ARTICLE IN PRESS

Seminars in Cancer Biology xxx (xxxx) xxx

Contents lists available at ScienceDirect

Seminars in Cancer Biology

journal homepage: www.elsevier.com/locate/semcancer

Targeting inflamed and non-inflamed melanomas: biological background and clinical challenges

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ARTICLE INFO

Keywords:
Melanoma
Inflamed
Non-inflamed
Immunotherapy
Gene expression profile

ABSTRACT

Immune checkpoint inhibitors (ICIs) have demonstrated impressive antitumor activity in patients with advanced and early stage melanoma, thus improving long-term survival outcomes. However, most patients derive limited benefit from immunotherapy, due to the development of primary, adaptive, or acquired resistance mechanisms. Immunotherapy resistance is a complex phenomenon that depends on genetic and epigenetic mechanisms which, in turn, drive the interplay between cancer cells and the tumor microenvironment (TME). Immunologically "cold" (i.e. non-inflamed) tumors lack or have few tumor infiltrating lymphocytes (TILs) as a result of low tumor mutational burden (TMB), defective antigen presentation, or physical barriers to lymphocyte migration, resulting in a minimal benefit from immunotherapy. In contrast, in most cases immunologically "hot" (i.e. inflamed) tumors display high TMB, implying a higher load of neoantigens and increased programmed cell death ligand 1 (PD-L1) expression, with a consequently higher rate of TILs. However, the presence of TILs does not necessarily denote the tumor as immunologically "hot", since the presence of tumor-specific $CD8^+$ T cells persistently exposed to antigenic stimulation induces a dysfunctional state called "exhaustion", which leads to a reduced response to immunotherapy. In recent years, efforts have been made to characterize mechanisms of resistance to immunotherapy, and to investigate strategies to overcome treatment resistance. Indeed, predictors of response and toxicity to immunotherapy are still lacking and, to date, there are no reliable predictive biomarkers to select patients according to baseline clinical, histological, or genomic characteristics. In this review, we will focus on the morphologic and immunohistochemical characteristics of the TME, and on the molecular determinants of resistance to immunotherapy, differentiating between inflamed and non-inflamed melanomas. Then, we will provide a thorough overview of preclinical data on genetic and epigenetic mechanisms with a potential impact on the immune response and patient outcome. Finally, we will focus our attention on the role of potential biomarkers in determining disease response to immunotherapy, in the adjuvant and metastatic setting, providing an insight into current and future research in this field.

1. Introduction

Immune checkpoint inhibitors (ICIs) have demonstrated impressive antitumor activity and durable response in patients with advanced and early stage melanoma, thus improving prognosis and long-term survival outcomes [1]. These drugs have also revolutionized the way we treat

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https://doi.org/10.1016/j.semcancer.2022.06.005
Received 5 January 2022; Received in revised form 30 May 2022; Accepted 18 June 2022
Available online 22 June 2022

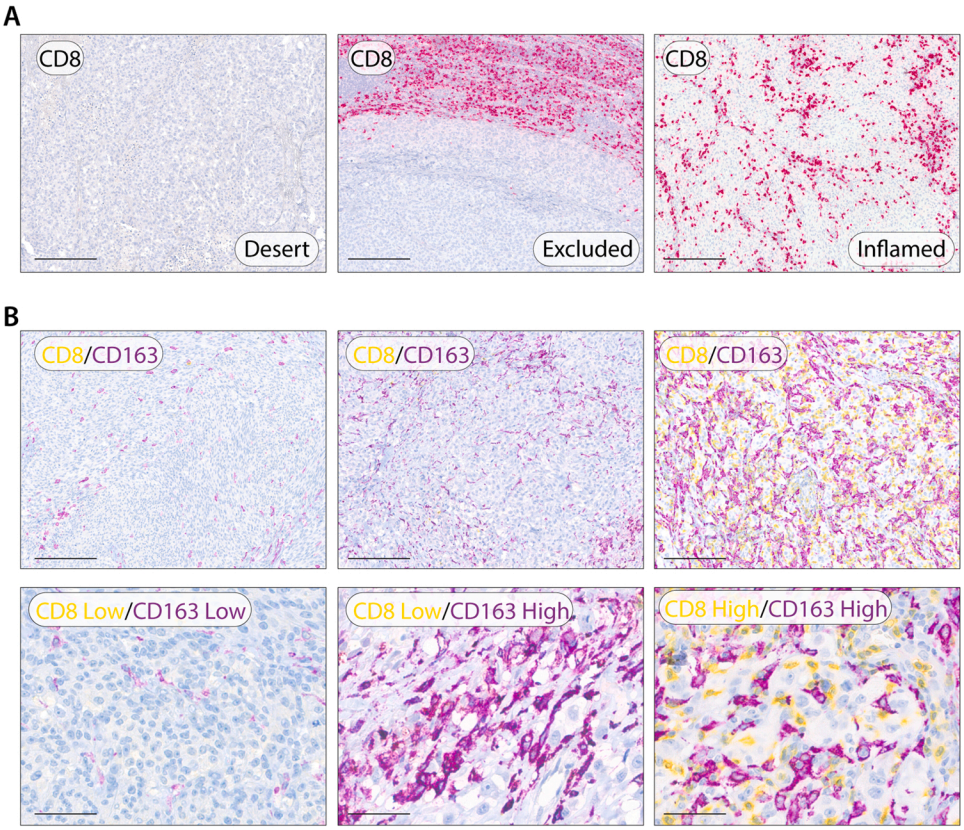

Fig. 1. A) Representative images of CD8 + T cells in relation to their immune phenotypes. Absence of CD8 + cells in immune desert tumors, accumulation of CD8 + cells at the margin or in the intratumoral stroma in immune excluded tumors and infiltration of CD8 + cells into the tumor parenchyma in inflamed tumors (scale bar 300 μm). B) Representative images of melanoma tissue stained with multiplex IHC for CD8/CD163, with low CD8 + low CD163, low CD8 high CD163 and high CD8 high CD163 positive cells (scale bar 200 μm, magnification 100 μm).

patients with limited disease, as they are able to reduce the relative risk of recurrence by approximately 45–50 %, with an absolute recurrence (RFS) and distant metastasis (DMFS) free survival benefit of approximately 20 % and 15 %, respectively [2–4]. However, most patients derive limited benefit from immunotherapy, due to the presence of primary, adaptive, or acquired resistance mechanisms [4]. Immunotherapy resistance is a complex phenomenon that depends on genetic, metabolic and epigenetic mechanisms which, in turn, drive the interplay between cancer cells and the tumor microenvironment (TME) [5]. Historically, a framework to stratify TMEs into different types, based on the presence or absence of tumor infiltrating lymphocytes (TILs) and programmed cell death ligand 1 (PD-L1) expression, has allowed the stratification of four subtypes of TMEs (i.e., type I, TIL+/ PD-L1 +: adaptive immune resistance; type II, TIL-/PD-L1-: immunological ignorance; type III, TIL-/PD-L1 +: intrinsic induction; type IV, TIL+/PD-L1-: tolerance) with different responses to ICIs and patient outcome [6]. Another classification based on the joint evaluation of tumor mutational burden (TMB) and a T cell–inflamed gene expression profile (GEP), has shown to independently predict response to anti–PD-1 therapy in several tumor types, thus reflecting an agnostic measure of distinct aspects of tumor immunobiology [7]. In particular, limited clinical responses to anti-PD1 occurred in patients with low levels of both TMB and T cell–inflamed GEP, whereas the greatest response rates were seen in patients with high levels of both biomarkers (median PFS among the melanoma cohort: 123 vs 504 days, respectively) [7].

Immunologically “cold” tumors lack or have few TILs as a result of low neoantigen load (i.e., tumor mutational burden, TMB), defective antigen presentation, or physical barriers to lymphocyte migration in the extracellular matrix or the tumor vasculature [8]. Other important features, as the infiltration of myeloid-derived suppressor cells (MDSC) and the tumor metabolic reprogramming, are known factors acting as major suppressors of immunotherapy mechanisms in the TME [9,10]. For this reason, the therapeutic impact of immunotherapy in immunologically “cold” tumors is generally minimal and prognosis is inevitably poor. Conversely, immunologically “hot” tumors in most cases display high TMB, implying a higher load of neoantigens and increased programmed cell death ligand 1 (PD-L1) expression, with a consequently higher rate of TILs [8]. However, the presence of TILs does not necessarily denote the tumor as immunologically “hot”, since the presence of tumor-specific $CD8^+$ T cells persistently exposed to antigenic stimulation induces a dysfunctional state called “exhaustion”, which leads to a reduced response to immunotherapy [11]. Similarly, there seems to be a higher benefit of immunotherapy for tumors displaying clonal TMB, rather than high TMB alone [12]. Conversely, the relative abundance of partially exhausted cytotoxic TILs seems to correlate with response to anti-PD1 antibodies, which aim to reinvigorate effector functions in TILs [13]. In this regard, designating “inflamed” versus “non-inflamed” melanomas might better reflect the morphologic and immunologic characteristics influencing response to immunotherapy on the basis of different immune cell populations within the TME.

In recent years, efforts have been made to characterize mechanisms of resistance to immunotherapy, to address the intrinsic resistance of immunologically “cold” (i.e., non-inflamed) melanomas, and to investigate strategies to overcome treatment resistance. The significant proportion of non-responsive patients, together with the financial burden of cancer immunotherapy and the risk of immune-mediated toxicities, are the main reasons for these efforts. Indeed, predictors of response and toxicity to immunotherapy are still lacking and, to date, there are no reliable predictive biomarkers to select patients according to baseline clinical, histological, or genomic characteristics. Moreover, there are limited treatment strategies for patients progressing after immunotherapy with conventional ICIs outside the context of clinical trials. There are two potential ways to increase tumor immunogenicity by operating a tumor “heating” process: the first involves direct modification of cancer cells, while the second indirectly enhances immunogenicity through altering the TME [14]. In this review, we will first focus on the morphologic and immunohistochemical characteristics of the TME, and on the molecular determinants of resistance to

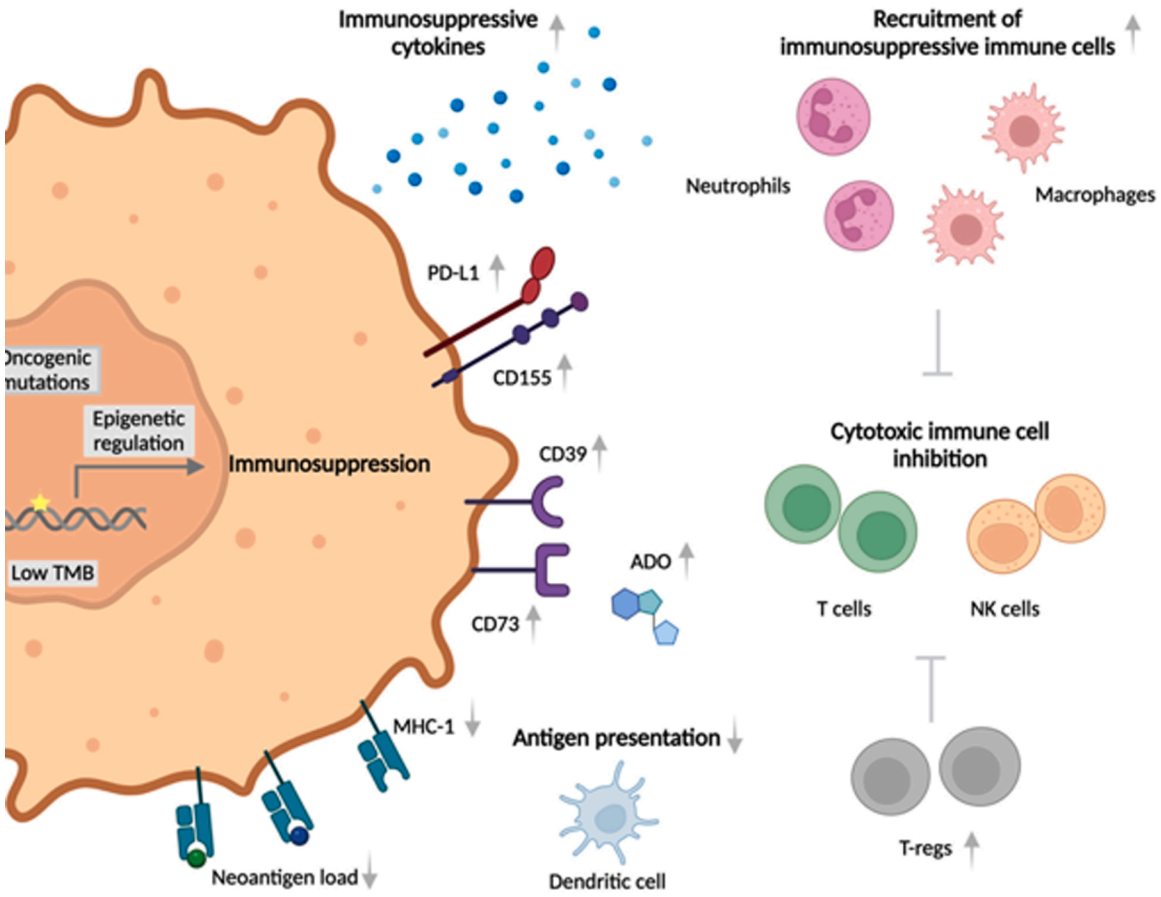

Fig. 2. Molecular and cellular principles of immune suppression in the melanoma microenvironment. Genetic and epigentic alterations in melanoma cells significantly contribute to the establishment of an immunologically cold TME. In addition, to hard-wired genomic changes can the expression of membrane-bound and/or soluble mediators drive immune suppression in melanoma. Ultimatlely these melanoma cell-derived molecules act on a variety of immune cells e.g. neutrophils, macrophages and regulatory T cells to limit the expansion and function of cytotoxic immune cells.

immunotherapy, differentiating between inflamed and non-inflamed melanomas. Then, we will provide a thorough overview of preclinical data on genetic and epigenetic mechanisms with a potential impact on the immune response and patient outcome. Finally, we will focus our attention on the role of potential biomarkers in determining disease response to immunotherapy, in the adjuvant and metastatic setting, providing an insight into current and future research in this field.

2. Histopathological features

Historically, pathologists have investigated TME features in melanoma tissues by conventional histopathological analysis and standard chromogenic immunohistochemistry (IHC), with the aid of specific antibodies for lymphocytes, other immune cells, and immune markers. Advantages of these well-established and readily available techniques include fast turn-around time, relatively low cost, and the possibility to evaluate the immune infiltrate with regard to the immune cell density and location (i.e., peritumoral or intratumoral) [15]. As most pathologists are more familiar with light microscopy, an additional benefit consists in the correlation with cytoarchitectural morphological features of the immune contexture, permanent staining, and an overall easier oversight, as compared to many fluorescent methods. However, visual semi-quantitative scoring of TILs suffers from interobserver variability and poor reproducibility. Differences in $CD8^+$ T cell counts may be also explained by the spatial and temporal heterogeneity of $CD8^+$ T cell distribution, especially if the assessed tissues are tissue microarrays (TMA) or small specimens (i.e., 1 mm) [16]. Moreover, semiquantitative assessment provides only a rough estimate of immune cell counts, and different cell types must be annotated in consecutive tissue sections, making it difficult to compare cells to each other.

Computational scoring by digital pathology is potentially a superior approach, as image-based analytic approaches help to overcome subjectivity, thus ideally replacing the currently employed light-microscopic methods in the clinical setting. Recently, a standardized diagnostic algorithm has been proposed in the routine pathology workflow based on a single chromogenic IHC with anti-CD8 antibody and HALO digital image analysis, providing an immune diagnosis of melanoma tissues based on the density of tumor-infiltrating $CD8^+$ T cell/μm/tumor compartment (both tumor center and invasive margin) [17]. Following the algorithm definition, inflamed (hot) melanomas are characterized by a high number of $CD8^+$ T cells in the stromal compartment and within the tumor parenchyma where they are in direct contact with tumor cells. Conversely, non-inflamed (cold) melanomas show a scarce immune infiltrate, whether as an immune desert or excluded pattern [17]. The immune desert pattern shows rare and isolated $CD8^+$ T cells in some of the tumor compartments. In contrast, even though present in the TME in the immune excluded pattern, $CD8^+$ T cells can be found at the invasive margin or within the stroma and, rarely, isolated intratumoral T cells are reported (Fig. 1) [17]. Expert review remains a prerequisite of digital image analysis algorithms, particularly for the assessment of unusual melanoma histotypes, tissues with evidence of tumoral regression and scarce viable cells, highly pigmented tissues, or challenging sites, including lymph-node metastases.

Novel technologies that enhance spatial mapping of the TME include multiplex quantitative pathology approaches and single-cell RNA sequencing. Multiplex immunofluorescence (mIF) staining using

immune system-based biomarkers has emerged as a novel potent tool for immune-profiling analysis, highlighting the simultaneous detection of multiple markers in a single formalin-fixed paraffin embedded (FFPE) tissue section [18]. Localization of multiple targets in the same tissue section provides unique insights into spatial co-localization of molecules of interest. The flexibility to create panels to different targets offers unprecedented opportunities for innovative digital image analysis approaches (e.g., proximity, 3D reconstruction). However, potential limitations of this approach include high costs and technical challenges. Some degree of autofluorescence and crosstalk between different fluorophores with overlapping emission spectra are critical issues that may be overcome with long-standing experience and the application of strict protocols [19,20]. Recent advances and the use of automated tissue strainers provide faster and higher throughput multiplex IHC (mIHC) of melanoma tissues, enabling simultaneous detection of melanoma and multiple immune subsets, and providing multi-parameter cell lineage assessment [21]. OPAL is one of the recently developed methods that allows robust high-throughput automated mIHC analyses of FFPE tissues [22,23]. Following antigen retrieval, melanoma tissue sections are stained by OPAL on an auto-stainer, including serial rounds of epitope labelling with monoclonal antibodies, followed by tyramide signal amplification (TSA), resulting in minimal or no background signal [24].

When measuring immune targets in melanoma tissues, spatial plotting and proximity analysis of each immune cell subset in relation to tumor cells can shape an immune contexture profile of clinical significance, which includes relative distances between immune cell subpopulations (e.g., CD8 + T cells, FOXP3 + regulatory T cells, PD1 +/PD-L1 +/SOX10- immune cells and SOX10 + tumor cells). The number of immune cells with each phenotype can be calculated at 20 μm intervals from the nearest SOX-10^{+} melanoma cell [23]. While mIHC is a significant advance in high-resolution tissue biomarker analysis, spatial transcriptomic for gene expression analysis at single-cell level is an innovative, groundbreaking method that can help to uncover cellular heterogeneity, as well as determine the dynamic interactions between tumor and immune cells at a multidimensional level. The combination and integration of spatial transcriptomic data with mIHC data can provide a better understanding of the functional interactions of complex tumor-immune cells in the TME and, in perspective, will drive biomarker discovery for patient selection in the clinic [25].

3. Pre-clinical melanoma models to study tumor–immune cell interactions in the TME

For decades, mouse models have been an essential tool to investigate the complexity of immune response against cancer. Only in sophisticated animal models can one study the molecular and cellular mechanisms of immune cell activation (e.g., sequential cell-cell interactions), trafficking (e.g., from the tumor draining lymph-node into the TME), and differentiation (e.g., priming, to activation, to exhaustion). Using transplantable syngeneic and genetically engineered mouse melanoma models (GEMMs), scientists have unraveled a multitude of mechanisms involved in tumor initiation, progression, metastasis, and response/resistance to different kinds of therapy.

The immune landscape of melanoma is primarily dictated by genetic and epigenetic alterations. While high TMB and neoantigen expression correlate with clinical benefit and promote cytotoxic anti-tumoral immunity, somatic mutations and epigenetic changes have been shown to contribute to an immunosuppressive melanoma microenvironment. In particular, oncogenic driver mutations frequently found in melanoma patients, such as $CDK4^{R24C}$, $BRAF^{V600E}$, $NRAS^{Q61K}$, etc., have been shown in mouse models to attenuate T cell responses by impairing antigen-presentation (through the down-regulation of the major histocompatibility complex [MHC]−1), and to promote an immunosuppressive microenvironment via the secretion of myeloid-recruiting chemokines (CCL2 and CXCL8) and pro-inflammatory cytokines (interleukin-1 [IL-1], IL-6, and vascular endothelial growth factor [VEGF]) [26–28] (Fig. 2). In support of those pre-clinical findings, Jerby-Arnon et al. nicely demonstrated a role for the CDK4/6 pathway in T cell exclusion and resistance to ICB in melanoma [29]. However, the impact of other oncogenic driver mutations in melanoma patients remains unclear.

3.1. The type I and II Interferon system - regulators of the melanoma immune microenvironment

Despite the necessity of pre-clinical animal models, it is important to consider that neither syngeneic transplantable melanoma models nor GEMMs fully represent the complexity of tissue- and organ-specific aspects, nor the context of the tumor immune system that is seen in human melanoma patients. In particular, these models do not represent the diverse immune phenotypes seen in patients, but often only represent a "non-inflamed" phenotype with low immune cell infiltration. As mentioned above, this can be explained in part by the presence of oncogenic driver mutations and a low tumor mutational and neoepitope burden. Thus, a majority of mouse melanoma models is resistant to various immunotherapies [30]. Therefore, mouse melanoma models have been extensively used to study the molecular and cellular mechanisms contributing to a "non-inflamed" TME, and to develop therapeutic strategies to turn on the heat. In addition to TMB and neoepitope expression, the type I interferon (IFN-I) pathway is critical for the recruitment and activation of innate and adaptive immune cells in melanoma. Many mouse melanoma models are poorly infiltrated with immune cells, which can be associated with a lack of IFN-I signaling [31–33]. This is in line with findings in melanoma patients in which activation of the IFN-I system correlates with cytotoxic immune cell infiltration and a favorable prognosis [31]. Thus, pre-clinical mouse models have facilitated the development of multiple therapeutic strategies to induce IFN-I signaling in "non-inflamed" melanomas. Activating innate RNA or DNA sensors by using synthetic agonists targeting STING, MDA-5, or TLRs, has shown promising therapeutic results in mouse models and paved the way for the clinical development of a variety of molecules [31,34,35]. Another strategy to turn-up the heat in the TME via the IFN-I system is the use of oncolytic viruses (OVs). In addition to the strong activation of the IFN-I system, OVs can be used as shuttles to introduce immunostimulatory cytokines to the TME, and to elicit immunogenic cell death in melanoma cells [8]. The most prominent example is Talimogene laherparepvec (T-Vec), a genetically modified type 1 herpes-simplex virus (HSV) encoding the granulocyte-macrophage colony-stimulating factor (GM-CSF), which has shown clinical activity in patients with advanced melanoma. However, the efficacy of T-VEC seems to be limited locally to the injection site, with little response in distant metastases. Thus, further refinement and the development of novel OVs is needed to generate stronger and more durable immune responses in melanoma patients [36]. Recently, Rider and colleagues developed an HSV-1 mutant, called VC2, unable to enter neurons via the axon termini, which might increase the safety profile of VC2 by preventing unwanted neuronal side effects. Using transplantable melanoma models, they showed that VC2 induces strong, durable, and T cell-dependent immune responses in mice [37]. In addition to HSV-1, other OVs are being extensively used, including Adenovirus and lymphocytic choriomenigitis virus (LCMV). A recent publication showed a novel aspect of viral vector-based immunotherapy, in which the reprogramming of stromal cells was critical for the eradication of murine melanoma [38].

Besides the type I IFN system, the type II IFN system also plays a critical role in innate and adaptive immune regulation in the TME. The role of interferon-γ (IFNγ) is usually associated with anti-tumoral and cytotoxic functions. IFNγ is mainly produced by natural killer (NK), CD4 helper T- and CD8 cytotoxic T cells and enhances cytotoxic immune cell function, differentiation as well as tumor cell apoptosis and senescence [39].

High IFNγ levels in the TME are associated with increased T cells

signatures, an inflamed (hot) microenvironment and better overall response to melanoma immunotherapy [40,41]. Furthermore, it is critically involved in the antigen processing machinery by inducing the upregulation of major histocompatibility (MHC) Class I on immune and non-immune cells, thus facilitating immune detection by effector T cells. The role of IFNγ for anti-melanoma immunity has been elucidated with the help of experimental mouse models in which deficiency of IFN-γ signaling is associated with aggressive tumor growth or resistance to immunotherapy [42–45].

Despite the importance of IFNγ for tumor immunity, it has a pleiotropic role and can also contribute to melanoma progression and resistance to immunotherapy. Pre-clinical studies have demonstrated that IFNγ can enhance melanoma growth and metastases and inhibit tumor cell apoptosis by facilitating a protumoral microenvironment through the enrichment of immunosuppressive polymorphonuclear leukocytes (PMN) and TGFβ-producing γδ T cells [46]. In addition, its well described that IFNγ signaling induces the expression of immune checkpoint receptors, such as PD-L1, on tumor and immune cells [47–49]. IFNγ secreted from activated T cell induces the expression of PD-L1 on neutrophils in lymph nodes and the TME. *Via* interaction with PD-1, expressed on activated melanoma-specific T cells, this in turn, inhibits T cell proliferation as well as effector functions and limits therapeutic efficacy of adoptive T cell transfer (ACT) immunotherapy in an experimental model of melanoma [48]. In addition, a sustained IFNγ-driven inflamed TME, also leads to the induction of PD-L1 on melanoma cells, thereby also contributing to melanoma immune evasion from ACT immunotherapy. However, this allows for effective salvage immunotherapy using PD-1/PD-L1 blocking antibodies [49].

Overall, both type I and II IFNs are critical for the orchestration and execution of effective anti-melanoma immune responses and the efficacy of various immunotherapies, however negative feedback loops must be considered as overshooting and chronic IFN responses will confer resistance.

3.2. Immunosuppressive molecules expressed on the cell surface of melanoma cells

In addition to the melanoma cell secretome, the expression of cell surface molecules by melanoma cells contributes to the regulation of immune cell activation, function, and phenotype (Fig. 2). The most prominent immunosuppressive cell surface molecule is PD-L1 (also known as B7-H1 or CD274) which, upon binding to its receptor, PD-1, effectively silences the effector functions of cytotoxic immune cells in the TME [50]. Blocking these receptor-ligand interactions has shown remarkable success in the treatment of melanoma and other cancer types [51]. However, we are still lacking reliable biomarker to predict response to PD-1/PD-L1 blocking antibodies. While the role of PD-L1 expression on melanoma cells is currently discussed, a recent study provided new evidences that PD-L1 expression also on antigen presenting cells contributes to impaired T cell immunity [52–54]. Taken together, these findings highlight that our understanding of the complex regulation of immune responses in the melanoma microenvironment by the PD-1/PD-L1 axis is still in its infancy.

Given that only a minority of patients experience durable response on PD-1/PD-L1 inhibitors, it is likely that additional immunosuppressive pathways have a role in melanoma immune evasion. In recent years, many such pathways have been identified with promising targets for novel immunotherapies. Among those molecules, various nectin family members and their immunoreceptors have been shown to shape anti-tumor immune responses in pre-clinical mouse models [55]. The nectin family consists of four nectins (Nectin-1–4, also known as CD111, CD112, CD113, and PRR4), and five nectin-like molecules (Necl1–5). They are type I integral membrane proteins and have ectodomains composed of three immunoglobulin (Ig) domains, which can form homophilic and heterophilic interactions. Nectin family members are expressed by different cell types within the TME [56]. Although primarily regulators of cell adhesion, migration, and proliferation, several nectins have been shown to modulate immune responses in the TME via interactions with the T cell activating receptor CD226 (DNAM-1), or with the inhibitory receptors T cell immunoglobulin and ITIM domain (TIGIT), CD96 and CD112R (PVRIG) [57–62]. While the role for CD96 in tumor immunity is controversially discussed, TIGIT is a well-established immune checkpoint [63]. On cytotoxic T cells, TIGIT can interfere with CD226 signaling rendering those T cells dysfunctional [64]. Furthermore, TIGIT is important for the suppressive activity of T regs in the TME and also inhibits the function of NK cells [65,66]. Thus, co-targeting of PD-1 and TIGIT represents a potential strategy to improve the survival of melanoma patients [63,67].

Another emerging player of the Nectin-Axis is the activating immune receptor CD226. Several preclinical tumor models show accelerated growth in mice lacking CD226 [68]. In contrast, functional signaling of CD226 is required for effective NK cell responses against melanoma metastasis [69,70]. CD226 expression in Tregs is thought to oppose the function of TIGIT and thus weakening the suppressive activity of tumor-infiltrating Tregs [60]. The role of CD226 in tumor-infiltrating CD8 + cytotoxic T cells has recently gained interest. Using mouse melanoma models, it was shown that loss of CD226 either induced by tumor cell CD155 or induction of EOMES renders tumor infiltrating T cells dysfunctional. Loss of CD226 impairs T cell receptor signaling and potentially affects the ability of T cells to form productive immunological synapses with DCs and/or target cells. Thus, CD226 negative T cells were dysfunctional and contributed to resistance to melanoma immunotherapy [60,71].

Despite the recent advances in understanding the biology and function of the Nectin-family in cancer, the complexity of receptor ligand interactions between nectins, nectin-like molecules, and their immunomodulatory receptors is huge and remains incompletely understood. Further pre-clinical and clinical studies are needed to fully exploit the therapeutic potential of this pathway [63,72].

Another group of immunomodulatory cell surface molecules are involved in the adenosine pathway [73]. Two key players and targets for biologicals are CD39 and CD73, both membrane-bound enzymes catalyzing the conversion of the immunogenic metabolite adenosine tri-phosphate (ATP) into the immunosuppressive molecule, adenosine (Ado) [74]. Adenosine, by binding to G-protein coupled receptors (A1, A2A, A2B and A3AR), may directly promote tumor growth and metastasis through enhanced angiogenesis and immune suppression [75]. Thus, significant efforts have been undertaken to study the therapeutic potential of targeting the adenosine pathway in cancer [74]. A large body of work using pre-clinical mouse models, demonstrated that indeed targeting the adenosine-axis improves anti-melanoma immunity and potentially the efficacy of cancer immunotherapies [76–79]. In line with these studies, several clinical studies showed that the adenosine pathway is associated with progression and resistance to ICB in melanoma patients [80–83]. Taken together, a wealth of data laid the foundation for the development of several lead-candidates that are currently being investigated in clinical trials.

3.3. Cellular immunosuppression in the melanoma microenvironment

Ultimately, all previously described molecular mechanisms (i.e., genetic, epigenetic, soluble, and membrane-bound molecules) require an appropriate cellular interaction partner to create an immunosuppressive melanoma microenvironment (Fig. 2). Several immune cell subsets and phenotypic states have been identified and associated with immune evasion in melanoma. Regulatory T cells (Tregs) are a highly immunosuppressive cell type, which is present in primary melanoma, infiltrated lymph-nodes, and distant metastases. Tregs can exert their immunosuppressive functions via a multitude of pathways, including the expression of immune checkpoints (e.g., CTLA-4, TIGIT, TIM-3) [84–86] and soluble mediators, including IL-10 and transforming growth factor β (TGFβ) [87]. Thus, targeting this population is of great

clinical interest. While depletion of Tregs is a dangerous strategy due to the induction of severe autoimmune reactions, the preferred target is in blocking Treg recruitment and function, which is extensively reviewed elsewhere [87].

Another important group of immunosuppressive cells in the TME of melanoma are myeloid cells. This diverse group of innate immune cells has been shown to promote tumor growth and metastasis as well as local and systemic immune suppression in preclinical mouse melanoma models. However, due to the enormous phenotypic plasticity of myeloid cells, the literature is full of contradicting findings. Therefore, a detailed and context-dependent assessment of cell states and function is required. While some studies have found that neutrophil granulocytes harbor the potential to eliminate cancer cells [88,89], other studies have demonstrated that these cells have pro-tumorigenic and immunosuppressive capacities [90–92]. A recent study showed that blocking the pro-inflammatory cytokine IL-1α in the context of a CD40-Ligand-based immunotherapy reduced the infiltration of melanoma with neutrophils, thereby promoting melanoma immunity [93]. In the context of melanoma progression, Daoud et al., discovered a novel role for the anti-apoptotic molecule X-linked inhibitor of apoptosis protein (XIAP). Using transplantable and primary mouse melanoma models, the authors showed that XIAP contributes to the secretion of neutrophil recruiting cytokines, leading to the accumulation of neutrophils and enhanced melanoma growth [94].

Similar to neutrophils, tumor associated macrophages (TAMs) also acquire context-dependent cell states that are either associated with anti- or pro-tumoral function. Recruited into the melanoma microenvironment by several cytokines (e.g., M-CSF, CCL2, CXCL2), TAMs can suppress T cell functions directly by the expression of PD-L1, or indirectly via the secretion of Treg-recruiting chemokines (e.g., CCL17 and CCL22) [95]. Additionally, myeloid immune cells can promote angiogenesis via the secretion of pro-angiogenic factors, including VEGF, platelet-derived growth factor (PDGF), TGFβ, and matrix metallo-proteinases (MMPs) [95]. In contrast, a recent study highlighted the importance of CD206 + macrophages as integral part of the anti-tumor orchestra. Similar to Clec9a+ cross-presenting dendritic cells, F4/80highCD206 + macrophages were shown to be important for the activation of anti-tumoral T cells by cross-presenting tumor antigens [96].

Overall, the immune system is one of the most complex networks with a high degree of redundancy and flexibility in mice and men. In order to understand these complex relationships in the context of melanoma progression and therapy, an immune competent model such as the mouse is needed. However, pre-clinical studies always need to be corroborated by translational studies to effectively identify the molecular and cellular mechanisms leading to immune evasion, which ultimately fuel the discovery of novel targets and therapeutic approaches.

3.4. Nanomedicine an emerging approach to target the immune system in melanoma

A novel approach to treat melanoma represents nanomedicine, which utilizes knowledge and tools from the nanotechnology field. Simply put, nanotechnology uses materials, devices or even systems at the nanometer scale. For the diagnosis and treatment of melanoma, several nanosystems, such as lipid or polymeric systems, natural nanosystems and inorganic nanoparticles have been tested in pre-clinical and clinical research [97]. Nanoparticle structures can be used as vehicles to directly deliver immunostimulatory, cytotoxic or imaging reagents into the TME. Thus, nanomedicine can be used for the diagnosis of melanoma, for direct killing of cancer cells or to stimulate innate and adaptive immune responses [98]. Recently, Chiang et al. demonstrated that, fucoidan-based magnetic nanoparticles could be used to specifically deliver immune checkpoint blocker and or T cell engager to the TME [99]. Another elegant approach combined nanoparticles with phototherapy. Here, intravenous injection of photosensitive nanoparticles coated with the adjuvant aluminum hydroxide effectively reduced tumor growth by inducing a potent anti-tumor immune response [100]. In addition, polymeric nanosystems used for the development of a theranostic agent, have been shown to significantly enhance melanoma CT imaging and efficiently mediate tumor-targeted chemotherapy in an experimental melanoma model [101]. Taken together, exploiting nanomedicine approaches could represent a promising strategy to modulate anti-melanoma immunity in the future.

4. What is the impact in early and advanced disease?

The presence of tumor-resident CD8 + T cells plays a critical role for immune control, and has a prognostic value in patients with melanoma, both during treatment with ICIs and with targeted therapies [50,102]. Specifically, the presence of CD103 + T lymphocytes in tumor tissue leads to response to anti-PD1 antibodies, due to a high expression of PD-1 and LAG-3 on this T cell subpopulation, which act as the initial targets of immunotherapy [103]. In fact, analysis of on-treatment tumor specimens of patients with melanoma has shown that CD103 + TILs are highly expanded during treatment with anti-PD1 upon increased local IL-15 levels, suggesting that these TILs are the first to be recruited after starting immunotherapy. Moreover, the persistence of tumor-resident CD8 + T cells might be an important determinant for long-term disease response, and the ability not only to recruit but also to retain these cells within the TME is necessary to grant long-lasting tumor control [104]. Tumeh et al. performed quantitative IHC, multiplex immunofluorescence, and next generation sequencing for T-cell receptors (TCR) on baseline and on-treatment biopsies of 46 patients with metastatic melanoma treated with pembrolizumab [50]. Serial IHC analyses have shown that patients with disease response had higher $CD8^+$ cell densities at the invasive margin at baseline, and a parallel increase in $CD8^+$ cell density at both the invasive margin and tumor center during anti-PD1 treatment, as compared with patients experiencing disease progression; the increase in CD8 + density from baseline significantly correlated with a decrease in radiologic disease response. Baseline and on-treatment staining for phospho-STAT1 (pSTAT1), a downstream effector of the IFN-γ pathway, showed a higher expression of $pSTAT1^+$ at the invasive margin, localized to the area of CD8 infiltrate in patients experiencing disease response, when compared to biopsies from patients with disease progression. Analysis of the TCR repertoire showed that a more restricted TCR beta chain usage, indicative of a more clonal T cell population, was correlated with better response to pembrolizumab [50]. Interestingly, the presence of TILs also has primary relevance in determining disease response to targeted therapy (i.e., BRAF and MEK inhibitors) in BRAF mutant melanomas. In fact, oncogenic BRAF induces T cell suppression by stimulating the production of inhibitory cytokines and the expression of co-inhibitory molecules, such as PD-L1 or PD-L2, and leads to an immune suppressive phenotype characterized by the presence of T regs, MDSC, or TAMs [105].

Although the presence of TILs is an essential condition for immune response, it is not the only determinant for response to ICIs. There is evidence that the spatial distribution of TILs and their proximity to tumor cells play a crucial role and impact on ICI response and outcome. Lepletier et al. analyzed pre-treatment tumor biopsies of patients with metastatic melanoma treated with ICIs and targeted therapy, showing not only that pre-treatment CD155 expression (an adhesion molecule and immune checkpoint ligand) was associated with anti-PD1 resistance, but also that there is a positive correlation with CD155 tumor levels and PD-1 positive T cells within the tumor parenchyma, but not in the stroma [106]. The presence of $PD\text{-}L1^+$ cells in proximity to tumor cells combined with intra-tumoral $CD8^+$ T cell density was predictive for better 12-month PFS among melanoma patients treated with anti-PD1 monotherapy [107]. Similarly, a study by Gartrell et al. demonstrated that close proximity of CD8 + T cells with non-activated macrophages was associated with poor survival of patients with stage II-III melanoma, supporting the hypothesis that the interaction of T cells with

macrophages promotes inflammation and impairs anti-tumor immunity [108]. Altogether, this evidence suggests that, although spatial proximity is a limited surrogate for cell-cell interaction, the spatial distribution of T cells with regard to tumor cells, but also to other immune cells, has an important impact in determining TIL activity.

The activation of $CD8^+$ T cells against the tumor might also be supported by B cells localized in tertiary lymphoid structures (TLS), improving antigen presentation, and cytokine production and signaling, thus contributing to increased immunotherapy efficacy [109]. Several studies that assessed the role of tumor-associated TLS in response to immunotherapy have shown that patients with TLS^{high} tumors had increased survival upon treatment with CTLA-4 blockade, anti-PD1 monotherapy, and combined anti-CTLA-4 and anti-PD1 therapy [109,110]. Interestingly, the TLS signature is independent of TMB among patients treated with both anti-CTLA4 and anti-PD1 antibodies [111,112]. Notably, differences in RNA-sequencing data from on-treatment biopsies performed on cycle 1 and at day 29 of anti-PD1 therapy suggest that TLS functionality is inducible and observed only in patients with clinical response to ICIs [111]. This provides evidence for therapeutic strategies aiming at enhancing TLS formation and function.

Other biomarkers associated with response and/or resistance to immunotherapy are those indicative of an inflamed TME, namely PD-L1 expression and gene expression signatures (GES) of activated T cells, and those related to tumor antigenicity, such as the TMB. However, the presence of high TMB alone does not always correlate with response and, conversely, patients with low TMB might experience durable disease response during immunotherapy, suggesting that a single biomarker might not be representative, since several genomic and non-genomic features contribute to ICI response patterns [113]. In a study by Hugo et al., high mutational load was significantly associated with improved survival, but not with better response rates, among melanoma patients treated with anti-PD1 antibodies [114]. Notably, a trend towards better survival was observed in patients with high mutational load, suggesting that high TMB is a prognostic factor that gains further relevance in the setting of anti-PD1 therapy [114]. In the same study, tumors enriched with the innate anti-PD1 resistance (IPRES) signature, indicating heightened epithelial to mesenchymal transition, angiogenesis, hypoxia and wound healing, showed reduced responses to ICIs [114]. This IPRES signature enrichment was observed in subjects with innate resistance to anti-PD1, but also in patients pre-treated with MAPKi therapy, suggesting that MAPKi-induced transcriptome-wide reprogramming might influence response to subsequent immunotherapy [114]. These findings suggest that targeting IPRES-related biological processes might enhance response to immunotherapy.

Cui et al. performed a combined analysis of an inflamed GEP signature and an immune-suppressive signature, obtained through the analysis of RNA sequencing data from a combined discovery cohort, among advanced melanoma patients treated with anti-PD1 and anti-CTLA4 antibodies [115]. The proposed immune-suppression signature comprised genes related to the activity of cancer-associated fibroblasts, macrophages, and epithelial to mesenchymal transition. Results from this study showed that the combination of these two signatures, namely the ratio of IFN-γ to immune-suppression signature, predicted response and survival to anti-PD1 antibodies [115]. Jiang et al. developed the Tumor Immune Dysfunction and Exclusion (TIDE), a computational framework aimed at identifying genome-wide scores of T cell dysfunction and exclusion, which are the two main factors of immune escape [116]. The TIDE signature was identified by combining transcriptome profiles of treatment-naïve melanoma with patient survival outcome, thus identifying the average expression of known regulators of T cell infiltration, such as *CD8A-B*, granzyme A and B (*GZMA-B*), and perforin 1 (*PRF1*), to estimate the level of T cells in a tumor, and transforming growth factor β 1 (*TGFB1*) and SRY-Box Transcription Factor 10 (*SOX10*), due to the interaction of these two genes' expression with overall survival [116]. In their work, the TIDE signatures predicted clinical response to immunotherapy based on pre-treatment tumor profiles. Moreover, the authors experimentally validated the Serpin Family B Member 9 (SERPINB9) as a potentially druggable target, as it inhibits the cytotoxic lymphocyte protease GZMB, thus playing a crucial role in the mechanisms of immune evasion and ICIs resistance [116].

The implementation of prognostic and predictive tools evaluating inflammation markers in melanoma has provided results both in the settings of advanced and early disease. High levels of TMB and a T-cell-inflamed GEP, called tumor inflammation signature (TIS), correlated with better response rates among patients with advanced solid tumors (including melanoma) treated with pembrolizumab from four clinical trials [7]. Interestingly, improved responses were seen in patients with high levels of both PD-L1 expression and TMB, reflecting the relationship between PD-L1, GEP, and a T-cell–inflamed TME [7]. KEYNOTE-028 was a basket trial of 20 different patient cohorts with PD-L1 positive, advanced solid tumors (excluding melanoma) treated with pembrolizumab. In this trial, the combination of a T-cell-inflamed GEP, PD-L1 expression, and TMB were associated with better ORR and longer PFS across all tumor types, suggesting that these three biomarkers alone or in combination could be valuable tools to select patients that could benefit from anti-PD1 therapy [117]. Recently, Newell et al. showed that combined high TMB, neoantigen load, IFNγ signature, PD-L1 expression, low PSMB8 methylation, and T cells in the TME are associated with response among melanoma patients treated with anti-PD1 with or without anti-CTLA4 antibodies [118]. In the multivariable model, tumors with high TMB and a high IFNγ signature showed the best response to immunotherapy.

The possibility to use biomarkers for selection and stratification of patients according to the risk of relapse, and to predict the potential benefit from immunotherapy, gains even more attraction in the context of neoadjuvant immunotherapy in locally advanced (i.e., stage III) melanoma. The phase 1b OpACIN and phase 2 OpACIN-neo studies have demonstrated impressive pathologic response rates of neoadjuvant ICIs, nivolumab plus ipilimumab, for the treatment of macroscopic stage III melanoma [119,120]. Biomarker analyses of patients treated in these two studies showed that high TMB and high IFN-γ signature were associated with pathologic response and low risk of disease relapse [121]. Conversely, patients with low IFN-γ signature were less likely to respond to neoadjuvant ipilimumab plus nivolumab. On the basis of this evidence, a phase 1b trial, the DONIMI study (NCT04133948) is ongoing, to evaluate the neoadjuvant combination of nivolumab with or without ipilimumab, with domatinostat, a class 1 histone deacetylase inhibitor, according to the IFN-γ signature in the tumor [122]. In this trial, IFN-γ signature high patients were randomized to 2 cycles nivolumab (arm A) or 2 cycles nivolumab + domatinostat (arm B), while IFN-γ signature low patients were randomized to arm C (same treatment regimen as arm B) or arm D (2 cycles nivolumab + ipilimumab + domatinostat) [122]. Preliminary data from this trial showed that pathologic response rate was 90 % in arm A, 80 % in arm B, 30 % in arm C and 40 % in arm D. After a median follow up of 8.9 months, the estimated 6-month RFS rate was 100 % in IFN-γ signature high patients and 79.4 % in IFN-γ signature low patients [123].

Based on the T cell-inflamed GEP, containing IFN-γ-responsive genes related to antigen presentation, chemokine expression, cytotoxic activity, and adaptive immune resistance previously described by Ayers et al. [124], the authors developed an IFN-γ signature algorithm to be used for patient classification in this prospective trial. This represents the first trial evaluating baseline GEP as a biomarker for patient selection and treatment tailoring and will provide important insight in this setting. Given the correlation of IFN-γ signature with disease response in neoadjuvant trials, the prognostic role of IFN-γ signature was also evaluated among patients with stage III melanoma treated with adjuvant anti-PD1 therapy. In this context, higher IFN-γ signature has been shown to be a prognostic biomarker both in patients receiving or not adjuvant immunotherapy: indeed, both patients with high and low IFN-γ signature showed benefit from adjuvant anti-PD1 therapy, meaning that IFN-γ signature can be considered a prognostic biomarker [125]. At the

same time, combined TMB and IFN-γ signature, as markers of an adaptive immune response, were predictors of benefit from adjuvant targeted therapy among patients with stage III melanoma treated in the COMBI-AD trial [126].

One of the main intrinsic limitations of tissue analysis and GES is the impossibility to capture the dynamic and multiple interactions of the immune system and the tumor cells. The analysis of circulating biomarkers can help integrate the information acquired with tissue analysis. Huang et al. analyzed the immune profiling of patients with stage IV melanoma receiving pembrolizumab, after disease progression on previous ipilimumab [127]. In this study, pembrolizumab exerted immunological response (i.e., increase in Ki67 expression in PD-L1 positive circulating T cells) in most patients; however, correlation with clinical response was observed in less than half of these patients. The T cell reinvigoration correlated with response to pembrolizumab; however, this variable itself strongly correlated with the TMB, suggesting that an effective T cell response may not be enough to provide disease response if the TMB is too high [127].

Epigenetic modifications have also been demonstrated to contribute to immune resistance during ICIs across different types of solid tumors. The first study demonstrating the role of epigenetic biomarkers in assessing response to immunotherapy in patients with melanoma showed that CTLA-4 promoter methylation was associated with better response to anti-PD1 combined with anti-CTLA4 antibodies, and improved OS [128]. Another study evaluating DNA methylation signatures of tissue samples of non-small cell lung cancer (NSCLC) and melanoma showed that global DNA methylation alterations are an important determinant of immune resistance during ICIs, with a higher predictive power for methylation loss than for mutational burden [129]. Kim et al. showed that chronic IFN-γ exposure induced melanoma cell dedifferentiation to a neural crest cell phenotype, thus improving responses to anti-PD1 therapy [130]. In this study, the biopsies of tumors respondent to anti-PD1 showed decreased expression of melanocytic markers and increased neural crest markers, suggesting treatment-induced dedifferentiation which, in vitro, was induced by an increased IFN-γ signature [130]. Several other morphologic features of the TME can contribute to maintaining melanomas "non-inflamed", including changes in tumor-associated vascular and lymphatic drainage, infiltration by immune-suppressive cells, such as MDSCs and T regs, and mechanisms of epithelial-mesenchymal transition [131].

Altogether, the complexity of the immune mechanisms operating within the TME and the influence of immune cells, tumor cells, soluble factors, and epigenetic mechanisms give reason for the challenge of identifying biomarkers of response and resistance to ICIs. Current research is focusing on how to combine different targets of resistance in order to obtain therapeutic results. The main therapeutic strategies presently under investigation are detailed in the next section.

5. Perspectives

The growing knowledge of the mechanisms of innate and adaptive resistance to immunotherapy has enabled us to identify possible points of intervention and has provided us with plausible reasons for treatment failures [131]. Patients with inflamed melanomas could reasonably benefit from single ICI, due to a certain grade of T cell infiltration in the TME. However, given the relatively low response rates ICIs given as single agent in a majority of patients (i.e., those with non-inflamed melanomas), several efforts have been made to identify treatment combinations that could increase the effectiveness of immunotherapy.

The activity of anti-PD1 inhibitors in non-inflamed melanomas can be enhanced by combining them with co-inhibitory receptors on T cells, such as the lymphocyte activation gene 3 (LAG3), TIM3, TIGIT, B and T lymphocyte attenuator (BTLA), V-domain Ig suppressor of T cell activation (VISTA), and sialic acid-binding Ig-like lection 9 (SIGLEC9) [132–135]. Recently, the phase III RELATIVITY trial showed that, in patients with locally advanced/metastatic melanoma, the combination of the anti-LAG3 antibody, relatlimab, with nivolumab, increases the response rate and doubles PFS as compared to nivolumab alone, regardless of LAG3 expression levels on tumor tissue [132]. Interestingly, the safety profile was more favorable than ipilimumab plus nivolumab given with the classical schedule [132].

Alternatively, ICIs can be combined with co-stimulatory checkpoint molecules enhancing T cell expansion and effector functions, but also inhibiting the suppressive functions of regulatory T cells (T reg), such as OX40, CD28, CD137, the glucocorticoid-induced TNFR-related protein (GITR), and the inducible T cell co-stimulator (ICOS) [136–138]. Feladilimab, an IgG4 ICOS agonist non-T-cell depleting monoclonal antibody, was the first ICOS agonist to show single-agent activity in patients with melanoma who had relapsed on previous immunotherapy, and has also shown promising clinical activity and manageable safety profiles in combination with pembrolizumab [139].

Most non-inflamed melanomas display T cell exclusion sustained by the physical inability of T cells to reach the tumor tissue. This might be due to lack of or reduced T cell recruiting signals to the presence of physical barriers protecting the tumor from T cell infiltration, or to the presence of an immune-suppressive TME. In the first case, preclinical studies have shown that epigenetic drugs modulating chemokine production and blocking β-catenin signaling could increase T cell recruitment, thereby turning cold tumors into hot tumors [140,141]. Abnormal TGFβ signaling combined with tumor fibrosis, may negatively interfere with the interaction between T cells and tumor cells and thereby contribute to resistance mechanisms by immune-exclusion, while increased tumor infiltration of M1-like macrophages enhances T cell activity. In fact, type III collagen and vimentin turnover have been demonstrated to contribute to resistance/response mechanisms to anti-PD1 inhibitors among patients with metastatic melanoma, and blood-based biomarkers reflecting excessive type III collagen turnover were associated with worse OS and PFS during treatment [142].

In cases where the tumor develops abnormal structural features, changes in the tumor-associated lymphatic and vascular structures lead to a hypoxic milieu, which contributes to the creation of an immune-suppressive TME [10]. The combination of pembrolizumab with the VEGF inhibitor, lenvatinib, has demonstrated durable responses in patients with advanced melanoma with confirmed disease progression on prior ICIs [143]; this combination is presently under investigation as first-line treatment in a phase III randomized clinical trial (NCT03820986). Blocking the enzymatic activity of two ectonucleotidases, CD73 and CD39, leading to reduced adenosine accumulation in the TME, thus impairing tumor growth and metastatic spread, is another promising therapeutic strategy to increase tumor immunogenicity [144]. However, to date, no agents targeting this pathway have reached regulatory approval.

Other potential targets in non-inflamed melanomas are represented by soluble factors, such as IL-2, IL-6, IL-10, and TGF-β, and cellular modulators of local adaptive immunity, such as MDSC and T reg. The CD122-preferential interleukin-2 pathway agonist, bempegaldesleukin, in combination with nivolumab, extended median PFS and was well tolerated in first-line metastatic melanoma in the phase II PIVOT-02 trial [145]. However, the phase 3 trial failed to meet its primary endpoints of PFS and ORR and the adjuvant trial was therefore closed based on these results. The combination of ipilimumab and nivolumab with the IL-6 inhibitor, tocilizumab, has recently yielded promising anti-tumor activity as first-line treatment of patients with advanced melanoma in a phase Ib/II trial, with a relevant reduction in the frequency of immune-related adverse events as compared with the combination of ipilimumab and nivolumab [146]. Several trials are currently investigating MDSC as a potential target by blocking their suppressive pathways (e.g., IDO, arginine, tryptophan, and nitric-oxide-related pathways), by modulating their tumor infiltration (e.g., through the blockade of colony-stimulating factor 1 receptor, CSF1R), or by reshaping them from a pro-tumoral M2 to an anti-tumoral M1 phenotype [147]. Still, the disappointing results of pembrolizumab combined

Table 1
Experimental agents and main therapeutic strategies currently under investigation in clinical trials, to turn non-inflamed into inflamed melanomas.

Strategy	Target (s)		Drugs (s)	Clinical trial (s)	
Enhancement of anti-PD1 activity	Co-inhibitory receptor molecules	LAG-3	Relatlimab	NCT03743766	Relatlimab plus nivo in immunotherapy naïve melanoma pts
			INCAGN02385	NCT04370704	INCAGN02385 plus anti-PD1 and anti-TIM-3 in pts with advanced solid tumors
			IMP321	NCT02676869	IMP321 plus pembro in melanoma pts progressed on anti-PD1
			LAG525	NCT03484923	Spartalizumab plus LAG525 in unselected and LAG-3 positive melanoma pts
			LBL-007	NCT04640545	LBL-007 plus toripalimab and axitinib in pts with advanced melanoma
			RO7247669	NCT04140500	PD1-LAG3 bispecific antibody, in pts with advanced solid tumors
		TIM-3	INCAGN02390	NCT04370704	INCAGN02390 plus anti-PD1 and anti-LAG-3 in pts with advanced solid tumors
			RO7121661	NCT03708328	PD1-TIM3 bispecific antibody, in pts with advanced solid tumors
			TSR-022	NCT02817633	TSR-022 combinatorial strategies in pts with advanced solid tumors
			MBG453	NCT02608268	MBG453 alone or combined with spartalizumab/ decitabine in pts with advanced solid tumors
		TIGIT	AB154	NCT05130177	AB154 plus antiPD1 in melanoma pts progressed on anti-PD1
			Vibostolimab	NCT04305054	Vibostolimab plus pembro in treatment naïve melanoma pts
			EOS-448	NCT05060432	EOS-448 plus pembro or inupadenant (A2AR) in pts with advanced solid tumors
		BTLA	JS004	NCT04773951	JS004 plus toripalimab in pts with advanced solid tumors
			TAB004	NCT04137900	TAB004 monotherapy or combined with toripalimab in pts with advanced solid tumors
Enhancement of T cell expansion and effector functions; inhibition of T reg suppressive functions	Co-stimulatory receptor molecules	OX40	mRNA-2752	NCT03739931	mRNA-2752 + /- durva in pts with advanced solid tumors
			INBRX-106	NCT04198766	INBRX-106 + /- pembro in pts with advanced solid tumors
			SL-279252	NCT03894618	SL-279252 (PD1-Fc-OX40L) in pts with advanced solid tumors
			PF-04518600	NCT02554812	PF-04518600 plus avelumab in pts with advanced solid tumors
		CD137	• BMS-663513	NCT02652455	BMS-663513, anti-PD-1 and adoptive cell therapy for advanced melanoma pts
		GITR	ASP1951	NCT03799003	ASP1951 alone or with pembro in pts with advanced solid tumors
		ICOS	KY1044	NCT03829501	KY1044 plus atezo in pts with advanced solid tumors
		CD27	CDX-1127	NCT03617328	Vaccination with 6MHP + /- CDX-1127 in pts with stage II-IV melanoma
		CD40	APX005M	NCT03502330	APX005M plus nivo and cabiralizumab (anti-CSF1R) in pts with untreated melanoma
Increased T cell recruitment	Chemokine production	β-catenin	Tegavivint	NCT04851119	Tegavivint, interfering with the binding of β-catenin to TBL1, in pts with advanced solid tumors
	Soluble factors	IL-1RAP	CAN04	NCT04452214	CAN04 plus pembro in pts with ICI refractory solid tumors
		IL-2	HD-IL2	NCT04562129	IL-2 plus ipi, and subsequent nivo in melanoma pts progressed on anti-PD1
			Bempegaldesleukin	NCT03635983	Bempeg plus nivo in treatment naïve melanoma pts
			Aldesleukin	NCT02748564	Aldesleukin plus pembro in treatment naïve melanoma pts
				NCT02500576	Pembro plus lymphodepletion, TIL, and High or Low Dose aldesleukin in pts with treatment naïve melanoma
			RO6874281	NCT03875079	RO6874281 plus pembro in immunotherapy naïve and pretreated melanoma pts (2 cohorts)
			hu14.18-IL2	NCT03958383	Intralesional hu14.18-IL2 plus RT, ipi and nivo in pts with advanced unresectable melanoma
			MDNA-11	NCT05086692	MDNA11 alone or combined with ICI in pts with advanced solid tumors
			IL-2	NCT03474497	Intralesional IL-2 plus pembro and RT in melanoma pts progressed on anti-PD1
			GI-101	NCT04977453	GI-101 + /- pembro, lenvatinib and RT in pts with advanced solid tumors
			Nemvaleukin alfa	NCT02799095	Nemvaleukin alfa + /- pembro in pts with advanced solid tumors

(*continued on next page*)

Table 1 (*continued*)

Strategy	Target (s)		Drugs (s)	Clinical trial (s)	
		IL-6	Tocilizumab	NCT03999749	Tocilizumab plus ipi and nivo in treatment naïve melanoma pts
		IL-12	DF6002	NCT04423029	DF6002 alone or combined with nivo in pts with advanced solid tumors
		IL-15	N-803	NCT03228667	N-803 plus ICI in pts with ICI refractory solid tumors
		IL-18	ST-067	NCT04787042	ST-067 in pts with advanced solid tumors
		TGF-β	Bintrafusp alfa	NCT04789668	Bintrafusp alfa plus pimasertib in melanoma pts with brain metastases
			PF-06952229	NCT03685591	PF-06952229 plus enzalutamide in pts with advanced solid tumors
	Cellular modulators of adaptive immunity	MDSC	SX-682	NCT03161431	SX-682 plus pembro in melanoma pts progressed on anti-PD1
			IPI-549	NCT02637531	IPI-549 alone or combined with nivo in pts with advanced solid tumors
			RGX-104	NCT02922764	RGX-104 alone or combined with ICI or CT in pts with advanced solid tumors
Reduction of T cell exclusion	Lymphatic and vascular structures	VEGF	Bevacizumab	NCT04356729	Bevacizumab plus atezo in treatment naïve melanoma pts
				NCT03175432	Bevacizumab + atezo or cobimetinib in treatment naïve melanoma pts with brain metastases
			Axitinib	NCT04493203	Axitinib plus nivo in melanoma pts progressed on anti-PD1
			Aflibercept	NCT02298959	Aflibercept plus pembro in pts with advanced solid tumors
			Lenvatinib	NCT04305054	Lenvatinib plus pembro and quavonlimab in treatment naïve melanoma pts
				NCT04305041	Lenvatinib plus pembro and quavonlimab in treatment naïve melanoma pts
				NCT04700072	Lenvatinib plus pembro + /- quavonlimab in pretreated melanoma pts
		Adenosine pathway	Inupadenant	NCT05060432	Inupadenant plus EOS-448 p in pts with advanced solid tumors
			NIR178	NCT03207867	NIR178 plus spartalizumab in pts with advanced solid tumors
Modulation of innate immune response in the TME	IFN pathway	STING	E7766	NCT04144140	Intratumoral E7766 in pts with advanced solid tumors
		TLR3	BO 112	NCT04570332	BO 112 plus pembro in melanoma pts progressed on anti-PD1
		TLR7	NKTR-262	NCT03435640	NKTR-262 plus bempeg in pts with advanced solid tumors
			LHC165	NCT03301896	LHC165 alone or combined with spartalizumab in pts with advanced solid tumors
		TLR9	SD-101	NCT04935229	Intrahepatic SD-101 + /- ipi and nivo in pts with metastatic uveal melanoma
increased production of TAAs and DAMPs	Tumor cells	TAA	TRK-950	NCT03872947	TRK-950 with ICI or CT in pts with advanced solid tumors
			IP-001	NCT03993678	Intratumoral IP-001after thermal ablation in pts with advanced solid tumors

Abbreviations: A2AR, adenosine A2A receptor; atezo, atezolizumab; bempeg, bempegaldesleukin; BTLA, B and T lymphocyte attenuator; CSF1R, colony-stimulating factor 1 receptor; CT, chemotherapy; DAMPs, damage-associated molecular patterns; durva, durvalumab; GITR, glucocorticoid-induced TINFR-related protein; HD-IL, high-dose interleukin; ICI, immune-checkpoint inhibitors; ICOS, inducible T cell co-stimulator; IFN, interferon; ipi, ipilimumab; LAG-3, lymphocyte activation gene 3; MDSC, myeloid-derived suppressor cell; nivo, nivolumab; pts, patients; PD1, programmed cell death 1; pembro, pembrolizumab; pts, patients; RAP, receptor accessory protein; RT, radiotherapy; STING, stimulator of IFN genes; TAA, tumor associated antigen; TBL1, Transducin Beta Like 1 X-Linked; TGF, transforming growth factor; TIGIT, T cell immunoglobulin and ITIM domain; TIM-3, T-cell immunoglobulin domain and mucin domain 3; TLR, Toll-like receptor; TME, tumor micro-environment; T reg, regulatory T cells; VEGF, vascular endothelial growth factor.
(source: clinicaltrials.gov, accessed December 11th 2021).

with the IDO inhibitor, epacadostat, in patients with melanoma suggest that this strategy needs to be further investigated or, at least, that better patient selection is needed to exploit this therapeutic strategy [148].

Modulation of the innate immune response within the TME is another strategy of intervention for non-inflamed melanomas. Intra-tumoral injection of the toll-like receptor 9 (TLR9) agonist, CMP-001, in combination with systemic pembrolizumab, has shown promising clinical activity in patients with melanoma progressing on previous anti-PD1 therapy, based on reverting PD-1 blockade resistance by triggering a strong IFN response [149]. Similarly, local injection of the stimulator of IFN genes (STING) agonist stimulates the production of type I and II IFNs, promotes vascular normalization and tertiary lymphoid structure formation, increases PD-L1 expression in the TME, and boosts dendritic cell (DC) accumulation and T cell mediated response [145]. Notably, the association of a PD-1 inhibitor is needed, since the STING-agonist-mediated IFN induction alone might not be successful in mounting an adequate T cell response in non-inflamed tumors [150].

Combination strategies aimed at increasing the tumor antigenicity and adjuvanticity include the association of immunotherapy with radiotherapy, chemotherapy, and targeted therapies. Together, such combinations lead to an increased production of neoantigens and damage-associated molecular patterns (DAMPs), and promotion of apoptosis and necroptosis, resulting in increased immune system activation [151,152]. Another way to elicit systemic immunity through DAMPs and tumor associated antigen (TAA) production is by using

oncolytic viruses [153]. T-VEC was the first virotherapeutic approach approved by the United States (US) Food and Drug Administration (FDA) as a local treatment for patients with unresectable cutaneous melanoma. Despite promising preliminary data, final analysis of the phase III randomized trial MASTERKEY-265 showed that the addition of intralesional T-VEC to systemic pembrolizumab did not add any survival advantage in treatment-naïve melanoma patients as compared with pembrolizumab monotherapy [154]. Several factors might have contributed to this result, including physical barriers impairing the intralesional spread of T-VEC and subsequent T cell trafficking (due to poor vascularization, hypoxia, and fibrosis), or poor antigenicity of cold tumors with reduced TAA production and reduced T cell priming [153]. Again, better patient selection or association of T-VEC and anti-PD1 with a third additional agent might lead to increased tumor heating and improved antitumor activity.

Several additional strategies are currently under evaluation in clinical trials, with the aim to remodel non-inflamed melanomas and convert them into inflamed melanomas, in order to increase disease response to ICIs. Table 1 summarizes the main therapeutic strategies and clinical trials ongoing in this setting.

6. Conclusions

Therapeutic advances in the field of immunotherapy and targeted agents have led to undisputed improvement in survival rates among patients with advanced melanoma. Still, several complex immune mechanisms operating within the TME involving immune cells, tumor cells, soluble factors, and epigenetic mechanisms give reason for the challenge of identifying biomarkers of response and resistance to ICIs. With increasing knowledge on the mechanisms of innate and adaptive resistance to immunotherapy, plausible reasons for treatment failures and potential points of intervention have been identified. Current research is focusing on how to combine different targets of resistance in order to increase the effectiveness of immunotherapy, delay the onset of treatment resistance, thus improving therapeutic results.

Acknowledgements

N.G. is funded by the Deutsche Krebshilfe within the Mildred Scheel School of Oncology (German Cancer Aid, Grant ID 70113307) T.B. is funded by the Deutsche Forschungsgemeinschaft (DFG, German Research Foundation) under Germany's Excellence Strategy – EXC2151–390873048 and the Melanoma Research Alliance (https://doi.org/10.48050/pc.gr.91568). Figures were created with BioRender.com. This work was funded by the Fondazione AIRC "Programma di ricerca 5 per Mille 2018— ID#21073" to EPICA investigators.

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