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Immune checkpoint inhibitor combinations: Current efforts and important aspects for success

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ABSTRACT

Immune checkpoint inhibitors (ICI) have emerged as a remarkable treatment option for diverse cancer types. Currently, ICIs are approved for an expanding array of cancer indications. However, the majority of patients still do not demonstrate a durable long-term response following ICI therapy. In addition, many patients receiving ICI therapy develop immune-related adverse events (irAEs) affecting a wide variety of organs. To increase the percentage of patients who benefit from ICI therapy and to reduce the occurrence of irAEs, there is an ongoing effort to combine current ICIs with novel checkpoints inhibitors or other therapeutic approaches to achieve a synergistic effect which is larger than the sum of its parts.

In this review we highlight the essential factors for more effective ICI combinations. We describe how the design of these strategies should be driven by the tumor's immunological context. We analyze current combination strategies and describe how they can be improved to unleash the immune system's full anti-cancer potential as well as convert immunologically "cold" tumors into "hot" ones. We examine the efforts to combine current ICIs (PD-1 and CTLA-4) with novel checkpoints (TIM-3, LAG-3, VISTA, TIGIT and others), immunotherapies (CAR-T cells and Cancer Vaccines) and delivery strategies (bispecific antibodies and other delivery platforms). Importantly, we outline how can one optimally combine ICIs with traditional pillars of cancer therapy such as radiation therapy (RT) and chemotherapy. We discuss the considerations regarding successful combination with RT and chemotherapy; these include fractionation schemes and selection of chemotherapeutics which can both directly eradicate cancer cells as well as increase the infiltration of immune cells into tumors. Finally, we critically assess these approaches and attempt to establish their strengths and weaknesses based on pre-clinical and clinical data.

1. Background

The consensus approach to cancer therapy is continuously evolving; the expanding body of knowledge, demand for more satisfactory efficacy points and prevention of toxicities are the main factors driving this process (Arruebo et al., 2011). Presently, we are at the epic of the immunotherapy era. The Cancer immunotherapy approach is to eradicate cancer cells either by targeting the tumor cells directly or by utilizing a holistic approach to manipulate the patients' immune system to kill the malignant cells. When the immunotherapeutic approach involves recruiting lymphocytes to the tumor site, such approaches are referred to as “Immune Oncology” (Arruebo et al., 2011; Diesendruck and Benhar, 2017; Peskov et al., 2019).

Historically, this promise is over 100 years old. Paul Ehrlich postulated in 1909 that in the majority of the population, aberrant cells remain completely latent thanks to the organism's positive mechanisms (Ribatti, 2017). This, paved the way for our understanding of the involvement of the immune system in cancer surveillance and how we can manipulate it for our therapeutic needs. The earliest demonstration of cancer cell's capability to stimulate a specific immune response dates back to 1953 (Ribatti, 2017). Initially, monoclonal antibodies were designed to target aberrant cells directly with the notion of Ehrlich's “magic bullet” in mind as a treatment which will reduce the side effects of chemotherapy (Cruz and Kayser, 2019; Winau et al., 2004). Ever since the approval of Rituximab, an FDA-approved anti-CD20 mAb for non-Hodgkin’s-Lymphoma (NHL), and the first monoclonal antibody (mAb) approved for cancer therapy, new mAbs have been approved for therapy in the US and Europe on an almost yearly basis (Arruebo et al., 2011; Dillman, 2018). Presently, mAbs are the largest class of biological therapeutics under development, representing a multi-billion dollar

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market worldwide (Arruebo et al., 2011). Currently however, most cancer targeting mAbs have limited efficacy and are considered "life extending" rather than "life-saving" (Diesendruck and Benhar, 2017).

Immune Checkpoint Inhibitors (ICIs) represent an alternative approach to cancer therapy. ICI antibodies do not directly target malignant cells, but prime the patients' immune system and recruit it to combat the disease. Chronologically, Ipilimumab, an anti-CTLA-4 checkpoint inhibitory mAb was the first to be approved by the FDA in 2011 for the treatment of metastatic melanoma (Sondak et al., 2011). Anti-PD1 Pembrolizumab and Nivolumab followed in (2014) as well as Cemiplimab in (2018), more recently the anti-PDL-1 Atezolizumab (2016) followed by Avelumab and Durvalumab (2017) (Chuk et al., 2017; Cruz and Kayser, 2019; Hazarika et al., 2017; Urquhart, 2018; Weinstock et al., 2017). These ICIs are approved for metastatic melanoma, advanced non-small cell lung cancer (NSCLC), Hodgkin's lymphoma, urothelial cancers, squamous cell cancer of the head and neck, Merkel cell carcinoma, kidney cancers, microsatellite high cancers, hepatocellular carcinoma and gastric cancer (Choi et al., 2019; Syn et al., 2017; Zappasodi et al., 2018). The ICI toolbox is expanding and the efficacy of targeting new checkpoints is constantly assessed (Diesendruck and Benhar, 2017). Eleven of thirty-three antibodies in late-stage clinical development, that were undergoing review for approval in 2019 are ICIs (Kaplon and Reichert, 2019).

Unfortunately, despite promising results in some indications, only a subset of patients initially responds to PD-1 and CTLA-4 checkpoint inhibitors. ICIs produce durable responses in about 20–40% of patients. Among these, Melanoma and Hodgkin's lymphoma patients demonstrate the greatest benefit regarding objective responses (Zappasodi et al., 2018). Clinical evidence reveals that a substantial percentage of initial responders will ultimately relapse with lethal, drug-resistant disease months or years after therapy (Syn et al., 2017). Mechanisms of resistance include lack of tumor infiltrating lymphocytes (TILs), enhancement of natural and regulatory T cells (Tregs), immune-suppressing tumor microenvironments (TME), defective tumor immune-recognition driven by lack of tumor cell antigenicity, insensitivity of cancer cells to immune effector molecules and upregulation of alternative immune checkpoints.

To render ICIs more effective for a larger fraction of patients and to reduce the occurrence of irAEs, current strategies focus on discovering predictive biomarkers for successful ICI therapy which are currently lacking, identifying novel druggable checkpoints, decreasing toxicities and developing combination approaches aimed at either targeting multiple checkpoints simultaneously or combining with other agents which will "prime" patients for a better response. Biomarkers for checkpoint inhibitor therapy prediction are not in the scope of this review, thorough reviews were recently published (Darvin et al., 2018; Gibney et al., 2016; Gorris et al., 2018; Maleki Vareki et al., 2017; Popovic et al., 2018).

Currently, there is a large clinical effort to devise novel ICI combination regimens. The hypothesis is that the combinatorial effect will elicit a strong synergistic effect, which will be more potent than the sum of its parts –i.e. monotherapies. To this extent, efforts are focused on combining approved ICIs with other approved ICIs, novel checkpoints, immune agonists, tumor microenvironment (TME) modulating agents, immunotoxin therapy, CAR-T therapies and cancer vaccines as well as with "old anchors" of cancer therapy – radiation and chemotherapy. These combinations carry a dual effect. First, most of them have direct cancer killing properties. Second, they may "prime" the immune context of tumors to bolster current ICI efficacy.

However, to achieve the optimal combinational effect, there are many aspects which need to be considered for each combination beyond agent selection. Therefore, in the next sections we will detail the rationale for these combinations, the preclinical and clinical evidence supporting it and will critically address open questions pertaining to the combination.

2. Checkpoint inhibitors and activators – brief biological introduction to immune checkpoint inhibitors and status update

ICI therapies are a product of extensive research efforts into the interactions that occur when immune cells meet their target cells or antigen-presenting cells (APCs), termed as the "immune synapse". The interactions that occur in this synapse enable specificity and control, thereby achieving a balanced reaction (Cruz and Kayser, 2019). In terms of the effect on T cells (which are the relevant immune cells when ICIs are discussed), the cumulative effect of the interactions between immune receptors and their ligands may be inhibitory or activating.

Cancer cells take advantage of these control mechanisms and manipulate the immune response in their favor, among others by suppressing the immune response via inhibitory immune checkpoint pathways. Of these checkpoints, Cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) and programmed cell death protein 1 (PD-1) are the most explored checkpoints for ICI-based therapeutics.

CTLA-4, a membrane protein, is a homologue of CD28, which has a higher binding affinity to B7 proteins. CTLA-4 competes with CD28 and prevents the second signal required for activation of T cells following engagement of the T cell receptor. This protein is upregulated after co-stimulation of TCR and the B7-CD28 axis to lull the immune response and prevent possible autoimmune reaction (Cruz and Kayser, 2019).

The CTLA-4 checkpoint was the first to be discovered (Popovic et al., 2018). CTLA-4 is upregulated in activated T cells, it is constitutively expressed on regulatory T cells (Tregs) and is relevant for T cell activation processes which occur in the secondary lymphoid organs (Choi et al., 2019; Diesendruck and Benhar, 2017). Anti-CTLA-4 antibodies have a dual function, they both lead to activation of effector cells by blocking the inhibitory axis as well as deplete inhibitory T cells (Tregs) at the tumor site through antibody-dependent cellular cytotoxicity (ADCC) or antibody-dependent cellular phagocytosis (ADCP) (Cruz and Kayser, 2019). This inhibition and depletion increases the $CD8^+$ to Treg ratio (Twyman-Saint Victor et al., 2015). This treatment also leads to an immune memory response. The pillars of successful immunotherapy are increased antigen recognition and activation of $CD8^+$ tumor-infiltrating lymphocytes (TILs) as well as induction of tumor-specific immunological memory. It is known that therapeutic response to the anti CTLA-4 mAb Ipilimumab in cancer patients can take up to several months after treatment (Diesendruck and Benhar, 2017). This is interesting considering that Ipilimumab has a terminal half-life of 15.4 days (van Bussel et al., 2019). This phenomenon is explained by a post treatment immune memory response, mostly attributed to an increase of central memory $CD4^+$ and $CD8^+$ T cells and effector memory T cells.

Following CTLA-4, the PD-1/PD-L1/PD-L2 axis was the next prime target that received more attention for ICI therapies with several approvals. The PD-1 receptor is expressed on activated T cells including Tregs, B cells, macrophages NK cells and several APC types (Cruz and Kayser, 2019). PD-1 can bind PD-L1 which is expressed on tissues after an inflammatory response or PD-L2 (Carter et al., 2002; Diesendruck and Benhar, 2017). Cancer cells take advantage of this and upregulate PD-L1 and PD-L2, two ligands which are overexpressed in many cancers. PD-L1 is more abundant in solid tumors, whereas PD-L2 is mostly expressed in B cell lymphomas and APCs (Daver et al., 2018; Diesendruck and Benhar, 2017; Labriola et al., 2019; Zappasodi et al., 2018). Overall, the outcome is the same, a controlled and soothed immune response. PD-1 is expressed on activated T cells and is not found on naïve or memory T cells. Rather, it is induced upon TCR stimulation or TGF-β and cytokine signaling (Cruz and Kayser, 2019). Therefore, compared to CTLA-4, PD-1 blockade is most relevant in the tumor microenvironment (TME) setting, where the T cells have already been activated (since PD-1 is primarily expressed by mature T cells in peripheral tissue). In patients, PD-1 blockade in cells circulating in peripheral blood was minor when compared to changes in TILs within tumors. Generally, two responses are noted for the PD-L1 upregulation

in cancer cells, either an innate upregulation whereby PD-L1 is a "passenger" mutation of overall mutations occurring within the cells or an adaptive PD-L1 upregulation which results from IFN-γ or other inflammatory cytokines within the TME. Eventually, PD-L1 binding to PD-1 reduces IFN-γ production by activated T cells and results in T cell exhaustion, anergy and eventually leads to differentiation of naïve $CD4^+$ T cells to Tregs (Labriola et al., 2019). Accordingly, Several studies point to a strong correlation between IFN-γ levels within the TME and PD-L1 expression in human tumors (Diesendruck and Benhar, 2017). Similar to CTLA-4, PD-1 blockade also results in an immune memory effect. In biopsies derived from cancer patients, $CD8^+$ memory T cells were the most prominent phenotype that expanded intra-tumorally upon therapy and are known to be indicative of better responses (Ribas et al., 2016). Natural Killer (NK) cells also have an important role in PD-1 blockade. PD-1 expression was noted on NK cells in cancer patients (Wang et al., 2019). Clinical data demonstrates how blocking PD-1 and PD-L1 can elicit a strong NK cell response that is indispensable for a complete immunotherapeutic effect. Furthermore, NK cells can stimulate DC recruitment to the TME which can result in tumor inhibition. Natural killer T (NKT) cells express markers of both T cells and NK cells yet are able to kill tumors via different NK and T cell associated mechanisms. NKT cells are abundant at highly immune infiltrated tumor sites and can also become overstimulated and anergic (Wang et al., 2019).

When comparing CTLA-4 and PD-1 inhibition, the main difference is in the site and stage of inhibition. PD-1 inhibition mostly occurs in the TME, whereas CTLA-4 inhibition occurs primarily in the secondary lymph nodes. Regarding the stage of inhibition, CTLA-4 blockade is considered more relevant for initial naïve T cell activation while PD-1 blockade is considered relevant for lymphocytes which have already been activated. Regarding the targeting antibodies, PD-1 inhibitors such as Nivolumab have a human IgG4 isotype, which has low to no potential to induce ADCC or complement-dependent cytotoxicity (CDC). This is key to prevent the killing of T cells upon binding of these antibodies. The Anti-CTLA-4 mAb, Ipilimumab, on the other hand has an IgG1k isotype which is necessary for its dual function of both inhibition and depletion of Tregs (van Bussel et al., 2019). It was noted that Nivolumab has FcRn binding capabilities which can enable antibodies to enter cells via endocytosis and possibly cross the blood brain barrier (BBB). Nivolumab has a half-life of 12–20 days regardless of the dose but occupies over 70% of PD-1 receptors on circulating T-cells for 2 months after infusion (van Bussel et al., 2019). Regarding toxicities, PD-1 inhibition demonstrates lower rates of immune-related adverse events (irAEs) compared to other standard therapies since $CD8^+$ T cells preferentially recognize neo-antigens rather than self-antigens (Chowdhury et al., 2018).

Predicting which patients will benefit from this treatment is not a simple task. Both PD-1/PD-L1 blockade as well as CTLA-4 inhibition are considered more effective for cancers with higher tumor mutational burden (TMB) (Diesendruck and Benhar, 2017; Popovic et al., 2018; Saleh et al., 2019). However, a recent Phase III clinical trial failed to demonstrate this correlation (Horn et al., 2018; Saleh et al., 2019). Adding to this, CheckMate 214, a Phase III clinical trial assessing PD-1 and CTLA-4 inhibitory combination in renal cell carcinoma, failed to demonstrate better survival outcome with PD-1/PD-L1 checkpoint inhibition therapy in $PD\text{-}L1^+$ compared to $PD\text{-}L1^-$ patients (Labriola et al., 2019; Motzer et al., 2018). Regarding efficacy, in a direct comparison between Nivolumab and Ipilimamab, Nivolumab outperforms Ipilimumab (Eggermont et al., 2018).

As monotherapies, current checkpoint inhibitors fail to achieve durable clinical responses in nearly 80% of cancer patients (Heidegger et al., 2019; Horn et al., 2018; Popovic et al., 2018). The effort is currently focused on increasing this durable response rate, one promising strategy is combination therapy. There is an increasing body of work devoted to the combination of ICIs with additional anti-cancer approaches (Heidegger et al., 2019). The working hypothesis is that with a combination approach, a synergistic effect may be achieved.

One crucial aspect to address in these combination approaches is untoward toxicity. Unfortunately, and similar to other immunotherapies, the strongest irAEs are correlated with effective therapy (Sato et al., 2019). The term immune-related adverse events (irAEs), was coined after ICI therapies (Diesendruck and Benhar, 2017). Most irAEs are mild or moderately severe, whereas grade 3–4 irAEs occur in approximately 8% of anti-PD-1/PD-L1 patients, 25% of anti-CTLA-4 patients, and 50% in patients undergoing anti-PD-1/PD-L1 and anti-CTLA-4 combination therapy (Simonaggio et al., 2019). Of ICIs, anti-CTLA-4 has been associated with the highest frequency of treatment related irAEs, in some clinical trials reaching 90% of patients with 43% exceeding grade 3 irAEs, where patients died of colitis, myocarditis or Guillain-Barre syndrome. This is compared to only 14% in patients treated with PD-1 axis inhibitors Nivolumab or Pembrolizumab (Eggermont et al., 2018). Toxicities range from diarrhea, colitis, dermatological side effects, thyroid imbalance, cardiac and coagulation disorders to neurological disorders (Antonov et al., 2019; Sato et al., 2019; Spain et al., 2016). Different irAE degrees demand different interventions; high grade 3–4 irAEs usually necessitate discontinuation of ICI therapy. A clinical trial assessed the option to re-administer ICIs after irAEs in cancer patients. This study aimed to measure the incidence of a second irAE in patients who following initial PD-1/PD-L1 blockade, demonstrated an initial grade 2 or higher irAE. It is established that patients harboring melanoma, lung, colorectal carcinoma and lymphoma who suffered from irAEs, displayed a shorter time after second treatment till the initial onset of irAE (9 vs. 15 weeks), yet the second irAE was not more severe than the first (Simonaggio et al., 2019).

3. Checkpoint combination opportunities - on hot and cold tumors

Grossly, primary tumors and metastases can be divided into "hot" and "cold" tumors. This “heat gradient” refers to the count and density of TILs within the tumor bed. “Hot” tumors are highly infiltrated by T cells and generally express high levels of some inhibitory checkpoint combination (PD-1, CTLA-4, TIM-3, LAG3) and respond better to ICIs. “Cold” tumors lack infiltrating T cells, due to lack of T cell activation on the account of a lack of tumor antigenicity and immunogenicity and rarely respond to ICIs. They are considered immunologically "ignorant", rarely express PD-L1 and are characterized by high proliferation with low mutational burden and low expression of neoantigens (Galon and Bruni, 2019). New approaches elaborate on this scheme and suggest further subdivision into “immunosuppressed hot tumors” and “excluded tumors”. Immunosuppressed hot tumors have limited T cell infiltration due to the presence of inhibitory cytokines (TGF-β, IL-10, and VEGF), lack of immune cell attracting chemokines such as CXCL 9/10/11 or CCL2/5 or presence of myeloid-derived immunosuppressive cells (MDSCs) and Tregs which repel T cells from infiltrating. In "Excluded tumors", T cells can be found at the periphery but not in the tumor bed due to physical blockade by aberrant ECM remodeling, tumor vasculature and hypoxia (Galon and Bruni, 2019). This gradient has important prognostic value and is standardized as what is known as an "Immunoscore". The immunoscore classifies cancers according to their immune infiltration ranked from I0 for low to I4 for high infiltration. An international effort established that the immunoscore-based prognosis not only successfully predicts risk assessment, but also outperformed all other clinical prognostic parameters regarding prognosis and was superior to the classical tumor size, node infiltration and metastasis (TNM) classification system. This was carried out in a multiple cancer center study by determining total tumor infiltrating T cell counts and time until recurrence after surgery in patients with stage I-III colon cancer (Jorissen et al., 2018; Pagès et al., 2018). This can carefully lead to the conclusion that immune context of tumors is more predictive for prognosis as well as treatment compared to the tumors physical aspects such as size and metastasis. However, in many instances, disease

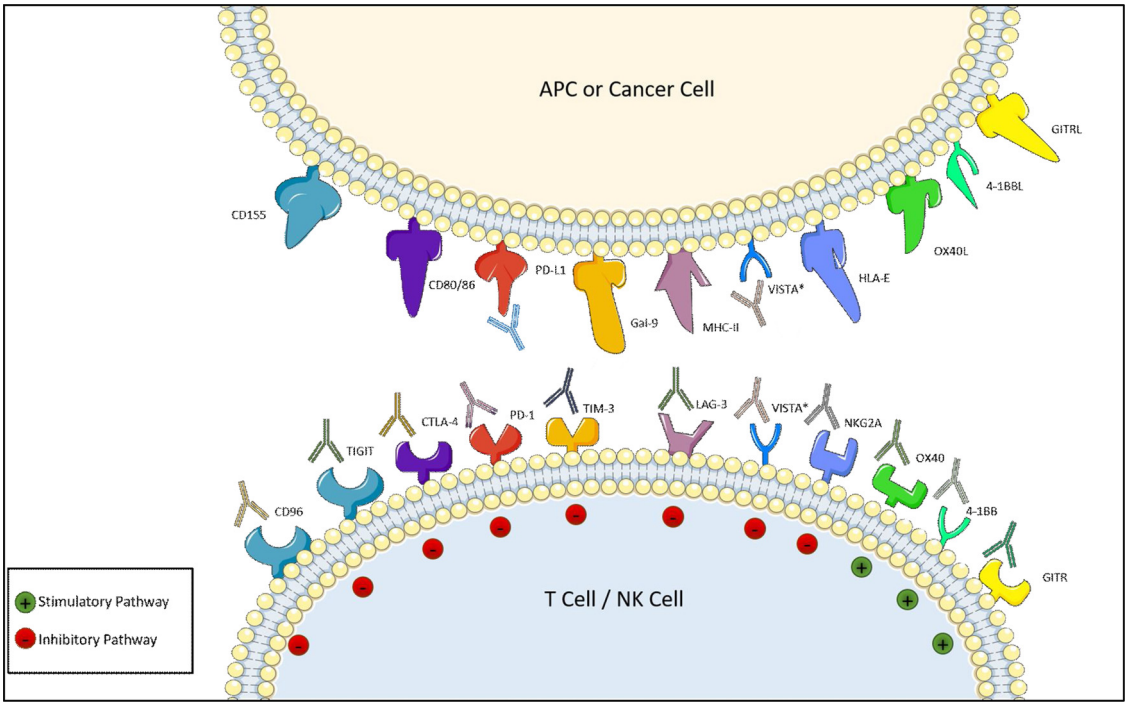

Fig. 1. Unleashing Multiple Breaks.
In the context of immunologically "hot" tumors, there is a large "target bank" of stimulatory, inhibitory receptors and ligands that can be targeted. Antibodies can either inhibit or stimulate receptors to reinvigorate exhausted T and NK cells. Due to compensatory upregulation of immune checkpoint molecules, blockade of one checkpoint will result in an upregulation of a different one. Therefore, there is a gradual shift towards targeting checkpoint combinations to achieve the maximal therapeutic effect. Inhibitory pathways include: PD-1; CTLA-4; TIM-3; LAG-3; TIGIT; CD96; NKG2A and VISTA (*expressed on effector T and NK cells as well as APCs). Stimulatory pathways include: OX40; 4-1BB and GITR among others.

progression and disease "coldness" go hand in hand as most stage IV cancers such as melanoma have poor to no TIL presence (Galon and Bruni, 2019).

Tumor "heat" gradient should guide considerations for all ICI strategies. PD-1 blockade will not be effective in a "cold" tumor context and will not be able to unleash antitumor T cell responses without fully primed T cells being present at the tumor site beforehand. Therefore, the presence of $CD8^+$ T cells at the tumor site is critical for therapeutic effectiveness in PD-1 blockade. CTLA-4 on the other hand is trickier to predict, since it is activated earlier in the lymph nodes (Galon and Bruni, 2019).

Therefore, treating "hot" tumors should focus on "lifting multiple brakes" and reinvigorating previously activated and exhausted T cells for a more potent immune response. It can be expected that "hot" tumors will benefit most from treatment with one or a combination of ICIs. Regarding "cold" tumors, we believe that a strategy of choice should be focused on attempts to "heat up" the tumor by different approaches before ICI intervention.

One way to render a tumor more "hot" is to cause Immunogenic Cell Death (ICD). ICD is a two-component process which includes antigenicity and adjuvanticity. ICD includes release of danger associated molecular patterns (DAMPs) in response to cellular stress and death as well as increased antigenicity such as display and release of neo-antigens (non-self-mutated and immunogenic antigens) which were not previously screened for peripheral tolerance (Galluzzi et al., 2017). In the past it was believed that the type of cell death (necroptosis or apoptosis) dictates an ICD, yet it is now realized that there is a requirement for antigenicity as well. This explains why tumors with high mutational burdens have better response rates.

Type I IFN, inflammasome and autophagy signaling pathways are all known to be involved in ICD. These pathways release or expose immune activating DAMPs in the TME. These include secretion of ATP, IL-1β, HMGB1, CXCL 9/10/16; and exposure of ER-related proteins such as chaperones and Calreticulin (CALR) on the cell surface (Galluzzi et al., 2017). For example, CALR exposure on the surface provides an "eat-me" signal which favors the transfer of Tumor associated antigens (TAA) to dendritic cells (DCs). Dying tumor cells can also stimulate Type-I IFN response which induces the local production of CXCL10 which attracts T cells and memory T cells to the tumor bed. HMGB1 is released from cells undergoing ICD, and acts as a robust adjuvant by binding to distinct PRRs including TLR2, TLR4 among others (Galluzzi et al., 2017). For these reasons, in "cold" tumors the second combinational arm should be focused on "heating up" tumors for a more potent ICI combination.

4. Releasing multiple breaks – combining ICIs

In "hot" tumors with a high immunoscore, TILs are already present at the tumor bed and metastases sites, yet they are exhausted and express some combination of inhibitory immune checkpoint molecules. Therefore, in this context, the combination strategy should be focused on "releasing" multiple breaks simultaneously, to obtain the most potent anti-cancer activity.

Naturally, the first two ICIs to be combined in a clinical trial were anti-PD-1 and anti-CTLA-4 ICI. CTLA-4 and PD-1 are activated via distinct non-redundant pathways. Preclinical studies have demonstrated that CTLA-4 blockade cannot restore exhausted T cells function and the PD-1 blockade cannot lead to anti-tumor responses if T cells are not fully primed and committed beforehand (Zappasodi et al., 2018). A phase III clinical trial demonstrated complementary activity of a Nivolumab and Ipilimumab combo therapy in metastatic melanoma (Larkin et al., 2015). The study concluded that the combined blockade of PD-1 and CTLA-4 was more effective than either target alone in PD-L1 negative melanoma. The overall survival in the combination arm did not seem very convincing at the 21 month mark yet demonstrated much more impressive results for the combination at the 57 month mark

(Hodi et al., 2018; Larkin et al., 2015). However, when examining the data, it seems like Ipilimumab underperformed compared to the combo or Nivolumab by itself. Eventually, ICIs produce durable responses in about 20–40% of metastatic melanoma patients as monotherapies yet up to 60% when combined (Zappasodi et al., 2018). This dual combination is also being tested for melanoma brain metastases; two phase II trials demonstrated intracranial activity of ICI inhibitors Nivolumab and Ipilimumab (Tawbi et al., 2018; van Bussel et al., 2019). Presently, dual ICI therapy combination (anti-PD-1 and anti-CTLA-4) has already gained approval for metastatic melanoma, renal cell carcinoma and subtypes of metastatic colorectal carcinoma (Cruz and Kayser, 2019).

Presently, novel checkpoint opportunities are being explored (Fig. 1). An increasing body of evidence demonstrates that there is a multitude of checkpoint axes in exhausted TILs, known as "compensatory upregulation of immune checkpoint inhibitors". It stipulates that blockade of one of the immune checkpoint molecules will lead to upregulation of the others, as commonly observed across different tumor types. These exhaustion markers can include CTLA-4, PD-1, TIM-3 and LAG-3 (Bialkowski et al., 2018). This might be the reason why single agent anti-PD-1/PD-L1 therapeutics have somewhat limited efficacy and motivate the attempt to apply multiple ICI combination. A preclinical study demonstrated that over 80% of TILs isolated from tumors *ex vivo* co-expressed PD-1, TIM-3 and LAG-3 (Bialkowski et al., 2018). In a murine GBM model it was found that the exhausted T cell phenotype corresponds to at least 2 markers (Kim et al., 2017). Since they are co-expressed, the "target bank" is enlarged and strategies to target multiple checkpoints simultaneously in "hot" tumor contexts are unfolding.

4.1. T cell immunoglobulin and mucin-domain containing molecule-3 (TIM-3)

TIM-3, an inhibitory receptor expressed on $CD4^+$, $CD8^+$, NK cells, Tregs, dendritic cells binds primarily to Galectin-9 on APCs to trigger T cell apoptosis (Popovic et al., 2018; Wang et al., 2019). It can also bind CEACAM-1, which is expressed by many tumors and is upregulated by IFN-γ (Dankner et al., 2017). TIM-3 has been described as a negative immune regulator which promotes peripheral tolerance, its expression is linked with chronic viral infections and its lack of involvement is correlated with autoimmune diseases (Koguchi et al., 2006; Yan et al., 2013). It has also been described as an infiltrating Treg marker. In one report, over 60% of TIL $TIM3^+$ $CD4^+$ T cells co-expressed CD25, and 80% were $CD127^{low}$ concomitant with expression of the FOXP3 transcription factor which together describe Tregs. Interestingly, this was found only in TILs and not in peripheral lymphocytes. This was explained by the Galectin-9:TIM-3 interaction which promotes Treg expansion in the tumor. In a characterization of 100 specimens of human hepatocellular carcinoma, cervical colorectal and ovarian cancer patient tissues, $TIM\text{-}3^+$ $CD4^+$ T cells were enriched in TILs compared to peripheral blood and non-TILs (Yan et al., 2013). However, contrasting reports claim Galectin-9 eliminates $TIM3^+$ effector cells by triggering their apoptosis and not by suppressing them. In melanoma patients, an anti-TIM-3 antibody restored T cell function of $TIM\text{-}3^+$ $CD4^+$ and $CD8^+$ cells which contribute to the immunosuppressive TME (Fourcade et al., 2010; Yan et al., 2013). In a murine glioblastoma multiforme (GBM) model, TIM-3 blockade concomitant with PD-1 inhibition and radiotherapy resulted in 100% overall survival which was not demonstrated in each of the other arms by itself (Kim et al., 2017). The long-term survivors demonstrated increased immune cell infiltration, activity and memory. This study demonstrated that TIM-3 blockage by itself did not yield a noticeable overall survival or $CD8^+$ or Treg cell population benefit within the TME. However, it increased IFNγ, TNFα, and IL17α inflammatory cytokine expression within these tumors. Furthermore, they have pinpointed the added benefit of the TIM-3 inhibition to be $CD4^+$ T cell dependent, since $CD4^+$ T cell depletion abrogated all anti-cancer effect of TIM-3 inhibition (Kim et al., 2017). Taken together, these findings mark $TIM\text{-}3^+$ cells as infiltrating intratumoral Tregs. Currently there are clinical trials which are assessing the combination of TIM-3 and PD-1/PD-L1 blockade combination in various advanced solid tumors (NCT02817633, NCT03099109, NCT02608268).

4.2. T cell immunoreceptor with immunoglobulin and ITIM domains (TIGIT) and CD96

TIGIT is expressed on $CD4^+$ and $CD8^+$ T cells upon activation, NK cells, follicular helper T cells, NKT cells and is highly upregulated on tumor tissue Tregs. TIGIT competes with the activating receptor CD226 for the immune activating receptor CD155 expressed on APCs and tumor tissue. TIGIT binding leads to both suppression as well as the direct killing of $TIGIT^+$ $CD8^+$ and NK cell (Dougall et al., 2017). The higher affinity of TIGIT for CD155 compared to CD226 results in competitive inhibition of co-stimulation, similar to CTLA-4 competitive inhibition. TIGIT binding shifts cytokine production from IL-12 to IL-10. Blockade of TIGIT synergizes with PD-1 blockade to increase production of IFN-γ and TNF-α by $CD8^+$ T cells. TIGIT is upregulated and co-expressed with PD-1 by the majority of tumor antigen specific $CD8^+$ T cells in the periphery and within metastatic tumor of advanced melanoma (Chauvin et al., 2015). Interestingly, $TIGIT^+$ $PD\text{-}1^+$ Tumor antigen-specific $CD8^+$ cells have an effector memory phenotype and are more differentiated than either $TIGIT^+$ or $PD\text{-}1^+$ cells. Therefore, dual blockade of TIGIT and PD-1 can lead to unleashing the effect of CD8 effector memory cells which are correlated with good prognosis. In preclinical studies, TIGIT PD-1 ICI combination enhanced $CD8^+$ T cell expansion of TILs and cytotoxic response to melanoma antigen stimulation compared to monotherapy checkpoint blockade (Dougall et al., 2017). TIGIT is currently evaluated in clinical trials both as a single agent as well as compared to combination with PD-1 blockade in advanced solid cancers (NCT03628677, NCT02913313).

CD96 is expressed primarily on α/β and γ/δ T cells, NK cells and NKT cells (Dougall et al., 2017). CD96 is another inhibitory component of the CD226/CD155/TIGIT axis which competes for CD155 binding (Georgiev et al., 2018). In various murine models, CD96 blockade suppressed primary tumor growth and promoted greater tumor control by increasing the population of IFN-γ secreting TILs (Mittal et al., 2019). CD96 blockade was found to be most effective when combined with PD-1, PD-L1, TIGIT and CTLA-4 blockade (Dougall et al., 2017). CD96 is co-expressed with PD-1 in TILs in both murine and human cancers. CD96, TIGIT and PD-1 represent non-redundant mechanisms of tumor induced immune suppression and T cell dysfunction. While TIGIT is highly enriched in Tregs, CD96 which is expressed on effector cells, this leads to an interesting option for checkpoint combinations by targeting non-redundant mechanisms (Dougall et al., 2017).

4.3. Lymphocyte-activation gene 3 (LAG-3)

LAG-3 is expressed on effector T cells, Tregs and dendritic cells among others (Lui and Davis, 2018; Popovic et al., 2018). As a structural homolog of CD4, it binds MHC-II and transmits inhibitory signals to reduce proliferation and effector function. Upregulation of both PD-1 and LAG-3 leads to T cell exhaustion and tolerance to self and tumor antigens. Inhibiting PD-1 and LAG-3 expression resulted in full tumor eradication in preclinical models. Currently, data from a clinical trial combining LAG-3 and PD-L1 inhibition demonstrated an overall response rate of 13% in patients who had previously relapsed on anti-PD1-monotherapy (Popovic et al., 2018). Dual inhibition of LAG-3 and PD-1 checkpoint blockade is currently assessed in clinical trials for solid tumors (NCT01968109).

4.4. V-domain Ig suppressor of T cell activation

VISTA, a structural homolog of PD-L1 is expressed on both myeloid

APCs and Tregs (Popovic et al., 2018). VISTA enhances Treg maturation, inhibits T cell activation and contributes to an immunosuppressive TME (Popovic et al., 2018). VISTA and PD-1 suppress T cells by non-redundant immune regulatory networks and can synergistically regulate T cell responses (Liu et al., 2015). VISTA blockade was found to increase T cell recruitment to the tumor bed, enhance anti-cancerous T cell functions and further differentiate $CD8^+$ T cells. Preclinical murine models demonstrate the added benefit of dual targeting of VISTA and PD-1 as well as CTLA-4 (Kondo et al., 2016; Liu et al., 2015). VISTA was found to be upregulated in TILs and M2 macrophages from localized and metastatic prostate cancer as well as prostate cancer patients after Ipilimumab treatment (Gao et al., 2017; Popovic et al., 2018).

4.5. CD94/NKG2A

NKG2A is an inhibitory receptor expressed on both T and NK cells. Targeting this checkpoint inhibits its signaling on both cell types and unleashes tumor specific T cell proliferation, memory and can assist in rendering cancer vaccines more effective (André et al., 2018; van Montfoort et al., 2018). This inhibition results in a greater population of effector memory $CD8^+$ T cells. NKG2A contributes to NK cell exhaustion and was noted in liver cancer and chronic lymphocytic leukemia among others (McWilliams et al., 2016; Sun et al., 2017). The NKG2A ligand is expressed in many tumors including lung, colon, pancreas, stomach, head and neck and liver (André et al., 2018). Monalizumab, a NKG2A inhibiting mAb is currently evaluated in clinical trials for various cancers either as monotherapy or in combination with PD-1/PD-L1 inhibitors (NCT02643550, NCT03822351) (Muntasell et al., 2017).

There is an increasing recognition of the contribution of NK and NKT cells to these therapies and their importance for ICI therapeutics. For example, NK cells have an important role in PD-1 blockade. Tumors with low MHC expression respond to PD-1 checkpoint blockade and clinical responses are also noted in cancers with extremely low mutational burdens such as Hodgkin's lymphoma. This suggests that immune cell types other than T cells may also play a role in PD-1 blockade (Hsu et al., 2018). In preclinical models, it was demonstrated that NK cell response is crucial for successful immunotherapy, and that PD-1 is expressed on NK cells similarly to T cells and marks exhausted cells. Furthermore, NK cells often infiltrate melanoma and lung tumors which can assist ICI therapies. For example, in Hodgkin's lymphoma, 79% of patients show decreased or no MHC-I expression, yet a large majority of patients still responds to PD-1 blockade (Hsu et al., 2018), suggesting that NK cells may be involved.

Testing multiple ICI combinations is a promising strategy for treating "hot" tumors via distinct non-redundant pathways. On the other hand, these combinations also carry the risk of increased toxicities. Lack of TIM-3 signaling for example can contribute to auto-immunity and is known to be associated with disease such as multiple sclerosis (Koguchi et al., 2006; Yang et al., 2014). Also, in the Nivolumab plus Ipilimumab combination, Ipilimumab treatment increased Anti-drug-antibodies (ADAs) against Nivolumab which were absent in the Nivolumab monotherapy arm (Sheng et al., 2017).

5. Turning up the "heat"

Unfortunately, not all tumors are highly infiltrated by immune cells. For these "cold" tumors, simply lifting the breaks will not be effective since there are no TILs in the tumor to activate. In these cases, the focus should be on combining "priming" for successful ICI therapy (Fig. 2). In the next section we will describe how this priming can be achieved with novel as well as "old" therapies. These old therapies can carry a dual effect by directly killing cancer cells and eliciting an immunogenic cell death to bolster ICI therapy.

5.1. Reprogramming the TME

The TME suppresses anti-cancer activities via a multitude of pathways. New combination approaches are attempting to target the tools by which the TME avoids immune-surveillance together with ICIs.

5.1.1. Vasculature and physical barriers

The aberrant tumor vasculature is a formidable challenge TILs need to overcome to reach the tumor and elicit their anti-tumor activities. This challenge is composed of multiple layers, first TIL extravasation is prevented by downregulation of T cell adhesion molecules such as ICAM1, VCAM1 and MAdCAM1. Next, the aberrant vasculature impedes the physical extravasation simply by physical exclusion.

Finally, once they do reach the tumor bed, the lack of oxygen creates a hypoxic environment which is tumor suppressive (Peske et al., 2015). VEGF-A is secreted from tumors to increase angiogenesis. VEGF-A was also noted to modulate TCR signaling and inhibit Th1 and CTL activity (Galon and Bruni, 2019). Th1-=directed activities by ICIs are correlated with vessel normalization. VEGF-A inhibition also promotes the differentiation and function of immune cells (Chowdhury et al., 2018). For these reasons, several clinical trials are assessing different combinations of anti-angiogenic agents with ICIs in metastatic renal cell carcinoma (mRCC). Contrastingly, anti-angiogenic agents increase hypoxia which upregulates the expression of PD-L1 as well as enhances adenosine accumulation in the TME which leads to several cancer promoting effects, making this combination less appealing than other combinations (Galon and Bruni, 2019). Unfortunately, initial VEGF and PD-1 combinations have resulted in high toxicities in clinical trials. Currently, there are clinical trials assessing the benefit of combining Bevacizumab (anti-VEGF) and Nivolumab (anti-PD-1) in renal and kidney carcinoma patients (NCT02210117).

The extracellular matrix protein (ECM) proteins are significantly deregulated during cancer progression. This inhibits immune processes and enhances metastatic spread (Galon and Bruni, 2019). In this respect, in preclinical melanoma models, hyaluronidase was utilized to degrade HA which is overexpressed in tumor tissues, to increase penetration and accumulation of PD-L1 targeting moieties and increase ICI efficacy (Guan et al., 2019).

5.2. Cytokines and immune modulators

Another weapon in the suppressive arsenal of the TME is the use of immunosuppressive cytokines and other immune modulators, the messengers by which they hijack local activities in their favor, to avoid immune surveillance. IL-10 and TGF-β for example, disrupt DC differentiation, migration and antigen presentation, increase Treg activation and tumor angiogenesis (Popovic et al., 2018). Another example, indoleamine-pyrrole 2,3-dioxygenase (IDO1), a tryptophan-metabolizing enzyme which is produced by cancer cells, tumor associated macrophages (TAMs) and MDSCs, diminishes T cell anticancer function and enhances Treg activity (Chowdhury et al., 2018; Popovic et al., 2018). IDO1 depletes tryptophan and leads to accumulation of kynurenine which results in T cell apoptosis (Austin and Rendina, 2015). Preclinical models in which both IDO as well as PD-1 were inhibited have demonstrated success in murine cancer models (Zhu et al., 2019). Currently IDO1 inhibitors are tested in combination with PD-1 inhibitors in metastatic melanoma and advanced solid cancers (NCT02178722, NCT02752074).

Secreted ATP is known as a damage-associated molecular pattern (DAMP) signal which can elicit ICD and recruit phagocytes as well as inhibit tumor cell proliferation. Extracellular ATP mediates both chemotactic and adjuvant like effects by interacting with purinergic receptors P2Y2 and P2 × 7 expressed on APCs and their precursors, respectively (Galluzzi et al., 2017). To remain invisible to immune cells, cancer cells secrete enzymes that metabolize ATP into adenosine which is not only unrecognized by immune cells but also attenuates immune

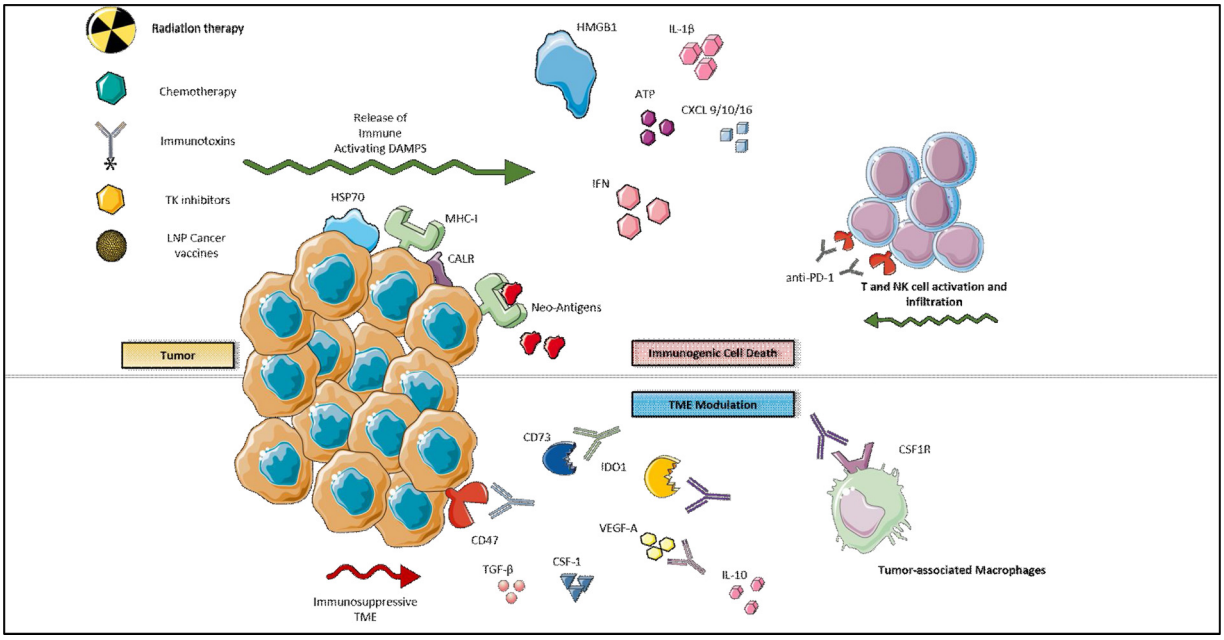

Fig. 2. Turning up the "heat".
In immunologically "cold" tumors, no TILs are present or activated to exert anti-cancer activity. Therefore, there is a need to create a proper immunogenic context by either eliciting immunogenic cell death (ICD) or directly modulating the immunosuppressive tumor microenvironment (TME) prior to immune checkpoint inhibition. Radiation therapy, chemotherapy, immunotoxins, small molecules and cancer vaccines among others, can both "heat" up the tumor context as well as directly kill cancer cells. This priming occurs by eliciting an ICD which releases immunostimulatory Danger-Associated-Molecular-Patterns (DAMPS) to the TME which include display of ER proteins on the plasma membrane (HSP70 and CALR among others), upregulation of MHC-I, release of neo-antigens, Type-I IFN molecules, ATP, HMGB1, IL-1β and CXCL 9/10/16 and more. Targeting CD47, CD73, VEGF-A, IDO1, and CSF1R can directly modulate the TME and dampen its immune inhibitory context.

effector cell function, inhibits T cell proliferation and activation as well as that of NK cells and stabilizes Tregs. Activation of the Adenosine A2A receptor (A2AR) leads to increased cell surface expression of PD-1 and CTLA-4 on T cells, inhibits their proliferation and secretion of pro-inflammatory cytokines (Wang et al., 2019). NK cells were also noted to express A2AR receptors at a higher level than T cells (Wang et al., 2019). Cancer immune evasion largely involves the generation of high amounts of immunosuppressive adenosine within the tumor environment. CD39 and CD73 are ecto-enzymes secreted into the TME, where their sequential activities hydrolyze immunogenic ATP into adenosine (Perrot et al., 2019). CD39 is a plasma membrane bound enzyme that cleaves ATP and ADP down into AMP which is converted into Adenosine by CD73 on the cell surface. Preclinical models of murine melanoma, fibrosarcoma and colon cancer have demonstrated that the proportion of $CD39^+$ T cells was higher in $PD\text{-}1^+$ $CD8^+$ TILs than in the PD-1 – populations (Perrot et al., 2019). The combined targeting promotes cancer patient T cell activation. Currently, there are several clinical trials assessing CD73 and A2AR agonist as monotherapies as well as combined with PD-1/PD-L1 inhibition in advanced solid tumors (NCT03454451, NCT02655822) (Hay et al., 2016).

CD47 a CALR antagonist is another interesting option to target. Calreticulin is exposed on the plasma membrane of ICD cells which is a known "eat me signal". CD47 binds CALR and yields a "don't-eat-me" signal and has been linked to dismal prognosis in individuals in various cancers such as AML, esophageal carcinoma and ovarian cancer (Liu et al., 2017). In breast cancer, CD47 is upregulated by HIF1, marks cancer stem cells and contributes to a lethal breast cancer phenotype (Zhang et al., 2015).

Recently, a Phase Ib clinical trial determined the efficacy of CD47 blockade together with Rituximab (anti CD20) in non-Hodgkin's B cell lymphoma with no clinically significant safety events (Advani et al., 2018). However, once again, toxicities need to be carefully assessed. Since CALR-specific antibodies have been associated with rheumatoid arthritis, coeliac and intestinal bowel disease and can be driven from robust DAMP signaling which might drive adaptive immune responses against self-antigens as a consequence of antigen spreading. Importantly, not all DAMPs released are immuno-stimulatory. For example, adenosine and prostaglandin E2 are also DAMPs which have a key function in immune toleration (Galluzzi et al., 2017).

5.3. Reprogramming TAMs and MDSCs

Another interest is to inhibit M2 tumor associated macrophage (TAM) activity. For example, CSF-1 is a macrophage secreted cytokine which maintains M2 polarization and induces TAM proliferation (Popovic et al., 2018). An anti-CSF1R antibody reprograms TAM polarization and is synergic with checkpoint blockade in pancreatic cancer (Popovic et al., 2018). Another approach is to inhibit TAM migration to tumor site by adding neutralizing antibodies to chemokine receptors. MDSCs promote tumor metastases by suppressing antigen-specific T cell proliferation and inducing Tregs in the TME and limit anti-CTLA-4 response in patients (Popovic et al., 2018). To this extent, researchers are trying to impair MDSC function to deplete or reprogram MDSCs to augment the efficacy of checkpoint blockades. For example, Etinostat, a histone deacetylase inhibitor (HDAC) which impairs MDSC function and contributes to anti-tumor responses when combined with anti-PD-1 antibody in lung and renal cell carcinoma models is currently assessed in clinical trials for solid cancers (NCT02437136). However, hypomethylating agents are a double-edged sword, as they can both enhance anti-tumor response as well as soften the immune response by upregulating immune checkpoint molecule expression (Daver et al., 2018). Another group demonstrated how targeted delivery of TLR7 and TLR8 in β-cyclodextrin nanoparticles which are FDA approved, resulted in an M1 phenotype. They have used FDA approved particles, reported to have TAM affinity *in-vivo* to control tumor growth and protect animals against tumor re-challenge (Rodell et al., 2018). Therefore, while some focus on depleting TAMs is in place, reeducating them can be a better strategy to use them in favor of ICI therapies by harnessing TAMs to the

anti-cancerous effort.

6. Combinations with radiation therapy

Radiation therapy (RT) is a veteran approach for treating some cancer types and is still a backbone of modern cancer medicine. It is estimated that more than 50% of all patients with solid tumors are treated with radiation therapy (Trommer et al., 2019). Radiation therapy confers an advantage over chemical approaches by limiting systemic toxicities and is easily accessible at most cancer centers. Regarding ICI combination, RT normalizes tumor vasculature, enhances expression of leukocyte adhesion molecules on endothelial cells and leads to secretion of $CD8^+$ T cell attracting chemokines (Choi et al., 2019; Dewan et al., 2009; Galon and Bruni, 2019; Zappasodi et al., 2018). An interesting result of RT is that it leads to ICD and the known abscopal effect following RT therapy. The abscopal effect depicts a scenario wherein ionizing radiation to one tumor focus results in regression of distant metastases. The abscopal effect following RT is attributed to the immune system which kicks in because RT induces ICD. Although the abscopal effect of RT alone has been reported many times, the overall occurrence rate is relatively low, probably due to the inability of the immune system to overcome the tumors and suppressive tumors which require ICI combination intervention (Liu et al., 2018). RT-induced damage serves as a type of vaccine by releasing cell fragments, neoantigens, DAMPs and secretion of cytokines (Chowdhury et al., 2018; Liu et al., 2018; Trommer et al., 2019). RT triggers ICD via exposure of CALR on the exterior membrane and ATP secretion driven by autophagy as well as type I IFN signaling, upregulation of MHC-I, exposure of HSP70, TLR3 signaling, HMGB1 release and IL-1β release (Galluzzi et al., 2017; Ko and Formenti, 2019). Therefore, one can expect synergistic effects when combined with ICIs, since RT has somewhat of a vaccine property. Following RT, some patients may express somatic mutations that generate neo-antigens which have the potential to serve as targets for more robust immune responses. Thus far, evidence shows that simultaneous administration of RT and ICI is considered safe and that the number of irAEs does not increase significantly (Trommer et al., 2019). In one study it was reported that combination RT in a metastatic focus with Ipilimumab treatment resulted in a complete response. The patient developed a $CD8^+$ response specific for a neo-epitope upregulated by RT (Ko and Formenti, 2019).

However, there is a need to fine-tune the radiotherapy dosage scheme which will be optimal for ICI combination. While a high-dose per fraction limits effective tumor immunity by minimizing the production of IFN-I in irradiated tumors, low dose high frequency will result in a diminished lymphocyte pool and eventually lead to immunotherapy ineffectiveness (Ko and Formenti, 2019). Interestingly, while high TMB rate is a generally accepted biomarker for immunotherapy RT combination may actually lead to better results in low TMB patients since radiotherapy can increase the TMB in patients (Ko and Formenti, 2019). In other words, RT may be viewed as an “adjuvant” for immunotherapy, which may turn “cold” into “hot” tumors. Furthermore, tumor regression following anti-CTLA-4 therapy combined with radiation was related to upregulation of PD-L1 on melanoma cells and T-cell exhaustion (Twyman-Saint Victor et al., 2015). An analysis of 24 metastatic cancer patients determined that RT within one month after the first or last application of Pembrolizumab or Nivolumab with at least one metastatic lesion outside the irradiation field demonstrated abscopal effects in non-irradiated lesions that were enhanced by PD-1 inhibition. Abscopal effects were noted in 29% of these patients, however optimal dosing and fractionation therapy have not been established at this stage (Trommer et al., 2019). For Hodgkin's lymphoma patients who have relapsed after chemotherapy and stem cell transplantation, combining RT with ICI lead to excellent outcomes (De Forceville et al., 2019). This is tested in a Phase II Clinical trial to assess the optimal Radiotherapy/Nivolumab regimen for non-responding Hodgkin's patients (NCT03495713). This approach was also tested in hepatocellular carcinoma (HCC) for tumors which are very suppressive since in many instances they are initialized due to chronic viral infections (Meyer et al., 2013). In a phase I clinical trial to assess the potential of anti-CTLA-4 + RT in melanoma metastases it was noted that the majority of patients did not respond to this treatment due to PD-L1 expression and T-cell exhaustion. Regarding irAEs, a study evaluating 133 patients with metastatic melanoma, NSCLC and renal cell carcinoma (RCC) treated with RT and anti-CTLA-4 or anti-PD-1 inhibition and radiation concluded that PD-1 inhibition and radiation therapy does not produce an increased risk of toxicity beyond the level of treatment with each agent as a monotherapy (Bang et al., 2017).

One factor that needs to be optimized is the RT regimen and scheduling when combination with ICI treatment is being considered. In murine preclinical models of carcinomas, it was demonstrated that an abscopal effect was noticed after anti-CTLA-4 combined with fractionated but not with single dose RT, which delivered the best effect (Dewan et al., 2009). This stresses the importance of designing a suitable RT regimen when combining the treatment with ICIs. Clinically, RT is given in multiple fractions to find a balance between tumor control and repair of damage to normal tissue in the area (Dewan et al., 2009). Interestingly, single dose and multiple fraction RT demonstrates differential gene expression levels, among genes amplified in all cells tested, there was a notable increase in IFN-related genes. On top of this, breast, prostate and gliosarcoma cancer cell lines demonstrated that a multiple fractioning regimen leads to a more robust induction of genes including both IFN -and TGF-β related genes (Tsai et al., 2007).

While this strategy seems promising, many tasks remain to be optimized such as optimal dose and fractionation timelines, scheduling ICIs with RT and identifying biomarkers which can predict treatment efficacy. Furthermore, fractionation may be ineffective since it may harm infiltrating TILs (Liu et al., 2018). Also, RT has been reported to induce signals to limit immune activation, promote tumor infiltration of MDSCs and lead to PD-L1 upregulation (Zappasodi et al., 2018). RT was reported to lead to secretion of cytokines which support tumor progression such as TGF-β, IL-10, CSF-1 which drives M2 polarization and, contradicting this, anti-cancerous such as IL-1β, CXCL 9, 10 and 16 which can attract anti-tumor effector T cells. RT can directly kill $CD8^+$ T cell lymphocytes and spare the less radiosensitive Tregs (Choi et al., 2019). Combination of RT and ICI is a fascinating approach and thus it will be interesting to see how it develops in clinical trials (NCT03495713, NCT02617589).

7. Combinations with chemotherapy

Presently, all oncology-related antibodies are combined with chemotherapeutics due to their low efficacy as monotherapies (Cruz and Kayser, 2019). Chemotherapy can assist ICI treatment by releasing neoantigens or reshaping the TME by depleting Tregs and MDSCs. Furthermore, chemotherapy is suggested to aid immunotherapy by downregulating PD-L2 expression on DCs and tumor cells, induce maturation of APCs, restore visibility of tumors and upregulate MHC-I expression (Garassino et al., 2018; Qiao et al., 2018). However, not all cytotoxic agents are the same, for example, taxol was reported to act as a TLR4 agonist and restore the anti-cancer activity of TAM by promoting an M1 profile and improving clinical efficacy of ICIs (Garassino et al., 2018). However, TLR4 agonist after prolonged exposure, drove M1 macrophages to an M2 response, stressing again the importance of the timing of treatments.

The notion here is to cause an ICD and turn "cold" into "hot" tumors, therefore selecting cytotoxic agents which are able to prime immunotherapies should be carefully considered. For example, cisplatin differs from oxaliplatin, its derivate, by an inability to trigger translocation of CALR to the outer leaflet of the plasma membrane of dying cells and therefore is a less potent ICD initiator (Galluzzi et al., 2017). In murine models, doxorubicin, mitoxantrone, and oxaliplatin lead to ICD (Galluzzi et al., 2017). This is due to exposure of DAMPs such as ER

chaperones which include CALR, PDIA3, HSP70 and HSP90 on the plasma membrane. As well as autophagy mediated secretion of ATP, activation of Type I IFN, and secretion of CXCL10, release of HMGB1 and annexin A1. Recently, a phase III clinical trial in small cell lung carcinoma (SCLC) patients, combined PD-L1 blockade with a chemotherapy regimen, demonstrated impressive results and is most likely to be approved by the FDA as a first-line-therapy regimen (Horn et al., 2018; Saleh et al., 2019; Trommer et al., 2019). ICI and chemotherapy combination is the only regimen that demonstrated a statistically significant increase in patient survival, interestingly, regardless of PD-L1 expression (Garassino et al., 2018). Pemetrexed and carboplatin, the chemotherapeutics which were approved with Pembrolizumab, induce upregulation of the IFNγ pathway and Type I IFN among other genes involved in T-cell recruitment and activation (Garassino et al., 2018; Popovic et al., 2018). Pembrolizumab does not have an overlapping toxicity profile with these agents (Langer et al., 2016). When comparing the treatment arms, the double platinum group demonstrated an ORR of 55% achieved on triple combination compared to only 29% in the non-immunotreated arm, with manageable irAE profile which was, as expected, enlarged in the pembrolizumab group (Langer et al., 2016).

Regarding scheduling, a phase II study assessed the effectivity of Ipilimumab, Paclitaxel and Carboplatin combination for stage IV NSCLC patients. To assess scheduling, two alternate regimens of drug administration were employed in the study. The first arm included a concomitant chemotherapy and ICI combination while the second arm was treated with the same combination yet in a sequential manner, chemotherapy was administered first followed by Ipilimumab treatment. Interestingly, an improvement in progression free survival was demonstrated for the phased but not the concurrent treatment regimens when compared to paclitaxel and carboplatin alone. Here there was a combination of taxanes and platinum-based compounds which induce the release of Tumor Specific Antigens (TSAs) from dying tumor cells resulting in T cell activation supplemented by an anti-CTLA-4 treatment (Lynch et al., 2012).

However, chemotherapy intervention should be calculated carefully since it can lead to lymphopenia and neutropenia which may interfere with the mechanism of action of checkpoint inhibitors by impairing clonal expansion of effector lymphocytes. One option is to plan the timeline of treatment to enable lymphoid regeneration. This is the probable reason for the greater success in the sequentially administered chemotherapy ICI combination trial (Lynch et al., 2012). Currently there are several trials assessing the potential of combining chemotherapy with ICIs (NCT03611556, NCT02768558).

8. Combinations with armed antibodies and with small molecule drugs (kinase inhibitors)

8.1. Armed antibodies

Antibodies conjugated to different drugs can enhance the therapeutic window of the targeting antibody by conjugating it to a drug or a toxin. Lysis of cells with armed antibodies (AAs) leads to an ICD and represents a potent stimulator of effector T cell recruitment to tumors (Gerber et al., 2016). AAs can also kill cells around the target, also known as the “by-stander effect”. This helps in the context of a heterogenic tumor site and hinders the development of therapy resistance mechanisms. Field experience points that this strategy is mainly hindered by AAs in the form of “off-target” toxicities (Cruz and Kayser, 2019). Mostly attributed to the potency of the cytotoxic payload, which stresses the importance of AA design to enhance the therapeutic index regarding both the linkage of the payload to the targeting antibody as well as the payload which it carries with it (Cruz and Kayser, 2019). Optimized combination of ICI and AAs can therefore lead to tumor specific immunological memory resulting in a durable response for more patients. This targeted therapy strategy can also circumvent the negative effects of chemotherapy use with checkpoint inhibitors, which include depletion of lymphoid cells and off-target toxicities. Therefore, AAs can induce antitumor immunity by directly killing cancerous cells and by eliciting an ICD which can turn cold into hot tumors.

Immunotoxins are protein drugs composed of a targeting domain fused to a protein toxin. Historically, the notion that certain infectious diseases can exert a therapeutic anti-cancerous effect dates back to the 1700s (Wiemann and Starnes, 1994). One such example of an immunotoxin are targeting antibodies linked to pseudomonas exotoxins (PE). Moxetumomab pasudotox, a PE based immunotoxin, was approved by the FDA in 2018 for the treatment of hairy cell leukemia (Leshem and Pastan, 2019). While the general consensus was that it kills cancer cells directly by irreversibly-inhibiting their targets ability to synthesize proteins, recent clinical observations claim that this alone cannot explain the anti-tumor activity. Historically, immunotoxins were studied in models which didn’t take into account possible interactions with the immune system such as tissue culture and immune deficient mice (Leshem and Pastan, 2019). The interest in the effect initiated after clinical trials with a PE immunotoxin demonstrated that several patients had a delayed complete response, six months after treatment (Leshem and Pastan, 2019), this suggests that in these patients the immunotoxin did something a bit more expanded than direct killing of the cancer cells. Toxins are expected to be active only while still in the body, therefore such a delayed response can hint at a second stage immune activation (Leshem and Pastan, 2019). Unfortunately, one of the main factors inhibiting PE-based immunotoxin treatment has been the development of neutralizing ADAs (Leshem and Pastan, 2019). The presence of ADAs has been the basis for the combination of immunotoxins with lympho-depleting chemotherapy in clinical trials (Leshem and Pastan, 2019). Regarding immunity, some researchers claim that inhibition of protein synthesis is a pathogen-associated damage pattern that promotes immunity (Leshem and Pastan, 2019).

Preclinical data demonstrates how both $CD4^+$ and $CD8^+$ T cells are crucial for regression of contralateral tumors and metastases in an IL13-R expressing D5 murine melanoma model treated with a PE immunotoxin (Kawakami et al., 2006). Furthermore, clinical trial data point to immunotoxin's ability to elicit ICD by increasing ATP secretion and CALR surface expression (Leshem and Pastan, 2019). In an astrocytoma model, the absence of $CD4^+$ and $CD8^+$ T cells antitumor activity was significantly inhibited using an EGFRvIII targeted PE-based immunotoxin (Ochiai et al., 2008).

Brentuximab vedotin (BV) is an antibody-drug conjugate (ADC) used to treat relapsed or refractory Hodgkin’s lymphoma and systemic anaplastic large cell lymphoma (ALCL). Brentuximab vedotin consists of the chimeric monoclonal antibody brentuximab (cAC10, which targets the cell-membrane protein CD30) linked with maleimide attachment groups, cathepsin cleavable linkers (valine-citrulline), and para-aminobenzylcarbamate spacers to three to five units of the anti-mitotic agent monomethyl auristatin E (MMAE). In a clinical trial where Hodgkin's lymphoma patients were treated with a combination of brentuximab vedotin and an anti-PD-1 ICI, a complete response rate of 61% was reported, compared to 33% BV alone or 28% with Nivolumab as a monotherapy. Interestingly, according to the report, a higher percentage of Tregs expressed CD30 compared with any other T cell subset examined (Herrera et al., 2018). This is an approach that should be carefully assessed since CD30 is also expressed on CTLs (Herrera et al., 2018). Interestingly, a clonal expansion of peripheral blood T cells was noted which was not demonstrated after Nivolumab monotherapy; it is possible that BV modulates the TME and promotes T cell clonal expansion. There are currently several trials assessing the potential of combining immunotoxins and ADCs with ICIs (NCT03258593, NCT03644550).

8.2. Small molecules

Small molecules are the most common drugs and are utilized for a wide variety of cancers by targeting different biological pathways.

8.2.1. BRAF inhibitors

BRAF oncogene mutations are dysregulated in approximately 5–10% of all human malignancies and result in a constitutive activation of the mitogen activated protein kinase (MAPK) pathway and activates mitogen-activated-protein kinases MEK1/2 (Morris and Kopetz, 2013). The BRAFV600E mutation is detected in 50% of all cutaneous melanomas. Melanoma patients with BRAF V600E mutation substantially benefit from a combination treatment of small molecules which inhibit BRAF and MEK1 (Eggermont et al., 2018). Constitutive BRAF activation can lead to anti-inflammatory cytokine expression which suppresses TILs. In addition, PD-L1 upregulation is associated with the development of resistance to BRAF inhibitors (Chowdhury et al., 2018; Sullivan et al., 2019). Therefore, there is a strong rationale to combine these small molecule inhibitors with checkpoint inhibitors which so far have yielded the most impressive results in metastatic melanoma patients. A recent phase Ib trial demonstrated the efficacy of combining BRAF and MEK inhibitors (cobimetinib and vemurafenib) with Atezolizumab (anti-PD-L1) in previously untreated metastatic melanoma patients with BRAF V600E mutations. The triple combo resulted in improved clinical results with manageable toxicities (Amaria et al., 2018). Another trial with Dabrafenib (BRAF inhibitor), Trametinib (MEK1 inhibitor) and Pembrolizumab (anti-PD-1) obtained a 100% response rate and reduced relapse rate compared with the current BRAF-MEK inhibitor, the standard of care (Eggermont et al., 2018; Ribas et al., 2019).

8.2.2. Tyrosine kinase inhibitors

Tyrosine kinases (TKs) have an important role in growth factor signaling. Activated forms of TKs can increase tumor cell proliferation, anti-apoptotic effects, angiogenesis and metastasis (Arora and Scholar, 2005; Kuusk et al., 2017). Currently there are many small molecule TK inhibitors (TKIs) approved for treatment of many malignancies. For advanced RCC, treatment regimens differ based on risk group. The good prognosis typically receive Sunitinib, an inhibitor of cellular signaling which targets multiple receptor tyrosine kinases including platelet-derived growth factor (PDGFRs), vascular endothelial growth factor receptors (VEGFRs) and CD117 (c-KIT). These play a key role in both tumor angiogenesis and tumor cell proliferation. In contrast, patients in intermediate and poor risk groups often receive Ipilimumab with Nivolumab (Rini et al., 2019). A recent phase III clinical trial demonstrated that RCC patients benefit from Pembrolizumab and Avelumab combined with the multi-TK inhibitor Axitinib (Brief, 2019). Treatment with Pembrolizumab and Axitinib resulted in significantly longer overall survival and progression-free survival. Regarding HCC, one has to consider that the liver is an anatomical challenge for immunotherapy. The liver is at the frontline of detoxification and elimination of orally taken toxins and pathogens and has somewhat of innate immune tolerant properties. PD-1 blockade may assist in this context and can also eliminate viral infections such as HCV and HBV as was demonstrated in a Phase I/II trial. For HCC, Sorafenib a pan-TKI targeting VEGF among other TKs is one of the only systemic therapies with proven survival benefits (Hato et al., 2016). Combining anti-angiogenic molecules with ICIs might not be a strong strategy since the former treatment can lead to hypoxia and limit TIL activity. However, Sorafenib combined with CXCR4 inhibition by the small molecule drug AMD3100 and an anti-PD-1 antibody enhanced the infiltration of activated CTL to the tumor and significantly delayed disease progression (Hato et al., 2016).

There are several approaches to utilize small molecules to boost ICI efficacy. For example, small molecules targeting c-KIT selectively reduce the immunosuppressive MDSCs and demonstrate good activity when combining treatment with anti-PD-1 or anti-CTLA-4 antibodies (Garton et al., 2017). Inhibition of c-Kit restores $CD8^+$ and $CD4^+$ T cell population percentages to normal level in a mouse colon cancer tumor model (Garton et al., 2017). PI3Kγ signaling is another attractive target since it is highly expressed in myeloid cells and promotes their migration. The small molecule drug IPI-549 selectively inhibits PI3Kγ. Inhibition with IPI-549 abolishes ICI resistance in murine models of breast cancer and melanoma (De Henau et al., 2016). For B cell malignancies, Ibrutinib, which is a covalent inhibitor of bruton's tyrosine kinase (BTK, a member of the BCR signaling pathway) is critical for the survival of malignant B cells, has been approved for therapy (Sagiv-Barfi et al., 2015). Interestingly, Ibrutinib also irreversibly inhibits IL-2-inducible kinase (ITK), an essential TH2 enzyme and can shift the balance to a Th1 response and result in Th1 T cell priming to increase immune responses (Dubovsky et al., 2013). This immune priming strategy was effective in both murine pre-clinical lymphoma models and surprisingly as well as in triple-negative breast cancer (TNBC) and colorectal cancer (CRC) which do not express BTK (Sagiv-Barfi et al., 2015). There is a multitude of small molecules which have been approved which have demonstrated a synergistic effect with ICIs and are currently assessed as combination treatments (NCT02853331, NCT01656642, NCT01472081).

9. Combinations with cancer vaccines

Cancer vaccines have been pursued for a few decades with little success, until recently. Cancer vaccines entail a dual effect of antigenicity and immunogenicity. For example, attempts have been made to induce cancer specific immune responses by introducing neoantigens encoded in DNA or mRNA or by loading cell lysates into DCs (Prins et al., 2013; Salcedo et al., 2006). The general approach to cancer vaccines is continuously evolving. Initially, autologous cancer cells were extracted, transduced *ex vivo* to secrete an immune stimulator such as GM-CSF, irradiated and reintroduced to the patient (Dranoff et al., 1993; Simons et al., 1999). Next, due to the lack of efficacy and development of strategies to cultivate primary DCs *ex vivo,* the strategy evolved to isolating autologous DCs, loading the antigens *ex vivo*, and reintroducing DCs to patients (Gilboa, 2007). The explanation for the initial failure has been described to be due to the lack of responsive immune context within cancer patients (Gilboa, 2007). Initially, the DCs were loaded *ex vivo* with peptides, whole protein, tumor lysates or apoptotic debris complexed with antibodies or by transfecting cDNA or mRNA (Gilboa, 2007). Currently, the strategy has been updated to directly inject naked or LNP encapsulated-mRNA based vaccines encoding neo-antigens directly into patients (Granot-Matok et al., 2019). This last step is again a direct result of lack of efficacy of the previous strategy as well as major developments in mRNA technologies which include delivery strategies as well as a dramatic increase in mRNA stability due to nucleic acid chemical modification (Defrancesco, 2017; Granot-Matok et al., 2019). The mRNA field, while still minor compared to the protein one, is gaining more attention recently. The majority of funds and clinical efforts in this field are currently aimed at prophylactic as well as cancer vaccines (Granot-Matok et al., 2019). A Cancer vaccine, supplies both antigenicity in the form of encoding neoepitopes as well as adjuvancy by utilizing for example alphavirus based self-replication (Brito et al., 2015; Geall et al., 2012). The neoantigen encoding cassettes can include self-replication domains based on non-structural protein machinery which includes an RNA-dependent-RNA polymerase. Self-replicating dsRNA in the cytoplasm is a very strong inducer of TLR 3 and leads to a very strong immune response which inhibits its own replication via protein synthesis inhibition as well as cell proliferation (Oberli et al., 2017; Pardi et al., 2018).

The mRNA-based vaccine approach has become highly personalized. Currently, patients can have their tumors sequenced for specific neoantigens which will be encoded on the mRNA vaccine they will receive (Sahin et al., 2017; Vormehr et al., 2015). Personalized vaccines are manufactured in a stepwise process. First, clinically-relevant non-synonymous mutations are computationally determined based on RNA and DNA isolated from tumor biopsies. Next, these mutations are encoded on mRNA and the personalized vaccine is administered to the patient (Vormehr et al., 2015). Some of these vaccines can encode up to 20 antigens in a single immunization for vaccinations which require a

certain combination of antigens to be expressed (Granot-Matok et al., 2019). In a first clinical trial, all melanoma patients administered with personalized mRNA cancer vaccines encoding multiple neo-epitopes displayed T cell responses against the multiple neo-epitopes encoded (Sahin et al., 2017). However, the introduction of these vaccines does not address the challenge of the suppressive TME and therefore combinations with ICI found on exhausted activated T cells can improve the treatment outcome. To bolster this, a DNA based vaccine with dual CTLA-4 and PD-1 blockade in murine melanoma, increased intratumoral infiltration of $CD8^+$ T cells (Bialkowski et al., 2018; Kos et al., 2019). Another regimen combining an mRNA vaccine with ICI of the IL-6/TGF-β pathway in a TC-1 (HPV-derived cancers) tumor cell mouse model, demonstrated efficacy and long-term immunity. This effect was noted only when treated with the combination (Bialkowski et al., 2018). Since the activated T cells can still be exhausted, there is room for ICI Cancer Vaccine therapy combinations. Currently, there are several clinical trials assessing mRNA cancer vaccines which are now carried out with arms that involve ICI combinations (NCT03313778, NCT03164772, NCT03291002, NCT03739931).

10. Future directions

10.1. Microbiome

The microbiome is also becoming increasingly relevant to the ICI field. In pre-clinical models, the absence of an intact gut microbiome negatively impacted ICI efficacy and these findings highlighted the importance of the commensal microbiota in immuno-oncology. Microbiome profiling can serve both as a marker for successful response as well as an alternative method to prime patients prior to ICI therapy (Gong et al., 2019). In preclinical studies, antibiotic treatment of mice comprised anti-tumor activity following anti-CTLA-4. On the flip side, anti-PD-L1 with *Bifidobacterium*, *A. muciniphila* and *E. hirae* reversed PD-1 resistance (Gong et al., 2019). Recently, there has been an understanding that bacteria in the TME can also affect ICI therapy. For example, *F. nucleatum* is present in the TME and can directly bind and inhibit NK and T cell activity via the immunosuppressive TIGIT receptor (Gur et al., 2015). This means that the microbiome can have both a predictive as well as personalized treatment opportunities which can directly affect ICI activity.

10.2. New targeting approaches

Targeting approaches can improve current ICI therapies by carrying out the effect specifically at the tumor site and mitigating systemic toxicities. For example, biodegradable scaffolds which can deliver ICIs to tumors specifically. One study reported on an *in situ* formed scaffold which leads to local release of Gemcitabine, an anchor chemotherapeutic agent, and an anti-PD-L1 antibody, with distinct release kinetics (Hu et al., 2018). In murine breast cancer and melanoma models, the scaffold which is constructed of a reactive oxygen species (ROS) degradable hydrogel distributed the agents in the ROS-rich TME. The idea is to promote an anti-cancer effect and avoid systemic side effects. Interestingly, another effect is that the scaffold uptakes the ROS which promotes M2 polarization, acting as a sponge in the TME (Hu et al., 2018). An alternative approach involves bispecific antibodies for ICI targeting (Davis et al., 2017; Koopmans et al., 2019). Bispecific antibodies contain two different antigen-binding sites in a single molecule. The rationale for evolving ICIs to bispecific antibodies is again to diminish the "on target/off tumor" binding of ICIs in non-inflamed regions, which will drastically improve the irAEs which accompany immunotherapies. In this respect, preclinical models demonstrate the added benefit of simultaneously targeting a checkpoint and a TSA (Davis et al., 2017; Koopmans et al., 2019). Other targeted approaches to target cells *in vivo* have the potential of finding other metastatic sites which cannot always be found or approached by an intratumoral approach. There are other challenges to address which are relevant for all mAb-based therapies. For example, antibody distribution is impeded by cellular internalization followed by endocytic clearance at the tumor edge also known as the “binding-site barrier”. This results in poor dispersion of antibody concentration at the tumor bed (Cruz and Kayser, 2019). As a result, antibodies with higher affinities may get stuck at the tumor periphery which has increased antigen expression (Cruz and Kayser, 2019). One way to overcome this barrier is to simply increase the dosage, however several studies suggest that even at higher doses the antibodies rarely reach the core hypoxic areas (Cruz and Kayser, 2019). Another option is to utilize small antibody fragments which demonstrate higher penetration efficacies, unfortunately at the price of poor stability, some of these fragments have half-lives of merely a few hours (compared to 2–3 weeks for IgGs). It will be interesting to determine if antibody fragments can be utilized for ICI-based therapies. Furthermore, there will be no issues with the lack of FC regions since many of the checkpoint inhibitors were designed to contain FCs which cannot elicit an immune response to prevent killing of the activated T cells.

10.3. CAR-T

Adoptive cell transfer (ACT) of chimeric antigen receptor (CAR) is a therapy approved for B cell malignancies. Currently, only CD19 targeting CAR-T cell therapies are approved for B-cell acute lymphoblastic leukemia (B-ALL) and non-Hodgkin's lymphoma (NHL) with hundreds of clinical trials trying to translate this success in liquid cancers to solid cancers (D’Aloia et al., 2018). CAR-T therapy involves the isolation of the patient's T cells, engineering them to express a chimeric receptor which enables non-MHC restricted killing of cells that express a selected antigen before returning the modified cells to the patient (Davila et al., 2012). To successfully enter the solid tumor field, CAR-T treatment will have to overcome two major obstacles. First, "on target/off tumor" toxicities can be improved by identifying new targets or by developing new targeting strategies such as dual targeting or rapid exchange of target by employing universal linkers (Chen et al., 2018; Lu et al., 2019). Second, for solid tumors, CAR-T cell activity can still be inhibited by an immunosuppressive TME (Flynn et al., 2017; Gay et al., 2017; Lim and June, 2017; Ninomiya et al., 2015; Zhang et al., 2017). Therefore, ICIs can assist CAR-Ts in the hostile TME. This challenge can be addressed via two approaches, combining mAb ICIs with CAR-T therapy or genetically engineering the T cells which are extracted to become resistant to a suppressive TME (Liu et al., 2019). Since the T cells are extracted and then engineered *ex vivo*, there is a stronger focus to genetically knockout (KO) the checkpoints rather than block them *in vivo* with a targeting antibody. This together with CARs which have dual epitope targeting schemes can mitigate systemic toxicities while exerting a strong effect (Chen et al., 2018; Mirzaei et al., 2016; Ren et al., 2017b). In preclinical testing, CRISPR-Cas9-mediated genome editing was utilized to demonstrate the efficacy of knocking out PD-1, CTLA-4 and LAG-3 individually or simultaneously by using multiple gRNAs at the same time (Mirzaei et al., 2016; Ren et al., 2017b, 2017a; Zhang et al., 2017). Since CAR-T and ICIs are currently the two most promising immune oncology approaches, it will be interesting to see how they converge. While both have serious irAEs which remain a major challenge, so far ICIs have gained clinical approval and displayed activity in solid cancers whereas CAR-T therapy, is currently still struggling to translate its success in liquid cancers to solid tumors. It will be interesting to see if combining ICIs can help bring success to the solid cancer field as well.

10.4. Combining immune stimulators with checkpoint inhibitors

Combining ICIs with immune activating agents can have synergistic properties which can lead to a stronger effect than each agent alone. In this respect, several clinical trials are assessing combinations of 4-1BB,

Table 1
Selected Immune Checkpoint Inhibitors in Clinical Trials.

Combination Type	Combination	Details	Indication	Phase	Performed by	NCT identifier
Multiple Checkpoint Targets	Pembrolizumab and Axitinib	PD-1 and TK small molecule inhibitor	Advanced/Metastatic renal cell carcinoma	III	Merck	NCT02853331
	Nivolumab and Ipilimumab	PD-1 and CTLA-4	Melanoma	III	Bristol-Myers Squibb	NCT01844505
	Nivolumab and Relatlimab	PD-1 and LAG-3	Solid tumors	I/II	Bristol-Myers Squibb	NCT01968109
	TSR-042 and TSR-033	PD-1 and LAG-3	Advanced Solid tumors	I	Tesaro	NCT02817633
	LY3321367 and LY3300054	PD-L1 mAb and TIM-3 Small molecule	Advanced Solid tumors	I	Eli Lilly and Company	NCT03099109
	MBG453 and PDR001	PD-L1 mAb and TIM-3 Small molecule	Advanced Solid tumors	Ib/II	Novartis	NCT02608268
	Nivolumab and BMS-986207	PD-1 and TIGIT	Advanced Solid tumors	I/II	Bristol-Myers Squibb	NCT02913313
	Nivolumab and Relatlimab	PD-1 and LAG-3	Metastatic Melanoma	II/III	Bristol-Myers Squibb	NCT03470922
	Durvalumab and Monalizumab	PD-1 and NKG2A	NSCLC	II	MedImmune LLC	NCT03822351
Checkpoint and TME	Durvalumab and Oleclumab	PD-L1 and CD73	Advanced Solid tumors	I	MedImmune	NCT02503774
	Atezolizumab and A2AR targeting	PD-L1 and A2AR	Advanced cancers	I	Corvus	NCT02655822
	PDR001, NZV930 and A2AR targeting	PD-1 and CD73 and A2AR	Advanced cancers	I	Novartis	NCT03549000
	Pembrolizumab and anti-CD73 antibody and small A2AR inhibitor	PD-1 and CD73 and A2AR	Solid tumors and non-Hodgkin's lymphoma	I/Ib	Corvus	NCT03454451
	Pembrolizumab and Etinostat	PD-1 and HDAC	Advanced Solid tumors	I/II	Merck Sharp & Dohme Corp.	NCT02437136
	Pembrolizumab and Epacadostat	PD-1 and IDO1	Advanced Solid tumors	I/II	Merck	NCT02178722
	Pembrolizumab and Epacadostat	PD-1 and IDO1	Metastatic Melanoma	III	Merck Sharp & Dohme Corp.	NCT02752074
	Nivolumab and Bevacizumab	PD-1 and VEGF-A	Renal and Kidney Carcinoma	I	NCI	NCT02210117
Checkpoint, TME and Chemotherapy	Durvalumab, Oleclumab and Chemotherapy combination	PD-L1 and CD73 and Chemotherapy	Pancreatic Cancer	III	MedImmune (now AstraZeneca)	NCT03611556
Checkpoint and Chemotherapy	Durvalumab and Chemotherapy combination	PD-L1 and Chemotherapy	TNBC	I/II	Astra Zeneca	NCT03616886
	Nivolumab and Cisplatin and Etoposide	PD-1 and Chemotherapy	NSCLC	III	Bristol-Myers Squibb	NCT02768558
Checkpoint and Radiation Therapy	Nivolumab and Low Dose Radiotherapy	PD-1 and RT	Hodgkin's lymphoma	III	Abramson Cancer center of the University of Pennsylvania	NCT03495713
	Nivolumab and Radiotherapy	PD-1 and RT	Brain cancer	III	Bristol-Myers Squibb	NCT02617589
Checkpoint and Targeted Therapy	Nivolumab and Brentuximab	PD-1 and Immunotoxin	Hodgkin's lymphoma	I/II	Seattle Genetics	NCT02572167
	Atezolizumab and Vemurafenib and Cobimetinib	PD-L1 and BRAF and MEK	Metastatic Melanoma with BRAFV600E Mutation	I	Genentech	NCT01656642
	Nivolumab and Sunitinib and Pazopanib	PD-1 and Tyrosine Kinases	Metastatic Renal Cell Carcinoma	I	Bristol-Myers Squibb	NCT01472081
	Monalizumab and Cetuximab and anti-PD-L1	PD-L1 and NKG2A and EGFR	Head and Neck Cancers	I/II	AstraZeneca	NCT02643550
	Durvalumab and Vicinium	PD-L1 and Immunotoxin	Bladder Cancer	I	NCI	NCT03258593
	Pembrolizumab and LMB-100	PD-1 and Immunotoxin	Malignant Mesothelioma	II	NCI	NCT03644550
Checkpoint and Cancer Vaccine	Nivolumab and Dendritic Cell vaccine therapy	PD-1 and Cancer Vaccine	Recurrent Primary Brain Tumors	I	Bristol-Myers Squibb	NCT02529072
	Nivolumab and Dendritic Cell vaccine therapy	PD-1 and Cancer Vaccine	Glioblastoma	II	Bristol-Myers Squibb/NCI/Northwest	NCT03014804
	Nivolumab and Dendritic Cell vaccine therapy	PD-1 and Cancer Vaccine	Recurrent Primary Brain Tumors	I	Bristol-Myers Squibb	NCT02529072
	Pembrolizumab and Personalized mRNA vaccine	PD-1 and Cancer Vaccine	Advanced Solid tumors	I	Moderna and Merck	NCT03313778
	Durvalumab and Tremelimumab and mRNA vaccine	PD-L1 and CTLA-4 and Cancer Vaccine	NSCLC	I/II	CureVac	NCT03164772

GITR and OX40 (Zahavi and Weiner, 2019). For example, 4-1BB as well as OX40 are expressed on both T and NK cells. In these settings there is an important aspect to the order of activation and inhibition. For example, OX40, a TNF family costimulatory receptor is upregulated on T and NK cells only after activation which leads to cytokine production and cytotoxicity (Messenheimer et al., 2017; Nuebling et al., 2018; Zahavi and Weiner, 2019). One study demonstrated that while concomitant anti-PD-1 and OX40 agonist treatment did not lead to a dramatic effect, sequential treatment by first treating with an OX40 agonist followed by anti-PD-1 yielded strong anti-cancer effect which was both CD4- and CD8-dependent (Messenheimer et al., 2017). Since OX40 is upregulated only on activated T cells, they are not tested as monotherapies, while there have been clinical trials assessing their activity as combinations (Zahavi and Weiner, 2019).

11. Conclusions and future perspectives

ICI have revolutionized the cancer immunotherapy field. These antibodies demonstrate full and durable responses and not only "life-extending" effects (Diesendruck and Benhar, 2017). These antibodies kick start the patients' own immune system to carry out an anti-cancer immune-based effect. This field is widely explored with multiple new antibodies and checkpoint targets seeking clinical approval (Table 1). Unfortunately, while they are approved for a wide array of clinical indications, only ˜20% of patients treated with ICIs demonstrate long-term clinical benefit. We predict that higher response rates will be driven by better understanding the tumor biology and determining relevant prognosis. Currently, there is a lack of definitive biomarkers which can predict successful treatment with ICIs for approved therapies. It was recently reported that even PD-L1 expression level does not necessarily predict successful treatment. Therefore, finding definitive markers is an urgent task. In the future, checkpoint inhibitors should be approved on the basis of these biomarkers to limit ineffective treatments. Beyond this, there are some limitations which are relevant to all mAb-based treatments and are pertinent to checkpoint inhibitor antibodies as well. These include optimal dosage, penetration, and emergence of intrinsic and acquired resistance mechanisms.

Currently there are concentrated efforts to combine and design more effective immune checkpoint combination approaches with many clinical trials pushing towards this direction. The hypothesis is that targeting multiple pathways can lead to a synergistic effect where the overall result will be larger than the sum of the individual parts. First, there are approaches to target multiple checkpoints simultaneously. This strategy can either target several co-expressed inhibition molecules on exhausted T cells in the same context such as PD-1, TIM-3 and LAG-3 or target non-redundant suppression pathways such as PD-1 and CTLA-4 which has already been clinically approved. In this respect, we must address a critical question. Unlike viral therapy, while cocktail combinations can afford to block each axis once due to evolution driving viruses to be lethal with a minimal amount of proteins, cells have many functionally redundant signaling pathways which can override and compensate for each other when needed. There is ample evidence for example that exhausted T cells upregulate several exhaustion markers and that targeting one of these will simply lead to an overexpression of another. Therefore, direct comparison of the success in the viral therapy field will not necessarily translate to the checkpoint inhibitors field. Considering this, combination strategies should be carefully designed and should take into account T cell activation or exhaustion status. Furthermore, combinations will have to be patient tailored since they are likely to be more toxic than single agents and more expensive (Cully, 2015). Also, it will be interesting to target checkpoints which are expressed on innate immune cells as well such as macrophages and NK cells simultaneously (Wang et al., 2019).

However, even before this, tumors should be assessed for their immune infiltration. For example, reinvigorating exhausted T cells will be effective only when the T cells have been activated and are present within the tumor bed. The presence of TILs within the tumor bed is known as tumor "heat" which was recently reported to be more predictive for disease progression compared to current standard of physical characterization of tumor size, node infiltration, metastasis (TNM) classification system. This symbolizes the epic of the immunotherapy era. For this reason, combination strategies should be designed to both kill cancer cells as well as convert "cold" tumors into "hot" ones by eliciting an ICD. In this review we shed light how this can be carried out with both "old" therapies which are still the backbone of cancer therapy such as radiotherapy and chemotherapy as well as novel TME reshaping approaches. While the combinations are important, data suggests that designing the therapy timeline is even more important. This timing refers to when each combination arm should be administered as well as the regimen itself. For example, multiple low-dose fractionation in radiotherapy vs. a few high doses as well as selecting the correct portfolio of chemotherapy which can elicit the most potent immunogenic cell death with limited toxicities and effector cell depletion. Unfortunately, while we have revealed important data on these aspects, there are still more challenges and questions to be answered. Regarding TME “hotness” or “coldness”, this is a complex scenario which is constantly changing as the TME evolves with disease progression and recurrence (Galon and Bruni, 2019). Further complicating this, different metastases can have distinct immunoscores and can even be considered as separate tumors. To determine the immunoscore and design the optimal immunotherapy strategy, a characterization should be ideally executed on resected tumors. Unfortunately, tumors are often non-resectable, while biopsies which are invasive, and don't necessarily represent the entire tumor landscape. Presently, efforts are being put into determining the immunoscores of different tumors and development of tools such as liquid biopsies, immune-PET imaging, tumor bulk deconvolution and more recently single cell approaches (Galon and Bruni, 2019). Regarding ICD, unfortunately, not all DAMPs released are immune-stimulatory. For example, immunosuppressive DAMPs such as adenosine and prostaglandin E2 seem to have a key function in immune tolerance (Galluzzi et al., 2017). For this reason, combination approaches should assess whether the stress elicited on tumor cells by the combination arm is truly an ICD, which demands antigenicity in the form of neo-epitopes as well as adjuvancy in the form of immuno-stimulatory DAMPs release to occur simultaneously.

Next, we will try to address the following question "Is the checkpoint inhibitor field exhausted?" From a historic perspective, the paradigm for most treatment approaches starts with an era of discoveries, followed by perfecting these discoveries and ends with optimizing combinations and field exhaustion (Arruebo et al., 2011). However, it is our opinion that this field is still far from exhaustion. This is evident by the investment in the field where 11 out of 33 antibodies which are currently in late stage clinical evaluation are ICIs. Adding to this, there is a plethora of new combination strategies which are already demonstrating clinical improvements over previous monotherapies in preclinical models and early-stage clinical trials. Finally, there is much preclinical data of novel checkpoints, checkpoint combinations and platforms to deliver them specifically to the tumor site. To sum up, we believe that the keys to increasing the percentage of checkpoint inhibitor responders lay in finding reliable biomarkers which will be predictive of successful treatment, novel delivery approaches to target the relevant immune cells and immune relevant ICI combination strategies. These combination strategies should both exert maximal cancer cell death as well as prime the body for a successful long-term immune response. It will be exciting to see how the ideas discussed in this manuscript actually unfold.

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