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Reprogramming lymphocytes for the treatment of melanoma: From biology to therapy

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ABSTRACT

This decade has introduced drastic changes in melanoma therapy, predominantly due to the materialization of the long promise of immunotherapy. Cytotoxic T cells are the chief component of the immune system, which are targeted by different strategies aimed to increase their capacity against melanoma cells. To this end, reprogramming of T cells occurs by T cell centered manipulation, targeting the immunosuppressive tumor microenvironment or altering the whole patient. These are enabled by delivery of small molecules, functional monoclonal antibodies, different subunit vaccines, as well as living lymphocytes, native or genetically engineered. Current FDA-approved therapies are focused on direct T cell manipulation, such as immune checkpoint inhibitors blocking CTLA-4 and/or PD-1, which paves the way for an effective immunotherapy backbone available for combination with other modalities. Here we review the biology and clinical developments that enable melanoma immunotherapy today and in the future.

Contents

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1. Introduction

1.1. Melanoma epidemiology

Malignant Melanoma, arising from epidermal melanocytes, is one of the deadliest skin cancers [1]. It is not restricted to the skin and can be found in other organs such as eyes, ears, gastrointestinal tract, oral and genital mucous membranes [2]. While melanoma accounts for <1% of skin cancer cases, it is responsible for the vast majority of skin cancer deaths. The global incidence of melanoma has been increasing over the last 30 years. According to data from Surveillance, Epidemiology, and End Results (SEER) in 2018, it is estimated that there will be 91,270 new cases of melanoma of the skin and an estimated 9320 people will die of this disease in the US [3]. The trends in melanoma incidence differ by age, as the rate in men and women over 50 years old increased by about 3%, the rate had stabilized among young adults [3]. The incidence also differs by sex, as incidence rates are higher in women before age 50, but by age 65, rates in men are double those in women, and by age 80 they are triple [3].

1.2. Melanoma development

According to the common dogma of melanoma progression, disrupted intracellular signaling in melanocytes can lead to a series of molecular steps resulting in malignant transformation and superficial spreading growth, which further progresses to local invasion and ultimately to metastasis [2]. The activating BRAF mutation in position V600 is a very common event, appearing in around 50% of melanomas, and may be responsible to the triggering of this molecular/pathological progression of disease. As melanoma is highly heterogenic [4] an alternative model for melanoma progression was proposed, the “phenotype switching model” [5]. According to this model, melanoma cells are clustered into two dominant transcriptional profiles; proliferative and invasive. The proliferative signature is characterized by a higher proliferation rate and high expression of melanocyte differentiation markers. When these differentiation antigens are lost, melanoma cells become more mesenchymal and display the invasive signature that is characterized by high invasion and increased expression of genes involved in microenvironment modulation.

1.3. Treatment of melanoma

Local melanoma is typically treated with wide excision surgery, combined with sentinel lymph node biopsy for Stage Ib or higher [6]. Melanoma is considered resistant to chemotherapy and standard radiotherapy, and until 2011 only little could be offered to advanced patients, Over the past 8 years dramatic advances were made in the systemic therapy of melanoma, These include *Targeted therapy,* which is aimed at the MAPK pathway, particularly mutated BRAF-V600 and MEK, and *Immunotherapy, which* harnesses the patient's own immune system, particularly the arm of cytotoxic lymphocytes, to eradicate the melanoma cells. These therapeutic modalities are recommended for the treatment of Stage IV patients [6], and have already increased their median survival from 6 to 8 months to almost 3 years. These treatments are already approved and recommended for secondary prevention in the adjuvant setting as well, after demonstrating efficacy in randomized controlled clinical trials [6]. Targeted therapies are reviewed elsewhere, as the focus of this review are the immunotherapeutic strategies used to treat melanoma.

1.4. Immunogenicity of melanoma

Melanoma is immunogenic by nature according to the following main lines of evidence: i) Up to 20–30% of primary melanomas undergo at least partial spontaneous regression [7], primarily due an incomplete immune response comprised of tumor infiltrating CD4+ and CD8+ T lymphocytes [8]; ii) Melanoma tumors strongly express a variety of tumor associated antigens, which are recognized by autologous T cells capable of inducing tumor-directed immune responses [9]; iii) Historically, 5% of metastatic melanoma patients were cured with high dose Interleukin-2 [10], or demonstrated long term benefit to adoptive transfer therapy of lymphocytes (will be described in this review). Another hint was the good prognosis associated with vitiligo [11], and iv) Current dogma affirms that the immune system monitors neoepitopes generated in self-proteins in the process of genomic instability and mutagenesis within the cancer cells. Thus, mutational burden seems to at least partly reflect antigenicity, and indeed, the highest mutational burden across different cancer types was documented in melanoma [12].

These collective observations position the T cells at the heart of the anti-melanoma immune response, and provide the rationale for the

development of multiple strategies aimed to harness them successfully. Here we review the different currently approved and future approaches for manipulation of lymphocytes used to treat melanoma.

2. Immune checkpoint inhibitors

2.1. Currently approved immune checkpoint inhibitors

To date, all of the FDA approved drugs targeting immune checkpoints are directed against Cytotoxic T lymphocyte antigen-4 (CTLA-4) or the Programmed cell death 1 (PD-1) axis. CTLA-4 is a critical checkpoint that functions mainly during the priming phase of T cell activation [13]. In resting T cells, CTLA-4 is an intracellular protein; after antigen-restricted engagement of the T cell receptor (TCR) with a costimulatory signal through CD28, the CTLA-4 protein translocates to the cell surface. CTLA-4 then competes with CD28 over the interaction with CD80/CD86 and mediates an inhibitory signal into the T cell. This inhibitory signal results in T cell arrest of proliferation and activation [14]. By raising the inhibitory threshold for activation, CTLA-4 plays a role in peripheral tolerance by limiting T cell repertoire to mitigate ignorant, autoreactive T cells [15]. Indeed, CTLA-4 knockout mice develop overt lethal autoimmune manifestations [16]. Melanoma can be treated by targeting CTLA-4 with blocking antibodies, which transiently lower the inhibitory threshold, to broaden T cell repertoire and unmask new anti-melanoma T cell clones [17]. Indeed, CTLA-4 blockade with Ipilimumab demonstrated overall survival benefit in metastatic melanoma patients [18], which is durable beyond 3 years in about 20% of the patients [19]. Importantly, it also demonstrated overall survival benefit of 11% for 5 years in the adjuvant setting [20], becoming the first undisputed effective adjuvant therapy in melanoma. The toxicity profile is of broad spectrum autoimmune manifestations, mainly of skin, gastrointestinal and endocrine systems [21], and is in direct link with the regimen dose [22], attesting that this is related to the proposed mechanism of action. Unfortunately, there are still no predictive biomarkers for benefit or failure of treatment with anti CTLA-4 antibodies.

PD-1 is a critical immune checkpoint that functions mainly during the effector phase of T cell activation [23]. PD-1 delivers inhibitory signals upon engagement through its ligands, PD-L1 or PD-L2, by recruitment of the tyrosine phosphatase SHP-2, which dephosphorylates signaling molecules downstream of the TCR [14]. PD-1 plays a physiological role in controlling the effector T cells, as evident by the immune dysregulation displayed by PD-1 knockout mice [24]. T cell inflamed tumors engage PD-1 for immune evasion, for example by expressing PD-L1 or PD-L2 [25]. Indeed, treatment of metastatic melanoma patients with PD-1 blocking antibodies, nivolumab or pembrolizumab, has dramatically extended overall survival when compared in first line to chemotherapy (Checkmate 066) [26] or Ipilimumab (Keynote 006) [27] respectively. Long-term data from early trials suggest that the overall survival of a third of the patients exceeds 5 [28]. Very recently, nivolumab and pembrolizumab met the primary endpoints also in the adjuvant setting, compared with Ipilimumab [29] or placebo [30], respectively. The risk reduction is around 50%, thereby becoming the new standard of care. The autoimmunity toxicity profile is milder than the CTLA-4 blockade, but while gastrointestinal manifestations are milder, hypothyroidism and pneumonitis are more pronounced [27]. The good safety profile and high response rate make PD-1 blockade an viable therapeutic option for elderly patients older than 80 years old [31]. In melanoma, PD-L1 has a prognostic association with benefit from PD-1 blockade, but it is not predictive and therefore it is not used in clinical practice [32]. Importantly, the combination of ipilimumab and nivolumab was first tested in metastatic melanoma, against single agent ipilimumab or nivolumab. The combination therapy clearly yields higher response rate and significantly higher immune toxicity [18]. Numerical superiority in overall survival as compared to nivolumab must be interpreted with caution as the study was not powered to test for this difference [18]. The higher overall survival over nivolumab single agent seems to persist over four years of follow up [33]. PD-L1 blocking antibodies, such as Atezolizumab, Durvalumab and Avelumab, are still being tested in clinical trials in melanoma patients. Results of all major clinical trials with ICI in melanoma are summarized in Table 1.

There are two types of resistance to immune checkpoint inhibitors (ICI): Innate resistance is characterized by progression of the disease despite of treatment, while in acquired resistance there is an objective clinical response at first, but later the tumor continues to progress. Clinical response to ICI is associated with several parameters: i) the presence of tumor reactive T cells within the tumor prior to therapy [34] or the T cell inflammation signature [35]; ii) expression of immune checkpoints and T cell exhaustion markers [36]; iii) ability of the tumor to respond to interferon, reflected by an active interferon pathway [37]. Therefore, resistance mechanisms to ICI include: i) mechanisms of immune desert that prevent elicitation of an effective immune response [38]; ii) immune exclusion mechanisms [36]; iii) expression of alternative immune checkpoints [36]; iv) metabolic suppression through the microenvironment [39]; v) recruitment of immune suppressive cells such as regulatory T cells or myeloid derived suppressive cells [36]; vi) loss of responsiveness to interferon [37]. Delineation of the all of these resistance mechanisms are at the heart of current research efforts, in order to develop the next generation of immunotherapy. Due to the dominant effect of PD-1 axis blockade, the main strategy today is to utilize it as a backbone in combination regimens with newly developed compounds.

2.2. Co-inhibitory molecules

One of the key evasion mechanisms is based on engagement of other immune checkpoints. This can play a role in primary resistance, where immune checkpoints other than PD-1 play a major role [40] or in acquired immune resistance, where the alternative immune checkpoints overcome PD-1 blockade [41]. Delineating the underlying biology of these co-inhibitory molecules is expected to lead to the development of effective medications that may broaden the cancer indication portfolio. Current clinical trials with the different co-inhibitory molecules are described in Table 1.

2.2.1. TIM-3

T cell immunoglobulin and mucin-domain (TIM) is a family of type I transmembrane proteins that are expressed by immune cells, both innate and adaptive. TIM proteins have a cytoplasmic tail and an extracellular domain consisting of an N-terminal immunoglobulin variable region-like (IgV) domain and a serine/threonine rich mucin-like region. The cytoplasmic tail contains tyrosines that can be phosphorylated, thereby enabling the interaction with proteins involved in signal transduction. However, the specific mechanisms by which TIM proteins regulate the function of immune cells are yet to be fully understood [42]. The human TIM family comprises of three members TIM-1, TIM-3 and TIM-4. TIM-3 is expressed on different immune cells, including T cells, natural killer (NK) cells, macrophages and dendritic cells. TIM-3 can be a direct negative regulator of T cells through induction of T cell apoptosis [43] or indirectly promote immunosuppression by enhancing secretion of pro-inflammatory cytokines from dendritic and NK cells [43] and inducing expansion of myeloid-derived suppressor cells [44]. TIM-3 interacts with both soluble ligands (galectin-9 and HMGB1) and cell surface ligands (CEACAM-1 and Phosphatidyl serine) [45] and in T cells, functions specifically to limit the duration and magnitude of Th1 and Tc1 T cell responses thus facilitating T cell exhaustion [46]. Indeed, low levels of TIM-3 have been associated with multiple sclerosis, diabetes and other autoimmune processes [44]. Accordingly, it was shown that the NY-ESO-1–specific $CD8^+$ T cells in advanced melanoma that co-express TIM-3 and PD-1 are the most dysfunctional. Blockade of the TIM-3 pathway alone restores IFN-γ and TNF-α production. Moreover, Co-blockade of Tim-3 and PD-1 restores IL-2 production as well [47]. These results suggest dual blockade of Tim-3 and PD-1 would be

Table 1
Ongoing clinical trials of novel immunotherapies in melanoma patients.

Trial number	Target	Intervention	Primary outcomes	status
Co-inhibitory immune checkpoints				
NCT02817633	TIM-3, PD-1	TSR-022 + anti PD-1 antibody	• Safety and tolerability • Anti-tumor activity • Recommended phase 2 dose	Recruiting
NCT03708328	TIM-3/PD-1	RO7121661	• Safety and tolerability • Anti-tumor activity	Recruiting
NCT02676869	LAG-3, PD-1	IMP321 + Pembrolizumab	• Safety and tolerability • Recommended phase 2 dose	Active, not recruiting
NCT01968109	LAG-3, PD-1	Relatlimab (BMS-986016) ± Nivolumab	• Safety and tolerability • Anti-tumor activity	Recruiting
Co-stimulatory immune checkpoints				
NCT02554812	PD-L1, 4-1BB, OX40, M-CSF	Avelumab ± Utomilumab ± PF-04518600 ± PD0360324	• Safety and tolerability • Pharmacokinetics and pharmacodynamics • Anti-tumor activity	Recruiting
NCT02737475	OX40, PD-1, CTLA-4	BMS-986178 ± Nivolumab ± Ipilimumab	• Safety and tolerability	Recruiting
NCT02410512	OX40, PD-L1	MOXR0916 + Atezolizumab	• Safety and tolerability	Active, not recruiting
NCT01239134	GITR	TRX518	• Safety and tolerability • Pharmacokinetics and pharmacodynamics	Recruiting
NCT02598960	GITR, PD-1, CTLA-4	BMS-986156 ± Nivolumab	• Safety and tolerability	Active, not recruiting
NCT03126110	GITR, PD-1, CTLA-4	INCAGN01876 ± Nivolumab ± Ipilimumab	• Safety and tolerability • Anti-tumor activity	Recruiting
Metabolic immune checkpoints				
NCT02073123	IDO, PD-1, CTLA-4	Indoximod ± Nivolumab ± Pembrolizumab ± Ipilimumab	• Safety and tolerability • Anti-tumor activity • Recommended phase 2 dose	Active, not recruiting
NCT03301636	PD-1, IDO	Pembrolizumab/Nivolumab ± Indoximod	• Safety and tolerability • Anti-tumor activity	Recruiting
NCT02903914	Arginase, PD-1	INCB001158 ± Pembrolizumab	• Safety and tolerability	Recruiting
Combination of MAPK inhibition and immune checkpoint blockade				
NCT02224781	PD-1, CTLA-4, BRAF, MEK	Nivolumab + Ipilimumab + Dabrafenib + Trametinib	• Anti-tumor activity	Recruiting
NCT02968303	PD-1, CTLA-4, BRAF, MEK	Nivolumab + Ipilimumab ± Vemurafenib + Cobimetinib	• Anti-tumor activity	Recruiting
NCT02968303	PD-1, CTLA-4, BRAF, MEK	Nivolumab + Ipilimumab ± LGX818 + MEK162	• Anti-tumor activity	Recruiting
NCT03235245	PD-1, CTLA-4, BRAF, MEK	Nivolumab + Ipilimumab ± Encorafenib + Binimetinib	• Anti-tumor activity	Recruiting
NCT02902029	BRAF, MEK, PD-L1	Vemurafenib + Cobimetinib + Atezolizumab	• Anti-tumor activity	Recruiting
Oncolytic viruses				
NCT02965716	T-VEC, PD-1	T-VEC + Pembrolizumab	• Anti-tumor activity	Recruiting
NCT03088176	T-VEC, BRAF, MEK	T-VEC + Dabrafenib + Trametinib	• Safety and tolerability	Recruiting
NCT03747744	T-VEC, Dendritic cells	T-VEC + autologous BDCA-1^+ myeloid dendritic cells	• Safety and tolerability	Recruiting
NCT02565992	CVA21, PD-1	CAVATAK + Pembrolizumab	• Safety and tolerability	Active, not recruiting
NCT02307149	CVA21, CTLA-4	CAVATAK + Ipilimumab	• Safety and tolerability • Anti-tumor activity	Active, not recruiting
Personalized cancer vaccines				
NCT01970358	Neoantigens	NeoVax (Neoantigen peptides + poly-ICLC)	• Safety and tolerability • Personalized vaccine feasibility	Active, not recruiting
NCT03480152	Neoantigens	NCI-4650 (mRNA-based Personalized Cancer Vaccine)	• Safety and tolerability • Anti-tumor activity	Recruiting
NCT03300843	Neoantigens	Peptide loaded dendritic cell vaccine	• Anti-tumor activity	Recruiting
NCT02035956	Neoantigens	IVAC MUTANOME (RNA vaccine)	• Safety and tolerability	Active, not recruiting
NCT02897765	Neoantigens, PD-1	NEO-PV-01 (peptide-based personalized cancer vaccine) + poly-ICLC + Nivolumab	• Safety and tolerability	Active, not recruiting
Specific Tregs targeting interventions				
NCT02281409	CCR4	Mogamulizumab	• Safety and tolerability	Recruiting
NCT02705105	CCR4, PD-1	Mogamulizumab + Nivolumab	• Safety and tolerability	Active, not recruiting
NCT02968303	CD25	ADCT-301 (anti-CD25 + Pyrrolobenzodiazepine)	• Safety and tolerability	Recruiting
NCT02646748	PD-1, JAK1, PI3K-δ	Pembrolizumab + Itacitinib or INCB050465	• Safety and tolerability	Recruiting
MDSC modulating drugs				
NCT02403778	CTLA-4, ATRA	Ipilimumab + VESANOID	• Safety and tolerability • MDSC frequency and suppressive function	Active, not recruiting

(continued on next page)

Table 1 (*continued*)

Trial number	Target	Intervention	Primary outcomes	status
	NCT03161431	CXCR1/2,	SX-682 ± Pembrolizumab	• Safety and tolerability
PD-1	• Anti-tumor activity	Recruiting		
NCT02637531	PI3K-γ, PD-1	IPI-549 ± Nivolumab	• Safety and tolerability	Recruiting
NCT02922764	LXR, PD-1	RGX-104 ± Nivolumab	• Safety and tolerability • Anti-tumor activity	Recruiting
Gut microbiota modulation				
NCT03353402	Gut microbiota	FMT from anti-PD-1 responder	• Safety and tolerability • Fecal implant engraftment	Recruiting
NCT03341143	Gut microbiota, PD-1	FMT from anti-PD-1 responder + Pembrolizumab	• Anti-tumor activity	Recruiting
NCT03637803	Gut microbiota, PD-1	MRx0518 (*Enterococcus gallinarum* strain) + Pembrolizumab	• Safety and tolerability • Anti-tumor activity	Recruiting
NCT03817125	Gut microbiota, PD-1	Nivolumab ± SER-401 (Oral bacterial cocktail)	• Safety and tolerability	Recruiting

synergistic. The role of TIM-3 in T cell exhaustion led to the development of TSR-022, an anti-TIM-3 monoclonal antibodies, which are currently being tested in early-phase clinical trials as a single agent and in combination with anti-PD-1 in patients with advanced malignancies (NCT02817633) (Table 1).

2.2.2. LAG-3

Lymphocyte activation gene-3 (Lag-3) is a transmembrane protein expressed by T cells and NK cells after major histocompatibility complex (MHC) class II ligation [44]. Structurally, LAG-3 resembles the CD4 co-receptor and binds to MHC class II as well as to LSECtin. LSECtin is a member of the DC-SIGN family of molecules and is expressed on the liver and on many tumors [45]. Though the exact mechanism remains unclear, LAG-3 has a negative regulatory effect over T cell functions. Moreover, LAG-3 and PD-1 are frequently co-expressed and upregulated on tumor infiltrating lymphocytes (TILs) leading to immune exhaustion and tumor growth [44]. Indeed, Experiments conducted on melanoma B16 cell-line showed that expression of LSECtin caused a reduction in IFNγ expression by CD8+ T cells. This reduction was restored with the blockade of LAG-3 [48].

Currently, there are two types of drugs targeting the LAG-3 pathway in early-phase clinical development, IMP321, a soluble LAG-3Ig fusion protein and anti-LAG3 mAb [44]. IMP321 binds to MHC class II with high avidity and mediates antigen presentation and activation of memory CD8+ T cells [49]. It is administered by injection either subcutaneously, intratumoral or intra-peritoneal, alone or as an adjuvant for anti-PD-1 therapy (NCT03252938). A phase I clinical trial studied the combination of chemotherapy (paclitaxel) and IMP321 in metastatic breast cancer patients and showed that IMP321 induced a higher number of antigen presenting cells (APCs), NK and cytotoxic effector-memory CD8+ T cells. The objective tumor response rate was 50% compared 25% in the control group and no significant specific toxicities were observed [49]. Currently there is a phase I clinical trial underway testing the safety and efficacy of IMP321 as an adjuvant to anti-PD1 therapy in unresectable or metastatic melanoma (NCT02676869). Targeting LAG-3 with antagonistic mAb interferes with the LAG-3 – MCH II interactions between the T cell and the tumor cell, thus promoting tumor cell apoptosis [44]. A phase I clinical trial is currently recruiting melanoma patients to determine the safety and efficacy of anti-LAG-3 mAb (BMS-986016) with and without anti-PD-1 (Nivolumab) (NCT01968109). Interim results show a safety profile similar to Nivolumab alone and promising efficacy with 16% objective response rate and 45% disease control rate [50] (Table 1).

2.2.3. CEACAM-1

Carcinoembryonic antigen-related cell adhesion molecule 1 (CEACAM1) is a cell-to-cell adhesion molecule that is expressed on a variety of cells, activated lymphocytes, epithelial cells, and melanoma cells but not on normal melanocytes. CEACAM1 can interact homophilically with CEACAM1 and heterophilically with CEACAM5 [51]. Engagement of CEACAM1 expressed by activated lymphocytes delivers inhibitory signals *via* its ITIM motifs in a SHP-1-dependent manner, which impairs proliferation, secretion of IFN and cytotoxic activity of NK cells and T cells [52,53]. Accordingly, CEACAM1-L is commonly expressed by melanoma tumors [54], thereby conferring poor prognosis [55], and on circulating lymphocytes in the peripheral blood of melanoma patients [56]. Furthermore, CEACAM1 plays a role in adaptive immune resistance, as it is induced by IFNγ in melanoma cells following T cell -mediated attack, which renders the surviving cells even more resistant to T cell killing [57].

Anti-CEACAM1 monoclonal antibody aimed to block CEACAM1 homophilic binding renders CEACAM1+ melanoma cells more susceptible to T cell mediated elimination in an antigen-restricted manner [58]. This antibody is expected to exert less immune-toxic effects seen in patients treated with anti-CTLA-4 and synergize with anti-PD-1.

2.3. Co-stimulating molecules

Another route to immune cell activation is through stimulatory pathways. Stimulatory molecules enhance survival and effector function activation thus promoting an immune reaction even in the presence of an inhibitory signal. Current clinical trials with the different co-inhibitory molecules are described in Table 1.

1.1.1 4-1BB

4-1BB is an inducible co-stimulatory receptor that is a member of the TNF receptor superfamily. It is expressed on T cells, NK cells, and APCs and upon ligand (4-1BBL) binding it triggers immune cell proliferation and activation [44], upregulates survival genes, cytokine secretion, cell division and prevents activation-induced cell death (AICD) in T cells [59]. Combination therapy of anti-4-1BB with other anticancer agents, such as radiation, has robust tumor-regressing abilities against non-immunogenic or poorly immunogenic tumors [59]. Furthermore, work on the poorly immunogenic B16F10 melanoma model showed that a combination of anti–PD-1 and anti–4-1BB resulted in a synergistic anti-tumor effect while a combination of anti–PD-1 and anti–LAG-3 was ineffective [60].

Currently there are several trials testing the safety and efficacy of anti-4-1BB agonist monoclonal antibody administered IV as a single agent and combined with other cancer immunotherapies. One phase I clinical study tested combination therapy of anti-4-1BB (Utomilumab) with anti-PD-1 (Pembrolizumab) in patients with advanced solid tumors (NCT02179918). The study revealed a trend with higher levels of activated memory/effector peripheral blood CD8+ T cells in responders *versus* non-responders. No dose-limiting toxicities were reported and 6 out of 23 patients had either complete response (CR) or partial response (PR) [61] (Table 1).

2.3.1. OX40

OX40 is a member of the TNFR super-family and is highly expressed by activated CD4, CD8 T cells, and Tregs, and in a lesser degree by neutrophils and NK cells [44]. Furthermore, there is a transient increase in OX40 expression following TCR/CD3 cross-linking, and by the presence of inflammatory cytokines [62]. OX40 has a pivotal role in T cell survival, activation and proliferation leading to enhanced anti-tumor immunity. Moreover, OX40 can also affect tumor immunity by inhibiting the suppressive activity of regulatory T cells (Tregs) either directly by interfering with their function and proliferation, or indirectly by antagonizing their inhibitory byproducts [44]. OX40 ligation can enhance IFN-γ production by T cells in response to TCR stimulation, which may lead to up-regulation of PD-L1 in the cancerous cell. This mechanism leads to a potentially synergistic therapy combining PD-1/PD-L1 blockade with OX40 agonist [62]. Indeed, A work on the poorly immunogenic murine ID8 ovarian cancer model showed that even though individual anti-PD-1 or OX40 mAb treatment were ineffective, combined anti-PD-1/OX40 mAb treatment markedly inhibited tumor outgrowth [63]. One possible way to enhance OX40 stimulatory affect is by an agonistic monoclonal antibody. These antibodies are a double-edged sword encompassing an anti-tumor activity along with TIL depletion through an antibody-dependent cell cytotoxicity by NK cells. Given that the antibody is injected into the tumor this depletion can only occur in tumors that have NK cells infiltrates. Another limitation promoting local injection rather than a systemic one is unwanted activation of peripheral lymphocytes [44].

Several clinical trials are currently testing the safety and efficacy of anti-OX40 agonist monoclonal antibody as a single agent and in combination with anti-PD-1 or anti-CTLA-4 in patients with advanced malignancies. One ongoing trial (NCT02410512) testing the safety and efficacy of agonist anti-OX40 monoclonal antibody (MOXR0916) along with anti PD-L1 (Atezolizumab) in patients with advanced malignancies reported preliminary results showing no dose-limiting toxicities. Objective response have been observed but yet to be published [64] (Table 1).

2.3.2. GITR

GITR is a member of the tumor necrosis factor receptor superfamily that acts as a co-stimulatory immune checkpoint molecule and is expressed on activated T cells, including T regulatory T cells. Interestingly, whereas activation of GITR by an agonistic antibody or its ligand suppresses effector Tregs immunosuppressive functions, it also provides costimulatory signaling for activated T cells [65,66]. Administration of agonistic anti-GITR antibody to tumor-bearing mice eradicated established tumors, increased intra-tumor interferon-γ secreting lymphocytes and tumor-specific CD8+ T cells activity, and decreased Treg infiltrates and FOXP3 expression within tumor infiltrating Tregs [67,68]. There are currently various agonistic anti-GITR antibodies or other GITR agonists that are being assessed in clinical trials as monotherapy or in combination with other immunotherapies including TRX-518 (NCT01239134), BMS-986156 (NCT02598960, NCT03335540), GWN323 (NCT02740270), INCAGN01876 (NCT02697591, NCT03126110), MEDI1873 (NCT02583165), OMP-336B11 (NCT03295942).

2.4. Metabolic suppressors

Upon T cell activation the availability of intracellular amino acids are constantly monitored to ensure sufficient energy. If the cytosolic amino acid resources are not sufficient amino acid starvation response will be activated leading to cell cycle arrest and apoptosis [69]. Depletion of essential amino acids in the tumor microenvironment facilitates immune suppression thus creating another possible route to cancer therapy [44,70]. Current clinical trials with the different co-inhibitory molecules are described in Table 1.

2.4.1. IDO-1

Indoleamine 2, 3-dioxygenase 1 (IDO1) is an interferon inducible, rate-limiting metabolic enzyme in the Kynurenine pathway that converts L-tryptophan into N-formylkynurenine. IDO1 expression is prevalent in many cancers and induces immune tolerance by suppressing T cell functions through tryptophan catabolism in the tumor microenvironment. The role of IDO1 in immunosuppression is comprised of three possible mechanism (i) Cell cycle arrest of T cells through local depletion of Tryptophan; (ii) Immune modulatory effect conducted by downstream Kynurenines causing effector T cell arrest or apoptosis, and (iii) Conversion of naive CD4+ T cells into immunosuppressive FOXP3-expressing Tregs through accumulation of Kynurenines [71].

Preclinical studies showed that a combination of immune checkpoint blockade and IDO1 pathway inhibition provides potent reactivation of tumor-infiltrating T cells [72]. Early phase clinical trials of IDO1 inhibitor (Epacadostat) in combination with anti PD-1 (Pembrolizumab) demonstrated encouraging results in melanoma, renal cell carcinoma and lung cancer [73]. Unfortunately, this combination did not prove to be superior to Pembrolizumab alone in Keynote-252, a randomized controlled Phase III trial in metastatic melanoma patients [74]. The limitations of this study included: i) uncertainty of IDO1 blocking adequacy, as there was no direct evidence regarding the degree of IDO1 inhibition within the tumor; ii) lack of an appropriate biomarker, as there are preclinical genetic evidence of tumors that bypass IDO1 blockade for example by Tryptophan-2,3-dioxygenase 2 (see below); and iv) lack of single agent activity in the preclinical models [75]. Therefore, this strategy should be further explored.

2.4.2. TDO

Tryptophan-2,3-dioxygenase 2 (TDO) is also a rate-limiting enzyme of the kynurenine pathway, converting L-tryptophan to N-formylkynurenine. In spite of the functional similarity, IDO1 and TDO do not share sequence homology, although there are some structural similarities around the catalytic site and the heme binding site [76]. TDO is physiologically expressed at high levels in the liver and in lowers levels in neurons [77]. It is expressed in some tumors, especially melanoma, hepatocellular carcinoma, bladder cancer and glioma [78,79]. A screen of cancer cell lines showed that that 19% of tumor cell lines are TDO positive, 16% are IDO1 positive and 15% express both TDO and IDO1 [78] suggesting that blocking only one pathway may be insufficient in arresting tryptophan depletion. The first TDO inhibitor that had high solubility, good inhibition potency and high bioavailability is LM10 [80] which also displayed anti-tumor activity in a preclinical model [78]. Two more TDO inhibitors were found since, N1-benzyl-1H-naphtho[2,3-d] triazole-4,9-dione and compound “1” that show promise, but all three inhibitors still need to be further evaluated before they can be tested clinically [80].

2.4.3. Arginase

Arginase is an enzyme that converts arginine and H2O into ornithine and urea. High levels of arginase are produced by tumor cells and myeloid derived suppressor cells (MDSCs) present in the tumor microenvironment thus leading to arginine depletion and T cell amino acid starvation response [44]. Moreover, tumor-associated macrophages (TAMs) use arginine either to support cytotoxic function through nitric oxide or to produce ornithine through arginase activity thus facilitating tumor cell proliferation [81]. Accordingly, the use of arginase inhibitors could negate the immunosuppressive effects of the tumor microenvironment and decrease the level of substances that favor tumor growth [44].

There is an ongoing phase I clinical trial testing a selective arginase inhibitor (CB-1158) alone or in combination with anti-PD-1 (Nivolumab) in patients with metastatic solid tumors (NCT02903914). Preliminary results show that the drug is well tolerated with no dose-limiting toxicities. At doses of 50 and 100 mg >90% of the arginase

was inhibited and there was a 2.4 and 4-fold increase in plasma arginine levels, respectively [82] (Table 1).

3. MAPK inhibitors as tumor microenvironment modulators

Around 40% of melanoma patients harbor BRAF V600E mutation [83], which constitutively activates BRAF, and consequently the MAPK/ERK pathway, to promote survival and proliferation of melanoma cells [84]. Therefore, there was a strong effort to develop inhibitors of the MAPK/ERK pathway for treatment of BRAF mutated melanoma patients. Since 2011, several BRAF inhibitors (BRAFi) were introduced (Vemurafenib and Dabrafenib), showing improved response and survival compared with chemotherapy in randomized controlled trials [85,86]. However, most patients developed resistance within 6–-9 months, mostly due to reactivation of the MAPK pathway [87]. A more significant survival improvement was observed when the MEK inhibitors (MEKi) were added to the BRAFi, increasing median PFS from 7.3 to 12.1 months and median OS from 17.8 to 26.1 months when compared with BRAFi alone [88,89]. Interestingly, 3 years after initiation of BRAFi and ERKi treatment, 25% of patient did not progressed, exhibiting a long term response potential with combined treatment, especially in patient with low tumor burden (LDH < ULN and < 3 organs with metastasis) [90] or patients that developed immune-related adverse events in melanocyte rich organs such as the skin and the eye [91].

Since the development of MAPK/ERK pathway inhibitors several papers investigated the effects of MAPK/ERK selective inhibition on the tumor microenvironment and tumor infiltrating lymphocytes function. Inhibition of the MAPK/ERK pathway increased melanoma differentiation antigens presentation [92,93], T cell infiltrates [94], and T cells cytotoxic markers [95] and sensitivity to T cells *in vitro* [96], while reducing immunosuppressive chemokines such as IL-6, IL-8, IL-10 & VEGF [93,97]. At present, there is no published data whether MAPK inhibition results in immunogenic cell death, a mechanism that could provide further synergy with immunotherapy. The improved efficacy of MAPK inhibition plus immunotherapy *vs.* immunotherapy alone was demonstrated in several mouse models [95,98,99] further solidifying the rationale to combine immunotherapy with MAPK inhibition.

Several factors must be considered when devising optimal regimen of such combination. Most notably, one should consider the sequence, timing, dosage and choice of drugs, and the risk for severe side effects or their frequency. For example, a phase I trial combining vemurafinib and ipilimumab was stopped due to high grade liver toxicities in >50% of patients [100]. At the moment, the first results from a small randomized Phase II study comparing Dabrafenib+Trametinib with or without Pembrolizumab did not show statistically significant differences, but the report is just over a short follow up period [101]. Based on the preclinical evidence, several phase III clinical trials combining immunotherapy and MAPK inhibition are underway (Table 1). An alternative approach to upfront triple drug combination could be a three staged approach: i) BRAFi/MEKi combination for 1–3 months to control tumor load and render the microenvironment more favorable to the immune system as described above ("induction"); ii) add PD-1 blockade (triple drug regimen) for 1–3 months ("consolidation"), and iii) continue only with PD-1 blockade ("maintenance"). This approach could have benefits in safety, tolerability, costs and maintaining future lines of therapy, over an upfront triple drug combination. Current clinical trials appear in Table 1.

4. Adoptive cell therapy

Another way to harness lymphocytes to eradicate metastatic melanoma is through adoptive cell transfer therapy (ACT). Lymphocytes are extracted from the patient, cultivated *in vitro* and later are infused back into the patient. The *ex vivo* cultivation stage allows for further manipulation by cell selection or genetic engineering.

4.1. T cell ACT

Tumor Infiltrating Lymphocytes (TILs) are a heterogeneous population of T cells, naturally occurring within the tumor. TILs play a crucial role in mediating the anti-cancer immune response as they identify cancer antigens and initiate a cytotoxic immune activity. In spite of this antitumor attack, when the cancer spreads, it is evident that the immune system has fallen short. Yet, autologous TIL can be harnessed through ACT protocols. The basic TIL ACT protocol consists of TIL extraction, *in vitro* expansion and administration of the TILs back into the patient with IL-2 boluses, after pre-conditioning with non-myeloablative lymphodepleting chemotherapy [102]. The TILs are extracted from a resected metastasis using either enzymatic digestion, fragmentation or mincing. Rapid expansion and activation *in vitro* is achieved using high concentration of IL-2 until the culture reaches a critical number of cells. Prior to the rapid expansion a selection process can be used in order to identify the T cells that recognize and react to the tumor cells. This could be done by co-culture with autologous tumor cells and screening for IFNγ release [102], or by other strategies, such as positive selection by 4-1BB(+) cells [103]. We have previously shown that using the entire population of minimally cultured TIL provides effective clinical regressions [104,105]. This therapy has shown to be an effective treatment for metastatic melanoma patients with remarkable clinical response rates of approximately 50% in the pre anti-PD-1 era [106].

Multiple challenges still face TIL ACT: i) Loss of key accessory molecules due to the *ex vivo* conditions leading to activation induced cell death. Stimulation of these cells with anti-4-1BB agonistic monoclonal antibody significantly inhibits activation induced cell death and increase their effector activity against melanoma cells [107]. Moreover, a murine model using Chimeric Antigen Receptor (CAR) T cells along with agonist anti–4-1BB showed a an enhancement of CAR T cell efficacy, an increase of IFNγ expression and a reduction in host immune-suppressive cells at the tumor site [108]; ii) Scalability and production. It is anticipated that the rapid development of bioreactor technologies such as Wave™ will eliminate this challenge; iii) Acute toxicity profile, which occurs mostly due to the accompanying IL-2 boluses. This may be solved with genetic engineering technology, for example to render TIL more sensitive to IL-2, or induce antigen-triggered IL-2 production [109]; Decreased efficacy in patients refractory to anti-PD-1, which may be due to accrual of patients that have already went through more effective lines of therapy, or due to cross-resistance mechanisms.

Current major efforts are invested in developing methods for recognizing neoantigen-specific TIL [110,111], as these represent the main effector cell population. Indeed, the first proof of concept was demonstrated by Tran and colleagues, that demonstrated clinical efficacy by infusing neoepitope-specific TIL in a patient with cholangiocarcinoma [112]. Identification of tumor specific antigens requires comparison between the exomic DNA from the tumor and from the normal tissue of the patient [113], or combining mass spectrometric peptidomics to focus on MHC-presented peptides [113]. The identified mutation can be synthesized as a polypeptide that can be expressed by the patients' APCs. T cells from the peripheral blood *co*-cultured with these APCs that recognize these polypeptides can be identified using activation markers such as OX40 and 41BB [114].

4.2. ACT with genetically engineered T cells

The current method of TIL ACT holds a number of limitations including the need for surgery to isolate the T cells, the significant time it takes to expand the culture, the unknown antigen specificity and the fact that TIL cultures cannot be generated for all of the patients. Those limitations could be addressed using genetically manipulated T cells [106].

4.2.1. TCR engineering

Genetic engineering T cell Receptors (TCR) could potentially allow for peripheral blood T cells to become tumor-identifying cells. Such a

TCR needs to target effectively all of the cells in a heterogeneous tumor while sparing normal tissues. An optimal TCR target would not only be tumor specific, but also important for tumor cell survival thus reducing the risk of tumor cell escape [115]. A few attempts have been made to engineer TCRs that target melanoma associated antigens. T cells expressing an affinity enhanced TCR targeting the melanoma/melanocyte differentiation antigen MART1 or the cancer/testis antigen NY-ESO-1 demonstrated encouraging results, albeit inducing autoimmunity against normal melanocytes [116–118]. Studies using TCRs targeting gp100 and MAGE-A3 observed significant adverse reactions thus emphasizing the need for a preclinical method to better evaluate potential toxicities [115]. Another approach could be to develop TCRs against neoepitopes derived from cancer driver mutations, as these are relatively common among different patients, as opposed to the discrete neoepitopes. A proof of concept study has successfully identified TIL directed against a peptide derived from mutated KRAS presented in the context of HLA-A3 [119]. Current efforts are invested in developing strategies for engineering TCRs with neoantigen-specificity, for example by utilizing single cell RNA seq [120].

4.2.2. CAR T cells

Chimeric antigen receptors (CARs) are engineered receptors that encode for transmembrane chimeric molecules with dual function – antigen recognition and T cell activation. This provides an alternative to HLA-mediated recognition and enables a workaround the tolerance acquired by tumor cells [121].

CAR T cells are classified as first, second, third and fourth generation. The first-generation consists of a single chain variable fragment and an immune-receptor tyrosine-based activation motif (ITIM). Both elements are sufficient for antigen recognition and T cell activation, but the activation and proliferation are short lived. The second-generation CAR T cells express co-stimulatory molecules such as CD28 and 4-1BB, which enhance expansion and prolong T cell activation. The third generation is based on the second generation adding another co-stimulatory molecule. This enhances activation, proliferation and elevates survival of T cells even more so. Along with the desired effect, the presence of multiple intracellular signaling could lead to an abundance of cytokines that could spiral into a life-threatening cytokine storm [122]. The fourth-generation CAR T cells includes expression of co-stimulatory ligands or pro-inflammatory cytokines that are released upon antigen recognition, thereby activating and attracting other components of the immune cells [123]. The breakthrough of CAR T cell therapy came into clinical reality against B-cell hematological malignancies by using anti-CD19 CAR. This therapy has demonstrated consistently high antitumor efficacy in pediatric and adult patients, with 70–04% of complete remissions [124].

Regrettably, clinical trials with CAR T cells targeting solid tumors failed so far to show similar results. The main obstacles are still the lack of a tumor specific antigen, improving tumor homing and enabling survival in the tumor microenvironment [121]. One possible antigen that can be used to target melanoma tumors is the high molecular weight melanoma associated antigen (HMW-MAA). HMW-MAA is expressed on >90% of human melanomas whereas the expression in normal tissues is restricted. In preclinical studies, HMW-MAA CAR-modified T cells showed the ability to recognize melanoma cell lines and elicit effector functions [106]. Another approach is to target antigens that are present on tumor stromal cells. One example is the vascular endothelial growth factor receptor 2 (VEGFR2) that is overexpressed in the tumor vasculature. Preclinical trials showed that VEGFR2 CAR T cells have an antitumor effect while sparing normal tissue but unfortunately, the results of a clinical trial using VEGFR-2 CAR T cells did not have the same premise. Among 24 recruited patients only one patient had a partial response (PR) while the other patients had progressive disease (PD) [125]. So far, the attempts to treat solid tumors with CAR T cells have been unsuccessful but the world of possibilities that CAR T cell engineering allows and the amazing success in treating B-cell malignancies still leaves an open door to further developments.

4.2.3. CRISPR

Clustered regularly interspersed short palindromic repeats (CRISPR) is a DNA targeting system evolved in some bacterial species to cleaves foreign genomes. The type II CRISPR system uses a single DNA nuclease, Cas9, to cleave the DNA strand at specific sites in the genome thus enabling targeted genome modifications [126]. The ability to knock out endogenous αβ T cell receptors and eliminate beta-2-microglobulin (β2M) with CRISPR-Cas9 system will allow patients to receive healthy donor-derived T cells who will not create a graph *versus* host disease (GVHD) [124]. This opens a world of possibilities allowing for "off the shelf" universal T cell based products for ACT treatments. Another approach in utilizing the CRISPR-Cas9 system is altering immune checkpoint genes allowing for improved T cell activation. It was demonstrated that directly disrupting genome PD-1 expression on human primary T cells, using the CRISPR-Cas9 system, enhanced IFN-γ production and improved tumor cells lysis [127]. This can be the next step in enhancing genetically modified T cells. Indeed, using anti-PD-1 antibody significantly boosts the anti-tumor efficacy of CAR T cells [128].

4.3. NK cell ACT

Adoptive cell therapy in melanoma is almost exclusively conducted with T cells. There is very limited data on ACT using other cell types. NK cells are lymphocytes that belong to the innate immune branch with cytotoxic abilities that can efficiently lyse tumor cells in a major histocompatibility complex (MHC) independent manner. This quality makes them elite candidates for treating patients whose tumor cells have lost their MHC expression thus making them resistant to T cell attack [106].

As any part of the immune system, NK cell functions depend on a balance between inhibitory and stimulating signals. In order to preserve tolerance to self, recognition of MHC class I alleles by Killer Ig-like Receptors (KIR) produces an inhibitory signal that allows for an escape mechanism by the tumor cells. Concordantly, ACT for metastatic melanoma patients using autologous NK cells has failed to yield a substantial clinical benefit. To bypass this hurdle it is possible to use allogeneic NK cells with HLA incompatibility or KIR-ligand mismatch, or alternatively, matching of NK lysis-receptors with NK lysis ligands. *In vitro* preclinical studies revealed that both HLA-mismatching or lysis receptor-ligand matching lead to efficient elimination of melanoma cells [129,130].

5. Oncolytic virus therapy

Oncolytic viruses represent a novel drug class in which viruses, either native or modified, mediate tumor regression. This is done through two distinct mechanisms (i) selectively replicating within tumor cells causing a cytolytic reaction [131], and (ii) priming of the adaptive immunity through the release of viral particles coupled with antigen release by dying tumor cells [132]. The overall aim is to generate a strong antitumor response both locally and systemic, while minimizing collateral damage of normal cells from either the virus itself or the immune response.

5.1. Talimogene laherparepvec (TVEC)

Talimogene laherparepvec (TVEC) is currently the only FDA approved oncolytic virus treatment for cutaneous, subcutaneous and nodal melanoma. It is delivered by direct intratumoral injections. It is a genetically modified herpes simplex virus type 1 with mutations in the infectious cell proteins $(ICP)_{34.5}$, ICP_{47} and added with human granulocyte-macrophage colony-stimulating factor (GM-CSF) [133]. $ICP_{34.5}$ is involved in inhibition of the host's defense response that

inhibits protein synthesis, thus the mutations protect normal cells and targeting malignant cells, in which the cells defense response has already been over ridden [134]. ICP_{47} inhibits the transports of antigens from the cytosol to the plasma membrane [135] and the production of human GM-CSF enhances antigen release, presentation and dendritic cells infiltration [133]. Collectively, all three modifications create a virus that attacks cancer cells selectively and elicits a T cell selective immune response through viral and tumor antigen presentation.

In a phase III clinical trial in patients with unresectable stage III or Stage IV M1a melanoma, there was a survival benefit for intratumoral injections of TVEC compared with subcutaneous injections of GM-CSF. The most common adverse events with TVEC were fatigue, chills, and pyrexia [136]. The results of this trial should be interpreted with caution, as subcutaneous GM-CSF is not a standard line of treatment. In line with the proposed mechanism of action, distant non-injected metastases regress as well, but at significantly lower rates than in the injected sites. This could be as a result of the immunosuppressive tumor microenvironment or the insufficient effector T-cell expansion that is expected after the dendritic cell stimulation [133].

The limited response in distant sites might be enhanced using combined therapy, TVEC and immune checkpoint inhibitors. Enhanced antitumor activity was demonstrated in a phase I study of TVEC combined with ipilimumab [137], which was confirmed in a randomized phase II trial comparing TVEC plus ipilimumab *vs.* ipilimumab alone. Objective response rates were 39% in the combination arm *vs.* 18% in the ipilimumab only arm. Regression of visceral lesions was observed in both arms, but the combination arm was significantly superior, evident in 52% of patients [138]. A phase Ib clinical trial testing the combination of TVEC and pembrolizumab following treatment with TVEC alone showed promising results with a confirmed objective response rate of 62% and a complete response rate of 33%. The response to combination therapy did not appear to be associated with baseline CD8+ T cell infiltration or baseline IFNγ signature suggesting that the use of an oncolytic virus alters the tumor microenvironment thus possibly improving the efficacy of anti-PD-1 therapy [139].

5.2. Other oncolytic viruses

HF10 is an HSV type 1 mutant oncolytic virus with high tumor selectivity and impaired neuro-invasiveness due to a loss of the UL56 gene, which is involved in HSV latency [140]. In a murine melanoma model, intra-tumoral administration of HF10 resulted in a reduction in tumor mass, and intraperitoneal injection was shown to produce a 100% survival rate, compared to 100% fatality in the control group [141]. A single arm open-label phase II clinical trial using HF10 in combination with ipilimumab in stage IIIB/IV unresectable melanoma showed best overall response rate of 41% at 24 weeks, and median overall survival of 21.8 months [142]. While these results are encouraging and exceeds historical efficacy of ipilimumab as a single agent, further investigation is necessary to understand the potential of using HF10 in the treatment of melanoma.

Another oncolytic virus whose full potential in treating melanoma is still not fully understood is the coxsackie virus A21 (CVA21). This is an unmodified wild-type virus that induces oncolysis of both *in vitro* cell lines and human melanoma xenografts [143]. The coxsackievirus has a great potential in treating melanoma because of its interactions with decay-accelerating factor (DAF) receptors and intercellular adhesion molecule-1 (ICAM-1) both of which have been shown to be overexpressed in malignant melanoma [144]. CVA21 (CAVATAK™) has completed phase II clinical trial studies in patients with stage IIIC and IV melanoma. 38.6% of the participants displayed immune-related progression free survival at 6 months with no serious adverse events attributable to CVA21 (ClinicalTrials.gov NCT NCT01227551). Combination of intratumoral CVA21 with other immune checkpoint inhibitors are being studied.

6. Cancer vaccines in the treatment of melanoma

The idea that exogenously administered components can elicit antitumor immune response and tumor regression first materialized in the 19th century, when William B. Coley reported that repeated injections of bacterial toxins led to tumor regression in a patient with advanced sarcoma [145]. In the second half of the 20th century, the discoveries of antigen presenting cells [146] and tumor specific antigens [147] further solidified the notion that a vaccine based on tumor specific antigens can activate dendritic cells (DCs) and induce durable anti-tumor immunity. However, with the exception of Sipuleucel T and oncolytic viruses (reviewed elsewhere in this paper), no therapeutic cancer vaccine has yet shown clinical efficacy in a phase 3 randomized trial [148].

Vaccines in general and cancer vaccines in particular, are composed from two major components – the antigen and the adjuvant. The optimal selection of both components is crucial for successful priming of anti-cancer T cells and induction of Th1 responses for tumor regression and protection against tumor recurrence or metastatic spread.

6.1. The optimal choice of the antigen and formulation

Cancer antigens can be classified to 2 major classes – tumor specific antigens (TSAs) and tumor associated antigens (TAAs). TSAs originate from two major sources – oncogenic viral antigens in virus induced cancers such as human papilloma virus (HPV)-associated cervical cancer; and neoantigens that arise from de-novo somatic mutations in the tumor. These antigens are solely expressed in cancer cells and therefore are not subjected to immune tolerance, making them highly suitable antigens for cancer vaccines construction. Tumor associated antigens are antigens that are expressed by the cancer cells and can induce an immune response but are also expressed in normal tissues. TAAs can be further classified to three main groups – overexpressed antigens, which are derived from overexpressed oncoproteins such as human epidermal growth factor 2 (HER2); differentiation antigens, which are derived from proteins expressed by the specific cell lineage from which the tumor arises; and oncofetal and cancer/testis (CT) antigens, which are derived from proteins preferentially expressed by cancer cells but also expressed in fetal or immune-privileged tissues [149]. Overexpression and differentiation antigens are expressed in normal tissues as well and thus can induce immune tolerance and autoimmunity [150]. Moreover, these antigens elicit low affinity T cell recognition due to negative selection of high-affinity T cells in the thymus [151]. Oncofetal and CT antigens are thought to be more potent in inducing T cell responses due to their higher tumor specificity, and thus are more suitable candidates for vaccine construction [152].

The main advantage of using TAAs as the immunogen in cancer vaccines is that they are shared between patients. Thus, a single TAA based vaccine can be used to treat multiple patients. However, although TAAs based melanoma vaccines have induced tumor regressions in some patients, none exhibited a significant survival benefit in clinical trials [153,154]. On the other hand, more evidence is accumulating that TSAs are the fuel that drives the anti-tumor immune response. Several papers have reported that high mutational burden is correlated with favorable prognosis to immunotherapy [155–157] and that recognition of specific neoantigens by T cells is associated with tumor regressions [119,158,159]. In addition, melanoma has the highest mutational load between all cancers [12], hence there should be a large pool of neoantigens that can be suitable for personalized vaccine development. With the advent and availability of next generation sequencing, the identification of patient specific neoantigens for personalized vaccines have become feasible, and thus, current efforts of therapeutic cancer vaccine development are focused on TSAs as the immunogens that propel cancer immunity.

An ideal cancer vaccine formulation should capture the highly individual antigen content of the tumor. The two main approaches are tumor antigen specific and whole tumor cell-based vaccines. Whole

tumor cell-based vaccines contain a broad spectrum of TSAs and TAAs. However, the tumor antigens are diluted by other self-antigens that are normally expressed within the tumor cells. These vaccines can be generated by irradiated tumor cells, tumor cell lysates from autologous tumor tissue or allogeneic cell lines, or DC-autologous tumor cell hybridization [160–163]. In melanoma, although some whole cell vaccines have shown increase in tumor immune cell infiltrates and tumor necrosis [164] their overall response rate was low [165] and none was superior even to past standard of care.

Tumor antigen specific formulations contain only the antigenic parts of the tumor cells that are required for immune response induction. These epitopes can be delivered as proteins, peptides, or nucleic acids. Peptides are the most commonly tested antigen formats being used for vaccination due to their superior *in vivo* stability and reduced costs [166]. Long multi-peptide vaccines have shown improved efficacy compared with short and single peptide vaccines, as they can induce both CD4+ and CD8+ T-cell response and are less likely to be subjected to immune escape due to loss of antigen expression [167,168]. Moreover, short peptides (<15 amino acids) can induce immune tolerance because they do not require processing by professional antigen presenting cells (APCs) for HLA class I presentation, and thus can be presented by all nucleated cells without the costimulatory signals required for T cell activation [169]. DNA and mRNA based vaccines encode antigenic peptides or proteins, while providing adjuvant function as result nucleic acids dependent activation of Toll-like receptors (TLRs) [170,171]. Even though some DNA based vaccines induced antigen specific immune response, none has shown survival benefit in melanoma patients [172,173]. Recently, a phase I clinical trial of intra-nodal administration of IVAC MUTANOME (a personalized RNA-based poly-neoepitope vaccine) to advanced melanoma patients demonstrated induction of T cell responses, reduced cumulative rate of metastatic events and vaccine-related objective responses [174], thereby providing hope that with proper selection of antigens and formulations, melanoma vaccines will be able to break through the “phase III clinical trial wall” which has seemed almost impenetrable so far.

6.2. Choice of adjuvant and delivery method

Effective vaccination requires co-administration of both antigens and immune adjuvants. Delivery of antigen without inflammation stimulating adjuvants results in insufficient activation of DCs with poor expression of costimulatory molecules, which can lead to T cell anergy and deletion [175]. Numerous adjuvants are used in cancer vaccines, of whom the most promising are TLR agonists, STING agonists, costimulatory agonists, and cytokines such as GM-CSF, IL-2 and interferons [176]. Several papers have demonstrated that a synergistic approach which combines several adjuvants, may enhance dendritic cell activation, T cell priming and tumor regression [177,178]. The choice of delivery material is also of tremendous importance, as it can either enhance or interfere with vaccine induced anti-tumor T cell reactions. Nanoparticle (NP) based delivery systems are an exponentially growing field in cancer vaccination, as they have many advantages - they can package several immune modulating agents into a single compound; act as immunostimulant adjuvant; protect the antigen from degradation; enhance antigen uptake by DCs; and promote antigen localization to the draining lymph nodes [148]. Although combination of various adjuvants and NP based delivery systems had demonstrated improved T cell responses and tumor regression in mice models and phase I & II clinical trials [179–181], none has shown significant survival benefit. Lack of comparative research between the different vaccine components hinders evidence-based selection of the optimal mixture.

6.3. Adoptive transfer of DCs

DCs antigen uptake, presentation and maturation is crucial for the clinical success of cancer vaccines. Thus, instead of using materials and adjuvants for enhancing DCs activation, *ex vivo* cultured autologous DCs directly loaded with antigens were developed. DC vaccines have shown increases in the breadth and diversity of melanoma neoantigen-specific T cells as well as objective responses in melanoma patients [162,182]. However, a large randomized phase III clinical trial comparing between DC vaccination and Dacarbazine failed to find survival difference between the groups [183].

6.4. Vaccine enhancement by overcoming immune suppression

Although therapeutic cancer vaccines can induce anti-tumor T cell responses, increase in tumor immune infiltration, and tumor regression, none has shown significant clinical benefit. In order to explain this gap, one can point to tumor induced immune suppression as the main culprit. The importance of overcoming immune suppression in order to enable prolonged anti-tumor immune responses is highlighted by the recent success of immune checkpoints blockade [184]. Indeed, several papers have demonstrated in mice models that vaccination with therapeutic cancer vaccines can lead to an increase in tumor PD-L1 expression, and that combination of immunotherapies that target the PD-1 pathway with therapeutic cancer vaccines is superior to either therapy alone [185,186]. Similarly, cancer vaccines in combination with CTLA-4 or PD-1 blockade were able to induce tumor regression, augmentation of tumor-reactive T cell responses and prolonged survival in the highly tumorigenic, poorly immunogenic B16 mice model [187,188].

6.5. Personalized melanoma vaccines

TSAs are ideal for cancer vaccine design as they do not elicit immune tolerance and are the drivers of anti-tumor immunity [189]. The vast genetic heterogeneity of tumor cells between cancer types and even within the individual patient [12,190], and the complexity and diversity of HLA molecules that affects the spectrum of presented peptides, suggests that therapeutic cancer vaccines should be tailored to capture the patient's unique landscape of tumor antigens. Candidate TSAs identified by differential exome sequencing tumor and germline DNA are prioritized by their probability of being presented on the patient's MHC molecules and by their ability to induce immune reactivity *in vitro*. Selected neoepitopes are synthesized as DNA, RNA or peptides [191]. In melanoma, preliminary results from several phase I clinical trials of personalized cancer vaccines exhibited robust neoantigen specific anti-tumor T-cell responses as well as objective responses and a significant reduction in the cumulative rate of metastatic events following vaccination [174,192,193]. Interestingly, two independent groups reported that most of the neoantigens that elicited a vaccine induced T cell responses were recognized by CD4+, and not CD8+, T cells [192,194]. These results further emphasize the importance of synthesizing long neoepitopes that can also be presented on MHC class II molecules. Encouraged by these preliminary results, there are currently five active phase I & II clinical trials that aim to test the safety and immunogenicity of personalized cancer vaccines in melanoma (NCT01970358, NCT03480152, NCT03300843, NCT02897765, NCT02035956). These trials vary in neoantigen formulation (peptide, RNA or DC vaccines), adjuvant selection, and administration protocol. In one of the above trials (NCT02897765), the personalized cancer vaccine is given in combination with nivolumab to investigate whether there is a synergistic effect to the two treatments.

7. Novel approaches

7.1. Targeting T regulatory cells

Regulatory T cells (Tregs) are a group of CD4+ CD25+ T cells that play an important role in maintaining self-tolerance and preventing autoimmune disorders [195]. They are characterized by their highly immunosuppressive capacity and the expression of the master

regulatory transcription factor FOXP3 [196]. Tregs can be generated centrally in the thymus or in the periphery by stimulation of conventional T cells with certain cytokines, such as TGF-β [197,198]. Naive Tregs possess weak immunosuppressive activity, but after activation in the periphery, they become strong immuno-suppressors [199]. Tregs exert their immunosuppressive functions by a few key mechanisms: i) contact dependent suppression by inhibitory molecules, most prominently CTLA-4; ii) IL-2 consumption by abundant expression of the high affinity IL-2 receptor α-chain; iii) secretion of immunosuppressive cytokines such as IL-10 and TGF-β; iv) conversion of the inflammatory molecule ATP to the inhibitory adenosine by CD39 and CD73, and v) secretion of cytotoxic molecules such as granzymes and perforin, which directly kill antigen presenting and effector T cells [200]. The important role of Tregs in suppressing anti-tumor T cell reactions was highlighted in 1999, when two papers demonstrated that Treg depletion in tumor-bearing mice resulted in tumor rejection [201,202]. In the pre-immunotherapy era, the presence of Tregs across different types of cancer, including melanoma was associated with poor prognosis [203]. However, the presence of Treg infiltrate does not predict response to immunotherapy, potentially as it is associated with pre-existing presence of intra-tumor activated T cells in general [204], among the most important favorable prognostic factors in cancer immunotherapy [205]. On the other hand, a low ratio of CD8+ effector T cells to Tregs in post-immunotherapy biopsies was shown to associated with poor clinical outcome [206]. Therefore, in light of all of the above, targeting Tregs to manipulate the effector T cell response is plausible. Interestingly, Tregs express a wide variety of inhibitory immune checkpoints including CTLA-4 and PD-1. Emerging evidences indicate that ipilimumab not only enhances antitumor effector T cell immunity, but also depletes Tregs within tumor lesion. This effect was shown to be Fcg-dependent in murine models [207], and clinical studies suggest this depletion is mediated by CD16+ macrophages, whose presence in the TME is associated with response to Ipilimumab and decreased Tregs infiltration after treatment [208,209]. The effect of PD-1 blockade on Tregs is still largely unknown, and evidence for a Treg depleting effect of PD-1 blocking antibodies is yet to be provided. Strategies that target Tregs include Treg depletion, modulation of suppressive functions and blocking of migration. Depletion of Tregs must be restricted to tumor infiltrating Tregs while preserving other Tregs which are critical for autoimmunity suppression.

7.1.1. CCR4

CCR4 is a chemokine receptor that is predominantly expressed by tumor infiltrating effector Tregs. Administration of anti-CCR4 antibody with ADCC activity to a few adult T-cell leukemia-lymphoma (ATL) patients markedly reduced effector Tregs population and augmented CD8 + effector T cell activity [210]. Mogamulizumab, a humanized anti-CCR4 antibody, yielded an overall response of 50% in relapsed patients with CCR4-positive ATL and was approved by the FDA for ATL treatment in 2018 [211,212]. Based on these results, multiple clinical trials based on Mogalizumab as a monotherapy (NCT02281409, NCT01929486) or in combination with immunotherapy (NCT02705105, NCT02476123, NCT02444793) are being conducted in advanced solid tumors, including melanoma [213].

7.1.2. CD25

High CD25 expression is one of the hallmarks of Tregs, however it is also expressed by other activated lymphocytes. In breast cancer patients receiving experimental cancer vaccine, administration of Daclizumab, a humanized anti-CD25 antibody authorized by the FDA for relapse multiple sclerosis, led to prolonged decrease in Tregs and boosted CD8+ and CD4+ T cells priming to all vaccine antigens [214]. However, in melanoma patients receiving dendritic cell vaccine, although Daclizumab depleted circulating effector Tregs, it did not enhance the efficacy of dendritic cell vaccine, due to effector functions suppression of *de novo* induced CD25+ antigen-specific T cells [215].

7.1.3. PI3K inhibitors

The PI3K pathway plays an instrumental role in Treg function, as selective inhibition or mutation of the p110δ isoform of PI3K in tumor-bearing mice resulted in reduced tumor growth, suppressed Tregs functions, and increased CD8+ T cell infiltrates. Although p110δ inhibition reduced the effectiveness of cytotoxic T lymphocytes (CTLs) *in vitro*, its inhibition of Tregs mediated suppression was more robust, enabling the weakened CTLs to attack more effectively the tumors. Thus, p110δ function is possibly more crucial to the immunosuppressive functions of Tregs than of the cytotoxic ability of T cells [216]. Currently, there is one ongoing clinical trial in that is assessing the safety and clinical outcome to combination therapy of a PI3K p110δ inhibitor with Pembrolizumamb in advanced solid tumors, including melanoma (NCT02646748).

7.2. MDSCs targeting in order to reduce immunosuppression

Myeloid-derived suppressor cells (MDSCs) are a heterogeneous population of immature myeloid cells (IMC) that vigorously suppress various T-cell functions [217]. Current nomenclature identifies three main types of human MDSCs: i) polymorphonuclear MDSC (PMN-MDSC), which represents >80% of all MDSCs in most cancers, are defined as CD11b + CD14- CD15+/CD66+ cells; ii) monocytic MDSCs (M-MDSCs) as CD11b + CD14+ CD15- HLA-DRlow/− cells, and iii) early-stage MDSCs (eMDSCs) as Lin- HLA-DR- CD33+ cells [218]. These IMC can accumulate within tumors and promote tumor progression by a broad range of suppressive molecules which inhibit the anti-tumor effector functions of immune cells, such as nitric oxide (NO), ARG1 and IDO [219–221]. MDSCs can further enhance immune suppression by secretion of immunosuppressive cytokines such as IL-10 and TGF-β, which recruit immunosuppressive regulatory T cells (Tregs) to the tumor [222]. In addition, MDSCs can promote tumor progression in an immunological independent manner, which are beyond the scope of this review. In melanoma, circulating MDSCs frequency is enhanced in advanced melanoma patients compared to healthy individuals, is associated with disease progression and decreased overall survival [223], as well as worse clinical outcome when treated with Ipilimumab [224,225]. Therefore, targeting MDSCs in order to alleviate the immune suppressive TME, might improve immunotherapy clinical outcomes.

Novel treatments aim to modulate MDSCs by inhibition of MDSC accumulation, migration, and suppressive functions [226]. Reduction in MDSC accumulation can be achieved by reducing IMC expansion or by boosting IMC differentiation. In several types of cancer, including melanoma, low dose chemotherapy demonstrated a reduction in MDSC numbers and immunosuppressive activity, resulting in improved clinical outcome [227,228]. Blockade of retinoic acid signal transduction by all-trans retinoic acid (ATRA) has enabled MDSCs differentiation into macrophages and dendritic cells in murine and human cell samples, and improved dendritic cell function and antigen specific T-cell responses in cancer patients [229,230]. In order to exert their immune suppressive function, MDSCs must migrate to the tumor site. Recent papers have delineated the importance of two chemokine receptors, CCR2 and CCR5, in MDSCs trafficking to the TME [231,232]. Blocking of the interaction between these receptors and their ligands resulted in reduced migration and immunosuppressive potential of MDSCs in tumor lesions, improved survival of tumor-bearing mice and enabled an influx of activated CD8+ T cells into the TME [231,232]. Lastly, inhibition of MDSCs immunosuppressive functions by inhibition of phosphodiesterase-5, class I histone deacetylase and STAT3 signaling, resulted in downregulation of iNOS and ARG1 expression and activity, activation of anti-tumor immunity, improved clinical outcome of advanced cancer patients including melanoma, and a synergistic effect with immunotherapy in tumor-bearing mice [233–238]. Of particular interest, a recent paper demonstrated that decoy oligodeoxynucleotids with STAT3 specific DNA sequences (dODNs) can be specifically delivered to TLR9+

immune cells, such as myeloid cells, by linkage of the dODNs to the Toll like receptor 9 (TLR9) ligand, cytosine guanine dinucleotide (CpG-dODNs) [238]. After administration, the CpG-dODNs conjugates are quickly internalized by TLR9+ immune cells and bind and sequester cytoplasmic STAT3, which results in downregulation of immunosuppressive functions, increase in T cell proliferation and regression of human MV4-11 AML in mice.

In melanoma, the benefit of targeting MDSCs in combination with immunotherapy was demonstrated in several murine models. Treatments that reduce the suppressive capacity of MDSCs or reduce MDSCs tumor migration increased the efficacy of immunotherapy, enhanced anti-tumor immune responses and prolonged survival of tumor-bearing mice [239–241]. Currently, there are several ongoing clinical trials that combine immunotherapy with MDSCs targeting drugs in advanced melanoma patients [226]. For example, combination of Ipilimumab and ATRA in a phase II trial (NCT02403778) demonstrated reduction in circulating MDSCs coupled with an increase in circulating mature myeloid cells and reduced MDSCs immunosuppressive functions compared to treatment with Ipilimumab alone [242]. Other clinical trials are utilizing the combination of immunotherapy with MDSC migration inhibitor (SX-682, NCT03161431), MDSC suppressive function inhibitor (RTA 408, NCT02259231) and MDSCs depletion (RGX-104, NCT02922764).

7.3. Modulating the gut microbiota to improve immunotherapy efficacy

The gut microbiota, which is defined as the collective genomes of micro-organisms the gut, is composed from genomes of trillions of micro-organisms (mostly bacteria but also viruses, fungi and protozoa) and encodes a 100-times more genes than the human genome, is now best thought of as a virtual organ of the body [243]. Microbial imbalance, also known as dysbiosis, is associated with a range of human pathologies, including gastrointestinal, auto-immune, metabolic and neurologic disorders, as well as carcinogenesis and immunotherapy-induced colitis [244,245]. The two first reports delineating the importance of gut microbiota in cancer immunotherapy were published in 2015 [246,247]. Vétizou et al. [246] demonstrated that tumor-bearing mice responsiveness to Ipilimumab is dependent upon a specific order of bacteria in the gut microbiota. Germ free or antibiotics-treated melanoma-bearing mice that were treated with Ipilimumab exhibited accelerated tumor growth and reduction in T-cell activation and TILs. This effect could be reversed by oral supplementation of bacteria from the Bacteroides species or by fecal microbial transplantation (FMT) harvested from malignant melanoma patients whose feces were enriched with bacteria of the Bacteroides species. Sivan et el [247]. observed differences in melanoma growth rate between two genetically similar mice populations with differing gut microbiota due to enhanced tumor specific T-cell responses and intratumoral CD8+ T cell accumulation in one group. This difference could be eliminated with cohousing or FMT from one group to the other. Moreover, Sivan et al. identified the Bifidobacterium genus as the main bacteria associated with increased anti-tumor T cell responses and diminished tumor growth, and supplementation of a cocktail of Bifidobacterium species augmented dendritic cell function, increased accumulation of antigen-specific CD8+ T cells in the tumor, and nearly abolished tumor outgrowth in combination with immunotherapy.

Following these reports, several papers have demonstrated the relation between gut microbiota and response to immunotherapy in melanoma patients. Alpha diversity as well as several genera of bacteria such as the Bacteroides and Bifidobacterium mentioned above, were associated with response to immunotherapy [248–251]. Furthermore, a few of these papers also demonstrated that FMT from responders, but not non-responders, ameliorated the response to immunotherapy of tumor-bearing mice [248,250,251]. These results prompted few pilot clinical trials that examine the effect of combination of FMT from immunotherapy responders (NCT03353402, NCT03341143) or specific bacterial strains (NCT03637803, NCT03817125) with immunotherapy on the host gut microbiota, trafficking and activation of immune cells in the gut and in the tumors and clinical outcomes of advanced melanoma patients. Our group made the first preliminary clinical report, demonstrating successful engraftment of the transplanted microbiome, immunomodulation both in the colon and CD8+ T cell infiltration into remote metastases, as well as clinical regressions [252]. Hopefully, future data will prove that modulation of the gut microbiota in combination with immunotherapy will allow for better dendritic cell function, improved T cell priming, trafficking of anti-tumor specific T cell into the tumors, and improved clinical outcome.

8. Discussion

The success of immunotherapy in melanoma has revolutionized the outcome of patients and set the bar for the next breakthrough. The currently available immune checkpoint inhibitors, anti CTLA-4 and PD-1, demonstrate two key principles on durability of response: i) a third of the stage IV patients reach the 5 year milestone. As evident from the survival curves, at that time point the tail of the curve plateaus, and ii) Treatment can be ceased among responding patients, enabling the patients to continue their lives with unmaintained response. According to the pemrolizumab approved protocols, treatment is ceased after two years. It was recently shown that >90% of patients with a partial or complete response maintain their response even after two years post treatment cessation [253]. These key principles have already been demonstrated in the past with IL-2 [10], but durable response was rarer whereas toxicity was substantially greater. Importantly, the underlying mechanisms are not entirely clear, but could involve immunological memory, T cell persistence or actual success in eradication of the last cancer cell. These phenomena, coupled with the relatively high proportion of patients that benefit from current immunotherapy, instill further optimism that the treatment of melanoma with immunotherapy aimed at T cell reinvigoration can be further on improved.

The landscape of melanoma immunotherapy described throughout this review are depicted in Fig. 1, according to their impact on the immune system or the tumor microenvironment, and eventually, the disease itself. However, they can also be categorized into three main types: i) T-cell centered; ii) Tumor microenvironment, and iii) Whole patient/Remote environment (Fig. 2). T cell centered strategies are aimed at direct enhancement of effector T cell function against melanoma and include, for example, targeting immune checkpoints, adoptive transfer of T cells (with or without engineering), and personalized melanoma vaccines. Microenvironment directed strategies are focused on rendering tumor cells more antigenic, changing the tumor vicinity towards immune favorable conditions, and on alleviating suppressive components of the stroma or regulatory immune cells. Whole patient modification to reprogram the entire immune system could be done, for example, by altering the microbiome with fecal microbial transplantation, or potentially by driving general aerobic metabolic state to improve T cell function [254]. Each one of these strategies has already yielded at least preliminary clinical evidence for efficacy, through advanced clinical development stages, all the way to FDA approvals.

The different categories cover the whole spectrum from fully personalized approach, particularly with the neoantigen-directed vaccines or T cell engineering, all the way to non-personalized, potentially generic strategies such as microbiome alteration or general metabolic modifications (Fig. 2). Personalization of treatment follows the philosophy of precision-medicine, and has the potential to achieve optimal anti-melanoma effect with minimal off-target side effects. The main hurdles currently are still technological, such as how to identify optimal neoantigens from available biologic material in a timely manner, and to create optimal vaccines or identify the optimal TCRs and clone them into vessel T cells. Other hurdles that should not be overlooked are the

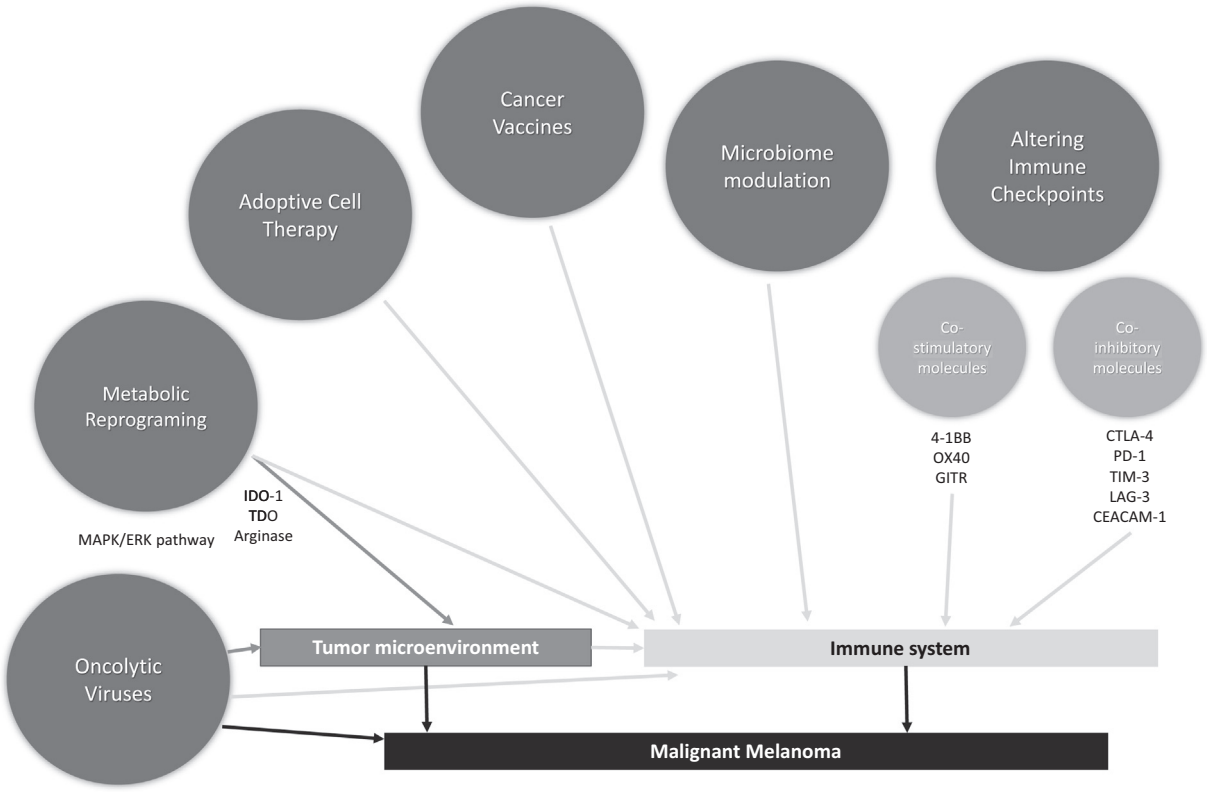

Fig. 1. Summary of the different immune-based strategies to treat melanoma.

economic implications of these approaches and their feasibility in terms of cost, and regulatory modifications, as each patient is in a sense a single-person clinical trial. Targeting the microenvironment is less selective, as it targets certain biological processes or suppressive cells. Nevertheless, these interventions should be coupled with an appropriate biomarker to indicate their relevance, for example the presence of target microenvironment enzymes, or certain immunosuppressive target cells. This strategy builds upon the presence of naturally selected tumor-specific T cells, and aims to alleviate restraining conditions. Finally, whole patient modifications are probably applicable to the majority of the patients, as this strategy bears fundamental implications on the entire immune system. Therefore, it is the least "personalized" approach and it also builds on the presence of naturally-selected tumor-specific T cells. From an economic perspective, this strategy is expected to be the least expensive.

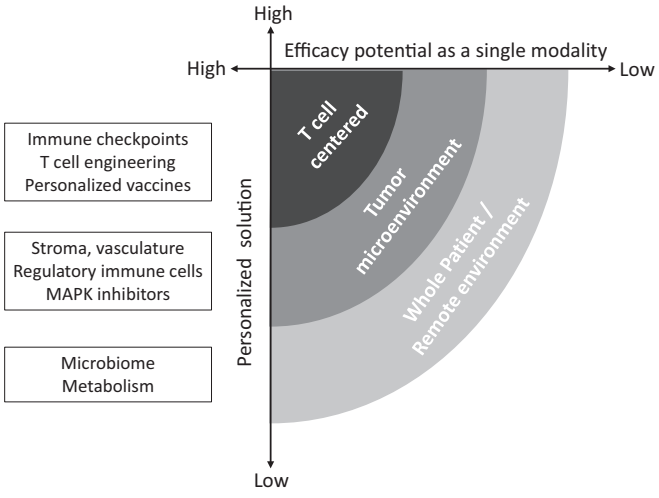

Fig. 2. Plotting of potential efficacy *versus* personalization level of different immune-based strategies.

The second axis by which these treatment categories can be classified is the potential for clinical efficacy as single agent or as part of a combination regimen (Fig. 2). T cell centered strategies have the potential to generate highly effective CD8+ T cells with strong anti-melanoma capacity. Therefore, these strategies have the potential for clinical efficacy as single agents, such as PD-1 blocking antibodies and TIL ACT have shown so far. Nevertheless, it is logical to assume that direct T cell manipulation has its limitations, which could be augmented with the other two strategies. Strategies that target the microenvironment or the entire patient are less likely to generate clinically meaningful immunological responses on their own, as observed for IDO1 or for most patients treated with BRAFi/MEKi, but have the potential to synergize with T cell centered strategies or even FDA approved immune checkpoint inhibitors. This is in fact a "reversed precision medicine" approach, as the objective is to manipulate and render the patient responsive to existing standard of care immunotherapy.

This framework puts in place the future efforts in melanoma immunotherapy, aiming to optimize precision-medicine like approaches focused directly on T cells, with reversed precision-medicine efforts, focused on enablement of T cell repertoire and function. As opposed to the hurdles for T cell centered strategies listed above, the main hurdles for microenvironment or whole patient strategies are to identify the optimal window of opportunities for combination therapy, particularly which intervention, timing, sequence, duration, dose, and with which T cell centered to combine them with. These question require substantial international efforts or basic and clinical scientists. As immunotherapy is in principle agnostic to tumor type, lessons learned from melanoma are expected to continue and affect the entire field of oncology.

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Declaration of Competing Interest

GM received honoraria from MSD, BMS, Roche and Novartis, serves on advisory board of MSD and Biond Biologics, received a research grant from Novartis, holds stock options in Biond Biologics and 4c Biomed, and is partially employed by 4c Biomed. NM and EM have no disclosures.

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