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REVIEW ARTICLE

# Rational combination of cancer immunotherapy in melanoma

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## Abstract

The recent advances in cancer immunotherapy with unprecedented success in therapy of advanced melanoma represent a turning point in the landscape of melanoma treatment. Given the complexity of activation of immunological system and the physiologic multifactorial homeostatic mechanisms controlling immune responses, combinatorial strategies are eagerly needed in melanoma therapy. Nevertheless, rational selection of immunotherapy combinations should be more biomarker-guided, including not only the cancer immunogram, PD-L1 expression, interferon gene expression signature, mutational burden, and tumor infiltration by CD8+ T cells but also intratumoral T cell exhaustion and microbiota composition. In this review, we summarize the rationale to develop combination treatment strategies in melanoma and discuss biological background that could help to design new combinations in order to improve patients' outcome.

**Keywords** Immunotherapy · Therapy · Combination · Melanoma · Outcome

## Introduction

The last decade can be considered a renaissance era in the treatment landscape of metastatic melanoma, with the approval of seven new drugs by the Food and Drug Administration (FDA) and the European Medical Agency (EMA) [1].

Clinical advances were built on five main discoveries: (i) the molecular basis of melanoma and the identification of *BRAFV600* mutation as target for systemic treatment, (ii) the biological understanding that cancer immunoediting is an important mechanism through which tumor cells avoid immune destruction and paradoxically promote cancer development and growth, (iii) the recognition that immunosurveillance plays a key role in the recognition and elimination of cancer cells, (iv) the identification of the genetic and immunologic heterogeneity in human tumors, and (v) the preclinical evidence that efficient anticancer strategies must focus on targeting different pathways and targets concurrently [2].

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In melanoma, two main strategies have been investigated, so far, partially in parallel: targeted therapy and immunotherapy. Approximately 40–50% metastatic melanoma patients harbor point mutations in B-Raf Proto-Oncogene (*BRAF*), over 95% of which are at V600 in *BRAF* exon 15 [3]. This mutation drives progression of melanomas through constitutive activation of the mitogen-activated protein kinase (MAPK) pathway. As a consequence, this led to identification of a truly druggable target with the consequent development of BRAF inhibitors. These drugs, initially introduced as monotherapies in patients with *BRAF*-mutant melanoma, demonstrated their clinical impact. Two prospective, randomized trials have demonstrated that in the metastatic setting, BRAF inhibitors (BRAFi) are associated with improved response rate, progression free survival (PFS), and overall survival (OS) compared with chemotherapy [4, 5]. However, responses are limited in duration mainly due to acquired resistance.

Dual MAPK pathway inhibition with addition of a MEK inhibitor (MEKi) to a BRAFi improved the efficacy and tolerability compared to BRAFi alone in well-designed prospective phase III randomized studies [6, 7]. Furthermore, unlike immune checkpoint inhibitors-related toxicities, which can occur late as well as after treatment discontinuation, BRAFi- and MEKi-related adverse events usually resolve with therapy withdrawal and late toxicities are uncommon after drug discontinuation.

Consequently, BRAF/MEKi combinations are recommended as first- or second-line therapies for advanced

*BRAFV600*-mutated melanoma. Nevertheless, PFS and OS are approximately 12 months and 24 months, respectively, reflecting development of resistance mechanisms in the majority of patients. From a molecular point of view, the acquired resistance is related to a re-activation of the MAPK pathway [8–10]. From a clinical point of view, on the other hand, a pooled analysis identified routinely used clinical parameters as predictors of outcome in patients treated with BRAFi and MEKi in the context of clinical trials [11, 12]. Specifically, a regression tree analysis identified three independent favorable prognostic factors: pretreatment LDH levels, presence of < 3 metastatic sites, and sum of lesion diameters < 66 mm. In the most favorable prognostic group, 3-year PFS was 42%, suggesting that a low disease burden at baseline can be prognostic for a long-term benefit with targeted therapies [11, 12].

Another strategy that has paved the way in cancer treatment has been the use of monoclonal antibodies that block immunologic checkpoints that are, in turn, important in controlling immune responses. Their ability to induce sustainable responses and their favorable tolerability has promoted studies with these drugs either as monotherapy or in different combinations both in advanced and adjuvant setting [13–18]. The objective response rates (ORRs) to treatment with ipilimumab monotherapy and anti-PD-1 alone (nivolumab, pembrolizumab) have been reported in 10% and 30–40% of metastatic melanoma patients, respectively [13–15]. More recently, a phase III prospective randomized clinical trial demonstrated a greater efficacy and a better tolerability of anti-PD-1 antibodies than ipilimumab [13]. Hence, anti-PD-1 antibodies are, by large, the most effective first-line immune checkpoint inhibitors, with a reported 3-year survival rate of 50% [13].

More recently, Talimogene laherparepvec (T-VEC; Imlygic™), a genetically modified herpes simplex virus, type 1, has been approved for the treatment of advanced melanoma by the US FDA and EMA.

One of the main clinical and biological characteristics peculiar of immunotherapy compared to targeted therapy is its capacity to elicit the immune system and to keep an immunological memory against cancer cells. This is supported by the maintenance of response after stopping anti-CTLA4 and anti-PD-1 therapy in the metastatic setting [19].

Nevertheless, although single-agent anti-PD-1/PD-L1 therapy has demonstrated promising clinical activity in a large proportion of metastatic melanoma patients, primary and acquired resistance to immunotherapies can develop [1]. In the adjuvant setting as well, 30% of patients receiving anti-PD-1 therapy still recur [16, 17]. Pursuing treatment strategies to overcome primary and acquired resistance is important in order to further improve patients' outcome.

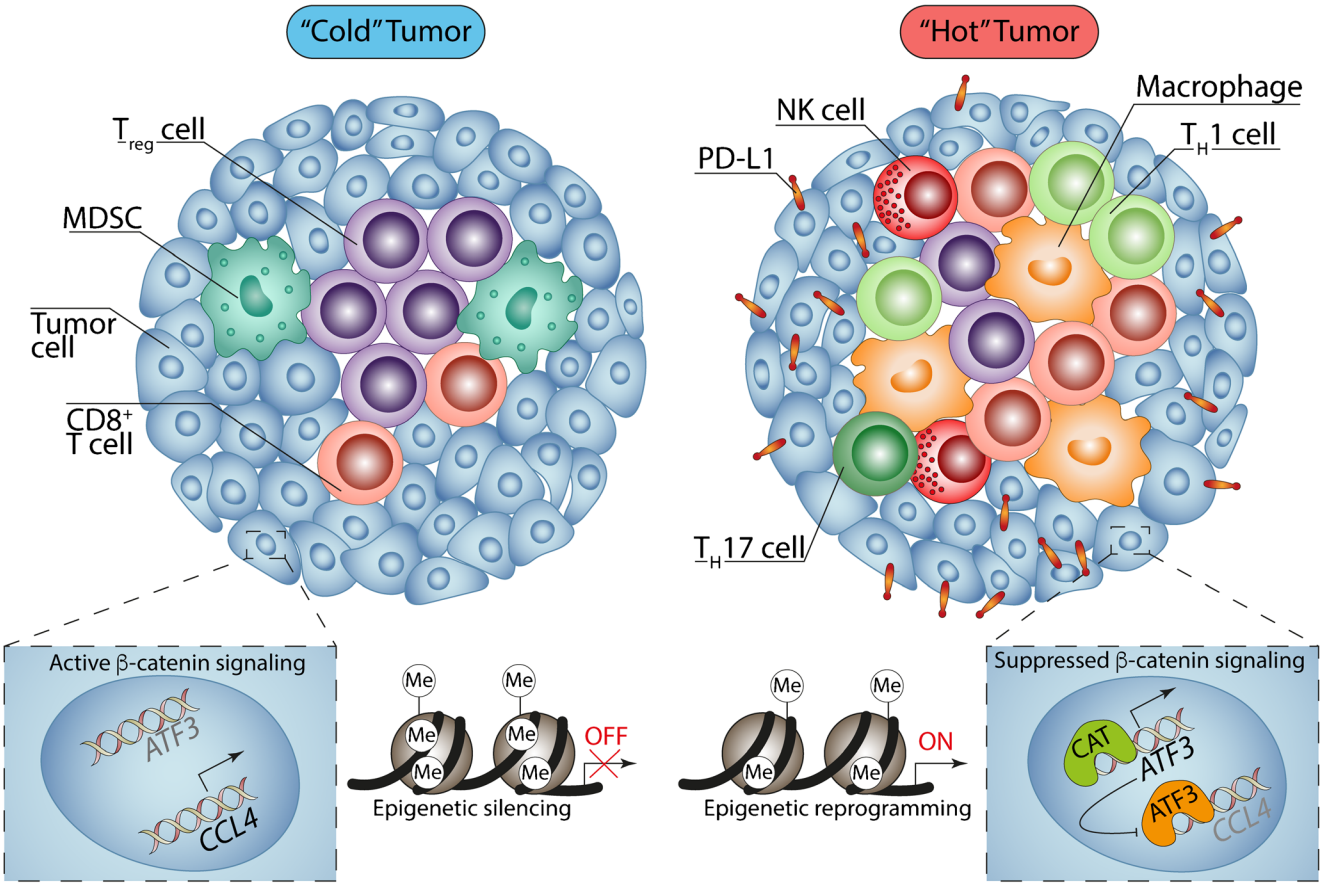
In this review, we summarize the rationale to develop combination treatment strategies in melanoma and discuss biological background that could help to design new combinations in order to improve patients' outcome.

## Rationale to combine immunotherapy in metastatic melanoma

Unprecedented progress in treatment of metastatic melanomas with immunotherapy increased the current long-term survival crossbar to 30–40%. However, this means that more than 60% of melanoma patients need more effective treatment. The sensitivity to monotherapy with anti-PD-1 drugs (nivolumab or pembrolizumab) is observed in 40% of patients, and with combination of nivolumab and ipilimumab, the activity increases further to 60% [20]. Nevertheless, toxicity is higher with the combination of nivolumab and ipilimumab compared to monotherapy. In the pivotal trial CA209-067, G3-G4 adverse events were reported in 55%, 16%, and 27% of metastatic melanoma patients, who received nivolumab plus ipilimumab, nivolumab, or ipilimumab alone, respectively [20]. Thirty-six percent of patients in the combination arm discontinued treatment, and among those, 70% maintained the previous response. The most common adverse events of any grade with the combination were gastrointestinal toxicity, asthenia, and pruritus followed by rash, loss of appetite, nausea, and pyrexia.

Overall, the combination treatment is, by large, more effective than ipilimumab alone, but the price to be paid is a higher toxicity. Trials so far conducted were not designed and powered to investigate the superiority of nivolumab plus ipilimumab over anti-PD-1 monotherapy, which represents the standard treatment for the majority of patients. Furthermore, we still do not know if the combination strategy should replace the sequencing approach of nivolumab/pembrolizumab monotherapy followed by ipilimumab at progression. Furthermore, some recent clinical data suggest that decreasing the ipilimumab dose in combination with nivolumab can lead to a decreased toxicity without compromising efficacy both in terms of PFS and OS [21].

The efficacy of anti-PD-1 drugs depends mostly upon the presence of immunocompetent antigen-specific lymphocytes T within tumor, as it happens in approximately 60% of melanomas, so-called inflamed ("hot") tumors (Fig. 1). Higher efficacy of anti-PD-1 drugs is linked to increased PD-L1 overexpression as well as the tumor mutational burden; nevertheless, both mutational burden and PD-L1 can be considered correlative and not predictive biomarkers of response to anti-PD-1 antibodies in melanoma. While acquired resistance usually occurs in patients treated with targeted therapies, durable responses suggestive of long-lasting immunologic memory are frequently seen in the vast majority of patients treated with immune checkpoint




| | | |
|---|---|---|
| Biological characteristics | • Epigenetic silencing<br>• Active β-catenin signaling<br>• Mesenchymal-like cells<br>• Stem cell-like cells<br>• Less differentiated cells<br>• Low PDL1 expression | • Epigenetic reprogramming<br>• Suppressed β-catenin signaling<br>• Epithelial cells<br>• High differentiated cells<br>• High PDL1 expression |
| Immunological characteristics | • Enriched immunosuppressive cytokines<br>• High numbers of $T_{reg}$ cells and MDSCs<br>• Few $T_H1$ cells, NK cells and $CD8^+$ T cells<br>• Few functional APCs | • Enriched in $T_H1$-type chemokines<br>• High numbers of effector immune cells ($T_H1$ cells, NK cells, and $CD8^+$ T cells)<br>• High numbers of functional APCs |
| Therapeutic implications | • Generate T cells (vaccines, T-cell therapy, adoptive cell transfer, β-catenin modulation ?, epigenetic modulation ?<br>• Prime the immune response and elicit the effector phase (bring T cells in tumors: CTLA4, sequence target and immuno | • Block adaptive immune resistance (anti-PD-1, anti-LAG-3, anti-TIM3, IDO) |

**Fig. 1** Biological phenotype, immunological characteristics of melanomas, and their therapeutic implications (modified with permission from Nisha Nagarsheth, Max S. Wicha and Weiping Zou Nature reviews immunology 2017 "Chemokines in the cancer microenvironment and their relevance in cancer immunotherapy")

inhibitors. However, at least 50% of patients treated with single-agent immune checkpoint inhibitors progress at a rate consistent with the natural history of disease. In addition, late disease progression is now emerging with longer follow-up of clinical trial populations, suggesting the emergence of acquired/secondary resistance.

There are several mechanisms of primary and secondary resistance to anti-PD-1 immunotherapy (Table 1) [22, 23] including:

1. Lack of immune cell infiltrations within tumor tissue ("cold" melanomas) or a microenvironment characterized by immunosuppressive features including high numbers of Treg cells and myeloid derived suppressive cells (MDSCs), few NK cells, CD8+ T cells, and functional APCs (Fig. 1).
2. T cell exhaustion during chronic exposure to cognate antigen, that is, in turn, related to overexpression of multiple inhibitory receptors on T lymphocytes including T cell immunoglobulin mucin 3 (TIM3), CLTA-4, lymphocyte activation gene 3 (LAG3), and B and T lymphocyte attenuator (BTLA) [24]
3. Expression of the indoleamine 2,3-dioxygenase (IDO), an enzyme driven by the interferon-inducible pathways, mostly in inflamed melanomas.
4. Increase of Foxp3+ Treg to CD8+ T cell ratio within tumor microenvironment.

Based on these mechanisms, we can hypothesize different strategies to overcome primary and secondary resistance by combining immunomodulating agents, which target different pathways in tumor (immune) microenvironment [25, 26]:

**Table 1** De novo and acquired mechanisms of resistance to immune checkpoint inhibitors

| Type of resistance | Mechanism of resistance | Molecular mechanism |
|---|---|---|
| Impaired immunorecognition | 1) Defects in tumor antigen presentation pathway | Impaired MHC class I complex (HLA and 𝛃-2-microglobulin) |
| | | MHC-I folding |
| | | Antigen processing and loading (MHC machinery) |
| | 2) Loss of neoantigen | Immunoselection pressure |
| | 3) Insufficient diversity and density of T cells | Inability of T cells to infiltrate and to diversify |
| | | Dysfunction of dendritic cells (homing and priming) |
| Insensitivity to immune effector molecules | 1) Loss of interferon-γ signaling | Inactivating mutations in JAK1 and JAK2 |
| | 2) Defects in interferon-γ pathway | Defects in interferon-γ receptor [IFNGR]1, interferon regulatory factor 1, JAK2, and IFNGR2 |
| | | STAT1-related epigenomic aberrations |
| Tumor microenvironment | 1) T cell exhaustion | Epigenenomic modifications |
| | 2) Immunosuppressive cells | Recruitment of myeloid-derived suppressor cells and tumor-associated macrophages |
| Immune metabolism | 1)Derangements of T cell immunometabolism | Serum LDH |
| | | Anaerobic glycolysis |
| | | Density and distribution of |
| | | Glucose uptake channels |
| | | Lactate export channels |
| | | Amino acid uptake channels |
| Angiogenesis | 2) Vascular and cytokine barriers to immune cells | VEGF signaling |
| | 3) apoptosis of CD8 T cells mediated by vascular mechanisms | FasL overexpression on vascular endothelial cells |
| Tumor plasticity | 1) Phenotypic heterogeneity<br>2) Epithelial to mesenchymal transition<br>3) "Inflammatory" state in TME | Genes involved in epithelial mesenchymal transition: AXL, TWIST2, WNT5A, LOXL2, ROR2, TAGLN, and FAP |
| | | Dedifferentiation and reprogramming induced by TNF-alpha |
| Enteric microbiome | 1) Microbiome diversity | Diminished titers of *Faecalibacterium prausnitzii* and Clostridiales |
| | | Diminished titers of *Bifidobacterium* spp. (including *Bifidobacterium breve* and *Bifidobacterium longum*) |
| Upregulation of alternative immune checkpoints | 1) Inhibitory checkpoints | PD-1, LAG3, TIM-3, CD160, and V domain immunoglobulin suppressor of T cell activation |

*PD-1* programmed death 1, *TCR* T cell receptor, *MHC* major histocompatibility complex, *JAK* Janus-activated kinase, *VEGF* vascular endothelial growth factor, *STAT* signal transducer and activator of transcription, *LDH* lactate dehydrogenase, *FasL* FAS ligand, *LAG3* lymphocyte activation gene 3,*TIM3* T cell immunoglobulin Mucin 3, *HLA* human leukocyte antigens

### Combination immunotherapy with dual immune checkpoint blockade, e.g., anti-CTLA-4 [20], anti-TIM-3 [27], anti-LAG3 [28], anti-TIGIT [29], anti-VISTA [30, 31], or T cell receptor agonists

Combination of anti-PD-1 and anti-CTLA-4 is the only combination tested in a prospective randomized phase III trial and approved in the clinic [20]. It is particularly effective in subpopulation of patients with PD-L1-negative melanoma, representing a method for breaking Treg-mediated anti-PD-1 resistance with an objective response rate of approximately 60% in patients with advanced melanoma and with a statistically significant improvement in terms of ORR over anti-CTLA4 monotherapy (19%) and numerically higher than anti-PD-1 monotherapy (44%). In the context of the CA209-067 clinical trial, the median OS in the nivolumab plus ipilimumab group was not reached, being 37.6 months in the nivolumab cohort and 19.9 months in the ipilimumab group, and the difference between combination therapy and nivolumab monotherapy was more evident in PD-L1-negative and BRAF-positive subgroups [20]. Anti-CTLA-4 therapy may lead to highly synergistic effect when combined with anti-PD-1 due to several mechanisms: anti-CTLA-4 antibodies enhance the efficacy of T cell-mediated antitumor-specific immunity and depletion of tumor-associated Tregs by an FccR-dependent mechanism [27] breaking down the peripheral immunotolerance. Furthermore, anti-CTLA-4 antibodies activate T cells via non-redundant and distinct pathway inhibiting CD-28 costimulatory signal, which is required of activation and function of T cell [32, 33].

Nevertheless, persistence of antigen together with continuous checkpoint engagement leads to the exhausted phenotype of T cell and secondary resistance to anti-PD-1 blockade. In this context, anti-LAG3 in combination with anti-PD-1 has demonstrated synergistic activity in a variety of preclinical models [34] and LAG-3 inhibition could reverse primary or acquired resistance. The combination of anti-LAG3 antibody relatlimab (BMS-986016) with nivolumab in melanoma patients who progressed on prior anti-PD-1/PD-L1 therapy showed disease control rate in 45% of patients with LAG3 expression ≥ 1% [28].

The efficacy of anti-LAG3 with anti-PD-1 is supported by solid preclinical data [26, 29, 33–35], and it is currently being investigated in a clinical trial for a number of solid tumors (ClinicalTrial.gov NCT01968109); furthermore, in untreated advanced melanoma, a phase III trial comparing anti-LAG3 with anti-PD-1 versus anti-PD-1 alone is currently planned in melanoma.

There is evidence that tumor-associated Tregs could be selectively depleted by using the combination of anti-PD-1 with costimulatory agonists anti-GITR [36] and anti-OX-40 [37]; in addition, anti-OX40 (CD134) monoclonal antibodies resulted in reduction of tumors growth in some studies [38–40]. Several trials evaluating anti-OX40 (CD134) monoclonal antibodies are currently ongoing (ClinicalTrials.gov; NCT02205333 and NCT01303705), and the results are eagerly awaited. Combination of the agonist antibody targeting OX40–MOXR0916 and atezolizumab in a phase I trial in patients with advanced solid tumors was well tolerated, and at least some objective responses were demonstrated [39]. Other anti-OX40 antibodies as PF-04518600 [40] and MEDI6383 have also entered early clinical development [41].

Another important target is the glucocorticoid-induced tumor necrosis factor receptor-related gene (GITR).

GITR is a costimulatory activating receptor that is upregulated on T cell activation. In the tumor microenvironment, Tregs express GITR at higher levels than T cell effectors. In a phase I/II clinical trial, BMS-986156–GITR agonist monoclonal antibody, with or without nivolumab, was well tolerated, with some clinical activity observed in patients treated with the combination [42].

The other agonistic antibodies targeting the costimulatory activating receptors CD40 or CD137 also demonstrated improvement of the effector functions of dendritic cells and in combination with anti-PD-1 drugs showed synergistic effects in models of melanoma, breast, and colon cancers [43]. Stimulation of CD137 (41BB) through its ligand 41BBL leads to coactivation and enhanced survival of T cells. Urelumab (BMS-663513), antibody targeting 41BB, showed acceptable tolerability at lower doses with preserved pharmacodynamic activity [44]. Utomilumab (PF-05082566)—anti-41BB—was evaluated in a phase Ib study in combination with pembrolizumab demonstrating manageable toxicity and early evidence of clinical activity [45].

### Acting on regulatory T cells/immunometabolism [46, 47] by inhibitors of IDO

IDO is the rate limiting enzyme that catalyzes the conversion of tryptophan to kynurenine in the de novo synthesis of nicotinamide adenine dinucleotide (NAD). This metabolic pathway creates an immunosuppressive milieu in tumors and in tumor-draining lymph nodes by inducing T cell anergy and apoptosis through depletion of tryptophan and accumulation of immunosuppressive tryptophan catabolites [46]. There is strong evidence that suppression of antitumor immune responses in precancerous lesions and established cancers by tryptophan catabolism promotes tumor growth, making such catabolism an attractive target for therapeutic interventions. Furthermore, there is evidence that upregulated expression of IDO-1 and PD-L1, as well as recruitment of Tregs, in the tumor microenvironment depends on the presence of $CD8^+$ T cells. As a corollary of this, an active immune response, including infiltration with $CD8^+$ T cells, can be found in a subset of patients, although these tumors are nonetheless not immunologically rejected.

Epacadostat, a selective oral IDO inhibitor, was well tolerated in monotherapy in phase I study; however, no objective response was generated [47]. Epacadostat was evaluated in combination with pembrolizumab in recently closed phase III clinical trial MASTERKEY-265 in first-line therapy of unselected advanced melanoma, and although it seemed very promising strategy after phase II trial results, a recent report showed no signs of improvement of progression-free survival in pembrolizumab + epacadostat combined therapy as compared to pembrolizumab monotherapy. In the context of melanoma, the development of BMS-986205 a selective IDO1 inhibitor, in combination with nivolumab, has been interrupted. Based on these results, it is unlikely that further studies with IDO inhibition will be planned.

## Converting "cold into hot melanomas" by combining immunotherapies

The development of antigen-specific effector T cells and sensitivity to anti-PD-1 therapy may be limited by poor immunogenicity of some tumors, so strategies aiming to liberate tumor antigen and attracting antigen-presenting cells and immunocompetent cells are likely to enhance sensitivity to anti-PD-1 therapy. One of such approaches includes oncolytic viruses [48].

Talimogene laherparepvec (T-VEC) is a herpes simplex virus type 1-derived oncolytic therapy designed for selective intratumoral replication and production of granulocyte macrophage colony-stimulating factor (GM-CSF), thus promoting lytic tumor death and migration, antigen presentation, and maturation of dendritic cells in tumor microenvironment [48, 49]. It has demonstrated significant activity in injectable metastases, and it is currently approved in therapy of stage IIIB/C-IVA melanomas [49]. The combination of T-VEC plus ipilimumab versus ipilimumab alone has been evaluated in a phase II randomized study [50]. Thirty-eight patients (39%) in the combination arm and 18 patients (18%) in the ipilimumab arm had an objective response. Responses were not limited to injected lesions; visceral lesion decreases were observed in 52% of patients in the combination arm and 23% of patients in the ipilimumab arm. Overall, the objective response rate was significantly higher with TVEC plus ipilimumab versus ipilimumab alone without additional safety concerns versus ipilimumab.

In vivo T-VEC demonstrated the utility for prevention of resistance to anti-PD-1 treatment, and it is currently evaluated in phase III trial in combination with pembrolizumab in advanced, unresectable melanomas. Another strategy that primes adaptive immune responses may be utilization of tumor-specific antigen autologous cancer vaccination or neoantigen vaccines to improve sensitivity to anti-PD-1 therapy [51].

The combination of CMP-001, an intratumoral toll-like receptor 9 (TLR9) agonist, and pembrolizumab showed clinical activity in reversing PD-1 checkpoint inhibition resistance in metastatic melanoma patients according to early results of phase Ib study. TLR9 pathway is one of the strongest inducer of the interferon gene expression signature, which in turn, correlates with response to anti-PD-1 therapy [52].

Another approach is the killer inhibitory receptor in natural killer (NK) cells; in this regard, one of the most promising drug is lirilumab (BMS-986015) [53].

Furthermore, in tumor microenvironment, the high concentration of extracellular adenosine is commonly observed, partly influenced by a hypoxic environment, which leads to suppression of T cell and NK cell activity [54]. In preclinical models, inhibition of A2A receptors in combination with anti-PD-1 antibodies demonstrated synergistic effect and A2A receptor inhibitors are already used in the clinic [55].

A further strategy is to target CD122 (Interleukin-2 receptor beta subunit; IL-2Rβγ) by its agonist NKTR-214, which elicits CD8+ effector T cells and NK cell populations. In preclinical studies, administration of NKTR-214 resulted in a rapid expansion of tumor-infiltrating lymphocytes (TILs) into the tumor microenvironment with a mean ratio of 450:1 within the tumor microenvironment of CD8-positive effector T cells compared with CD4-positive Tregs [56]. The dose escalation phase 1/2 PIVOT clinical trial (NCT02983045) incorporated a Fleming 2-Stage design, with efficacy targets separately predefined for each tumor type using a historical objective response rate (for single-agent checkpoint inhibitor) [57]. In the melanoma cohort phase 1 study, prespecified efficacy criteria were met for ORR with 11/13 85% of patients achieving either a partial response or complete response (CR). Median time on study for 28 patients in phase II was 4.6 months. Responses were observed in 14/28 (50%) patients. Among the 25 patients with known PD-L1 status, ORR in PD-L1-negative patients was 5/12 (42%) and in PD-L1-positive patients was 8/13 (62%). One patient with unknown PD-L1 baseline status experienced a CR. The phase II study suggests a decrease in response rate over time; hence, translational studies investigating the biological effect of this combination on $CD8^+$ T cell clonal expansion and memory cells against melanoma antigens will be needed to better understand the synergistic effect.

Tumor microenvironment may be modified by radiotherapy, which may lead to upregulation of tumor-associated antigens and MHC class I and synergistic effects in combination with immunotherapy [58, 59]. Radiotherapy combined with anti-CTLA-4 therapy increases PD-L1 [60]. In mice, PD-L1 expression is increased when radiotherapy is combined with anti-TGFb (tumor growth factor beta) or stimulator of interferon gene (STING) agonists. In small phase II trial, 50% of stage IV melanoma patients experienced benefit by combining radiotherapy and ipilimumab [61].

### Epigenetic modulation and tumor immunogenicity

Epigenetic changes constitute an alternative strategy for reestablishment of tumor immunogenicity; these agents include histone deacetylases (HDAC) and DNA methyltransferases (DNMT) inhibitors [62]. It has been shown that these agents have broad immunomodulatory properties, acting on expression of oncogenes and tumor suppressor genes [63, 64] and immune-related genes as IFN-response genes or PD-L1/L2 expression [65]. Moreover, promoting demethylation may act on reversal of T cell exhaustion [63, 64].

### Myeloid suppressor cells as target of TME immunomodulation

Acting on myeloid suppressor cells, e.g., CSF1R inhibitor/antibodies may interfere with tumor microenvironment especially with high content in tumor-associated macrophages (TAM) improving response to checkpoint inhibitors [66].

Considering the above data, we need to investigate further combined therapies for three reasons: (i) the toxicity profile with the standard nivolumab and ipilimumab combination is challenging for routine use; (ii) there is an intrinsic complexity of the tumour microenvironment (TME) that requires further combinations; and (iii) a TME-based immunotherapy should be tested in prospective clinical trials.

## Rationale to combine immunotherapy with targeted therapy in metastatic melanoma

Although new targeted and immune therapies have improved response and/ or survival in advanced melanoma patients ][6, 7], data from randomized clinical trials show that the majority of advanced melanoma patients receiving targeted therapies develop resistance. Among patients receiving BRAFi and MEKi or BRAFi alone, respectively, approximately 70–80% and 95% progress at 3 years [6, 7].

Several genomic mechanisms of acquired resistance to MAPKi therapies have been reported including *V600EBRAF* amplification and single nucleotide variants (SNVs) in *NRAS*, *KRAS*, *MEK1/2*, *PTEN*, *CDKN2A*, and *DUSP4*. Nevertheless, non-genomic mechanisms of resistance have been described and among them immunological mechanisms play an important role. This is translationally relevant because in the current therapeutic landscape, salvage therapies for patients with disease progression on targeted therapies involve immunotherapies, including anti-CTLA-4 and anti-PD-1 antibodies or their combination.

There is now enough evidence that TME plays a role in the developing of resistance upon treatment with BRAFi or BRAFi and MEKi.

It is well known that BRAFi and MEKi enhance in vitro and in vivo melanoma derived antigens (MDAs) expression and promote the immune response against tumor cells [67]. Tumor infiltration by CD4+ and CD8+ lymphocytes increases following BRAF inhibitor treatment, and the increased intratumoral CD8+ lymphocyte expression correlates with a reduction in tumor size and an increase in necrosis in post-treatment matched biopsies. Nevertheless, within 2 weeks upon starting BRAFi, an increased expression of PD-1 and PD-L1 occurs, a finding that suggests a potential immune-mediated resistance mechanism to BRAF inhibition in the early phase upon starting treatment [67].

Recently, Hugo et al. reported that LEF1 down-expression and ß-catenin modulation cause acquired resistance to BRAFi and MEKi [68]. Tumor-intrinsic ß-catenin pathway activation is mechanistically involved in T cells as well as CD103 dendritic cell exclusion via inhibition of CCL4 secretion [69]. Accordingly, Massi et al. showed that the presence of CD8+ T cell infiltration as well as the subset of CD8+CD103+ T cells in melanoma samples obtained before starting treatment with MAPKi correlates with the therapeutic response [70]. By multivariate analysis, progression-free survival PFS and OS were longer in patients with high density of CD8+ T cells and β-catenin < 10% than those without CD8+ T cells infiltration and β-catenin ≥ 10%. The prognostic impact of CD8+ T cells was validated in a recent study [71]. These results underline the importance of the preexisting immunological status in determining the response and outcome of MM patients treated with MAPKi.

About 50% of MM patients with acquired MAPKi resistance exhibit a profound CD8 T cell deficiency and/or an exhaustion of the phenotype [68]. There is evidence that CD8+ T cells in MM patients progressing during BRAFi treatment decrease compared to patient-matched baseline expression level. Overall, there is strong evidence of both CD8 T cell depletion and exhaustion upon progression to BRAFi.

In addition to T cell depletion, the stromal fibroblast-derived hepatocyte growth factor (FDHGF) and the tumor necrosis alpha (TNFα) originating from the stroma have been reported to play an important role as mechanisms of resistance [72]. Macrophages have been reported to contribute to MAPKi resistance. These cells release TNFα, which, in turn, increases resistance due to its ability to enhance the expression of the melanoma survival factor MITF [73].

Overall, these studies provide a framework for considering the TME as a target to be exploited in combination strategies between targeted therapies and immunotherapy. However, in clinical practice, the choice of treatment is influenced by clinical determinants including the rapidity of progression of disease, presence or absence of symptoms, medical history, comorbidities, and other factors (e.g., patient preference).

The best sequencing or combination of these two different systemic treatments is currently unknown. Among the several

open questions, there are at least six issues to be addressed: (i) which form of immunotherapy should be used in combination with targeted therapy, (ii) the optimal sequence and timing of targeted therapy and immunotherapy, (iii) which schedule of targeted therapy should be used in combination with immunotherapy, (iv) the toxicity of combination treatment, (v) how to tailor treatment according to patients need, and (vi) novel biomarkers to drive treatment strategies.

Initial efforts to combine a BRAFi, vemurafenib, with ipilimumab were limited by toxicity. Specifically, hepatotoxicity was observed in a substantial proportion of patients, consisting mainly of grade 2 or 3 elevations in liver function tests (LFT and bilirubin level) [74].

A phase I, open-label, multicenter, dose-finding study (NCT01767454) in unresectable or metastatic melanoma patients and *BRAF* V600E/K mutations was undertaken to investigate the safety of dabrafenib and ipilimumab with and without trametinib. The study consisted of two treatment arms: a doublet combination treatment arm of dabrafenib and ipilimumab, and a triple combination treatment arm of dabrafenib, trametinib, and ipilimumab, with the potential for expansion of each treatment arm. In the doublet combination treatment arm, the starting dose of dabrafenib was the recommended monotherapy dose of 150 mg twice daily. Ipilimumab was administered at the approved dose of 3 mg/kg every 3 weeks for a total of four doses in this study.

The study was closed due to DLTs in two subjects in the triplet combination treatment arm, as both subjects developed colitis with colonic perforation that raised the possibility of added toxicity with the triplet combination over ipilimumab as a single agent.

In KEYNOTE-022 (NCT02130466) study, dabrafenib and trametinib are currently being evaluated in combination with pembrolizumab in a phase I/II study in *BRAF* V600 mutant melanoma patients [75]. Based on the phase I results, the recommended regimen for phase II was pembrolizumab 2 mg/kg Q3W plus dabrafenib 150 mg BID with trametinib 2 mg QD. In the double-blind phase II part of KEYNOTE-022, patients were randomized to pembrolizumab 2 mg/kg Q3W + dabrafenib 150 mg BID + trametinib 2 mg QD or placebo + dabrafenib + trametinib [76]. Primary end point was PFS. The triple combination did not improve significantly the response rate, PFS or OS. The triple combination was more toxic than the combo target. Based on these results, the role of this triple combination is not clear in the current landscape of melanoma treatment.

Additionally, the combination of dabrafenib and trametinib with the anti-PD-L1 antibody durvalumab in *BRAF* V600 mutant melanoma was tested (NCT02027961). Two doses of durvalumab were tested. Initially, 6 patients received the labeled dose of dabrafenib and trametinib and durvalumab (3 mg/kg Q2W) and 20 patients received 10 mg/kg of durvalumab Q2W. Both doses were tolerated and were manageable. Combining data from both doses, the ORR was 69.2% (95% CI 48.2–85.7), median DOR was 67.1 weeks, and 55.6% of responders had an ongoing response at the time of the data cutoff. [68].

Finally, Sullivan et al. reported preliminary results of a phase 1 study investigating vemurafenib in combination with atezolizumab, a PD-L1 antibody. Triple-combination therapy with atezolizumab + vemurafenib and cobimetinib (MEK inhibitor) in this patient population is under investigation [77]. Table 2 summarizes ongoing clinical trials exploring targeted therapies in combination with immunotherapy.

## Challenges to combine targeted therapy to immunotherapy

In the last decade, the medical oncology community has witnessed a dramatic paradigm shift in the treatment of metastatic melanoma. One of the most fascinating fields of investigation remains how to integrate immunotherapy with targeted therapies in *BRAF*-mutated melanoma patients. There is indeed now strong evidence supporting the observations that the therapeutic efficacy of BRAFi and MEKi relies on other factors including the immunomodulation of microenvironment. Nevertheless, several unanswered questions still remain.

## Which immunotherapy is the best promising candidate to be combined with targeted therapy?

Initial and ongoing efforts focused on combining BRAF-targeted therapy with FDA and EMA licensed immunomodulating agents (such as anti-PD-1, PD-L1 antibodies, and anti-CTLA4), but subsequent trials should combine drugs targeting new checkpoint inhibitors such as LAG-3 and TIM-3, which offer great promise in melanoma and in other cancer types. This introduces two main potential problems, acute and late toxicities, as well as long-term benefit with these new agents is still unknown, and combination therapies will be challenged by the lack of definitive estimates, which are important for clinical trials' design. Several options could be pursued including that of lower starting doses and up-titrating dose as tolerated, given the potential for additive toxicity.

## Which schedule is most appropriate to combine immunotherapy and targeted therapies?

The combination of targeted therapies with immunotherapy may result in an increased severe toxicity. This can impair

**Table 2** Ongoing clinical trials investigating concomitant or sequencing of targeted therapy and immunotherapy in advanced melanoma patients (from www.clinicaltrials.gov, updated on May 2018)

| Drugs (clinical trial) | Phase | Pathology | Primary end point(s) | Status |
|---|---|---|---|---|
| Targeted therapy + immune checkpoint inhibitors | | | | |
| Dabrafenib ± trametinib ± ipilimumab (NCT01767454) | 1 | Unresectable stage IIIc/IV melanoma | AEs<br>MTD | Completed |
| Durvalumab (anti-PD-L1) and trametinib ± dabrafenib (NCT02027961) | 1 | Unresectable stage IIIc/IV melanoma | AEs<br>MTD | Active not recruiting |
| Atezolizumab + vemurafenib ± cobimetinib (NCT01656642) | 1b | Unresectable stage IIIc/IV melanoma | AEs<br>MTD | Active not recruiting |
| Vemurafenib and cobimetinib ± atezolizumab (NCT02908672) | 3 | Unresectable stage IIIc/IV melanoma | PFS | Active not recruiting |
| Dabrafenib ± trametinib ± pembrolizumab (NCT02130466) | 1/2 | Unresectable stage IIIc/IV melanoma | AEs<br>MTD<br>PFS | Active not recruiting |
| Dabrafenib + trametinib ± pembrolizumab (NCT02130466) | 3 | Unresectable stage IIIc/IV melanoma | PFS | Active not recruiting |
| Anti-PD-1 antibody PDR001, in combination with dabrafenib and trametinib<br>Part 3 (NCT02967692) | 3 | Unresectable stage IIIc/IV melanoma | PFS | Recruiting |
| Anti-PD-1 antibody PDR001, in combination with dabrafenib and trametinib<br>Part 1/2(NCT02967692) | 1/2 | Unresectable stage IIIc/IV melanoma | AEs<br>MTD<br>Biomarkers | Active not recruiting |
| MEDI4736 + trametinib ± dabrafenib (NCT02027961) | 1/2 | Unresectable stage IIIc/IV melanoma | AEs<br>MTD | Active not recruiting |
| Ipilimumab + imatinib mesylate (NCT01738139) | 1 | C-KIT positive metastatic or unresectable GIST, melanoma, other tumor histotypes | MTD | Recruiting |
| Vemurafenib + ipilimumab (NCT01400451) | 1 | Stage IV melanoma with BRAF V600E/K mutation | AEs<br>Hepatic DLT<br>MTD | Closed |
| Vemurafenib + cobimetinib + pembrolizumab (NCT02818023) | 1 | Unresectable stage III/IV melanoma with BRAF V600E/K mutation | DLT<br>ORR* | Recruiting |
| Ipilimumab ± dabrafenib ± trametinib ± nivolumab (NCT01940809) | 1 | Unresectable stage III/IV melanoma with BRAF V600E/K mutation | G3 or higher irAEs | Recruiting |
| Ipilimumab + dabrafenib (NCT02200562) | 1/2 | Unresectable stage III/IV melanoma with BRAF V600E/K mutation | AEs<br>MTD | Stop enrolling, trial withdrawn |
| Dabrafenib + trametinib + pembrolizumab (NCT02625337) | 2 | Stage IV melanoma with BRAF V600E/K mutation | AEs<br>MTD<br>Feasibility | Active not recruiting |
| Imatinib + pembrolizumab (NCT02812693) | 1/2 | Unresectable stage III/ IV melanoma with c-KIT mutation/amplification | BORR | Withdrawn (poor accrual) |
| Targeted therapy + cytokines | | | | |
| Vemurafenib + IL-2 (NCT01754376) | 2 | Stage IIIc/IV melanoma with BRAF V600E mutation | PFS | Terminated (changes in available treatments for melanoma) |
| Vemurafenib + high dose IL-2 (NCT01683188) | 4 | Stage IV melanoma with BRAF V600E/K mutation | CR rate | Terminated (a shift in the melanoma treatment landscape adversely affected accrual) |
| Vemurafenib + IL-2 (infusional 96 h) + INF alfa-2b (NCT01603212) | 1/2 | Unresectable stage III/IV melanoma with BRAF V600E/K mutation | AEs<br>MTD<br>PFS | Completed |
| Vemurafenib + pegylated IFN (NCT01959633) | 1/2 | | AEs | Recruiting |

**Table 2** (continued)

| Drugs (clinical trial) | Phase | Pathology | Primary end point(s) | Status |
|---|---|---|---|---|
| | | Unresectable stage III/IV melanoma with BRAF V600E/K mutation | | |
| Vemurafenib + high-dose INF alfa-2b (NCT01943422) | 1/2 | Unresectable stage III/IV melanoma with BRAF V600E/K mutation | AEs | Completed |
| Targeted therapy + T cell | | | | |
| Vemurafenib + cyclophosphamide and fludarabine + TIL + high dose IL-2 (NCT 01585415) | 1 | Stage IV melanoma with BRAF V600E/K mutation | AEs<br>MTD | Terminated |
| Vemurafenib + ACT with TIL infusion + high-dose IL-2 (NCT01659151) | 2 | Unresectable stage III/IV melanoma with BRAF V600E/K/D mutation | ORR<br>Dropout rate | Active not recruiting |
| Vemurafenib + ACT + TIL infusion (NCT02354690) | 1/2 | Unresectable stage III/ IV melanoma with BRAF V600E/K mutation | AEs<br>MTD | Recruiting |

*DLT* dose-limiting toxicity, *ORR* overall response rate, *AEs* adverse events, *SAEs* serious adverse events, *BOR* best overall response, *OS* overall survival, *PFS* progression-free survival, *ORR* objective response rate, *MTD* maximum tolerated dose, *DR* duration of response, *PK* pharmacokinetic, *CR* complete response, *IL-2* Interleukin-2, *IFN* interferon, *irAEs* immune-related adverse events, *TTP* time to progression, *OR** overall response, *TIL* tumor Infiltrating lymphocytes, *ACT* adoptive cell transfer

the dose intensity and the maintenance of the triple combination for the majority of patients. The use of intermittent schedules seems to be promising; however, larger studies are needed.

## How to combine immunotherapy and what schedule should be used in cold melanomas?

An important area of investigation consists in exploring new strategies in melanoma lacking TILs, PD-L1 expression, and interferon signature (immune ignorance). This group represents a not negligible fraction of melanoma patients (~41%), with poor prognosis based on their lack of detectable immune reaction. In this group of patients, single-agent checkpoint blockade would most likely not to be successful given the lack of preexisting T cell infiltrates. In this adverse clinical scenario, BRAFi and MEKi in association with stimulator of interferon gene (STING) protein agonists, basic leucine zipper transcriptional factor ATF-like 3 (*BATF3*) modulators, or epigenetic drugs could be a promising strategy to modulate this hostile immune microenvironment.

## Rationale of combination therapy in adjuvant setting

At early stages of disease, surgical excision of melanoma is the treatment of choice and this strategy is initially curative for the majority of patients. However, only approximately 40–60% of AJCC stage III melanoma patients who undergo surgical excision are disease-free after 5 years of follow-up [78]. These patients can experience locoregional or distant recurrence. The primary aim of adjuvant therapies is to reduce the recurrence rate on radically operated patients and potentially to improve survival.

Since new treatments have appeared in the landscape of metastatic melanoma, new potential scenarios in the adjuvant setting have been opened. In particular, recent results with immune checkpoint inhibitors and target therapies have been recently published showing their potential benefit. In the adjuvant setting, for patients with resected high-risk melanoma, three prospective randomized clinical trials evaluating the efficacy of immune checkpoint inhibitors have been recently reported [16–18]. Ipilimumab at the dose of 10 mg/kg was the first drug approved by FDA in the adjuvant setting based on the results of the E18041 clinical trial that showed an absolute improvement of 11% in terms of PFS and OS in radically resected, stage III cutaneous melanoma patients [16]. In 2017, an anti-PD-1 antibody, nivolumab, independently demonstrated better efficacy and tolerability than Ipilimumab in resected stage IIIB/C-IV melanoma patients and has been recently approved by FDA [17]. Finally, pembrolizumab showed similar benefit in resected stage IIIA/B/C melanoma patients [18], with a 43% relative risk reduction of recurrence compared to placebo. Updated results of "Combi AD" study have been recently reported as well [79]. With a median follow-up of 44 months, PFS was not reached in combo target arm versus 16.6 months in the placebo arm. Estimated cure rate was 54% in the combo target arm versus 37% in the placebo arm.

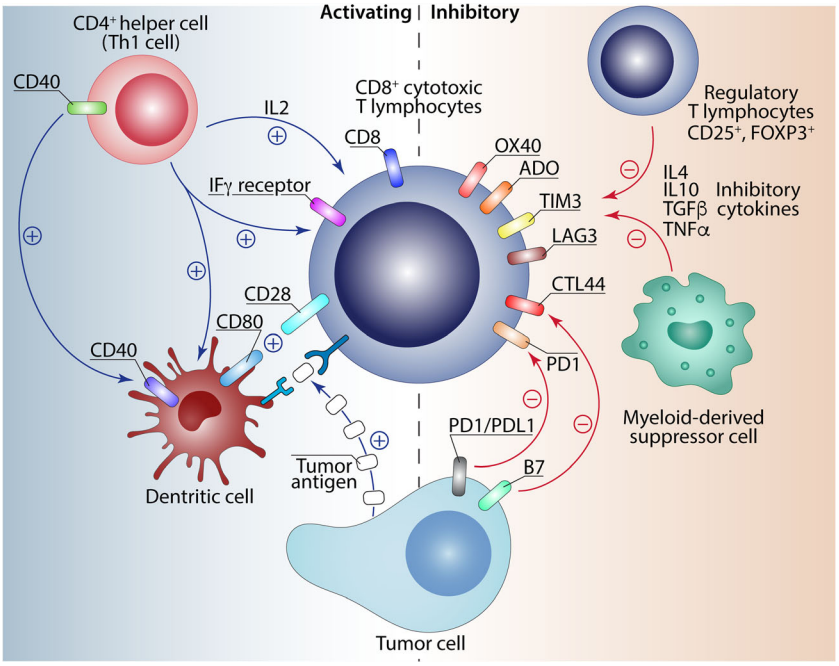



**Fig. 2** The complex interplay between CD8+ T cytotoxic lymphocytes and the tumor microenvironment (modified from Tristan A Barnes and Eitan Amir BJC 2017 "HYPE or HOPE: the prognostic value of infiltrating immune cells in cancer")

Nevertheless, several questions remain open in the field. First, the role of combination therapy in the adjuvant setting is complicated by changing surgical management based on the recent publication of the MSLT-2 [80] and DeCOG results [81]. All the above reported adjuvant clinical trials included patients after complete lymphadenectomy (CLND), which is not more a standard in patients with positive sentinel lymph node. In our opinion, this paradigm shift in surgical management will not change the recommendation to implement these new strategies in the adjuvant setting for four reasons: (1) the regulatory approval by FDA and EMA does not require lymphadenectomy; (2) adjuvant therapy are provided in order to eradicate the microscopic disease that is present in 18–20% of patients with positive sentinel lymph node; hence, these new strategies should be able to eradicate microscopic disease in the majority of patients; (3) recent data showed that CLND leads to AJCC upstaging in the only 5–6% of patients; and (4) in the CA209-915 trial, which randomized patients to receive nivolumab or nivolumab plus ipilimumab, patients without lymphadenectomy were allowed to enter the clinical trial. Another important issue is patient selection for adjuvant therapy, since some patients could experience severe long-term toxicities. Biomarkers so far evaluated in the metastatic disease, including PD-L1, tumor mutation burden, metabolic signatures, T cell infiltration, and gene expression signatures, can be considered only correlative and not predictive biomarkers, hence cannot help to select patients who receive benefit from adjuvant therapy.

From a clinical point of view, there is evidence that patients with non-ulcerated primary melanoma and small microscopic metastasis in single sentinel lymph node (< 1 mm in maximal diameter; stage IIIA disease) can achieve a long-term survival in more than 80% of cases. These patients were not included in modern clinical trial and should not receive adjuvant therapy. Furthermore, it is important to mention that although stage III patients are those at greatest risk of recurrence, most patients are diagnosed in stage I and II. In particular, stage II

**Table 3** The spectrum of biological biomarkers for response and/or resistance to immune checkpoint inhibition

| Biomarkers | Source of detection | Effect on response |
|---|---|---|
| CD8+ T cells | Tumor | Positive |
| PD-L1 | Tumor | Positive |
| TCR clonality | Tumor | Positive |
| Tumor mutation burden | Tumor | Positive |
| Neoantigen heterogeneity | Tumor | Negative |
| MHC I/II expression | Tumor | Positive |
| JAK 1–2 or ⍦-2-microglobulin | Tumor | Negative |
| *NRAS* mutations | Tumor | Positive |
| *PTEN* loss | Tumor | Negative |
| Interferon signal | Tumor | Positive |
| Serum LDH | Serum | Negative |
| Serum PD-L1 | Serum | Positive |
| Circulating tumor DNA | Serum | Negative |
| MDSCs | Blood-PBMC | Negative |
| Microbiome | Stool | Positive/negative |

*PD-L1* programmed death-ligand 1, *TCR* T cell receptor, *MHC* major histocompatibility complex, *JAK* Janus-activated kinase, *NRAS* neuroblastoma ras viral oncogene homolog, *PTEN* phosphatase and tensin homolog, *MDSCs* myeloid-derived suppressor cell, *LDH* lactate dehydrogenase

patients are those who in absolute terms fall into stage IV. Therefore, to reduce melanoma mortality, it is important to impact on this group of patients. An ongoing prospective phase III study is evaluating the efficacy of pembrolizumab to placebo in stages IIB and IIC (KEYNOTE MK3475-076). Nevertheless, selection of patients is important and biological driven strategies to include only AJCC stage II patients at high risk of recurrence should be pursued to minimize patient exposed to adjuvant therapy and to focus on very high-risk patients who deserve an adjuvant therapy.

## Conclusion

The recent advances in cancer immunotherapy with unprecedented success in therapy of advanced melanoma, including perioperative approaches, have necessitated deeper insight in mechanism of antitumoral activity of immunotherapy and resistance to checkpoint blockade. It must constitute the rationale for design of new immunotherapy-based combinations. Activation of T cells and their cytotoxic functionality are complex biologic processes regulated by several mechanisms with many immunoregulatory molecules; some of them can be upregulated on lymphocytes and tumor cells. Several studies have focused on the interaction between cytotoxic T lymphocytes and tumor cells. However, it is now clear that in the tumor microenvironment, other cells contribute to the complex immune response in cancer, some of which promote tumor surveillance and control while others facilitate tumor growth (Fig. 2). Moreover, immune contexture, including the location, density, and type of immunocompetent cells within the tumor microenvironment, has relevance to the sensitivity of patients to therapy with checkpoint inhibitors (Fig. 1); thus, in addition to targeting immune cells directly through stimulatory and checkpoint pathways, several modalities are developed with the aim of altering the tumor microenvironment to create more favorable conditions for different combinations. Given the complexity of activation of immunological system and the physiologic multifactorial homeostatic mechanisms controlling immune responses, combinatorial strategies are eagerly needed in melanoma therapy (Fig. 1) [1, 2]. Nevertheless, rational selection of immunotherapy combinations should be more biomarker-guided (Table 3), including not only immunogram factors, as PD-L1 expression, interferon gene expression signature, mutational burden, and lymphocyte tumor infiltration by CD8+ T cells [82] but also intratumoral T cell exhaustion with higher expression of LAG3 or TMI3, tumor burden (probably related to the proportional immune competition), and microbiota composition [83].

**Authors' contributions** Dr. Mandalà planned the workflow of the manuscript. Dr. Mandala and Prof. Rutkowski wrote the manuscript and approved the final version.

## Compliance with ethical standards

**Conflict of interest** Dr. Mandala received honoraria for invited speeches, consulting, and advisory board from Novartis, Roche, BMS, MSD, Pierre Fabre, and Incyte.

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