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Molecular basis and rationale for combining immune checkpoint inhibitors with chemotherapy in non-small cell lung cancer

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ABSTRACT

Immunotherapy has prompted a paradigm shift in advanced non-small cell lung cancer (NSCLC) treatment, by demonstrating superior efficacy to chemotherapy alone both in second- and in first-line setting. Novel insights on molecular mechanisms and regimens to enhance the efficacy of immunotherapy are warranted, as only a minority of patients (~20%) respond to checkpoint blockade. Taking into account the multiple mechanisms adopted by tumor cells to evade the immune system through cancer immunoediting, the frontline combination of immune checkpoint inhibitors with chemotherapy appears to be a successful strategy as: 1) it enhances the recognition and elimination of tumor cells by the host immune system (immunogenic cell-death), and 2) it reduces the immunosuppressive tumor microenvironment. Remarkably, the immune checkpoint inhibitors pembrolizumab and atezolizumab have already been approved by the FDA in combination with chemotherapy for the first-line treatment of advanced NSCLC and many other chemo-immunotherapeutic regimens have been evaluated as an initial therapeutic approach in metastatic NSCLC. Concurrently, several preclinical studies are evaluating the molecular mechanisms underlying immunomodulation by conventional chemotherapeutic agents (platinum salts, antimitotic agents, antimetabolites and anthracyclines), unraveling drug- and dose/schedule-dependent effects on the immune system that should be exploited to achieve synergistic clinical activity. The current review provides a detailed overview of the immunobiological rationale and molecular basis for combining immune checkpoint inhibitors with chemotherapy for the treatment of advanced NSCLC. Moreover, current evidence and future perspectives towards a better selection of patients who are more likely to benefit from chemo-immunotherapy combinations are discussed.

Introduction

Lung cancer is a major contributor to cancer-related deaths (Siegel et al., 2018). Worldwide, non-small cell lung cancer (NSCLC) accounts for 80–85% of all lung cancer cases. Despite the development and use of innovative treatments, the estimated 5-year survival rate of metastatic NSCLC remains only 18% (Siegel et al., 2018). Both histologically and molecularly, NSCLC comprises a group of heterogeneous diseases (Inamura, 2017). Histological features, molecular characteristics, and tumor stage determine the first-line therapeutic approach, which consists of surgery with a curative intent only in the presence of a localized disease (Reck and Rabe, 2017). However, the majority of NSCLC cases are diagnosed at an incurable advanced state with a dramatic impact on patient prognosis. The molecular background of these tumors may vary considerably, even within the same subtype. Several molecular alterations, defined as driver mutations, such as mutations in epidermal growth factor receptor (EGFR), Kirsten rat sarcoma (KRAS) and translocations in genes encoding for anaplastic lymphoma kinase (ALK)

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(Reungwetwattana et al., 2012), have been recognized.

Approximately 85–90% of advanced NSCLC patients have no oncogenic alterations suitable for a targeted treatment. Before the advent of immunotherapy and immune check point inhibitors (ICI) (Kon and Benhar, 2019), the standard of care was systemic doublet platinum-based chemotherapy, when feasible (Hellmann et al., 2016; Reck and Rabe, 2017). Unfortunately, the outcome of standard chemotherapeutics are underwhelming, as durable disease control is rarely achieved (Lazzari et al., 2018; Sui et al., 2018). Indeed, the clinical efficacy of chemotherapy is limited by either intrinsic or acquired drug resistance (Huang et al., 2017; Maione et al., 2015). Although a complete understanding of drug resistance in NSCLC is still lacking, some key mechanisms have been identified and it has been showed that additional mutations in tumor-driving genes may lead to drug insensitivity and tumor progression after treatment. Thus, surmounting drug resistance (Bar-Zeev et al., 2017; Cui et al., 2018; Livney and Assaraf, 2013; Mudduluru et al., 2016; Shapira et al., 2011; Zhitomirsky and Assaraf, 2016) remains an open challenge and underscores the urgent need for innovative treatment strategies for NSCLC.

Recent breakthroughs brought about the development of cancer immunotherapy have dramatically changed the treatment algorithm of NSCLC patients, significantly improving their prognosis (Attili et al., 2017; Meyers et al., 2018; Rebuzzi et al., 2019). It is now recognized that the immune system plays a central role in the control of tumor growth and progression, a process known as cancer immunoediting (Hanahan and Weinberg, 2011; Vesely et al., 2011). Cancer cells are able to evade immune destruction by dysregulating the host immune response (Hanahan and Weinberg, 2011; Kon and Benhar, 2019). Specifically, the activation of immune checkpoints including programmed death 1 (PD-1), PD ligand 1 (PD-L1) or cytotoxic T cell lymphocyte antigen-4 (CTLA-4), originally evolved to prevent T cells from attacking healthy tissues, may lead to inhibition of T cell signaling and contribute to the generation of an immunosuppressive tumor microenvironment (TME) (Meyers et al., 2018). The key role of ICI, targeting either the PD-1/PD-L1 axis and CTLA4, relies on the recognition and elimination of tumor cells by the host immune system itself, by turning on an effective antitumor immune response (Hoos, 2016). Following the very promising results of different clinical trials, two PD-1 inhibitors (nivolumab and pembrolizumab) and one PD-L1-targeted antibody (atezolizumab), have been introduced in the therapeutic landscape of metastatic NSCLC (“NCCN Clinical Practice Guidelines in Oncology (NCCN Guidelines®) - Non-Small Cell Lung Cancer - Version 2.2019,” 2019NCCN Clinical Practice Guidelines in Oncology (NCCN Guidelines®) - Non-Small Cell Lung Cancer - Version 2, 2019“NCCN Clinical Practice Guidelines in Oncology (NCCN Guidelines®) - Non-Small Cell Lung Cancer - Version 2.2019,” 2019). In addition, the effectiveness of the CTLA4 inhibitor ipilimumab, which has already been approved for the treatment of advanced melanoma, is currently under intense investigation also in NSCLC (Lazzari et al., 2018). However, since only a limited fraction of NSCLC patients will benefit from the ICI treatment as monotherapy (Califano et al., 2018), it is advisable to develop strategies that broaden the use of these agents beyond accurate selection criteria. Combining ICI-based immunotherapy with chemotherapy, appears to be a viable strategy which has demonstrated encouraging results in several preclinical and clinical studies (Gadgeel et al., 2016; Gandhi et al., 2018; Govindan et al., 2017; Langer et al., 2016; Paz-Ares et al., 2018; Rizvi et al., 2016; Socinski et al., 2018a). Clinical trials involving chemo-immunotherapy approaches in the first line setting are the most promising and advanced; nivolumab, pembrolizumab and atezolizumab are all being investigated in combination with chemotherapy. To date, while the role of nivolumab still remains debated (Borghaei et al., 2018; Bristol-Myers Squibb Provides Update on Part 2 of CheckMate -227 | BMS Newsroom, 2019), the other two ICI have been approved by both the US Food and Drug Administration (FDA) and European Medicines Agency (EMA), in combination with chemotherapy for the first line treatment of advanced NSCLC, hence shifting the paradigm of NSCLC treatment. Pembrolizumab in combination with platinum and pemetrexed is the new standard first-line therapy for non-squamous NSCLC, regardless of PD-L1 expression (Gandhi et al., 2018). In addition, pembrolizumab is currently the best choice for the first-line treatment of metastatic squamous NSCLC in association with carboplatin and either paclitaxel or nab-paclitaxel (Paz-Ares et al., 2018). Atezolizumab, variably combined with carboplatin/paclitaxel ± the antiangiogenic drug bevacizumab (Socinski et al., 2018a; West et al., 2019), or in association with pemetrexed and platinum-based chemotherapy (Papadimitrakopoulou et al., 2018), constitutes another frontline option for advanced non-squamous NSCLC. The latter anti-PD-L1 antibody has also been recently demonstrated as an effective therapy in squamous NSCLC when combined with carboplatin plus paclitaxel/nab-paclitaxel (“NCCN Clinical Practice Guidelines in Oncology (NCCN Guidelines®) - Non-Small Cell Lung Cancer - Version 2.2019,” 2019NCCN Clinical Practice Guidelines in Oncology (NCCN Guidelines®) - Non-Small Cell Lung Cancer - Version 2, 2019“NCCN Clinical Practice Guidelines in Oncology (NCCN Guidelines®) - Non-Small Cell Lung Cancer - Version 2.2019,” 2019; Socinski et al., 2018b). However, the molecular mechanisms underlying synergistic interactions in immunotherapy/chemotherapy combinations are not always completely understood, which may impact the efficacy and clinical relevance of these therapeutic approaches.

In this review, the molecular basis and preclinical rationale of combining ICI with chemotherapy for the treatment of NSCLC is explored and discussed. A schematic representation of the principal effects of the combination of ICI-based immunotherapy with chemotherapy on the host immune system is depicted in Fig. 1.

Overview of anti-tumor immune responses in NSCLC

Cancer - immunity cycle: innate and adaptive immune response

An effective anticancer immune response leading to cancer cell death implies the participation of the host immune system in a series of stepwise events, which constitute the cancer-immunity cycle, as detailed by Chen and Mellman (Chen and Mellman, 2013). In this scenario, the adaptive and innate immune system cooperate to identify and eradicate tumor cells.

The adaptive immune system consists of B and T lymphocytes, implicated in the detection of tumor neoantigens via their B- and Tcell receptors (TCR). The activated T cell population comprises CD8+ cytotoxic T lymphocytes (CTLs) and CD4+ helper T cells, which require at least two signals to be activated. The first antigen-specific signal is provided when the TCR recognizes antigens presented on cancer cell surface by the major histocompatibility complex (MHC) class I molecules. The second signal, which is antigen non-specific, is mediated by T cell co-stimulatory receptors, such as CD28 (Alegre et al., 2001). CD4+ helper T cells are required for the activation of B-cells and the modulation of CD8+ cell activity (Wang et al., 2018a, 2018b). However, the vast majority of NSCLC tumor cells is typically killed by the activation of a CTL response, through the release of perforin-1 (PRF1) and granzyme B (GrzB) (Chen and Mellman, 2013; Domagala-kulawik, 2015; Mathew et al., 2018; Ramsay, 2013; Zitvogel et al., 2008). Moreover, CTLs hinder tumor growth by unleashing interferon (IFN)-γ.

On the other hand, the innate immune system is involved in the presentation of (neo)antigens to effector cells by antigen presenting cells (APCs), such as macrophages, monocytes, and dendritic cells (DCs), and contributes to the anti-tumor attack by natural killer (NK) cell-mediated immune surveillance, which is a crucial step in NSCLC carcinogenesis (Aktaş et al., 2018; Remark et al., 2015).

The abovementioned cancer-immunity cycle, involving both adaptive and innate immune system, entails three distinct phases resulting in neoplastic cell death (Chen and Mellman, 2013). First, DCs migrate to the tumor and are subsequently activated by the released neoantigens created by the oncogenic process. Next, DCs present the captured

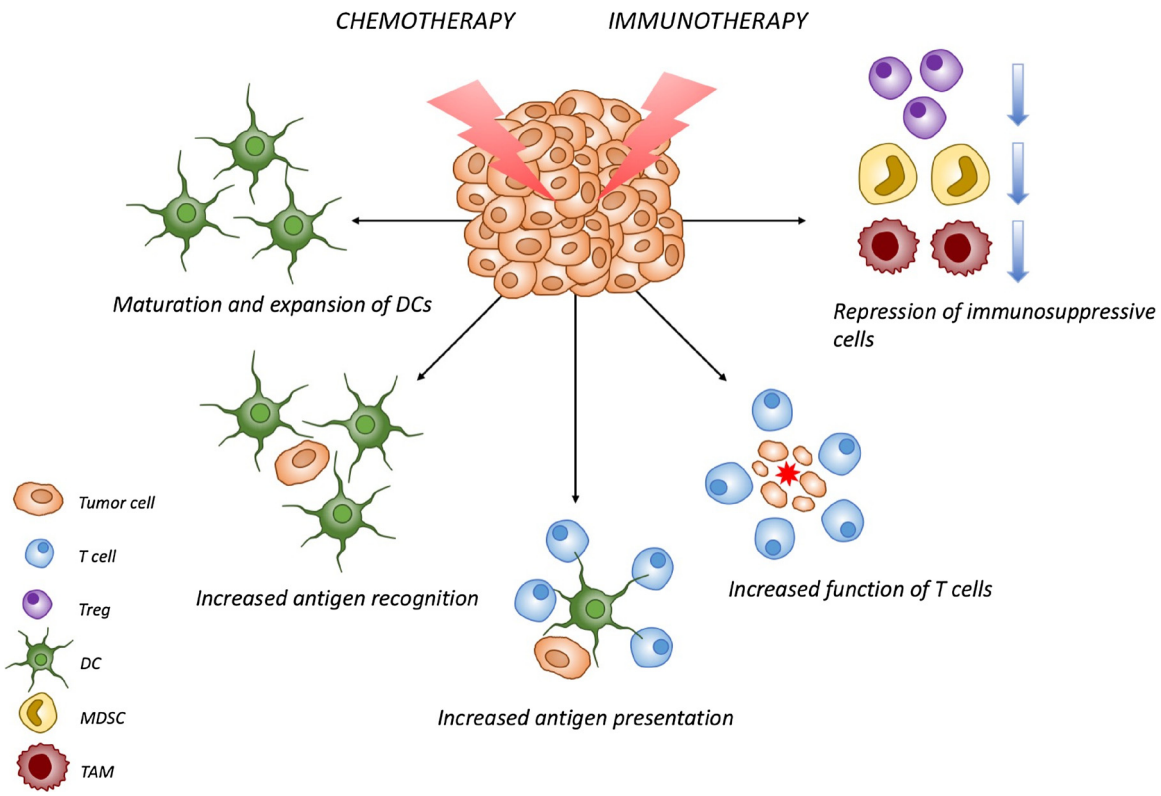

Fig. 1. Principal effects of ICI-based immunotherapy combined with chemotherapy on the host immune system. Abbreviations: DC, dendritic cell; MDSC, myeloid-derived suppressor cell; TAM, tumor associated macrophage; Treg, Regulatory T cell.

antigens on MHC class I and class II molecules to T cells and activate antigen-specific T cells. The final step consists of activated tumor-specific CTL trafficking towards the tumor site, ultimately leading to the killing of cancer cells. In turn, tumor antigens are released, so that a new cancer-immunity cycle can be triggered (Chen and Mellman, 2013; Gotwals et al., 2017).

Cancer immunoediting: elimination, equilibrium and escape

The complex relationship between the immune system and cancer has been repeatedly demonstrated in several neoplasms including NSCLC. This prompted the development of the cancer immunoediting hypothesis (Dunn et al., 2004), that strengthens and partially transcends the traditional concept of immune surveillance (Burnet, 1957; Lawrence, 1959). Cancer immunoediting has been found to play a crucial role in NSCLC development (Domagala-kulawik, 2015; Remark et al., 2015) and is considered a highly dynamic process composed of three distinct phases: elimination, equilibrium and escape. Elimination exemplifies the classical notion of cancer immunosurveillance based on recognition of tumor neoantigens by immune cells and subsequent induction of an anticancer response. Equilibrium is the time frame of immune-mediated latency following incomplete tumor destruction, where cancer editing definitely occurs. Lastly, in the escape phase, tumor growth proceeds unrestrained by immune pressure, becoming clinically detectable (Domagala-kulawik, 2015; Gajewski et al., 2017). The process of evading immune destruction is one of the well-known hallmarks of cancer, as described by Hanahan and Weinberg (Hanahan and Weinberg, 2011), and comprises multiple mechanisms (Ramsay, 2013). Indeed, edited tumor cells surviving the equilibrium phase of the cancer immunoediting process, enter the escape phase where they carry out distinct immune evading strategies including: *i.* reduction of immune recognition due to the absence of immunogenic tumor antigens, or the loss of MHC class I, class I-like, and co-stimulatory molecules (Vinay et al., 2015); *ii.* increased resistance or survival through upregulation of STAT-3 or anti-apoptotic molecules such as Bcl2; *iii.* development of an immunosuppressive TME through the release of cytokines (VEGF, TGF-β) or immunoregulatory molecules (IDO, PD-1/PD-L1, Tim-3/ galectin-9, LAG-3) (Dunn et al., 2004; Gajewski et al., 2017; Vesely et al., 2011).

Tumor immune microenvironment

The critical role of tumor immune microenvironment (TIME) in the antitumor reaction against lung cancer, as well as in the response to novel immune targeting drugs, has been clearly demonstrated (Binnewies et al., 2018; Hanahan and Coussens, 2012). Historically, NSCLC was considered a non-immunogenic tumor unable to generate profound and durable host immune responses. This assumption relied on the ability of lung tumors to escape immunological eradication, by creating a suppressive cancer immune contexture, which displays high density of immune suppressor phenotypes, upregulation of specific immune checkpoint proteins and increased production of anti-inflammatory cytokines.

Among immune suppressive subpopulations, myeloid-derived suppressor cells (MDSCs), CD4 + CD25 + FOXP3 + regulatory T cells (Tregs) and tumor associated macrophages (TAMs) were found to be overrepresented in NSCLC TIME (Carbone et al., 2015; Lazzari et al., 2018), strongly conditioning tumor immune escape. In particular, MDSCs contribute to the immunosuppressive environment by expressing several molecules, including the metabolic mediator indoleamine 2,3-dioxygenase (IDO) that might lead to the expansion of Treg cells while depleting tryptophan, which is crucial for proper tumor-specific T cell function (Carbone et al., 2015). Along the same lines, Tregs inhibit anti-tumor immune surveillance in healthy individuals as well as in NSCLC patients via the secretion of inhibitory cytokines, such as interleukin-10 (IL-10) (Vahl et al., 2017) and transforming growth factor-β (TGF-β) (Carbone et al., 2015; Eser and Jänne, 2018), as well as the direct cytolysis of natural killer and cytotoxic T cells (Sakaguchi et al.,

2008). It is noteworthy that TGF-β can also directly stimulate NSCLC growth (Carbone et al., 2015). Finally, TAMs, mostly characterized by an M2-like phenotype, may support tumor progression at different levels, such as by promoting genetic instability, cancer stem cell growth, angiogenesis, epithelial-to-mesenchymal transition and tumor spreading at distant sites, while mitigating protective adaptive immunity (Mantovani et al., 2017).

In addition to these specific cell phenotypes, another key factor driving the TME towards an immune suppressive fate is represented by the activation of immune checkpoint pathways (Catacchio et al., 2018; Vinay et al., 2015). Specifically, both PD-1 and CTLA-4 checkpoint receptors reduce T cell proliferation and function in different ways. PD-1, expressed on the surface of activated T, B and NK cells, binds to PD-L1, which is expressed both on tumor and immune cells, and results in reduced cytokine production, proliferation, and cell survival signaling, while enhancing Tregs proliferation. CTLA-4, which is also expressed on activated T cells, competes with CD28, a co-stimulatory TCR, in B7-1/B7-2 ligands binding on APCs, thus, mediating opposite signals on T cell activation. Indeed, the interaction between inhibitory CTLA-4 and its ligand, determines T lymphocyte anergy (Alegre et al., 2001; Carbone et al., 2015).

The rationale for combining ICI with chemotherapy

The remarkable clinical efficacy observed with ICI is still dealing with several critical issues. The latest clinical data indicate that ICI are therapeutically active only in a limited fraction of NSCLC patients, suggesting the lack of a deep understanding of the molecular mechanisms implicated in sensitivity or insensitivity towards immunotherapy. In this regard, given the dynamic nature of immune responses to tumors and the complex regulation of immune checkpoint expression, it may be difficult to rely on any single immunologic biomarker to select patients for the proper targeted treatment. Thus, the analysis of multiple components within the TME of NSCLC is warranted. This approach is indeed crucial in order to distinguish between an immunogenic ("hot") context that is comprised of infiltrating T cells, cytokines such as granzyme B, and PD-L1 expression, versus a non-immunogenic ("cold") TME which lacks these features (Gibney et al., 2016; Seo et al., 2018; Sharma and Allison, 2015; Taube et al., 2014). Patients whose tumors are immunogenic would be treated with ICI as frontline therapy; conversely, patients displaying cold immune features represent good candidates to receive combination therapies designed to activate immunogenic processes and boost the immune response (Sharma and Allison, 2015). According to this scenario, conventional chemotherapeutic agents are emerging as a valuable tool to elicit an effective anti-tumor response in a broader spectrum of NSCLC patients (Attili et al., 2017; Flynn and Larkin, 2017; Gadgeel et al., 2016; Giaccone et al., 2015; Gotwals et al., 2017; Govindan et al., 2017; Grabosch et al., 2015; Hato et al., 2014; Hellmann et al., 2016; Hodge et al., 2012; Huang et al., 2017; Langer et al., 2016; Lazzari et al., 2018; Mathew et al., 2018; Peng et al., 2015; Puri et al., 2017; Rizvi et al., 2016; Zitvogel et al., 2008). Indeed, chemotherapy increases tumor immunogenicity even when used at low or ultra-low-dose, defined as 1/3 – 1/10 and 1/10 – 1/30, respectively, of the maximum tolerated dose (Landreneau et al., 2015; Ramakrishnan and Gabrilovich, 2011). Furthermore, combining chemotherapy and ICI has a proven synergistic effect, since chemotherapy aids in fast tumor regression and reduction of tumor burden, while immune checkpoint blockade may prolong this effect inducing a long-lasting anti-tumor response (Champiat et al., 2014). Finally, the non-overlapping toxicity profiles of ICI and chemotherapy render them good candidates for combination strategies (Champiat et al., 2014).

Typically, tumor heterogeneity is a critical determinant for treatment outcome (Chen et al., 2014; Jamal-Hanjani et al., 2013; Lazzari et al., 2018), since resistance to standard therapy is often due to the presence of different subclones with distinct characteristics. The temporary response to single-agent anti-cancer therapies can be explained by the persistence of not targeted subpopulations of cancer cells. Surviving tumor cells may thus facilitate repopulation of the original tumor and initiate tumor progression (Chen et al., 2014). The limited efficacy of single-agent immunotherapy may partly be clarified by this phenomenon, due to the broad tumor antigen heterogeneity that prevents the immune system from instigating an effective attack. Against this background, a logical approach would be the use of a combination of drugs in order to target a wider subset of tumor cells. Moreover, the emergence of different subclones creates diverse immunogenicity across different tumor sites and areas of the same tumor (Attili et al., 2017). In this respect, chemotherapy appears to have the potential to create a more active immune response against the poorly immunogenic domains of the tumor, by contributing to antigen shedding and presentation (Attili et al., 2017; Lake and Robinson, 2005).

Molecular basis for combining ICI and chemotherapy

Cytotoxic chemotherapy mainly operates by blocking cell division and promoting tumor cell killing through deregulation of DNA replication, cellular metabolism or microtubule assembly (Gotwals et al., 2017). However, during the last decades, there has been a renewed interest in the restoration of immune surveillance by chemotherapeutics. It has long been thought that chemotherapeutic agents act in an immunosuppressive manner exerted by a direct inhibition or killing of effector cells as well as indirectly by inducing anergy (i.e. functional inactivation) or immune paralysis (Zitvogel et al., 2008). An overview of specific immunomodulatory mechanisms according to different chemotherapy agent used in the treatment of NSCLC is provided in Table 1. Cyclophosphamide was the first chemotherapeutic agent that was shown to have, when used at specific doses, an immune modulating effect by a selective depletion of Tregs. This observation prompted other studies to address immunomodulating features of other chemotherapeutics (Lutsiak, 2005). Nowadays, accumulating evidences have revealed the positive immunologic effects of several chemotherapeutics (Lazzari et al., 2018; Zitvogel et al., 2008). The underlying molecular mechanisms vary between chemotherapeutic agents. In summary, conventional chemotherapy can boost the immune system in different ways. Some agents elicit cellular rearrangements that render dying tumor cells visible to the immune system, while other drugs may determine a transient lymphodepletion, overturn immunosuppressive mechanisms, or exert direct or indirect stimulatory effects on immune effectors. The relevant modifications of the TME of NSCLC exerted by chemotherapeutics are illustrated in Fig. 2.

Induction of immunogenic cell death

A variety of chemotherapeutics investigated in NSCLC, including doxorubicin, platinum compounds and cyclophosphamide, can indirectly prompt an effective anti-cancer immune response via the induction of ICD (Mathew et al., 2018). ICD is known as the induction of tumor cell apoptosis and the concurrent appearance of specific damage-associated molecular patterns (DAMPs) on the surface of the apoptotic cells, able to trigger an anti-tumor immune response. Essential characteristics of ICD are pre-apoptotic calreticulin (CRT) exposure from the endoplasmic reticulum (ER), adenosine-triphosphate (ATP) secretion from lysosomes during the blebbing phase of apoptosis, release of high mobility group box protein 1 (HMGB1) from dying cancer cells and stimulation of type I IFN responses. Collectively, the presence of these DAMPs enhances the anti-tumor immune response by the promotion of DC maturation and activation of CTLs (Gotwals et al., 2017; Mathew et al., 2018; Wang et al., 2018a, 2018b). ICD is associated with an adaptive stress response that promotes the increased synthesis of inducible heat shock proteins (HSPs). Besides their regular function as a chaperone (i.e. refolding or degrading damaged proteins), HSPs are involved in the activation of the innate and adaptive immune system (Zitvogel et al., 2008). Exposure to HSP70 and HSP90 can stimulate

Table 1
Immune modulating properties of chemotherapeutic agents commonly used for NSCLC treatment.

Type	Agent	Immune modulation mechanism	Reference
Platinum alkylating agents	Cisplatin	Increased T cell recognition, CTL-mediated attack (when combined with vinorelbine)	(De Biasi et al., 2014; Goss and Tsvetkova, 2012; Hato et al., 2014; Mathew et al., 2018; Wang et al., 2018a, 2018b; Wu and Waxman, 2018; Zitvogel et al., 2008)
	Carboplatin	Increased T cell recognition	(Bezu et al., 2015; De Biasi et al., 2014; Goss and Tsvetkova, 2012; Hato et al., 2014; Mathew et al., 2018; Roselli et al., 2013; Wang et al., 2018a, 2018b; Wu and Waxman, 2018; Zitvogel et al., 2008)
Spindle poisons (taxanes)	Paclitaxel	Enhanced expression of MHC class II, DC maturation, decreased Tregs, increased function of CD4+ and CD8+	(John et al., 2010; Zhang et al., 2008; Zitvogel et al., 2008)
	Docetaxel	Depletion of Tregs, activation of STAT3 signaling, reduced MDSCs	(Champiat et al., 2014; John et al., 2010; Kodumudi et al., 2010; Zhang et al., 2008; Zitvogel et al., 2008)
Antimetabolites	Pemetrexed	Increased T cell infiltrate and antigen presentation, activation of CD45RO + memory T cells and IFN-γ producing NK cells	(Mathew et al., 2018; Novosiadly et al., 2018; Tomasini et al., 2016)
	Gemcitabine	Enhanced expression of tumor-antigens, selective MDSC repression	(Galluzzi et al., 2017; Mathew et al., 2018; Novosiadly et al., 2018; Tomasini et al., 2016; Weir et al., 2011)
Anthracyclines	Doxorubicin	Increased presence of T cells and T cell chemokines, ICD-inducer	(Hopewell et al., 2013; Mi et al., 2008; Wang et al., 2018a, 2018b; Wu and Waxman, 2018; Zitvogel et al., 2008)

CTL = cytotoxic T lymphocytes; DC = dendritic cell; ICD = immunogenic cell death; MDSC = myeloid-derived suppressor cell; NK cell = natural killer cell; STAT = signal transducers and activators of transcription, Treg = regulatory T cell.

maturation of DCs via interaction with the CD91 receptor on their surface (Pawaria and Binder, 2011). Furthermore, HSPs can bind to tumor-antigens and guide antigen presentation for T cell activation (Zitvogel et al., 2008). Similarly, chemotherapeutic agents trigger ER stress which initiates translocation of CRT from the ER to the cell surface (Wang et al., 2018a, 2018b; Zitvogel et al., 2008). CRT is a chaperone involved in antigen presentation via MHC class I (Galluzzi and Kroemer, 2017), whose membrane exposure attracts DCs and macrophages to the dying tumor cell, interacting with their CD91 receptor (Pawaria and Binder, 2011). Recognition of this phagocytic signal is followed by engulfment and antigen presentation, which may lead to an effective anti-tumor T cell response. Moreover, binding of CRT to the CD91 receptor of DCs, stimulates the release of pro-inflammatory cytokines, such as TNF-α and IL-6, into the TME (Wang et al., 2018a, 2018b). CRT exposure is followed by ATP release from dying tumor cells. ATP release is associated with the induction of the autophagic self-degradative process, which represents another stress response. Of note, autophagy is frequently repressed in tumor cells and might possess anti-tumor potential (Wang et al., 2018b). However, the effect of autophagy on apoptosis in NSCLC remains controversial, as autophagy can either counteract or act synergistically with apoptosis (Liu et al., 2017). Additionally, ATP functions as a chemotactic signal for DCs (Müller et al., 2010) and macrophages (Kronlage et al., 2010) by binding to their purinergic P2Y2 receptor. Besides recruitment of DCs and macrophages, ATP also stimulates DCs and macrophage maturation (Wang et al., 2018b).

Next, dying cancer cells start to release the chromatin binding protein HMGB1 (Gotwals et al., 2017; Vinay et al., 2015; Zitvogel et al., 2008). When present extracellularly (Wu et al., 2018), this protein provokes an anti-tumor immune response through interaction with TLR4 (Apetoh et al., 2007). Binding of HMGB1 to TLR4 results in the generation of an inflammatory environment and stimulation of tumor-antigen presentation by DCs. HMGB1 is indeed needed for optimal processing of phagocytosed tumor fragments (Gotwals et al., 2017; Vinay et al., 2015; Zitvogel et al., 2008). Another binding partner of HMGB1 is the receptor for advanced glycation end products (RAGE). Their interaction activates the mitogen-activated protein kinase (MAPK) and nuclear factor kappa B (NF-κB) signaling pathways, which further stimulate DC maturation and subsequent migration (Wang et al., 2018b). Finally, the stressed cancer cells exhibit a type I IFN response (Galluzzi et al., 2017; Galluzzi and Kroemer, 2017; Sistigu et al., 2014; Wang et al., 2018b), thus promoting the secretion of the CXC chemokine ligand 10 (CXCL10) by the tumor. CXCL10 exerts its chemotactic effect by binding to the CXC-chemokine receptor 3 (CXCR3), which is highly expressed on effector T cells (Sistigu et al., 2014). It remains unclear, however, whether this is the mechanism responsible for the immunogenic effects of the therapy-driven type I IFN response (Galluzzi et al., 2017). What is currently known, is that the type I (and II) IFN response can be suppressed by active c-Myc, which is frequently overexpressed in NSCLC (Kim et al., 2017), as well as by signal transducers and activators of transcription (STAT) 1 signaling. Notably, a subset of chemotherapeutics, including doxorubicin (Hannesdóttir et al., 2013), can downregulate c-Myc signaling. Consequently, type I IFN signaling will be restored together with a viable anti-tumor response (Wang et al., 2018b).

Chemotherapy-specific immune modulating mechanisms

Platinum alkylating agents

The classic anti-tumor efficacy of platinum alkylating agents, including cisplatin and carboplatin, relies on their covalent binding to the DNA which leads to the formation of inter-and intra-strand platinum–DNA crosslinks. The formed crosslinks disrupt DNA replication and transcription, which eventually induces apoptosis in cancer cells. Besides this mechanism of action, it has been documented that platinum agents partly exert their anti-tumor effect via modulation of the immune system (Goss and Tsvetkova, 2012; Wang et al., 2018b; Zitvogel et al., 2008). Platinum agents attenuate STAT6 signaling by blocking STAT6 phosphorylation. Loss of STAT6 phosphorylation results in the downregulation of PD-L2 on DCs and tumor cells. This, in turn, triggers increased tumor cell recognition by T lymphocytes (Hato et al., 2014; Mathew et al., 2018). Another immunogenic effect mediated by platinum agents is the stimulation of a CTL-mediated attack (Hato et al., 2014). When using the combination of cisplatin and vinorelbine for NSCLC treatment, it has been demonstrated that tumor cells become more sensitive to a MHC-guided perforin and granzyme B-mediated CTL attack. The increased sensitivity is mainly attributed to the upregulation of MHC class I expression (De Biasi et al., 2014). Furthermore, it has been found that the ratio of effector CD4+ and Tregs might increase following cisplatin treatment when combined with vinorelbine (Roselli et al., 2013).

Although ICD is widely discussed above, it is important to note that cisplatin is not able to comprehensively trigger this stress response due to its inability to stimulate CRT release from the ER, which appears to be a critical factor for ICD induction (Wu and Waxman, 2018). Similarly, carboplatin is not able to induce ICD, since it causes only a partial release of CRT and HMGB1. On the other hand, platinum compounds induce the release of ATP from dying cells, thus recruiting and

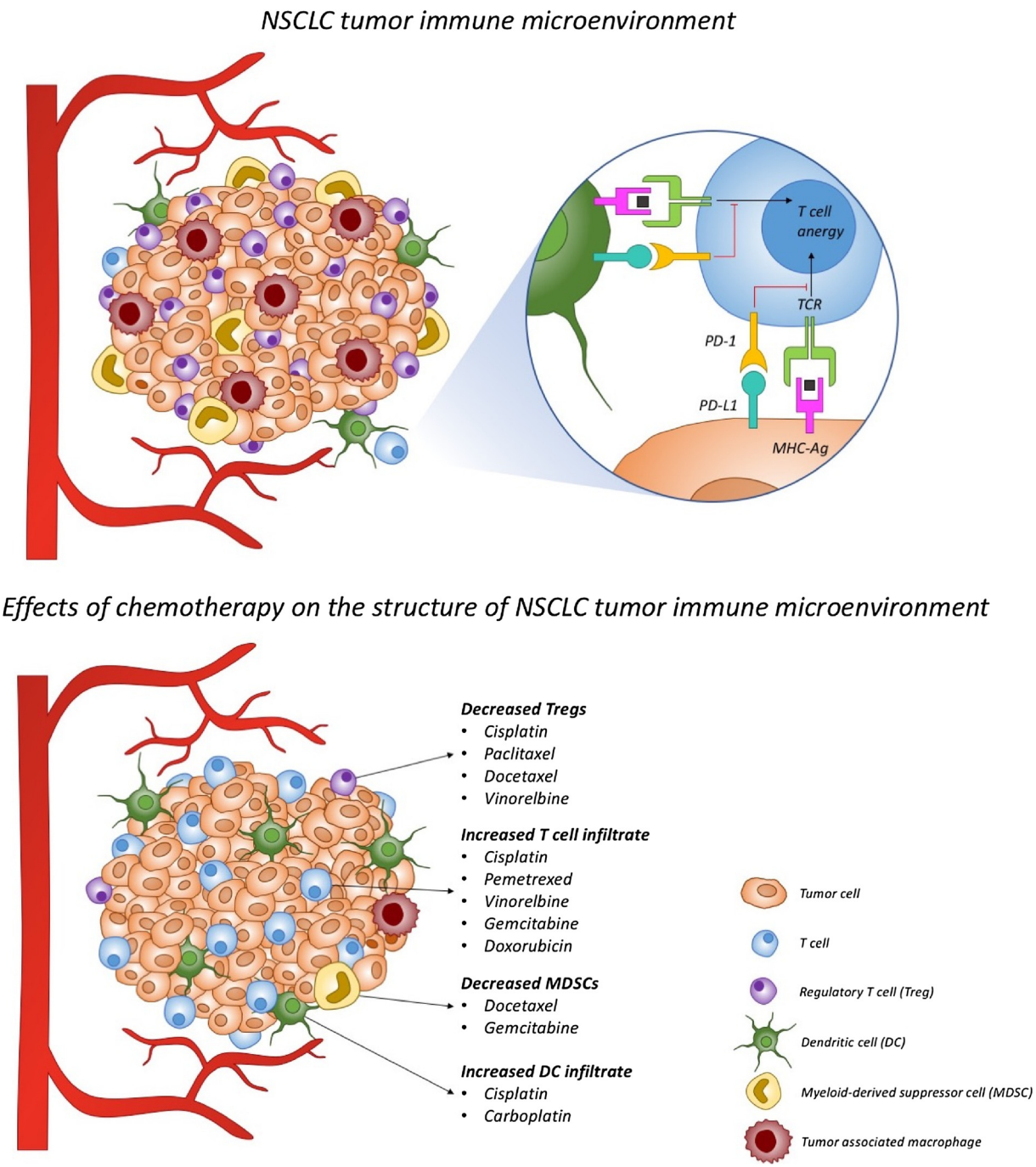

Fig. 2. Tumor immune microenvironment in NSCLC and its modification after chemotherapy.
NSCLC generally displays a suppressive TME, with abundance in immunosuppressive cell populations, among which Tregs, MDSCs and TAMs are most represented. The upregulation of immune checkpoints contributes to the suppressive immune context in NSCLC. The selective effects provided by different chemotherapeutics for NSCLC treatment on the TME is depicted in the lower part of the figure.
Abbreviations: MDSC, myeloid-derived suppressor cells; MHC-Ag, Major Histocompatibility Complex-Antigen; NSCLC, non-small cell lung cancer; PD-1, programmed death 1; PD-L1, PD ligand 1; TAM, tumor associated macrophage; TCR, T cell receptor; Treg, Regulatory T cell.

activating DCs to the tumor site (Hato et al., 2014). However, it is unknown whether both cisplatin and carboplatin administration triggers an immunogenic IFN I response (Bezu et al., 2015), and further studies on the effects of these compounds on immune response are warranted.

Antimitotic agents

Spindle poisons commonly used in NSCLC include the taxanes paclitaxel and docetaxel. More recently, a phase II trial revealed encouraging feasibility and activity of concurrent chemoradiation with nab-paclitaxel in patients with locally advanced NSCLC (Kawano et al., 2018). The mechanism of action of these drugs is attributed to arresting cell division via stabilization of microtubules (Calderoni and Cerny, 2001). Both taxanes show additional properties related to the modulation of specific immune cell subsets (Zitvogel et al., 2008). At clinically relevant doses, paclitaxel plays a role in the induction of DC maturation through enhanced expression of MHC class II (John et al., 2010). Paclitaxel also reduces the number of Tregs by increasing

apoptosis through upregulation of Fas cell death receptor. Conversely, CD4+ and CD8 + T cell function is enhanced after paclitaxel treatment (Zhang et al., 2008). Likewise, docetaxel treatment contributes to immune modulation by selectively depleting Tregs. Docetaxel also reduces the amount of MDSCs present at the tumor sites, partially via activation of STAT3 signaling (Champiat et al., 2014; Kodumudi et al., 2010).

Antimetabolites

Pemetrexed and gemcitabine are both antimetabolites widely used for NSCLC treatment. Pemetrexed is an antifolate that exerts its function by inhibiting enzymes involved in the folate pathway predominantly thymidylate synthase but also dihydrofolate reductase and glycinamide ribonucleotide formyltransferase (Assaraf, 2007; Gonen and Assaraf, 2012; Tomasini et al., 2016). Even though this pathway is crucial for proper T cell activation, it has been reported that pemetrexed leads to increased T cell infiltratration and antigen presentation at tumor sites (Novosiadly et al., 2018). More specifically, pemetrexed is able to selectively activate CD45RO + memory T cells (Mathew et al., 2018) and IFN-γ producing NK cells. Gemcitabine is a pyrimidine analogue that interferes with RNA and DNA synthesis (Ciccolini et al., 2016). This antimetabolite modulates the anti-tumor immune response by selectively repressing MDSCs (Weir et al., 2011), while enhancing the expression of tumor-antigens, thus rendering cancer cells more detectable by the immune system. Despite the effects on components of the innate and adaptive immune response, gemcitabine fails to kill cancer cells in an immunogenic manner, more likely because of its inability to elicit cellular responses required for proper induction of ICD. Another potential explanation is that the immunomodulatory effects of gemcitabine are off-target (Galluzzi et al., 2017). Nevertheless, when combined with anti-PD-1 in preclinical NSCLC models, gemcitabine increased the CD8 + T cell infiltration and enhanced the effect of the ICI given alone (Lu et al., 2017). Interestingly, Lin and colleagues demonstrated that both pemetrexed and gemcitabine, but not cisplatin, paclitaxel or vinorelbine, induced PD-L1 expression in NSCLC cell lines at concentrations below the IC_{50} values, indicating that they might be good candidates to be individually combined with PD-L1-targeted therapy in NSCLC (Lin et al., 2017).

Anthracyclines

Doxorubicin is an anthracycline that acts through inhibition of DNA replication and RNA synthesis by intercalating between base pairs and subsequent hindrance of replication as well as by inhibiting topoisomerase II. Doxorubicin damages DNA and cell membranes by generating free oxygen radicals. Doxorubicin has limited clinical efficacy in NSCLC, due to the rapid onset of acquired chemoresistance, likely ascribed to upregulation of NF-κB signaling. Activation of NF-κB stimulates oncogenic pathways, by regulating genes involved in tumor cell proliferation, DNA damage response, anti-apoptosis and angiogenesis (Mi et al., 2008). Contrary to its oncogenic effects, NF-κB promotes a T cell-mediated anti-tumor response promoting a favorable TME. A study by Hopewell and collaborators linked NF-κB signaling to an increased presence of T cells and T cell chemokines (Hopewell et al., 2013). An additional study found that doxorubicin induced an anti-tumor response by upregulation of the STAT1 signaling axis. Moreover, doxorubicin is known as an ICD-inducer, with favorable immunomodulatory properties which have been described above (Wang et al., 2018b; Wu and Waxman, 2018).

Challenges

Most studies presented in the current review have clarified that the combination of ICI-based immunotherapy with chemotherapy has a strong rationale for NSCLC treatment. Nevertheless, questions regarding the dose, drug treatment sequence and most optimal combinations remain unanswered. More ingenious therapeutic strategies and rational combinations are needed to obtain the maximum anti-tumor response towards curative cancer treatment (Mathew et al., 2018).

Dose dependency and sequence strategy

A precise dosing schedule is desirable in order to obtain the maximum anti-tumor immune response or disease control, while minimizing untoward side effects (Sui et al., 2018). As previously mentioned, chemotherapy may exert immunogenic effects also when administered at low doses (Ramakrishnan and Gabrilovich, 2011). A study by Wu and collaborators proposed an original dosing strategy to obtain a more effective immune response, which entails a modified metronomic schedule for drug delivery, defined as medium-dose intermittent chemotherapy (MEDIC). During this schedule, chemotherapeutic drugs are administered intermittently at an intermediate dose. The main goal of the MEDIC approach is to augment anti-cancer immunity through the initiation of a repetitive cytotoxic damage to tumor cells likely favoring a continuous anti-tumor immune response (Wu and Waxman, 2018).

Beyond proper dosing, the impact of the sequence of each regimen also seems to play a key role in achieving a stronger anti-tumor effect. When using anti-PD-1 first, response rates were found to be 23–25% (Garon et al., 2015; Gettinger et al., 2015), as compared to 18–20% (Borghaei et al., 2015; Brahmer et al., 2015; Herbst et al., 2016) when initially using chemotherapy. However, it is unclear whether this difference can be ascribed to the positive effects of chemotherapy. A possible explanation could be that chemotherapy unleashes the anergy of immune cell subsets and concomitantly mediates the killing of tumor cells (Dwary et al., 2017). To date, compelling evidence demonstrating that it is advantageous to administer ICI before, during or after chemotherapy, is still lacking (Hellmann et al., 2016).

Choice for the best combinations

An in-depth understanding of which chemo-immunotherapy combinations are most ideal for NSCLC treatment remains unaddressed. The rapid development of novel immunotherapies makes it even more challenging to select the most optimal strategy. Besides, the exact influence of chemotherapeutic agents on the immune system within combination regimens is not fully clear yet (Wu and Waxman, 2018). Some chemotherapeutic drugs are known as ICD-inducers, but the contribution of this specific property to their clinical efficacy has to be fully elucidated (Hato et al., 2014). A more profound knowledge on chemo-immunotherapy combinations is needed to avoid unnecessary high toxicity of combinations that exert only a low anti-tumor activity (Mathew et al., 2018). A novel approach consists of dual chemotherapy-based chemo-immunotherapy. This dual approach combines immunotherapy with two chemotherapeutics to enhance anti-tumor efficacy. Two chemotherapeutic agents with distinct mechanisms of action may complement each other. For instance, one drug may reduce the tumor burden by its cytotoxic activity, while the other induces ICD. Another advantage is that the dual use of chemotherapeutics may target a broader spectrum of drug-resistant tumor cell clones. Furthermore, different classes of chemotherapeutics may modify different immune cell subsets. This should increase the extent of tumor immunogenicity compared to that achieved individually (Wu and Waxman, 2018).

Optimal patient selection

Due to the heterogeneous nature of both NSCLC and the immune system, the identification of specific biomarkers or well-defined criteria for patient selection for chemo-immunotherapy, is extremely challenging. In combination trials using chemotherapy and anti-PD-(L)1, a significant proportion of patients did not respond, prompting new studies to define a proper selective approach. It has been found that patients with targetable oncogenic drivers should only receive anti-PD-1/PD-L1 treatment when all other treatment options have been

unsuccessful (Califano et al., 2018). Indeed, immunotherapy proved ineffective in NSCLC with targetable driver mutations, due to their low tumor mutational burden (TMB) and immunogenicity (Tsakonas and Ekman, 2018). Similarly, patients with untreated brain metastasis, severe kidney or liver dysfunction, or an active autoimmune disease which requires systemic treatment, are also not suitable for single anti-PD-1/PD-L1 therapy. However, several other patient characteristics, including age, smoking status, tumor histology or treatment history, poorly predicted the response to anti-PD-1/PD-L1 single-agents (Califano et al., 2018).

PD-L1 expression is a deeply investigated biomarker in NSCLC, and it is currently used to guide patient selection for single-agent anti-PD-1/PD-L1. In general, increased response rates are typically observed in patients with high PD-L1 expression (Kerr and Hirsch, 2016). Despite these results, PD-L1 does not always accurately perform as a predictive factor, since, in some cases, durable responses are also observed in NSCLC patients with low or even no PD-L1 expression. Potential explanations for these discrepancies include the heterogeneous and dynamic expression pattern of PD-L1 at the baseline and the variety of detection assays (Califano et al., 2018). In addition, it remains questionable whether or not the combinatorial approach of immunotherapy and chemotherapy is most beneficial for tumors with high, low or no PD-L1 expression (Santana-Davila and Chow, 2018). As clearly documented in several clinical trials (Rocco et al., 2019), the benefits from the ICI-chemotherapy combination have been granted irrespective of the PD-L1 levels, although with a trend towards a better effectiveness in highly expressing PD-L1 tumors.

The presence of TILs at the tumor site could also be considered a possible biomarker to guide patient selection, since both ICI and chemotherapy can be used to reactivate the anti-tumor ability of immune cells. TILs have been found to have good prognostic significance for disease-specific survival and OS in NSCLC (Al-Shibli et al., 2008; Hiraoka et al., 2006; Kawai et al., 2008; Schalper et al., 2015; Zeng et al., 2016), however their significance as predictive factors of response to systemic treatments is still controversial and has to be clarified for the combination treatment in NSCLC (Zeng et al., 2016). Of note, TILs phenotype, density, and location are all factors that can influence the response to treatment in NSCLC patients (Zeng et al., 2016).

TMB, defined as the measurement of mutations carried by tumor cells, constitutes an emerging predictive biomarker of the response to combined ICI-based chemo-immuno treatment in NSCLC (Hellmann et al., 2018). A high TMB results in an increased number of tumor-associated antigens that in turn increase the probability of an efficient immune response. However, assays and definition of high TMB should be standardized and more broadly evaluated in the chemo-immuno combinations in NSCLC (Hendriks et al., 2018).

The role of genomic, transcriptomic and epigenetic signatures as well as other circulating biomarkers, such as immune cells phenotypes, sPD-L1 levels or miRNAs with immune-regulatory effect, are under intense investigation (Havel et al., 2019).

Current ICI + chemotherapy combinations in advanced NSCLC

The synergistic effects of combining chemotherapy with immunotherapy have been demonstrated in clinical trials, thus providing the proof-of-concept that chemo-immunotherapeutic associations can be more beneficial than either treatments alone. Improved progression-free survival (PFS) and/or OS have been documented in both NSCLC histologies, following nivolumab/pembrolizumab/atezolizumab-based first line combinations. Specifically, on the basis of encouraging results of the phase I CheckMate 012 trial, involving 56 treatment-naïve patients with advanced NSCLC (Rizvi et al., 2016), the combination of nivolumab plus standard platinum-based chemotherapy, has been further explored and validated in a cohort of the randomized phase III CheckMate 227 study. Nonetheless, while chemo-immunotherapy greatly performed compared to chemotherapy alone in terms of PFS in NSCLC with high TMB, regardless of PD-L1 expression (Borghaei et al., 2018), the second part of the trial did not meet the primary endpoint of OS (Bristol-Myers Squibb Provides Update on Part 2 of CheckMate -227 | BMS Newsroom, 2019

Conversely, pembrolizumab has repeatedly demonstrated outstanding results in both squamous and non-squamous histotypes of NSCLC. In detail, encouraging data from KEYNOTE-021 phase II randomized trial cohort G, favoring pembrolizumab-chemotherapy combination over chemotherapy alone (overall response rate [ORR]: 56.7% vs 30.2%, PFS HR: 0.53, OS HR: 0.56), have led to the launching of the randomized phase III KEYNOTE-189 trial. 616 untreated non-squamous advanced NSCLC patients were enrolled hence becoming candidates to receive a platinum-based chemotherapy plus either pemetrexed or placebo. The benefit in terms of ORR, OS and PFS was observed across all the assessed PD-L1 categories, thus leading to FDA and EMA approval of this strategy regardless of PD-L1 staining level (Gandhi et al., 2018). Notably, according to the positive results of KEYNOTE-407 trial in which pembrolizumab was administered in combination with carboplatin plus paclitaxel/nab-paclitaxel in 559 advanced squamous NSCLC patients, the more effective chemo-immunotherapy combination has been granted full approval of the FDA and EMA as first line therapy also for squamous NSCLC (Paz-Ares et al., 2018).

Finally, along the same vein, anti PD-L1 atezolizumab has gained even more attention in metastatic NSCLC setting, due to its remarkable efficacy in both squamous and non-squamous histologies, independently of PD-L1 levels. Specifically, two different atezolizumab-based combinations, including carboplatin plus nab-paclitaxel and cisplatin/carboplatin plus pemetrexed, have been tested in IMpower130 and IMpower132 studies, respectively. In both clinical trials, chemo-immunotherapy combination proved more effective than chemotherapy alone, although it reached statistical significance only in terms of PFS (Papadimitrakopoulou et al., 2018; West et al., 2019). Another phase III randomized trial, IMpower150, has evaluated atezolizumab-chemotherapy combination, in which bevacizumab was introduced in addition to atezolizumab-carboplatin-paclitaxel regimen. Once again, the chemo-immunotherapy group performed better when compared to the chemotherapy (± bevacizumab) arm, also displaying an acceptable safety profile (Socinski et al., 2018a). Of note, the survival benefit was confirmed also in patients with tumors harboring EGFR or ALK genetic alterations, so that EMA approved this combination in non-squamous metastatic NSCLC, including EGFR and ALK positive tumors. Lastly, on the basis of the IMpower131 study, in which atezolizumab has been tested in combination with carboplatin plus paclitaxel/nab-paclitaxel, chemo-immunotherapy has become a valuable option also in squamous cell lung cancer (Socinski et al., 2018b). Nonetheless, the beneficial effect in terms of PFS and duration of response has not been paralleled by a significant increase in OS (Jotte et al., 2018).

Conclusions

Despite the remarkable recent advances in therapeutic options, the mortality rate of NSCLC remains high. Immunotherapy has demonstrated promising results, however durable disease control is only observed in a minority of patients. This emphasizes the urgent need for the majority of advanced-staged NSCLC patients of more effective frontline treatment strategies, among which combining ICI with chemotherapy appears to be promising. The synergistic effect of chemo-immunotherapy is attributed to the induction of ICD as well as reduction of immunosuppression in the TME. Although pre-clinical and clinical results of combining immunotherapy with chemotherapy have been encouraging, several main challenges remain unmet. The understanding of treatment dosing, sequence and most optimal combinations, is of paramount importance. Besides, the dynamic and heterogeneous nature of both the immune system and NSCLC tumors complicate the achievement of baseline predictive biomarkers. Valuable insights on the immune responsiveness during combination treatment are currently

missing. The validation of predictive parameters for the use of chemo-immunotherapy would be helpful to select patients who will most likely benefit from this treatment. Monitoring the immune responsiveness would aid in the development of optimal dosing and sequence schedules of chemo-immunotherapy.

In summary, combining ICI-based immunotherapy with chemotherapy has the potential to enhance the recognition and elimination of tumor cells by the immune system. This strategy appears to have a strong cancer biology rationale for the establishment of durable and potent anti-tumor immune responses towards curative NSCLC treatment. Future clinical trials undertaking a parallel evaluation of both the clinical outcome and of several immunologic endpoints (such as lymphocytes phenotype) will be essential to validate the mechanisms underlying the synergistic interaction between chemotherapy and ICI-based immunotherapy, and select the most suitable NSCLC patients for this chemo-immuno combinatory treatment.

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