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Non-Thermal Plasma-Induced Immunogenic Cell Death in Cancer: A Topical Review

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Abstract

Recent advances in biomedical research in cancer immunotherapy have identified the use of an oxidative stress-based approach to treat cancers, which works by inducing immunogenic cell death (ICD) in cancer cells. Since the anti-cancer effects of non-thermal plasma (NTP) are largely attributed to the reactive oxygen and nitrogen species that are delivered to and generated inside the target cancer cells, it is reasonable to postulate that NTP would be an effective modality for ICD induction. NTP treatment of tumors has been shown to destroy cancer cells rapidly and, under specific treatment regimens, this leads to systemic tumor-specific immunity. The translational benefit of NTP for treatment of cancer relies on its ability to enhance the interactions between NTP-exposed tumor cells and local immune cells which initiates subsequent protective immune responses. This review discusses results from recent investigations of NTP application to induce immunogenic cell death in cancer cells. With further optimization of clinical devices and treatment protocols, NTP can become an essential part of the therapeutic armament against cancer.

Cancer and conventional therapies:

Cancer development is partly attributed to cells acquiring multiple mutations that remain unrecognized by the immune system due to low immunogenicity of these mutated cells. Failure of the immune system to recognize cancer cells contributes to cancer progression by selecting for tumor cells that can survive in an immunocompetent host. Unchecked growth is further facilitated within the tumor microenvironment by the production of immunosuppressive factors by these cancerous cells, stromal cells, and local immune cells. [1-3]. To successfully check the development and progression of cancer, the innate and adaptive immune system must act together to identify and destroy mutated cells [1]. The innate arm of the immune system generates a rapid, non-specific inflammatory response on recognition of a "foreign" cell (mutated) which serves to initiate development of specific immune responses. The innate cells have the difficult job of differentiating between normal cells (self) and mutated cells (altered mutated self), often a key step in the failure of immune recognition. The adaptive arm of the immune system, on the other hand, allows for specific immunity to mutated cells via T cells, but takes days to weeks to develop. It is the T lymphocytes that selectively destroy cancerous cells that carry the specific mutation throughout the body. Furthermore, immunologic memory, the hallmark of the adaptive immune system, allows for a more rapid immune response upon re-exposure to a specific antigen that defines its "foreignness" [4].

Modern cancer treatment typically entails a multimodal approach involving a combination of systemic chemotherapies, surgery, and radiation; it is associated with a broad spectrum of inherent toxicities and in many cases, only modest patient survival. The evolution of immunotherapeutic treatment strategies has offered newer treatment modalities for cancer patients with better clinical outcomes. These strategies are based on methods that break immune tolerance of self-antigens (or 'altered mutated self') to

overcome ignorance and tolerance to potential antigenic targets on cancer cells as a way to destroy tumor cells by immunological mechanisms, hence improving selectivity and reducing toxicity[5, 6]. Recent historic advances in immunotherapy highlighted in this review underscore our continued quest for novel approaches to the treatment of cancer [7-11].

Non-thermal plasma in cancer therapy

Non-thermal plasma (NTP), also known as cold atmospheric plasma or non-equilibrium, atmospheric pressure plasma, has emerged as a promising anti-cancer approach. NTP is formed by the application of an electric field to ionize surrounding gas (ambient air or a specific gas), creating a discharge consisting of charged molecules, ultraviolet light, reactive oxygen species (ROS), and neutral molecules [12, 13] Mounting evidence demonstrates that NTP can change the oxidative status of cells through stimulation of intracellular ROS production [14, 15]. ROS are known to influence multiple signaling pathways regulating cell processes including proliferation, differentiation, and cell death [16]. Because NTP has the advantage of being a controlled source of incremental ROS, it is therefore being employed for the treatment of various diseases, including cancer. Two distinct methods are used to generate NTP [13, 15, 17-19]. Dielectric barrier discharge (DBD) devices generate plasma directly on the treatment target while plasma jets generate the bulk of the plasma remotely and utilize a gas, such as helium or argon, for transport of plasma components to the treatment target [13, 20-23]. The two main strategies employed for experimental, biomedical delivery of plasma involve (1) direct exposure of cultured cells *in vitro* or tissue *in vivo* or, more recently, (2) indirect exposure to plasma components using plasma-activated liquids in standard cell culture for *in vitro* studies or via perfusion of affected organs [13]. The anti-cancer effects of indirectly and directly applied NTP appear to be similar. Direct treatment exposes the

target tissue to all the components of plasma. Yan et al demonstrated that directly applied NTP induced an “activation” state, resulting in increased sensitivity of cells to ROS and RNS [24]. With indirect treatment, active species dissolve into a liquid when exposed to plasma from different sources. The “treatment dose” is determined by time of plasma-exposure of the liquid as well as the period for which the cells or tissue is allowed to remain in contact with this liquid. While the differences between the interaction of NTP with liquid media (indirect) or the skin (direct) are complex and not fully clear, an important distinction between the two treatment modalities is the presence of short lived reactive species such as OH during direct treatment [25]. These short lived species may trigger production of intracellular, long lived reactive species such as H_2O_2, potentially resulting in H_2O_2-mediated lymphocyte activation[26]. In a study comparing indirect and direct application of NTP against metastatic melanoma, Saadati *et al* demonstrated significantly greater cancer cell death and reduction in tumor growth with direct treatment [25]. In the same study, they recognized the potential advantage of plasma activated liquids for the treatment of tumors deeper than skin level in combination with chemotherapy [25]. Other studies have demonstrated that application of plasma-treated medium decreases peritoneal tumor burden in mice and prolongs survival [27-29]. Although direct treatment with NTP is more effective than indirect treatment, indirect treatment is believed to be associated with less toxicity [24-26, 30].

NTP, whether generated from DBD or jet-based devices, results in somewhat selective toxicity to cancer cells both *in vitro* and *in vivo* (Table 1), although the subject of selectivity is open to question. *In vitro,* anticancer efficacy of NTP has been demonstrated in several distinct, established cancer cell lines: brain cancer, skin cancer, breast cancer, colorectal cancer, lung cancer, cervical cancer, leukemia, head and neck cancer, and hepatoma (Table 1) [13, 31-44]. The majority (70%) of these studies employed jet devices

[13]. In a pioneering study demonstrating cancer growth inhibition *in vivo*, Vandamme et al. directly applied NTP to subcutaneous U87-Luc malignant glioma tumors in nude mice. They reported safety of their treatment protocol as well as anti-tumor activity [17, 45, 46]. Subsequent *in vivo* investigations looking at NTP for treatment of subcutaneous xenograft tumors or orthotopic melanomas in mice have consistently demonstrated tumor cell death/growth inhibition and increased animal survival [13, 37, 45-53]. Other mouse model studies have demonstrated anticancer capacity of plasma in bladder cancer [37], head and neck cancer [48], ovarian cancer [54], pancreatic cancer [47, 55], neuroblastoma [49], melanoma [51, 52], and breast cancer [50]. Local tumor regression was in seen in all cases, and in some cases, improved animal survival was documented. Since these studies employed different NTP devices, it is difficult to judge if the inconsistent animal survival is because of differences in tumor types or the nature and delivery of plasma generated. Furthermore, studies variously used immunocompetent or immunocompromised animals making it difficult to delineate the contribution of immune pathways in NTP efficacy. Finally, the mechanism of tumor cell death or the organismal level pathways activated were not investigated in depth in the aforementioned studies. These topics are subjects of ongoing studies in many labs around the world.

Anti-cancer mechanism of non-thermal plasma

It is fair to state that the anti-cancer mechanism of NTP is incompletely understood. The effects and importance of individual NTP components (radiation, electromagnetic fields, and reactive species) in cancer cell death continue to be investigated and it seems unlikely that any single component will emerge as the critical factor. Nevertheless, the direct anti-cancer potential of NTP has been ascribed to a rise in reactive species inside cancer cells, changing the cellular redox balance. Although there is evidence that other components of NTP contribute to its therapeutic role, the cocktail of RONS, both

originating from NTP and those produced by NTP-exposed cells, are postulated to be the most important active constituent for the effects on cancer cells [56]. The resulting challenge to the cancer cell's anti-oxidant response ultimately pushes the cell toward cell cycle arrest and apoptosis [13]. While other therapies function through a similar pathway, one of the primary advantages of NTP is selective toxicity against cancer cells by the ability to controllably deliver ROS such that there is relatively little impact on non-cancerous cells in the tumor microenvironment and the surrounding tissues. Although defining the mechanism of selectivity against cancer cells continues to be a key challenge in the field, it has been attributed to greater rise in ROS in cancer cells compared to normal cells [13, 57, 58].

Another challenge in the understanding of NTP mechanism of action is the penetration of plasma components in treated tissue. The physical effects of plasma are believed to be only a few hundred micrometers, but biological effects been observed several millimeters deep within tissue. The biological effects of NTP extend beyond the superficial layers, evidenced by significant shrinkage of subcutaneous tumors with diameters of ~ 1 cm following surface NTP treatment [17, 47, 49]. However, the specific plasma-produced RONS delivered to biological targets and the depth to which they are delivered remain uncertain. Furthermore, there remain questions as to how RONS interact with various components of the target tissue [59]. Experimental and simulation studies have shown that deeper in the tissue (greater than millimeters below the surface), most plasma-derived RONS are converted to more stable RONS with longer half-lives [60-64]. Since the initial interaction between plasma-derived RONS and the tissue remains largely unknown, the reasons why biological effects permeate beyond physical reach of NTP components remain a key area of research interest.

Recent studies suggest that immune activation may also contribute to NTP's anti-

cancer effects [1, 65-67]. In that context, the efficacy of plasma treatment, with respect to life-expectancy, was greater in studies performed on immunocompetent mice compared to those using immunodeficient mice. These particular findings warrant further investigations of the immunogenic capacity of plasma [1, 37, 49]. More recently, studies have demonstrated macrophage augmentation and immunogenic cell death in response to NTP-induced ROS and reactive nitrogen species (RNS) [65-70]. Importantly, Mizuno et. al. demonstrated abscopal effects of NTP on B16-F10 melanoma growth. This was evidenced by tumor growth suppression at sites distal to site of NTP treatment in CD2F1 and C57BL/6 murine models [71], further endorsing the immunogenic potential of NTP. It is important to reiterate that more in depth longitudinal studies of plasma interaction with cancers, especially in animal models, are critically needed to define the anti-cancer mechanism of NTP.

Recent advances in immunotherapy for cancer:

Cancer immunotherapy has gained popularity and momentum in recent years. In fact, the 2018 Nobel Prize in Physiology or Medicine was awarded to cancer immunotherapy researchers James P. Allison and Tasuku Honjo for their historic work on immune check-point inhibitors. Indeed, the ultimate goal of cancer immunotherapy is to induce immunologic memory against tumor cells. However, it remains challenging to induce immunologic memory against a tumor cell that continues to acquire mutations, thus changing its antigenic nature and allowing for immune escape. Allison and Honjo established that by inhibiting cellular “off” signals [i.e., cytotoxic T lymphocyte associated protein 4 (CTLA-4), which is vital for maintaining homeostasis during normal immune responses, and programmed death protein 1 (PD-1) of T lymphocytes (the foot soldiers of the immune system)], the T lymphocyte response would be unleashed to eliminate tumor cells. Today, ipilimumab, the antibody to CTLA-4, is an FDA approved treatment for

melanoma [9, 10] (Figure 1a). Furthermore PD-1 blocking agents (nivolumab, pembrolizumab, durvalumab, atezolizumab and avelumab) remove the brakes on T cells, allowing them to effect tumor regression and improved survival in patients with advanced and metastatic tumors [11] (Figure 1b).

Chimeric antigen receptors (CARs) are another novel class of drugs in the field of cancer immunotherapy. CAR is a fusion protein consisting of an antigen recognition domain and a T cell signaling domain. It is the antigen recognition domain that recognizes and binds tumor proteins to provide the specificity against the tumor being targeted. T cells can be genetically engineered in the laboratory to express specific CARs against cancer cells, resulting in direct, targeted anti-cancer immune response. The signaling domain initiates killing pathways once the antigen recognition domain is bound to the antigen – in this case, the cancer cell expressing the antigen. Two CAR T cell therapy agents are now approved for the treatment of hematologic malignancies for pediatric acute lymphoblastic leukemia (ALL) and adult diffuse large B-cell lymphoma (DLBCL) [72, 73] (Figure 1c).

While there have been significant breakthroughs made in the field of cancer immunotherapy, these agents have, at times, been subject to side effects and lack of success against all tumors. Many patients are refractory to these treatments and others develop resistance. They are also extremely expensive and time intensive to generate. Importantly, these advances provide strong support for the importance of harnessing the immune system for cancer control as a vital part of cancer treatment regimens. They provide a platform for continued development of novel immunotherapeutic strategies.

Rationale for use of physical methods in immunotherapy

In addition to these immunotherapeutic agents, more general, non-specific physical methods known to activate the immune system, including photodynamic therapy (PDT) and radiation therapy (RT), have been employed to treat cancer [1]. The anti-cancer properties of PDT are attributed to the combination of non-toxic photosensitizers and visible light, which, upon interaction with oxygen, produce cytotoxic ROS that kill malignant cells via apoptosis/necrosis, shut down tumor vasculature, and subsequently stimulate the immune system [74]. Immunogenicity of PDT is attributed to induction of acute inflammation and the release of cytokines/stress response proteins, with the resultant leukocyte influx contributing to tumor destruction and, importantly, immune memory [1, 74, 75]. RT is part of the traditional anti-cancer armament being used for tumor regression through direct cytotoxicity. In recent years, the immuno-stimulatory potential of RT, another redox stress-based therapy, in conjunction with other immunotherapeutic agents has been and continues to be investigated in ongoing clinical trials involving several types of cancer [1, 76, 77].

Immunogenic cell death-based immunotherapy and rationale for plasma-based immunotherapy

Immunogenic cell death (ICD), initially described by the Kroemer/Zitvogel model, is a form of programmed cell death that has the potential to stimulate the adaptive immune system [67, 78, 79]. Figure 2a demonstrates the proposed role of NTP in induction of ICD. Various anti-cancer agents, including select systemic chemotherapies, PDT, RT, high hydrostatic pressure, small molecules, and oncolytic viruses, have been described as having the capacity to elicit ICD. When ICD occurs, immunostimulatory molecules collectively called damage associated molecular patterns (DAMPs) are released from or displayed by the dying cell [78]. Figure 2b demonstrates the proposed mechanism of NTP-induced ICD. Several surrogate markers suggestive of ICD can be detected both *in vitro*

and *in vivo* [78, 79]. These surrogate markers, or critical DAMPs, include surface-expressed calreticulin (ecto-CRT), secreted ATP, high mobility group box 1 (HMGB1), heat shock protein 70 (HSP70), heat shock protein 90 (HSP90), and type I interferons (IFNs) [78, 80]. These molecules are harmless when inside the cell and perform vital functions for maintaining cellular integrity and function. They become immunogenic only when outside the cell, whether membrane-bound or released. Once externalized, DAMPs initiate an immunologic response by attracting innate cells, also called antigen presenting cells (APCs) (e.g., macrophages, dendritic cells) to the tumor. ATP and HMGB1 act as "find me" signals for APCs and chemotactically recruit APCs to the area of DAMP emission. Ecto-CRT acts as an "eat me" signal for APCs, promoting phagocytosis of DAMP-emitting cells and thus causing activation of APCs. The activated APCs travel to lymphatic organs and present tumor antigens to T cells resulting in the expansion of effector and memory T cells that are specific for that tumor [78, 80].

In vitro emission of DAMPs serves as a useful indicator of ICD. However, the gold standard for verifying the functional ICD notably requires a vaccination protocol involving immunocompetent animal models and syngeneic cancer cells [78] (Figure 3). This vaccination protocol involves *in vitro* exposure of malignant cells to an ICD inducer under evaluation. Following thorough washing of malignant cells to remove the ICD-inducing agent but not the DAMPs, these dying malignant cells are inoculated subcutaneously into a flank of immunocompetent mice. One week later, the mice are challenged with subcutaneous inoculation of the same living malignant cells in the opposite flank. The mice are then monitored for development of subcutaneous tumors at the challenge site. The proportion of mice that do not develop tumors at the challenge site reflects the immunogenicity of cell death induced by the ICD agent being tested [78]. NTP has now emerged as a *bona fide* ICD inducer. [67, 81]

Existing inducers of ICD have drawbacks as well as advantages. Although ICD-mediated immuno-stimulatory effects of radiation have been identified, there is little evidence of direct immune cell stimulation. To the contrary, immune cells are highly radiosensitive [1, 82]. Additionally, there is mounting evidence suggesting that RT can contribute to reversion of the tumor suppressive barriers of the tumor microenvironment [83]. Similarly, while PDT is an ICD inducer, it has also been linked to immunosuppression in mice and occurrence of late metastases in patients [84].

Similar to RT and PDT, NTP is an attractive candidate as an ICD inducer with the added benefit of stimulating, or at the very least, preserving immune cells. NTP for cancer treatment would compete in this landscape and the comparison is summarized in Table 2. NTP is unique in that it induces ICD in tumor cells and simultaneously stimulates immune cells at the same treatment regimen. Studies have demonstrated enhanced migratory activity in macrophages exposed to NTP *in vitro* [1, 66]. Furthermore, increased macrophage-mediated, tumor cell killing of radioresistant, human CNE-1 nasopharyngeal carcinoma cells subsequent to NTP exposure has been demonstrated [1, 65]. Thus, NTP may confer a significant advantage over RT, PDT, and other ICD inducers that have demonstrated concomitant immunosuppression. Further work is required to confirm and characterize this direct immunostimulatory effect of NTP within plasma-exposed tumors.

***In vitro* and *In vivo* data demonstrating NTP-induced ICD**

NTP-induced ICD has been investigated *in vitro* in lung cancer and nasopharyngeal carcinoma via detection of DAMPs, which are surrogate markers of ICD. Specifically, intracellular ROS-induced expression of DAMPs (ecto-CRT and ATP) was shown in human A549 lung carcinoma cells and human CNE-1 nasopharyngeal carcinoma cells [65, 68]. In addition, there was simultaneous activation of anti-tumor

effects of macrophages. This was evidenced by trans-well experiments demonstrating increased CNE-1 cell loss when CNE-1 cells were co-cultured with plasma-exposed macrophages [65]. Tumor cell toxicity and immunogenicity were also demonstrated following exposure to NTP in cultured murine metastatic B16F10 melanoma cells [70]. In this study, NTP treatment resulted in decreased metabolic activity, decelerated cell growth, and increased cell death. Immunogenicity was confirmed by expression of surrogate ICD markers, including major histocompatibility complex I, ecto-CRT, and melanocortin receptor I [70]. ICD resulting from NTP treatment has also been demonstrated *in vitro* in CT26 colon cancer cells. These cells were found to undergo apoptosis accompanied by calreticulin externalization upon exposure to NTP [69]. Studies with newer plasma devices and diverse treatment regimens are ongoing in many laboratories around the world to induce ICD in different tumor cell types.

In vivo investigations of the anticancer capacity of NTP have been undertaken for brain cancer, skin cancer, breast cancer, colorectal cancer, lung cancer, cervical cancer, leukemia, head and neck cancer, and hepatoma [12]. The role of ICD in plasma-mediated cancer immunotherapy has been specifically investigated in multiple studies using murine models. These studies have introduced a novel paradigm of plasma onco-immunotherapy to engage the immune system via ICD induction. In the seminal study demonstrating NTP-induced ICD *in vivo,* a CT26 murine colorectal tumor model was employed [67]. The generation of DAMPs (ATP and externalized-CRT) along with recruitment of APCs to the tumor area was clearly demonstrated. Most importantly, the capacity of NTP to function as a *bona fide* ICD inducer using the prescribed vaccination protocol was demonstrated. This vaccination assay demonstrated protective immunity against CT26 tumor challenge in Balb/c mice immunized with NTP-treated CT26 cells. Ninety percent of the mice in the plasma-immunized group had tumor volumes smaller than the mean tumor volume of the

control group. Furthermore, three of the ten mice in the plasma-immunized group did not develop subcutaneous tumors at the challenge site, signifying complete protection [67].

This vaccination protocol has also been successfully employed in a melanoma mouse model [85]. C57BL/6 mice were vaccinated with melanoma cells treated with DBD plasma. Of the eight mice in the NTP-treated group, five were completely protected against challenge with live tumor cells, comparable to cells treated with a known ICD-inducer (Mitoxantrone) [85]. Furthermore, it was demonstrated that the observed ICD-inducing effects of DBD plasma were from the short-lived ROS (e.g. OH, NO, O/O) produced, and not from more persistent species (H_2O_2, NO_2^-, NO_3^-, $ONOO^-$). Vaccines created with equivalent amounts of persistent species did not significantly improve protection of mice against tumor challenge (37.5%) compared to the negative control group (25%) [85]. These two *in vivo* studies offer conclusive evidence that direct application of NTP induces ICD in multiple cancer cell lines of different tissue origins. Similar observations have been reported with the perfusion of plasma-activated liquids in the abdominal cavities of mice bearing metastatic pancreatic cancer masses [27, 53]. Here, the effect is largely due to the more stable species, in contrast to the observations of Lin *et al* [85]. In-depth, mechanistic studies and longitudinal studies to determine the effect on disease progression/remission and animal survival are needed. Further investigations into plasma delivery and administration protocols with other cancer-cell lines are also warranted for potential clinical translation.

Clinical translation of NTP

Modern approaches to cancer treatment include multimodality strategies that typically involve combinations of surgery, radiation, systemic chemotherapy, and more recently, immunotherapy. These therapeutic modalities are frequently associated with minimal or incomplete treatment responses, disease progression/recurrence, inherent

toxicity, and limited patient survival. NTP provides an important platform, rationale, and applicability in the arena of cancer treatment. We propose several avenues for using NTP alone as a cancer therapy or in combination with currently available therapeutic strategies.

Previous applications of NTP in oncology have focused on direct tumor cell killing, ignoring NTP's impact on host immunity. Future studies will extend recent observations to further elucidate the immunogenic cascade and therapeutic response induced in the host in response to NTP treatment. If successful, NTP can provide a safer alternative or adjunct to existing ICD mediators, such as RT, chemotherapy, and PDT. NTP used in conjunction with current therapeutic strategies may synergistically increase antitumor responses, hence decreasing therapeutic levels of chemotherapy administered (dose sparing) while converting ICD transforming "cold" metastatic lesions into inflammatory "hot" spots prone to immune recognition and tumor immunity. The therapeutic potential of NTP is particularly promising in the treatment of cancers refractory to conventional therapies due to development of resistance.

Another challenge faced by the clinician is the all-too-often clinical scenario of patients with unresectable or borderline resectable locally advanced tumors. We propose that NTP can potentially transition/downstage these tumors to resectable tumors making patients better candidates for operative removal. To accomplish this, NTP can be directly applied to tumors both by laparoscopic and robotic as well as open laparotomy approaches for *in situ* delivery and treatment. Additionally, NTP may be used to directly treat microscopic positive surgical tumor margins in order to prevent local disease recurrence. Moreover, after surgical resection, NTP may also be applied indirectly to treat clinically occult micro metastatic peritoneal and serosal disease bearing surfaces to halt significant disease progression. Likewise, indirect application may be used in the case of peritoneal metastases whereby NTP-treated peritoneal fluid is infused into the peritoneal cavity by placing indwelling catheters, similar to application frequently used for delivery of

intraperitoneal chemotherapy. This expanding treatment paradigm may also be applied similarly to treating recurrent disease. As our understanding of mechanism of NTP action improves, so too will the clinical applications available for the use of NTP in cancer therapy.

Conclusion

Herein we have summarized the therapeutic potential of NTP application for cancer immunotherapy via ICD. Previous applications of NTP in oncology have focused on direct anti-cancer (ablative) approaches, ignoring the potential impact of plasma therapy on host immunity. NTP-induced ICD can promote lasting immunity against tumor antigens. Several animal studies have, in part, elucidated the mechanisms and potential efficacy of this approach. Further evaluation of mechanism of action of NTP, optimization of NTP devices, clinical delivery systems, and treatment protocols, will facilitate application of NTP-induced ICD to the clinical setting.

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In Vivo	In Vitro	Plasma Source Used	Article
Benign Melanocytic Tumors		Ar Plasma Jet	Yajima et al
Bladder Ca		He Plasma Jet	Keidar et al
Breast		He Plasma Jet	Mirpour et al
	Breast	He Plasma Jet	Mirpour et al
	Breast	He Plasma Jet	Wang et al
	Cervical	Microplasma Jet Device	Tan et al
	Cervical	Plasma Jet with Air Flow	Ahn et al
	Colorectal	Ar Plasma Jet	Bekeschus et al
	Colorectal	FE-DBD	Vandamme et al
	Colorectal	He Plasma Jet	Ishaq et al
Colorectal	Colorectal	DBD	Lin et al*
Glioblastoma		FE-DBD	Vandamme et al
	Glioblastoma	DBD	Kaushik et al
	Glioblastoma	Indirect PAM treated with Ar gas flow	Tanaka et al
Glioblastoma	Glioblastoma	FE-DBD	Vandamme et al
	Hepatoma	Microplasma Jet Device	Tan et al
	Hepatoma	He Plasma Jet	Zhao et al
	HNSCC	He Plasma Jet	Guerrero-Preston et al
HNSCC	HNSCC	He/O_2 Plasma Jet	Kang et al
	Leukemia	Atmospheric Air Plasma System	Thiyagarajan et al
	Leukemia	DBD	Thiyagarajan et al
	Lung	DBD	Lin et al*
	Lung	He Plasma Jet	Keidar et al
	Lung	Microplasma Jet Device	Kim, JY et al
Melanoma		DBD	Chernets et al
Melanoma		DBD	Lin et al*
Melanoma		N_2/O_2 Plasma Jet	Mizuno et al
	Melanoma	Ar Plasma Jet	Bekeschus et al
	Melanoma	DBD	Kim, G et al
	Melanoma	FE-DBD	Fridman et al
Melanoma	Melanoma	He Plasma Jet	Keidar et al
	Neuroblastoma	He Plasma Jet	Walk et al
Ovarian	Ovarian	Indirect PAM treated Ar Plasma Jet	Utsumi et al
Pancreatic		He Plasma Jet	Brulle et al
	Pancreatic	He Plasma Jet	Brulle et al
Pancreatic	Pancreatic	Direct Ar Plasma Jet, Indirect PAM treated with Ar Plasma Jet	Liedtke et al*
Pancreatic	Pancreatic	Ar Plasma Jet	Pertecke et al

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Table 1: Studies Investigating Anti-Cancer Potential of Non-Thermal Plasma (NTP)

A list of *in vivo* and *in vitro* studies reviewed, including type of cancer investigated in each study.

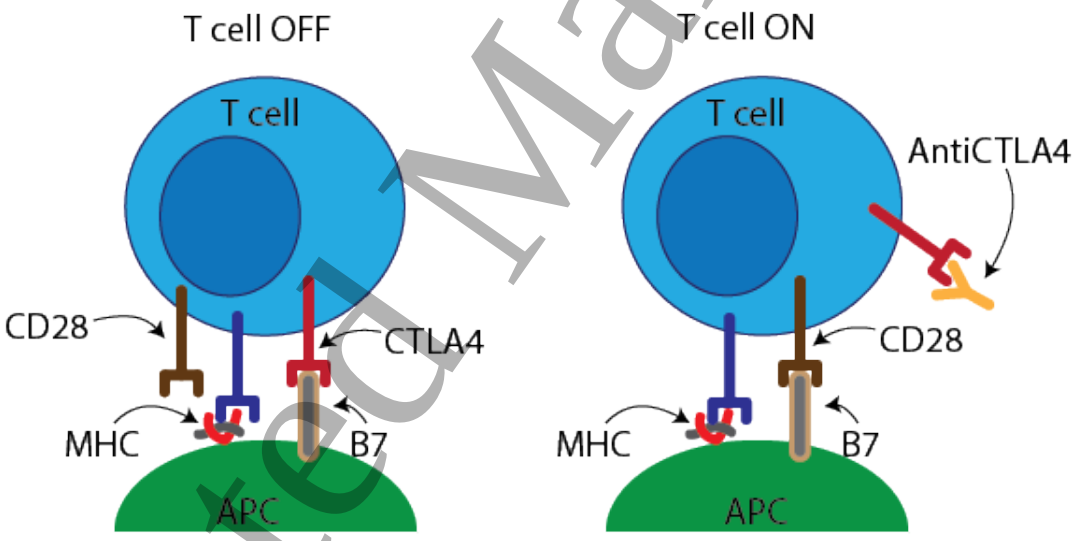
* indicated studies investigating NTP-induced immunogenic cell death (ICD).

NTP- non-thermal plasma, RT- radiation therapy, PDT- photodynamic therapy

	NTP	**RT**	**PDT**
Targeted	Yes	Yes	Yes
May require Invasive procedure for application	Yes	No	Yes
Side effects	Unknown No major side effects reported	Skin changes, second cancer, site specific side effects caused by damage to nearby organs	Skin changes
Mechanism of action	Oxidative stress [13]	DNA breakage [82]	Oxidative stress, damage to tumor blood vessels [74]
Depth of effect	Superficial	Deep	Superficial
Causes ICD	Yes [66]	Yes [76]	Yes [74]
Direct effects on immune cells	Preservation/stimulatory [1]	Suppressive [82]	Suppressive [84]

Table 2: Comparison of Nonthermal Plasma vs Radiation Therapy vs Photodynamic Therapy

Fig 1: Current Immunotherapy Approaches: When the T cell receptor encounters a foreign antigen presented in the MHC cleft of an APC, several checks and balances are put in place to avoid inappropriate immune responses. T cell proliferation and activation occurs only if the next step of CD28 (a co-stimulatory molecule) binding with B7 ensues. CTLA4 is another natural ligand for B7 but this binding does results in T cell inhibition. It is the fine-tuned balance between CD28/B7 and CTLA4/B7 that determines the activated (T cell on) and inactivated (T cell off) status and hence the nature of immune responses. PD1 is another negative regulator of T cell function that functions later during an immune response. Another approach being used in the clinic is the production of genetically engineered T cells (CAR T cells) that are designed to attack specific tumor antigens. (MHC, major histocompatibility complex; APC, antigen presenting cell)



1a: Cytotoxic T Lymphocyte Associated Protein 4 (CTLA4) Blockade

CTLA4 functions as a checkpoint during immune responses to foreign antigens by sending inhibitory signals and down regulating T cell responses. This function is necessary for preventing autoimmunity. Normally, T-cell activation is attenuated by the regulatory mechanism of CTLA4/B7 interaction (T cell OFF). Release of negative regulation by using anti-CTLA4 antibodies is achieved by making CTLA4 unavailable for B7 interaction (T cell ON).

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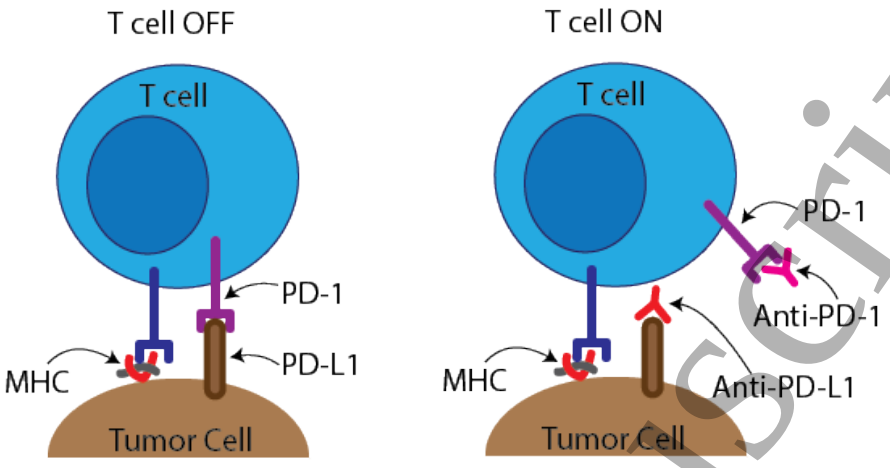
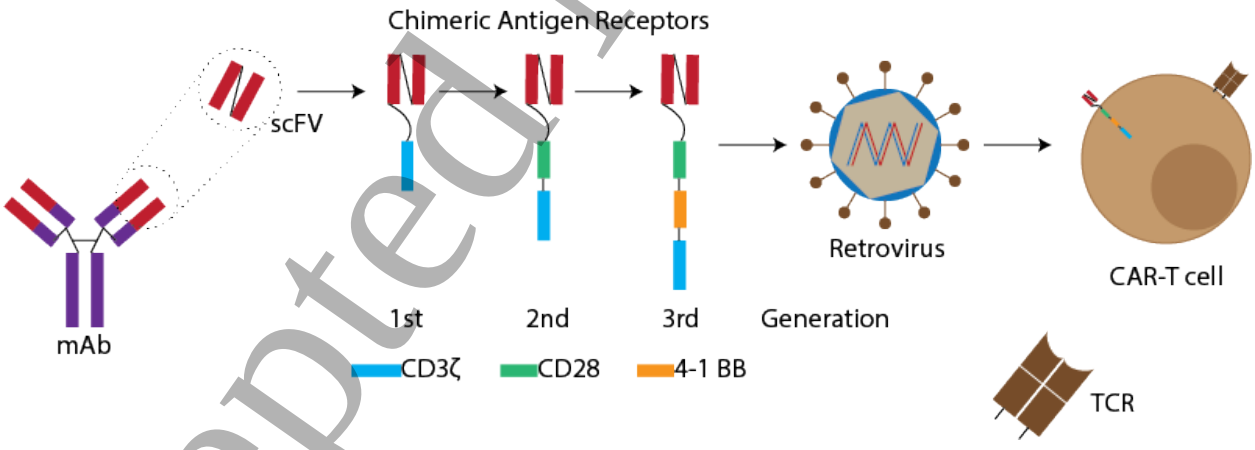


1b: Programmed Death 1/ Programmed Death – Ligand 1 Blockade

T-cell proliferation and activation is attenuated by the normal regulatory mechanisms of PD-1/PD-L1 interaction (T cell OFF). PD1 expression is a hallmark of T cell exhaustion. When this interaction is blocked by antibodies, these inhibitory signals are overcome and the T cell becomes functional again (T cell ON).



1c: Chimeric Antigen Receptor-T Cell Production

CARs are fusion proteins consisting of antigen recognition domain and T cell signaling domain. T cells can be made to express CARs, resulting in a targeted anti-cancer immune response. The antigen-recognizing variable regions (scFVs) are from monoclonal antibodies (mAB) with an intracellular T-cell signaling domain. The CD3 ζ chain of the T-cell receptor (TCR) was used in

first generation receptors. Second and third generation receptors utilize costimulatory signals such as CD28 or 4-1 BB (which is derived from the TNF family). CARs are then inserted into T-cells via a retrovirus-mediated gene transfer.

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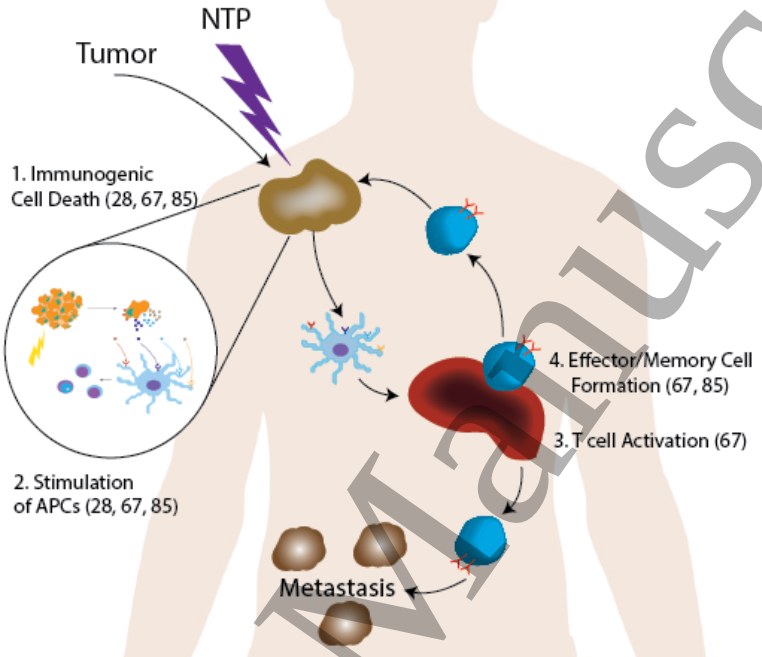

Figure 2a: Potential Action of Non-thermal Plasma (NTP) in Cancer Immunotherapy

NTP via proposed exposure both directly stimulates immune cells and induces immunogenic cell death. Immunogenic cell death (Ref 28, 67, 85) results in recruitment and stimulation of APC (antigen presenting cells) (Ref 28, 67, 85), memory cell formation, and T-cell development (Ref 67, 85). These circulating cells can then target other non-NTP exposed metastatic tumors of the same origin.

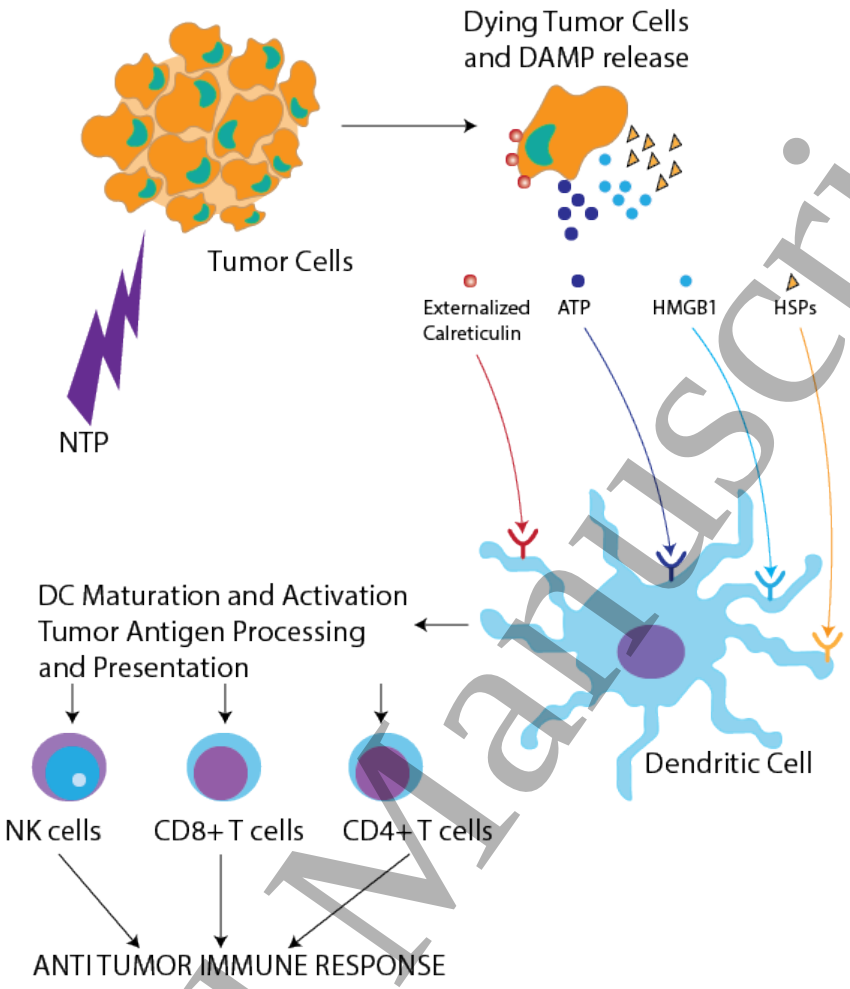

Figure 2b: Proposed Mechanism of Non-thermal Plasma Induced Immunogenic Cell Death (ICD)

Immunogenic cell death is a specific cell death mechanism that stimulates cells of the adaptive immune system. When ICD occurs, damage associated molecular patterns (DAMPs) are emitted; calreticulin is externalized on the cell membrane and ATP, HMGB1, and HSPs (heat shock proteins 70 and 90) are released by the dying cell (Ref 65, 67, 68, 69, 70). These DAMPs then initiate an immunologic response by attracting innate dendritic cells to the tumor. DAMPs also aid in the maturation of antigen carrying dendritic cells, resulting in a tumor-specific immune response by activated T cells (Ref 28, 67).

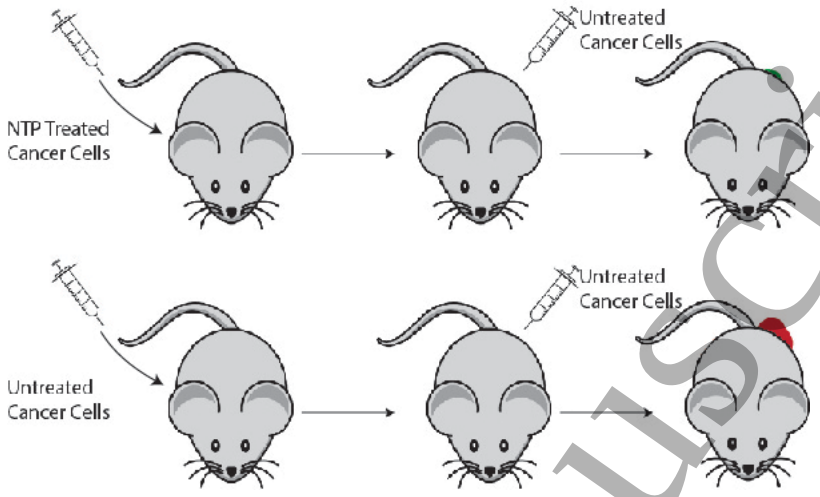

Figure 3: Validation of Non-Thermal Plasma-Induced (NTP) ICD Using a Vaccination Protocol

Vaccination protocol demonstrating protective immunity against a tumor challenge in mice immunized with non-thermal plasma (NTP) treated tumor cells. Vaccination with tumor cells undergoing ICD causes development of a robust, protective immune response against the specific tumor in the animals. When challenged with live tumor cells at a different site, none or smaller tumor nodes should be observed, if the vaccination was successful. In mice immunized with NTP treated tumor cells, several animals developed no tumors. Of those that did, a significantly smaller mean tumor volume at the distal challenge site was observed as compared to the control group.