti-tumor responses initiated by tumor ablation.

HIFU treatment can cause the release of endogenous danger signals (ATP and hsp60) and exposure of dendritic cells (DCs) and macrophages to the supernatants of HIFU-treated tumor cells leads to an increased expression of co-stimulatory molecules (CD80 and CD86) with enhanced secretion of IL-12 by the DCs and elevated secretion of TNF-alpha by the macrophages (134). Loading of DCs with tumor debris from ultrasound ablated tumors induced maturation of DCs, and increased cytotoxicity and TNF-α and IFN-γ secretion by CTL, thus initiating host specific immune response after tumor cell challenge in the vaccinated mice (135). It was suggested that the efficacy of HIFU cancer treatment in enhancing the host's anti-tumor immunity is closely related to dendritic cell activation (136). Furthermore, tumour destruction by radiofrequency ablation and cryoablation, elevated the numbers of antigen containing DC in the draining lymph node (LN), and both destruction methods were able to induce DC maturation (137).

Treatment of local murine Lewis lung carcinoma, D122-luc-5.5. tumors with cryoablation and inoculation of immature DCs with administration of the immune adjuvant, CpG oligodeoxynucleotides resulted in reduced tumor growth, low metastasis and significantly prolonged survival (138). RFA of subcutaneous colon cancer cell (MC38) tumors, and then OK-432-stimulated DCs injected locally, strongly inhibited tumor growth as compared to mice treated with RFA alone or treatment involving immature DC transfer. The antitumor effect of this treatment depended on both CD8-positive and CD4-positive cells (139).

4.1.3. Cytokines and growth factors

Immunostimulation can be achieved by various cytokines and growth factors. Thus, several cytokines, mainly IL-2, were used to boost anti tumor immunity triggered by ablation.

In view of the reports that tumor ablation by electrochemotherapy (ECT) can trigger anti-tumor

immune responses, attempts were made to enforce this response by IL-2 in order to achieve higher cure rates. An increase of the rate of completely cured animals was achieved by injecting mice with interleukin-2 (IL-2) and ECT (140). The next step was to use ECT, which causes short-term complete regressions of treated tumors but no resistance to challenge, was combined with plasmid delivery encoding for IL-2. The combination treatment resulted in the induction of long-term immunity to recurrence and resistance to challenge in up to 25% of mice (141). Another cytokine, which increased the systemic antitumor effectiveness of electrochemotherapy, was IL-12. Therefore, it was proposed to treat by electrochemotherapy with peritumoral IL-12 electrotransfer (142).

Enhanced systemic antitumor immunity was also achieved by RFA + IL-2 therapy of human head and neck cancer in a murine orthotopic model. The combined treatment induced the highest levels of macrophage recruitment and dendritic cell migration resulting in enhanced CTL activity, increased tumor apoptosis, and the best inhibition of tumor growth among all groups (143). Similar effects were observed by treatment of mice bearing subcutaneous tumors with RFA and huKS-IL2. The combination attained significantly greater tumor growth suppression and enhanced survival compared with mice treated with RFA or huKS-IL2 alone (144).

A combination treatment of radiation and IL-3 gene-transduced irradiated tumor cell vaccines of established immunogenic (FSAR) and non-immunogenic (FSAN) tumors enhanced the efficacy of the IL-3 vaccine by decreasing tumor burden. Systemic IL-3 vaccine treatment increased intratumoral levels of intercellular adhesion molecule-1, Mac-1, EB22/5.3., tumor necrosis factor-alpha, and IL-1 mRNA in irradiated tumors, indicating that cellular infiltration was part of the response (145). In another study a combination of radiotherapy and growth factor Flt3-Ligand (Flt3-L) of 67NR tumors, impaired the growth of non-irradiated tumors in the same animal. This abscopal effect was shown to be tumor specific, and immunologically controlled since no growth delay of non-irradiated 67NR tumors was observed when T cell deficient (nude) mice were treated with RT plus Flt3-L (32).

Ablation and combinations immunostimulatory measures were also tested. Local hyperthermia (43.7.°) and intratumoral dendritic cell and/or systemic granulocyte macrophage colony-stimulating factor (adenovirus-expressing murine GM-CSF), applied in a syngeneic murine model of prostate cancer (RM-1) resulted in significant tumor growth delays when compared with animal cohorts that received hyperthermia alone (146).

4.1.4. Tumor vaccines

Tumor ablation and tumor vaccines were also combines in an attempt to achieve a better control of metastatic cancer. RFA induced immunogenic modulation on the surface of tumor cells and increased T-cell responses to CEA and additional TAAs. Combination therapy with RFA and a poxviral vaccine expressing CEA and a TRIad of COstimulatory Molecules (CEA/TRICOM) in CEA-transgenic mice induced a synergistic increase in CD4(+) T-cell immune responses to CEA and eradicated both primary CEA(+) and distal CEA(-) subcutaneous tumors. Sequential administration of low-dose and high-dose RFA with vaccine decreased tumor recurrence compared to RFA alone (147). In a recent review it was suggested that the immune-mediated distant bystander (abscopal) effects of RT could be enhanced when combined with autologous whole-tumor-cell-based vaccines generated by high hydrostatic pressure technology (148).

4.2. Agents that inhibit immunosuppressive cells and molecules

Most of the anti tumor immune reactions are rather weak and are counteracted by immunosuppressive activities. In order to rescue the anti tumor immune responses attempts are made to deplete the immunosuppressive functions by the use of agents, which inhibit the function or deplete immune suppressor cells such as myeloid derived suppressor cells (MDSC) or regulatory T cells (Tregs), or inhibitors of the suppressive function of immunological checkpoint molecules.

4.2.1. Inhibition or depletion of MDSC and/or tregs

Host immune cells with a suppressive phenotype represent a significant hurdle to successful immunotherapy of metastatic cancer. The function of suppressor cells, which facilitate tumor growth and confer immune tolerance against the tumor, was already suggested in 1972 (for review see 149), and their elimination by radiation was postulated to boost anti-tumor immunity (150). Among the suppressor cells, Tregs and MDSC are significantly increased in hosts with advanced malignancies (151, 152). Tumor-derived immunosuppression constantly diminishes anti-tumor immune responses; therefore, therapies neutralizing immunosuppression should be given before vaccination and continued throughout treatment.

Tregs, in most cancers, play a central role in contributing to the progression of the disease (153). Thus, suppression mechanisms mediated by Tregs are thought to contribute significantly to the failure of current therapies that rely on induction or potentiating of anti-tumor responses (154). MDSCs are a heterogeneous population of immature myeloid cells that are increased in many cancer types. MDSCs play a central role in suppression of host immune system through mechanisms such as arginase1, release of immune-suppressive factors such as ROS, NO and cytokines (151). Elimination of Tregs (155, 156) or MDSCs (157, 158)

was found to be essential for an effective anti-tumor immunotherapy. MDSC depletion was associated with restoration of immune dysfunction in hepatocellular carcinoma patients (159) suggesting that their inhibition might improve the control of cancer development.

Thus, inhibition of MDSC or Tregs or both in combination with ablation methods, which stimulate anti-tumor immunity, was tested for improved control of residual local and systemic disease, and enforcement of anti tumor immunity.

Inhibition of MDSC with a selective blocker of CSF1 receptor together with gamma radiotherapy suppressed tumor growth more effectively than irradiation alone (160). The possible role of MDSC in the outcome of tumor ablation was pointed out by the study, which showed that increased MDSC-related functions are an early indicator for incomplete radiofrequency ablation of NSCLC (161).

Inhibition of Tregs was applied in combination of different ablation methods. Photodynamic therapy in combination with low-dose cyclophosphamide (CY) (but not high-dose CY) increased long-term survival and complete cure of tumor bearing animals. Low-dose CY alone gave no permanent cures but did provide a survival advantage and was shown to reduce CD4+FoxP3+ Tregs in lymph nodes, whereas high-dose CY reduced other lymphocyte classes as well. A high percentage of the cured animals rejected a tumor rechallenge (162, 163). Treg targeting together with tumor gamma-irradiation significantly reduced tumor burden and improved overall survival (164). When combined with Treg depletion, cryoablation was significantly more effective than either surgical excision or cautery at inducing systemic antitumor immunity, resulting in the cure of a fraction of animals with established metastatic disease and resistance to reinjection of tumor cells (165). In view of the findings that ablation-triggered anti tumor immunity can be improved by inhibition of Tregs or by immunoadjuvants led to combination treatments of the above treatments. Gamma-irradiation in combination with an immunostimulant and inhibitor of Tregs was reported to be the most effective treatment of a breast cancer (166). Regulatory T-cells (CD25+, Foxp3+) removal by low dose cyclophosphamide can also potentiate the PDT-induced immune response (163).

The observation that synergistic suppression of Tregs and MDSCs cells in mice with tumors promoted their anti-tumor immunity (167), and our previous demonstration that CpG can augment anti-tumor immunity in combination with alpha radiation mediated ablation (DaRT) (37), led us to attempt to maximize the anti–tumor immunity following ablation of the low immunogenic DA3 primary tumor by DaRT by adding CpG to inhibition of Tregs blow-dose cyclophosphamide (CP) and MDSCs by phosphodiesterase-5 (PDE-5) inhibitor, sildenafil (168). This combined treatment was very effective in eliminating both primary tumors and lung metastases, was significantly more effective than CP alone or DaRT alone in blocking tumor growth and extending overall survival (unpublished results). These findings are corroborated by a clinical study which claims that the key mechanisms of augmenting the functions of adoptively transferred T cells by total body irradiation in melanoma patients include the depletion of Tregs and MDSCs and the activation of the innate immune system via Toll-like receptor 4 signaling (169).

4.2.2. Inhibitors of immune inhibitory pathways-checkpoint blockade

In recent years cancer immunotherapy gained momentum when the therapeutic benefit of monoclonal antibodies against immune checkpoints (CTLA-4/CD80/CD86 and PD-1/PDL-1) was reported. Activating the immune system was demonstrated by the ability of the anti-CTLA4 antibody, ipilimumab, to achieve a significant increase in survival for patients with metastatic melanoma, for which conventional therapies have failed. These successes suggest that active immunotherapy represents a path to obtain a durable and long-lasting response in cancer patients (For review see 26, 170). As a follow up the beneficial anti-tumor effects of combining checkpoint inhibitors with various ablation modalities were examined.

CTLA-4 blockade was the most tested treatment and was used in combination with radiofrequency ablation, cryoablation, thermo ablation, radiation and chemotherapy.

Immune responses induced by thermoablation are mostly weak and not sufficient for complete eradication of established tumors or durable prevention of disease progression. The following studies reveal that combination therapies with immunomodulating drugs and thermal ablations were evaluated with promising results.

An early report stated that treatment of murine B16-OVA melanoma cell derived tumors by RFA and a blocking monoclonal antibody against cytotoxic T-lymphocyte-associated antigen 4 augmented the anti tumor effect of splenocytes from the treated animals in adoptive transfer experiments, and resulted in long-lasting tumor protection (60). Further studies showed that RFA and cryoablation can be efficiently combined with immune modulation by anti-CTLA-4 antibodies or regulatory T-cell depletion. These combination treatments protected mice from the outgrowth of tumour challenges, and led to *in vivo* enhancement of tumour-specific T-cell numbers, which produced more IFN-gamma upon activation (126). Similarly, the co-administration of microwave thermal ablation, GM-CSF microspheres, and anti-CTLA-4 rejected tumour rechallenge in 90% of treated mice in

a subcutaneous murine Hepa 1-6 model, and cured established distant tumour in 50% of the treated mice. This anti-tumour immune response was tumour-specific and mediated by natural killer (NK), CD4+, and CD8+ T cells (171). Cryoablation of TRAMP C2 mouse model of prostate cancer did not confer protection against a tumor challenge but a combination treatment with anti-CTLA-4 was sufficient to slow growth or trigger rejection. In addition, secondary tumors were highly infiltrated by CD4(+) T cells and CD8(+) T cells, and there was a significant increase in the ratio of intratumoral T effector cells to CD4(+)FoxP3(+) T regulatory cells, compared with monotherapy (172).

Radiation potentiates the effect of immune therapy via induction of autophagy and resultant trafficking of mannose-6-phopsphate receptor (MPR) to the cell surface. Radiation-induced MPR up-regulation was the result of redistribution of the receptor to the cell surface. This effect was caused by autophagy with redirection of MPR to autophagosomes in a clathrin-dependent manner. Down-regulation of MPR in tumor cells with shRNA completely abrogated the combined effect of XRT and immunotherapy (CTLA4 antibody) in B16F10-bearing mice without changes in the tumor-specific responses of T cells (173). A recent review summarizes preclinical and clinical data demonstrating that radiation acts in concert with antibodies targeting the immune checkpoint cytotoxic T-lymphocyte antigen-4 (CTLA-4), to induce therapeutically effective anti-tumor T cell responses in tumors otherwise non responsive to anti-CTLA-4 therapy (174).

The data indicating that chemotherapy killed tumour cells, engage with anti tumour immune responses (as discussed in section 3) suggested the development of new protocols combining chemotherapy with inhibition of immunological checkpoint molecules. Chemotherapy was used with inhibitors of various immunological checkpoints with promising results.

It was suggested to combine chemotherapy with anti-CD40 antibodies, to gain therapeutic synergy with general applicability to many cancer types (22). A combination of immunotherapy (CD40 ligation using FGK45) with gemcitabine to treat established solid tumors induced long-term cures in < or =80% of mice, and all of the cured mice were resistant to tumor rechallenge. It was associated with an increase in both CD4 and CD8 T-cell infiltration of the tumor. CD8 T cells but not CD4 T cells were required for the success of this treatment regimen (175). Further studies indicated that partial surgical debulking followed by combination chemotherapy (gemcitabine) and anti CD-40 antibodies elicited the same proportion of cured animals as complete resection, but in contrast to complete resection, a memory response was invoked (176). Thus, anti-CD40 antibody provides an ideal therapy for combination with traditional cancer treatments (i.e., chemotherapy, surgery) in order to elicit immune-mediated anti-tumor effects. The mechanisms of action of agonistic anti-CD40, the use of mouse models to investigate its effects and combinations with other therapies *in vivo*, and current clinical trials combining humanized anti-CD40 antibody with chemotherapy and/or other immunotherapies were hence summarized (177).

Treatment with chemotherapy (gemcitabine) in combination with CTLA-4 blockade also exhibited tumor regression and long-term protective immunity in mice bearing non-immunogenic murine tumor. Depletion experiments demonstrated that both CD4(+) and CD8(+) T cells are required for optimal therapeutic effect (178).

CD47 is another candidate for collaboration with ablation treatments to boost anti tumor immunity and control metatstatic cancer. CD47 is a widely expressed cell surface protein that functions as a regulator of phagocytosis mediated by cells of the innate immune system, such as macrophages and dendritic cells. CD47 serves as the ligand for a receptor on these innate immune cells, SIRP-alpha, which in turn delivers an inhibitory signal for phagocytosis. It was suggested that CD47 might serve as another checkpoint molecule and as a target for checkpoint inhibition therapy (179). An extensive study reported that blocking CD47 in the context of radiotherapy enhances antitumor immunity by directly stimulating CD8(+) cytotoxic T cells, with the potential to increase curative responses. Combining CD47 blockade with irradiation did not affect fibrosarcoma growth in T cell-deficient mice, whereas adoptive transfer of tumor-specific CD8(+) T cells restored combinatorial efficacy. In CD47-deficient syngeneic hosts, engrafted B16 melanomas were 50% more sensitive to irradiation, establishing that CD47 expression in the microenvironment was sufficient to limit tumor radiosensitivity. Mechanistic investigations revealed increased tumor infiltration by cytotoxic CD8(+) T cells in a CD47-deficient microenvironment, with an associated increase in T cell-dependent intratumoral expression of granzyme B. Correspondingly, an inverse correlation between CD8(+) T-cell infiltration and CD47 expression was observed in human melanomas (180).

Lately appeared a report about a humanized anti-CD47 antibody, 5F9, with potent efficacy and favorable toxicokinetic properties as a candidate therapeutic. Hu5F9-G4 induced potent macrophage-mediated phagocytosis of primary human AML cells *in vitro* and completely eradicated human AML *in vivo*, leading to long-term disease-free survival of patient-derived xenografts (181).

4.3. Adoptive transfer of T cells

Bear and colleagues (182) characterized the immune effects of AuNP mediated photothermal therapy (PTT) and explored this modality in combination with

adoptive T cell therapy. PTT was delivered by optically tuned gold nanoshells that generate heat upon exposure to near infrared radiation. PTT of B16 melanoma induced an immune response with anti-tumor activity. Importantly, however, the anti-tumor activity appeared dependent on distant tumor location; tumors located subcutaneously shrunk while those in the lungs grew after PTT of a primary site. PTT of the primary tumor promoted the infiltration of secondary tumor sites by CD11b(+)Ly-6G/C(+) myeloid-derived suppressor cells, consequently failing to slow the growth of poorly immunogenic B16-F10 tumors and enhancing the growth of distant lung metastases. This growth appeared to be induced by an inflammatory response to PTT that caused a systemic increase in immune suppressive myeloid-derived suppressor cells (MDSCs), and this effect was counter-acted with the combination with adoptive T cell therapy. The combination of local control by PTT and systemic antitumor immune reactivity provided by adoptively transferred gp100-specific pmel T cells, prevented primary tumor recurrence post-ablation, inhibited tumor growth at distant sites, and abrogated the outgrowth of lung metastases (182).

5. CLINICAL MANIFESTATION OF ABLATION AND IMMUNOSTIMULATION

5.1. Radiation therapy mediated ablation and anti tumor immunity

Radiation therapy (RT) is widely used with curative or palliative intent in the clinical management of multiple cancers. Although mainly aimed at direct tumor cell killing, mounting evidence suggests that radiation can alter the tumor to become an immunostimulatory milieu (the abscopal effect). Clinically, if RT treatments can be optimized to promote anti-tumor immunity, this could increase the odds of achieving local cancer control and combat growth of micrometastases.

Data suggest that the immunogenic effects of radiation can be exploited to promote synergistic antitumor effects in combination with immunotherapeutic agents in cancer patients (183). Recent papers review the concepts associated with the immunogenic consequences of RT and how preclinical findings are translated into clinical benefit for patients receiving combination regimens of RT and therapeutic cancer vaccines such as dendritic cell vaccines, whole tumor cell vaccines, viral vaccines, peptide or protein vaccines, and nucleic acid vaccines (184), checkpoint inhibitors or cytokines (185). Clinical trials combining RT and immunotherapy, two modalities yet to be used in combination in routine practice, are summarized in recent extensive reviews (50, 186). These trials include careful immune monitoring of the patients enrolled and will generate important data about the pro immunogenic effects of radiation in combination with a variety of immune modulators, in different disease settings.

Role of human myeloid-derived suppressor cell (MDSC) subsets and of T-cell-mediated immune responses in clinical outcomes in patients with oligometastases treated by stereotactic body radiotherapy (SBRT) and sunitinib have been evaluated. Sunitinib treatment increased the efficacy of SBRT in patients with oligometastases by reversing MDSC and Treg-mediated immune suppression and may enhance cancer immune therapy to prevent tumor recurrence post-SBRT. Sunitinib treatment resulted in a significant reduction in monocytic MDSC, phosphorylated STAT3, and arginase levels in monocytic MDSC (CD33(+)CD14(+)CD16(+), and an increase in T-cell proliferative activity in cancer patients. SBRT synergized the therapeutic effects of sunitinib, especially as related to decreased numbers of monocytic MDSC, Treg, and B cells, and augmented Tbet expression in primary CD4 and CD8 T cells. These effects were not observed in patients receiving radiation therapy alone. The responders, defined by sunitinib-mediated reduction in CD33(+)CD11b(+) myeloid cell populations, tend to exhibit improved progression-free survival and cause-specific survival (187).

5.2. Thermal ablation

5.2.1. RF ablation and anti-tumor immunity

Radio-Frequency Ablation (RFA) is a minimally invasive technique, which is used as standard local therapy of primary and metastatic liver tumors in patients. RFA is one of the treatments for hepatocellular carcinoma (HCC) or liver metastases of colon carcinomas (CRC) and is known to enhance host immune response. Following RFA, changes can be detected in immune-related cells and molecules in the serum of patients. Such agents might be involved in modulating the immune responses towards tumor cells which express the *in vivo* released tumor antigens.

Since hepatocellular carcinoma (HCCs) are in general only weakly immunogenic, cell injury induced by local tumor ablation, by ethanol injection (PEI) or radiofrequency thermal ablation (RFTA), may increase HCC immunogenicity and may release endogenous adjuvants that activate dendritic cells (DC). HCC ablation induced a functional transient activation of myeloid DC but not of plasmocytoid DC associated with increased serum levels of TNF-alpha and IL-1beta (188). RFA treated patients with liver metastases of colorectal cancer or with hepatocellular carcinoma show also a significant tumor-specific cytotoxic T-cell stimulation, assessed by an interferon gamma (IFNgamma) secretion assay, and manifest a dramatic increase in tumor specific cytolytic activity of CD8(+) T cells against human CaCo colorectal cancer and HepG2 HCC cells (189).

In cancer patients, only few studies have described the induction of specific immune responses after RFA. Napoletano *et al.* reported that naïve and memory $CD62L^+$ T cells translocate to the tissues and

that T cells produced IFN-γ in response to the tumor-associated MUC1 antigen, while humoral immune responses were unaffected by RFA treatment (190). HCC thermal ablation can create an antigenic source along with stimuli appropriate for maturation of APCs to induce HCC-specific T-cell responses. Expression of costimulatory molecules, lymph-node homing chemokine receptor, antigen presentation, and cytokine secretion were enhanced in monocytes from RFA treated patients after incubation with RFA treated HCC tissue and granulocyte macrophage colony-stimulating factor (GM-CSF), or GM-CSF plus IL-4, as compared with untreated HCC and non tumor liver tissue. Moreover, HCC-specific T-cell responses could be induced by monocytes activated with GM-CSF and incubated with thermally ablated HCC tissue (191). Tumor ablation by RFA induces effects important for boosting anti-tumor immune responses. Thus, tumor cell necrosis generates a permanent immunogenic source of tumor antigens, which are captured, processed and presented by dendritic cells for effective immunization without requirement for *ex vivo* antigen loading (192). These authors were also able to show increased IFN-g production and cytotoxic activity of NK cells 4 weeks after RFA. By dividing the patients into high and low responders these parameters gained predictive value on the efficacy of the ablative treatment. These data suggest the involvement of NK cells in tumor control after RFA (193).

Since hepatocellular carcinoma (HCC) recurs frequently after minimally invasive therapy, adoptive immunotherapy was considered helpful in lowering recurrence and metastasis rates. A combined therapeutic regimen for HCC patients, composed of transfusion of autologous RetroNectin activated killer (RAK) cells and radiofrequency ablation reported no severe adverse events, recurrences or deaths in all 7 HCC patients during a seven-month follow-up (194).

Univariate analyses of parameters in 20 HCC patients treated by RFA identified the number of TAA-specific $CD8^+$ T cells as a significant prognostic factor for recurrence-free survival (195). A similar extensive study by Mizukoshi and colleagues also suggested that RFA stimulates anti-tumor immunity. They analyzed immune responses before and after RFA in 69 HCC patients using 11 tumor-associated antigen (TAA)-derived peptides. An increase in the number of TAA-specific T cells occurred in 62.3.% of patients after RFA. The antigens and their epitope to which enhanced T cell responses occur were diverse, and some of them were newly induced. The number of TAA-specific T cells after RFA was associated with the prevention of HCC recurrence, and it was clarified to be predictive of HCC recurrence after RFA. The number of TAA-specific T cells after RFA was inversely correlated with the frequency of CD14+ HLA-DR(-/low) myeloid-derived suppressor cells (MDSCs) (196). Further evidence was presented that RFA might extend survival by reducing myeloid derived suppressor cells and thus promote anti-tumor immunity. The frequency of MDSCs in 123 HCC patients was significantly increased compared to patients with chronic hepatitis and healthy controls. The serum concentrations of IL-10, IL-13, and vascular endothelial growth factor were significantly increased in patients with high MDSCs and correlated with the frequency of MDSCs. In 33 HCC patients who received curative radiofrequency ablation therapy, the frequency of MDSCs after treatment showed various changes and was inversely correlated with recurrence-free survival time (197).

RFA, which is also applied for treatment of renal cell carcinoma (RCC) can cause changes in the peripheral blood lymphocyte population after RFA of RCC patients. In 5 out of 6 patients, the proportion of activated (CD3(+)DR(+)) cells increased over the whole follow-up period with the highest values in the second week after RFA, while the percentage of NK cells (CD56(+) CD16(+)) was decreased in most of the patients. The proportion of CD4(+) and CD8(+) lymphocytes changed but no consistent pattern was observed. In all patients, the changes were most pronounced 2 weeks after the procedure (198).

In a comprehensive study the activation of tumor antigen-specific antibodies, as well as antigen-specific CD4(+) and CD8(+) T cells was assessed in 49 patients suffering from various primary or secondary malignancies (CRC liver metastases, lung carcinoma, breast carcinoma, melanoma HCC or RCC), and treated by radiofrequency ablation with or without concomitant chemotherapy. An increase of antibodies (in 4 patients with CRC metastases, RCC or melanoma), CD4(+) T cells or CD8(+) T cells (in 2 patients of 49) could be detected several weeks to months following intervention (199).

RFA may promote anti tumor immunity by causing the release of danger signals, which promote dendritic cell maturation and function. To this end a significant increase in serum levels of heat shock protein 70 was detectable in a patient cohort with mainly CRC (liver metastases) 1 day after radiofrequency ablation. More than a twofold increase was observed in nine out of 22 patients, which tended to correlate with favorable clinical outcome. No patient of the control group revealed a comparable increase. Thus, elevated heat shock protein 70 serum levels may constitute a biomarker for favorable clinical outcome (200).

5.2.2. Laser and microwave ablation (MWA)

Thermal ablation by laser or microwaves of patients with various tumors yielded activation of anti tumor immunity, which was hence enforced by immunomodulating treatments.

Patients with advanced melanoma in which combined intratumoral injection of DC and local hyperthermia led to immunostimulation and decreased

tumor progression (201). Laser-induced thermotherapy (LITT) of colorectal cancer liver metastases induced a specific cytotoxic T cell response in patients. CD3+, CD4+ and CD8+ T cells triggered by autologous tumor tissue secreted elevated IFNg levels, and a significantly increased cytolytic activity of CD3+, CD4+ and CD8+ T cells after LITT against an allogeneic tumor (CaCo cell line) was observed (202). Li and collaborators applied photohermal therapy using lasers with the imiquimod immune adjuvant for *in situ* photoimmunotherapy (ISPI) of metastatic melanoma. The treatment induced a complete response in six out of eleven patients and resulted in a 12-month survival probability of 70% (203).

5.2.3. High intensity focused ultrasound (HIFU)

The immunological consequences of HIFU ablation were studied in preclinical and clinical settings. HIFU treatment triggered systemic cellular immune responses in cancer patients with posterior choroidal melanoma (204), late-stage pancreatic cancer (205), osteosarcomas, HCC and RCC (206). Clinical evidences suggest that HIFU treatment may also enhance local antitumor immunity in prostate cancer patients (207). HIFU upregulated expression of HSP70 in breast cancer tumor debris (208).

Immune cell infiltration, mainly APCs, was observed along the margins of the ablated regions in all HIFU-treated tumors, in breast cancer patients, and numbers of tumor-infiltrating DCs, macrophages and B lymphocytes increased significantly in the HIFU group (209). Furthermore, HIFU could induce significant infiltration of TILs in human breast cancer, including CD3, CD4, CD8, B lymphocytes and NK cells (210).

Immunosuppression in a patient with malignant tumor is a major obstacle in cancer treatment. Study on the effect of HIFU treatment on the circulating level of immunosuppressive cytokines in patients with malignancy revealed that HIFU could decrease tumor-secreted immunosuppressive cytokine production in addition to its direct tumor destruction. There were also significant decreases of VEGF, TGF-beta1, and TGF-beta2 before and after HIFU treatment. Compared with the values in the metastatic patients, serum levels of immunosuppressive cytokines were significantly lower in the non-metastatic patients after HIFU treatment. This change may lessen tumor-induced immunosuppression and renew antitumor immunity after HIFU in cancer patients (211).

5.2.4. Cryoablation

Treatment of cancer patients by cryoablation in combination with immunotherapy improved survival in various tumors. Either cryosurgery or topical imiquimod have been used to treat patients with lentigo maligna (a melanoma *in situ* that consists of malignant cells but does not show invasive growth) in cases where surgery is not feasible. A patient with lentigo maligna, who was treated with the combination of topical imiquimod and cryosurgery, showed Sustained clearance of lentigo maligna for 26 months after treatment (212). In another study percutaneous cryoablation of lung metastasis from renal cell carcinoma (RCC) in combination with aerosolized granulocyte-macrophage colony stimulating factor induced systemic cellular and humoral immune responses in patients. The treatment induced robust and brisk tumor specific cytotoxic T lymphocytes, specific *in vitro* antitumor antibody responses, and enhanced Th1 cytokine production in 4 of 6 patients. The magnitude of cellular and humoral antitumor response seems to be associated with clinical responses (213).

Cryotherapy and, especially, comprehensive cryosurgery (ablation) plus dendritic cell-cytokine-induced killer cell immunotherapy (cryo-immunotherapy) significantly increased overall survival in 45 patients with metastatic hepatocellular cancer. Multiple cryo-immunotherapy was associated with a better prognosis than single cryo-immunotherapy. After an 8-year follow-up Median overall survival was higher following cryo-immunotherapy (32 mo) or cryotherapy (17.5. mo; $P < 0.0.5$) than in the untreated group (3 mo) (214).

5.3. Electric based cancer ablation

High voltage electrochemotherapy (ECT) is currently used for the treatment of melanoma patients. In a phase I dose escalation trial patients with metastatic melanoma received ECT into metastatic melanoma lesions immediately after plasmid interleukin (IL)-12 injection. Post-treatment biopsies showed plasmid dose proportional increases in IL-12 protein levels as well as marked tumor necrosis and lymphocytic infiltrate. Two of 19 patients with no other systemic therapy showed complete regression of all non-electroporated distant metastases, whereas eight additional patients showed disease stabilization or partial response (215). To find if the destruction of the tumor by ECT stimulates immune response components, the presence of dendritic cells (DCs) in the inflammatory infiltrate of ECT-treated lesions from melanoma patients was determined. The data showed that ECT promotes LCs migration from the tumour to draining lymph nodes and plasmacytoid DCs and dermal DCs recruitment at the site of the lesion (216).

5.4. Photodynamic therapy

Photodynamic therapy (PDT) has become a well-established treatment modality, which has been shown to be effective and safe for many skin and mucosal disorders. Pre-clinical and clinical studies demonstrate that, in addition to the direct local cytotoxicity and vascular effects, PDT can induce various host immune responses.

As early as 2001 Abdel-Hady and colleagues contended that high-risk HPV infection and lack

of cell-mediated immunity may play a role in the observed poor response of lower genital lesions of vulval intraepithelial neoplasia (VIN) to topical PDT. They assessed immune infiltrating cells in VIN biopsies from responders and non-responders and found that compared with normal vulval skin, VIN lesions showed increased infiltration by CD4 (T-helper) and CD68 (macrophages) but not CD1a (Langerhans cells) or CD8 (CTLs). However, in PDT responders a significant increase of CD8 infiltration was observed post treatment compared with non-responders (217).

Clinical data show that improved clinical outcomes can be obtained through the sequential use of PDT and the immunostimulant, Imiquimod. Imiquimod can activate monocytes, macrophages, and dendritic cells by binding to Toll-like receptor 7 and 8 (TLR-7, TLR-8) on the cell surfaces. Women with high-grade VIN were treated with topical imiquimod and PDT sequentially and clinical response was assessed by measuring lesion size. The non-responders showed a significantly higher level of T regulatory cells in the lesions after imiquimod treatment, which may obstruct any possible anti-tumor immune responses (218). A summary and discussion of various clinical studies on PDT treatment for VIN and the clinical and immunological responses were also reported (219). PDT is also an effective treatment for non-melanoma skin premalignant and malignant lesions, and can be augmented by imiquimod to achieve better tumor control (220).

5.5. Chemical and biological ablation

The “immunogenic cell death” induced by certain cytotoxic drugs claims that immunostimulation occurs and facilitates the elimination of residual disease, as found in preclinical studies and discussed in Section 3.5. Yet, in a clinical setting it is difficult to demonstrate stimulation of anti tumor immunity after chemotherapy due to the immunosuppressive nature of many chemotherapeutic drugs. An indirect correlation between chemotherapy and immunostimulation was presented in a study performed by Tesniere and collaborators (221). They showed that the anthracycline, oxaliplatin (OXP) triggered the exposure of the danger signals, high-mobility group box 1 protein (HMGB1) and calreticulin (CRT) in a series of murine and human colon cancer cell lines. In patients with advanced (stage IV, Duke D) CRC, who received an OXP-based chemotherapeutic regimen, the loss-of-function allele of TLR4, reducing its affinity for HMGB1 was as prevalent as in the general population. However, patients carrying the TLR4 loss-of-function allele exhibited reduced progression-free and overall survival, as compared with patients carrying the normal TLR4 allele (221). Results in this line indicated that the progression-free survival of anthracycline treated patients is reduced in breast cancer patients bearing loss-of-function alleles of TLR4 or P2RX7. In contrast, loss-of-function TLR4 and P2RX7 alleles do not affect overall survival in non-small cell lung cancer (NSCLC) patients, irrespective of the administration and type of chemotherapy. The intrinsic characteristics of NSCLC which is highly chemoresistant and/or the drug of choice for treatment employed to treat this malignancy (cisplatin) may explain why two genes that affect the immune response to dying cells fail to influence the clinical progression of NSCLC patients (222).

6. COMPARISON OF IMMUNOSTIMULATORY EFFICACY OF DIFFERENT ABLATION MODALITIES

There is a lack of direct information on the relative efficacy of different ablation methods to stimulate anti tumor immunity. Most of the studies compared radiofrequency ablation with cryoablation or electric based treatments. Most of the studies were performed by ablation of normal liver tissue, and compared the resulting inflammatory reactions.

A review of the literature claimed that in cancer patients the immune responses elicited by cryotherapy, both cellular and cytokine, seem far greater than those produced by radiofrequency or microwave ablation, probably as a consequence of the peculiar mechanism of cell death of the former (disruptive necrosis) (223).

den Brok and collaborators directly compared the ability of radiofrequency and cryoablation to provide an antigen source for DC and compared this with an *ex vivo*-loaded DC vaccine. Their study revealed that upon tumour destruction by radiofrequency ablation, up to 7% of the total draining lymph node (LN) DC contained antigen, while after cryoablation the amount of antigen-loaded DC is almost doubled. Only few DC from the conventional vaccine reached the LN (137).

Multisystem injury, including acute lung injury, is a severe complication associated with hepatic cryoablation of 30% to 35% or more of liver parenchyma, but this complication has not been reported with RFA. Hepatic cryoablation, but not RFA, induced NF-kappaB activation in the non-ablated liver and lung and was associated with acute lung injury. Histologic lung sections from rats after cryoablation showed multiple foci of perivenular inflammation, with activated lymphocytes, foamy macrophages, and neutrophils. In animals undergoing RFA, inflammatory foci were not present. NF-kappaB activation was detected at 1 hour in both liver and lung tissue samples of animals undergoing cryoablation but not after RFA, and serum cytokine levels were significantly elevated in cryoablation versus RFA animals. Lung inflammation is associated with the thawing phase of cryoablation and may be related to soluble mediator(s) released from the cryoablated tissue. These findings correlate with the clinical observation of an increased incidence of multisystem injury, including adult

respiratory distress syndrome (ARDS), after cryoablation but not RFA (224).

Other preclinical studies compared the ability of different ablation methods to induce inflammation in normal swine liver. Different hepatic procedures (RFA, cryotherapy, hepatic pedicle ligation, and hepatectomy) were performed on the medial lobe of the liver (30% of the liver volume) of 23 domestic swine and systemic responses in terms of systemic inflammatory marker changes and end-organ functions were determined. During the early postoperative period, the systemic inflammatory marker concentrations (tumor necrosis factor-alpha and interleukin-1beta) in the RFA group were significantly lower than in the cryotherapy group. However, the increase in serum inflammatory markers and pneumonitis after RFA was substantial when compared with hepatectomy (225). A comparative study investigated whether there are different inflammatory and coagulative responses between cryoablation (CA), radiofrequency ablation (RFA), and laser induced thermotherapy (LITT) techniques applied on rat livers. Transaminase levels as well as the inflammatory response upon CA, as reflected by white blood cell count and IL-6 and IL-10 cytokine levels, were significantly higher than following RFA or LITT (226). The levels of the pro-inflammatory cytokines interleukin (IL)-1β and IL-6 were also significantly higher after cryotherapy (CRYO) and RFA compared with MTA, hepatic resection, or controls. Transitional zones produced after RFA were larger than those after CRYO or MTA, but no correlation was present with the amount of cytokines (227).

Evaluation of the safety and effectiveness of Electrolytic therapy (ECT) in comparison with radiofrequency ablation (RFA) was performed in tumor mimics created by injecting a gel into the pig liver. ECT produced predictable and reproducible necrosis in pig livers and was as effective as RFA at destroying a defined target lesion. After ECT but not RFA infiltrating lymphocytes often surrounded necrotic zones. A local inflammatory reaction after ECT may favor the development of a systemic immune response (228). Comparison of the effects of electrochemical treatment (ECT) and Radiofrequency ablation (RFA) in pigs, showed that both ECT and RFA were associated with a reversible increase in monocyte, C-reactive protein (CRP) and aspartate aminotransferase (AST) levels. There was no significant increase in interleukin-1β (IL-1β), tumour necrosis factor-α (TNF-α) and IL-6 (229).

It can be concluded that radiofrequency ablation induced weaker inflammatory responses compared with cryoablation electric based ablation or laser induced thermal ablation. Whether this is also manifested as a weaker anti tumor immunity induced by RFA was not yet proven.

7. SUMMARY AND CONCLUSIONS

In this article are reviewed and discussed preclinical and clinical studies on different ablation methods used to treat a large spectrum of tumors. As indicated, ablation has been utilized to destroy the tumors and as a result create inflammation and upregulate expression of immunomodulatory surface molecules and secretory molecules in the tumor, and its microenvironment.

The delivery of radiation therapy (RT) for cancer with intent to cure has been optimized and standardized over the last 80 years. External beam gamma radiation is the most prevalent *in situ* ablation treatment and its use exposed the “abscopal effect”. Both preclinical and clinical work emphasized the observation that radiation destroys the tumor and exposes it’s components to the immune response in a mode, which facilitates the induction of anti-tumor immunity or reinforces such a response. Different types of radiation such as gamma, alpha or particles can carry out this activity. Radiation may also directly affect the distribution and function of immune cells such as T ccelle, Tregs, and mononuclear phagocytes.

Heat based tumor ablation is also frequently used by elevating the temperature over a wide range (37°-80°) either in the whole body or locally in tumors. Physical measures such as radiofrequency, microwaves, high intensity focused ultrasound, and lasers are used to generate high temperatures which produce a wide range of effects in tumor bearing hosts and have been used in cancer therapy. Data from animal models and human patients indicate that whole body and locoregional high temperature treatment of cancer destroys tumoral tissue generating a local necrosis followed by marked inflammatory response. It also exerts many biological and therapeutic effects on immune competent cells and cytokines, and the immune effects may depend on the type of treatment. In the ablation range cancer cell necrosis dominates and tumor specific immunity is observed, an effect that may play an important role in the outcome of treatment. Tumor destruction can be also achieved by Cryoablation, which involves the use of freezing temperatures to kill cells and destroy tissue.

Electric-based cancer ablation was developed for *in situ* ablation of solid tumors. These range from measures of low electric currents or fields to high and very high electric fields with a similar intent to destroy the tumors and a similar result of induction of anti tumor immunity.

Photodynamic therapy (PDT) uses non-toxic photosensitizers and light in combination with oxygen to produce cytotoxic reactive oxygen species that kill malignant cells, and damage the tumor

microvasculature and create rapid dramatic changes in tumor microenvironment. PDT, which is used mainly for superficial tumors, induced inflammation following cell death, debris elimination and resolution of the inflammation.

Cytotoxic chemotherapy, the principal treatment modality for advanced cancer, may also be considered as an *in situ* ablation treatment. Certain conventional chemotherapeutic drugs cause cell death that can elicit a specific antitumor immune response driven by dendritic cells, by increasing tumor immunogenicity and by triggering 'danger signals'. Chemotherapy can also exert other immune modulatory effects on a number of immune cells such as regulatory T cells. Systemic treatment by biological agents, such as imatinib, which kill tumor cells, may also result in activation of anti tumor immunity, and this topic has not been studied yet extensively.

The results indicate that the immune response to local ablation treatments is complex, and potential combinations can be tailored to address both immune stimulatory and suppressive elements. It is therefore important to further understand the immune response that follows a local ablative treatment and the immune effector cells that are involved.

To day we have a more profound understanding of the function and intercellular interactions of immune cells. As noted anti tumor immunity requires the presence of tumor antigens, the proper MHC molecules, the involvement of antigen presenting cells and cross presentation mechanisms to trigger helper and cytotoxic T lymphocytes, and danger signals for proper activation of APC, and expression of costimulatory molecules.

The involvement of DCs and macrophages are critical to the initial response and to inducing T cell activity against other sites. A big effort was made to show that immunoadjuvants, cytokines and dendritic calls can take the weak responses triggered by ablation and promote them to effective tumor eradicating combination treatments. However, immune suppressive cells and molecules such as tumor-associated macrophages (TAMs), Tregs, MDSCs, and immunological checkpoint molecules can inhibit the response to local treatment and should be targeted. The results show that down regulation of the function of Tregs, MDSC and the use of immunological checkpoint blockade strengthened the anti tumor reactivity induced by *in situ* ablation.

An interesting and important question pertains to the relative capacity of the different ablation methods to induce anti tumor immunity, but very limited information can be found in the literature about it. Several studies compared radiofrequency ablation with cryoablation stating that cryoablation is better in inducing inflammation and might be a better immunostimulatory measure. Yet, the data is scares and more studies on this topic are required.

The overall conclusion is that the use of different ablation methods on different tumors resulted in most of the cases in stimulation of anti tumor immunity. The understanding of the interactions between ablation therapies and the immune system and the tumor microenvironment is crucial for the rational development of combination treatments of immunotherapy with conventional or targeted therapies to achieve a synergistic antitumor effect and improved treatment outcomes. Such information is also required in order to estimate the possible collateral damage of such treatment modalities.

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