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UNIVERSIDADE DO PORTO

# Immunotherapy in colorectal cancer: is there a role?

Artigo de Revisão Bibliográfica

**ANA RITA FERREIRA PACHECO QUENTAL**

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- Artigo de Revisão Bibliográfica

# Immunotherapy in colorectal cancer: is there a role?

**Autor**:
Ana Rita Ferreira Pacheco Quental
Estudante do 6º ano do Mestrado Integrado em Medicina do Instituto de Ciências Biomédicas Abel Salazar, Universidade do Porto
Número de aluno: 200000865
Endereço eletrónico: quentalrita@gmail.com
**Afiliação:** Instituto de Ciências Biomédicas Abel Salazar, Universidade do Porto
Rua de Jorge Viterbo Ferreira, 228, 4050-313 Porto, Portugal

**Orientador**:
Professor Doutor António Manuel Ferreira Araújo
Diretor do Serviço de Oncologia Médica do Centro Hospitalar do Porto; Professor Auxiliar Convidado do Instituto de Ciências Biomédicas Abel Salazar, Universidade do Porto
**Afiliação:** Centro Hospitalar do Porto (CHP); Instituto de Ciências Biomédicas Abel Salazar, Universidade do Porto

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## Abstract

Colorectal cancer is the third most commonly diagnosed cancer in men and the second in women, and represents one of the leading causes of cancer-related deaths. Despite progresses in treatment options, prognosis for advanced disease with recurrence and metastasis is still poor, emphasizing the need for new therapeutic strategies.

In recent years, increasing knowledge about the interaction between the immune system and tumour cells, as well as about the tumour microenvironment, led to the development of novel anti-cancer approaches globally known as cancer immunotherapy. Immunotherapies are designed to enhance antitumour immune response and to avoid immunosuppression, and comprise several different strategies like cancer vaccines, adoptive T-cell therapy and immune checkpoint inhibitors. Recently, the use of checkpoint inhibitors such as specific antibodies for cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) and programmed cell death protein 1 (PD-1) have shown to be an effective treatment in several types of cancers. Although the importance of the immune system in colorectal cancer has already been recognized, the potential significance of immunotherapy in this cancer is still at an early stage. Currently, several immunotherapeutic approaches, particularly those with immune checkpoints inhibitors, are being evaluated in various clinical trials.

This paper reviews the most relevant clinical data concerning such immunotherapeutic approaches for colorectal cancer, pointing out their possible advantages and disadvantages.

Despite the research using different immunotherapy strategies in the treatment of colorectal cancer, thus far none has been approved for use in clinical practice. The most promising results have been achieved in the subset of patients with microsatellite instable tumours using an immune checkpoint inhibitor; however, the majority of patients have microsatellite stable cancers. Therefore, several clinical trials are in progress, either with single agents or via combination regimens, to test whether viable treatment options arise.



**Keywords:** immunotherapy, colorectal cancer, immune checkpoint inhibitors, cancer vaccines, adoptive T-cell therapy.

## Resumo

O cancro colorretal é o terceiro tipo de cancro mais diagnosticado nos homens e o segundo nas mulheres, e representa uma das principais causas de morte relacionadas com o cancro. Apesar dos avanços obtidos nas opções terapêuticas, o prognóstico nos casos de doença avançada ainda é fraco, o que demonstra a necessidade de novas estratégias terapêuticas.

Nos últimos anos, o conhecimento crescente sobre a interação entre o sistema imunitário e as células tumorais, bem como sobre o microambiente tumoral, levou ao desenvolvimento de novas estratégias no combate ao cancro, genericamente conhecidas como **imunoterapia oncológica**. As imunoterapias têm como objetivo aumentar a resposta imunitária antitumoral e evitar a imunossupressão, e incluem opções como vacinas, terapia adoptiva com células T, e inibidores de *checkpoints* imunológicos. Recentemente, o uso destes inibidores, com anticorpos para o antigénio 4 associado a linfócitos T citotóxicos (CTLA-4) e para a proteína de morte celular programada 1 (PD-1), revelou ser eficaz em vários tipos de cancro. Embora a importância do sistema imunitário no cancro colorretal já tenha sido reconhecida, a relevância da imunoterapia neste tipo de cancro ainda se encontra numa fase inicial. Atualmente, vários ensaios clínicos estão a ser realizados, particularmente com inibidores de *checkpoints* imunológicos, para avaliar a eficácia destas terapêuticas.

Este artigo tem como objetivo fazer uma revisão dos resultados clínicos mais relevantes no que diz respeito às diferentes imunoterapias em estudo no cancro colorretal, salientando as suas possíveis vantagens e desvantagens.

Apesar da investigação feita com diferentes estratégias imunoterapêuticas nos doentes com cancro colorretal, até ao momento nenhuma delas foi aprovada para uso na prática clínica. Os resultados mais promissores foram obtidos no subgrupo de doentes com tumores com instabilidade de microssatélites utilizando um inibidor de *checkpoint* imunológico; porém, a maioria dos doentes apresenta tumores com estabilidade de microssatélites. Vários ensaios clínicos estão a ser realizados, quer com agentes isolados quer com regimes combinados, para avaliar novas opções de tratamento.



**Palavras-chave:** imunoterapia, cancro colorretal, inibidores de *checkpoints* imunológicos, vacinas anti-cancro, terapia adoptiva com células T.

## Abbreviations

| | |
|---|---|
| ASI | Active specific immunotherapy |
| APC | Antigen presenting cell |
| BCG | Bacillus Calmette-Guérin |
| CAR | Chimeric antigen receptor |
| CEA | Carcinoembryonic antigen |
| CIMP | CpG island methylator phenotype |
| CIN | Chromosomal instability |
| CRC | Colorectal cancer |
| CTL | Cytotoxic T lymphocyte |
| CTLA-4 | Cytotoxic T lymphocyte-associated antigen 4 |
| DC | Dendritic cell |
| DFS | Disease-free survival |
| FDA | Food and Drug Administration |
| HLA | Human leukocyte antigen |
| MDSC | Myeloid-derived suppressor cells |
| MFS | Metastases-free survival |
| MHC | Major Histocompatibility Complex |
| MMR | Mismatch repair |
| MSI | Microsatellite instability |
| NSCLC | Non-small cell lung cancer |
| OS | Overall survival |
| PD-1 | Programmed cell death protein 1 |
| PD-L1 | Programmed death-ligand 1 |
| PFS | Progression-free survival |
| RFI | Recurrence-free interval |
| RFS | Recurrence-free survival |
| SLN | Sentinel lymph node |
| TAA | Tumour associated antigens |
| TCR | T-cell receptor |
| TIL | Tumour-infiltrating lymphocyte |
| Treg | Regulatory T-cells |

# Table of Contents


**1. Introduction** **1**

1.1 Epidemiology 1

1.2 Molecular Basis of Colorectal Cancer 1

**2. Objectives** **3**

**3. Material and Methods** **4**

**4. Results** **5**

4.1 The immune system and cancer 5

4.2 Immunotherapy in Colorectal Cancer 7

4.2.1 Cancer vaccines 8

*Autologous Tumour Cell Vaccines* 8

*Peptide Vaccines* 9

*Dendritic cell vaccines* 10

*Viral-vector vaccines* 11

4.2.2 Adoptive T-cell Therapy 13

4.2.3 Immune Checkpoint Inhibitors 15

*CTLA-4* 15

*PD-1* 16

**5. Discussion and Conclusions** **19**

**6. References** **22**

# 1. Introduction

## 1.1 Epidemiology

Colorectal cancer (CRC) is the third most commonly diagnosed cancer in men (746,000 cases, 10.0% of the total) and the second in women (614,000 cases, 9.2% of the total) worldwide. Variations in incidence rates are observed across the world, with the highest rates in Australia/New Zealand and lowest in West Africa; mortality rates present less variability, with highest rates in Central and Eastern Europe and lowest in Western Africa (Ferlay et al., 2015).

In the United States (US) in 2017, the estimated number of newly diagnosed individuals with CRC is 135,430 and the estimated number of deaths from the disease is 50,260. Although most of the cases (58%) are diagnosed in people of 65 years or older, 39% of women and 45% of men are younger than that (Siegel et al., 2017a). A downward trend in mortality rates has been observed in the last decades in US, falling 51% from 1976 to 2014 (28.6 to 14.1 per 100,000 cases) (Siegel et al., 2017a). Similarly, most European countries have also experienced a decline in CRC mortality, with the average rate dropping from 35.3 to 31.3 deaths per 100,000 between 2003 and 2013 (OECD/EU, 2016). Mortality reductions are attributed to changes in risk factors (e.g. diet and life-style related aspects such as smoking and alcohol consumption), the introduction of screening tests, and improvements in treatment (OECD/EU, 2016). In spite of that, CRC is still one of the most common cause of cancer-related death both in men and women. In fact, although 39% of the patients are diagnosed with localized CRC, for whom the 5-year relative survival rate is 90%, about 25% of patients present with metastases at diagnosis and almost half of the patients will develop metastases during the course of the disease (Van Cutsem et al., 2014; Siegel et al., 2017b). Current treatment for metastatic CRC is generally based on cytotoxic chemotherapy regimens in combination with targeted therapies (e.g. anti-EGFR antibodies). Despite progress in treatment options, 5-year relative survival rate decreases to 71% and 14% for patients diagnosed with regional and distant metastasis, respectively (Siegel et al., 2017b).

Thus, prognosis for patients with advanced disease is still poor, highlighting the need of novel therapeutic strategies.

## 1.2 Molecular Basis of Colorectal Cancer

Colorectal cancer occurs in three different patterns: sporadic, familial and inherited. The sporadic form accounts for most of CRC cases (~70%) and derives from somatic

mutations. Inherited CRC occurs in 10% of cases and comprises cancer predisposition syndromes such as Lynch Syndrome and Familial Adenomatous Polyposis (FAP), whereas familial CRC accounts for 25 % of CRCs and presents without precisely defined Mendelian inheritance patterns or genetic etiology (Roper & Hung, 2013).

Different molecular mechanisms can give rise to CRC, namely chromosomal instability (CIN), microsatellite instability (MSI), and CpG island methylator phenotype (CIMP) (Tariq & Ghias, 2016). Most of CRCs (65-70%) arise via the CIN pathway, which is characterized by aneuploidy and loss of heterozygosity. Microsatellite instability, found in about 15% of all CRCs, is characterized by length alterations in repetitive DNA regions called microsatellites, and derives from defects in the DNA mismatch repair (MMR) system (Tariq & Ghias, 2016). Most MSI CRCs are sporadic cases due to hypermethylation of *MLH1* gene promotor, whereas 3% are caused by germline mutations in *MSH2*, *MSH6*, *MLH1* and *PMS2* genes or *EpCAM* deletion, causing Lynch syndrome (Zhang & Li, 2013). The CIMP pathway is characterized by promoter hypermethylation of various tumour suppressor genes. These three mechanisms, however, are not mutually exclusive, as tumours can sometimes demonstrate features of multiple pathways (Tariq & Ghias, 2016).

The different pathways are associated with distinct clinical features and pathologic behavior. Indeed, MSI tumours are often associated with the proximal colon, have higher levels of tumour-infiltrating lymphocytes (TIL) and are associated with a better prognosis (Smyrk et al., 2001; Benatti et al., 2005).

## 2. Objectives

The conventional treatments for advanced CRC cancer, including radiation, chemotherapy and targeted agents are designed to act directly on tumours, inhibiting their growth or destroying them. However, resistance to these therapies eventually develops and toxicities may limit their administration at effective levels. These facts highlight the need to develop alternative strategies in order to prolong patients' survival. In this context, immunotherapy is emerging as a promising approach in oncology, intended to stimulate and enhance the ability of a patient's immune system to recognize and destroy tumours. Cancer immunotherapies may therefore overcome some of the resistance mechanisms that occur with traditional agents.

Immunotherapy strategies are currently therapeutic options in several types of cancer, including melanoma, non-small cell lung cancer (NSCLC), and renal cell carcinoma. It remains however in the experimental field in the case of CRC.

This paper reviews the antitumour immune response and the most relevant immunotherapeutic approaches under investigation in CRC, such as cancer vaccines, adoptive T-cell therapy, and immune checkpoint inhibitors.

## 3. Material and Methods

References for this review were identified by searches of PubMed database using the keywords: “immunotherapy”, “colorectal cancer”, “cancer vaccines”, and “immune checkpoints”. The selection of articles was based on the relevance to the topic of the review, date of publication, and journal’s impact factor. In addition, further articles were identified from references of selected studies.

# 4. Results

## 4.1 The immune system and cancer

The immune system primarily functions to protect the host from damage caused by pathogens, but it has also the ability to recognize and destroy cancer cells based on their expression of tumour antigens, in a process historically known as cancer immunosurveillance. However, it is now well established that the immune system can have a dual role in cancer development and progression. In fact, although it can inhibit tumour growth by destroying malignant cells, it can also promote its progression either by selecting tumour cells with reduced immunogenicity or by establishing conditions within the tumour microenvironment that facilitate tumour outgrowth (Schreiber et al., 2011). These findings prompted the refinement of the immunosurveillance concept to the formulation of the cancer immunoediting hypothesis, which refers to a dynamic process including three distinct phases: elimination, equilibrium, and escape. Elimination refers to cancer immunosurveillance, in which innate and adaptive immune systems cooperate to detect an emerging tumour and destroy it before it turns clinically visible. In spite of that, some tumour cells may still persist and enter an equilibrium phase, in which they co-exist with the effector cells of the immune system in a state of tumour dormancy. During this phase, the immune system restricts outgrowth of malignant cells but also shapes their immunogenicity by selecting for less immunogenic variants. These cells eventually acquire the ability to evade immune recognition and destruction, allowing tumour progression and clinical expression. This represents the last step in the immunoediting process, known as the escape phase (Schreiber et al., 2011).

To mount an effective antitumour immune response several different processes are required. First, dendritic cells (DSs) must capture and process tumour antigens and present them on MHC class I and class II molecules. In the presence of appropriate activation and/or maturation signals, DCs will differentiate and migrate to lymph nodes where they present these antigens to naïve T-cells. This leads to priming and activation of effector T-cells against tumour-specific antigens. Finally, the activated effector T-cells traffic to and infiltrate the tumour, specifically recognizing and binding to tumour cells through the interaction between its T-cell receptor (TCR) and its cognate antigen bound to MHC class I, ultimately destroying target cancer cells (Figure 1) (Chen & Mellman, 2013).

Nevertheless, malignant cells can exploit numerous mechanisms to evade recognition and destruction by the immune system. One of these mechanisms is the ineffective

presentation of antigens to immune cells by reducing the expression of MHC class I molecules in the cell surface, which have been associated with a poor prognosis in CRCs (Watson et al., 2006). Recruitment of immunosuppressive cells (regulatory T-cells - Tregs, and myeloid-derived suppressor cells - MDSCs) is also an important mechanism of escape from the host immune system. In normal physiologic conditions, Tregs prevent autoimmunity disorders by secreting immunosuppressive cytokines. In cancer patients, however, Treg can block immune response against cancer cells and therefore Treg infiltration of tumours is generally associated with a poor outcome (Halvorsen et al., 2014), although contradictory results have been reported in CRC (Clarke et al., 2006; Ling et al., 2007; Salama et al., 2009; Frey et al., 2010). In addition, it was demonstrated that CRC patients have high levels of MDSCs in the primary tumour and in peripheral blood, which also correlates with advanced disease stages and lymph node metastases (OuYang et al., 2015). Other escape strategy includes the dysregulation of T-cell activity. This can occur either by the activation of inhibitory pathways (known as immune checkpoints), such as cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) and programmed cell death protein 1 (PD-1), or through the inhibition of T-cell activation pathways, for example CD137, OX-40, CD40, GITR, and HVEM (Pennock & Chow, 2015). A deep understanding of these escape mechanisms is essential and must be taken into consideration in the development of novel immunotherapeutic strategies.

As previously mentioned, there is an increasing body of evidence suggesting that immune cells play a significant role in tumours progression. Indeed, high lymphocytic infiltration has been associated with favorable clinical outcome in several types of tumours, such as melanoma, head and neck, breast, renal, prostate and lung cancer (Fridman et al., 2012). In line with these data, Galon et al. (2006) have also demonstrated that CRC patients without recurrence had higher immune cell densities in the center and in the invasive margin of the tumour than patients whose cancer had recurred. The authors concluded that the level of lymphocytic infiltration within the tumour is a strong prognostic factor that predicts recurrence and longer overall survival (Galon et al., 2006). Subsequent studies reported similar results, reinforcing the importance of immunity in this type of cancer (Ogino et al., 2009; Mlecnik et al., 2011).

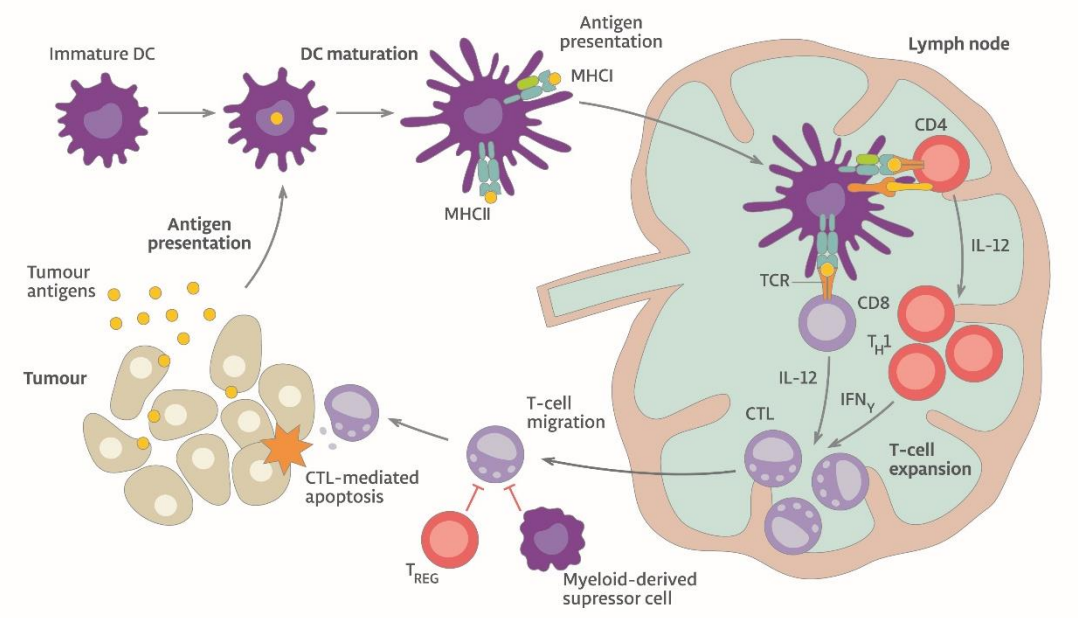


**Figure 1: Phases in the development of a cellular immune response against tumour antigens.** The antitumour immune response is initiated by immature DCs, which capture and process tumour antigens and present them on MHC class I and MHC class II molecules. DCs then migrate to tumor-draining lymph nodes where they present tumour antigens to naïve T-cells. Activation of T-cells involves not only the interaction between the antigen–MHC complex on DCs and TCRs, but also costimulatory molecules. The immune response proceeds with the infiltration of activated cytotoxic T-cells into the tumour, eventually killing malignant cells. CTL, cytotoxic T-cell; DC, dendritic cell; MDSC, myeloid-derived suppressor cell; MHC, major histocompatibility complex; TCR, T-cell receptor; TH1, type 1 T helper cells; $T_{REG}$, regulatory T-cells. Adapted from Melero et al. (2014).

## 4.2 Immunotherapy in Colorectal Cancer

Cancer immunotherapy is a therapeutic approach designed to generate or enhance a host immune response against malignant cells, and include active, passive or immunomodulatory strategies. Whereas active immunotherapy increases the ability of patient's immune system to generate effective antitumour immune responses, passive immunotherapy involves the administration of immunologic effectors to patients, such as lymphocytes or antibodies, to mediate an immune response. Immunomodulatory agents, in turn, improve general immune responsiveness and are intended to increase antitumour immune responses, but are not targeted at specific antigens (Melero et al., 2014).

Several immunotherapy strategies for treatment of CRC patients are currently under research in clinical trials, including cancer vaccines, adoptive T-cell therapy, and immune checkpoint inhibitors, as described in the following sections.

### 4.2.1 Cancer vaccines

Cancer vaccines are active therapeutic approaches used to generate immunologic responses against tumour cells through the induction of effective cellular and humoral responses. Cancer vaccines require dendritic cell activation and presentation of the targeted tumour antigens to T-cells in order to establish an immune response.
The most important types of cancer vaccines include autologous tumour cell vaccines, peptide vaccines, dendritic cell vaccines, and viral-vector vaccines.

***Autologous Tumour Cell Vaccines***

Autologous tumour cell vaccines are derived from cancer cells isolated from a patient, which are processed *ex vivo* to a vaccine formulation and then re-administered to the patient, usually in combination with an adjuvant immunostimulant. Typically, these vaccines are produced after surgical resection of the tumour, which is irradiated or treated with reagents to create an inactive lysate (Patel et al., 2014). Whole tumour cell vaccines have the advantage of comprising all tumour antigens, so they can potentially induce adaptive antitumour immunity against several antigens. However, these vaccines are specific to each patient and their preparation is relatively costly and time consuming. Furthermore, it has been demonstrated that the expression level of tumour-associated antigens by tumour cell vaccines is probably lower than that of other vaccine types, resulting therefore in a less effective immune response (Xiang et al., 2013).
Whole cancer cell vaccines have shown some promise in CRC. **OncoVax**, an irradiated autologous tumour-cell vaccine with bacillus Calmette-Guérin (BCG) adjuvant, is one of the most intensively studied in CRC. Harris et al. (2000) have conducted a phase III clinical trial with an autologous tumour cell–BCG vaccine in stage II (n=297) and stage III (n=115) colon cancer patients to determine if surgical resection combined with active specific immunotherapy (ASI) was more beneficial than resection alone. Results did not show a statistically significant difference in disease-free survival (DFS) and overall survival (OS) between groups. However, patients with delayed-type hypersensitivity (DTH) reaction had increased 5-year survival which correlated with the order of the induration response, suggesting that patients who develop a greater local reaction may benefit from the vaccine (Harris et al., 2000). A similar study also in stage II and III colon cancer patients (n=254) using OncoVAX in an adjuvant setting demonstrated a clinical

benefit only in stage II patients, with a significant longer recurrence-free interval (RFI) and 61% risk reduction for recurrences (Vermorken et al., 1999). Using part of this cohort of patients, de Weger et al. (2012) investigated whether the beneficial effects correlate with microsatellite status of the primary tumour. The analysis revealed that patients with microsatellite stable stage II tumours who received adjuvant vaccine treatment had a significant improved recurrence-free survival (RFS) as compared to controls. In addition, patient group with microsatellite instability did well overall, irrespective of tumour stage and treatment arm (de Weger et al., 2012).

Hanna et al. (2001) performed a meta-analysis of three phase III studies using OncoVAX again in stages II and III colon cancer patients. In the three studies, patients were randomized either to a vaccine-treatment arm or to a control arm after surgical resection of the primary tumour. The results showed that OncoVAX-treated patients had a significant improvement in RFI and in RFS. The beneficial effect in these clinical outcomes was superior in stage II patients. A confirmatory phase IIIb clinical trial in patients with stage II colon cancer is currently ongoing (NCT02448173).

In addition to BCG, autologous CRC vaccines have also been produced using Newcastle disease virus (ATV-NDV). A phase III trial evaluated the clinical efficacy of ATV-NDV after resection of liver metastasis from CRC (n=50). Although no differences in the OS, DFS and metastases-free survival (MFS) were observed between the immunized and control CRC groups, a significant advantage for vaccinated colon cancer patients (not for rectal cancer patients) was observed with respect to OS and MFS (Schulze et al., 2009).

To date, research concerning autologous cell vaccines in CRC has shown modest clinical activity. The small proportion of tumour-specific antigens expressed by cancer cells and the generally poorly immunogenicity of these type of vaccines can explain, at least in part, the moderate results obtained in most trials (Signorini et al., 2016).

### *Peptide Vaccines*

Peptide vaccines are based on the identification and synthesis of epitopes unique to cancer cells which can induce tumour antigen-specific immune responses. These vaccines have therefore the potential to generate a more specific antitumour response when compared to autologous tumour cell vaccines. Although considered simple, safe, economical and able to target tumour-specific antigens, peptide vaccines have some disadvantages that have restricted their effectiveness. These include poor immunogenicity, HLA-restriction limiting the peptide vaccines to specific HLA haplotypes, cancer recurrence due to antigenic escape and an excessively long lag between the first infusion and the clinically relevant response (Signorini et al., 2016).

Several tumour-associated antigens have been targeted by peptide vaccines in CRC including carcinoembryonic antigen (CEA), mucin 1, β-human chorionic gonadotropin (β-hCG), EpCAM, 5T4, EpHA2, gastrin, survivin, SART3, and p53 (Patel et al., 2014). Phase I clinical trials have generally shown that these vaccines are safe and able to elicit antigen-specific immune responses.

More recently, Okuno et al. (2011) used a peptide vaccine derived from ring finger protein 43 (RNF43) and 34-kDa translocase of the outer mitochondrial membrane (TOMM34) that was administered in combination with chemotherapy in patients with metastatic CRC. Cytotoxic T lymphocyte (CTL) responses were induced against one or both antigens in 57% (12/21) and 38% (8/21) of the patients, respectively. Interestingly, patients with CTL responses against both antigens had the longest survival, followed by patients who developed a response to one peptide.

Additional phase II and III trials are needed to establish the efficacy of peptide vaccines in CRC treatment.

***Dendritic cell vaccines***

As previously mentioned, DCs are potent antigen-presenting cells (APC) and also express costimulatory molecules necessary for an effective immune response. It is therefore expected that DC vaccines are able to elicit antitumour immune responses against tumour-specific antigens. A DC-based cancer vaccine (Sipuleucel-T) has been approved by FDA for the treatment of metastatic castrate-resistance prostate cancer, demonstrating the potential of this approach (Cheever & Higano, 2011).

Several strategies have been used to deliver tumour antigens *ex vivo* to DC, including pulsing DC with synthetic peptides derived from tumour-associated antigens, tumour cell lysates, apoptotic tumour cells, tumour RNA, and physically fuse DCs with whole tumour cells (Signorini et al., 2016). The selection of the tumour antigens to be loaded in DC is a critical step to achieve best clinical results.

In CRC, previous studies have shown that CEA-DC vaccines are safe, well tolerated and able to induce a CEA-specific immune response (Morse et al., 1999; Fong et al., 2001; Itoh et al., 2002; Lesterhuis et al., 2006). A phase II study evaluated the clinical benefit of a DC vaccine consisting of autologous DCs pulsed with allogenic melanoma cell lysate in patients with advanced CRC expressing MAGE-antigens (Toh et al., 2009). Twenty patients were included in the study and 17 of them received immunizations: one patient experienced a partial response and seven achieved stable disease, representing a clinical benefit rate of 40%. The median OS from inclusion was 7.4 months and the median progression-free survival (PFS) was 3.4 months. Five patients (25%) experienced a prolonged PFS for more than 6 months. Using a similar vaccine, Burgdorf

et al. (2008) performed a study in patients with advanced CRC who had no indication for further treatments (radio-, chemo- or surgical therapy). The clinical responses were limited: no partial or complete responses were observed; 4 patients (out of 17) achieved stable disease, of which two remained stable through the entire study period, rendering a clinical benefit rate of 24%.

More recently, the clinical benefit of a DC vaccine combined with cytokine-induced killer cells (CIK) was assessed in a study with CRC patients (n=13) disease-free after surgical resection and chemo/radiotherapy (Gao et al., 2014). The results demonstrated a significantly prolonged DFS and OS in CRC patients of the treatment group compared to the CRC controls (5-year DFS rate: 66% vs 8%; OS rate: 75% vs 15%; $P<0.01$). Despite the small patient cohort, these data demonstrate that the DC-CIK vaccine might be a useful approach to improve survival and tumour recurrence rates in post-surgical CRC patients (Gao et al., 2014).

***Viral-vector vaccines***

Viral vectors can be genetically modified to express tumour antigens to produce cancer vaccines, taking advantage of the natural immunogenicity of the viruses that acts as adjuvant to improve the immune response (Signorini et al., 2016). These vaccines include viruses with high transfection efficiency and immunostimulatory capacity such as lentiviruses, poxviruses, adenoviruses and retroviruses. As disadvantages, viral-vector vaccines are expensive, potentially pathogenic and can lead to insertional mutagenesis (Xiang et al., 2013).

Several virus-based vaccines have been tested in CRC patients, of which the most significant are described below:

- **ALVAC** is a nonreplicating canarypox virus modified to express CEA and sometimes also B7.1 costimulatory molecule, which has been used in CRC patients. A phase I trial showed that ALVAC expressing CEA was safe and resulted in increased CEA-specific T-cell responses (Marshall et al., 1999). More recently, Kaufman et al. (2008) performed a phase II trial in patients with metastatic CRC to assess the effect of chemotherapy on ALVAC-CEA/B7.1. Patients were randomized to receive either ALVAC before and concomitantly with chemotherapy (n=39), ALVAC with tetanus adjuvant before and concomitantly with chemotherapy (n=40), or chemotherapy followed by ALVAC (n=39). The results demonstrated that all patients developed antibody responses against ALVAC, but increased anti-CEA antibody titers were detected in only three patients, and increases in CEA-specific T-cells was detected in 30% to 50% of the patients. In addition, objective clinical responses were observed

in 40% of the vaccinated patients. Overall, there were no differences in clinical or immune responses between the treatment groups. Therefore, this study demonstrated that systemic chemotherapy did not affect vaccine-mediated immunity.

- **TroVax** is a highly-attenuated strain of vaccinia virus encoding the human oncofetal antigen 5T4. The 5T4 antigen is rarely detected on normal tissues and is expressed at high levels on trophoblastic cells and most adenocarcinomas, including CRC, in which it is associated with poor prognosis (Starzynska et al., 1994). A phase I study in patients with advanced CRC demonstrated that TroVax was well tolerated and able to induce anti-5T4 cellular and humoral immune responses. In addition, a positive association was found between the development of 5T4 antibodies and patient survival or time to disease progression (Harrop et al., 2006). In a different study, Elkord et al. (2008) administered TroVax to CRC patients, pre- and post-surgical resection of liver metastases. Most patients developed a 5T4-specific cellular and/or humoral responses, and patients with above median 5T4-specific antibody responses tend to survive longer than those with below median responses, although this difference was not statistically significant. TroVax has also been evaluated alongside two different chemotherapy regimens in CRC patients, establishing its safety and ability to induce specific immune responses without increased toxicity (Harrop et al., 2007; Harrop et al., 2008).

- **PANVAC** is a recombinant poxvirus based vector encoding CEA, MUC1, and three costimulatory molecules (ICAM-1; LFA-3; B7.1). In a phase II trial by Morse et al. (2013b), patients with metastatic CRC, disease-free after metastectomy and perioperative chemotherapy, were randomized to immunization either with DC modified with PANVAC (DC/PANVAC) or PANVAC with GM-CSF (PANVAC/GM-CSF). No significant differences in PFS and OS were observed between the two vaccine strategies, but vaccinated patients as a group exhibited prolonged survival compared to unvaccinated controls (Morse et al., 2013b). Similarly, a fowlpox virus-vector vaccine also expressing CEA, MUC1, and three costimulatory molecules (TRICOM: ICAM-1; LFA-3; B7.1) has been tested in patients with different CEA expressing tumours, including 35 patients with advanced CRC. The results demonstrated that this vaccine was safe and induced CEA-specific T-cell response in most of the patients, as well as stabilization of disease in 40% patients for at least 4 months (Marshall et al., 2005).

- An adenoviral vector encoding the CEA antigen, Ad5 [E1-, E2b-]-CEA(6D), has been studied in a phase I/II clinical trial and shown to induce cell-mediated immunity in 61% of the patients with advanced CRC. The efficacy of Ad5 vaccines can be limited by pre-existing Ad5-specific neutralizing antibodies; however, OS (48% at 12 months) was similar across patients regardless of pre-existing Ad5 neutralizing antibody titres (Morse et al., 2013a).

### 4.2.2 Adoptive T-cell Therapy

Adoptive T-cell therapy is a form of passive immunotherapy that involves the isolation of autologous T-cells with antitumour activity, their activation and expansion to large numbers *ex vivo*, and their re-infusion into the patient, frequently together with IL-2. In most cases, lymphodepleting regimens are used prior to cells administration given their association with an increase persistence of the transferred T-cells (Restifo et al., 2012). An important advantage of this therapy is that T-cells are activated *ex vivo* so some the immunosuppressive factors that occur *in vivo* are overcome. On the other hand, limitations of this approach include possible lack of immune memory, reduced persistence of adoptive T-cells *in vivo*, high costs, and extensive time required to prepare T-cells, in addition to the risk of severe adverse effects (Xiang et al., 2013).

Adoptive T-cell therapy with autologous TILs has been shown to be an effective immunotherapy to induce complete durable responses in patients with metastatic melanoma (Rosenberg et al., 2011).The effectiveness of this approach has also been investigated in CRC patients in some previous studies. In a clinical trial performed by Gardini et al. (2004), 14 patients received adjuvant immunotherapy with TILs in combination with IL-2 after resection of liver metastasis from CRC. The results did not demonstrate a significant improved long-term survival or reduced risk of recurrence compared to the control group (Gardini et al., 2004).

Adoptive T-cell therapy using sentinel lymph node (SLN) acquired lymphocytes has also been investigated in two different studies with CRC patients. In a pilot study, Karlsson et al. (2010) reinfused SLN acquired $CD4^+$ Th1-lymphocytes expanded *ex vivo* in 16 patients with CRC (5 patients with stage II CRC and characteristics of high-risk tumour; 2 with stage III; and 9 with stage IV CRC). All patients with stage IV disease responded to treatment, four with stable disease, one with a partial response, and four with complete tumour regression. In addition, the median survival of these 9 stage IV CRC patients was 2.6 years compared with 0.8 years of conventionally treated controls. Four of stage II patients and all stage III patients were classified as stable disease. None of the patients experienced adverse side-effects (Karlsson et al., 2010). More recently, a phase I/II

study involving 71 stage I-IV CRC patients subjected to surgery demonstrated that transfusion of SLN-T lymphocytes expanded *ex vivo* was not associated with significant toxicity and represents a safe adjuvant immunotherapy. In addition, evaluation of the clinical response in stage IV patients revealed that the 24-month survival rate of the treated group was significantly higher than that of the control group (55.6% versus 17.5%), and the median OS was 28 and 14 months, respectively (Zhen et al., 2015). These two studies demonstrated that SLN-T lymphocyte is a viable and safe immunotherapy option for patients with advanced CRC.

The antitumour potential of the adoptive therapy can be improved with genetic engineered T-cells expressing TCRs with high-affinity to tumour-associated antigens (TAA). A disadvantage of this approach is that these TCRs are MHC-restricted and thus limited to patients with the corresponding MHC haplotype. An alternative approach is the use of chimeric antigen receptors (CARs), which are composed of an extracellular TAA-specific single-chain antibody variable fragment (scFv) linked through hinge and transmembrane domains to an intracellular T-cell signalling domain. CARs can mediate non-MHC-restricted recognition of tumour antigens on the surface of a malignant cell. This approach, using CD19-targeted CAR T-cells, has demonstrated great success in the treatment of hematologic malignancies such as acute lymphoblastic leukemia (Maude et al., 2014). In this context, three patients with metastatic CRC refractory to conventional treatments have been treated with autologous T-cells genetically engineered to express a murine TCR against CEA (Parkhurst et al., 2011). All patients experienced decreases in serum CEA levels (74% to 99%) and one patient had a partial response with regression of cancer metastatic to the lung and liver. However, the three patients developed a severe transient inflammatory colitis which implies a dose limiting toxicity. This adverse event was probably due to the expression of CEA on normal epithelial cells of colonic mucosa that could have been recognized by CEA-reactive T lymphocytes (Parkhurst et al., 2011). A severe adverse event was also reported by Morgan et al. (2010) in a CRC patient with metastasis treated with Her2-specific CAR T-cells. In this case, minutes after cells infusion the patient experienced severe respiratory distress and displayed a dramatic pulmonary infiltrate on chest X-ray, eventually resulting in patient's death in 5 days. The authors hypothesized that this patient' death resulted from a cytokine-release-syndrome caused by the recognition of ERBB2 on normal lung epithelial cells by the very high dose of cells administered (Morgan et al., 2010).

In general, the clinical studies using adoptive T-cell therapy reported modest success and involved small numbers of CRC patients. Further studies are needed to determine the safety and efficacy of this strategy in CRC.

### 4.2.3 Immune Checkpoint Inhibitors

The immunotherapy approach that is getting most attention and progress in recent years is the immune checkpoint blockade.

Immune checkpoints refer to complex inhibitory pathways that mediate immunological self-tolerance and modulate the duration and amplitude of physiological immune responses in order to prevent damage of peripheral tissues (Pardoll, 2012). However, it is now well established that tumour cells can develop mechanisms to evade the host immune system, namely by exploiting these inhibitory checkpoints, thus limiting antitumour immune response (Poschke et al., 2011).

CTLA-4 and PD-1 are two immune checkpoint molecules that downregulate T-cell activity and have been intensively studied in several types of cancer such as melanoma and NSCLC (Pardoll, 2012). Immunotherapies using antibodies designed to inhibit these immune checkpoints are under intense investigation and encouraging clinical results have been achieved.

***CTLA-4***

As previously described, an immune response against pathogens or tumour cells is triggered by the recognition and binding of a TCR to an antigen displayed in the context of an MHC molecule on the surface of an APC. An effective immune response requires in addition costimulatory signals - typically the interaction of CD28 expressed on T-cells with either B7-1 (CD80) or B7-2 (CD86) on the APCs. CD28 signalling induces cytokine production (IL-2) and T-cell activation. Once activated, T-cells up-regulate CTLA-4 transcription and expression in the cell surface, where it is able to bind B7 molecules with much more affinity than CD28, thereby preventing CD28 costimulation and T-cell activation (Figure 2, Panel A) (Walunas et al., 1994; Krummel & Allison, 1995). Furthermore, previous studies have demonstrated that CTLA-4 can also remove B7 ligands from APCs via transendocytosis (Qureshi et al., 2011). CTLA-4 therefore down-modulates the amplitude of early T-cell activation, limiting their antitumour activity (Pardoll, 2012).

The essential role of CTLA-4 in maintaining immunologic tolerance is demonstrated by the lethal phenotype of *Ctla-4* null mice, which developed early lymphoproliferative disease with lymphocytic infiltration into multiple tissues, and died within 3-4 weeks of age (Tivol et al., 1995; Waterhouse et al., 1995). In humans, germline heterozygous mutations in *CTLA-4* gene have been identified in patients with severe immune impairments, characterized mainly by dysregulation of $FoxP3^+$ Treg cells, hyperactivation

of effector T-cells and lymphocytic infiltration of target organs (Kuehn et al., 2014; Schubert et al., 2014).

In line with these data, Leach et al. (1996) showed that the *in vivo* administration of antibodies against CTLA-4 induced rejection of pre-stablished tumours in mouse and provided immunity against secondary exposure to tumour cells. These pre-clinical data prompted the development of two monoclonal antibodies targeting CTLA-4 – **ipilimumab** and **tremelimumab** – in order to increase antitumour immune response (Figure 2, Panel A). Ipilimumab was the first checkpoint inhibitor to be approved by FDA (2011) for the treatment of unresectable or metastatic melanoma (Hodi et al., 2010).

Despite the successful clinical results achieved with CTLA-4 inhibitors in melanoma, clinical studies with CRC patients have been quite disappointing. A phase II study with the anti-CTLA-4 antibody tremelimumab in patients with refractory metastatic CRC did not demonstrate significant antitumour activity. In fact, from the 45 patients evaluable for response, 43 received just one dose of tremelimumab due to disease progression, and only one patient had a partial response and received a total of five doses, upon which disease progression was documented. Thirty of the patients (63.8%) experienced adverse events, including diarrhea, colitis, fatigue, nausea, pyrexia, vomiting, skin rash, and autoimmune thrombocytopenia (Chung et al., 2010).

***PD-1***

In contrast to CTLA-4, which regulates the early activation of T-cells predominantly in the lymph nodes, PD-1 suppresses T-cells primarily in peripheral tissues at a later phase of the immune response (Pardoll, 2012). PD-1 is expressed on the surface of activated and exhausted T-cells, B-cells, NK cells, and macrophages. It interacts with two ligands, programmed death ligand-1 (PD-L1) and PD-L2, which are expressed on the surface of APCs and malignant cells (Figure 2, Panel B) (Baumeister et al., 2016).

Blockade of this pathway using monoclonal antibodies directed to PD-1 or PD-L1 has demonstrated favorable clinical responses in several type of tumours, including melanoma, NSCLC, renal-cell carcinoma, Hodgkin's lymphoma, and head and neck cancers (Brahmer et al., 2012; Topalian et al., 2012; Ferris et al., 2016; Younes et al., 2016). Based on these data, since 2014 two anti-PD-1 monoclonal antibodies – **nivolumab** and **pembrolizumab** – have been approved by FDA as treatment options in several malignancies. In addition, in 2015 the combination of ipilimumab and nivolumab was approved by FDA for the treatment of patients with advanced melanoma (Larkin et al., 2015).

In what concerns CRC, initial clinical trials have demonstrated very low response rates to PD-1 inhibitors. A phase I trial to evaluate the safety, tolerability and pharmacokinetics of nivolumab included patients with advanced metastatic melanoma (n=10), CRC (n=14), castrate resistant prostate cancer (n=8), NSCLC (n=6), and renal cell carcinoma (n=1). Two patients (melanoma, renal cell carcinoma) exhibited partial responses and one patient with CRC had a complete response which persisted 3 years after treatment (Brahmer et al., 2010; Lipson et al., 2013). Nivolumab was well tolerated, with only one serious adverse event documented (inflammatory colitis). Topalian et al. (2012), in a study with 296 patients with different types of cancer, observed partial or complete responses in those with NSCLC (18%), melanoma (28%) and renal-cell cancer (27%), but no objective responses were reported in CRC and prostate cancer patients. Grade 3 or 4 treatment-related adverse events occurred in 14% of the patients and three of them died from pneumonitis. Similar results were observed in a phase I trial with an anti-PD-L1 antibody, with objective responses reported in patients with metastatic NSCLC, melanoma, renal-cell cancer and ovarian cancer, but not in CRC, gastric, pancreatic nor breast cancer patients (Brahmer et al., 2012).

Interestingly, a recent study has demonstrated that the microsatellite instability status of the tumour could predict the clinical benefit of the immune checkpoint blockade (Le et al., 2015). This phase II trial, designed to evaluate the clinical activity of pembrolizumab (anti-PD-1), found that patients with mismatch repair (MMR)-deficient CRC (n=10) had significantly improved response rates (40%) and immune-related PFS rates (78%) than those who had MMR-proficient CRC (0% and 11%, respectively). More importantly, in those patients with MMR-deficient CRC, the median PFS and median OS were not reached contrasting with the cohort of patients with MMR-proficient tumours, in which the median PFS was 2.2 months and the median OS was 5.0 months. The rationale behind this study was that tumours with MMR deficiency would exhibited a large number of mutations. These generate more neoantigens resulting in a tumour that is “more visible” to patient’s immune system. Indeed, whole-exome sequencing revealed a mean of 1782 somatic mutations per tumour in patients with MMR deficiency, contrasting to 73 in patients with MMR-proficient tumours. In addition, membranous PD-L1 expression was only observed in patients with MMR-deficient cancers. Remarkably, additional analysis of the single CRC patient with a durable complete response to nivolumab in the study by Brahmer et al. (2010) previously mentioned revealed that the tumour was MMR-deficient (Lipson et al., 2013). These results are in agreement with other studies indicating that MSI tumours present a microenvironment rich in activated Th1 cells and cytotoxic T lymphocyte (CTL) (Dolcetti et al., 1999; Smyrk et al., 2001; Llosa et al., 2015). Llosa et al. (2015) have also detected an overexpression of several immune checkpoints

in the MSI tumours microenvironment (including PD-1, PD-L1, and CTLA-4, among others) as compared to microsatellite-stable tumours. The authors conclude that MSI tumours upregulate inhibitory molecules to compensate the CTL-rich microenvironment, thereby preventing elimination by the immune system.

Taken together these data suggests that the subset of CRC patients with MSI could be a particularly good candidate to immunotherapy with checkpoint inhibitors. Currently, several ongoing clinical trials are evaluating the clinical activity of combinations of ipilimumab with PD-1/PD-L1 antibodies as well as combinations of different checkpoint inhibitors with chemotherapy or radiation in CRC.

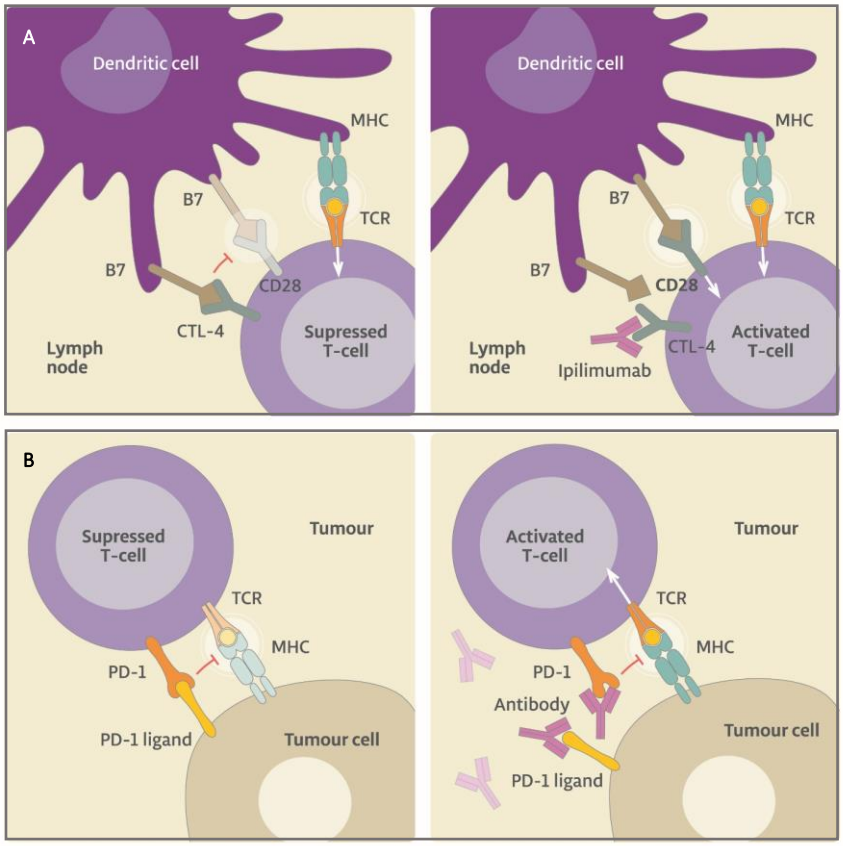


**Figure 2: Immune checkpoints (CTLA-4 and PD-1) signaling in an antitumour immune response.** CTLA-4 is expressed by T-cells shortly after their activation and competes with CD28 for B7 costimulatory molecules on DCs. CTLA-4 binding to B7 provides inhibitory signals to T-cells. Antibody blockade of CTLA-4 (e.g. ipilimumab) prevents this inhibition (Panel A). PD-1 is expressed by T-cells during long-term antigen exposure and its interaction with PD-L1 or PD-L2 on tumour cells suppresses the effect of the TCR on T-cell activation. Blockade of PD-1 or PD-L1 with antibodies (e.g. nivolumab or atezolizumab, respectively) releases the inhibition over TCR signaling, allowing T-cell activation (Panel B). Adapted from Ribas et al. (2005).

# 5. Discussion and Conclusions

To date, immunotherapy in CRC demonstrated modest clinical benefits, but several trials are ongoing in order to evaluate additional immune-based therapies either as single agents or in combination regimens.

Active specific immunotherapy using therapeutic cancer vaccines has been investigated over decades in different types of tumors, but only Sipuleucel-T has been approved by the FDA for the treatment of prostate cancer patients. This reflects a possible limiting step of cancer vaccines which is its reliance on previous identification of tumour antigens that are able to elicit an effective cytotoxic T-cell response against cancer cells. The ideal antigens must be highly expressed on tumour cells but not in healthy tissues, and must have minimally homology with other host proteins to ensure specificity of the response and reduce off-target toxicities.

In what concerns the adoptive T-cell therapy, clinical trials performed in CRC patients revealed that more studies are required to improve both safety and efficacy of this strategy. Thus far, adoptive T-cell therapy using CD-19-targeted CAR T-cells showed great success in the treatment of B-cell malignances such as acute lymphoblastic leukemia. CD19 is a suitable antigen because it is expressed on a broad range of differentiated B-cells, but is not present on hematopoietic stem cells or other cell types (Khalil et al., 2016). The selection of similar antigens in solid tumors could be challenging, not only because these malignancies are in general more heterogeneous than liquid tumors, but also because target antigens are more likely to be also expressed in normal tissues (Khalil et al., 2016). Concerning CRC, some studies have identified other molecules that may be potentially suitable as cancer-specific antigens, like GUCY2C and OR7C1 (Snook et al., 2011; Morita et al., 2016).

Immune checkpoint inhibitors are the immunotherapy agents that are receiving most attention in the last few years. Monoclonal antibodies against CTLA-4, PD-1 and PD-L1 have been evaluated in several trials and approved to be used in the clinical setting for the treatment of melanoma and NSCLC, among other tumours. In CRC, studies have demonstrated that the subgroup of patients with microsatellite instable tumors benefits from therapy with pembrolizumab (PD-1 inhibitor) and, based on this finding, a phase II study (NCT02460198) and a phase III study (NCT02563002) are underway in MMR-deficient advanced CRC to confirm such results. However, this group represents a small subset of patients, so clinical trials are currently in progress to evaluate other immunotherapy agents that could have an impact on clinical outcome also in CRC patients with microsatellite stable tumours. In particular, the combination of immune checkpoint inhibitors with other anticancer agents that may increase tumour sensitive to

immunotherapy is being tested. Recently, preliminary data from a phase Ib trial (NCT01988896) demonstrated for the first time a clinical value for PD-L1 inhibition in patients with microsatellite-stable tumours (Bendell et al., 2016). This study is evaluating the safety and clinical activity of atezolizumab (anti-PD-L1 antibody) in combination with cobimetinib (MEK inhibitor) in patients with locally advanced or metastatic solid tumours. The rationale underlying this approach was based on preclinical studies which demonstrated that targeted inhibition of MEK results in MHC class I upregulation on cancer cells, promotes intratumoral T-cell infiltration, and enhances anti-PD-L1 activity. Provisional results from this trial showed that from the 23 CRC patients, 4 had a partial response and 5 had stable disease; most importantly, three of the responders had MSS tumours.

Similarly, numerous combination strategies comprising agents with different mechanisms of action are being investigated in order to achieve higher response rates and prolonged overall survival. For instance, CTLA-4 and PD-1 inhibitors have been used together in patients with metastatic melanoma with positive results (Larkin et al., 2015). Checkpoint inhibitors may also exhibit synergistic effects when combined with antigen-directed therapies (e.g. cancer vaccines) and even with conventional cytotoxic agents or radiation. Although these last two anticancer therapies were initially considered immunosuppressive, it is presently known that some agents can induce an immune response. Radiation, for example, by destroying tumor cells, releases antigens that can be captured by antigen presenting cells, triggering T-cell activation. Once again, checkpoint blockade would prevent inhibition of T-cells activation, thereby allowing for an effective anticancer immune response.

The modest results obtained thus far in studies of CRC immunotherapy could also rely on the fact that most of them have been carried out in patients with advanced-stage tumours, with a likely compromised immune system that is unable to mount an appropriate immune response. It could be speculated, therefore, that the best candidates for immunotherapy are patients at initial-stage disease with a risk of recurrence after tumour resection.

While cancer immunotherapy has emerged as an important treatment option, it presents relevant features that differentiate it from conventional therapies and of which physicians should be aware of. In this context, the introduction of checkpoints inhibitors in clinical practice revealed a unique toxicity profile known as “immune-related adverse events” (irAE) (Wolchok et al., 2009). These irAE are mainly related to inflammatory conditions such as dermatitis, colitis, hepatitis, pancreatitis, pneumonitis and hypophysitis, as it could be expected from the mechanism of action of immunotherapies. Adverse events

can range from moderate to severe but are usually manageable with immunosuppressive agents like corticosteroids, which do not seem to alter the antitumor effect of the therapy (Sharma & Allison, 2015). Although the profile of irAE observed with CTLA-4 and PD-1/PD-L1 inhibitors is comparable, anti-CTLA4 therapy is associated with more frequent and severe toxicity. Another important distinction of cancer immunotherapy compared to conventional treatments has to do with the kinetics of clinical response, which comprises three progressive steps (Hoos et al., 2010): firstly, the immune activation and proliferation of T-cells occurs; secondly, after weeks to months, an antitumour immune response becomes evident; thirdly, months after the initial treatment, an effect on patient survival could be evident. This means that, contrasting with conventional cytotoxic therapies that directly attack cancer cells, clinical response to immunotherapy may take several months to be detectable. In fact, some patients may even experience an initial phase of tumour and/or metastatic lesions growth due to infiltration by immune cells. All these aspects are disregarded by the Evaluation Criteria in Solid tumors (RECIST) traditionally used to evaluate the response to cytotoxic agents, so new criteria, better adapted to immunotherapy, have been established to incorporate these findings (the immune-related response criteria - irRC) (Hoos et al., 2010).

In conclusion, immunotherapy represents a novel and promising approach to fight cancer. While individual agents have shown high response rates and durable response in some types of cancer, it is likely that in the future combined strategies will improve clinical outcomes and be effective in the treatment of other types of cancer, such as colorectal cancer.

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