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Pharmacological Combination of Nivolumab with Dendritic Cell Vaccines in Cancer Immunotherapy: an Overview

João Calmeiro[a,b], Mylène A. Carrascal[b,c], Adriana Ramos Tavares[a,b], Daniel Alexandre Ferreira[d] , Célia Gomes[d,e], Maria Teresa Cruz[a,b], Amílcar Falcão[a,f*], and Bruno Miguel Neves[g*]

[a] Faculty of Pharmacy – FFUC, University of Coimbra, 3000-548, Coimbra – Portugal

[b] Center for Neuroscience and Cell Biology – CNC, University of Coimbra, 3004-504, Coimbra – Portugal

[c] Tecnimede Group, 2710-089, Sintra – Portugal

[d] Coimbra Institute for Clinical and Biomedical Research – iCBR, Faculty of Medicine, University of Coimbra, 3000-548, Coimbra – Portugal

[e] Center for Innovation in Biomedicine and Biotechnology – CIBB, University of Coimbra, 300-504, Coimbra – Portugal

[f] Coimbra Institute for Biomedical Imaging and Translational Research – CIBIT, University of Coimbra, 3000-548, Coimbra – Portugal

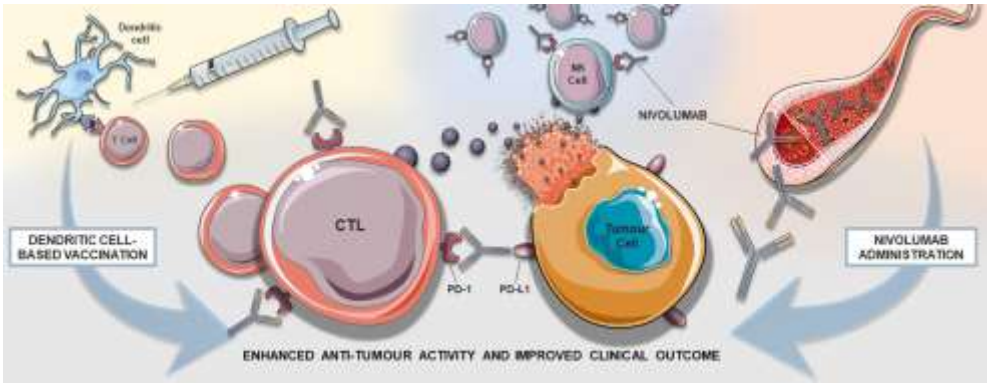
[g] Department of Medical Sciences and Institute of Biomedicine – iBiMED, University of Aveiro, 3810-193, Aveiro – Portugal

*** Corresponding authors:**

Amílcar Falcão: Faculty of Pharmacy – FFUC, University of Coimbra, 3000-548, Coimbra – Portugal (e-mail: acfalcao@ff.uc.pt; Telephone: +351-239 488 400)

Bruno Miguel Neves: Department of Medical Sciences and Institute of Biomedicine (iBiMED), University of Aveiro, Agra do Crasto - edifício 30, 3810-193 Aveiro - Portugal (e-mail: bruno.neves@ua.pt; Telephone: +351-964182278)

Graphcial abstract t



Abstract

In the last decade, immunotherapy led to a paradigm shift in the treatment of numerous malignancies. Alongside with monoclonal antibodies blocking programmed cell death receptor-1 (PD-1)/PD-L1 and cytotoxic T- lymphocyte antigen 4 (CTLA-4) immune checkpoints, cell-based approaches such as CAR-T cells and dendritic cell (DC) vaccines have strongly contributed to pushing forward this thrilling field. While initial strategies were mainly focused on monotherapeutic regimens, it is now consensual that the combination of immunotherapies tackling multiple cancer hallmarks can result in superior clinical outcomes. Here, we review in depth the pharmacological combination of DC-based vaccines that boost tumour elimination by eliciting and expanding effector immune cells, with the PD-1 inhibitor Nivolumab that allows blocking key tumour immune escape mechanisms. This combination represents an important step in cancer therapy, with a significant enhancement in patient survival in several types of tumours, paving an important way in establishing combinatorial immunotherapeutic strategies as first-line treatments.

Abbreviations

APC: Antigen-presenting cell; B2M; beta-2-microglobulin; CTL: Cytotoxic T lymphocyte; CTLA-4: Cytotoxic T- lymphocyte antigen 4; CTP: Cytoplasmic transduction peptide; DC: Dendritic cell; EMA: European Medicine Agency; FDA: Food and Drug Administration; ICB: Immune checkpoint blockade; ICI: Immune checkpoint inhibitor; IFN: Interferon; IgG4: Immunoglobulin G4; IV: Intravenous; mAb: monoclonal antibody; mCPA: metronomic cyclophosphamide; MDSC: Myeloid-derived suppressor cells; MHC: Major histocompatibility complex; myDC: myeloid dendritic cells; NK: Natural Killer cells; ORR: Objective response rate; OS: Overall survival; PD-1: Programmed death-1 receptor; PD-L1: Programmed cell death ligand 1; PFS: progression-free survival; PS: Performance status; RCC: Renal cell carcinoma; TAA: Tumour-associated antigens; TGF: Transforming growth factor; Th1: T helper cell type 1; Th2: T helper cell type 2; TIL: Tumour infiltrating lymphocytes; TME: Tumour microenvironment; Treg: Regulatory T cell; TRP: Tyrosinase related protein;

Keywords: Antitumour immunotherapy; Immune checkpoint inhibitors; Nivolumab, Dendritic cell vaccines; Combinatory therapies

1. Introduction

Cancer is a global leading cause of death, with 9.6 million fatalities in 2018 and increasing prevalence in the following decades in part due to the growing human lifespan [1]. This represents a major social, economic, and scientific problem, with the establishment of effective therapies being continually pursued. Considering that the central function of the immune system is to recognise and destroy potential threats, immune cells ought to identify tumour cells as foreign and efficiently eradicate them before dissemination to distant organs

[2]. Strategies to boost this immune response are the underlying principle of antitumour immunotherapy, a field that revolutionized cancer treatment in the last decades.

Tumours establish a complex and dynamic interplay with immune cells, subverting or escaping their patrolling effects. This concept termed cancer immunoediting [3–5] is widely accepted and assumes great importance for the rational establishment of effective immunotherapies. It can be defined in three sequential steps: elimination, equilibrium, and escape. The first phase refers to effective and stable control on cancer cell growth due to tumour-specific immune response [3,4,6]. Nevertheless, in this phase, certain cancer cell variants lacking individual tumour specific immunogenic antigens can be opposed to eradication. This conflict launches the establishment of the second phase, the equilibrium phase. This phase can last through decades and is characterized by the control of tumour outgrowth by immune system effectors, such as cytotoxic T cells (CTL), T helper cells type 1 (Th1) and Natural Killer (NK) cells. However, the genetic instability of cancer cells held in equilibrium originates modifications that imbalance the game in favour of tumour growth. These modifications are related to the decline of perception by adaptive immunity, with flaws in antigen processing and presentation, generation of an immunosuppressive state within the tumour microenvironment (TME) and insensitivity of cancer cells to immune deleterious mechanisms. Hence, these changes give rise to the escape phase where tumours start to grow gradually, becoming clinically evident and eventually metastasize [3–5]. Thus, the main goal of cancer immunotherapy can be defined as to alter the balance from escape and equilibrium to elimination. With that in mind, multiple strategies have been developed, namely dendritic cell (DC)-based vaccines and monoclonal antibodies (mAbs) blocking immune checkpoints used by tumours to evade the immune system [7].

DCs are innate immune sentinels that sense danger signals coming from microorganisms or damaged/aberrant host cells. They are the most specialized and proficient

antigen-presenting cells (APCs), acting at the interface of innate and adaptive immunity with an unmatched capacity to prime immunogenic or tolerogenic immune responses [8,9]. These characteristics have made DC-based vaccines an attractive approach to boost antitumour immunity. Extensive pre and clinical research culminated in 2009 with the approval by the Food and Drug Administration (FDA) of Sipuleucel-T, a DC vaccine targeting hormone-refractory prostate cancer [10,11]. This represents a milestone in the immunotherapy field since it was the first antitumour cell therapy reaching the market.

The more than 350 completed or ongoing clinical trials on antitumour DC-based vaccines have proven the safety of this approach, with the majority of adverse events reported being short-lived grade 1 or 2, such as flu-like symptoms and local injection site reactions [12]. However, clinical responses have been inconsistent or disappointing, rarely exceeding 15% of the objective tumour response rate [13]. To overcome this lack of robustness, recent efforts focused on the combination of DCs vaccines with other immunotherapies, namely immune checkpoint inhibition [7]. The rational of these strategies is to simultaneously address multiple aspects of the tumour immunoediting course: the DC vaccine enhances the elimination phase by priming or boosting tumour antigen-specific T cells, while checkpoint inhibitors create a favourable milieu for the action of these effector T cells, through blockade of tumour immune escape mechanisms [7,14,15].

Major immune checkpoint inhibitors (ICI) include mAbs targeting programmed cell death receptor-1 (PD-1), its ligand (PD-L1) and cytotoxic T- lymphocyte antigen 4 (CTLA-4). Both immune checkpoints inhibit T cells, with CTLA-4 acting as a negative co-stimulator during T cell-DC interaction [16,17] and PD-1 as an inhibitory signal transducer in activated T cells [18,19].

In this review, we highlight the pharmacological combination of Nivolumab, an anti-PD-1 mAb, with DC-based vaccines. We analyse this combination from the drug

characterization and mechanism of action to the pharmacodynamics, pharmacokinetics, and pre-clinical and clinical data available for different types of cancer.

2. Nivolumab, the drug

2.1 Chemistry and production

Nivolumab (BMS-936558, ONO-4538, or MDX1106) is a human immunoglobulin G4 (IgG4) mAb, highly selective for the blockade of PD-1. It was developed by the biopharmaceutical company Medarex, later acquired by Bristol-Myers Squibb (BMS) that marketed the drug under the commercial name OPDIVO [20,21]

The antibody is structurally characterized by having two identical heavy polypeptide chains with 440 amino acids and two light chains consisting of 214 amino acids, which are connected via disulphide bonds. With a molecular mass of approximately 146 kDa, Nivolumab is obtained by immunization of transgenic mice for human Ig loci in an endogenous IgH and Igκ knockout background with Chinese hamster ovary (CHO) cells expressing full human PD-1 receptor followed by boosting with PD-1 (amino acids 1–167)/human IgG1 Fc fusion protein [20–22]. Hybridomas were formed by fusing myeloma cells with spleen cells from the mice carrying detectable anti-PD-1 antibodies. Then, a screening was performed on the hybridomas and the process was continued using the clone with the most promising characteristics: the highest PD-1 binding affinity and specificity and the enhancement of T cell proliferation and cytokine production. The variable regions of selected clone and human kappa and IgG4 Fc region were grafted together, with a S228P mutation present on the latter, which accounts for enhanced stability and reduced therapeutic variability [23]. Nivolumab is thereby produced at a manufacturing-scale by the expansion of Chinese hamster ovary cells transfected with an expression vector containing coding

sequences for the heavy and light chains of the IgG. The biological drug is finally purified across a series of chromatography, viral inactivation, filtration and ultrafiltration/diafiltration processes. Nivolumab is packaged in type I flint glass tubing vial sealed with butyl stopper and presents a 24-month shelf life when stored at 2°C to 8°C and protected from light.

Great efforts have been made to develop alternative cost-effective production platforms that would lower the price of Nivolumab. Indeed, a recent study reports the production of an anti-PD1 IgG4 mAb resorting to a plant platform, with comparable characteristics to commercial Nivolumab. This strategy included introducing the heavy and light chains of the antibody into geminiviral vectors, later used to transform *Agrobacterium tumefaciens*, which were delivered into the leaves of *Nicotiana benthamiana* using vacuum infiltration. The process was shown to be efficient and scalable with a yield of 140 μg antibody/g of fresh leaf [24]. The plant-derived Nivolumab showed expected molecular weight and monomer form, as well as mammalian-like N-linked glycosylation patterns. Functional activity was similar to the commercial form, showing high affinity to human PD-1, successful inhibition of PD-1/PD-L1 binding and activation of T cell response [24]

2.2 Mechanism of action

Nivolumab acts by disrupting the interaction of the PD-1 receptor with its ligands programmed death-ligand 1 (PD-L1) and programmed death-ligand 2 (PD-L2). PD-1 (CD279) is a transmembrane inhibitory co-receptor highly expressed on activated and exhausted T and B cells.Particularly, when engaged, it transduces inhibitory signals limiting T-cell proliferation, cytokine production and cytotoxic activity [18,19,25]. The ligands PD-L1 (B7-H1) and PD-L2 (B7-DC) are constitutively expressed in both immune cells and several

tissues, after induction by inflammatory mediators such as IFN-γ [26–28]. PD-L1 and PD-L2 are also found in multiple human tumours where they are thought to play an important role in immune escape processes [18,29,30]. Hence, PD-1 blockade by Nivolumab unleashes pre-existing antitumour immunity and has become prominent cancer immunotherapy [31].

2.3 Pharmacokinetics & Pharmacodynamics

Nivolumab is administered by IV infusion, allowing for full bioavailability. The indicated dosage depends on the tumour type and practical considerations, being either 3 mg/kg, 240 mg every 2 weeks or 480 mg every 4 weeks. When administered in monotherapy, the drug has linear pharmacokinetics in the posology range of 0,1-10 mg/kg [21]. Maximum concentration and area under the curve are directly dose-related, being the peak concentration reached 1 to 4 hours after starting IV infusion [32]. The determined serum half-life (t1/2) was of 12 days for 0,3 mg/kg, 1 mg/kg and 3 mg/kg or of 20 days for the dose of 10 mg/kg [21]. The central and peripheral volumes of distribution of the antibody are 3.63 L and 2.78 L, respectively (normalized to an 80-kg, White female) [33]. Body weight and sex were found to account for 21% of the variation in the volume of the central compartment [33]. Furthermore, infusion of 3mg/kg every 2 weeks over a 30- or 60-minute period leads to a geometric mean clearance of 7,9 mL/h, terminal half-life of 25 days and average exposure stands at 86,6 µg/mL. Finally, when used at 1 mg/kg in combination with Ipilimumab, no clinically relevant increases are noted in clearance [34,35].

At pharmacodynamics level, Nivolumab binds to the PD-1 receptor by interacting with high affinity and specificity with its N-loop, which account for its effectiveness and general low off-targeted effects. In a single-dose administration regimen, the median peak receptor occupancy on circulating $CD3^+$ T cells was found to be 85% after 4-24h, with a plateau of 72% reached at and over 57 days, and a decay observed after 85 days [21]. In another study

where Nivolumab was given at a dose of 0.1–10.0 mg/kg every 2 weeks, the mean receptor occupancy varied from 64-70%. The antibody has an EC50 of 64 nmol/L and a dissociation constant of 2,06 nmol/L, as determined by Scatchard analysis [32].

2.4 Therapeutic uses of Nivolumab

Nivolumab is well tolerated with a significantly low risk of severe adverse effects but with an increased risk of thyroid dysfunction, vitiligo and pruritus [36]. At this time, there are more than 800 clinical trials involving the use of Nivolumab in the treatment of cancer, in which 45 are already completed, around 430 new studies are ongoing recruitment and 184 are now active but not recruiting. From the completed studies, most are in combination with other therapies, such as other inhibitors or blocking antibodies (20 trials), chemotherapy (8 trials) and radiotherapy (2 trials).

Nivolumab is approved by both the European Medical Agency (EMA) and FDA for the treatment of several oncologic conditions, as aforementioned below, either as a monotherapy, combination therapy or in an adjuvant setting. In clinical cases of advanced-stage melanoma, current guidelines indicate the use of Nivolumab as monotherapy or in combination with Ipilimumab. Additionally, it is indicated as an adjuvant monotherapy following complete tumour resection in adults, specifically in metastatic or lymph node-involving cases. In the case of non-small cell lung cancer, Nivolumab is indicated for adults with locally advanced or metastatic disease who have undergone prior adequate chemotherapy. As for renal cell carcinoma (RCC), it is approved as a single agent for adults in an advanced stage, provided that a prior treatment has been administered. Moreover, the combination of Nivolumab with Ipilimumab stands as the first-line therapy for adults with intermediate or poor-risk advanced RCC. Furthermore, Nivolumab is also the indicated monotherapy to treat adults with urothelial carcinoma following unsuccessful platinum-based treatment of unresectable or

metastatic tumours (EMA and FDA guidelines), or if there is disease progressing in the 12 months following administration of adjuvant or neoadjuvant platinum-containing chemotherapy (FDA).

As a monotherapy, Nivolumab is additionally indicated to treat classical Hodgkin Lymphoma in adults with a relapsed or refractory form that have undergone previous autologous stem cell transplant and treatment with brentuximab vedotin (EMA and FDA guidelines), or after administration of at least three lines of systemic therapy including stem cell transplantation (FDA-specific guideline). Lastly, this medication can be prescribed to treat adult patients with recurrent or metastatic forms of squamous cell cancer of the head and neck, with progression on platinum-containing chemotherapy.

Accelerated approval is also being sought by the FDA to treat other cancer settings, as the agency contemplates Nivolumab to treat: 1) metastatic small cell lung cancer that progresses after undergoing at least platinum-based chemotherapy and one other treatment; 2) hepatocellular carcinoma patients that have previously undergone treatment with kinase inhibitor Sorafenib; 3) monotherapy or combination therapy with Ipilimumab for metastatic colorectal cancer in adult or paediatric patients with microsatellite instability or mismatch repair deficiency following previous unsuccessful therapies.

2.5 How tumour characteristics and microenvironment affect response to Nivolumab

Despite the effective clinical results observed in a subset of patients [37–40], primary or acquired resistance to Nivolumab, as for other ICI, has been recurrently reported [41–46]. Anti-PD-1/PD-L1 therapies rely on the unrestraint of the effector functions of pre-formed tumour antigen-specific CTLs. Therefore, factors that interfere with the priming of these CTLs, their adequate migration to the tumour site and capacity to recognize and destroy tumour cells are pointed as the basis for the observed difference in the effectiveness of PD-

1/PD-L1 blockade in T-cell-inflamed and non-T-cell-inflamed cancers, the so-called "hot" and "cold" tumours, respectively (Figure 1) [47].

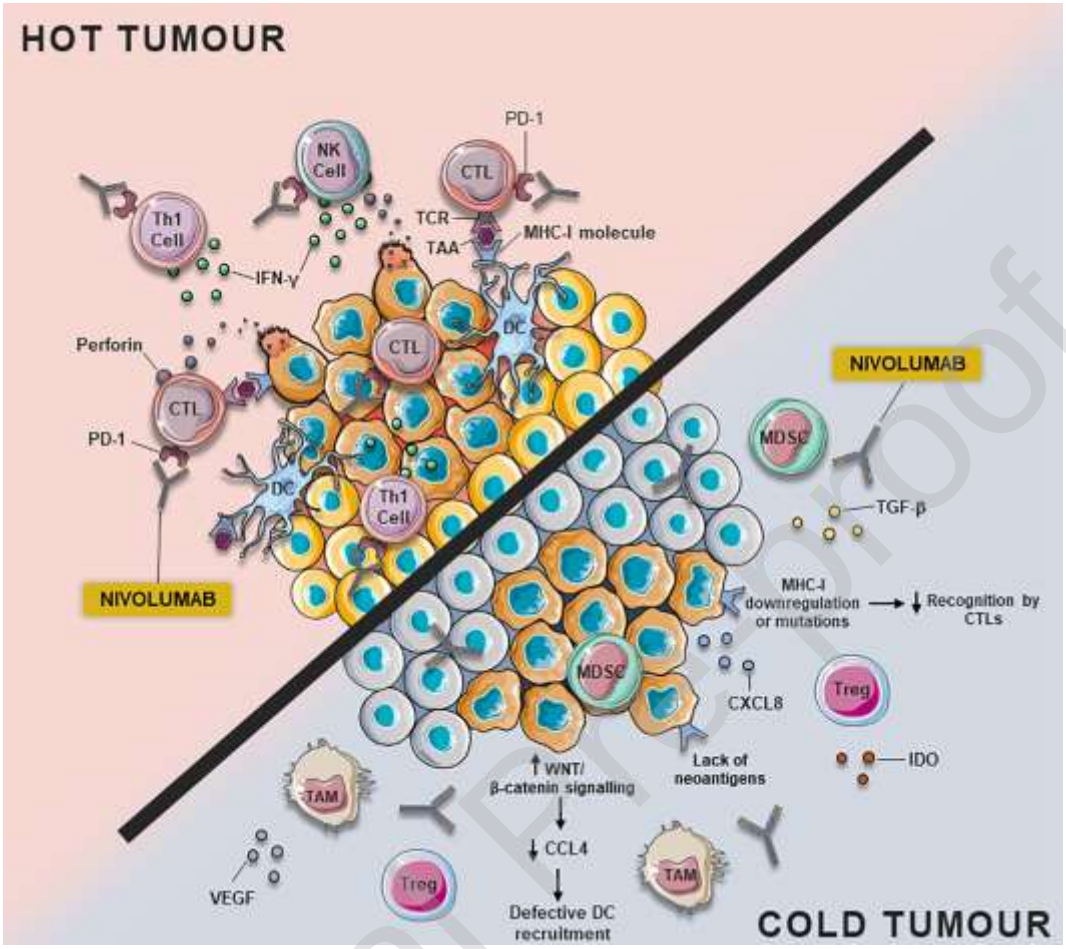


Figure 1 – Nivolumab therapeutic efficacy on "Hot" vs. "Cold" tumours. Therapeutic effectiveness with Nivolumab is highly dependent on the overall tumour immune infiltration. As this therapy functions by blocking the tumour immune suppressive mechanisms caused by the PD-1-PD-L1 axis, tumours with high immune infiltration, as seen on the left side of the image, will take maximum advantage of this strategy, while non-T-cell-inflamed cancers, the so-called "cold" tumours, shown on the right side, will not take benefit from this approach.

Key: CTL: cytotoxic T lymphocyte; DC: dendritic cell; IDO: indoleamine 2,3-dioxygenase; IFN: interferon (IFN-γ in green); MDSC: myeloid-derived suppressor cells; MHC: major histocompatibility complex (MHC-I, in blue); PD-1: programmed cell death receptor-1 (in dark purple); TAA: tumour associated antigens; TAM: tumour associated macrophage; TCR: T cell receptor (in purple); TGF: transforming growth factor; Th1, T helper cell; Treg: regulatory T cell; VEGF: vascular endothelial growth factor

The absence of a clinical response from the beginning of the therapy, termed as primary resistance, is attributed to low tumour antigen immunogenicity, defective DC-T cell interaction, T cell exhaustion, MHC-I malfunctions in tumour cells, resistance to IFN-γ, and immunosuppressive microenvironment (reviewed in [48]). In fact, the lack or weak tumour immunogenicity is a major factor contributing to unresponsiveness to immune checkpoint inhibition, being the response rates proportional to tumour mutational burden and inherent to the higher immunogenicity of neoantigens that are recognized as foreign by CTLs [49,50]. Recently, effective DC-T cell crosstalk has shown to be not only critical for the priming of antitumour CTLs but also to license their killing activity at the tumour site [51]. Garris and collaborators demonstrated that the success of PD-1 blockade depends on the production IFN-γ by activated T cells that in turn stimulates the release of interleukin (IL)-12 by DCs infiltrating the tumour. This DC-derived IL-12 ultimately boosted antitumour T cell immunity [51]. Therefore, immunosuppressive mechanisms affecting either DC or effector T cells functions could strongly impact the effectiveness of Nivolumab. Among these mechanisms, increased tumour infiltration of Treg, myeloid-derived suppressive cells (MDSCs) and tumour-infiltrating macrophages (TAMs) was found to be particularly detrimental to anti-PD-1/PD-L1 therapies [52–55].

The nature of immune cell infiltration and polarization is strongly conditioned by soluble factors such as cytokines, chemokines and metabolites present at TME. Elevated production of transforming growth factor (TGF)-β and CXCL8 by tumours results in the recruitment of Tregs, MDSCs and TAMs, contributing to anti-PD-1 resistance [56,57]. In turn, the downregulation of Th1 type chemokines such as CXCL9 and CXCL10 leads to limited effector T-cell trafficking to the tumour site, thereby hampering anti-PD1/PDL1 efficacy [58]. Also limiting immune cell trafficking, tumour-intrinsic active β-catenin

signalling in melanoma cells was shown to downregulate the expression of the chemokine CCL4, leading to defective DC recruitment to TME [59]. This results in limited T cell infiltration and priming being partially responsible for observed resistance to anti-PD-L1/anti-CTLA-4 antibody therapy [59]. Of note, analysing The Cancer Genome Atlas, Luke and co-workers found that tumour-intrinsic WNT/β-catenin signalling is enriched in 90% of non-T-cell-inflamed tumours [60]. This explains, at least in part, the lack of clinical response to immune checkpoint blockade (ICB) observed in this type of cold tumours. Vascular endothelial growth factor and metabolites such as Indoleamine 2,3-dioxygenase and extracellular adenosine are well known immunosuppressors, promoting the generation and activation of Tregs and MDSCs while hampering DC maturation and causing T cell exhaustion. Therefore, increased levels of these molecules at TME were shownto contribute to anti-PD-1/PD-L1 mAbs resistance [61–64].

Finally, characteristics of tumour cells that limit the recognition by effector T cells are also important factors hampering ICB therapies. Accordingly, in a cohort of melanoma patients treated by PD-1 blockade, the frequency of beta-2-microglobulin (B2M) mutations was found to be three folds higher in non-responders compared with responders [65]. B2M is an essential component of major histocompatibility complex (MHC) class I antigen presentation machinery, with point mutations, deletions or loss of heterozygosity in *B2M* gene in tumour cells causing downregulation of antigen presentation and subsequent attenuation of CTL cytotoxicity.

Regarding acquired resistance to ICB, the process is still not fully understood but is known to be multifactorial and partially dependent on immunoediting mechanisms where tumour subclones presenting superior immune escape activity are selected by the pressure caused by the therapy [66]. Briefly, acquired resistance to PD-1/PD-L1 blockade has been attributed to decreasing in PD-L1 expression, upregulation of other immune checkpoints such

as LAG3, TIM3 and CD73 [67–71], exhaustion of $CD8^+$ T cells [54,72], Treg expansion [73], disruption of antigen presentation [65,74–77], and development of resistance to IFN-γ signalling [78].

Alternative therapeutic options for resistant tumours are scarce, with regimen combination on the forefront of potential approaches for these patients [79]. Hence, co-administration of Nivolumab alongside DC-based antitumour vaccines may stand as a valuable strategy to obtain more robust outcomes in tumours failing to mount antigen-specific immunity.

3. Blockade of PD-1 plus DC-based vaccines combinatorial regimens - Pre-clinical data

The potential of combining DC-based vaccines with ICI has been addressed and highlighted in many pre-clinical studies. The hypothetical synergistic effects arise from the expansion of tumour antigen-specific effector T cells (CTLs and Th1) caused by DC vaccines and the attenuation of immunosuppressive mechanisms achieved with checkpoint inhibitors [80–82]. Furthermore, considering that poorly immunogenic tumours are less susceptible to ICB, combination regimens with therapies that increase the number and the cytotoxic activity of infiltrating tumour-specific T cells (such as DC vaccination) are a current major therapeutic interest [82].

Kodumudi and collaborators studied the combination of anti-PD-1 mAb with both MHC class I and II HER2-pulsed DC1 vaccine, in a preclinical model of $HER2^+$ breast cancer (TUBO-bearing mice) [83]. Both class I and class II HER2-DC1 vaccines delayed tumour growth and enhanced T cell infiltration within the tumours. Class II HER2-DC1 led to a significant increase in tumour-infiltrating $CD4^+$ and $CD8^+$ T cells, whereas class I HER2-DC1 only caused a significant increase in $CD8^+$ T cells. Importantly, these tumour-infiltrating

$CD8^+$ T cells were found to express high levels of PD-1 receptor. It was also observed by testing different administration regimens that mice receiving a class I HER2-DC1 vaccine, concurrently with anti-PD-1 or anti-PD-L1 mAbs, had no significant delay in tumour growth when compared to mice treated with a single vaccine [83]. However, when anti-PD-1 was given the following 3 weeks of HER2-DC1 vaccination (twice a week), a significant delay in tumour growth and increase survival rate was observed compared to animals receiving DC1 or mAb alone. These results highlighted the sequence and timing of ICB as critical factors in designing combinatorial strategies [83]. Additionally, the study underlined the crucial role of $CD4^+$ T cells in the antitumour activity of the tested therapeutic strategy. When combined with anti-PD-1 mAb, class II HER2-DC1 increased anti-HER2 $CD4^+$ Th1 immune response and augmented tumour infiltration of $CD4^+$ and $CD8^+$ T cells, resulting in the quadruplication of survival rate. However, depletion of $CD4^+$ T cells completely abolished the antitumour efficacy of the vaccination, both alone or in combination with anti-PD-1 therapy [83].

In a murine model of myeloma, the combination of DC vaccination with lenalidomide and PD-1 blockade revealed to be able to control tumour growth in a more effective manner, when compared to isolated therapies [84]. This effect was associated with a reduction of immune suppressor cells such as MDSC, M2 macrophages, and Tregs and with an increase of $CD4^+$ and $CD8^+$ T cells, NK cells and M1 macrophages in the TME [84]. This combinatory therapy improved the cytolytic activity of CTLs and induced Th1 polarization, leading to the production of high levels of IFN-γ while diminishing the production of immunosuppressive cytokines, such as TGF-β and IL-10, through suppression of Th2 immune responses [84]. Of note, a similar increase in circulating tumour reactive $CD8^+$ T cells was observed by Rosenblatt and coworkers when examining the combination of PD-1 blockade and DC/tumour fusions vaccine in patients with active myeloma [85].

The synergistic effects of PD-1 blockade and DC vaccines were also demonstrated in a proof-of-concept study using two genetically engineered mouse melanoma models (RET and BRAFV600E transgenic mice) [86]. After vaccination, with DCs presenting tyrosinase-related protein (TRP)-1, TRP-2, tyrosinase and human glycoprotein 100, melanoma-bearing mice exhibited an augmented $CD8^+$ T cell activation. Additionally, it was reported higher production of IFN-γ along with a reduced immunosuppressive response by MDSCs and Tregs [86]. This pattern was further amplified when the vaccination was combined with ultra-low doses of anti-PD-1 antibodies, inducing potent antitumour effects and significantly prolonging mice survival [86]. Additionally, in mice implanted with B16/BL6 melanoma and Lewis lung carcinoma cells, the combination of x-ray irradiation with DC vaccination and anti-PD-1 administration has proven to be superior relatively to those therapies applied separately [87]. *In vivo* analyses indicated that the triple combination led to a reduction in tumour growth, an extended survival rate associated with T-cell proliferation and increased IFN-γ production [87]. This strategy also inhibited the growth of metastatic tumours, an effect that radiation alone was not able to prevent due to an insufficient systemic immune response [87].

Using B16 melanoma and B16-specific TCR-transgenic T-cells (pmel-1), a study compared DC-based vaccines with short-peptide vaccines for induction of antitumour immunity, to select the best vaccine strategy to combine with checkpoint blockade [88]. DC-based vaccines were found to efficiently prime and expand pmel-1 cells with an active effector and central memory phenotype. Authors also determined that vaccine-primed cells were metabolically distinct from naïve cells, with DC based-primed pmel-1 cells displaying better effector function and higher proliferation rates, being able to inhibit tumour growth both in prophylactic and therapeutic models [88]. Regarding the combination with checkpoint blockade, only DC-based vaccines, but not peptide vaccines, showed improved antitumour activity when combined with anti-PD-1 therapy [88]. These results highlight the promising

ability of DC-based vaccines of activating the immune effector response as well as the combinatorial potential of these approaches with anti-PD-1 inhibitors for the accomplishment of optimal anti-cancer activity [88].

In a series of *in vitro* experiments, the combination of DCs pulsed with cytoplasmic transduction peptide (CTP)-fused with WT1 or BIRC5 tumour antigens and PD-1 blockade was tested against glioblastoma cells [89]. The study demonstrated an enhancement in glioblastoma antigen-specific CTL activity after blocking with anti-PD1 antibodies [89]. Anti-PD1 therapy resulted in increased IFN-γ-producing effector T cells, leading to a stronger anti-tumour effect with single CTP-fused BIRC5 or in combination with CTP-fused WT1 and CTP-fused BIRC5, against U87 cell line and primary glioblastoma cells [89]. Similar results were obtained using an intracranial glioma tumour–bearing mice treated with PD-1 blockade following DC vaccination [90]. Treatment with combinatorial therapy resulted in long-term survival, while no single agent improved the survival in animals with larger and established tumours [90]. This effect was linked to an upregulation in $CD8^+$ T cell activity and an increased expression of integrin homing and immunologic memory markers on TILs [90].

Another study explored the combination of class I restricted peptide-based cancer vaccine (DPX), anti-PD-1 therapy and metronomic cyclophosphamide (mCPA) in a murine tumour model expressing HPV16 E7 (C3), highlighting the efficacy of combining potent T cell-activating therapies such as DC or DC-targeting vaccines with PD-1 inhibition [91]. Anti-PD-1 therapy alone had no benefit in reducing tumour growth as C3 tumours had low expression of PD-L1 [91]. However, tri-therapy with DPX/mCPA/anti-PD-1 improved systemic antigen-specific immune responses, expanding antigen-specific clones of $CD8\alpha^+$ T cells within the TME and increasing IFN-γ production, providing long-term tumour control [91].

In a proof of concept, an injectable, self-assembled and nonimmunogenic nanofibrous hydrogel was tested for the delivery of exogenous DCs loaded with RADA16 peptides together with anti-PD-1 antibodies [92]. The approach was designed to overcome the problematics that DC adoptive transfer immunotherapy usually faces, namely poor cell viability, durability and weak drainage to lymph nodes while maintaining DC's biological functions [92]. Moreover, due to the presence of anti-PD-1 antibodies, the immune suppressive mechanisms connected with therapeutic resistance were minimized. A superior antitumour response was achieved in both prophylactic and therapeutic settings, with the vaccine being able to recruit resident DCs and increase proliferation and infiltration of activated $CD8^+$ T cells, while reducing Tregs. This resulted in delayed tumour growth and prolonged mice survival [92]. Even though the authors assume that there are still some optimizations to be accomplished, the ability of this vaccine to powerfully amplify tumour-specific effector T-cell responses suggests great potential in the cancer treatment field and supports the synergistic effect of combining DC vaccines with PD-1 blockade for reaching greater clinical outcomes [92].

Overall, pre-clinical studies revealed that combinations of DC-based vaccines with anti-PD-1 antibodies have superior antitumour effects than monotherapy (summarized in Table 1). The PD-1/PD-L1-axis exerts negative effects on T and NK cell effector functions, causing T-cell exhaustion and inhibiting IFN-γ mediated cytotoxicity [93]. Therapeutically targeting this axis can circumvent tumour-induced suppressive mechanisms, rendering the TME to be more favourable for effector immune cell infiltration and sensitizing tumour cells for cytotoxicity, unleashing the full potential of DC-therapy (Figure 2).

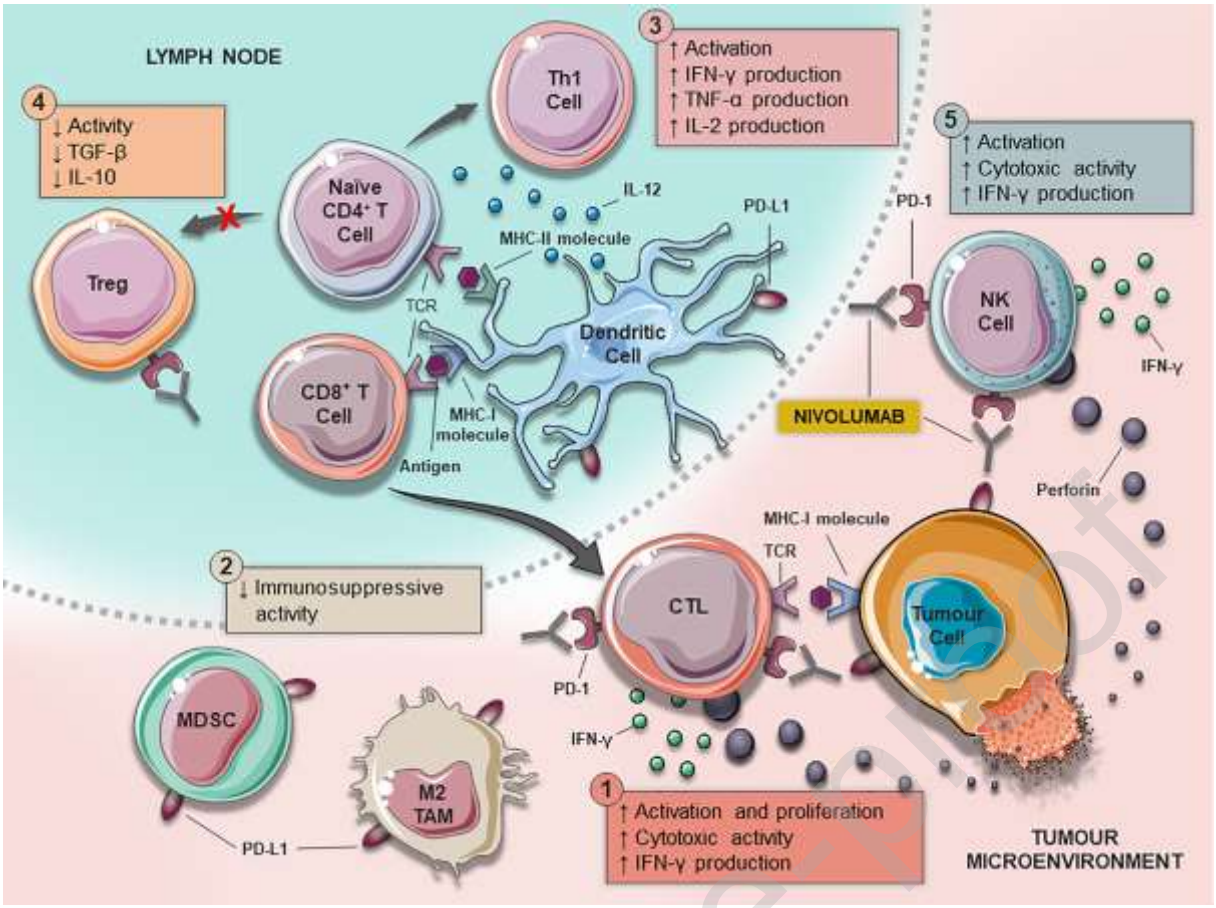


Figure 2 – Antitumour immune response promoted by combinatorial treatment with DC-based vaccination and Nivolumab administration. After injection, DCs migrate towards the lymph nodes where they present the loaded TAAs to T lymphocytes. Through MHC-I molecules, DCs cross-present the antigens to CD8+ T cells, leading to the generation of antigen-specific CTLs which specifically recognize the tumour cells through surface antigens also presented by MHC-I molecules. (**1**) As a defence mechanism against the host immune response, tumour cells express the inhibitory molecule PD-L1, which upon binding to its cognate ligand PD-1, on the T cell, silences its antitumour activity. However, due to the blockade of this axis by Nivolumab, CTL's function is restored, leading to their activation, proliferation, IFN-γ production and cytotoxic activity against tumour cells. (**2**) Downregulating the CTL activity through the PD-1-PD-L1 axis are also several immunosuppressive cells present in the TME, such as MDSCs and TAMs (more specifically M2 TAMs), which in normal conditions limit the exacerbated immune response. In this scenario, ICB also prevents the physiological regulation of the immune response, reducing the immunosuppressive effect of immune regulatory cells and, therefore, increasing the overall antitumour activity. (**3**) Via MHC-II molecules, DCs also present TAAs to CD4+ Naïve T cells, which under the influence of IL-12 are polarized into Th1 cells that secrete IFN-γ, TNF-α and IL-2. (**4**) As Th1 polarization is promoted, Treg differentiation from CD4+ T cells is downregulated, and their

immunosuppressive effect over DCs through cytokine production (TGF-β and IL-10) is repressed as a result of Nivolumab administration. (**5**) Moreover, the antitumour activity of NK cells is also boosted under the therapeutic combination of DC vaccines, which lead to their massive activation, and the effect of Nivolumab, which stops the ligation of the PD-1 receptor on the NK surface to its ligand on the DC or tumour cell membrane leading to NK cell anergy. This further promotes NK cell activity and their unparalleled cytotoxic activity, as well as their ability to highly produce IFN-γ.

Key: CTL: cytotoxic T lymphocyte; ICB: immune checkpoint blockade; IFN: interferon; IL: interleukin; MDSC: myeloid-derived suppressor cells; MHC: major histocompatibility complex; TAA: tumour associated antigens; TAM: tumour associated macrophage; TCR: T cell receptor; TGF: transforming growth factor; Th1: T helper cell; TME: tumour microenvironment; TNF: tumour necrosis factor; Treg: regulatory T cell.

Table 1: Summary of the main pre-clinical studies combining Anti-PD-1 and antitumour DC vaccines

Disease	Model	Treatment combination			Results	References
		Dendritic cells	PD-1 Antibodies	Others		
HER2 breast cancer	TUBO breast cancer cell line injected subcutaneously in female Balb/C mice	MHC class I or class II HER2 peptide pulsed DC1 (type I polarized dendritic cells)	Anti-PD-1 (clone RMP1-14)	Monoclonal antibody anti-PDL-1 (clone 10F.9G2)	- Both class I and class II HER2-DC1 vaccination increased the infiltration of TILs. - Class II HER2-DC1 combined with anti-PD-1 antibody increased survival rate, anti-HER2 CD4+ Th1 immune response and CD4+ and CD8+ T cells infiltration within the tumour.	[83]
Myeloma	MOPC-315 cell lines injected subcutaneously in female BALB/c mice	Dying myeloma cell-loaded bone marrow-derived DCs	Anti-PD-1	Lenalidomide	- Combination of DC vaccination with lenalidomide and PD-1 blockade was more effective in controlling tumour growth, improving the cytolytic activity of CTLs and inducing Th1 polarization, with high levels of IFN-γ and low levels of TGF-β and IL-10.	[84]
Melanoma	*RET* and $BRAF^{V600E}$ transgenic mice	DCs presenting TRP-1, TRP-2, tyrosinase and glycoprotein 100	Anti-PD-1 (clone 2A3)	Paclitaxel	- DC vaccine leads to augmented CD8+ T cell activation, higher production of IFN-γ and reduced Treg response - The combination of DC vaccination with anti-PD-1 antibodies improved mouse survival with a stronger reduction of Treg immunosuppressive phenotype.	[86]
Melanoma and lung cancer	B16/BL6 melanoma and Lewis lung carcinoma cells implanted subcutaneously in C57BL/6 mice	Bone marrow-derived DCs	Anti-PD-1 (clone J43)	X-ray irradiation	- The triple combination led to a reduction in tumour growth and an extended survival rate associated with T cell proliferation and increased IFN-γ production. - The triple combination also significantly inhibited the growth of metastatic tumours.	[87]
Melanoma	B16F10 cells injected subcutaneously	Bone marrow-derived DCs pulsed with	Anti-PD-1 (clone RMP1-14)	hgp100 peptide vaccine	- DC-based vaccines were found to efficiently prime and expand pmel-1 cells and inhibited tumour growth in prophylactic and therapeutic	[88]

	in C57BL/6 mice with B16-specific TCR-transgenic T-cells (pmel-1)	hgp100 peptide			settings. - DC-based vaccines combined with anti-PD-1 showed improved antitumour activity.	
Glioblastoma	U87 cell line and primary glioblastoma cells	CTP-fused WT1 and CTP-fused BIRC5 pulsed VaxDCs	Anti-PD-1 (clone J110)	-	- The combination of DC with anti-PD1 enhanced antigen-specific T cell reactivity such as IFN-γ release and T cell proliferation.	[89]
Glioblastoma	GL261 glioma cells injected intracranially in C57BL/6 mice	Lysate-pulsed BM-derived DCs	Anti-PD-1 (clone RMP1-14)	-	- Combinatorial therapy resulted in long-term survival, which was completely dependent on CD8+ T cells activity. - Each treatment alone was not able to improve the survival in animals with larger, established tumours.	[90]
- Tumour-induced by human papillomavirus type 16-transformed cells	C3 tumour cell line (HPV16-transfected mouse embryo cells) injected subcutaneously in C57BL/6 mice	Endogenous DCs	Anti-PD-1 (clone RMP1-14)	Class I restricted HPV16 $E7_{49-57}$ peptide-based cancer vaccine (DPX) and metronomic cyclophosphamide (mCPA)	- Anti-PD-1 therapy alone had no benefit in reducing tumour growth. - Tri-therapy with DPX/mCPA/anti-PD-1 improved systemic antigen-specific immune responses, expanding antigen-specific clones of CD8α+ T cells and increasing IFN-γ production.	[91]
Lymphoma	EG7-OVA cells injected subcutaneously in C57BL/6 mice	Bone marrow-derived DCs loaded with OVA or tumour cell lysates	Anti-PD-1	Encapsulation of therapies into a self-assembled peptide nanofibrous hydrogel	- Hydrogel with DCs and OVA or tumour cell lysates in combination with anti-PD-1 increased proliferation and infiltration of activated CD8+ T cells while reducing Tregs, which results in delayed tumour growth and prolonged mice survival.	[92]

4. Nivolumab plus DC-based vaccines combinatorial regimens - Clinical data

Currently, the majority of more than 80 ongoing clinical trials involving antitumour DCs vaccines are being performed in combination with chemotherapy, radiotherapy or with another immunotherapeutic approach. Supported by strong pre-clinical evidence demonstrating complementary or synergistic effects, several clinical trials are being performed combining the use of DC vaccines and ICI, specifically PD-1 inhibitors such as Nivolumab, Pembrolizumab and Pidilizumab.

Regarding the combination of DC-based vaccines and Nivolumab, there are 5 ongoing clinical trials, 1 withdrawn and 2 completed studies where both immunotherapies have been used to treat cancer patients (Table 2).

Table 2: Summary of the existing clinical trials combining Nivolumab and antitumour DC vaccines

NTC number	Interventions	Condition or disease	Phase	Status
NCT02529072	Human CMV pp65-LAMP mRNA-pulsed autologous DCs + Nivolumab	Recurrent Grade III and Grade IV Brain Tumours (Malignant Glioma, Astrocytoma, Glioblastoma)	1	Completed
NCT02775292	NY-ESO-1 TCR-transduced autologous peripheral blood lymphocyte + NY-ESO-1(157-165) Peptide-pulsed Autologous Dendritic Cell Vaccine + Nivolumab	Advanced Solid Tumours Expressing NY-ESO-1	1	Completed
NCT03707808	Autologous CD1c (BDCA-1)+ myDC + Ipilimumab and Avelumab + Nivolumab	Solid Tumour, Metastases to Soft Tissue	1	Recruiting
NCT04199559	Autologous dendritic cells pulsed with antigen peptides + Nivolumab	Advanced Non-Small Cell Lung Cancer	1 and 2	Recruiting
NCT03406715	Dendritic Cell-based p53 Vaccine (Ad.p53-DC) + Nivolumab + Ipilimumab	Relapsed Small Cell Lung Cancer	2	Recruiting
NCT03782064	Dendritic Cell (DC)/Myeloma fusions vaccine/GM-CSF + Nivolumab	Multiple Myeloma	2	Recruiting
NCT03014804	DCVax-L (Autologous Dendritic Cells Pulsed With Tumour Lysate Antigen) + Nivolumab	Giant Cell Glioblastoma, Gliosarcoma, Oligodendroglioma, Recurrent Glioblastoma, Small Cell Glioblastoma	2	Withdrawn
NCT04203901	Autologous Dendritic Cell Therapy (CMN-001) + Nivolumab+Ipilimumab + Lenvatinib+Everolimus	Advanced Renal Cell Carcinoma	2	Not yet recruiting

The safety of administering DC vaccines with Nivolumab has been tested for the treatment of 6 patients with recurrent grade III and IV brain tumours (trial NCT02529072). In this study, the use of Nivolumab in monotherapy (Group I) was compared to the combination of Nivolumab and DC vaccine therapy (Group II) until surgical resection. After surgery, both groups of patients received combined DC vaccine and Nivolumab therapy. DC vaccination consisted on the intradermal injection of human CMV pp65-LAMP mRNA-pulsed autologous DCs, which has already been reported to induce long-term progression-free survival (PFS) and overall survival (OS) in patients with glioblastoma [94]. The results have shown that the combination of the DC vaccine and Nivolumab was well tolerated, with no unacceptable toxicity or grade 3 or 4 adverse events reported. Comparing both groups of patients, those receiving the combination regimen before surgery presented an increased PFS and OS. Following this study, a new phase 2 clinical trial was planned combining DCVax-L (autologous dendritic cells pulsed with tumour lysate antigen) and Nivolumab to treat patients with recurrent glioblastoma multiform (NCT03014804), however, it was withdrawn in the final contract negotiations. Still in the treatment of central nervous system (CNS) tumours, there is a report of a patient with recurrent primary CNS lymphoma in which a complete tumour remission was achieved after multiple administrations of Nivolumab and DC vaccination [95]. Interestingly the authors found that PD-1/PD-L1 immunosuppressive axis in that patient was mainly dependent on PD-L1 produced not by tumour cells but by tumour-associated macrophages [95]. Finally, among completed clinical trials, the safety and feasibility of the combination of NY-ESO-1 transduced T cell therapy with Nivolumab and NY-ESO-1 peptide-pulsed DCs were tested in advanced solid cancers expressing MY-ESO-1 (NCT02775292). Although completed in April 2019, results of this study have not yet been disclosed.

The use of autologous CD1c (BDCA-1^{+}) myeloid dendritic cells (myDC) in combination with intratumoural injection of Ipilimumab (anti-CTLA-4 mAb) and Avelumab (anti-PD-L1 mAb), alongside with intravenous administration of Nivolumab, is under investigation in phase 1 clinical trial (NCT03707808). Here, myDC cells obtained by immunomagnetic isolation from leukapheresis product are injected intratumourally in metastasis soft tissues to boost anti-tumour immune response. Ipilimumab and Avelumab are also injected intratumourally while Nivolumab is administrated intravenously. The study is primarily focused on the incidence and severity of treatment-related adverse events and secondarily on the objective response rate (ORR) of combinatorial therapy.

In clinical trial NCT04199559, the combination of Nivolumab and autologous DCs pulsed with antigen peptides is being tested to treat advanced NSCLC. Specifically, patients receive intradermal injections of autologous DCs pulsed with WT1-H/K-HELP, Survivin-H/K-HELP, MAGE-A4-H／K-HELP and MUC1-22 peptides and intravenous administration of Nivolumab. The study will evaluate the changes in the PFS, ORR, disease control rate and OS. Furthermore, another phase 1/2 clinical trial is addressing the treatment of relapsed small-cell lung cancer with the combination of Nivolumab, Ipilimumab and a DC-based p53 vaccine (Ad.p53-DC) (NCT03406715). The adenovirus-p53 transduced DC vaccine (Ad.p53-DC) used in this study is obtained by the insertion of the p53 gene into monocyte-derived DCs and was shown to be safe and able to induce significant immune response [96]. The primary objectives of the study are the evaluation of disease control rate, PFS, OS, ORR and elicited immune response.

For the treatment of advanced renal cell carcinoma, a phase 2 clinical trial using the autologous DC therapy (CMN-001) with Nivolumab plus Ipilimumab and Lenvatinib plus Everolimus (NCT04203901) is being planned. Specifically, Nivolumab plus Ipilimumab will be administrated as a first-line therapy, and Lenvatinib plus Everolimus will be used as

second-line therapy after progression, until discontinuation criteria are met. In the combination arm of the study, CMN-001 will be administered throughout the first line of therapy in combination with Nivolumab plus Ipilimumab. CMN-001 is an autologous tumour antigen-loaded DC immunotherapy where cells will be co-electroporated with both *in vitro* transcribed RNA from an autologous tumour specimen and CD40L RNA. The main objectives of this trial are the determination of patient's OS, PFS, tumour response and to monitor treatment-emergent adverse events between both arms of the study.

The combination of Nivolumab with DC/ multiple myeloma (MM) fusion and GM-CSF is under investigation in phase 2 clinical trial for the treatment of relapsed MM (NCT03782064). DC/(MM) fusions are obtained by chemically fusing patient-derived myeloma cells obtained from marrow aspirates with autologous DCs differentiated from monocytes cultured with IL-4, GM-CSF and TNF-α . This DC/MM fusion vaccine allows the presentation of a broad spectrum of myeloma-associated antigens in the context of DC-mediated T cell costimulation. Moreover, it was already tested in a phase 1 trial displaying a good safety profile and the capacity to expand circulating myeloma antigen-specific $CD4^+$ and $CD8^+$ T cells, leading to disease stabilization in most of the patients enrolled [97].

Finally, the feasibility of producing a personalized autologous DC vaccine for further administration with Nivolumab was tested in a proof of concept study for the treatment of resected pancreatic adenocarcinoma [98]. The authors identified personalized neoantigens in three pancreatic ductal adenocarcinoma patients through new proteo-genomics antigen discovery pipeline, followed by their loading into autologous monocyte-derived DCs. The process was conducted according to good manufacturing practices, being the base for a future phase 1 clinical trial combining this personalized DC vaccine with gemcitabine/capecitabine and enteric-coated aspirin, followed by Nivolumab administration [98].

5. Concluding remarks

Our growing knowledge on the immunobiology of cancer indicates that simultaneously tackling several cancer hallmarks could be the key for more effective therapies. Among assessed combinatory immunotherapeutic regimens, the blockade of PD-1 with Nivolumab plus DC-based vaccines were shown to notably enhance clinical efficacy in several types of tumours. Collected pre-clinical and clinical data favours a synergistic effect of both immunotherapies, contributing to the increase antitumour immune responses and patient survival, with minimal toxicity. In the future, results from the ongoing clinical trials will further elucidate the contribution of combining different DC-based vaccines with Nivolumab, shedding light on the real value of this combination.

Author contributions

J.C., M.A.C., M.T.C., and B.M.N. designed and structured the manuscript. J.C., M.A.C., A.R.T., and D.A.F. performed the literature search and wrote the first draft of the manuscript. C.G., A.F., M.T.C., and B.M.N. revised and edited the final version of the manuscript. All authors read and approved the final manuscript.

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Declarations of interest:

The authors declare that they have no competing interests. Mylène Carrascal is an employee of Tecnimede Group. Tecnimede Group had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

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