Nanotechnology is an important strategy for combinational innovative chemo-immunotherapies against colorectal cancer

Ana I. Matos, Barbara Carreira, Carina Peres, Liane I.F. Moura, João Conniot, Thibaut Fourniols, Anna Scomparin, Águeda Martínez-Barriocanal, Diego Arango, João P. Conde, Véronique Préat, Ronit Satchi-Fainaro, Helena F. Florindo

PII: S0168-3659(19)30327-X
DOI: https://doi.org/10.1016/j.jconrel.2019.06.017
Reference: COREL 9822

To appear in: *Journal of Controlled Release*

Received date: 30 April 2019
Revised date: 12 June 2019
Accepted date: 16 June 2019" role="suppressed

Please cite this article as: A.I. Matos, B. Carreira, C. Peres, et al., Nanotechnology is an important strategy for combinational innovative chemo-immunotherapies against colorectal cancer, Journal of Controlled Release, https://doi.org/10.1016/j.jconrel.2019.06.017

Nanotechnology is an important strategy for combinational innovative chemo-immunotherapies against colorectal cancer

Ana I. Matos[a], Barbara Carreira[a], Carina Peres[a], Liane I. F. Moura[a], João Conniot[a], Thibaut Fourniols[b], Anna Scomparin[c,d], Águeda Martínez-Barriocanal[e], Diego Arango[e], João P. Conde[f,g], Véronique Préat[b], Ronit Satchi-Fainaro[c,h,*] ronitsf@tauex.tau.ac.il, and Helena F. Florindo[a,*] hflorindo@ff.ulisboa.pt

[a]Research Institute for Medicines (iMed.ULisboa), Faculty of Pharmacy, Universidade de Lisboa, Av. Prof. Gama Pinto, 1649-003 Lisbon, Portugal
[b]Louvain Drug Research Institute, Advanced Drug Delivery & Biomaterials, Université Catholique de Louvain, 1200 Brussels, Belgium
[c]Department of Physiology and Pharmacology, Sackler Faculty of Medicine, Tel Aviv University, Tel Aviv 69978, Israel
[d]Department of Drug Science and Technology, University of Turin, Via P. Giuria 9, 10125 Turin, Italy
[e]Group of Biomedical Research in Digestive Tract Tumors, CIBBIM-Nanomedicine, Vall d'Hebron Research Institute (VHIR), Universitat Autònoma de Barcelona (UAB), Barcelona 08035, Spain
[f]Instituto de Engenharia de Sistemas E Computadores – Microsistemas e Nanotecnologias (INESC MN) and IN – Institute of Nanoscience and Nanotechnology, Rua Alves Redol, 9, 1000-029 Lisbon, Portugal;
[g]Department of Bioengineering, Instituto Superior Tecnico, University of Lisbon, 1049-001, Lisbon, Portugal
[h]Sagol School of Neuroscience, Tel Aviv University, Tel Aviv, Israel

[*]Corresponding authors.

Abstract

Colorectal cancer (CRC) is among the five most commonly diagnosed cancers worldwide, constituting 6% of all cancers and the third leading cause of cancer death. CRC is the third and second most frequent cancer in men and women worldwide, accounting for 14% and 13% of all cancer incidence rates, respectively. CRC incidence is decreasing in older populations, but it has been significantly rising worldwide in adolescents and adults younger than 50 years old.

Significant advances in the screening methods and surgical procedures have been underlying the reduction of the CRC incidence rate in older populations. However, there is an urgent demand for the development of alternative effective therapeutic options to overcome advanced metastatic CRC, while preventing disease recurrence.

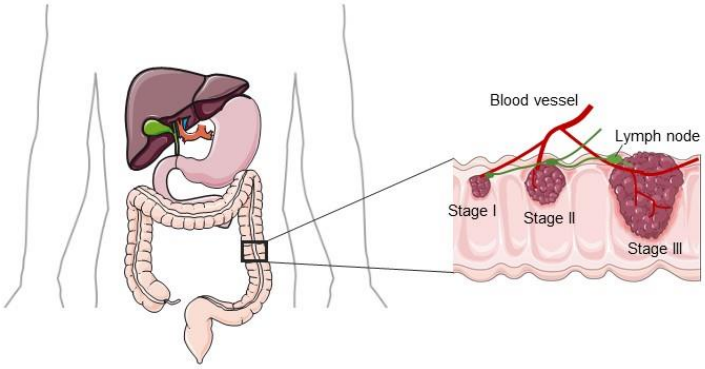
This review addresses the immune and CRC biology, summarizing the recent advances on the immune and/or therapeutic regimens currently in clinical use. We will focus on the emerging role of nanotechnology in the development of combinational therapies targeting and thereby regulating the function of the major players in CRC progression and immune evasion.

Keywords: Colorectal cancer; nanotechnology; immunotherapy; combinational schemes; tumor immune microenvironment

1. Introduction

Colorectal cancer (CRC), also referred as bowel cancer, occurs when abnormal cells grow in the colon or rectum [1]. Approximately 96% of CRC are adenocarcinomas that result from the prolonged and slow growth of precancerous adenomatous polyps or adenomas, in the inner wall of the colon and rectum [2, 3]. Among those, 10% may progress to invasive cancer [4, 5]. Once established, those malignant cells can spread into the colorectum wall and potentially invade blood or lymph vessels, leading to metastases in distant organs and tissues, such as the liver, lungs, or peritoneum [1].

According to the American Joint Committee on Cancer, CRC can be classified into five stages (Figure 1) [1, 6].



CRC STAGES	DESCRIPTION
0	Tumor confined to the mucosa (carcinoma *in situ*), without colon or rectum wall invasion. These are considered 100% cured after surgical excision.
I	Tumor formed in the mucosa grows across colon or rectum wall invading submucosa and *muscularis propria* without spreading to adjacent tissues. After surgical resection, patients present a 5-year survival of 90%.
II	It can be divided into IIA once tumor invades pericolorectal tissues through the *muscularis propria*, penetrating the visceral peritoneum surface (IIB) and other organs or structures (IIC). After surgical resection, patients have a 5-year survival rate of 67%, 59% and 37%, among IIA-IIC stages.
III	Tumor extended through the colon or rectum walls invading *muscularis propria* or submucosa (IIIA), visceral peritoneum surface or pericolorectal tissues through *muscularis propria* (IIIB), or other organs or structures with metastases in the regional lymph nodes (LN; in the range of 1-3 to more than 7, according to sub-stages) and nearby tissues. Standard treatments include surgical resection followed by adjuvant chemotherapy.
IV	Patients present distant metastases confined to other body organs, such as liver or lungs, and a 5-year survival rate of 10%. Although not often curable, stage IV CRC is treatable. Standard treatments include surgical resection followed by adjuvant chemotherapy.

Figure 1. CRC stage according to the American Joint Committee on Cancer. Adapted from [1, 6].

A 5-year relative survival rate of 65% was obtained for patients diagnosed from 2006 to 2012 [7, 8]. Even though, these survival rates change according to illness stage, being 90, 71 or 14% for CRC patients diagnosed with localized (39% of patients), regional or distant-stage disease, respectively [9].

Despite the recognized advances in the screening methods, surgical procedures and chemotherapeutic treatments currently available [10, 11], approximately 20% of CRC patients at the time of the first diagnosis, and 30-50% after the surgical resection of the primary tumor, are diagnosed with the metastatic disease [12, 13]. These patients with metastatic CRC present a median survival time lower than 8 months, in the absence of treatment.

This review summarizes the CRC biology and immunology, focusing on the immune and stromal cells with major role on the progression of this disease. It presents also an overview of the therapies approved for the CRC treatment, as well as the preclinical and clinical data available for the emerging approaches. We focus on the potential of nanotechnology-based technologies as cutting-edge combinational platforms to regulate the tumor-immune-stromal microenvironment, and thereby overcome CRC evasion and proliferation.

1.1.Risk factors and causes of CRC

The discrepant CRC incidence rates worldwide are related to genetic and environmental factors, in addition to gender, age and ethnicity [14].

Genomic instability of several forms (Table 1) plays a significant role in the development of sporadic or inherited CRC, by facilitating the acquisition of genetic and epigenetic mutations in specific oncogenes and/or tumor suppressor genes by normal epithelial cells, thereby potentiating the colorectal epithelial cell transformation into adenocarcinoma and metastasis [15-17].

Table 1. Genomic instability forms in CRC [16, 17].

GENOMIC INSTABILITY FORMS	ALTERED GENES/PATHWAYS
Chromosomal instability of tumor suppressor genes	*APC*, *P53*, and *SMAD4*
MSI	-
Epigenetic gene silencing induced by aberrant DNA methylation within certain promotor associated CpG islands	*MLH1*
Inactivation of DNA MMR genes	*MLH1*, *MSH2*, *MSH6*, and *MUTYH*
Altered signaling pathways	*Wnt/APC/β-catenin*
	MAPK/RAS/BRAF

	PI3K/AKT/GSK-3β
	TGF-β/SMAD
	NF-κB

Notes: *APC*: adenomatous polyposis coli; CpG: cytosine-phosphate-guanine; DNA: deoxyribonucleic acid; *GSK-3β*: glycogen synthase kinase-3β; MAPK: mitogen-activated protein kinase; *MLH1*: MutL homolog 1; MMR: mismatch-repair; *MSH2*: MutS protein homolog 2; *MSH6*: MutS protein homolog 6; MSI: microsatellite instability; *MUTYH*: MutY homolog; *NF-κB*: nuclear factor kappa B; *P53*: tumor suppressor protein *P53*; *PI3K*: phosphoinositide 3-kinase; *SMAD4*: SMAD family member 4 or mothers against decapentaplegic homolog 4; *TGF-β*: transforming growth factor-β.

Seven tenths of all CRC are sporadic and usually derive from somatic mutations and dysfunctional *Wnt/β-catenin* signaling pathway [17]. But, 5% of CRC cases are a consequence of characterized hereditary syndromes derived from specific gene mutations [18].

The most common autosomal dominant inherited syndromes are the Lynch syndrome or hereditary nonpolyposis CRC (HNPCC), and the classical familial adenomatous polyposis (FAP). The first is associated with mutations in the genes *MLH1* and *MSH2*, which are involved in the DNA mismatch-repair pathway, while the FAP is caused by mutations in the tumor suppressor gene *APC* [19]. HNPCC and FAP are responsible for 2-4% and less than 1% of all CRC cases, respectively [20, 21]. Although less clinically defined, *MUTYH*-associated polyposis (MAP), usually associated with attenuated FAP and caused by autosomal recessive mutations in the base excision repair gene *MUTYH*, seems to promote the development of less adenomas in the large bowel [19, 22].

About 30% of CRC patients present family CRC history, where the lifestyle risk factors add to the accumulation of usual genetic alterations over generations [18]. When compared to people without CRC family history, people with a first-degree relative present a higher risk of developing this disease [22].

In addition to the hereditary and family CRC history, individuals who have a personal medical history of adenomatous polyps, chronic inflammatory bowel disease (ulcerative colitis and Crohn's disease) characterized by a prolonged inflammation of the colon and rectal mucosa or full thickness of bowel wall, and type 2 diabetes have also an increased risk of developing CRC and other intestinal neoplasms than general individuals [23].

Several behaviors strictly related to sedentary lifestyle, long-term smoking and alcohol addiction, are known as CRC modifiable risk factors. Physically active or less sedentary people have a risk of colon cancer (but not rectal cancer) 25% lower than sedentary people [24]. Independently of the physical activity, obesity increases the risk

in 50% and 20% for colon cancer, and 20% and 10% for rectal cancer, for men and women respectively, being responsible for nearly a third of CRC and lower survival likelihood [25].

Formation and growth of polyps have been associated with cigarette smoking [26], and some mutations caused by tobacco are less effectively repaired in the presence of alcohol intake [27]. CRC incidence is also strongly influenced by the diet, which has a large impact on the microbiome environment in the large intestine, and consequently, in the immune response, inflammation and tumor development [28]. In contrast to the reduced CRC risk associated with dietary fiber intake (vegetables and fruits) [29], diets rich in fat, red and processed meat are related with a raised risk [30].

1.2. CRC metastasis: role of tumor immune microenvironment

Metastatic CRC (mCRC) is a prolonged and multifaceted process involving several cellular and molecular pathways. CRC metastases can be found in the peritoneum [31], lungs [32], bone [33, 34] and brain [35, 36]. However, liver is usually the most affected organ in mCRC and often the single site of metastasis. Liver metastases affect 20-25% of patients at initial diagnosis time and 40% of individuals after primary tumor resection [37, 38]. High levels of the carcinoembryonic antigen (CEA), associated to several symptoms, such as nausea, jaundice, weight loss and pain in the right upper quadrant, may be related to the hepatic metastatic form of CRC [39]. Additionally, in contrast to lung metastasis that are commonly asymptomatic [32], peritoneal metastasis are associated to abdominal swelling and distress, nausea, vomiting, weariness and weight loss [31].

Most of the metastases presented by these mCRC patients cannot be addressed surgically, being rather treated with chemotherapy, alone or in combination with biological agents. Even though, a limited success has been achieved by these therapeutic combinations, which may be explained by the so-called “tumor immune microenvironment” (TIME) [40].

Similarly to other solid tumors, CRC TIME is a complex network of bidirectional interactions established between a complete set of stromal and immune cells, along the extracellular matrix, which can suppress and/or promote tumorigenesis *via* individual or collective functions. It has been associated with the multistep process from normal colonic epithelium to an adenomatous polyp, and ultimately to an invasive colon carcinoma [41].

The stroma plays an essential role in tumor architecture, providing a physical support for the functions of residing cells [42, 43]. Stromal cells, such as endothelial cells (EC) (vascular or lymphatic), pericytes, and cancer-associated fibroblasts (CAF) have been identified as having an active role in CRC microenvironment (Figure 2) [44].

The immune system itself is also an important contributor to the suppressive TIME. Despite the ability of the immune system to regulate the tumor biology and inhibit tumor development, both innate and adaptive immune cells can polarize from their "tumoricidal" form to their "tumorigenic" one within TIME, further influencing the growth, proliferation, and infiltration of other immune cells into the site of injury [45]. These cells include mostly the tumor-infiltrating lymphocytes (TIL; T cells, B cells, and natural killer (NK) cells), tumor-associated macrophages (TAM), mast cells, myeloid-derived suppressor cells (MDSC), granulocytes (neutrophils, eosinophils and basophils), and dendritic cells (DC) (Figure 2) [46, 47].

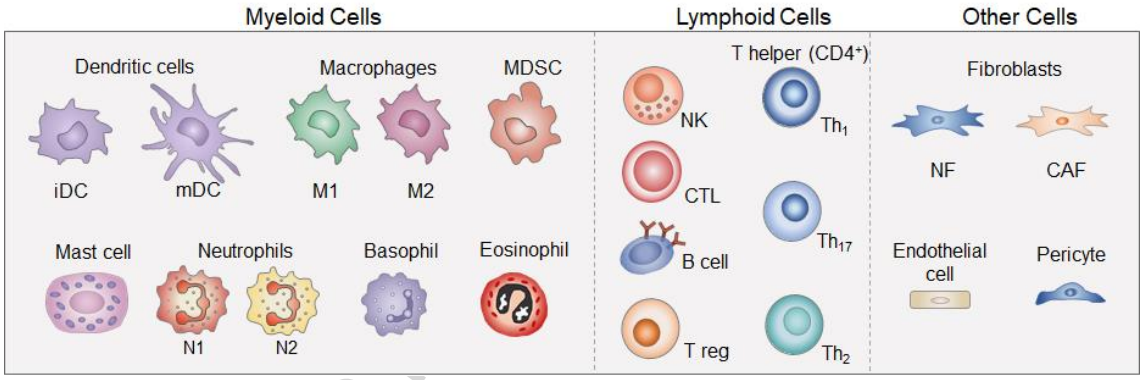


Figure 2. Set of stromal and immune cells involved in the CRC microenvironment.
Notes: CAF: cancer-associated fibroblasts; CTL: cytotoxic T lymphocytes; DC: dendritic cells; iDC: immature DC; MDSC: myeloid-derived suppressor cells; NF: normal fibroblasts; NK: natural killer cells; rDC: regulatory DC; sDC: stimulatory DC; Treg: regulatory T cells. Adapted from [46].

The major roles of each of the main individual components of the TIME is described in Table 2.

Table 2. Key players of the CRC TIME and their major role.

CELL TYPE	ROLE WITHIN THE TIME	REF
Cytotoxic T lymphocytes (CTL)	CTL promotes the apoptotic death of recognized target cells, by releasing cytotoxins, such as perforins and granzyme B, or through the engagement of the Fas/Fas-ligand (FasL), which activate different pathways leading to apoptosis and target cell death. CTL also employ nonlytic effector mechanisms including the production of IFN-γ, a cytokine with several direct and indirect anti-tumor properties. High density of CTL in the CRC TIME predicts better patient survival.	[48, 49]
T helper cells	$CD4^+$ T cells act as helper cells and modulate the activation of CTL. Depending on the amount and type of cytokines present in the environment, Th cells may differentiate into four	[50, 51]

(Th cells)	main classes: Th1, Th2, Th17 and regulatory T cells (Treg). Th1 cells' response is associated with the anti-tumor activity through the IFN-γ secretion and the recruitment and activation of macrophages. IFN-γ can also remarkably induce the IL-12 production by mature dendritic cells (maDC), which, in turn, triggers the polarization of more naïve T cells into the Th1 phenotype, contributing to their own growth and maintenance. CRC patients displaying high levels of Th1-associated gene expression in tumor tissues have better prognosis. Th2 cells favor the tumor growth and may be the predominant subpopulation among the infiltrating lymphocytes of some tumors. The Th2-type cytokines in the CRC microenvironment have no prognostic significance for patient survival. Th17 cells' role in the TIME is controversial, since they can either promote or prevent cancer cell growth and metastasis. However, a poor prognosis has been reported for CRC patients with high expression of the Th17 cluster. Treg cells suppress the activation, proliferation and effector functions of a wide range of immune cells, including $CD4^+$ and $CD8^+$ T cells, NK, B cells and APC. Treg can regulate the immune response by different mechanisms: i) secretion of immunosuppressive molecules, as IL-10, and TGF-β; ii) cytolytic functions *via* a variety of mediators like granzyme B, the TNF-related apoptosis-inducing ligand (TRAIL) pathway and galectin-1; iii) IL-2 deprivation-mediated apoptosis of effector $CD4^+$ T cells. Treg prevent effector T cells activation through the inhibition of co-stimulatory molecules or suppression of DC maturation via IL-10/TGF-β signaling. In addition, the accumulation of Treg at TME has also been associated with faster angiogenesis.	
B cells	B cell infiltrates have been found in many different tumors, including CRC. These cells may play both positive and negative roles in tumor immunity. At one hand, B cells were shown to promote anti-tumor T cell-mediated immune responses, but also presented a highly cytotoxic activity mediated by the IgG2b. On the other hand, Regulatory B cells (Breg) are a newly designated subset of B cells, identified both in murine and human tumor samples. Breg cells have been shown to promote tumor progression by attenuating the anti-tumor immunity *via* the suppression of the T cell immune response through the secretion of anti-inflammatory mediators, such as IL-10, TGF-β and IL-35, and by facilitating the generation of Treg.	[52, 53]
Natural killer (NK) cells	NK cells have cytotoxic functions and shape a proinflammatory microenvironment. NK can recognize the altered expression of tumor cell surface ligands through the activation of specific receptors. Moreover, the downregulation or lack of MHC class I molecules on tumor cell surfaces promotes NK cell activation and induces their cytotoxic activity through granzyme B- and perforin-mediated apoptosis, FasL-associated apoptosis or via TRAIL. Recently, NK cells have been shown to stimulate the recruitment of conventional DC 1 (cDC1) into the TIME, which is critical for an anti-tumor immunity, but also to prevent metastasis by controlling tumor architecture.	[54, 55]
Tumor-associated macrophages (TAM)	Activated macrophages are usually classified as M1 (anti-tumorigenic) or M2 (pro-tumorigenic) TAM. In the TIME, TAM are more likely differentiated into M2-TAM. M1-TAM secrete high levels of IL-12 and Th1 cell-attracting chemokines, also displaying a tumoricidal capacity. M2-TAM down-regulate the MHC class II expression, promote tumor progression through the secretion of immunosuppressive cytokines, and promotion of Treg differentiation, angiogenesis, wound healing and therapeutic resistance via several mediators. A strong infiltration of M2-TAM is associated with poor prognosis, while the infiltration of M1-TAM stands as an independent prognostic factor. Infiltration of TAM in CRC seems to be related with a poor outcome.	[56, 57]
Myeloid-derived suppressor cells (MDSC)	MDSC exhibit remarkable immunosuppressive and tumorigenic activities. MDSC mediate the suppression of T cell functions through the upregulation of immune suppressive factors, such as arginase and nitric oxide synthase isoform (iNOS), and the increased production of reactive oxygen species (ROS), promotion of Treg functions through the secretion of IL-10 and TGF-β, as well as the regulation of NK cell functions.	[58]
Mast cells (MC)	Although MC consistently infiltrates tumors, their role as pro- or anti-tumorigenic players is controversial. While some studies have shown a protective role, recent evidences indicate that MC enhance blood and lymphatic vessel formation, which results from their degranulation and release of pro-angiogenic and growth stimulatory factors. In CRC, high	[59]

	mast cell density has been related to tumor aggressiveness and reduced survival.	
Tumor-associated neutrophils (TAN)	TAN are divided into anti-tumor and pro-inflammatory N1 type or tumor-progressive and immunosuppressive N2 type. TAN are more likely to be polarized to the N2 type within the TIME. N1-TAN anti-tumor effects result from the expression of immunoactive cytokines and chemokines, the generation of oxidative damage caused by ROS and the induction of FasL-associated apoptosis. N2-TAN secrete pro-tumorigenic factors and affect other immune cells in an immunosuppressive manner, for example, by inducing T cell tolerance. However, the specific role of tumor-infiltrating neutrophils in the local CRC TIME remains to be fully elucidated.	[60]
Eosinophils	Their role in TIME remains controversial. However, eosinophils' CRC infiltration has been correlated with favorable prognoses. When co-cultured with CRC cells *in vitro*, eosinophils were able to kill tumor cells due to the release of TNF-α, eosinophil cationic protein, eosinophil-derived neurotoxin and granzyme A.	[61, 62]
Basophils	Their role in CRC TIME remains unclear. Clinical studies have demonstrated that the presence of basophils in tumor-draining LN and tumors of patients with pancreatic ductal adenocarcinoma or chronic myeloid leukemia favored a Th2 environment that is pro-tumoral and, therefore, correlated with reduced survival.	[63, 64]
Dendritic cells (DC)	Depending on the signals present in the TIME, maDC can be subdivided in stimulatory (sDC) and regulatory (rDC) DC. Exposure to pro-inflammatory signals generates sDC, thus promoting the stimulation of T cell proliferation and impairment of Treg function. In contrast, tolerogenic signals, such as TGF-β, IL-10 and prostaglandins, generate rDC that suppress T cell activation and proliferation, and provide signals that enable Treg differentiation and expansion.	[65, 66]
Endothelial cells (EC)	EC play a key role in the development and function of blood and lymph vessels. In the TIME, EC are essential to transport nutrients and oxygen for tumor survival and growth. EC have an irregular shape and size with ruffled margins in the TIME. The tips of some branched EC may penetrate the vessel lumen creating small intercellular gaps in the wall, which contribute to the metastatic spread.	[67, 68]
Pericytes	Pericytes are cells of mesenchymal origin that provide important support for blood vessel formation and function. Pericytes are capable of tumor homing and are important cellular components of the TIME. The immunosuppressive phenotype acquired by these cells once in the TIME is of great relevance, since they may act in synergy with tumor cells to inhibit local immune response, contributing to tumor angiogenesis, growth, and metastasis.	[69, 70]
Cancer-associated fibroblasts (CAF)	CAF are involved in cancer progression and metastasis through their ability to enhance tumorigenicity, angiogenesis, and metastatic dissemination of cancer cells. The expression of the fibroblast activation protein (FAP)-α, which is not detected in normal fibroblasts, has been associated with an overall poorer prognosis in several cancer types, including CRC.	[71-73]
Extracellular matrix (ECM)	ECM anomalies deregulate the behavior of stromal cells, facilitate tumor-associated angiogenesis and inflammation, and thus lead to the generation of a tumorigenic microenvironment. ECM can affect the fate of tumor by establishing the direct contact with newly forming cancer cells. In addition, ECM can promote the conversion to malignant tumor and metastasis by secreting different cytokines.	[74-76]

Notes: APC: antigen presenting cells; Breg: regulatory B cells; CAF: cancer-associated fibroblasts; cDC1: conventional type 1 DC; CRC: colorectal cancer; CTL: cytotoxic T lymphocyte; DC: dendritic cell; EC: endothelial cells; ECM: extracellular matrix; FAP: fibroblast activation protein; FasL: Fas-ligand; IFN: interferon; iNOS: nitric oxide synthase isoform; IgG2b: immunoglobulin G2b; IL: interleukin; LN: lymph nodes; maDC: mature DC; MC: mast cells; MDSC: myeloid-derived suppressor cells; MHC: major histocompatibility complex; NK: natural killer; rDC: regulatory DC; ROS: reactive oxygen species; sDC: stimulatory DC; TAM: tumor-associated macrophages; TAN: tumor-associated neutrophils; TGF: tumor growth factor; Th cells: T helper cells; TIME: tumor immune microenvironment; TNF: tumor necrosis factor; TRAIL: TNF-related apoptosis-inducing ligand; Treg: T regulatory cell.

Another important component to take into consideration within the CRC microenvironment is the inflammation. The activation of this process is a major contributor to the TIME and subsequent tumorigenesis [46, 47]. Currently, the chronic inflammation is well recognized as both a tumor initiator and promoter [77]. Additionally, inflammatory cells release several biomolecules, such as cytokines, chemokines, free radicals, prostaglandins, enzymes, and matrix metalloproteinases (MMP), that can induce genetic and epigenetic changes, which in turn may lead to tumor development and progression, resistance to apoptosis and angiogenesis [45, 78]. Moreover, several intracellular signaling pathways are often dysregulated during chronic inflammation, which leads to abnormal expression of pro-inflammatory genes involved in malignant transformation [45, 79]. On the other hand, inflamed stroma has been shown to promote the progression of colonic adenomas to adenocarcinomas *in vivo* [80]. However, it has also been observed that tumors that do not progress as a direct consequence of chronic inflammation (sporadic tumors) are also characterized by an inflammatory microenvironment [81].

2. Current chemotherapeutic-based established therapies against colorectal cancer

Chemotherapy is one of the major modalities of cancer treatment that uses cytotoxic drugs to suppress abnormal cell proliferation and induce apoptosis of damaged cancer cells.

CRC chemotherapy was initiated with the discovery of 5-fluorouracil (5-FU) by Heidelberger and colleagues in 1957 [82]. The anti-tumor efficacy, as well as the cytotoxicity of 5-FU administered as an intravenous bolus was demonstrated to be potentiated by the addition of the reduced folate leucovorin (LV; 5-formyl tetrahydrofolate [THF]) [83-85]. The topoisomerase I inhibitor irinotecan (IRI) plus the intravenous bolus 5-FU/LV (IFL regimen) was considered the standard of care in 2000 [86, 87]. However, the undesired toxicity profile of the IFL regimen led to the development of the IFL infusion or FOLFIRI regimen (IRI plus infusional 5-FU/LV) [88, 89], especially as a second-line chemotherapy for patients with good status and organ function [90, 91].

Discovered by Yoshinori Kidani in 1976 [92], oxaliplatin seems to be active in DNA mismatch repair deficient (dMMR) tumors [83]. The FOLFOX (oxaliplatin plus infusional 5-FU/LV) regimen can overcome resistance to 5-FU [93]. It was associated with a significant decrease of several chemotherapy-derived side effects (diarrhea,

nausea, vomiting, dehydration), as well as with an improved overall survival (OS), time to progression (TTP) and response rate (RR), when compared to the IFL regimen [94, 95]. Moreover, the FOLFOX regimen has been successfully applied as a perioperative combination chemotherapy (3 months before and after metastasis resection) in patients with resectable liver metastasis [96, 97]. Similar efficacies in terms of RR, OS, TTP and progression free survival (PFS) were demonstrated for both FOLFIRI and FOLFOX regiments, as well as, for FOLFIRI followed by FOLFOX *vs.* FOLFOX followed by FOLFIRI [98, 99]. From a safety perspective, FOLFIRI can be associated with gastrointestinal toxicity and alopecia, while FOLFOX is correlated with polyneuropathy and hematologic toxicity [90, 98, 99]. As previously reported, FOLFOX and FOLFIRI regimens demonstrated a greater efficacy in the adjuvant treatment of patients at stage III [100, 101] and stage IV [102, 103], when compared with the IFL regimen.

The efficacy of capecitabine combined with oxaliplatin (CapeOX) was demonstrated to be non-inferior to FOLFOX in the first-line treatment of mCRC, presenting similar activity and safety profiles including PFS and OS [104, 105]. In contrast, capecitabine plus IRI (CapeIRI) presented significantly lower efficacy and more severe side effects comparing to FOLFIRI [88].

Table 3 summarizes the major findings obtained in clinical studies using the currently recommended chemotherapeutic strategies against CRC.

Table 3. Summary of the results of clinical trials developed using current CRC chemotherapeutic.

CYTOTOXIC DRUG(S)	BIOLOGICAL EFFECT	REF.
5-FU-based adjuvant therapy	Improved OS rate of patients within stage III or high-risk stage II disease.	[106]
High/low-dose 5-FU/LV	Enhanced 5-FU cytotoxicity, RR (18.8-48 *vs*. 10-12%) and OS (11.7-12 *vs*. 7.7-10.5 months) for mCRC patients using high/low-dose 5-FU/LV, when compared to 5-FU alone, in phase I, II and III studies.	[107-112]
IFL regimen	IRI plus intravenous bolus 5-FU/LV (IFL regimen) improved the RR (35-49 vs. 22-31%), PFS (7 *vs*. 4.3 months) and OS (14.8-17.4 *vs.* 12.6-14.1 months) in mCRC patients compared to 5-FU/LV alone.	[86, 87]
IFL infusion or FOLFIRI regimen	Offered as a second-line chemotherapy for patients with good status and organ function; improved PFS and OS.	[88, 89] [90, 91]
FOLFOX	According to the Intergroup N9741 trial, FOLFOX decreased several chemotherapy-derived side effects (diarrhea, nausea, vomiting, dehydration) and an improved OS (19.5 *vs*. 15 months), TTP (8.7 *vs.* 6.9 months) and RR (45 *vs*. 31%) compared to IFL regimen.	[94, 95]
	Improved PFS (9 *vs.* 6.2 months) and RR (50.7 *vs.* 22.3%) compared with therapy alone, 5-FU/LV or oxaliplatin.	[113-116]
FOLFIRI &	According to the Gruppo Oncologico Dell'Italia Meridionale (DOIM) 9901 trial, FOLFIRI and FOLFOX regiments induced a similar efficacy in terms of	[98]

FOLFOX regimens	RR (31 *vs*. 34%), OS (14 *vs*. 15 months) and TTP (7 months).	
	Similar efficacy of FOLFIRI followed by FOLFOX *vs.* FOLFOX followed by FOLFIRI in terms of RR (56% *vs.* 54%), OS (21.5 *vs.* 20.6 months) and PFS (8.6 *vs.* 8 months), according to the randomized crossover GERCOR study.	[99]
FOLFOXIRI or FOLFIRINOX	Increased toxicities, predominantly severe neutropenia, neurotoxicity, and diarrhea. However, greater RR (60 *vs.* 34%), PFS (9.8 *vs.* 6.9 months) and OS (22.6 *vs.* 16.7 months) for mCRC patients treated with FOLFOXIRI compared to the FOLFIRI regimen, in a randomized phase III study.	[117]
	Phase III trial did not show differences in the efficacy of FOLFOXIRI when compared to the FOLFIRI regimen.	[118]
Capecitabine	Similar efficacy to the one induced by 5-FU/LV in mCRC, besides causing less myelosuppression and stomatitis.	[83, 104, 119]

Notes: FOLFIRI: IRI plus infusional 5-FU/LV; FOLFOX: oxaliplatin plus infusional 5-FU/LV; FOLFOXIRI or FOLFIRINOX: triplet combination of infusional 5-FU/LV, IRI and oxaliplatin; 5-FU/LV: 5-fluorouracil/leucovorin; IFL regimen: IRI plus intravenous bolus 5-FU/LV; IRI: irinotecan; LV: leucovorin; mCRC: metastatic colorectal cancer; OS: overall survival; PFS: progression free survival; RR: response rate; TTP: time to progression.

According to the international guidelines (Table 4), most patients with CRC present a localized or restricted tumor, which treatment is based on the surgical resection followed by an adjuvant therapy (chemotherapy or radiotherapy) for high-risk patients in stage II or stage III [120-125]. In addition, approximately 20-30% of CRC patients present unresectable metastasis (Table 5), and about 40-50% of previously treated patients develop recurrent disease, requiring systemic chemotherapy to improve survival and palliation through the control of tumor size and symptoms (Table 6) [120, 121, 126].

The most used active cytotoxic drugs approved by the US Food and Drug Administration (FDA) to control tumor growth and to improve life expectancy of CRC patients include 5-FU, IRI, oxaliplatin, and capecitabine [127]. In two randomized phase III trials, the triplet combination of infusional 5-FU/LV, IRI and oxaliplatin (FOLFIRINOX or FOLFOXIRI) appeared as an interesting option as the first-line treatment of mCRC, but remain controversial [117, 118]. On the other hand, the 5-FU oral prodrug capecitabine was developed to increase 5-FU bioavailability and efficacy over a prolonged period, as well as, to replace the uncomfortable infusion regimen [83, 104, 119].

Table 4. Adjuvant treatment in nonmetastatic colon cancer according to the National Comprehensive Cancer Network® (NCCN®) guidelines® [120].

TNM (DISTANT METASTASIS) **PATHOLOGIC STAGING SYSTEM**	**ADJUVANT TREATMENT**
T1, N0, M0 T2, N0, M0; T3, N0, M0 (MSI-H or dMMR)	Observation
T3, N0, M0 (MSI-L or MSS and no high-risk features)	• Observation • Capecitabine or 5-FU/LV

T3, N0, M0 at high risk for systemic recurrence T4, N0, M0	• Capecitabine or 5-FU/leucovorin • FOLFOX or CapeOX • Observation
T1-3, N1 (Low-risk stage III)	• CapeOX (3 months) • FOLFOX (3–6 months) • Capecitabine (6 months) or 5-FU (6 months)
T4, N1-2 T Any, N2 (High-risk stage III)	• CapeOX (3–6 months) • FOLFOX (6 months) • Capecitabine (6 months) or 5-FU (6 months)

Notes: CapeOX: capecitabine plus oxaliplatin; dMMR: deficient DNA mismatch repair; FOLFOX: oxaliplatin plus infusional 5-fluorouracil/leucovorin (5-FU/LV); M: distant metastasis; MSI-H: high microsatellite instability; MSI-L: low microsatellite instability; MSS: microsatellite stable; N: regional lymph nodes; T: stage.

Table 5. Primary and adjuvant treatments in unresectable metastatic colon cancer according to the National Comprehensive Cancer Network® (NCCN®) guidelines® [120].

UNRESECTABLE METACHRONOUS METASTASIS	PRIMARY TREATMENT	CONVERSION TO RESECTABLE
Previous adjuvant FOLFOX/CapeOX within past 12 months	• FOLFIRI or irinotecan ± bevacizumab (preferred) or ziv-aflibercept or ramucirumab • FOLFIRI or irinotecan ± cetuximab or panitumumab (*KRAS/NRAS-wt* gene only) • Nivolumab or pembrolizumab (dMMR/MSI-H only) • Irinotecan + (cetuximab or panitumumab) + vemurafenib (*BRAF V600E* mutation positive)	• **Yes:** Systemic therapy ± biologic therapy • **No:** Systemic therapy
• Previous adjuvant FOLFOX/CapeOX >12 months • Previous 5-FU/LV or capecitabine • No previous chemotherapy	Systemic therapy	

Notes: CapeOX: capecitabine plus oxaliplatin; dMMR: deficient DNA mismatch repair; FOLFIRI: irinotecan plus infusional 5-FU/LV; FOLFOX: oxaliplatin plus infusional 5-fluorouracil/leucovorin (5-FU/LV); *KRAS*: kirsten rat sarcoma; MSI-H: high microsatellite instability; *NRAS*: neuroblastoma rat sarcoma; *wt*: wild-type.

Table 6. Systemic therapy used as continuum of care for advanced or metastatic disease according to the National Comprehensive Cancer Network® (NCCN®) guidelines® [120].

	SYSTEMIC THERAPY	SUBSEQUENT THERAPY
Previous oxaliplatin-based therapy without irinotecan	• FOLFIRI or irinotecan • FOLFIRI + bevacizumab (preferred) or ziv-aflibercept or ramucirumab • Irinotecan + bevacizumab (preferred) or ziv-aflibercept or ramucirumab	• Irinotecan + cetuximab or panitumumab (*KRAS/NRAS-wt* only) • Regorafenib • Trifluridine + tipiracil1 • Nivolumab or pembrolizumab (dMMR/MSI-H only)
	• FOLFIRI + cetuximab or panitumumab (*KRAS/NRAS-wt* only) • Irinotecan + cetuximab or panitumumab (*KRAS/NRAS-wt* only) • Irinotecan + cetuximab or panitumumab + vemurafenib (*BRAF V600E* mutation positive)	• Regorafenib • Trifluridine + tipiracil • Nivolumab or pembrolizumab (dMMR/MSI-H only)
	• Nivolumab or pembrolizumab (dMMR/MSI-H only)	Subsequent therapy

Notes: dMMR: deficient DNA mismatch repair; FOLFIRI: irinotecan plus infusional 5-fluorouracil/leucovorin (5-FU/LV); *KRAS*: kirsten rat sarcoma; MSI-H: high microsatellite instability; *NRAS*: neuroblastoma rat sarcoma; *wt*: wild-type.

Although the great advances in CRC chemotherapy, non-specific drug biodistribution and adverse side effects on healthy tissues present the main concerns for patient's life quality [128].

Depending on the drug pharmacokinetics, dictated by the release and distribution profiles, absorption rate, metabolism, half-life and excretion, the most suitable administration route will be selected. The most common are the intravenous and oral routes of administration [129].

The cytotoxic drugs are indeed used to destroy cancer cells. Even though, normal cells are also susceptible and are commonly negatively affected. Most frequent side effects of chemotherapy resultant from that non-specific cytotoxic effect include fatigue, nausea and vomiting, appetite and hair loss, diarrhea, swelling, rashes and mouth sores [1]. Immunosuppression through the repression or death of effector immune cells and bone marrow damage are among the worst consequences of chemotherapy that increase the probability of infections and compromise self-immunity. Although most side effects of chemotherapy are temporary and reversible after treatment terminus, some of them persist for several years. Moreover, cancer cells acquire mutations that make them resistant to chemotherapeutic drugs [1].

Thus, improved and targeted therapies to protect and direct cytotoxic drugs to the site of interest are needed to minimize these undesired effects.

3. Modulation of host immune system against colorectal cancer

Immunotherapy has been adopted as a therapeutic approach that harnesses host immune system to reduce or eliminate tumor cells [130]. Immunotherapy can be divided in active and passive approaches [131]. A temporary anti-tumor effect is normally obtained using a passive immunotherapeutic strategy, and therefore constant administrations are needed due to the nonexistence of immunological memory. Examples include the monoclonal antibodies directed to a specific target on a cancer cell, or against a tumor growth-related enzyme or protein, such as the immune checkpoints or cytokine network, and immune cells engineered *ex vivo* that are injected in the patients to induce an immune response [132] (Figure 3). In opposite, the active immunotherapy relies on the activation of host´s immune system to achieve a specific destruction of targeted cancer cells. Cytokines and other cell signaling molecules, or vaccines are examples of non-specific and specific active immunotherapy, respectively, which once successful will result in an immunological anti-tumor memory [131, 132].

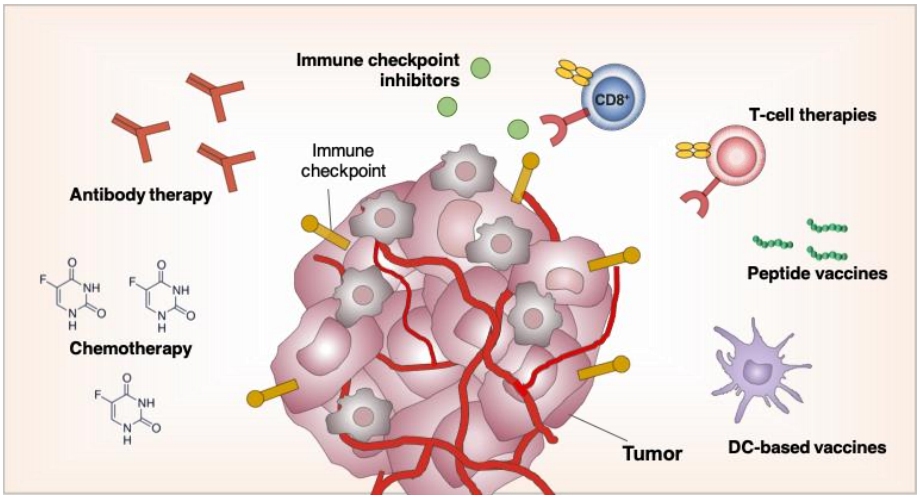


Figure 3. Examples of different approaches explored to modulate the tumor-immune-stromal cross-talk towards the induction of host immunity against CRC.

Different immunotherapeutic strategies have been used to cause tumor destruction in CRC, namely through the increase of effector T cells, the suppression of negative immune checkpoints, as well as the elimination of cytokines and cells related to tumor development [132-135].

The following sections cover the major immunotherapeutic approaches currently used to treat CRC patients, as well as those that are under investigation, namely the T-cell therapy, immune checkpoint inhibitors and DC-based cancer vaccines. Specific examples are discussed and summarized in Tables 7 and 8.

3.1.Modulation of T cell function within tumor microenvironment

T lymphocytes have a crucial role as effector cells in the immune response against tumor cells, as they recognize and generate a cytotoxic response against tumor antigens [136]. The amplitude and duration of that anti-tumor T-cell response is dependent on the balance of co-stimulatory and inhibitory signals.

Checkpoint inhibitors

Novel therapies have been developed to inhibit tumor-immune escape, namely the checkpoint inhibitors. These are blocking antibodies that inhibit the interactions between immune checkpoint molecules on T cells and its ligands on tumor cells and/or antigen presenting cells (APC).

Anti-cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) and anti-programmed cell death protein 1 (PD-1) / programmed death ligand 1 (PD-L1) monoclonal

antibodies have been approved for the treatment of metastatic melanoma, advanced small-cell lung carcinoma, triple negative breast carcinoma and head and neck cancer [137]. Monoclonal antibodies against the CTLA-4 and PD-1/ PD-L1 immune checkpoints were approved by the US FDA in 2018 to address the CRC disease (Table 5) [133, 138]. CTLA-4 is a co-inhibitory molecule expressed by T cells instead of the co-stimulatory CD28. It induces a suppressive signal by binding to its ligand CD80/86 on APC [139]. PD-1 is an immunosuppressive receptor on T cells and it has an important role on the suppression of antigen-specific T-cell responses through binding to its receptor PD-L1 [139]. PD-L1 is highly expressed in CRC tumor cells and APC, and its upregulation is associated with poor prognosis [133]. Specific examples can be found in Table 7.

Other antibodies have been developed against immune checkpoints, such as mucin-domain containing-3 (TIM-3) and OX40 [140]. TIM-3 inhibition has an important role in protecting Th1 responses and promoting cell death. Xu *et al.*, 2015 [141] analyzed peripheral blood samples from CRC patients and verified high levels of circulating $TIM\text{-}3^{+}PD\text{-}1^{+}CD8^{+}$ T cells compared to control patient's blood. They concluded that the blockage of TIM-3 and the restoration of T-cell responses may be a potential therapeutic approach for CRC patients. OX40 is a co-stimulatory immune checkpoint of the tumor necrosis factor (TNF) receptor superfamily member 4 (TNFRSF4) that promotes effector T-cell expansion. OX40 agonists are able to modulate Treg cells, promoting anti-tumor $CD8^{+}$ T-cell responses [142]. Petty *et al.*, 2002 [143] showed that high levels of $OX40^{+}$ lymphocytes are present in primary colon cancers and this increased expression in tumors significantly correlates with better survival.

Antibodies against cytokine network

Interferon (IFN), interleukins (IL) and granulocyte macrophage colony-stimulating factor (GM-CSF) are cytokines that have an important role in non-specific immunotherapy, increasing the immunity of the host against the tumor [136]. Particularly in human CRC, there are high levels of Th17 cell-derived cytokines (IL-17 and IL-22), Th17 cell-polarizing cytokines (IL-1β, IL-6, IL-21 and TGF-β) and pro-inflammatory cytokines (TNF) related to poor clinical outcomes and advanced stage of disease; and surprisingly low levels of IL-10 and IFN-ɣ [144]. Several agents were developed to block cytokines determinant in the CRC progression, such as the etanercept and infliximab (both antibodies against TNF-α), anakinra (recombinant IL-1

receptor antagonist) and canakinumab (IL-1β specific antibody) [144, 145], and those are currently being evaluated under preclinical and clinical settings (Table 7).

T-cell therapy

Adoptive T-cell therapy is based on the collection of autologous T cells from patient´s tumors, LN or peripheral blood, to get them activated and expanded *in vitro*. After, activated T cells are reintroduced into the patient body to modulate the immune response. However, this procedure is complex, time consuming and expensive for a sustainable health care system. In addition, it is often unsuccessful since they need to overcome the immunosuppressive TIME within the solid mass [133]. To address these limitations of process, genetic modifications of T cells to express tumor specific T cell receptor (TCR) genes have been performed. This promising strategy is denominated as chimeric antigen receptor (CAR) T-cell therapy and it allows an increased specificity and activation on binding [146]. CAR-T cell therapy has been approved by the European Medicines Agency (EMA) and FDA for pediatric relapsed or refractory (r/r) B-cell acute lymphoblastic leukemia (ALL) and r/r diffuse large B-cell lymphoma (DLBCL) in adult patients (Kymriah® Novartis) in 2018.

Regarding the CRC, adoptive cell therapies have shown some potential by modifying genetically T cells to express high-affinity receptors for CRC-associated antigens [138]. Besides the potency demonstrated by the high-affinity engineered T cells in some studies that used adoptive cell transfer to treat patients with mCRC, these cellular therapies failed due to the induction of severe autoimmune effects [147, 148]. However, some clinical trials are ongoing (Table 7) in order to overcome these drawbacks.

Table 7. Summary of the main outcomes of clinical trials focused on the modulation of the TIME against CRC.

STRATEGY	COMPOSITION	IMMUNE/BIOLOGICAL EFFECT	REF.
Immune checkpoint targeting by monoclonal antibodies	Ipilimumab® (anti-CTLA-4 antibody)	Promising antibody against CRC since it improves the anti-tumor immune response and deplets Treg cells, which has been correlated to poor clinical results in CRC patients.	[140]
	Tremelimumab® (anti-CTLA-4 antibody)	In a phase II study, 47 patients with mCRC were treated for 2.3 months. Most of patients experienced disease progression.	[149]
	Nivolumab® (anti-PD-1 antibody) alone or in combination with Ipilimumab® (CTLA-4 blocking antibody), Relatlimab®, or	Nivolumab alone and Nivolumab® combined with Ipilimumab were well tolerated in most patients and demonstrated encouraging clinical activity and survival of mCRC patients. In 2018, US FDA approved the combination of Ipilimumab® (Yervoy®) and Nivolumab® (Opdivo®) for MSI-H or dMMR mCRC patients previously treated with standard chemotherapy	[150-153]

	Metformin	drugs, supported by phase II CheckMate-142 trial. (ClinicalTrials.gov identifier: NCT02060188) Phase II trials using Nivolumab® combined with Relatlimab® or Metformin to treat MSS advanced/refractory CRC patients, are under recruitment status. (ClinicalTrials.gov identifiers: NCT03642067 and NCT03800602)	
	Pembrolizumab® (anti-PD-1 antibody) or in combination with Regorafenib®, Binimetinib and Bevacizumab, or Grapiprant (ARY-007)	In a phase II study, patients with previously-treated locally-advanced unresectable, MSI-H or dMMR mCRC were treated with Pembrolizumab® (MK-3475, Keytruda®) monotherapy, after previously treated with standard therapies. Study is ongoing. (ClinicalTrials.gov identifier: NCT02460198) In 2017, Pembrolizumab (Keytruda®) was approved by the US FDA for the treatment of patients with MSI-H or dMMR tumors, according solely with the presence of a genetic feature in a tumor, rather than the patient's cancer type. Phase I/II studies, where mCRC, refractory, or advanced/progressive MSS CRC patients will be treated with Pembrolizumab® combined with Regorafenib®, Binimetinib and Bevacizumab, or Grapiprant (ARY-007), are under recruitment status. (ClinicalTrials.gov identifier: NCT03657641, NCT03475004, and NCT03658772)	[154-157]
	OX-40 (CD134)	High levels of OX-40$^+$ tumor-infiltrating lymphocytes found in primary colon tumors from 15 of 72 patients correlates with better survival (mean survival high OX-40, 47 months, low OX-40, 35 months, *P* _0.05), although this correlation was not stage-independent. Thirty-one cases had prominent lymphocytic infiltrates at the invasive margin of the tumor expressing OX-40. Overall, 50% of primary tumors showed high expression of OX-40. Nearly all mesenteric lymph nodes expressed OX-40, whether tumor was present or not. Low levels of OX-40 in the normal margins of colon.	[143]
		High CRC infiltration by OX40$^+$/CD8$^+$ cells significantly associated with an independent, favorable, prognostic marker in CRC with an overall survival similar to stage I cancers. Freshly excised CRC with OX40^{high}/CD8^{high} infiltration present a prolonged overall survival, as compared to tumors with OX40^{low}/CD8^{high}, OX40^{high}/CD8^{low} or OX40^{low}/CD8^{low} infiltration. Irrespective of TNM stage, CRC with OX40^{high}/CD8^{high} density infiltrates showed an overall survival similar to that of all stage I CRC. In contrast, OX40$^+$ and FOXP3$^+$ cell infiltration presents a poor prognostic significance.	[158]
Antibodies against cytokine network (TNF-α)	Infliximab®	Well tolerance in patients with advanced or metastatic solid tumors, including CRC, with no dose-limiting toxic effects. Additionally, no evidence of tumor progression was observed in any patient.	[159]
T-cell therapy	CAR T-EGFR cells (CAR T-cells transduced with the anti-EGFR Lentivirus vector)	Phase I/II study in patients with EGFR positive advanced/unresectable solid tumors, such as lung cancer and CRC. Study ongoing with no results posted. (ClinicalTrials.gov identifier: NCT01869166)	[160]
	Multi-target CAR-T/TCR-T Cell	Phase I/II study to assess the safety and effectiveness of CAR-T/TCR-T cells to treat different malignancies patients, including CRC. One of the ten distinct tumor-specific antibody on CAR-T/TCR-T cells is the anti-C-met against hepatoma, CRC, ovarian and renal cancers. Study ongoing with no results posted. (ClinicalTrials.gov identifier: NCT03638206)	[161]
	EGFR-/IL12-CAR T-	Phase I/II studies of chimeric antigen receptor EGFR (EGFR-	[162,

	cells	CAR T or EGFR-IL12-CAR T) cells in patients with EGFR-positive mCRC. Studies ongoing with no results posted. (ClinicalTrials.gov identifiers: NCT03542799 / NCT03152435)	163]
	Autologous NK cells with bortezomib	Adoptive infusion of *ex vivo* expanded autologous NK cells against metastatic solid tumors (pancreatic, CRC, lung) or hematological malignancies (chronic myeloid leukemia and multiple myeloma) sensitized to NK TRAIL cytotoxicity with Bortezomib. Phase I trial ongoing with no results posted. (ClinicalTrials.gov identifier: NCT00720785)	[164]

Notes: CAR T: chimeric antigen receptor T; CRC: colorectal cancer; CTLA-4: cytotoxic T-lymphocyte-associated protein 4; dMMR: deficient DNA mismatch repair; EGFR: epidermal growth factor receptor; FDA: Food and Drug Administration; IL: interleukin; mCRC: metastatic colorectal cancer; dMMR: deficient DNA mismatch repair; MSI-H: high microsatellite instability; MSS: microsatellite stable; NK: natural killer cells; PD-1: programmed cell death protein 1; TNF-α: tumor necrosis factor-α; Treg: regulatory T cells; US: United States.

3.2.Vaccines

Cancer vaccination is a promising strategy for cancer treatment due to its ability to induce tumor-specific cytotoxic T lymphocytes (CTL) and humoral responses specifically against tumor-associated antigens (TAA) [146]. The principal aim of vaccination is to elicit an anti-tumor immune response to eradicate tumor cells and potentiate ongoing surveillance and thereby avoid tumor recurrence [134]. Different vaccine strategies based on autologous whole tumors, peptides/proteins, viral vectors and DC-based vaccines have been explored for CRC treatment in several clinical studies, summarized in Table 8.

Whole tumor vaccines

Whole tumor vaccines are based on the use of autologous whole tumor cells or cell lysates (to obtain multiple TAA) mixed with an immune adjuvant. This mixture is then reinjected into the patients [135]. The use of whole TAA reduce the opportunity to tumor escape, compared with the use of a single epitope peptide to induce an adaptive anti-tumor immunity against several TAA [135, 165]. The principal disadvantage of this approach comes from the difficulty in obtaining an universal vaccine viable to all patients with a certain cancer [165]. Examples can be found in Table 8.

Peptide vaccines

To overcome the limitations of whole tumor vaccines, many research groups started to use peptide vaccines since they have the potential to induce a more specific anti-tumor response by using known antigens that are ideally overexpressed only by tumor cells [134]. Peptide-based vaccines use whole proteins or fragments of proteins generated from tumor-specific proteins that are administered to patients with immune adjuvants.

In CRC, many TAA have been identified and used in vaccination, such as CEA, mucin-1, squamous cell carcinoma antigen recognized by T cells 3 (SART3), survivin-2B or p53 [166-169]. CEA is the most common antigen targeted in CRC vaccines already tested in clinical trials. Some examples are described on Table 8.

Viral vector vaccines

The viral vector vaccines were developed to overcome the reduced efficacy of peptide-based vaccines. Recombinant lentiviruses, poxviruses, adenoviruses and retroviruses are the principal viruses with high transfection efficacy and immunostimulatory activity used in the production of these vaccines [135]. The use of recombinant virus to express CRC TAA is promising once virus are naturally immunogenic and could infect DC to induce boost anti-CRC immune responses [134, 136, 170]. Nevertheless, the high production costs and the potential pathogenicity and mutagenicity of these vectors are the principal disadvantages for their administration [170].

DC-based vaccines

The use of DC as vaccines has been considered of major relevance for cancer immunotherapy. DC are professional APC that efficiently activate $CD4^+$ and $CD8^+$ T cells through the presentation of endogenous and exogenous immunogenic peptides [132, 136, 139]. DC-based vaccines use autologous DC (from peripheral blood or bone marrow) that are loaded with peptides derived from TAA and then injected back into cancer patients [138]. Once inside the body, DC migrate into the lymphoid tissue and induce a peptide reactive CTL response [132]. Concerning antigen delivery, DC can be pulsed with synthetic peptides derived from TAA, tumor cell lysates, tumor RNA or physically fused with whole tumor cells [134].

In case of CRC patients, CEA peptides and CEA-expression vectors are the most used in DC-based vaccines [134, 165].

Table 8. Summary of the main outcomes of clinical trials involving different vaccination strategies against CRC.

STRATEGY	COMPOSITION	IMMUNE/BIOLOGICAL EFFECT	REF.
Whole tumor vaccines	OncoVAX (autologous tumor cell vaccine) combined with BCG	No significant alteration of the disease-free interval or OS compared to control group in CRC patients.	[171]
	FANG vaccine (autologous whole tumor-based product) incorporating a plasmid encoding for GM-CSF and a	Patients with advanced cancer received up to 12 monthly intradermal injections of FANG vaccine. Safe immune response correlated to prolonged	[172]

	bifunctional short hairpin RNAi (bi-shRNAi) targeting furin convertase.	survival.	
Peptide vaccines	Vaccine with CEA and the adjuvant GM-CSF	Administration in 24 resected CRC patients without macroscopic disease for 12 months. Nontoxic effect able to induce potent and persistent antigen-specific IgG and T-cell responses.	[173]
	Survivin-2B peptide vaccine	In a phase I clinical study, it induced no severe adverse events in 15 patients with advanced or recurrent CRC. In 6 patients, the tumor marker levels (CEA and CA19-9) decreased during the period of vaccination. Slight reduction of the tumor volume in a minor responder patient. No changes in three patients while the remaining eleven patients experienced tumor progression. Besides these results, this vaccine was safe and is a potential candidate to increase the immune and clinical efficacy in HLA-A24^{+} CRC patients.	[174]
	p53-SLP® vaccine	In a phase I/II clinical trial, a safe and immunogenic p53-SLP® vaccine was administrated to 10 patients with mCRC. In 6 tested patients, p53-specific T-cell reactivity persisted at least for six months. The isolated p53-specific T cells were CD4^{+} T cells producing Th1 and Th2 cytokines upon stimulation with the p53 peptide.	[168]
	VRP vaccine expressing CEA(6D) (AVX701)	T cell responses associated to longer survival predominantly in the stage IV than stage III CRC patients post the same regimen immunization with AVX701. Greater T cell and antibody responses were obtained in the stage III than stage IV patients, thus reflecting less immunosuppressive milieu for stage III patients. ClinicalTrials.gov Identifier: NCT01890213	[175]
Viral vector vaccines	Autologous DC modified with CEA and MUC1 (PANVAC) compared with vaccines based on poxvectors encoding PANVAC and GM-CSF	Results showed a similar survival for DC and poxvector vaccines in patients with resected CRC metastasis.	[167]
	Pexa-Vec (JX-594), an oncolytic and immunotherapeutic vaccinia	In a phase Ib study, 15 patients with refractory CRC received at least one dose of intravenous Pexa-Vec and all showed to be safe and effective. A phase II studies of Pexa-Vec in combination with immune checkpoint inhibitors are ongoing in patients with metastatic CRC (Table 9).	[176]
DC-based vaccines	Autologous tumor lysate and control protein (KLH)-pulsed DC-based vaccine	Autologous tumor-specific T-cell proliferation in 63% of patients subjected to CRC metastasis resection. CD40L maturation induced CD86 and CD83 expression on DC but had no effect on immune responses.	[177]
	DC-based vaccine pulsed with HLA-A*0201- or HLA-A*2402-restricted CEA peptides	mCRC patients were immunized and did not show any toxicity or autoimmune reactions. The number of CEA-specific T cells was stimulated in 70% of tested patients.	[178]

Notes: BCG: Bacillus Calmette Guérin; BLS: bright-light surgery; CD40L: CD40 ligand; CEA: carcinoembryonic antigen; CRC: colorectal cancer; DC: dendritic cell; FGS: fluorescence-guided surgery; GM-CSF: granulocyte-macrophage colony-stimulating factor; HLA: human leukocyte antigen; IgG: Immunoglobulin G; KLH: keyhole limpet hemocyanin; mCRC: metastatic CRC; MUC1: mucin 1; OS: overall survival; Pexa-Vec: pexastimogene devacirepvec; RNAi: ribonucleic acid interference; shRNAi: short hairpin RNAi; SLP: synthetic long peptides; Th: T helper; VRP: alphaviral replicon particle vaccine.

Despite the high number of studies based on cytokines, peptide, viral vectors and DC-based vaccines, a very small number of immunotherapeutic strategies, such as nivolumab and pembrolizumab or the combination of ipilimumab with nivolumab, have been recently approved by FDA for CRC treatment.

4. Combinational approaches for CRC destruction

Approximately 20% of these patients present metastases, especially in the liver and lungs. Chemotherapy, radiation, targeted therapies and immunotherapeutic strategies may be given alone or in combination to relieve symptoms and prolong survival in mCRC cases [179].

4.1. Combination of chemotherapeutic drugs with targeted strategies

Recent evidences point out that chemotherapy is more effective when co-administered with other targeted therapeutic drugs that work by different molecular mechanisms within the complex TIME, showing additive or synergistic effects. These combinatorial approaches increased tumor's cell death without intolerable side effects [179]. Targeted therapy is a relatively new area of drug development based on the molecular understanding of cancer cell progression, invasion and metastasis [83]. Drugs are designed to target specific molecules with crucial role at different stages of cancer, such as tumor growth and progression, with significantly less side effects than chemotherapeutic drugs [179]. This approach aims to direct anti-cancer agents to the tumor site, avoiding adjacent tissue targeting. Unlike classical cytotoxic agents, these molecules tend to work in tumors with specific genetic defects, allowing for a personalized therapeutic approach with stronger response [1]. These agents can either be small molecule inhibitors or monoclonal antibodies designed to target cell surface receptors or intracellular enzymes [83, 180].

According to National Comprehensive Cancer Network® (NCCN®) guidelines® [120], many targeted therapies have been approved by the US FDA and EMA for the management of mCRC, such as bevacizumab, cetuximab, panitumumab, ziv-aflibercept, ramucirumab, regorafenib, and vemurafenib.

All these different drugs can be used in different regimens alone or with a variety of combinations as first-, second- and third-line of treatments, to achieve an enhanced and effective disease regression (Table 5 and 6) [120, 181].

Anti-VEGF strategies

Several monoclonal antibodies and small molecules have been developed to inhibit the growth of new tumor blood vessels by targeting the vascular endothelial growth factor (VEGF).

Bevacizumab is a recombinant humanized antibody that binds circulating VEGF-A. This targeted antibody improved survival as first- and second-line therapies, increasing the activity of any active cytotoxic agent [182-184]. It was the first antiangiogenic agent approved by the US FDA as a first-line treatment for mCRC patients, based on a randomized phase III clinical trial [185, 186]. This trial evaluated the combination of bevacizumab (Avastin) with standard IFL regimen (Tables 5 and 6). The addition of bevacizumab to IFL compared to IFL alone significantly improved the RR, PFS, and OS of mCRC patients [187, 188]. In other three studies, Kabbinavar *et al.* investigated the influence of adding bevacizumab to 5-FU/LV-based chemotherapy, which resulted in a significant improvement of PFS and a better OS [189-191]. In a different study, Shmiegel *et al*. evaluated the efficacy of CapeOX and dose-modified CapeIRI (mCapeIRI) both plus bevacizumab in mCRC patients, as a first-line treatment [192]. Both combinations, CapeOX-bevacizumab and mCapeIRI-bevacizumab, revealed promising activity with reduced toxic profiles. Moreover, in this study, the combination with bevacizumab allowed the dose reduction of capecitabine and IRI, with no apparent loss of efficacy, which is a major step for reducing unfavorable side effects.

Several other studies consistently reported an improvement in the primary endpoint PFS, but failed in showing significant changes on the OS when bevacizumab was integrated in first-line treatments [193-196]. In a phase III study, Stathopoulos *et al.* studied bevacizumab in combination with FOLFIRI. The results showed no significant differences on OS by adding bevacizumab when compared with chemotherapy alone [193] (Tables 5 and 6). In another study comparing the efficacy of bevacizumab when added to first-line oxaliplatin-based chemotherapy, Saltz *et al.* demonstrated that bevacizumab significantly improved the primary endpoint PFS, but the OS was not significantly better [194]. Although in these studies the OS was not improved, a consistent improvement in the PFS was mostly pronounced in the studies where bevacizumab was combined with the fluoropyrimidine therapy [195, 196].

Tebbutt *et al.* analyzed the effect of bevacizumab on capecitabine-based chemotherapy, as a first-line treatment. The results demonstrated that bevacizumab improved the PFS, but the secondary endpoint OS was not affected [196]. In a similar study, Cunningham *et al.*, assessed the efficacy of adding bevacizumab plus

capecitabine on elderly patients. Once more, PFS was improved as well as the RR, in the presence of bevacizumab, with no significant changes on the OS [195]. Based on these studies, the combination of bevacizumab with fluoropyrimidine therapy became a first-line treatment of mCRC patients [194, 195, 197].

Additionally, it was shown that continuing to use bevacizumab as a second-line treatment after progression in first-lines, it improves both the PFS and OS. In the phase III Eastern Cooperative Oncology Group (ECOG) 3200 clinical trial, it was determined the effect of bevacizumab on survival of mCRC patients previously treated with FOLFOX. The addition of bevacizumab improved the RR, PFS and OS in bevacizumab naïve-patients with mCRC after progression in first-line therapy [198]. Another study, the ML18147 trial provided evidences of the advantage of adding bevacizumab after disease progression in first-line treatments [184]. In this study, the efficacy of standard second-line chemotherapy (FOLFOX, FOLFIRI, CapeOX, or CapeIRI) was evaluated in the presence and absence of bevacizumab, whereas the choice of chemotherapy regimen depended on the first-line treatment. An improvement in the PFS and OS was noted in the presence of bevacizumab [184].

All these studies confirmed the advantage of using angiogenesis inhibitors, such as bevacizumab in mCRC, both as first- and second-line treatments in combination with standard chemotherapeutic agents. However, this biological agent has specific side effects, such as hypertension, bleeding, thromboembolic events, and proteinuria/albuminuria.

Aflibercept (also known as ziv-aflibercept in the US) is another antiangiogenic agent that has been investigated to treat mCRC in combination with chemotherapy. This biological agent is a recombinant fusion protein that targets the VEGF-A, VEGF-B, and the placental growth factor. In the phase III VELOUR trial, Cutsem *et al.* studied the effect of adding aflibercept to FOLFIRI in mCRC patients previously treated with oxaliplatin [199]. The addition of aflibercept improved the OS and PFS relative to placebo plus FOLFIRI; however adverse symptoms were experienced, including the characteristic anti-VEGF and increased chemotherapy-related adverse effects. In a subgroup analysis from the VELOUR trial, patients previously treated with oxaliplatin in first-line treatment, still benefit from adding aflibercept to FOLFIRI in second-line treatments, including patients with prior bevacizumab treatment [200].

All these studies confirm the real worth of combining antiangiogenic agents with standard chemotherapeutic regimens for CRC treatment. According to NCCN®

guidelines® (Table 5 and 6) [120], bevacizumab can either be used as first- or second-line in fluoropyrimidine-based treatment. Aflibercept plus FOLFIRI may be an alternative to bevacizumab in second-line treatments.

Anti-EGFR strategies

Cetuximab and panitumumab are two antibodies that target the EGFR and inhibit its dimerization and activation, thus interfering with cancer cell growth. According to the NCCN® guidelines® [120], these anti-EGFR antibodies are under clinical use in both early and later stages of the CRC disease. The administration of these antibodies improved the outcome on mCRC patients either as a monotherapy or in combination with chemotherapy (Table 5 and 6). They constitute the first therapeutic strategy for mCRC treatment, especially in patients with specific molecular profiles, such as patients whose tumors are wild-type (*wt*) for Kirsten rat sarcoma (*KRAS*) and neuroblastoma rat sarcoma (*NRAS*) [182, 186, 201, 202].

Cetuximab and panitumumab have been used as active single agents in chemorefractory mCRC patients. In this late stage of treatment, cetuximab enhanced the survival of patients, compared with best supportive care [203]. Panitumumab improved the RR and PFS, but did not result in significant survival differences [204]. Both antibodies were compared in a phase III clinical study for the treatment of chemorefractory *KRAS-wt* patients, and similar clinical activity was obtained for both antibodies [205]. The BOND clinical trial revealed that the combination of cetuximab with irinotecan was more effective than cetuximab monotherapy, establishing a crucial reference treatment for chemorefractory *KRAS-wt* mCRC patients [206].

Both anti-EGFR antibodies, cetuximab and panitumumab, showed to increase the activity of cytotoxic agents in the first-line treatment of *KRAS-wt* patients. Different studies evidenced that the combination of cetuximab with FOLFIRI increased the PFS, RR and OS of treated CRC patients, when compared to FOLFIRI alone [207, 208]. Additionally, cetuximab combined with FOLFOX improved RR and PFS of *KRAS-wt* CRC patients as first-line treatment [209-212]. However, these results were not consistently confirmed when using different oxaliplatin-based regimens, as first-line treatment [213-215]. The PRIME clinical trial showed that the combination of panitumumab with FOLFOX chemotherapy increased the RR, PFS and OS in *KRAS-wt* patients, whereas a detriment was noted in patients with a *KRAS* mutation [202, 216].

In second-line treatment of mCRC patients, three different clinical trials confirmed that the addition of the anti-EGFR antibodies improved RR and PFS, but failed to translate into a prominent survival [217-219]. Accordingly, Sobrero *et al.* assessed the effect of adding cetuximab to irinotecan in the survival of mCRC patients, previously treated with fluoropyrimidine and oxaliplatin [218]; a phase III clinical trial evaluated the combination of panitumumab with FOLFIRI to address PFS in *KRAS-wt* population [217]; and in Seymour *et al*., panitumumab was paired with irinotecan in pre-treated advanced CRC [219].

Overall, these studies demonstrate the value of combining anti-EGFR antibodies with chemotherapeutic agents in all lines of treatment, particularly to treat refractory patients in late stages of cancer.

Tyrosine kinase inhibitors

Regorafenib, an antiangiogenic agent, is an orally multi-target tyrosine kinase inhibitor approved by the US FDA in 2012, for the treatment of patients with mCRC. Regorafenib significantly improved the survival and PFS in refractory patients compared to other treatment options, including the cytotoxic drugs 5-FU, IRI and oxaliplatin, as well as the antibodies bevacizumab and anti-EGFR [182, 220, 221]. A large phase III clinical trial evidenced that Regorafenib improved the OS and PFS in chemotherapy refractory patients, compared to placebo. However, it led to significant adverse effects, including skin reaction, fatigue, and hypertension [220].

Vemurafenib, a *BRAF* kinase V600E-mutated form inhibitor, is approved by the FDA for the treatment of *BRAF* V600E–mutated, unresectable, or metastatic melanoma [222]. However, in mCRC patients, vemurafenib has shown to be ineffective alone due to the EGFR feedback activation [223]. Preclinical studies showed a successful dual inhibitory effect by blocking EGFR and *BRAF* V600E together [223, 224]. Although the combinatorial effect of vemurafenib with cetuximab- or panitumumab-based therapies have shown to be poorly effective in early clinical trials [225], the addition of irinotecan to *BRAF* and EGFR inhibition was suggested to possibly improve the antitumor activity [226]. The phase II SWOG S1406 trial, in which 99 patients with *BRAF* V600E-mutated mCRC were treated with a combination of irinotecan and cetuximab, with or without vemurafenib, showed that the introduction of the vemurafenib improved the PFS (4.3 *vs* 2.0 months), RR (4% *vs* 16%), and disease control rate (22% *vs* 67%) [227, 228]. However, severe side effects were felt by patients

treated with the vemurafenib/cetuximab/irinotecan treatment. Thus, the vemurafenib/panitumumab/irinotecan treatment was added as an alternative for *BRAF* V600E–mutant mCRC patients to improve safety [229].

4.2. Combinatorial immunotherapeutic strategies

Multi-targeted approaches combining the tumor cell destruction using chemotherapeutic drugs or radiation, with the modulation of immune cells within the TIME have been explored to rationally devise more efficient tools to overcome highly aggressive tumors.

Besides being an immunosuppressive treatment, chemotherapy has been shown to promote immune responses in certain cancers, by eliciting an immunogenic tumor cell death (ICD) that further enhances the anti-cancer activity [230]. Accordingly, a pilot study suggested that the combination of chemotherapy and immunotherapy, by treating stage III colon cancer patients with the simultaneous combination of the standard adjuvant CapeOX-based chemotherapy with the anti-tumor vaccination, keyhole limpet haemocyanin (KLH) and CEA-pulsed DC, resulted in enhanced anti-cancer immune responses and therapeutic efficacy [231]. Additionally, the shrinkage of the tumor obtained after the radiotherapy treatment led to the release of specific TAA following the destruction of tumor cells, and consequently activating the immune system [232].

Besides the approval of the ipilimumab (Yervoy®) and nivolumab (Opdivo®) combination for the treatment of MSI-H or dMMR mCRC patients previously treated with standard chemotherapeutic drugs, broader combinations of multiple immune modulators are also under evaluation. Some immune effectors enhance tumor killing by stimulating T cells with greater and specific cytolytic activity, while others are designed to interfere with immunomodulatory or immunosuppressive mechanisms [233]. Table 9 summarizes several preclinical and clinical studies involving different combinatorial immunotherapeutic strategies for CRC treatment.

Table 9. Preclinical and clinical studies concerning different combinatorial immunotherapeutic strategies against CRC.

STRATEGY	COMPOSITION	IMMUNE/BIOLOGICAL EFFECT	REF.
CLINICAL TRIALS			
Chemotherapy & Vaccines	Chemotherapy (5-FU/LV/IRI: IFL or FOLFIRI) + ALVAC-CEA/B7.1 vaccine with or without tetanus toxoid adjuvant	The combination was safe in half of mCRC patients since serious gastrointestinal (n = 30) and hematologic (n = 24) adverse effects were observed. Increased CEA-specific T cells were detected in 50, 37 and 30% of patients treated with ALVAC followed by chemotherapy and booster	[234]

		vaccination (group 1), ALVAC and tetanus toxoid followed by chemotherapy (group 2), or chemotherapy alone followed by ALVAC in patients without disease progression (group 3), respectively. Systemic chemotherapy did not change the CEA-specific T-cell responses following vaccination.	
	Chemotherapy (UFT and leucovorin) + HLA-A24^{+}/HLA-A2^{+} peptide vaccine	The therapy was well tolerated. Increased peptide-specific IFN-γ production or peptide-specific IgG secretion. The median time of PFS was 10.7 weeks.	[235]
	Chemotherapy (folinic acid, oxaliplatin, and fluorouracil) + MVA-5T4 vaccine (TroVax®)	10 patients had 5T4-specific antibody responses. 1 patient had a complete response, 6 had partial response, and 5 had stable disease.	[236]
	Chemotherapy (folinic acid, irinotecan, and fluorouracil) + MVA-5T4 vaccine (TroVax®)	10 patients had 5T4-specific antibody responses. 6 patients had either complete response or stable disease.	[237]
	Chemotherapy (oxaliplatin and capecitabin) + DC-based vaccine with KLH and CEA peptides	4 out 7 patients induced functional CEA-specific T-cell responses. After oxaliplatin administration, an enhanced non-specific T-cell reactivity was observed. KLH-specific T-cell responses were not affected by the chemotherapy, and B-cell responses were diminished.	[231]
	Chemotherapy (cyclophosphamide) + 5-HLA-A2402-restricted epitope peptide vaccine (KOC1, TTK, URLC10, DEPDC1 and MPHOSPH1)	No adverse events above grade 3. The number of Treg dropped from baseline after cyclophosphamide administration. TAA-specific T-cell responses were associated with longer OS.	[238]
	Chemotherapy + DC-based vaccine with CIK cells	The median survival time was significantly longer in patients treated with immunotherapy plus chemotherapy than with chemotherapy alone.	[239]
	Chemotherapy (UFT and leucovorin) + 2-peptide vaccine (RNF43 and TOMM34 peptides)	CTL responses were induced against both peptides in 8 patients, and against one peptide in 12 patients. 1 patient had no CTL response. The group with CTL responses to both antigens presented the highest survival.	[240]
	Chemotherapy (UFT and leucovorin) + 7-peptide vaccine (RNF43 TOMM34, FOXM1, MELK, HJURP, VEGFR-1, and VEGFR-2 peptides)	3 patients had a partial response, 15 patients had stable disease, and 12 patients had progressive disease. Patients who exhibited positive CTL responses to all 7 peptides had longer OS compared to other patients.	[241]
	Chemotherapy (5-FU derivate, TS-1) + personalized peptide vaccine	An increase in peptide-specific IgG was observed in most patients regardless of the dose of TS-1. An increased peptide-specific IFN-γ production by CTL was more prominent in patients treated with the highest dose of TS-1.	[242]
	Chemotherapy (cyclophosphamide) + MVA-5T4 vaccine (TroVax®)	Cyclophosphamide depleted Treg cells in 24 of the 27 patients receiving the MVA-5T4 vaccine. The same patients had higher PFS and OS.	[243]
	Chemotherapy (cyclophosphamide) + whole tumor vaccine (irradiated, allogeneic human colon cancer cells and GM-CSF-	6 out of 9 patients survived longer than 36 months, and 4 of these 6 without disease recurrence. GM-CSF-producing colon cancer vaccine enhanced the production of anti-MUC1 antibodies.	[244]

	producing bystander cells)		
Vaccine & Immunomodulators	Pexa-Vec (JX-594), an oncolytic and immunotherapeutic vaccinia + anti-CTLA-4 and anti-PD-L1 antagonist antibodies	Phase I/II studies in refractory and advanced CRC patients. Study ongoing with no results posted. (ClinicalTrials.gov identifier: NCT03206073 and NCT02977156)	[245, 246]
PRECLINICAL STUDIES			
Chemotherapy & Cytokines	Chemotherapy (oxaliplatin) + IL-2	Oxaliplatin 3 days before starting the induction regime of IL-2 achieved efficient elimination of liver metastasis and improved the protection against tumor re-challenge. It was also observed a shift in the TIME towards a more pro-immunogenic phenotype, with an increase in the $CD8^+$/Treg ratio and a reduction in MDSC.	[247]
	Chemotherapy (oxaliplatin) + IL-7	*In vivo* results showed that IL-7 combined with oxaliplatin inhibited tumor growth in lung and abdomen metastasis models of colon cancer (CT26). The combination inhibited the tumor growth by immunoregulation, resulting in a higher number of $CD8^+$T cells and reduced levels of Treg.	[248]
	Chemotherapy (cyclophosphamide) + IL-12	50% of mice treated with the combined therapy had complete tumor regression and prolonged survival. Treg were significantly reduced, increasing the number of active DC and inducing IFN-γ-secreting $CD4^+$ T cells.	[249]
Radiotherapy & Immune checkpoint inhibitors	Fractionated radiotherapy + monoclonal antibody against CTLA-4 (clone 9H10)	Mice models were injected at two separate sites – a primary site that was irradiated and a secondary site outside the radiotherapy field. The combination of 9H10 and radiotherapy allowed for an enhanced tumor response at the primary site. A significant growth inhibition of the tumor outside the radiotherapy field was observed in mice treated with the combination.	[250]
	Ionizing irradiation + anti-PD-L1 antibody (clone 10F9G2)	Anti-PD-L1 enhanced the efficiency of irradiation through a CTL-dependent mechanism in mice. Activation of CTL by the combination therapy mediated the reduction of MDSC in tumors through the cytotoxic actions of TNF-α.	[251]
Vaccine & Immunomodulators	CTLA-4 blockade + anti-CD25 + DC-based vaccine (CEA and HLA-A2)	An improved tumor-free survival against CEA-expressing tumors compared with mice immunized with DC-based vaccine alone. The combined strategy led to an increased secretion of IFN-γ and enhanced HLA-A2-restricted CEA-specific CTL responses.	[252]
	DC-based vaccine + 4-1BB (CD137) antibody	Significant increase on tumor rejection. $CD4^+$ T-cell levels were reduced but tumor antigen IFN-γ secreting $CD8^+$ CTL were significantly induced.	[253]
	IFN-α + poxvirus vaccine targeting CEA	IFN-α improved cellular cytotoxicity (NK and $CD8^+$ T cells) and antigen presentation. It inhibited tumor growth, improved survival, and elicited CEA-specific CTL responses.	[254]
	IFN-α-transduced tumor cell vaccine + anti-PD-L1 antagonist antibody	The tumor growth was significantly reduced. Marked infiltration of $CD4^+$ and $CD8^+$ T cells. The blockade of PD-L1 ligand enhanced the Th1-type anti-tumor immune responses induced by IFN-α.	[255]
Immunomodulators	IL-15 + anti-PD-L1 and anti-CTLA-4 antibodies	Higher CTL killing and IFN-γ secretion. Surface expression of PD-1 on $CD8^+$ T cells was reduced, as well as IL-10 secretion. Triple combination induced longer survival rate, compared to mice	[256]

		treated with IL-15 alone, or combined singularly with anti-PD-L1 or anti-CTLA-4.	
Chemotherapy & Vaccine & Immunomodulators	Cyclophosphamide + MYB cDNA-vaccine + anti-PD-1 antibody	Cyclophosphamide was used to overcome Treg immunosuppression, and anti-PD-1 antibody was used to block T-cell exhaustion. This combination therapy elicited protection when tumor burden was higher and promoted tumor-specific cell killing.	[257]
Radiotherapy & Vaccine & Immunomodulators	Ionizing radiation + iDC-based vaccine + anti-CTLA-4 antibody	The growth of distant tumors was inhibited by radiation/vaccine and this effect was significantly increased by the combination treatment. The synergistic effect was related to IFN-γ-secreting T cells and CTL activity.	[258]

Notes: ALVAC: canary pox virus; cDNA: complementary DNA; CEA: carcinoembryonic antigen; CIK: cytokine-induced killer; CTL: cytotoxic T lymphocyte; CTLA-4: cytotoxic T-lymphocyte-associated protein 4; DC: dendritic cell; DEPDC1: DEP domain containing 1; FOLFIRI: irinotecan plus infusional 5-FU/LV; FOXM1: forkhead box protein M1; 5-FU/LV: 5-fluorouracil/leucovorin; GM-CSF: granulocyte macrophage colony-stimulating factor; HJURP: holliday junction recognition protein; HLA: human leukocyte antigen; iDC: immature DC; IFL regimen: irinotecan plus intravenous bolus 5-FU/LV; IFN: interferon; IgG: immunoglobulin G; IL: interleukin; KLH: keyhole limpet hemocyanin; KOC1: outer chloroplast membrane 1; MDSC: myeloid-derived suppressor cell; MELK: maternal embryonic leucine zipper kinase; MPHOSPH1: M-phase phosphoprotein 1; MUC1: mucin 1; MVA: modified vaccinia virus Ankara; NK: natural killer; OS: overall survival; PD-1: programmed cell death protein 1; PD-L1: programmed death ligand 1; PFS: progression free survival; RNF43: ring finger protein 43; TAA: tumor associated antigens; Th: T helper; TIME: tumor immune microenvironment; TNF: tumor necrosis factor; TOMM34: translocase of outer mitochondrial membrane 34; Treg: T regulatory cell; TTK: TTK protein kinase; UFT: tegafur/uracil; URLC10: up-regulated lung cancer 10; VEGFR: vascular endothelial growth factor receptor.

5. Nanotechnology as a promising strategy for CRC therapy

Nanomedicine comprises the application of nanotechnology to biomedicine and health sciences to diagnose, prevent and treat diseases, while allowing for a better understanding of the complexity of disease pathophysiology, yielding more effective therapies and improving patients' life quality. In this context and according to the European Science Foundation (ESF), nanomedicine uses nanoscale functional systems engineered with distinct materials and shapes, and developed within a controlled size range, from one to hundreds of nanometers, similar in scale to bioactive molecules and functional constituents of living cells [259].

Deepening in oncology field, nanomedicines present a great potential for cancer therapy as powerful miniaturized nanosystems that carry the therapeutic agent to desired target sites, increasing the therapeutic outcomes [260].

Nanocarriers have been highlighted as favorable platforms for vaccine and drug delivery due to their ability to i) entrap, encapsulate or embed different types of active constituents into the same carrier, including cytotoxic agents, chemosensitizers, antiangiogenic agents, TAA, immunomodulatory and/or immunostimulatory molecules; ii) protect active constituents from enzymatic degradation *in vivo*, prolong their systemic circulation lifetime and release them in a sustained manner avoiding the need for repeated administrations; iii) modify their surface with biological targeting

multivalent ligands as peptides, proteins or antibodies to provide an effective, selective and site-specific cargo delivery to target cells, subcellular compartments or body sites; and iv) arrive at a particular site by overcoming extra and intracellular physiological barriers in the body, in a size-dependent and surface-dependent manner.

The direct administration through the rectum or the oral route have been explored to deliver active agents site-specifically to colon and rectum, increasing drug concentration at the target site and thereby reducing drug-related side effects [261, 262]. Oral delivery is a preferable route to administer biologically active agents compared to parenteral methods, due to the great expediency of self-medication and less discomfort, contributing to a better patient compliance [261, 262]. Moreover, oral delivery of vaccines can induce both mucosal and systemic immunities. Delivery systems allow the protection of antigens and/or adjuvants from the harsh environment of the gastrointestinal tract (low pH, presence of digestive enzymes, and the detergent activity of bile salts) until their delivery to the targeted immune cells. The nanocarriers are favorably taken up by specialized epithelial cells, called microfold cells (M cells), present in the follicle-associated epithelium of the Peyer's patches of the gut associated lymphoid tissue (GALT). These promote a direct gateway for the induction of localized immune responses [262, 263], thus fostering the regulation of immune cell function at the mucosal surfaces.

Nanomedicines thus contribute to overcome adverse reactions related to the off-target effects, to suppress dose-limiting toxicities and to ensure improvements in stability, solubility, bioavailability, pharmacokinetics, non-specific biodistribution and targeting of delivered cargo. This can lead to better therapeutic efficacy, survival and patient's quality of life [264, 265].

Over the last few years, several types of delivery systems (Figure 4), such as lipid nanoparticles (NP) [266, 267], liposomes [268-271], polymeric NP [272-274], polymeric micelles [275, 276], dendrimers [277], mesoporous silica particles [278, 279], as well as gold [280], silver [281] and magnetic [282] NP, have been explored among different CRC therapeutic applications including drug [283-287], vaccine [288, 289] and gene delivery [290]. Moreover, some nanomedicines such as CPX-1 liposome (Phase II; NCT00361842), Etirinotecan Pegol or NKTR-102 (Phase II; NCT00598975; NCT00856375) and Aroplatin or Liposomal NDDP (Phase I/II; NCT00081536; NCT00057395; NCT00043199) are under clinical evaluation. Also important, the use of fluorescent NP in image-guided surgery and intraoperative fluorescence imaging has

emerged to provide real-time imaging of tumor site and metastases, taking advantage of the enhanced permeability and retention (EPR) effect, the increased circulation time, and the active targeting of nanocarriers [291, 292]. cRGDY-targeted silica NP are under clinical evaluation for real-time image-guided intraoperative mapping of nodal metastases (sentinel lymph nodes) directly link to CRC (Phase I; NCT02106598). Moreover, nanomedicines have also been used as an adjuvant/post-surgical therapy for CRC. ThermoDox comprises thermally sensitive liposomes encapsulating doxorubicin. It was clinically tested (Phase II; NCT01464593) as an adjuvant/post-surgical therapy combined with radiofrequency thermal ablation in the treatment of recurrent or refractory colorectal liver metastases [293]. Even if this study is concluded, the results are not yet publicly available.

However, despite the promising chemical compositions and morphologies already developed for different delivery systems, the clinical approval of nanomedicines for CRC treatment and their translation into routine clinical practice remains limited and requires a multidisciplinary analysis of clinical, ethical and societal aspects. Accordingly, Cimzia®, a PEGylated antibody (Certolizumab pegol) against Crohn's disease is the only nanomedicine in the market approved by the US FDA (UCB company, 2008) related to gastrointestinal tract diseases [294].

The following sections review the major nanotechnology-based approaches explored to deliver chemo and immunotherapeutics isolated, as an effort to increase efficacy, while overcoming severe adverse effects. It will give focus on advanced approaches currently under investigation for CRC treatment to demonstrate how nanotechnology can add into the combined delivery of chemotherapeutic agents with other classes of therapeutics such as, drugs, antivascular agents, small molecule inhibitors, gene regulators, antibodies, checkpoint inhibitors and radiotherapy.

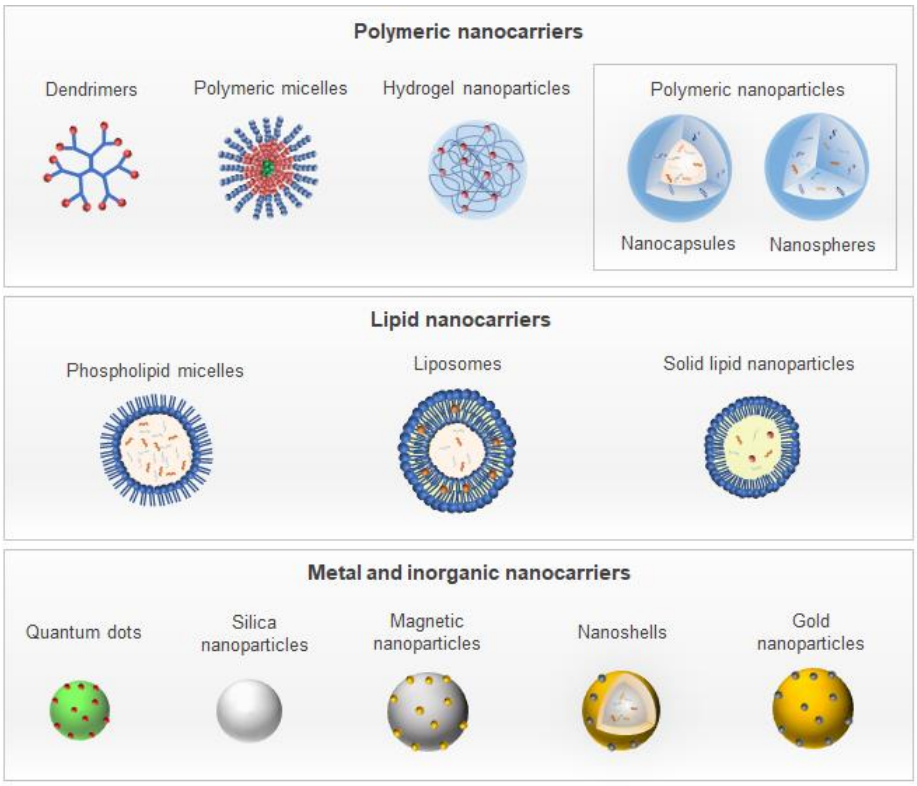


Figure 4. Schematic representation of polymeric, lipid, metal and inorganic nanocarriers. Adapted from [295].

5.1.Nanosystems for drug delivery

Passive targeting is the most common mechanism reported for the delivery of drugs to tumors through conventional NP (no surface modification), taking advantage of NP size and tumor vasculature properties. Due to anatomical and functional defects of cancer cells and pathophysiological features of blood vessels surrounding the tumors, such as disorganized, irregular, leaky, dilated and porous shape, enlarged gap junctions among EC are formed [296]. More specifically, tumors whose volumes reach 2 mm^3 undergo difficulties on nutrients and oxygen intake. To overcome those issues, cancer cells endorse angiogenesis by increasing vascular mediator levels (e.g. bradykinins, nitric oxide, VEGF, prostaglandins) to promote the creation of new blood vessels to meet tumor needs. However, the compromised leaky tumor vasculature, with pore cutoff size of 100 nm to 2 μm (depending on cancer type) among EC, and negligible lymphatic drainage, due to high interstitial pressure on tumor core, creates a perfect gap that promotes the "Enhanced Permeability and Retention (EPR) effect", which enables the extravasation through these pores and accumulation of NP (up to 400 nm) within interstitial solid tumors [297]. Due to the high retention ability of tumor tissues and reduced lymphatic network when compared to the normal ones, NP accumulate and

remain longer within the tumor site to release *in situ* the carried active substances, thus improving their therapeutic efficacy and reducing their exposure to healthy tissues, overcoming drug side effects and widespread toxicity [298].

However, CRC are poorly vascularized and do not exhibit a considerable vascular permeability or EPR effect, becoming passive targeting a limited option [299]. Among the controversial behind the relative input of passive (EPR effect) and active (receptor-mediated) targeting on the NP accumulation at tumor sites [300], Li and co-workers showed that the active targeting appeared to contribute three times more than the EPR effect over time, specifically for the urokinase-type plasminogen activator receptor (uPAR)-mediated targeting and the human serum albumin (HSA)-mediated EPR effect [301].

As expected, the nature of the targeting agents and tumor receptors is a critical variable for both passive or active effects [300]. The active targeting strategy encompassing the covalent conjugation of targeting moieties onto NP surface has been developed to promote the selective and efficient delivery of drugs, or other bioactive molecules to tumor site, thus preventing the contact with undesired sites and thereby improve the efficacy of NP drug delivery systems as anticancer therapeutics [302]. This targeting approach comprises the use of ligands, such as peptides, proteins, aptamers, monoclonal antibodies, antibody fragments, enzymes, carbohydrates, nuclei acids-based ligands, and small molecules. The ligands ideally are specifically directed to an antigen or receptor exclusively overexpressed at the tumor cell surface and vasculature, to enhance NP internalization by tumor cells via receptor-mediated endocytosis [303-305]. These targeting moieties can be conjugated pre- or post-NP synthesis [306, 307]. The ligands mostly explored as CRC targeting moieties are addressed on Table 10.

Table 10. Targeted CRC molecules and most considered respective targeting moieties.

CRC TARGETS	EXPRESSION IN CRC	TARGETING MOIETIES	REF.
EGFR	32.8% more expressed in CRC than in normal tissues and is directly correlated to a metastatic form of CRC.	EGF, HB-EGF, TGF-α, betacellulin, amphiregulin, epiregulin; and bevacizumab, cetuximab and panitumumab antibodies	[6, 272, 285]
VEGF	VEGF expression is positive in ~50% of CRC cases and associated with tumor angiogenesis and poor prognosis.	Aflibercept (VEGF-A and -B), bevacizumab (VEGF-A)	[308, 309]
MT1-MMP	Overexpression on EC and CRC cells, and directly involved in metastasis and angiogenesis.	Angiogenic peptide GPLPLR	[310, 311]
VCAM-1	VCAM-1 is overexpressed on endothelial CRC cells (57%) and during inflammation.	Anti-VCAM mAb	[312, 313]
$\alpha_v\beta_3$	$\alpha_v\beta_3$ is an EC receptor overexpressed in CRC	Proteins harboring the RGD	[264, 314-

	cases, and directly related to EC migration and VEGFR-2 signaling.	sequence (von Willebrand factor, fibrinogen, fibronectin, vitronectin, plasminogen, thrombospondin, prothrombin, MMP-2, laminin, osteopontin) and anti-$\alpha_v\beta_3$-specific mAb	316]
TR	TR overexpressed on metastatic and drug-resistant cells	Transferrin	[317]
FRα	FRα is overexpressed in CRC, 33-44% more than in normal tissue.	Folic acid	[318-320]
CD44 receptor	CD44 receptor is overexpressed in most of CRC cases. Prominent expression of CD44 is a hallmark of highly tumorigenic CRC cells.	Hyaluronic acid	[286, 321]
CEA	CEA is the most consistently marker overexpressed in 98.8% of CRC cells.	Humanized anti-CEA mAb (A5B7, hPR1A3)	[322, 323]
A33 antigen	A33 antigen is expressed in >95% of human colon cancers.	Humanized anti-A33 mAb	[324]
DR5	DR5 is significantly up-regulated in CRC tissues (stages II and III), compared to normal pairs.	Conatumumab (AMG 655)	[325, 326]
TAG-72	TAG-72 is more highly expressed in tumor tissue than in matched normal tissue in 79.0% of CRC cases.	Humanized anti-TAG-72 mAb (B72.3)	[327]

Notes: CEA: carcinoembryonic antigen; CRC: colorectal cancer; DR5: death receptor-5; EC: endothelial cell; EGF: epidermal growth factor; EGFR: epidermal growth factor receptor; FRα: folate receptor-α; GPLPLR: Gly-Pro-Leu-Pro-Leu-Arg; HB-EGF: heparin-binding EGF; mAb: monoclonal antibody; MMP: matrix metalloproteinase; MT1: membrane type 1; RGD: Arginine-Glycine-Aspartic acid; TAG-72: tumor-associated glycoprotein-72; TGF-α: transforming growth factor-α; TR: transferrin receptor; VCAM: vascular cell adhesion molecule; VEGF: vascular endothelial growth factor.

Clinical studies involving the passive and active drug delivery against CRC are summarized in Table 11.

Table 11. Clinical studies of passive and active targeted drug delivery systems against CRC.

DRUG DELIVERY SYSTEM	ACTIVE MOLECULE	TARGETING MOIETIES	CLINICAL TRIAL STAGE	REF.
Micellar nanoparticles based on PEG/Polyamino acid (NC 6004)	Cisplatin derivatives linked via coordination bond to the Poly aminoacid Free Gemcitabine	NA	Phase Ib/II (ClinicalTrials.gov identifiers: NCT02240238; NCT02043288)	[328]
Polyethylozaxoline (PEOX)-based Polymer FID-007	Encapsulated paclitaxel	NA	Phase I (ClinicalTrials.gov identifiers: NCT03537690)	[329]
NanoLiposome (CNL)	C_6-ceramide entrapped in NanoLiposomes	NA	Phase I (ClinicalTrials.gov identifiers: NCT02834611)	[330]
NanoLiposome (PEP02, MM-398, nal-IRI)	Encapsulated Irinotecan Free Trifluridine and Tipiracil Hydrochloride	NA	Phase I/II (ClinicalTrials.gov identifiers: NCT03368963)	[331]
NanoLiposome (PEP02, MM-398, nal-	Encapsulated Irinotecan Free eucovorin calcium and	NA	Phase II (ClinicalTrials.gov	[332]

IRI)	fluorouracil		identifiers: NCT01375816)	
Liposome LE-SN38	Encapsulated SN-38	NA	Phase II (ClinicalTrials.gov identifiers: NCT00311610)	[333]
Liposome CPX-1	Encapsulated irinotecan:floxuridine	NA	Phase II (ClinicalTrials.gov identifiers: NCT00361842)	[334]
Lipid Nanoparticle	mRNA-2752 encoding Human OX40L, IL-23, and IL-36γ Free Immune Checkpoint Blockade	NA	Phase I (ClinicalTrials.gov identifiers: NCT03739931)	[335]
Thermally sensitive Liposome (Thermodox)	Encapsulated Doxorubicin	NA	Phase II (ClinicalTrials.gov identifiers: NCT01464593)	[336]
Antibody-Drug-Conjugate Trastuzumab (DS-8201a)	Conjugated Deruxtecan	HER2-targeting Trastuzumab	Phase II (ClinicalTrials.gov identifiers: NCT03384940)	[337]
Antibody-Drug-Conjugate Trastuzumab (T-DM1)	Conjugated emtansine	HER2-targeting Trastuzumab	Phase II (ClinicalTrials.gov identifiers: NCT02465060)	[338]

5.2. Exploring nanotechnology for CRC immunotherapy

Nano-based immunotherapeutic approaches can be rationally developed to selectively target DC, as well as, to modulate the function of many other populations of cells within TIME, such as cancer cells, T cells, CAF, TAM, among other stromal cells [339]. However, the anatomical location of these cells, as well as their particular functional features must be taken into account while developing these nanotechnology-based immunotherapies in order to achieve a sustained and robust anticancer effect [340].

In response to an external attack, a non-specific pro-inflammatory response is triggered by innate immune cells, such as granulocytes (neutrophils, basophils, eosinophils, mast cells), phagocytic macrophages, antigen-presenting DC, and cytotoxic NK cells, to protect the body against harmful stimuli [341]. Among them, DC are known as the most professional APC in inducing T-cell responses, by bridging the

innate and adaptive immunity. The priming of strong innate and adaptive immune responses is essentially dependent on the direct recognition of immune stimulators or danger signals stated as pathogen-associated molecular patterns (PAMP) by a broad spectrum of pattern recognition receptors (PRR) on innate immune cells [342]. After PAMP recognition by PRR, several intracellular signal transduction pathways are activated to promote an immune response through the induction of optimal antigen processing and presentation, the expression of co-stimulatory molecules (CD80, CD86 and CD40) and the secretion of a wide range of pro-inflammatory molecules, including chemokines, cytokines (IL-6 and TNF-α), and type-I IFN [66, 343].

Nanoscale vaccine delivery systems hold a great potential to improve vaccine efficacy and modulate anti-tumor immune responses *in vivo*. Even though, an efficient DC activation and maturation stimuli induced by nanosystems usually requires three main components: (i) one or several antigens that are recognized by the immune system and thereby promote a specific anti-tumor adaptive immunity; (ii) one or multiple adjuvants/immunopotentiators to trigger the activation and maturation of APC and potentiate antigen-specific responses; and (iii) a carrier to ensure an optimal cargo delivery [344].

TLR ligands for immunoregulation

Improved cancer immunotherapeutic outcomes thus require the co-delivery of both TAA and PAMP/adjuvants within a single nanosystem [345]. As shown in Table 12, there are several PAMP, such as proteins, lipids, lipoproteins, carbohydrates and nucleic acids, as well as PRR, including the transmembrane (toll-like receptors (TLR), CLR, mannose and scavenger receptors) and the less explored cytoplasmic proteins, such as the nucleotide-binding oligomerization domain (NOD), leucine-rich repeat-containing receptors (NLR), retinoic acid-inducible gene (RIG)-I like receptors (RLR) and the AIM2 (absent in melanoma 2)-like receptors [346].

Table 12. Human PRR and their natural and synthetic ligands. Adapted from [346, 347].

CELL LOCATION	PRR	PAMP	SYNTHETIC AGONIST	IMMUNE RESPONSE
Cell membrane	TLR 1 & 2	Triacylated lipoproteins	Pam3Cys	Inflammatory cytokine production
	TLR 2 & 6	Diacylated lipoproteins; LTA	Pam2Cys; MALP-2	
	TLR 4	LPS; HSP	MPLA; LPS analogs	Type 1- IFN synthesis
	TLR 5	Flagellin	-	TNF-α production
	TLR 10	Profillin-like proteins	-	-

Endosomal membrane	TLR 3	dsRNA	Poly(I:C); Poly(A:U)	Type 1- IFN synthesis
	TLR 7 & 8	ssRNA	Resiquimod (R848); Imiquimod (R837); Gardiquimod; Loxoribine (Guanosine analogs)	Anti-viral response
	TLR 9	CpG DNA motifs; DNA; Malaria hemozoin	CpG ODN	CpG type A/D: IFN-α induction CpG type B/K: IL-12 & TNF- α induction
Cytosol (RLR)	RIG-I	Short dsRNA	5'ppp-dsRNA; short Poly(I:C)	Type 1- IFN synthesis
	MDA5	Long dsRNA	Long Poly(I:C)	
	LGP2	RNA	-	RIG-I and MDA5 modulator
Cytosol (NLR)	NOD1	DAP	DAP analog	Inflammatory cytokine production
	NOD2 & NLRP3	MDP, DNA, RNA, ATP	MDP analog	Inflammasome activity (caspase-1, IL-1β, IL-18)

Notes: ATP: adenosine triphosphate; CpG ODN: cytosine-guanine rich oligodeoxynucleotide; DAP: diaminopimelic acid; DNA: deoxyribonucleic acid; HSP: heat shock proteins; IFN: interferon; IL: interleukin; LGP2: laboratory of genetics and physiology 2; LPS: lipopolysaccharide; LTA: lipoteichoic acid; MALP-2: macrophage-activating lipoprotein-2; MDA5: melanoma differentiation-associated gene 5; MDP: muramyl dipeptide; MPLA: monophosphoryl lipid A; NOD/NLR: nucleotide-binding oligomerization domain (NOD) and leucine-rich repeat-containing receptors (NLR); Pam2Cys: dipalmitoyl-S-glyceryl cysteine; Pam3Cys: tripalmitoyl-S-glyceryl cysteine; Poly(A:U): poly(adenylic–uridylic) acid; Poly(I:C): polyribo(inosinic-cytidylic) acid; RIG-I/RLR: retinoic acid-inducible gene (RIG)-I-like receptors (RLR); ss/dsRNA: single/double stranded ribonucleic acid; TLR: toll-like receptor; TNF: tumor necrosis factor.

In mammals, TLR are the most studied and best characterized class of PRR predominantly expressed by DC and another APC. Twelve different TLR were identified in mice and ten in humans. TLR can be divided into two subfamilies according to their subcellular localization. Nucleic acid-sensing TLR are expressed intracellularly on endosomal membranes and comprise the mouse TLR7 and its homologous human TLR8, TLR3, and TLR9, which bind to single stranded RNA, double stranded RNA and unmethylated DNA CpG-based oligodeoxynucleotide (CpG ODN), respectively. The TLR1, 2, 4-6 and TLR10 in humans, and the TLR11 in mice are expressed on the plasma membrane at the cell surface, and these identify exclusively bacterial products [348, 349]. Monophosphoryl lipid A (MPLA) (TLR4 ligand) and imiquimod (TLR7 ligand) are the only TLR agonists approved by FDA for human use [350, 351]. Over the past decades, CpG (TLR9 agonist) and poly(I:C) (TLR3 agonist) adjuvants have emerged as important inductors of robust immune responses induced against cancers, when combined with vaccine formulations [352]. Despite CpG has been a fairly studied adjuvant for vaccine purposes due to its ability to induce strong type-I IFN-dependent T-cell responses in mouse models, the expression of TLR9 is limited in human pDC, when compared to those levels found in mice, which have restricted their approval for human use [353]. However, recent clinical studies strongly

support the anti-tumor immune modulatory effect obtained with the intratumoral administration of CpG in lymphoma and CRC mouse models, as well as, in lymphoma and melanoma patients [354-358]. These studies are revolutionizing the application of this TLR agonist for cancer treatment, which can certainly be expanded upon their delivery by nanosystems, namely those specifically targeting subpopulations of DC. Since TLR are differently expressed among distinct DC populations, the use of different TLR agonists will activate different DC. While human mDC are activated by TLR1-6 and TLR8 agonists [359], the pDC in LN are activated by TLR7 and 9 [360]. The amount of TLR ligands can also be decreased 100 fold when delivered in nanosystems, overcoming the need for high doses or repeated immunizations and minimizing their systemic toxicity [361].

The nanotechnology-based carrier

Although some similarities, the design of nanovaccines will certainly differ from the nanosystems used for drug delivery. While nanovaccines should transport immunomodulatory adjuvants and antigens to APC to elicit T-cell activation against tumor cells, drug carries are designed to release their cargo within TIME avoiding APC. In addition, nanocarriers must promote a continuous, prolonged and sustained release of antigens and adjuvants to APC (depot effect), avoiding the need for booster doses, thus overcoming tolerance and T-cell exhaustion, or anergy induced by a continuous T-cell stimulation [362].

Peripheral LN are the most important organs for vaccine purpose since they represent the site where APC, especially DC, communicate with T cells to induce specific anti-tumor adaptive immune responses [363]. Since DC easily internalize foreign substances, DC passive targeting can be achieved by directing nanovaccines to DC-rich locals, such as LN and peripheral tissues (skin), by controlling mostly two different essential parameters, NP size and administration route [364]. Besides the multiple advantages concerning the use of nanovaccines to target LN and promote a more efficient recognition and antigen presentation by phagocytic cells when compared to soluble antigens, many factors must be considered while designing these immunotherapeutic tools.

The nature and type of the carrier used to deliver TAA and immunoregulators should be carefully considered. For example, carbon nanotubes, quantum dots, and

superparamagnetic iron oxide were reported as immunogenic and toxic at some extent for animals [365, 366].

NP physicochemical features, as size, shape, surface charge, hydrophobicity, rigidity and biodegradability will deeply affect the extension of anti-tumor immune responses. LN targeting can be attained by two different size-dependent pathways, which differ in the dependence or independence on cell transportation using larger or smaller nanocarriers, respectively; however, the optimal NP size to target LN remains a utopian matter. Several studies have demonstrated that larger carriers (> 500 nm) stay physically imprisoned in the skin and are efficiently internalized via phagocytosis by APC, predominantly by monocytes and skin iDC at the dermis, epidermis and to a lesser extent at the subcutis, being subsequently drained to the LN within 18 h [367]. A faster pathway and independent on cell transport is attained by smaller nanovaccines (< 100 nm), which travel directly into the lymphatic drainage by interstitial flow reaching lymphoid organs to interact with LN-resident DC, within 2-3 h after administration [367, 368]. Although the rapid LN targeting by smaller NP, a compromise between NP size and retention in LN must be attained to maximize LN trafficking and retention, since larger-sized NP are retained more efficiently into LN and very small NP can bypass them [369]. Nano-sized particles ranged 100-500 nm are able to cross efficiently some physiological barriers (i.e. intestinal tract and pulmonary system path) and follow both independent or dependent cell-based pathways, endorsing a stronger immunostimulation by promoting the antigen delivery to both peripheral and LN-resident iDC [367, 370].

Although size-dependent, most of the nanocarriers administered subcutaneously and intradermally persist at site of injection making these specific routes the most commonly used to target DC in peripheral tissues. On the other hand, intraperitoneal and intranodal administrations can be used to target LN-resident DC [371]. Less size-dependent, intravenous injection is frequently used to target blood and splenic DC.

Cellular internalization mechanisms are also dependent on NP size. While larger NP (> 500 nm) are usually internalized via macropinocytosis or phagocytosis, inducing a predominant humoral immune response, the uptake of smaller NP (20-200 nm) is mediated via clathrin-dependent endocytosis (size < 200 nm) [372] or via caveolae-dependent endocytosis (size range 50 – 100 nm) [373], promoting both cellular and humoral immunities.

Regarding NP surface charge, anionic or neutral NP appear to be desirable. There is an apparent improved internalization of positively charged NP due to the ionic interactions established with negatively charged cell membranes. However, NP with a positively charged surface leads to NP aggregation with serum blood proteins, particularly when administered intravenously, and subsequent conformational changes (local fluidity) induced on cell membranes can be responsible for hypersensitivity reactions, toxicity and cell metabolic destabilization [374, 375]. Neutral or negatively charged NP are more readily internalized by APC, are usually more biocompatible and travel at a higher extent to LN due to repulsion of proteins of the interstitial milieu [376].

To avoid aggregation of hydrophobic NP at the hydrophilic biological medium that further affects NP internalization and retention by LN, NP surface coating by conjugating, grafting or adsorbing hydrophilic agents (e.g. non-ionic polymer poly(ethylene glycol), PEG) should be considered. On the other hand, hydrophilic coating achieved through "PEGylation" process will also improve bloodstream circulation half-life *in vivo* of hydrophobic NP, by especially avoiding their immediate recognition by reticuloendothelial system as foreign or non-self systems [295, 377].

To reach more effectively and selectively the DC and prevent antigen tolerance induced by premature antigen presentation on DC surface before reaching the LN, NP uptake can be mediated by receptor-ligand interactions *via* NP surface decoration with anchoring molecules with adhesive properties (e.g. lectins) or specific ligands able to target DC surface receptors [378]. Notwithstanding, "PEGylation" can stabilize functional ligands onto NP surface *via* terminal end of PEG-grafted polymers during the conjugation process [377]. Some molecules are exclusive of DC, such as the Clec9A expressed by mouse $CD8^+$ DC and plasmacytoid DC (pDC), and the langerin receptor expressed in mouse splenic $CD8^+$ DC [379, 380]. However, most of DC surface targets are also expressed by other APC, such as macrophages, monocytes and lymphocytes. Besides being non-specific DC surface receptors, such as β2 integrins (CD11c/CD18) [381], C-type lectin receptors (CLR, such as DEC-205 [382], mannose receptor (CD206) [383-385], and DC-SIGN [386]), and Fc receptors (FcγRI (CD64), FcγRIIa(CD32a), FcγRIIIa/b(CD16a/b), and FcγRIIb(CD32b), [381, 387-389]), their targeting has promoted the induction of specific immune responses. NP surface conjugation with binding and endocytosis ligands with a terminal sugar, such as mannose [390, 391], against lectin receptors and DC-specific antibodies anti-CD11c

[392, 393] and anti-CD40 [392, 394-396], have been shown to improve the targeted delivery to DC *in vivo* and overall vaccine effectiveness.

Besides LN targeting and cellular internalization, NP intracellular trafficking is also extremely important for nanovaccines' efficiency as it impacts antigen processing pathways and therefore dictates the extension of humoral and cytotoxic immune responses. As schematized on Figure 5, NP are internalized by DC and usually follow the endolysosomal pathway, where TAA are complexed with MHC class II molecules to be presented to $CD4^+$ T cells. Usually the cytokine priming favors a T helper (Th)2 profile, which results in an extensive humoral immune response, with B cell activation and antibody production against TAA. Furthermore, through the secretion of IL-2, IL-12 and IFN-ɣ, Th1 can also be activated to help enhancing CTL functions [362]. However, the NP escape from the endolysosomal pathway to reach the cytosol is extremely important in cancer nanovaccination and must be promoted. In this process termed "cross-presentation", NP accumulate in the endoplasmic reticulum and TAA processed by proteasomes are complexed with MHC class I molecules to be presented to $CD8^+$ T cells, inducing directly the activation of CTL and the recruitment of players from the innate immune system, such as NK cells, granulocytes and macrophages, able to kill tumor cells [397]. Particularly some DC populations, such as human $BDCA3C^+$ (CD141C) blood myeloid DC (mDC) [398] and murine $CD8\alpha^+$/$DEC205^+$ DC [399], present the unique capacity to efficiently cross-present antigens. Cross-presentation can be promoted by several mechanisms, such as the use of positively charged agents (e.g. chitosan and cell penetrating peptides) that endorse the pore formation on destabilized endosomal membranes and consequently antigen endosomal escape.

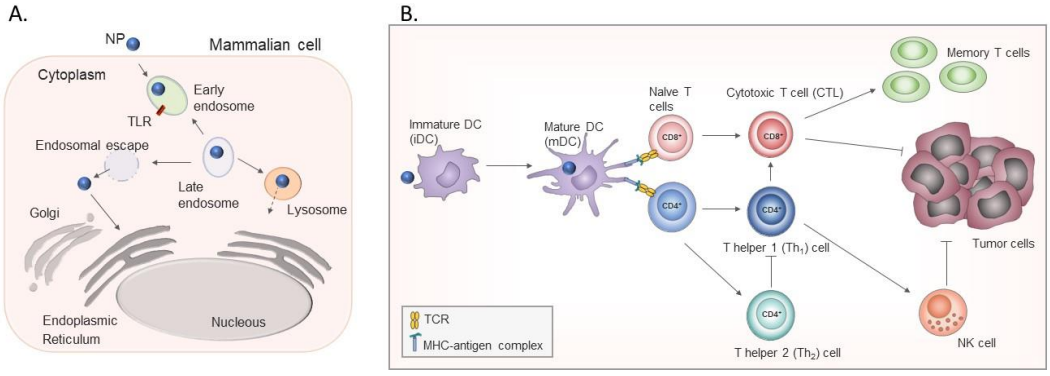


Figure 5. A. Schematic representation of the hypothetical intracellular trafficking of the NP within DC [400]. B. Nanoparticulate cancer vaccines (adapted from [295, 362]).

Preclinical and clinical development of nanotechnology-based immunotherapies

Although the great advantages of using nanotechnology to improve immunotherapeutic efficiency and safety, the clinical development of NP for immunotherapy against CRC remains limited. Some *in vivo* studies have demonstrated a highly improvement of anti-tumoral immune responses based on antigen-specific CTL when antigen and adjuvants are delivered by particulate nanovaccines, in contrast to soluble molecules and other controls [345].

Going deeper with specific examples, the adjuvanticity inherent to NP to act as an intrinsic "danger signal" [344] was reported to help in the induction of potent immune responses capable of suppressing colon adenocarcinoma growth and improving animal survival [169, 288, 401-403]. Luo *et al.* reported a simple synthetic polymeric based-nanovaccine (29 nm), generated from the modest physical mixture among antigen and code-named PC7A NP, able to induce strong cytotoxic T-cell responses [401]. PC7A NP enabled an efficient delivery of tumor antigen especially to DC at draining LN, an enhanced antigen cross-presentation *via* pH-dependent membrane disruption at early endosomes, as well as the innate cellular immunity stimulation by stimulator of interferon genes (STING)-type I interferon pathway. Most importantly, this nanovaccine comprising a cocktail of three tumor neoantigens ($Reps1_{P45A}$, $Adpgk_{R304M}$, $Dpagt1_{V213L}$) into PC7A NP inhibited tumor growth in MC38 colon cancer mouse model in contrast to peptides alone. Moreover, a significant improved tumor growth inhibition and prolonged survival was also reported for melanoma and human papilloma virus-E6/E7 models [401].

A DC-based nanovaccine was also tested in MC38 tumor model. Mice immunized with DC loaded with iron oxide-zinc core(ZnO)-shell-binding peptide(RPHRKGGDA) (ZBP) NP (15 nm) carrying CEA tumor antigen (NP/ZBP-CEA) showed strong cytotoxic T-cell responses and IFN-ɣ secretion by $CD8^+$ T cells, in contrast to control groups (NP/CEA, ZBP-CEA, CEA, NP or DC only) [402].

Furugaki and co-workers also developed a gene vaccine platform based on polyplex micelles carrying genes encoding for SART3, adjuvant CD40L, and GM-CSF to test its efficacy against peritoneal dissemination in subcutaneous CT26 CRC mouse models [169]. The intraperitoneally administered SART3/CD40L+GM-CSF triplet gene-loaded nanovaccine was mainly biodistributed within the lymphatic organs (LN and spleen) and liver. It sub-localized predominantly within DC, and accumulated in macrophages to a lesser extent, being able to improve significantly the survival of mice holding CT26 peritoneal dissemination, compared to mock controls. In CT26 subcutaneous model, the

triplet DNA nanovaccine also inhibited tumor growth and metastasis. Moreover, long-term surviving mice that were re-challenged with CT26 tumors, exhibited complete tumor rejection. This anti-tumoral effect and prolonged survival was especially due to high stimulation of DC/CTL/NK cell activities in the lymphatic organs, and a consequent improved infiltration of $CD11c^+$ DC and $CD4^+$/$CD8a^+$ T cells into tumor regions, elicited by the triplet DNA nanovaccine in contrast to mock controls. This triplet DNA nanovaccine thus constitutes a new versatile platform to elicit anti-tumoral immunity [169].

More recently, new generation adjuvants able to target cytosolic receptors have also been explored. STING signaling pathway agonists, such as cyclic GMP-AMP (cGAMP), have been reported as TLR-independent mediators that significantly inhibit tumor growth, especially in combination with anti-PD-L1 antibody [404]. Moreover, STING pathway is not restricted to pDC, but can be extended to all APC, such as pDC, mDC, and macrophages, which are related with strong anti-tumor responses [405]. In contrast to CpG or poly(I:C) that present long base sequences, small cyclic dinucleotides as STING agonists present an improved stability and resistance against conformational changes and enzymatic degradation. However, effective delivery into the cytosol constitutes a challenge and autoimmune diseases have been reported as major concerns when working with STING agonists. Goodwin and co-authors [288], evaluated the therapeutic efficacy of three potent cancer nanovaccines based on mannose-targeted lipid calcium phosphate (DOPA, CaP, DOTAP, cholesterol, DSPE-PEG2000, mannose) NP (35 nm) co-encapsulating the colon cancer peptide antigen, p-AH1-A5, and each phosphorylated adjuvant, CpG, 2'3'cGAMP, or 5'pppdsRNA, respectively, in an orthotopic CRC liver metastasis CT26 FL3 model. In contrast to unvaccinated controls, and CpG (TLR9 ligand) or 2'3'cGAMP (STING ligand) adjuvant nanovaccines, a potent growth inhibition of primary colon tumor and liver metastasis was obtained when mice were subcutaneously immunized mice with RIG-1 receptor ligand (5'pppdsRNA)-based adjuvant nanovaccine. This potent therapeutic effect was due to RIG-1 ligand adjuvant effect which helps to break the cancer immune tolerance by increasing the CTL infiltration within tumor, as well as, decreasing immunosuppressor cells, such as Treg and MDSC. Here, it is important to highlight that although the three adjuvant nanovaccines could induce a potent pro-inflammatory response through IFN-ɣ and antigen-specific CTL production, 5'pppdsRNA-based adjuvant nanovaccine was the most promising in promoting the recruitment and

infiltration of CTL while maintaining lower populations of immunosuppressive cells within tumor. Despite the great efficacy of this adjuvant nanovaccine, it is a single part of a complex process crucial to achieve a full regression of tumor burden. Therefore, the investigation of a potential synergistic anti-tumor effect of this model adjuvant/vaccine formulation combined with immune modulating therapies focused on blocking immunosuppressive factors and cells involved in cancer progression was suggested by authors to promote a more robust treatment against liver metastatic CRC model [288]. Zhu *et al.* also reported tumor immunotherapeutic nanovaccines development based on intertwining DNA-RNA nanocapsules (iDR-NC) used to co-deliver DNA-RNA adjuvants and tumor-specific neoantigens for a synergistic APC activation in LN, following a sustained and optimal antigen presentation and the induction of a robust anti-tumor T-cell response [403]. These polymeric-based nanovaccines (252 nm) were engineered for cancer immunotherapy by shrinking DNA CpG and Stat3-silencing shRNA microflowers into iDR-NC using PEG-grafted cationic (glutamic acid-based) polypeptide (PPT-g-$(PEG)_6$) copolymer and physically loading the hydrophobic MC38-specific neoantigen Adpgk generated from a specific mutation (ASMTNRELM→ASMTNMELM) in MC38 tumor. CpG/shRNAStat3-iDR-NC/Adpgk complexes enhanced higher levels of Adpgk-specific peripheral $CD8^+$ CTL when compared to the free vaccine CpG/Adpgk, induced Adpgk-specific memory $CD8^+$ T cells, and suppressed significantly the tumor progression on MC38 syngeneic colon adenocarcinoma tumor mouse model. Altogether, these results suggest that the potent and durable immunity was especially elicited by the strong synergistic immunostimulatory effects of Stat3 silencing and CpG immunostimulation of iDR-NC/Adpgk complexes. Particularly in this study, a nanovaccine exploring the synergistic effect of the co-delivery of immunomodulatory DNA-RNA adjuvants and neoantigens elicited strong and prolonged anti-tumor immunity allowing tumor growth inhibition. However, tumors were not regressed by iDR-NC/Adpgk complexes and an upregulated expression of immune checkpoint inhibitor PD-1 on $CD8^+$ T cells was observed, suggesting that by exploring a synergistic combination of this nanovaccine with other therapies, such as immune checkpoint inhibitors, anti-tumor cytokines or chemotherapy can possibly have a great potential by improving iDR-NC/Adpgk therapeutic efficacy and inducing the complete tumor remission [403].

Optimized nanovaccines alone have been able to efficiently trigger anti-tumor T cell responses, but these immunotherapeutic strategies fail to maintain the activity and

tumor-infiltration capacity of those T cells. To attain their huge potential, nanovaccines must be combined with other immune modulators.

Cytokines are also potent enhancers and regulators of innate and adaptive immunities. Cytokine-based immunotherapy has been reported as highly promising in inducing immune responses against tumor in several mouse models; however, when free cytokines are intravenously injected, even with lower doses, limited cytotoxic effects are observed. To improve the cytokine half-life *in vivo*, the transduction efficacy and therapeutic levels of cytokine delivery as well as decrease the concerns about the toxicity induced by free viral plasmids, non-viral gene carriers were explored to deliver exogenous DNA for the potential expression of cytokines able to stimulate strong humoral and cellular immune responses. Exploring anti-tumoral immune responses against the same CRC mouse model, two different delivery systems were developed to safely transfect CT26 cancer cells to secrete IL-12 [406] and IL-21 [290]. Liu and co-authors developed a non-toxic hybrid nanosystem by self-assembly method, DOTAP/MPEG-PLA-pIL12 (DMP-pIL12) (37.5 nm), able to inhibit tumor growth *in vivo* at both subcutaneous or peritoneal model. By increasing the secretion of the anti-proliferative and angiogenic mediators IL-12, IFN-ɣ, and TNF-α, which promote the increase of CTL and apoptosis, as well as the inhibition of proliferative and angiogenic effects within tumor tissue, DMP-pIL12 demonstrated higher anti-tumoral effects than the other tested groups (DMP-pVax: the vector plasmid, DMP, and NS: normal salt), being considered a promising system for CRC therapy in a clinical setting [406]. On the other hand, in the study reported by Tan *et al.* [290], tumor-bearing mice treated with chitosan-based dsNKG2D-IL.21 gene NP (200-400 nm) showed reduced tumor sizes and increased life expectancy. These plasmid NP bridged tumor cell eradication and lymphocyte infiltration within tumor by targeting tumor cells *in vivo* through the NKG2D moiety and activated NK and $CD8^+$ T cells through the IL-21 moiety, simultaneously. Although the therapeutic effect of dsNKG2D-IL-21 NP had been worse than the one obtained for the recombinant IL-21, the amounts of activated $CD4^+$ and $CD8^+$ T cells within the tumor were higher.

5.3.Combined therapies based on nanotechnology-based approaches

Combinatorial and complementary approaches concerning chemotherapy, radiotherapy, immunotherapy and TIME modulation, may be the key to modulate multiple pathways involved in tumorigenesis and dissemination. Therefore, the potential

of CRC therapies may be based on rationally designed combinatorial nanomedicine(s) that target multiple signaling pathways promoting a better and durable therapeutic efficacy and thereby attaining the cure, overwhelming disease resistance and severe toxicity.

Chemotherapy containing nanomedicines have also their place in the TIME modulation. Indeed, chemotherapy can induce immunogenic cell death (ICD). 5-FU or Oxaliplatin-induced ICD can lead to CTL infiltration in CRC [407-409]. Nanomedicines could also be used to inhibit tumor infiltrated cells, as it has been shown in other cancer types against CAF [410, 411] and against immunosuppressive cells [412]. For example, the anti-PD1 antibodies with Doxorubicin, loaded in synthetic high-density lipoprotein (sHDL)–like nanodiscs induced a 80% cure on MC38 tumor growing in the cecum and 3-fold CD8+ lymphocytes increase compared to free DOX treatment [413].

Combinatorial nanomedicine regimens are emerging as promising solutions due to their ability to improve the therapeutic efficacy and prevent recurrence through a synergistic activity, allowing a decrease on dosages of individual active compounds and toxicity [414]. The ratio of administered agents also determines the clinical outcome of a combinatorial therapy once it regulates the degree of synergy or antagonism [415]. Because different therapeutic agents present dissimilar cellular uptake and pharmacokinetic profiles, their entrapment within nanoscale delivery systems able to keep synergistic cargo ratios through pharmacokinetic control is essential to overcome the exposure of suboptimal or antagonistic cargo ratios at the tumor and improve the therapeutic index of combined active agents [415-417]. Ideally, the correct ratio of multiple therapeutic agents should also be simultaneously delivered using a single nanoconstruct to decrease manufacturing variability and costs [277, 325, 415, 416, 418-429]. Alternatively, the delivery of therapeutic agents with different pharmacokinetics, biodistribution and clearance profiles using different vehicles have also been explored against CRC [414, 430-433]; however, it can induce more variability [434]. Also, highly important is the choice of carrier material once it affects the pharmacokinetics and pharmacodynamics of active cargo.

According to the different studies on combinatorial nanomedicines against CRC available in the literature and presented on Tables 13 and 14, different nanomaterials, such as liposomes, solid lipid NP, polymeric NP, polymeric micelles, NP-drug conjugates, and dendrimers, have been explored. Different active agents (drugs, genes,

antiangiogenic agents, kinase signaling pathway inhibitors, antibodies) against CRC were entrapped inside a single nanoconstruct among different combinations [277, 415, 416, 418-422, 424, 426]. On the other hand, the combinatorial administration of individual nanosystems entrapping each one a single drug was also described in the literature for CRC [414, 430-433]. NP entrapping a single agent were also combined with other free active agents against CRC [434-438]. Finally, the combination of chemotherapy using nanosystems and radiotherapeutic treatment was also explored [439-441]. A few nanomedicine combinations have entered into clinic. We can highlight liposomal irinotecan approved and marketed under the name of Onivyde® in association with free 5-FU/LV for locally advanced or metastatic pancreatic adenocarcinoma, has been tested in phase II clinical trial for metastatic colorectal cancer, with comparable results to optimized FOLFIRI-3 treatment [416].

In general, all these combinatorial therapies based on nanotechnology against CRC attained a better response and improved overall survival compared to single-agent therapies, in both preclinical and clinical studies, and allow multiple combinatorial strategies to tackle TIME-related resistances.

Table 13. Examples of clinical combinatorial nanotechnology-based therapeutic strategies against CRC.

Composition of nanomedicine	Association	Patients	Results	Ref.
Liposome PEP02 (MM-398) with IRI	Free bevacizumab and 5-FU/LV	Oxaliplatin-pretreated mCRC (Phase II)	Regimen active as the optimized FOLFIRI3 and more active than the standard FOLFIRI regimen. Acceptable safety profile and best overall response rate.	[435]
Liposome (CPX-1) with IRI & floxuridine	Co-encapsulation in 1:1 ratio	33 advanced solid tumors, including 15 CRC (Phase I)	1 patient died of persistent diarrhea; PR occurred in 12%, PFS lasting > 6 months occurred in 36% of patients. Among CRC patients, the calculated median PFS was 5.4 months; 73.3% achieved disease control and 13.3% had PR.	[416]
		59 CRC (Phase II)	In 26 IRI-naïve patients: ORR 8%; DCR=ORR+SD 65%. Median PFS 3.2 months with 35% > 4 months PFS. In 33 IRI-refractory patients: 0% responses; DCR 38%. Median PFS 1.8 months, with 18% > 4 months PFS. Safety data: 67% grade 3/4 toxicities. 1 death treatment related. CPX-1 appears to be more active than FOLFIRI used after FOLFOX (Tournigand).	[442]
NP–drug conjugate CRLX101 with CPT	Surgery, Capecitabin & radiotherapy	32 Rectal Cancer (Phase I/II)	19% of pathological complete response; 56% of moderate response; 22% of minimal response. (ClinicalTrials.gov identifier: NCT02010567)	[441]

Notes: CPT: camptothecin; CRC: colorectal cancer; DCR: disease control rate; FOLFIRI: IRI plus infusional 5-FU/LV; FOLFOX: oxaliplatin plus infusional 5-FU/LV; 5-FU/LV: 5-fluorouracil/leucovorin; IRI: irinotecan; mCRC: metastatic CRC; NP: nanoparticles; ORR: objective response rate; PFS: progression free survival; PR: partial response; SD: stable disease.

Table 14. Examples of preclinical combinatorial nanotechnology-based therapeutic strategies against CRC.

Strategy	Composition	Association	Disease model	*In vitro* outcomes	*In vivo* outcomes	Ref.
Associated Chemotherapies	Liposome with CUR	Free Oxaliplatin	Colo205 & LoVo; Subcutaneous xenografts on female nude mice	Similar antiproliferative effect of equimolar combination compared to single CUR. Synergy of liposomal CUR and oxaliplatin at a 4:1 molar ratio.	No synergy for the combination. Highest tumor growth inhibition for liposomal CUR with or without oxaliplatin, but higher growth inhibition than oxaliplatin alone in Colo205 xenografts. Antiangiogenic effect including the attenuation of CD31, VEGF, and IL-8 expression.	[443]
	SLN with cholesteryl butyrate, DOX or PTX	Co-administration	HT29	Synergistic antiproliferative effect of cholesteryl butyrate SLN with free DOX		[418]

				or PTX.		
	Mannosylated Albumin NP with disulfiram/copper complex and regorafenib	Co-encapsulation	HCT8/ADR drug-resistant colon tumor & Mouse macrophage Raw 264.7; HCT8/ADR subcutaneous xenograft	Better efficacy of encapsulation and dual targeting, and simultaneous action on cancer cells and M2 macrophages *vs.* free drug.	Tumor growth inhibition; improved apoptosis, upregulation of intracellular ROS, anti-angiogenesis, and TAM "re-education" vs. free drug.	[417]
	CS-based NP with CUR or 5-FU	Co-administration	HT29	Enhanced anticancer effects *vs.* bare drugs.	Improved drug bioavailability vs. bare drugs.	[430, 431]
	N-succinyl CS NP with PTX and Gemcitabine	Co-encapsulation	HT29 subcutaneous xenograft	Swelling at colonic pH and sequential release pattern of drugs. Increased antiproliferative effect with the combination.	Prolonged survival time up to 45 days wherein 50% of mice treated with the combination was still alive.	[414]
	Pullulan acetate NP with PTX; CS-based NP with all-trans retinoic acid	Co-administration	CT26	Synergistic antiproliferative effect, increased by NP encapsulation. Significant decrease of MMP-2 activity.		[432]
	PCL-CS polymeric micelles with doxifluridine (5-FU prodrug) and SN38	Co-encapsulation	HT29	Synergistic antiproliferative activity enhanced by addition of SN38.		[419]
	Non-targeted or HA-functionalized, PLGA/CS-based NP with CPT and CUR	Co-encapsulation at different weight ratios of CPT:CUR	Colon-26	Strong synergistic antiproliferative effects by CPT:CUR in a single HA-functionalized NP at ratios 1:1 or 4:1.		[420] [415]
	Micelles of PEG-poly(-benzyl l-glutamate) (GEG) with DOX or ETP; Micelles of PLA-b-PEG with PTX	Co-administration	CT26 subcutaneous syngeneic tumor	Synergy for free drugs.	Improved synergistic anti-tumor effects of DOX-GEG + PTX-PLA-PEG micelles or DOX-GEG + ETP-GEG micelles compared with single therapy.	[433]

	PEG-*b*-PLA micelles with PTX, 17-allylamino-17-demethoxygeldanamycin, and rapamycin	3-in-1 Co-encapsulation	LS180 human colon xenograft model		Synergy for blocking tumor growth *vs.* single encapsulation of PTX. Improved further uptake of dye-containing micelles.	[422]
Chemotherapy & Antivascular/ Antiangiogenic Agents	SQ-NC with gemcitabine and isoCA-4	Co-encapsulation	LS174-T human colon carcinoma xenograft nude mice model	Better cell uptake of SQ-NC with isoCA-4 than without; comparable cytotoxic and antiproliferative effects than free drug.	Complete tumor regression (by 93%) with the co-delivery. Overall tolerance better than free drugs.	[423]
	PEG-PLGA NP with PTX and PEDF plasmid gene	Co-encapsulation	Colon-26 & HUVEC; Colon-26 subcutaneous tumor	Higher antiangiogenic and antitumoral effects of PEDF and PTX simultaneously encapsulated in the same NP than either of single drug-loaded NP.	Improved anti-cancer effect, micro-vessel density reduction and tumor cell apoptosis vs. controls including co-administration.	[424]
Chemotherapy & Small Inhibitors	LDH with 5-FU	Free BEZ-235 (PI3K/Akt inhibitor)	HCT116	Synergy with improved antiproliferative effect *vs.* free BEZ-235 and LDH-loaded 5-FU.		[436]
	Hyaluronic acid cloaked oleic acid NP with AZD6244 (ERK inhibitor) and cisplatin	Co-encapsulation	HCT116 & DLD-1	Synergy with inhibition of ERK1 and ERK2, DNA damage inducing cell death, in contrast to free drug cocktail.		[425]
Chemotherapy & Gene Therapy	NCP with miR-655-3p and oxaliplatin	Co-encapsulation with miRNA at the surface and oxaliplatin prodrug in the core	HCT116 xenogeneic hepatic metastasis model		Tumor growth suppression by co-delivery of miR-655-3p with oxaliplatin, suggesting additive or synergistic interactions.	[426]
	Polymeric NP based on cysteine trimethyl CS and carboxymethyl dextran, with SN38 and hSET1 antisense ODN	Co-encapsulation	HT29	Significantly higher cytotoxicity of hSET1/SN38 NP compared with NP containing SN38, free SN38 or		[428]

				hSET1.		
	PLGA-based MLNP with CPT and pTRAIL	Co-encapsulation with pDNA at the first external layer, and penetrating peptide at the surface	HCT116 xenografts	Synergistic antiproliferative effect of co-delivered CPT and pTRAIL.	Significant tumor growth inhibition using the combination therapy vs. monotherapy.	[429]
	PCL NP with 5-FU	Free doxycycline to activate suicide gene *E* (phage φX174)	SW480	Synergistic antiproliferative effect. *E* gene expression sensitizes cancer cells to the cytotoxic action of 5-FU.		[437]
	CS/Carboxymethyl dextran NP with Snail siRNA and DOX	Co-encapsulation	HCT116	Significant changes of EMT genes (down-regulation of MMP-9 and vimentin, and up-regulation of E-cadherin), apoptosis cell death and migration inhibition.		[427]
	PLGA NP with PVA and chitosan, encapsulating CPT and siRNA anti-CD98, embedded into a hydrogel for oral administration	Co-encapsulation	Orthotopic, duced by oxymethaned dextranlfate sodium	Improved cytotoxicity and migration/invasion inhibition *vs.* single drug NP	Reduction in tumor numbers and of inflammation, slight improvement *vs.* untargeted NP	[444]
Chemotherapy & Antibodies	PLGA NP with CPT	NP grafted with Conatumumab death receptor 5 antibody	HCT116	Enhanced cytotoxic effects through simultaneous drug delivery and apoptosis induction with targeted NP.		[325]
	Self-assembled Panitumumab linked to dichloro(1,2-diaminocyclohexane) Pt(II) (NANO-Pt-Pan)	Co-encapsulation	HT29 & Caco-2 subcutaneous xenografts	Improved cytotoxicity *vs.* combination of free drug.	Complete inhibition of Caco-2 and 5-time reduction of HT29 tumor growth at day 35 with good tolerance.	[445]
	sHDL-mimicking nanodiscs with DOX (sHDL-DOX)	Anti-PD-1 immunotherapy	CT26 & MC38 subcutaneous CT26 orthotopic		Induced potent antitumor CD8+ T cell responses against established tumors. Complete regression of established tumors in 80 to 88% of mice,	[413]

					CT26 liver metastasis inhibition, and long-term survival against tumor recurrence compared with free DOX or sHDL-DOX treatment.	
Vaccine & Checkpoint Inhibitor	sHDL-mimicking nanodiscs with Adpgk neoantigen and cholesterol-CpG ODN	Anti-PD-1 immunotherapy	MC38 syngeneic tumor		Combinative treatment shows 7/8 mice with complete remission in curative pattern vs. 2/8 for the combined therapy with a classical vaccine (soluble Adpgk, CpG, and anti-PD-1) and 0/8 for monotherapy. Immunological memory against recurrence.	[434]
Checkpoint Inhibitors	Immunoswitch iron-dextran NP	NP coated with agonistic antibodies against 4-1BB (co-stimulation of effector T cells) and antagonistic antibodies against PD-L1 (inhibitory checkpoint blockade on cancer cells)	MC38-OVA subcutaneous xenograft		Significant delay of tumor growth and survival extend *vs.* no treatment and isotype NP. Complete remission of palpable tumors in 5/10 mice.	[446]
Gene Therapy Synergism	PAMAM dendrimer with anti-EGFR and c-Src specific antisense ODN	Co-encapsulation	HT29	Reduction of EGFR and c-Src protein expression and enhanced antiproliferative effect.		[277]
	PLGA-based NP with siRNA anti-DCAMKL-1	Free DAPT (γ-secretase inhibitor)	HCT116 xenograft nude mice model		Inhibition of tumor growth and Notch-1 pathway via a miR-144 dependent mechanism. No synergy.	[438]
Chemo & Radiotherapy	PEGylated liposome (Promitil) with MMC	5-FU-based chemoradiotherapy	HT29 & SW480; HT29 mouse xenograft	Increased MMC release from Promitil, which potently radiosensitized irradiated HT29 (but no SW480).	Increased efficacy of radiotherapy and 5-FU combination. Improved antitumor efficacy	[439, 440, 447]

					compared to equitoxic doses of MMC.	
	NP-drug conjugate CRLX101 with CPT	5-FU-based chemoradiotherapy	HT29 & SW480; Mouse xenografts	Equivalent to free CPT in radiosensitizing cells.	CRLX101 combined with 5-FU-based chemoradiotherapy showed better therapeutic efficacy than combinations with oxaliplatin replacing CRLX101.	[441]
Chemo & Gene & Phototherapy	Gold nanospheres with thiol-siRNA−DY647, HA1 peptide, and TCP-1 peptide (GNS); Gold nanorods with Avastin Thiol-PEG-COOH and TCP-1 peptide (GNR)	In a single hydrogel, co-delivery of both GNR (phototherapy + anti-VEGF) & GNS (phototherapy + anti-Kras gene therapy)	LoVo-6-Luc-1 SCID mice		Complete regression of tumor in 5 mice by the combination.	[448]

Notes: CRC: colorectal cancer; CS: chitosan; CpG ODN: cytosine-guanine rich ODN; CPT: camptothecin; CUR: curcumin; DNA: deoxyribonucleic acid; DOX: doxorubicine; EGFR: epidermal growth factor receptor; EMT: epithelial mesenchymal transition; ETP: etoposide; 5-FU: 5-fluorouracil; HA: hyaluronic acid; sHDL: high-density lipoprotein; IL: interleukin; isoCA-4: isocombretastatin A-4; LDH: layered double hydroxide; miRNA: micro ribonucleic acid; MLNP: multi-layered polymer NP; MMC: mitomycin-C; MMP: matrix metalloproteinase; NCP: nanoscale coordinator polymers; ODN: oligodeoxynucleotide; OVA: ovalbumin; PD-1: programmed cell death protein 1; PEDF: pigment epithelium-derived factor; PEG: poly(ethylene glycol); PLA: poly(lactic acid); PLGA: poly(lactic-co glycolic) acid; pTRAIL: plasmid encoding TNF related apoptosis inducing ligand; PTX: paclitaxel; ROS: reactive oxygen species; SLN: solid lipid nanoparticles; SQ-NC: squalene-based nanocomposites; TAM: tumor associated macrophages; TNF: tumor necrosis factor; VEGF: vascular endothelial growth factor.
Human CRC cell lines: HT29, Caco-2, HCT116, SW480, Colo205, LoVo, Colon-28, LS180, LS174, DLD-1
Murine CRC cell lines: CT26, MC38

Conclusion

CRC prevention and screening programs have greatly contributed for the reduction of CRC incidence levels and death rates. However, these numbers are not so optimistic for patients under the 50's and limited effective therapeutic options are currently available to stop the progression of the metastatic disease.

Tumorigenesis is a complex and dynamic process that involve different cellular and non-cellular elements, and their interaction contribute to tumor development, progression, metastasis and drug resistance. Significant progress has been achieved in uncovering several key cellular and molecular pathways involved in mCRC. This deep knowledge is crucial to guide the development of innovative strategies targeting multiple TIME players that dictate the CRC differentiation, proliferation and cell dissemination. These combinational therapies enable tumor destruction and overcome low response rate of responders, also by preventing the premature interruption of the CRC treatment due to off-target effects.

Nanotechnology holds great promise for the development of chemo-, immuno-, and combinatorial-based therapies against CRC. However, in addition to improved manufacturing technologies enabling the synthesis of reproducible systems, it is fundamental to understand and predict the biological impact that these sophisticated and complex nano-based immune/therapeutics. Related to that it is the urgent need for the validation of models in appropriate settings resembling a specific disease stage or disease evolution conditions. Importantly, those validated models of the disease must reflect the complex immune-related mechanisms in order to successfully improve the translation of the most promising emerging combinational therapies into the clinic.

Acknowledgements

This project has been supported by the Fundação para a Ciência e Tecnologia-Ministério da Ciência, Tecnologia e Ensino Superior (FCT-MCTES), under the frame of EuroNanoMed-II (ENMed/0003/2015: HF, VP, DA; ENMed/0051/2016: HF, RS-F). AIM, BC, CP, JC and LM are supported by the FCT-MCTES (Fellowships PD/BD/113959/2015, SFRH/BD/131969/2017, SFRH/BD/87591/2012, SFRH/BD/87150/2012, SFRH/BPD/94111/2013). This project has received funding from European Structural & Investment Funds through the COMPETE Programme and from National Funds through FCT under the Programme grant SAICTPAC/0019/2015 (HF).

References

1. Society, A.C., *Colorectal Cancer Facts & Figures 2017-2019*, A.A.C. Society, Editor. 2017.
2. Pickhardt, P.J., et al., *Assessment of volumetric growth rates of small colorectal polyps with CT colonography: a longitudinal study of natural history.* The lancet oncology, 2013. **14**(8): p. 711-720.
3. Stewart, S.L., et al., *A population‐based study of colorectal cancer histology in the United States, 1998–2001*. Cancer, 2006. **107**(S5): p. 1128-1141.
4. Levine, J.S. and D.J. Ahnen, *Adenomatous polyps of the colon.* New England Journal of Medicine, 2006. **355**(24): p. 2551-2557.
5. Risio, M., *Reprint of: the natural history of adenomas.* Best practice & research Clinical gastroenterology, 2010. **24**(4): p. 397-406.
6. Cisterna, B.A., et al., *Targeted nanoparticles for colorectal cancer.* Nanomedicine, 2016. **11**(18): p. 2443-2456.
7. Howlader N, N.A., Krapcho M, Garshell J, Neyman N, Altekruse SF, Kosary CL, Yu M, Ruhl J, Tatalovich Z, Cho H, Mariotto A, Lewis DR, Chen HS, Feuer EJ, Cronin KA (eds), *SEER Cancer Statistics Review, 1975-2010, National Cancer Institute.* Bethesda, MD, 2013. **http://seer.cancer.gov/csr/1975_2010/, based on November 2012 SEER data submission**.
8. Siegel, R.L., et al., *Colorectal cancer statistics, 2017.* CA Cancer J Clin, 2017. **67**(3): p. 177-193.
9. DeSantis, C.E., et al., *Cancer treatment and survivorship statistics, 2014.* CA Cancer J Clin, 2014. **64**(4): p. 252-71.
10. Jawed, I., et al., *Colorectal cancer survival gains and novel treatment regimens: A systematic review and analysis.* JAMA Oncology, 2015. **1**(6): p. 787-795.
11. Murphy, C.C., et al., *Patterns of Colorectal Cancer Care in the United States: 1990-2010.* J Natl Cancer Inst, 2015. **107**(10).
12. Edwards, M., et al., *A systematic review of treatment guidelines for metastatic colorectal cancer.* Colorectal Disease, 2012. **14**(2): p. e31-e47.
13. Kekelidze, M., et al., *Colorectal cancer: current imaging methods and future perspectives for the diagnosis, staging and therapeutic response evaluation.* World J Gastroenterol, 2013. **19**(46): p. 8502-8514.
14. Gulbake, A., et al., *Insight to drug delivery aspects for colorectal cancer.* World J Gastroenterol, 2016. **22**(2): p. 582-99.
15. Ewing, I., et al., *The molecular genetics of colorectal cancer.* Frontline Gastroenterology, 2014. **5**(1): p. 26-30.
16. Linnekamp, J.F., et al., *Colorectal cancer heterogeneity and targeted therapy: a case for molecular disease subtypes.* Cancer Res, 2015. **75**(2): p. 245-9.
17. De Rosa, M., et al., *Genetics, diagnosis and management of colorectal cancer (Review).* Oncol Rep, 2015. **34**(3): p. 1087-96.
18. Patel, S.G. and D.J. Ahnen, *Familial colon cancer syndromes: an update of a rapidly evolving field.* Curr Gastroenterol Rep, 2012. **14**(5): p. 428-38.
19. Roncucci, L. and F. Mariani, *Prevention of colorectal cancer: How many tools do we have in our basket?* Eur J Intern Med, 2015. **26**(10): p. 752-6.
20. Win, A.K., et al., *Risks of primary extracolonic cancers following colorectal cancer in lynch syndrome.* J Natl Cancer Inst, 2012. **104**(18): p. 1363-72.
21. Jasperson, K.W., et al., *Hereditary and familial colon cancer.* Gastroenterology, 2010. **138**(6): p. 2044-58.

22. Valle, L., *Genetic predisposition to colorectal cancer: where we stand and future perspectives.* World J Gastroenterol, 2014. **20**(29): p. 9828-9849.
23. Lutgens, M.W., et al., *Declining risk of colorectal cancer in inflammatory bowel disease: an updated meta-analysis of population-based cohort studies.* Inflammatory bowel diseases, 2013. **19**(4): p. 789-799.
24. Campbell, P.T., et al., *Associations of recreational physical activity and leisure time spent sitting with colorectal cancer survival.* J Clin Oncol, 2013. **31**(7): p. 876-85.
25. Campbell, P.T., et al., *Impact of body mass index on survival after colorectal cancer diagnosis: the Cancer Prevention Study-II Nutrition Cohort.* J Clin Oncol, 2012. **30**(1): p. 42-52.
26. Botteri, E., et al., *Cigarette smoking and adenomatous polyps: a meta-analysis.* Gastroenterology, 2008. **134**(2): p. 388-95.
27. Poschl, G. and H.K. Seitz, *Alcohol and cancer.* Alcohol Alcohol, 2004. **39**(3): p. 155-65.
28. Brennan, C.A. and W.S. Garrett, *Gut Microbiota, Inflammation, and Colorectal Cancer.* Annu Rev Microbiol, 2016. **70**: p. 395-411.
29. Aune, D., et al., *Dietary fibre, whole grains, and risk of colorectal cancer: systematic review and dose-response meta-analysis of prospective studies.* BMJ, 2011. **343**.
30. Zhao, Z., et al., *Red and processed meat consumption and colorectal cancer risk: a systematic review and meta-analysis.* Oncotarget, 2017. **8**(47): p. 83306-83314.
31. Klaver, Y.L., et al., *Peritoneal carcinomatosis of colorectal origin: Incidence, prognosis and treatment options.* World J Gastroenterol, 2012. **18**(39): p. 5489-94.
32. Zisis, C., et al., *The management of the advanced colorectal cancer: management of the pulmonary metastases.* J Thorac Dis, 2013. **5 Suppl 4**: p. S383-8.
33. Baek, S.-J., et al., *The Characteristics of Bone Metastasis in Patients with Colorectal Cancer: A Long-Term Report from a Single Institution.* World journal of surgery, 2016. **40**(4): p. 982-986.
34. Santini, D., et al., *Natural history of bone metastasis in colorectal cancer: final results of a large Italian bone metastases study.* Annals of oncology, 2012: p. mdr572.
35. Tan, W.-S., K.-S. Ho, and K.-W. Eu, *Brain metastases in colorectal cancers.* World journal of surgery, 2009. **33**(4): p. 817-821.
36. Damiens, K., et al., *Clinical features and course of brain metastases in colorectal cancer: an experience from a single institution.* Current Oncology, 2012. **19**(5): p. 254-258.
37. Weledji, E.P., *Centralization of Liver Cancer Surgery and Impact on Multidisciplinary Teams Working on Stage IV Colorectal Cancer.* Oncol Rev, 2017. **11**(2): p. 331.
38. Valderrama-Treviño, A.I., et al., *Hepatic Metastasis from Colorectal Cancer.* Euroasian J Hepatogastroenterol, 2017. **7**(2): p. 166-175.
39. Lee, J.H. and S.W. Lee, *The Roles of Carcinoembryonic Antigen in Liver Metastasis and Therapeutic Approaches.* Gastroenterol Res Pract., 2017. **2017**: p. 7521987.

40. Kroemer, G., et al., *Colorectal cancer: the first neoplasia found to be under immunosurveillance and the last one to respond to immunotherapy?* Oncoimmunology, 2015. **4**(7): p. e1058597.
41. Ocaña, M.C., et al., *Metabolism within the tumor microenvironment and its implication on cancer progression: An ongoing therapeutic target.* Medicinal Research Reviews, 2018.
42. Valkenburg, K.C., A.E. de Groot, and K.J. Pienta, *Targeting the tumour stroma to improve cancer therapy.* Nature reviews Clinical oncology, 2018: p. 1.
43. Gascard, P. and T.D. Tlsty, *Carcinoma-associated fibroblasts: orchestrating the composition of malignancy.* Genes & Development, 2016. **30**(9): p. 1002-1019.
44. Bussard, K.M., et al., *Tumor-associated stromal cells as key contributors to the tumor microenvironment.* Breast Cancer Research : BCR, 2016. **18**: p. 84.
45. Ribatti, D., *Inflammation and Angiogenesis*. 2017: Springer, Cham.
46. Colangelo, T., et al., *Friend or foe? The tumour microenvironment dilemma in colorectal cancer.* Biochim Biophys Acta, 2017. **1867**(1): p. 1-18.
47. Peddareddigari, V.G., D. Wang, and R.N. Dubois, *The tumor microenvironment in colorectal carcinogenesis.* Cancer Microenviron, 2010. **3**(1): p. 149-66.
48. Church, S.E. and J. Galon, *Regulation of CTL Infiltration Within the Tumor Microenvironment.* Adv Exp Med Biol, 2017. **1036**: p. 33-49.
49. Weigelin, B., M. Krause, and P. Friedl, *Cytotoxic T lymphocyte migration and effector function in the tumor microenvironment.* Immunology Letters, 2011. **138**(1): p. 19-21.
50. Dobrzanski, M.J., *Expanding Roles for CD4 T Cells and Their Subpopulations in Tumor Immunity and Therapy*. Frontiers in Oncology, 2013. **3**: p. 63.
51. Kiraz, Y., Y. Baran, and A. Nalbant, *T cells in tumor microenvironment.* Tumor Biology, 2016. **37**(1): p. 39-45.
52. Sarvaria, A., J.A. Madrigal, and A. Saudemont, *B cell regulation in cancer and anti-tumor immunity.* Cellular and Molecular Immunology, 2017. **14**(8): p. 662-674.
53. Tsou, P., et al., *The Emerging Role of B Cells in Tumor Immunity.* Cancer Res, 2016. **76**(19): p. 5597-5601.
54. Böttcher, J.P., et al., *NK Cells Stimulate Recruitment of cDC1 into the Tumor Microenvironment Promoting Cancer Immune Control.* Cell, 2018. **172**(5): p. 1022-1037.e14.
55. Dyck, L. and L. Lynch, *New Job for NK Cells: Architects of the Tumor Microenvironment.* Immunity, 2018. **48**(1): p. 9-11.
56. Kim, J. and J.S. Bae, *Tumor-Associated Macrophages and Neutrophils in Tumor Microenvironment.* Mediators Inflamm, 2016. **2016**: p. 6058147.
57. Xiang, W., et al., *Monoacylglycerol lipase regulates cannabinoid receptor 2-dependent macrophage activation and cancer progression.* Nature communications, 2018. **9**(1): p. 2574.
58. Kumar, V., et al., *The Nature of Myeloid-Derived Suppressor Cells in the Tumor Microenvironment.* Trends in Immunology, 2016. **37**(3): p. 208-220.
59. Aponte-López, A., et al., *Mast cell, the neglected member of the tumor microenvironment: role in breast cancer.* Journal of immunology research, 2018. **2018**.
60. Fridlender, Z.G. and S.M. Albelda, *Tumor-associated neutrophils: friend or foe?* Carcinogenesis, 2012. **33**(5): p. 949-955.
61. Reichman, H., D. Karo-Atar, and A. Munitz, *Emerging Roles for Eosinophils in the Tumor Microenvironment.* Trends Cancer, 2016. **2**(11): p. 664-675.

62. Legrand, F., et al., *Human eosinophils exert TNF-alpha and granzyme A-mediated tumoricidal activity toward colon carcinoma cells.* J Immunol, 2010. **185**(12): p. 7443-51.
63. De Monte, L., et al., *Basophil Recruitment into Tumor-Draining Lymph Nodes Correlates with Th2 Inflammation and Reduced Survival in Pancreatic Cancer Patients.* Cancer Res, 2016. **76**(7): p. 1792-803.
64. Matsushima, T., et al., *Prevalence and clinical characteristics of myelodysplastic syndrome with bone marrow eosinophilia or basophilia.* Blood, 2003. **101**(9): p. 3386-3390.
65. Hsu, Y.-L., et al., *Interaction between Tumor-Associated Dendritic Cells and Colon Cancer Cells Contributes to Tumor Progression via CXCL1.* International journal of molecular sciences, 2018. **19**(8): p. 2427.
66. Palucka, K. and J. Banchereau, *Cancer immunotherapy via dendritic cells.* Nat Rev Cancer, 2012. **12**(4): p. 265-77.
67. Maishi, N. and K. Hida, *Tumor endothelial cells accelerate tumor metastasis.* Cancer Science, 2017. **108**(10): p. 1921-1926.
68. Chouaib, S., et al., *Endothelial cells as key determinants of the tumor microenvironment: interaction with tumor cells, extracellular matrix and immune killer cells.* Critical Reviews™ in Immunology, 2010. **30**(6).
69. Ribeiro, A.L. and O.K. Okamoto, *Combined Effects of Pericytes in the Tumor Microenvironment.* Stem Cells International, 2015. **2015**: p. 868475.
70. Meng, M.B., et al., *Pericytes: a double-edged sword in cancer therapy.* Future Oncol, 2015. **11**(1): p. 169-79.
71. Tao, L., et al., *Cancer associated fibroblasts: An essential role in the tumor microenvironment.* Oncology Letters, 2017. **14**(3): p. 2611-2620.
72. Xing, F., J. Saidou, and K. Watabe, *Cancer associated fibroblasts (CAFs) in tumor microenvironment.* Frontiers in bioscience : a journal and virtual library, 2010. **15**: p. 166-179.
73. Jahangiri, B., et al., *Cancer-associated fibroblasts enhance cell proliferation and metastasis of colorectal cancer SW480 cells by provoking long noncoding RNA UCA1.* J Cell Commun Signal, 2019. **13**(1): p. 53-64.
74. Lu, P., V.M. Weaver, and Z. Werb, *The extracellular matrix: a dynamic niche in cancer progression.* J Cell Biol, 2012. **196**(4): p. 395-406.
75. Giussani, M., et al., *Tumor-extracellular matrix interactions: Identification of tools associated with breast cancer progression.* Seminars in Cancer Biology, 2015. **35**: p. 3-10.
76. Crotti, S., et al., *Extracellular Matrix and Colorectal Cancer: How Surrounding Microenvironment Affects Cancer Cell Behavior?* J Cell Physiol, 2017. **232**(5): p. 967-975.
77. Wang, D. and R.N. DuBois, *Pro-inflammatory prostaglandins and progression of colorectal cancer.* Cancer Lett, 2008. **267**(2): p. 197-203.
78. Patel, M., et al., *The relationship between right-sided tumour location, tumour microenvironment, systemic inflammation, adjuvant therapy and survival in patients undergoing surgery for colon and rectal cancer.* British journal of cancer, 2018. **118**(5): p. 705.
79. Slattery, M.L., et al., *The NF-κB signalling pathway in colorectal cancer: associations between dysregulated gene and miRNA expression.* Journal of cancer research and clinical oncology, 2018. **144**(2): p. 269-283.
80. Maywald, R.L., et al., *IL-33 activates tumor stroma to promote intestinal polyposis.* Proc Natl Acad Sci U S A, 2015. **112**(19): p. E2487-96.

81. Quante, M., et al., *The Gastrointestinal Tumor Microenvironment.* Gastroenterology, 2013. **145**(1): p. 63-78.
82. Heidelberger, C., et al., *Fluorinated pyrimidines, a new class of tumour-inhibitory compounds.* Nature, 1957. **179**(4561): p. 663-666.
83. Lind, M.J., *Principles of systemic anticancer therapy.* Medicine, 2016. **44**(1): p. 20-24.
84. Palaghia, M., et al., *Metastatic Colorectal Cancer: Review of Diagnosis and Treatment Options.* Jurnalul de Chirurgie, 2015. **1**(10): p. 269-276.
85. Park, J.-G., et al., *Enhancement of fluorinated pyrimidine-induced cytotoxicity by leucovorin in human colorectal carcinoma cell lines.* Journal of the National Cancer Institute, 1988. **80**(19): p. 1560-1564.
86. Saltz, L.B., et al., *Irinotecan plus fluorouracil and leucovorin for metastatic colorectal cancer.* New England Journal of Medicine, 2000. **343**(13): p. 905-914.
87. Douillard, J., et al., *Irinotecan combined with fluorouracil compared with fluorouracil alone as first-line treatment for metastatic colorectal cancer: a multicentre randomised trial.* The Lancet, 2000. **355**(9209): p. 1041-1047.
88. Fuchs, C.S., et al., *Randomized, controlled trial of irinotecan plus infusional, bolus, or oral fluoropyrimidines in first-line treatment of metastatic colorectal cancer: results from the BICC-C Study.* Journal of Clinical Oncology, 2007. **25**(30): p. 4779-4786.
89. Köhne, C.-H., et al., *Phase III study of weekly high-dose infusional fluorouracil plus folinic acid with or without irinotecan in patients with metastatic colorectal cancer: European Organisation for Research and Treatment of Cancer Gastrointestinal Group Study 40986.* Journal of Clinical Oncology, 2005. **23**(22): p. 4856-4865.
90. Rougier, P., et al., *Randomised trial of irinotecan versus fluorouracil by continuous infusion after fluorouracil failure in patients with metastatic colorectal cancer.* The Lancet, 1998. **352**(9138): p. 1407-1412.
91. Seymour, M.T., et al., *Different strategies of sequential and combination chemotherapy for patients with poor prognosis advanced colorectal cancer (MRC FOCUS): a randomised controlled trial.* The Lancet, 2007. **370**(9582): p. 143-152.
92. Kidani, Y., N. Masahide, and T. TASHIRO, *Antitumor activity of platinum (II) complexes of 1, 2-diaminocyclohexane isomers.* GANN Japanese Journal of Cancer Research, 1980. **71**(5): p. 637-643.
93. Des Guetz, G., et al., *Microsatellite instability and sensitivitiy to FOLFOX treatment in metastatic colorectal cancer.* Anticancer research, 2007. **27**(4C): p. 2715-2719.
94. Goldberg, R.M., et al., *A randomized controlled trial of fluorouracil plus leucovorin, irinotecan, and oxaliplatin combinations in patients with previously untreated metastatic colorectal cancer.* Journal of Clinical Oncology, 2004. **22**(1): p. 23-30.
95. Goldberg, R.M., et al., *Randomized controlled trial of reduced-dose bolus fluorouracil plus leucovorin and irinotecan or infused fluorouracil plus leucovorin and oxaliplatin in patients with previously untreated metastatic colorectal cancer: a North American Intergroup Trial.* Journal of Clinical Oncology, 2006. **24**(21): p. 3347-3353.
96. Nordlinger, B., et al., *Perioperative chemotherapy with FOLFOX4 and surgery versus surgery alone for resectable liver metastases from colorectal cancer*

(EORTC Intergroup trial 40983): a randomised controlled trial. The Lancet, 2008. **371**(9617): p. 1007-1016.

97. Nordlinger, B., et al., *Perioperative FOLFOX4 chemotherapy and surgery versus surgery alone for resectable liver metastases from colorectal cancer (EORTC 40983): long-term results of a randomised, controlled, phase 3 trial.* The lancet oncology, 2013. **14**(12): p. 1208-1215.
98. Colucci, G., et al., *Phase III randomized trial of FOLFIRI versus FOLFOX4 in the treatment of advanced colorectal cancer: a multicenter study of the Gruppo Oncologico Dell'Italia Meridionale.* Journal of Clinical Oncology, 2005. **23**(22): p. 4866-4875.
99. Tournigand, C., et al., *FOLFIRI followed by FOLFOX6 or the reverse sequence in advanced colorectal cancer: a randomized GERCOR study.* Journal of Clinical Oncology, 2004. **22**(2): p. 229-237.
100. André, T., et al., *Oxaliplatin, fluorouracil, and leucovorin as adjuvant treatment for colon cancer.* New England Journal of Medicine, 2004. **350**(23): p. 2343-2351.
101. Van Cutsem, E., et al., *Randomized phase III trial comparing biweekly infusional fluorouracil/leucovorin alone or with irinotecan in the adjuvant treatment of stage III colon cancer: PETACC-3.* Journal of Clinical Oncology, 2009. **27**(19): p. 3117-3125.
102. Maindrault-Goebel, F., et al., *Oxaliplatin added to the simplified bimonthly leucovorin and 5-fluorouracil regimen as second-line therapy for metastatic colorectal cancer (FOLFOX6).* European Journal of Cancer, 1999. **35**(9): p. 1338-1342.
103. Andre, T., et al., *CPT-11 (irinotecan) addition to bimonthly, high-dose leucovorin and bolus and continuous-infusion 5-fluorouracil (FOLFIRI) for pretreated metastatic colorectal cancer.* European Journal of Cancer, 1999. **35**(9): p. 1343-1347.
104. Cassidy, J., et al., *Randomized phase III study of capecitabine plus oxaliplatin compared with fluorouracil/folinic acid plus oxaliplatin as first-line therapy for metastatic colorectal cancer.* Journal of Clinical Oncology, 2008. **26**(12): p. 2006-2012.
105. Cassidy, J., et al., *XELOX (capecitabine plus oxaliplatin): active first-line therapy for patients with metastatic colorectal cancer.* Journal of Clinical Oncology, 2004. **22**(11): p. 2084-2091.
106. Sargent, D., et al., *Evidence for cure by adjuvant therapy in colon cancer: observations based on individual patient data from 20,898 patients on 18 randomized trials.* Journal of Clinical Oncology, 2009. **27**(6): p. 872-877.
107. Machover, D., et al., *Treatment of advanced colorectal and gastric adenocarcinomas with 5-FU combined with high-dose folinic acid: a pilot study.* Cancer treatment reports, 1982. **66**(10): p. 1803.
108. Madajewicz, S., et al., *Phase I-II trial of high-dose calcium leucovorin and 5-fluorouracil in advanced colorectal cancer.* Cancer research, 1984. **44**(10): p. 4667-4669.
109. Petrelli, N., et al., *A prospective randomized trial of 5-fluorouracil versus 5-fluorouracil and high-dose leucovorin versus 5-fluorouracil and methotrexate in previously untreated patients with advanced colorectal carcinoma.* Journal of Clinical Oncology, 1987. **5**(10): p. 1559-1565.

110. Petrelli, N., et al., *The modulation of fluorouracil with leucovorin in metastatic colorectal carcinoma: a prospective randomized phase III trial. Gastrointestinal Tumor Study Group.* Journal of Clinical Oncology, 1989. **7**(10): p. 1419-1426.
111. Thirion, P., et al., *Modulation of fluorouracil by leucovorin in patients with advanced colorectal cancer: an updated meta-analysis*. 2004.
112. O'Connell, M.J., *A phase III trial of 5- fluorouracil and leucovorin in the treatment of advanced colorectal cancer. A mayo clinic/north central cancer treatment group study.* Cancer, 1989. **63**(S6): p. 1026-1030.
113. de Gramont, A.d., et al., *Leucovorin and fluorouracil with or without oxaliplatin as first-line treatment in advanced colorectal cancer.* Journal of Clinical Oncology, 2000. **18**(16): p. 2938-2947.
114. Rothenberg, M.L., et al., *Superiority of oxaliplatin and fluorouracil-leucovorin compared with either therapy alone in patients with progressive colorectal cancer after irinotecan and fluorouracil-leucovorin: interim results of a phase III trial.* Journal of Clinical Oncology, 2003. **21**(11): p. 2059-2069.
115. Giacchetti, S., et al., *Phase III multicenter randomized trial of oxaliplatin added to chronomodulated fluorouracil–leucovorin as first-line treatment of metastatic colorectal cancer.* Journal of Clinical Oncology, 2000. **18**(1): p. 136-136.
116. Grothey, A., et al., *Phase III study of bolus 5-fluorouracil (5-FU)/folinic acid (FA)(Mayo) vs weekly high-dose 24h 5-FU infusion/FA+ oxaliplatin (OXA)(FUFOX) in advanced colorectal cancer (ACRC).* Onkologie, 2002. **25**: p. 13.
117. Falcone, A., et al., *Phase III trial of infusional fluorouracil, leucovorin, oxaliplatin, and irinotecan (FOLFOXIRI) compared with infusional fluorouracil, leucovorin, and irinotecan (FOLFIRI) as first-line treatment for metastatic colorectal cancer: the Gruppo Oncologico Nord Ovest.* Journal of Clinical Oncology, 2007. **25**(13): p. 1670-1676.
118. Souglakos, J., et al., *FOLFOXIRI (folinic acid, 5-fluorouracil, oxaliplatin and irinotecan) vs FOLFIRI (folinic acid, 5-fluorouracil and irinotecan) as first-line treatment in metastatic colorectal cancer (MCC): a multicentre randomised phase III trial from the Hellenic Oncology Research Group (HORG).* British journal of cancer, 2006. **94**(6): p. 798-805.
119. Van Cutsem, E., et al., *Oral capecitabine vs intravenous 5-fluorouracil and leucovorin: integrated efficacy data and novel analyses from two large, randomised, phase III trials.* British journal of cancer, 2004. **90**(6): p. 1190-1197.
120. Benson, A.B., et al., *NCCN guidelines insights: colon cancer, version 2.2018.* Journal of the National Comprehensive Cancer Network, 2018. **16**(4): p. 359-369.
121. Engstrom, P.F., et al., *NCCN Clinical Practice Guidelines in Oncology: colon cancer.* J Natl Compr Canc Netw, 2009. **7**(8): p. 778-831.
122. Labianca, R., et al., *Primary colon cancer: ESMO Clinical Practice Guidelines for diagnosis, adjuvant treatment and follow-up.* Annals of Oncology, 2010. **21**(suppl 5): p. v70-v77.
123. Jonker, D., K. Spithoff, and J. Maroun, *Adjuvant systemic chemotherapy for Stage II and III colon cancer after complete resection: an updated practice guideline.* Clinical Oncology, 2011. **23**(5): p. 314-322.
124. Benson, A.B., et al., *Metastatic colon cancer, version 3.2013 featured updates to the NCCN guidelines.* Journal of the National Comprehensive Cancer Network, 2013. **11**(2): p. 141-152.

125. Kuipers, E.J., et al., *Colorectal cancer.* Nat Rev Dis Primers, 2015. **1**: p. 15065.
126. Gill, S., A.W. Blackstock, and R.M. Goldberg, *Colorectal cancer.* Mayo Clin Proc, 2007. **82**(1): p. 114-29.
127. Maroun, J., et al., *A Phase I Study of Irinotecan, Capecitabine (Xeloda), and Oxaliplatin in Patients With Advanced Colorectal Cancer.* Clin Colorectal Cancer, 2018. **17**(2): p. e257-e268.
128. Shabaruddin, F.H., et al., *A systematic review of utility values for chemotherapy-related adverse events.* Pharmacoeconomics, 2013. **31**(4): p. 277-288.
129. Corrie, P.G., *Cytotoxic chemotherapy: clinical aspects.* Medicine, 2004. **32**(3): p. 25-29.
130. O'Donnell, J.S., M.W.L. Teng, and M.J. Smyth, *Cancer immunoediting and resistance to T cell-based immunotherapy.* Nat Rev Clin Oncol, 2019. **16**(3): p. 151-167.
131. Galluzzi, L., et al., *Classification of current anticancer immunotherapies.* Oncotarget, 2014. **5**(24): p. 12472.
132. Amin, M. and A.C. Lockhart, *The potential role of immunotherapy to treat colorectal cancer.* Expert opinion on investigational drugs, 2015. **24**(3): p. 329-344.
133. Lapeyre-Prost, A., et al., *Immune therapy in colorectal cancer.* Future Medicine, 2017.
134. Markman, J.L. and S.L. Shiao, *Impact of the immune system and immunotherapy in colorectal cancer.* Journal of gastrointestinal oncology, 2015. **6**(2): p. 208.
135. Sanchez-Castañón, M., et al., *Immunotherapy in colorectal cancer: What have we learned so far?* Clinica Chimica Acta, 2016. **460**: p. 78-87.
136. Singh, P.P., et al., *Immune checkpoints and immunotherapy for colorectal cancer.* Gastroenterology report, 2015. **3**(4): p. 289-297.
137. Sharma, P. and J.P. Allison, *Immune checkpoint targeting in cancer therapy: toward combination strategies with curative potential.* Cell, 2015. **161**(2): p. 205-214.
138. Oh, D.Y., A.P. Venook, and L. Fong, *On the verge: immunotherapy for colorectal carcinoma.* Journal of the National Comprehensive Cancer Network, 2015. **13**(8): p. 970-978.
139. Ohtake, J., et al., *Personalized immunotherapy in colorectal cancer.* Expert Review of Precision Medicine and Drug Development, 2016. **1**(3): p. 267-277.
140. Jacobs, J., et al., *Immune checkpoint modulation in colorectal cancer: What's new and what to expect.* Journal of immunology research, 2015. **2015**.
141. Xu, B., et al., *Circulating and tumor-infiltrating Tim-3 in patients with colorectal cancer.* Oncotarget, 2015. **6**(24): p. 20592.
142. Linch, S.N., M.J. McNamara, and W.L. Redmond, *OX40 agonists and combination immunotherapy: putting the pedal to the metal.* Frontiers in oncology, 2015. **5**.
143. Petty, J.K., et al., *Survival in human colorectal cancer correlates with expression of the T-cell costimulatory molecule OX-40 (CD134).* The American journal of surgery, 2002. **183**(5): p. 512-518.
144. West, N.R., et al., *Emerging cytokine networks in colorectal cancer.* Nature Reviews. Immunology, 2015. **15**(10): p. 615.
145. Dinarello, C.A., A. Simon, and J.W. Van Der Meer, *Treating inflammation by blocking interleukin-1 in a broad spectrum of diseases.* Nature reviews Drug discovery, 2012. **11**(8): p. 633-652.

146. Bever, K.M. and D.T. Le, *An Expanding Role for Immunotherapy in Colorectal Cancer*. Journal of the National Comprehensive Cancer Network, 2017. **15**(3): p. 401-410.
147. Parkhurst, M.R., et al., *T cells targeting carcinoembryonic antigen can mediate regression of metastatic colorectal cancer but induce severe transient colitis.* Molecular Therapy, 2011. **19**(3): p. 620-626.
148. Morgan, R.A., et al., *Case report of a serious adverse event following the administration of T cells transduced with a chimeric antigen receptor recognizing ERBB2.* Molecular Therapy, 2010. **18**(4): p. 843-851.
149. Chung, K.Y., et al., *Phase II study of the anti-cytotoxic T-lymphocyte–associated antigen 4 monoclonal antibody, tremelimumab, in patients with refractory metastatic colorectal cancer.* Journal of clinical oncology, 2010. **28**(21): p. 3485-3490.
150. Overman, M.J., et al., *Nivolumab ± ipilimumab in treatment (tx) of patients (pts) with metastatic colorectal cancer (mCRC) with and without high microsatellite instability (MSI-H): CheckMate-142 interim results.* Journal of Clinical Oncology, 2016. **34**(15_suppl): p. 3501-3501.
151. NCT02060188, C.g.I., *An Investigational Immuno-therapy Study of Nivolumab, and Nivolumab in Combination With Other Anti-cancer Drugs, in Colon Cancer That Has Come Back or Has Spread (CheckMate142).*
152. NCT03642067, C.g.I., *Study of Nivolumab and Relatlimab in Patients With Microsatellite Stable (MSS) Advanced Colorectal Cancer.*
153. NCT03800602, C.g.I., *Nivolumab and Metformin in Patients With Treatment Refractory MSS Colorectal Cancer.*
154. NCT02460198, C.g.i., *Study of Pembrolizumab (MK-3475) as Monotherapy in Participants With Previously-Treated Locally Advanced Unresectable or Metastatic Colorectal Cancer (MK-3475-164/KEYNOTE-164).*
155. NCT03657641, C.g.I., *Regorafenib and Pembrolizumab in Treating Participants With Advanced or Metastatic Colorectal Cancer.*
156. NCT03475004, C.g.I., *Study of Pembrolizumab, Binimetinib, and Bevacizumab in Patients With Refractory Colorectal Cancer.*
157. NCT03658772, C.g.I., *Grapiprant and Pembrolizumab in Patients With Advanced or Progressive MSS Colorectal Cancer.*
158. Weixler, B., et al., *OX40 expression enhances the prognostic significance of CD8 positive lymphocyte infiltration in colorectal cancer.* Oncotarget, 2015. **6**(35): p. 37588.
159. Brown, E.R., et al., *A clinical study assessing the tolerability and biological effects of infliximab, a TNF-α inhibitor, in patients with advanced cancer.* Annals of Oncology, 2008. **19**(7): p. 1340-1346.
160. NCT01869166, C.g.i., *Treatment of Chemotherapy Refractory EGFR(Epidermal Growth Factor Receptor) Positive Advanced Solid Tumors (CART-EGFR) (CART-EGFR).*
161. NCT03638206, C.g.I., *Autologous CAR-T/TCR-T Cell Immunotherapy for Malignancies.*
162. NCT03542799, C.g.I., *EGFR-IL12-CART Cells for Patients With Metastatic Colorectal Cancer (EGFRCART).*
163. NCT03152435, C.g.I., *EGFR CART Cells for Patients With Metastatic Colorectal Cancer.*
164. NCT00720785, C.g.I., *Natural Killer Cells and Bortezomib to Treat Cancer.*

165. Koido, S., et al., *Immunotherapy for colorectal cancer.* World Journal of Gastroenterology: WJG, 2013. **19**(46): p. 8531.
166. Bilusic, M., et al., *Phase I trial of a recombinant yeast-CEA vaccine (GI-6207) in adults with metastatic CEA-expressing carcinoma.* Cancer Immunology, Immunotherapy, 2014. **63**(3): p. 225-234.
167. Morse, M.A., et al., *A randomized phase II study of immunization with dendritic cells modified with poxvectors encoding CEA and MUC1 compared with the same poxvectors plus GM-CSF for resected metastatic colorectal cancer.* Annals of surgery, 2013. **258**(6).
168. Speetjens, F.M., et al., *Induction of p53-specific immunity by a p53 synthetic long peptide vaccine in patients treated for metastatic colorectal cancer.* Clinical Cancer Research, 2009. **15**(3): p. 1086-1095.
169. Furugaki, K., et al., *Intraperitoneal administration of a tumor-associated antigen SART3, CD40L, and GM-CSF gene-loaded polyplex micelle elicits a vaccine effect in mouse tumor models.* PloS one, 2014. **9**(7): p. e101854.
170. Pardieck, I.N., et al., *Novel avenues in immunotherapies for colorectal cancer.* Expert review of gastroenterology & hepatology, 2016. **10**(4): p. 465-480.
171. Hanna, J., Michael G, *Immunotherapy with autologous tumor cell vaccines for treatment of occult disease in early stage colon cancer.* Human vaccines & immunotherapeutics, 2012. **8**(8): p. 1156-1160.
172. Senzer, N., et al., *Phase I trial of "bi-shRNAi furin/GMCSF DNA/autologous tumor cell" vaccine (FANG) in advanced cancer.* Molecular Therapy, 2012. **20**(3): p. 679-686.
173. Ullenhag, G.J., et al., *Durable Carcinoembryonic Antigen (CEA)-Specific Humoral and Cellular Immune Responses in Colorectal Carcinoma Patients Vaccinated with Recombinant CEA and Granulocyte/Macrophage Colony-Stimulating Factor.* Clinical Cancer Research, 2004. **10**(10): p. 3273-3281.
174. Tsuruma, T., et al., *Phase I clinical study of anti-apoptosis protein, survivin-derived peptide vaccine therapy for patients with advanced or recurrent colorectal cancer.* Journal of Translational Medicine, 2004. **2**(1): p. 19.
175. Morse, M.A., et al., *Phase I study of alphaviral vector (AVX701) in colorectal cancer patients: comparison of immune responses in stage III and stage IV patients.* Journal for immunotherapy of cancer, 2015. **3**(2): p. P444.
176. Park, S.H., et al., *Phase 1b trial of biweekly intravenous Pexa-Vec (JX-594), an oncolytic and immunotherapeutic vaccinia virus in colorectal cancer.* Molecular Therapy, 2015. **23**(9): p. 1532-1540.
177. Barth, R.J., et al., *A randomized trial of ex vivo CD40L activation of a DC vaccine in colorectal cancer patients: tumor-specific immune responses are associated with improved survival.* Clinical Cancer Research, 2010.
178. Liu, K.-J., et al., *Generation of carcinoembryonic antigen (CEA)-specific T-cell responses in HLA-A* 0201 and HLA-A* 2402 late-stage colorectal cancer patients after vaccination with dendritic cells loaded with CEA peptides.* Clinical cancer research, 2004. **10**(8): p. 2645-2651.
179. Al-Lazikani, B., U. Banerji, and P. Workman, *Combinatorial drug therapy for cancer in the post-genomic era.* Nature biotechnology, 2012. **30**(7): p. 679-692.
180. Slastnikova, T.A., et al., *Targeted Intracellular Delivery of Antibodies: The State of the Art.* Front Pharmacol, 2018. **9**: p. 1208.
181. Goldstein, D.A., et al., *Metastatic colorectal cancer: a systematic review of the value of current therapies.* Clinical colorectal cancer, 2016. **15**(1): p. 1-6.

182. Van Cutsem, E., et al., *Metastatic colorectal cancer: ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up.* Annals of oncology, 2014. **25**(suppl_3): p. iii1-iii9.
183. Welch, S., et al., *Bevacizumab combined with chemotherapy for patients with advanced colorectal cancer: a systematic review.* Annals of oncology, 2009. **21**(6): p. 1152-1162.
184. Bennouna, J., et al., *Continuation of bevacizumab after first progression in metastatic colorectal cancer (ML18147): a randomised phase 3 trial.* The lancet oncology, 2013. **14**(1): p. 29-37.
185. Fakih, M.G., *Metastatic colorectal cancer: current state and future directions.* Journal of clinical oncology, 2015. **33**(16): p. 1809-1824.
186. Cartwright, T.H., *Treatment decisions after diagnosis of metastatic colorectal cancer.* Clinical colorectal cancer, 2012. **11**(3): p. 155-166.
187. Hurwitz, H., et al., *Bevacizumab plus irinotecan, fluorouracil, and leucovorin for metastatic colorectal cancer.* New England journal of medicine, 2004. **350**(23): p. 2335-2342.
188. Hurwitz, H.I., et al., *Bevacizumab in combination with fluorouracil and leucovorin: an active regimen for first-line metastatic colorectal cancer.* Journal of Clinical Oncology, 2005. **23**(15): p. 3502-3508.
189. Kabbinavar, F., et al., *Phase II, randomized trial comparing bevacizumab plus fluorouracil (FU)/leucovorin (LV) with FU/LV alone in patients with metastatic colorectal cancer.* Journal of Clinical Oncology, 2003. **21**(1): p. 60-65.
190. Kabbinavar, F.F., et al., *Combined analysis of efficacy: the addition of bevacizumab to fluorouracil/leucovorin improves survival for patients with metastatic colorectal cancer.* Journal of clinical oncology, 2005. **23**(16): p. 3706-3712.
191. Kabbinavar, F., et al., *Bevacizumab improves the overall and progression-free survival of patients with metastatic colorectal cancer treated with 5-fluorouracil-based regimens irrespective of baseline risk.* Oncology, 2008. **75**(3-4): p. 215-223.
192. Schmiegel, W., et al., *Capecitabine/irinotecan or capecitabine/oxaliplatin in combination with bevacizumab is effective and safe as first-line therapy for metastatic colorectal cancer: a randomized phase II study of the AIO colorectal study group.* Annals of oncology, 2013. **24**(6): p. 1580-1587.
193. Stathopoulos, G., et al., *Treatment of colorectal cancer with and without bevacizumab: a phase III study.* Oncology, 2010. **78**(5-6): p. 376-381.
194. Saltz, L.B., et al., *Bevacizumab in combination with oxaliplatin-based chemotherapy as first-line therapy in metastatic colorectal cancer: a randomized phase III study.* Journal of clinical oncology, 2008. **26**(12): p. 2013-2019.
195. Cunningham, D., et al., *Bevacizumab plus capecitabine versus capecitabine alone in elderly patients with previously untreated metastatic colorectal cancer (AVEX): an open-label, randomised phase 3 trial.* The lancet oncology, 2013. **14**(11): p. 1077-1085.
196. Tebbutt, N.C., et al., *Capecitabine, bevacizumab, and mitomycin in first-line treatment of metastatic colorectal cancer: results of the Australasian Gastrointestinal Trials Group Randomized Phase III MAX Study.* Journal of clinical oncology, 2010. **28**(19): p. 3191-3198.

197. Loupakis, F., et al., *Initial therapy with FOLFOXIRI and bevacizumab for metastatic colorectal cancer.* New England Journal of Medicine, 2014. **371**(17): p. 1609-1618.
198. Giantonio, B.J., et al., *Bevacizumab in combination with oxaliplatin, fluorouracil, and leucovorin (FOLFOX4) for previously treated metastatic colorectal cancer: results from the Eastern Cooperative Oncology Group Study E3200.* Journal of Clinical Oncology, 2007. **25**(12): p. 1539-1544.
199. Van Cutsem, E., et al., *Addition of aflibercept to fluorouracil, leucovorin, and irinotecan improves survival in a phase III randomized trial in patients with metastatic colorectal cancer previously treated with an oxaliplatin-based regimen.* Journal of Clinical Oncology, 2012. **30**(28): p. 3499-3506.
200. Tabernero, J., et al., *Aflibercept versus placebo in combination with fluorouracil, leucovorin and irinotecan in the treatment of previously treated metastatic colorectal cancer: prespecified subgroup analyses from the VELOUR trial.* European journal of cancer, 2014. **50**(2): p. 320-331.
201. De Roock, W., et al., *Effects of KRAS, BRAF, NRAS, and PIK3CA mutations on the efficacy of cetuximab plus chemotherapy in chemotherapy-refractory metastatic colorectal cancer: a retrospective consortium analysis.* The lancet oncology, 2010. **11**(8): p. 753-762.
202. Douillard, J.-Y., et al., *Panitumumab–FOLFOX4 treatment and RAS mutations in colorectal cancer.* New England Journal of Medicine, 2013. **369**(11): p. 1023-1034.
203. Jonker, D.J., et al., *Cetuximab for the treatment of colorectal cancer.* New England Journal of Medicine, 2007. **357**(20): p. 2040-2048.
204. Amado, R.G., et al., *Wild-type KRAS is required for panitumumab efficacy in patients with metastatic colorectal cancer.* Journal of clinical oncology, 2008. **26**(10): p. 1626-1634.
205. Price, T.J., et al., *Panitumumab versus cetuximab in patients with chemotherapy-refractory wild-type KRAS exon 2 metastatic colorectal cancer (ASPECCT): a randomised, multicentre, open-label, non-inferiority phase 3 study.* The Lancet Oncology, 2014. **15**(6): p. 569-579.
206. Cunningham, D., et al., *Cetuximab monotherapy and cetuximab plus irinotecan in irinotecan-refractory metastatic colorectal cancer.* New England journal of medicine, 2004. **351**(4): p. 337-345.
207. Van Cutsem, E., et al., *Cetuximab and chemotherapy as initial treatment for metastatic colorectal cancer.* New England Journal of Medicine, 2009. **360**(14): p. 1408-1417.
208. Van Cutsem, E., et al., *Cetuximab plus irinotecan, fluorouracil, and leucovorin as first-line treatment for metastatic colorectal cancer: updated analysis of overall survival according to tumor KRAS and BRAF mutation status.* Journal of Clinical Oncology, 2011. **29**(15): p. 2011-2019.
209. Bokemeyer, C., et al., *Fluorouracil, leucovorin, and oxaliplatin with and without cetuximab in the first-line treatment of metastatic colorectal cancer.* Journal of clinical oncology, 2008. **27**(5): p. 663-671.
210. Bokemeyer, C., et al., *Efficacy according to biomarker status of cetuximab plus FOLFOX-4 as first-line treatment for metastatic colorectal cancer: the OPUS study.* Annals of Oncology, 2011. **22**(7): p. 1535-1546.
211. Bokemeyer, C., et al., *Addition of cetuximab to chemotherapy as first-line treatment for KRAS wild-type metastatic colorectal cancer: pooled analysis of*

the CRYSTAL and OPUS randomised clinical trials. European journal of cancer, 2012. **48**(10): p. 1466-1475.

212. Ye, L.-C., et al., *Randomized controlled trial of cetuximab plus chemotherapy for patients with KRAS wild-type unresectable colorectal liver-limited metastases.* Journal of Clinical Oncology, 2013. **31**(16): p. 1931-1938.
213. Tveit, K.M., et al., *Phase III trial of cetuximab with continuous or intermittent fluorouracil, leucovorin, and oxaliplatin (Nordic FLOX) versus FLOX alone in first-line treatment of metastatic colorectal cancer: the NORDIC-VII study.* Journal of clinical oncology, 2012. **30**(15): p. 1755-1762.
214. Maughan, T.S., et al., *Addition of cetuximab to oxaliplatin-based first-line combination chemotherapy for treatment of advanced colorectal cancer: results of the randomised phase 3 MRC COIN trial.* The Lancet, 2011. **377**(9783): p. 2103-2114.
215. Alberts, S.R., et al., *Effect of oxaliplatin, fluorouracil, and leucovorin with or without cetuximab on survival among patients with resected stage III colon cancer: a randomized trial.* Jama, 2012. **307**(13): p. 1383-1393.
216. Douillard, J.-Y., et al., *Randomized, phase III trial of panitumumab with infusional fluorouracil, leucovorin, and oxaliplatin (FOLFOX4) versus FOLFOX4 alone as first-line treatment in patients with previously untreated metastatic colorectal cancer: the PRIME study.* Journal of clinical oncology, 2010. **28**(31): p. 4697-4705.
217. Peeters, M., et al., *Randomized phase III study of panitumumab with fluorouracil, leucovorin, and irinotecan (FOLFIRI) compared with FOLFIRI alone as second-line treatment in patients with metastatic colorectal cancer.* Journal of clinical oncology, 2010. **28**(31): p. 4706-4713.
218. Sobrero, A.F., et al., *EPIC: phase III trial of cetuximab plus irinotecan after fluoropyrimidine and oxaliplatin failure in patients with metastatic colorectal cancer.* Journal of clinical oncology, 2008. **26**(14): p. 2311-2319.
219. Seymour, M.T., et al., *Panitumumab and irinotecan versus irinotecan alone for patients with KRAS wild-type, fluorouracil-resistant advanced colorectal cancer (PICCOLO): a prospectively stratified randomised trial.* The lancet oncology, 2013. **14**(8): p. 749-759.
220. Grothey, A., et al., *Regorafenib monotherapy for previously treated metastatic colorectal cancer (CORRECT): an international, multicentre, randomised, placebo-controlled, phase 3 trial.* The Lancet, 2013. **381**(9863): p. 303-312.
221. Kirstein, M.M., et al., *Targeted therapies in metastatic colorectal cancer: a systematic review and assessment of currently available data.* The oncologist, 2014. **19**(11): p. 1156-1168.
222. Sharma, A., et al., *Vemurafenib: targeted inhibition of mutated BRAF for treatment of advanced melanoma and its potential in other malignancies.* Drugs, 2012. **72**(17): p. 2207-22.
223. Prahallad, A., et al., *Unresponsiveness of colon cancer to BRAF(V600E) inhibition through feedback activation of EGFR.* Nature, 2012. **483**(7387): p. 100-3.
224. Corcoran, R.B., et al., *EGFR-mediated re-activation of MAPK signaling contributes to insensitivity of BRAF mutant colorectal cancers to RAF inhibition with vemurafenib.* Cancer Discov, 2012. **2**(3): p. 227-35.
225. Pietrantonio, F., et al., *Predictive role of BRAF mutations in patients with advanced colorectal cancer receiving cetuximab and panitumumab: a meta-analysis.* Eur J Cancer, 2015. **51**(5): p. 587-94.

226. Hong, D.S., et al., *Phase IB study of vemurafenib in combination with irinotecan and cetuximab in patients with metastatic colorectal cancer with BRAFV600E mutation.* Cancer discovery, 2016. **6**(12): p. 1352-1365.
227. Kopetz, S., et al., *Randomized trial of irinotecan and cetuximab with or without vemurafenib in BRAF-mutant metastatic colorectal cancer (SWOG S1406)*. 2017, American Society of Clinical Oncology.
228. NCT02164916, C.g.I., *S1406 Phase II Study of Irinotecan and Cetuximab With or Without Vemurafenib in BRAF Mutant Metastatic Colorectal Cancer.*
229. Silkin, S.V., et al., *Complete clinical response of BRAF-mutated cholangiocarcinoma to vemurafenib, panitumumab, and irinotecan.* Journal of gastrointestinal cancer, 2016. **47**(4): p. 502-505.
230. Chang, C.-L., et al., *Dose-dense chemotherapy improves mechanisms of antitumor immune response.* Cancer research, 2013. **73**(1): p. 119-127.
231. Lesterhuis, W.J., et al., *A pilot study on the immunogenicity of dendritic cell vaccination during adjuvant oxaliplatin/capecitabine chemotherapy in colon cancer patients.* Br J Cancer, 2010. **103**(9): p. 1415-21.
232. Gandhi, S.J., et al., *Awakening the immune system with radiation: Optimal dose and fractionation.* Cancer Lett, 2015. **368**(2): p. 185-90.
233. Morse, M.A. and H.K. Lyerly, *Checkpoint blockade in combination with cancer vaccines.* Vaccine, 2015. **33**(51): p. 7377-7385.
234. Kaufman, H.L., et al., *Combination Chemotherapy and ALVAC-CEA/B7.1 Vaccine in Patients with Metastatic Colorectal Cancer.* Clinical Cancer Research, 2008. **14**(15): p. 4843-4849.
235. Hattori, T., et al., *Immunological evaluation of personalized peptide vaccination in combination with UFT and UZEL for metastatic colorectal carcinoma patients.* Cancer Immunol Immunother, 2009. **58**(11): p. 1843-52.
236. Harrop, R., et al., *Vaccination of colorectal cancer patients with TroVax given alongside chemotherapy (5-fluorouracil, leukovorin and irinotecan) is safe and induces potent immune responses.* Cancer Immunol Immunother, 2008. **57**(7): p. 977-86.
237. Harrop, R., et al., *Vaccination of colorectal cancer patients with modified vaccinia ankara encoding the tumor antigen 5T4 (TroVax) given alongside chemotherapy induces potent immune responses.* Clin Cancer Res, 2007. **13**(15 Pt 1): p. 4487-94.
238. Murahashi, M., et al., *Phase I clinical trial of a five-peptide cancer vaccine combined with cyclophosphamide in advanced solid tumors.* Clin Immunol, 2016. **166-167**: p. 48-58.
239. Niu, J., et al., *Retrospective comparative study of the effects of dendritic cell vaccine and cytokine-induced killer cell immunotherapy with that of chemotherapy alone and in combination for colorectal cancer.* Biomed Res Int, 2014. **2014**: p. 214727.
240. Okuno, K., et al., *Phase I clinical trial of a novel peptide vaccine in combination with UFT/LV for metastatic colorectal cancer.* Exp Ther Med, 2011. **2**(1): p. 73-79.
241. Okuno, K., et al., *Clinical Trial of a 7-Peptide Cocktail Vaccine with Oral Chemotherapy for Patients with Metastatic Colorectal Cancer.* Anticancer Research, 2014. **34**: p. 3045-3052.
242. Sato, Y., et al., *Immunological evaluation of personalized peptide vaccination in combination with a 5-fluorouracil derivative (TS-1) for advanced gastric or colorectal carcinoma patients.* Cancer Sci, 2007. **98**(7): p. 1113-9.

243. Scurr, M., et al., *Effect of Modified Vaccinia Ankara-5T4 and Low-Dose Cyclophosphamide on Antitumor Immunity in Metastatic Colorectal Cancer: A Randomized Clinical Trial.* JAMA Oncol, 2017. **3**(10): p. e172579.
244. Zheng, L., et al., *A safety and feasibility study of an allogeneic colon cancer cell vaccine administered with a granulocyte-macrophage colony stimulating factor-producing bystander cell line in patients with metastatic colorectal cancer.* Ann Surg Oncol, 2014. **21**(12): p. 3931-7.
245. NCT03206073, C.g.I., *A Phase I/II Study of Pexa-Vec Oncolytic Virus in Combination With Immune Checkpoint Inhibition in Refractory Colorectal Cancer*.
246. NCT02977156, C.g.I., *Immunization Strategy With Intra-tumoral Injections of Pexa-Vec With Ipilimumab in Metastatic / Advanced Solid Tumors. (ISI-JX).*
247. Gonzalez-Aparicio, M., et al., *Oxaliplatin in combination with liver-specific expression of interleukin 12 reduces the immunosuppressive microenvironment of tumours and eradicates metastatic colorectal cancer in mice.* Gut, 2011. **60**(3): p. 341-9.
248. Gou, H.-F., et al., *Chemo-Immunotherapy with Oxaliplatin and Interleukin-7 Inhibits Colon Cancer Metastasis in Mice.* PloS one, 2014. **9**(1): p. e85789.
249. Malvicini, M., et al., *A novel synergistic combination of cyclophosphamide and gene transfer of interleukin-12 eradicates colorectal carcinoma in mice.* Clin Cancer Res, 2009. **15**(23): p. 7256-65.
250. Dewan, M.Z., et al., *Fractionated but not single-dose radiotherapy induces an immune-mediated abscopal effect when combined with anti-CTLA-4 antibody.* Clin Cancer Res, 2009. **15**(17): p. 5379-88.
251. Deng, L., et al., *Irradiation and anti-PD-L1 treatment synergistically promote antitumor immunity in mice.* J Clin Invest, 2014. **124**(2): p. 687-95.
252. Saha, A. and S.K. Chatterjee, *Combination of CTL-associated Antigen-4 Blockade and Depletion of CD25+ Regulatory T Cells Enhance Tumour Immunity of Dendritic Cell-based Vaccine in a Mouse Model of Colon Cancer.* Scandinavian Journal of Immunology, 2010. **71**(2): p. 70-82.
253. Lee, H., et al., *Combinatorial therapy for liver metastatic colon cancer: dendritic cell vaccine and low-dose agonistic anti-4-1BB antibody co-stimulatory signal.* J Surg Res, 2011. **169**(1): p. e43-50.
254. Hance, K.W., et al., *The antitumor and immunoadjuvant effects of IFN-alpha in combination with recombinant poxvirus vaccines.* Clin Cancer Res, 2009. **15**(7): p. 2387-96.
255. Omori, R., et al., *Effects of interferon-alpha-transduced tumor cell vaccines and blockade of programmed cell death-1 on the growth of established tumors.* Cancer Gene Ther, 2012. **19**(9): p. 637-43.
256. Yu, P., et al., *Simultaneous blockade of multiple immune system inhibitory checkpoints enhances antitumor activity mediated by interleukin-15 in a murine metastatic colon carcinoma model.* Clin Cancer Res, 2010. **16**(24): p. 6019-28.
257. Cross, R.S., et al., *Therapeutic DNA vaccination against colorectal cancer by targeting the MYB oncoprotein.* Clin Transl Immunology, 2015. **4**(1): p. e30.
258. Son, C.-H., et al., *CTLA-4 Blockade Enhances Antitumor Immunity of Intratumoral Injection of Immature Dendritic Cells into Irradiated Tumor in a Mouse Colon Cancer Model.* J Immunoyher, 2014. **37**(1): p. 1-7.
259. European Science Foundation. 2004. Nanomedicine – An ESF–European Medical Research Councils (EMRC) Forward Look Report. Strasbourg cedex, F.E.

260. Park, Y.-M., et al., *Nanoparticle-based vaccine delivery for cancer immunotherapy*. Immune network, 2013. **13**(5): p. 177-183.
261. Krishnaiah, Y.S. and M.A. Khan, *Strategies of targeting oral drug delivery systems to the colon and their potential use for the treatment of colorectal cancer*. Pharmaceutical Development and Technology, 2012. **17**(5): p. 521-540.
262. Lu, L., et al., *Nanoparticle-based oral delivery systems for colon targeting: principles and design strategies.* Science bulletin, 2016. **61**(9): p. 670-681.
263. Gaspar D, P.C., Florindo H, Almeida AJ, *Mucosal immunization using polyester-based particulate systems. In: Ravi Kumar MNV (ed), Handbook of polyester drug delivery systems*. 2016: Pan Stanford Publishing Pte. Ltd., Singapore
264. Mishra, B. and S. Chaurasia, *Design of novel chemotherapeutic delivery systems for colon cancer therapy based on oral polymeric nanoparticles.* Therapeutic delivery, 2017. **8**(1): p. 29-47.
265. Zupancic, E., et al., *Translational Peptide-associated Nanosystems: Promising Role as Cancer Vaccines.* Curr Top Med Chem, 2016. **16**(3): p. 291-313.
266. Ngwuluka, N.C., D.J. Kotak, and P.V. Devarajan, *Design and characterization of metformin-loaded solid lipid nanoparticles for colon cancer.* AAPS PharmSciTech, 2017. **18**(2): p. 358-368.
267. Yassin, A.E.B., et al., *Optimization of 5-flurouracil solid-lipid nanoparticles: a preliminary study to treat colon cancer*. International journal of medical sciences, 2010. **7**(6): p. 398.
268. Garg, A., et al., *Targeting colon cancer cells using PEGylated liposomes modified with a fibronectin-mimetic peptide.* International journal of pharmaceutics, 2009. **366**(1): p. 201-210.
269. Kono, K., et al., *Highly temperature-sensitive liposomes based on a thermosensitive block copolymer for tumor-specific chemotherapy.* Biomaterials, 2010. **31**(27): p. 7096-7105.
270. Ogawara, K.-i., et al., *In vivo anti-tumor effect of PEG liposomal doxorubicin (DOX) in DOX-resistant tumor-bearing mice: Involvement of cytotoxic effect on vascular endothelial cells.* Journal of Controlled Release, 2009. **133**(1): p. 4-10.
271. Schroeder, A., et al., *Ultrasound triggered release of cisplatin from liposomes in murine tumors.* Journal of controlled release, 2009. **137**(1): p. 63-68.
272. Maya, S., et al., *Actively targeted cetuximab conjugated γ-poly (glutamic acid)-docetaxel nanomedicines for epidermal growth factor receptor over expressing colon cancer cells.* Journal of biomedical nanotechnology, 2014. **10**(8): p. 1416-1428.
273. Chaurasia, S., et al., *Curcumin-polymeric nanoparticles against colon-26 tumor-bearing mice: cytotoxicity, pharmacokinetic and anticancer efficacy studies.* Drug development and industrial pharmacy, 2016. **42**(5): p. 694-700.
274. de Mattos, A.C., et al., *Polymeric nanoparticles for oral delivery of 5-fluorouracil: Formulation optimization, cytotoxicity assay and pre-clinical pharmacokinetics study.* European Journal of Pharmaceutical Sciences, 2016. **84**: p. 83-91.
275. Tsai, M.-H., et al., *Enhanced efficacy of chemotherapeutic drugs against colorectal cancer using ligand-decorated self-breakable agents.* RSC Advances, 2015. **5**(112): p. 92361-92370.
276. Feng, S.-T., et al., *pH-sensitive nanomicelles for controlled and efficient drug delivery to human colorectal carcinoma LoVo cells.* PloS one, 2014. **9**(6): p. e100732.

277. Nourazarian, A.R., et al., *Combined EGFR and c-Src antisense oligodeoxynucleotides encapsulated with PAMAM Denderimers inhibit HT-29 colon cancer cell proliferation.* Asian Pacific Journal of Cancer Prevention, 2012. **13**(9): p. 4751-4756.
278. Chen, L., et al., *Overcoming acquired drug resistance in colorectal cancer cells by targeted delivery of 5-FU with EGF grafted hollow mesoporous silica nanoparticles.* Nanoscale, 2015. **7**(33): p. 14080-14092.
279. Nguyen, C.T., et al., *Bifunctional Succinylated ε-Polylysine-Coated Mesoporous Silica Nanoparticles for pH-Responsive and Intracellular Drug Delivery Targeting the Colon.* ACS Applied Materials & Interfaces, 2017. **9**(11): p. 9470-9483.
280. Hosseinzadeh, H., et al., *SN38 conjugated hyaluronic acid gold nanoparticles as a novel system against metastatic colon cancer cells.* International Journal of Pharmaceutics, 2017. **526**(1): p. 339-352.
281. Devanesan, S., et al., *Rapid biological synthesis of silver nanoparticles using plant seed extracts and their cytotoxicity on colorectal cancer cell lines.* Journal of Cluster Science, 2017. **28**(1): p. 595-605.
282. Kuo, C.-Y., et al., *Magnetically triggered nanovehicles for controlled drug release as a colorectal cancer therapy.* Colloids and Surfaces B: Biointerfaces, 2016. **140**: p. 567-573.
283. Patel, M.N., et al., *Characterization and evaluation of 5-fluorouracil-loaded solid lipid nanoparticles prepared via a temperature-modulated solidification technique.* AAPS PharmSciTech, 2014. **15**(6): p. 1498-1508.
284. Yang, C., et al., *Oxaliplatin long-circulating liposomes improved therapeutic index of colorectal carcinoma.* BMC biotechnology, 2011. **11**(1): p. 21.
285. Maya, S., et al., *Cetuximab conjugated O-carboxymethyl chitosan nanoparticles for targeting EGFR overexpressing cancer cells.* Carbohydrate polymers, 2013. **93**(2): p. 661-669.
286. Liu, K., et al., *Hyaluronic acid-tagged silica nanoparticles in colon cancer therapy: therapeutic efficacy evaluation.* International journal of nanomedicine, 2015. **10**: p. 6445.
287. Gonzalez-Ballesteros, N., et al., *Green synthesis of gold nanoparticles using brown algae Cystoseira baccata: Its activity in colon cancer cells.* Colloids and Surfaces B: Biointerfaces, 2017. **153**: p. 190-198.
288. Goodwin, T.J. and L. Huang, *Investigation of phosphorylated adjuvants co-encapsulated with a model cancer peptide antigen for the treatment of colorectal cancer and liver metastasis.* Vaccine, 2017. **35**(19): p. 2550-2557.
289. Yamaguchi, S., et al., *EphA2-derived peptide vaccine with amphiphilic poly (γ-glutamic acid) nanoparticles elicits an anti-tumor effect against mouse liver tumor.* Cancer immunology, immunotherapy, 2010. **59**(5): p. 759-767.
290. Tan, L., et al., *Chitosan nanoparticle-based delivery of fused NKG2D-IL-21 gene suppresses colon cancer growth in mice.* Int J Nanomedicine, 2017. **12**: p. 3095-3107.
291. Blau, R., et al., *Are nanotheranostics and nanodiagnostics-guided drug delivery stepping stones towards precision medicine?* Drug Resistance Updates, 2016. **27**: p. 39-58.
292. Kim, D.-H., *Image-guided Cancer nanomedicine.* Journal of imaging, 2018. **4**(1): p. 18.

293. Chen, K.-J., et al., *Hyperthermia-mediated local drug delivery by a bubble-generating liposomal system for tumor-specific chemotherapy.* ACS nano, 2014. **8**(5): p. 5105-5115.
294. Sainz, V., et al., *Regulatory aspects on nanomedicines.* Biochemical and biophysical research communications, 2015. **468**(3): p. 504-510.
295. Conniot, J., et al., *Cancer immunotherapy: nanodelivery approaches for immune cell targeting and tracking.* Frontiers in chemistry, 2014. **2**: p. 105.
296. Hashizume, H., et al., *Openings between defective endothelial cells explain tumor vessel leakiness.* Am J Pathol, 2000. **156**(4): p. 1363-80.
297. Matsumura, Y. and H. Maeda, *A new concept for macromolecular therapeutics in cancer chemotherapy: mechanism of tumoritropic accumulation of proteins and the antitumor agent smancs.* Cancer Res, 1986. **46**(12 Pt 1): p. 6387-92.
298. Acharya, S. and S.K. Sahoo, *PLGA nanoparticles containing various anticancer agents and tumour delivery by EPR effect.* Advanced drug delivery reviews, 2011. **63**(3): p. 170-183.
299. Anitha, A., et al., *Combinatorial nanomedicines for colon cancer therapy.* Wiley Interdisciplinary Reviews: Nanomedicine and Nanobiotechnology, 2016. **8**(1): p. 151-159.
300. Lammers, T., et al., *Cancer nanomedicine: is targeting our target?* Nat. Rev. Mater, 2016. **1**(9).
301. Li, R., et al., *Be Active or Not: the Relative Contribution of Active and Passive Tumor Targeting of Nanomaterials.* Nanotheranostics, 2017. **1**(4): p. 346.
302. Subbiah, R., M. Veerapandian, and K. S Yun, *Nanoparticles: functionalization and multifunctional applications in biomedical sciences.* Current medicinal chemistry, 2010. **17**(36): p. 4559-4577.
303. Danhier, F., O. Feron, and V. Préat, *To exploit the tumor microenvironment: passive and active tumor targeting of nanocarriers for anti-cancer drug delivery.* Journal of controlled release, 2010. **148**(2): p. 135-146.
304. Hochwald, S.N., *Molecular-targeted therapy for cancer and nanotechnology*, in *Cancer Nanotechnology*. 2010, Springer. p. 11-23.
305. Bertrand, N., et al., *Cancer nanotechnology: the impact of passive and active targeting in the era of modern cancer biology.* Advanced drug delivery reviews, 2014. **66**: p. 2-25.
306. Yu, D.-H., et al., *Peptide-conjugated biodegradable nanoparticles as a carrier to target paclitaxel to tumor neovasculature.* Biomaterials, 2010. **31**(8): p. 2278-2292.
307. Arruebo, M., M. Valladares, and Á. González-Fernández, *Antibody-conjugated nanoparticles for biomedical applications.* Journal of Nanomaterials, 2009. **2009**: p. 37.
308. MacDonald, D.A., et al., *Aflibercept exhibits VEGF binding stoichiometry distinct from bevacizumab and does not support formation of immune-like complexes.* Angiogenesis, 2016. **19**(3): p. 389-406.
309. Troiani, T., et al., *Beyond bevacizumab: new anti-VEGF strategies in colorectal cancer.* Expert Opin Investig Drugs, 2012. **21**(7): p. 949-59.
310. Sardinha, T.C., et al., *Membrane-type 1 matrix metalloproteinase mRNA expression in colorectal cancer.* Dis Colon Rectum, 2000. **43**(3): p. 389-95.
311. Kondo, M., et al., *Anti-neovascular therapy by liposomal drug targeted to membrane type-1 matrix metalloproteinase.* Int J Cancer, 2004. **108**(2): p. 301-6.

312. Maurer, C.A., et al., *Over-expression of ICAM-1, VCAM-1 and ELAM-1 might influence tumor progression in colorectal cancer.* International journal of cancer, 1998. **79**(1): p. 76-81.
313. Gosk, S., et al., *VCAM-1 directed immunoliposomes selectively target tumor vasculature in vivo.* Biochimica et Biophysica Acta (BBA) - Biomembranes, 2008. **1778**(4): p. 854-863.
314. Sengupta, S., et al., *Activation of tumor suppressor LKB1 by honokiol abrogates cancer stem-like phenotype in breast cancer via inhibition of oncogenic Stat3.* Oncogene, 2017. **36**(41): p. 5709-5721.
315. Chen, J., et al., *Association between integrin αvβ3 expression and malignancy lymph node metastasis: A meta-analysis.* Biomedical Research, 2017. **28**(7).
316. Danhier, F., A. Le Breton, and V. Preat, *RGD-based strategies to target alpha(v) beta(3) integrin in cancer therapy and diagnosis.* Mol Pharm, 2012. **9**(11): p. 2961-73.
317. Varshosaz, J., et al., *Transferrin-targeted poly (butylene adipate)/terephthalate nanoparticles for targeted delivery of 5-fluorouracil in HT29 colorectal cancer cell line.* Journal of Bioactive and Compatible Polymers, 2017. **32**(5): p. 503-527.
318. Sharma, M., et al., *Folic acid conjugated guar gum nanoparticles for targeting methotrexate to colon cancer.* Journal of biomedical nanotechnology, 2013. **9**(1): p. 96-106.
319. Ebrahimnejad, P., et al., *Preparation and in vitro evaluation of actively targetable nanoparticles for SN-38 delivery against HT-29 cell lines.* Nanomedicine: Nanotechnology, Biology and Medicine, 2010. **6**(3): p. 478-485.
320. Wang, Y., et al., *Targeted delivery of 5-fluorouracil to HT-29 cells using high efficient folic acid-conjugated nanoparticles.* Drug delivery, 2015. **22**(2): p. 191-198.
321. Choi, K.Y., et al., *Theranostic nanoparticles based on PEGylated hyaluronic acid for the diagnosis, therapy and monitoring of colon cancer.* Biomaterials, 2012. **33**(26): p. 6186-6193.
322. Tiernan, J., et al., *Carcinoembryonic antigen is the preferred biomarker for in vivo colorectal cancer targeting.* British journal of cancer, 2013. **108**(3): p. 662.
323. Conaghan, P., et al., *Targeted killing of colorectal cancer cell lines by a humanised IgG1 monoclonal antibody that binds to membrane-bound carcinoembryonic antigen.* British journal of cancer, 2008. **98**(7): p. 1217.
324. Cortez, C., et al., *Influence of size, surface, cell line, and kinetic properties on the specific binding of A33 antigen-targeted multilayered particles and capsules to colorectal cancer cells.* Acs Nano, 2007. **1**(2): p. 93-102.
325. Fay, F., et al., *Conatumumab (AMG 655) coated nanoparticles for targeted pro-apoptotic drug delivery.* Biomaterials, 2011. **32**(33): p. 8645-8653.
326. Devetzi, M., et al., *Death receptor 5 (DR5) and a 5-gene apoptotic biomarker panel with significant differential diagnostic potential in colorectal cancer.* Scientific reports, 2016. **6**: p. 36532.
327. Xu, F., et al., *Expression profile of mucin-associated sialyl-Tn antigen in Chinese patients with different colorectal lesions (adenomas, carcinomas).* Int J Clin Exp Pathol, 2015. **8**(9): p. 11549-54.
328. Subbiah, V., et al., *Phase Ib/II Trial of NC-6004 (Nanoparticle Cisplatin) Plus Gemcitabine in Patients with Advanced Solid Tumors.* Clinical Cancer Research, 2018. **24**(1): p. 43-51.

329. Yin, R., et al., *Evaluation of the toxicity and efficacy of paclitaxel nanoencapsulated with polyethyloxazoline polymers.* Journal of Clinical Oncology, 2013. **31**(15_suppl): p. e13538-e13538.
330. Morad, S.A.F., et al., *Short-chain ceramides depress integrin cell surface expression and function in colorectal cancer cells.* Cancer Letters, 2016. **376**(2): p. 199-204.
331. NCT03368963, C.g.I., *TAS102 in Combination With NAL-IRI in Advanced GI Cancers.*
332. Chibaudel, B., et al., *PEPCOL: a GERCOR randomized phase II study of nanoliposomal irinotecan PEP02 (MM-398) or irinotecan with leucovorin/5-fluorouracil as second-line therapy in metastatic colorectal cancer.* Cancer medicine, 2016. **5**(4): p. 676-683.
333. NCT00311610, C.g.I., *Liposomal SN-38 in Treating Patients With Metastatic Colorectal Cancer.*
334. NCT00361842, C.g.I., *Multicenter Study Of CPX-1 (Irinotecan HCl: Floxuridine) Liposome Injection In Patients With Advanced Colorectal Cancer.*
335. Hewitt, S.L., et al., *Durable anticancer immunity from intratumoral administration of IL-23, IL-36γ, and OX40L mRNAs.* Science Translational Medicine, 2019. **11**(477): p. eaat9143.
336. NCT01464593, C.g.I., *Phase 2 Study of Thermodox as Adjuvant Therapy With Thermal Ablation (RFA) in Treatment of Metastatic Colorectal Cancer(mCRC) (ABLATE).*
337. Yoshino, T., et al., *146TiPA multicenter, multicohort, phase II study of trastuzumab deruxtecan (DS-8201a) in subjects with HER2-expressing metastatic colorectal cancer.* Annals of Oncology, 2018. **29**(suppl_9).
338. Parikh, A., et al., *Prolonged Response to HER2-Directed Therapy in a Patient With HER2-Amplified, Rapidly Progressive Metastatic Colorectal Cancer.* Journal of the National Comprehensive Cancer Network : JNCCN, 2017. **15**(1): p. 3-8.
339. Sun, Q., et al., *Nanomedicine and macroscale materials in immuno-oncology*. Chemical Society Reviews, 2019. **48**(1): p. 351-381.
340. Yameen, B., et al., *Insight into nanoparticle cellular uptake and intracellular targeting.* J Control Release, 2014. **190**: p. 485-99.
341. Medzhitov, R., *Origin and physiological roles of inflammation.* Nature, 2008. **454**(7203): p. 428-35.
342. Maisonneuve, C., et al., *Unleashing the potential of NOD- and Toll-like agonists as vaccine adjuvants.* Proc Natl Acad Sci U S A, 2014. **111**(34): p. 12294-9.
343. Pulendran, B. and R. Ahmed, *Immunological mechanisms of vaccination.* Nat Immunol, 2011. **12**(6): p. 509-17.
344. Silva, J.M., et al., *Immune system targeting by biodegradable nanoparticles for cancer vaccines.* J Control Release, 2013. **168**(2): p. 179-99.
345. Zupancic, E., et al., *Rational design of nanoparticles towards targeting antigen-presenting cells and improved T cell priming.* J Control Release, 2017. **258**: p. 182-195.
346. Kawai, T. and S. Akira, *The roles of TLRs, RLRs and NLRs in pathogen recognition.* Int Immunol, 2009. **21**(4): p. 317-37.
347. Silva, A.L., et al. *Nanoparticle impact on innate immune cell pattern-recognition receptors and inflammasomes activation*. in *Seminars in immunology*. 2017. Elsevier.

348. Diebold, S.S., *Activation of dendritic cells by toll-like receptors and C-type lectins.* Handb Exp Pharmacol, 2009(188): p. 3-30.
349. Chaturvedi, A. and S.K. Pierce, *How location governs toll-like receptor signaling.* Traffic, 2009. **10**(6): p. 621-8.
350. Basith, S., et al., *Toll-like receptor modulators: a patent review (2006-2010).* Expert Opin Ther Pat, 2011. **21**(6): p. 927-44.
351. Johnson, D.A., *Synthetic TLR4-active glycolipids as vaccine adjuvants and stand-alone immunotherapeutics.* Curr Top Med Chem, 2008. **8**(2): p. 64-79.
352. Zaks, K., et al., *Efficient immunization and cross-priming by vaccine adjuvants containing TLR3 or TLR9 agonists complexed to cationic liposomes.* J Immunol, 2006. **176**(12): p. 7335-45.
353. Lynn, G.M., et al., *In vivo characterization of the physicochemical properties of polymer-linked TLR agonists that enhance vaccine immunogenicity.* Nat Biotechnol. , 2015. **33**(11): p. 1201-10.
354. Houot, R. and R. Levy, *T-cell modulation combined with intratumoral CpG cures lymphoma in a mouse model without the need for chemotherapy.* Blood, 2009. **113**(15): p. 3546-52.
355. Wang, S., et al., *Intratumoral injection of a CpG oligonucleotide reverts resistance to PD-1 blockade by expanding multifunctional CD8+ T cells.* Proc Natl Acad Sci U S A, 2016. **113**(46): p. E7240-e7249.
356. Sagiv-Barfi, I., et al., *Eradication of spontaneous malignancy by local immunotherapy.* Sci Transl Med., 2018. **10**(426).
357. Frank, M.J., et al., *In situ vaccination with a TLR9 agonist and local low-dose radiation induces systemic responses in untreated indolent lymphoma.* Cancer discovery, 2018. **8**(10): p. 1258-1269.
358. Ribas, A., et al., *SD-101 in combination with pembrolizumab in advanced melanoma: results of a phase Ib, multicenter study.* Cancer discovery, 2018. **8**(10): p. 1250-1257.
359. Liu, Y.J., *IPC: professional type 1 interferon-producing cells and plasmacytoid dendritic cell precursors.* Annu Rev Immunol, 2005. **23**: p. 275-306.
360. Krug, A., et al., *Identification of CpG oligonucleotide sequences with high induction of IFN-alpha/beta in plasmacytoid dendritic cells.* Eur J Immunol, 2001. **31**(7): p. 2154-63.
361. Diwan, M., et al., *Dose sparing of CpG oligodeoxynucleotide vaccine adjuvants by nanoparticle delivery.* Curr Drug Deliv, 2004. **1**(4): p. 405-12.
362. Peres, C., et al., *Poly(lactic acid)-based particulate systems are promising tools for immune modulation.* Acta Biomater, 2017. **48**: p. 41-57.
363. Irvine, D.J., et al., *Synthetic nanoparticles for vaccines and immunotherapy.* Chemical reviews, 2015. **115**(19): p. 11109-11146.
364. Mogrão, J., et al., *Modulation of dendritic cells by nanotechnology-based immunotherapeutic strategies.* Journal of biomedical nanotechnology, 2016. **12**(3): p. 405-434.
365. Chen, C. and H. Wang, *Biomedical applications and toxicology of carbon nanomaterials*. 2016: John Wiley & Sons.
366. Mashinchian, O., et al., *Impacts of quantum dots in molecular detection and bioimaging of cancer.* BioImpacts: BI, 2014. **4**(3): p. 149.
367. Thomas, S.N., et al., *Targeting the tumor-draining lymph node with adjuvanted nanoparticles reshapes the anti-tumor immune response.* Biomaterials, 2014. **35**(2): p. 814-24.

368. Reddy, S.T., et al., *In vivo targeting of dendritic cells in lymph nodes with poly (propylene sulfide) nanoparticles.* Journal of Controlled Release, 2006. **112**(1): p. 26-34.
369. Yan, S., W. Gu, and Z.P. Xu, *Re-considering how particle size and other properties of antigen–adjuvant complexes impact on the immune responses.* Journal of colloid and interface science, 2013. **395**: p. 1-10.
370. Bachmann, M.F. and G.T. Jennings, *Vaccine delivery: a matter of size, geometry, kinetics and molecular patterns.* Nature Reviews Immunology, 2010. **10**(11): p. 787.
371. Kim, J. and D.J. Mooney, *In vivo modulation of dendritic cells by engineered materials: towards new cancer vaccines.* Nano today, 2011. **6**(5): p. 466-477.
372. Ehrlich, M., et al., *Endocytosis by random initiation and stabilization of clathrin-coated pits.* Cell, 2004. **118**(5): p. 591-605.
373. Wang, Z., et al., *Size and Dynamics of Caveolae Studied Using Nanoparticles in Living Endothelial Cells.* ACS Nano, 2009. **3**(12): p. 4110-4116.
374. Albanese, A., P.S. Tang, and W.C. Chan, *The effect of nanoparticle size, shape, and surface chemistry on biological systems.* Annu Rev Biomed Eng, 2012. **14**: p. 1-16.
375. Knudsen, K.B., et al., *In vivo toxicity of cationic micelles and liposomes.* Nanomedicine, 2015. **11**(2): p. 467-77.
376. Frohlich, E., *The role of surface charge in cellular uptake and cytotoxicity of medical nanoparticles.* Int J Nanomedicine, 2012. **7**: p. 5577-91.
377. Cruz, L.J., et al., *The influence of PEG chain length and targeting moiety on antibody-mediated delivery of nanoparticle vaccines to human dendritic cells.* Biomaterials, 2011. **32**(28): p. 6791-803.
378. Leleux, J. and K. Roy, *Micro and nanoparticle-based delivery systems for vaccine immunotherapy: an immunological and materials perspective.* Adv Healthc Mater, 2013. **2**(1): p. 72-94.
379. Caminschi, I., et al., *The dendritic cell subtype-restricted C-type lectin Clec9A is a target for vaccine enhancement.* Blood, 2008. **112**(8): p. 3264-73.
380. Sancho, D., et al., *Tumor therapy in mice via antigen targeting to a novel, DC-restricted C-type lectin.* J Clin Invest, 2008. **118**(6): p. 2098-110.
381. Van Broekhoven, C.L., et al., *Targeting dendritic cells with antigen-containing liposomes: a highly effective procedure for induction of antitumor immunity and for tumor immunotherapy.* Cancer research, 2004. **64**(12): p. 4357-4365.
382. Wang, W.W., D. Das, and M.R. Suresh, *A versatile bifunctional dendritic cell targeting vaccine vector.* Molecular pharmaceutics, 2008. **6**(1): p. 158-172.
383. Thomann, J.-S., et al., *Antitumor activity of liposomal ErbB2/HER2 epitope peptide-based vaccine constructs incorporating TLR agonists and mannose receptor targeting.* Biomaterials, 2011. **32**(20): p. 4574-4583.
384. Ghotbi, Z., et al., *Active targeting of dendritic cells with mannan-decorated PLGA nanoparticles.* Journal of drug targeting, 2011. **19**(4): p. 281-292.
385. Hamdy, S., et al., *Activation of antigen-specific T cell-responses by mannan-decorated PLGA nanoparticles.* Pharmaceutical research, 2011. **28**(9): p. 2288.
386. Unger, W.W., et al., *Glycan-modified liposomes boost CD4+ and CD8+ T-cell responses by targeting DC-SIGN on dendritic cells.* Journal of Controlled Release, 2012. **160**(1): p. 88-95.
387. Kawamura, K., et al., *Dendritic cells that endocytosed antigen-containing IgG-liposomes elicit effective antitumor immunity.* Journal of immunotherapy, 2006. **29**(2): p. 165-174.

388. Suzuki, R., et al., *Development of effective antigen delivery carrier to dendritic cells via Fc receptor in cancer immunotherapy.* Yakugaku zasshi: Journal of the Pharmaceutical Society of Japan, 2007. **127**(2): p. 301-306.
389. Serre, K., et al., *Liposomes Targeted to Fc Receptors for Antigen Presentation by Dendritic Cells In Vitro and In Vivo⁎*, in *Methods in enzymology*. 2003, Elsevier. p. 100-118.
390. Silva, J.M., et al., *In vivo delivery of peptides and Toll-like receptor ligands by mannose-functionalized polymeric nanoparticles induces prophylactic and therapeutic anti-tumor immune responses in a melanoma model.* J Control Release, 2015. **198**: p. 91-103.
391. Yang, R., et al., *Cancer Cell Membrane-Coated Adjuvant Nanoparticles with Mannose Modification for Effective Anticancer Vaccination.* ACS nano, 2018.
392. Cruz, L.J., et al., *Targeting nanoparticles to CD40, DEC-205 or CD11c molecules on dendritic cells for efficient CD8+ T cell response: a comparative study.* Journal of Controlled Release, 2014. **192**: p. 209-218.
393. Lewis, J.S., et al., *Microparticle surface modifications targeting dendritic cells for non-activating applications.* Biomaterials, 2012. **33**(29): p. 7221-7232.
394. Rosalia, R.A., et al., *CD40-targeted dendritic cell delivery of PLGA-nanoparticle vaccines induce potent anti-tumor responses.* Biomaterials, 2015. **40**: p. 88-97.
395. Broos, S., et al., *Synergistic augmentation of CD40-mediated activation of antigen-presenting cells by amphiphilic poly (γ-glutamic acid) nanoparticles.* Biomaterials, 2012. **33**(26): p. 6230-6239.
396. Gu, L., et al., *Multivalent porous silicon nanoparticles enhance the immune activation potency of agonistic CD40 antibody.* Advanced materials, 2012. **24**(29): p. 3981-3987.
397. Varkouhi, A.K., et al., *Endosomal escape pathways for delivery of biologicals.* J Control Release, 2011. **151**(3): p. 220-8.
398. Cohn, L., et al., *Antigen delivery to early endosomes eliminates the superiority of human blood BDCA3+ dendritic cells at cross presentation.* J Exp Med, 2013. **210**(5): p. 1049-63.
399. Heath, W.R., et al., *Cross-presentation, dendritic cell subsets, and the generation of immunity to cellular antigens.* Immunol Rev, 2004. **199**: p. 9-26.
400. Silva, J.M., et al., *Development of functionalized nanoparticles for vaccine delivery to dendritic cells: a mechanistic approach.* Nanomedicine (Lond), 2014. **9**(17): p. 2639-56.
401. Luo, M., et al., *A STING-activating nanovaccine for cancer immunotherapy.* Nature Nanotechnology, 2017. **12**(7): p. 648-654.
402. Cho, N.H., et al., *A multifunctional core-shell nanoparticle for dendritic cell-based cancer immunotherapy.* Nat Nanotechnol, 2011. **6**(10): p. 675-82.
403. Zhang, H.J., et al., *Visible-light-sensitive titanium dioxide nanoplatform for tumor-responsive Fe2+ liberating and artemisinin delivery.* Oncotarget, 2017. **8**(35): p. 58738-58753.
404. Wang, H., et al., *cGAS is essential for the antitumor effect of immune checkpoint blockade.* Proc Natl Acad Sci U S A, 2017. **114**(7): p. 1637-1642.
405. Merad, M., et al., *The dendritic cell lineage: ontogeny and function of dendritic cells and their subsets in the steady state and the inflamed setting.* Annu Rev Immunol, 2013. **31**: p. 563-604.
406. Liu, X., et al., *Modified nanoparticle mediated IL-12 immunogene therapy for colon cancer.* Nanomedicine, 2017. **13**(6): p. 1993-2004.

407. Halama, N., et al., *Localization and density of immune cells in the invasive margin of human colorectal cancer liver metastases are prognostic for response to chemotherapy.* Cancer Res, 2011. **71**(17): p. 5670-7.
408. Lim, S.H., et al., *Effect of neoadjuvant chemoradiation on tumor-infiltrating/associated lymphocytes in locally advanced rectal cancers.* Anticancer Res, 2014. **34**(11): p. 6505-13.
409. Wang, Y.J., et al., *Immunogenic effects of chemotherapy-induced tumor cell death.* Genes Dis, 2018. **5**(3): p. 194-203.
410. Guo, S., et al., *Co-delivery of cisplatin and rapamycin for enhanced anticancer therapy through synergistic effects and microenvironment modulation.* ACS Nano, 2014. **8**(5): p. 4996-5009.
411. Miyashita, T., et al., *Neoadjuvant Chemotherapy with Gemcitabine Plus Nab-paclitaxel Reduces the Number of Cancer-associated Fibroblasts Through Depletion of Pancreatic Stroma.* Anticancer Res, 2018. **38**(1): p. 337-343.
412. Torres Andón, F. and M.J. Alonso, *Nanomedicine and cancer immunotherapy – targeting immunosuppressive cells.* Journal of Drug Targeting, 2015. **23**(7-8): p. 656-671.
413. Kuai, R., et al., *Elimination of established tumors with nanodisc-based combination chemoimmunotherapy.* Science advances, 2018. **4**(4): p. eaao1736.
414. Guo, X.Y., et al., *Paclitaxel and gemcitabine combinational drug-loaded mucoadhesive delivery system in the treatment of colon cancers.* Drug Res (Stuttg), 2015. **65**(4): p. 199-204.
415. Xiao, B., et al., *Co-delivery of camptothecin and curcumin by cationic polymeric nanoparticles for synergistic colon cancer combination chemotherapy.* Journal of Materials Chemistry B, 2015. **3**(39): p. 7724-7733.
416. Batist, G., et al., *Safety, pharmacokinetics, and efficacy of CPX-1 liposome injection in patients with advanced solid tumors.* Clin Cancer Res, 2009. **15**(2): p. 692-700.
417. Zhao, P., et al., *Dual-Targeting to Cancer Cells and M2 Macrophages via Biomimetic Delivery of Mannosylated Albumin Nanoparticles for Drug-Resistant Cancer Therapy.* Advanced Functional Materials, 2017. **27**(44): p. 1700403.
418. Serpe, L., et al., *Cytotoxicity of anticancer drugs incorporated in solid lipid nanoparticles on HT-29 colorectal cancer cell line.* Eur J Pharm Biopharm, 2004. **58**(3): p. 673-80.
419. Wang, J. and C.-A. Peng, *Anticancer Effectiveness of Polymeric Drug Nanocarriers on Colorectal Cancer Cells.* 33rd Annual International Conference of the IEEE EMBS, 2011.
420. Xiao, B., et al., *Hyaluronic acid-functionalized polymeric nanoparticles for colon cancer-targeted combination chemotherapy.* Nanoscale, 2015. **7**(42): p. 17745-55.
421. Liu, D., et al., *Self-assembled nanoscale coordination polymers with trigger release properties for effective anticancer therapy.* Nat Commun, 2014. **5**: p. 4182.
422. Cho, H. and G.S. Kwon, *Polymeric micelles for neoadjuvant cancer therapy and tumor-primed optical imaging.* ACS Nano, 2011. **5**(11): p. 8721-9.
423. Maksimenko, A., et al., *Therapeutic Modalities of Squalenoyl Nanocomposites in Colon Cancer: An Ongoing Search for Improved Efficacy.* ACS Nano, 2014. **8**(3): p. 2018-2032.

424. Xu, B., et al., *Polymeric Nanomedicine for Combined Gene/Chemotherapy Elicits Enhanced Tumor Suppression.* Mol Pharm, 2016. **13**(2): p. 663-76.
425. Palvai, S., M.M. Kuman, and S. Basu, *Hyaluronic acid cloaked oleic acid nanoparticle inhibits MAPK signaling with sub-cellular DNA damage in colon cancer.* Journal of Materials Chemistry B, 2013.
426. Oshima, G., et al., *In Vivo Delivery and Therapeutic Effects of a MicroRNA on Colorectal Liver Metastases.* Mol Ther, 2017. **25**(7): p. 1588-1595.
427. Sadreddini, S., et al., *Chitosan nanoparticles as a dual drug/siRNA delivery system for treatment of colorectal cancer.* Immunol Lett, 2017. **181**: p. 79-86.
428. Dinarvand, M., et al., *Oral delivery of nanoparticles containing anticancer SN38 and hSET1 antisense for dual therapy of colon cancer.* Int J Biol Macromol, 2015. **78**: p. 112-21.
429. Ediriwickrema, A., et al., *Multi-layered nanoparticles for combination gene and drug delivery to tumors.* Biomaterials, 2014. **35**(34): p. 9343-54.
430. Anitha, A., et al., *In vitro combinatorial anticancer effects of 5-fluorouracil and curcumin loaded N,O-carboxymethyl chitosan nanoparticles toward colon cancer and in vivo pharmacokinetic studies.* Eur J Pharm Biopharm, 2014. **88**(1): p. 238-51.
431. Anitha, A., et al., *Combinatorial anticancer effects of curcumin and 5-fluorouracil loaded thiolated chitosan nanoparticles towards colon cancer treatment.* Biochim Biophys Acta, 2014. **1840**(9): p. 2730-43.
432. Hong, G.Y., et al., *Combination of paclitaxel- and retinoic acid-incorporated nanoparticles for the treatment of CT-26 colon carcinoma.* Arch Pharm Res, 2011. **34**(3): p. 407-17.
433. Na, H.S., et al., *Combination antitumor effects of micelle-loaded anticancer drugs in a CT-26 murine colorectal carcinoma model.* Int J Pharm, 2010. **383**(1-2): p. 192-200.
434. Kuai, R., et al., *Designer vaccine nanodiscs for personalized cancer immunotherapy.* Nat Mater, 2017. **16**(4): p. 489-496.
435. Chibaudel, B., et al., *PEPCOL: a GERCOR randomized phase II study of nanoliposomal irinotecan PEP02 (MM-398) or irinotecan with leucovorin/5-fluorouracil as second-line therapy in metastatic colorectal cancer.* Cancer Med, 2016. **5**(4): p. 676-83.
436. Chen, J., et al., *Effective inhibition of colon cancer cell growth with MgAl-layered double hydroxide (LDH) loaded 5-FU and PI3K/mTOR dual inhibitor BEZ-235 through apoptotic pathways.* Int J Nanomedicine, 2014. **9**: p. 3403-11.
437. Ortiz, R., et al., *5-Fluorouracil-loaded poly(epsilon-caprolactone) nanoparticles combined with phage E gene therapy as a new strategy against colon cancer.* Int J Nanomedicine, 2012. **7**: p. 95-107.
438. Sureban, S.M., et al., *Nanoparticle-based delivery of siDCAMKL-1 increases microRNA-144 and inhibits colorectal cancer tumor growth via a Notch-1 dependent mechanism.* Journal of Nanobiotechnology, 2011. **9**(40): p. 1-13.
439. Gabizon, A., et al., *Therapeutic efficacy of a lipid-based prodrug of mitomycin C in pegylated liposomes: studies with human gastro-entero-pancreatic ectopic tumor models.* J Control Release, 2012. **160**(2): p. 245-53.
440. Tian, X., et al., *Preclinical Evaluation of Promitil, a Radiation-Responsive Liposomal Formulation of Mitomycin C Prodrug, in Chemoradiotherapy.* Int J Radiat Oncol Biol Phys, 2016. **96**(3): p. 547-55.

441. Tian, X., et al., *CRLX101, a Nanoparticle-Drug Conjugate Containing Camptothecin, Improves Rectal Cancer Chemoradiotherapy by Inhibiting DNA Repair and HIF1alpha.* Cancer Res, 2017. **77**(1): p. 112-122.
442. Batist, G., et al., *A multicenter, phase II study of CPX-1 liposome injection in patients (pts) with advanced colorectal cancer (CRC).* Journal of Clinical Oncology, 2008. **26**(15_suppl): p. 4108-4108.
443. Li, L., et al., *Liposomal curcumin with and without oxaliplatin: effects on cell growth, apoptosis, and angiogenesis in colorectal cancer.* Mol Cancer Ther, 2007. **6**(4): p. 1276-82.
444. Xiao, B., et al., *Silencing of Intestinal Glycoprotein CD98 by Orally Targeted Nanoparticles Enhances Chemosensitization of Colon Cancer.* ACS Nano, 2018. **12**(6): p. 5253-5265.
445. Tsai, M.H., et al., *Panitumumab‐Conjugated Pt‐Drug Nanomedicine for Enhanced Efficacy of Combination Targeted Chemotherapy against Colorectal Cancer.* Advanced healthcare materials, 2017. **6**(13): p. 1700111.
446. Kosmides, A.K., et al., *Dual targeting nanoparticle stimulates the immune system to inhibit tumor growth.* ACS nano, 2017. **11**(6): p. 5417-5429.
447. NCT01705002, C.g.I., *Intravenously Administered Pegylated Liposomal Mitomycin-C Lipid-based Prodrug (PROMITIL) in Cancer Patients With Solid Tumors.*
448. Conde, J., et al., *Local triple-combination therapy results in tumour regression and prevents recurrence in a colon cancer model.* Nat Mater, 2016. **15**(10): p. 1128-38.

Highlights

- Colorectal cancer (CRC) is within the 5 most commonly diagnosed cancers worldwide.
- Promising clinical development of immune and chemotherapeutic regimens against CRC.
- Nanotechnology can improve CRC combinational chemo-immunotherapies.
- Urgent need for the validation of models resembling a specific CRS disease stage.