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Topical Review

Plasma, cancer, immunity

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Abstract

Albeit heavily investigated for several decades already, the importance of the immune system in targeting cancer has received wide clinical attention only in recent years. This is partly because of long-standing rather traditional concepts on tumor biology on the one hand and the complexity of the immune system and its processes on the other. The viewpoint of evaluating existing and emerging approaches in oncology based on toxicity to tumors and the ability to engage antitumor-immunity is gaining ground across several disciplines. Along those lines, cold physical plasma was suggested as potential anticancer tool more than a decade ago, but solid evidence of the immune system playing a role in plasma cancer treatment only emerged in recent years. Moreover, plasma may support cancer immunotherapies in the future. Cancer immunotherapies are systemic treatments with biologicals that were reported to synergize with existing local physical modalities before, such as radiotherapy and photodynamic therapy. This review outlines key concepts in oncology, immunology, and tumor therapy, links them to plasma research, and discusses immuno-oncological consequences. Finally, promising future clinical applications are summarized. Synoptically, first scientific evidence supports an immuno-oncological dimension of plasma cancer treatment in selected instances, but robust clinical evidence is still lacking. More basic and clinical research is needed to determine the immuno-molecular mechanisms and detailed plasma application modalities to facilitate real patient benefit in the long term.

Keywords: cold physical plasma, immunity, oncology, reactive oxygen species, redox biology, T-cells

(Some figures may appear in colour only in the online journal)

1. Introduction

Cancer is a highly prevalent and often fatal disease. In 2020, an estimated 19.3 million new cancer cases and almost 10 million cancer deaths occurred [1]. The burden of cancer incidence and mortality is growing worldwide, and the World Health Organization described cancer as the first to second leading cause of death before the age of 70. High mortality in male patients is most often caused by lung cancer, followed by prostate cancer. In women, breast and cervical cancer are leading causes of cancer death. In addition to the overall frequency of different tumor entities, the ability of tumors to metastasize and their aggressiveness are linked to poor clinical outcomes and shorter life expectancy [2, 3].

A specific set or combination of therapies is suggested for each type of cancer, consisting of a single or combined use of surgery, chemotherapy, radiotherapy, or immunotherapy. Yet, not all tumors respond to therapy [4]. This is due to, for instance, the tumor's genetic and functional heterogeneity and

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its need for individual treatments. In addition, the human (and animal) body has an inbuilt system to address the individual's defense needs in a spatiotemporal fashion, the immune system. Lately, the importance and understanding of the immune system in tumor control has increased, as it is involved in both cancer development and demise [5].

Some processes during tumor growth and the immune response are regulated by reactive oxygen species (ROS), which stimulate cells at low concentrations and contribute to cell death in higher concentrations [6] (see section 2.3). Cold physical plasmas generate ROS and have been used for medical purposes, including skin disinfection and treatment of chronic wounds in the clinics since 2013 [7] and within clinical evaluation studies since 2009. Despite a few early and optimistic viewpoints [8], utilizing ROS as anticancer agents has always been deemed too unspecific and general to induce durable responses by the biomedical community when applied in a pharmacological sense systemically [9]. However, local and high levels of ROS, especially when complemented with other types of therapies, have achieved compelling medical success, as evident for decades already using photodynamic therapy (PDT), for instance [10]. Considering the widespread reports on the immunomodulatory action of PDT [11], it was and is plausible to consider immuno-oncological effects of plasma treatment, too. However, PDT's success depends on oxygen in the tumor micro milieu to generate radicals, and therapy fails without tissue oxygenation [12]. In contrast, cold physical plasma creates a plethora of reactive species on its own, for instance, if admixing oxygen to the feed gas of plasma jets [13].

Notwithstanding, history teaches that there is no one-size-fits-all solution in oncology. Clinical plasma antitumor effects will likely depend on many factors, and the amplitude of contribution of each of them is unclear. For instance, some aspects depend on tumor features, such as cancer type, stage, and mutational load, while others are linked to the plasma generation, such as parameters, excitation frequency, electrode geometry, feed gas utilized, or total ROS generation potency, for example. Albeit there is a general agreement that—in principle—plasma sources achieve similar biomedical effects, these will still be reached on different spatial (treated area) and time (exposure) scales for each device. This is meaningful for oncology if plasma cancer treatment is to be combined with standard oncological therapy schemes (see section 4), which also depend on factors like the patients' immune fitness [14]. Having said that, evidence is accumulating that plasma affects anticancer immunity [15, 16]. In the present work, the primary hallmarks and mechanisms, as well as potential links between plasma, cancer, and immunity are outlined. Additionally, the main routes and findings of immunostimulation in plasmas cancer treatment are summarized.

2. Cancer

Albeit it is tempting to subsume all known malignancies under the term *cancer*, cancer is a dreadfully heterogeneous disease. This is reflected in the circumstance that approval of new cancer therapies is always achieved only for the specific type of cancer investigated in the respective clinical trial. Later, approval can be extended to other types of cancers, but this again requires extensive clinical research. Moreover, comprehensive molecular characterization of individual tumors, along with documented therapy failure in the same patient (metastasis therapy resistance), exemplifies the high level of tumor heterogeneity in cancer patients [17]. To this end, modern oncology aims at predicting patient-specific cancer treatment that considers the individual tumor genetics and the immune status [18–20]. Another goal is time-resolved tumor characterization, i.e. re-defining the tumors of the same patient after therapy failure for subsequent adjustments. Yet, there is considerable genetic heterogeneity within a single tumor of a patient. The issue of tumor heterogeneity will not be discussed in detail here, and the interested reader is referred to eminent reviews on this topic [21, 22]. However, this issue should be kept in mind when discussing existing and new cancer therapies and antitumor immunity, considering the highly dynamic nature of these processes and the inability of most single treatment approaches to provide a cure to a broad set of malignancies and patients.

2.1. Characteristics

Malignant neoplasms can originate from different types of cells in various organs in the body, e.g. from the blood-forming bone marrow and lymphatic tissues such as leukemia and lymphoma. Others, such as adenocarcinomas, squamous cell carcinomas, malignant tumors of the transitional epithelium (urothelial carcinomas), and small cell carcinomas in the lungs, have their origin in the inner and outer surfaces of the body (epithelia). In addition, malignant tumors can also have their origin in the connective and supporting tissue of bones and joints (e.g. sarcomas), in the supporting cells of the nervous system (gliomas and neuroblastomas), or in pigment-forming cells (melanomas) [23].

Cancer cells have slight but significant genetic differences compared to non-malignant cells, and each person's cancer has individual genetic alterations. An estimated 5%–10% of all cancers are caused by an inherited genetic mutation (hereditary). The others emerge by spontaneous mutations that accumulate throughout life that are generated randomly or via intrinsic or extrinsic factors (e.g. UV light, genotoxic and carcinogenic molecules, and chronic inflammation) [24–26]. Humans have 23 chromosomes, which are present in each cell in duplicate. Most cancers develop when the same genes of both chromosomes are mutated (two-hit hypothesis) [27]. This means two rare mutation events are necessary for cells to become cancerous. Genes control how cells function, drive metabolism, and grow and divide. Changes in those genes, which carry the instructions to make proteins, can cause a wrong regulation or faulty instruction, leading to dysfunctional proteins or signaling pathways. Those mutations are one of the hallmarks of cancer [28], as they result in less controlled or uncontrolled cell division and promote growth (figure 1).

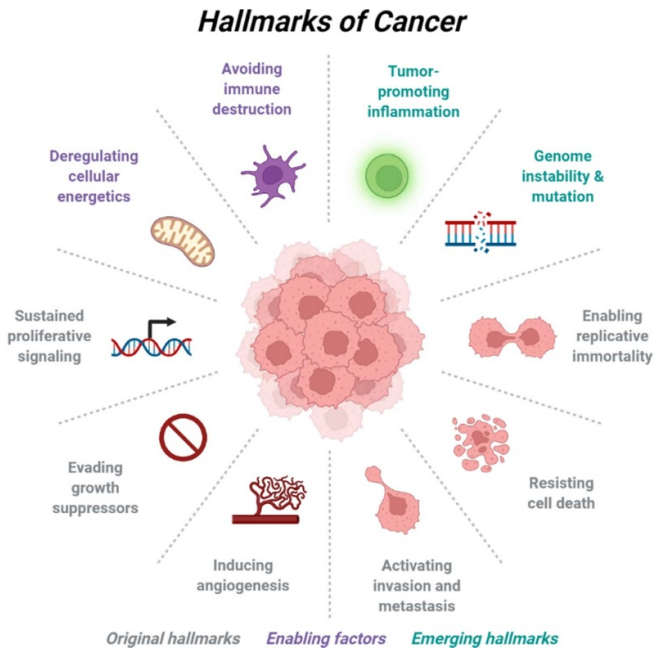

Figure 1. Hallmarks of cancer from based on [28].

For instance, cancer-causing gene mutations can increase the production of proteins that actively spur cell growth [29]. Other mutations may cause non-functional proteins otherwise responsible for repairing DNA damage, resulting in accumulating DNA damage, mutations, and excessive cell proliferation. Further discussions on molecular details are not the scope of this work, and the presence of various proto-oncogenes and tumor suppressor genes in different ethnic tumor groups has been adequately summarized for the interested reader [30–32]. Notably, spontaneous gene mutations occur frequently during cell proliferation, but regulatory processes control cancer development through repair mechanisms and the detection of individual degenerated cells. With about five DNA double-strand breaks per cell replication, the body faces about three Mio DNA double-strand breaks per second, corresponding to 1.5–2 gray of ionizing radiation [33]. The numbers illustrate that by chance, harmful mutations are not the exception but the rule and that the vastly active DNA repair machinery safeguards the body from its proliferative capacity on a daily basis [34].

Once a cancer cell grows and forms a bulk of cells (tumor), there often is an intrinsic risk of developing metastases. Herein, tumor cells leave their primary tumor bulk site and spread in a complex and highly selective process via blood and lymph vessels to other bodily sites.

For this to work for the tumor, the tumor cells need to be genetically unstable with, e.g. DNA mutations, chromosome rearrangements, and epigenetic changes to promote such metastasis via dysregulation of migratory proteins, for instance [35]. Other such characteristics, also called hallmarks of cancers, are, for example, relative resistance to regulated and inducible cell death, unrestrained replicative potential, deregulated cellular energetics and metabolism, and evasion of immune control. In the latter, tumor cells actively downregulate all phases of the anti-tumor immune response by utilizing a spectrum of different strategies and mechanisms [36]. These mechanisms counteract the detection by and action of the immune system, which usually recognizes and eliminates tumor cells based on their degenerated and pathological character. In addition, there are other historic and emerging hallmarks of cancer, which have been extensively described elsewhere [28].

The tissue in which the tumor is embedded (or has embedded itself into) is the so-called tumor microenvironment (TME) (see section 3), a complex network consisting of tumor cells, stromal cells, and (infiltrated) immune cells, among others [37]. It is hoped that the mentioned gene mutations in cancer cells, metabolic changes, growth behavior, single-cell migration, metastasis, and TME help characterizing and describing tumors based on cellular and molecular features to predict the success of the therapy to a certain degree in the future. Experimentally, especially in experimental rodent tumor models, the feasibility of characterizing and describing the correlating TME composition with survival prognosis and therapy success is evident [38–40]. The same has been shown to a great degree for many types of human cancers within dedicated studies [41–43]. Still, highly detailed molecular TME fingerprinting requires expensive technologies, with many of them not approved for diagnostic purposes. In pathology institutes of hospitals worldwide, chromogenic staining of ultrathin tumor biopsies slices and microscopic investigation by highly experienced examiners have been the gold standard for decades already. The examiner discriminates tumor tissue from normal tissue by classifying cells and describing the degree to which tumor cells differ from healthy cells (grading). The less differentiated the cancer cells are within a tumor, the less it resembles normal tissue and the more malignant it is. Aggressive, undifferentiated tumors can grow faster, metastasize faster, or recur more quickly [44]. This is done for diagnosis in a process called tumor staging. Medicine divides cancers into four stages based on their progress and ability to penetrate and invade into healthy, non-malignant tissue. All tumors start at stage 0, when the first tumor mass forms. Most stage 0 tumors are small and often remain undetected. They can but not have to progress through stages I–IV. How long this will take depends on the type of tumor, its mutational load, the patient's genetics, and the amplitude of the other cancer hallmarks' presence. Different therapeutic approaches are taken depending on the tumor type, grade, stage, localization, and original site of cancer development. In the following, only the main therapeutic modalities are described.

2.2. Therapeutic approaches

The main therapeutic approaches are briefly outlined (figure 2) and summarized below (more complex explanations, other therapeutic options, examples, and therapeutic success can be found in detail elsewhere [45–47]). Essentially, a given therapy can be applied locally and specifically to the tumor site. By contrast, a given agent can be injected, usually into the bloodstream, and is thus active within the entire body, which

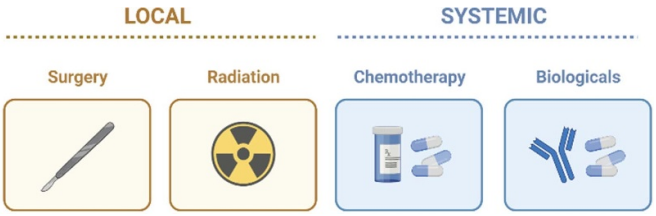

Figure 2. Local and systemic anticancer therapies.

is called systemic treatment. Systemic treatments also affect tumor metastases while usually having more side effects, whereas local treatments are generally well tolerated but limited in terms of metastases reach.

An early cancer diagnosis, often defined by a tumor stage of 0 or I–II, is often associated with a high survival rate (for some cancer types, nearly 100% [48]). Much fewer therapies are successful in many patients with advanced disease courses (stages III and IV). In particular, treatments applied locally, such as surgical removal, are often not complete or curative if metastases have spread across different bodily sites. Frequently, combination therapy is used, for example, chemotherapy or immunotherapy before (neo-adjuvant) or after (adjuvant) surgical removal of the primary tumor or tumor nodes. Another challenge to achieving promising clinical outcomes is the TME, a potential barrier that ultimately decides the therapeutic success. For instance, a hostile TME (e.g. low oxygen supply (hypoxia), immunosuppressive milieu, extensive tissue necrosis) can slow or inhibit antitumor immune cells, regardless of their activation state [49, 50]. Hence, the TME and heterogeneity of tumors contribute to the complexity and individuality of cancers and still represent a challenge for successful therapy. Unfortunately, if cancer progression cannot be halted, the primary goal of treatment is to extend life with medicines exhibiting only few side effects, a process called palliation. The main therapies that are administered at different stages of the disease are explained below:

2.2.1. Surgery (local). The spectrum of surgical intervention ranges from gentle to pervasive and stressful options. For example, surgery can be minimal with only a small incision site to remove, e.g. a suspicious skin lesion. However, major surgical interventions are often needed in oncology, in which the surgeons remove the tumor and adjacent tissue and the associated lymph nodes if metastasis is expected or diagnosed. The advantage is the most efficient removal of primary tumors of stage I–III, but some remnants may be left behind that regrow later on (tumor recurrence).

2.2.2. Chemotherapy (systemic). Cancer cells quickly proliferate. Classic chemotherapeutic agents halt cell cycle progression and hence stop cell division [51]. They do so by, for example, blocking control processes during cell division (e.g. intercalatents, mitosis inhibitors, taxanes), alkylating DNA (e.g. cisplatin), blocking proteins that regulate metabolism and proliferation (e.g. tyrosine kinase inhibitors, mitotane), acting as antimetabolites (e.g. 5-fluorouracil), and interfering with DNA repair (e.g. nitrosoureas) [52, 53]. The drugs affect all cells with a higher proliferation rate, including highly proliferating non-malignant cells in the gut, bone marrow, vascular system, and skin and hair follicles, leading to the typical side effects. In contrast to traditional chemotherapy, which essentially only slows the growth of all body cells to a certain extent, targeted chemotherapy is active against cancer cells by exploiting their over-dependence on specific signaling pathways [54] (e.g. sorafenib inhibits AKT signaling pathway [55]).

2.2.3. Biologicals (systemic). Biopharmaceuticals, also called biologicals, are extracted or semi-synthesized from biological sources, such as blood products, tissues, bone marrow, and cell cultures. Major anticancer biologicals are interleukins and interferons [56], potent cytokines that affect immune-related processes. Cell therapies, injections of large amounts of specific types of immune cells, are also considered biologicals applied successfully in oncology [57]. Antibodies are the by far most prominent biological with the greatest application range across many types of cancers due to specific binding to surface molecules for blocking cell growth mechanism (e.g. pertuzumab), angiogenesis (e.g. Avastin), or inducing apoptosis (e.g. rituximab). One antibody category is so-called checkpoint inhibitors. They block (by binding to receptors or ligands and thereby sterically inhibiting their interaction) immunosuppressive molecules on cancer or immune cells, thereby promoting anti-tumor immunity (e.g. ipilimumab) [58]. Cell therapies, are somewhat more complex. Herein, immune cells are isolated from the patient's body, expanded in the laboratory, stimulated with cancer cell material, and injected back to the patient hoping the cells will elicit potent antitumor immunity [59]. A similar approach is to isolate immune cells, genetically modify them for tumor-lytic activity, and expand them in a laboratory before returning them to the patient [60, 61]. Despite both approaches' variable but undeniable success, the therapies can only be delivered at enormous economic costs (>10 000–100 000 € per injection), which potentially limits their global and widespread use.

2.2.4. Physical modalities (local). Physical treatments, such as radiotherapy and PDT, are applied locally. Radiation therapy uses large doses to kill cancer cells or slow their growth by damaging DNA. Cancer cells whose DNA is irreparably damaged stop dividing or die. With this therapy, the killing of non-carcinogenic cells in the surrounding tissue cannot be ruled out, and conversely, metastases are not affected directly. The photosensitizers for PDT are locally excited at a wavelength between 630 and 635 nm and triggers an inflammatory reaction and oxidative stress in tumor cells [62]. Mechanistically, this occurs via reactive species generation and potentially strengthens anticancer immunity [63]. However, many solid tumors are characterized by a hypoxic environment, leading to a limited therapeutic effect of PDT [12, 64, 65]. A more recent method, namely cold physical plasmas, is a physical treatment applied locally and mainly based on reactive species generation and oxidative stress induction [66]. Multi-ROS generation

and their trajectories, reactions, and biological effects are highly complex. Linking specific plasma-derived ROS to specific biological responses in tissues has so far not been successful. It is technically impossible to shield the target from all but one single reactive species type being generated [7]. By contrast, species concentrations and individual effects can—*in vitro*—often be attributed to a few particular types of species, thanks to the ample presence of liquid that is simpler to investigate compared to tissues [67]. Yet, *in vitro* models are not complex enough to predict biomedically relevant effects of plasma treatment. Nevertheless, plasma technology can be used and optimized to apply multi-ROS to tissues, thereby creating different micromilieus. The exogenous ROS generated by plasma are mostly already known to body cells, as these generate ROS constantly by endogenous enzymes for different purposes, as outlined in the following.

2.3. ROS

Exogenous and endogenous reactive species are relevant for regulating molecular processes in non-malignant as well as malignant cells [6, 68]. Lower ROS levels promote cellular processes, such as proliferation, migration, or angiogenesis, through so-called oxidative eustress. Conversely, higher ROS concentrations trigger stress responses leading to oxidative distress, characterized by cell damage and death beyond redox signaling [6]. In order to maintain a balance of intracellular ROS production and removal, many enzymes contribute to such homeostasis, even though elevated ROS levels are sometimes inevitable [69]. In contrast, dysregulation of these proteins and mechanisms leads to chronically high ROS levels and oxidative stress. Several diseases are linked to oxidative stress and ROS, such as diabetes, arteriosclerosis, neurodegenerative diseases, macular degeneration, and cancer [70, 71]. The recent view on intracellular ROS includes findings that suggest different ROS levels in different cellular compartments, indicating an absence of an overall redox balance and focusing on compartment-specific ROS regulation [72].

The most important enzymes for ROS homeostasis are nicotinamide adenine dinucleotide phosphate oxidase, xanthine oxidase, superoxide dismutase (SOD), nitric oxide synthase (NOS), and myeloperoxidase (MPO) [73, 74]. High or low levels and/or activity of one or several redox enzymes are often found to correlate with disease onset or severity [75]. For example, SOD catalyzes the dismutation of the radical superoxide anion into hydrogen peroxide (H_2O_2) or ordinary molecular oxygen (O_2), thereby preventing the formation of peroxynitrite from superoxide with nitric oxide endogenously generated by NOS [76]. Intracellular compartments such as mitochondria, peroxisomes, and the endoplasmic reticulum produce endogenous by-product ROS and, at the same time, antioxidants to counterbalance oxidative damage [77]. Conversely, oxidative stress caused by ROS dysregulation promotes damage to cell structure, including proteins, lipids, membranes, and DNA, which play a key role in cancer development. For example, the tumor suppressor protein BReast Cancer 1, early-onset (BRCA1) can inhibit ROS and estrogen-mediated DNA damage [78]. However, as a mutated tumor suppressor, it increases estrogen and oxidative stress production, potentially enabling cancer development [79].

Immune cells are also critical in the production and regulation of ROS. For instance, granulocytes and macrophages can form reactive species through enzymatically catalyzed processes (e.g. MPO converts chloride and hydrogen peroxide to hypochlorous acid) [80]. The produced ROS are microbicidal, which is important to kill bacteria, viruses or parasites during an injury or infection. Furthermore, the ROS induce oxidative stress in degenerated cells, and ensure the recruitment of other immune cells to kill pathogens and sick or atypical endogenous cells [81, 82]. ROS are also critical for the immune cells because they are necessary for redox-based energy and signal transport, as very low ROS levels also promote lymphocyte proliferation [83]. Subsequently, ROS has specific effects on tumor cells and immune cells during an inflammatory process and can be simulated and examined by cold physical plasma technology.

3. The immune system

The two arms of an immune response, called innate and adaptive, enable higher organisms to protect themselves efficiently against pathogenic structures (more detailed immune response explanations can be found in detail elsewhere [84, 85]). Pathogens are disease-causing microorganisms such as bacteria, viruses, parasites, fungi, and pathogenic proteins. This includes infected body cells that are also pathogenic to the host, as they contribute to the spread of bacteria and viruses. Along similar lines, strongly mutated cells, such as cancer cells, can be immune-recognized and eliminated as well. The innate immune system consists of physiological barriers, inflammatory processes, and the uptake of particulate substances to contribute to the defense reaction from the first day of life. The innate immune system cannot use immunization processes to learn to attack new structures, such as mutated virus entry proteins in the case of the corona virus. Still, it is fast in the detection of and reaction to well-known structures. By contrast, the adaptive immune system can detect new structures, but it is slow as it is based on molecular learning. It can detect new structures that were not present in our evolutionary history and thus enables an efficient defense through an extraordinarily complex interaction of many types of immune cells, the production of antibodies, and the specific recognition of molecules by immune cells. This is the basis of successful vertebrate evolution, the quick response to known pathogenic agents by innate immunity together with the ability to adapt to pathogens that circumvent innate immunity's defense and extend its defense repertoire, which is done by the adaptive immunity. The core job of the immune system is to defend from pathogens every second of our lives, e.g. on the skin, lung, and gut surfaces. Anticancer immunity is an important, but in relation tiny part of our immune competence repertoire compared to pathogen defense, which is why the immune system is best understood from the infection perspective.

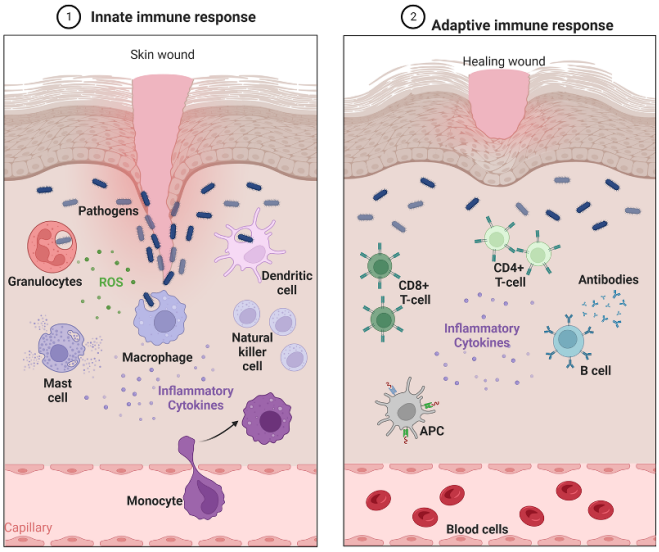

Figure 3. Innate and adaptive immune response.

3.1. Innate immunity

The innate immune system includes natural barriers such as skin and mucous membranes and innate immune cells (figure 3). The barriers make it difficult for pathogens to penetrate the tissue. At the same time, the innate cells recognize evolutionary conserved pathogenic structures (based on receptors encoded in our germline DNA) to attack foreign structures (pathogens and their molecules). Together they build a frontline defense to eliminate pathogens and prevent their spread. This fast but non-specific reaction is accompanied by an inflammatory response—a process called inflammation. It is characterized primarily by the development of heat, redness, and swelling. Furthermore, inflammation is also critical for successful anticancer immunity and, hence, plasma cancer treatment, as we will see later.

During this first defense, inflammation is carried out by different immune cell subpopulations with various tasks. Tissue-resident phagocytes, such as macrophages, granulocytes, and dendritic cells (DCs), take up microbes intracellularly in the phagosome, followed by the microbe killing after fusion with lysosomes. In the phagolysosome, antimicrobial proteins and ROS are generated at a low pH to facilitate efficient microbial killing [80, 86]. Meanwhile, the phagocytes secrete cytokines to communicate with other immune cells. Those small molecules are necessary to recruit more immune cells and signal 'danger' (pro-inflammatory) to alarm the tissue or 'peace' to dampen local (immune) reactions (anti-inflammatory). The most relevant molecules expressed and released by monocytes, macrophages, and DCs to signal, e.g. for immune cell recruitment, are members of the interferon (IFN), interleukin (IL), and chemokine (CCL and CXCL) families [87, 88]. Another prominent member, the name already tells its initially identified effect, is the tumor-toxic tumor necrosis factor (TNF). Different subclasses have different functions. For instance, IL-1α, IL-6, INF-γ, and TNF-α promote inflammatory responses, whereas IL-12 can stimulate the growth and function of T-cells. The released cytokines can attract innate lymphoid cells, such as mast cells, which secrete cytotoxic granules to kill pathogens. There are also cytokines to recruit other immune cells, such as natural killer cells (NK) that recognize and attack infected, degenerated, or cancer cells [89]. DCs and other so-called professional antigen-presenting cells (APCs) can be activated to do so after making contact with 'danger' signals [90] (more on this process in section 3.1). After their activation, APCs stop absorbing substances and migrate towards the draining lymph nodes. During this migration, the engulfed fragments, mainly proteins, are digested intracellularly. After intracellular protein breakdown, the resulting peptides are bound to major histocompatibility complex (MHC) molecules and transported in vesicles to the membrane. The peptide-loaded MHC complex is then embedded in the membrane and enables the activation of adaptive immune cells. Focusing on the T-cells, which are pivotal in antitumor immunity, as we will see later, each T-cell briefly contacts the APCs in the lymph node to find its cognate antigen. If none of the APCs show the peptide the T-cell receptor (TCR) is specific against and the T-cell would react to, the T-cell leaves into the bloodstream to enter the next lymph node. If the cognate peptide is presented, the TCR docks to the MHC-peptide complex to proliferate [91, 92]. Strong immunity always requires both innate and adaptive responses. It takes two to tango.

3.2. Adaptive immunity

Without adaptive immunity, life would not be possible. Yet, life is long, and a single highly destructive bacteria or virus species able to fully evade innate immune detection would be sufficient to wipe out a given higher species. Luckily, most vertebrates, including humans, have a so-called adaptive immune system. As the name says, it can adapt an immune attack against any protein, provided the new protein is embedded in a 'danger' context to signal the immune system to focus on targeting such detrimental structures (e.g. virus-related proteins). Teaching cells to distinguish 'danger' signals from 'peace' situations is vital to life as most proteins—including our own and the many that are not part of our body (food)—are harmless. Their immunological ignorance is critical for survival, as can be seen with critical allergic and autoimmune reactions [93]. The differentiation between 'danger' and 'peace' is made by the context of protein uptake. Simply put, damage- and microbe-associated molecules help mount immune responses [94, 95], while their absence leads to tolerance (more in section 3.2).

The cellular adaptive immune response is mediated by antigen-specific immune cells such as T-lymphocytes (T-cells) and B-lymphocytes (B-cells). Humoral adaptive immune responses are the B-cell product and lifesaver in the current pandemic [96], antibodies (figure 3). Specific subtypes of T-cells either eliminate infected or cancer cells ($CD8^+$ T-cells, also called cytotoxic T-cells) or interact with and support other immune cells ($CD4^+$ T-cells, also called T-helper cells). T-cell activation via a specific TCR (one TCR type per single T-cell, with $>10^5$–10^6 different TCRs across all T-cells in the human body [97]) leads to proliferation and differentiation,

which activates other adaptive immune cells to elicit immune responses.

In a way, immunity randomly spawns an army of wildcard warriors, not knowing if their weapon will ever be needed. T-cells mature and are selected in the thymus gland. Only those not identifying body structures ('self') can leave the thymus; all others die in the thymus to avoid unspecific binding and attacking the body structures (autoimmunity). Most immature T-lymphocytes die in the thymus, while the few surviving share two properties. First, they strongly bind to the host's MHC-I or MHC-II receptors. Second, they do not (strongly) bind to peptides of proteins that the body produces to avoid autoimmunity. The cells reaching both criteria mature further and are released into the periphery. Each T-cell carries only one type of TCR. Millions of T-cells (i.e. millions of different TCRs) patrol the body daily, searching for that one or those few APCs sitting in a lymphoid organ (such as lymph nodes) presenting its cognate antigen. If the T-cell finds the right (called cognate) antigen on the APC, it produces millions of TCR-identical subclones that flush the bloodstream and body. The positive interaction with APCs licenses them to become active and divide; negative interaction leaves the T-cell non-activated. During the activation process, the T-cell requires three signals: antigen recognition: the TCR interacts with peptide-loaded MHC complex it is primed to target on APCs in a lymphoid organ (signal 1), co-stimulation: besides showing the correct peptide to the T-cell, the APC has to present high levels of co-stimulating ligands indicating alert (signal 2), and cytokine priming: additional alarming by APC (signal 3). Signal 1 is made via the TCR binding to MHC that carries the peptides of the proteins taken up nearby in the body. $CD4^+$ T-cells bind to MHC-II, and $CD8^+$ T-cells bind to MHC-I.

Generally speaking, $CD8^+$ T-cells recognize peptides that have originated from intracellular proteins. If a cell becomes infected with a virus, the virus hijacks the cells' intracellular protein production machinery to produce viral proteins. All body cells are constantly required to provide MHC-I (their 'ID-card') on their cell membrane, which is continuously loaded with peptides from intracellular protein production. The virus protein and its fragment, the peptide on MHC-I, are unknown to the body. The $CD8^+$ T-cell bears a receptor that is specific for this peptide learns of its target by seeing the peptide on an APC, which has previously eaten a dead body cell that had been producing viral proteins. The $CD8^+$ T-cell becomes alarmed, replicates million-fold, and the clones swarm through the body to find the site of virus replication. Once arrived at the site of infection, the specific $CD8^+$ cells scan the MHC-I molecules on all cells in the present tissue. If $CD8^+$ cells detect the same peptide as initially presented by the APC in the lymph node, the cytotoxic $CD8^+$ T-cell will kill the target cell via different mechanisms. The target cell can no longer produce viral particles. In simple words, this is how viral infections, including COVID19, are cleared. Albeit it appears surprising at this stage: the mechanism of tumor control by $CD8^+$ T-cells is very similar [98]. Whether the novel protein is from a viral or tumor protein mutation is irrelevant. If it is new and causes trouble, it will be attacked. This process is supported by $CD4^+$ T-helper cells that have an analog activation cycle (activation in the lymph node, activity in the target tissue) with two distinct differences. First, they engage with MHC-II, which is only present in APCs and carry protein peptides of extracellular origin. The job of the activated $CD4^+$ cells is to support other immune cells, such as B-cells. B-cells cannot produce antibodies *per se*. They need a confirmation that the target its antibody binds to is new to the body and causes trouble. The activated $CD4^+$ T-cell provides such help.

Humoral immune responses are mounted when T-cells and B-cells connect via the B-cell receptor (a prototype of an antibody) with the ultimate goal of producing antigen-specific antibodies. These antibodies bind to the target, marking them for other immune processes and cells for destruction. Antibodies can also mask structures, preventing the structure from interacting with the target. For instance, anti-corona virus antibody sera are given to patients with severe disease courses [99]. The antibodies bind and mask the viral entry proteins with the consequence of many viruses being unable to enter the target cells (e.g. in the lung). In the context of cancer, antibodies can be given *in vivo* to inhibit structures [100, 101] or be used *in vitro* as a diagnostic tool, mark cancer-related structures (e.g. tumor-specific antigens), or observe disease progression [102–104]. In immunological research, immune responses were generated in animal models against newly synthesized chemical substances that had never existed on earth before [105, 106]. This demonstrates the beautiful power of our immune system. Its protection unambiguously is key to our modern lives and societies.

In summary, the immune system is a highly active molecular communicator and can be activated, showing new structures to induce an antigen-specific immune response. Unchecked immune responses would quickly kill its host. Sepsis (heavy infections in the blood) and COVID19 deaths (often caused by overshooting immune responses) are prime examples of why multiple checkpoints and cell types are needed before the immune system attacks a target. On the contrary, tumor cells actively suppress immunity, and cancers evolve mechanisms to escape immune responses.

3.3. Cancer immune evasion

Tumors evolve under immune pressure. This often leads to cancers hijacking innate immune cells, leading to wound-healing-like growth stimulation and, ultimately, increased tumor growth [107]. Simultaneously, as early as 1963, Frank Macfarlane Burnet described the idea of immunological tumor surveillance, in which immune cells control the organism [108]. This immunosurveillance is the elimination phase during immunoediting, a dynamic process between tumor cells and immune cells (figure 4). Similar to infected cells, tumor cells can be attacked because immune cells identify them as abnormal due to mutations in the peptides they constantly present on their 'ID cards' (MHC-I) [109]. Cancer cells that present abnormal peptides are highly immunogenic as they are easily recognized and eliminated by immune cells. At this point, it is critical to mention that body cells that express no or little MHC-I are killed by NK-cells. Still, it remains a good

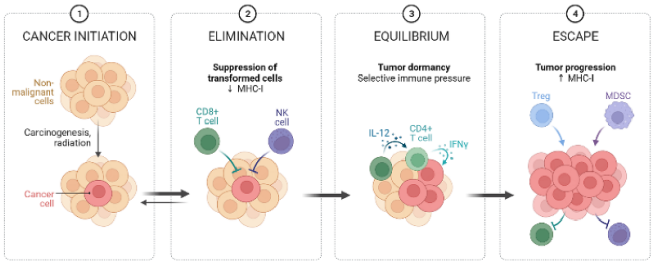

Figure 4. Cancer immuno-editing. By killing tumor cells with high immunogenicity, the immune system puts pressure on cancers for tumor cell variants with low immuno-stimulating profiles to emerge and proliferate preferentially.

tumor strategy to downregulate their MHC-I levels as much as possible to remain undetected from T-cells [110].

As the immune system pressures cancer cells by inactivating immunogenic variants, only the tumor cells with the best hiding and growth strategies escape immune control (figure 4, (2)). It is hypothesized that cancer frequently appears in most humans but that the few malignant cells are quickly identified and eradicated by immune cells. Vice versa, tumors develop better in immunosuppressed patients than in immunocompetent people, illustrating constant immune control from cancer. Immune fitness declines with age, while cancer incidence rises with age, a correlation with a putative degree of cause-consequence relationship. Tumor cells develop different mechanisms to hide and escape the immune response (immune escape) [110]. These mechanisms include the expression of receptors, ligands, and/or a section of molecules with immunosuppressive functions. In addition, tumor cells avoid antigen presentation by reducing MHC expression, as explained. As a result, cancer cells with reduced antigenicity or immunogenicity have an advantage because they evade immune cells and T-cell-induced killing (figure 4, (3)). Subsequently, the growth of those cells that remained undetected by the immune system is promoted. Tumor cell clones are indirectly selected under pressure for these clones that have the best evasion strategy. Through this 'cancer immuno-editing' process, cancer clones evolve to avoid immune-mediated elimination by leukocytes with anti-tumor properties. Again, there are requirements for this to happen. The tumor cells should be genetically unstable to a certain degree to allow mutations, which will create a pool of tumor cells that are similar but not the same. This diversification enhances the chance that one of the clones is the fittest to withstand immune control. However, too high genetic lability leads to dysfunctional regular cellular processes and growth disadvantages due to cellular senescence (inability to proliferate) or death. Hence, 'successful' tumors are often balanced in their features to survive and grow by a try-and-error strategy, similar to the immune system. Of course, there is no intention in the system but only the random biological variances and responses of the cellular environment that select some properties over others. The tumor bulk comes with another critical aspect that influences immune cells—the tumor microenvironment.

3.4. The TME

Being understudied for decades, intensive research and new laboratory methods and technologies led to detailed profiling of the TME across many cancer entities. The TME is considered today a main bottleneck for many types of cancer therapy, including immunotherapy. There are many reasons for this, which are outlined in detail in other reviews [111–113].

The TME is a complex system that includes cellular and non-cellular components, such as tumor and immune cells, stromal cells, blood vessels, extracellular matrix, tumor metabolites, redox reactions, and cytokines [114]. Due to spontaneous mutation of the tumor cells and the interaction with other cells (e.g. immune cells), different mechanisms can take effect, so the tumor heterogeneity entails a diversity of the TME. Additionally, most solid tumors have hypoxic regions and show elevated extracellular acidification, leading to tumor progression and drug resistance. Such TME promotes the property of tumor cells to evade elimination by suppressing the activity of other immune cells, e.g. by recruiting immunosuppressive leukocytes, including regulatory T-cells [115, 116]. The receptors on the tumor cell surface that stimulate or suppress immune cells are highlighted below. First, as mentioned earlier, the MHC complex, which represents intracellular metabolites and processes, is downregulated to reduce the chance of being recognized by tumor-toxic $CD8^+$ T-cells. At the same time, due to high mutational loads or antigens from overexpressed, misfolded, or cell-unspecific proteins are presented on the surface. They are called neoantigens or tumor-associated antigens as they differentiate between tumor cells and non-tumor cells [117], even though these antigens are often not recognized as pathological or foreign due to the immunosuppressive microenvironment and other escape mechanisms. In addition, some tumors tend to be more immunogenic than others, such as melanoma, characterized by high numbers of neoantigens compared to other types of cancer [118]. However, an additional tumor immune-escape mechanism is the down-regulation of these neoantigens by selecting and eliminating tumor subclones that do not carry these neoantigens. Different tumor cells with various escape mechanisms support the generally immune suppressive milieu as part of the TME.

In addition to MHC, other regulatory molecules on the cell surface contribute to immune escape mechanisms. Tumor cells express molecules that suppress the immune response, such as CTLA-4, CD95L, or programmed death receptor ligand 1 (PD-L1). These actively inhibit the cytotoxic function of immune cells ($CD8^+$ cytotoxic T-cells in particular) or induce tolerance by activating regulatory T-cells. Depending on the tumor type, some cancer cells and cancer-supporting immune cells secrete immunosuppressive molecules. These include inhibitory cytokines such as IL-10, TGF-β, and the enzyme indoleamine 2,3-dioxygenase. The latter is strongly immunosuppressive by producing specific metabolic products [119, 120]. PD-L1, which is expressed on tumor cells and non-malignant cells, also has an inhibitory effect on T-cells by down-regulating TCR expression [121]. Thus, inhibited

or even killed immune cells can promote the development of an anti-inflammatory, growth-supportive TME. There are many ways tumors render their microenvironment to favor their growth, and many of these are intertwined with immune cell regulation, polarization, differentiation, migration, and inhibition [122, 123]. The advent of cancer immunotherapies helped address such and other hurdles in immuno-oncology.

4. Cancer immunotherapy

Immunotherapy aims to trigger the body's immune response to attack tumor cells. By definition, immunotherapy is a systemic therapy. Local therapies, such as physical modalities, can support (adjuvant effect) immunotherapies [124, 125]. When discussing antitumor immune cells, it is mainly referred to cytotoxic $CD8^+$ T-cells as these are the effectors eventually killing the tumors (figure 5).

Basically, there are two main focal points when discussing the immune system in cancer. The first relates to the systemic immune status and action, such as the ability of the patient to produce functional immune cells in the bloodstream, the ability of immune cells to become activated, and the levels of immuno-suppressants in the blood. Novel and costly approaches rely on genetic engineering of patient-derived T-cells with antigen-specific TCRs [126]. The exact receptor properties need to be identified beforehand for each patient based on their bio-material and computer modeling to ensure that the TCR binds to the specific tumor-associated peptide in the MHC binding groove. The second concerns the tumor's local characteristics (TME); e.g. whether it is immunosuppressive or immunostimulatory.

4.1. Cancer immunotherapy approaches

Essentially, cancer immunotherapies can be subdivided into two main classes: cellular and non-cellular. During cellular therapies, specific immune cells, such as DCs or T-cells, are grown and amplified in the laboratory and injected into the patient. Non-cellular immunotherapies concern injecting small to large molecules (biologicals) into the patient, including cytokines, antibodies, single-domain-antibodies (a special type of antibodies originally isolated from alpacas/llamas), and vaccines. A related method is the injection of oncolytic viruses, which are genetically engineered to kill tumors. Because this process is thought to stimulate antitumor immunity, oncolytic viruses are often mentioned along with cancer immunotherapies (figure 6).

There are several ways to modulate T-cell activity through immunotherapy; the main three key signals to modulate are 'antigen recognition,' 'co-stimulation,' and 'cytokine priming.' The signal for antigen recognition can be increased through vaccination applications with a protective or therapeutic function. In addition, administering different vaccines with or without adjuvant substances to increase immunization enables a wide range of applications. For example, vaccines that contain tumor-associated antigens stimulate the immune system and generate antigen-specific T-cells that recognize and attack cancer cells. This could involve injecting tumor-specific structures such as proteins, peptides [127], tumor lysates [128], or mRNA [129] to generate T-cells with tumor-specific TCRs. Furthermore, to increase the

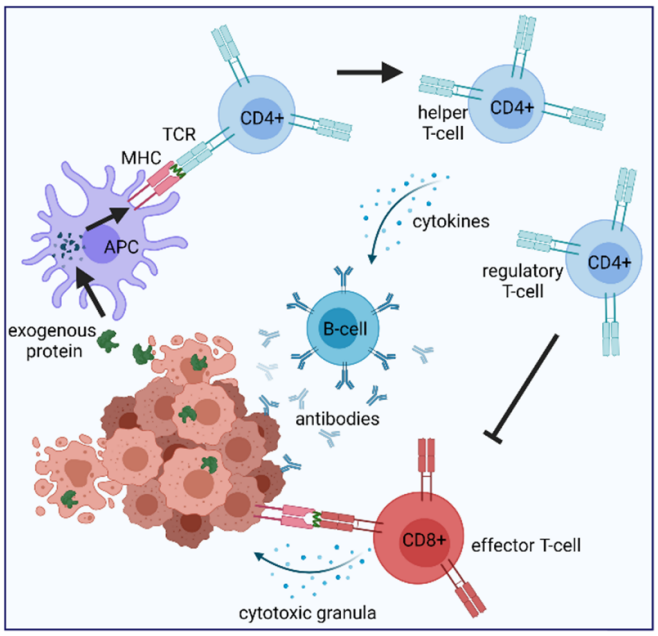

Figure 5. Selected immune cells and their function during an antitumor immune response. $CD4^+$ (and $CD8^+$) find their cognate antigen (protein peptide) by contact with professional APCs, such as DCs. Subsequently activated $CD4^+$ T-cells provide co-stimulatory signals to other immune cells, such as B-cells (middle) for antibody production. Activated $CD8^+$ T-cells kill tumor cells that present the specific protein peptide the $CD8^+$ T-cell is armed against. Killing is performed, for instance, via cytotoxic granules that create pores in the tumor cell membranes. $CD4^+$ regulatory T-cells (T_{reg}) and also tumor cells can suppress tumor-toxic immune cell activities.

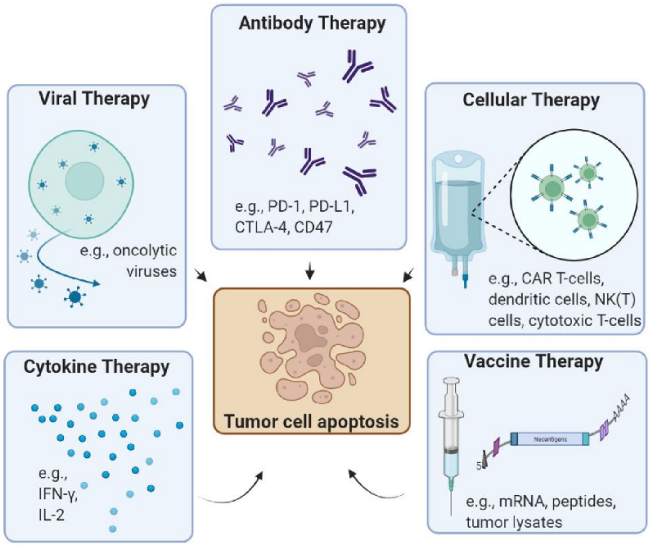

Figure 6. Several relevant cancer immunotherapies. Antibody therapies are the by far mostly applied immunotherapy in the clinics. Much hope lies on anticancer mRNA vaccines, as these have shown remarkable clinical results [135].

tumor antigens' immunogenicity and the chance of inducing antigen-specific T-cells, adjuvants can be added to the vaccine, or DCs are loaded with antigens. Herein, APCs are isolated from patients, expanded in the laboratory, loaded with the tumor antigen or lysate or mRNA, and returned to the host [130]. Another application for generating antigen-specific T-cells is chimeric antigen receptor (CAR) T-cells. Like the DC-loaded vaccine, lymphocytes are isolated from the patient, expanded in the laboratory, and genetically modified before being returned to the host [131].

The by far most promising and currently applied cancer immunotherapy is antibody therapy. Monoclonal antibodies are highly specific and readily mask negative immune checkpoint surface molecules on tumor or immune cells [132], such as PD-1, PD-L1, or CTLA 4. In 2018, the Nobel Prize in medicine or physiology was awarded to James P. Allison (USA) and Tasuku Honjo (Japan) for identifying this concept in oncology. As a result, the activity of lymphocytes is promoted to secrete cytotoxic molecules and eliminate tumor cells. Antibodies are also used to target tumor growth signaling receptors, for example, epidermal growth factor receptor in head and neck cancer [133]. Also, angiogenesis, a process of forming new blood vessels to supply tumor cells with nutrition and oxygen, is frequently targeted using antibody therapy [134].

An effective antitumor immune response requires specific cytokine profiles. At the same time, some cytokines, such as IFN, are increasing the visibility of tumor cells to the immune system via, e.g. MHC-I increase. Accordingly, cytokine administration has been used for long in cancer immunotherapy, including the lymphocyte-survival-enhancer IL-2 [136] and IFN-γ. Another reason for cytokine administration is their relatively cheap production and simple intravenous administration routine within existing pharmacological schemes.

The activation of immune cells or modulation of tumor cells by injecting cytokines, activated immune cells, or immune checkpoint inhibitors promote an anti-tumor immune response. However, this leads to further selection pressure on tumor cells and their TME, and successful therapy requires the presence of immune cells within the TME, which is not always the case in strongly immunosuppressive environments. Therefore, some immunotherapeutic approaches indirectly activate immune cells by targeting tumors to turn them into immunogenic structures. This is achieved by, for instance, induction of immunogenic cell death (ICD).

4.2. Immunogenic (cancer) cell death (ICD)

The TME influences the body's antitumor defense through its heterogeneity and modulates immune cell responses through the secretion of various cytokines and chemokines. In general, tumor cells try to avoid the immune response with the help of their immune escape mechanisms and keep immune cells away. Conversely, a successful endogenous immune response is likely increased when more immune cells reach the tumor tissue. Therefore, tumors are categorized into 'cold,' non-inflamed tumors, characterized by a lack of immune cells, and 'hot' inflamed tumors, characterized by ample immune

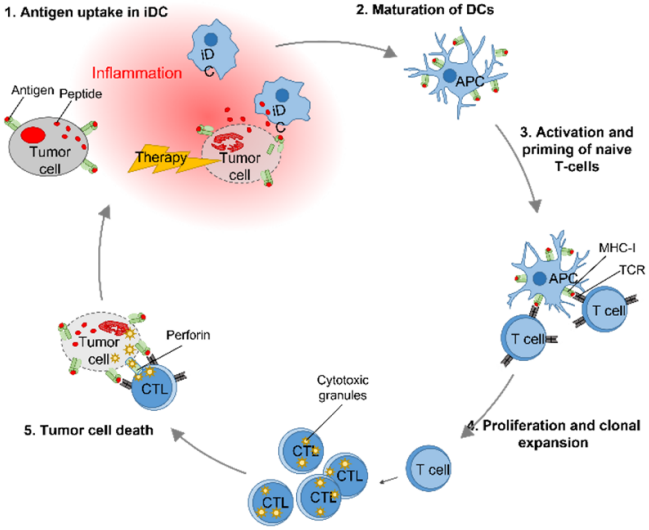

Figure 7. The cancer-immunity cycle, describing how ICD promotes eliciting antitumor immune responses. Reprinted from [137]. Copyright (2018), with permission from Elsevier.

cells. In mixed form, 'ignored tumors,' immune cells accumulate at one or several specific sites in or at the tumor without spreading into the entire tumor area [139]. Various stimulants are needed to attract immune cells into the tissue to turn a 'cold' tumor into a 'hot' tumor. One of them is a regulated form of cell death that stimulates immune cells and causes sterile inflammation, the act of alarming the immune system in the absence of pathogens such as bacteria and viruses [140]. This so-called ICD is characterized by the expression of several surface markers and the secretion of proteins [140, 141]. These ICD-specific markers include, for example, calreticulin (CRT), high mobility group box 1 protein (HMGB1), heat shock proteins (HSP), and the energy currency of cells, adenosine triphosphate (ATP). Adaptive immune cells recognize these ICD molecules as damage-associated molecular patterns (DAMPs) while routinely phagocytosing and taking up dead tumor material in the TME. Under normal conditions, this would happen in a non-inflammatory, 'silent' fashion because dead cells appear million-fold per second in the body and must not mount immune responses as the clearance of the dead cell is biological routine. If, however, the tumor material uptake occurs in the presence of inflammatory mediators and DAMPs, the phagocytes become activated and migrate to the draining lymph node where they can meet T-cells to (cross) present the cargo (protein-peptides). Suppose an antitumor T-cell with suitable TCR comes in contact with a cross-presenting phagocyte, the former can become activated, start replicating, and swarm into the bloodstream to migrate through the body tissues to attack distant cancer sites (figure 7).

If the TME is not too hostile, e.g. low pH and oxygen, and if immunosuppressive signals from tumor cells or tumor-hijacked-immune cells do not dominate, the $CD8^+$ T-cell will strike and kill. This process is called the cancer immunity cycle, with ICD being a central point of setting the cycle in motion. Further information on this can be found elsewhere

[142, 143]. Many molecular details of ICD have been clarified as well already [144].

ICD can be induced by different physical, chemical, and biological therapies, such as ionizing radiation [145], PDT [146], and specific chemotherapeutics [147]. Cold plasma is a recent technology among the physical modality family of ICD inducers, as explained in the following.

5. Plasma, cancer, immunity

5.1. Plasma cancer treatment

Plasma treatment of cancer cells has been the subject of intense research for over a decade now [148, 149]. The idea follows the principle of other physical modalities that are applied locally and topically (i.e. on the skin or bodily surfaces), with some of them also relying on ROS to mediate biomedical effects, such as photodynamic therapy, UV therapy, cryo-therapy, hyperthermia, and laser therapy [150]. Many studies have re-iterated the principle that high concentrations of plasma-derived ROS lead to cellular demise, such as cell cycle arrest, declined metabolic activity, and cell death [151]. Surprisingly, similar effects are observed across different plasma devices, albeit there are differences in, e.g. total energy deposited or treatment time required to achieve similar treatment efficacy. However, detailed effects may differ nonetheless, and studies thoroughly comparing different plasma devices based on congruent laboratory protocols and matched cytotoxicity conditions are rare, if not absent. One putative study protocol approaching a plasma jet head-to-head comparison was suggested by us [152] and should be extended by molecular analyses and the inclusion of more plasma sources. Nevertheless, most effects observed in cell culture systems after treatment with different plasma sources are similar. Those effects can be explained by H_2O_2 [153] or HOCl [154], which dominate the long-lived species chemistry [155], and are generated predominantly in cell culture experiments in the liquid phase. These findings may be entirely different from effects on tissues, as in the case of clinical settings or animal experiments. The current critical knowledge gap in plasma medicine is to identify the specific treatment settings that dominate anticancer reactive species formation and plasma effectors such as electric fields in plasma systems. It is hoped that modulating feed gas fluxes in plasma jets and thereby modulating the reactive species profiles in terms of quality and quantity will help decipher those (sets of) species that are mainly responsible for anticancer effects. We have recently applied such an approach in a melanoma mouse model, and the findings suggested atomic and singlet delta oxygen among such potent anticancer species in question [156]. However, changing the feed gas admixture also possibly leads to changes in other plasma parameters, such as UV, temperature, and electric fields, besides dozens of species that usually remain undetected if not complex tools such as two-photon absorption laser-induced fluorescence [157] or molecular beam mass spectrometry [158] are utilized. Knowing the key antitumor plasma effectors would allow optimizing plasma sources for such applications.

In parallel, clinical evidence is needed to underline the practical relevance of plasma cancer treatment. For detailed studies of experimental (non-patient) plasma cancer treatment, the reader is referred to recent reviews on this topic [159, 160]. In Greifswald, Germany, the visionary oncologist Hans-Robert Metelmann has tested the kINPen plasma jet for the palliative treatment of 20 patients suffering from incurable ulcerative head and neck cancer. Several of these patients responded well to the plasma treatment, as evidenced by tumor growth deceleration or even tumor mass decline [161]. Unfortunately, nothing is known about why the plasma treatment worked in some but not all patients. Was the reactive species composition optimal in all cases? Should plasma treatment exposure time, frequency, and onset and offset of plasma treatment be adjusted on a case-to-case basis? Would some tumor areas benefit from lengthy plasma treatments while others only needed low 'doses'? There still is much to learn on clinical plasma cancer treatment, not only regarding the 'how' but also the 'if'. It is hoped that a frequent and less deadly disease, actinic keratosis, will help answer some of these questions. Actinic keratosis is a low-grade carcinoma (*in situ*), potentially developing into cutaneous squamous cell carcinoma. Several case report series [162–164] and one randomized clinical trial [165] showed promising effects of plasma application, and we have provided mechanistic evidence of plasma-treated lesions in a mouse model recently [166]. The different responses depend on the cell type, as some cells are more sensitive to exogenous ROS than others [167]. It was also observed that the plasma treatment had a selective toxic effect on tumor cells but not on non-malignant cells. Plasma treatment is safe, showing no long-term consequences in mice after 1 year [168, 169] and humans after 1–5 years [170, 171], at least for the kINPen. Furthermore, genotoxicity is not observed [172, 173]. DNA damage was observed since it is a secondary event of apoptosis rather than a consequence of short-lived highly-reactive species [174]. However, the secondary effects of plasma-treated tumors raise the question about ICD-induction and other immune-stimulatory properties. Recently, this question has been increasingly investigated, and some studies are explained in the following.

5.2. Immune-related effects in plasma-treated cancer cells

In vitro, several ICD markers, such as secreted and membrane-bound molecules, have been identified in response to plasma treatment in different cell lines. Increased ATP release has been determined after plasma treatment in, for instance, murine and human melanoma cells [175, 176], human glioblastoma cells [177], human nasopharyngeal carcinoma [178], human lung cancer cells [179], and murine colon cancer cells [180]. Increased HMGB1 translocation or release was identified in several plasma-treated cancer cell lines as well, such as murine and human colorectal cancer cells and human gliomas and pancreatic cancer cells [152, 177, 181–183]. There are also chemokines, such as CXCL1 and CXCL10, that are thought to contribute to pro-immunogenic effects, similar to IFN-γ. During the cytokine screening campaigns in supernatants, we

have found one or several of the factors increased many times, e.g. in melanoma [175, 176] and pancreatic [184, 185] and prostate cancer [186].

Moreover, a range of membrane-bound molecules is linked to ICD. These surface markers either have translocated to the surface after stress of the endoplasmic reticulum such as CRT or HSPs, or are related to antigen presentation (MHC), phagocytosis (CD47), DAMP degeneration (CD39/CD73), and immune checkpoints (PD-1, PD-L1, PD-L2, CTLA-4, TIM-3, LAG-3, etc.). Most studies in plasma medicine focused on CRT, finding increased expression of this chaperon on the membrane of plasma-treated cells [152, 156, 177, 179–182, 187]. As mentioned, these data hint toward increased immunogenicity and ICD. Following this, the ability of plasma-treated tumor cells to be phagocytosed by macrophages or DCs may be increased. This hypothesis can be strengthened by investigating professional phagocytes' uptake of plasma-treated tumor cells.

Human, monocyte-derived APCs (differentiated from peripheral blood mononuclear cells) are the gold standard for analyzing tumor cell uptake, as these cells derive from primary human blood cells and are, therefore, the *in vitro* model with the highest relevance. Increased tumor material uptake by DCs was observed following the kINPen tumor cell treatment [152] and kINPen-treated phosphate-buffered saline [182], along with enhanced DC stimulation profiles [188]. Also, cell lysates exposed to plasma-derived ROS increased the co-stimulatory DC profile and ability to interact with T-cells [189]. Strikingly, ICD cannot only be induced in cell lines from solid tumor cells but also in leukemia cells, as recently shown [190]. Notably, in this study, we also provided evidence that plasma-induced cell stress changes the protein expression of leukemia cells. This altered the peptide spectrum being presented on MHC-I, which would potentially amplify the recognition of cancer cells by immune cells that were not directly killed by the plasma process.

5.3. Immune-responses in plasma cancer treatment—experimental evidence

Before discussing plasma cancer studies in the context of immune-related effects, a few things need to be clarified. First, the most relevant models to thoroughly study the immune system's impact on cancer therapeutics are syngeneic murine tumor models (table 1). Syngeneic means the injected tumor cells have the same biomolecular equipment as the mouse strain, i.e. the tumor cell line was initially retrieved from that mouse strain. One of these biomolecular pieces of equipment is the ID-card (MHC molecules) because not only the peptide plays a role in recognition by immune cells. The TCR interacts with an MHC complex like a key–lock principle, so these building blocks must also belong to the same species/strain. Injected tumor cells whose MHC molecules are different from the mouse strain grow only in immune-deficient animals called xenografts, with a few rare exceptions [191]. Naturally, xenograft models are less suitable for studying the immune effects of tumor treatment. Second, immune-related effects can be suggested *in vitro* but are obviously of limited real-world relevance. They can, however, provide useful information about the inflammatory context. These are, for instance, the release of cytokine and chemokines and the expression and release of ICD molecules. A series of syngeneic plasma cancer animal experiment reports are available to inform on immuno-oncological consequences.

5.3.1. Abscopal effects of direct plasma treatment. The first reports came from Ryo Ono's laboratory using a syngeneic orthotopic mouse model, where melanoma cells were injected subcutaneously, similar to a naturally occurring skin tumor. The group investigated the abscopal effect, placing two B16-tumors in mice, each beneath the skin of one hind leg. Plasma treatment of one tumor site led to the tumor growth reduction on the plasma-treated as well as the non-treated site (abscopal effect) [138]. However, the conclusion was only reached by merging data from several experiments and two tumor models, all with different sub-conditions; the raw tumor weight data, which would allow transparent conclusions, are not shown. Re-stimulation of splenocytes with melanoma cells induced IFN-γ production, suggesting activated tumor antigen-specific T-cells. In a follow-up study, the authors surgically removed the tumor after plasma treatment. Then, they measured the time and extent of tumor regrowth at the incision site (assuming that some tumor mass was left behind), which was found to be reduced in the plasma group [192]. Interestingly, the reduction was also observed if two tumors were set per mouse, one was treated, both were removed, and the untreated tumor site was observed. In immunohistochemistry analysis of the tumor tissue, the authors did not find a significant difference in proliferation (using the marker Ki67), indicative that the tumor growth kinetics were not altered strongly. However, significantly greater numbers of tumor-infiltrating $CD8^+$ but not $CD4^+$ T-cells were found. Using the 4T1 syngeneic breast cancer model in an abscopal effect setting similar to the studies from Ryo Ono, a true abscopal effect was found for the first time in a breast cancer model and using a helium multi-jet plasma device [187]. Moreover, we thoroughly investigated the TME using algorithm-driven quantitative fluorescence immune-population imaging of whole-section tumor slices. Elevated levels of T-cells, DCs, and CRT were found in both the treated and the untreated abscopal-affected tumors. However, we did not investigate the expression of CD47. CD47 is currently targeted in clinical trials using humanized monoclonal antibodies because the molecule inhibits tumor cell phagocytosis by immune cells [193]. Its expression levels were studied after DBD-plasma treatment of B16 melanomas *in vivo*. Despite tumor reduction and lower levels in *in vitro* and *in ovo* plasma-treated tumors as well as in silico data, no significant change in intratumoral CD47 levels in mice was found [194]. This, however, does not exclude that effects may be achieved with other plasma devices or tumor models.

5.3.2. Immunization approaches in plasma medicine. An increased amount of infiltrated immune cells in the tumor tissue enables T-cells' activation, proliferation, and

Table 1. Syngeneic murine tumor models with plasma-mediated growth reduction and reported immune-related effects.

Syngeneic model	Plasma source/ feed gas	Main findings	References
B16-melanoma two flanks	Nanosecond-pulsed streamer discharge/humidified O_2 or N_2	• Tumor growth reduction on treated site; calculated reduction on non-treated site (abscopal effect). • IFN-γ and TNF-α production of splenocytes co-cultured with melanoma cells in vitro.	[138]
PDA6606 peritoneal pancreatic cancer	kINPen MED plasma jet-treated RPMI1640 cell culture medium/Ar	• Reduced growth (MRI), improved survival, increased apoptosis (TUNEL), safe application (hematology). • Increased CRT, $CD11c^+$ cells (DCs), macrophages.	[201, 202]
B16-melanoma one flank	Nanosecond-pulsed streamer discharge/humidified O_2	• Tumors were plasma-treated and resected, tumor recurrence was delayed at plasma-treated and non-plasma-treated site but overall proliferation similar. • Increased $CD8^+$ but not $CD4^+$ T-cell infiltration.	[192]
CT26 colorectal cancer one flank	Nanosecond-pulsed dielectric barrier discharge/ambient air	• Vaccination with *in vitro* plasma-killed CT26 cells protected 1/3 of animals from subcutaneous growth of subsequently injected live cells, non-protected animals had bigger tumors than medium or cisplatin (drug) control. • Direct tumor treatment increased CRT, HMGB1, and immune cell infiltrate.	[180]
B16-melanoma one flank	Nanosecond-pulsed dielectric barrier discharge/ambient air	• Vaccination with *in vitro* plasma-killed B16 cells protected 2/3 of animals from subcutaneous growth of subsequently injected live cells, similar to positive control (mitoxantrone), reactive species + pulsed fields alone protected only 1/3 of animals. • No TME investigation to infer on immunological effects *in vivo*.	[195]
CT26 peritoneal colorectal cancer	kINPen MED plasma jet-treated 0.9% NaCl/Ar	• Repeated peritoneal lavage with plasma-treated NaCl reduced tumor burden by >80%. • Increased splenocytes' T-cell activity upon re-stimulation with CT26 cells ex vivo; increased intratumoral macrophages.	[181]
B16-melanoma one flank	kINPen plasma jet/dry Ar, Ar/O_2, He, He/O_2	• Ar-plasma is potent and He/O_2 plasma was most potent in reducing tumor growth, possibly due to atomic and singlet oxygen. • Strong combination anti-melanoma effect with imiquimod (Aldara) achieved highest tumor immune-infiltrates and T-cell activation, He/O_2 and argon plasma with similar effects. • Vaccination with Ar kINPen-killed B16 cells protected 50% of mice from tumor growth after re-challenge with live cells; positive control (mitoxantrone) was 85%, negative control (mitomycin C) was 20%.	[156]
B16-melanoma one flank	Hollow-structure microneedle plasma patch/He (16.5 slm)	• Tumor growth reduction. • Combination with checkpoint (anti-PD-L1) immunotherapy improved anti-melanoma effect. • Increased intratumoral T-cells and activated DCs. • Abscopal effect to distant, untreated tumors.	[198]
MX-7 rhabdo-myosarcoma	Plasma jet at 40 kHz/He	• Plasma treatment increases blood serum HMGB1 levels 1 h after exposure. • Similar HMGB1 serum levels 24 h after treatment; no reported effect on tumor size. • No TME investigation to infer on immunological effects *in vivo*.	[200]
CT26 peritoneal colorectal cancer	kINPen MED plasma jet or wINPlas DBD-treated 0.9% NaCl/Ar	• Peritoneal tumor mass reduction with kINPen better than wiNPlas. • Peritoneal lavage cytokines: wINPlas: increased TNF-α; reduced IFN-γ, IL-2, IL-6, IL-10; kINPen: reduced IFN-γ, IL-6, IL-10; $\rightarrow$ liquid should not be too acidic.	[203]
4T1-breast cancer two flanks	Plasma multi-jet at 20 kHz/He	• Abscopal effect (growth reduction of untreated tumors if tumor on different site of the animal was plasma-treated). • In tissue sections, increased apoptosis (TUNEL), $CD8^+$ and $CD4^+$ T-cells, CRT, and $CD11c^+$ DCs in the plasma-treated and untreated contra-lateral tumor of the same animal (abscopal immunogenicity).	[187]

(Continued.)

Table 1. (Continued.)

Syngeneic model	Plasma source/ feed gas	Main findings	References
B16-melanoma one flank	Nanosecond-pulsed dielectric barrier discharge/ambient air	• Reduced tumor growth. • Plasma showed significant changes of CD47 expression in silico, *in vitro*, and *in ovo*, but not in murine tumors *in vivo*.	[194]
4T1-breast cancer and B16-melanoma one flank	Portable plasma device (16.5 slm)/He	• 4T1/B16 tumors were incompletely removed to leave tumor mass behind. • Plasma treatment induced ICD *in situ*, decreasing tumor recurrence. • exposure induced strong T-cell responses and cytokine release.	[204]
SB-28-glioblastoma one flank	Nanosecond-pulsed dielectric barrier discharge/ambient air	• No tumor-reducing plasma effect alone. • Modest but significant tumor reduction in combination with Auranofin. • No TME investigation to infer on immunological effects *in vivo*.	[177]
B16-melanoma one flank	kINPen plasma jet/dry Ar or He/O_2	• Plasma-treated Ova increased T-cell immune-reactivity compared to untreated Ova. • Vaccination with plasma-treated Ova increases protection from B16-Ova-melanoma growth, and increases T-cell activity and cytokine release. • Feed gas composition matters, He/O_2 more effective than Ar.	[197]
CT26 peritoneal colorectal cancer	kINPen MED plasma jet-treated 0.9% NaCl/Ar	• Peritoneal tumor mass reduction with kINPen non-significantly different from administering the same concentration of hydrogen-peroxide loaded sodium chloride. • *In vitro* ICD induction was similar in both treatment regimens. • No TME investigation to infer on immunological effects *in vivo*.	[205]

differentiation. Thereby, memory T-cells differentiate and can recognize tumor cells, a process that can also be achieved through immunization. A subsequent study that used a syngeneic CT26 colorectal cancer model confirmed the increased activity of cytotoxic immune cells [180]. The tumor cells were killed with plasma in the laboratory; as controls, vehicle liquids and a clinical drug (cisplatin) were used. After injection of the dead tumor cells into mice, the animals were re-challenged with viable tumor cells. The hypothesis was that a vaccine with killed tumor cells activates immune cells and generates antigen-specific T-cells to kill the live tumor cells injected later. Interestingly, in the plasma-vaccine group, the highest percentage of mice (30%) was protected from subsequent tumor growth after live-cell injection of CT26 cells. Moreover, direct plasma treatment of tumors increased CRT expression and DC infiltration into the tumor tissue. In a similar approach, the study was repeated using the B16-melanoma model, revealing that 2/3 of mice were protected from tumor growth by the plasma-treated B16-cell vaccine when live tumor cells were administered later following the vaccine prime-boost-scheme [195].

Interestingly, a combination of long-lived ROS and pulsed-electric field application (both matched to the concentrations and intensity of the DBD plasma device used) did not lead to successful immunization, underlining the unique ability and necessity of short-lived plasma-derived reactive species to perform its immunogenic action. What was unknown so far were two points: first, to what extent is the cancer growth-reducing and tumor-immunological effect dependent on the composition of reactive oxygen and species? Second, is the vaccine generation also working with a plasma jet, such as the kINPen? Data that the latter is true were shown already before in the two mentioned vaccination studies at an international plasma cancer meeting in early 2018 [196]. The vaccination efficacy with the kINPen was 50%, the positive control (mitoxantrone) provided tumor protection to more than 80%, and the negative control (mitomycin C) only about 15% [156].

Finally, an entirely novel field of research is introduced: the plasma-optimized immunogenicity of proteins. This could be helpful in, for instance, tumor vaccine development. We were the first to provide comprehensive data on the proof of concept of such an approach. Using ovalbumin (Ova), a well-characterized protein, and Ova-reactive T-cells, we found plasma treatment of Ova to increase the T-cell response when compared to untreated (native) Ova [197]. Injecting the plasma-treated Ova into mice also generated stronger T-cell responses. We then chose a translational approach by employing B16-melanoma cells genetically engineered to stably express Ova. Ova-specific T-cells attack these cells. Vaccinating wild-type mice with plasma-treated Ova provided significantly greater immune protection from melanoma growth and increased T-cell activity. Importantly, one plasma treatment mode was more potent than another, re-iterating the previously mentioned power that lies in optimizing plasma sources, especially jets, via the feed gas compositions. Using mass spectrometry and circular dichroism (CD) spectroscopy, oxidative post-translational protein modifications with a per-aminoacid-resolution as well as structural protein changes were investigated that depended on the ROS mixture utilized [197].

5.3.3. Plasma as an adjuvant in combination treatment. ROS mixtures were critical to antitumor efficacy and attracting or repelling critical immune cells into the TME using plasma

treatment [156]. Specifically, we identified the helium–oxygen plasma mode, rich in atomic and singlet delta oxygen, besides the argon plasma mode, to critically provide tumor control and elevated tumor-infiltrating leukocytes. Strikingly, this study was the first to show a combination therapy approach of plasma treatment with the toll-like-receptor agonist imiquimod in a syngeneic, orthotopic tumor model by finding increased immune infiltration. Detailed T-cell subpopulation analysis and cytokine profiling provided more immuno-oncological evidence for plasma treatment of tumor cells for the first time. Using a so-called hollow cathode plasma patch and the B16 melanoma model, another report investigated plasma-mediated tumor control and activation of DCs and elevated intratumoral T-cell infiltration and combination therapy with PD-L1 checkpoint inhibitors [198]. This is the first study of its kind using checkpoint antibodies together with plasma as an adjuvant treatment. From a clinical perspective, such a setup is highly likely since malignant melanoma was the first tumor entity the checkpoint immunotherapy was approved for, but some patients still respond poorly [199]. Plasma treatment could aid in providing additional ICD-induced tumor antigen, while T-cell suppression is halted simultaneously due to the checkpoint antibody therapy so that new T-cell clones may emerge. An additional study of note used a syngeneic model of MX-7 subcutaneous rhabdomyosarcoma tumors [200]. Tumor size was not reported to shrink after plasma treatment, but *in vitro*, the cells had already shown large levels of HMGB1 release after plasma treatment. Blood was collected one hour after plasma exposure of the tumors *in vivo*. Markedly elevated levels of HMGB1 were detected in blood serum. This was not the case 24 h after treatment, arguing for a short-term effect, relying on the immediate tumor-toxicity of the plasma treatment. Levels of several other cytokines and cytokines in blood serum were essentially unchanged. Yet, much is unknown on the mechanisms so far, although especially the melanoma models showed critical proof-of-concept experiments on plasma, supporting the idea of enhanced antitumor-immunity and tumor growth reduction.

Using a DBD plasma source and a syngeneic but not orthotopic (subcutaneous injection) glioblastoma model using SB-28 cells, a toxic effect of DBD treatment alone was not found [177]. In combination with Auranofin, a substance targeting thiol redox-homeostasis, additive cytotoxicity was observed *in vivo*; the TME, which could have supported a role of immune cells in this additive effect, was, however, not investigated. The model could be interesting for future studies since the immune system's role in glioblastoma treatment has received increasing interest in the past years [206]. In 2021, a fascinating report emerged, re-iterating the Ryo Ono study from the incomplete tumor resections published in 2018. The idea remained the same: many tumor surgeries either intentionally (e.g. in the presence of vital blood vessels) or involuntarily leave (micro)tumor tissue behind, and such tumor wound is prone to cancer recurrence. The question was whether plasma treatment would induce ICD *in situ*, thereby empowering antitumor T-cell immunity and reducing disease recurrence. The authors could demonstrate the feasibility of such an approach by using two syngeneic and orthotopic models, B16 melanoma and 4T1 breast cancer [204]. They suggest a plasma setup to be feasible as an intraoperative routine following cancer excision, which in principle would also be possible with the kINPen as an approved medical product, as evident by the tumor wound treatments of Hans-Robert Metelmann and colleagues in Greifswald, Germany [161].

The mentioned direct treatment options are suitable for tumors that grow on the surface and are easily accessible (e.g. melanoma). In contrast, as discussed below, solid tumors cannot be plasma-treated directly due to physiological barriers (at least not in a repeated fashion) but may be targeted by injecting plasma-treated (also referred to as plasma-conditioned or plasma-activated) liquids.

5.3.4. Plasma-treated liquids for solid tumors. As mentioned above, only syngeneic models are discussed here as these can be used to learn about anticancer immune processes. The general efficacy of plasma-treated liquids was recently reviewed [207]. In 2017, a study investigated the ability of kINPen plasma-treated cell culture medium to treat experimental peritoneal carcinomatosis. In a syngeneic and orthotopic (i.e. the tumor occurs at the site in mice where it would naturally occur in humans as well) model, pancreatic cancer was induced by injecting PDA6606 cells intraperitoneally [201]. The repeated injection of the plasma-oxidized liquid (cell culture medium) reduced tumor growth as measured via magnetic resonance imaging, induced apoptosis in cancer cells, and significantly increased mice's survival. Intriguingly, a subsequent analysis of the tumor immune infiltrates revealed substantially elevated levels of macrophages in the plasma group, which, however, had no increased expression of the tumor-supportive M2 macrophage marker CD206 [202]. In addition, significantly more T-cells, DCs, and expression of the ICD-marker CRT were observed in the plasma group. These results already suggested that oxidative tumor cell death is immunogenic. In parallel, we provided evidence of plasma-treated sodium chloride (0.9% NaCl), which has the advantage over cell culture medium that it is a medical product and thereby would have fewer regulatory hurdles to take on the way to the clinics [207]. Plasma-oxidized NaCl reduced CT26 colorectal tumor burden by more than 80%. This was accompanied by elevated levels of macrophages in the tumor tissue and increasingly activated splenic T-cells after *ex vivo* co-culture with CT26 cells [181].

The generation of plasma-treated NaCl with the kINPen plasma jet is a lengthy process. For 50 ml, about 30 min of treatment time were needed. Searching for alternatives, we compared the kINPen head-to-head against another type of discharge, the wINPlas [208], capable of generating 500 ml in 120 min. Interestingly, however, the wINPlas did not significantly reduce CT26 peritoneal tumor burden compared to the kINPen [203]. This was hypothesized to be due to the high acidification with the wINPlas and extremely tutorial for designing future plasma-based peritoneal lavage applications. To exemplify the effect of the high acidification on cells, we mention the different concentrations of the cytokine IL-2

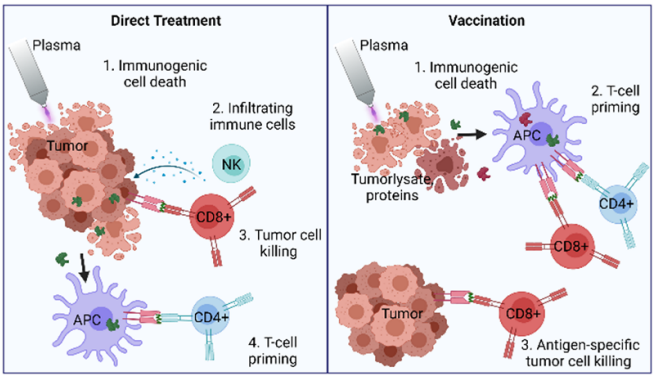

Figure 8. Two main modes and hypotheses in supporting anticancer immunity using plasma technology. In the vaccination approach, an anticancer vaccine is prepared with the help of plasma in vitro/ex vivo, and added to the host thereafter to elicit anticancer immunity.

relevant for the survival of T-cells. For example, the average level of IL-2 in animals without tumors was 2 pg ml^{-1}, which was elevated by tumor inoculation 400-fold to 800 pg ml^{-1}. Therapeutically inactive lavage with wiNPlas-generated saline decreased peritoneal IL-2 concentrations in tumor-bearing mice to 30 pg ml^{-1}. This suggests that acidification eradicated intraperitoneal T-cell activity almost completely. By contrast, therapeutically-effective lavage with kINPen-generated saline decreased IL-2 levels to about 720 pg ml^{-1}. Therefore, plasma-treated liquids may play a role in targeting cancer, especially peritoneal carcinomatosis, in an immunogenic fashion but, similar to direct plasma exposure, the details on the generation and application frequency of plasma-treated liquids can be possibly optimized. However, these studies also provided evidence that the anticancer and potentially immunogenic effects strongly relied on the long-lived oxidant H_2O_2, which we could show in our latest study [205].

In summary, there is ample evidence from *in vitro* and *in vivo* studies for several tumor models and plasma devices that plasma treatment reduces tumor growth in an immunogenic fashion, which could potentially be transferred to clinical settings.

5.4. Scientific concepts for plasma supporting anticancer immunity

From the mentioned studies, three main concepts can be distilled where plasma treatment could support anticancer immunity.

The first concept involves the direct plasma treatment of tumors. Local damage will induce ICD and release tumor antigens to promote anticancer T-cell immunity further. At the same time, plasma treatment elevates inflammatory markers, leading to the influx of additional immune cells into the tumor, which can aid in tumor killing or dampen the immunosuppressive features of the TME (figure 8, left panel).

However, definitive evidence is missing that this process contributes to the anticancer effect in the plasma setting. The issue is that the plasma treatment performs anticancer action by itself, so it is hard to distinguish how much of the effect comes from the immune system (at least in the models where only one subcutaneous tumor was injected). Animal studies are needed to control for this. Using antibodies, T-cells should be blocked and eventually removed from the system to infer on their role. If the long-term (in the sense of a few weeks in a mouse model) anticancer plasma effects are more potent in the presence of T-cells, their antitumor contribution is clearly shown. In the case of sustained tumor growth retardation upon plasma treatment also in the absence of T-cells, this could be a result of the treatment to change, e.g. the metabolic profiles or other TME features of the tumor. In addition, tumor models with moderate growth kinetics should be chosen to investigate immune-related processes better. Another unknown factor is the composition of the TME in the tumor model in question. It is known that tumor cells can shape the TME in their favor so that even the most dedicated T-cells will become immunosuppressed quickly in the TME. Although this is still a question in tumor immunology in general, plasma may be a tool to study how local tumor damage affects anticancer immunity. Another point is to unambiguously show that immune-related molecules, such as DAMPs and ICD markers, are increased within the tumor after plasma treatment. The unavoidable nature of most studies is repeated plasma exposure, followed by animal euthanization at given endpoint days to weeks later. To study immediate ICD or DAMPs effects, proof of concept experiments should terminate animal experiments e.g. already 24 h after plasma treatment. This timepoint also allows for studying the TME in detail. An inspiring approach is blood collection, as was done in one study 1 h after plasma treatment, to monitor blood serum HMGB1 levels [200]. However, it is unclear whether such findings might occur with other tumor cell types, too. Another elegant method would be the injection of genetically modified tumor cells with, for instance, an increased expression of ICD markers *per se*. The highlight would be the expression under a joint promoter with a fluorescent protein or luciferase to follow the intratumoral expression of such a marker via *in vivo* imaging methods.

The second approach is vaccination (figure 8, right panel). We recently provided the first study of its kind that plasma-supported vaccination approaches not only of cells but also of proteins are, in principle, feasible and could be effective [197]. However, it needs to be shown that effects rely on their action by, e.g. using anti-T-cell antibodies to support this idea conceptually. If not, and improved efficacies of plasma-supported anticancer vaccination are still shown, other mechanisms than T-cells need to be considered, e.g. antibody-producing B-cells. A textbook prime-boost (the COVID19 pandemic news made these terms clear to everyone today) immune response in the mouse takes at least two weeks for T-cell immunity and at least three weeks for proper antibody production. The Ova antigen model has already shown increased immunogenicity and successful vaccination properties as a plasma-treated model antigen. It further enables studies to clarify fundamental questions. Nevertheless, proof-of-concept vaccination experiments are needed to show that plasma treatment can increase the immunogenicity of a tumor or tumor-associated antigen (e.g. MART1 in the case of melanoma) [209]. It is also unclear which plasma treatment mode, ROS composition, and exposure time are

needed to increase such immunogenicity to a maximum. In addition, such an approach may work best in the presence of an adjuvant, with the best adjuvant needing to be determined. Moreover, to unambiguously show that the plasma process increased the width and/or depth of T-cell responses to a given antigen, TCR sequencing is needed, a technically ambitious and costly intent. Our recent review can offer more in-depth information on this approach [15].

The third approach to induce toxic effects in the tumors and potentially promote anticancer immunity is using plasma-treated liquids. However, conceptual evidence lacks that such toxicity is due to the short-lived ROS that makes plasma unique. What is observed, to the best of our knowledge, mainly is the presence of H_2O_2, nitrite, and nitrate only [210–212]. These three molecules could be easily added to liquids with higher pharmaceutical accuracy and without using technologically elaborated plasma processes that in clinical scenarios would succumb to strict regulatory and quality management constraints. Some studies claim the presence of some secret ingredient in such liquids because they cannot replicate the effect of plasma-treated liquid if using concentration-matched (chemically generated) analogs of such liquids. This, however, may be a consequence of inaccurate species quantification such as using spectroscopy; the standard methods used and proposed in redox biology, the community working with reactive species for decades, should be used instead. The simplest method to underline the expected dominant effect of H_2O_2 in such liquid is the addition of the H_2O_2 degrading enzyme catalase in the H_2O_2 detection method (to show that the catalase is functional) and the cells exposed to the plasma-treated liquids or the liquid directly [213]. Moreover, H_2O_2 deteriorates over time, and nitrite converts to nitrate; hence, timing in measuring and using such liquids is important. Furthermore, we recently demonstrated *in vivo* the similar efficacy of plasma-treated saline and a concentration-matched control against peritoneal carcinomatosis in mice [205], underlining the dominant role of H_2O_2.

There is also the possibility of plasma oxidizing molecules or biomolecules in liquids. Yet, evidence is lacking that these oxidized molecules are the primary mediators of biological effects induced by plasma-treated liquids apart from a detailed study on Ringer's lactate by Tanaka and colleagues, which, however, was not yet confirmed by other groups or for other plasma devices [214]. Besides the question of the scientific soundness of using plasma-treated liquids, their anticancer effects *in vitro* and *in vivo* are evident and summarized in detail elsewhere [215, 216]. In terms of hard evidence on their immune-stimulating role, all of the mentioned knowledge gaps are also present here. T-cell depletion, thorough investigation of the TME, and more profound exploration of the TCR clonality are vital to unambiguously demonstrate the oxidative (likely H_2O_2-induced) tumor cell death to enable additional immune protection to a certain degree. Conceptionally, our head-to-head study comparing the kINPen vs. the wINPlas treated liquids *in vivo* [203] raised many more questions on whether we understand the generation, storage, characteristics, and applications of such liquids and the potential differences that may appear when testing several tumor models. Some of these aspects were investigated in a very elegant approach, in our hands, by Jinthe van Loenhout. van Loenhout and colleagues compared several cell lines, utilizing human primary monocyte-derived DCs, and assaying several aspects of immunogenicity, such as surface marker, activation marker, cytokine secretion, and tumor cell phagocytosis in the context of plasma-treated liquids [182]. A similar, partially less exhaustive study by Tomic and colleagues extending the investigation to T-cell activation by using tumor cell lysates exposed to plasma-treated liquids *in vitro* provides additional clues to test the immunogenic consequences of such liquids [189]. Analogous studies are needed to understand better the relationship between the types of liquids, plasma sources, H_2O_2, nitrite, and nitrate levels, and cell types investigated. It is also required to comprehend the immuno-oncological consequences of plasma-treated liquids within suitable *in vivo* models.

5.5. Clinic-related concepts for plasma supporting anticancer immunity

Plasma is still a relatively recent therapeutic concept in oncology. Concerning the pyramid of evidence-based medicine, the clinical benefit of plasma cancer treatment is markedly questionable. Randomized and controlled clinical studies with larger patient cohorts on high-grade tumors are absent in the literature. Notwithstanding, it is outlined in the following what are the main clinical applications where plasma cancer treatment might be helpful to stimulate immunity. It should be mentioned that many new therapies are tested in palliative cancer patients, i.e. patients that failed all standard therapies. This is because it would be unethical to deny patients existing and clinically proven therapies in favor of novel but even less tested approaches of unknown efficacy, even if safety testing was provided.

The first application is the direct and topical treatment of superficial tumors. In terms of potential targets for gas plasma treatment in patient-derived samples, this includes skin cancer, such as melanoma [217] and cutaneous squamous cell and basal cell carcinoma [218], as well as ulcerating cancers breaking through the skin, such as breast cancer [219–221] and oral and head and neck squamous cell carcinoma [161, 222, 223] and oral precancerous lesions [224]. In addition, for most of these tumor types, *in vivo* studies had shown pro-immunogenic effects of plasma treatment as indicated above. Combining the plasma treatment as an adjuvant with checkpoint therapy would appear plausible, allowing potentially novel or supported existing T-cell clones to amplify their antitumor activities. However, checkpoint therapy is less used in the palliation of tumor patients or patients who have already failed to respond to checkpoint inhibitors. Regarding ulcerating tumors, we have previously speculated that their infectious cargo, usually bacteria and fungi, may function as immune boosters during plasma treatment [225]. Specifically, cancer cells and pathogens are killed in such infected ulcerating tumors side by side. The strong immune-stimulating action of microbial products amplifies anti-pathogen and anticancer immune responses. Preclinical evidence proving this hypothesis in

those tumor models is scarce, but microbes have long been hypothesized to support antitumor immunity involuntarily [226]. For instance, the long-standing application of BCG bacteria intentionally instilled into bladder cancer patients' bladders promotes inflammation [227]. For non-ulcerating melanoma and cutaneous squamous cell carcinoma, the skin metastases exposed to plasma would serve as a tumor antigen pool freed by the plasma treatment to promote immune responses. Most lesions could be easily removed via surgery. Still, one of the primary ideas of plasma cancer treatment supporting immunity is not to debulk the tumor (i.e. eliminating tumor mass via plasma exposure). Instead, tumor cell killing should occur in a specific immunogenic fashion in conjunction with inflaming the TME to provide *in situ* antitumor vaccination. Similarly, any tumor wound in the surgerical setting could be subjected to plasma treatment, e.g. internal tumors. The idea would be to remove micro-metastases and induce immunogenic effects in the residual tumor cells. The caveat of such an approach is that plasma could be applied only once (i.e. during surgery). While the idea of treating tumor wounds has been around for more than 10 years, two *in vivo* studies provided experimental evidence that such treatment improves antitumor immunity [192, 204]. In the case of non-ulcerating tumors, the human skin is too thick to permit long-ranged plasma effects, as this was non-successfully tried before using the kINPen [228]. The relevance of mouse models here is relative, as their skin is much thinner than human skin. A promising approach seems the hollow microneedle technology combined with plasma treatment provided by Richard Wirz and Zhen Gu's laboratories [198]. Most clinical evidence with regular plasma exposures is present for treating actinic keratosis, a grade 0 carcinoma *in situ* that—by itself—is not fatal. Nothing is known concerning plasma-treated actinic keratosis's role in endorsed anticancer immunity.

As a second clinical approach, the anti-tumor effect of the plasma could be increased through vaccination (figure 9). As with the previously explained plasma-oxidized tumor-associated antigens or plasma-treated tumor lysate, the tumor could be reduced successfully *in vivo* [180, 197]. Furthermore, the studies show evidence of the generation of memory T-cells after vaccinating oxidized tumor lysates and antigens, which are essential for detecting cancer cells. The increased immunogenicity of the plasma-treated antigens and lysates is based on an adjuvant (supporting) effect. In tumor cells, plasma induces immunogenic cell death, which causes increased immunogenicity. It could also be speculated that the oxidized differ from native antigens. While a native protein is probably recognized as the body's own, plasma-induced modification could lead to an alternative reaction of the T-cells and enables an expansion of the TCR repertoire [229].

Cell transfer therapy with antigen-loaded DCs bypasses this complicated process and has already been shown for DCs loaded with plasma-treated lysates *in vitro* [189]. Yet, such an approach has not been used *in vivo* or in clinics. It would require extensive efforts because the plasma-treated tumor lysate would be categorized as a medical product, subsiding specific and extensive regulatory protocols. Moreover, the detailed treatment protocols and optimal plasma process have not yet been worked out. However, if sufficient proof-of-concept data are available, the method could, in principle, be applied to all tumor types and stages, sufficient immunogenicity provided. It should be mentioned that tumor vaccination is an extensive field of research, and no consensus has been reached in tumor vaccination immunology on optimal vaccination strategies. Hence, albeit the approach of plasma tumor vaccination is encouraging, the clinical application of this strategy probably is a long-term rather than short-term achievement.

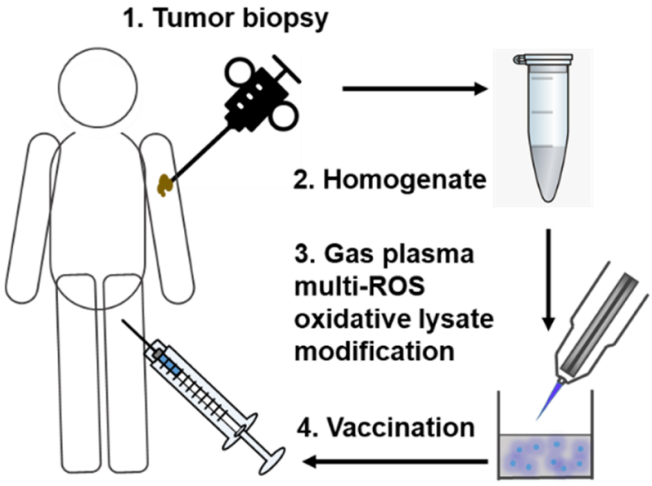

Figure 9. Hypothetical scheme of generating plasma-aided anticancer vaccines. Reproduced from [15]. CC BY 4.0.

The third clinical approach envisioned is immunostimulation using plasma-treated liquids (figure 10). So far, clinical evidence is not present. It must be mentioned that only liquids approved as medical products, such as sodium chloride and Ringer's lactate, can be used as plasma-treated liquid for clinical application [215]. Despite the promising results seen with using cell plasma-treated culture media, such solutions have no practical or clinical relevance. Therefore, those solutions should be avoided to focus research capacities on clinically promising approaches. Plasma-treated liquids could be used in combination with hyperthermic intraperitoneal chemotherapy [230] or pressurized intraperitoneal aerosol chemotherapy (PIPAC) [231]. Here, drug-loaded liquids are pumped or sprayed into the peritoneal cavity to reduce tumor burden if the number of locations of metastases are difficult to remove by surgery. There is not much known about the immunostimulating effects of HIPEC and PIPAC. Combination with plasma-treated liquid may reduce side effects of HIPEC and PIPAC and promote immunogenic oxidative cell death, as suggested in some of our studies [181, 202]. As of now, it is questionable whether clinical evidence will be generated to investigate combination treatment with HIPEC or PIPAC and plasma-treated liquids aiming at increasing anticancer efficacy and promoting immunity. Both treatments are offered only by a few specialized centers at relatively high costs and efforts. Related to the field of plasma-treated liquids is the application of plasma-treated hydrogels to treat surgical tumor margins [232, 233]. However, clinical application has

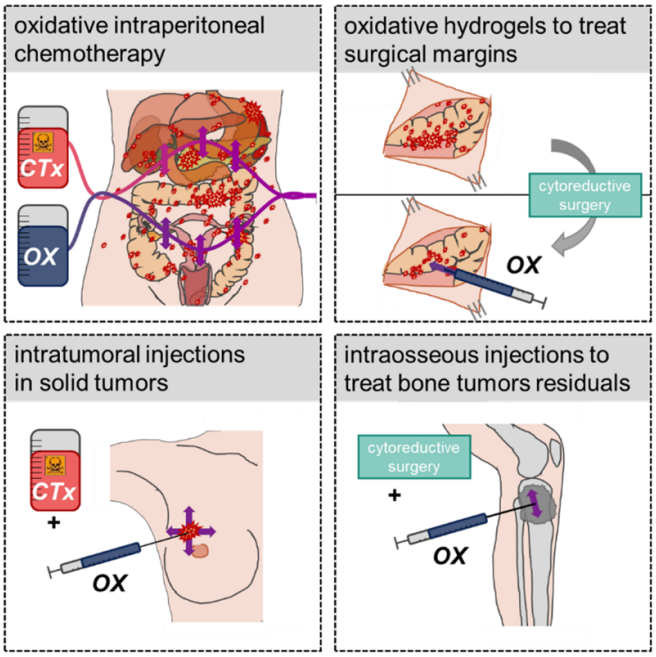

Figure 10. Putative clinical treatment routes of plasma-treated liquids or hydrogels. The type of liquid needs to be a medical product if clinical application is envisaged. CTx = chemotherapy; Ox = oxidized liquid, generated using e.g. a plasma process. Reproduced from [215]. CC BY 4.0.

not been reported for this plasma approach yet. The same is true for intratumoral intraosseous injection of plasma-treated liquids to targeted cancer cells, albeit some promising reports are available on preclinical evaluations of this application [234, 235].

6. Conclusion

Compelling evidence suggests plasma-generated ROS to induce oxidative cancer cell death, which can have inflammatory and immunogenic properties. Several syngeneic tumor models provided evidence of altered immune cell activity and intratumoral infiltration. However, the amplitude by which immune cells contribute to anticancer activity is less clear as of now. Abscopal effects observed in plasma-treated animals indicate unleashed antitumor immune responses. Yet, final proof using T-cell depletion models is awaited. Clinically, the dedicated utilization of plasma processes as antitumor immune-stimulants is not of particular medical interest yet. This is because the use of plasma technology as an anticancer tool is still in its infancy. However, first case reports series in palliative and heavily infected head and neck cancer tumor wounds and using an approved argon plasma jet provide evidence of an anticancer efficacy and suggest potential immune-simulative effects. In general, the vaccination with *in situ* plasma treated tumor cells of surface tumors or resected tumor margins during surgery are leading concepts of how antitumor immunity may be engaged using plasma systems in oncology. Clinical efficacy might be complemented using dedicated plasma-assisted autologous tumor vaccine preparations.

In summary, plasma cancer treatment and immunostimulation is a promising field, but further studies *in vivo* and clinical investigations are awaited to provide more scientific evidence for its rational use for helping future patients.

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