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IMMUNE MODULATION OF CANCER: MECHANISMS AND RESISTANCE

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SUMMARY

The development and clinical application of immune modulation represent one of the most extraordinary achievements in the treatment of cancer. Monoclonal antibodies against checkpoint inhibitors are actually used in clinical practice, but other antibodies that stimulate co-accessory molecules or kill regulatory cells are being studied. Interestingly, some conventional chemotherapeutics and tyrosine kinase inhibitors not only kill cancer cells but also modulate the immune response against tumors in that the induced immunogenic cell death can modulate tumor microenvironment (TME) cells. However, the tumor can hijack response to treatment and can be helped by the tolerogenic nature of the TME, resulting in an unexpected resistance that allows tumor progression in many patients.

In this review, after presenting how immune cells in TME are modulated by anticancer treatments, we analyze the mechanisms responsible for primary and secondary resistance to immunomodulatory drugs highlighting the urgent need to discover ways to overcome resistance. One such way is the association of drugs that modulate the activity of immune cells in the TME, particularly when they stimulate the immune system through different mechanisms.

Key words

Immune checkpoints inhibitors; immunogenic cell death; drug combination; drug resistance; tumour microenvironment.

Impact statement

The most promising approach to overcome drug resistance in cancer is the combination of treatments performed both in a biomarker-selected mode and as a first-line treatment.

INTRODUCTION

The tumor mass is heterogeneous in composition in that it comprises not only cancer cells, but also resident and infiltrating immune cells (including macrophages and T lymphocytes), secreted pro- and anti-inflammatory factors, growth factors, and extracellular matrix proteins, collectively known as tumor microenvironment (TME). The influence of TME on cancer cells is nowadays recognized as indispensable for tumor establishment and progression. The fine interaction of cancer cells with their environment can determine whether the primary tumor is eradicated, metastasized, or established as dormant micrometastases. The nature of the TME can also shape both

therapeutic responses and resistance, justifying the recent impetus to target components of the TME, which is exemplified by the success of immune checkpoint inhibitors in the clinic (1-3).

The development and clinical application of immune checkpoint inhibitors represent one of the most extraordinary accomplishments in cancer treatment. Anti-programmed cell death protein-1 (Anti-PD-1)/anti-programmed cell death ligand-1 (anti-PD-L1) or anti-cytotoxic T lymphocyte-associated protein 4 (anti-CTLA-4) antibodies are currently attractive anti-cancer immune checkpoints inhibitors (ICI), approved as monotherapy or in combination with conventional chemotherapeutics in different kinds of cancer such as melanoma, non-small cell lung cancer, head and neck squamous cell carcinoma, renal cell carcinoma and Hodgkin lymphoma (4). However, the overall response rate to ICIs is observed in less than one-third of the patients due to the establishment of primary or acquired resistance (4).

The mechanisms of immune escape and drug resistance in tumor immunotherapy include tumor cell-internal and external factors. The internal factors, determined by tumor immunoediting and epigenetic changes, include 1) loss of tumor antigens, 2) intratumor heterogeneity, 3) defects in antigen presentation, 4) increased expression of constitutive PD-L1. The external factors concern cells, structure, and factors of the TME, including 1) structural organization and, 2) several immune cell types favoring immunosuppression, including regulatory T cells (Treg) and tumor-associated macrophages; 3) lack of natural killer (NK) cells, activated $CD8^+$ (CTL) cells and conventional $CD4^+$ T cells or their anergy/exhaustion; 4) non-tumoral, non-immune cells (such as cancer-associated fibroblasts and endothelial cells); 5) tumor metabolites in the immune microenvironment; 6) intestinal microflora (5).

Conventional cancer treatment and target therapy, such as kinase inhibitors act mainly through cytotoxic mechanisms but it was recently shown their immune potentiating mechanisms of action (6). In particular, certain chemotherapeutic drugs trigger immunogenic cell death (ICD), a form of regulated cell death that is sufficient to activate an adaptive immune response in an immunocompetent setting, thus eliciting long-term efficacy of anticancer drugs by combining direct cancer cell killing and antitumor immunity (6). In other experimental models, antitumor drugs kill cells of the TME, such as Tregs.

In the present review, we focus on the effects of older standard-of-care antitumor treatment and target therapy on TME immune cells, the immunomodulating effects of ICI and antibodies binding co-activating molecules. In addition, an overview of mechanisms of primary resistance to immunomodulating agents is reported.

IMMUNOGENIC CELLULAR DEATH DUE TO CONVENTIONAL ANTITUMOR TREATMENT AND TARGET THERAPY

ICD is an emerging type of cancer cell death triggered by several drugs. This type of cell death is preceded by premortem stress responses and emission of signals, called damage-associated molecular patterns (DAMPs), from cancer cells that render them detectable for the immune system. Among those signals, cells release ATP as well as an endoplasmic reticulum (ER) stress response inducing exposure of calreticulin (CRT) and heat-shock proteins (HSP70 and HSP90), the release of high-mobility group box-1 (HMGB1), type I IFNs, and members of the IL-1 cytokine family. Those molecules represent "find me" and "eat me" signals to phagocytic cells of the immune system, which can allow the immune recognition of cancer cells and reverse immunoevasion. Accordingly, subcutaneous injection of dying tumor cells undergoing ICD to animal models has been found to lead to anticancer vaccine effect (7).

Several findings indicate a complex interplay between traditional anticancer drugs and the anticancer immune response. Chemothera-

peutic agents like anthracyclines, oxaliplatin and cyclophosphamide are known ICD inducers and exert part of their therapeutic effects through immune-dependent mechanisms (8, 9) (**figure 1**). Similarly, natural compuonds (*i.e.* antracyclines, vinca alkaloids, taxanes) and their semi- or synthetic derivatives can induce ICD due to their activity on altering the stability of microtubules (10), leading to the release of DAMPs, responsible for the increased anti-tumor immunogenicity (11). To note, the induction of ICD underlies their prolonged clinical responsiveness even after treatment discontinuation due to the induction of an antitumor immune response following ICD (12).

Immunogenic Cell death (ICD) by anthracyclines

Anthracyclines induce both cytostatic and cytotoxic effects on cancer cells. They are able to lead cancer cells not only to apoptosis but also to ICD (7, 8). Pro-apoptotic caspase activation seems to play a critical role in the immune response, as demonstrated by the evidence that caspase inhibition through the broad-spectrum caspase inhibitor Z-VAD-fmk abrogated the immunogenicity of doxorubicin-induced cell death in mice (9). A relevant observation is that the immunogenic effects of doxorubicin-treated cancer cells were recorded in the absence of any adjuvant or costimulus (8, 9).

Induction of ICD by a doxorubicin-liposome-microbubble complex was observed both in vitro and in vivo. The complex induced apoptosis, membrane exposure, and release of ER stress-derived factors and DAMPs in lung and colon cancer cells and increased the maturation of murine myeloid (mDCs) and plasmacytoid dendritic cells (pDCs). In particular, Terlizzi and coauthors found that the administration of doxorubicin in a mouse model of lung cancer induced tumor cell death associated with a higher influx of pDCs to the lung,

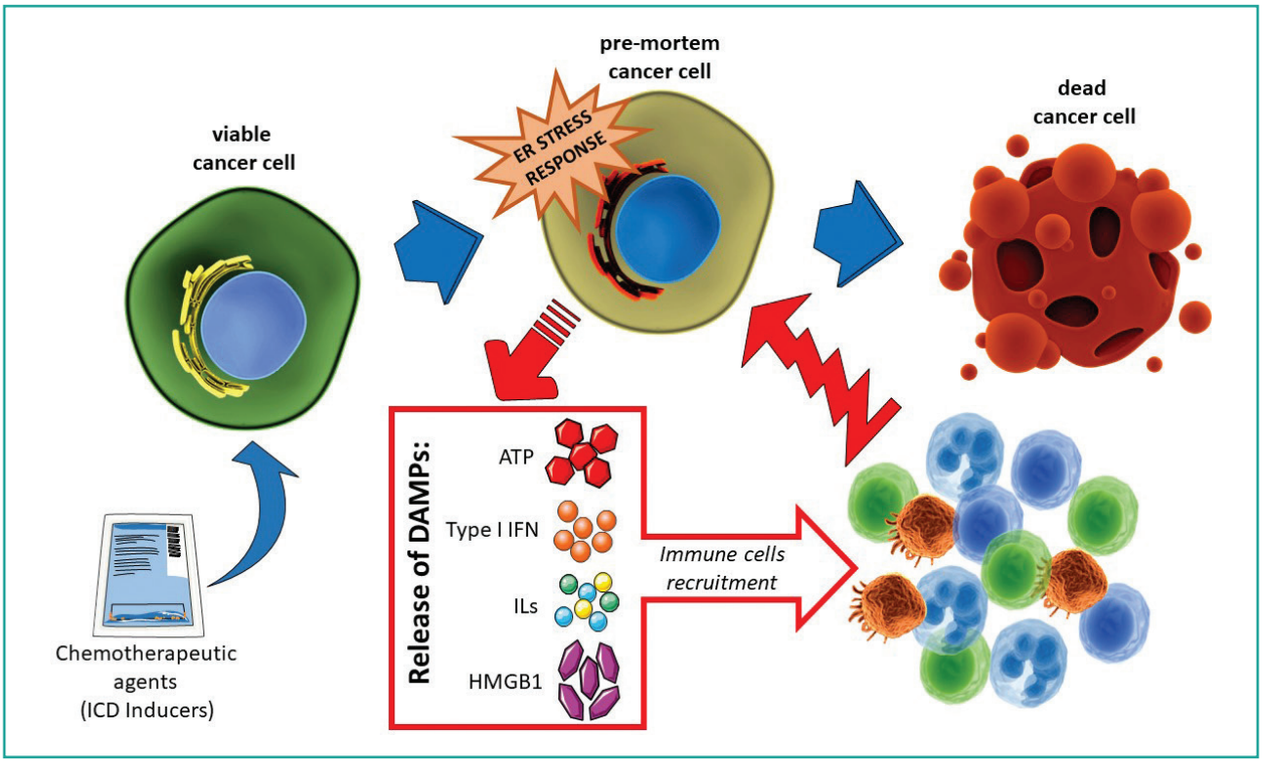

Figure 1. The main mechanism of action of chemotherapeutic drugs acting as ICD inducers. Treatment of tumors with ICD inducers can promote endoplasmic reticulum (ER) stress and the release of DAMPs (*i.e.* ATP, type I IFN, ILs, and HMGB1) from dying tumor cells, strengthening the cytotoxic activity of the immune system.

which had higher levels of MHC I and MHC II that well correlated with the higher proliferation rate of $CD4^+$ and $CD8^+$ T cells compared with the control group of mice (13). Besides, treatment with the doxorubicin-liposome-microbubble complex increased the percentage of IFN-γ-producing $CD8^+$ T cells and reduced the percentage of $CD4^+CD25^+FOXP3^+$ Treg cells in tumor tissues from mice vaccinated with complex-treated tumor cells compared to mice vaccinated with only microbubble (14).
Cancer cells undergoing ICD can activate the immune system not only by DAMPS emission but also by mimicking pathogen-induced host defense response. As an example, doxorubicin-induced dead cells release dsRNA. dsRNA interacts with TLR3 and leads to the release of type I IFN cytokines (15). The activation of TLR3 and the ensuing type I IFN signature in DCs are responsible for lung metastasis arrest (16).

Immunogenic Cell death (ICD) by alkylating agents, tyrosine kinase inhibitors, and other antitumor drugs

Another classical antitumor drug endowed with the ability to induce ICD is cyclophosphamide. This alkylating agent (100 mg/kg) has been found to induce the hallmarks of ICD in EG7 lymphoma cell-bearing mice. In particular, it led to the expansion of $CD8\alpha^+$ dendritic cells and to dead cells able of favoring tumor infiltration and $CD8^+$ T cell cross-priming by $CD8\alpha^+$ dendritic cells (17).
Despite the similarities between oxaliplatin and cisplatin, only oxaliplatin has been identified as an ICD inducer (18). Cisplatin induces HMGB1 release, but it was unable to provoke CRT exposure and anticancer vaccination (19). The reason for the different activity of oxaliplatin and cisplatin is not clear at all. Thus, it has been postulated that the difference in their ability to induce ICD may reside in the interaction with non-DNA targets, such as proteins. Due to its higher hydrosolubility, oxaliplatin can have higher interaction with proteins than cisplatin (20). This aspect is important since ER stress and exposure of CRT originate from damaged protein accumulation in the cell (20, 21).
Recent studies report that several tyrosine kinase inhibitors (TKI) are inducers of ICD. Crizotinib is a TKI approved for the treatment of tumors carrying activated tyrosine kinases such as ALK, MET, or ROS1 (22, 23), which clinical efficacy is due to immune mechanisms as well. Indeed, crizotinib induced CRT exposure and ATP and HMGB1 release in different cancer cell lines. Interestingly, the combination of high-dose crizotinib (10 μM) with a non-ICD inducer like cisplatin (150 μM) triggered ICD in non-small cell lung carcinoma (NSCLC) cells and inhibited the growth of carcinogen- or oncogene-induced orthotopic NSCLC models through the increased T lymphocyte infiltration of tumors. Besides, the association boosted the expression of PD-1 and PD-L1 and strongly sensitized NSCLC to PD-1 antibodies (24).
Pt-N-heterocyclic carbene (NHC), a novel anticancer metal compound, is an ICD inducer: it triggers CRT exposure, ATP secretion, extracellular HMGB1 release, and ROS-mediated ER stress (25). Since ER stress plays a critical role in all the scenarios where ICD occurs, it is not surprising that proteasome inhibitors such as bortezomib, which provokes a potent ER stress response, have been found to induce ICD and elicit effective antitumor immunity (22, 23).
Remarkably, there are more than 50 clinical trials currently exploring the induction of ICD by anticancer drugs in various types of tumors. The involved drugs are anthracyclines, oxaliplatin, cyclophosphamide, and bortezomib (26). These data highlight the interest in identifying ICD-based optimal treatment interventions to improve cancer management.

TREG KILLING BY STANDARD ANTITUMOR TREATMENT AND TARGET THERAPY

ICD is not the only effect of standard chemotherapeutic agents on immune cells present in the TME. Even before the description of

thymus-derived Treg cells (tTregs) (27), some papers demonstrated the effect of cyclophosphamide on the immune system (27, 28). In the early 2000s, several authors described the deleterious role of tTreg in TME and the effects of anti-cancer drugs on tTregs. In particular, cyclophosphamide, 5-fluorouracil, and neo-adjuvant treatment of patients with breast cancer using drugs such as anthracyclines, cyclophosphamide, and 5-fluorouracil, had cytotoxic effects on tTreg in the TME (29-33). However, the effects of cyclophosphamide are dose-dependent, since at high doses even conventional $CD4^+$ T cells are killed (30). More recently, the role of selective depletion of tTregs by aromatase inhibitors, docetaxel, fludarabine, and gemcitabine has been described (34-36). Finally, some target therapies, including TKIs such as sunitinib, sorafenib, and imatinib, have been reported to downregulate the suppressive activity of tTregs.

IMMUNOMODULATING EFFECTS OF IMMUNE CHECKPOINT INHIBITORS (ICIS) AND ANTIBODIES BINDING CO-ACTIVATING MOLECULES

A promising anticancer strategy consists of the use of immunomodulating agents, which have as their ultimate goal the activation of the patient's immune system against the tumor, eliciting the activation of TME resident CTLs and NK cells. To achieve this goal, immunomodulating agents may act on immune components of the TME in several ways.

Break the brakes of the immune system: the immune checkpoint inhibitors

In the last decades, many ICIs have been developed against CTLA-4 and PD-1 systems, and, in the last ten years, some of them have been approved for clinical practice, such as CTLA-4 antagonist ipilimumab monoclonal antibody (mAb) (2011), PD-1 antagonists pembrolizumab (2014), nivolumab (2014), and cemiplimab (2018) and PD-L1 antagonists atezolizumab (2016), avelumab (2017), and durvalumab (2017) (37-43). Moreover, mAbs against other immune checkpoint molecules, such as TIGIT, VISTA, TIM-3, and LAG3 are under study (44-47).

The immune checkpoints are the main switches of the immune system and, when stimulated, can restrain the immune responses to an immunologic stimulus (48). Activation of these receptors is one of the primary mechanisms by which several tumors defend themselves from the host immune system (49). Therefore, the use of mAbs hampering the effect of the inhibitory checkpoints has improved the prognosis of some cancers, with particular reference to some lucky patients that achieve a complete response (50, 51). The clinical success of such mAbs represented the proof of concept that appropriate modulation of the TME cures cancer without a treatment directly promoting tumor cell killing.

The actual clinical efficacy of these drugs is still unsatisfactory because of the low response rate (52) and the presence of adverse effects, not only due to the hamper of the immune system but also to some off-target effects (53). These adverse effects my be occasionally severe and life-threatening (53).

Awake the immune response: the use of agonist antibodies

When the balance between stimulatory and inhibitory signals is in favor of the stimulatory ones, CTLs are activated and exert their function. Agonist mAbs trigger costimulatory receptors expressed by $CD4^+$ conventional T cells and CTL resident in the TME, providing costimulatory signals able to defeat the immunosuppressive signals and intensifying effector cell function against the tumor cells.

4-1BB, OX40, GITR, CD27, ICOS, and CD40 are the actively studied costimulatory targets (54-59). However, to date, no mAb is approved for clinical use. In our opinion, three aspects have slowed their entry into the clinic: 1) the safety issue; 2) the difficulty in preparing true agonist mAbs (60); 3) their expression in

cells different from NKs and CTLs, such as, for example, Tregs.
Regarding safety, we must remember that treatment with agonist antibodies may determine a generalized immune system activation in the brief and long term. A severe warning is the development of a severe cytokine storm as it occurred for six healthy volunteers who died during phase I clinical trial using an anti-CD28 agonist mAb (61). The other crucial issue in using mAbs that target costimulatory molecules concerns the expression of the same markers by Treg cells and other cells of the TME. Therefore, while boosting the anti-cancer activity of CTLs and NK cells, agonist mAbs may increase the number and/or activity of Treg cells and other cells of the TME with suppressive activity, at least in the long term, vanishing the antitumor effect of the treatment.

Reverse the immunosuppression: annihilate Treg cells

It is well known that Treg cells play a crucial role in supporting tumor growth by providing a suppressive TME, mainly due to the expression of suppressive cytokines. Therefore, killing the tumor-resident Tregs may represent an effective therapeutic approach, particularly in heavily immune-infiltrated cancers (hot tumors). Antibody-dependent cell-mediated cytotoxicity (ADCC), and antibody-dependent cellular phagocytosis (ADCP) by which myeloid and NK cells eliminate mAb-coated cells (62, 63), are two mechanisms that can be exploited to achieve this goal. The activation of ADCC/ADCP requires the binding of the mAb to both the cell marker and the activating Fcγ receptors (aFcγRs) expressed on myeloid and NK cells, which are induced to activation with an ensuing killing of mAb-coated cells. This is the way by which some therapeutic mAbs (*e.g.*, rituximab or trastuzumab) kill tumor cells (64), ipilimumab kills Tregs in humans (65, 66), and mAbs against GITR and OX40 kill Tregs, exerting anti-tumor activity in preclinical studies (67, 68).
The efficacy and safety of such an approach may be interesting. Indeed, some papers suggest that the effect of such treatments may be tumor-centered (69, 70), considering that ADCC/ADCP is elicited only if myeloid and NK cells are next to mAb-coated cells (68) and that aFcγR-expressing cells are present at very low levels in lymph nodes.
The crucial point to fully exploit this approach concerns the choice of the target that must be expressed at high levels on Tregs and low levels on the other cells of the TME that participate in tumor rejection. Many studies demonstrated that the markers of tumor-infiltrating Tregs are different according to the type of cancer (71) and that several subpopulations of Tregs are tissue- and TME-specific (72-76).

MECHANISM(S) OF PRIMARY RESISTANCE TO IMMUNOMODULATING AGENTS

As mentioned before, several patients do not respond to ICI treatment (primary resistance), and others undergo acquired resistance in that long-term establishment/development of specific cellular/molecular mechanisms render the patient refractory. ICI resistance is multi-factorial and may be induced by genetic and epigenetic changes in cancer cells or by immune cells that are recruited or populate the TME.

Primary resistance

Patients who have primary resistance to ICIs do not respond to the first-line treatment. Several mechanisms have been proposed uncovering both tumor cell-intrinsic and extrinsic factors.
The most reasonable mechanisms underlying tumor cell-intrinsic changes are described by the absence of antigenic proteins, antigen presentation, and genetic T cell exclusion. The lack of tumor antigens cross-presented to T cells is primarily due to a low tumor mutational burden (TMB) (77, 78), which renders tumor patients eligible for the sole conventional chemotherapy. To note, though, in some clinical trials, regardless of the low grade of PD-L1

staining, patients were still treated with ICIs and showed benefit from therapy, implying novel still unknown molecular mechanism/s underlying ICI activity.

Alternatively, the genetic alterations of the antigen-presenting machinery which include MHC silencing, beta-2 microglobulin (B2M) loss, and transporter associated with antigen processing (TAP) gene mutation (79) are responsible for the primary resistance to all ICIs, mechanisms that can evolve into therapeutic ineffectiveness due to the incapability of APCs to educate the adaptive immune system against the tumor cells. This phenomenon is better known as adaptive resistance. Other mechanisms that have been proposed for ICI adaptive resistance are:

1. induction of IL-8 and VEGF in a MAPK-dependent manner after oncogenic signaling, culminating in the inhibitory effect of IL-8 on T cell recruitment and function (80);
2. reduced activity of IFNγ and granzyme B together with reduced $CD8^+$ T cell infiltration due to PTEN loss or gene mutation (81);
3. a higher expression of PD-L1, PD-L2, and IFNγ receptor, associated with PTEN deletion, PI3K/Akt mutations, EGFR mutations, Myc overexpression, and CDK5 disruption. In this case, the alteration in IFNγ receptor signaling, as in the case of JAK1/2 and STAT3-dependent downstream signaling, gives an advantage to tumor cells to escape, which according to the ensuing downregulation of PD-L1 due to overstimulation by IFNγ, can evade the mechanism of action of the pharmacological tool (80). Alternatively, the expression of two splicing-derived PD-L1 variants, which lack the transmembrane domain, can mask the efficacy of atezolizumab and durvalumab as they can behave as a decoy (82);
4. another molecular mechanism underlying primary resistance is the enrichment of genes overexpressed in anti-PD-1 treated patients. These genes have been identified as IPRES (innate anti-PD-1 resistance signature) (83) and are correlated to ICI-related non-responsiveness due to epithelial-mesenchymal transformation, stemness in tumors, aberrant activation of cell proliferation genes (*i.e.*, Myc), and expression of other immune checkpoints (*i.e.*, LAG-3, TIM-3).

Primary resistance to ICIs is also explained by tumor cell-extrinsic mechanisms that favor tumor immune evasion according to the recruitment of Treg, myeloid-derived suppressor cells (MDSCs), and M2 macrophages to the TME. Indeed, the blockade of the CTLA-4 and/or PD-1/PD-L1 axis could be overcome by the presence of other immunosuppressive cells, such as MDSC and M2 macrophages. Arginase-1, indolamine-2,3-deoxygenase (IDO), IL-33, iNOS activity, CCL2, associated with higher expression of IFNγ, following the treatment with ICIs, may additionally promote the expression of immunosuppressive molecules in tumor cells and in the same M2 and MDSCs that can contribute to peripheral tolerance and have a direct negative effect on effector T-cell function (84). Similarly, IFNγ-induced carcinoembryonic antigen cell adhesion molecule-1 (CEACAM1) release can act as another inhibitory molecule in that it can inhibit T-cell-APC interaction, inhibit B cells, and NK cytotoxicity (85). Therapeutic antibodies blocking CEACAM1 and TIM-3, another immune-checkpoint that is overexpressed after ICI treatment (acquired resistance), have resulted in enhanced anti-tumor immune responses (86, 87).

In this scenario, other biomarkers in both the TME and immune cells have been identified as opposing to ICI effectiveness. Increased levels of the immunosuppressive and pro-fibrotic TGFβ as well as CD73, a soluble and membrane enzyme that converts AMP into adenosine that can interact with the pro-tumor receptors A2A and A2B, are recognized as potential biomarkers, which higher levels are predictive of bad prognosis after ICI treatment. Of relevance, tumor-associated cytokines and chemokines, such as CCL5, CCL7, and CXCL8, bind to their receptors CCR1 or CXCR2 expressed on

MDSCs (88), recruited to the TME, and can further subvert the effectiveness of ICIs. Similarly, CCR4, a receptor for the chemokine CXCL12, is highly expressed by the immunosuppressive Tregs (89). Indeed, the use of monoclonal antibodies against CCR4 has proved to reduce Treg recruitment as well as to promote ADCC, facilitating Treg apoptosis (90).

Figure 2 summarizes the factors involved in ICI primary resistance.

Acquired resistance

In paragraphs 2 and 3 we have reported the immunomodulatory effects of standard chemotherapeutic drugs. However, it has been demonstrated that the homeostatic response of the tumor may counteract these effects. For example, carboplatin, doxorubicin, gemcitabine, and paclitaxel induce a relevant upregulation of PD-L1, CD47, and CD73 on tumor cells (91). CD47 enables cancer cells to evade killing macrophages, whereas CD73 and PD-L1 inhibit T cell activation, directly or indirectly (92). The upregulation of immunosuppressive molecules is determined by hypoxia-inducible factors (HIFs), implicated in the regulation of innate and adaptive immunity (93).

Several homeostatic responses have been described in response to ICIs. Indeed, one-third of ICI-treated patients develop acquired resistance that occurs after a variable period of treatment. The mechanisms potentially determining relapse include loss of T cell function, lack of T cell recognition by downregulation of tumor antigen presentation, and development of escape mutation variants in cancer cells. These mechanisms are similar to those observed for primary resistance, although they occur at a later time point after the pharmacological treatment. The genetic alteration of HLA class I due to a truncating mutation in B2M (94) and loss-of-function of JAK1/JAK2, which facilitates cancer cells to escape IFNγ anti-proliferative activity, may underlie genetic

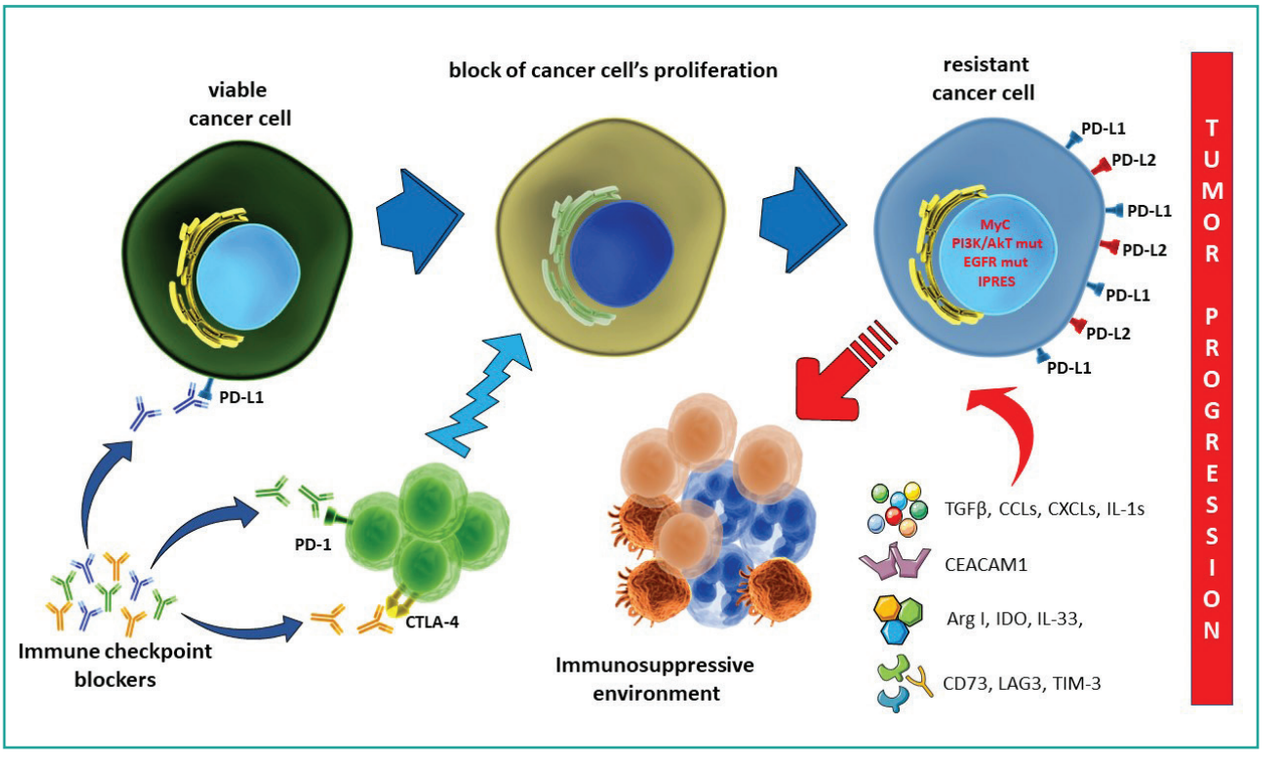

Figure 2. Main cellular factors involved in ICI primary resistance. The blockade of immune-checkpoints (*i.e.* CTLA-4, PD-1, or PD-L1) through ICIs fosters the cytotoxic activity of the immune system. However, circumventing mechanism/s can promote the expression of other immunosuppressive mediators to favor tumor progression.

mechanisms that alter the antigen-presenting machinery and IFNγ signaling.

Besides genetic and epigenetic alterations, other molecular mechanisms of acquired resistance have been proposed. SHP-2, a ubiquitous tyrosine phosphatase protein, is described as an oncogene in that it regulates cancer cell survival and proliferation via the activation of the RAS-ERK signaling pathway. Indeed, k-Ras mutated tumor cells require SHP-2 for their growth. At the same time, SHP2 is activated downstream PD-1 activation in T cells, whereas in NK cells it is downstream IFNγ-induced activation of STAT-1 phosphorylation, resulting in suppression of the anti-tumor immunity (95). In addition, oncogenic Ras signaling via MEK increases PD-L1 mRNA stability, opposing to the therapy (96).

In nivolumab-treated lung cancer patients with low $CD8^+$ T and high TGF-β1 cells have a poorer prognosis than those who present lower levels of TGFβ1 (97). Another mechanism of acquired resistance to ICIs correlated to poor prognosis is the expression of CEACAM-1, which forms a heterodimer with TIM-3 in human and murine $CD4^+$ T cells, favoring the immunosuppressive pro-tumor arm (97). Very recently, Zhang *et al.* demonstrated that aneuploid circulating tumor endothelial cells (CTECs) are characterized by PD-L1 expression, which interacts with the systemic ICIs and acts as a decoy that masks and blocks ICI pharmacological activity in the tumor site, allowing tumor immune escape (98). To note, more than half of the advanced-stage tumor patients who are not responsive to ICIs develop anti-drug antibodies (ADAs). The reported frequency of ADA-positive patients following treatment with immune checkpoint inhibitors varies from as low as 1.5% for pembrolizumab to 54% for atezolizumab (99).

In conclusion, although the introduction of ICIs has revolutionized the oncological armamentarium, many cellular and molecular mechanisms underlying primary and acquired resistance still need further investigation, which could give diagnostic tools to predict the response rate/prognosis and, more importantly, set up treatments avoiding resistance.

THE NEW CHALLENGE: FINDING SYNERGIC TREATMENTS AGAINST APPROPRIATE TUMOR MICROENVIRONMENT (TME)

In the TME the immunosuppressive signals evolve during tumor development, due to immunoediting and immune subversion. The immunosuppressive microenvironment is so firmly established that the immunomodulating treatment must not only favor the reversal of immune suppression but also resist the homeostatic attempt of the tumor to maintain the immunosuppressive microenvironment.

If conventional antitumor treatments and target therapy have as the primary aim to kill tumor cells and the effects of these drugs on TME immune cells can be considered as an add-on effect synergizing with the first one, ICIs aim to promote tumor rejection by the immune system. If treatment does not reach the aim, it fails. Therefore, ICIs and other immunomodulating approaches must be used to treat primary sensitive tumors in appropriate patients. Indeed, several pieces of evidence suggest that the TME not only is peculiar to each tumor type but also is peculiar to each patient (100). In this context, despite some interesting results linking PD-L1 expression with tumor response, more studies are needed to find biomarkers of sensitivity and resistance to immunotherapy reaching a personalized approach. In a recently published article (101), Hegde and Chen have identified the key points to be explored to improve the effectiveness of ICIs, highlighting the need to deepen our knowledge regarding the organ- and patient-specific features of the tumor immune infiltrate as well as the molecular and cellular regulators of primary and secondary immune escape processes. Old and new approaches, including tumor immunostaining, immunophenotyping of tumor infiltrate and bioinformatic analysis must participate in find-

ing the best approach for a patient. In the context of bioinformatic analysis, the use of reliable cell signatures may help in defining the characteristic of hot tumors, considered to be the most responsive to immunomodulating treatments (102).

It is reasonable to suppose that combining different immunostimulatory treatments is a promising approach. However, as reviewed by Meric-Bernstam et al. despite the hundreds of combinations that have been tested only a few treatments show synergic effects (103). This finding suggests that the combination of treatments must be set on a solid rational basis. Despite the demonstration that antibodies modulating the PD-1/PD-L1 system improve their effects in some tumors when combined with antibodies targeting CTLA-4, we believe that combining different strategies to potentiate the immunostimulating effect of the treatment is the most promising approach. In our opinion, the combination of treatments performed in a biomarker-selected mode and performed as a first-line treatment can increase the likelihood of response. In particular, the association of ICI and another immunomodulating agent, such as conventional chemotherapeutics, may offer more chances of success by acting on different components of the TME, avoiding more serious adverse effects which are usually observed when combining drugs with a similar mechanism of action (104, 105).

CURRENT UNDERSTANDING AND FUTURE DIRECTIONS

The immunomodulation of cancer has so far exploited its clinical effectiveness in the usage of ICIs, but occurrence of both primary and acquired resistance along with immune-related adverse events (irAEs) has limited their clinical application.

The actual guidelines for using ICIs as first-line treatment is based upon the expression of the immune checkpoints (*i.e.* PD-1/PD-L1 and CTLA-4) that are actually druggable and which pharmacologically inhibition is approved by both the European Medicinal Agency (EMA) and Food and Drug Administration (FDA). Other mAbs that block different immune checkpoints (*i.e.* LAG-3, CD-73, *etc.*) are actually under investigation to clinically validate them either when the expression of PD-1/PD-L1axis is elusive/too low or to overcome the ICI's resistance, thus proposing them as alternative approaches in second-line treatment.

So far, the alternative approaches to bypass ICI's primary or secondary resistance could be considered as in the following points:

1. modification of the phenotype of the immune cells that are recruited or populate the TME by using agonist antibodies (*i.e.* CD28), which unfortunately present discordant efficiency;
2. elicit the phenomenon of ADCC/ADCP by ensuring both the inhibition of the biological immune target and the induction to death of mAb-coated tumor cells via the recognition of the Fc portion of the Ig by myeloid and NK cells. This aspect needs further evaluation according to the potential cytotoxicity on T effector cells;
3. render the tumor a "hot" tumor by using conventional chemotherapeutics (i.e. anthracyclines, paclitaxel, oxaliplatin, 5-FU, *etc.*) in order to promote ICD that could synergize with ICIs. In this respect, the release of DAMPs could increase the immune response boosting ICIs' activity.

Our current understanding is that both the immune system at first line and then the entire TME as second line can be pharmacologically manipulated in order to achieve the desired clinical effectiveness in terms of lifestyle and overall survival of cancer patients. However, both irAEs and secondary resistance have to be taken into consideration. Aware of these phenomena, several ongoing studies are taking into consideration differential approaches. For example, preclinical evidence highlighted that resistance to combinatorial immune checkpoint blockade could

be bypassed by inhibiting the chronic interferon response (103). Other future approaches stand in the manipulation of the microbiome, actually well known for colon cancer and melanoma patients, herein defined as "good bacteria" (*i.e. Akkermansia muciniphila* or *Faecalibacterium genus*), which can enhance anti-tumour efficacy with immunotherapy combination.

Another approach could be the modulation of the innate immune system, which actually represents a limiting factor for mounting an effective adaptive anti-tumor immune response. The activation of TLR7 or TLR9 or the stimulator of interferon genes (STING) pathway are actually being investigated (106). In the latter case, the activtion of STING underlies the activation of the enzyme cGAS which recognizes citoplasmic DNA, which could derive by conventional chemotherapeutic-induced ICD. The activation of STING increases type I IFN which on the other side could lead to higher PD-L1 expression in both tumor and immune cells, allowing in this way the pharmacological activity and efficiency of ICIs. In support, preclinical studies proved that the injection of a STING agonist in combination with PD-L1 blockade and OX40 activation resulted in tumor arrest/regression due to the ensuing break of the immunotolerant arm, rendering STING agonist/s potential immune adjuvant (103, 104), similarly to the activity of "older" drugs, such as paclitaxel.

In our opinion, future directions include both re-purposing of "older" drugs or targeting novel immune-related pathways (*i.e.* STING or microbiome) to boost ICIs' clinical effectiveness.

CONCLUSIONS

The introduction of ICIs as well as the combination of ICIs with conventional chemotherapeutics or ICD inducers has the merit not only to ameliorate clinical outcomes for cancer patients, but also to evidence that preclinical and clinical data, these two defined as pre-marketing phases, together with the post-marketing data (which has highlighted in the case of ICIs the phenomenon of clinical resistance) need to stay continuously bound either to re-purpose "older" drugs or to target novel pathways. In this context, defined that clinical trials remain the gold standard to study new therapeutic strategies, we would like to underline that the real word evidence (RWE) is necessary to integrate the evidence generated in the pre-marketing (pre-clinical and clinical) setting/s (107, 108). Therefore, in the oncological field as well as in other pathologies, the generation of RWE in the post-marketing phase represents an arm to complete and verify experimental studies especially because of a different sample dimension that could explore different outcomes compared to the clinical trials. The origin of this opinion comes from the heterogenous nature of cancer cells in that once resistance to a first-line treatment is developed, it can be overcome by alternative options that can easily derive by the interconnection between the RWE and the preclinical settings, helping to move towards future therapeutic options/directions either by re-purposing or combining already known anti-tumor agents or by investigating novel pathways.

CONFLICT OF INTERESTS

The authors declare that they have no conflict of interests.

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