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CDR CANCER DRUG RESISTANCE

Resistance to Cancer Immunotherapy: Mechanisms, Advances, Challenges and Opportunities

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Topic: Resistance to Cancer Immunotherapy: Mechanisms, Advances, Challenges and Opportunities

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Dr. Michael Lahn, currently serving as the Chief Medical Officer at iOnctura SA in Geneva, Switzerland, is a distinguished medical professional with a robust background in oncology. Graduating with a Medical Doctorate from the University of Mainz in 1990, Dr. Lahn then served as an Assistenzarzt at Albert-Ludwigs-Universität Freiburg im Breisgau. In 2019, Dr. Lahn brought over two decades of industry expertise, garnered from Eli Lilly and Company, AstraZeneca, and Incyte Corporation, to iOnctura. His impactful career involves shaping the development of diverse molecular entities, from small molecule inhibitors like the ALK5 inhibitor galunisertib to the successful registration of osimertinib in non-small cell lung cancer (NSCLC). Passionate about pioneering cancer therapies, Dr. Lahn is equally dedicated to mentoring colleagues in the intricate domain of clinical drug development.

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Dr. Jordi Rodón is a distinguished expert in early drug development, biomarker discovery, personalized cancer medicine, and neuro-oncology. He earned his medical and doctoral degrees from the Universitat Autonoma de Barcelona. Currently holding the position of Associate Professor in the Department of Investigational Cancer Therapeutics at The University of Texas MD Anderson Cancer Center in Houston, TX, Dr. Rodón serves as the Associate Medical Director in the Department of Institute for Personalized Cancer Therapy. Additionally, he holds a dual/joint/adjunct appointment as an Associate Professor in the Department of Genomic Medicine. Dr. Rodón's commitment to advancing cancer therapeutics is underscored by his role as the Clinical Co-Director of the Precision Oncology Decision Support Team at MD Anderson. He has also contributed significantly to the academic community as the Co-chair of the Clinical Trials Task Force for Cancer Core Europe.

Prof. Hendrik Tobias Arkenau
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Professor Tobias Arkenau stands as a distinguished Consultant Medical Oncologist, renowned for his expertise in Gastrointestinal Cancers, Drug Development, and Immunotherapy. Graduating with a medical degree from Hanover Medical School in Germany in 2000, Prof. Arkenau completed his oncology training at esteemed institutions, including the Royal Marsden Hospital. Recognized for his substantial research contributions, he earned a Fellowship from the Royal College of Physicians. He previously held a professorship at the University of London, and as the founding Medical Director and Executive Medical Director of the Sarah Cannon Research Institute UK. Currently holding the position of Chief Medical Officer and Global Head of Drug Development at Ellipses Pharma, Prof. Arkenau continues to contribute significantly to the evolution of cancer care and drug development. His medical practice encompasses a wide range of cancers, with a focus on gastrointestinal and skin cancers.

Special Issue introduction:

Despite the clinical benefit obtained with current cancer immunotherapy, there is a large group of patients who do not respond or respond poorly to current therapies. In fact, some even show hyperprogression once immunotherapy is started. Underlying mechanisms of resistance are thought to be driving this lack of response to immunotherapy.

In the present Special Issue, the various concepts of resistance mechanisms will be summarized. This will include mechanisms of primary resistance, various barriers to immunotherapies, the selection of tumour clones that lead to resistance and finally the molecular changes within tumour cells rendering them resistant to therapy.

An important pillar for studying resistance is the available tools to detect and monitor resistance in patients. Biomarker tests that can assess emerging or pre-existing intrinsic or extrinsic factors leading to resistance are perhaps more than ever needed to direct physicians towards seeking novel therapies.

This Special Issue aims to compile a collection of updated research, review and expert opinion articles on resistance mechanisms, the available strategies to detect such developments and propose potential novel therapeutic approaches.

Sub-topics:

Concept of resistance to immunotherapy

Potential mechanisms of resistance to immunotherapy

Targets for overcoming resistance to immunotherapy

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Cancer Drug Resistance (***CDR***) was founded with Professor Godefridus J. Peters as the Editor-in-Chief in March 2018. In a testament to its growing prominence and scholarly impact, ***CDR*** has achieved several notable milestones in recent years. The journal's recognition journey began in March 2021 with its inclusion in the esteemed **Scopus** indexing. This was followed by another significant accolade in May 2022, as ***CDR*** secured its place in the **PubMed Central®** (**PMC**) database, further amplifying its reach and accessibility to the global research community. The year 2023 marked a series of remarkable achievements for ***CDR***, with Scopus bestowing a CiteScore of 5.5, which subsequently surged to an impressive 6.3 as per the latest CiteScoreTracker update as of December 5, 2023. Furthermore, June 2023 emerged as a landmark month for ***CDR***, as it achieved its first **Impact Factor** of 3.7, a recognition conferred by **Clarivate Analytics** in the esteemed Journal Citation Report (JCR). These milestones not only underscore the journal's commitment to advancing the field of cancer drug resistance but also reflect its growing influence and stature in the scientific community.

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Cancer Drug Resistance

Review

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Tumor-intrinsic metabolic reprogramming and how it drives resistance to anti-PD-1/PD-L1 treatment

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Abstract

The development of immune checkpoint blockade (ICB) therapies has been instrumental in advancing the field of immunotherapy. Despite the prominence of these treatments, many patients exhibit primary or acquired resistance, rendering them ineffective. For example, anti-programmed cell death protein 1 (anti-PD-1)/anti-programmed cell death ligand 1 (anti-PD-L1) treatments are widely utilized across a range of cancer indications, but the response rate is only 10%-30%. As such, it is necessary for researchers to identify targets and develop drugs that can be used in combination with existing ICB therapies to overcome resistance. The intersection of cancer, metabolism, and the immune system has gained considerable traction in recent years as a way to comprehensively study the mechanisms that drive oncogenesis, immune evasion, and immunotherapy resistance. As a result, new research is continuously emerging in support of targeting metabolic pathways as an adjuvant to ICB to boost patient response and overcome resistance. Due to the plethora of studies in recent years highlighting this notion, this review will integrate the relevant articles that demonstrate how tumor-derived alterations in energy, amino acid, and lipid metabolism dysregulate anti-tumor immune responses and drive resistance to anti-PD-1/PD-L1 therapy.

Keywords: Immunotherapy resistance, tumor-immune microenvironment, immune checkpoint blockade, energy metabolism, amino acid metabolism, lipid metabolism

INTRODUCTION

The development of immune checkpoint blockade (ICB) therapies revolutionized cancer treatment across a variety of indications. Immune checkpoints are necessary for the controlled initiation and termination of immune responses as well as for the maintenance of self-tolerance, which are critical in preventing autoimmunity[1]. However, tumors leverage this checkpoint system to inappropriately dampen the immune response and facilitate immune escape[1]. Continuous antigen stimulation drives the upregulation of checkpoint receptors on $CD8^+$ T cells[2], while tumor cells exploit a variety of mechanisms to upregulate checkpoint ligands. Therefore, blocking the interaction between immune checkpoint receptors and ligands reinvigorates $CD8^+$ T cell function to elicit tumor cell killing. There are several ICB therapies that are currently utilized in the clinic, but the most well-studied are anti-programmed cell death protein 1 (anti-PD-1), which is predominantly found on T cells, and anti-programmed cell death ligand 1 (anti-PD-L1), which is expressed on tumor and myeloid cells[3]. While anti-PD-1/PD-L1 treatments are widely used, a substantial number of patients are resistant to this type of therapy[4], prompting researchers to identify resistance mechanisms that drive inadequate outcomes. Response to ICB is largely dependent on the existing profile and infiltration of immune cells within the tumor, specifically $CD8^+$ T cells, because they are the main contributors to anti-tumor effects[4]. Therefore, modulating the tumor-immune microenvironment (TIME) to enhance $CD8^+$ T cell infiltration and function, in combination with current ICB therapies, serves as an attractive approach to increase efficacy and overcome resistance.

The intersection of cancer and metabolism has been at the forefront of oncology research for several decades. Otto Warburg and his identification of the Warburg effect, wherein malignant cells exhibit a metabolic shift from oxidative phosphorylation to glycolysis[5], ignited massive research efforts towards uncovering the metabolic reprogramming that occurs in tumors. These efforts led to the classification of dysregulated tumor cell metabolism as one of the hallmarks of cancer in 2022[6]. Therefore, altered metabolism of lipids, amino acids, carbon, and nucleotides, to name a few, are highly implicated in the development and progression of cancer[7]. More recently, this field of onco-metabolism has expanded to include the immune system, given its role in regulating tumorigenesis. Immune cells and their subtypes have different metabolic requirements during activation, differentiation, and expansion[8], wherein alterations in the extrinsic metabolome at any of these stages can lead to immune cell dysfunction. The TIME is an objectively harsh environment for many cell types due to its acidity, hypoxia, nutrient deprivation, and accumulation of inhibitory metabolites[9]. To the advantage of the tumor, malignant and immunosuppressive cells, such as T regulatory cells (Tregs), myeloid-derived suppressor cells (MDSCs), and macrophages, are better adapted to this oppressive environment compared to anti-tumor $CD8^+$ T cells[10]. These conditions, which are largely facilitated by cancer cells, heavily contribute to decreased $CD8^+$ T cell infiltration and function.

There is mounting evidence that tumor-intrinsic metabolic reprogramming has a profound effect on the recruitment and function of various immune cell types within the TIME. As such, it is necessary to identify ways to specifically target malignant cell metabolism to enhance the efficacy of ICB. The scope of this review article will aim to cover the current literature that demonstrates how tumor-derived alterations in energy, amino acid, and lipid metabolism within the TIME mediate $CD8^+$ T cell dysfunction and how targeting these pathways combats resistance to anti-PD-L1/PD-1 treatment.

ENERGY METABOLISM

Energy metabolism includes a complex network of biochemical pathways that contribute to sustained cellular function through the production of adenosine triphosphate (ATP). Some of these processes include glycolysis, the tricarboxylic acid (TCA) cycle, and fatty acid b-oxidation. A shift in energy metabolism

towards the Warburg effect in malignant cells generates high levels of lactic acid, while consuming and producing ATP/adenosine diphosphate (ADP) and oxidizing and reducing nicotinamide adenine dinucleotide (NAD). While concurrently studying lactate, adenosine, and NAD^+ in the context of energy metabolism is important, each individual metabolite uniquely influences the function of malignant and immune cells within the TIME. Therefore, this section will focus on how the altered metabolism of lactate, adenosine, and NAD^+ by tumor cells impacts the anti-tumor immune response by $CD8^+$ T cells and contributes to anti-PD-1/PD-L1 resistance.

Lactate

Lactate is predominantly formed through glycolysis, wherein lactate dehydrogenase (LDH) reduces pyruvate to lactic acid, which then dissociates into hydrogen (H^+) and lactate ions [Figure 1]. To a lesser extent, glutaminolysis also drives pyruvate formation, resulting in lactic acid production[11]. Lactate and H^+ are exported through proton-linked monocarboxylate transporters 1-4 (MCT1-4)[12], wherein export is highly dependent on the existing concentration of extracellular lactate[13]. Intracellular lactate levels are also modulated by import through MCT1[14]. Extracellular lactate facilitates intracellular signaling by binding to hydroxycarboxylic acid receptor 1 (HCAR1), which regulates a variety of downstream oncogenic pathways, such as cell proliferation, migration, and invasion[15]. Accumulation of H^+ via lactic acid production contributes to the acidity of the TIME, which promotes an immunosuppressive milieu[16]. Conversely, lactate ions have both tumor-promoting and -inhibiting effects in $CD8^+$ T cells.

T cells require adequate levels of lactic acid for proper development and function[17,18], but excess amounts in the TIME and intracellularly promote dysfunction. Tumor-derived lactic acid accumulation within the TIME inhibits T cell proliferation and cytokine production by altering redox homeostasis[19]. Specifically, lactic acid downregulates T cell production of both reactive oxygen species (ROS) and the antioxidant glutathione[19]. While excess amounts of ROS promote oxidative stress, low levels are important for T cell activation and signaling[20], suggesting that tumor-derived lactic acid inhibits T cell functions by ablating ROS formation. Additionally, overabundance of lactic acid in the TIME prevents T cell export of lactate and H^+ ions because of the unfavorable concentration gradient, and subsequent accumulation promotes intracellular acidification and decreases effector function[21]. In particular, intracellular acidification in T cells due to tumor-derived lactic acid production prevents the expression of nuclear factor of activated T cells (NFAT)[22], a family of transcription factors that mediate T cell development[23]. In $CD8^+$ T cells, decreased NFATC1 expression reduces IFNg production, whereas inhibiting lactate dehydrogenase A (LDHA) reduces intracellular acidification and restores $CD8^+$ T cell function and tumor infiltration[22]. Similarly, the hypoxic nature of the TIME drives upregulation of LDHA in $CD8^+$ tumor-infiltrating lymphocytes (TILs), leading to excess intracellular lactic acid, which then inhibits IFNg and granzyme B production[24] and T cell expansion[18]. Upon chronic antigen stimulation, $CD8^+$ T cells will progress through progenitor exhausted and terminally exhausted states, with the latter resulting in dysfunction and the inability to elicit anti-tumor effects[25]. Therefore, there has been a significant focus on promoting the expansion of non-exhausted states and inhibiting the progression into terminal exhaustion to reinvigorate the anti-tumor response. Researchers found that treatment of $CD8^+$ T cells with IL-21 promotes expansion but does not drive T cells towards an exhausted state, like IL-2[18]. Moreover, IL-2, but not IL-21, induced metabolic reprogramming in T cells to favor glycolysis and shunt pyruvate towards lactic acid formation[18]. Treatment with IL-2 and LDH inhibitor invoked a shift from glycolysis towards oxidative phosphorylation, and IL-2 or IL-21 treatment in combination with LDH inhibitor increased stem cell memory T cell formation and reduced tumor growth[18]. These data demonstrate that tumor-derived lactic acid can directly or indirectly inhibit T cell function and anti-tumor immune response.

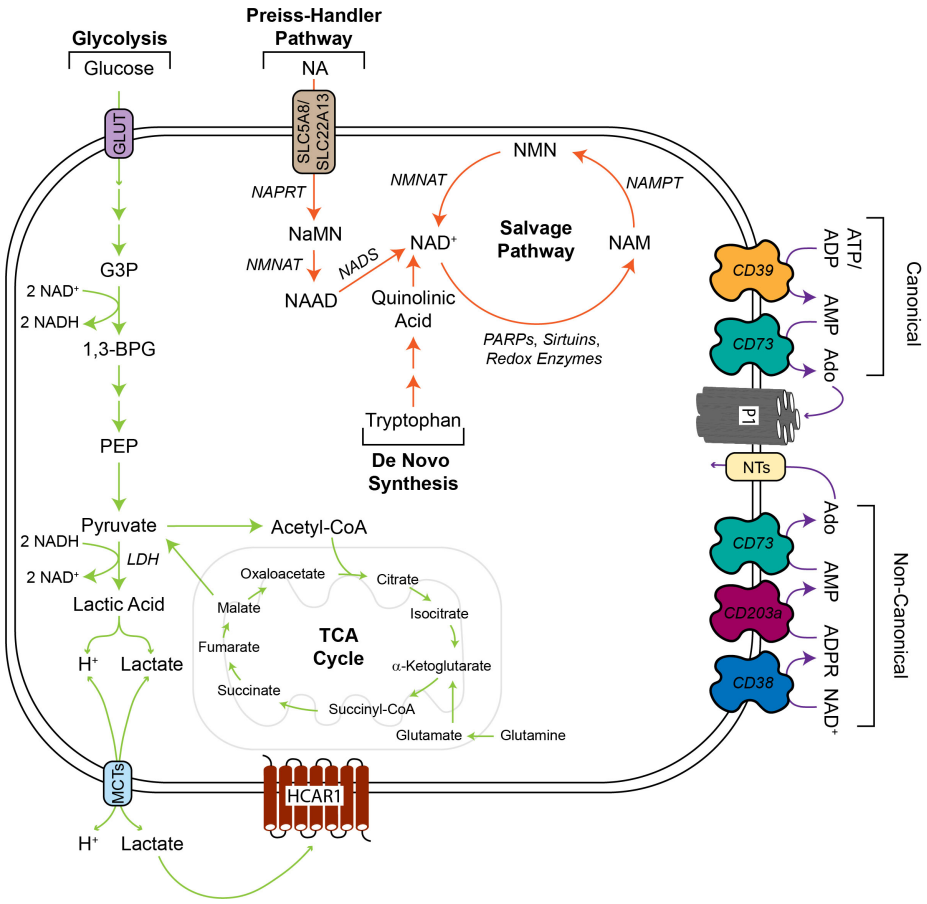

Figure 1. Energy metabolism pathways for lactate, adenosine, and NAD^+. Pyruvate is generated predominantly through glycolysis, but the TCA cycle also contributes to pyruvate production via conversion from malate. LDH catalyzes the reaction to convert pyruvate to lactic acid, which dissociates into H^+ and lactate ions that are exported and imported through MCTs. Alternatively, pyruvate can be converted to acetyl-CoA to participate in the TCA cycle to drive energy metabolism. In the TIME, H^+ contributes to the low pH and lactate facilitates a variety of intracellular signaling pathways by binding to HCAR1. Extracellular adenosine is formed through both the canonical and non-canonical pathways. The canonical pathway utilizes CD39 to convert ATP or ADP to AMP and CD73 to convert AMP to adenosine. The non-canonical pathway metabolizes NAD^+ to ADPR through CD38, ADPR to AMP through CD203a, and finally, AMP to adenosine via CD73. Extracellular adenosine binds to P1 to initiate intracellular signaling pathways or is imported through NTs. Note: adenosine generated by the canonical and non-canonical pathways participates in both P1 signaling and NT import. NAD^+ is formed through the Preiss-Handler pathway, de novo synthesis, salvage pathway, and various enzymatic reactions in energy metabolism, such as PEP to pyruvate. The Preiss-Handler pathway imports NA and forms NAD^+ through a series of enzymatic reactions. Do novo synthesis of NAD^+ results from the metabolism of tryptophan and the salvage pathway recycles NAM to regenerate intracellular NAD^+ levels. NAD^+ serves as a co-factor for many enzymes and participates in redox reactions, such as pyruvate to lactic acid. Ado: Adenosine; ADP: adenosine diphosphate; ADPR: adenosine diphosphate ribose; AMP: adenosine monophosphate; ATP: adenosine triphosphate; GLUT: glucose transporter; G3P: glycerol-3-phosphate; H^+: hydrogen; HCAR1: hydroxycarboxylic acid receptor 1; LDH: lactate dehydrogenase; MCTs: monocarboxylate transporters; NA: nicotinic acid; NAAD: nicotinic acid adenine dinucleotide; NAD^+: nicotinamide adenine dinucleotide; NADS: NAD^+ synthetase; NAM: nicotinamide; NaMN: nicotinic acid mononucleotide; NAMPT: nicotinamide phosphoribosyltransferase; NAPRT: nicotinic acid phosphoribosyltransferase; NMNAT: nicotinamide mononucleotide adenylyltransferase; NMN: nicotinamide mononucleotide; NTs: nucleoside transporters; PARP: poly (ADP-ribose) polymerase; PEP: phosphoenolpyruvate; P1: type 1 purinergic receptors; TCA: tricarboxylic acid; 1,3-BPG: 1,3-Bisphosphoglycerate.

Lactate serves as a carbon source in both tumor and T cells[26-28], but like with any metabolite, overabundance dampens cellular functions. In T cells, increased lactate metabolism depletes NAD^+ levels by reducing it to NADH, preventing the downstream glycolytic processes that rely on NAD^+[29]. Similarly, reduced glycolytic flux in T cells diminishes serine production, which is critical for T cell proliferation[29]. Moreover, tumor-derived lactate promotes depletion of NAD^+ in naïve T cells, resulting in translation inhibition of FIP200, which forms one subunit of the ULK kinase complex that regulates autophagy[30]. FIP200 is selectively lost in naïve T cells from ovarian cancer patients, wherein autophagy is suppressed,

leading to mitochondrial dysfunction and, ultimately, apoptosis[30]. Genetic ablation of FIP200 in naïve T cells reduced $CD8^+$ and $CD4^+$ T cell infiltration and IFNg production[30]. Recently, tumor-derived lactate was also found to diminish TCA-intermediate recycling in $CD8^+$ T cells by inhibiting pyruvate carboxylase, which shunts pyruvate to oxaloacetate[31]. Pyruvate carboxylase is exceedingly important to maintain TCA cycle anaplerosis in $CD8^+$ T cells because succinate is diverted from the TCA cycle to participate in autocrine signaling[31]. In addition to tumor-derived lactate suppressing $CD8^+$ T cell function, it also drives the expansion and function of immunosuppressive cells. Tregs inhibit the function of anti-tumor immune cells and require lactate to maintain their suppressor functions in the harsh TIME[32,33]. Moreover, lactate produced by cervical cancer cells supports immunosuppressive macrophages by regulating anti-inflammatory cytokine production and HIF1a expression[34]. Taken together, these data highlight that tumor-derived lactate not only directly inhibits effector T cell functions, but also indirectly through supporting immunosuppressive cell populations. As such, multiple reports have examined the feasibility of inhibiting tumor-intrinsic lactate metabolism in combination with anti-PD-1/PD-L1 therapy.

Several correlative studies through bioinformatic analyses have demonstrated that targeting lactic acid metabolism might overcome ICB resistance and yield better patient outcomes. High LDH expression has been evaluated as a selection criterion for and predicting response to ICB therapy[35-39]. Similarly, other lactate-related genes have been correlated with the expression of immune checkpoint proteins, $CD8^+$ T cell infiltration, and resistance to ICB in breast cancer[40]. Moreover, decreased glycolytic flux in melanoma patients treated with anti-PD-1 therapy was associated with increased probability of progression-free survival[41].

In addition to bioinformatics studies, numerous reports indicate that inhibiting tumor-intrinsic lactic acid metabolism in combination with anti-PD-1/PD-L1 therapies combats resistance and increases efficacy. MCT4 is regulated at the mRNA level by the demethylase alkB homolog 5 (ALKBH5)[42]. Genetic or pharmacologic inhibition of ALKBH5 reduces intratumoral lactate concentration and the number of Tregs and MDSCs, but has no effect on the number of infiltrating cytotoxic T cells[42]. Furthermore, utilizing a small molecular inhibitor of ALKBH5 significantly improved the efficacy of anti-PD-1 treatment in murine melanoma tumors[42]. Consistent with the findings that lactic acid benefits immunosuppressive cells, researchers found that lactic acid produced by high-glycolytic tumors drove expression of PD-1 on Tregs, but not $CD8^+$ T cells, leading to anti-PD-1 resistance[43]. However, inhibiting either LDHA in tumors or MCT1 in Tregs combined with anti-PD-1 therapy reversed these effects[43]. In addition to inhibiting lactic acid production and/or lactate import, antagonizing intracellular lactate signaling in malignant cells through HCAR1 also promotes anti-tumor effects[44]. Abrogating HCAR1-mediated lactate signaling sensitized tumors to anti-PD-1 and metformin treatment, leading to reduced tumor volume and increased $CD8^+$ T cell infiltration and IFNg production[44].

While a plethora of evidence supports the notion that lactic acid production by tumors and accumulation in T cells drives oncogenesis, a few reports contradict this idea. In mouse melanoma tumors, blocking the export of lactate and H^+ ions through MCT1 and MCT4 reduced the acidification of the TIME[41]. While blocking MCT1 and 4 in T cells decreased lactate secretion and glucose uptake, it surprisingly did not impair IFNg production[41], which contrasts with other findings that accumulation of intracellular lactic acid promotes acidification and dampens effector functions[21,22,24]. The authors found that inhibiting MCT1 and 4 activities in T cells increased glucose flux through the TCA cycle and increased oxygen consumption, thus providing an explanation as to why $CD8^+$ T cell effector functions were preserved[41]. Moreover, pharmacologically inhibiting MCT1 and 4 in combination with anti-PD-1 treatment resulted in increased efficacy and decreased tumor volume[41]. The results from these findings are indeed surprising given the

mounting evidence that accumulation of lactic acid within T cells dampens their function. Researchers have also found that lactate, when studied separately from H^+ in the form of sodium lactate, induces stemness and tumor infiltration, and reduces apoptosis in $CD8^+$ T cells[45]. Moreover, sodium lactate supplementation in three mouse tumor models showed synergistic effects with anti-PD-1 treatment[45]. A plausible explanation for these somewhat contradictory findings is that variations between the TIMEs of different tumor types metabolically reprogram $CD8^+$ TILs in distinct ways, wherein some tumors drive increased sensitivity of $CD8^+$ TILs to lactic acid. Therefore, it is exceedingly important to delineate the metabolic changes in $CD8^+$ TILs from different tumor types to identify the most effective therapy.

Additional research is needed to tease apart the intricate relationship between lactate, lactic acid, tumor cells, $CD8^+$ T cells, and immunosuppressive cells. Inhibiting tumor-derived lactic acid production seems to generally have anti-tumor effects, due to the detrimental effects of high acidity on the anti-tumor immune cells within the TIME. While lactate ions serve as a carbon source and promote $CD8^+$ T cell stemness, they also benefit immunosuppressive cells and excess amounts can dampen T cell effector functions. Collectively, these data demonstrate that tumor-derived alterations in lactic acid metabolism contribute to ICB resistance and modulating these pathways may augment efficacy, prompting the need for continued research efforts in this field.

Adenosine

Adenosine is formed through two major pathways [Figure 1]. In the canonical pathway, ectonucleoside triphosphate diphosphohydrolase-1 (CD39) hydrolyzes ATP or ADP to adenosine monophosphate (AMP)[46], which is subsequently converted to adenosine by ecto-5′-nucleotidase (CD73)[47]. The non-canonical pathway involves the conversion of NAD^+ to adenosine diphosphate ribose (ADPR) through cyclic ADP ribose hydrolase (CD38); ADPR is then metabolized to AMP via ectonucleotide pyrophosphatase/phosphodiesterase 1 (CD203a), and finally to adenosine through CD73[48]. Extracellular adenosine has several fates; it is converted to inosine via adenosine deaminase, converted back to AMP through adenosine kinase, or binds to type 1 purinergic receptors, which include A1, A2A, A2B, and A3. Both A2A and A2B receptors (A2AR and A2BR) are important for mediating adenosine signaling in immune cells within the TIME[49]. High affinity A2AR is more broadly expressed on immune cells, while low affinity A2BR facilitates the expansion of MDSC populations[50].

Within the TIME, adenosine formation is predominantly mediated by malignant and immunosuppressive cells[51] and the impact of this metabolite on immunosuppression and cancer progression was recently comprehensively reviewed[52]. Under physiological conditions, extracellular ATP and adenosine levels are low[53]. However, during cellular stress, such as hypoxia and nutrient deprivation, intracellular ATP is released and serves as a strong pro-inflammatory mediator by recruiting immune cells[53,54]. On the other hand, adenosine is a potent immunosuppressive metabolite[50]. As such, it is not surprising that tumor cells highly upregulate CD73 and immunosuppressive cells, such as cancer-associated fibroblasts (CAFs), Tregs, and MDSCs, highly upregulate CD39 to facilitate adenosine accumulation within the TIME[52,55-59]. Further, terminally exhausted $CD8^+$ T cells exhibit increased CD39 expression, therefore contributing to the elevated adenosine levels within the TIME[60], and adenosine drives the expansion of Treg populations[61].

Tumor-derived adenosine inhibits $CD8^+$ T cell functions in a myriad of ways. Adenosine triggers IL-10 secretion from cervical cancer cells, leading to downregulation of MHC-I expression and subsequent immune evasion from $CD8^+$ T cells[62]. Increased adenosine production also favors tumor growth, as indicated by the negative correlation between CD73 expression and survival in pancreatic adenocarcinoma human cohorts[63]. Moreover, loss of CD73 in pancreatic ductal adenocarcinoma cell lines leads to increased

activation and IFNg production in $CD8^+$ T cells[63], highlighting the inverse relationship between adenosine and $CD8^+$ T cell function. Adenosine production within the TIME is also regulated by cancer exosomes, which are endosomal-derived extracellular vesicles[64,65]. Specifically, cancer exosomes were found to express CD39 and CD73, leading to inhibition of T cell activation and proliferation in human neuroblastoma samples[66] and bladder, colorectal, prostate, and breast cancer cell lines[67]. Accumulation of adenosine within the TIME also severely hinders tumor infiltration by $CD8^+$ T cells due to adenosine-mediated dysfunction of KCa3.1 channels[68,69]. KCa3.1 is a potassium channel that regulates Ca^{2+} influx, which affects T cell gene expression, activation, and differentiation[70]. Inhibition of KCa3.1 by adenosine reduced T cell migration and cytokine production[69], and decreased KCa3.1 channel activity, but not protein expression, resulting in decreased tumor infiltration[68]. Building on this, the same group later found that anti-PD-1 therapy increased the activity of ion channels KCa3.1 and Kv1.3, leading to enhanced $CD8^+$ T cell infiltration in head and neck squamous cell carcinoma (HNSCC) patient samples[71]. While not the focus of this section, it is important to mention that Treg-derived adenosine also drives $CD8^+$ T cell dysfunction[56,57,72,73]. On the other hand, increased IL-7 signaling in $CD8^+$ T cells inhibits FoXO1 activation, which is a transcription factor that controls T cell proliferation, to overcome the suppressive effects of the adenosine-rich TIME and promote tumor infiltration and expansion[74]. Leveraging these mechanisms might be a viable therapeutic strategy to be used in conjunction with current ICB therapies to overcome resistance.

Adenosine within the TIME engages with the A2A receptor (A2AR) on $CD8^+$ T cells to drive adenosinergic signaling that results in impaired anti-tumor effects[75]. Early studies found that A2AR signaling inhibited T cell activation and proliferation[76], and in the context of cancer, many studies have shown that A2AR signaling promotes immune evasion and T cell dysfunction. In mouse melanoma and fibrosarcoma models, pharmacological inhibition or genetic deficiency of A2AR increases $CD8^+$ T cell tumor infiltration and IFNg production, and reduces tumor growth[77,78]. Moreover, targeted knockdown or antagonizing A2AR increases $CD8^+$ T cell infiltration[79] and decreases Treg infiltration and tumor volume in mouse models of HNSCC[80]. Similarly, administering A2AR agonists during T cell activation impaired cytotoxic function, although proliferative capacity was maintained, and these effects persisted after A2AR agonists were removed[81]. These data demonstrate that even if $CD8^+$ T cells infiltrate the adenosine-rich TIME, adenosinergic signaling reduces their effector functions and renders them incapable of eliminating tumor cells. However, one study showed that complete abrogation of the A2AR gene in $CD8^+$ T cells inhibited expansion and effector functions[75]. In this way, it is important to preserve some degree of A2AR signaling in $CD8^+$ T cells to maintain proper cell function, highlighting that complete deletion of immunosuppressive targets might not produce the most efficacious results.

The studies thus far have demonstrated that tumor-intrinsic adenosine metabolism adversely affects $CD8^+$ T cell function; therefore, it is not surprising that these metabolic alterations also contribute to anti-PD-1/PD-L1 resistance. To date, there are many drugs in the pre-clinical and clinical stages that target CD39, CD73, and A2AR, either alone or in combination with anti-PD-1/PD-L1 therapies[82]. Because it is not feasible to cover all these data, we have chosen to focus on the relevant articles from 2020 until now to demonstrate that modulating adenosine metabolism helps overcome resistance to ICB therapies. Using bioinformatics approaches, researchers showed that adenosine signaling gene signatures are inversely correlated with survival and efficacy of anti-PD-1 treatment across multiple cancer indications[83]. The first-in-human study using an A2AR antagonist with anti-PD-L1 treatment improved the probability of progression-free survival and overall survival, and monotherapy or combination with anti-PD-L1 increased $CD8^+$ T cell infiltration[84]. However, current A2AR antagonists do not perform well in the adenosine-rich TIME, so multiple groups have developed novel A2AR antagonists to increase effectiveness[85,86]. Both

compounds have shown limited toxicity in Phase I clinical trials[85,86], with iTeos Therapeutics' compound demonstrating initial signs of clinical benefit[86]. Dizal Pharmaceuticals' compound was also evaluated in murine models of prostate cancer, where treatment with the novel antagonist and anti-PD-1 significantly reduced tumor volume compared to monotherapy[85].

There are several pre-clinical and clinical studies that demonstrate promising results for targeting CD39 or CD73 in combination with anti-PD-1 or PD-L1. Cancer exosomes expressing CD39 and CD73 drive adenosine accumulation and were also found to promote CD39 expression on macrophages[87]. Macrophage-derived CD39 cooperates with tumor-derived CD73 to increase adenosine levels in the TIME, which drives anti-PD-1 resistance[87]. Targeting CD39 on macrophages in combination with anti-PD-1 therapy abrogated therapeutic resistance and synergistically reduced the volume of murine hepatocellular carcinoma tumors and increased $CD8^+$ T cell infiltration and granzyme B production[87]. Moreover, a first-in-human Phase I clinical trial was conducted in 2020 to assess the efficacy of an anti-CD39 antibody (IPH5201) in combination with anti-PD-L1 treatment[88], and the first patient for the Phase II study was dosed in June 2023[89]. A poster presentation at the European Society for Medical Oncology Immuno-Oncology Summit in 2022 showed pre-clinical data for IPH5201, wherein treatment alone reduced adenosine levels in the TIME of mouse fibrosarcoma tumors[90]. The data also demonstrated that combining anti-CD39, the chemotherapeutic agent gemcitabine, and anti-PD-L1 controlled tumor growth and increased survival better than monotherapy or anti-PD-L1 with gemcitabine in murine colorectal carcinoma tumors[90]. In a clinical study of 44 patients, researchers found no major toxicities when combining an anti-CD39 monoclonal antibody with anti-PD-1 and the chemotherapy regimen FOLFOX for the treatment of gastric cancer or gastroesophageal junction adenocarcinoma[91]. These data are critical first steps in the approval and use of anti-CD39 therapies in combination with anti-PD-1/PD-L1 treatment. The results from a first-in-human Phase I clinical trial with anti-CD73 and anti-PD-L1 recently reported tolerable safety and moderate efficacy[92]. Further, targeting CD73 has also recently been shown to be a promising therapeutic strategy, wherein Phase II clinical trials combining anti-CD73 with anti-PD-L1 elicit increased response rate and progression-free survival compared to anti-PD-L1 monotherapy in patients with non-small cell lung cancer[93]. One thing to consider when targeting CD39 or CD73 is that anti-CD39 treatments not only inhibit adenosine production, but also promote accumulation of immunostimulatory ATP.

In addition to more conventional treatment methods, several unique approaches for inhibiting adenosine metabolism and PD-1 have recently been discovered. Because of the ubiquitous expression of A2AR on T cells, localizing inhibition of A2AR signaling to tumor-infiltrating $CD8^+$ T cells would likely mitigate off-target effects. In this approach, researchers increased tumor oxygenation to relieve the hypoxic conditions that promote tumor-derived adenosine production[94]. Using a photo-modulated nanoreactor, hydrogen peroxide is converted to oxygen within the TIME, leading to decreased adenosine production and abrogated A2AR signaling in $CD8^+$ T cells[94]. Moreover, combination with anti-PD-1 therapy synergistically reduced tumor growth and increased $CD8^+$ T cell infiltration in triple-negative murine breast cancer tumors[94]. In another tumor-targeting approach, researchers utilized cancer-derived exosomes packaged with both a CD39 antagonist and AMPK agonist to inhibit adenosine and promote ATP production, respectively[95]. This method increased $CD8^+$ T cell infiltration and production of granzyme B and IFNg, reduced intratumoral adenosine and Treg populations, and synergized with anti-PD-1 treatment in mouse melanoma models[95]. The final targeted approach used ROS-producing nanoparticles to deliver a CD39 inhibitor[96]. Inducing ROS accumulation in the TIME seems counterintuitive, but like hypoxia, ROS trigger the release of ATP. Therefore, ROS would increase ATP concentration and inhibiting CD39 would prevent adenosine formation, thus remodeling the TIME away from an immunosuppressive state[96]. This method

alone decreased tumor volume and increased $CD8^+$ T cell production of IFNg and, together with anti-PD-1, elicited a more robust anti-tumor effect in murine mammary carcinoma tumors[96].

Collectively, these data strongly demonstrate that tumor-derived adenosine has detrimental effects on $CD8^+$ T cell infiltration and effector functions, thereby contributing to anti-PD-1/PD-L1 resistance mechanisms. As such, there is a compelling need for the continued development of adenosine-targeting drugs that can synergize with current anti-PD-1/PD-L1 therapies to prevent resistance and evoke better patient response.

NAD^+

NAD^+ is comprised of adenosine monophosphate linked to nicotinamide mononucleotide. NAD^+ can be reduced to form NADH or phosphorylated and subsequently reduced to form $NADP^+$ or NADPH, respectively. NAD^+ is synthesized through three pathways: de novo biosynthesis, Preiss-Handler pathway, or the salvage pathway, the latter of which is the predominant way that cells restore NAD^+ levels[97] [Figure 1]. NAD^+ is a co-factor that is involved in a variety of redox and non-redox reactions. In energy metabolism, NAD^+ and its derivatives are indispensable for cellular function because they accept and donate electrons in a variety of metabolic pathways, such as glycolysis, pentose phosphate pathway, TCA cycle, and fatty acid b-oxidation[98]. NAD^+ also acts as a substrate for multiple enzyme families, including sirtuins, PARPs, and ADP-ribosyl cyclases[97]. Moreover, the metabolic pathways of adenosine and NAD^+ are tightly linked through CD38, an ectoenzyme present on the surface of tumor and immune cells, which depletes NAD^+ levels, which ultimately results in adenosine formation[99].

High NAD^+ levels are required in malignant cells to meet their increased energetic demands for rapid growth and proliferation. Therefore, malignant cells will upregulate NAD^+ biosynthesis to replenish intracellular stores, leading to depletion of this metabolite within the TIME. Several enzymes involved in anabolic NAD^+ pathways, such as nicotinamide phosphoribosyltransferase (NAMPT), have been heavily implicated in cancer progression and severity[100]. Moreover, drugs targeting these enzymes have shown promising results in pre-clinical and clinical studies[101]. Targeting tumor-intrinsic NAD^+ metabolism is a promising therapeutic approach because it would restore NAD^+ levels in the TIME, thus allowing T cells to utilize this metabolite to maintain proper function.

NAD^+ is highly important for anti-tumor immune functions and NAMPT is an important regulator of NAD^+ availability. As previously mentioned, NAD^+ and adenosine metabolism are highly linked due to the ability of NAD^+ to be converted to adenosine. Inhibiting NAMPT in tumor cells reduces levels of intracellular NAD^+ and extracellular adenosine, thereby enhancing $CD8^+$ T cell functions[102]. Further, NAMPT expression in $CD8^+$ T cells is necessary to produce NAD^+ and induce anti-tumor effects[103]. In tumor-infiltrating lymphocytes (TILs), NAMPT and NAD^+ levels are lower compared to peripheral T cells[103], suggesting that the TIME induces NAD^+ depletion in TILs, leading to impaired function. Mechanistically, NAD^+ deficiency in TILs drives mitochondrial dysfunction and reduces ATP production, whereas supplementation with nicotinamide (NAM), the substrate of NAMPT, reverses these effects to promote a strong anti-tumor immune response *in vivo*[103]. Interestingly, TCR stimulation in $CD8^+$ T cells leads to a 16-fold upregulation of NAMPT, compared to 1.3-fold upregulation in Tregs[104]. This suggests that $CD8^+$ T cells rely more heavily on NAMPT expression and NAD^+ levels compared to Tregs, giving these immunosuppressive cells an advantage in the NAD^+-depleted TIME. Consistently, Tregs are particularly sensitive to NAD^+-induced cell death[105], and systemic NAD^+ treatment preferentially depleted Tregs, leading to decreased tumor volume[106]. To date, there are several pre-clinical and clinical studies investigating the use of NAMPT inhibitors in both solid and hematologic malignancies[107]. However, systemic inhibition of NAMPT might have profound adverse effects on $CD8^+$ T cell function, decreasing the drugs' efficacy. Perhaps these types of drugs are more effective in cancers that do not have high T cell infiltration but overexpress NAMPT.

In immune cells, CD38 is inversely correlated with NAD^+ levels because it degrades NAD^+ to NAM and ADP-ribose[108,109]. These derivates of NAD^+ are important secondary messengers that regulate intracellular calcium levels and storage, which in turn mediates T cell differentiation and activation[109]. CD38 expression is a marker of T cell exhaustion that contributes to adverse epigenetic modifications in $CD8^+$ TILs[110]. Further, high expression of CD38, PD-1, and CD101 correlates with the inability of $CD8^+$ T cells to undergo epigenetic reprogramming to reverse the exhausted state[110]. Conversely, inhibiting CD38 expression in Tregs and B-regulatory cells induced cell death, but drove proliferation of cytotoxic T cells, likely due to depletion of the immunosuppressive populations[111]. Consistently, mice deficient in CD38 expression exhibited lower Treg numbers as a result of increased NAD^+ levels[106]. CD38 expression on tumor cells has also been implicated in a variety of solid and hematologic malignancies[112-116]. Increased CD38 expression on malignant cells results in acquired resistance to anti-PD-1/PD-L1 therapy by driving $CD8^+$ T cells towards an exhausted state[114]. Moreover, $CD8^+$ T cell function was found to be inhibited by CD38-mediated adenosine production, and anti-PD-L1 and CD38 combination therapy synergistically inhibited the growth of murine lung adenocarcinoma tumors[114]. Currently, there are two approved anti-CD38 monoclonal antibody treatments (Daratumumab and Isatuximab) and one in clinical trials (MOR202) to treat multiple myeloma; however, these drugs do not inhibit the ectoenzymatic activity of CD38, rather they induce antibody-dependent cell-mediated cytotoxicity[117-119]. There are several drugs in pre-clinical stages that target the ectoenzymatic activity of CD38 to increase NAD^+ levels for different diseases[120-122]. While these drugs are not yet being evaluated in the oncologic space, it would be advantageous because inhibiting CD38 is both beneficial for T cells and detrimental for malignant and immunosuppressive cells, thus eliminating the need for cell-specific drugs.

Taken together, these data demonstrate an important role for lactate, adenosine, and NAD^+ in regulating immune cell function and ultimately controlling cancer development and progression. Further, pre-clinical studies show promising results that combining these treatments with existing ICB therapies can remodel the TIME to boost the anti-tumor immune response. Thus, continued pre-clinical and clinical efforts are needed to determine whether resistance to anti-PD-1/PD-L1 therapy is ablated when combined with approved anti-CD39/CD73/A2AR/CD38 treatments.

AMINO ACID METABOLISM

Amino acid metabolism is widely implicated in oncogenesis due to the necessity of amino acids in protein synthesis, epigenetic modifications, and fueling energetic processes. Of the 20 amino acids, only a handful are well-studied in the context of immuno-oncology metabolism and resistance to ICB. Because tryptophan is thoroughly researched in this space and was recently comprehensively reviewed[123], we wanted to focus on amino acids that are sometimes overlooked but still immensely important in regulating cancer development and progression. As such, this section will discuss how tumor-derived alterations in arginine, glutamine, and methionine metabolism contribute to anti-tumor immunity and how modifying the metabolism of these amino acids helps diminish resistance to anti-PD-1/PD-L1 therapy.

Arginine

Arginine is considered a non-essential amino acid in normal cells because it can be imported or synthesized through citrulline metabolism in the urea cycle[124] [Figure 2]. Conversely, arginine is also catabolized through the urea cycle to form urea and ornithine through arginase (ARG) enzymes[124]. Extracellular arginine also participates in the activation of intracellular signaling pathways by binding to G protein-coupled receptor family C group 6 member A (GPRC6A)[125]. While arginine itself is important for many

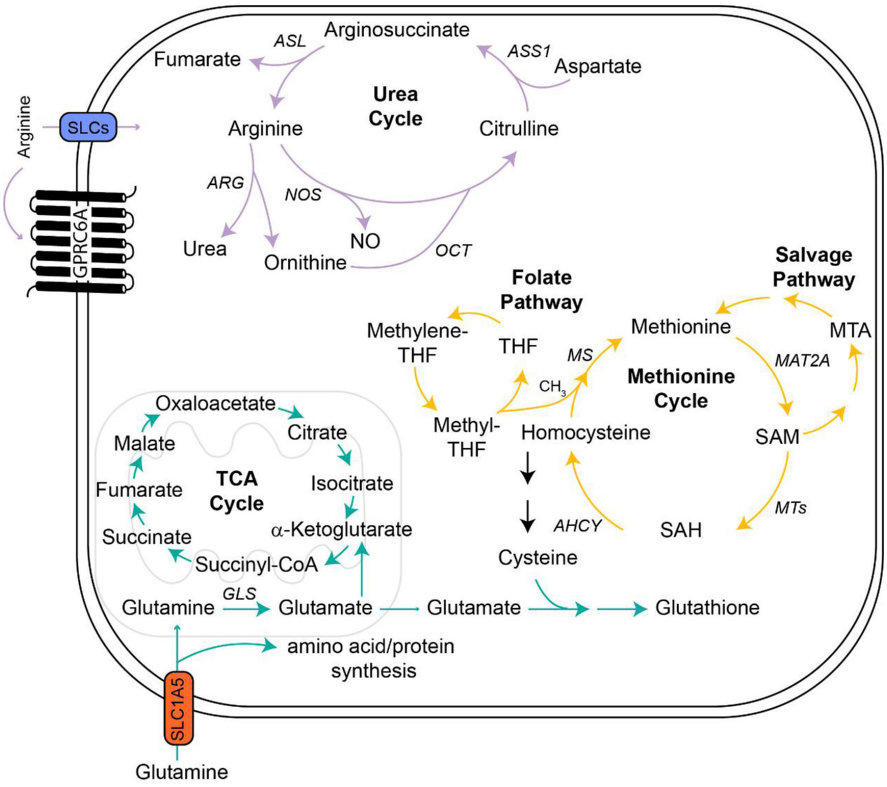

Figure 2. Metabolic pathways of arginine, glutamine, and methionine. Extracellular arginine binds to GPRC6A to drive intracellular arginine signaling or it is imported through various SLC transporters depending on the cell type. Arginine can also be formed through metabolism of citrulline in the urea cycle. Once inside the cell, arginine is catabolized through NOS to form NO or ARG into urea and ornithine, the latter of which is converted back into citrulline to fuel the urea cycle. Glutamine is similarly imported through a variety of SLCs, with SLC1A5 being the predominant transporter on T cells. Intracellular glutamine is used for amino acid/protein synthesis or transported to the mitochondria and converted to glutamate via GLS. In the mitochondria, glutamate is converted to a-Ketoglutarate to fuel the TCA cycle. In the cytosol, glutamate combines with cysteine to form glutathione to combat oxidative stress. Cysteine is generated in part through metabolism of homocysteine in the methionine cycle, which generates methionine for various cellular processes. Methionine is generated by re-methylation of homocysteine through donation of CH_3 by methyl- THF in the folate cycle. Methionine is then converted to SAM, an indispensable methyl donor, and subsequently SAH following loss of the methyl group. SAM is also involved in the methionine salvage pathway that restores intracellular methionine levels. AHCY: Adenosylhomocysteinase; ARG: arginase; ASL: argininosuccinate lyase; ASS1: argininosuccinate synthase 1; CH_3: a methyl group; GLS: glutaminase; GPRC6A: G protein-coupled receptor family C group 6 member A; MAT2A: methionine adenosyltransferase 2A; MS: methionine synthase; MTA: 5′-methylthioadenosine; MTs: methyltransferases; NO: nitric oxide; NOS: nitric oxide synthase; OCT: ornithine transcarbamoylase; SAH: S-adenosyl-L-homocysteine; SAM: S-adenosylmethionine; SLC: solute carrier; TCA: tricarboxylic acid; THF: tetrahydrofolate.

cellular processes, it is also a precursor for the synthesis of polyamines, which are organic compounds that facilitate cell proliferation and are upregulated in a variety of cancers[126-128]. Similarly, nitric oxide synthase (NOS) metabolizes arginine to nitric oxide (NO), which promotes angiogenesis and metastasis, and dampens the immune response[129].

In malignant cells, arginine helps sustain tumor-promoting functions, and arginine starvation results in detrimental effects, such as ROS formation, mitochondrial dysfunction, and cell death[130-135]. Despite this, metabolic rewiring of the urea cycle in tumor cells results in increased ornithine and proline synthesis and decreased arginine synthesis[131]. Moreover, many cancer types have decreased expression of arginosuccinate synthase 1 (ASS1), which catalyzes the penultimate step in arginine synthesis[130]. As such, arginine is considered an essential amino acid in malignant cells, and they must rely on exogenous uptake to sustain their metabolic demands[130-132]. On the other hand, T cells are completely reliant on exogenous arginine

because they do not express ASS1[136,137], meaning they must compete with tumor cells and immunosuppressive cells for arginine.

T cell function is highly disrupted by arginine depletion within the TIME, which is mediated by both malignant cells[138-141] and immunosuppressive cells[142-147]. In T cells, arginine is important in regulating CD3z expression, which is necessary for proper antigen recognition by the TCR-CD3 complex[148-151]. For example, ARG2-dependent depletion of arginine by murine renal cell carcinoma cells leads to decreased expression of CD3z in T cells[139]. Sufficient arginine levels are also necessary during T cell activation because arginine is quickly metabolized to fuel downstream processes[152]. Moreover, decreased systemic arginine levels in Lewis lung carcinoma[150] and arginine depletion via ARG1 from cancer-derived exosomes in ovarian carcinoma[153] inhibit antigen-specific proliferation of $CD8^+$ TILs. Arginine depletion also impairs the effector function of $CD8^+$ T cells by preventing the secretion of IFNg and granzyme B[154,155]. On the other hand, arginine supplementation in $CD8^+$ T cells induces metabolic rewiring from glycolysis towards oxidative phosphorylation to promote proliferation, survival, and anti-tumor responses[152].

Several promising pre-clinical studies have demonstrated that targeting arginine metabolism in combination with anti-PD-1/PD-L1 treatment increases efficacy in overcoming resistance. Employing anti-PD-1 treatment in combination with vaccine inhibition of ARG1 synergistically impaired tumor growth and led to increased $CD8^+$ T cell infiltration in mouse models of colorectal carcinoma and fibrosarcoma[156]. Further, systemic arginine supplementation with anti-PD-1 or PD-L1 treatment increased $CD8^+$ T cell infiltration and exhibited more efficacious results than monotherapy in mouse models of colon carcinoma[157] and osteosarcoma[158]. Utilizing a unique approach, researchers engineered an *E. coli* strain that localizes to the TIME and converts ammonia to arginine[159]. This innovative method promoted continuous arginine supplementation in murine colorectal carcinoma tumors, leading to increased $CD8^+$ T cell infiltration and synergistic anti-tumor effects when combined with anti-PD-L1 treatment[159]. Extensive pre-clinical studies for a novel ARG1/2 inhibitor (OATD-02) have shown promising results alone and in combination with both anti-PD-1 and -PD-L1, and researchers are hopeful this drug will enter first-in-human clinical trials soon[150,160-162]. Moreover, the ARG1 inhibitor CB-1158 entered first-in-human clinical trials in 2017 and was evaluated with anti-PD-1 treatment[163-165]. The results indicate that CB-1158 monotherapy and combination with anti-PD-1 are well-tolerated and elicit a response in solid tumors[163-165].

A considerable amount of evidence demonstrates that tumor-mediated depletion of arginine negatively impacts $CD8^+$ T cell function and the anti-tumor response. Additionally, the enhanced anti-tumor effects seen by combining anti-PD-1/PD-L1 with ARG inhibitors or arginine supplementation demonstrate that altering tumor metabolism could have profound effects on the efficacy of ICB. However, continued pre-clinical and clinical efforts are necessary to identify additional ways to target tumor-derived arginine metabolism and reinvigorate the anti-tumor immune response to improve ICB.

Glutamine

Glutamine has many essential functions, such as supporting the formation of nucleotides and non-essential amino acids, protein synthesis, energy metabolism, and maintaining intracellular redox states[166]. Import of glutamine is facilitated by many transporters, predominantly SLC1A5[136,167] [Figure 2]. Once inside the cell, glutamine is transported to the mitochondria to be converted to glutamate via glutaminase enzymes[166]. In the cytosol, glutamate serves as a precursor for glutathione synthesis, which is a strong antioxidant[166]. The metabolism of glutamine also drives the formation of NADPH, which is critical for restoring the intracellular redox balance by reducing oxidized glutathione[168]. In the mitochondria, glutamate is converted to a-Ketoglutarate to drive the TCA cycle[166].

Many cancers exhibit a dependence on or addiction to glutamine. As such, increased glutaminolysis is highly important for ATP production, redox homeostasis, and activation of various oncogenic signaling pathways in tumor cells[168-170]. Glutamine fuels KRAS signaling in pancreatic adenocarcinoma[168], mTORC1 signaling in osteosarcoma and cervical cancer cells[170], and promotes lipid biogenesis under hypoxic conditions to provide additional energy sources[171]. Hypoxia also drives the mitochondrial import of glutamine to support ATP and glutathione production to combat oxidative stress and promote uncontrolled cell growth[172]. Interestingly, data suggest that some cancers will adapt to the glutamine-deprived TIME and will cease to rely on glutamine. In patient-derived melanoma tumors, for example, excess dietary glutamine inhibits cell growth[173].

T cells require glutamine for a variety of functions during differentiation and development[174]; thus, there is stiff competition between tumor cells and T cells for glutamine consumption. Ligation of CD3 and CD28 on T cells induces glutamine uptake via ERK and calcineurin pathways to sustain T cell activation, proliferation, and cytokine production[175,176]. Interestingly, glutamine is also required for glucose uptake and glycolysis in activated $CD8^+$ T cells, and proper effector functions were dependent on both glucose and glutamine[177]. As such, increasing glutamine availability for T cells, while depriving tumor cells and immunosuppressive cells, has strong anti-tumor effects. For example, selectively inhibiting glutamine uptake in triple-negative breast cancer cells increased $CD8^+$ T cell activation and effector function by promoting glutathione production[178]. On the other hand, non-specific intracellular depletion of glutamine leads to impaired mitochondrial function and $CD8^+$ T cell apoptosis[179], likely due to increased oxidative damage from reduced glutathione production. Data also suggest the temporal importance of glutamine availability in driving T cell function. During TCR stimulation, glutamine deprivation decreases PD-1 and increases Ki67 expression[180], suggesting that glutamine abundance needs to be tightly regulated at various stages of T cell development to ensure proper functionality. As discussed in previous sections, immunosuppressive cells largely thrive in the nutrient-deprived TIME. Specifically, tumor-associated macrophages respond to low glutamine levels by secreting IL-23 to promote Treg proliferation and activation, resulting in diminished $CD8^+$ T cell function[181].

Several reports have demonstrated that inhibiting tumor-associated glutamine metabolism in combination with anti-PD-1/PD-L1 therapies may be a promising approach to restore $CD8^+$ T cell function and overcome resistance. Because glutamine deprivation promotes T cell dysfunction, specifically inhibiting glutamine metabolism in tumor cells would yield the most efficacious results. Two separate groups found that glutamine deprivation in cell lines of human clear cell renal carcinoma[182], human non-small cell lung carcinoma[183], and mouse colorectal carcinoma[183] induced PD-L1 expression, which would theoretically boost anti-PD-L1 response. Byun *et al.* found that anti-PD-L1 monotherapy had almost no effect on tumor volume in murine colorectal carcinoma models[183]. However, tumor-specific inhibition of glutamine uptake and glutaminase activity in combination with anti-PD-L1 therapy strongly induced $CD8^+$ T cell proliferation and granzyme B production, while abating tumor growth[183]. Similarly, another group targeted tumor-derived glutamine enzymes by creating a prodrug that is only activated by TIME-restricted enzymes to limit the cytotoxic effects of systemic glutamine antagonism[184]. This treatment method decreased glycolysis in malignant cells, decreased hypoxia, acidosis, and nutrient depletion within the TIME, and increased activation of and oxidative phosphorylation in $CD8^+$ T cells[184]. In combination with anti-PD-1 therapy, tumor-specific glutamine antagonism synergistically reduced tumor growth and increased survival in murine colorectal carcinoma tumors[184]. Conversely, employing a non-tumor cell specific glutaminase inhibitor does not yield the same efficacious results. Serine/threonine kinase 11 (STK11) phosphorylates AMPK to regulate a variety of downstream pathways, such as cell growth and proliferation, lipid metabolism, and PD-L1 expression[185]. Several studies have shown that STK11 mutations, resulting in loss

of function, are associated with resistance to anti-PD-1 treatment[186-188]. Building on this, one group found that STK11-mutated lung adenocarcinomas from both patient samples and cancer cell lines exhibited increased glutamate production, so they hypothesized that targeting glutaminase would be a viable way to overcome resistance to anti-PD-1 treatment[189]. However, they found that using a non-tumor cell-specific glutaminase inhibitor in combination with anti-PD-1 severely impeded $CD8^+$ T cell clonal expansion and anti-tumor functions, and anti-PD-1 efficacy was dependent on intact $CD8^+$ T cell glutaminase activity[189].

These data demonstrate a promising future for targeting glutamine metabolism to bolster $CD8^+$ T cell effector function and combat ICB resistance. However, it also highlights the importance of finding ways to specifically target malignant cells due to the highly conserved nature of these metabolic pathways.

Methionine

Methionine is an essential amino acid that is involved in a variety of metabolic pathways, such as methylation reactions, homocysteine synthesis, and the folate pathway [Figure 2]. This metabolite also cooperates with arginine and glutamine to promote polyamine and glutathione synthesis, respectively[190]. In the methionine pathway, methionine is converted to S-adenosyl methionine (SAM), which is critical for the methylation of histones, DNA, RNA, proteins, and various metabolites[191]. The loss of a methyl group converts SAM to S-adenosyl homocysteine (SAH), and subsequently homocysteine, which is ultimately metabolized to glutathione[192]. Methionine regeneration is supported by the metabolism of SAM through the salvage pathway[192] and through the re-methylation of homocysteine via intermediates in the folate pathway[193].

The role of methionine in malignant transformation and growth is not as well-studied as other metabolites, but its wide consumption in cancer cells suggests its importance[194,195]. In tumor-initiating cells, exogenous methionine is consumed at extreme rates, leading to pro-tumorigenic epigenetic modifications through methionine adenosyltransferase 2A (MAT2A), which metabolizes methionine to SAM to promote histone methylation[196]. In the presence of methionine, malignant cells activate c-MYC, leading to increased MAT2A activity and tumorigenic genome modifications[197]. On the other hand, tumor overexpression of nicotinamide N-methyltransferase (NNMT), which converts SAM to NAD^+ and 1-Methylnicotinamide, leads to increased NAD^+ levels, hypomethylation, and tumor progression[198], highlighting that altered methionine metabolism can drive oncogenesis in multiple ways.

In T cells, proper metabolic regulation of methionine and its derivatives is necessary for epigenetic reprogramming during activation and differentiation[199], as evidenced by increased expression of methionine transporters during antigen recognition[175]. However, dysregulated methionine metabolism by tumor cells alters the abundance of SAM and 5-methylthioadenosine (MTA)[200], both of which drive the methionine salvage pathway[201]. Increased abundance of SAM and MTA within the TIME are associated with T cell exhaustion and expression of inhibitory checkpoint markers[200]. These two metabolites decrease chromatin accessibility in $CD8^+$ T cells for genes involved in TCR signaling, lymphocyte proliferation and differentiation, and increase the accessibility of PD-1[200]. Together, these data indicate that tumor-derived alterations in methionine metabolism have a substantial impact on the anti-tumor immune functions of $CD8^+$ T cells, but much remains to be discovered.

Despite the limited studies in this field, two recent reports demonstrate that restricting tumor methionine increases $CD8^+$ T cell effector functions and overcomes resistance to anti-PD-1/PD-L1 treatment. The first study shows that dietary restriction of methionine reduces SAM levels in murine colorectal carcinoma tumors[202]. Mechanistically, SAM controls the expression of immune inhibitory markers PD-L1 and VISTA

through m^{6}A methylation, whereby the RNA-binding protein YTHDF1 enhances the translation efficiency of RNA containing m^{6}A methylation[202]. While anti-PD-1 treatment alone in mouse colorectal carcinoma tumors did not significantly alter tumor volume or CD8$^+$ T cell infiltration, depletion of YTHDF1 or restricting methionine in the diet synergized with anti-PD-1 treatment to significantly increase survival probability and CD8$^+$ T cell infiltration, while decreasing tumor volume[202]. Similarly, the second study found that methionine-dependent histone methylation regulates CD8$^+$ T cell anti-tumor activities. Methionine deprivation in CD8$^+$ T cells resulted in reduced H3K79me2 methylation and subsequent STAT5 expression[203], which is a critical transcription factor that maintains CD8$^+$ T cell effector functions[204]. *In vitro*, methionine supplementation increased CD8$^+$ T cell survival and IFNg and TNFa production, while inhibiting murine melanoma tumor growth[203]. The authors also found that SLC43A2 and SLC7A5 import methionine in malignant cells, but T cells are predominantly dependent on SLC7A5[203]. As such, genetic ablation of SLC43A2 in mouse melanoma cells restored CD8$^+$ T cell polyfunctionality and survival *in vitro*, and decreased tumor growth *in vivo*[203]. While anti-PD-1 treatment or pharmacological inhibition of SLC43A2 alone did not elicit significant anti-tumor effects, combination treatment synergistically increased CD8$^+$ T cell function and infiltration, and decreased growth of mouse melanoma and ovarian tumors[203]. These data demonstrate that resistance to anti-PD-1 treatment can be negated by restricting methionine availability and metabolism in tumors.

Taken together, the studies in this section have undoubtedly established that targeting amino acid metabolism is an efficacious way to improve the response to anti-PD-1/PD-L1 treatment. Targeting these metabolic pathways proves to be challenging because, unlike the immunosuppressive metabolites that have been discussed, amino acids are beneficial for both T cells and tumor cells. Therefore, therapeutic strategies have to promote amino acid supplementation in T cells but restriction in tumor cells, which is no easy feat. Despite these challenges, researchers have made great strides in pre-clinical settings towards identifying how to alter amino acid metabolism in a way that impedes ICB resistance.

LIPID METABOLISM

The TIME is enriched with various lipid classes[205-207], which is in contrast to other metabolites that are predominantly depleted. Lipids are ubiquitously important for structural support, energy supply, and signaling, making them essential for the malignant properties of tumors and for the proper function of anti-tumor immune cells. Specifically, cholesterol is indispensable for cell membrane integrity and facilitating cell-to-cell and intracellular signaling, while fatty acids (FAs) are the most abundant lipid intermediate, so they are more readily detectable and their role in cancer biology is better understood. Therefore, this section will highlight how tumor-mediated cholesterol and FA dysregulation within the TIME affects CD8$^+$ T cell function and anti-PD-1/PD-L1 resistance.

Cholesterol

Cholesterol serves as an important component in cellular membranes and regulates membrane fluidity and cell signaling through the formation of lipid rafts[208] [Figure 3]. Moreover, cholesterol is a precursor for steroid hormones, bile acids, and vitamin D[208]. Intracellular cholesterol levels are maintained through biosynthesis via the mevalonate pathway, which converts acetyl-CoA to cholesterol through a series of enzymatic reactions. Additionally, cholesterol is imported as low-density lipoproteins, which are small lipid-enclosed particles that facilitate the systemic transport and cellular import of cholesterol[209]. On the other hand, cholesterol is exported through ATP-binding cassette transporters[210]. Excess intracellular free cholesterol is converted to cholesteryl esters and stored in lipid droplets, which promote oncogenic signaling and cancer growth[211].

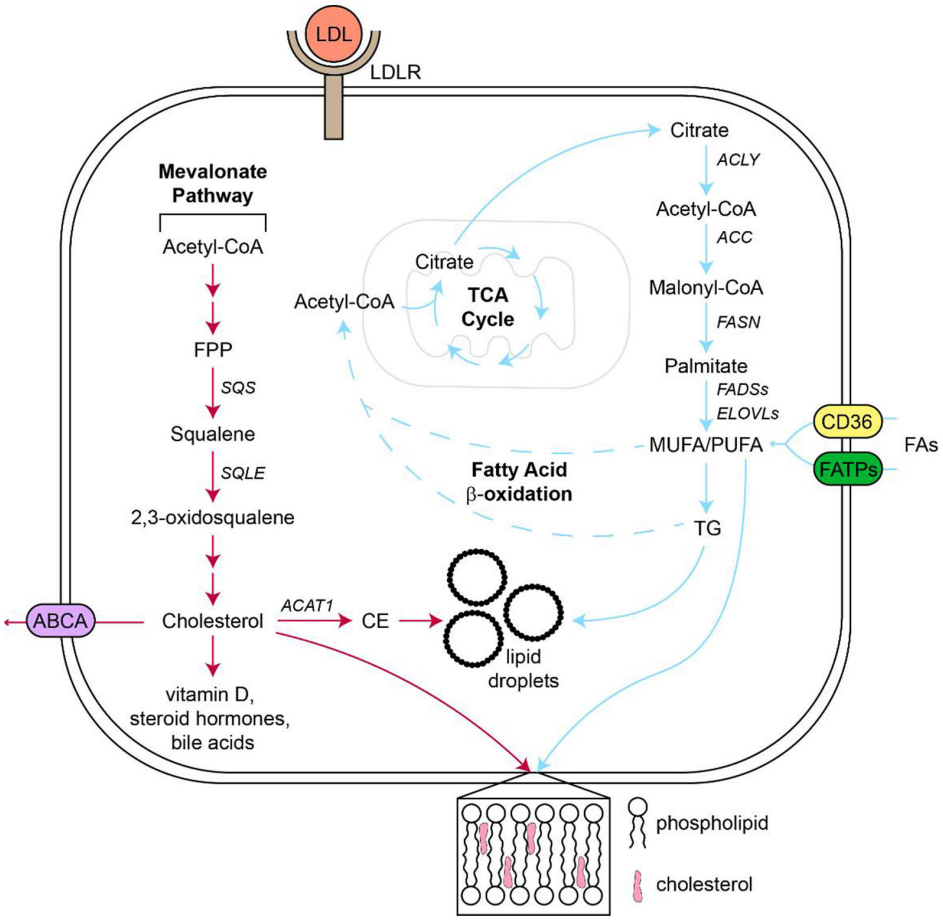

Figure 3. Diagram of cholesterol and FA metabolic pathways. Cholesterol is either imported as LDL through LDLR or it is synthesized through the mevalonate pathway. From there, cholesterol serves as a precursor to vitamin D, steroid hormones, and bile acids or it integrates into the cellular membrane to regulate membrane fluidity and cell signaling. Excess intracellular cholesterol is exported through ABCA or esterified to form CE, which are stored in lipid droplets. FAs are imported via CD36 and fatty acid transport proteins or synthesized through citrate from the TCA cycle. Palmitate, the initial FA that is formed, undergoes elongation and desaturation by ELOVL and FADS enzymes, respectively, to form a variety of FAs with varying chain lengths and degrees of unsaturation. FAs participate in energy metabolism through the FA b-oxidation pathway that generates acetyl-CoA to drive the TCA cycle. Similar to cholesterol, fatty acids are important components of cellular membranes via the formation of phospholipids and excess fatty acids are converted to TG and stored in lipid droplets. ABCA: ATP-binding cassette transporters; ACAT1: Acyl-CoA cholesterol acyl transferase 1; ACC: acetyl-CoA carboxylase; ACLY: ATP citrate lyase; ATP: adenosine triphosphate; CE: cholesteryl esters; ELOVL: elongation of very long chain fatty acids protein; FA: fatty acid; FADS: fatty acid desaturase; FATP: fatty acid transport protein; FASN: fatty acid synthase; FPP: farnesyl diphosphate; LDL: low-density lipoprotein; LDLR: low-density lipoprotein receptor; MUFA: mono-unsaturated fatty acid; PUFA: poly-unsaturated fatty acid; SQS: squalene synthase; SQLE: squalene epoxidase; TCA: tricarboxylic acid; TG: triglyceride.

Malignant cells utilize excess cholesterol to sustain their rapid growth and proliferation[212-214] and elevated intracellular cholesterol levels are maintained by increasing import and synthesis and decreasing export[215,216]. Altered cholesterol content in malignant cell membranes regulates apoptosis[217], proliferation, metastasis[218], and killing by cytotoxic T cells[219]. Cholesterol and its derivatives are also involved in various oncogenic signaling pathways and protein modifications[220]. Unsurprisingly, these metabolites are sequestered by tumor cells to promote malignant growth, and dysregulation of cholesterol in the TIME by tumor cells affects the cytotoxic functions of $CD8^+$ T cells.

There are multiple ways in which tumor cells directly alter cholesterol metabolism within the TIME to inhibit $CD8^+$ T cell function. Protein convertase subtilisin/kexin type 9 (PCSK9) is a secreted enzyme that

regulates cholesterol levels by facilitating the degradation of low-density lipoprotein receptors (LDLR)[221-224], which imports low-density lipoprotein cholesterol. Tumor-secreted PCSK9 promotes intratumoral accumulation of cholesterol[225], prevents LDLR and TCR recycling in $CD8^+$ TILs[226], and inhibits MCH-1 recycling on tumor cells[227], leading to immune evasion in multiple ways. Further, several reports demonstrate that intratumoral cholesterol accumulation promotes PD-L1 expression[228-231], thereby contributing to immune evasion. Mechanistically, cholesterol binds to the transmembrane domain of PD-L1 to stabilize cell surface expression[231]. Cholesterol-derived metabolites produced by malignant cells also dictate anti-tumor response. For example, cholesterol sulfate creates a chemical barrier within the TIME to prevent $CD8^+$ T cell infiltration[232]. Moreover, cholesterol sulfate-producing tumors are more resistant to ICB therapy[232] than tumors that do not produce this metabolite, demonstrating that targeting tumor-intrinsic cholesterol metabolism could enhance ICB outcomes.

In addition to cholesterol biochemical pathways regulating $CD8^+$ T cell function, mechanical forces driven by altered cholesterol levels within tumor cells also influence anti-tumor immune response. Cancer cells accumulate cholesterol within the cell membrane, leading to increased membrane fluidity, or "cell softening"[219]. This phenomenon is associated with cancer development and progression because cancer cell softening impairs the cytotoxic effects of T cells, leading to immune escape[219]. By reversing these effects and promoting cancer cell stiffening, increased T cell forces and actin accumulation at the immunological synapse enhance tumor killing[219]. Notably, cancer cell stiffening did not alter TCR signaling or cytokine production, demonstrating that these effects were purely through mechanical forces[219].

In T cells, maintaining a proper balance between membrane and intracellular cholesterol levels is important for development, activation, and effector functions. Cholesterol in the cell membrane is essential for the intricate formation of lipid rafts which regulate TCR signaling[233]. In TILs, several studies report that the allocation of cholesterol towards cell membrane formation instead of storage as cholesterol esters promotes anti-tumor activities. Pharmacologic inhibition in tumor cells and $CD8^+$ T cells of acyl-CoA cholesterol acyltransferase 1 (ACAT1), which promotes cholesterol esterification, inhibits cancer cell growth[234]. Similarly, another group found that RORa, a nuclear hormone receptor, promotes $CD8^+$ T cell membrane cholesterol accumulation by inhibiting cholesterol esterification, thus enhancing anti-tumor functions[235]. On the other hand, intracellular cholesterol accumulation in $CD8^+$ T cells due to cholesterol enrichment in the TIME leads to endoplasmic reticulum (ER) stress, which causes T cell exhaustion and increased expression of immune checkpoint markers[207]. Mechanistically, ER stress promotes upregulation of the ER stress sensing protein XBP1, which drives the expression of immune inhibitory markers, namely PD-1 and 2B4[207]. As a result, inhibiting XBP1 or reducing cholesterol in $CD8^+$ T cells or the TIME boosts the anti-tumor functions of $CD8^+$ T cells[207]. These studies demonstrate that shifting cholesterol away from intracellular stores towards membrane formation in T cells might be an effective therapeutic strategy to diminish resistance to ICB therapy.

Given the profound effect of tumor-derived cholesterol on $CD8^+$ T cell function, it is no surprise that targeting this altered metabolic pathway inhibits resistance to anti-PD-1 treatment. Building on the idea that allocating cholesterol towards cellular membranes in $CD8^+$ T cells is beneficial for the anti-tumor response, researchers found that pharmacologic inhibition of ACAT1 in combination with anti-PD-1 treatment synergistically reduced the growth of mouse melanoma tumors[236]. Further, slight anti-tumor effects were observed in four mouse tumor models following genetic ablation of PCSK9, but combination of genetic or pharmacologic inhibition of PCSK9 with anti-PD-1 resulted in robust synergistic effects to increase MHC-I expression and survival and reduce growth of murine melanoma and colorectal carcinoma tumors[227]. Another emerging target is squalene epoxidase (SQLE), which catalyzes one of the rate-limiting steps in

sterol synthesis [Figure 3]. Bioinformatics approaches have identified a negative correlation between SQLE expression in human pancreatic adenocarcinoma and immune cell infiltration and immunotherapy response[237], prompting the need for further validation of this potential target. While the intersection of tumor-mediated cholesterol metabolism and ICB response is not as robust as other metabolic programs, these recent studies hint at how this relationship can be exploited to overcome ICB resistance.

Fatty acids

Similar to cholesterol, FAs have a variety of cellular functions, including cell membrane formation through phospholipids, energy metabolism, and precursors for signaling lipids [Figure 3]. Intracellular FA abundance is regulated by import through CD36 or FA transport proteins and synthesis via fatty acid synthase (FASN) from acetyl-CoA or malonyl-CoA[238]. FAs undergo modifications to chain length to form long-chain FAs (LCFAs) or very long-chain FAs (VLCFAs) and saturation to form mono-, di-, and poly-unsaturated FAs. Saturation and chain length dictate FA function and their role in oncogenesis[239]. In energy metabolism, FAs are subject to fatty acid b-oxidation (FAO) in the mitochondria to generate FADH, NADH, and acetyl-CoA to fuel a variety of energetic processes[240].

The increased demand for FAs in malignant cells sustains their rapid proliferation by serving as an energy source via FAO and as an indispensable component for cell membrane formation. Moreover, certain FAs are important precursors for a variety of oncogenic signaling mediators[241-243]. To meet these metabolic demands, cancer cells will increase the uptake and synthesis of fatty acids, while also inducing lipolysis of neighboring adipocytes[244-248]. Continuous evidence is emerging that altered FA metabolism by tumor cells alters the lipidome in the TIME, contributing to $CD8^+$ T cell dysfunction. However, the effect of tumor-derived FA metabolic alterations on ICB resistance is not well-studied.

Malignant cells exploit the increased lipid availability in patients with obesity and remodel the TIME to inhibit $CD8^+$ T cell function and promote cancer growth. High-fat diet-induced obesity in multiple mouse models of cancer alters the metabolic profile of malignant cells to increase FA uptake and utilization and creates an immunosuppressive TIME that inhibits $CD8^+$ T cell infiltration and function[249]. Moreover, inhibiting obesity-induced metabolic rewiring in murine colorectal carcinoma tumors restores $CD8^+$ TIL function and increases anti-tumor immune function[249]. Mechanistically, researchers found that $CD8^+$ T cells in obesity-associated breast cancer tumors exhibit ligation of leptin and PD-1 to reduce effector functions through activation of STAT3, which promotes FAO and inhibits glycolysis[250]. PD-1 ligation also promotes FAO in T cells through upregulation of carnitine palmitoyltransferase 1A (CPT1A), an essential enzyme involved in FAO[251]. Further, obesity in mice, humans, and non-human primates leads to increased PD-1 expression and $CD8^+$ T cell exhaustion[252]. These data are consistent with the notion that $CD8^+$ T cells exhibit a shift from glycolysis to FAO as they become exhausted, highlighting the need to further explore targeting metabolic reprogramming as a way to reinvigorate $CD8^+$ T cells and abate ICB resistance.

Similar to obese models of cancer, non-obese models show that $CD8^+$ T cell function is inhibited by an overabundance of FAs within the TIME. In response to excess lipid content within the TIME, $CD8^+$ TILs exhibit increased intracellular lipid levels compared to peripheral $CD8^+$ T cells[205]. Exhaustion in $CD8^+$ TILs is characterized by the expression of CD36, which imports oxidized low-density lipoproteins, oxidized phospholipids, and long-chain fatty acids[205]. Increased uptake of oxidized low-density lipoproteins promotes lipid peroxidation in $CD8^+$ TILs, leading to decreased cytokine production and effector function[205]. Moreover, the accumulation of VLCFAs within the TIME drives the uptake of LCFAs in $CD8^+$ T cells, and instead of serving as an energy source, they promote mitochondrial dysfunction, lipotoxicity, and exhaustion[253]. Like cancer cells, immunosuppressive cells, such as Tregs, macrophages, and MDSCs,

rely heavily on exogenous FAs to sustain their increased rate of FAO[254-257]. In this regard, increased FA abundance within the TIME hinders $CD8^+$ T cell function, while benefiting malignant and immunosuppressive cells.

FAs are the building blocks for a variety of bioactive lipids, which are involved in signaling pathways. Tumor cells, and to a lesser extent CAFs[258], secrete the enzyme autotaxin (ATX) that converts ubiquitously available lysophosphatidylcholine (LPC) to the bioactive lipid lysophosphatidic acid (LPA)[259]. LPA modulates numerous signaling pathways through lysophosphatidic acid receptors 1-6 (LPAR1-6), which are present on a variety of cell types[259]. In malignant cells, the ATX/LPA axis also functions in an autocrine manner by promoting oncogenic signaling through LPAR1[260]. On $CD8^+$ T cells, tumor-derived LPA binds to LPAR6 and prevents tumor infiltration by inhibiting migration[260]. LPA also signals through LPAR5 on $CD8^+$ T cells to induce cytoskeletal dysfunction, immunological synapse malformation, and impaired cytokine secretion and intracellular calcium release[261-263]. LPAR5 signaling on $CD8^+$ T cells also induces an exhausted-like state by promoting metabolic stress through ROS production and ultimately impairing antigen-specific killing[264]. The recent development of a first-in-class ATX inhibitor demonstrated tumor growth inhibition in mouse models of breast cancer[265,266]. The safety of this compound was tested in Phase I clinical trials in 2021, where the drug was well-tolerated with no significant clinically adverse effects[266]. These promising results demonstrate the previously unexplored capacity to target ATX in solid tumors, with the future potential to combine this treatment with pre-existing ICB therapies.

There is very limited research on targeting FA metabolism in combination with anti-PD-1/PD-L1 therapy, but more evidence is emerging that supports this approach to overcome ICB resistance. Bioinformatics methods have identified that FASN expression in patients with bladder cancer, melanoma, and non-small cell lung carcinoma is linked to immune infiltration and ICB response[267,268]. Interestingly, ICB is more efficacious in obese patients with melanoma compared to non-obese patients[252,269-272]. While this may seem contradictory, obesity drives PD-1 expression on $CD8^+$ T cells, thus eliciting a more robust response. On the other hand, $CD8^+$ TILs in pancreatic adenocarcinoma exhibit increased expression of checkpoint inhibitors, but ICB therapy largely fails[273-275]. The variability in ICB response between cancer types prompts the need for a deeper understanding of the mechanisms that contribute to resistance. To further complicate things, under hypoxic and hypoglycemic conditions, pharmacologically enhancing FA catabolism in $CD8^+$ T cells promotes effector function[206]. Moreover, anti-PD-1 treatment, in combination with increased FA catabolism, synergistically reduced the volume of murine melanoma tumors and promoted anti-tumorigenic metabolic reprogramming in $CD8^+$ T cells[206]. These data suggest that under stressful conditions, i.e., oxygen and glucose depletion, increased FAO is required for $CD8^+$ T cell function, but this contradicts other studies that demonstrate a shift towards FAO promotes exhaustion.

Together, these research efforts have laid the groundwork to further characterize the intricate relationship between tumor-mediated cholesterol and FA metabolism and $CD8^+$ T cell function within the TIME. To date, it is not clear whether inhibiting cholesterol or FA metabolism is a viable treatment option to improve response to anti-PD-1/PD-L1 therapies. As new data emerges, researchers will have a better understanding of the tumor-specific cholesterol and FA metabolic programs that are exploited by cancer cells and if these can be targeted to prevent ICB resistance.

CONCLUSION

While ICB therapies have been an imperative advancement in cancer treatment, a majority of patients exhibit resistance, prompting the need for researchers to identify and target these resistance mechanisms. This review has provided a multitude of examples wherein tumor-intrinsic alterations to energy, amino

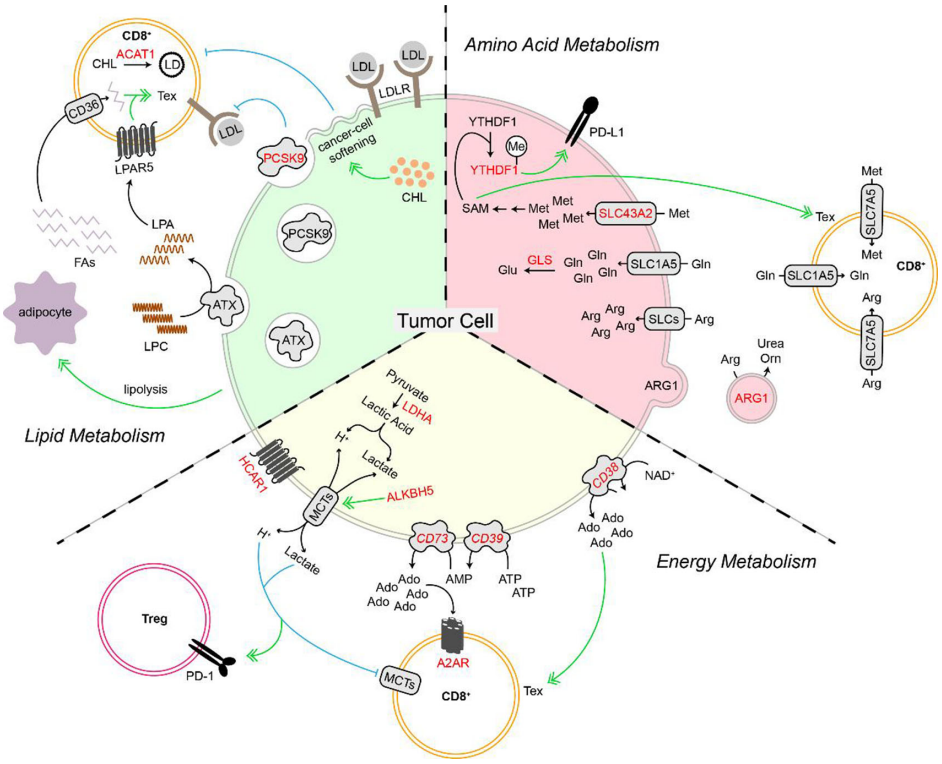

Figure 4. Summary schematic of how altered tumor-intrinsic energy, amino acid, and lipid metabolism drive $CD8^+$ T cell dysfunction and resistance to anti-PD-1/PD-L1 treatment. Targets in red are described in the previous sections and modulating these targets overcomes resistance to anti-PD-1/PD-L1 therapy. ACAT1: Acyl-CoA cholesterol acyl transferase 1; Ado: adenosine; ALKBH5: alkB homolog 5, RNA demethylase; Arg: arginine; ARG1: arginase 1; ATX: autotaxin; A2AR: adenosine A2A receptor; $CD8^+$: CD^+ T cell; CHL: cholesterol; FAs: fatty acids; Gln: glutamine; GLS: glutaminase; Glu: glutamate; HCAR1: hydroxycarboxylic acid receptor 1; LD: lipid droplet; LDHA: lactate dehydrogenase A; LDL: low-density lipoprotein; LDLR: low-density lipoprotein receptor; LPA: lysophosphatidic acid; LPAR5: lysophosphatidic acid receptor 5; LPC: lysophosphatidylcholine; MCT: monocarboxylate transporter; Me: methyl; Met: methionine; NAD^+: nicotinamide adenine dinucleotide; Orn: ornithine; PCSK9: proprotein convertase subtilisin/kexin type 9; PD-L1: programmed cell death ligand 1; PD-1: programmed cell death protein 1; SAM: S-adenosylmethionine; SLC: solute carrier; Tex: $CD8^+$ T cell exhaustion; Treg: T regulatory cell; YTHDF1: YTH N6-methyladenosine RNA binding protein F1.

acid, and lipid metabolism have a significant impact on $CD8^+$ T cell function and resistance to anti-PD-1/PD-L1 therapies [Table 1 and Figure 4]. In many of the studies presented here, anti-PD-1/PD-L1 therapy alone elicits limited anti-tumor effects but, when combined with targeting metabolic pathways, the response is significantly more robust. Nevertheless, there are a limited number of metabolism-targeting drugs that make it to the clinic because these pathways are highly conserved and not tumor-cell specific. As such, this warrants either unique ways to mitigate systemic effects, some of which have been provided in this review, or continued efforts to identify tumor-specific pathways. However, the extreme heterogeneity of the TIME, metabolome, and lipidome between cancer types necessitates large research efforts to uncover these distinct metabolic programs.

Future directions for the fields of immuno- and onco-metabolism are rooted in the utilization of metabolomic and lipidomic analyses to understand the metabolic landscape of cancer and develop efficacious cancer treatments. Taking a true multi-omics approach by incorporating proteomics, transcriptomics/spatial transcriptomics, and metabolomics/spatial metabolomics will greatly advance our understanding of targetable pathways, both within malignant cells and T cells. These methods are gaining more traction within the oncology research space and hopefully will be more widely utilized in the coming years.

Table 1. Tumor-intrinsic metabolic targets, the resulting metabolites, and the drug or compound used against the target that have been evaluated pre-clinically and/or clinically in combination with anti-PD-1/PD-L1 therapy

Target (metabolite)	Drug/Compound	Pre-clinical or clinical	Combination with anti-PD-1/PD-L1	Ref.
ALKBH5 (lactate)	ALK-04	Pre-clinical	Anti-PD-1	[42]
LDHA (lactate)	GSK2837808A	Pre-clinical	Anti-PD-1	[43]
HCAR1 (lactate)	3-OBA	Pre-clinical	Anti-PD-1	[44]
A2AR (adenosine)	CPI-444	Clinical	Anti-PD-L1	[84]
A2AR (adenosine)	DZD2269	Pre-clinical	Anti-PD-1	[85]
CD39 (adenosine)	IPH5201	Clinical	Anti-PD-L1	[89]
CD39 (adenosine)	IPH5201	Pre-clinical	Anti-PD-L1	[88-90]
CD39 (adenosine)	TTX-030	Clinical	Anti-PD-1	[91]
CD73 (adenosine)	MEDI9447 (oleclumab)	Clinical	Anti-PD-L1	[92,93]
A2AR (adenosine)	Nanoreactor	Pre-clinical	Anti-PD-1	[94]
CD39 (adenosine)	POM-1	Pre-clinical	Anti-PD-1	[95]
CD39 (adenosine)	ARL67156	Pre-clinical	Anti-PD-1	[96]
CD38 (NAD^+)	Anti-CD38 and RHein	Pre-clinical	Anti-PD-L1	[114]
ARG1 (arginine)	Vaccine	Pre-clinical	Anti-PD-1	[156]
ARG1/2 (arginine)	OATD-02	Pre-clinical	Anti-PD-1	[150,160-162]
ARG (arginine)	CB-1158	Clinical	Anti-PD-1	[163-165]
SLC1A5 (glutamine)	V-9302	Pre-clinical	Anti-PD-L1	[183]
Glutamine-utilizing enzymes (glutamine)	JHU083	Pre-clinical	Anti-PD-1	[184]
YTHDF1 (methionine)	Short-hairpin knockdown of YTHDF1	Pre-clinical	Anti-PD-L1	[202]
SLC43A2 (methionine)	BCH	Pre-clinical	Anti-PD-L1	[203]
ACAT1 (cholesterol)	CI-1011	Pre-clinical	Anti-PD-1	[236]
PCSK9 (cholesterol)	AMG-145 and D10335	Pre-clinical	Anti-PD-1	[227]

ACAT1: Acyl-CoA cholesterol acyl transferase 1; ALKBH5: alkB homolog 5; ARG1: arginase 1; A2AR: adenosine A2A receptor; HCAR1: hydroxycarboxylic acid receptor 1; LDHA: lactate dehydrogenase A; PCSK9: proprotein convertase subtilisin/kexin type 9; PD-L1: programmed cell death ligand 1; PD-1: programmed cell death protein 1; SLC: solute carrier; YTHDF1: YTH N6-methyladenosine RNA binding protein F1.

PERSPECTIVES

In recent years, immense strides have been made in studying the intersection of metabolism, cancer, and the immune system. In addition to the metabolites and pathways covered in this review, there are a plethora of others waiting to be linked to $CD8^+$ T cell dysfunction and ICB resistance. For example, other amino acids and lipid classes, metabolites produced by the gut microbiome, and a closer look at the metabolites associated with oxidative phosphorylation and ATP production. Moreover, there is much to uncover about how tumor-derived metabolic alterations affect other immune and non-immune cell types. Continued research efforts in this field will provide a more comprehensive understanding of tumor-intrinsic metabolic alterations and reveal nuanced ways to target tumor metabolism and overcome resistance to ICB therapies.

DECLARATIONS

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Cancer Drug Resistance

Review

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A review of strategies to overcome immune resistance in the treatment of advanced prostate cancer

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Abstract

Immunotherapy has become integral in cancer therapeutics over the past two decades and is now part of standard-of-care treatment in multiple cancer types. While various biomarkers and pathway alterations such as dMMR, *CDK12,* and AR-V7 have been identified in advanced prostate cancer to predict immunotherapy responsiveness, the vast majority of prostate cancer remain intrinsically immune-resistant, as evidenced by low response rates to anti-PD(L)1 monotherapy. Since regulatory approval of the vaccine therapy sipuleucel-T in the biomarker-unselected population, there has not been much success with immunotherapy treatment in advanced prostate cancer. Researchers have looked at various strategies to overcome immune resistance, including the identification of more biomarkers and the combination of immunotherapy with existing effective prostate cancer treatments. On the horizon, novel drugs using bispecific T-cell engager (BiTE) and chimeric antigen receptors (CAR) technology are being explored and have shown promising early efficacy in this disease. Here we discuss the features of the tumour microenvironment that predispose to immune resistance and rational strategies to enhance antitumour responsiveness in advanced prostate cancer.

Keywords: Prostate cancer, immunotherapy, immune checkpoint inhibitor, immune resistance, tumour microenvironment

INTRODUCTION

Prostate Cancer has the second highest cancer incidence worldwide and is the 5th leading cause of cancer death in men[1]. The cornerstone treatment of locally-advanced and metastatic prostate cancer centres upon androgen deprivation therapy. Patients who experience disease progression while having castrate levels of testosterone are considered castration-resistant. In the advanced prostate cancer setting, additional treatment modalities include novel hormonal agents (NHAs), chemotherapy, radioligand therapy, poly(ADP)-ribose polymerase (PARP) inhibitors, and immunotherapy. Successive waves of clinical trials in the past decade have brought these treatment modalities forth from the castration-resistant setting into the hormone-sensitive setting, showing improved survival with early introduction of chemotherapy, NHAs, or combinations of these[2]. Despite these advances in prostate cancer treatment, the 5-year survival for metastatic prostate cancer patients in 2022 remains low at 32.3%[3].

Immunotherapy, in the form of sipuleucel-T, received FDA approval in 2010 for the treatment of patients with asymptomatic or minimally symptomatic metastatic castration-resistant prostate cancer (mCRPC). In patients with deficient mismatch repair or microsatellite-high (dMMR/MSI-H) tumours, pembrolizumab and dostarlimab are FDA-approved options[4,5]. However, the prevalence of dMMR/MSI-H in prostate cancer is dismal at 1%, with *MSH2* being the most frequently implicated (other MMR genes being *MSH6, MLH1, PMS2*)[6]. Owing to an immunologically "cold" microenvironment in unselected acinar prostate adenocarcinoma, to date, no other immunotherapeutic agents have shown to be beneficial in the current treatment of advanced prostate cancer. In this review, we look at the current treatment paradigm, the role of immunotherapy, and existing and up-and-coming methods to overcome immune therapy resistance in prostate cancer.

IMMUNE REGULATION IN THE TUMOUR MICROENVIRONMENT (TME) OF PROSTATE CANCER

Immuno-oncology has changed the treatment paradigm of multiple tumour types, including melanoma, renal cell carcinoma, and lung carcinoma. The cancer-immunity cycle is depicted in Figure 1, explaining how the innate immune system fends off cancer cells and the various points at which therapeutic targets act. Despite successes in these typically immunogenic tumours, prostate cancer has traditionally been considered to have an immunologically "cold" tumour microenvironment (TME) characterized by T cell exclusion, low neoantigen load, and a highly immunosuppressive microenvironment comprising a high proportion of myeloid-derived suppressor cells (MDSCs)[7,8]. Factors that suggest a maladaptive immune response against tumour cells include lack of tumour-infiltrating lymphocytes (TILs), presence of M2-polarized tumour-associated macrophages (TAMs) and MDSCs, with evidence that increment in such cell populations within the TME is correlated with tumour progression[9]. MDSCs are immune cells that are activated in cancers and display potent immunosuppressive effects leading to prostate cancer resistance to anti-hormonal therapy[10]. Furthermore, CRPCs frequently exhibit *PTEN* loss, which is associated with increased MDSC infiltration[11] and may interact with the interferon-1 pathway required for innate immune activation[12]. Other immune-suppressive factors within the TME, such as soluble tumour necrosis factor (sTNF), interleukin-1 beta (IL-1β), TGF-β, and IL-10, promote chronic inflammation and increase myeloid cell differentiation into MDSCs[13,14].

Reduced immune stimulatory factors can also contribute to the immunologically cold TME in prostate cancer. CRPC patients have decreased peripheral natural killer (NK) cell pools, and this may be due to increased NK cell group 2 member D (NKG2D) serum receptor levels from the tumour[15]. This phenomenon is more pronounced with metastatic disease[9]. NK cells are lymphocytes that have roles in innate and adaptive immunity, whereas NKG2D is an activating cell surface receptor expressed on NK cells,

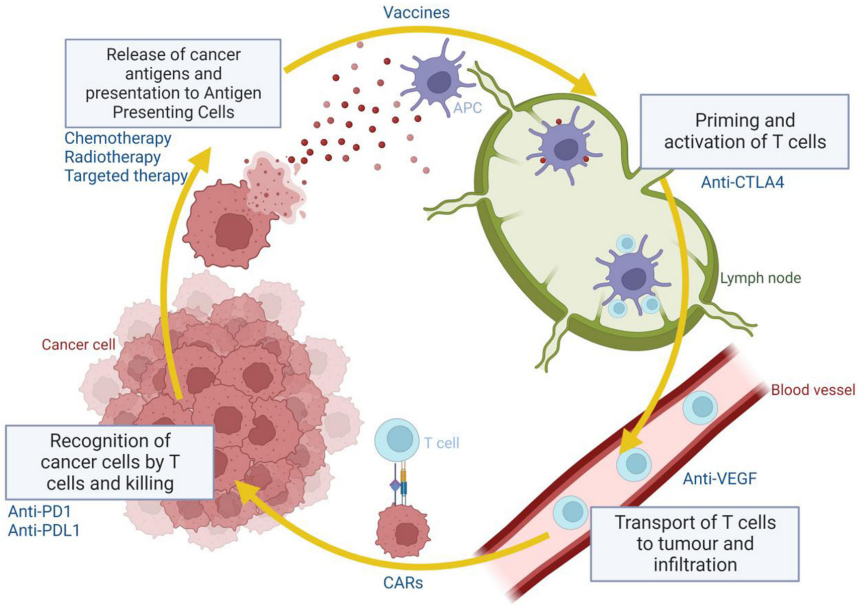

Figure 1. The cancer immunity cycle and where various classes of drugs act on.

NKT cells, and subsets of γδ T cells. Although initially thought to enhance immune responses against cancer, it appears that when NKG2D ligands are expressed chronically, this can instead lead to inhibition of immune cell function[16]. Low tumour mutational burden (TMB) in prostate cancer is associated with reduced neoantigen load recognised by the immune system[17]. These mechanisms enable immune evasion by cancer cells and directly impact the therapeutic response to anti-PD(L)1/anti-CTLA4 immune checkpoint inhibitors (ICIs)[18]. Figure 2 illustrates the interplay amongst the immune cells, cancer cells and vascular supply within the TME.

Potential biomarkers for ICI response include dMMR/MSI-H as mentioned above and tumours with DNA damage repair (DDR) pathway deficiencies. Tumours with DDR pathway deficiencies have increased mutational load as a result of decreased DNA repair capacity, leading to genomic instability[19]. Patients with somatic alterations in genes involved in DNA replication or repair have been shown to express higher neoantigen load, higher mutational burdens, higher levels of CD3+ and CD8+ TILs and higher PD-1/PD-L1 levels, all of which correlate with sustained ICI responses[20-24]. Despite this, dMMR and *CDK12*-altered prostate cancers have more aggressive biology[25,26]. A retrospective study of prostate cancer patients from the Royal Marsden Hospital showed that 8.1% of the patients had dMMR, which was correlated with decreased survival (median OS 4.1 years for dMMR *vs.* 8.5 years for proficient MMR)[26]. *CDK12* alterations were found in 6% of advanced prostate cancer in one study[25], and were typically linked to poor prognosis as well as insensitivity to PARP inhibitors[27]. However, these tumours have increased neoantigen load and tumoural lymphocyte infiltration, which may increase their response to ICIs[27].

ICI MONOTHERAPY IN THE UNSELECTED PROSTATE CANCER PATIENT

Cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) is a receptor found on the surface of T lymphocytes. When APCs activate T cells in response to the presence of foreign antigens, there is involvement of costimulatory molecules such as CD28 and B-7, which enhance the immune response. CTLA-4 acts as an immune checkpoint by binding to B-7, counteracting the costimulatory effect of CD28 and overall cause suppression of the immune response[28,29]. Cancer cells can downregulate the immune

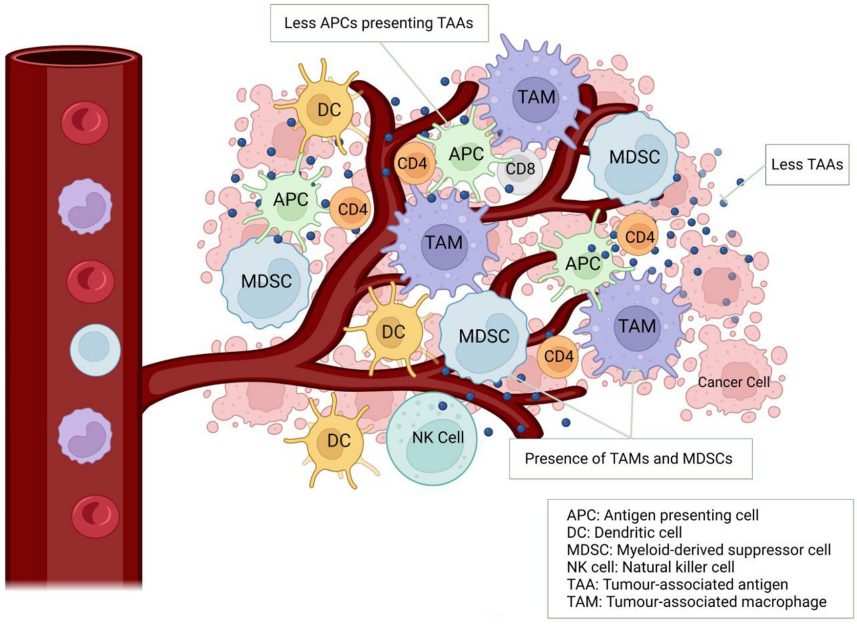

Figure 2. The immunologically "cold" tumour microenvironment in prostate cancer.

response by exploiting CTLA-4, and this forms the basis of targeting CTLA-4 with monoclonal antibodies such as ipilimumab. Inhibition of CTLA-4 activity causes activation and proliferation of cytotoxic T cells against tumour cells[30,31]. To date, two phase 3 trials have looked at the activity of ipilimumab in mCRPC patients. The first study, CA 184-043, recruited 799 mCRPC patients with at least one bone metastasis and have progressed on docetaxel chemotherapy. Patients were randomised to receive either one fraction of bone-directed radiation therapy followed by ipilimumab at 10 mg/kg or placebo. There was no overall survival benefit seen in this study (median OS 11.2 *vs.* 10 months, HR 0.85, 95% CI 0.72-1.00), but a progression-free survival (PFS) benefit (4.0 *vs.* 3.1 months, HR 0.70, 95% CI 0.61-0.82) was seen[32]. The second study by Beer *et al.* (2017) randomised 602 mCRPC patients who were chemotherapy-naive and had no visceral metastases to ipilimumab at 10 mg/kg *vs.* placebo. The study showed no overall survival benefit (median OS 28.7 *vs.* 29.7 months; HR 1.11, 95% CI 0.88-1.39), although a PFS benefit (median PFS 5.6 *vs.* 3.8 months; HR 0.67; 95% CI 0.55-0.81) was observed. Exploratory analyses further showed a higher prostate-specific antigen (PSA) response rate with ipilimumab (23%) than with placebo (8%)[33]. Taken together, the PFS and PSA response with ipilimumab suggests antitumour activity despite the lack of survival benefit.

PD-1 is a transmembrane glycoprotein found on the surfaces of activated cytotoxic T cells, B cells, dendritic cells, NK cells, and macrophages[34]. The binding of PD-1 to its ligands programmed death ligands 1 and 2 (PD-L1 and PD-L2) found on cancer cells delivers inhibitory signals for T-cell activation, suppressing an immune response[35,36]. Monoclonal antibodies targeting PD-1/PD-L1, such as nivolumab and pembrolizumab, have shown activity in multiple cancer types, leading to regulatory approval for their use[37,38]. Pembrolizumab was studied in the phase 1b KEYNOTE-028 and phase 2 KEYNOTE-199 trials as monotherapy in mCRPC, showing poor responses[39,40]. The objective response rate (ORR) was 5% in PD-L1 combined positive score (CPS) ≥ 1 patients in KEYNOTE-199, compared with 3% for patients with a negative PD-L1 expression[39]. Three phase 1 dose-escalation trials of nivolumab monotherapy in mCRPC patients likewise showed no objective response[41-43]. As mentioned, the paucity of PD-L1 expression in the TME in prostate cancer patients could account for this. Despite the glaringly low response rates for anti-PD(L)1/anti-CTLA4 monotherapies in unselected prostate cancer, the expression of immune

checkpoints has been reported to be dynamic, and various agents such as ipilimumab, sipuleucel-T and enzalutamide can increase T cell infiltration into the TME and modulate response to anti-PD(L)1 therapy[44]. This sets the stage for combination of various therapies with ICIs to improve immunotherapeutic response in prostate cancer.

ONGOING STRATEGIES TO OVERCOME IMMUNE RESISTANCE

Several strategies have been examined to modulate antitumour immunity in advanced prostate cancer.

PARP inhibitors and ICIs

PARP inhibitors are small molecules that prevent the repair of single-strand DNA breaks. Pathogenic DDR gene alterations are found in 23% of mCRPCs[45], with *BRCA2*, *ATM*, *CHEK2*, and *BRCA1* being the most frequently implicated genes[46]. The resulting homologous recombination deficiency (HRD) leads to sensitivity to PARP inhibition as a result of synthetic lethality[47]. Presently in mCRPC patients, the FDA has approved rucaparib for use in those with germline/somatic *BRCA* mutation and olaparib for those with germline/somatic homologous recombination repair (HRR) gene mutations. This is based on a high ORR of 50.8% seen with rucaparib use in the phase 2 TRITON2 study and improved radiologic PFS with olaparib use over enzalutamide/abiraterone in the phase 3 PROfound study[48,49]. The phase 3 TRITON3 study showed similar results[50]. Furthermore, efforts made in examining PARP inhibition in unselected patients have been successful as well, with the phase 3 PROpel trial showing improvement in radiologic PFS with combination abiraterone plus olaparib over abiraterone plus placebo as first-line treatment of mCRPC patients, overall suggesting an increasing role in PARP inhibition[51].

Increased micronuclei and cytosolic double-stranded DNA release after PARP inhibitor treatment as a result of PARP-DNA trapping and DNA damage leads to increased neoantigen formation, increased PD-L1 expression, increased intra-tumoural CD8 T cell infiltration and increased interferon production in the TME, forming the basis for ICI-PARP inhibitor combinations, and potentially expanding the benefit of PARP inhibitors beyond tumours harbouring alterations[52,53]. A phase 2 open-label clinical trial combining durvalumab with olaparib in men with mCRPC showed a response (radiographic or biochemical) in 9 out of 17 patients. Five of the 9 responders were found to have dysfunctional DDR genes based on genomic analysis and the presence of mutated DDR genes was associated with significantly higher 12-month PFS than those without (83.3% *vs*. 36.4%). Interestingly, patients with fewer peripheral MDSCs were more likely to respond[54]. This study showed early evidence of combining PARP inhibitors and ICIs, and other ongoing studies looking at similar combinations are listed in Table 1.

As mentioned, *CDK12*-altered prostate cancers typically carry poor prognosis and do not respond well to PARP inhibition, yet they present increased neoantigen load and lymphocytic infiltration, which may increase responsiveness to anti-PD1 therapy[25,27]. A retrospective study of 60 men with *CDK12*-altered advanced prostate cancer showed that of the 9 men who received PD-1 inhibitor therapy, 33% had a PSA response and the median PFS was 5.4 months[27,55]. Similarly, the ongoing phase 2 IMPACT trial has shown a 21.4% PSA response with ipilimumab-nivolumab combination in these patients[55].

Vaccines and ICIs

Anti-cancer vaccines can be classified into four groups: cell-based, viral-based, DNA/RNA-based, and peptide-based vaccines[56,57]. The goal of vaccine therapy is to stimulate the host's adaptive immune response against tumour-associated antigens (TAA). Prostate cancer is suitable for vaccine therapy because it has many TAAs such as PSA, prostate-specific membrane antigen (PSMA), prostate acid phosphatase (PAP), prostate stem cell antigen (PSCA), prostate cancer antigen 3 (PCA3), mucin-1, and six-transmembrane epithelial antigens of the prostate (STEAP)[58].

Table 1. Trials looking at ICI combinations in treatment of advanced prostate cancer

Trial number	Phase	Intervention arm(s)	Population	Outcome	Status
ICIs + PARP inhibitor					
NCT02484404	2	Durvalumab + Olaparib	mCRPC after progression with 1 NHA or Docetaxel	ORR, safety, DOR, PSA response	Completed
NCT04336943	2	Durvalumab + Olaparib	Recurrent prostate cancer with immunogenic signature	PSA response	Active, recruiting
NCT03834519	3	Pembrolizumab + Olaparib NHA (Abiraterone or Enzalutamide)	mCRPC after progression with 1 NHA and chemotherapy	OS, rPFS	Active, not recruiting
NCT02861573	1/2	Pembrolizumab + Olaparib Multiple cohorts	mCRPC	ORR, safety, PSA response	Active, recruiting
NCT05568550	2	Pembrolizumab + Olaparib + RT Pembrolizumab + RT	High-risk localised PC	PSA response	Not yet recruiting
NCT03338790	2	Nivolumab + Rucaparib Nivolumab + Docetaxel Nivolumab + Enzalutamide	mCRPC	ORR, PSA response	Active, not recruiting
NCT04592237	2	Maintenance Cetrelimab + Niraparib Maintenance Niraparib	Aggressive variant mPC given induction Cabazital + Carboplatin + Cetrelimab	PFS	Active, recruiting
ICIs + vaccines					
NCT03024216	1	Atezolizumab + Sipuleucel-T	mCRPC	Safety	Completed
NCT01832870	1	Ipilimumab + Sipuleucel-T	CRPC eligible to receive Sipuleucel-T in accordance to FDA-approved labeling	Antigen-specific T cell response, antibody response	Completed
NCT00113984	1	MDX-010 (anti-CTLA-4) + PROSTVAC-V/TRICOM (virus vaccine)	mCRPC after progression with anti-androgens and ≤ 1 chemotherapy	Safety	Completed
NCT02933255	1/2	Nivolumab + PROSTVAC-V/F	mCRPC Neoadjuvant therapy for localised PC planned for surgery	Safety, changes in T-cell infiltration	Active, recruiting
NCT03315871	2	M7824 (anti-PD-L1/TGFβ) + PROSTVAC + CV301 (virus vaccine)	CRPC	PSA response	Active, recruiting
NCT03532217	1	Ipilimumab + Nivolumab + PROSTVAC-V/F + Neoantigen DNA vaccine	mHSPC	DLT, safety, immune response	Completed
NCT03493945	1/2	M7824 (anti-PD-L1/TGFβ) + BN-Brachyury (virus vaccine)+ N-803 (IL-15 superagonist complex) + Epacadostat (IDO1 inhibitor)	CRPC	CBR	Active, recruiting
NCT02325557	1/2	Pembrolizumab + ADXS31-142 (bacteria vaccine)	mCRPC after progression on ≤ 3 systemic therapies	Safety	Unknown
NCT02499835	1/2	pTVG-HP + concurrent Pembrolizumab pTVG-HP + sequential Pembrolizumab	mCRPC	ORR, safety, PSA response, PFS	Active, not recruiting
NCT04090528	2	Pembrolizumab + pTVG-HP (DNA vaccine) + pTVG-AR HP (DNA vaccine) Pembrolizumab + pTVG-HP	mCRPC	PFS	Active, recruiting
NCT04382898	1/2	Cemiplimab + BNT112 BNT112 (RNA vaccine)	mCRPC after progression on 2-3 therapies including NHA and/or chemotherapy	DLT, ORR, Safety	Active, recruiting
ICIs + tyrosine kinase inhibitors					
NCT04446117	3	Atezolizumab + Cabozantinib + NHA (Abiraterone or Enzalutamide)	mCRPC after progression on 1 NHA	PFS, OS	Active, recruiting
NCT03170960	1/2	Atezolimab + Carbozantinib	mCRPC after progression on ≤ 1 NHA	DLT, ORR	Active, not recruiting
NCT04477512	1	Nivolumab + Cabozantinib + Abiraterone	mHSPC	DLT	Active, recruiting
NCT04159896	2	Nivolumab + ESK981 (Pan-VEGFR/TIE2 TKI)	mCRPC after progression on 1 NHA and 1 chemotherapy	Safety, PSA response	Unknown
Combination ICIs					
NCT04717154	2	Ipilimumab + Nivolumab	mCRPC with immunogenic signature	DCR	Active, recruiting
NCT03570619	2	Ipilimumab + Nivolumab	mCRPC with CDK12 aberration	ORR, PSA response	Active, not recruiting

NCT03061539	2	Ipilimumab + Nivolumab	mCRPC with immunogenic signature after progression on 1 systemic therapy	ORR, PSA response	Active, not recruiting
NCT02985957	2	Ipilimumab + Nivolumab Ipilimumab Cabazitaxel	mCRPC	ORR, rPFS	Active, not recruiting
NCT03333616	2	Ipilimumab + Nivolumab	Non-adenocarcinoma PC	ORR	Active, recruiting
NCT02788773	2	Durvalumab + Tremelimumab	mCRPC with prior exposure to 1 NHA	ORR	Active, not recruiting
ICIs + androgen receptor antagonist					
NCT03016312	3	Atezolizumab + Enzalutamide Enzalutamide	mCRPC with prior exposure to 1 NHA and 1 chemotherapy	OS	Completed
NCT02787005	2	Pembrolizumab + Enzalutamide	mCRPC progressing on Enzalutamide	ORR	Completed
NCT04191096	3	Pembrolizumab + Enzalutamide Enzalutamide	mHSPC	rPFS, OS	Active, not recruiting
NCT03834493	3	Pembrolizumab + Enzalutamide Enzalutamide	mCRPC, allows for prior Abiraterone exposure	rPFS, OS	Active, not recruiting
NCT02312557	2	Pembrolizumab + Enzalutamide	mCRPC after progression on Enzalutamide	PSA response	Active, not recruiting
NCT03338790	2	Nivolumab + Rucaparib Nivolumab + Docetaxel Nivolumab + Enzalutamide	mCRPC	ORR, PSA response	Active, not recruiting
NCT01688492	1/2	Ipilimumab + Abiraterone	mCRPC	Safety, PFS	Active, not recruiting
ICIs + chemotherapy					
NCT03338790	2	Nivolumab + Docetaxel	mCRPC	ORR, PSA response	Active, not recruiting
NCT04100018	3	Nivolumab + Docetaxel Nivolumab	mCRPC after progression on 1-2 NHAs	rPFS, OS	Active, recruiting
NCT03834506	3	Pembrolizumab + Docetaxel Docetaxel	mCRPC with prior exposure to 1 NHA	rPFS, OS	Active, not recruiting
NCT02861573	1/2	Pembrolizumab + Docetaxel Multiple cohorts	mCRPC	ORR, safety, PSA response	Active, recruiting
NCT03409458	1/2	Avelumab + PT-112 (Platinum + Pyrophosphate ligand)	mCRPC	Safety, PSA response	Active, not recruiting
NCT02601014	2	Nivolumab + Ipilimumab	AR-V7-expressing mCRPC	PSA response	Completed
NCT02788773	2	Durvalumab + Tremelimumab Durvalumab	mCRPC with prior exposure to 1 NHA	ORR	Active, not recruiting
ICIs + radiopharmaceuticals					
NCT02814669	1	Atezolizumab + Radium-223	mCRPC after progression on 1 NHA and 1 chemotherapy	ORR, safety	Completed
NCT04109729	1/2	Nivolumab + Radium-223	mCRPC with symptomatic bone metastases	Safety, ctDNA reduction	Active, recruiting
NCT03658447	1/2	Pembrolizumab + 177Lu-PSMA	mCRPC after progression on 1 NHA	Safety, PSA response	Completed

CBR: Clinical benefit rate; CRPC: castration-resistant prostate cancer; DCR: disease control rate; DLT: dose limiting toxicity; DOR: duration of response; ICIs: immune checkpoint inhibitors; mCRPC: metastatic castration-resistant prostate cancer; mPC: metastatic prostate cancer; NHA: novel hormonal agent; ORR: objective response rate; OS: overall survival; PARP: poly(ADP)-ribose polymerase; PC: prostate cancer; PFS: progression-free survival; PSA: prostate-specific antigen; rPFS: radiologic progression-free survival; RT: radionuclide therapy.

Sipuleucel-T is a therapeutic dendritic cell-based vaccine that has received FDA approval for use in the treatment of patients with asymptomatic or minimally symptomatic mCRPC, based on overall survival (OS)

benefit seen from the phase 3 IMPACT trial[59]. It is prepared from autologous peripheral blood mononuclear cells obtained by leukapheresis, and pulsed *ex vivo* with PAP2024, a unique fusion protein of granulocyte-macrophage colony-stimulating factor (GM-CSF) and prostatic acid phosphatase (PAP). GM-CSF fosters the maturation of dendritic cells and other APCs to present PAP to the patient's T cells, resulting in PAP-specific T-cell proliferation targeting the PAP-expressing prostate cancer cells for killing. Both humoural and cellular responses have been reported, with peripheral immune responses to PAP and measures of APC activation correlating with improvements in OS[60,61]. Despite success with the use of sipuleucel-T, other vaccines studied have not been as successful. G-VAX is another cell-based GM-CSF-secreting vaccine that utilises irradiated TAAs[62]. The TAAs are derived from two cell lines: one hormone-sensitive (LNCaP) and one hormone-resistant (PC3)[63]. Despite initially promising results in asymptomatic mCRPC, the phase 3 VITAL 1 and VITAL 2 trials in asymptomatic mCRPC and symptomatic mCRPC patients, respectively, failed to show the OS benefit of G-VAX plus docetaxel against docetaxel alone. Both studies were terminated early based on futility assessments. A viral-based vaccine, PROSTVAC, utilizes recombinant poxviruses that express PSA with immune-enhancing costimulatory molecules to stimulate immune response[64,65]. In addition to induced modified human PSA, they contain three costimulatory domains for T cells (B7.1, leukocyte function-associated antigen-3, and intercellular adhesion molecule-1), called TRICOM[66]. The phase 3 PROSPECT trial was unable to demonstrate the OS benefit of PROSTVAC against placebo control[67].

Given the increase in T cell infiltration and inflammation within TME with sipuleucel-T[60,61], it is therefore postulated that synergy might be observed with the combined use of vaccines and ICIs. Ipilimumab and PROSTVAC were combined in a phase 1 dose-escalation trial, showing evidence of improved clinical and immunologic outcomes. The median OS was 34.4 months[68], which appears to be numerically larger than PROSTVAC alone in its original study[67]. There was a PSA reduction in 54% of patients and a PSA decline of more than 50% was seen in 25% of patients. ADXS31-142 is a live, attenuated, bioengineered listeria-based vaccine targeting PSA. It is being studied as part of the KEYNOTE-046 trial, with current results showing a median OS of 33.7 months for patients treated with combination vaccine and pembrolizumab[69]. Other ongoing studies of vaccine therapy with ICIs are listed in Table 1.

Tyrosine kinase inhibitors and ICIs

Prostate cancers have dysregulated vasculature that promotes an immunosuppressive TME[7,8]. These include promoting a shift in TAMs toward M2-like immunosuppressive phenotype, reduced maturation of dendritic cells which results in reduced antigen presentation, and increased PD-L1 expression[70]. Vascular endothelial growth factor (VEGF) overexpression has been found to prevent the differentiation of monocytes into dendritic cells[71]. Meanwhile, an improvement in the regulation of local vascular in preclinical models was associated with the assimilation of TAMs with M1-like immune-stimulatory phenotype, increased CD4+ and CD8+ T-cell infiltration into the TME, and reduction of MDSCs[72-75]. These suggest that targeting angiogenesis in tumours can inhibit tumour-induced dysregulation of local vasculature and promote immunogenicity in the TME, forming the basis of combining antiangiogenesis agents with ICIs. Indeed, it has been shown in renal cell carcinoma that anti-VEGF therapy leads to a reduction in immune inhibitory stimuli such as regulatory T-cells and MDSCs[76,77]. Aside from VEGFR targeting, the TAM family of receptor tyrosine kinases comprising TYRO3, AXL and MER has been shown to promote immune suppression as well, making it an attractive target[78,79].

Cabozantinib is a multi-kinase inhibitor targeting MET, VEGFR-1, -2 and -3, AXL, RET, ROS1, TYRO3, MER, KIT, TRKB, FLT-3, and TIE-2[80]. Preclinical data suggests that it has an effect on the TME by reprogramming M2 TAMs to "pro-inflammatory" M1 macrophages, in addition to reducing MDSCs and T regulatory cells[81]. A dose-expansion cohort in the phase 1b COSMIC-021 trial evaluated the combination

of cabozantinib with atezolizumab (anti-PD1) in mCRPC patients who have had disease progression following treatment with novel hormonal agents such as abiraterone or enzalutamide. An ORR of 32% was observed in 132 patients treated with the combination, with a disease control rate (DCR) of more than 80%. This effect was consistent in patients with visceral disease as well[82]. Due to promising results from this study, this combination is now being evaluated in a phase 3 clinical trial for mCRPC patients. Other ongoing studies looking at combination anti-VEGF therapy with ICIs are listed in Table 1.

Combination ICIs

CheckMate-650 is a phase 2 study looking at various dosing combinations of nivolumab with ipilimumab in asymptomatic or minimally symptomatic mCRPC patients who have progressed on novel hormone therapy in two cohorts (chemotherapy-naive and chemotherapy-exposed). In the chemotherapy-naive cohort, nivolumab/ipilimumab achieved an ORR of 25% with a median radiological PFS of 5.5 months and a median OS of 19.0 months. In the chemotherapy-exposed cohort, the ORR was 10%, with a median radiological PFS of 3.8 months and a median OS of 15.2 months[83]. Exploratory analyses revealed that PD-L1 ≥ 1%, the presence of DDR or homologous recombination deficiency (if at least one gene in the relevant gene panel had a deleterious mutation/homozygous deletion) were associated with higher ORR[83]. In this study, 44 patients had quality-controlled whole-exome sequencing data, giving rise to a median TMB of 74.5 mutations/patient. Tumours harbouring TMB exceeding this median were associated with higher ORR, PSA response rate, radiologic PFS, and median OS[83].

Combination nivolumab and ipilimumab has been examined in AR-V7 expressing mCRPC patients as well. Androgen receptor splice variant 7 (AR-V7) expression is found in approximately 20% of mCRPC patients and is associated with alterations in a greater number of DDR genes, which could increase susceptibility to ICIs[84]. The STARVE-PC trial is a phase 2 non-randomised study that evaluated the activity of nivolumab and ipilimumab in 15 AR-V7 expression mCRPC patients, showing an ORR of 25%, PSA response rate of 13% and OS of 8.2 months[85]. Responses were more pronounced in six of the patients who were found to have mutations in DDR genes (three in *BRCA2*, two in *ATM,* and one in *ERCC4*)[86]. Lastly, an ongoing phase 2 randomised study is looking at mCRPC patients following progression on novel hormonal agents, randomising them to receive durvalumab or combination durvalumab plus ipilimumab. The ORR with combination ICI was 16% *vs.* 0% with durvalumab monotherapy in this study[87]. Other ongoing trials evaluating the efficacy of combination ICIs are listed in Table 1.

Androgen receptor antagonists and ICIs

How prostate cancer treatment impacts the immune response is variable. ADT enhances lymphopoiesis, which can mitigate immune tolerance to prostate cancer antigens[88]. On the other hand, androgen receptor antagonists have been shown to inhibit T cell responses[89].

ADT and anti-androgens can both target the AR signalling pathway and have been shown to result in an increase in the number of TILs, and a decrease in the number of regulatory T cells supporting an antitumour response to ADT[90,91]. Animal models confirm that while ADT induces pro-inflammatory conditions initially, the subsequent development of castration resistance and immune tolerance to prostate cancer antigens reduces this[92,93]. Therefore, the combination of AR-signalling blockade with ICIs, especially during its pro-inflammatory state, may be beneficial in the treatment of advanced prostate cancer.

The phase 2 IMbassador250 trial examined 759 advanced CRPC patients who had progressed on abiraterone and docetaxel, randomising them to receive combination enzalutamide and atezolizumab *vs.* enzalutamide alone. The study was closed prematurely due to futility (combination therapy *vs.* enzalutamide monotherapy, 15.2 *vs.* 16.6 months; HR 1.12, 95% CI 0.91-1.37). However, pre-planned

exploratory analyses showed a longer PFS with combination therapy in patients with high PD-L1 IC2/3, CD8 expression[94]. The study also performed an unbiased RNA sequencing-based analysis of immune-related gene expression that had previously correlated with mCRPC responses to immunotherapy[95], and found longer PFS with combination therapy in patients harbouring genes related to pre-existing immunity such as TAP-1, CXCL9, interferon signalling[94]. The multicohort phase 2 KEYNOTE-199 trial examined combination pembrolizumab with enzalutamide in mCRPC patients whose disease were refractory to enzalutamide. In the cohorts with measurable disease and bone-predominant disease (cohorts 4 and 5), the disease control rate was 51% and ORR was 12%. The duration of response was almost 6 months in 60% of responders[96]. This strategy is being evaluated further in an ongoing phase 3 trial [Table 1].

Systemic chemotherapy and ICIs

Chemotherapy may potentiate antitumour immunity by various mechanisms, including the release of TAAs and enhancing antigen presentation, stimulating the activity of cytotoxic T lymphocytes[97,98]. Importantly, chemotherapy may reduce immunosuppressive cell populations such as MDSCs and regulatory T cells, known to maintain prostate cancer immune evasion[99,100]. Preclinical studies have suggested that chemotherapy does improve antitumour immune responses, showing that the addition of taxanes can cause a shift in macrophage populations toward the M1-like (immune-activating) phenotype and reduce regulatory T cell and MDSC populations in mouse models[101,102]. The multicohort phase 2 trial CheckMate 9KD showed that combination nivolumab and docetaxel in 41 chemotherapy-naive mCRPC patients who have progressed on novel hormonal agents achieved an ORR of 36.8%, radiologic PFS of 8.2 months and PSA response of 46.3%[103]. KEYNOTE-365 is an ongoing multicohort phase 1b/2 study examining combination pembrolizumab and docetaxel in mCRPC patients, yielding an ORR of 18%, PSA response of 28%, radiologic PFS of 8.3 months, and OS of 20.4 months[104]. Ongoing phase 3 trials (CheckMate7DX and KEYNOTE-921) evaluating the superiority of combination chemotherapy with immunotherapy over chemotherapy alone will shed light in this area [Table 1].

Radiopharmaceuticals and ICIs

177Lu-PSMA-617 has gained regulatory approval for the treatment of mCRPC patients who have been treated with androgen receptor (AR) pathway inhibition and taxane chemotherapy, based on positive results on a phase 3 trial[105]. In murine models, targeted radionuclide therapy (TRT) may increase PD-L1 expression on T cells and the combination of TRT with ICIs leads to increased infiltration of CD8 T cells[106]. There is, hence, interest in combining radionuclide therapy with ICIs. Despite low clinical response (ORR 6.8%, PSA response 4.5%, radiologic PFS 3 months) seen on a phase 1b trial combining Atezolizumab and Radium-223 in mCRPC[107], the interim results of another phase 1b/2 PRINCE trial are relatively promising. In this study, 37 mCRPC patients who have progressed on a novel hormonal agent and docetaxel were treated with pembrolizumab and 177Lu-PSMA-617, yielding an ORR of 78%, PSA response of 73%, and 24-week radiologic PFS of 65%[108] [Table 1].

FUTURE DIRECTIONS AND CONCLUSIONS

Research is ongoing to identify more immunogenic targets and pair them with the multiple TAAs that prostate cancer expresses. Amongst these, cellular-based therapy is an area that deserves special mention. Adoptive cell therapy involves the engineering of patients' T lymphocytes to target specific viruses or tumours. The use of chimeric antigen receptors (CAR) allows for the creation of artificial T-cell receptors used in adoptive cell therapy[109]. A first-in-human phase 1 study of 13 CRPC patients tested PSMA-targeting CAR T cells armoured with a dominant-negative TGF-β receptor. TGF-β is an inhibitory factor found at high levels within the prostate TME. In this study, 4 patients had a ≥ 30% reduction in PSA and 1 patient had a > 98% reduction in PSA. Five patients experienced grade 2 or higher cytokine-release syndrome (CRS)[110]. Another CAR T therapy using P-PSMA-101, which targets PSMA, was evaluated in 10

heavily-pre-treated CRPC patients, yielding PSA decline in 7 patients, with 4 patients having > 50% reduction in PSA. CRS was seen in 60% of patients[111]. Other CAR T products targeting Epithelial cell adhesion molecule (EpCAM) and Natural Killer Group 2D (NKG2D) have shown activity in prostate cancer patients as well[112,113]. Other potential targets of interest with adoptive cell therapy include PSA, PAP, PSCA, and B7-H3[114], and Table 2 shows a list of ongoing clinical trials.

Bispecific T cell engager (BiTE) antibodies is another technology that has been developed to target TAAs such as PSMA in prostate cancer cells. Structurally, these are bispecific monoclonal antibodies that can crosslink TAAs with the coreceptors on T cells, generating an antitumour immune response. Pasotuxizumab is a bispecific monoclonal antibody that crosslinks CD3 and PSMA, and its efficacy has been studied in 16 mCRPC patients on a phase 1 trial, showing ≥ 50% decline in PSA in 3 patients, of which two were long-term responders treated for 14.0 and 19.4 months, respectively. 81% of the patients had adverse events of grade ≥ 3[115]. The efficacy of AMG 160, a BiTE product that binds CD3 on T cells and PSMA on cancer cells, was evaluated in mCRPC patients on a phase 1 trial. In the preliminary report, 27% of patients had confirmed PSA responses and 84% of patients experienced CRS (10% grade ≥ 3)[116]. The study also had a subset of patients who received AMG 160 with pembrolizumab, and such a combination will likely be examined in future studies as well. Other potential BITE targets including STEAP, CEACAM5, DLL3, HER2 are being studied[117,118], and a list of ongoing trials can be seen in Table 2. Figure 3 shows a schematic diagram of BiTE therapy.

On the horizon, relevant and novel targets to modulate antitumour immunity in prostate cancer may include the targeting of relevant immune-metabolic pathways, such as the adenosine receptor[119-121], or cytokine-directed efforts, such as IL-8 involved in the differentiation of TAM to M2 phenotype (promotes immune resistance and tumour metastasis)[122,123], IL-23 which is a cytokine secreted by MDSCs[124] and TGF-β which promotes tumour growth and immunosuppression in the TME[81]. Targeting cell signalling pathways such as the phosphoinositide 3-kinase/mammalian target of rapamycin (*PI3K/mTOR*) pathway has also been shown to downregulate immunosuppressive cells such as T regulatory cells and may have a role in improving ICI efficacy in prostate cancer[125,126]. For example, in prostate cancer mouse models, intermittent *PI3K* inhibition was able to alleviate *PTEN*-null cancer cell-intrinsic immunosuppressive activity and turn "cold" tumours into T cell-inflamed ones[127]. Novel immune checkpoints may be worth exploiting in prostate cancer. Increased expression of V domain Ig suppressor of T Cell activation (VISTA) was found to promote immune resistance following Ipilimumab treatment, which may serve as a new immunotherapeutic target in advanced prostate cancer[128].

There are presently limited biomarkers that can identify prostate cancer patients who may benefit from ICI therapy. It appears that combination strategies to promote immunogenicity within the "cold" TME of prostate cancer can increase the effect of ICIs. We recognise that the majority of the existing efforts are presently in the preclinical or early phase setting and may not be ready for use in the clinics yet. It would nevertheless be interesting to monitor this space for future developments.

Table 2. Trials looking at novel therapies in advanced prostate cancer

Trial number	Phase	Intervention arm(s)	Population	Outcome	Status
CAR T					
NCT04227275	1	CART-PSMA-TGFβRDN	mCRPC after progression on 2 NHAs	DLT, safety	Active, not recruiting
NCT03089203	1	CART-PSMA-TGFβRDN	mCRPC after progression on ≥ 1 systemic therapy	Safety	Active, recruiting
NCT04053062	1	LIGHT-PSMA-CART	mCRPC after progression on Abiraterone and chemotherapy	Safety	Suspended
NCT04249947	1	P-PSMA-101 CAR-T	mCRPC	ORR, DLT, safety	Active, not recruiting
NCT03873805	1	Anti-PSCA-CAR-4-1BB/TCRzeta-CD19t-expressing T-lymphocytes	PSCA+ mCRPC	DLT, safety	Active, recruiting
NCT02744287	1/2	BPX-601 (PSCA-specific CAR-T cells)	PSCA+ mCRPC	DLT, safety	Active, recruiting
NCT03013712	1/2	EpCAM-specific CAR T Cells	EpCAM+ mCRPC	Safety	Unknown
BiTE					
NCT04104607	1	CC-1 (PSMAxCD3)	mCRPC after progression on ≥ 3 systemic therapies	Safety	Active, recruiting
NCT03792841	1	Acapatamab (PSMAxCD3)	mCRPC after progression on 1 NHA and 1 chemotherapy	DLT, safety	Active, not recruiting
NCT01140373	1/2	HPN424 (PSMAxCD3)	mCRPC after progression on ≥ 2 systemic therapies	ORR, DLT	Active, not recruiting
NCT03972657	1/2	REGN5678 (PSMAxCD28) + Cemiplimab	mCRPC after progression on ≥ 2 systemic therapies	ORR, DLT, safety	Active, recruiting
NCT04221542	1	AMG 509 (STEAP1xCD3)	mCRPC after progression on 1 NHA and 1 chemotherapy	DLT, safety	Active, recruiting
NCT03406858	2	HER2Bi-armed activated T cells (HER2xCD3) + Pembrolizumab	mCRPC	PFS	Active, not recruiting

DLT: dose limiting toxicity; EpCAM: Epithelial cell adhesion molecule; mCRPC: metastatic castration-resistant prostate cancer; NHAs: novel hormonal agents; ORR: objective response rate; PFS: progression-free survival; PSCA: prostate stem cell antigen.

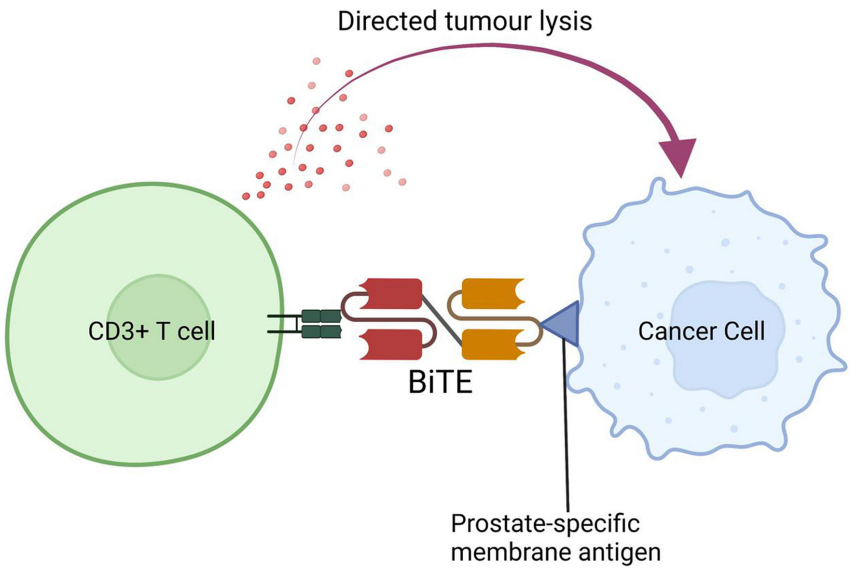

Figure 3. Bispecific T cell engager binding CD3 on T cell with PSMA on prostate cancer cell. BiTE: Bispecific T-cell engager; PSMA: prostate-specific membrane antigen.

DECLARATIONS

Authors' contributions

Conceptualisation: Sooi K, Wong A, Ngoi N
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Visualisation: Sooi K

Availability of data and materials

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All authors declared that there are no conflicts of interest.

Ethical approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

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Cancer Drug Resistance

Review

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Unveiling T cell evasion mechanisms to immune checkpoint inhibitors in acute myeloid leukemia

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Abstract

Acute myeloid leukemia (AML) is a heterogeneous and aggressive hematologic malignancy that is associated with a high relapse rate and poor prognosis. Despite advances in immunotherapies in solid tumors and other hematologic malignancies, AML has been particularly difficult to treat with immunotherapies, as their efficacy is limited by the ability of leukemic cells to evade T cell recognition. In this review, we discuss the common mechanisms of T cell evasion in AML: (1) increased expression of immune checkpoint molecules; (2) downregulation of antigen presentation molecules; (3) induction of T cell exhaustion; and (4) creation of an immunosuppressive environment through the increased frequency of regulatory T cells. We also review the clinical investigation of immune checkpoint inhibitors (ICIs) in AML. We discuss the limitations of ICIs, particularly in the context of T cell evasion mechanisms in AML, and we describe emerging strategies to overcome T cell evasion, including combination therapies. Finally, we provide an outlook on the future directions of immunotherapy research in AML, highlighting the need for a more comprehensive understanding of the complex interplay between AML cells and the immune system.

Keywords: Acute myeloid leukemia, T cells, immune checkpoint, immune evasion

INTRODUCTION

Acute myeloid leukemia (AML) is a devastating blood cancer and is the most common form of acute

leukemia in adults. Long-term outcomes for AML have not significantly improved over the past few decades, with a suboptimal 5-year overall survival rate of 30% for AML patients ages 20 and older and less than 10% for AML patients ages 65 and older[1]. The current standard of care approaches for AML, including induction chemotherapy, combinations of venetoclax with hypomethylating agents, and stem cell transplantation, still yield high relapse rates with significant toxicities. Therefore, new less toxic therapeutic approaches need to be developed to improve survival and prevent relapse in this disease.

Hematopoiesis is the process through which all mature blood cell lineages are generated from hematopoietic stem cells (HSCs), which have the capacity to both self-renew and differentiate. Without proper regulation of their cell-intrinsic and cell-extrinsic cues (primarily signaling pathways, transcription factors, and epigenetic regulators), HSCs and downstream progenitors can acquire unlimited self-renewal potential at the expense of differentiation, as well as increased proliferation and survival, leading to AML development[2-5]. AML blasts develop from aberrant HSCs or progenitors - termed the leukemic stem cell (LSC). LSCs are undifferentiated blood cells that have pathologic self-renewal properties and lead to abnormal blood production. Phenotypically, LSCs share some of the same cell surface markers as HSCs, but unique LSC and pre-LSC gene expression signatures have been identified by high throughput sequencing[6-9]. Like HSCs, LSCs are primarily quiescent and are therefore resistant to chemotherapy and other therapies that target actively cycling cell populations[3,10]. Yet, the standard induction "7 + 3" chemotherapy regimen remains the preferred up-front treatment strategy for AML patients who are fit enough to tolerate intensive induction therapy, which, in addition to sparing LSCs, results in various toxicities, such as pancytopenia and infection[11,12]. This has led to enhanced research efforts to identify novel therapies that target the LSC population while sparing healthy HSCs to improve AML patient outcomes.

However, in addition to the cell-autonomous mechanisms AML cells have employed to persist despite the cytotoxic effects of chemotherapy, AML cells have developed additional ways to persist despite treatment, including resistance mechanisms to targeted therapies and immune evasion. Notably, AML cells employ several mechanisms, such as reliance on immune cells, to establish an immunosuppressive environment to ensure their survival. This is accomplished through the reduction of cytotoxic and effector T and NK cells, increased T cell exhaustion, and recruitment of immunosuppressive populations such as regulatory T cells, myeloid-derived suppressor cells (MDSCs), and M2 macrophages[13]. Importantly, it has been reported that the number of effector and cytotoxic T cells, termed tumor-infiltrating lymphocytes (TILs), present in the bone marrow can be a prognostic marker for overall survival and leukemia-free survival[14]. In addition, increased numbers and function of regulatory T cells in both the peripheral blood and bone marrow of AML patients have been reported, with bone marrow-resident regulatory T cells exhibiting more immunosuppressive effects on CD4+ effector T cell proliferation[15]. A lower frequency of regulatory T cells was found to correlate with complete remission rates in AML patients, while a higher frequency was observed in patients who relapsed[15].

Despite advances in immunotherapies in solid tumors and some lymphoid malignancies, AML has been particularly difficult to treat with immunotherapies, primarily due to poor T cell recruitment to the bone marrow and because LSCs are immune privileged. Because even with the current therapeutic options, AML remains a lethal disease with a suboptimal long-term survival rate, it is imperative to identify and exploit the mechanisms by which AML cells evade immune detection to unleash the potential benefits of immunotherapy in AML treatment. This review summarizes the roles of T cells in the immune response, and highlights the challenges that AML cells pose to the efficacy of ICIs by evading T cell detection.

T CELLS FRONT THE ADAPTIVE IMMUNE RESPONSE

The adaptive immune system plays an essential role in eliminating a variety of threats to our bodies, including cancer and infection. Key players in the adaptive immune response are B lymphocytes (B-cells) and T lymphocytes (T cells). They are distinguishable from cell types that primarily function in the innate immune response because they have antigen-specific receptors - B-cell receptor (BCR) and T cell receptor (TCR), respectively[16]. T cells can differentiate into three different cell types: effector T cells, cytotoxic T cells, and regulatory T cells. Effector T cells, also known as "helper T cells", which express the cell-surface protein CD4, function through cytokine signaling, such as interferon gamma (IFNγ) and tumor necrosis factor alpha (TNFα), which stimulate other immune cells[16]. Cytotoxic T cells, which express the cell-surface protein CD8, program invading cells to undergo apoptosis via the secretion of granzyme B, perforin, and IFNγ[16]. Unlike effector and cytotoxic T cells, regulatory T cells function to suppress immune cells to mitigate any possible damage from a prolonged immune response, and to prevent auto-immunity[16]. They can be identified through flow cytometry by the expression of CD4, CD25, and FoxP3[16].

In order to activate a T cell-mediated immune response, two different signals are required. The first signal occurs when the disease-causing cell presents an antigen, or host-derived protein molecule, to a T cell [Figure 1]. Specifically, short peptide fragments of an antigen are presented on the surface of host cells, termed antigen-presenting cells (APCs), by major histocompatibility complex (MHC) molecules. There are two classes of MHC molecules, MHC class I and MHC class II. Notably, CD8+ T cells selectively recognize MHC class I molecules, while CD4+ T cells selectively recognize MHC class II molecules. MHC class II molecules are often expressed on dendritic cells and macrophages, which engulf the antigen and process it for presentation. MHC class II molecules can also be present on the surface of foreign APCs. The Class II transactivator (CIITA) is a master regulator of MHC gene expression[17]. CIITA responds to IFNγ activation, where it then acts as a transcriptional activator to turn on MHC gene expression[17].

The second signal required for T cell activation is termed the co-stimulatory signal, and co-stimulation is thought to occur through the interaction between the CD28 molecule on T-lymphocytes and either CD80 (B7.1) or CD86 (B7.2) molecules on the APC[18] [Figure 1]. The discovery of CD28 and its essential role in T cell activation has led to further discovery of other cell-surface molecules that regulate T cell activity. Interestingly, the discovery of cytotoxic T lymphocyte antigen 4 (CTLA4) on T cells identified another binding partner of B7-1. However, CTLA4 expression is induced following T cell activation, where it can out-compete CD28 binding to B7.1 to dampen the T cell response[18].

This has led to the discovery and categorization of other cell-surface molecules that positively (referred to as co-stimulatory receptors) and negatively (co-inhibitory receptors) modulate T cell activity. Other co-inhibitory receptors on T cells include programmed cell death protein 1 (PD1), which binds to its ligands programmed death-ligand 1 (PD-L1, also known as B7-H1) or programmed death-ligand 2 (PD-L2, also known as B7-H2) on APCs; T cell immunoglobulin and mucin domain-containing protein 3 (TIM3), and lymphocyte activation gene-3 (LAG3)[19,20]. TIM3 binds to various ligands (including Galectin-9, Ceacam-1, and HMGB-1), while LAG3 binds to MHC class II molecules with higher affinity than the CD4+ TCR[19,20]. Other co-inhibitory ligands on APCs include B7-H3, B7-H4, and B7-H5[21].

MECHANISMS OF IMMUNE EVASION IN AML

There are currently several different immunotherapy strategies being investigated in hematologic malignancies, including in AML[11,21]. Immune checkpoint inhibitors, such as antibodies targeting CTLA4 and PD1, have been approved for the treatment of some types of lymphoma and some solid tumors, including melanoma, lung cancer, kidney cancer, head and neck cancer, bladder cancer, and colorectal

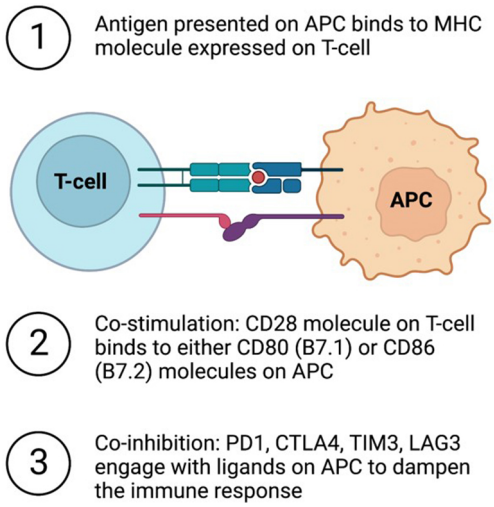

Figure 1. T cell-mediated immune response. Overview of the steps required for full T cell activation. Figure created with Biorender.com. APC: Antigen-presenting cell; CTLA4: cytotoxic T lymphocyte antigen 4; LAG3: lymphocyte activation gene-3; MHC: major histocompatibility complex; PD1: programmed cell death protein 1; TIM3: T cell immunoglobulin and mucin domain-containing protein 3.

cancer[22]. However, in AML, the use of immune checkpoint inhibitors has been more challenging, and there are no FDA approvals of this class of agents in AML to date. This is in part due to the various cell-autonomous and cell non-autonomous mechanisms that leukemic cells employ to reprogram themselves and the bone marrow microenvironment to render them immune privileged [Figure 2]. Additionally, ICIs are often most effective in cancers with a high mutation burden (i.e., melanoma, lung cancer), which is often not as high in AML[23]. For example, many AML patients have a defined blast population with 1-2 driver mutations and/or cytogenetic alterations, with sub-clones that may not arise until disease progression or relapse[24].

Increased immune checkpoint expression

Immune checkpoints are known to be a key mechanism that mediates T cell immunosuppression in AML. Interesting work using PD1 knockout mice delineated the importance of this axis in regulatory T cells and CD8+ cytotoxic T cells. Specifically, AML development was impeded when AML cells were injected into PD1 knockout mice[25]. This was dependent on the ability of regulatory T cells to suppress CD8+ T cells via enhanced PD1 expression on T cells and PD-L1 expression on APCs[25]. Interestingly, treating mice that developed AML with IL-2 linked to diphtheria toxin (IL-2DT), followed by anti-PDL1 monoclonal antibody treatment, markedly reduced the AML tumor burden[25]. Therefore, this work suggests that strategies to deplete regulatory T cells and inhibit the PD1/PD-L1 interaction could be effective in overcoming the AML-privileged microenvironment.

T cell exhaustion is also a mechanism for immune evasion and is often phenotypically characterized by the expression of the immune checkpoint TIM3. In one study evaluating the role of exhausted T cells in AML relapse following transplantation, the frequency of PD1-high TIM3-positive T cells was significantly correlated with AML relapse[26]. These T cells were confirmed to be exhausted, as they exhibited reduced production of IL-2, TNFα, and IFNγ[26]. The impact of this study was clinically significant, as the expression of exhaustion markers on T cells could be detected before the diagnosis of relapse[26]. These results were echoed in a subsequent study that characterized the exhausted T cell population following AML relapse post-transplantation, which, despite displaying specific leukemic blast recognition (determined by CDR3

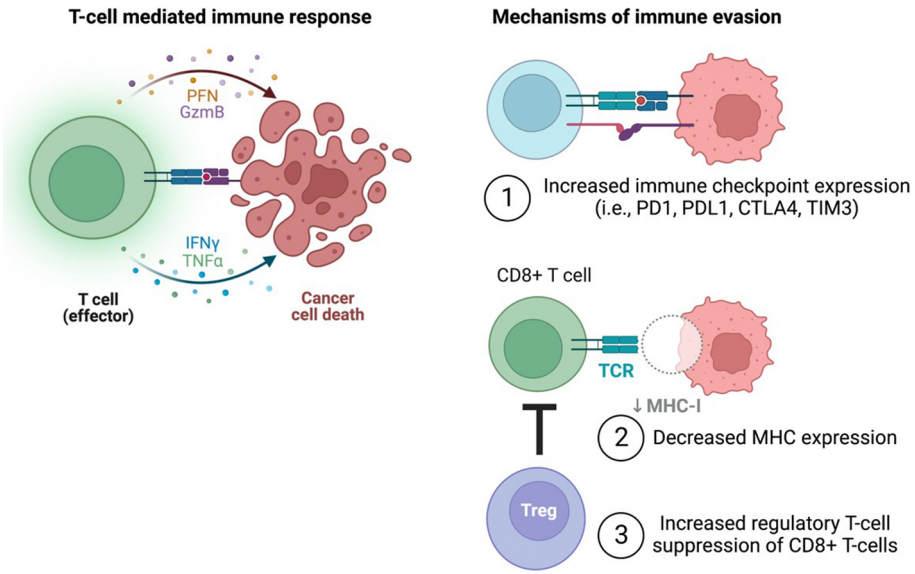

Figure 2. Mechanisms of T cell evasion in AML. T cells engage with and kill cancer cells through the presentation of MHC molecules and subsequent T cell co-stimulation (left). Mechanisms to evade T cell detection employed by AML cells include (1) increased expression of co-inhibitory immune checkpoints; (2) decreased MHC expression; and (3) suppression of cytotoxic CD8+ T cell function through increased regulatory T cells (right). Figure created with Biorender.com. AML: Acute myeloid leukemia; CTLA4: cytotoxic T lymphocyte antigen 4; IFNγ: interferon gamma; MHC: major histocompatibility complex; PD1: programmed cell death protein 1; PFN: perforin; TCR: T cell receptor; TIM3: T cell immunoglobulin and mucin domain-containing protein 3; TNFα: tumor necrosis factor alpha.

sequencing of TCR-α and TCR-β chains), had impaired effector T cell function[27]. As the prognosis for patients who relapse after transplantation is poor, early detection of T cell exhaustion markers could be a useful predictive tool[26,27].

Modulation of checkpoint expression on AML cells themselves is another key driver of immune evasion. For example, increased PD-L1, PD-L2, and CTLA4 expression on AML cells has been shown to correlate with poor overall survival[28,29]. PD-L1 expression was found to be elevated in AML patient blasts, both at diagnosis and at relapse[30]. Furthermore, CTLA4 was previously discovered to not be restricted to the lymphoid lineage, as AML cells from both diagnostic and relapsed patients, but not healthy CD34+ cells, were found to express CTLA4[31,32]. Therefore, the upregulation of inhibitory immune checkpoints on AML cells is another potential mechanism for immune evasion in AML.

Downregulation of MHC expression

Dampening of MHC expression on AML cells is also an important mechanism of immune evasion. Specifically, RNA sequencing analysis of paired AML samples collected at diagnosis and at relapse post-transplantation identified altered expression of immune-related genes, including decreased expression of CIITA, the master regulator of MHC-II expression, and of MHC-II molecules at relapse[33]. *Ex vivo* treatment of AML blasts isolated from relapse patients with IFNγ was able to restore MHC-II expression[33]. The clinical significance of this is revealed by the differences in CD4+ effector T cell activation, as measured by IFNγ production, following co-culture of either diagnostic or post-transplantation relapsed AML samples with CD4+ T cells, as CD4+ T cell activity was diminished in post-transplantation relapse co-culture assays[33].

Accordingly, in a recent transcriptome analysis of AML cells from patients who relapsed following transplantation, a transcription factor complex consisting of IRF8, MYB, MEF2C, and MEIS1 was found to regulate MHC expression in AML, and combinatorial changes in their expression are essential for reduced MHC expression at relapse[34]. Interestingly, the authors found a small cell population with silenced MHC expression at leukemia diagnosis, and concluded that, similar to resistant LSC populations, this population may be selected after transplantation and can contribute to relapse[34]. Overall, these mechanisms are plausible explanations for why the treatment of patients who relapse post-transplantation is particularly challenging. Identifying ways to overcome decreased MHC expression following transplantation is underway. For example, a recent study using AML xenograft mouse models reported that MDM2 inhibition can increase MHC-II production, and CD8+ T cells isolated from MDM2 inhibitor-treated primary AML mice can eradicate disease in secondary recipients[35].

The role of regulatory T cells in the immunosuppressive microenvironment

The increased number and activity of regulatory T cells (Tregs) in the leukemic bone marrow renders the bone marrow an immunosuppressive microenvironment due to their effects on effector and cytotoxic T cell populations. Recent insights have identified mechanisms for increased Treg function in the AML microenvironment, such as via increased expression and production of IFNγ by AML cells, leading to upregulation of genes that promote differentiation into Tregs[36]. Recognizing the correlation between increased Treg numbers and poor AML outcomes, one group investigated the effects of Treg ablation on leukemogenesis[37]. Using Foxp3-DTR to ablate Tregs in mice, they observed prolonged survival in MLL-AF9-induced AML mouse models and increased CD8+ T cell activity[37]. As Treg ablation is likely not easily transferrable to the clinic, they also identified additional ways to impede Treg accumulation in the leukemic microenvironment in mice, including CCL3-CCR1/CCR5 and CXCL12-CXCR4 blockade[37]. Importantly, as increased regulatory T cell populations are also a predictor of AML relapse following transplantation[27], it is critical to exploit mechanisms that decrease Treg numbers and function.

NK cell-mediated immune evasion mechanisms

AML cells can also evade detection by NK cells, which are canonically activated by the recognition of stress-induced ligands on foreign cells[38]. Similar to their evasion of T cells, AML cells can also evade NK-cell recognition and elimination through multiple mechanisms, including (1) the reduced expression of stress-induced ligands on AML cells; (2) increased expression of inhibitory receptors on NK cells to suppress NK cell function; (3) the induction of the immunosuppressive environment to limit NK cell numbers and function; and (4) activation of anti-apoptotic pathways to resist NK-cell induced cell death[39,40]. These NK-cell evasion mechanisms, as well as strategies to target them, have been extensively reviewed elsewhere[39,41-44]. For example, it was shown that epigenetic mechanisms mediate the silencing of NKG2D ligands in AML, and that treatment with hypomethylating agents can increase their expression and subsequent NK-cell recognition[45]. Furthermore, pivotal work demonstrated that LSCs are immune privileged through their lack of expression of NKG2D ligand, which is essential for NK-cell detection and subsequent clearance[46]. As NK2GD remains a hot target for immunotherapy in AML[47-49], it is important to appreciate that other mechanisms may be required to eliminate the LSC population.

Another mechanism that can mediate NK-cell evasion is CD48 silencing[50-52]. It was demonstrated that high CD48 expression on AML cells is correlated with a favorable prognosis. However, in a subset of AML patients, CD48 expression can be suppressed through enhanced methylation[52]. Therefore, treatment with hypomethylating agents may be able to increase CD48 expression to increase NK-cell killing[53]. Overall, understanding NK-cell evasion mechanisms is critical to overcoming immunotherapy resistance and identifying targets for immunotherapy.

CLINICAL INVESTIGATION OF IMMUNE CHECKPOINT INHIBITORS IN AML

Several strategies that incorporate checkpoint inhibitors have been tested in AML in clinical trials, and several more clinical trials are underway [Table 1]. In a phase 1/1b clinical trial of ipilimumab (anti-CTLA4) in patients with hematologic malignancies that relapsed after allogeneic stem cell transplantation, analysis of the AML subset (12/28 patients) showed that 5/12 patients achieved complete remission following treatment, and this was accompanied by a reduction in the frequency of circulating Tregs compared to non-responders[54].

Furthermore, in a Phase II study investigating the combination of high-dose cytarabine and pembrolizumab (anti-PD1) in relapsed/refractory AML patients, 14 out of 37 patients achieved complete remission (CR). Interestingly, of the patients that achieved a CR, TCR signaling identified a trend towards increased TCR diversity in these patients, as well as decreased regulatory T cell and increased CD8+ T cell frequencies[55]. Of note, RNA-seq analysis of AML blasts from these patients revealed that increased MHC expression was significantly upregulated at baseline in patients who achieved CR compared to non-responders[55].

Interestingly, recent data suggests that PD1 signaling may be implicated in the poor response to hypomethylating agents (HMAs), including azacitidine and decitabine, as patients who are resistant to HMAs show higher expression of PD-L1, PD-L2, and CTLA4[56-58]. On the other hand, preclinical findings from single-agent immune checkpoint inhibitor trials in AML have demonstrated limited efficacy. This has prompted the investigation of checkpoint inhibitors in combination with HMAs[57]. In a phase 1b trial investigating the combination of ipilimumab with decitabine in relapsed/refractory AML, patients who were transplant naïve (N = 23) observed a higher response rate than those who relapsed following stem cell transplantation (N = 20) (CR + CRi + mCR 52% *vs.* 20%, P = 0.034; median overall survival 16.2 months *vs.* 8.6 months)[59]. Not surprisingly, when performing integrative transcriptome-based analysis of bone marrow infiltrating cells from participating patients, a high baseline ratio of T cells to AML cells was associated with higher response rates[60]. The authors speculated that the inadequate clearance of the immature LSC population triggered relapse in patients following stem cell transplantation, but also noted that ipilimumab exposure resulted in increased memory T cell bone marrow infiltration and high expression of CTLA4 and FOXP3, suggesting that the efficacy of ipilimumab and decitabine may be impacted by these immune evasion mechanisms employed by LSCs[60]. The results of the ipilimumab and decitabine combination studies also highlight the limitations of ICIs in AML. A comparison of the memory and exhaustion gene scores associated with CD8+ T cells from AML bone marrow with those from CD8+ TILs isolated from solid tumors, in which ipilimumab demonstrates high clinical activity, revealed higher exhaustion profiles and checkpoint expression in solid tumor-derived T cells[60].

In two ongoing trials testing the combination of pembrolizumab and decitabine in relapsed/refractory AML, interim results showed a tolerable safety profile with promising efficacy data[56,61]. Furthermore, through the generation of RNA expression datasets from patients who were treated with conventional cytotoxic chemotherapy or with pembrolizumab and azacitidine in relapsed/refractory AML, Rutella *et al.* revealed a newly defined CD8+ T cell senescent gene population with a distinct gene expression signature[62]. These cells were impaired in their ability to kill AML blasts isolated from the same patient sample, and their frequency negatively correlated with overall survival[62]. However, there is still promise for the combination of PD1 blockade and HMA, as results from the Phase II trial investigating nivolumab and azacitidine in relapsed/refractory AML yielded a 33% overall response rate, with a higher response rate in HMA naïve *vs.* HMA pre-treated patients (58% *vs.* 22%)[63]. Based on these clinical trials, the possible predictors of response to immune checkpoint inhibitors are summarized in Table 2. Overall, given these data, the field is anxiously awaiting the results of additional clinical trials currently that are investigating

Table 1. Overview of ongoing clinical trials of immune checkpoint inhibitors in AML

Target	Agent	Regimen	Population	Phase	NCT identifier	Primary endpoints
PD-1	Pembrolizumab	IC ± Pembrolizumab	ND AML	2	NCT04214249 (BLAST MRD AML-1)	MRD-CR
		VEN + AZA ± Pembrolizumab	ND AML	2	NCT04284787 (BLAST MRD AML-2)	MRD-CR
		HiDAC followed by Pembrolizumab	R/R AML	2	NCT02768792	CR
		Decitabine + Pembrolizumab ± VEN	ND or R/R AML	1	NCT03969446	Incidence of AE, MTD, CR
	Nivolumab	Nivolumab	AML patients in remission after IC	2	NCT02275533 (REMAIN TRIAL)	PFS
		Nivolumab	AML patients in remission after IC	2	NCT02532231	Recurrence-free survival
		AZA + Nivolumab ± Ipilimumab	ND or R/R AML	2	NCT02397720	MTD, ORR
		Decitabine + VEN + Pembrolizumab	ND *TP53*-mutant AML	1	NCT04277442	Incidence of AE, CR
		Nivolumab ± Ipilimumab	AML patients post-HSCT	1	NCT03600155	Optimal dose
CTLA-4	Ipilimumab	Decitabine + Ipilimumab	R/R AML	1	NCT02890329	MTD
		Ipilimumab + CD25hi Treg-depleted DLI	R/R AML post-HSCT	1	NCT03912064	MTD

AE: Adverse event; AML: acute myeloid leukemia; AZA: Azacitidine; CR: complete remission; DLI: donor lymphocyte infusion; HSCT: hematopoietic stem cell transplant; HiDAC: high dose cytarabine; IC: intensive chemotherapy; MRD-CR: minimal residual disease negative complete remission; MTD: maximum-tolerated dose; ND: newly diagnosed; ORR: overall response rate; PFS: progression-free survival; R/R: relapsed/refractory; VEN: Venetoclax. Source: clinicaltrials.gov.

immune checkpoint inhibitors in AML.

Further investigation into the molecular mechanisms that both AML cells and T cells employ to evade immune detection may help to identify novel combination strategies for ICIs in AML. For example, altered signaling and expression of cellular proteins due to genetic alterations are hallmarks of AML cells. With both approved and investigational therapies available to target oncogenes (e.g., FLT3, IDH1/2, NPM1c/Menin inhibitors) responsible for regulating the expression and/or post-translational modifications (e.g., methylation, acetylation, glycosylation, ubiquitination) of proteins in AML cells, it is critical to determine if targetable driver mutations are important for the increased expression of immune checkpoints in AML cells.

Alternatively, further investigation into the mechanisms that T cells employ to increase checkpoint expression or to increase Treg function is warranted to improve ICI outcomes in AML. For example, a recent study analyzing the transcriptome of CD8 T cells from the bone marrow of AML patients demonstrated the downregulation of genes responsible for T cell activation, differentiation, and function (e.g., NF-KB, FOXO, cytokine/chemokine signaling)[64]. With several of these genes being involved in epigenetic regulation, the authors postulate that epigenetic changes to T cells may impair TCR activation and overall T cell function[64]. However, additional studies are necessary.

Lastly, additional studies are underway to identify mechanisms that increase the frequency of Tregs, with some insights regarding tumor necrosis factor receptor-2 (TNFR2) and the TNFα pathway playing an important role in increasing the frequency of Tregs in AML patient samples[65], in addition to increased expression of IFNγ via *IDO1* overexpression in mesenchymal stem cells[36]. Importantly, the mechanisms

Table 2. Possible predictors of response to immune checkpoint inhibitors in AML or MDS

Immune checkpoint inhibitor	Clinical setting	Possible predictors of response	Response assessment	Ref
Ipilimumab	Post-HSCT	-Baseline donor T cell chimerism of > 99% -Lower frequency of CD4+ Tregs -Increase in plasma CXCL2, CXLC5, CXCL6, IL1R, ANGPT-1 and -2, VEGF	CR or stable disease	[54]
Pembrolizumab	R/R AML, post-HiDAC	-Trend towards higher TCR diversity at baseline -Higher frequency of senescent T cells in BM -Higher frequency of terminally differentiated effector T cells in PB -Increased frequency of CD8+ T cells expressing CD28, PD-1, and TIGIT in BM -Presence of pre-treatment CD8+ T cells co-expressing TCF-1 and PD-1 -Transcriptional upregulation of PI3K/AKT/MTOR signaling pathway in BM blasts	CR	[55]
Ipilimumab	In combination with decitabine in AML or MDS before and after HSCT	-No clear predictors of response	Leukemic cell burden, frequency of infiltrating lymphocytes	[59]
Ipilimumab	In combination with decitabine in AML or MDS before and after HSCT	-Lower VAF of recurrent AML/MDS-associated mutations -Higher T cell to AML ratio -Increased T cell to myeloid ratio -Donor-derived myeloid cells present at higher % in responders -Higher circulating expression of CCL17, CXCL1, CXCL5, EGF, LAMP3, and PDGF subunit B	CR/CRi	[60]
Pembrolizumab	In combination with decitabine in R/R AML	-Trend towards increased CD3+ infiltrates in BM during treatment -No association of TCRb sample clonality with response	CR	[61]
Pembrolizumab	In combination with azacitidine in newly diagnosed AML *vs.* cytotoxic chemotherapy	-Increased proportion of CD3+CD8+CD57+KLRG1+ senescent T cells in baseline BM associated with worse OS -Increased proportion of senescent T cells in BM post-treatment associated with worse OS -High IED signature score associated with worse OS	OS	[62]
Nivolumab	In combination with azacytidine in R/R AML	-Trend towards association with improved response: no prior HMA, presence of ASXL1 mutation -Higher frequency of pre-treatment BM % CD3+ T cells in responders -Trend towards higher frequency of CD4+ T effector cells and CD8+ T cells in pre-treatment BM in responders	ORR	[63]

AML: Acute myeloid leukemia; BM: bone marrow; CR: complete remission; CRi: incomplete remission; HiDAC: high-dose cytarabine; HMA: hypomethylating agent; HSCT: hematopoietic stem cell transplantation; IED: immune effector dysfunction; MDS: myelodysplastic syndrome; ORR: overall response rate; OS: overall survival; PB: peripheral blood; R/R AML: relapsed or refractory acute myeloid leukemia; TCR: T cell receptor; Tregs: regulatory T cells; VAF: variant allele frequency.

employed by AML cells and T cells may be interrelated, as suggested by recent evidence collected in AML cell lines that induced expression of PD-L1 on AML cells could result in the conversion and subsequent expansion of CD4+CD25+FoxP3+ Tregs from CD4+ T cells[66].

CONCLUSION

In summary, through antigen recognition and co-stimulation, T cells front the adaptive immune response, causing AML cells to employ both cell-autonomous and cell non-autonomous mechanisms to create an immunosuppressive microenvironment and evade detection and killing by T cells. These mechanisms include (1) reduced expression of antigens and MHC molecules on the cell surface of AML cells; (2)

immune checkpoint activation to suppress T cell responses, both on T cells and on AML cells themselves; (3) induction of T cell exhaustion; and (4) the induction of an immunosuppressive environment by increasing the numbers of regulatory T cells and other immunosuppressive populations in the bone marrow to inhibit effector and cytotoxic T cell activity. All of these mechanisms ultimately promote AML cell survival. This review complements several other recent review articles in this field, which illustrate the importance of understanding the mechanisms of immune evasion in AML to overcome immunotherapy resistance and improve AML outcomes[13,53,67-69].

In our review of the current ICI landscape for hematologic malignancies, evident frustrations arise when comparing the success of checkpoint inhibitors in solid tumors to the more limited progress made with these agents in AML. The mechanisms highlighted above undoubtedly contribute to the slow adoption of ICIs in AML. With many clinical trials underway in this space, continued research efforts identifying ways to overcome immunotherapy resistance, such as combining ICIs with targeted therapies against components of signaling pathways notoriously activated in AML, as seen in solid tumors[70], are warranted. Furthermore, while not a major focus of this review, it remains a challenge to identify tumor-specific targets for personalized immunotherapies for AML, such as CAR T cells and bispecific antibodies[71-73].

While this review provides some insights into the roles of immune evasion mechanisms in relapse following stem cell transplantation, as well as the clinical trials underway utilizing ICIs for this patient population, the poor prognosis rates for AML patients who relapse after transplantation highlight the need for a review focused on this specifically. Some groups have taken this initiative already, including a summary of the current understanding of the downregulation of HLA molecules and inhibitory checkpoints between T cells and AML cells[74]. Additionally, recent insights into novel mechanisms by which an altered immune landscape following transplantation – characterized by increased expression of TIGIT and CD161 within the CD4+ T cell population post-transplantation – has begun to identify predictors of relapse[75]. A more recent review focuses on epigenetic mechanisms that underlie T cell evasion in the relapse post-transplant setting, and is also a good source for this topic[76].

Lastly, this review does not cover the advances and limitations of emerging immunotherapy treatment modalities in AML- notably chimeric antigen receptor (CAR) T- and NK-cell therapies, bispecific antibodies, dual affinity re-targeting (DART) molecules, monoclonal antibodies, and antibody-drug conjugates. While these agents are approved in other cancers [e.g., acute lymphocytic leukemia (ALL), non-Hodgkin lymphoma (NHL) subtypes, and multiple myeloma (MM)], their adoption in AML has been slow, due to the difficulty of finding AML-specific antigens that are not also expressed on HSCs or myeloid progenitors. Furthermore, mechanisms of antigen escape, the AML immunosuppressive environment, and the impaired quality of autologous cells are also potential problems with these approaches, as reviewed elsewhere[72]. Nonetheless, current clinical trials underway in relapsed/refractory AML include CD33, CD38, CD123, and CD19 CAR-T cell therapies, allogenic CAR NK-cells, and CD33xCD3 and CD123xCD3 bispecific antibodies[11,41].

Overall, as we continue to uncover the mechanisms underlying immune evasion in AML, exploiting these mechanisms will be of high priority to unleash the potential of immunotherapy in this disease. This is exemplified by the pivotal work done already, identifying a niche for immune checkpoint inhibitors after observing increased checkpoint expression in AML cells following HMA treatment[58]. Additionally, it will be important to identify strategies to suppress regulatory T cell activity in AML to allow for the unleashing of effector and cytotoxic and T cell activity. Thinking ahead, continued efforts to identify patient populations at higher risk for immune evasion during available treatments or following stem cell

transplantation, such as characterizing TIL populations prior to and during treatment, or examining T cell and NK cell numbers and function in specific molecular or cytogenetic subgroups of AML will pave the way for more personalized AML treatment plans.

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Authors' contributions

Wrote, reviewed, and edited the manuscript: Gurska L, Gritsman K.

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Cancer Drug Resistance

Review

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Drug resistance in glioblastoma: from chemo- to immunotherapy

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Abstract

As the most common and aggressive type of primary brain tumor in adults, glioblastoma is estimated to end over 10,000 lives each year in the United States alone. Stand treatment for glioblastoma, including surgery followed by radiotherapy and chemotherapy (i.e., Temozolomide), has been largely unchanged since early 2000. Cancer immunotherapy has significantly shifted the paradigm of cancer management in the past decade with various degrees of success in treating many hematopoietic cancers and some solid tumors, such as melanoma and non-small cell lung cancer (NSCLC). However, little progress has been made in the field of neuro-oncology, especially in the application of immunotherapy to glioblastoma treatment. In this review, we attempted to summarize the common drug resistance mechanisms in glioblastoma from Temozolomide to immunotherapy. Our intent is not to repeat the well-known difficulty in the area of neuro-oncology, such as the blood-brain barrier, but to provide some fresh insights into the molecular mechanisms responsible for resistance by summarizing some of the most recent literature. Through this review, we also hope to share some new ideas for improving the immunotherapy outcome of glioblastoma treatment.

Keywords: Glioblastoma, immunotherapy, drug resistance, tumor microenvironment, immunosuppression

INTRODUCTION

Brain tumors affect more than ~17,000 people in the United States each year, where gliomas are considered

the most common type of primary brain tumor[1]. Glioblastoma is a grade IV astrocytoma that was initially categorized into four molecular subtypes, termed neural, proneural, classical, and mesenchymal subtype[2]. Transcriptional profiling and genetic modeling in mice showed that glioblastoma originated from neural stem cells (NSC), NSC-derived astrocytes, and oligodendrocyte precursor cells (OPCs)[3-5]. Besides the four molecular subtypes based on their transcription profiling, glioblastoma tumors can also be classified by the status of the isocitrate dehydrogenase gene (IDH) as IDH wild-type and IDH-mutant tumors. Similarly, epigenetics factors, such as CpG island methylation phenotype of O6-methylguanine-DNA methyltransferase (MGMT) promoter, are also commonly used for glioblastoma tumor stratification[6,7].

Since the approval of Temozolomide (TMZ) for newly diagnosed glioblastoma treatment by the FDA in early 2000, surgery followed by radiotherapy and TMZ treatment has remained the first-line glioblastoma treatment[8]. However, none of these therapies eliminate cancer cells entirely because of challenges marred by high infiltration rate, tumor heterogeneity, blood-brain barrier (BBB), and immunosuppressive environment factors[9,10]. The highly infiltrative nature of glioblastoma does not allow the removal of cancerous cells using resection; self-renewing cells followed by resection become more prone to radioresistance and chemoresistance. Similarly, cellular heterogeneity and BBB prevent targeted drug delivery in glioblastoma[11,12].

COMMON DRUG RESISTANCE MECHANISMS IN BRAIN CANCERS

Blood-brain barrier

Although the BBB in glioblastoma is compromised to some extent, tumor BBB still presents a great challenge for therapeutics to reach glioblastoma cells. As the intrinsic barrier for brain cancer, BBB is a microvasculature structure surrounding the central nervous system (CNS), tightly regulating the movement of molecules and cells between the CNS and blood. Normally, BBB maintains the homeostasis of CNS and prevents infiltration of toxins, pathogens, inflammation, and harmful metabolites[13-15]. Disruption of the neurovascular unit (NVU) is associated with blood-brain dysfunction in neurodegenerative disease and brain tumors[16]. The NVU consists of vascular cells (endothelial, pericytes, and vascular smooth muscle cells), glia (astrocytes, microglia, and oligodendroglia), and neurons, and it plays an important role in maintaining BBB functional integrity and regulating the volume of cerebral blood flow[17,18]. The endothelial cells in neurovascular parenchyma form capillary beds connected through tight junctions (TJs), surrounded by a specialized basal lamina shared with pericytes and astrocytic end feet. They are sparsely interconnected by neuronal endings and microglia[19,20]. Astrocytes and pericytes, an essential constituent of NVU, release Sonic Hedgehog and vitronectin and angiopoietin I, respectively, acting on endothelial cells for their survival and maintaining BBB.

Overexpression of efflux pumps

Efflux transporters on the BBB membrane also contribute to cerebrospinal fluid homeostasis by protecting it from potentially harmful endogenous and exogenous substances[21,22]. These transporters also pose challenges by blocking therapeutic compounds from entering the brain parenchyma. Efflux transporters on compartments of the BBB belong to either ATP-binding cassette (ABC) or the solute carrier (SLC) superfamilies[23,24]. Organic anion-transporting polypeptides (OATP) are a superfamily of solute carrier organic anionic (SLCO) transmembrane transporters that are known for cancer drug resistance[25,26]. These peptide transporters regulate a variety of xenobiotic and endogenous substrates, including endogenous hormones, their conjugates, and anticancer drugs[27]. OATP1A2 is a sodium-independent uptake transporter family member and is highly expressed on the luminal membrane of BBB in tumors and adjacent healthy tissues[28]. A study by Cooper *et al.* in glioblastoma patients showed significant over-expression of all the OATP isoforms (OATP1A2, 2B1, 1C1, and 4A1) in tumor tissues compared to non-neoplastic brain[29].

Enhanced DNA damage repair pathways (MGMT) and abnormal activation of survival signaling pathways

As part of the glioblastoma standard treatment regimen, TMZ is a potent DNA alkylating agent that leads to DNA damage in cancer cells and cell death[30]. However, TMZ treatment often results in drug resistance in ~50% of glioblastoma patients due to overexpression of MGMT, which reverses the methylation of the O6 position of guanine. In addition to upregulated MGMT expression, glioblastoma often exhibits enhanced DNA damage repair capacity through several related mechanisms. For instance, poly(ADP-ribose) polymerase (PARP) was shown to interact with MGMT and enhance MGMT function in the removal of O6-methylation of DNA[31]. Interestingly, even in MGMT-deficient glioblastoma, TMZ resistance may still arise due to the loss of mismatch repair (MMR) pathway in tumor cells. Recent work by Lin *et al.* developed a new class of compound (KL-50) to achieve MMR-independent glioblastoma cell killing. It demonstrated a promising strategy to exploit cancer-specific deficiencies in DNA repair pathways[32]. Glioblastoma tumors also have elevated levels of receptor tyrosine kinases, such as EGFR gene amplification or mutation (EGFRvIII), PDGFR and FGFR, and aberrant activation of PI3K/ATK signaling and other growth factors (e.g., IGF-1, CTGF, and TGFβ)[33-39], with a potential contribution to the drug resistance phenotype.

Role of glioma stem cells

Glioma stem cells (GSCs) represent a subpopulation of relatively undifferentiated cells capable of self-renewal while also generating clonal populations of differentiated tumor cells in glioblastoma. These cells are increasingly recognized as a driving force supporting glioma genesis, therapy resistance, and recurrence[40]. GSCs have high regenerative capacity and can differentiate into cells expressing several lineage markers such as CD133, SOX2, CD15, CD44, integrin α6, and CD36[41]. Along with heterogeneity, various factors contribute to the chemoresistance of GSCs. Intrinsic factors include upregulated MGMT, higher anabolic capacity, and autophagy-mediated clearance of ROS induced by chemotherapy. Extrinsic factor is mainly hypoxic tumor microenvironment (TME). Hypoxia promotes the expression of GSC markers and a cancer stem-like phenotype[42]. Hypoxia-response genes, such as hypoxia-inducible factor HIF-2α and VEGF, are highly expressed in GSCs. Intriguingly, two reports have demonstrated that hypoxia-associated transcriptional signatures can be used as prognostic markers for glioblastoma patients[43,44].

Epigenetic modulations

Epigenetic dysregulation has been increasingly recognized as one of the significant drivers of oncogenesis, and several subtypes of glioblastoma are associated with epigenetic alterations[45,46]. These epigenetic modifications may serve as valuable biomarkers for tumor stratification and prognostic prediction. For instance, the glioblastoma resistance to receptor tyrosine kinase (RTK) inhibitors has been found to involve both genetic and epigenetic mechanisms[47], resulting in subclones with a gain of copy number in the insulin receptor substrate-1(IRS1) and substrate-2 (IRS2) loci. Another study identified a long non-coding RNA (LINC00021) that promotes TMZ resistance through Notch signaling and epigenetically silenced p21 expression via recruiting EZH2[48], one of the methyltransferases responsible for histone methylation. Epigenetic modifications in glioblastoma are also exploited as drug targets. Among the promising epigenetic interventions for glioblastoma are the histone deacetylase (HDAC) inhibitors[49], which have been extensively tested in various cancers[50]. HDAC inhibitors can block cancer cell proliferation by inducing cell cycle arrest, cell differentiation, and/or apoptosis[51]. With a large amount of supportive preclinical data, various HDAC inhibitors in glioblastoma clinical trials are underway.

DRUG RESISTANCE TO IMMUNOTHERAPY IN GLIOBLASTOMA

Current status of immunotherapy trials in glioblastoma

Although immune checkpoint inhibitors have greatly improved cancer treatment today, the clinical trials in glioblastoma treatment have been largely unsuccessful.

We summarized the most common immunotherapies that have been evaluated in glioblastoma in either preclinical or clinical trials [Figure 1]. The most widely tested immunotherapies in glioblastoma (like in all other cancers) are immune checkpoint inhibitors (ICIs). Immune checkpoint molecules are typically expressed on the surface of immune cells, and they play a crucial role in maintaining immune balance, preventing excessive immune activation, and avoiding auto-immune response. This function of immune regulation is achieved through the interaction of immune checkpoints with their corresponding ligands on other cells, and cancer cells often hijack this communication mechanism to suppress the anti-tumor immunity and evade immune surveillance[53-55]. A common working mechanism of ICIs is to block the inhibitory signal to the immune cells (usually from cancer cells) through an antibody binding to the checkpoint or its ligand to disengage their interaction. Since the discovery of the first immune checkpoint, cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), more than a dozen of these checkpoint molecules have been identified to date, such as PD1 and its ligand PD-L1/L2, TIM3, lymphocyte activation gene-3 (LAG3), and TIGIT[54]. Among various ICIs, α-PD-1 has been widely studied as a monotherapy[56] or in a combination of either radiation or radiation plus TMZ in multiple trials (CheckMate 143, 498 and 548)[57-59]. Overall, the clinical outcome has been rather murky in both primary and recurrent glioblastoma due to multiple resistance mechanisms, including high tumor heterogeneity, low mutational burden, systemic immunosuppression, and local immune dysfunction[60].

CAR-T therapy has been studied in glioblastoma[61]. The targets of these CARs in clinical trials span from growth signaling receptors (EGFR/EGFRvIII, Her2), cytokine receptors (IL13Rα2), immune checkpoint (B7-H3) to even matrix metalloproteinase (MMP2), and disialoganglioside (GD2)[62]. Besides very limited responders, including pediatric patients with diffuse intrinsic pontine glioma (DIPG)[63,64], most trials failed to demonstrate a sustained clinical benefit, mainly due to CAR-T-associated severe side effects, including cytokine release syndrome and high grade of neurotoxicity[65,66].

Cancer vaccines have also been explored in glioblastoma trials with minimal success. A peptide vaccine targeting EGFRvIII called rindopepimut has been tested in various trials, with only one trial (phase II) reporting a marginal increase in median overall survival of 12.0 months with rindopepimut plus bevacizumab compared to 8.8 months with bevacizumab plus vaccine placebo[67]. The main limitation of EGFRvIII vaccine is that the expression of EGFRvIII is only limited in some glioblastoma patients, and there is also an intra-tumoral heterogeneous pattern of EGFRvIII expression, which further hinders the overall immune response to the tumor. Another cancer vaccine strategy is to use patient-derived dendritic cells with ex vivo exposure to glioblastoma neoantigens. For instance, ICT-107 and DCVax-L both used patient autologous dendritic cells with pulse to either peptides designed based on patient tumors (ICT-107) or autologous tumor lysates (DCVax-L). Both trials have reached phase 3 and had an acceptable safety profile, though the efficacy was minimal[68,69].

Oncolytic virus (OV) can be viewed as a gene & immuno-hybrid therapy. Typically, an OV exerts its anti-tumor function through a dual mode of action - tumor cell killing (lysis) and induction of systemic anti-tumor immunity[70]. An OV can selectively infect and lyse cancer cells, and various viruses have been employed to develop oncolytic viruses[71]. Upon lysis of tumor cells due to OV replication, many tumor antigens will be released, leading to a local and systemic anti-tumor reaction[72]. One of the main issues associated with OV therapy is the host's anti-viral immune response to the OV[73]. Currently, a modified herpes simplex virus type 1, named teserpaturev or G47Δ, is the only OV that received conditional approval (in Japan) for glioblastoma treatment[74], and many more oncolytic viruses are currently in clinical trials for glioblastoma treatment (reviewed by Suryawanshi & Schulze[75]). Among them, a retroviral OV called Toca511 reached phase III clinical trial, but was terminated due to its failure to improve survival and meet

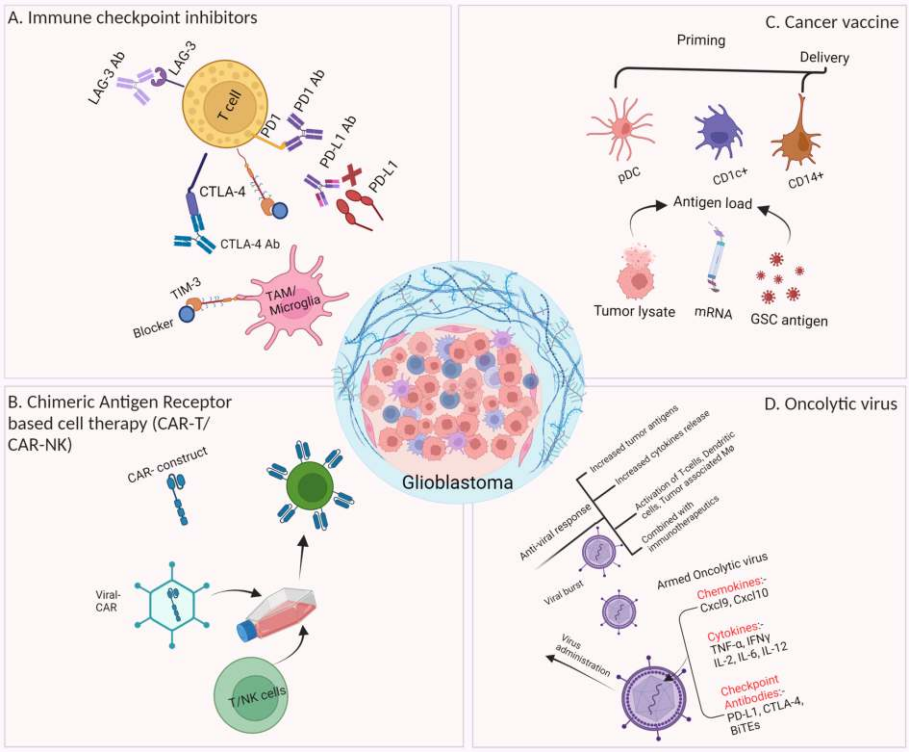

Figure 1. Various forms of immunotherapy in preclinical and clinical trials for glioblastoma treatment. (A) Various checkpoint inhibitors, including α-PD1, α-PD-L1, α-CTLA-4, and α-TIM-3, have been studied in glioblastoma treatment; (B) CAR-based adoptive cell therapies have attained immense success against hematopoietic cancer, but have shown limited effects on glioblastoma; (C) Cancer vaccine has been tested in glioblastoma treatment by priming antigen-presenting cells (e.g., Dendritic Cells) with tumor antigens/lysate or synthetic antigen peptides, followed by infusion back to the patients; (D) An OV can lyse tumor cells through replication. OV can be armed with immunotherapy in which a virus is genetically modified to carry checkpoint inhibitors (e.g., α-PD-L1 and α-CTLA-4), therapeutic proteins, chemokine (Cxcl9, Cxcl10) or cytokines genes (IFNγ, IL-6, IL-12). Those armed OVs are more potent in killing cancer cells[52]. (Created with BioRender.com). CTLA-4: Cytotoxic T-lymphocyte-associated protein 4; GSC: glioma stem cell; LAG-3: lymphocyte activation gene-3; OV: oncolytic virus; TAM: tumor-associated macrophage.

other endpoints[75].

Immunosuppressive TME

Glioblastoma tumors generally have a low to moderate mutation rate, especially compared to other solid tumors such as melanoma, non-small cell lung cancer, GI cancer, and head and neck cancer[76]. The tumor mutation burden was found to be correlated with immunotherapy treatment response[77]. In addition, glioblastoma also has a highly immune-suppressive microenvironment with a large amount of infiltrating myeloid cells, including bone marrow-derived macrophages (MΦ), myeloid-derived suppressor cells (MDSCs), dendritic cells (DCs), and neutrophils[78]. T lymphocyte dysfunction in the glioblastoma is very severe and was found to be mediated partially by IL-10 produced by the myeloid cells[79]. Additionally, within the TME, prolonged antigen exposure to T cells leads to the expression of LAG3, which in turn causes T cell exhaustion[80]. More strikingly, patients with glioblastoma also have systemic immune suppression. For instance, glioblastoma patients have lower numbers of circulating T cells due to the sequestration of T cells in the bone marrow, possibly due to loss of sphingosine-1-phosphate receptor 1 (S1P1) expression[81]. S1P1 is a GPCR that binds the lipid second messenger, sphingosine-1-phosphate (S1P), and the S1P-S1P1 axis plays a pivotal role in lymphocyte trafficking[82]. Typically, surface S1P1 affords T cell egress from the spleen, lymph node, and thymus. In a mouse glioblastoma model, the T cells from tumor-bearing mice were found to have lost surface expression of S1P1, leading to T cells sequestered

mainly in bone marrow[81]. This may partially explain the T cell lymphopenia in glioblastoma patients. However, treatment (radiation and TMZ) associated T cell lymphopenia was also very common[83,84].

Glioblastoma tumors can produce IL-6 and drive myeloid immunosuppression by inducing PD-L1 expression on MDSCs[85]. Glioblastoma can also utilize the natural immune tolerance mechanisms to recruit regulatory T cells (Tregs) through the expression of indoleamine 2,3-dioxygenase (IDO)[86], as well as the tumor-associated macrophages (TAMs) expression of TIM4[87]. Besides soluble factors, extracellular vesicles containing various signaling molecules, including growth factors, non-coding RNAs, cytokines, and other functional proteins, have been found to play an important role in the regulation of glioblastoma TME[88]. Those mechanisms involve an extensive network of DCs, TAMs, MDSCs, and T lymphocytes with complex and dynamic crosstalk [Figure 2].

Heterogeneity in tumor microenvironment

Tumor heterogeneity has been well-known in glioblastoma biology at multiple levels[89], including genetics/epigenetics (molecular subtypes), molecular signaling (tumor driver mutations), cellular components (clonal and subclonal tumor cells *vs.* tumor microenvironment), and temporal (primary *vs.* secondary). scRNAseq analysis of infiltrating neoplastic cells in human glioblastoma revealed vast genomic and transcriptomic heterogeneity[90]. Another work in brain endothelial cells derived from human glioblastoma using a similar approach (scRNAseq) showed five distinct endothelial cell phenotypes representing different states of EC activation and BBB impairment and association with different anatomical locations within and around the tumor[91].

With the advancement of multi-omics platforms, tumor heterogeneity at both inter- and intra-tumoral levels has been much better depicted in glioblastoma[92-94]. The inter-tumoral heterogeneity can be readily appreciated by the molecular subtyping of human glioblastoma tumors by their transcriptional profile and phenotypical response to therapy[2,95,96]. Consistent with the four molecular subtypes of glioblastoma, a more recent scRNAseq analysis showed that glioblastoma cells can differentiate into four principal states, including astrocyte-like, oligodendrocyte progenitor-like, neural progenitor cell-like, and mesenchymal-like state[97]. These four cellular states are influenced by the tumor microenvironment and oncogenic drivers with certain plasticity[97].

The intra-tumoral heterogeneity in glioblastoma is characterized by the presence of clonal and subclonal differentiated tumor cells, glioma stem cells (GSCs), and various components of the tumor microenvironment (stromal, endothelial, and infiltrating immune cells). A recent study by Schaettler *et al.* using scRNAseq revealed the differences between primary and secondary glioblastoma in their genomic abnormality and neoantigen formation, as well as the spatially differential T cell clones within the glioblastoma[98]. The authors used TCR β-chain CDR3 sequences as unique barcodes of individual T cell clones, as TCR β-chain CDR3 is highly diverse with a significant role in antigen recognition[99]. Their results demonstrated a topological clonal diversity of T cells in glioblastoma[98]. Besides microglia, another representative cell population that further complicates glioblastoma heterogeneity is a large variety of myeloid cells in the TME[100]. They mainly comprise TAMs, MDSCs, DCs, neutrophils, and undifferentiated monocytes[78,101]. Another study using scRNAseq and multiplexing tissue-imaging techniques demonstrated a spatially differential tumor microenvironment characterized by inflammatory signaling and hypoxia in glioblastoma[102]. The authors revealed that CD73, a critical regulator of local purinergic signaling with an essential role in inflammatory response[103], was mainly expressed in glioblastoma cells with a positive correlation between levels of CD73 and HIF1α expression in the hypoxic tumor regions, where the CD73+ glioma cells co-localize with CD39+ microglia to form a spatially compartmentalized microenvironment to

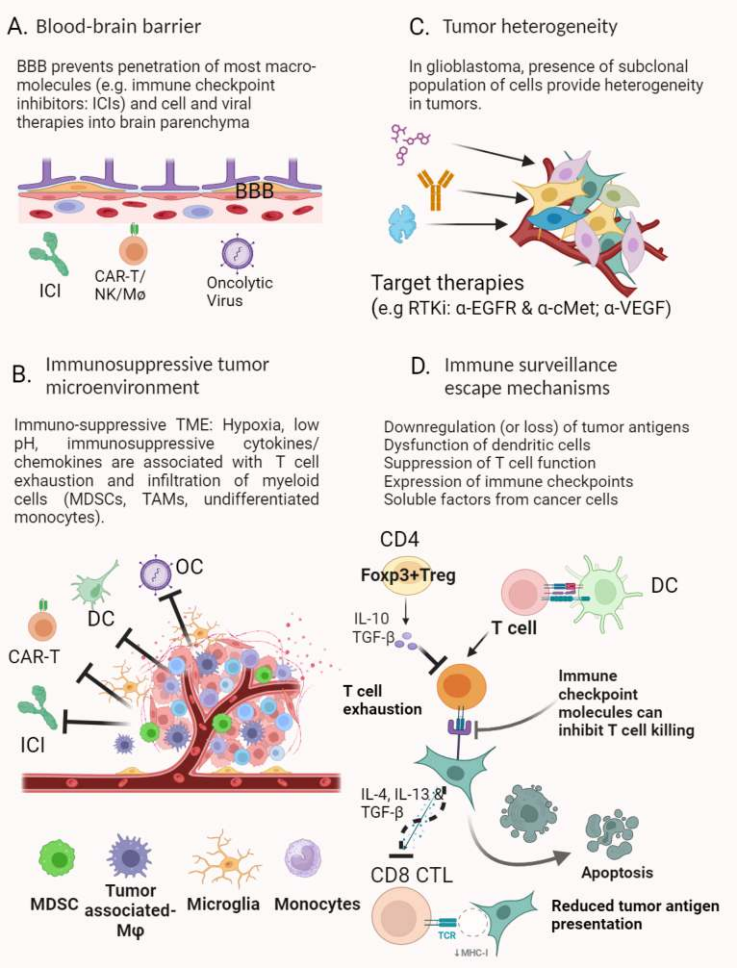

Figure 2. Main determinants of therapeutic failures in glioblastoma. (A) BBB can prevent the transport of most macromolecule therapeutics (e.g., immune checkpoint inhibitors), cell-based therapies, and most oncolytic viruses; (B) Within the glioblastoma, TME is a severely immunosuppressive local environment that can inhibit the function of most immunotherapies; (C) Clonal heterogeneity represents a complex problem for targeted therapeutics (e.g., receptor tyrosine kinase inhibitors and α-VEGF) to attack glioblastoma tumor cells effectively; (D) Various mechanisms for glioblastoma tumor cells to evade immune attack: tumor cells derived soluble factors (e.g., IL-4, IL-13, prostaglandin E2, and TGF-β) can suppress T cell proliferation; T cell exhaustion induced by prolonged antigen exposure can severely diminish CD8 CTL mediated cancer killing; FOXP3+ CD4 Tregs also block T cell activation. (Created with BioRender.com). BBB: Blood-brain barrier; CTL: cytotoxic T cell; DC: dendritic cell; ICIs: immune checkpoint inhibitors; MDSCs: myeloid-derived suppressor cells; TAMs: tumor-associated macrophages; TME: tumor microenvironment.

regulate the production of adenosine, a potent immunosuppressive metabolite[102].

Immune surveillance escape mechanisms

The crosstalk between glioblastoma and the TME through which glioblastoma tumors escape immune surveillance is very complex and highly dynamic, involving many signaling mechanisms, including both soluble factors and cell-cell interactions. Besides the BBB, which prevents drugs from reaching their target sites, these mechanisms include various immune-suppressive mechanisms, such as secretion of immunosuppressive cytokines (IL-10, TGF-β, and IL-6)[104,105], expression of immune checkpoints[106], and recruitment of regulatory T cells (Tregs)[107], induction of M2-like phenotype of tumor-associated MΦ and microglia[106], reduced tumor antigen presentation through downregulation of MHC expression, and the ability to evade immune through soluble ligands[108,109] [Figure 2].

T cell exhaustion

T cell exhaustion is exceptionally severe in glioblastoma[110], resulting in poor therapeutic efficacy of immunotherapy. Most immunotherapies focus on eliciting an anti-tumor T cell response that requires a collaboration of at least CD4 T Helper cells and CD8 cytotoxic T cells (CTLs). CD4 T Helpers can modulate antigen-specific immune response through their high plasticity and cytokine production, while CD8 CTLs exert cancer cell killing through direct cell-cell interaction and targeted release of effector molecules (perforin and granzymes)[111]. T cell exhaustion is mainly induced by persistent antigen exposure, and it is commonly seen in chronic infections and cancers. It is generally characterized by elevated expression of various immune checkpoints (PD-1, CTLA-4, LAG-3, and TIM-3). Remarkably, T cell exhaustion was also found to correlate with hypoxia in glioma, and both the number of exhausted T cells and the associated exhaustion markers (PD-L1, FOXO1, and PRDM1) correlated with HIF1α levels[112].

The presence of regulatory T cells (Tregs) is another contributing factor for the dysregulation of T cell function in glioma TME [Figure 2]. Tregs are a subset of CD4 T cells that usually prevent autoimmunity response via suppression of inflammation and maintenance of self-tolerance[113]. Tregs (CD4+ Foxp3+) naturally arise from thymic differentiation[114] or are induced in the already differentiated Foxp3- CD4+ T cells in the periphery[115]. A recent study showed that Tregs promote CD8 T cell exhaustion and restrict clonal diversity of tumor-infiltrating CD8 CTLs[116]. Therefore, strategies to eliminate Tregs have been developed to restore anti-tumor immunity in glioblastoma, including activation of glucocorticoid-induced tumor necrosis factor-related protein (GITR). GITR is an immune checkpoint constitutively expressed in Tregs, and its activation through ligand binding leads to the depletion of Tregs and reduced immunosuppression. A preclinical study by Amoozgar *et al.* demonstrated that targeting Tregs with anti-GITR antibodies can relieve resistance to immunotherapy (e.g., anti-PD1) in mouse glioblastoma models[117].

Immunosuppression by myeloid cells

A large number of myeloid cells, such as monocytes, macrophages and MDSCs, in the glioblastoma TME impose another great challenge for immunotherapy to function [Figure 2]. Among the tumor-infiltrating myeloid populations in glioblastoma, TAMs play a pivotal role in tumor progression, immunosuppression, and therapy resistance. TAMs are usually found to exhibit a tumor-promoting phenotype by producing immune suppressive cytokines such as IL-6, IL-10, and TGF-β[104,105], and they represent a large population of cells with immunosuppressive function in TME. Various approaches have been proposed to target TAMs for glioblastoma treatment. For instance, by dual targeting IL-6 and CD40, Yang *et al.* showed that they could reverse TAMs-mediated tumor immunosuppression and sensitize the glioblastoma tumor to immune checkpoint inhibitors (anti-PD1 and anti-CTLA-4) in mouse tumor models[118]. In addition, the relatively undifferentiated monocytic MDSCs have been found to play a significant role in glioblastoma-associated immunosuppression. Domenis *et al.* demonstrate that CD14+ monocytic MDSCs were the primary mediators of the T cell suppression induced by the GSC-derived exosomes containing various immune suppressive cytokines[119].

Glioblastoma can also evade immune attack by down-regulating tumor antigen expression. Tumor antigen loss during immunotherapy treatment, especially by CAR-T therapy, has been frequently reported[120]. Migrating or invading glioblastoma cells were found to have reduced expression of major histocompatibility complex (MHC) class I and II genes, resulting in significant down-regulation of tumor antigen presentation[121]. Additionally, glioblastoma TME is quite a hypoxic and acidic environment. Both hypoxia and acidosis are essential environmental cues for maintaining GSCs, especially in a HIF1α-dependent manner[122,123]. GSCs are believed to be primarily responsible for tumor resistance to chemotherapy and radiotherapy[124,125]. More importantly, GSCs have also been shown to have a significant role in the evasion of

immune function[126].

Resistance to ICIs

ICIs are currently the most prevalent immunotherapy for cancer treatment. Since the approval of the first ICI (α-CTLA-4) by the FDA in 2011, these antibodies have been studied in an increasingly growing number of clinical trials, including those cancers with low response rates, such as breast cancer, cervical cancer, and brain cancer[60,127,128]. Despite the success of ICIs in treating hematopoietic cancers, the clinical trials in glioblastoma have been underwhelming. Besides the BBB, several contributing factors that render ICIs ineffective in glioblastoma treatment have been identified.

Low tumor mutational burden in glioblastoma tumor

Glioblastoma is generally considered an immunologically "cold" tumor type with a relatively lower tumor mutational burden (TMB). Thus, the neoantigen levels are also lower[129,130]. Higher TMB often leads to the formation of a greater number of neoantigens and a greater potential for T-cell repertoire against tumor-specific antigens[131]. TMB has been found to be correlated with the clinical outcome of cancer immunotherapy[76]. Compared with the immunologically "hot" tumor types such as melanoma and NSCLC, glioblastoma shows a much lower neoantigen burden[132].

T cell dysfunction

Glioblastoma patients are often found to have T cell dysfunction in both CNS and peripheral blood, and T cell exhaustion is pervasive and severe in glioblastoma TME. CD8 T cell exhaustion usually starts with the loss of IL-2 production, a cytokine crucial for T cell proliferation, followed by loss or decreased production of TNF-α, IFN-γ, and granzyme B[133]. Tregs also make a significant contribution to the T cell dysfunction in glioma. Both natural and induced Tregs can suppress the cytotoxicity of CD8 CTLs. Tregs were found to be associated with worse prognosis in glioblastoma patients[134], and it seems that the natural Tregs are the dominant subpopulation of Tregs in glioblastoma. Besides dysregulated T cell function, surprisingly, neurons have been shown to play a role in the ICI therapy resistance in glioblastoma. A recent study reported neuronal calmodulin-dependent kinase kinase-2 (CaMKK2) as a driver for the resistance to ICIs in glioblastoma[56], in which CaMKK2 increased CD8 T cell exhaustion, reduced CD4 effector cell expansion, and played a role in the maintenance of immunosuppressive phenotype of tumor-associated microglia[135].

Deficits in antigen presentation by microglia

In glioblastoma TME, antigen presentation machinery is dysregulated in almost all types of antigen-presenting cells. The immunosuppressive microenvironment in glioblastoma leads to the downregulation of MHC expression in microglia[136,137]. The decreased MHC expression significantly impairs the ability of microglia to effectively present antigens, limiting the activation of other immune cells and undermining the immune response against the tumor. Similarly, TAMs were found to be deficient in antigen presentation, lacking costimulatory molecules CD86, CD80, and CD40 critical for T-cell activation[138]. In fact, although glioblastoma tumor-infiltrating dendritic cells seemed more efficient than both MΦ and microglia in priming T-cells with exogenous antigens[139], data from a preclinical study demonstrated that a better anti-tumor immunity is associated with both tumor-infiltrating dendritic cells and microglia[140].

TAMs

A new study using patient-derived recurrent glioblastoma tumors with neoadjuvant PD-1 antibody treatment showed that α-PD-1 activated T cells and dendritic cells, but was unable to reverse the immunosuppressive phenotype in TAMs[141]. Work by Chen *et al.* analyzed scRNAseq data from a combined of >19,000 individual macrophages from 66 human glioma cases (50 glioblastomas and 16 low-

grade gliomas) and discovered a pro-tumor subset of bone marrow-derived macrophages with the expression of a scavenger receptor MARCO[142]. More interestingly, this subpopulation of MARCO+ TAMs was found almost exclusively in the IDH-WT glioblastoma, and they exhibited a completely oppositive dynamic in α-PD-1 responders *vs.* non- responders[142]. Park *et al.* studied the immune landscape of mouse glioblastoma with α-PD-1 treatment, and found that chemokine CCL5 induced by α-PD-1 treatment seemed to recruit the anti-inflammatory TAMs into the glioblastoma TME[143]. A CyToF-based high-plexing immune profiling approach revealed that ICI-sensitivity in both human and mouse tumors was associated with a higher number of T cells and dendritic cells (DCs) and a lower number of PD-L1 positive TAMs[144].

Anti-inflammatory glucocorticoids

Glucocorticoids have been used to control certain adverse effects associated with cancer immunotherapy. Interestingly, concurrent administration of dexamethasone, a potent corticosteroid frequently used in glioblastoma patients to decrease tumor-associated edema, has been shown to be detrimental to immunotherapy for patients with glioblastoma[145]. Though the clinical data in this study was limited to a subset of patients with wild-type IDH-1 glioblastoma under α-PD-L1 treatment, the concurrent dexamethasone diminished the response to α-PD-1 therapy in two different mouse glioma models[145]. It is worth mentioning that glioblastoma patients under standard (radiation plus TMZ) treatment who received dexamethasone treatment also showed a worse outcome[146]. However, this is likely because MGMT promoter contains two nonconsensus glucocorticoid-responsive elements and glucocorticoids can upregulate MGMT expression[147]. A comprehensive study of MGMT promoter activity in glioblastoma cell lines further clarified that dexamethasone, but not TMZ or irradiation, can induce the upregulation of MGMT expression via a SP-1 dependent fashion[148], while not through altering the epigenetic status (i.e., methylation) of the MGMT promoter.

Role of non-coding RNAs

Long non-coding RNAs (LncRNAs) have been increasingly recognized for their essential role in cell growth, survival, proliferation, pluripotency, and immune functions correlating to the malignant transformation of normal cells into cancerous cells[149-151]. MALAT1, NEAT1, and H19 are among the common LncRNAs that influence the response of glioblastoma/glioma to chemotherapeutics[152]. Another lncRNA, LINC00021, was significantly upregulated in glioblastoma, especially in the TMZ resistance cells or tissues, enhancing resistance to TMZ through Notch pathway and epigenetically silencing p21 expression[48]. A study also showed that LncRNA SNHG15 promotes pro-glioblastoma cytokines TGF-β and lL-6 in TMZ-resistance cells via M2-polarization of microglial cells[153].

Micro RNA (miRNA) also plays a role in the regulation of glioblastoma TME. One example is the miR-15/16 cluster, which was found to be differentially expressed in various human cancers such as glioma and prostate cancer[154,155]. In a mouse glioblastoma model, Yang *et al.* demonstrated that loss of miR-15/16 in mice carrying GL261 tumors resulted in improved survival, enhanced CD8 T cell infiltration, and reduced expression of T cell exhaustion markers (PD1, TIM-3, and LAG-3)[156]. An *in vitro* study by Hubner *et al.* identified miR-93 as an anti-inflammatory tumor suppressor in glioblastoma[157]. Their data showed that miR-93 was downregulated in human glioblastoma cell lines, and restoration of miR-93 levels in glioblastoma cells led to a decreased expression of an array of inflammatory genes (HIF-1α, MAP3K2, IL-6, G-CSF, IL-8, LIF, and IL-1β)[157]. More interestingly, TCGA data mining confirmed that high expression of miR-93 was associated with better survival in the MGMT-methylated cohort of glioblastoma patients.

OPPORTUNITIES

Approaches to alter immuno-suppression in glioblastoma TME

Many great efforts have been made to overcome the difficulty of immunotherapy applications in neuro-oncology. For example, a clinical trial found that neoadjuvant PD-1 blockade resulted in significantly improved overall survival and progression-free survival in patients with recurrent glioblastoma[158]. In this study, patients received anti-PD1 treatment ~2 weeks before surgery, and the PD1 antibody was able to elicit both systemic and local anti-tumor immunity. Other attempts are primarily focused on modulating the immune suppression in the glioblastoma tumor microenvironment by targeting various components of the TME, such as TAMs and MDSCs (summarized in a recent review by Wang *et al.*[159]). In the meantime, new targets have been identified for future immunotherapy development. For instance, TAMs associated CD73 was found to be a promising target with potentially synergistic effects along with dual inhibition of PD1 and CTLA-4[160]. CD47/SIRPα axis is another exciting target to consider. SIRPα governs the phagocytosis activity of MΦ. When CD47 on the cancer cell surface engages with SIRPα on MΦ, it sends a "Don't-eat-me" signal to prevent phagocytosis of cancer cells by MΦ. Treatment with anti-CD47 plus TMZ was shown to activate both innate and adaptive anti-tumor immunity in a preclinical study[161].

A single-cell RNA-seq study of patient glioma infiltrating T cells revealed CD161 (KLRB1) as a promising immunotherapy target. Depleting CD161 led to T cell activation and anti-tumor immunity both *in vitro* and *in vivo*[162]. An independent study using data from a large cohort of glioma patients confirmed that CD161 might play an important role in promoting glioma progression via inhibition of T cell function[163].

Besides checkpoint inhibition, a deeper understanding of the resistance mechanism to CAR-T therapy in solid tumors was achieved through a genome-wide CRISPR knockout screen in glioblastoma[164]. A recent study using a genome-wide CRISPR knockout screen in glioblastoma revealed a functional requirement of IFN-γ receptor in glioblastoma for sufficient adhesion of CAR-T cells to mediate productive cytotoxicity[164]. This study suggests that strategies to enhance the binding of CAR-T cells to the solid tumor will likely result in a better treatment response. Another strategy to enhance the infiltration of CAR-T cells into glioblastoma tumors by combining CAR-T with a CXCL11-armed oncolytic virus also demonstrated an improved anti-tumor immunity in a syngeneic mouse glioma model[165].

Combinatorial approaches and new forms of immunotherapies

Combination therapy has been extensively explored to improve glioblastoma treatment. For instance, resistance to α-VEGF monotherapy was common in glioblastoma. A new study reported that combined blockade of VEGF, Angiopoietin-2, and PD1 could reprogram glioblastoma endothelial cells into quasi-antigen-presenting cells and induced a durable anti-tumor T cell response[166]. A recent review has nicely summarized the current status of combinatorial approaches, including both chemo- and immunotherapies, for glioblastoma treatment[167]. Additionally, many new forms of immunotherapy are emerging with great hope to shift the paradigm of glioblastoma treatment. A recent study reported a nanoporter (NP)-hydrogel complex for local induction of CAR-macrophages (CAR-MΦ) targeting CD133+ glioblastoma stem cells in tumor resection cavity with promising results[168]. This nanomicelle complex consists of a self-assembled peptide-based hydrogel loaded with the CD133-targeting CAR construct and then was coated with a citraconic anhydride–modified dextran with the ability to bind to CD206, a typical surface marker of M2 macrophages. Different from the *ex vivo* engineering of CAR-MΦ developed by Klichinsky *et al.*[169], the nanoporter-hydrogel-based in situ induction of strategy CAR-MΦ largely simplified the process of CAR-MΦ preparation and minimized potential systemic toxicity from CAR-MΦ.

The CAR-NK cells have also been explored to treat glioblastoma either by Her2 targeting monotherapy[170] or in combination therapy. For instance, the Off-the-Shelf EGFR-targeting CAR-NK cells have been tested in combination with an oncolytic virus expressing the IL15/IL15Ralpha complex and the combinatorial therapy demonstrates a strong anti-tumor immunity[171]. A significant problem associated with CAR-NK cell therapy is the shedding or down-regulation of the ligands in cancer cells that bind natural killer group 2D (NKG2D) receptors on the natural killer (NK) cells. NKG2D is an activating receptor widely expressed in NK cells as well as in some subsets of T cells[172]. To overcome the limitation of NKG2DL heterogeneity in the tumor, a recent study using a bispecific antibody with two ScFv fragments (linked with a IgG4-Fc) that target Her2 (tumor) and NKG2D (NK cells), respectively, in combination with human NK-92 cells, showed synergistic tumor cell killing effects in both *in vitro* and *in vivo* conditions[173]. Although the syngeneic tumor model they used represents a situation of a heterogenous expression of NKG2DLs in tumor cells, the flank tumors they used did not address the difficulty in delivery of the combination therapy across the BBB[173].

Another interesting phenomenon is the sex difference in response to immunotherapy in glioma. The sex disparity in brain cancer has been reported by several groups[174-177]. A recent meta-analysis revealed that female patients with glioblastoma treated with immunotherapy had a statistically significant survival advantage in overall survival over their male counterparts[178]. They also found that female patients exhibited a more robust survival advantage with cancer vaccine treatment. Another study by Bayik *et al.* discovered that two subsets of myeloid-derived suppressor cells (MDSCs) have a sex-specific tumor-promoting phenotype in both mouse and human glioblastoma[179]. All these data suggest that a more personalized approach, which at least considers sex differences in glioblastoma treatment, will more accurately evaluate the efficacy of immunotherapy.

New drug delivery technologies to overcome BBB limitation and activate glioblastoma TME

Various new technologies have demonstrated promising progress in overcoming BBB, and we summarized a few new approaches with great potential to improve the glioblastoma treatment outcome [Figure 3]. Among those new approaches, the use of ultrasound to open BBB for glioblastoma treatment has been applied in several areas, including immunotherapy delivery. Using low-intensity pulsed ultrasound to temporarily disrupt BBB, Sabbagh *et al.* demonstrated a significantly improved BBB penetration of both anti-PD1 antibody and EGFRvIII targeting CAR-T cells, as well as significantly improved survival in mouse glioblastoma models[180]. Another study by Sheybani *et al.* applied MRI-guided focused ultrasound with systemic injection of microbubbles and studied the impact of this approach on temporary BBB disruption in a mouse glioma model[181]. This approach caused a transient local inflammatory phenotype in the mouse glioblastoma, with an increased number of dendritic cells and the upregulated maturation marker. However, they did not see a significant increase in CD8 T cells in the TME[181].

Another technology to modulate BBB function is photodynamic therapy (PDT). Conventionally, PDT relies on a photosensitizer, such as 5-aminolevulinic acid (5-ALA)[182], that can accumulate in tumor tissue, plus a laser that can stimulate the photosensitizer, followed by energy transfer to generate reactive oxygen species, leading to damages to the cancer cells[183]. It is noteworthy that PDT has shown promise in temporary opening of BBB, possibly through modulating certain components of TJs[184]. Interestingly, PDT can also induce an acute inflammatory response in which both innate and adaptive immune systems are activated[185]. Recently, BBB opening was shown to affect the meningeal lymphatic system characterized by an anti-tumor effect of talaporfin sodium (TS)-PDT as well as its synergy with the immune checkpoint inhibitor[186]. *In vitro* studies have demonstrated that targeted TS-PDT triggers various forms of cell death, including apoptosis, necrosis, and autophagy-associated cell death. Furthermore, TS-PDT induces the acute activation of lymphatic drainage in the brain and the clearance of unwanted molecules from the CNS[187,188].

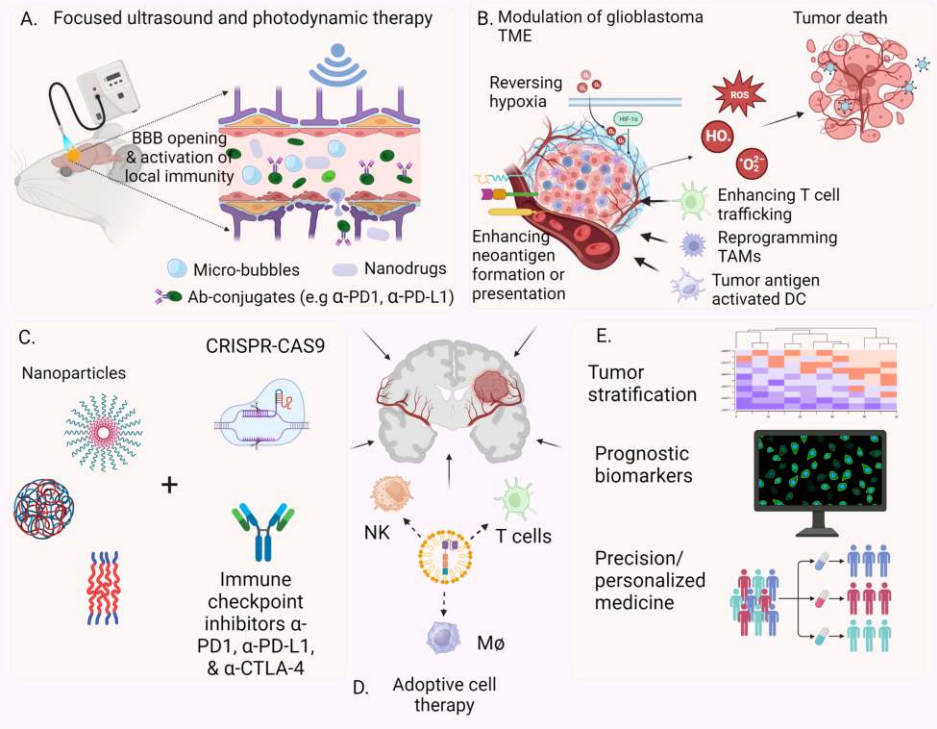

Figure 3. Potential new approaches to improve glioblastoma treatment. (A) The focused ultrasound in combination with micro-bubbles and photodynamic therapy (PDT) can temporarily open BBB to allow therapeutics crossing. PDT can also activate local immunity in TME; (B) New approaches to modulate glioblastoma TME by targeting hypoxia, activating suppressed local immunity, or enhancing cancer neoantigen formation in tumor cells; (C) Novel nanodrug delivery technologies in combination with CRISPR/Cas9-based gene editing and immune checkpoint inhibitors; (D) Various forms of adoptive cell therapies; (E) Better strategies for tumor stratification, prognostic prediction and personalized medicine would enhance the clinical outcome of glioblastoma treatment. (Created with BioRender.com). BBB: Blood-brain barrier; DC: dendritic cell; TAMs: tumor-associated macrophages; TME: tumor microenvironment.

The approval of 5-ALA by the FDA for fluorescence-guided glioblastoma resection has sparked a renewed interest in its potential application for PDT[182].

Nanotechnology has also made significant advancements in the field of glioblastoma treatment. Various forms of nanomedicines have exploited the features of the glioblastoma tumor microenvironment for efficient BBB crossing and release of payloads[189-191]. Fan *et al.* engineered an MMP-2-activated nanoparticle to carry anti-CD276 & CD3 bispecific antibodies and demonstrated that this strategy enhanced IFN-γ-induced tumor cell ferroptosis[192]. A polylactic-co-glycolic acid (PLGA) nanoparticle encapsulated disulfiram was used to block hypoxia-induced NF-κB signaling and glioma stem cells[193]. Zou *et al.* devised a polymer-based CRISPR-Cas9 nano-capsule for systemic gene therapy delivery to glioblastoma[194]. This nano-capsule has both the BBB crossing and tumor targeting functions mediated through an angiopep-2 peptide[195]. By targeting polo-like kinase (PLK-1) via a sgRNA, the strategy demonstrated a significant survival advantage over the control mice[194].

CONCLUSION

Despite advances in surgical technologies and therapeutics development, there has been limited improvement in the long-term survival rate of glioblastoma patients, with a 5-year survival still around 5%-10%. Many lessons have been learned in glioblastoma drug resistance mechanisms, especially with cutting-edge scRNAseq, spatial biology, and other-omics platforms. Efforts are needed to overcome BBB and tumor heterogeneity, targeting glioma stem cells and their niches, enhancing T cell trafficking and preventing their

exhaustion, and modulating the immunosuppressive TME in glioblastoma. A complex disease, such as glioblastoma, would require a complex solution. Multidisciplinary approaches involving nanodrug carriers, focused ultrasound, plus temporary BBB permeability enhancement technologies (micro-bubbles, phototherapy) in combination with gene and immuno-therapy will likely lead to an improved outcome [Figure 3]. In addition, a much less traveled path is to enhance glioblastoma neoantigen formation. Glioblastoma tumors have a relatively lower TMB, which was shown to correlate with immunotherapy outcomes in solid tumors[76,196]. Lower TMB results in lower neoantigen generation, which enables a stealth mode of glioblastoma cells. Therefore, increasing the formation of neoantigens may significantly promote tumor recognition and clearance by the immune system[197]. Besides T cells, strategies to activate other infiltrating immune cells (TAMs, microglia, and MDSCs) that reside in the glioma TME in large abundance may effectively reverse the local immunosuppression. Finally, a more precise tumor stratification approach and improved prognostic biomarkers will help determine the most effective combinatorial therapies for glioblastoma treatment.

DECLARATIONS

Authors' contributions

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Cancer Drug Resistance

Review

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Unlocking antitumor immunity with adenosine receptor blockers

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Abstract

Tumors survive by creating a tumor microenvironment (TME) that suppresses antitumor immunity. The TME suppresses the immune system by limiting antigen presentation, inhibiting lymphocyte and natural killer (NK) cell activation, and facilitating T cell exhaustion. Checkpoint inhibitors like anti-PD-1 and anti-CTLA4 are immunostimulatory antibodies, and their blockade extends the survival of some but not all cancer patients. Extracellular adenosine triphosphate (ATP) is abundant in inflamed tumors, and its metabolite, adenosine (ADO), is a driver of immunosuppression mediated by adenosine A2A receptors (A2AR) and adenosine A2B receptors (A2BR) found on tumor-associated lymphoid and myeloid cells. This review will focus on adenosine as a key checkpoint inhibitor-like immunosuppressive player in the TME and how reducing adenosine production or blocking A2AR and A2BR enhances antitumor immunity.

Keywords: Immunotherapy, adenosine, adenosine receptors, adenosine A2A receptors (A2AR), adenosine A2B receptors (A2BR), tumor cells, immune cells, tumor microenvironment

INTRODUCTION

Deadly tumors have the ability to resist the body's formidable immune defenses. They create protective micro-environments that limit antigen presentation, inhibit T and natural killer (NK) cell responses, and

induce T cell exhaustion, effectively escaping immune surveillance. These mechanisms allow some tumors to grow unchecked and resist conventional cancer therapies.

Checkpoint inhibitors that block immunosuppressive signaling molecules such as PD-1, T lymphocyte antigen 4 (CTLA4), and lymphocytic activation gene 3 protein (LAG-3) have ushered in a new era of cancer immunotherapy, offering hope for prolonged survival and enhanced quality of life for many patients. However, the beneficial effects of these therapies are not universal due to the ability of some tumors to maintain an immunosuppressive environment. The interplay between cancer cells and immune cells within the tumor microenvironment (TME) is a critical determinant of the therapeutic response.

A key driver of immunosuppression within the TME is extracellular adenosine (ADO), an adenosine triphosphate (ATP) metabolite. ADO formation and its immunosuppressive signaling play a pivotal role in maintaining the immunosuppressive state of the TME, promoting tumor growth, and facilitating resistance to other therapies. This review explores the role of ADO signaling in the TME. Inhibiting ADO receptors on immune cells reduces immunosuppression and, in some cases, has an additive antitumor effect when combined with other cancer treatments.

UNDERSTANDING NON-RESPONDERS TO IMMUNOTHERAPY IN SOLID TUMORS

Despite having antitumor effects, cancer immunotherapy often fails. One prominent reason is that most tumor proteins are recognized as self-proteins and fail to activate T cells, which serve as the frontline warriors of the adaptive immune response[1]. To the extent that tumors are recognized by the immune system, their activation is muted by immunosuppressive signals like adenosine.

Antigen-presenting cells (APCs) such as macrophages and dendritic cells (DCs) also regulate the immune response to tumors[2]. The function of APC is to recognize, engulf, and present tumor antigens on their surface. Their failure to optimally phagocytose and present antigens can undermine the initiation of adaptive immunity. The host's baseline immune response also significantly influences the success or failure of cancer immunotherapy. The baseline immune response is crucial in determining treatment efficacy, represented by the association between increased T cell infiltration into tumors and improved patient survival and immunotherapy response rates[3]. However, the factors that dictate the extent of T cell infiltration into tumors, whereby an extensively infiltrated tumor is considered "hot", and a sparsely infiltrated tumor is considered "cold", are just beginning to be elucidated. The factors that influence infiltration vary across tumor types and subtypes due to immune cell heterogeneity[4]. The complexity and dynamics of the immune system, in conjunction with the adaptability of tumor cells, create a challenging landscape for the successful deployment of cancer immunotherapy.

The complexity of the TME in solid tumors

The TME in solid tumors is complex, consisting of various immune cells, cytokines, chemokines, and metabolites. Specific features of the TME depend on the tumor type and the location within the patient. Some tumors develop an extracellular matrix (ECM) of fibrous proteins and stromal cells that define and isolate the TME from the surrounding tissue[5,6]. Within "cold" solid tumors, very few antitumor CD8+ T cells, NK cells, and DCs are present, due to failure by immune cells to enter through the ECM[7]. Immune cells that contribute to the immunosuppressive state are tumor-associated macrophages (TAMs) and myeloid-derived suppressor cells (MDSCs). Monocytes that enter tumors can polarize into M1 (proinflammatory) or M2 (immuno-suppressive) cells. In cancer, most become M2 and MDSC[8] and function to secrete vascular endothelial growth factor (VEGF) and transforming growth factor β (TGF-β), which stimulates angiogenesis within the tumor[9]. M2-TAMs and MDSCs also suppress CD8+ T cell

infiltration and increase regulatory T cell (Treg) differentiation from precursor CD4+ T cells[10]. Tregs release suppressive cytokines such as interleukin-10 (IL-10) and TGF-β that inhibit CD8+ T cell function and enhance cancer cell escape from the immune attack[11]. In some solid tumors, such as pancreatic ductal adenocarcinoma (PDAC), increased IL-10 is correlated with reduced survival[12-14].

MDSCs are considered immature myeloid cells and secrete reactive oxygen species (ROS), IL-10, IL-13, TGF-β, arginase-I, inducible nitric oxide synthase (iNOS), and other immunosuppressive factors[15-17].

The TME is usually hypoxic due to a poorly developed tumor vasculature. Most cytotoxic immune cells (CD8+ T cells and NK cells) function poorly in hypoxic states, while suppressive cells (M2 TAMs and Tregs) thrive[18]. The hypoxic TME favors the production of ADO and the induction of immunosuppressive A2A receptors (A2AR) and A2B receptors (A2BR) in immune cells. A shift in tumor metabolism from oxidative phosphorylation to primarily glycolysis also suppresses immune cell infiltration due to increased lactate in the TME. The increase in lactate lowers tumor pH[19]. This decrease in pH drives M2 polarization while inhibiting the nuclear factor of activated T cells (NFAT) within T cells[20,21]. This suppression of NFAT inhibits chemotaxis into tumors and reduces T cell activation.

The role of immune checkpoint inhibitors (ICIs) in cancer immunotherapy

Immune checkpoint inhibitors (ICIs) have greatly advanced immunotherapy, especially in some hard-to-treat tumors. The two most studied checkpoints are CTLA4 and PD-1[1,22,23]. Although antibodies that block these inhibitory signals have improved survival, most patients with solid tumors eventually develop either primary or secondary resistance to ICI. In primary resistance, tumors display early resistance to ICI and progress soon (within six months) after ICI treatment. In secondary resistance, patients respond to treatment initially but develop resistance later[1]. Studies have demonstrated that the tumor mutational burden (TMB) influences the response to ICI[24-26]. Reduced TMB within tumors treated with ICI can result in acquired resistance to ICI immunotherapy.

Contributions to therapy resistance by suppressive state and hypoxia

Suppressive immune cells within the TME contribute to checkpoint inhibitor resistance. Tregs, MDSCs, and M2-TAMs secrete immunosuppressive cytokines (TGF-β, CXCL8, CCL5, and IL-10) that prevent cytotoxic infiltrating immune cells from entering the tumor[27-31]. An increase in VEGF due to the activation of the mitogen-activated protein kinase (MAPK) pathway can also stimulate tumor angiogenesis[27-31] and inhibit immune cell infiltration[32].

Hypoxia results from various physiological and pathological conditions, including solid tumors, ischemia-reperfusion injury, stroke, and chronic obstructive pulmonary disease (COPD)[33]. A hypoxic TME contributes to an increase in extracellular ADO. The molecular mechanisms underlying hypoxia-driven responses include *Adora2a* and *Adora2b* via HIF-1α and HIF-2α[34,35]. HIFs also stimulate angiogenesis, vasodilation, and attenuation of inflammation[34,36]. HIF-1α induces CD73 and CD39 and increases the conversion of ATP into ADO, leading to T cell inhibition, metastasis, and increased angiogenesis[37-40]. The accumulation of ADO within tumor suppresses cytotoxic immune cells and APCs, such as CD8+ T cells and DCs, while enhancing the accumulation of immunosuppressive cells[41,42]. When ADO encounters its receptors, it can affect the activity of neutrophils and macrophages, reducing the release of IL-12, tumor necrosis factor-alpha (TNFα), and ROS[43-45].

The role of cancer-associated fibroblasts (CAFs) in tumor development

Cancer-associated fibroblasts (CAFs) have a different morphology than other cells within the tumor. They lack epithelial, endothelial, and leukocyte markers, and do not have the same mutations as tumor cells[46].

CAF development within tumors occurs when there is an increase in inflammatory markers such as IL-6 and TGF-β due to cancer cell DNA damage[47,48]. When IL-6 and TGF-β are increased in the TME, they tend to reduce the number of T cells, limiting the extent of the antitumor response[49]. These cytokines increase JAK-STAT signaling and ECM transition, promoting CAF formation[50-52]. Breast cancers increase Notch signaling within the TME to increase CAFs[53]. Patients who receive chemotherapy and radiation to treat solid tumors experience DNA breaks, and this stress can promote fibrosis or CAF accumulation and function. This change in CAF function causes resistance to therapy in various solid tumors[54-56].

Solid tumor CAFs may exhibit different phenotypes depending on the TME. The different phenotypes exhibit different cell surface markers, but identifying these can be challenging[46]. Breast cancers increase expression of fibroblast activation protein (FAP) to cause high immunosuppression through Treg activation[57]. Pancreatic cancers express both myofibroblastic CAFs (myCAFs) and inflammatory CAFs (iCAFs) at different locations in the tumor. MyCAFs have high expression of TGF-β and αSMA and are located close to tumor cells, while iCAFs have high IL-6 secretion and are more distal in the TME[58,59]. iCAFs can recruit TAMs and MDSCs to the TME to increase the immunosuppressive state[13,60]. Targeting the MAPK/STAT pathways in iCAFs through inhibitors in combination with checkpoint inhibitor therapy (e.g., anti-PD-1) can lead to increased survival of patients with solid tumors like PDAC[60]. McAndrews *et al.* discovered that in early-stage PDAC, iCAFs tend to be more abundant, while myCAFs have a higher abundance in late-stage cancer. When FAP+ CAFs were depleted, there was an increase in mouse survival. Conversely, when αSMA+ CAFs were depleted, there was a decrease in survival. In the TME, when FAP+ CAFs were depleted, there was a decrease in macrophages and B cells. However, αSMA+ CAF loss showed a decrease in effector T cells (Teff) and increased Tregs. A loss in IL-6 production in FAP+ CAFs increased responses in mice to gemcitabine therapy and combination therapy of gemcitabine + checkpoint inhibitors[57].

THE ADENOSINE PATHWAY: A NEW APPROACH TO OVERCOMING THERAPEUTIC RESISTANCE TO CHECKPOINT INHIBITORS

Understanding the interaction between cells in the TME is crucial to overcoming therapeutic resistance. The concept of targeting ADO biosynthesis or inhibiting its receptors has garnered increased interest from the scientific community[58]. Targeting extracellular ADO and its receptors opens opportunities for increasing the antitumor immune response in innate and adaptive immune cell populations normally suppressed within the TME[59]. Since high ADO levels in the TME are predictive of immunosuppressive responses[60], combining current immunotherapies with ADO blockade may help to overcome ICI resistance in solid tumors.

Chemotherapies and various cancer treatments result in elevated cell death and heightened ATP release[61]. ATP is rapidly converted to ADO within solid tumors. This process is mediated by ectonucleotidases CD39 and CD73 and contributes to the formation of an immunosuppressive TME[62]. CD39 acts on ATP to produce adenosine monophosphate (AMP), which is subsequently converted into ADO by CD73. The resulting extracellular ADO interacts with one of four G-protein-coupled receptors (A1R, A2AR, A2BR, and A3R) found in tumor cells, immune cells, and endothelial cells. This increase in ADO levels within the TME hinders the activity of effector immune cells and promotes the expansion of immunosuppressive regulatory T cells[63]. Exosomes released into the TME during cell death also express CD73 and CD39[62].

The A2AR is associated with elevated levels of checkpoint molecules like PD-1, CTLA4, and LAG-3 on T cells[64]. Activation of this receptor tends to inhibit the antitumor functions of macrophages and the proliferation and cytokine production of cytotoxic T cells[65]. However, increased expression of CD39 and

CD73 within the TME leads to an upsurge in MDSCs and Tregs[59].

The influence of adenosine receptor expression in key immune cells

Innate immunity: the role of adenosine receptors in macrophages, dendritic cells, and natural killer cells

Innate immunity plays a critical role in the body's defense against cancer by providing the first line of protection against malignant cells [Figure 1]. This system, comprising various immune cells such as NKs, TAMs, DCs, and soluble factors like cytokines and chemokines, acts to identify and eliminate transformed cells. Recent studies have highlighted the dynamic interplay between innate immunity and cancer progression, shedding light on the delicate balance between tumor-promoting and tumor-suppressing functions of the innate immune system. An essential aspect of the innate immune system's interaction with cancer cells involves the A2BR, which modulates immune cell functions and significantly impacts the balance between tumor-promoting and tumor-suppressing activities.

Tumor-associated macrophages

TAMs exhibit substantial plasticity that can play a part in tumor progression and drug resistance[66]. The two main classes of TAMs within the TME are activated M1 and alternatively activated M2[67]. M1 is known to be the proinflammatory subset within the tumor, while M2 is considered to be suppressive. However, the classes are not static; the cells can change their state based on the cytokines present. There are also subsets of M2 within the TME, and each class plays a role in tumor formation and progression[67,68].

The TME regulates M1 and M2 macrophages to regulate the immune response to tumors. TAM precursors are derived from embryogenic or bone marrow-derived monocytes[66]. TAMs tend to differentiate primarily into M2-like phenotypes. These cells express high levels of VEGF (pro-angiogenic), mannose receptor (CD206), and scavenger receptor (CD163). They release suppressive cytokines such as IL-10 and promote immunosuppression within the tumor. M1 plays a key role in vaso-proliferation through the secretion of inflammatory cytokines such as IL-6, IL-8, TNF-α, and IL-1β[67,69,70]. The presence of TAMs with high levels of IL-1β within the TME contributes to neovascularization and is a predictor[69].

Apoptotic cells phagocytosed by TAMs (aka efferocytosis) release ATPs into the extracellular space in tissues[71]. ATP derivatives, specifically ADO, affect the immune activation of TAM through ADO receptors[71-73]. The A2BR is upregulated on TAMs in response to interferon-gamma (IFN-γ). When activated, A2BR suppresses the production of TNFα in infiltrating TAMs, inhibiting their capacity to secrete cytokines that are crucial for antitumor immunity. This process ultimately promotes tumor growth[71].

Dendritic cells

DCs are important APCs that present antigens to T cells on MHC proteins. T cells that recognize self-proteins abundant in tumors do not survive selection in the thymus. Intra-tumoral injections of DCs that initiate CD8+ T cell activation have been used to increase responses to immune checkpoint blockade immunotherapies[74-76]. Damage-associated molecular patterns (DAMPs) are used to determine if an immune response needs to be stimulated or if there is immune tolerance to that antigen[74,77]. DAMPs initiate a CD8+ T cell response within tumors but can also help re-prime effector CD8+ T cells to continue the adaptive immune response. However, when tumor cells overcome immune surveillance, DCs may have altered antigen processing and defective T cell activation[78].

Within the TME, there are numerous subsets of DCs along with migratory/tissue-resident DCs[79]. Classical DCs are derived from common myeloid progenitors that differentiate into common DC progenitors. Plasmacytoid DCs (pDC) are believed to be derived from lymphoid cells but can also be derived from

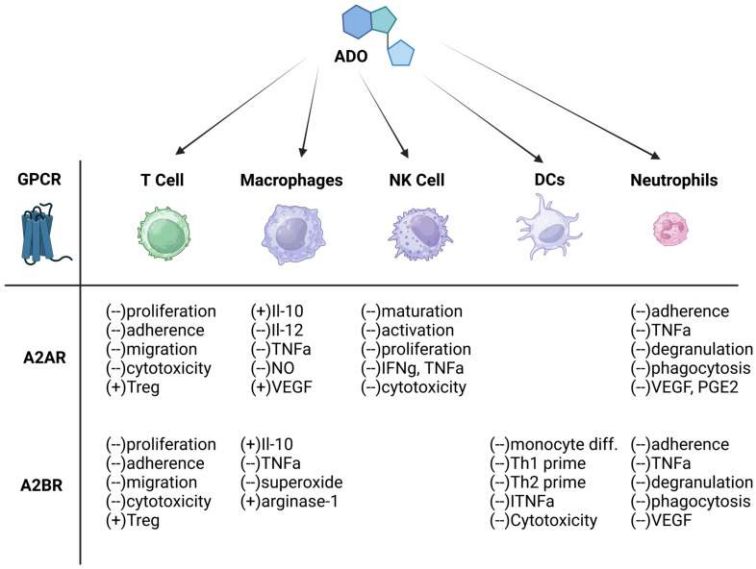

Figure 1. Adenosine's pleiotropic effects on immune cells. ADO facilitates the evasion of tumor cells from immune detection by restricting the activity of T cells, DCs, NK cells, macrophages, and neutrophils. Concurrently, adenosine amplifies the functionality of immunosuppressive cell types like MDSCs and Tregs. ADO: Adenosine; A2AR: A2A receptors; A2BR: A2B receptors; DCs: dendritic cells; MDSCs: myeloid-derived suppressor cells; NK: natural killer; TNFα: tumor necrosis factor-alpha; Tregs: regulatory T cells; VEGF: vascular endothelial growth factor.

myeloid precursors. Common monocyte precursors differentiate into a third major subtype of DCs[80]. Classical DCs have two major states: type 1 and type 2 (cDC1 and cDC2). cDC1 acts to recognize apoptotic and necrotic cell debris presented on its MHC-I receptors to activate CD8+ effector T cells. Their function helps to drive an antitumor response within the TME[75]. cDC2 are more heterogenous than cDC1 cells in tumors but are believed to play a role in recognizing exogenous tumor antigens and presenting them to CD4+ T cells on MHC-II[81]. Within cDC2 populations, there are two further subtypes: anti-inflammatory (cDC2A) and proinflammatory (cDC2B). Classical DCs are now emerging as a potential target for PD-1/PD-L1 immune checkpoint blockade. It has been demonstrated that the proper functioning of checkpoint blockade requires cis interactions with CD80 and PD-L1, as well as PD-1 and PD-L1, between T cells and the DCs[82,83].

DCs that have differentiated during exposure to ADO display diminished activity. Moreover, these DCs express high levels of angiogenic, immunosuppressive, proinflammatory, and tolerogenic factors, such as cyclooxygenase-2 (COX-2), indoleamine 2,3-dioxygenase (IDO), interleukin-6 (IL-6), interleukin-8 (IL-8), IL-10, TGF-β, and VEGF[84,85]. These DCs depend on the upregulation of A2BR when producing these factors to promote ongoing tumor growth and increased angiogenesis for metastasis[85]. As a result, blocking A2BR can preserve the activity of DCs to present neoantigens to T cells, thereby facilitating the process of tumor cell destruction.

NK cells

NKs are derived from CD34+ hematopoietic stem cell progenitors in bone marrow. These cells kill targets that express either no or extremely low MHC-I on their surface[86,87]. NK cells have a specific killer immunoglobulin-like receptor (KIR) on their surface that recognizes MHC-I molecules. When the KIRs recognize MHC-I in self-cells, NK cells are downregulated to prevent an immune response[86,88,89]. Tumor cells tend to have low MHC-I, which can stimulate suppressed NK cells to become activated[90]. NK cells often recognize specific cancer ligands upregulated within tumors, such as MHC-I polypeptide-related

sequence A (MICA), MICB, UL-16 binding proteins, complement factor P, and platelet-derived growth factor DD[91-93].

NK cells may have an important role in tumor immunosurveillance. In some cancers, such as colon cancer and gastrointestinal stromal tumors, low NK cell expression is associated with poor outcomes[94,95]. NK cells can also kill circulating tumor cells that are implicated in metastasis[96]. Tumors may become resistant to NK cell effects by suppressing immune cell activation. Like other immune cells, the hypoxic and low-nutrient environment in tumors can decrease NK cell activation[97]. Tumors increase the interactions of activating ligands with their receptors on NK cells to stimulate later resistance to the cells. This increased stimulation can suppress the NK cell function[98].

When NK cells are activated and encounter adenosine through the A2BR, it triggers the cyclic adenosine monophosphate (cAMP) pathway. This activation subsequently blocks cytotoxic activity and cytokine production, diminishing antitumor activity[99,100]. NK cells can also increase CD73 on their surface after encountering mesenchymal stromal cells, thereby contributing to an increase in ADO and tumor growth[101]. This increase in ADO associated with high levels of A2AR contributes to suppressing NK antitumor function[102,103].

Adaptative immunity - the influence of adenosine receptors on t cell function

Adaptive immunity is the second line of defense in the immune system during infection or cancer. These immune responses are cytotoxic to tumor cells. The main two tumor-infiltrating lymphocyte (TIL) cell populations that mediate adaptive immunity are T cells (CD4+ and CD8+) and B cells. T cell infiltration within tumors depends on the tumor chemokine profile and how easily immune cells can enter through the tumor extracellular matrix. Over the last decade, improved methods have been developed to engineer T cells to be better at avoiding cancer immunosuppression. These techniques have resulted in clinical trials of blood-derived tumors and some sarcomas. Very little progress has been made for solid tumors. However, targeting the A2AR on T cells may help overcome the difficulties of T cell immunotherapies in solid tumors. Since ADO causes an increase in Tregs among infiltrating T cells, blocking A2AR can help maintain a high amount of CD4+ T cells in solid tumors. Relative to T cells, B cells have very low levels of A2BR, and limited studies have investigated its role in A2BR activation from ADO[104]. A2AR are more abundant in human than mouse B cells, but their role in immunotherapy is unknown.

T cells

T cells have been the main target of immunotherapy. CAR T cells and immune checkpoint inhibitors have been used to enhance T cell-mediated tumor killing. Dangaj *et al.* demonstrated that CCL5 must be present within the TME for TILs to enter solid tumors. The macrophages and DCs within the tumor also need to produce CXCL9 to aid in T cell infiltration[105]. Anti-PD-1 therapies have only shown limited success in solid tumors. In metastatic head and neck squamous cell carcinoma, only 15% of patients responded to anti-PD-1 treatment, and very few responses have been seen in microsatellite-stable colorectal cancer[106,107]. Duhen *et al.* discovered a subset of CD4+ T cells in tumors that are double positive for PD-1 and inducible costimulator (ICOS)[106]. These cells can have a tumor tissue-resident phenotype that allows them to recognize both tumor antigens and neoantigens on MHC-II. CD8+ TILs, on the other hand, are more heterogeneous in their response to tumor antigens[108]. The presence of PD-1 and ICOS on CD4+ T cells may work in conjunction with CD8+ T cells to stimulate a robust antitumor response.

Co-expression of CD39 and CD103 on CD8+ TIL within solid tumors has shown promise in targeting tumor cells. CD8+ T cells that have high expression of CD39 and CD103 can be identified in both primary

tumors and metastatic sites but not in the periphery[109]. The level of CD39 and CD103 double positive (DP) cells determines how well patients will respond to immunotherapy[110,111]. However, tumors can still escape these DP TILs through exhaustion mechanisms. All DP TILs express high levels of PD-1 and other exhaustion markers[112]. Checkpoint inhibitors may be useful, but there are also additional ADO pathways linked to CD39+ cells. Blocking the ADO pathway and using immune checkpoint inhibitors may help keep the DP CD8+ T cells active and prevent tumor growth.

Tumor cells increase their expression of CD39 to suppress both CD4+ and CD8+ T cell proliferation and cytotoxicity in the TME[100]. Activation of A2AR in T cells causes increased CD4+ differentiation into Treg cells. There is also an increase in additional suppressive receptors such as PD-1, LAG-3, CTLA4, and T cell immunoglobulin and mucin domain-containing protein 3 (TIM3) on the T cells. Increases in ADO may have a negative effect on immunotherapies when checkpoint inhibitors are given to patients alone[113].

The importance of ADO biosynthesis in the TME

Extracellular ADO is found at low levels in unstressed tissues[114]. It is produced in response to the breakdown of adenine nucleotides and AMP outside injured cells [Figure 2][115]. In response to cancer initiation, ADO levels rapidly increase within tissues due to hypoxic, inflammatory, and/or ischemic conditions. Stressed cells release ATP into the extracellular space as a distress signal that transiently signals via P2 purinergic receptors. Ectoenzymes CD39 and CD73 can rapidly break down extracellular ATP on cell surfaces to produce extracellular ADO[116,117]. Initially, CD39 converts ATP into adenosine diphosphate (ADP) and AMP, followed by CD73-mediated conversion of AMP into ADO[118]. The accumulation of ADO in the TME helps create the suppressive niche.

Inhibition of critical immune mechanisms stimulates the formation of the pro-adenosine niche and fibrotic remodeling

The formation of solid tumors in tissues begins with an increase in cell death, inflammation, and hypoxia. This leads to an increase in extracellular ATP and ADO within the TME. When the proinflammatory metabolite extracellular ATP is cleaved into extracellular ADO and is recognized by the A2AR and A2BR within tumors, there is suppression of immune functions[59]. Endothelial cells within the forming tumor and infiltrating immune cells express CD39 and CD73 on their surface. This allows for an increase in ADO within the TME. Endothelial and immune cells also express A2BR on their surface, and when activated by ADO, the tumor can suppress immune cell infiltration. Solid tumors become hypoxic, which feeds back to increase ATP, CD73, and CD39 in the TME to further suppress the immune infiltration[115]. Tumor cells interact with suppressive immune cells to increase A2BR expression, leading to metastasis, proliferation, and VEGF production[119].

CAF increases within solid tumors, forming a dense tumor stroma. These CAFs express high levels of CD39 and CD73 on their surface in various solid tumors such as ovarian, pancreatic, colorectal, and breast cancer, which contribute to ADO production[120-122]. A dense fibrotic stroma allows ADO to remain in high concentration to drive immunosuppressive signaling throughout the tumor. An increase in A2BR on CAFs increases the secretion of IL-6 into the TME, which can convert epithelial cells to a more mesenchymal phenotype[63]. This remodeling of the TME leads to increased metastasis and therapy resistance.

Increases in the ADO pathway cause resistance to immune checkpoint inhibitor therapies

Immune checkpoint inhibitors such as anti-PD-1 and anti-CTLA4 have shown great promise for improving the survival of patients with solid tumors. This form of therapy targets PD-1 and CTLA4 on CD8+ T cells. Tumor cells inhibit CD8+ T cell function by targeting these checkpoint molecules. By blocking PD-1 and

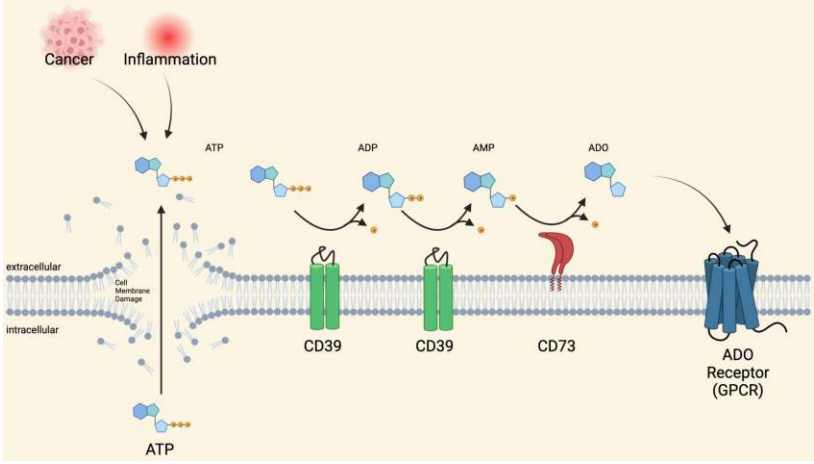

Figure 2. Adenosine Biosynthesis in Inflamed Tissues. The accumulation of extracellular ATP, driven by stress-induced conditions, stimulates extracellular ADO production by the enzymes CD39 and CD73. ADO binds four G protein-coupled receptors, A1, A2A, A2B, and A3. ADO: Adenosine; ADP: adenosine diphosphate; AMP: adenosine monophosphate; ATP: adenosine triphosphate.

CTLA4 from being recognized, the cytotoxic function of the CD8+ T cells is increased to clear the tumor cells[113]. However, patients with solid tumors tend to relapse and become resistant to checkpoint therapy. Maj *et al.* discovered that when checkpoint therapy is given, there is an increase in the death of cancer cells and Tregs. The sudden death of these cells releases a high amount of ATP in the TME, which is then converted to adenosine by CD39 and CD73. This increase in ADO in the TME counteracts checkpoint therapy and suppresses the antitumor immune response[123].

While an increase in CD73 and CD39 is associated with poor prognosis in patients, increasing A2AR and A2BR expression also contributes to an increased risk of resistance to checkpoint therapy. It has been found that having an increase in A2AR in non-small cell lung cancer (NSCLC) or A2BR in triple-negative breast cancer (TNBC) contributes to poor survival[119,124]. Chalmin *et al.* showed that the adenosine pathway is involved in resistance to anti-PD-1 therapies. They demonstrated that when patients were given checkpoint therapy, there was an increase in CD73, leading to resistance[125]. Combination therapies targeting CD73, and checkpoint inhibitors may help overcome early resistance in solid tumors. Studies have shown that targeting CD73 and PD-1 in murine colon tumors can inhibit tumor growth[126]. The idea that targeting both the adenosine pathway and checkpoint inhibitors may overcome resistance in solid tumors gives promise to advancing immunotherapy.

Endothelial cells increase CD39 and CD73 levels during hypoxia in tumors, leading to angiogenesis

Hypoxia occurs in solid tumors and suppresses immune cell infiltration by activating hypoxia-inducible factor 1/2 (HIF1/2), IL-6, TGFβ, and TNF. Under these conditions, endothelial, tumor, and various suppressive immune cells increase CD73 and CD39 to increase ATP conversion to AMP and ADO[39,127]. Tumor cells that increase CD73 expression can generate ADO to interact with A2ARs on the tumor cells to stimulate an increase in VEGF secretion[128]. VEGF works to increase angiogenesis within the tumor and provides nutrients and oxygen for growth and metastasis [Figure 3].

Endothelial cells express CD39 within tumors to degrade ATP and promote increased neovascularization and tumor growth[129]. To have the ADO concentration needed to sustain the tumor-protective endothelial barrier, CD73 and A2BR are needed within the TME[130]. Feng *et al.* and Sun *et al.* demonstrated that inhibiting CD39 on solid tumor endothelial cells decreased angiogenesis and tumor growth[129,131]. CD39 on endothelial cells and the vascular is highly expressed within pancreatic and rectal carcinoma. High

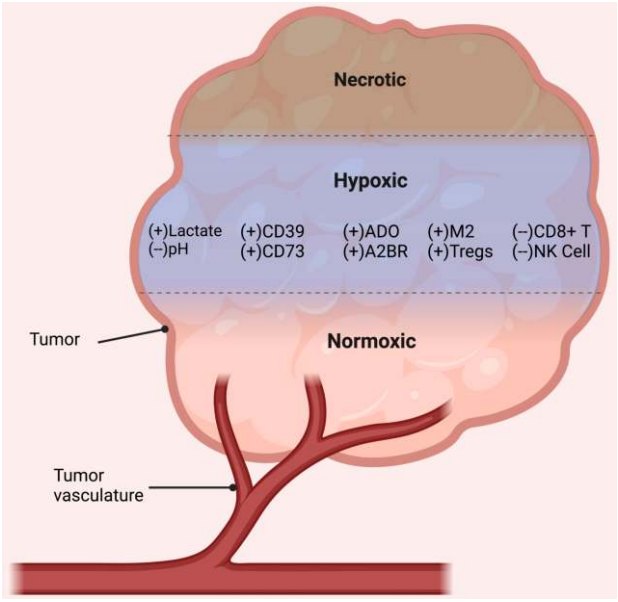

Figure 3. Hypoxia in the TME. This figure illustrates a region of a tumor devoid of vasculature, leading to an oxygen-starved environment. This hypoxic zone is characterized by low pH and a predominance of lactate. Within this environment, TAMs tend to adopt an M2 phenotype, and Tregs become the predominant T cell population. Hypoxic conditions also encourage the expression of the ectoenzymes CD39 and CD73, leading to a surge in extracellular ADO. This increase in adenosine, in turn, helps to sustain an immunosuppressive environment within the tumor. Note: this illustration does not depict the anatomical structure of the tumor, but rather represents the phenomena occurring at different levels of tumor oxygenation. ADO: Adenosine; A2BR: A2B receptors; NK: natural killer; TAM: tumor-associated macrophage; TME: tumor microenvironment; Tregs: regulatory T cells.

expression of these cells in these tumors is correlated with early TNM and better survival after tumor resection[132,133]. Studies have shown that having high levels of CD73 in many solid tumors is associated with worse outcomes. High CD73 levels correlate with higher adenosine concentrations in the tumor, leading to a sustained immunosuppressive TME. With a high expression of both CD39 and CD73 within solid tumors, future combination therapies targeting CD39/CD73, PD-1/CTLA4, and A2BR may allow for better survival in patients.

ADO receptors play various roles in cancer growth

An increase in adenosine within the TME allows for immunosuppression that promotes tumor growth. Receptors for ADO on tumor cells, endothelial cells, and immune cells are drivers of tumor growth and metastasis. The ADO G-protein coupled receptors have four subtypes: A1, A2A, A2B, and A3 [Figure 4]. The A1R, A2AR, and A2BR proteins are highly conserved, while the A3R varies among species. These receptors interact with MAPK pathways to promote proliferation. A2AR and A2BR also increase activation of the mTOR and ERK pathways[134]. However, receptor signaling is dependent on the concentration of extracellular ADO. This level of ADO is primarily dictated by ATP and ADP metabolism by CD39 and CD73 on cells to make AMP and then ADO. In the following, we will focus only on A2AR and A2BR, which appear to play major roles in tumor immunosuppression.

A2AR and A2BR increase immunosuppression in solid tumors

Both A2AR and A2BR are involved in ADO's immunosuppressive functions. A2AR and A2BR are expressed in most immune and tumor cells[59,135]. A2BR on myeloid cells normally has lower expression than A2AR on other immune cells. However, this receptor increases in the presence of pathological responses such as infections or cancer[136]. A2BR upregulation and activation signal macrophages towards the

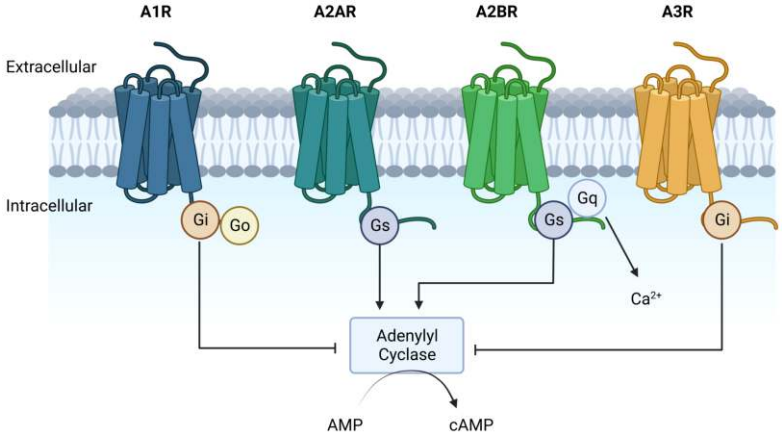

Figure 4. Adenosine receptors. Adenosine interacts with four distinct receptors - A1R, A2AR, A2BR, and A3R. Each of these receptors is linked to a G protein-coupled receptor. The A1R and A3R couple with the Gi protein to inhibit Adenylyl Cyclase. In contrast, the G protein coupled to A2AR and A2BR activates Adenylyl Cyclase, leading to an increased formation of intracellular cyclic AMP. A2BRs also couple to Gq which mobilizes Ca^{2+} upon activation. AMP: Adenosine monophosphate; A2AR: A2A receptors; A2BR: A2B receptors; cAMP: cyclic adenosine monophospha.

suppressive M2-like phenotype and secrete IL-10 and VEGF to promote angiogenesis and tumor growth[43]. A2ARs on infiltrating T cells within the TME are activated when ADO increases in the surroundings. This activation suppresses the effector CD8+ T cells in the TME while signaling CD4+ T cell differentiation into Tregs[137-139]. IFN-γ production decreases in NK cells when the A2AR on these cells is activated by ADO[99]. Decreases in antitumor cytotoxic cells and cytokines allow for increased suppressive factors that drive tumor growth and resistance to antitumor therapies.

A2BR activation due to TME hypoxia works to maintain the epithelial barrier of the tumor[39]. Maintaining this barrier prevents antitumor immune cells from penetrating the tumor. A2AR and A2BR-mediated immunosuppression also allow for an increase in metastasis amongst solid tumors[58].

A2BR is key for immunosuppression in solid tumors caused by ADO

Since A2BRs are expressed at low levels under normal conditions, this receptor may be key for triggering immunosuppression within solid tumors. The A2BR has the lowest potency for ADO under normal physiological conditions. However, during inflammation and sudden increases in apoptosis, the A2BR is activated to create an immunosuppressive niche. ADO drives immunosuppression in solid tumors by binding to the A2BR on immune and tumor cells. Once activated, the A2BR increases the secretion of VEGF and IL-8 into the TME[140,141]. This secretion from immune cells comes mainly from monocyte-derived immune cells. When activated by ADO and A2BR interactions, these cells contribute major driving factors in tumor immunosuppression. Ben Addi *et al.* discovered that the A2BR, not the A2AR, on bone marrow-derived DCs decreased the production of IL-12p70 in mice[84]. A2BR knock-out lung carcinoma cells produced lower VEGF levels in this model when stimulated with adenosine than wildtype controls[140].

Hypoxia increases A2BR expression

As described earlier, hypoxia is a hallmark of solid tumors. Recent studies have expanded our understanding of the role of hypoxia in solid tumors, emphasizing its impact on angiogenesis and tumor growth and its influence on other aspects of tumor biology, such as immune evasion and therapy resistance[18,142]. Hypoxia-driven upregulation of A2BRs and ADO signaling contributes to tumor progression, angiogenesis, and immune escape[44,113].

The hypoxic response also modulates the immune system, affecting innate and adaptive immunity. Hypoxia can alter the functions of immune cells such as TAMs, neutrophils, DCs, T cells, and NK cells[18,143]. Hypoxia-driven changes in the TME, for example, can create an immunosuppressive milieu, impairing the ability of immune cells to target and eliminate cancer cells[144]. Novel therapeutic strategies targeting hypoxia and adenosine signaling pathways, including A2BRs, are currently being investigated to improve the efficacy of existing cancer treatments and overcome treatment resistance[14].

The role of ADO and its receptors blockade to overcome resistance

ADO and its receptors are critical in maintaining immunosuppression in the TME, contributing significantly to immunotherapy resistance. Several clinical trials are exploring the potential of ADO receptor blockade as a novel strategy to counteract this resistance.

Several A2AR antagonists are currently being explored in clinical trials [Table 1], and two of the more advanced therapies are discussed further as part of this review: Corvus Pharmaceutical's ciforadenant and AstraZeneca's AZD4635[145].

In a first-in-human Phase 1 dose-escalation study in patients with advanced refractory cancers (NCT02655822), ciforadenant (either monotherapy or in combination with atezolizumab) was administered to 502 patients. Of those, a cohort of 68 renal cell carcinoma (RCC) patients yielded clinical responses, including partial responses (PR) in 11% of patients treated with a combination of A2AR antagonists and anti-PD-L1 antibodies, and in 3% of patients treated with A2AR antagonists alone[146]. Further, tumor regression was observed in an additional 24% of patients, although the regression was not significant enough to be classified as PR by RECIST criteria. These findings are noteworthy, especially considering that the patients involved in the study were not only resistant to PD-1 blockade but were also deemed untreatable prior to the trial.

According to Michail Sitkovsky[147], the observed tumor regressions in patients with RCC, who were previously untreatable and refractory to PD-1 blockade, likely occurred in patients meeting specific criteria: their tumors were immunogenic, developed tumor-reactive effector T cells, retained a significant number of effector cells post-toxic cancer chemotherapies, and were protected by immunosuppressive extracellular ADO to A2AR signaling. Ciforadenant appears to have facilitated the invasion and tumor-rejecting functions of T and NK cells in these patients; however, the levels of antitumor immunity in responsive patients were not high enough to achieve a complete response. The major limitation appears to be the lack or low numbers of tumor-reactive T and NK cells in refractory patients, either due to the tumor's poor immunogenicity or past toxic chemotherapies.

A2AR antagonists are anticipated to be most efficacious in patients with sufficient aggressive, multifunctional tumor-reactive T cells. Without these cells, it could be expected that A2AR antagonists would only have antitumor effects when combined with cancer vaccines or T-cell transfers that increase the number of tumor-reactive T cells. Future treatments combining A2AR antagonism with adoptive cell transfer (ACT) are promising, especially for refractory patients, as ACT ensures the presence of sufficient T-cells and NK-cells in patients, enhancing the potential for A2AR antagonism as an immunotherapy.

These antagonists have been able to show, both *in vitro* and *in vivo*, that blocking the adenosine pathway at the A2AR increases cytotoxic T cells within the TME, increases cytokine production, and reverses T cell inhibition.

Table 1. List of current clinical trials evaluating A2AR and A2BR antagonists alone or in combination with cancer immunotherapies

Drugs	Combinations	Clinical trial information				
		Phase	Indications	Enrollment	NCT number	Completion date
EOS448 (A2AR antagonist)	· EOS-448, a small molecule, combined with pembrolizumab, an anti-PD-1 antibody · EOS-448 combined with inupadenant, an investigational adenosine A2AR antagonist · EOS-448 combined with dostarlimab, an anti-PD-1 antibody · Inupadenant combined with dostarlimab · EOS-448 combined with inupadenant and dostarlimab · EOS-448 combined with dostarlimab and standard-of-care chemotherapies in participants with NSCLC	1/2	Lung/H&N cancers/Melanoma	376	NCT05060432	2024-09
NIR178 (Taminadenant)	· DFF332, a small molecule targeted to HIF-2α · DFF332 in combination with everolimus, an mTOR inhibitor · DFF332 in combination with spartalizumab (an anti-PD-1 antibody) plus taminadenant (A2AR antagonist)	1	RCC	180	NCT04895748	2025-04
TT-10 (A2AR antagonist)	· TT-10, a small molecule, as a single agent	1/2	Prostate/NSCLC/RCC	90	NCT04969315	2025-08
ILB2109 (A2AR antagonist)	· ILB2109, a small molecule, as a single agent	1	Advanced solid tumor	48	NCT05278546	2024-01
AZD4635 (A2AR antagonist)	· AZD4635 as monotherapy · Combination with durvalumab · Combination with durvalumab plus oleclumab · Combination with docetaxel · Combination with either abiraterone acetate or enzalutamide	1	Solid tumor	313	NCT02740985	2021-04
AZD4635 (A2AR antagonist)	· AZD4635 with durvalumab, an anti-PDL-1 antibody · AZD4635 with oleclumab, an anti-CD73 antibody	2	Prostate tumor	59	NCT04089553	2023-04
CPI-444 (A2AR antagonist)	· CPI-444, a small molecule, in combination with ipilimumab, an anti-CTLA4 antibody · CPI-444 in combination with nivolumab, an anti-PD-1 antibody	1/2	RCC	15	NCT05501054	2026-11
CPI-444 (A2AR antagonist)	· CPI-444 (ciforadenant) as a single · Combination with atezolizumab, a PD-L1 inhibitor	1	RCC	502	NCT02655822	2021-07
PBF-1129 (A2BR antagonist)	· Combination of adenosine A2BR antagonist PBF-1129 (mAb) and nivolumab, an anti-PD-1 antibody	1	Metastatic NSCLC	30	NCT05234307	2025-12
	· PBF-1129 as a single agent	1	Metastatic NSCLC	18	NCT03274479	2023-12
M1069 (Dual A2AR/A2BR antagonist)	· M1069, a small molecule, as a single agent	1	Unresectable solid tumors	30	NCT05198349	2023-12
AB928 (Dual A2AR/A2BR antagonist)	· Combination of SRF617, an anti-CD39 antibody, etrumadenant (AB928), and zimberelimab (AB122), an anti-PD-1 antibody	2	Prostate cancer	15	NCT05177770	2023-04
TT-4 (A2BR antagonist)	· TT-4, a small molecule, as a single agent	1/2	GI cancers	69	NCT04976660	2023-09

Data obtained from ClinicalTrials.gov. A2AR: A2A receptors; A2BR: A2B receptors; CTLA4: T lymphocyte antigen 4; GI: gastrointestinal; H&N: Head & Neck; NSCLC: non-small cell lung cancer; RCC: renal cell carcinoma.

Another A2AR antagonist in development is AstraZeneca's AZD4635. In a monotherapy phase 1 trial (NCT02740985), observed adverse events included nausea, fatigue, and vomiting. In addition, one patient with colorectal cancer had sudden death 15 days after the last dose of AZD4635, which was considered treatment-related by the investigator. However, AZD4635 was well tolerated both as a monotherapy and in combination with durvalumab in all patients. There were patients with responses such as stable disease, partial response, and complete response and RNA analysis confirmed that in 5 of 7 patients, intertumoral adenosine signaling decreased. Four of these 7 patients also had increases in gene-expression signatures of cytolytic activity and IFN-γ signaling. These findings suggest that there were observable positive responses to the treatment in some patients.

A2BR antagonists are also under investigation in several clinical trials. Arcus' etrumadenant, a dual A2AR and A2BR antagonist, is being evaluated in several cancers and was recently discontinued in mCRPC due to a lack of efficacy (NCT05177770). Palofarma's PBF-1129, an A2BR antagonist, is being evaluated in metastatic NSCLC (NCT03274479) and EMD Serono's M1069 (NCT05198349), another A2AR and A2BR antagonist, is currently undergoing a first-in-human trial in patients with advanced malignancies. In addition, Portage is evaluating TT-4, an A2BR antagonist, as a single agent in gastrointestinal cancer (NCT04976660). Further investigation will likely provide more insights into the clinical potential of these promising strategies. Overall, compounds exhibiting the highest water solubility tend to possess increased bioavailability, making them more effective[148]. With the advancement in the development of ADO receptor antagonists, enhancing the solubility of these promising compounds while preserving their selectivity emerges as an avenue for improvement.

CONCLUSION

In the complex battleground of cancer, it is necessary to understand the adaptations tumors employ to resist therapies. This review has emphasized the role of ADO, a significant player in the TME, in driving immunosuppression and fostering cancer drug resistance. The importance of ADO and its receptors, particularly the A2AR and A2BR subtypes, in promoting an immune-escaping environment was thoroughly explored.

Current research endeavors focus on various approaches to counteract immunosuppression, including monoclonal antibodies against CD73 and the blockade of ADO receptors. Ongoing clinical trials investigate combinations of these approaches with existing therapies, aiming to stimulate immune responses and improve patient outcomes.

The results of ongoing clinical trials will inform new ways of overcoming cancer drug resistance. However, further research is required to understand and fully exploit ADO's pathway. Targeting ADO could improve cancer treatments, providing hope for patients who previously had limited treatment options. This underscores the importance of ongoing research in this area, aiming to improve the prognosis for all cancer patients.

DECLARATIONS

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Authors' contributions

Made substantial contributions to the conception, outline, and writing: Remley VA, Dimastromatteo J
Performed critical review/editing for scientific accuracy: Linden J, Bauer TW

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Conflicts of interest

Drs. Dimastromatteo and Linden are members of Adovate, a for-profit company dedicated to improving patient health using a new generation of adenosine analogs.

Ethical approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

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Cancer Drug Resistance

Review

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Immune checkpoint inhibitors in breast cancer: development, mechanisms of resistance and potential management strategies

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Abstract

The use of immune checkpoint inhibitors (ICIs) has increased exponentially in the past decade, although its progress specifically for breast cancer has been modest. The first U.S. Food and Drug Administration approval for ICI in breast cancer came in 2019, eight years after the first-ever approval of an ICI. At present, current indications for ICIs are relevant only to a subset of patients with triple-negative breast cancer, or those displaying high microsatellite instability or deficiency in the mismatch repair protein pathway. With an increasing understanding of the limitations of using ICIs, which stem from breast cancer being innately poorly immunogenic, as well as the presence of various intrinsic and acquired resistance pathways, ongoing trials are evaluating different combination therapies to overcome these barriers. In this review, we aim to describe the development timeline of ICIs and resistance mechanisms limiting their utility, and summarise the available approaches and ongoing trials relevant to overcoming each resistance mechanism.

Keywords: Immunotherapy, immune checkpoint inhibitors, resistance mechanisms, breast cancer

INTRODUCTION

Breast cancer is the most common cancer worldwide, accounting for 12.5% of all new cancer cases globally, and is the leading cause of cancer mortality in women[1]. In the year 2020, an estimated 2.3 million female breast cancers were diagnosed globally, and about 685,000 women died from their disease[2]. This number is expected to grow to more than 3 million new cases diagnosed and 1 million deaths by the year 2040[2].

With advances in our understanding of cancer biology, immuno-oncology has become an area of great interest and extensive research. Cancer immunotherapy employs the use of cutting-edge technologies, including immune checkpoint inhibitors (ICIs) such as those targeting Programmed Cell Death Protein-1 (PD-1), Programmed Cell Death Ligand-1 (PD-L1), and Cytotoxic T-Lymphocyte-Associated Antigen 4 (CTLA-4), and more recently, chimeric antigen receptor (CAR) T cell therapies. Other frontiers being pushed in the realms of immunotherapy include the use of cancer vaccines[3], for cancer prevention, such as vaccines for Human Papilloma Virus and Hepatitis B[4], as well as in cancer treatment, as in the case of Sipuleucel-T for prostate cancer[5].

Since the first U.S. Food and Drug Administration (FDA) approval of ipilimumab, a CTLA-4 monoclonal antibody, in 2011 for the treatment of metastatic melanoma[6], ICIs have transformed the treatment landscape across multiple tumour types[7]. There are now eleven FDA approvals for ICIs: two CTLA-4 inhibitors (ipilimumab, tremelimumab), five PD-1 inhibitors (pembrolizumab, nivolumab, cemiplimab, dostarlimab, retifanlimab), three PD-L1 inhibitors (atezolizumab, avelumab, durvalumab), and one lymphocyte-activation gene 3 (LAG-3) blocking antibody (retatlimab)[6-9]. However, amongst the numerous available approvals for ICIs, there are currently only two specific FDA approvals in the setting of breast cancer, both for pembrolizumab in the subgroup of triple-negative breast cancer (TNBC), given in combination with chemotherapy in the metastatic[10] and neoadjuvant[11] settings. Additional FDA approvals that are tumour agnostic and apply to breast cancer include pembrolizumab[12] and dostarlimab[13] in breast cancers displaying high microsatellite instability (MSI-H) or a deficiency in the mismatch repair protein (dMMR) pathway. While one of the hallmarks of treatment with ICIs is its durable response that translates to prolonged survival of these patients, admittedly, only a very small subset of patients benefit. In this review article, we will first describe the evolution of ICI in the TNBC subtype, focusing on its approved indications, before delving into the understanding of the resistance mechanisms towards ICIs, and how we can harness such knowledge to develop new combination strategies.

EVOLUTION OF ICIS IN BREAST CANCER

Monotherapy ICIs in TNBC

Evidence for the use of ICIs in breast cancer first came from single-agent immunotherapy trials in the metastatic setting, including the KEYNOTE-012 and KEYNOTE-086 studies. KEYNOTE-012 was a phase I study that aimed to evaluate the role of single-agent pembrolizumab in patients with various advanced solid tumours. In the cohort of TNBC who had progressed on a median of 2 lines of treatment, the objective response rate (ORR) was 18.5% and 6-month progression-free survival (PFS) was 24.4%, 6-month and 12-month overall survival (OS) were 66.7% and 43.1%, respectively[14]. The investigators observed that there was a suggestion of response with increasing expression of PD-L1, albeit within a small sample size (n = 32).

KEYNOTE-086 was designed specifically to look at the role of pembrolizumab monotherapy in patients with metastatic TNBC. This phase II multicohort study included all comers with ≥ 1 prior systemic treatment for metastatic disease regardless of PD-L1 status (Cohort A)[15], and also patients with no prior systemic treatment in the metastatic setting who had PD-L1 positive tumours defined as combined positive score (CPS) ≥ 1 based on the Dako PD-L1 IHC 22C3 platform (Cohort B)[16]. Comparing across cohorts,

there was a suggestion of improved ORR in less heavily pre-treated patients (ORR 21.4% *vs.* 5.3%) in Cohort B *vs.* all-comers in Cohort A. This was consistent with other similar phase 1 trials evaluating avelumab (JAVELIN study)[17] and atezolizumab (PCD4989g trial)[18] as monotherapy in metastatic TNBC, suggesting clinical benefit when used in earlier lines of treatment and PD-L1 expressing tumours.

A subsequent KEYNOTE-119 randomised phase III trial compared pembrolizumab monotherapy *vs.* single agent physicians' choice chemotherapy in patients who progressed on 1 or 2 prior lines of treatment for metastatic TNBC[19]. While the trial was negative for its primary endpoint of OS in all subgroups, there was a positive trend to survival benefit in patients with PD-L1 CPS ≥ 10 (12.7 *vs.* 11.6 months, HR 0.78; $P = 0.057$).

The limited efficacy of single-agent immunotherapy observed in breast cancer might be due to intrinsic tumour resistance due to its complex and enigmatic relationship with the immune system. Breast cancer was traditionally thought to be poorly immunogenic, also known as a "cold tumour". Immunogenicity, or the ability to elicit an antitumoural response by the body's immune system, is dependent upon the formation of neo-antigens that are derived from gene mutations, viral oncogenes alternative splicing, or gene rearrangement[20-22]. It is assessed by the antigenicity of a cancer, which in turn is evaluated by its mutagenicity[23]. One measure of the antigenicity of cancer is its mutational load or tumour mutational burden (TMB), which refers to the average number of somatic mutations per (Mb)[23,24]. Cancers like melanoma and lung cancer are known to be "hot tumours", as observed in a study by Chalmers *et al.* who reported their median TMB levels to be 13.5 mut/Mb and 7.2 mut/Mb, respectively[25]. In contrast, the TMB in breast cancer is generally much lower. In a study by Barroso-Sousa *et al.*[26] of 3,969 patients with breast cancer, the median TMB reported was 2.63 mut/Mb, while another Chinese study of 196 breast cancer patients demonstrated a higher median TMB of 4.03 mut/Mb[27]. Due to the poor efficacy observed with the use of single-agent immunotherapy treatment, further efforts were directed at exploring combination treatment.

Combining ICIs with chemotherapy in TNBC

The rationale for combination treatment with chemotherapy was that chemotherapeutic agents had been shown to have synergistic effects with ICIs by inducing immunogenic cell death, causing the release of tumour-associated neoantigens as well as its ability to stimulate immune surveillance[28,29]. Indeed, this has proven to be an effective strategy in several subgroups of TNBC.

The initial results of several phase I studies evaluating this combination in the setting of metastatic TNBC were promising, reporting response rates ranging between 23.4%-39%[30,31]. Several phase III trials confirmed these positive preliminary findings, leading to the first FDA-approved indication for an ICI for use in breast cancer treatment.

Atezolizumab

The first FDA accelerated approval of an ICI for breast cancer was with the anti-PD-L1 inhibitor atezolizumab, which was granted on 8 March 2019[32] based on the IMpassion 130 trial[33]. This phase III placebo-controlled randomised trial evaluated 902 patients with treatment naïve, unresectable locally advanced or metastatic TNBC. Patients were randomised to receive either atezolizumab or its placebo, in combination with albumin-bound paclitaxel (nab-paclitaxel). In patients whose tumours expressed PD-L1 based on the VENTANA PD-L1 SP142 assay, there was a significant median PFS benefit: 7.5 months in patients receiving atezolizumab *vs.* 5.0 months with placebo (HR 0.62, $P < 0.001$). The final approval of this combination was contingent upon the results of the IMpassion 131 trial evaluating atezolizumab with paclitaxel in TNBC in the same setting, which unfortunately failed to meet its primary endpoint of superior

PFS[34]. This led to Genentech voluntarily withdrawing the previously granted accelerated FDA approval for atezolizumab on 27 August 2021. Eventually, when the final OS was read out for the IMpassion 130 trial, the addition of atezolizumab to nab-paclitaxel failed to meet statistical significance, precluding further testing[35].

Pembrolizumab

Pembrolizumab is currently FDA-approved for use in TNBC in the first-line metastatic and neoadjuvant settings, both in combination with chemotherapy. It first received FDA approval on 13 November 2020 as combination therapy with chemotherapy for patients with unresectable locally-advanced or metastatic TNBC whose tumours have a PD-L1 CPS ≥ 10 based on the Dako 22C3 assay[10]. This was based on KEYNOTE-355, a phase III randomised placebo-controlled study evaluating the role of pembrolizumab in combination with chemotherapy in patients in the above-mentioned setting. It reported a median OS (mOS) benefit of about 7 months in patients whose tumours expressed PD-L1 CPS ≥ 10 (mOS 23.0 *vs.* 16.1 months; HR 0.73, $P = 0.0185$). In patients whose tumours expressed PD-L1 CPS ≥ 1 or in the intention-to-treat population, there was no survival benefit shown.

In addition, pembrolizumab also has tumour-agnostic FDA approval for advanced unresectable or metastatic solid tumours that are dMMR or MSI-H[12]. This was based on the combined results of 5 single-arm trials where a total of 149 patients with dMMR/MSH-H solid tumours achieved an ORR of 39.6%, with 78% of patients having responses lasting 6 months or more. It should be noted, however, that only 2 out of the 149 patients had breast cancer. They both achieved partial responses, with duration of response (DoR) of 7.6 and 15.9 months[36].

Dostarlimab

Most recently, on 17 August 2021, dostarlimab also received accelerated FDA approval for recurrent or advanced solid tumours that are dMMR based on the GARNET trial[13]. This was an open-label, non-randomised, multicohort phase I trial evaluating dostarlimab as monotherapy in the above-mentioned clinical setting. In these patients, there was an ORR of 41.6%, with 9.1% complete responses and 32.5% partial responses. The median DoR was 34.7 months, with 95.4% of patients still showing continued response at 6 months. In cohort F, which enrolled 106 non-endometrial solid tumours, 1 patient had dMMR breast cancer and reported a complete response[37].

With the promising results of a combination of ICI therapy and chemotherapy in the metastatic setting, efforts were then shifted to study it in the earlier curative stages of breast cancer. One of these trials is the phase II I-SPY 2 trial, which adopted an adaptive trial design to evaluate various novel therapeutics in combination with chemotherapy, comparing that to standard treatment as in the neoadjuvant setting for early-stage breast cancer[38]. Pembrolizumab was included in one of the study arms, where patients were randomised to receive 4 cycles of pembrolizumab given in combination with weekly paclitaxel *vs.* weekly paclitaxel alone, followed by doxorubicin/cyclophosphamide and then definitive surgery. Compared to standard chemotherapy alone, the addition of pembrolizumab improved pathologic complete response (pCR) rates in all breast cancer subtypes: 44% *vs.* 17% in HER2-negative breast cancers, 30% *vs.* 13% in HR-positive/HER2-negative breast cancers, and 60% *vs.* 22% in TNBC[39].

Focusing on the TNBC subtype, the role of pembrolizumab in the neoadjuvant setting was proven in the confirmatory phase III KEYNOTE-522 trial, which subsequently led to pembrolizumab receiving its second breast cancer-specific FDA approval on 26 July 2021[11]. In this phase III randomised controlled study, 1,174 patients with previously untreated stage II or III TNBC were randomised in a 2:1 ratio to receive pembrolizumab or a placebo, respectively, in combination with chemotherapy , before undergoing surgery.

Pembrolizumab or its placebo was continued post-operatively for up to 9 cycles. Both primary endpoints of the trial were met; there was a significant improvement in pCR of 64.8% *vs.* 51.2%; $P = 0.00055$, although this had reduced by the third interim analysis[40] to 63.0% *vs.* 55.6%. There was also an improvement in 3-year event-free survival (EFS) 84.5% *vs.* 76.8%; $P < 0.001$[41]. Interestingly, contrary to data in the metastatic setting, PD-L1 expression was not predictive of benefit[11], and consequently the FDA approval in the neoadjuvant setting was granted irrespective of PD-L1 expression.

The benefit of ICIs in combination with chemotherapy in the neoadjuvant setting was also echoed in the IMpassion 031 study evaluating atezolizumab. In IMpassion 031, atezolizumab was evaluated in the neoadjuvant setting in patients with stage II-III TNBC treated for curative intent. This was a double-blind phase III randomised trial where patients received either atezolizumab or its placebo, in combination with nab-paclitaxel, followed by doxorubicin and cyclophosphamide. The investigators found an increase in pCR rates from 58% *vs.* 41% in the all-comers population; $P = 0.0044$, (significance boundary 0.0184), and 69% *vs.* 49% in PD-L1 positive patients; $P = 0.021$, (significance boundary 0.0184). As it did not hit the prespecified boundary of significance for its second co-primary endpoint, the study is not formally powered for further survival analyses[42].

However, not all trials evaluating the addition of ICIs in combination with chemotherapy in the neoadjuvant setting for TNBC have yielded similar results. Both the NeoTRIP and GeparNeuvo evaluating atezolizumab and durvalumab, respectively, in the neoadjuvant setting were negative for pCR benefit. Patients in the NeoTRIP study were randomised to receive neoadjuvant carboplatin and nab-paclitaxel with or without 8 cycles of atezolizumab. Anthracyclines were given in the adjuvant setting after definitive surgery. The addition of atezolizumab resulted in numerically higher but nonsignificant pCR rates: 48.6% *vs.* 44.4%; $P = 0.48$[43]. Similarly, the GeparNuevo trial studied the addition of durvalumab to neoadjuvant chemotherapy with paclitaxel followed by epirubicin and cyclophosphamide, which found a nonsignificant but numerically superior pCR rates of 53.4% *vs.* 44.2%; $P = 0.224$[44]. Interestingly, a survival benefit with the addition of durvalumab compared to placebo was observed; 3-year invasive disease-free survival (iDFS) was 84.9% *vs.* 76.9% (HR 0.54; $P = 0.0559$) and 3-year OS 95.1% *vs.* 83.1% (HR 0.26; $P = 0.0076$)[45].

While there is general consensus for the use of ICIs in combination with chemotherapy in the neoadjuvant setting for TNBC, its optimal duration is currently still widely discussed. In both KEYNOTE-522 and IMpassion 031, the ICI was continued post-operatively for a total of 1 year, while NeoTRIP and GeparNuevo only administered ICI in the neoadjuvant setting. GeparNuevo is the only study that has shown survival benefits with the use of ICI despite being administered only in the neoadjuvant context without continuation in the adjuvant setting, leading to questions of whether there is a need for continual ICI in the adjuvant setting. Additionally, the pCR benefit that was observed in the durvalumab group in GeparNuevo was exclusively seen in the cohort of patients who received a 2-week lead-in of durvalumab prior to chemotherapy, although the reason for this observation is currently unclear. We have summarised the trials evaluating the use of ICI both as monotherapy and in combination with chemotherapy in Table 1.

In the adjuvant setting, there are ongoing trials such as the A-BRAVE trial[46] investigating the use of avelumab in the treatment of high-risk TNBC, as well as the ALEXANDRA/IMpassion030 trial[47] evaluating standard chemotherapy with or without atezolizumab in patients with early-stage TNBC. Additionally, the use of ICIs in early relapsing TNBC is also being investigated in the IMpassion 132 trial, a phase III randomised trial evaluating the role of combining atezolizumab with chemotherapy in patients with locally recurrent inoperable or metastatic TNBC within 12 months from receiving curative-intent treatment[48].

Table 1. Summary of trials evaluating the use of ICI as monotherapy and in combination with chemotherapy

Trial name/ID	Phase	Population	Arms	Results
KEYNOTE-012 NCT01848834	I	Advanced TNBC, PD-L1 + ve; pre-treated	Pembrolizumab	ORR 18.5% 6-mo PFS 24.4% 6-mo OS 66.7%, 12-mo OS 43.1%
KEYNOTE-086 NCT02447003	II	Metastatic TNBC; pre-treated Cohort A: all-comers Cohort B: PD-L1 + ve	Pembrolizumab	Cohort A: ORR 5.3%, mPFS 2.0 mo, mOS 9.0 mo Cohort B: ORR 21.4%, mPFS 2.1 mo, mOS 18.0 mo
JAVELIN NCT01772004	I	Metastatic breast cancer; pre-treated	Avelumab	ORR: 3.0% (overall population), 5.2% (TNBC), 16.7% (PD-L1 + ve), 1.6% (PD-L1-ve)
PCD4989g NCT01375842	I	Metastatic TNBC; any-line	Atezolizumab	ORR 24% (1st line), 6% (≥ 2nd line) ORR 12% (1st line), 0% (≥ 2nd line) mOS 10.1 mo (PD-L1 + ve), 6.0 mo (PD-L1-ve)
KEYNOTE-119 NCT02555657	III	Metastatic TNBC; 1 or 2 prior lines	Pembrolizumab *vs.* chemotherapy	mOS 9.9 mo *vs.* 10.8 mo HR 0.97 (overall population) mOS 12.7 mo *vs.* 11.6 mo HR 0.78; *P* = 0.057 (PD-L1 CPS ≥ 10)
IMpassion 130 NCT02425891	III	Metastatic TNBC; untreated	Nab-paclitaxel +/- atezolizumab	mPFS 7.2 mo *vs.* 5.5 mo, HR 0.79; *P* = 0.002 (ITT) mOS 21.0 mo *vs.* 18.7 mo HR 0.87; *P* = 0.077 (ITT) mPFS 7.5 mo *vs.* 5.0 mo HR 0.63, *P* < 0.0001 (PD-L1 + ve) mOS 25.4 mo *vs.* 17.9 mo HR 0.67; (PD-L1 +ve)
IMpassion 131 NCT03125902	III	Metastatic TNBC; untreated	Paclitaxel +/- atezolizumab	mPFS 6.0 mo *vs.* 5.7 mo, HR 0.82; *P* = 0.20 (PD-L1 + ve) mPFS 5.7 mo *vs.* 5.6 mo, HR 0.86 (ITT)
KEYNOTE-355 NCT02819518	III	Metastatic TNBC; untreated	Chemotherapy +/- pembrolizumab	mPFS 9.7 mo *vs.* 5.6 mo HR 0.66 (CPS ≥ 10) mPFS 7.6 mo *vs.* 5.6 mo HR 0.75 mOS 23.0 *vs.* 16.1 mo HR 0.73; *P* = 0.0185 (CPS ≥ 10) mOS 17.6 mo *vs.* 16.0 mo HR 0.86 *P* = 0.1125 (CPS ≥ 1)
I-SPY 2 NCT01042379	II	High-risk stage II/III breast cancer	Chemotherapy +/- pembrolizumab	pCR 44% *vs.* 17% (ERBB2-negative), 30% *vs.* 13% (HR- + ve/ERBB2-ve), 60% *vs.* 22% (TNBC)
KEYNOTE-522 NCT03036488	III	Stage II/III TNBC	Chemotherapy +/- pembrolizumab	pCR 64.8% *vs.* 51.2%; *P* = 0.00055 3yr EFS 84.5% *vs.* 76.8% HR 0.63; *P* < 0.001
IMpassion-031 NCT03197935	III	Stage II/III TNBC	Chemotherapy +/- pembrolizumab	pCR 58% *vs.* 41%; *P* = 0.0044 (all-comers) pCR 69% *vs.* 49% *P* = 0.021 (significance boundary 0.0184) (PD-L1 + ve)
NeoTRIP NCT002620280	III	Early high-risk and locally advanced TNBC	Chemotherapy +/- atezolizumab followed by surgery, then adjuvant anthracyclines	pCR 48.6% *vs.* 44.4% OR 1.18; *P* = 0.48
GeparNuevo NCT02685059	II	Non-metastatic TNBC	Chemotherapy +/- durvalumab *window phase included 2 weeks of durvalumab/placebo	pCR 53.4% *vs.* 44.2% OR 1.45; *P* = 0.224 3yr iDFS 84.9% *vs.* 76.9% HR 0.54; *P* = 0.0559 3yr OS 95.1% *vs.* 83.1% HR 0.26; *P* = 0.0076

CPS: Combined positive score; EFS: event-free survival; ICI: immune checkpoint inhibitor; iDFS: invasive disease-free survival; mOS: median OS; ORR: objective response rate; OS: overall survival; pCR: pathologic complete response; PD-L1: programmed cell death ligand-1; PFS: progression-free survival; TNBC: triple-negative breast cancer.

ICIs in other subtypes of breast cancer

While there have also been efforts to evaluate the use of ICIs in HER2-positive and hormone-positive/HER2-negative breast cancers, none of the studies have led to conclusive evidence for its use in these settings at present. In particular, HER2-positive breast cancer is thought to share certain similarities with TNBC that might suggest a benefit from ICI therapy. This includes the presence of higher tumour infiltrating lymphocytes (TILs) and PD-L1 expression. The presence of TILs in the tumour and its

surrounding microenvironment is thought to be a reflection of pre-existing antitumour immunity[49,50], and its presence is thought to be predictive of response to systemic anti-cancer treatment[50], as well as a prognostic biomarker[24]. TNBC and HER2-positive breast cancers have been observed to have a higher number of TILs compared to hormone-positive breast cancers[51,52]. PD-L1 expression has also been observed to be upregulated in HER2-positive breast cancer[53], and be predictive of response to ICIs in the PANACEA and KATE2 studies[54,55]. Further in-depth discussion of ICIs in HER2-positive and hormone-positive/HER2-negative breast cancers is beyond the scope of our current article, but has been extensively reviewed[56-58].

UNDERSTANDING AND OVERCOMING RESISTANCE MECHANISMS TO ICIS

Given that the earliest approval for ICI use in breast cancer came on 8 March 2019 for atezolizumab in combination with nab-paclitaxel in metastatic TNBC based on the IMpassion 130 trial[33], the experience and evidence available on resistance mechanisms specific to immunotherapy in breast cancer is scarce. In addition, discounting tumour agnostic approvals, which form a very small proportion of breast cancer patients as discussed above[36,37], the approval for ICIs in breast cancer is now only limited to the TNBC subtype, which constitutes only 15%-20% of all patients with breast cancer[59], and even so, only a subset of them with high risk early-stage and metastatic disease. Hence, much of our understanding of resistance to ICIs comes from the available data and research on ICI treatment as a whole from various other tumour types.

Resistance pathways to ICIs can be tumour-intrinsic, e.g., alteration of certain genes or signalling pathways within the tumour, or tumour-extrinsic, e.g., changes in components within the tumour microenvironment (TME) other than the tumour cell itself[60]. This can happen either from the outset, conferring primary resistance whereby no response to treatment is noted, or after a period of observed response, highlighting the concept of acquired resistance. As previously mentioned, breast cancers are known to be immunogenically cold tumours, which contributes to their primary resistance to ICI. We will discuss the various mechanisms of resistance by looking at both tumour-intrinsic and tumour-extrinsic pathways, and how each of them might potentially be harnessed to overcome drug resistance [Figure 1].

TUMOUR INTRINSIC RESISTANCE MECHANISMS TO ICIS

Alteration of signalling pathways

There are several critical signalling pathways that control cell-cycle progression, apoptosis, and cell growth. Alterations in any of these pathways can sometimes be exploited by cancer cells to escape immune surveillance, leading to resistance to ICIs. Some of these pathways are known to be more commonly mutated in breast cancer, for example, the mitogen-activated protein kinase (MAPK) pathway, phosphatidylinositol 3-kinase (PI3K)/protein kinase B (AKT)/mammalian target of rapamycin (mTOR) pathway, Wnt/β-catenin pathway and Janus kinase (JAK) and signal transducers and activators of transcription (STAT) pathway[61]. Hence, various combination therapies of ICIs with other therapeutic agents to target each of these specific pathways are gaining traction and have shown promising preliminary activity.

MAPK pathway

Signalling via the MAPK pathway induces the expression of various proteins such as vascular endothelial growth factor (VEGF) as well as interleukin (IL)-8 that inhibit T cell recruitment and function[62]. Inhibiting the MAPK pathway can also upregulate major histocompatibility complex (MHC)-I, MHC-II, and PD-L1 expression, and enhance infiltration of TILs[63]. Loi *et al.* had confirmed this observation in an analysis of 111 patients with TNBC who had been treated with neoadjuvant chemotherapy, and demonstrated that

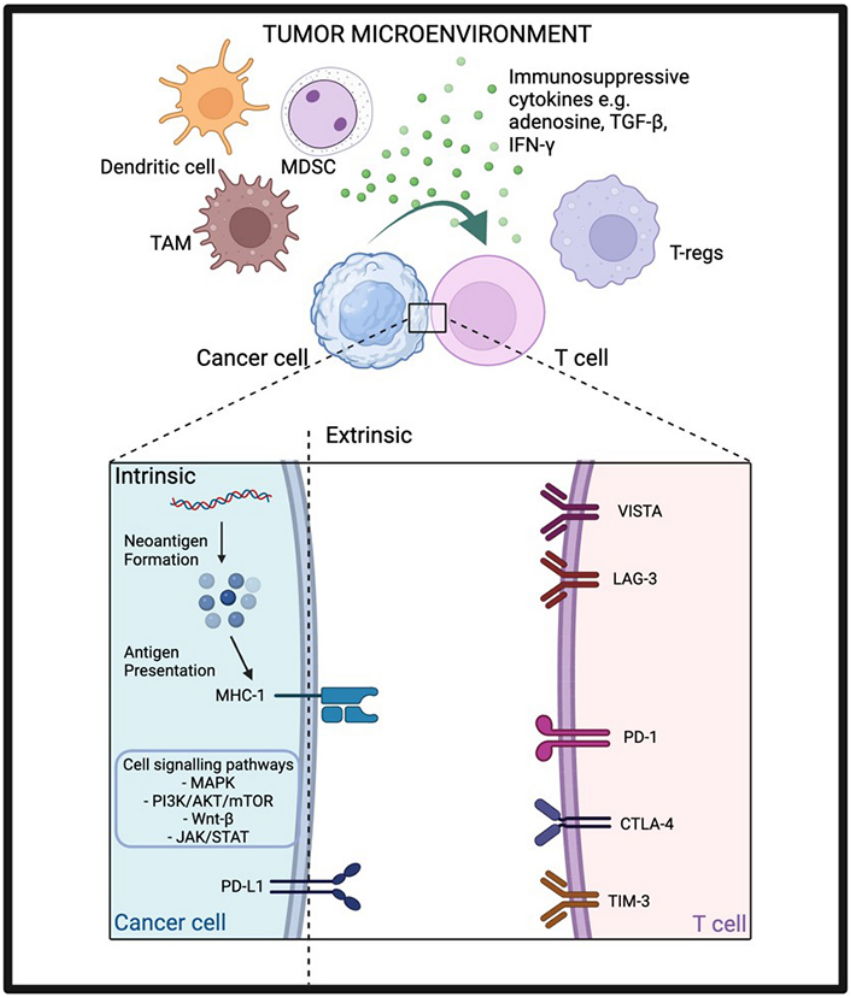

Figure 1. Tumour intrinsic and extrinsic resistance pathways to ICIs. Created with BioRender.com. AKT: Protein kinase B; CTLA-4: cytotoxic T-lymphocyte-associated antigen 4; IFN-γ: interferon-γ; JAK: janus kinase; LAG-3: lymphocyte-activation gene 3; MAPK: mitogen-activated protein kinase; MDSC: myeloid-derived suppressor cell; MHC-I: major histocompatibility complex-I; mTOR: mammalian target of rapamycin; PD-1: programmed cell death protein-1; PD-L1: programmed cell death ligand-1; PI3K: phosphatidylinositol 3-kinase; STAT: signal transducers and activators of transcription; TAM: tumour-associated macrophage; TGF-β: transforming growth factor-β; TIM-3: T-cell immunoglobulin, mucin domain-3 protein; T-reg: regulatory T cell; VISTA: V-domain immunoglobulin suppressor of T-cell activation.

alterations in the MAPK signalling pathway can suppress the expression of both MHC-I and MHC-II[64].

Hence, trials evaluating the combination of mitogen-activated protein kinase kinase (MEK or MAP2K) inhibitors with ICIs are ongoing. The COLET trial[65] was a phase II trial that investigated cobimetinib, a MEK inhibitor, in combination with atezolizumab and taxane chemotherapy in untreated metastatic TNBC. It demonstrated a numerical but nonsignificant increase in ORR of 34.4% and 29% in patients treated with paclitaxel *vs.* nab-paclitaxel, respectively. Exploratory biomarker analysis suggested that patients with PD-L1-positive disease (defined as IC ≥ 1% by the SP142 IHC assay) had numerically higher ORR compared to those with PD-L1 negative disease (39% *vs.* 19%), as well as median PFS (7.0 *vs.* 3.7 months).

PI3K/AKT/mTOR pathway

Abnormalities in the PI3K/AKT/mTOR pathway are also another well-known mechanism of resistance in breast cancer[66]. The protein phosphatase and tensin homolog (PTEN) tumour suppressor is a negative regulator of PI3K signalling and deletions in PTEN result in the enhancement of PI3K signalling[66,67]. PTEN loss has also been associated with resistance to T cell-mediated immunotherapy by increasing the expression of immunosuppressive cytokines, particularly VEGF[68]. VEGF can contribute further to immunosuppressive TME by recruiting suppressive immune cells such as myeloid-derived suppressor cells (MDSC) and regulatory T cells (Tregs)[69].

Based on these preclinical findings, AKT inhibitors have been combined with ICIs to overcome this resistance pathway. The phase Ib study evaluating the triplet combination of ipatasertib, atezolizumab, and a taxane as first-line treatment for locally advanced/metastatic TNBC reported a promising ORR of 73% irrespective of their PD-L1 status or PIK3CA/AKT1/PTEN alteration status[70]. The BEGONIA study (NCT03742102) is a phase Ib/II trial evaluating the combination of durvalumab with different novel oncologic therapies designed for immune modulation, with or without paclitaxel as first-line treatment in patients with metastatic TNBC. In arm 2[71], the addition of capivasertib was studied, yielding an ORR of 53.3%. Importantly, there was a relatively high rate of G3/4 treatment-related adverse events of 73%, although only 6.7% discontinued treatment due to adverse events.

Wnt/β-catenin pathway

The Wnt/β-catenin pathway is an important oncogenic signalling pathway involved in many essential cellular processes[72]. The activation of Wnt results in the accumulation of the transcriptional co-activator β-catenin to initiate the transcription of several cell cycle genes and oncogenes such as Myc[73]. The high levels of β-catenin via the canonical pathway have also been shown in a murine study by Spranger *et al.* to decrease the presence of CD103+ dendritic cell (DC) by reducing the expression of chemokine that attracts CD103+ DC (CCL4), preventing the migration of DC into the TME[74]. Consequently, this results in the blocking of adaptive antitumour immunity[75]. A study of TNBC by Castagnoli *et al.* showed that TNBC stem cells are able to upregulate PD-L1 expression via the Wnt pathway[75].

JAK/STAT pathway

Interferon γ (IFN-γ) is a cytokine produced by activated T cells and antigen-presenting cells (APCs) that is critical in immune cell activation via the Janus kinase 1 and 2 (JAK1/2) as well as signal transducers and activators of transcription-1 (STAT1) pathway[76]. Any mutation or epigenetic silencing of molecules in this pathway allows tumours to escape its apoptotic or cytostatic effect[77]. A study analysing melanoma patients who were treated with ICI therapy and subsequently developed resistance noted that resistance was associated with defects such as loss-of-function mutations in the JAK1/2 pathway[78]. Another study of 16 melanoma patients observed that those who were non-responders to CTLA-4 inhibition harbor a much higher rate of genomic changes in the IFN-γ pathway genes compared to those who responded[79].

Antigen presentation

A crucial feature of adaptive immunity is its ability to recognise antigens that are foreign or not "self". Cancer cells generally harbour accumulated somatic mutations and genomic instability within DNA coding regions. Antigen peptide sequences that distinguish tumour cells are classified based on their unique cell expression patterns[73]. Tumour-specific antigens (TSA) refer to novel peptide sequences, i.e., neo-antigens, that develop via mutations and are not present in normal healthy cells. Examples of mutations that result in TSAs usually involve oncogenic driver mutations, such as mutations in the *BRCA1/2* gene[80]. The presentation of these neoantigens by APCs via MHC-I molecules is critical in priming specific cytotoxic

CD8+ T cells, thereby triggering an immune response towards the tumour. Indeed, studies have shown that increasing neoantigen formation helps to improve response to ICIs[81-83].

Antibody-drug conjugates

Similar to the rationale for combining ICIs with chemotherapy which was discussed earlier, antibody-drug conjugates (ADCs) also increase tumour neoantigen formation via immunogenic cell death[84]. An ADC consists of an antigen-specific monoclonal antibody bound to a cytotoxic payload via a molecular linker. The binding of an ADC via its antigen-binding portion induces its internalisation via endocytosis. Once inside the tumour cell, cleavage of its linker through proteolysis results in the release of the cytotoxic payload. This allows for target-dependent activation and selective cytotoxicity[85]. Of note, two ADCs, namely trastuzumab deruxtecan (T-DXd) and sacituzumab govitecan, have received FDA approvals for the treatment of specific breast cancer subtypes. T-DXd is approved for unresectable or metastatic HER2-positive breast cancer based on the results of DESTINY-Breast 03, confirming significant PFS benefit (HR 0.28; $P < 0.0001$)[86], as well as for unresectable or metastatic HER2-low breast cancer based on DESTINY-Breast 04 showing both promising PFS (HR 0.50; $P < 0.001$) and OS (HR 0.64; $P = 0.001$) benefit[87]. Sacituzumab govitecan, on the other hand, has been approved both for unresectable or metastatic TNBC as well as hormone-positive, HER2-negative breast cancer based on the ASCENT and TROPiCS-02 trials, respectively, both confirming PFS and OS benefit[88,89].

Preclinical data have suggested that the combination of ADCs with ICIs may improve the efficacy of ICIs via increasing neoantigen formation and presentation, as well as by activating DCs and increasing the expression of PD-L1[85]. There are currently several ongoing trials evaluating the combination of different ADCs with ICIs. In the earlier described BEGONIA study, two ADCs, T-DXd and datopotamab deruxtecan (Dato-DXd), are being studied in arms 6 and 7 of the trial, respectively. Preliminary data for both arms were promising; ORR with the addition of T-DXd was 66.7%[90] and 74% with the addition of Dato-DXd[91]. Other ongoing trials in this space are summarised in Table 2.

Poly(ADP-ribose) polymerase inhibitors

Poly(ADP-ribose) polymerase (PARP) inhibitors increase DNA damage, leading to more TSAs and also increased MHC-I expression, thereby causing increased antigen presentation[92]. The increase in DNA damage associated with breast cancer patients who harbour the *BRCA1/2* mutation occurs via a process known as synthetic lethality[93]. The use of PARP inhibitors blocks the repair of single-stranded DNA breaks via base excision repair. This allows single-stranded breaks to accumulate, leading to the generation of double-stranded breaks (DSBs). These DSBs can usually be restored by either the high-fidelity homologous repair pathway or the error-prone non-homologous end-joining method. As *BRCA1/2* mutant breast cancer patients already have existing defects in homologous repair, they are unable to effectively repair DNA damage, resulting in the generation of TSAs. In addition to increasing antigen presentation, PARP inhibitors have also been shown in preclinical studies to alter the TME by activating intra-tumoural dendritic cells and increasing CD8+ T cell infiltration via the STING (stimulator of interferon genes) pathway[94]. It also enhances the upregulation of PD-L1 expression by reducing the PARylation of STAT3[95]. The latter two mechanisms help to overcome tumour extrinsic mechanisms of resistance to immunotherapy that will be expounded upon later.

Consequently, there have been several studies evaluating the combination of PARPi together with ICIs. The TOPACIO/KEYNOTE-162 trial[96] studied the efficacy of niraparib together with pembrolizumab in 55 patients with metastatic TNBC. In the subgroup of patients with *BRCA1/2* mutations, the ORR was 47% and mPFS 8.3 months. In contrast, patients who were non-*BRCA1/2* mutants had an ORR of 11% and mPFS of

Table 2. Summary of ongoing trials evaluating the addition of ADC to ICI therapy

Trial name/ID	Phase	Patients enrolled	ICI	ADC	Primary endpoint(s)
ASCENT-04 NCT05382286	III	Treatment naïve advanced/metastatic TNBC	Pembrolizumab	Sacituzumab Govitecan	PFS
NCT04448886	II	Metastatic HR+/HER2- breast cancer who have progressed on or within 12 months of adjuvant endocrine or ≥ 1 endocrine therapy in the metastatic setting	Pembrolizumab	Sacituzumab Govitecan	PFS
NCT03310957	I/II	Advanced/Metastatic TNBC	Pembrolizumab	SGN-LIV1A	ORR, DLT, adverse events
Morpheus-TNBC NCT03424005	Ib/II	Metastatic TNBC	Atezolizumab	Sacituzumab Govitecan or SGN-LIV1A	ORR, adverse events
InCITe NCT03971409	II	Metastatic TNBC	Avelumab	Sacituzumab Govitecan	ORR
Astefania NCT04873362	III	Patients with residual invasive disease in breast/axillary lymph nodes following neoadjuvant chemotherapy	Atezolizumab	Trastuzumab emtansine	Invasive disease-free survival
KATE3 NCT04740918	III	Metastatic PD-L1-positive cancer after progression on H +/- P and taxane	Atezolizumab	Trastuzumab emtansine	PFS, OS
NCT03032107	I	Metastatic breast cancer on progression on prior H and a taxane	Pembrolizumab	Trastuzumab emtansine	Safety and tolerability
NCT04042701	Ib	Metastatic HER2 positive or HER2 low breast cancer	Pembrolizumab	Trastuzumab deruxtecan	DLT and ORR
NCT03523572	I	Metastatic breast cancer progressed on ≥ 2 anti-HER2-based regimens	Nivolumab	Trastuzumab deruxtecan	DLT, ORR
DESTINY-Breast07 NCT04538742	Ib/II	Metastatic 2nd line and beyond (Part 1) and 1st line (Part 2)	Durvalumab	Trastuzumab deruxtecan	Safety and toxicity
DESTINY-Breast08 NCT04556773	I	Advanced or metastatic HER2-low breast cancer	Durvalumab	Trastuzumab deruxtecan	Safety and toxicity

ADC: Antibody-drug conjugate; DLT: dose-limiting toxicities; ICI: immune checkpoint inhibitor; ORR: objective response rate; OS: overall survival; PD-L1: programmed cell death ligand-1; PFS: progression-free survival; TNBC: triple-negative breast cancer.

2.1 months. The MEDIOLA trial[97] studied the combination of olaparib and durvalumab as first or second-line therapy in germline *BRCA1/2* mutant metastatic TNBC, noting an ORR of 63%, mPFS of 8.2 months and mOS 21.5 months. Table 3[96-101] summarises some of the available trials evaluating this combination.

Tumour cells can also evade immune surveillance by altering any step in the antigen presentation pathway, thereby conferring resistance to treatment with ICIs. Several studies involving patients with breast cancer have reported the downregulation of expression of the transporters TAP1, TAP2, and TAPBP, which are necessary for transporting antigens to be loaded onto MHC molecules[102-104]. Other mechanisms that have been observed include loss of heterozygosity and epigenetic suppression of certain MHC-I molecules[105] or alterations in the expression of beta-2-microglobulin (B2M) which is essential for the transport and subsequent expression of MHC-I on the cell surface[105,106]. Luo *et al.* reported the potential use of DNA methyltransferase inhibitors to overcome resistance to immunotherapy in breast cancer patients[107].

TUMOUR EXTRINSIC MECHANISMS OF RESISTANCE TO IMMUNOTHERAPY

Alteration of the tumour microenvironment

The TME comprises various components that are constantly evolving, with ongoing crosstalk between tumour and stromal cells, all of which can influence the immune response and drive resistance to ICIs[73]. The presence of TILs in the tumour and its surrounding microenvironment is thought to be a reflection of pre-existing antitumour immunity[49,50], and its presence is thought to be predictive of response to systemic anti-cancer treatment[50], and a prognostic biomarker[24]. TNBC and HER2-positive breast cancers have a

Table 3. Summary of ongoing trials evaluating the addition of PARPi to ICI therapy

Trial name/ID	Phase	Patients enrolled	ICI	PARPi	Primary endpoint(s)	Results (if any)
TOPACIO/ KEYNOTE-162 NCT02657889	I/II	Advanced or metastatic TNBC	Pembrolizumab	Niraparib	DLT and ORR	ORR 21%, 47%, 11% (overall, BRCA mutant, BRCA wild-type)[96]
NCT04683679	II	Metastatic TNBC or HR+/HER2- breast cancer	Pembrolizumab	Olaparib	ORR	
NCT03101280	Ib	Previously treated metastatic TNBC with BRCA mutation or BRCA-like molecular signature	Atezolizumab	Rucaparib	Number of dose modifications due to adverse events	
NCT02849496	II	Advanced or metastatic non-HER2-positive breast cancer with homologous DNA repair deficiency	Atezolizumab	Olaparib	PFS	
NCT04690855	II	Germline BRCA1/2 negative, PD-L1 positive metastatic TNBC	Atezolizumab	Talazoparib	ORR	
MEDIOLA NCT02734004	I/II	Germline BRCA mutated metastatic HER2-negative breast cancer	Durvalumab	Olaparib	DCR, safety, and tolerability	DCR at 12 weeks 80%, 28 weeks 50% ORR 63.3%[97]
DORA NCT03167619	II	Platinum-treated metastatic TNBC	Durvalumab	Olaparib	PFS	Combination arm: mPFS 6.1 mo, DCR 68.2%[98]
DOLAF NCT04053322	II	Advanced ER+, HER2- breast cancer with BRCA mutation, alteration in homologous recombination repair or MSI	Durvalumab	Olaparib	PFS	
PHOENIX NCT03740893	II	Post-neoadjuvant chemotherapy with residual TNBC	Durvalumab	Olaparib	Biomarker study pre-surgery and post-surgery	
NCT03801369	II	Metastatic TNBC	Durvalumab	Olaparib	ORR	
NCT03544125	I	Metastatic TNBC	Durvalumab	Olaparib	Safety and efficacy	
NCT02484404	I/II	Advanced TNBC	Durvalumab	Olaparib	Dose finding and toxicities	
JAVELIN PARP Medley NCT03330405	Ib/II	Advanced/ metastatic TNBC or HR+/HER2- breast cancer	Avelumab	Talazoparib	DLT and ORR	ORR 18.2% and 34.8% (TNBC, HR+/HER2-)[99]
JAVELIN BRCA/ATM NCT03565991	II	BRCA or ATM mutant advanced or metastatic solid tumour	Avelumab	Talazoparib	ORR	ORR 26.4% (BRCA) 4.9% (ATM)[100]
TALAVE NCT03964532	I/II	Advanced breast cancer	Avelumab	Talazoparib	Safety and toxicities	
NCT03945604	Ib	Recurrent, metastatic TNBC	Camrelizumab (anti-PD-1)	Fluzoparib	DLT	mPFS 5.2 mo, 12 mo OS 64.2%[101]

DCR: Disease control rate; DLT: dose-limiting toxicities; ICI: immune checkpoint inhibitor; MSI: microsatellite instability; ORR: objective response rate; OS: overall survival; PARPi: poly(ADP-ribose) polymerase inhibitors; PD-L1: programmed cell death ligand-1; PD-1: programmed cell death protein-1; PFS: progression-free survival; TNBC: triple-negative breast cancer.

higher number of TILs[51,52]. Other components of the TME include Tregs, MDSCs, tumour-associated macrophages (TAMs), and cytokines.

Tregs suppress effector T cells and APC via secretion of inhibitory cytokines, direct contact, and limiting inflammation[108]. The increased infiltration of Tregs into tumour cells has been observed in several other tumour types[109,110], and murine studies have demonstrated that depleting Tregs from the TME can help to restore antitumour immunity[109].

The presence of MDSCs in the TME has also been shown to promote angiogenesis, immune evasion, tumour growth and metastasis[108]. A study of patients with melanoma treated with CTLA-4 inhibitors

suggested that the increase in MDSCs was associated more often with non-responders[111]. Interestingly, the γ isoform of PI3K has been noted to be highly expressed in MDSC cells in a study of several cancer types, including breast cancer[112], and selectively inhibiting it can help to re-establish sensitivity to ICIs[113].

Another important group of cells present in the TME that promote immunosuppression and play a role in resistance to immunotherapy are TAMs, which consist of M1 and M2 macrophages[114]. M1 macrophages are mainly involved in antitumour immunity, while M2 macrophages are pro-tumourigenic. The accumulation of TAMs is regulated by cytokines, such as chemokine ligand 2 (CCL2), which was demonstrated by Qian *et al.* in their study using breast cancer- bearing murine model[115], as well as colony-stimulating factor-1 (CSF-1). It was observed to be correlated with increased macrophage infiltration and more frequent metastases in breast cancer patients[116]. Indeed, studies that evaluated CSF-1 receptor inhibition in combination with ICI treatment showed synergy of both agents and promising tumour regression, suggesting that CSF-1 receptor inhibitors can help to overcome tumour resistance to immunotherapy[117,118].

Besides individual populations of cells, the make-up of various cytokines present in the TME is also important in immune cell recruitment, activation, and proliferation by its balance of both stimulatory and suppressive effects[119]. For example, cytokines such as transforming growth factor β (TGF-β) induce immunosuppression by upregulating Tregs and inhibiting cytotoxic T lymphocytes[120]. Tumour cells also express ecto-5'-nucleotidase (CD73), which is an enzyme that dephosphorylates adenosine monophosphate (AMP), forming adenosine[121]. Adenosine is a potent immunosuppressor that binds to A2A receptors found on lymphocytes and suppresses its function[122]. Breast cancer cells have been shown to express CD73[123], and its expression appears to be regulated by the estrogen receptor (ER), whereby the loss of ER enhances the expression of CD73[124]. A proof of concept study confirmed that anti-CD73 antibody therapy can trigger adaptive antitumour immunity and inhibit metastasis in breast cancer[125].

Upregulation of other immune checkpoints

Resistance to ICIs can also be achieved via upregulation of other immune checkpoints such as T-cell immunoglobulin, mucin domain-3 protein (TIM-3), LAG-3, V-domain immunoglobulin suppressor of T-cell activation (VISTA), B and T lymphocyte attenuator (BTLA), and T-cell immunoreceptor tyrosine-based inhibition motif domain (TIGIT)[108,126-128]. The co-expression of multiple immune checkpoints has been demonstrated to be associated with T cell exhaustion, and subsequently resistance to ICIs[129]. Targeting these alternative pathways represents potential therapeutic options for overcoming drug resistance to ICIs. Although most studies evaluating such combination strategies have been in other tumour types such as melanoma and NSCLC[130-132], these are still relevant in breast cancers as epigenetic modifications resulting in upregulation of multiple immune checkpoints such as PD-L1, CTLA-4, TIM-3, and LAG-3 have been observed, and correlated with poorer patient prognosis in a study of breast cancer patients[133]. A study that specifically included breast cancer patients was a phase I study of LAG525, a monoclonal antibody blocking the binding of LAG-3 to MHC-II in combination with spartalizumab (an anti-PD-1 antibody) in patients with advanced malignancies, which showed durable responses[134]. In particular, 2 out of 5 patients with advanced TNBC showed a response, and in TNBC tumour biopsies, a trend in the conversion of immune-cold to immune-activated biomarker profiles was reported[134].

CONCLUSION: CHALLENGES AND FUTURE DIRECTIONS

Aside from resistance mechanisms to ICIs, there are also many unresolved and unanswered questions that have limited the use of ICIs in breast cancer. These include identifying the best predictive and prognostic biomarkers to guide treatment, evaluating the optimal duration of ICIs in the neoadjuvant/adjuvant setting,

and chemotherapy backbone in the metastatic setting, just to name a few. Recent review articles have discussed some of these topics[135,136].

Further advancement in this field needs to be led by sound science with good preclinical evidence from appropriate murine tumour models that can reflect the human immune environment. While this has conventionally largely been restricted due to a limited selection of murine tumour models, novel syngeneic tumour murine models have been better able to mirror the genomic heterogeneity of human cancer, and recapitulate the TME so as to provide accurate results. It is hoped that the use of appropriate novel syngeneic tumour murine models will allow us to further study ICI combinations effectively and accurately[137].

Lastly, studies looking beyond immunotherapy-based treatments are also being investigated. One such area is the study of the human gut microbiome, a host factor that influences not only the biology of tumour development but also the modulation of its response and resistance to immunotherapy[138-140]. Consequently, there are ongoing studies looking at modifying the gut microbiota in order to increase the efficacy of immunotherapy treatment. These include interventions such as the use of antibiotics, probiotics, faecal microbiota transplantation, and diet and prebiotics[141].

There is much to be anticipated in this evolving field of immunotherapy in breast cancer. While previously thought to be an immunologically "cold" cancer with limited responses to ICI, this is certainly set to change. The numerous ongoing trials evaluating ICIs in combination with novel therapies to overcome resistance and exploit the immune system, as well as the development of innovative immunomodulatory strategies, will allow us to further harness and expand the role of immunotherapy in breast cancer.

DECLARATIONS

Authors' contributions

Conceptualization: Wong RSJ, Ong RJM, Lim JSJ
Original draft writing: Wong RSJ, Ong RJM
Manuscript review and editing: Wong RSJ, Lim JSJ
All authors contributed to the article and approved the submitted version.

Availability of data and materials

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Conflicts of interest

JSJ Lim has received honoraria from Astra Zeneca, Novartis, Roche, DKSH, MSD, Eisai, Pierre Fabre; has advisory activity with Astra Zeneca, Novartis, Roche, DKSH, Pfizer and MSD; received research funding from CTI biopharma, Daiichi Sankyo and Synthon pharmaceuticals; and has received travel grants from Astra Zeneca and MSD.

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Not applicable.

Consent for publication

Not applicable.

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Cancer Drug Resistance

Review

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Targeting T regulatory (T_{reg}) cells in immunotherapy-resistant cancers

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Abstract

Primary or secondary (i.e., acquired) resistance is a common occurrence in cancer patients and is often associated with high numbers of T regulatory (T_{reg}) cells ($CD4^+CD25^+FOXP3^+$). The approval of ipilimumab and the development of similar pharmacological agents targeting cell surface proteins on T_{reg} cells demonstrates that such intervention may overcome resistance in cancer patients. Hence, the clinical development and subsequent approval of Cytotoxic T Lymphocyte Antigen-4 (CTLA-4) targeting agents can serve as a prototype for similar agents. Such new agents aspire to be highly specific and have a reduced toxicity profile while increasing effector T cell function or effector T/T regulatory (T_{eff}/T_{reg}) ratio. While clinical development with large molecules has shown the greatest advancement, small molecule inhibitors that target immunomodulation are increasingly entering early clinical investigation. These new small molecule inhibitors often target specific intracellular signaling pathways [e.g., phosphoinositide-3-kinase delta (PI3K-δ)] that play an important role in regulating the function of T_{reg} cells. This review will summarize the lessons currently applied to develop novel clinical agents that target T_{reg} cells.

Keywords: Primary and secondary resistance, T regulatory cells, flow cytometry, mass cytometry, hyperprogression

INTRODUCTION

Immunotherapy with immune checkpoint inhibitors (ICI) has become the backbone of several treatment regimens for cancer and has resulted in unprecedented benefits for patients[1]. Notwithstanding this progress, many patients eventually experience disease progression while undergoing treatment with ICI, and the mechanisms of the underlying resistance remain elusive[2]. One important contributor to such resistance is the immunosuppressive tumor microenvironment[3-5]. Based on the state and quality of immune cells, the tumor microenvironment has been classified as immune-inflamed, immune-excluded, and immune-deserted[6,7]. A second classification incorporates the role of cancer-associated fibrosis to describe the response to ICI[8,9]. A third classification integrates the role of epithelial-mesenchymal transition (EMT) as a key factor for resistance to ICI[10]. T regulatory (T_{reg}) cells emerge as key contributors of resistance to ICI and are included in each of the three above-mentioned classifications, primarily in immune-excluded or immune-enriched fibrosis conditions [Figure 1]. Considering that T_{reg} cells play an important function in tissue homeostasis, responses to infections, and the control of autoimmunity, their involvement in immune-excluded or immune-enriched fibrosis conditions is perhaps expected[11]. Furthermore, T_{reg} cells are no longer recognized as a single group of T cells, but instead consist of different subgroups with varied immunosuppressive properties against which distinct inhibitors can be developed[12]. This review will discuss the advances in drug development of large and small molecule agents to overcome T_{reg} cell-mediated resistance to ICI.

BIOLOGY AND CHARACTERIZATION OF T_{reg} CELLS

Early discovery of T_{reg} cell biology

Originally described as T suppressor cells[13-16], T_{reg} cells play a specific role in different phases of immune responses[17]. T_{reg} cells were first identified as a subset of $CD4^+$ T cells by their cell surface expression of CD25 (alpha chain of the IL-2 receptor) and consequently labeled as $CD4^+CD25^+$ T_{reg} cells[18]. Functionally, T_{reg} cells were initially characterized by the production of interleukin (IL)-10 and Transforming Growth Factor beta (TGF-β1)[19]. Ongoing studies have demonstrated that T_{reg} cells have a high degree of diversity[17]. In humans, of all circulating $CD4^+$ T cells, approximately 1%-3% are $CD4^+CD25^+$ T_{reg} cells[20]. They are often overlooked in clinical studies with respect to their contribution to treatment outcomes of new agents.

Ontogeny of T_{reg} cells [Figure 2]

T_{reg} cells were defined by their anatomical site of differentiation and the detection of the Forkhead box protein P3 (FOXP3)[21]: (1) natural T_{reg} cells (nT_{reg}) are T_{reg} cells that develop in the thymus and subsequently migrate to the periphery[22]; (2) induced T_{reg} cells (iT_{reg}) are those that evolve from naïve $CD4^+FOXP3^-$ T cells upon stimulation in the periphery[21,23]. Unfortunately, T_{reg} cells induced *in vitro* were also labeled as iT_{reg} (i.e., inducible T_{reg}). This has led to some confusion regarding the nomenclature of T_{reg} cells. Therefore, the 3rd International Conference on regulatory T cells[24] has recommended the following nomenclature to resolve the existing confusion:

1. Thymus-derived T_{reg} cells (tT_{reg}) - in lieu of nT_{reg}.
2. Peripherally-derived T_{reg} cells (pT_{reg} - i.e., $FOXP3^+$ T_{reg} cells that differentiate in the periphery) - in lieu of induced or adaptive T_{reg} cells.
3. *In vitro*-iT_{reg} - i.e., to differentiate T_{reg} cells derived *in vitro* studies from those investigated during *in vivo* studies.

The above-mentioned classifications of T_{reg} cells are based on ontogeny studies and two models are used to describe the generation of T_{reg} cells. The first model is called “instructive model”. According to the

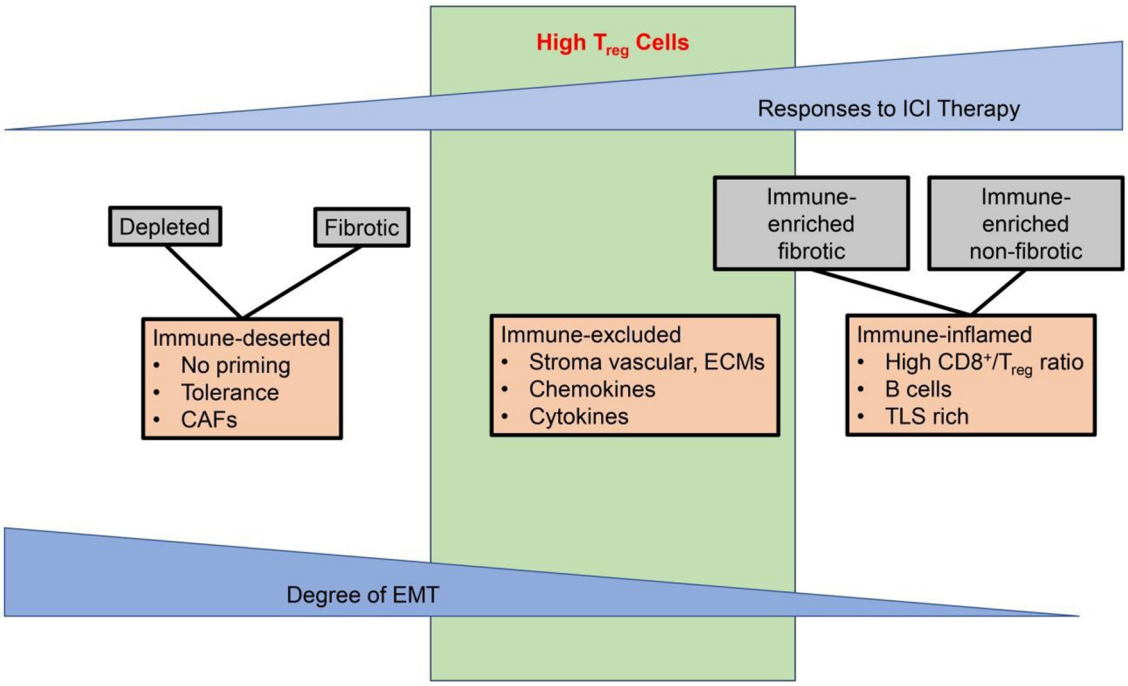

Figure 1. Main Mechanisms of Resistance (primary or secondary) to ICI. There are three different classifications or models summarizing the main mechanisms of resistance to ICI. The first classification (blue triangles) describes the response to ICI in relationship to markers of EMT[10]: the more tumors show a status of EMT, the lesser they respond to ICI. The second classification associates the degree and type of fibrosis with responses to ICI (grey boxes)[8]: response to ICI is generally observed in conditions with immune-enriched fibrotic and non-fibrotic conditions. By contrast, immune-depleted or fibrotic conditions are not responsive to ICI. The third classification is based on the presence of specific immune cells or markers (red boxes)[5,6]: responses to ICI are commonly observed in patients with immune-inflamed conditions (characterized by a high $CD8^+/T_{reg}$ cell ratio, B cells and TLS-rich tissues); conversely, responses are reduced in immune-excluded conditions (characterized by high vascular stroma content with fibrosis, chemokines, such as CCL, CCL2, CCL5, CCL13, CCL22, or cytokines TGF-β). Limited or no responses to ICI are observed in patients with an immune-deserted tumor microenvironment (lacking T cell priming, exhibiting tolerance, and displaying CAF-related markers). While T_{reg} cells (green box) can be found in each of these conditions, their highest quantity and functional role are observed in either immune-excluded conditions or in immune-enriched fibrotic tissues. ICI: Immune checkpoint inhibitor; EMT: epithelial-mesenchymal transition; TLS: tertiary lymphoid structure; CCL: chemokine c-c-motif ligand; TGF-β: transforming growth factor beta; CAF: cancer-associated fibrosis.

"instructive model", T cells are being "instructed" after T cell receptor (TCR) selection in the thymus. Intermediate TCR stimulation (in contrast to negative and positive selection) leads to the intracellular gene expression of FOXP3, which subsequently determines the generation of T_{reg} cells. The second model is called "selection model". According to this model, T_{reg} cells are being "selected" rather than "instructed" from a pool of pre-formed T cells. According to this model, *FOXP3* gene expression is independent of the strength of TCR stimulation and further assumes the presence of $FOXP3^-$ and $FOXP3^+$ T cells in the thymus. Upon exposure to self-antigens, the $FOXP3^+$ T cells are resistant to negative selection and form the majority of T_{reg} cells[25].

Independent of the thymus, which is a key organ for the development of T_{reg} cells, secondary lymphoid organs also appear to play a prominent role in generating $CD4^+FOXP3^+$ T cells from $CD4^+FOXP3^-$ T cells[26]. Such pT_{reg} cells can originate from sub-immunogenic stimuli, non-inflammatory conditions, long-lasting or chronic infections, and inflammation. Furthermore, they are frequently present in various cancers where they contribute to an immunosuppressive environment[27-30].

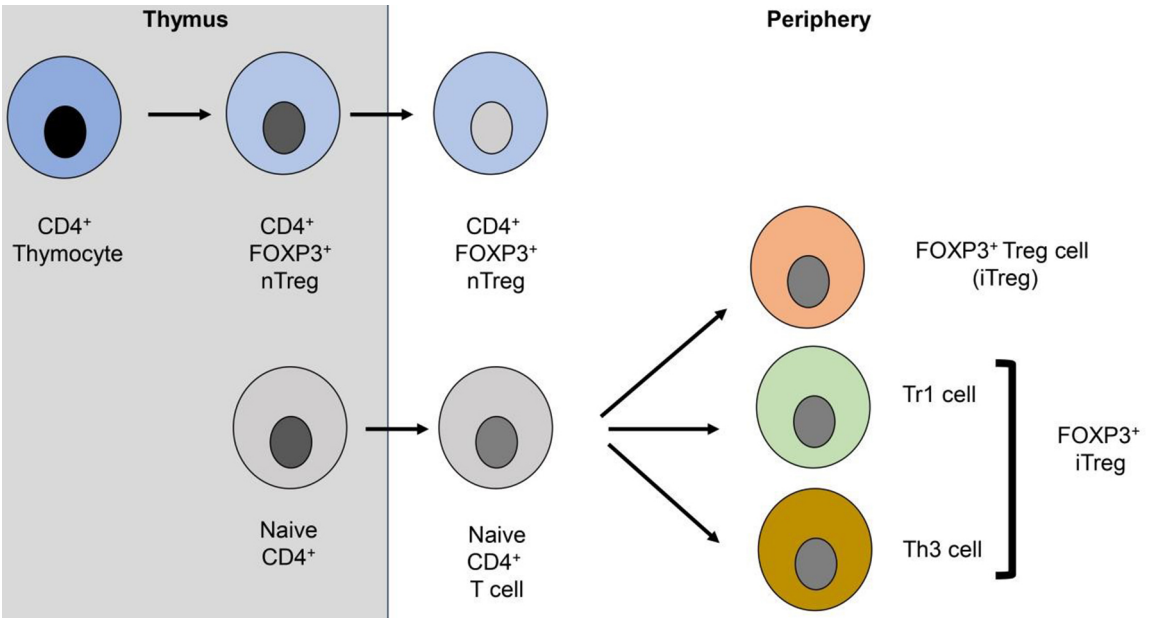

Figure 2. Characterization of T_{reg} cells and subsets: $CD4^+$ T cells egress from the thymus and differentiate in blood and tumor tissue. Depending on the degree of CD45RA and FOXP3 expression, $CD4^+$ are defined as nT_{reg} cells. $CD4^+$ or nT_{reg} cells egress into the periphery, where either cell population is subsequently altered and selected for different types of T_{reg} cells. Based on the "selection model", $CD4^+$ naïve cells are selected to transition into iT_{reg} cells, differing in their functional status as "Tr1 cells" or Th3 cells. FOXP3: Forkhead box protein P3; nT_{reg}: natural T regulatory cells; iT_{reg}: induced T_{reg} cells; Tr1 cells: type 1 T_{reg} cells; Th3 cells: T helper 3 cells.

Classification of T_{reg} cells

In general, $CD4^+CD25^+$ T_{reg} cells are characterized by FOXP3[28-31]. Additionally, low expression of the IL-7 receptor alpha chain (CD127) on the cell surface of T_{reg} cells often coincides with the intracellular presence of FOXP3[32]. Therefore, some classifications use the low expression of CD127 as an alternative marker to FOXP3, recognizing that this may not reflect the entire T_{reg} cell population[33]. Using a composite of intracellular and cell surface proteins, four major subsets of $CD4^+$ T cells, from which T_{reg} cells are derived, are classified as non-T_{reg}, naïve T_{reg}, effector T_{reg} and tumor-associated effector T_{reg} cells [Table 1]. Each subset is further characterized by additional surface markers[31,34].

Another nomenclature defines T_{reg} cells as "fractions" [Table 1][34,35]. This nomenclature also takes into consideration elements of functionality. Each T_{reg} cell fraction has distinct functions depending on the type of organ and anatomical location within the organ[36] [Table 1].

Some authors have preferred to define T_{reg} cells based on their function. For example, "type 1 T_{reg} cells" (Tr1) and T Helper (Th)3 cells are T_{reg} cells that produce immunosuppressive factors[23,37]. In contrast to the tT_{reg} cells, Tr1 and Th3 T_{reg} secrete the immunosuppressive cytokines IL-10 and TGF-β[38]. Others used HELIOS, a member of the Ikaros family of zinc-finger transcription factors, to identify precursors of peripheral T_{reg} cells emerging from the thymus and designated them as nT_{reg} [39]. Moreover, the expression of neuropilin-1 is used to distinguish T_{reg} cells selected from iT_{reg} in peripheral or extrathymic tissues[29]. Recently, the expression of programmed death 1 (PD-1) on T_{reg} cells was found on a highly immune-suppressive subset of T_{reg} cells, especially in patients previously exposed to ICI therapy[40]. In summary, these observations underscore the plasticity of T_{reg} cells and the selection of T_{reg} cell subsets in the periphery or extrathymic tissues[41].

Table 1. Two different classifications of T_{reg} cells

Classification of T_{reg} cells[31]

T_{reg} cells subsets	Phenotype markers	Characteristics
Non T_{reg}	CD45RA$^-$ CD4$^+$CD25$^+$FOXP3^{low} CTLA-4$^+$PD-1$^+$	No suppressive activity
Naïve T_{reg}	CD45RA$^+$ CD4$^+$CD25$^+$FOXP3^{low} CTLA-4^{low}PD-1$^-$	Weak suppressive activity Differentiate into effector T_{reg} cells
Effector T_{reg}	CD45RA$^-$ CD4$^+$CD25^{++} FOXP3^{++} CTLA-4^{++}PD-1$^+$ GITR$^+$LAG3$^+$CD127$^-$	Strong suppressive activity Prone to apoptosis
Tumor Effector T_{reg}	CD45RA$^-$ CD4$^+$CD25^{++} FOXP3^{++} CTLA-4^{+++}PD-1^{++} GITR^{++}LAG3^{++} CD127$^-$	High activation and proliferation

Classification of T_{reg} cells based on the concept of "fractions (Fr)"[34,35]

Fraction	Classification	Definition/Phenotype	Characteristics
Fr 1 (= naïve or resting)	rT_{reg}	CD45RA$^+$ CD4$^+$CD25^{low}FOXP3^{low} CTLA-4^{low}CD127$^{low/-}$Ki67$^-$	Derived from the thymus Weak suppressive activity Proliferation and differentiation into effector T_{regs} by TCR stimulation
Fr 2 (= effector or activated)	eT_{reg}	CD45RA$^-$ CD4$^+$CD25^{hi}FOXP3^{hi} CTLA-4^{hi}, PD-1$^+$, ICOS$^+$, GITR$^+$, OX40$^+$, CD15s$^+$, CCR4$^+$, CCR8$^+$, IL-10$^+$, TGF-β$^+$	Terminal differentiation status Strong suppressive activity Prone to apoptosis Tend to increase in peripheral blood with aging
Fr 3 (= non-T_{reg} cells)	Non-T_{reg}	CD45RA$^-$ CD4$^+$CD25^{low}FOXP3^{low} IL-2$^+$, IFN-γ$^+$, IL-17$^+$	Heterogenous population No suppressive activity

T_{reg} cells: T regulatory cells; FOXP3: forkhead box protein P3; CTLA-4: cytotoxic T lymphocyte antigen-4; PD-1: programmed death 1; GITR: glucocorticoid-induced TNFR-related protein; LAG-3: lymphocyte-activation gene 3; TCR: T cell receptor; ICOS: inducible T-cell costimulator; CCR: C-C chemokine receptor; IL: interleukin; TGF-β: transforming growth factor beta; IFN-γ: interferon gamma.

Molecular mechanisms generating T_{reg} cells and their function [Figure 3]

As highlighted above, FOXP3 is an important intracellular transcription factor determining the fate of T_{reg} cells. The myocyte enhancer factor 2D (MEF2D) is a transcription factor that influences the function of T_{reg} cells[29,42,43]. The role of MEF2D is important for two reasons: first, its presence is required for the expression of IL-10, Cytotoxic T Lymphocyte Antigen-4 (CTLA-4), and inducible T-cell costimulator (ICOS) and consequently for the acquisition of the effector T_{reg} cell function. Second, MEF2D acts synergistically with FOXP3[42]. Such discoveries point to multiple molecular regulators to generate or maintain T_{reg} cells[44]. Consistent with this hypothesis, recent studies have found additional master regulators of human tumor T_{reg} cells[45]. By comparing the transcriptional profile of tumor associated with matched peripheral T_{reg} cells from 36 patients with four different malignancies (i.e., glioblastoma, bladder cancer, renal cell carcinoma, prostate adenocarcinoma), 17 master regulators (MRs) were identified[45]. *In vivo* CRISPR-cas9 screening with gRNA against these MRs identified Transcriptional Repressor GATA Binding 1 (TRPS-1) as an essential transcription factor for tumor-associated T_{reg} cells. Genetic depletion of TRPS-1 in mice delayed tumor growth by inhibiting infiltration and function of tumor-associated T_{reg} cells, while preserving tolerance in the periphery.

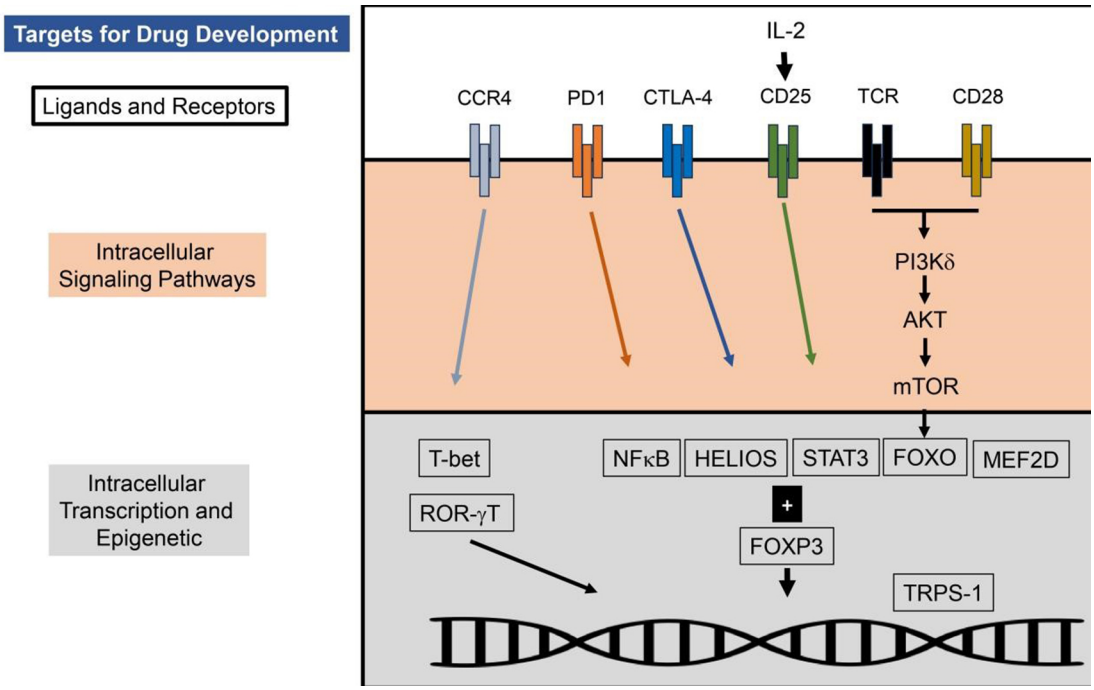

Figure 3. General Concept of Developing Drugs Blocking Activity of T_{reg} cells: In general, there are three main compartments enriched in T_{reg} cells, which are currently being targeted with drugs: (1) Extracellularly by blocking Ligands (white background), such as IL-2. Alternatively, blocking specific receptors on T_{reg} cells, e.g., CTLA-4, CCR4, with monoclonal antibodies, such as ipilimumab or mogamulizumab, can arrest the activity of T_{reg} cells; (2) Intracellularly (red background), signaling pathways can be blocked with small molecule inhibitors, e.g., targeting PI3K-δ; (3) Transcription, gene modification is targeted with different pharmacological agents, such as antisense oligonucleotides, molecular glue, and small molecules. These pharmacological interventions are mainly in non-clinical or early clinical investigations. They target a variety of factors, of which HELIOS and FOXP3 are perhaps the most unique to T_{reg} cells. T_{reg} cells: T regulatory cells; IL: interleukin; CTLA-4: cytotoxic T lymphocyte antigen-4; CCR4: C-C chemokine receptor; PI3K-δ: phosphoinositide-3-kinase delta; FOXP3: forkhead box protein P3.

In addition to intracellular transcription factors and the interaction with TCR, chemokines such as C-C motif chemokine ligand (CCL22) can induce the formation of T_{reg} cells[46]. CCL22, secreted by dendritic cells (DC) and macrophages, engages with its receptor C-C chemokine receptor (CCR4), which is predominantly expressed on T_{reg} cells[47]. Blocking this CCL22/CCR4 axis and consequently removing T_{reg} cells leads to anti-tumor immune responses[48]. Recent studies further show that FOXP3 is required to increase the expression of CCR4 on T_{reg} cells[49]. This co-regulation underscores that soluble and molecular events determine the fate of T_{reg} cells.

Epiregulation

The function or the generation of T_{reg} cells can also be influenced by mechanisms of epiregulation[50]. In murine models, complement factors determined the methylation of the FOXP3 in T_{reg} cells. Since complement is part of the innate immune system, epigenetic regulation of T_{reg} cells appears to occur early during an immune response. Hence, interventions of blocking complement activation may have an impact on the generation of T_{reg} cells.

Immunosuppressive function of T_{reg} cells

The classifications of T_{reg} cells can be based on functional studies for all T_{reg} cells or their subsets. Generally, T_{reg} cells exert their suppressive function in three ways: (1) soluble factors; (2) inhibitory receptors; (3) competition for activation or growth factors[51]. In recent years, the list of such mechanisms has expanded, and the following examples for each mechanism are presented to illustrate the basis for novel anti-cancer

therapies targeting T_{reg} cells.

1. Soluble Factors: IL-10 is secreted by T_{reg} cells and is one of the key cytokines contributing to immune suppression in cancer[52]. IL-10 also acts on T_{reg} cells themselves by expanding their number and increasing CTLA-4 expression[53]. TGF-β signaling is another cytokine that is associated with immunosuppression by T_{reg} cells[54,55]. Like IL-10, TGF-β signaling can also induce T_{reg} cells[56]. Its significance might surpass that of IL-10 in the function of T_{reg} cells, as it also inhibits the differentiation and function of Th1 and Th2 cells. TGF-β signaling promotes the differentiation of Th17 and Th9 cells, differentiation of tissue-resident memory $CD8^+$ T cells, generation of natural killer (NK) cells, and other tissue-resident cells, e.g., γδ T cells, innate lymphoid cells, and gut intraepithelial lymphocytes[57]. Given the tissue distribution of TGF-β signaling proteins and its feedback loop on T_{reg} cells, it may be one factor contributing to the tissue-dependent functionality of T_{reg} cells [Table 2].

2. Inhibitory Receptors: Perhaps the most recognized inhibitory receptor expressed on T_{reg} cells is the CTLA-4[35,58]. Because of its role in competing with CD28 for the co-stimulatory molecules CD80 (B7.1) and CD86 (B7.2) on antigen presenting cells (APCs), CTLA-4 can induce cell cycle arrest, inhibit the production of IL-2, and down-regulate ligands needed for the activation of T effector cells. Hence, it was termed an immune checkpoint inhibitor (ICI) and this critical discovery was recognized through the Nobel Prize awarded to James Allison and Tasuku Honjo[59]. This observation led to the discovery of similar receptors with inhibitory function, such as CD73[60,61]. The expression of CD73 in conjunction with TGF-β signaling contributes to a significant increase in T_{reg} cells and renders ICI therapies ineffective.

3. Competition for Growth Factors: Interleukin-2 (IL-2) is not only produced by activated $CD4^+$ and $CD8^+$ T cells, but also by Dendritic Cells (DCs) and thymic cells[62]. IL-2 engages with the IL-2R, which consists of IL-2Rα (=CD25), IL-2Rβ and common γ-chain[62]. T_{reg} cells express CD25 constitutively in contrast to T effector cells[63,64]. Persistent IL-2 signaling is needed to sustain the T_{reg} cell inhibitory function and survival[65]. Insulin Growth Factor was found to act synergistically with IL-2 to achieve persistent T_{reg} cell activity, which suggests that pro-inflammatory conditions support T_{reg} cells[66]. Other pro-inflammatory conditions are observed in patients with glioblastoma after receiving a single administration of a Chimeric Antigen Receptor T cell (CAR-T) directed against Epithelial Growth Factor Receptor III[67]. After the administration of the CAR-T in patients with glioblastoma, an increase of T_{reg} cells in the tumor microenvironment was observed, which was associated with a lack of treatment response. In another study, children receiving an IL13 CAR-T intracranially showed no reduction in T_{reg} cells in their cerebrospinal fluid[68]. Other soluble drivers may originate from metabolic pathways. For example, the fatty acid transporter CD36 sustains mitochondria fitness and the suppressive function of T_{reg} cells in the tumor microenvironment[69]. Therefore, T_{reg} cells may not only be influenced by soluble factors, such as cytokines or chemokines, but indirectly affected by factors from the metabolic pathways embedded in the microenvironment.

Overall, these few examples demonstrate that T_{reg} cell function can be induced and maintained by a variety of factors. Hence, activating or blocking these functions is relevant to therapeutic drug development. To appropriately assess the responses to therapies directed against T_{reg} cells, it is necessary to detect and monitor the T_{reg} cells in either tumor tissue or peripheral blood. This assumes that most T_{reg} cells are selected in the periphery and that, regardless of their ontogeny, they share similar mechanisms of action.

METHODS TO MEASURE T_{reg} CELLS

There are several methods to determine T_{reg} cells in cancer patients. Multiparametric cellular flow cytometry

Table 2. Phenotype characteristics of T_{reg} cells based on tissue distribution highlights the plasticity of T_{reg} cells

Tissue	T_{reg} cell phenotype and function
Brain	IL-10, IL-33, IL-35, ST2, CTLA-4, TGF-β, IDO, 5-HT_7, AREG
Lung	COX-2, PGE_2, TGF-β, AREG, IL-33, CD103, PHD, HIFα
Liver	IL-10, IL-35, CTLA-4, TGF-β, SCFAs, AREG, RA, IDO1, COX2, PGE2, GITR, LAG3, ICOS, CD39/CD73, ST2
Adrenal gland	β1-adrenergic receptors, Glucocorticoid receptor α
Lymph node	IDO, TGF-β, CTLA-4, ICOS, CXCR5, IL-2, CD28, CD103
Skin	IL-10, TGF-β, GITR, CTLA-4, Jag1, IDO, $OX40^+$, ARG2, CCR4, CCR6, CLA
Bone	CD39/CD73, RANK, PGE3, TGF-β, IDO, HIF1α, CXCR4

T_{reg} cells: T regulatory cells; IL: interleukin; CTLA-4: cytotoxic T lymphocyte antigen-4; TGF-β: transforming growth factor beta; IDO: indoleamine-pyrrole 2,3-dioxygenase; AREG: amphiregulin; GITR: glucocorticoid-induced TNFR-related protein; LAG3: lymphocyte-activation gene 3; CCR: C-C chemokine receptor.

(FC) was historically used to evaluate the T_{reg} cells and their subsets[70,71]. Even today, the main advantage of flow cytometry is the quick turn-around time (i.e., generally within hours), and thus can be used to monitor T_{reg} cells before and after novel treatments. An alternative tool to monitor T_{reg} cells is mass cytometry[72,73]. Mass cytometry has a reduced risk of signal spill-over, thus improving background noise, and is a highly dimensional method to assess several complex markers simultaneously. The disadvantage of mass cytometry lies in the longer turn-around time, destruction of the specimen at the end of the examination, and the subsequent bioinformatic analyses of high-volume data[74]. The power of mass cytometry to measure small subsets of immune cells in blood is exemplified in an ongoing clinical study with the phosphoinositide-3-kinase delta (PI3K-δ) inhibitor roginolisib (IOA-244). In this study, mass cytometry detected a reduction in blood T_{reg} cells across dose cohorts, which was only marginally detected with standard FC[75].

In tumor specimens, standard immunohistochemistry has also provided early insights into changes in T_{reg} cells before and after treatment with standard or novel therapies[76-78]. Multiplex immunohistochemistry using a wide range of fluorochromes has increased the ability to simultaneously assess T_{reg} cells and their interaction with adjacent cells, such as $CD8^+$ T cells[79]. Like standard immunohistochemistry, multiplex studies retain the anatomical features of the specimen and the spatial relationship of cells and stroma, for example, the interaction of T_{reg} cells with APC, $CD8^+$ T cells, or tumor cells[80].

Transcriptomics provides another high-dimensional approach to assess T_{reg} cells along with other changes in the tumor or blood[81]. Gene expression profiles can describe the T_{reg} cells along with other immune cells using whole tissue extracts[82]. Under such conditions, the anatomical structure is lost for the benefit of detecting low signal events. A modification of this technique is single-cell transcriptomics approaches, which have revealed new functions of T_{reg} cells[83]. Using this technology, the destruction of the tumor specimen is kept to a minimum while the detection of cellular events is increased. The disadvantage of this technology primarily lies in the processing and evaluation of high-volume data, which leads to long turn-around times.

Like Transcriptomics, Proteomics is a collection of high-dimensional data of proteins either within tumor tissue or proteins shed from tumors to the blood[84,85]. Thus, a wide range of secreted proteins can be evaluated, including chemokines (e.g., CCL22) or cytokines (e.g., IL-2, TGF-β) associated with T_{reg} cells[86]. For drug development, Proteomics offers a broad discovery tool to study the effect of novel agents. From this discovery platform, specific diagnostic tools can also be developed, such as companion diagnostics or laboratory developed tests.

In vivo imaging has been used to describe the dynamics of T_{reg} cells in animals[87]. While such studies in animals have shown important insights into T cell regulation in the presence of CTLA-4 inhibition, there are no such specific imaging tools available for appropriate clinical investigation. The most advanced imaging tool uses CD8-labeled PET imaging and reveals significant heterogeneity in $CD8^+$ T cell distribution during immunotherapy in patients[88]. Therefore, to date, such imaging tools still need to prove their value to guide the drug development of novel agents.

While there are no regulatory-approved tests for assessing T_{reg} cells or their function, FC is the most widely used laboratory test in clinical studies. In contrast to tissue-based tests, T_{reg} cells in the blood can be monitored longitudinally either alone or in comparison to other blood-based immune cells.

T_{reg} CELLS DURING IMMUNOTHERAPY AND THEIR ROLE IN RESISTANCE

Background

T_{reg} cells play an important role in tissue homeostasis and co-regulation of other immune cell subsets[89]. In the following section, the role of T_{reg} cells during immunotherapy will be reviewed and their potential as either prognostic (i.e., relevant to the disease progression and independent of therapies) or predictive (i.e., in assessing possible response to therapies) biomarkers[90].

Baseline levels of T_{reg} cells in malignancies and their potential role as prognostic marker [Table 3]

The prognostic value of T_{reg} cells was examined by a systematic meta-analysis using data from 76 articles, which included 17 different types of cancers and 15,512 cancer cases[91]. This study evaluated T_{reg} cells as part of tumor-infiltrating lymphocytes (TILs). High numbers of T_{reg} cells were associated with shorter overall survival (OS) in most tumor types (e.g., cervical, renal, melanoma, and breast cancer), but were associated with longer OS in colorectal, head and neck, and esophageal cancer. The main parameters that influenced the prognostic value included tumor location, stage of disease, and molecular subtype.

In addition to this meta-analysis, studies assessed the prognostic role of T_{reg} cells in specific tumor types and a few important examples are described below.

In Non-small Cell Lung Cancer (NSCLC), the frequency of T_{reg} cells in peripheral blood increases with the stage of NSCLC[96,97]. In 156 NSCLC patients, naïve T_{reg} cells and not terminal T_{reg} cells were correlated with poor outcomes[101]. These naïve T_{reg} cells produced TGF-β and IL-10, indicating an immunosuppressive function. A study in the perioperative setting also found that T_{reg} cells in peripheral blood increased with the stage of disease[100]. This increase in T_{reg} cells was independent of histology such as squamous and adenocarcinoma. The postoperative T_{reg} cell frequency was not reduced to levels comparable to healthy subjects, suggesting that the immunosuppressive condition remained intact after surgery. Therefore, some investigators proposed to use the presence of T_{reg} cells in tumor tissue to assess the risk for relapse. For example, the T_{reg}/TIL Combination Risk Index identified that patients with Stage I NSCLC and a high count of T_{reg} cells were at risk of relapsing[95].

While another study also reported that T_{reg} cells increased with the stage of NSCLC, it found that serum levels of IL-17 and not IL-10 were negatively correlated with T_{reg} cells[98]. Gene expression of IL17 in lymphocytes was correlated with numbers of circulating T_{reg}, suggesting that IL-17 is being produced by lymphocytes[99]. Thus, serum levels of immunomodulatory factors may not always reflect the function of T_{reg} cells in patients. Consequently, for NSCLC patients receiving PD-1 therapies, counts of T_{reg} cells need to be combined with functional assays[111].

Table 3. Examples of malignancies with elevated T_{reg} cells associated with treatment resistance

Indication	Number of patients	Method and panel	Clinical observation	Ref.
T_{reg} cells at baseline				
Pan-cancer	15,512	Meta-analysis of studies assessing FOXP3 in tumor tissue and OS	Influence factors for prognosis included tumor location, molecular subtype, tumor stage For most solid tumors, T_{reg} cells correlated with poor OS	Shang *et al.* 2015[91]
Endometrial cancer	82	Flow cytometry using $CD4^+CD25^+CD127^-$	Baseline associated with treatment resistance	Li *et al.* 2019[92]
Endometrial cancer	275	IHC with FOXP3 Flow cytometry using $CD4^+CD25^+CD127^-$	Tumor tissue enriched for T_{reg} cells at baseline and associated with poor OS Endometrial cancer cells expanded $CD4^+CD25^+CD127^-$ cells *ex vivo*	Kolben *et al.* 2022[93]
Breast cancer	164	Flow cytometry using $CD4^+CD25^+FOXP3^+$	High T_{reg} cells in tumor tissue and draining lymph nodes associated with invasiveness Associated with CCL5 and increased expression of CCR5 on T_{reg} cells	Qiu *et al.* 2022[94]
NSCLC	64	IHC CD3 and FOXP3	High T_{reg} cells in tumor tissue of patients with stage I are at risk of relapse	Petersen *et al.* 2006[95]
NSCLC	28	Peripheral blood and flow cytometry using $CD4^+CD25^+$	$CD4^+CD25^+$ is higher compared to healthy subjects Increased $CD8^+CD28^-$ lymphocytes	Karagoez *et al.* 2010[96]
NSCLC	23	Peripheral blood and flow cytometry using $CD4^+CD25^+FOXP3^+$	T_{reg} cells elevated compared to healthy subjects T_{reg} cells increase depending on the stage of NSCLC High intracellular CTLA-4 expression	Erfani *et al.* 2012[97]
NSCLC	36	Peripheral blood and flow cytometry using $CD4^+CD25^+FOXP3^+$	T_{reg} cells elevated compared to healthy subjects T_{reg} cells were negatively correlated with serum IL-17	Hu *et al.* 2018[98]
NSCLC	26	Peripheral blood and flow cytometry using $CD4^+CD25^+FOXP3^+$	T_{reg} cells elevated compared to healthy subjects Correlation of Th17 cells with T_{reg} cells High levels of TGF-β, IL-17, IL-23	Li *et al.* 2014[99]
NSCLC	49	Peripheral blood and flow cytometry using $CD4^+CD25^+FOXP3^+$	T_{reg} cells increase depending on the stage of NSCLC T_{reg} cells decreased after surgery	Chen *et al.* 2014[100]
NSCLC	156	Peripheral blood and flow cytometry using $CD4^+CD25^+FOXP3^+$	T_{reg} cells produce TGF-β and IL-10 Naïve T_{reg} cells elevated and correlated with poor outcome High frequency of terminal T_{reg} cells correlated with improved outcome	Kotsakis *et al.* 2016[101]
NSCLC (EGFR mutation)	323 (164 with EGFR mutation)	IHC for FOXP3 (clone 236A/E7)	Significant High FOXP3 expression in EGFR mutation-positive NSCLC Association with poor survival	Luo *et al.* 2021[102]
NSCLC (EGFR mutation)	19 (6 EGFR-mutated and 13 EGFR-wildtype)	Flow cytometry with $CD45RA^-FOXP3^+CD4^+$ (=Fraction 2)	EGFR mutation is non-inflamed (no presence of $CD8^+$ T cells) High presence of T_{reg} cells EGFR mutation induces CCL22, which induces T_{reg} cells	Sugiyama *et al.* 2020[103]
T_{reg} cells response during treatment (possible predictive value)				
Cutaneous melanoma	40	Flow cytometry using $CD4^+CD25^{high}CD127^-Foxp3^+$	High baseline levels Reduction after 3 consecutive doses of ipilimumab Enrichment of $CD39^+HELIOS^+$ T_{reg} cells	Bjoern *et al.* 2016[104]
Cutaneous melanoma	32	Flow cytometry using $CD4^+CD25^+CD127^-PD\text{-}1^+$	Reduction after nivolumab or pembrolizumab treatment observed in patients responding to PD-1 inhibitors No reduction observed in patients with no response	Gambichler *et al.* 2020[105]
NSCLC	31	IHC using FOXP3 for tumor tissue and flow cytometry using $CD4^+CD25^+$ $FOXP3^+$ for blood	Neo-adjuvant treatment with cetuximab/docetaxel/cisplatin showed a correlation of reduction in T_{reg} cells and response T_{reg} cells at diagnosis did not predict clinical response with therapy	Pircher *et al.*[106]
NSCLC	132	Flow cytometry using $CD4^+CD25^+CD45RA^-FOXP3^+$	High T_{reg} cells and TGF-β1 levels after 1 week of treatment with PD-1 inhibitors are associated with increased OS High T_{reg} cells at baseline associated with longer OS and PFS	Koh *et al.* 2020[107]
NSCLC	27	IHC and mass cytometry T cell subsets	Ratio of $PD1^+$ on $CD8^+/PD1^+$ on T_{reg} cells was predictive of outcomes	Kumagai *et al.* 2022[108]

			Ratio was predictive in other tumor types as well, i.e., gastric cancer and melanoma	
Renal cell carcinoma	43	Flow cytometry using $CD4^+$ $CD25^+CD127^-FOXP3^+$	Treatment with nivolumab reduced T_{reg} cells only in responders when assessed after 3 months Inhibition with CXCR4 antagonist blocked T_{reg} cell function *in vitro* Treatment with nivolumab reduced T_{reg} cells only in responders when assessed after 3 months	Santagata *et al.* 2020[109]
Uveal melanoma	9	Mass cytometry using $CD4^+$ $CD25^+CD127^-$	T_{reg} cells reduced within 3 months, while $CD8^+$ and NK cells increased	Di Giacomo *et al.* 2022[110]

T_{reg} cells: T regulatory cells; FOXP3: forkhead box protein P3; OS: overall survival; IHC: immunohistochemistry; CCL: chemokine c-c-motif ligand; CCR: C-C chemokine receptor; NSCLC: non-small cell lung cancer; CTLA-4: cytotoxic T lymphocyte antigen-4; IL: interleukin; Th17 cells: T helper 17 cells; TGF-β: transforming growth factor beta; EGFR: epidermal growth factor receptor; PD-1: programmed death 1; NK: natural killer.

In 275 tumor specimens from patients with endometrial cancer, high FOXP3 expression was correlated with poor OS[93]. A similar observation was reported for patients with primary breast cancer, where T_{reg} cells and CCL5 were co-expressed with standard prognostic markers for breast cancer[94]. The authors postulated that CCL5 engages the CCR5 on T_{reg} cells and subsequently induces the production of TGF-β[94]. Like the CCL5/CCR5 axis, the chemokine receptor CCR8 (its ligand being CCL1) also plays a critical role in upregulating genes of intra-tumoral T_{reg} cells as observed in patients with breast, colorectal, and lung cancer[112,113]. In each of these tumor types, the expression of CCR8 correlated with T_{reg} cell signature and was associated with poor prognosis[114].

Oncogenic driver mutations are associated with a tumor microenvironment rich in immunosuppressive mediators and T_{reg} cells. For instance, Epidermal Growth Factor Receptor (EGFR) mutations in NSCLC are associated with high levels of T_{reg} cells[102]. The microenvironment of patients with EGFR-mutated NSCLC is immune-suppressed, as indicated by tissue expression of FOXP3 and PD-L1[102]. Furthermore, high numbers of Fraction 2 T_{reg} cells, low numbers of $CD8^+$ T cells (i.e., non-inflamed condition), and high levels of CCL22 (the main ligand for CCR4) are observed in EGFR-mutated NSCLC patients[103]. This immunosuppressive state was reversed during combination treatment of EGFR inhibitors and PD-1 monoclonal antibodies, leading to a reprogramming of the immune subsets, and consequently overcoming the resistance. Kirsten Rat Sarcoma Virus (KRAS) mutated tumors are also associated with high numbers of T_{reg} cells, for example, in KRAS-mutated colorectal cancers[115]. KRAS-mutated tumors produce the immune suppressive mediators IL-10 and TGF-β1 and thus drive a phenotype switch from naïve to T_{reg} cells[116]. Because of these observations in EGFR- and KRAS-mutated tumors, it is possible that other mutations are associated with similar immunosuppressive mediators and T_{reg} cells[117].

In contrast to solid tumors, lymphoma patients may harbor four functionally distinct T_{reg} cell groups: (1) Suppressor T_{reg} cells: similar to solid tumors, this group of T_{reg} cells is immunosuppressive; (2) Malignant T_{reg} cells: the malignant clone derived from precursors of T cells expresses FOXP3 as a marker for adult T cell leukemia/lymphoma (ATLL) and cutaneous T-cell lymphomas (CTCL); (3) Direct tumor-killing T_{reg} cells: T_{reg} cells with suppressive cytotoxicity capable of killing tumor cells; (4) Incompetent T_{reg} cells: mostly observed in angioimmunoblastic T-Cell lymphoma (AITL), and their presence is associated with autoimmune symptoms[118]. These different groups with distinct functions were not considered in a recent meta-analysis of 23 lymphoma studies. In this meta-analysis, high numbers of T_{reg} cells at baseline were associated with improved survival[119]. However, in some subsets of T cell lymphoma and follicular lymphoma, the high T_{reg} cell counts were not associated with improved OS. Hence, additional differentiation markers are needed to accurately assess the functional role of T_{reg} cells in lymphoma and its sub-types.

While the above-mentioned examples show how T_{reg} cells are associated with survival, it remains unclear whether the presence of T_{reg} cells is merely an epiphenomenon or a key driver of immune suppression in cancer patients. Therefore, changes in T_{reg} cells after clinically meaningful responses to therapies may help to recognize where T_{reg} cells are key drivers of tumor progression.

T_{reg} cells as potential drivers of tumor progression and their potential role as predictive biomarkers [Table 3]

Studies of immunotherapy and other anti-cancer treatments were selected to determine whether T_{reg} cells are potentially related to treatment outcomes, either as a negative or positive predictive marker[120]. For example, patients with hyperprogression during immunotherapy have elevated T_{reg} cells, which is associated with treatment failure[121,122]. In such patients, T_{reg} cells expand and copious amounts of immune suppressive cytokines (e.g., TGF-β1, IL-10) are secreted. Furthermore, T_{reg} cells upregulate PD-1 expression during PD-1/PD-L1-targeting therapies, generating highly immunosuppressive T_{reg} cells[123]. This observation is not limited to peripheral blood T_{reg} cells. PD-1 expression on T_{reg} cells is also observed in the tumor microenvironment of patients with NSCLC[108]. While the expression of PD1 on T_{reg} cells is already predictive for PD-1-based therapies, the ratio of $PD1^+$ T_{reg} cells and $CD8^+$ T effector (T_{eff}) has a superior predictive value than PDL-1 staining alone[108]. Hence, detecting $PD1^+$ T_{reg} cells by either FC in blood or IHC in tissue can predict the efficacy of ICI therapies.

T_{reg} cell dynamics are not always associated with poor outcomes. For example, PD-L1-treated patients with NSCLC had high frequencies of circulating T_{reg} cells one week after therapy. These levels were correlated with a high response rate, longer progression-free survival, and overall survival[107]. At the same time, TGF-β levels were elevated and associated with a favorable response to anti-PD-1 immunotherapy. A second study in patients with cutaneous melanoma also reported an association of high levels of T_{reg} cells with improved outcomes after adjuvant PD-1-based therapies[124]. Several reasons may explain this difference between T_{reg} cells as a predictive marker of poor or improved outcomes. First, the mere phenotypic description of T_{reg} cells may ignore certain functional characteristics of T_{reg} cells, which can miss the degree of immune suppression. For instance, T_{reg} cells expressing signal transducer and activator of transcription 3 (STAT3) appear to be less immune suppressive[124]. By adding a STAT3 inhibitor to such T_{reg} cells, their suppressive function was enhanced[124]. Hence, it is possible that studies reporting increased T_{reg} cells are capturing a broader T_{reg} cell population, including T_{reg} cells, with reduced immunosuppressive function. Second, levels of T_{reg} cells may differ between early and later stages of immunotherapy. Most studies assessed the levels of T_{reg} cells several weeks after starting immunotherapies. Patients with renal cell carcinoma (RCC) treated with nivolumab had a reduction in peripheral T_{reg} cells once they were treated for 3 months, indicating a response to the therapy[109]. Similarly, patients with cutaneous melanoma had a significant reduction in T_{reg} cells after three consecutive doses of ipilimumab[104]. In uveal melanoma, the peripheral T_{reg} cell population began to decrease after approximately 2 months of treatment with the PI3K-δ inhibitor roginolisib[110]. Patients with endometrial cancer who did not respond to immunotherapy had increased T_{reg} cells after several treatment cycles in their blood, indicating a treatment failure[92]. Given these differences, it is important to characterize the T_{reg} cell population during a novel therapy before drawing a conclusion on whether T_{reg} cells can serve as a prediction marker. Third, an increase in T_{reg} cells early in therapy may represent a mobilization of the T_{reg} cells from the tumor tissue into the periphery and consequently have limited value for a prediction. Using *in vitro* co-cultures of peripheral blood mononuclear cells (PBMCs) from healthy volunteers, adding them to endometrial cancer cell lines led to an increase of T_{reg} cells within a few hours, suggesting a prompt migratory response of T_{reg} cells[93]. Hence, it is possible that once tumor cells are prevented from producing chemoattractant factors as a result of therapeutic intervention, T_{reg} cells may migrate away from the tumor tissue and subsequently be detected in peripheral blood. As mentioned previously, a numerical increase in T_{reg} cells needs to be accompanied by appropriate functional tests to

determine whether a change is clinically meaningful.

In hematologic malignancies, T_{reg} cells play a role in the regulation of bone marrow progenitor cells, in controlling the development of malignant clones (e.g., either by transcriptional changes in the malignant B- or T cell), and in influencing the immune cell composition. Some examples are used to illustrate the complexity of targeting T_{reg} cells in hematologic malignancies. Patients with chronic lymphocytic leukemia (CLL) and responding to PI3K inhibitors idelalisib or duvelisib show a reduction in T_{reg} cells[125]. Interestingly, this reduction in T_{reg} cells seemed to coincide with toxicities reminiscent of autoimmune toxicities observed in patients receiving ICI[126]. Therefore, treatments with oral PI3K-δ inhibitors have offered new insights into the role of T_{reg} cells or their mediators, such as the underappreciated role of IL-17[127-129]. Whether this effect of PI3K-δ inhibitors is uniquely related to the reduction in T_{reg} cells remains to be determined, because a reduction or inhibition of the function of T_{reg} cells is not always associated with autoimmune toxicities. One example of T_{reg} cell reduction without autoimmune toxicities is observed in patients receiving Janus kinase (JAK) 1/2 inhibitors in Primary Myelofibrosis (PMF). Patients who respond to the treatment with the JAK 1/2 inhibitor ruxolitinib show a decrease in T_{reg} cells[130]. Interestingly, the highest frequency of T_{reg} cells was observed in patients with the highest allele frequency of the JAK2 V617F mutation. Furthermore, long-term treatment with ruxolitinib was associated with disease control and reduction in T_{reg} cells[131]. In contrast to the experience with CTLA-4 targeting agents and PI3K inhibitors, the reduction in T_{reg} cells was not associated with autoimmune toxicities. There are at least two factors that may explain the autoimmune toxicities in patients treated with anti-CTLA-4 antibodies or PI3K-δ inhibitors, while they are absent in patients receiving agents while reducing T_{reg} cells. First, common among both drug groups is the question about specificity and selectivity. For example, monoclonal antibodies with a modified Fc framework have an altered response and perhaps also a reduced autoimmune-toxicity profile[132,133]. Additionally, for the designated PI3K-δ inhibitors, such as idelalisib[134], parsaclisib[135] and duvelisib[136], the selectivity profile in humans is less clear. All known PI3K-δ inhibitors are not as selective as originally assumed with some important safety implications as recently evaluated[137]. Second, in addition to specificity or high selectivity, the immune competency of patients may play a role. For example, in patients with CLL, the B cell function is disrupted. Hence, it is possible that the reduction in T_{reg} cells induces the elevation of cytotoxic Th17 T cells[125].

Examples of drugs targeting T_{reg} cells and T_{reg} cell-mediated resistance [Table 4]

The success of the CTLA-4 targeting agents such as ipilimumab has provided important lessons for future drug development concepts. Herein, we review drug candidates with specific inhibition profiles for T_{reg} cells. Furthermore, the novel agents intend to provide a greater benefit/risk profile. Drugs designed to increase the T_{reg} cells, such as for improving transplantation outcomes, will not be reviewed.

The lessons from the drug development of such agents support the hypothesis that T_{reg} cells are key players in the resistance mechanisms of immunotherapy[157]. This explains the increasing number of drug candidates targeting T_{reg} cells with an aim to rebalance the overall immune cell compartment[12,158].

Large Molecules: Because of the preferential expression of CTLA-4 on T_{reg} cells, CTLA-4 inhibitors, such as ipilimumab or tremelimumab, are perhaps the prototype of selective T_{reg} cell inhibitors, although a reduction in T_{reg} cells cannot always be detected[138-140,159]. Both ipilimumab and tremelimumab have received approvals for a wide range of indications and form the backbone of many standard treatments[160]. With a greater understanding of dose and dose schedule, the use of CTLA-4 targeting agents is evolving. For example, it appears that continuous dosing may not be required to achieve the full effect of CTLA-4 targeting agents[104,161,162]. This is best observed in the neo-adjuvant setting, where limited doses of

Table 4. Examples of drugs targeting T_{reg} cells

Drug/Intervention	Observation	Ref.
Large molecules		
CTLA-4 targeting agents	Intra-tumoral T_{reg} cells unchanged after ipilimumab or tremelimumab therapy In neo-adjuvant setting, ipilimumab transiently increased T_{reg} cells In patients with metastatic melanoma, T_{reg} cells are reduced after extended treatment time (> 3 months)	Sharma *et al.* 2019[138] Retseck *et al.* 2018[139] Bjoern *et al.* 2016[104] Patel *et al.* 2023[140]
PD1 targeting agents	Ratio of expression on T_{reg}/T_{eff} cells after immunotherapy potentially predicts response $PD1^+$ T_{reg} cells may be dysfunctional	Kumagai *et al.* 2020[108] Lowther *et al.* 2016[141]
CCR-4 targeting agents	Monoclonal antibody mogamulizumab (NCT02705105) showed limited activity (ORR or 10%) either as monotherapy or in combination with nivolumab Blood and tumor T_{reg} show a reduction for patients with ORR	Hong *et al.* 2022[142]
CCR-8 targeting agents	Subpopulation of T_{reg} cells express CCR-8 Blocking CCR-8 appears not to be associated with autoimmune adverse events in animal studies Monoclonal antibody GS-1811 in early phase clinical trials (NCT05007782)	Kidani *et al.* 2022[143] Weaver *et al.* 2022[144]
CD25 targeting agents	CD25 high-affinity subunit alpha Monoclonal antibody RO7296682 (RG6292) had no overt adverse events in animals RO7296682 in clinical trials (NCT04158583)	Solomon *et al.* 2020[145]
IL-2 targeting agents	Selective inhibition of trimeric and not dimeric CD25 leads to T_{reg} cell reduction	Wyant *et al.* 2023[146]
CEACAM-5 targeting agents	CEACAM-5 and 6 is expressed on highly suppressive T_{reg} cells NEO201 reduces T_{reg} cells	Cole *et al.* 2023[147]
Small molecules		
Chemotherapies	Low-dose cyclophosphamide and vaccines Low-dose cyclophosphamide in CRC Docetaxel in NSCLC Sunitinib in RCC	Le *et al.* 2012[148] Ghiringhelli *et al.* 2007[149] Scurr *et al.* 2017[150] Roselli *et al.* 2013[151]
STAT3 (FOXP3) inhibition	T_{reg} cell reduction	Revenko *et al.* 2022[152]
ATP-competitive PI3K-δ inhibitors	Drug-related Grade 3/4 toxicities limiting continuous dosing and reducing potential efficacy T_{reg} cell reduction in tumor tissue Chemokines inducing T_{reg} cells reduced in lymphoma patients	Eschweiler *et al.* 2022[153] Tarantelli *et al.* 2021[154]
Non-ATP competitive PI3K-δ inhibitor roginolisib (IOA-244)	Low grade 3/4 toxicity with no requirement of drug modifications Safety in long-term treated uveal melanoma Reduction in T_{reg} cells, increase in $CD8^+$ T and NK cells	Di Giacomo *et al.* 2022[110]
JAK1/2 inhibitors	Reduction in T_{reg} cells in patients with PMF responding to ruxolitinib	Massa *et al.* 2014[130]
CDK4/6	Reduction in T_{reg} cells and increase in T_{eff} cells, with a greater reduction in patients with responses to therapy	Scirocchi *et al.* 2022[155]
BCL2 (e.g., Venetoclax)	Reduction in peripheral T_{reg} cells and enhancement of immune cells	Kohlhapp *et al.* 2021[156]

T_{reg} cells: T regulatory cells; CTLA-4: cytotoxic T lymphocyte antigen-4; CCR: C-C chemokine receptor; NSCLC: non-small cell lung cancer; RCC: renal cell carcinoma; STAT3: signal transducer and activator of transcription 3; FOXP3: forkhead box protein P3; PI3K-δ: phosphoinositide-3-kinase delta; NK: natural killer; JAK: Janus kinase; PMF: primary myelofibrosis.

ipilimumab have contributed to a greater disease-free survival and revolutionized treatment for high-risk melanoma patients[163].

In addition to the approved anti-CTLA-4 agents, the group of approved anti-PD-1 targeting agents, such as pembrolizumab and nivolumab, can reduce T_{reg} cells. In contrast to CTLA-4, PD1 is not preferentially expressed on T_{reg} cells. Therefore, the ratio of PD1 expressing T_{eff} and T_{reg} cells can be used as a monitor for response[108,164,165]. Whether the $PD1^+$ T_{reg} cells are functionally immunosuppressive or have reduced functional activity remains a topic of ongoing research[141,165]. Since both main groups of ICI (i.e., CTLA-4 and PD1 targeting agents) affect T_{reg} cells, clinical studies evaluating T_{reg} cells during ICI therapies may provide valuable information for the development of novel inhibitors of T_{reg} cells.

The chemokine receptor CCR4 is expressed on T_{reg} cells. For example, the monoclonal antibody against CCR4, mogamulizumab, is approved for relapsed or refractory mycosis fungoides (MF) or Sézary syndrome (SS)[166]. Although mogamulizumab achieved an ORR of 10% in a diverse population with solid tumors[142], T_{reg} cells in tumor tissue and circulating blood were reduced in patients with tumor responses, while there were no changes or even increases in T_{reg} cells for patients who progressed.

In addition to the above-mentioned approved monoclonal antibodies, there are several drug development candidates designed to target specific proteins on T_{reg} cells. One such drug is GS-1811, a monoclonal antibody blocking CCR8 on T_{reg} cells[143,144]. This antibody is designed to remove the highly immune suppressive T_{reg} cells, which express CCR8. This approach of reducing a specific subset of T_{reg} cells may address the toxicity concerns otherwise observed with the CTLA-4 targeting agents. Furthermore, it appears that the expression of CCR8 is highly restricted to tumor-infiltrating T_{reg} cells[144].

Targeting CD25 on T_{reg} cells is another selective approach to block T_{reg} cells. RO7296682 (also known as RG6292), a monoclonal antibody designed to specifically block the CD25-mediated function on T_{reg} cells, is currently under clinical investigation (NCT04158583)[145]. Due to its design, RO7296682 promises to be more selective and less toxic than prior anti-CD25 monoclonal antibodies, such as daclizumab or basiliximab. As with GS-1811, the anticipated benefit is the reduced toxicity profile compared to the approved CTLA-4 targeting monoclonal antibodies.

Early non-clinical and clinical development efforts are currently targeting the ligand of CD25. This approach relies on blocking IL-2 or modifying the binding of IL-2. Recent technologies can generate multivalent, asymmetric IL-2-Fc fusions with different binding properties (including variable forms to either block or activate T_{reg} cells)[167]. A more traditional approach consists in the generation of specific IL-2 blocking antibodies, such as AU-007[146]. AU-007 binds to the CD25-binding epitope of IL-2, which prevents the interaction with the trimeric IL-2R expressed on T_{reg} cells, while not affecting the dimer of the IL-2R on memory or naïve T and NK cells. Patients receiving AU-007 had a decrease in T_{reg} cells, with an increase in $CD8^+$ T cells. This approach may overcome the known drug resistance in triple-negative breast cancer, where $CD25^+$ T_{reg} cells are associated with resistance to immunotherapy[168].

The surface protein CD38 is present on a wide range of immune cells, including T_{reg} cells. The reduction in T_{reg} cells following dosing of the anti-CD38 monoclonal antibody isatuximab plus atezolizumab in patients with advanced solid tumors was evaluated[169]. Surprisingly, isatuximab plus atezolizumab was not associated with a reduction in T_{reg} cells, although nearly all patients showed a reduction in $CD38^+$ T cells. The low overall response rate, diverse patient population, and low immune cell population at baseline may explain the lack of detectable changes in T_{reg} cells.

The carcinoembryonic antigen-related cell adhesion molecules (CEACAM)-5 and CEACAM-6 are expressed on tumor cells and T_{reg} cells with a profound immunosuppressive function[170]. The monoclonal

antibody NEO20, which targets CAECAM-5 and -6, reduced T_{reg} cells only in patients with long-term stable disease (SD)[147]. Therefore, the observations from the early clinical trials with the anti-CD38 and anti-CEACAM-5 monoclonal antibodies suggest that factors other than selectivity are important in the design of novel T_{reg} cell inhibitors.

Small Molecules: In addition to the large molecules, small molecules are being used to target signaling pathways uniquely or preferentially present in T_{reg} cells. There is an increasing list of small molecules that have been associated with the regulation of T_{reg} cells[171]. Perhaps the most common treatments associated with a reduction in T_{reg} cells are chemotherapies, such as cyclophosphamide, either as a therapy alone or in combination with vaccines[148]. In particular, the low dose cyclophosphamide (50 mg twice a day for a 2-week of a 4-week cycle) is associated with a reduction in T_{reg} cells and an increase in T_{eff} cells[150]. A variation of this administration is the metronomic regimen which also generates reproducible changes in T_{reg} cells[149]. Other chemotherapies with immunomodulatory effects include regimens containing docetaxel in NSCLC[151,172], sunitinib in renal cell carcinoma[151], and cisplatin plus vinorelbine in breast and lung cancer[151].

Chemotherapies are not sufficiently selective for T_{reg} cells and their subsets. Hence, more specific inhibitors may target unique pathways of T_{reg} cells, such as targeting FOXP3. Recently, a screen from different compounds found potential candidates that would directly degrade FOXP3, such as derivatives of gallic acid[173]. AZD8701 is an antisense oligonucleotide (ASO) blocking STAT3 and thus indirectly FOXP3[152]. During the Phase 1 study of AZD8701 in combination with durvalumab (NCT00637039), the FOXP3 expression was reduced with a concurrent reduction in T_{reg} cells.

Following the drug development experience of large molecules targeting CCR4, small molecule inhibitors of CCR4 are being investigated in patients[174]. For example, CCR4-351 is a small molecule inhibitor of CCR4, which reduces T_{reg} cells in animal and *in vitro* models[174]. CCR4 small molecule inhibitors block the migration of T_{reg} cells and therefore keep T_{reg} cells from entering the tumor microenvironment[175]. Despite a wide range of different CCR4 small molecule inhibitors, their clinical development has not led to an approved agent to this date[176].

Another approach is blocking signaling pathways downstream of T cell receptors or co-stimulatory molecules. One such pathway is the PI3K-δ signaling pathway[177]. By blocking PI3K-δ signaling, T_{reg} cells show reduced proliferation and, in patients' plasma, chemokines such as CCL2, CCL3, CCL5, and CCL22 are decreased[125,154]. In solid tumors, blocking PI3K-δ signaling modulated immune homeostasis and reinforced PD-1 blockade[178]. Based on this observation, the combination of pembrolizumab with parsaclisib (a designated PI3K-δ inhibitor) was investigated in patients who had progressed on prior immunotherapies[179]. Unlike the combination of pembrolizumab with the JAK1 inhibitor itacitinib, parsaclisib rebalanced the immune environment towards an interferon (IFN)-γ signature. Patients receiving the combination of parsaclisib and pembrolizumab also showed responses in both ICI-naïve and ICI therapy-resistant tumors (8/28 patients; 28%). Another designated PI3K-δ inhibitor, AMG-319, was investigated in patients with head and neck cancers[153]. In post-treatment biopsies, T_{reg} cells were reduced only in patients who tolerated AMG-319 for approximately 2 weeks, and thus were able to complete their scheduled treatment period. The tumor responses were minor and transient, most likely because the treatment was relatively short. These adenosine triphosphate (ATP)-competitive and designated PI3K-δ inhibitors, such as AMG-319 or idelalisib, have limitations due to their toxicity profile in patients with solid malignancies[180]. By contrast, the non-ATP, allosteric modulator and highly selective PI3K-δ inhibitor, roginolisib (IOA-244), has a lower rate of severe toxicity, which allows for treatments lasting greater than 6

months[75,181,182]. This well-tolerated profile is associated with a reduction in T_{reg} cells and a simultaneous increase of $CD8^+$ T and NK cells[183]. In patients with metastatic uveal melanoma, these changes in immune cell composition were associated with longer-than-expected overall survival (median OS of 20.8 compared to historic OS of 7.8 months)[110]. Whether roginolisib has the potential to overcome resistance to immunotherapy or prevent disease hyperprogression will be the objective of future investigation.

"Molecular glue" compounds, which are derived from cyclosporin A and FK506, are an emerging class of agents for clinical investigation[184]. Targeting IKZF2 (the gene that encodes for the zinc finger protein HELIOS, a member of the Ikaros family of transcription factors), the novel glue degrader NVP-DKY709 (=DKY709) reduces tumor resident and circulating T_{reg} cells[185]. Because HELIOS is uniquely expressed in a subset of T_{reg} cells[39], this approach promises a selective depletion of T_{reg} cells. DKY709 has been under clinical investigation in a Phase 1 study since 2019, either as a monotherapy or in combination with the PD1 inhibitors PDR001 (NCT03891953; accessed 3rd December 2023). Results on the biomarker responses are soon to be presented.

Reprogramming of T_{reg} cells provides an additional approach to reduce or alter the function of T_{reg} cells[186-188]. One such agent is the MALT1 inhibitor, MPT-0118, which in murine models showed a change in tumor-resident T_{reg} cells while not affecting T_{reg} cells in healthy tissue[189]. This approach can reduce the anticipated toxicity associated with global T_{reg} cell inhibition. In the first-in-human dose clinical trial, a low toxicity rate was observed along with some functional re-programming of T_{reg} cells[190].

Lastly, there are a growing number of approved small molecules that seem to affect T_{reg} cells, although they were not specifically designed to target T_{reg} cell pathways. We will highlight a few examples to illustrate such underappreciated drugs and their potential as immunotherapeutics. CDK4/6 inhibitors can reduce T_{reg} cells and improve immune responses in patients with breast cancer[155]. Similarly, breast cancer patients treated with trastuzumab, either alone or in combination with chemotherapy, showed a reduction in T_{reg} cells[191]. The JAK1/2 inhibitor ruxolitinib is associated with a reduction in T_{reg} cells in patients with primary myelofibrosis[130,131]. The FLT3 inhibitor midostaurin reduced T_{reg} cells in PBMCs from patients with AML[192]. Whether this effect is mediated via Dendritic Cells is being investigated[193]. The BCL2 inhibitor venetoclax, alone and in combination with pembrolizumab, improves immune responses and is associated with the reduction in T_{reg} cells in animal studies[156]. SRC inhibition represents another target for T_{reg} cell modification. The SRC inhibitor dasatinib seems to reduce T_{reg} cells and enhance immune responses in preclinical models[194]. While these aforementioned approved small molecule inhibitors do not specifically target signaling pathways in T_{reg} cells, they seem to have clinical benefits associated with a reduction in T_{reg} cells. This opens a new avenue for the rapid development of new immunotherapies with established agents as pursued by clinical research initiatives[195,196].

CONCLUSION

Lessons from the drug development of CTLA-4 inhibitors may provide valuable insights to successfully develop new therapies targeting T_{reg} cells. The research on T_{reg} cells has uncovered a T cell population with great plasticity. Despite their relatively small size, T_{reg} cells play a critical role in modulating immune responses to tumors. Hence, for novel drugs to be successfully developed in the clinic, the appropriate methods to assess the function of T_{reg} cells need to be evaluated alongside the standard measures of clinical benefit. The discovery of the precise pharmacologic platform (i.e., large or small molecule) that will deliver the greatest advantage is currently an exciting area of drug development.

DECLARATIONS

Authors' contributions

Reviewed literature, and critically reviewed, analyzed and interpreted the current state-of-the-art data: Spiliopoulou P, Kaur P, Hammett T, Di Conza G, Lahn M

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Spiliopoulou P declared that there are no conflicts of interest. Kaur P, Hammett T, and Lahn M are full-time employees of iOnctura SA; Di Conza G and Lahn M holds stocks in iOnctura SA.

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AUTHOR INSTRUCTIONS

1. Submission Overview

Before you decide to publish with *Cancer Drug Resistance* (*CDR*), please read the following items carefully and make sure that you are well aware of Editorial Policies and the following requirements.

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The topic of the manuscript must fit the scope of the journal. Please refer to Aims and Scope for more information.

1.2 Open Access and Copyright

The journal adopts Gold Open Access publishing model and distributes content under the Creative Commons Attribution 4.0 International License. Copyright is retained by authors. Please make sure that you are well aware of these policies.

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The publication fee for each submission is $2000. There are no additional charges based on color, length, figures, or other elements. OAE provides expense deduction for authors as appropriate. For more details, please refer to OAE Publication Fees.

1.4 Language Editing

All submissions are required to be presented clearly and cohesively in good English. Authors whose first language is not English are advised to have their manuscripts checked or edited by a native English speaker before submission to ensure the high quality of expression. A well-organized manuscript in good English would make the peer review even the whole editorial handling more smoothly and efficiently.

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If an accepted manuscript was funded by National Institutes of Health (NIH), the authors may inform Editors of the NIH funding number. The Editors are able to deposit the paper to the NIH Manuscript Submission System on behalf of the authors.

2. Submission Preparation

2.1 Cover Letter

A cover letter is required to be submitted accompanying each manuscript. It should be concise and explain why the study is significant, why it fits the scope of the journal, and why it would be attractive to readers, *etc.*

Here is a guideline of a cover letter for authors' consideration:

In the first paragraph: include the title and type (e.g., Original Article, Review, Case Report, *etc.*) of the manuscript, a brief on the background of the study, the question the author sought out to answer and why;

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If the manuscript was presented partly or entirely in a conference, the author should clearly state the background information of the event, including the conference name, time and place in the cover letter.

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The journal publishes Original Article, Review, Meta-Analysis, Case Report, Commentary, *etc.* For more details about paper type, please refer to the following table.

Manuscript Type	Definition	Word Limit	Abstract	Keywords	Main Text Structure

Original Article	An Original Article describes detailed results from novel research. All findings are extensively discussed.	5000 max	Structured abstract including Aim, Methods, Results and Conclusion. No more than 250 words.	3-8 keywords	The main content should include four sections: Introduction, Methods, Results and Discussion.
Review	A Review paper summarizes the literature on previous studies. It usually does not present any new information on a subject.	7000 max	Unstructured abstract. No more than 250 words.	3-8 keywords	The main text may consist of several sections with unfixed section titles. We suggest that the author include an "Introduction" section at the beginning, several sections with unfixed titles in the middle part, and a "Conclusion" section in the end.
Case Report	A Case Report details symptoms, signs, diagnosis, treatment, and follows up an individual patient. The goal of a Case Report is to make other researchers aware of the possibility that a specific phenomenon might occur.	2500 max	Unstructured abstract. No more than 150 words.	3-8 keywords	The main text consists of three sections with fixed section titles: Introduction, Case Report, and Discussion.
Meta-Analysis	A Meta-Analysis is a statistical analysis combining the results of multiple scientific studies. It is often an overview of clinical trials.	5000 max	Structured abstract including Aim, Methods, Results and Conclusion. No more than 250 words.	3-8 keywords	The main content should include four sections: Introduction, Methods, Results and Discussion.
Systematic Review	A Systematic Review collects and critically analyzes multiple research studies, using methods selected before one or more research questions are formulated, and then finding and analyzing related studies and answering those questions in a structured methodology.	3000 max	Structured abstract including Aim, Methods, Results and Conclusion. No more than 250 words.	3-8 keywords	The main content should include four sections: Introduction, Methods, Results and Discussion.
Technical Note	A Technical Note is a short article giving a brief description of a specific development, technique or procedure, or it may describe a modification of an existing technique, procedure or device applied in research.	3500 max	Unstructured abstract. No more than 250 words.	3-8 keywords	/
Commentary	A Commentary is to provide comments on a newly published article or an alternative viewpoint on a certain topic.	2500 max	Unstructured abstract. No more than 250 words.	3-8 keywords	/
Editorial	An Editorial is a short article describing news about the journal or opinions of senior editors or the publisher.	1000 max	None required	None required	/
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2.3 Manuscript Structure

2.3.1 Front Matter

2.3.1.1 Title

The title of the manuscript should be concise, specific and relevant, with no more than 16 words if possible. When gene or protein names are included, the abbreviated name rather than full name should be used.

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Authors' full names should be listed. The initials of middle names can be provided. Institutional addresses and email addresses for all authors should be listed. At least one author should be designated as corresponding author. In addition, corresponding authors are suggested to provide their Open Researcher and Contributor ID upon submission. Please note that any change to authorship is not allowed after manuscript acceptance.

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Original research, systematic reviews, and meta-analyses require structured abstracts. The abstract should provide the context or background for the study and should state the study's purpose, basic procedures (selection of study participants, settings, measurements, analytical methods), main findings (giving specific effect sizes and their statistical and clinical significance, if possible), and principal conclusions. It should emphasize new and important aspects of the study or observations, note important limitations, and not overinterpret findings. Clinical trial abstracts should include items that the CONSORT group has identified as essential. It is not allowed to contain results which are not presented and substantiated in the manuscript, or exaggerate the main conclusions. Citations should not be included in the abstract.

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Three to eight keywords should be provided, which are specific to the article, yet reasonably common within the subject discipline.

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Manuscripts of different types are structured with different sections of content. Please refer to Types of Manuscripts to make sure which sections should be included in the manuscripts.

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The introduction should contain background that puts the manuscript into context, allow readers to understand why the study is important, include a brief review of key literature, and conclude with a brief statement of the overall aim of the work and a comment about whether that aim was achieved. Relevant controversies or disagreements in the field should be introduced as well.

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Methods should contain sufficient details to allow others to fully replicate the study. New methods and protocols should be described in detail while well-established methods can be briefly described or appropriately cited. Experimental participants selected, the drugs and chemicals used, the statistical methods taken, and the computer software used should be identified precisely. Statistical terms, abbreviations, and all symbols used should be defined clearly. Protocol documents for clinical trials, observational studies, and other non-laboratory investigations may be uploaded as supplementary materials.

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This section contains the findings of the study. Results of statistical analysis should also be included either as text or as tables or figures if appropriate. Authors should emphasize and summarize only the most important observations. Data on all primary and secondary outcomes identified in the section Methods should also be provided. Extra or supplementary materials and technical details can be placed in supplementary documents.

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This section should discuss the implications of the findings in context of existing research and highlight limitations of the study. Future research directions may also be mentioned.

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It should state clearly the main conclusions and include the explanation of their relevance or importance to the field.

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Journal articles not in English	Zhang X, Xiong H, Ji TY, Zhang YH, Wang Y. Case report of anti-N-methyl-D-aspartate receptor encephalitis in child. *J Appl Clin Pediatr* 2012;27:1903-7. (in Chinese)
Journal articles ahead of print	Odibo AO. Falling stillbirth and neonatal mortality rates in twin gestation: not a reason for complacency. BJOG 2018; Epub ahead of print [PMID: 30461178 DOI: 10.1111/1471-0528.15541]
Books	Sherlock S, Dooley J. Diseases of the liver and billiary system. 9th ed. Oxford: Blackwell Sci Pub; 1993. pp. 258-96.
Book chapters	Meltzer PS, Kallioniemi A, Trent JM. Chromosome alterations in human solid tumors. In: Vogelstein B, Kinzler KW, editors. The genetic basis of human cancer. New York: McGraw-Hill; 2002. pp. 93-113.
Online resource	FDA News Release. FDA approval brings first gene therapy to the United States. Available from: https://www.fda.gov/NewsEvents/Newsroom/PressAnnouncements/ucm574058.htm. [Last accessed on 30 Oct 2017]
Conference proceedings	Harnden P, Joffe JK, Jones WG, Editors. Germ cell tumours V. Proceedings of the 5th Germ Cell Tumour Conference; 2001 Sep 13-15; Leeds, UK. New York: Springer; 2002.
Conference paper	Christensen S, Oppacher F. An analysis of Koza's computational effort statistic for genetic programming. In: Foster JA, Lutton E, Miller J, Ryan C, Tettamanzi AG, editors. Genetic programming. EuroGP 2002: Proceedings of the 5th European Conference on Genetic Programming; 2002 Apr 3-5; Kinsdale, Ireland. Berlin: Springer; 2002. pp. 182-91.
Unpublished material	Tian D, Araki H, Stahl E, Bergelson J, Kreitman M. Signature of balancing selection in Arabidopsis. *Proc Natl Acad Sci U S A*. Forthcoming 2002.

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Authors must keep data anonymized. If participants' details are not to be anonymized, authors must ensure that written informed consent, including consent for publication, was obtained from each participant, and consent statement must be included in the manuscript.

3.1.5 Ethical Approval and Informed Consent for Survey Studies

Researchers must ensure the participant's right to confidentiality has been considered, and they must inform all participants about the aims of the research and if there are any possible risks, and how the collecting data is being stored. The voluntary consent to participate of participants should be recorded and any legal requirements on data protection should be adhered to. Same with all research studies, ethics approval from IRB/local ethics committee for survey studies must be obtained before performing study. If ethics approval for certain survey study is not required, authors must include a statement to explain this clearly in the manuscript.

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assigns human participants or groups of humans to one or more health-related interventions to evaluate the effects on health outcomes. Interventions include but are not restricted to drugs, cells, other biological products, surgical procedures, radiologic procedures, devices, behavioral treatments, process-of-care changes, preventive care, *etc.*".

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9.1 Initial check

9.1.1 Initial manuscript check

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