# Chapter 14
# Perspectives of Immunotherapy in Advanced Melanoma: Combinations and Sequencing

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## Immunotherapy: The Fourth Pillar of Cancer Treatment

Therapeutic intervention with monoclonal antibodies (mAb) that target immune checkpoint(s) inhibitors (ICI) is a novel and rapidly evolving anticancer strategy that is providing meaningful clinical efficacy in a proportion of cancer patients with different tumor histotypes [1]. The prototype approach of this therapeutic modality relies on the inhibition of negative signals delivered by cytotoxic T lymphocyte-associated protein (CTLA)-4 expressed on activated T lymphocytes. Ipilimumab, the first anti-CTLA-4 mAb approved by regulatory agencies, has profoundly changed the therapeutic landscape of patients with cutaneous metastatic melanoma (MM), significantly improving their survival. However, objective clinical responses with ipilimumab are limited, and only ~20% of patients achieve long-term disease control [2]. Since these initial results, an improved understanding of the molecular mechanisms regulating

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host's immune response to tumor has led to the expansion of the repertoire of checkpoint signaling pathways; among these, one of the most crucial is the programmed cell death-1 (PD-1) pathway.

Immunomodulatory mAb against PD-1, like nivolumab and pembrolizumab, have significantly increased the survival of MM, with ~40% of subjects achieving a long-term survival [3]. However, despite these unprecedented results, a significant proportion of MM patients fail to respond to ICI therapy either upfront (primary resistance) or after an initial benefit (acquired/secondary resistance) [1]. Therefore, identifying new mechanism(s) underlying treatment failure and designing novel therapeutic combinations and/or sequences to overcome primary and acquired resistance are mandatory to improve the overall efficacy of ICI therapy.

## Resistance to ICI Therapy and Rationale for PD-1-Based Combinations

First-line therapy with anti-PD-1 mAb nivolumab and pembrolizumab has significantly improved the survival of MM patients [3]. Unfortunately, 40–65% of MM patients treated with anti-PD-1 mAb develop a primary or acquired resistance to PD-1 therapy. The mechanisms leading to resistance to PD-1 inhibition can occur at any phase of the cancer immunity cycle, are multifactorial, and can be overlapping in an individual patient. Among others they can include (1) alterations in the antigen-processing pathway; (2) lack of tumor antigen expression; (3) loss of Human Leukocyte Antigen (HLA) expression; and (4) constitutive expression by tumor cells of the ligands for Immune Checkpoints (IC) [e.g., PD-1 ligand (PD-L1)]. Besides these mechanisms, neoplastic cells can utilize immune-evasive strategies to prevent T-cell trafficking and infiltration into tumors, including overexpression of vascular endothelial growth factor (VEGF) that downregulates T-cell adhesion to the endothelium, and upregulation of endothelin B receptor, controlling T-cell trafficking through the tumor and lymph nodes. Additionally, the expression of a specific subset of genes, called the innate anti-PD-1 resistance signature or IPRES, has been identified as a mechanism of primary resistance. IPRES is associated with the transition of melanoma cells to a mesenchymal subtype, a reversion back to a more stem cell-like phenotype [4]. Upregulation of these genes may be produced by inflammation in the tumor microenvironment (TME), driving increased tumor plasticity, and angiogenesis. Other factors driving resistance to PD-1 therapy are tumor cell extrinsic and involve the TME [4]. Indeed, the migration of immunosuppressive cells into the TME can inhibit local immune cells from exerting their effector functions. Furthermore, increased numbers of regulatory T cells (Treg) and of myeloid-derived suppressor cells (MDSC), mediated by indoleamine 2,3-dioxygenase (IDO) that is expressed in a wide range of human cancers, have all been linked to primary resistance to immunotherapy. The expression of IC (including PD-1 and CTLA-4) at the surface of these immune suppressive cells provides them with the ability to inhibit local T-cell activation directly. Additionally, immunosuppressive mediators

produced by Treg and MDSC, including Interleukin (IL)-10 and Transforming Growth Factor (TGF)-β, can enhance the establishment of a local network of immunosuppressive cells in the TME. For instance, TGF-β can induce differentiation of neutrophils into a pro-tumor, "N2-like" phenotype, thereby limiting the anticancer activity of N1-like neutrophils. Similarly, IL-10 and TGF-β can polarize monocytes to protumor M2-like tumor-associated macrophages (TAM), which, among their immune-suppressive actions, can also fight with local dendritic cells (DCs) for tumor antigens and consequently inhibit T-cell priming [4].

Most of the factors responsible of primary resistance drive also the occurrence of acquired immune escape. In this regard, truncating mutations in JAK 1 and 2 were recently shown to result in a lack of responsiveness to Interferon (IFN)-γ in tumor cells and consequently in a secondary resistance to ICI [4]. Alterations of JAK1 and JAK2 were also found to correlate with tumor relapse, providing initial evidence that acquired resistance to ICI therapy may involve substantial alteration and evolution of cancer cells and immune cells in the TME [4]. Furthermore, the loss of beta-2-microglobulin (B2M) expression observed in melanoma cell lines from patients treated with immunotherapy, resulted in a loss of Major Histocompatibility Complex (MHC) class I expression, and thus in a subsequent decrease in recognition by CD8+ T cells [4]. Notably, other immune IC pathways, such as lymphocyte activation gene 3 (LAG-3) and T-cell immunoglobulin and mucin domain 3 (TIM-3), have also been revealed to interfere with the effector activity of T cells, resulting in acquired resistance to immunotherapy (Table 14.1) [4].

**Table 14.1** Mechanisms of resistance to ICI therapy

| Phase of immunity cancer cycle | Mechanisms of resistance | Contributing factors |
|---|---|---|
| Antigen presentation and T-cell activation | Insufficient antigen presentation and recognition | Low tumor mutational burden<br>Lack of neoantigen recognition<br>Loss of B2M<br>Loss of MHC class I<br>Loss of function of transporters associated with antigen-processing (TAP) proteins |
| T-cell trafficking and tumor infiltration | Absence of T cells from TME | VEGF overexpression<br>Upregulation of endothelin B receptor |
| T-cell killing activity within TME | Presence of immunosuppressive molecules within the TME | Expression of IPRES<br>Induction of IDO<br>Upregulation of PD-L1<br>Upregulation of Tregs<br>Upregulation of MDSCs<br>Upregulation of immune-checkpoint markers (LAG-3, TIM-3) |

*TME* tumor microenvironment, *B2M* beta-2-microglobulin, *MHC* major histocompatibility complex, *VEGF* vascular endothelial growth factor, *IDO* indoleamine 2,3-dioxygenase, *IPRES* innate anti-PD-1 resistance signature, *Tregs*, regulatory T cells, *MDSCs* myeloid-derived suppressor cells, *LAG-3* lymphocyte activation gene 3, *TIM-3* T-cell immunoglobulin and mucin domain 3
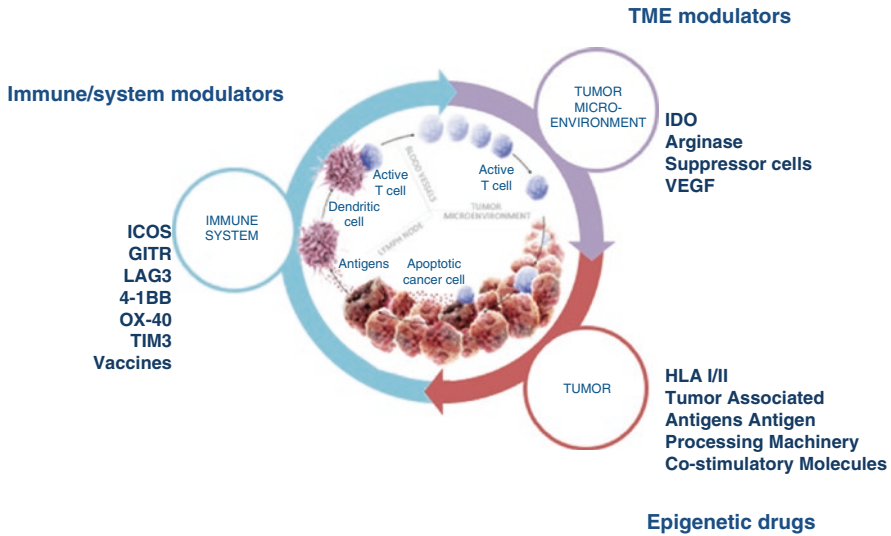
**Fig. 14.1** The future of immunotherapy: targeting and modulating multiple compartments. The initiation of a successful antitumor immune response requires (1) effective antigen presentation and T-cell activation, (2) T-cell trafficking and tumor infiltration, and (3) T-cell killing activity within the tumor microenvironment. The mechanisms triggering both primary and acquired resistance to PD-1 inhibition can happen at any phase of cancer immunity cycle. Potential therapeutic strategies targeting immune system, tumor, and TME can be utilized at each stage of the cancer immune cycle to overcome immunotherapy resistance. *ICOS* inducible T-cell co-stimulatory, *GITR* glucocorticoid-induced TNFR family-related gene, *LAG-3* lymphocyte activation gene 3, *TIM-3* T-cell immunoglobulin and mucin domain 3, *IDO* indoleamine 2,3-dioxygenase, *VEGF* vascular endothelial growth factor, *HLA* human leukocyte antigen

All these recent insights into the mechanisms of ICI resistance support the investigation into novel combination strategies, using multiple treatment modalities such as new IC agonist/antagonists, TME modulators, targeted agents, and epigenetic drugs (Fig. 14.1; Table 14.2).

## *Combinations or Sequencing with Anti-CTLA-4 mAbs*

The rational to combine an anti-CTLA-4 and an anti-PD-1/PD-L1 mAb stems from their non-redundant functional activity, acting at different sites and at different stages of T-cell activation: CTLA-4 on naïve T cells typically in the lymph nodes; PD-1 on antigen-experienced T cells, primarily in peripheral tissues [5]. From preclinical experiences to early-phase studies, combination therapy has shown to be more effective than monotherapy in terms of melanoma control by increasing T-cell infiltration and the presence of effector T cells in the TME; also INF-γ and other pro-inflammatory cytokines were upregulated in the course of combination therapy,

**Table 14.2** Selected immunotherapy combination trials in melanoma[a]

| Trial number | Trial name | Status |
|---|---|---|
| Dual monoclonal antibody therapies | | |
| NCT02599402 (CheckMate 401) | Nivolumab Combined with Ipilimumab Followed by Nivolumab Monotherapy as First-Line Treatment for Patients with Advanced Melanoma | Active, not recruiting |
| NCT03470922 | A Study of Relatlimab Plus Nivolumab Versus Nivolumab Alone in Participants with Advanced Melanoma | Recruiting |
| Anti-PD-1 in combination with oncolytic viral therapy | | |
| NCT04068181 (Masterkey-115) | Talimogene Laherparepvec with Pembrolizumab in Melanoma Following Progression on Prior Anti-PD-1 Based Therapy | Recruiting |
| Anti-PD-1/PD-L1 in combination with BRAF and MEK inhibitors | | |
| NCT02224781 | Dabrafenib and Trametinib Followed by Ipilimumab and Nivolumab or Ipilimumab and Nivolumab Followed by Dabrafenib and Trametinib in Treating Patients with Stage III–IV BRAFV600 Melanoma | Recruiting |
| NCT03625141 (TRICOTEL) | A Study Evaluating the Safety and Efficacy of Cobimetinib Plus Atezolizumab in BRAFV600 Wild-Type Melanoma with Central Nervous System Metastases and Cobimetinib Plus Atezolizumab and Vemurafenib in BRAFV600 Mutation-Positive Melanoma with Central Nervous System Metastases | Recruiting |
| Anti-PD-1 in combination with co-stimulatory molecules and cytokines | | |
| NCT02253992 | An Investigational Immuno-therapy Study to Determine the Safety of Urelumab Given in Combination with Nivolumab in Solid Tumors and B-Cell Non-Hodgkin's Lymphoma | Completed |
| NCT02528357 (ENGAGE-1) | GSK3174998 Alone or with Pembrolizumab in Subjects with Advanced Solid Tumors | Completed |
| NCT02554812 (JAVELIN Medley) | A Study of Avelumab In Combination with Other Cancer Immunotherapies in Advanced Malignancies | Recruiting |
| NCT02723955 (INDUCE-1) | Dose Escalation and Expansion Study of GSK3359609 in Participants with Selected Advanced Solid Tumors | Recruiting |
| NCT02983045 (PIVOT-02) | A Dose Escalation and Cohort Expansion Study of NKTR-214 in Combination with Nivolumab and Other Anti-Cancer Therapies in Patients with Select Advanced Solid Tumors | Active, not recruiting |
| NCT03635983 | A Study of NKTR-214 Combined with Nivolumab vs Nivolumab Alone in Participants with Previously Untreated Inoperable or Metastatic Melanoma | Recruiting |
| Immune-checkpoint inhibitors and TME modulators | | |
| NCT03589651 | INCMGA00012 in Combination with Other Therapies in Patients with Advanced Solid Tumors | Recruiting |
| NCT03459222 | An Investigational Study of Immunotherapy Combinations in Participants with Solid Cancers That Are Advanced or Have Spread | Recruiting |

**Table 14.2** (continued)

| Trial number | Trial name | Status |
|---|---|---|
| NCT02903914 | Arginase Inhibitor INCB001158 as a Single Agent and in Combination with Immune Checkpoint Therapy in Patients with Advanced/Metastatic Solid Tumors | Recruiting |
| Epigenetic-based combinations | | |
| NCT04250246 (NIBIT-ML1) | A Study of NIVO Plus IPI and Guadecitabine or NIVO Plus IPI in Melanoma and NSCLC Resistant to Anti-PD1/PDL1 | Not yet recruiting |
| NCT02437136 (ENCORE-601) | Ph1b/2 Dose-Escalation Study of Entinostat with Pembrolizumab in NSCLC with Expansion Cohorts in NSCLC, Melanoma, and Colorectal Cancer | Active, not recruiting |

*TME* tumor microenvironment, *NSCLC* non-small cell lung cancer
[a]As of Jul 26, 2020. Source: clinicaltrials.gov

with the creation of an inflammatory rather than immunosuppressive TME. Furthermore, blockade of both molecules supports the expansion of tumor-infiltrating CD8(+) T cells; however, at variance with PD-1 blockade, CTLA-4 targeting triggers a powerful CD4(+) effector T-cell response via the expansion of an Inducible T-cell co-stimulator (ICOS) + T helper (Th)1-like CD4 subset, therefore sustaining long-term antitumor immune responses [5]. All these lines of evidence suggested that combination therapies may act in a complementary or even synergistic fashion, and this hypothesis was confirmed by the higher response rates and improved survival of cancer patients treated with the combination of PD-1 and CTLA-4 blockers [6]. More in detail, the combination of nivolumab and ipilimumab has been investigated as sequential and combination approaches in MM, in several clinical trials.

In the phase II CheckMate 064 study, patients with unresectable stage III or IV MM were randomized to receive a sequential induction treatment with nivolumab followed by ipilimumab (Cohort A) or ipilimumab followed by nivolumab (Cohort B). Following induction treatment, both cohorts received nivolumab until progression or unacceptable toxicity. Objective Response Rate (ORR) at week 25 was higher in the nivolumab–ipilimumab group vs the ipilimumab–nivolumab group (41.2% vs 20%), with a lower progression rate (38.2% vs 60%). Notably, the group receiving nivolumab followed by ipilimumab exhibited a greater 12-month overall survival rate compared with the group treated with ipilimumab followed by nivolumab (76%; 95% CI 64–85 vs 54%; 42–65). Treatment-related grade 3–4 Adverse Events (AEs) occurred in 50.0% in the nivolumab–ipilimumab group and in 42.9% in the ipilimumab–nivolumab group [7]. Given the similar results in terms of clinical outcomes and toxicity, sequential treatment does not appear to offer any significant improvement over concurrent combination therapy. However, it should be noted that the study design was not optimal, with a different time interval between sequential treatments (2 weeks for Cohort A and 3 weeks for Cohort B), thus not answering the question of the optimal sequence [7].

In the phase III, randomized CheckMate 067 study, 945 treatment-naïve cutaneous and mucosal melanoma MM patients were randomly assigned 1:1:1 to receive

ipilimumab (3 mg/kg), nivolumab (1 mg/kg), or ipilimumab *plus* nivolumab (3 mg/kg + 1 mg/kg). The long-term follow-up of the study has shown a median overall survival (OS) of more than 60.0 months in the nivolumab *plus* ipilimumab group, 36.9 months in the nivolumab group, and 19.9 months in the ipilimumab group. Overall survival at 5 years was 52% in the nivolumab *plus* ipilimumab group and 44% in the nivolumab group, as compared with 26% in the ipilimumab group. Median progression-free survival (PFS) was 11.5 months (95% CI, 8.7–19.3) for nivolumab *plus* ipilimumab, 6.9 months (95% CI, 5.1–10.2) for nivolumab, and 2.9 months (95% CI, 2.8–3.2) in the ipilimumab arm. Progression-free survival rate at 5 years was 36% in the nivolumab *plus* ipilimumab group, 29% in the nivolumab group, and 8% in the ipilimumab group. The rate of objective response among treated patients was 58% in the nivolumab *plus* ipilimumab group, 45% in the nivolumab group, and 19% in the ipilimumab group. The median duration of response had not been reached in the nivolumab *plus* ipilimumab and nivolumab groups and was 14.4 months in the ipilimumab group, with ongoing responses at 5 years in 62%, 61%, and 40% of the patients with a response, in nivolumab *plus* ipilimumab, nivolumab, and ipilimumab groups, respectively. The duration of response was sustained across stratification subgroups (according to BRAF mutation status, PD-L1 status, and metastasis stage). These long-term data clearly showed that patients with MM treated with nivolumab, delivered either as monotherapy or in combination with ipilimumab, continued to show superior OS, PFS, and response rates compared with those on ipilimumab. Combination therapy was more toxic with grade 3 or worse AEs in 59% of patients, compared with 21% for nivolumab and 28% for ipilimumab; however, managing patients with established safety guidelines, AEs usually resolved within 3–4 weeks. Notably, the 5-year survival rate was similar between patients who discontinued nivolumab *plus* ipilimumab due to treatment-related adverse events and the overall population [8]. These data suggest that combined treatment elicited higher rates of toxicity than either monotherapies, but that benefit from dual therapy was conferred even despite discontinuation of treatment.

A separate consideration deserves mucosal melanoma. Although objective response rate was lower than in the overall population, limited short-term data indicated clinical benefit with nivolumab *plus* ipilimumab, nivolumab, and ipilimumab in patients with mucosal melanoma [9]. In detail, a pooled analysis, that included also data from CheckMate 067, reported, among mucosal melanoma patients who received nivolumab monotherapy, a median PFS of 3.0 months (95% CI, 2.2–5.4 months, with ORR of 23.3% (95% CI, 14.8%–33.6%). Median PFS in patients treated with nivolumab combined with ipilimumab was 5.9 months (95% CI, 2.8 months to not reached), with ORR of 37.1% (95% CI, 21.5%–55.1%). The incidence of grade 3 or 4 treatment-related adverse events was 8.1% for nivolumab monotherapy and 40.0% for combination therapy [9]. Thus, nivolumab combined with ipilimumab seemed to have greater efficacy than either agent alone also in mucosal melanoma and, although the activity was lower than in cutaneous melanoma, the safety profile was similar between the two subtypes. The 5-year outcomes of mucosal melanoma patients treated in CheckMate 067

were also recently reported, confirming that patients with mucosal melanoma treated with nivolumab *plus* ipilimumab have more favorable survival outcomes than those treated with nivolumab or ipilimumab alone. However, the 5-year analysis showed that patients with mucosal melanoma in the CheckMate 067 had poorer long-term efficacy vs ITT [10].

In order to define the optimal dosage of the combination of ipilimumab *plus* nivolumab, clinical trials have explored a lower dose of ipilimumab that would possibly have lower toxicity rates. Regarding this evidence, the phase IIIb/IV CheckMate 511 study has investigated the combination of nivolumab 3 mg/kg *plus* ipilimumab 1 mg/kg. In part 1 of the study, MM patients received either nivolumab 3 mg/kg *plus* ipilimumab 1 mg/kg (NIVO3 + IPI1) or nivolumab 1 mg/kg *plus* ipilimumab 3 mg/kg (NIVO1 + IPI3) once every 3 weeks for four doses [11]. Patients who discontinued combination therapy as a result of toxicity did not enter the maintenance phase (part 2 of the study) in which nivolumab was administered at a flat dose of 480 mg once every 4 weeks until disease progression or unacceptable toxicity. At a minimum follow-up of 12 months, incidence of treatment-related grade 3–5 AEs was 34% with NIVO3 + IPI1 versus 48% with NIVO1 + IPI3 ($P = 0.006$). In descriptive analyses, ORR was 45.6% in the NIVO3 + IPI1 group and 50.6% in the NIVO1 + IPI3 group, with complete responses in 15.0% and 13.5% of patients, respectively. Median PFS was 9.9 months in the NIVO3 + IPI1 group and 8.9 months in the NIVO1 + IPI3 group. Median OS was not reached in either group [11]. The CheckMate 511 study met its primary end point, demonstrating a significantly lower incidence of treatment-related grade 3–5 AEs with NIVO3 + IPI1 versus NIVO1 + IPI3. Descriptive analyses showed that there were no significant differences between the groups for any efficacy end point, even if a longer follow-up may help to better characterize clinical efficacy outcomes [11].

Based on these results, the combination of ipilimumab and nivolumab is an effective strategy in MM, though the identification of the right patient, dosage, and duration of treatment remains a challenge.

## Role of ICI Combination in Brain Metastases

Although melanoma brain metastases are the third-most common origin of metastases to the brain after lung and breast cancers, melanoma shows the highest level of cerebral tropism of all cancer types. Brain metastases affect 25% of patients at diagnosis of advanced melanoma, and up to 75% of melanoma patients have brain metastases at the time of death [12]. In light of this evidence, the American Joint Committee on Cancer (AJCC) has acknowledged the negative impact of brain metastases on the prognosis of patients with MM in its latest eighth edition staging system, by defining this subgroup as M1d. Moreover, until recently, most of the systemic chemotherapeutic agents had limited activity on brain metastases, due to their acknowledged limitation to effectively cross the blood–brain barrier (BBB). In light of this notion and of their association with a poorer prognosis, patients with brain metastases were generally excluded from clinical trials with

chemotherapeutic agents in the past, and also from the initial studies with ICI. Nevertheless, in the last years, the better comprehension of the interactions between the immune system and the TME in brain metastases has led to recognize the TME of brain metastases as one of the most important factors responsible for response or resistance to treatment. TME is the environment around a tumor and it is composed of neoplastic and non-neoplastic cells (i.e., endothelial cells, pericytes, fibroblasts, and immune cells) [13]. It was reported that the alteration in the pericyte subpopulation in brain metastases causes a remodeling of the BBB favoring a great infiltration of multiple immune suppressive cell types from the peripheral circulation, thus contributing to resistance to therapy. Additionally, it was shown that brain-metastasizing melanoma cells can promote astrocytes to express the pro-inflammatory cytokine IL-23, which induces the production of matrix metalloproteinase-2 (MMP-2) that enhances the degradation of the extracellular matrix, thus promoting the extravasation and consequent spreading of tumor cells in the brain [13]. Moreover, the recruitment of type 2 TAM, MDSC, T-reg, and cancer-associated fibroblasts (CAF), with their pro-tumorigenic features, reduced the expression of co-stimulatory molecules (i.e., CD80, CD86, CD40) involved in T-cell activation, resulting thereby in an impairment of antigen presentation, and deregulation of the homeostasis of the brain microenvironment [13]. In this highly immune-suppressive TME, tumor-infiltrating lymphocytes (TIL) are poorly represented and functionally impaired. About this latter evidence, different studies reported a downregulation of T-cell activity in brain metastases, resulting from tumor-induced T-cell exhaustion. Indeed, PD-1 expression was detected on >60% of TIL, although the correlation with clinical outcomes has yet to be fully understood. In light of this evidence and based on the upcoming clinical results, the use of immunotherapeutic agents should be encouraged also in patients with brain metastases [13].

The initial clinical evidence of ICI activity used in combination with other therapeutic agents in MBM was generated in the Italian Network for Tumor Biotherapy (NIBIT)-M1 study [14]. In this phase II trial, 86 patients with MM were assigned to receive ipilimumab at 10 mg/kg combined with fotemustine; among the 20 patients who had asymptomatic brain metastases at study enrollment, the immune-related Disease Control Rate (ir-DCR) was 50%, as compared with 46.5% in the whole population. Notably, the 3-year survival rate was 27.8% in patients with brain metastases and 28.5% in the whole population, suggesting for a long-term clinical benefit also in patients with asymptomatic brain metastases [15]. A more recent follow-up of this study has shown that 5 complete regressions of brain disease were obtained, with a duration of brain complete response (CR) of 16, 28, 39, 80+, 94+ months; notably, the 2 patients still alive, in the absence of subsequent treatment, had achieved a CR both intra- and extra-cranial [13]. In light of these intriguing clinical data and of available results showing the therapeutic efficacy of ipilimumab combined with nivolumab in melanoma, the multicenter, phase III, randomized, open-label NIBIT-M2 study (NCT02460068), sponsored by the NIBIT Foundation, was activated. This three-arm study was designed to assess the OS of previously untreated metastatic melanoma patients with asymptomatic brain metastases who received fotemustine, its combination with ipilimumab, or the combination of

ipilimumab and nivolumab. In this study, 76 patients with active, untreated, and asymptomatic brain metastases were randomly assigned to ARM A (fotemustine), ARM B (ipilimumab *plus* fotemustine), or ARM C (nivolumab *plus* ipilimumab). With a median follow-up of 39 months, median OS was 8.5 months for ARM A, 8.2 months for ARM B, and 29.2 months for ARM C. The ir-ORR was 0%, 19.2%, and 44.4% in ARMs A, B, and C, respectively [16].

Other two studies have recently investigated the dual blockade of CTLA-4 and PD-1 molecules in MBM. The phase II, single-arm, CheckMate 204 study enrolled patients into two cohorts: those with asymptomatic brain metastases (cohort A) and those with neurologic symptoms (cohort B). In both cohorts, patients received nivolumab (1 mg/Kg) *plus* ipilimumab (3 mg/Kg) every 3 weeks for up to four doses, followed by nivolumab (3 mg/kg) every 2 weeks until progression of unacceptable toxic effects. Among the 94 asymptomatic enrolled patients, the intracranial and extracranial ORR were 55% and 50%, respectively, with a global ORR of 51%, and with 90% ongoing objective responses at a relatively short median duration of follow-up of 14 months [17]. An updated analysis of cohort A (with a follow-up of 20.6 months) reported an intracranial and extracranial ORR of 54% and 49%, respectively, with a global ORR of 51%, among the 101 evaluable patients; the 18-month survival rate was 75%. In cohort B, at a median follow-up of 5.2 months, intracranial ORR was 16.7%, with a 6-month survival rate of 66%. The safety profile of the regimen was similar to that reported in patients with melanoma who do not have brain metastases [18].

In line with these results are those from the Australian Brain Collaboration (ABC) study, a phase II, prospective trial enrolling 3 cohorts of patients with asymptomatic or symptomatic brain metastases. Patients with no prior local brain treatment were randomized to receive nivolumab 1 mg/kg *plus* ipilimumab 3 mg/kg followed by nivolumab 3 mg/kg (Cohort A) or nivolumab 3 mg/kg (Cohort B), whereas patients with brain metastases progressed after local therapy, or who had neurological symptoms or leptomeningeal spreading disease were enrolled in non-randomized cohort C (nivolumab 3 mg/kg). At a median follow-up of 17 months, the intracranial ORR was 46%, 20%, and 6% in Cohorts A, B, and C, respectively, with complete intracranial response in 17%, 12%, and 0% patients in each cohort. Among patients enrolled in Cohort A, those with treatment-naïve brain disease achieved a 56% ORR while it was 16% in BRAF mutant patients pretreated with BRAF and MEK inhibitors [19]. In a more recent analysis with a median follow-up of 34 months, the intracranial ORR in Cohorts A, B, and C were 51%, 20%, and 6%, respectively, with complete intracranial response in 26%,16%, and 0% patients in each cohort. The 24-month intracranial PFS rate was 49% in Cohort A, 15% in Cohort B, and 6% in Cohort C, with a 24-month survival rate of 63%, 51%, and 19% in Cohorts A, B, and C, respectively [20]. Consistent with the safety results from CheckMate 204 study, treatment-related grade 3/4 adverse events in Cohorts A, B, and C were 54%, 20%, and 13%, respectively, with no treatment-related deaths [19]. Altogether, these results supported the safety and tolerability of nivolumab utilized alone or in combination with ipilimumab in MM patients with brain metastases.

Notably, a recent systematic literature review and meta-analysis suggested that combined immunotherapy increased long-term OS and PFS of MM patients with brain metastases, compared with anti-PD1 mAb monotherapy or targeted therapy [21]. Taken together, consistent with those reported in extracranial disease, available data show a considerable efficacy and with a good safety profile of combination therapy with CTLA-4 *plus* PD-1 in melanoma patients with brain metastases, that should now represent the standard of care in this clinical setting. Furthermore, several ongoing clinical trials are exploring novel combinations also with radiotherapy in this subset of melanoma patients.

## Combinations with Other ICI

The increasing knowledge about inhibitory molecules whose mechanisms may act within the TME has led to the development of new therapeutic agents that could have complementary functions to those of approved immunotherapeutic agents. Currently, multiple clinical trials are underway examining the activity and safety of combined immunotherapies, in particular using an anti-PD-1 mAb in combination with agents that target novel emerging checkpoints. Among these, ICI directed at lymphocyte-activation gene 3 (LAG-3), a cell surface molecule expressed on Teff and Tregs, are among the most deeply investigated. At least 60 clinical trials are presently ongoing targeting LAG-3 both alone and in combination with other immune checkpoints, in melanoma and other different tumor types. Specifically, LAG-3 is an additional immune checkpoint pathway known primarily to be expressed on exhausted T cells which have less potent effector functions [22]. It may downregulate T-cell responses via interaction with MHC-II on DC. As result of continuous melanoma antigen expression, LAG-3 expression on T cells is increased, thereby inhibiting T-cell action and reducing IFN-γ production within the TME under the influence of PD-1 co-stimulation [22]. Moreover, in vivo studies in murine cancer models have shown that when expressed at high levels, concomitant LAG-3/PD-1 expression is mostly restricted to infiltrating TILs [23]. This may indicate that a combined immunotherapy targeting LAG-3 and PD-1 may elicit tumor-specific responses, avoiding nonspecific or self-antigen-specific immune responses, possibly improving safety profile as compared with PD-1 and CTLA-4 blockade combination. Indeed, preclinical evidence, suggesting that LAG-3 has a synergistic activity with anti-CTLA-4 or anti-PD-1 mAbs, is driving its clinical development [24]. Immuno-modulating mAbs targeting LAG-3 is being tested in several clinical trials, and new combinations of anti-LAG-3 and anti-PD-1 mAbs have shown encouraging activity in fighting PD-1 resistance. In detail, preliminary results from the ongoing phase 1/2a study which is testing the combination ofanti-LAG-3 mAb relatlimab with nivolumab (NCT01968109) have shown encouraging initial clinical activity in patients who were refractory to a previous anti-PD-1/PD-L1 therapy. Furthermore, this combination showed a good safety profile, comparable with nivolumab monotherapy, with uncommon grade 3/4 AEs. Moreover, the

combination therapy can increase objective response rates from 5% to 18% in patients with LAG-3-positive tumors [25]. In light of these results, the ongoing phase 2/3 CA224-047 (NCT03470922) clinical trial will hopefully assess efficacy and safety of relatlimab with nivolumab versus nivolumab monotherapy as first-line treatment in advanced melanoma.

Additionally, TIM-3, a co-inhibitory receptor expressed on T cells, has both inhibitory and activating properties. It induces T-cell apoptosis, anergy, and exhaustion through the interaction with galectin-9 on immune cells [26]. Since TIM-3 has been established as an exhaustion marker in cancer, it can represent an interesting immunotherapy target. The combination of TIM-3/PD-1 blockade led to superior tumor regression than single-agent PD-1 blockade in murine cancer models and the combination of anti-TIM-3 plus anti-PD-1 mAbs is currently being investigated in phase I/II trials (NCT02817633, NCT02608268) [26].

B7–H3 (CD276) is a receptor of the CD28 (a co-stimulatory molecule) and B7 (a co-inhibitory molecule) family molecules found on Antigen-Presenting Cells (APCs). B7–H3 has found to be over-expressed in melanoma, favoring tumor growth and conferring resistance to apoptosis induction [26]. Enoblituzumab, a first in class mAb targeting B7–H3, has been tested in phase I trials in combination with pembrolizumab in refractory cancers (NCT02475213) and also with ipilimumab (NCT02381314) [26]. Final results of these studies are awaited.

V-domain Ig suppressor of T-cell activation (VISTA) is a PD-L1 homolog and a co-inhibitory receptor of the B7 family, expressed primarily within the hematopoietic compartment (MDSCs, TAMs, and DCs) and on leukocytes such as naïve T cells. VISTA may contribute to the suppression of effector T-cell (T-eff) responses and T-reg induction via interaction with its ligand V-Set and immunoglobulin domain containing 3 (VSIG-3). VSIG-3 can inhibit T-cell function and, in the presence of T-Cell Receptor (TCR) signaling, it may impair T-cell proliferation via the VSIG-3/VISTA pathway. Preclinical experience has indicated that VISTA blockade with a monoclonal antibody (13F3) enhanced effector T-cell response within the TME through the production of cytokines such as IFN-y and TNF-alpha. Concurrent blockade of VISTA and PD-1 checkpoints is emerging as a therapeutic option, therefore the small oral molecule antagonist CA-170 electively targets PD-L1/2 and VISTA has been investigated in a phase Idose escalation trial (NCT02812875) in advanced hematologic and solid tumors, with acceptable safety [26].

## *Combinations with Oncolytic Viral Therapy*

Oncolytic virus therapy is an antitumor approach that utilizes native or genetically modified viruses that selectively replicate within cancer cells. Even if its mechanism of action is not completely understood, oncolytic viruses seem to mediate anticancer activity through the combination of two distinct mechanisms of action: a direct cancer cell lysis resulting from the selective viral replication within

neoplastic cells and indirect induction of systemic antitumor immune response [27]. Moreover, immunosuppressive TME, such as in melanoma, is ideal for viral replication. Upon infection with an oncolytic virus, cancer cells initiate an antiviral response that leads to the upregulation of reactive oxygen species (ROS) and the initiation of antiviral cytokine production. ROS and cytokines, specifically type I IFNs, are released from the infected cancer cell and stimulate immune cells [i.e., APCs, CD8(+) T cells, and natural killer (NK) cells] [27]. Subsequently, the virus causes oncolysis, that triggers the release of viral progeny, pathogen-associated molecular patterns (PAMPs), danger-associated molecular pattern signals (DAMPs), and tumor-associated antigens (TAAs), including neo-antigens [27]. The release of viral progeny propagates the infection with the oncolytic virus, but, on the other hand, the PAMPs (consisting of viral particles) and DAMPs (comprising host cell proteins) stimulate the immune system by triggering activating receptors such as Toll-like receptors (TLRs). In the context of the resulting immune-stimulatory environment, TAAs and neo-antigens are released recognized by APCs. Altogether, these events result in the activation of immune responses against virally infected cancer cells, as well as de novo immune responses against TAAs/neo-antigens displayed on un-infected cancer cells [27].

Talimogene laherparepvec (T-VEC) is a herpes simplex virus type 1 derived oncolytic immunotherapy [28]. Preclinical studies have shown that T-VEC elicits antitumor activity by selectively replicating within cancer cells and thereby destroying them, as well as through the release of TAAs and the production of granulocyte-macrophage colony-stimulating factor (GM-CSF), which enhances antitumor immune response.

T-VEC was approved in the United States in 2015 for the local treatment of unresectable MM with cutaneous, subcutaneous, and nodal recurrent lesions, based on data from the phase III, open-label, randomized OPTiM, trial [25]. In this study, intratumoral administration of T-VEC was compared with subcutaneous administration of GM-CSF in patients with stage IIIB–IVM1 melanoma. Overall response rates were 31.5% and 6.4%, with a median OS of 23.3 and 18.9 months (hazard ratio 0.79; $p = 0.0494$) for T-VEC and GM-CSF, respectively. With grade 3–4 events in less than 2% of the 436 treated patients, the durable response rate (>6 months) was higher with T-VEC (19%) than GM-CSF (1.4%). Talimogene laherparepvec efficacy was more marked in stage IIIB–IVM1a melanoma [28].

Moreover, in the OPTiM study T-VEC has considerable local immune activity, with intralesional administration resulting in responses (regression ≥50%) in 64% of injected lesions. A 50% reduction in tumor size was also seen in 34% of non-injected, non-visceral lesions and in 15% of visceral lesions, indicating that T-VEC also induces systemic antitumor immunity and response. While activity was observed at distant metastases, it has been hypothesized that combining T-VEC with other systemic immunotherapies may further enhance the activity of both agents. It has been also shown that TVEC contributes to anti-PD1 mAb activity by augmenting the inflammatory state of the TME, which results in the increased homing and activation of tumor-reactive T cells [29]. Promoting the influx of T cells into the tumor is extremely important for patients with low intratumoral TILs, thus limiting

response to PD-1 blockade [29]. Indeed, intratumoral administration of single-agent T-VEC resulted in increased levels of circulating and tumor-infiltrating T cells [29]. In light of this evidence, the complementary mechanism of action of talimogene supports its use in combination with different immunomodulatory agents within clinical trials.

Along this line, T-VEC was evaluated in combination with pembrolizumab in the phase Ib part of the MASTERKEY-265 clinical trial [30]. Pembrolizumab was administered intravenously at 200 mg every 2 weeks, after the third dose of T-VEC [30]. This sequential treatment was associated with a confirmed ORR of 57% and a confirmed CR rate of 24% [30]. In a follow-up efficacy analysis after a median follow-up of 38.6 months, ORR was 67% with a CR rate increased to 43% [31]. As previously reported, an increase in circulating cytotoxic T cells as well as an upregulation of PD-1 on these cells was observed after T-VEC monotherapy administration, suggesting a priming effect of T-VEC on the immune response during the subsequent pembrolizumab therapy [30]. Additional data from the MASTERKEY-265 clinical trial might confirm the role of this strategy in advanced melanoma. Furthermore, clinical studies combining T-VEC with BRAF and MEK inhibitors in BRAF-mutated advanced melanoma (NCT03088176), or with pembrolizumab, following progression on prior anti-PD-1-based therapy (NCT04068181) are recruiting. Finally, a trial of T-VEC with or without radiotherapy (NCT02819843) is currently ongoing, and T-VEC will be also tested in neoadjuvant setting in combination with nivolumab for resectable early metastatic (stage IIIB/C/D–IV M1a) melanoma with injectable disease (NIVEC) (NCT04330430).

## *Combinations with BRAF and MEK Inhibitors*

BRAF and MEK inhibitors as well as ICI have significantly improved treatment outcomes of patients with BRAF-mutant melanoma. Although BRAF and MEK inhibitors are associated with a higher ORR as compared with immunotherapy, acquired resistance results in relapse within months, with a median progression-free survival of 11.5 months [32]. However, preclinical and translational data have shown that BRAF and MEK inhibition has an immune-modulating effect, augmenting antitumor immunity [32]. For instance, BRAF inhibition alone (vemurafenib) or BRAF+MEK inhibition (dabrafenib+trametinib) are associated with increased tumor infiltration by CD8(+) lymphocytes and consequently with tumor shrinkage and increased necrosis in posttreatment biopsies [32]. Furthermore, BRAF inhibition or BRAF+MEK inhibition are correlated with an enhanced expression of melanoma antigens at least in the first weeks after treatment initiation. Moreover, a decrease in immunosuppressive cytokines like IL-6 and IL-8 and an increase in markers of T-cell cytotoxicity were observed [32]. Intriguingly, BRAF V600E mutation downregulates the expression of IFN-α-receptor-1 (IFNAR-1), while BRAF inhibition upregulates the expression of most of the HLA class I

antigen-processing machinery components, enhancing thereby the recognition of melanoma cells by relative T cells.

Regarding the potential overlapping efficacy from combined BRAF and immune checkpoint inhibitor, evidences from patients treated with BRAF inhibitors showed increased expression PD-1 and its ligand, PD-L1, suggesting potential benefit from this combinatorial approach. Of note, some preclinical experiences have also reported the efficacy of the triple combination therapy with dabrafenib, trametinib, and anti-PD1 in increasing the expression of melanoma antigens and MHC, as well as of the global immune-related gene upregulation in tumors with BRAF V600E mutation. Interestingly, the amount of circulating MDSCs, which repress antitumor immunity, decreased in response to vemurafenib [32].

Taken together, these findings support a combinatorial approach in BRAF-mutated melanoma by the testing of triple combination of BRAF and MEK inhibitors with immunotherapy. Notably, the combination of the BRAF inhibitor vemurafenib and the anti-CTLA-4 ipilimumab was associated with an unacceptable rate of grade 3–4 hepatitis, which led to subsequent discontinuation of the phase I study [33]. Similarly, a phase I trial with dabrafenib and ipilimumab was prematurely closed due to the occurrence of severe colitis in three patients [34]. In contrast, early-phase studies have shown promising anti-melanoma activity and manageable safety profile with combinations of BRAF-inhibitors, MEK-inhibitors, and anti-PD-1 leading thereby to develop phase II and III clinical trials [35].

In detail, Keynote-022 study is a double-blind, randomized, phase II study, comparing the efficacy of pembrolizumab *plus* dabrafenib and trametinib with dabrafenib and trametinib *plus* placebo, in patients with BRAF V600 E/K mutant melanoma. Initial results at a 9-month follow-up demonstrated improved PFS in the triplet group, 16.0 months, compared with 10.3 months in the doublet group (hazard ratio, 0.66; $P = 0.043$) without reaching statistical significance [32]. A more recent analysis (with a follow-up of 24 months) reported a median PFS of 16.9 (95% CI, 11.3–27.9) months with pembrolizumab and 10.7 (95% CI, 7.2–16.8) months with placebo (hazard ratio, 0.53; 95% CI, 0.34–0.83), with a survival rate at 24 months of 63.0% and 51.7% with pembrolizumab and placebo, respectively [36]. Of note, the combination of dabrafenib, trametinib, and pembrolizumab has led to higher rates of grade 3/4 AEs than would be expected for targeted therapy alone. Indeed, grade 3/4 treatment-related AEs occurred in 58.3% of patients in the triplet group and 26.7% in the doublet group. The most common adverse events were pyrexia, increased transaminase level, and rash. One patient receiving triplet therapy died of pneumonitis [35].

The COMBI-i phase III trial investigating dabrafenib, trametinib, and the anti-PD-1 agent PDR001 in patients with advanced BRAF V600 mutant melanoma has yielded encouraging preliminary results. Indeed, a first analysis, with a median follow-up of 15.2 months, of part 1 and part 2 reported a DCR of 94% and a CR rate of 33% [37]. The full results of these trials are eagerly awaited.

Furthermore, IMspire150 is a randomized, double-blind, phase 3 study testing the efficacy of atezolizumab *plus* vemurafenib and cobimetinib compared with

vemurafenib and cobimetinib *plus* placebo, in previously untreated BRAFV600E/K mutant advanced melanoma patients. The primary endpoint PFS was significantly prolonged with atezolizumab compared with placebo (15.1 vs 10.6 months; hazard ratio 0.78; $p = 0$–025), while overall response rates in the atezolizumab (66%) and control groups (65%) were similar. Moreover, the prevalence of treatment-related grade 3 or 4 AEs was 182 (79%) of 230 in the atezolizumab arm and 205 (73%) of 281 in the placebo arm [38].

All these data suggest that the combination of anti-PD-1 mAb with BRAF and MEK inhibitors as first-line therapy in patients with advanced BRAFV600-mutant melanoma induced durable response with an encouraging PFS. Although triplet therapy led to a higher incidence of grade 3/4 treatment-related adverse events, most resolved with treatment interruption or dose reduction. In light of these results, the Food and Drug Administration (FDA) has recently approved the combination of atezolizumab with cobimetinib and vemurafenib for patients with BRAF V600 mutation-positive unresectable or MM.

However, the role of the triple combination of PD-1/PD-L1 *plus* BRAF and MEK inhibitors in the rapidly evolving melanoma treatment scenario will have to be established, mainly due to the increasing use of combined CTLA-4–PD-1 therapy. Ongoing trials [i.e., Immuno-CobiVem (NCT02902029), SECOMBIT (NCT02631447), DREAMseq (NCT02224781), and part 3 of COMBI-i)] will certainly advise the better therapeutic algorithm with regard to optimal combination or sequencing for the first-line treatment of BRAF-mutated MM [38].

## Combinations with Co-stimulatory Molecules and Cytokines

T-cell activation is controlled by two sets of signals mediated by TCR and T-cell co-signaling receptors. Positive (co-stimulatory) and negative (co-inhibitory) signals from T-cell co-signaling receptors regulated T-cell function in response to TCR stimulation. Several studies have shown that activating T-cell co-stimulatory receptors, such as OX40, CD137 (4-1BB), and ICOS, can enhance T cell-mediated antitumor immunity. Thus, they emerged as novel targets for immunotherapeutic strategies.

CD137 and OX40 are members of the tumor necrosis factor receptors (TNFR) super family, expressed on T and NK cell surface and they act through a complex interplay of cytolytic T lymphocytes, helper T cells, regulatory T cells, dendritic cells, and vascular endothelium in tumors. Their stimulation promotes a high antitumoral immunity in a variety of murine tumor models. Furthermore, preclinical evidence suggests that combining agonist mAbs specific for TNFR members with conventional cancer therapies or additional immunotherapeutic agents may be particularly effective. Indeed, T-cell responses elicited by tumor antigens released through immunogenic tumor cell death are enhanced by these immunostimulatory agonist mAbs. Combinations with other immunomodulatory mAbs such as CTLA-4 and PD-1 are under investigation and seem to be promising [39].

More in detail, the clinical development of the anti-CD137 mAb urelumab started in 2005. Urelumab was evaluated as a monotherapy in two studies, CA186-001 (NCT00309023) and CA186-006 (NCT00612664). In December 2008, urelumab development program was put on hold due to the occurrence of two hepatotoxicity-related deaths. Subsequent detailed analysis of the clinical safety data showed that urelumab dose was the most important factor contributing to the development of the reported severe immune-related liver inflammation. Thus, in February 2012, the urelumab clinical development program was restarted with CA186-011 study (NCT01471210) to investigate monotherapy doses <1 mg/kg and it has been established that the optimal dosage seems to be 0.1 mg/kg every 3 weeks. Afterwards, a clinical trial was conducted that combined urelumab at this dose with nivolumab (NCT02253992) and its results are awaited [40].

In addition, early-phase clinical trials evaluating agonist antibodies targeting the OX40 pathway alone or in combination with ICI in cancer patients are ongoing. Among these, ENGAGE-1 (NCT02528357) is testing the combination of OX40 agonist mAb and pembrolizumab, JAVELIN Medley (NCT02554812) is investigating the combination of OX40 agonist mAb and avelumab, while INDUCE-1 study is testing the combination of OX40 agonist mAb and an anti-ICOS receptor agonist mAb (NCT02723955).

ICOS is a member of the CD28 superfamily that is expressed on activated T cells and regulates a lot of T-cell functions, including effector T-cell activation, interactions with B cells, and Treg infiltration. Additionally, preclinical work reports that an ICOS agonistic aptamer enhances the efficacy of anti-CTLA-4 therapy against melanoma in vivo. Thus, ICOS agonist mAbs are currently tested in early-phase clinical trials alone and in combination with ICI, in solid tumors [41].

Lastly, cytokines are soluble proteins acting as strong but complex mediators of immune activation. Due to the discovery of their potent antitumor activities in animal models, some of the earliest immunotherapeutic strategies have involved exogenous administration of interferon and IL-2. Both drugs exhibited only modest efficacy and produced significant toxicity, limiting their clinical value [42]. However, a renovated interest in the antitumor properties of cytokines has led to an exponential increase in the clinical studies that investigate the safety and efficacy of cytokine-based drugs, not only as monotherapy, but also in combination with other immunomodulatory drugs. These second-generation drugs under clinical development include known molecules with novel mechanisms of action, new targets, and fusion proteins that increase half-life and target cytokine activity to the TME or to the expected effector immune cells [42]. They could represent key molecules to overcome primary and acquired resistance mechanisms to anti-PD(L)-1 immunotherapies in light of their power to expand and reactivate effector NK and T lymphocytes, and promote tumor infiltration by lymphocytes, as well as due to their persistence in the TME. In this scenario, cytokines are being investigated in combination with other immunotherapeutic agents, mainly with anti-PD-1 and anti-PD-L1 mAbs.

We here report initial data about ICOS agonists and, among second-generation IL-2, about bempegaldesleukin (NKTR-214), a pegylated (PEG)-IL-2 designed to improve safety profile as recently reported in the phase I/II trial PIVOT-02 [43].

## ICOS Agonists

In light of the demonstrated efficacy of CTLA-4 and PD-1 antagonists in blocking inhibitory pathways, great interest surrounds the targeting of T-cell co-stimulatory molecules, such as ICOS. ICOS is a co-stimulatory immune checkpoint expressed on activated T cells. Its ligand, ICOSL, is widely expressed on APCs and somatic cells, including cancer cells in the TME. ICOS and ICOSL expression is linked to the release of cytokines, induced by activation of the immune response. ICOS and ICOSL binding promotes either antitumor T-cell responses when activated in Th1, CD4(+) and CD8(+) cells, or pro-tumor responses when triggered in Tregs. Thus, mAbs targeting this pathway are being tested for cancer immunotherapy [41]. In preclinical studies, ICOS agonistic mAbs enhance the efficacy of anti-CTLA-4. ICOS knockout mice do not respond well to anti-CTLA-4 indicating that ICOS signaling is required for successful antitumor responses, possibly mediated by effector T cells. Hence, concomitant CTLA-4 and ICOS stimulation had a superior antitumor effect in comparison with anti-CTLA-4 alone. Interestingly, ICOS (+) T cells were described to be increased posttreatment with ipilimumab and to correlate with clinical responses in terms of DCR and OS in MM patients. Thus, changes in the number of circulating ICOS(+), CD4(+), and CD8(+) T cells assessed at baseline and during treatment with ipilimumab may be considered as early biomarkers of clinical response [44]. Even though ICOS alone seems to be less active in comparison with other pathways targeted by immunotherapeutic agents, especially due to the predominance of CD4(+) Tregs, the combination of ICOS agonistic mAbs and anti-CTLA-4 or PD-1/PD-L1 mAbs might have the potential to generate robust synergistic effects [41, 44]. The first-in-human, INDUCE-1 trial (NCT02723955), is testing an ICOS agonist mAb administered alone (part 1) or in combination (chemotherapy or pembrolizumab or an anti-OX40 mAb or dostarlimab, a novel anti-PD-1, or dostarlimab plus an anti-TIM-3 mAb or a bifunctional fusion protein targeting TGF-β and PD-L1) (part 2) in patients with advanced solid tumors, including melanoma. The study has shown promising results in terms of tolerability, safety profile, and clinical activity. The most frequent treatment-related AEs were fatigue (15%), fever (8%), transaminitis (5%, representing also the most frequent grade 3–4 AE) and diarrhea (3%).One dose-limiting grade 3 pneumonitis occurred, no related deaths were reported [45]. Final analysis of the INDUCE trial and additional data from new ongoing clinical trials evaluating the combination with the anti-CTLA-4 mAb tremelimumab (e.g., NCT03693612) or with an anti-PD1 mAb (e.g., NCT04128696) will confirm the role of this strategy.

## PEG-IL-2

IL-2 represents a key cytokine in promoting the expansion of NK cells and T lymphocytes [42].The administration of this cytokine at high doses is currently approved by the FDA for the treatment of metastatic Renal Cell Carcinoma (RCC) and MM [42]. However, the systemic administration of this cytokine at the

recommended dose is associated with high-grade toxicity, which often includes grade 3 and 4 adverse events. Along this line, second-generation IL-2-based drugs, with improved pharmacokinetic and pharmacodynamic profiles, are being developed [42]. Improvement of the pharmacokinetic profile is achieved through covalent binding of IL-2 to Conjugating Polyethylene Glycol (PEG) molecules that increases the half-life in circulation. IL-2 is recognized by three types of receptor complex expressed on NK and T lymphocytes: low-, medium-, and high-affinity IL-2 receptor, that are highly expressed on Treg cells. Therefore, the high-affinity IL-2 receptor shifts IL-2 activity toward the expansion of Treg cells and reduces the bioavailability of the cytokine that can stimulate antitumor effector NK and T lymphocytes [42]. Several of the second-generation IL-2-based compounds, designed to avoid binding to the high-affinity IL-2 receptor, were tested within clinical trials. Among these, bempegaldesleukin (NKTR-214) is composed of a recombinant IL-2 and multiple molecules of PEG. Directed PEGylation generates an inactive cytokine with a long half-life in circulation; the PEG groups are progressively released, yielding IL-2 molecules with double or single PEGylation that can interact with the medium affinity- but not with the high-affinity IL-2 receptor [42]. Improvement of the pharmacodynamic properties is reached by using biotechnology modifications to reduce binding to the high-affinity IL-2 receptor, while maintaining binding to the medium-affinity IL-2 receptor to increase the amount of cytokine available to stimulate NK and T cells. NKTR-214 has undergone dose-escalation studies and has also been used in combination with nivolumab, with encouraging response rates in immunotherapy-naive melanoma, RCC or non-small-cell lung cancer (NSCLC) patients [43]. Indeed, results of the phase I/II PIVOT-02 (NCT02983045) study, that investigated NKTR-214 combined with nivolumab, are very promising, remarkably for treatment-naive melanoma patients, with a ORR and DCR of 63.6% and 90.9%, respectively, without signals of overlapping or unexpected toxicity [43]. Moreover, part 3 and part 4 of the PIVOT-02 trial have investigated the combination of NKTR-214 with nivolumab plus ipilimumab. Furthermore, an ongoing phase III study (NCT03635983) is testing the efficacy, safety, and tolerability of NKTR-214, when combined with nivolumab versus nivolumab given alone in patients with previously untreated unresectable or metastatic melanoma. NKTR-214 is also being evaluated in clinical trials in combination with pembrolizumab (NCT03138889). Final results of these trials are awaited.

## ICI in Combinations with TME Modulators

Growing data are providing evidence that TME is critical for the efficacy of immunotherapy. TME consists of nonmalignant cells such as immune cells (e.g., myeloid cells, including macrophages, MDSC, DCs, and neutrophils), cells of mesenchymal origin (e.g., fibroblasts, myofibroblasts, mesenchymal stromal cells), and vascular cells (e.g., endothelial cells and pericytes) which create a tumor-promoting milieu,

producing multiple factors including reactive oxygen species (ROS), cytokines (IL-10, TGF-β), PD-L1, as well as IDO and arginase [46].

IDO is an enzyme that often overexpresses in tumor, with special interest in immuno-oncology because of the immunosuppressive effects that result from its role in tryptophan catabolism [46].

An additional pathway that plays an important role in the regulation of immune cell reactivity is arginine metabolism, mediated by arginase and responsible for impairment of T-cell functions. Inhibition of also arginase could represent another target to improve the efficacy of cancer immunotherapy [47].

Finally, Toll-Like Receptors (TLRs) are a family of pattern-recognition receptors. They recognize molecules that are broadly shared by pathogens but distinguishable from host molecules, collectively referred to as PAMPs, thereby inducing potent innate and adaptative immune response. TLRs are widely expressed on TME immune cells, including monocytes, DCs, macrophages, etc. Activation of TLRs on DCs stimulates maturation of the APC, induction of inflammatory cytokines and the subsequent priming of naive T cells for adaptive immunity [48, 49]. In light of this evidence, the activation of TLRs is becoming an interesting target for cancer treatment. TLR agonists, administered intratumorally, due to the upregulation of IC genes including IDO-1, PD-L1, and CTLA-4 in injected and uninjected lesions, in combination with ICI, may suppress tumor growth and reshape the TME. Indeed, preclinical experiences have shown the ability of TLR agonists to increase the ratio of M1/M2 macrophages, T-cell clonality, and recruitment of CD8(+) T cells [48, 49]. We describe TLR9 agonists.

## Combinations with IDO Inhibitors

IDO is expressed in tumor cells, T-regs, DCs, macrophages, and endothelial cells in the TME. It is an enzyme responsible for the degradation of tryptophan into kynurenine. Depletion of local tryptophan by IDO can induce naive CD4(+) T cells toward differentiation into Treg cells. In addition, IDO produces soluble factors (kynurenine and downstream metabolites) that bind and activate the aryl hydrocarbon receptor (AhR) that can induce Treg cell differentiation and can also induce DCs and macrophages toward an immunosuppressive phenotype [46]. This inducible counter-regulation is helpful when IDO is controlling dangerous inflammation or creating tolerance to apoptotic cells but is highly unfavorable when it is suppressing the immune system's attempted response against cancer [46]. In light of its function, blocking IDO emerged as a potential target to enhance immunity against cancer. Intriguingly, preclinical evidence in a melanoma mouse model reported IDO overexpression after treatment with anti-CTLA-4 and anti-PD-1 mAbs [46]. Moreover, IDO overexpression conferred resistance to anti-CTLA-4 and anti-PD-1 mAbs, promoting thereby tumor growth. This property was found to be reversible by combination treatment with anti-CTLA-4 and IDO inhibitors. Studies conducted in the B16.SIY melanoma mouse model have shown that combinations of CTLA-4

or PD-1/PD-L1 with IDO blockade restored both IL-2 production and CD8(+) T-cell proliferation within the TME, underlying the potential ability of a combinatorial targeting approach. Furthermore, overexpression of isoform 1 (IDO1) is associated with poor patient survival in several tumor types [46]. Despite these findings and the promising antitumor activity shown by the anti-PD-1 inhibitor/IDO inhibitor combination therapy in phase I/II trials, the results of the phase III study (ECHO-301) combining the IDO1-selective inhibitor epacadostat with pembrolizumab did not show improved PFS and OS, in comparison with pembrolizumab alone [50]. Unfortunately, these results have led to the stoppage of the ongoing phase III trials with IDO1 inhibitors in different tumor histotypes [50], despite this failure it should be considered with caution, first of all due to the uncertainty of the appropriate target inhibition. In this regard, no direct evidence exists about the degree of IDO1 inhibition within the tumor, and previous data suggested that a sufficient drug exposure may not have been reached at the dose tested in ECHO-301 [46]. Thus, the optimal dose of epacadostat in combination with a novel anti-PD-1 mAb (retifanlimab) continues to be explored in an ongoing clinical trial (NCT03589651). Furthermore, the evaluation of IDO1 expression was not an eligibility criterion and no subgroups of interest based on clinical features or biomarkers were identified [43]. In light of these limitations and given the potential of IDO1 to enhance immunologic function, it would be desirable to continue to design clinical trials combining an anti-PD-1 inhibitor *plus* IDO1 inhibitors, tailoring them for specific subset of melanoma patients.

## TLR 9 Agonists

Among the TLR family, Toll-like receptor 9 (TLR 9) recognizes unmethylated cytosine–phosphate–guanine (CpG) dinucleotide motifs present in bacterial and viral deoxyribonucleic acid (DNA) and synthetic oligodeoxynucleotides and is expressed in endosomal compartments of DCs and B cells. Signaling mediated by TLR 9 triggers cytokine production and release, including interferon (IFN)-α and T helper 1 (Th1)-type cytokines, B-cell proliferation, and upregulation of co-stimulatory molecules. Accordingly, TLR 9 agonists are being widely investigated not only in the treatment of infectious diseases, allergy, asthma, but also in the treatment of cancer [48, 49]. Along this line, IMO-2125 is a synthetic phosphorothioate oligonucleotide that acts as a direct agonist of TLR 9 to stimulate the innate and adaptive immune systems. IMO-2125 induces high levels of IFN-α from DCs along with an array of endogenous cytokines and chemokines. IMO-2125 also induces B-cell proliferation and differentiation and it can activate TLR 9 on B cells and dendritic cells in the TME to initiate and potentiate a Th1-polarized local and systemic immune response when administered by intratumoral injection [48, 49]. In vivo studies in mouse models of colon carcinoma, lymphoma, and melanoma indicate that intratumoral IMO-2125 monotherapy has been shown to produce effects both in injected and uninjected lesions, including antitumor activity associated with an increase in

infiltrating CD8(+) T cells, and durable and specific cytotoxic T-cell responses against tumor antigens. Intratumoral administration was more effective than subcutaneous administration. Although intratumoral delivery of pattern recognition receptor agonists like TLR 9 is an effective means of creating an adaptive antitumor immune response, this can still be attenuated by dampening mechanisms such as immunosuppressive tumor-infiltrating regulator T cells and anergic/exhausted tumor-infiltrating or peritumoral cytotoxic T cells [48, 49]. Therefore, combining a TLR 9 agonist with checkpoint inhibitors or other modulators of the immune response to enhance systemic immunity is a compelling strategy. In vivo studies in mouse models have indeed shown that the combination of intratumoral IMO-2125 with either an anti-CTLA-4 or anti-PD-1 antibody results in improved tumor control compared with either agent alone. Preliminary clinical experience is also promising as the combination of IMO-2125 with ipilimumab is well tolerated and shows encouraging clinical activity in the setting of PD-1 refractory melanoma [51]. In detail, clinical trials are currently evaluating IMO-2125 monotherapy or combination with ipilimumab, or pembrolizumab, in previously treated metastatic melanoma patients. A phase 1/2 clinical study in patients with advanced melanoma that is refractory to PD-1 inhibitors (NCT02644967) has investigated intratumoral IMO-2125 in combination with ipilimumab or pembrolizumab in melanoma. At the time of the first analysis, tilsotolimod with ipilimumab was well tolerated and associated with an ORR in 3 out of the 6 evaluable patients, including complete response lasting >21 months [51]. Interestingly, dendritic cell activation, type I interferon response, CD8(+) T-cell proliferation was also reported in responding patients [51]. In light of this evidence, it has been designed the ILLUMINATE 301 trial (NCT03445533), a randomized phase 3 multicenter, open-label study of intratumoral tilsotolimod (8 mg) in combination with ipilimumab (3 mg/kg) versus ipilimumab monotherapy in patients with advanced melanoma who progressed on or after anti-PD-1 therapy [52]. Results of these trials are highly expected.

## Combinations with Arginase Inhibitors

Recent studies have also demonstrated that specific enzymes in the TME are able to inhibit the immune response by limiting amino acid availability. Among them, there are two arginase isoforms (ARG1 and ARG2) that catalyze degradation of semi-essential L-arginine to L-ornithine and urea. Besides their fundamental role in the hepatic urea cycle, arginases have been shown to impair T-cell functions [47]. ARG1 is a cytosolic protein, while ARG2 is mostly located in the mitochondria. High arginase levels, either ARG1 or ARG2, have been reported in several cancer types, including breast cancer, NSCLC, head and neck squamous cell carcinoma, RCC, colorectal cancer, skin cancer, and cervical cancer. Arginases are mainly produced by MDSCs that are widely represented in the TME, and the role of ARG1-expressing MDSCs in altering T-cell responses in cancer patients has been well established. Depletion of L-arginine from the microenvironment arrests T-cell cycle progression

and inhibits IFN-γ production. Arginase activity also leads to the downregulation of the expression of MHC class II molecules essential for antigen presentation [47]. Inhibitors of arginine degradation are thus being studied as monotherapy or combination with ICI in an early-phase clinical trial (NCT02903914). In detail, this is an open-label phase 1 trial, which has evaluated INCB001158 as a single agent and in combination with pembrolizumab in patients with advanced/metastatic solid tumors, including melanoma. Patients have been enrolled into monotherapy or combination cohorts. Interestingly, this trial has enrolled melanoma patients resistant to anti-PD-1 therapy. Final results of this trial are awaited and might define the role of this combination also in metastatic melanoma treatment.

## Epigenetic-Based Combinations

Epigenetic alterations play a crucial role in cancer development and progression. Pharmacologic reversion of such alterations is feasible, and "epigenetic drugs" have demonstrated significant immunomodulatory properties, thus representing a promising strategy to overcome ICI resistance. Both DNA methylation and posttranslational histone modifications have been described to regulate the expression of different molecules of the antigen-processing and presentation machinery (APM), and to impair cellular immunity by modulating Th1 chemokines and IFN-related genes [53].

In detail, epigenetic modifications require the activity of specific cellular enzymes to be generated and maintained: DNA methyl transferases (DNMT) for DNA methylation, and the opposite activities of histone acetyl transferases (HAT)/histone deacetylases (HDAC) and histone methyltransferases (HMT)/histone demethylases in determining the status of histone acetylation and methylation, respectively. Epigenetic gene regulation is finally delivered by the cooperation of promoter DNA methylation, histone deacetylation, and by specific patterns of histone methylation that trigger chromatin condensation leading to gene silencing [53].

Epigenetic alterations are well acknowledged to be used by tumor cells to impair their immunogenicity and immune recognition. The latter occurs through the downregulation, either direct or indirect, of the expression of key molecules required for the efficient interaction of cancer cells with the host's immune system. All steps of antigen processing and presentation, including suppression of TAA expression, generation of intratumor TAA heterogeneity, downregulation of TAP1/2 and chaperone molecules, reduced MHC expression, as well as reduced levels of accessory/co-stimulatory molecules and of surface-exposed stress-induced ligands can be affected by epigenetic silencing. These molecular events finally lead to an increased uptake and immunogenic presentation of tumor antigens by professional APCs, which is compulsory for the induction of antitumor T-cell immune responses [53].

It has also been reported that epigenetic alterations can modulate Th1-type chemokines and IFN-related genes and impair CD8(+) T-cell activation and

proliferation and the cytolytic activity of human IFN-γ + T cells, which correlated with decreased antitumor responses and survival of patients with solid tumors [53].

In light of this evidence, different epigenetic drugs that can revert epigenetic modifications are developed and they are currently tested within clinical trials. Among these, the best known are DNMT inhibitors (DNMTi) and HDAC inhibitors (HDACi). However, second-generation DNMTi [e.g., guadecitabine (SGI-110)] has become more recently available, showing a higher in vivo stability and a better safety profile. The significant role of epigenetics in cancer immune escape provides a strong rationale for the use of epigenetic modifiers to improve immunologic targeting of cancer cells and to design novel clinical trials to improve immunotherapy efficacy and overcome ICI resistance. Combined treatment with the CTLA-4-blocking mAb and either first- or next-generation DNMTi5-aza-CdR or guadecitabine, respectively, significantly reduced the growth of poorly immunogenic syngeneic grafts of murine mammary carcinoma and of mesothelioma as compared to single agents [54]. Consistent with these data, combined treatment with the DNMTi 5-azacytidine, the HDACi entinostat, and ICI (anti-PD-1 and anti-CTLA-4 mAb) markedly improved survival and tumor regression in syngeneic mammary (i.e., 4T1) and colorectal (i.e., CT26) carcinoma mouse models [53].

Along this line, based on the preclinical evidence gained on the broad immunomodulatory activity of the DNA hypomethylating agents (DHAs), the proof-of-concept phase 1 NIBIT-M4 combination study has been designed to provide evidence to the immunologic and clinical activity of an epigenetic immune-sequencing strategy with CTLA-4 blockade combined with DHA in metastatic cutaneous melanoma [55].

## *Epigenetic Immune Remodeling: The NIBIT-M4 Study*

The Investigator Initiated Trial (IIT) NIBIT-M4 is a phase Ib study, sponsored by the NIBIT Foundation, that has evaluated for the first time safety, clinical and immunobiologic activities of the epigenetic priming with the second-generation DHAs, guadecitabine, followed by CTLA-4 blockade with ipilimumab in melanoma patients. In detail, patients with unresectable stage III/IV melanoma received escalating doses of guadecitabine at 30, 45, or 60 mg/m$^2$/day subcutaneously on days 1–5 every 3 weeks, followed by ipilimumab 3 mg/kg intravenously on day 1 every 3 weeks, starting 1 week after guadecitabine, for four cycles. Primary endpoints were safety, tolerability, and Maximum Tolerated Dose (MTD) of treatment; secondary were ir-DCR, ir-ORR, OS, and PFS; exploratory endpoints included the pharmacokinetic profile of guadecitabine and decitabine at cycle 1, day 1, patient-wise genome-wide DNA methylation and RNA sequencing, and analysis of the tumor immune contexture, using neoplastic samples obtained by surgical removal at baseline, week 4, and week 12. Nineteen melanoma patients were treated; 84% had grade 3/4 adverse events, and neither dose-limiting toxicities nor overlapping toxicities were observed [55]. Treatment-related AEs of any grade were observed in 18

(95%) patients, and grade 3 or 4 events in 15 (79%) patients [55]. The most common treatment-related AEs of any grade were myelotoxicity in 17 (89%) patients, and ir-AEs in 12 (63%) patients. Myelotoxicity events were grade 3 or 4 in 79% of cases and were more frequent in patients treated with guadecitabine at 60 mg/m$^2$/day; no febrile neutropenia was observed. All ir-AEs were grade 1 or 2 and were most commonly skin or gastrointestinal toxicities. No DLTs were observed at any investigated dose of guadecitabine. Treatment-related AEs and ir-AEs were generally manageable and reversible as per protocol management guidelines [55].

The ir-ORR was 26% (95% CI, 10.1–51.4) and the ir-DCR was 42% (95% CI, 21.1–66.0). At a median follow-up of 26.3 months, median PFS was 5.6 months (95% CI, 4.5–6.6) and median OS was 26.2 months (95% CI, 3.5–48.9); 1- and 2-year OS rates were 80% (95% CI, 59.2–100.0) and 56% (95% CI, 29.0–83.0), respectively [54].

Genome-scale analysis of DNA methylation of tumor samples showed a wide demethylating effect of guadecitabine during therapy in comparison with pretreatment levels. RNA sequencing data analysis displayed that immune-related pathways were mainly activated by treatment; frequent activation of pathways related to T-cell function/activation indicated intratumoral enhancement of the T-cell compartment. Even if the relative contributions of guadecitabine and ipilimumab to this finding cannot be unequivocally established, CTLA-4 blockade possibly plays an active role due to its effect on T-cell function. In turn, upregulation of HLA class I molecules described on melanoma cells in the majority of investigated tumor samples supports their specified upregulation, formerly reported in vitro and in syngeneic mouse models with various DHAs, comprising guadecitabine [55].

Tumor contexture analysis has shown an increase in median values of CD8(+) and PD-1(+) T-cell densities in tumor core specimens at week 12, but not at week 4, compared with baseline, suggesting that longer exposure to guadecitabine and ipilimumab may be required to generate high levels of tumor-infiltrating CD8(+) T cells. Notably, median values of CD8(+) and PD-1(+) T-cell densities were higher in responding compared with non-responding [55].

The comprehensive results of the NIBIT-M4 study provide initial support to the efficacy of tumor remodeling by epigenetic drugs in metastatic disease and support the notion that DHA represents ideal "partner drug" to improve the therapeutic efficacy of immune-checkpoint blockade, including their foreseeable role in reverting primary resistance to treatment [55].

## Epigenetic and ICI Combination in PD-1/PD-L1-Resistant Patients: The NIBIT-ML1 Study

The lack of adequate therapies for patients resistant to ICI therapy remains a critical unmet need in melanoma and NSCLC patients. Therefore, identifying mechanism(s) underlying treatment failure(s) and designing novel therapeutic approaches to overcome primary/secondary resistance are mandatory to improve the overall efficacy of

anti-PD-1 therapy. We have firstly demonstrated the clinical and immunological activity of the combination of ipilimumab *plus* guadecitabine in the NIBIT-M4 study. These results provided a scientific rationale to develop novel immunotherapeutic approaches combining guadecitabine with ICI in patients with primary resistance to anti PD-1/PDL-1 therapy, even due to epigenetic drugs' potential role in reverting resistance to treatment. Along this line, we have hypothesized that priming the tumor with DHA might improve the therapeutic efficacy of CTLA-4 blockade combined with anti-PD-1 in patients with MM and NSCLC resistant to PD-1 treatment; therefore, the NIBIT-ML1 study was designed. The NIBIT-ML1(NCT04250246) is a randomized, phase II study designed according to a two-stage optimal design by Simon, in unresectable Stage III or Stage IV MM (Cohort A) or NSCLC (Cohort B) patients who failed therapy with anti-PD-1/PDL-1. Primary objective of the study was immune(i) ORR according to iRECIST criteria. Secondary objectives included safety, iDCR, PFS, median OS, and survival rate at 1 and 2 years. Exploratory endpoints will investigate immuno-biologic correlates. Following a safety run-in phase in 6 subjects *per* cohort, eligible patients will be randomized to receive guadecitabine *plus* ipilimumab and nivolumab (ARM A) or ipilimumab and nivolumab (ARM B). Sample size will range from 6 to 92 patients *per* cohort [56]. The first patient first visit is foreseen in August 2020.

Additionally, initial evidence of clinical activity of epigenetic drugs in combination with ICI was reported in patients with melanoma and NSCLC who have progressed following treatment with prior PD-1 and PDL-1 blockade. In detail, preliminary results of the ENCORE-601 (NCT02437136), open-label phase Ib/II study evaluating entinostat, a HDACi, (5 mg PO weekly) plus pembrolizumab (200 mg IV Q3W) in patients with unresectable or metastatic melanoma, NSCLC, and colorectal cancer who have progressed to prior PD-1 blockade, CTLA-4 blockade, showed significant clinical activity and acceptable safety profile [57]. The confirmed objective response rate with entinostat plus pembrolizumab was 19%, while grade 3/4 related AEs occurring in >5% of patients included neutropenia, fatigue, and hyponatremia. Five patients (9%) experienced a grade 3/4 immune-related AEs (2 events of rash, 1 each of colitis, pneumonitis, and immune-related hepatitis) [57].

Results from these ongoing clinical trials might define the role of an epigenetic-based immune combination to overcome resistance to anti-PD-1 blockade in melanoma and NSCLC.

# Conclusions

The last decade has witnessed a dramatic shift in the care of cancer patients from a focus on cytotoxic therapies toward approaches that enhance antitumor immunity through IC targeting. Immunotherapy with ICI has significantly extended the survival of cancer patients, though a proportion of patients do not achieve durable disease control yet. Therefore, identifying novel mechanism(s) underlying treatment failure(s) and designing new IC-based combinations/sequences to overcome

primary/secondary resistance are mandatory to achieve the full potential of cancer immunotherapy. Along this line, given the complexity of the immune activation and the considerable variability in tumor biology across patients and tumor types, the identification of biomarkers to warrant patient selection needs to be further explored.

In summary, combined immunotherapies have undoubtedly shown significant clinical results in cancer patients, but efforts are required to identify the optimal combinations, dosages, and timing of therapy. Ongoing clinical trials will hopefully shed light on the treatment paradigm with regard to the ideal combination and sequencing of immunotherapeutic strategies.

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