Chapter 10

Current Immunotherapeutic Treatments in Colon Cancer

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Additional information is available at the end of the chapter

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Abstract

The immune system is able to act against cancer cells and consequently these cells have developed a range of responses to evade or suppress the immune systems anticancer responses. The concept of cancer immunotherapy is based on techniques developed to restore or boost the ability of the immune system to recognize and target tumor cells. It is known that colon cancer does initiate an immune response and that this type of cancer initiates pathways and responses to evade or suppress the immune system. This chapter will discuss some of the dominant therapies being developed to treat colon cancer based on the concept of cancer immunotherapy. Cancer vaccines are based on the concept of providing the immune system with antigen targets derived from tumor-specific molecules, while monoclonal antibodies involve the development of antibodies specifically targeting proteins expressed on the surface of tumor cells. Antibody-based immunotherapy has further applications in the use of bispecific antibodies (BsAb), which are synthetic antibodies designed to be able to recognize two different antigens or epitopes and in this way can increase the immunoresponse and limit immune evasion observed in mono-targeted therapy. Immune checkpoint inhibitors target proteins that are responsible for keeping immune responses in check. Tumor cells overexpress these proteins in order to evade the immune response. Blocking these proteins will lead to an increased immune response against these cells. Cytokine-based immunotherapies involve the use of the immune systems' own molecular messengers that are responsible for a robust immune response, to boost the antitumor response of the immune system. Oncolytic viral therapy is based on the use of viruses that selectively infect and replicate in cancer and associated endothelial cells and subsequently kills these cells. Adoptive immunotherapy involves the use of immune cells from the patient to be cultured and altered in the laboratory and then reintroduced to boost the immune response. This is normally performed with T cells. Immunotherapy may be the next logical step in the development of an effective therapy for colon cancer and other cancers. The combination of these therapies with traditional chemotherapy or radiotherapy has shown promise in cancer treatment.

Keywords: Cancer vaccines, Monoclonal antibodies, Bispecific antibodies, Cytokines, Immune checkpoint inhibitors, Adoptive therapy, Immunotherapy

1. Introduction: immunotherapy

Tumor-associated antigens (TAAs) are antigens that can elicit a specific immune response. Immune cells and immune-related components such as macrophages, neutrophils, complement components, γδ T cells, natural killer (NK) cells, NKT cells, and certain cytokines (interleukin (IL)-12, interferon gamma (IFN-γ)) and cells of the adaptive immune system, including B lymphocytes, helper T cells (Th cells), and cytotoxic T lymphocytes (CTLs), are all active against cancer cells [1]. TAAs are presented to the cells of the adaptive immune system by cells such as dendritic cells (DCs) or other antigen-presenting cells (APCs). These antigens are processed and presented by major histocompatibility complex (MHC) class I and class II molecules leading to the activation of antigen-specific lymphocytes, resulting in antibody production [1].

Colon cancer evades the immune system through the shift from Th1 to Th2 immune responses [2] loss/downregulation of human leukocyte antigen (HLA) class I antigen processing and presentation [3], defective DC function [4, 5], T-cell loss of signaling molecules [6, 7], escaping death receptors, HLA G expression, alterations in transforming growth factor (TGF) beta signaling [8], Increased vascular endothelial growth factor (VEGF) expression, impaired NK activity, regulatory T-cell downregulation [9], and complement decay accelerating factor CD55 [2]. A shift is known to occur in the white blood cell composition with elevated numbers of CD8 T cells in the initial stages, but an overall reduction in the numbers of circulating immune-related cells. At the same time the levels of cytokines such as IFN-γ and tumor necrosis factor-α (TNFα) are reduced during vascular invasion [10]. Antitumor T cells can be inhibited through NO production by the enzyme arginase [11].

Immunotherapy can be divided into two main categories: passive and active immunotherapy. Passive immunotherapy makes use of *in vitro* produced immunologic effectors that are capable of influencing tumor cell growth. This includes monoclonal antibody (mAb) therapy and adaptive transfer of antigen-specific effector cells. Active immunotherapy aims at inducing or boosting immune effector cells [1].

2. Cancer vaccines

Cancer vaccines are active immunotherapeutic approaches that are intended to activate and expand tumor-specific T cells to induce an antitumor response. Conventional vaccines are preventative in nature, but current cancer vaccines activate the immune system to destroy tumors once present. A range of tumor antigens have been identified. These include T-cell

epitope peptides, defined carbohydrates of glycoproteins and glycolipids, antibody-based anti-idiotype vaccines, plasmid DNA and recombinant viral vector vaccines, allogeneic or autologous whole tumor cell vaccines, DC-based vaccines, oncolysates, or autologous heat-shock protein (HSP)-peptide complex vaccines. An ideal prophylactic cancer vaccine would be affordable, stable, and safe. It would induce effective immunity rapidly and require few immunizations (ideally one) to induce protection [12]. This section aims to address advances made in developing vaccines against colon cancer. This will include vaccines that are currently in use and vaccines still undergoing clinical trials. It will report on the safety, side effects, and efficacy of these vaccines.

Colon cancers express multiple immunogenic proteins, all of which may serve as targets for the development of T-cell-mediated adaptive immune responses [10]. It is also known that colorectal cancers do activate the immune system, leading to the attenuation of metastasis and increasing the survival of patients [10]. In order to evade the immune response, colorectal cancer suppresses the immune response or displays only weak immunogenicity. Additionally, studies have shown that restoration or supplementation of the immune function toward these tumors is possible [10, 13, 14]. Peptides used to inoculate a patient suffering from colon cancer will be degraded and the resulting fragments will be endocytosed by APCs. These cells will then present the antigen to the T cells. CTLs or CD8+ T cells induce apoptosis in tumor cells through the release of granzymes and perforins and through the Fas death receptor pathway. Type 1 CD4+ T cells and T-helper cells secrete cytokines leading to the recruitment of CTLs, macrophages, and NK cells. These secrete cytokines that activate cytotoxic pathways [11] (**Figure 1**). It is also known that colorectal cancers do activate the immune system, leading to the attenuation of metastasis and an increase in the survival rate and time of patients [10]. The effectiveness of peptide vaccines can be enhanced by altering the amino acid sequence of the peptide to enhance the interaction with the T-cell receptor (TCR), to improve binding to MHC, and finally to improve biostability and reduce degradation by proteases [11].

2.1. Evasion of the immune system by altered ligand expression

One mechanism utilized by cancer cells to evade the immune system involves the expression of FasL. This ligand binds to the Fas receptor present on CTL, leading to the CTL to undergo apoptosis. The expression of FasL leads to the increased resistance to Fas-induced apoptosis. Altered peptide ligands (APLs) are analogs of immunogenic peptides which are ligands for TCRs. These altered ligands bind the TCR, but does not lead to lysis of the tumor cell [11]. Regulatory T cells can inhibit antitumor immunity by upregulating cell membrane molecules that lead to the inhibition of effector T-cell activation and function. Cancer cells have defective antigen presentation allowing them to avoid recognition by the immune system. This is accomplished by reduced expression of MHC I, antigen-processing machinery, or Tumor-associated antigens themselves [11].

2.2. Tumor-associated antigens (TAAs)

Defined TAA epitopes can be used to vaccinate cancer patients. The peptide fragments are presented by the two MHC proteins, MHC classes I and II (HLA I and II) to the TCRs. MHC

class I presents the vaccine-derived peptide to naive CTLs. Primed CTLs recognize the tumor antigen on the surface of the tumor and send out a death signal to the tumor. Helper T cells are generated by MHC class II proteins [11] (**Figure 1**). However, there are not many specific peptides that can be targeted as cancer specific. In order to increase uptake and presentation of the antigens by APCs, an adjuvant is added [1]. Another strategy is to inject the DNA sequences coding for specific TAAs to be taken up. The target ill be transcribed into mRNA, translated into a protein, and processed into peptides by APCs. This can be done by using viruses engineered to express TAAs. However, the immune system may preferentially react to the viral antigens rather than the TAAs, leading to the attenuation of the antitumor immune response [1]. The earliest example of the therapeutic use of tumor antigens was in the form of crude tumor lysates being administered to patients. These lysates are still used as a means to prime DCs, facilitating peptide presentation [11]. This is because the ideal source of TAAs is all the TAAs the tumor itself expresses. By incubating DCs with dead tumor cell lysate, these antigens will all be presented by MHC class I (cross-presentation) and MHC class II pathways. This will result in a diversified immune response involving CTLs as well as CD4+ T-helper cells [1].

The use of tumor lysates has largely been superseded by the use of synthetic peptides. These have certain advantages over tumor lysates. They provide a higher amount of specific antigen and allow for modification of the target peptide. It is also easy to monitor the immune response to vaccination with a single peptide as only one CTL type requires evaluation [11]. Tumor-specific antigens (TSAs) are mutated or virus-derived epitopes and contain unique immunogenic neo-antigens that can be recognized by the immune system. These include N-RAS and p53 [1].

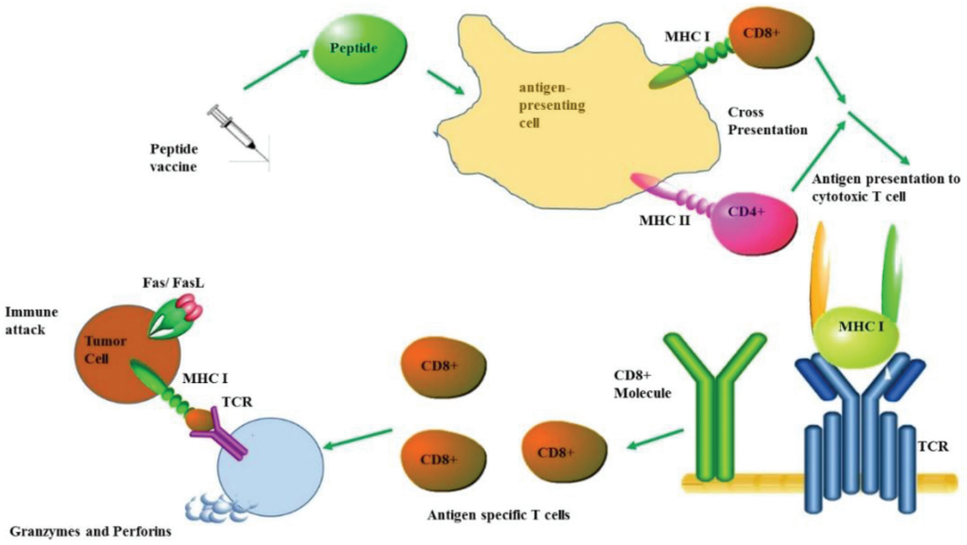

Figure 1. Antitumor effect of peptide vaccine therapy: following introduction of peptide vaccine to the bloodstream, it is processed and presented by the APC leading to the activation of CD4+ helper T cells and CD8+ cytotoxic T cells. Interaction between MHC I molecules on APC and TCR during antigen presentation facilitated by CD8 molecule leads to the generation of tumor-specific CTLs capable of lysing tumor cells.

Peptides	Mechanism	Study details	References
Tyrosine kinase receptor ephrin type-A receptor 2 (EphA2-derived peptide)	EphaA2 EphA2-specific CTL	High level of immunity against colorectal cancer in murine model	[15]
RNF43-721		Phase 1 clinical trial	[16]
ABT-737	Inhibition of antiapoptotic Bcl-2 family	Sensitized cancer cells in mouse colon cancer model	[17]
Epitopes of HER2, MVF, GMP, and n-MDP	Multiple targets	Phase 1 clinical trial	[18]
Endoglin	Inhibition of angiogenesis	Inhibition of tumor growth in mouse model	[19]
CEA CEA691	Induction of tumor-specific CTLs	Increase in survival rate in colon carcinoma mouse model	[20]
OX40L – TNF family protein		Inhibition of tumor growth in mouse model	[21]
Mucin 1: MUC1 a cell surface-associated protein		Stimulation of antigen-specific CTL, abundant secretion of IFN-γ. Tumor burden was significantly reduced in colon cancer mouse model	[22]
Heat-shock protein Gp96	Induction of tumor-specific CTLs	Two-year overall survival and disease-free survival were significantly improved	
SART3-tumor-rejection antigen	Induction of tumor-specific CTLs	Increased cellular immune responses to the tumor. No improved clinical outcome	[23, 24]
Lck-derived peptides	Induction of tumor-specific CTLs		[24]
Survivin-2B	Induction of HLA-A24-restricted cytotoxic T cells resulting in high toxicity against HLA-A24-positive survivin-2B-positive cancer in vitro	Increased proportion of peptide-specific CTL. No significantly improved clinical outcome	[25]
βHCG CTP37-DT (Avicine)		Phase II trials showed improved patient survival	[26]
CDX 1307	Fusion between βHCG and an antibody against the mannose receptor	Phase I trial. Inoculation leads to DC activation as well as cytotoxic T-cell activity against tumor cells	[27]
p53 (SLP)	p53-specific CD4+ Th cell SLP is a p53 synthetic long peptide	Antitumor response against p53-overexpressing tumors. The p53-SLP vaccine induces p53-specific T-cell responses	[10]

Peptides	Mechanism	Study details	References
EGFR2 gefitinib or erlotinib	EGFR mutations enhance tyrosine kinase activity in response to EGF, increasing the efficacy of anti-EGFR	In a phase I trial, the vaccine elicited antibody response phase II cancer	[28]
Gastrin: G17DT (gastroimmune)	Antigastrin-17 immunogen, raising antibodies that blockade gastrin-stimulated tumor growth	Phase II trials showed gastroimmune combined with irinotecan chemotherapy increased patient survival	[29]

Examples of peptide-based vaccine targets, their mechanism, as well as the current results of any trials performed using the vaccines to treat colon cancer.

Table 1. Peptide targets and mechanism of action.

Discussed below are examples of peptide-based vaccines and their targets that have been used to treat colon cancer. More examples are listed in **Table 1**. Beta human chorionic gonadotropin (βHCG) is not produced by normal colorectal cells. The increase in the expression of this antigen in colon cancer cells leads to an increase in tumor invasiveness, higher metastatic incidence and promotion of tumor growth, neovascularization, and immune system suppression. This makes it an attractive target for the development of an antibody-based vaccine [10]. Carcinoembryonic antigen (CEA) is an oncofetal antigen that can serve as a target for vaccine development. It is found overexpressed on the surface of colon cancer cells, with very low levels of expression on normal cells. Unfortunately this protein is normally expressed during fetal development and is therefore tolerated by the immune system. This led to the creation of an artificial CEA. CeaVac is based on anti-idiotypic antibodies and mimics CEA [10]. Another oncofetal protein 5T4 is a leucine-rich membrane glycoprotein. Once again it is nearly absent in normal tissues but is overexpressed in colon cancer cells and developing cells. Its presence is associated with poor survival. The drug TroVax uses 5T4 with a pox virus vector and a modified vaccinia virus. Preclinical trials in mouse models resulted in a 90% reduction of tumor burden [10].

Onyvax-105 is another anti-idiotype antibody mimicking the glycosylphosphatidylinositol-anchored protein CD55. CD55 regulates complement activation protecting cells against complement attack thereby enhancing tumor cell survival. The gastric acid–stimulating hormone gastrin is a hormone the precursors of which are overexpressed in colon cancer, where they act as growth factors. This leads to increases in angiogenesis and cell proliferation. Vaccines raised against this protein would therefore result in inhibition of cell growth, proliferation, and metastasis [10]. Onartuzumab is a mAb that targets human growth factor receptor (HGFR). It is a monovalent HGF antagonist antibody against MET Proto-Oncogene, Receptor Tyrosine Kinase that benefits patients who overexpress HGFR [30].

The FANG ™ vaccine consists of tumor cells from the patient and a plasmid expressing granulocyte-macrophage colony-stimulating factor (GM-CSF) and bifunctional short hairpin RNAfurin (bi-shRNAfurin). The growth and production of DCs are induced by GM-CSF. The

enzyme furin transforms precursor proteins into active proteins and the presence of bi-shRNAi furin inhibits the production of active proteins. This particularly inhibits the production of TGF β1 and 2 (TGFβ). Overexpression of TGFβ is associated with cancer progression and immune suppression by inhibiting GM-CSF and the consequent production of dendritic and other APCs. The vaccine therefore prevents the overexpression of TGFβ and leads to immune cell activation and the inhibition of cancer cell proliferation [30]. The vaccine was manufactured using GM-CSF and IL-13 to generate DCs from monocytes. The DCs were loaded with 6HLA-A*0201-binding peptides derived, among others, from CEA, MAGE-2 (melanoma antigen overexpressed in gastrointestinal cancer), and HER2/neu [10, 30].

TroVax is an attenuated strain of vaccinia virus that encodes the 5T4 protein. This protein is an oncofetal antigen and is a transmembrane glycoprotein. It is highly expressed in colon cancers and is virtually absent in normal tissue. The receptor is thought to play a role in metastasis and the expression level increases with the advancement of the stage of the cancer. This vaccine is able to induce an effective immune response, as it results in the formation of antibodies for both the 5T4 antigen and the viral particle [31].

2.3. Heat-shock proteins (HSPs)

HSPs are widely expressed in tumors, where they promote cancer progression. HSP 72 and glucose-regulated protein 96 (gp96) are two of these proteins that are highly expressed in colon cancer [32]. These proteins are thought to play a role in cell growth and signal transduction and expression of these proteins is higher in tumors undergoing metastasis. This makes them useful as diagnostic and prognostic markers. However, this expression is not related to patient survival [32].

HSPs enhance antigen-specific tumor immunity as they play an important role in the presentation of antigens to $CD8^+$ T cells through the MHC I pathway. This is because of the roles HSP 70s play as chaperones and in the transport of peptides to the heterodimeric transporters associated with antigen processing [33]. Similarly gp96 is a major chaperone involved in the lumen of the endoplasmic reticulum (ER), where it facilitates the folding of the MHC Iβ-2 microglobulin-peptide complexes in the ER [34].

Vaccines based on HSPs have been tested in animal trials and been found to be highly effective in the treatment of cancers [32, 35]. The function of the HSP in transporting and presenting other peptides as surface antigens has led many researchers to propose that HSPs can be used to create a HSP target protein fusion. This booster strategy would therefore enhance the ability of the target protein to be used as an antigen by T cells [36]. Two of these proteins that can be coupled to HSPs to improve their immunogenicity and usefulness as a cancer vaccine are alpha-fetoprotein (AFP) in hepatocellular carcinoma and CD44 in colonic carcinomas [32].

3. Targeted therapy: monoclonal antibodies

Recently, a new class of targeted agents have been identified, which bind to the ligand or the extracellular domain of a receptor. This results in alteration of intracellular signal transduction

pathways which will affect cell proliferation, dedifferentiation, inhibition of apoptosis, and stimulation of neoangiogenesis [37]. This section will look into VEGF-targeted drugs (e.g., ramucirumab (Cyramza®) and bevacizumab (Avastin®)), EGFR-targeted drugs (e.g., cetuximab (Erbitux®) and panitumumab (Vectibix®)), and others such as those that target kinases [37].

3.1. Bevacizumab and ramucirumab

VEGF is a potent angiogenic factor and functions by binding to one of three VEGF receptors located on endothelial cells and angioblasts. The VEGF receptor-2 is overexpressed on up to 50% of colorectal cancer cell surfaces. VEGF-A and other proangiogenic factors promote the degradation of the extracellular matrix. This enables proliferation and migration of endothelial cells [37]. The ligands of the VEGF family include VEGF-A, VEGF-B, VEGF-C, VEGF-D, and VEGF-E; and the receptors are VEGFR-1, R-2, and R-3. In colon cancer the ligand that is most abundant is VEGF-A [38]. Sustained angiogenesis is a hallmark of cancer; and targeted inhibition of blood vessel development is an established strategy for antitumor therapy [38]. Anti-VEGF therapies have been associated with a survival benefit across multiple malignancies including colon cancer [38].

Bevacizumab is a humanized mAb against VEGF and it acts by preventing ligand binding by binding to VEGF. This prevents downstream intracellular signal transduction; however, the response to bevacizumab appears to be independent of VEGF expression or high microvessel density (MVD) [37]. MVD assessment is a good predictor of metastasis, with selective antibodies, such as endoglin, distinguishing between tumor neovascularization and preexisting vessels. VEGF expression is highest in patients with metastatic tumors and the level is associated with cancer stage [38]. Bevacizumab is typically used in combination with other chemotherapeutic agents, and it is also indicated in improving the delivery of chemotherapy by changing tumor vasculature and decreasing the elevated interstitial pressure in tumors. The combination of therapies results in improved survival [38].

Ramucirumab is a fully humanized IgG1 mAb targeting the extracellular domain of VEGF receptor 2 (VEGFR2). Large-scale trials have indicated that ramucirumab shows promising antitumor effects and is well tolerated. The origin of this antibody was through the use of a large phage display library with tailored *in vitro* selection methods to identify a high-affinity antibody [39]. Measurement of VEGFA and soluble VEGFR1/2 during phase I trials of the antibody indicated that there is an increase in the expression of VEGF as well as a decrease in VEGFR1/2 levels. These changes were not dose related, which suggests that the receptor was saturated [39]. Phase I trial results were promising and phase II trials resulted in a high percentage of patients presenting with progression free survival at 6 months. Phase III trials showed an increase in overall patient survival [39]. Adverse reactions to ramucirumab included hypertension, vascular thrombotic events, and proteinuria [40].

Aflibercept is a recombinant fusion protein consisting of the second immunoglobulin (Ig) domain of VEGFR-1 and the third Ig domain of VEGFR-2, fused to human IgG1. It exhibits affinity for VEGF-A, VEGF-B, and PlGF. The antibody displayed effective activity against colon cancers with improvements in the primary endpoint of overall survival and overall response

rate, as well as displaying a high degree of tolerability in patients [40]. VEGFR-1 also plays a role in colon cancer and inhibiting its signaling could also play a role in cancer treatment. An antibody developed to target this receptor named IMC-18F1 has been developed. This is a high-affinity human VEGFR-1-neutralizing antibody that specifically binds the extracellular domain of VEGFR-1. It exhibits antiangiogenic and antiproliferative activity [40].

3.2. Panitumumab and cetuximab

The epidermal growth factor receptor (EGFR) is a target for the therapeutic monoclonal antibodies panitumumab and cetuximab the treatment of metastatic colorectal cancer. Panitumumab is a fully human Ig G2 mAb that binds the EGFR extracellular domain with high affinity and inhibits ligand-induced EGFR tyrosine phosphorylation, tumor cell activation, and tumor cell proliferation (**Figure 2**). Cetuximab is a chimeric human-mouse IgG1 mAb [41]. Cetuximab and panitumumab are both Food and Drug Administration (FDA) approved for advanced colorectal cancer therapy, and both have clear benefits for colon cancer treatment of most patients. The exception is those patients that carry *KRAS* mutations at codons 12 and 13 [42]. *KRAS* mutations occur in approximately 35–40% of colorectal tumors, and *KRAS* is a member of the rat sarcoma virus (Ras) gene family of oncogenes and is involved in integrating the signaling cascades controlling gene transcription, including many EGFR-mediated pathways [41]. The ligands of the EGFR transmembrane tyrosine kinase receptor include EGF, TGFα, epiregulin, amphiregulin, β-cellulin, and heparin. EGFR activates downstream signaling pathways such as the Ras/Raf/mitogen-activated protein kinase (MAPK) pathway, the phosphatidylinositol 3-kinase (PI3K)/AKT pathway, and the signal transducer and activator of transcription (STAT) pathway. These downstream pathways activate cellular survival, proliferation, invasion, metastasis, and angiogenesis. Abnormal activation of the EGFR signaling network due to excessive overexpression is common in colon cancer (**Figure 2**). EGFR is composed of an extracellular ligand-binding domain, a hydrophobic transmembrane region, and an intracellular domain with tyrosine kinase activity [41, 43].

Cetuximab competes with EGFR ligands, such as EGF or TGFα, with a high affinity ($K_d = 1 \times 10^{-10}$ M). This results in the inhibition of cell cycle progression and arrest of cell cycle in G1 phase, inhibition of angiogenesis, inhibition of metastasis by reduction of production of matrix metalloproteinase, inhibition of apoptosis, and potentiation of antitumor activities of chemotherapy and radiotherapy [43]. Panitumumab treatment results in improved clinical outcomes in patients with chemotherapy-refractory colon cancer [41]. Panitumumab also has a high affinity for EGFR ($K_d = 5 \times 10^{-11}$ M) and acts to arrest cell cycle progression and block cancer growth; however, it is an IgG2 and does not act through antibody-dependent cell cytotoxicity [43].

Using a single type of mAb to block a single transduction pathway may only have a limited effect, as tumors can shift to other alternate pathways. One solution is to combine monoclonal antibodies to block two signaling transduction pathways [44]. Use of multiple monoclonal antibodies has other advantages including limited overlapping toxicity and few to no pharmacokinetic interactions between antibodies. However, some studies indicate that certain combinations such as bevacizumab with cetuximab or panitumumab lead to shorter survival

and increased toxicity in advanced colorectal cancer compared to therapy with a single antibody [44]. The addition of panitumumab to chemotherapy improves clinical outcomes among patients with wild-type *KRAS* [41]. Other biomarkers that affect the efficacy of panitumumab include mutations in the *BRAF* and *PIK3CA* genes [41]. An important side effect of panitumumab treatment is the occurrence of skin toxicity; however, occurrences of skin toxicity correlate with favorable outcomes for patients [41, 43].

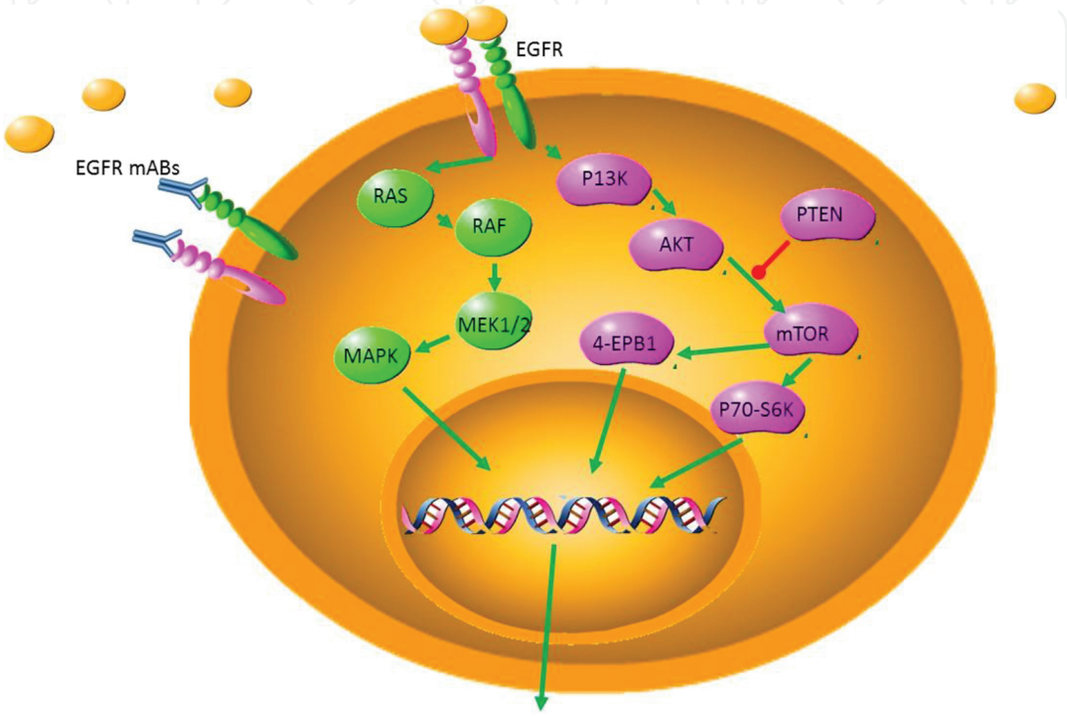

Figure 2. Mechanism of action of anti-EGFR mAbs. The binding of these mAbs on EGFR prevents the dimerization and the activation of EGFR.

4. Immune checkpoint inhibitors

During the progression of tumor development, the cancer cells undergo changes to escape immune surveillance. In order to accomplish this, a large enough number of the tumor cells must escape in order for there to be an equilibrium between tumor growth and tumor killing [1]. Normally, tumor-infiltrating lymphocytes (TIL) would control the progression of cancers; however, cancer cells can evade this response using a process known as T-cell exhaustion. This occurs due to the expression of inhibitory receptors. Blocking these receptors through the use of inhibitory molecules or monoclonal antibodies is known as immune checkpoint inhibition [45]. The immune system depends on multiple checkpoints to avoid overactivation in healthy cells. These inhibitory molecules expressed by the cancer cells take advantage of these checkpoints to escape detection by the immune system. They will often express molecules that serve as "immune checkpoints," by so doing; a message is sent to the immune system that an

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immune response is not necessary. Drugs are being developed to block immune checkpoint molecules from binding to their molecular partners, thus allowing the body to elicit an immune response and therefore attack cancer cells. An analysis of the expression patterns in colon cancers revealed a large overexpression of immune checkpoint-related proteins [46].

The programmed death-1 (PD-1) checkpoint is blocked in most cancers and blocking the pathway with antibodies to reactivate this checkpoint is a viable cancer therapy [47]. Recent insights indicate that blockade of the PD-1 checkpoint exists in many cancer patients and a repertoire of tumor-specific or tumor-selective T cells can be reactivated to achieve tumor therapy [48, 49]. Blocking the PD-1 pathway with antibodies results in durable tumor regression. Programmed death-ligand 1 (PD-L1) is a transmembrane receptor that plays a role in suppressing the immune system by suppressing the proliferation of CD8+ T cells and to lower the level of antigen particles by regulating apoptosis. PD-1, expressed on T cells, B cells, and other immune effector cells, interacts with this receptor, resulting in a negative signal to the T cell. Expression of PD-L1 in tumor biopsies shows that this pathway acts to block antitumor immune responses [46]. PD-1 has two ligands, PD-L1 and PD-L2. Tumor cells expressing PD-L1 are associated with poor outcomes for patients. Targeting these ligands prevent T-cell exhaustion and promotes T-cell recognition of tumors [45]. PD-L1 also binds to the co-stimulatory molecule CD80, implying that CD80 has the potential to facilitate antitumor immunity by inhibiting the PD-1 suppressive pathway [47].

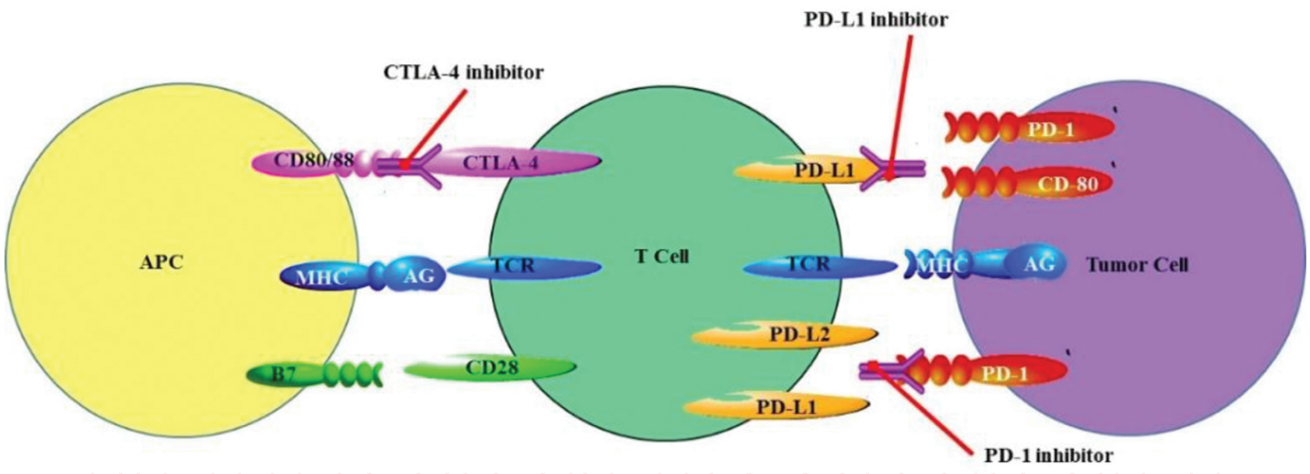

Figure 3. Immune checkpoint interactions and antibody-based inhibition on T cells. CTLA-4 inhibition can be performed by inhibiting CD28 co-stimulation (through binding with its ligands CD80 or CD86) that is required to complete T-cell activation. The PD-1/PD-L1 pathway can be inhibited by targeting its inhibitory role. Here it interacts with PD-L1 on the tumor cell. Inhibiting this interaction results in a more robust targeted antitumor immune response.

Ipilimumab is a mAb that targets CTLA-4 (cytotoxic T-lymphocyte-associated protein 4), which normally negatively regulates the activity of T cells. This antibody was the first checkpoint-blocking antibody to be approved by the US FDA for cancer treatment; CTLA-4 is a member of the Ig superfamily of receptors, which also includes PD-1, TIM-3 (T-cell Ig and mucin domain-containing protein 3), BTLA (B and T-lymphocyte attenuator), and VISTA (V domain Ig suppressor of T-cell activation). Use of this drug enhances the antitumor activity of

CD8 T cells and inhibits the suppressive function of Tregs [45]. This was followed by a second antibody, pembrolizumab, which targets the programmed death 1 (PD-1; CD279) molecule [50] (**Figure 3**). Trials of these antibody therapies have only shown modest clinical benefits, and this may indicate that tumors use multiple and nonoverlapping immunosuppressive mechanisms to evade the immune response [45]. Multiple studies indicate that effective therapy involves the targeting of multiple immunosuppressive pathways [51].

Tumor cells can also evade the immune system through the production of extracellular adenosine by CD73 which is expressed on lymphocytes and endothelial and epithelial cells. CD73 performs an endothelial cell barrier function, protecting cells from ischemia and regulating immune responses. This receptor is overexpressed in many types of cancer, with high CD73 expression being associated with poor outcomes for patients due to increases in tumor immune escape and metastasis [45]. Blocking CD73 can induce potent antitumor immune responses. Additionally the inhibition of molecular pathway components upstream of CD73 such as CD39 also has similar therapeutic effects. This treatment can also be used to supplement immune checkpoint therapies that make use of anti-CTLA-4 and anti-PD-1 mAbs, to increase the effectiveness of such therapies [45].

Another strategy to target immune checkpoints is the use of small molecule drugs that target critical survival pathways. These include Gleevec and ibrutinib, both of which are tyrosine kinase inhibitors. Ibrutinib is a covalent inhibitor of BTK (Bruton's tyrosine kinase), a key enzyme in B-cell receptor signaling [49].

Controlling the immune response to cancer cells through the use of anti-inflammatory drugs to control the inflammatory components reduces the risk of developing certain types of cancer. Aspirin is able to reduce the incidence of colon cancer and slow down tumor progression. Cyclooxygenase (Cox) enzymes 1 and 2 are the targets of aspirin. These enzymes are overexpressed in tumor cells. Immunosuppressive drugs such as cyclosporine A (CsA) and tacrolimus (FK506) inhibit the calcium/calmodulin-dependent phosphatase calcineurin, which acts upon members of the nuclear factor of activated T cells (NFAT) [52]. These transcription factors are important for cytokine production by T cells and are required for the normal function of B cells, DCs, and mast cells. Expression of NFAT family members leads to tumor suppression [53]. Treatment of tumor cells with CsA is capable of inducing necroptosis and a mild G0/G1 cell cycle arrest [52].

5. Cytokines

Cytokines are signaling proteins produced by white blood cells that help control the growth and activity of immune system cells. The two types of cytokines that are used in the treatment of cancer are IFNs and ILs. Cytokines stimulate a broad-based immune response as opposed to generating a targeted response to a specific antigen [54]. Tumors secrete factors to recruit inflammatory cells and/or activate stromal cells. Inflammation plays a major role in tumor promotion and progression. The soluble factors that drive inflammation are cytokines and

chemokines produced by tumor cells themselves and by the cells recruited to the tumor microenvironment [55].

Several cytokines are capable of activating and recruiting specific immune cells that can enhance antitumor immunity; these include IL-2, IL-12, IL-15, TNFα, and GM-CSF. These cytokines can be used as single-agent therapies or in combination with other immunotherapeutic strategies. GM-CSF immunization leads to APC recruitment. Tumors activate Stat3 and Braf, which leads to the release of IL-10, inhibiting the tumoricidal activity of NK cells. Stat3 activation in DCs leads to these cells becoming tolerogenic DC [11].

Additionally, TNFα, hepatocyte growth factor, PDGF, and FGF19 activate Wnt/β-catenin signaling in tumor cells. This is the oncogenic pathway activated in the majority of colon cancers. This pathway results in β-catenin accumulation in the cytoplasm, which activates cell growth and differentiation pathways. IL-1β is a potent activator of Wnt signaling in colon cancer cells leading to increased survival of colon cancer cells [55].

Oncogenic signaling through the Wnt and NF-kB pathways is activated through TNFα. Pharmacological inhibition of TNFα by neutralizing TNFα antibodies has been used to treat both irritable bowel disorders and colon cancer. Results from trials using enbrel or remicade suggest that these neutralizing antibodies have activity against colon cancer cells. TNFα signaling initiates NF-kB signaling. NF-kB is continuously expressed in certain tumors, leading to enhanced survival by protecting the tumor cells from apoptosis. Treatment with the TNFα antagonist, etanercept led to inhibition of Wnt/β-catenin6 signaling as seen by the , reduced expression of active β-catenin [55].

5.1. Interleukins 1β, IL-6, and IL-1

The proinflammatory cytokine IL-1β is produced by activated macrophages. In turn, IL-1β induces the expression of TNFα, IL-6, IL-8, IL-17, Cox-2, and PGE2, promoters of tumor cell growth. Inducing the expression of IL-1β leads to increased incidence of cancer in wild-type mice. The IL-1β signaling pathway functions through the receptors IL-1RI and IL-1RII to induce NF-kB activity. The pathway involves the two adaptor proteins, MyD88 and IRAK. Macrophages are stimulated to release IL-1β and activate NF-kB and Wnt pathways, but IL-1β signaling requires STAT1. The silencing of STAT1 expression leads to decreased IL-1β release and prevented cancer cell growth [55].

IL-6 is secreted by stimulated monocytes, fibroblasts, and endothelial cells, macrophages, T cells, and B lymphocytes. Macrophages are stimulated by colon cancer cells to produce IL-6 and activate STAT3 in tumor cells. Inhibition of IL-6 signaling interferes with the growth of tumor cells and protects them from apoptosis. Research indicates that decreasing the expression or inhibiting the activity of STAT3 may have adverse effects on tumor promotion. Targeting STAT3 will affect the expression of β-catenin and the co-expression of STAT3 and β-catenin is associated with poor survival of colon cancer patients [55].

A subset of T-helper cells produces the cytokines IL-17, IL-22, and TNFα (Th17 cells). Paneth cells also produce IL-17. Th17 cells require IL-6, TGFβ, IL-1β, and IL-23, while IFN-γ and IL-4 negatively regulate differentiation of Th17 cells. IL-17 induces IL-6 and STAT3, promoting the

survival of cancer cells. This cytokine may also have an anticancer function by enhancing antitumor immunity [55].

5.2. Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)

TRAIL (also known as Apo2L) activates the apoptotic cascade. Tumor cells can evade this apoptosis signal through the action of β-catenin. TRAIL's role in tumor surveillance has been confirmed in knockdown experiments and is a promising candidate to be used in cancer therapy, because it selectively kills cancer cells while leaving normal cells unharmed [55].

Sorafenib a Raf kinase inhibitor sensitized A TRAIL –resistant colon cancer line to TRAIL-induced apoptosis by preventing NF-kB-dependent expression of the antiapoptotic genes, IAP2 and MCl-1.

6. Oncolytic virus (OV) therapy

Over a century ago, researchers observed that viral infection both in human and animal models results in the expression of targets that can be recognized by T cells and/or antibodies. Subsequently, vaccination has been used to treat an array of diseases such as hepatitis B virus and human papillomavirus 15 which can cause liver and cervical cancer respectively. Vaccination against infections is used to induce neutralizing antibodies that act prophylactically. However, with regard to cancer vaccination, cancer vaccine candidates should induce and expand immune responses that can cause disruption of biological pathways that support cancer growth.

The concept of cancer immunotherapy is based on the ability of the immune system to recognize cancer cells and affect their growth and replication. Researchers have observed that cancer regression would occur spontaneously in patients after viral infection [56, 57]. For example, studies conducted by Lindeman and Klein 1967 showed that oncolysis of tumor cells by influenza virus increased immunogenicity of tumor cell antigens. The recent advances in successful sequencing of the cancer genome together with insights into how tumors evade the immune system have led cancer research to evolve from searching for a gene that causes individual cancer to one that blocks or disrupts biological pathways that support cancer growth [58, 59]. As a result, cancer vaccines are now being designed with the aim to boost the immune system to protect itself from carcinogenesis and progression of cancer. In 2010, the FDA approved Provenge which is a therapeutic vaccine for cancer [60]. It is designed to treat advances in prostate cancer and has shown to increase the survival rate. The success of Provenge resulted in stimulating the interest in the development of other therapeutic cancer vaccines.

In recent years, OV has been shown to be effective in treating cancer in both preclinical models and clinical trials. Toda and coworkers showed that genetically modified oncolytic HSV G207 is a potential cancer vaccine for induction of specific antitumor immunity in CT26 colon cancer cells [64, 61, 62]. This type of immunotherapy is largely dependent on the network of the host

immune system to fight cancer by (i) boosting the patient's immune system, (ii) decreasing cancer-induced immunosuppression, and (iii) increasing the immunogenicity of the tumor itself [63, 64]; OVs can be RNA- or DNA-based virus derived from human or animals.

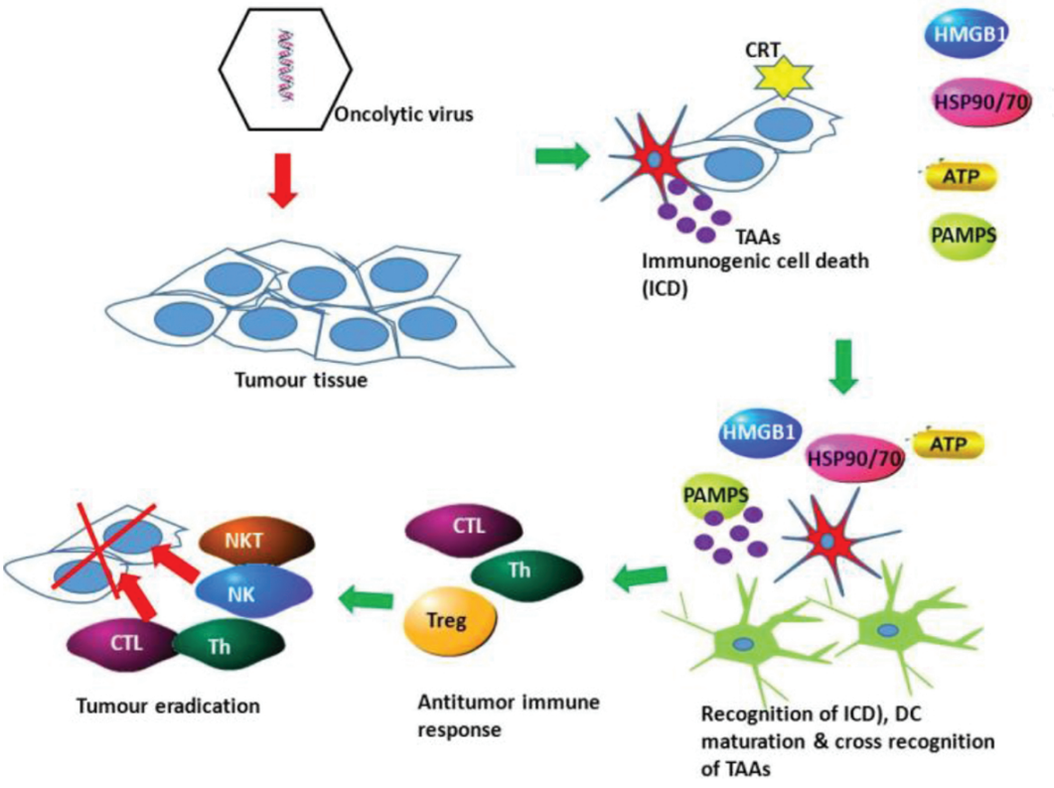

Figure 4. Immunogenic cell death of cancer cells induced by oncolytic viruses. An oncolytic virus selectively replicates in tumor cells, leading to induction of the death of these cells, presenting destruction signals on the cell surface and consequent release of danger signals from necrotic cells. Apoptotic bodies are engulfed by APC, and TAAs are processed and presented along with MHC complex and co-stimulatory molecules. The released DAMPs (and PAMPs) activate and mature DCs, and TAAs are cross-presented to naive T cells. This process can be further enhanced at different steps by other immunomodulatory agents (in a combination strategy). The resulting cytotoxic immune response against tumor and associated stromal cells, involving CD4+ and CD8+ T cells, may help in complete eradication of tumor mass. Additional immunotherapies targeting DCs, T cells, and the immunosuppressive TME can further enhance this antitumor immune response.

OV selectively infects and replicates in cancer and associated endothelial cells and subsequently kills these abnormal cells without harming the normal cells. The selectivity of OV can be an inherited feature of the virus or due to genetic engineering [56, 65]. OV therapy has multiple antitumoral activities including direct effect by cytotoxic cytokines released upon infection by tumor residents or infiltrating immune cells [66, 67]. The lysis-dependent cytoreductive activity activates innate immune receptors when immunogenic cell debris is taken up and cross presented by APCs (**Figure 4**).

OVs also directly affect various signaling pathways which are implicated in cancer such as Ras, Wnt, anti-apoptosis, and EGFR [66, 68, 69]. The altered signaling pathway creates a favorable environment for OV replication resulting in Cells infected with these viruses

showing sustained proliferation, resisting cell death, evading growth suppressors and escaping immune surveillance. Cancer cells also show increased genomic instability and DNA damage stress, which is favorable to OV replication [70–72]. Genetic manipulation of OV enables these viruses to be (i) safe for use as a vaccine, (ii) highly selective for specific cancer type, and (iii) altering virus tropism. In comparison with current regimes for cancer treatment, OVs are advantageous because (i) they have a low chance for generation of resistance because they use multiple ways to exert cytotoxicity and (ii) virus dose in a tumor increases with time due to in situ virus replication whereas in the classical drug Pharmacokinetics, dose decreases with time [71, 73].

The major drawbacks in the use of OV include nonimmune human serum, development of anti-OV antibodies resulting from the use of human virus, and appropriate delivery into the tumor. Various delivery mechanisms have been explored to enable delivery of OV to tumor cells. For an example, cell carriers such as neural stem cells and myeloid-derived suppressor cells have been used to deliver OV to specific tumor cells. The cells protect the virus from anti-OV antibody neutralization, thereby facilitating virus deliver [71, 73]. In using OV to treat colon cancer, ONYX-015 has advanced to phase II clinical trials and is used in combination with chemotherapy [74, 75]. Recently, adenovirus 5 (PSE-EA1 and E deleted) has been approved to treat prostate cancer in China giving hope to development of OV as an alternative cancer treatment.

7. Bispecific antibody

Antibody-based therapy has been explored in treating a range of diseases and is promising to be a success with the FDA having approved more than 13 monoclonal antibodies for treatment of cancer (see Section 2). Furthermore, over 100 antibodies are at different stages of clinical trials. Medical researchers have explored the properties of antibodies which are (i) highly specific in binding their targets and (ii) are nontoxic for medical application using technologies such as hybridoma and phage display for antigen targeting [76–79]. Also, new technologies have been employed to manipulate antibodies for wide application. For example, the conventional antibody which is made up of two identical pairs of heavy and light chain linked together by disulfide bonds is monospecific and bivalent. Using a hybridoma technology, a fusion can be created between two hybridoma resulting in quadromas with two different heavy and light chains as a result of random pairing, thus forming molecules that do not occur in nature (**Figure 5A**). Antibodies produced by these methods have an ability to bind different species but could also be nonfunctional [77, 79, 80].

Conventional antibodies posed various challenges to therapeutic application due to inadequate exposure to the tumor as a result of their size (150 kDa) and impaired interactions with the immune system. Using enzyme-based antibody digestion, full antibodies may be truncated into Fc and Fab regions (**Figure 5A**). The Fab region which has the antigen-binding domain of the antibody is then used for therapeutic application. The drawback in using the Fab region of the antibody is its reduced half-life due to renal clearance [81, 82]. In addressing these

challenges, bispecific antibodies (BsAb) were developed in 1961 [83]. Just as their name implies, this class of antibodies binds two different antigens or two different epitopes on the same region.

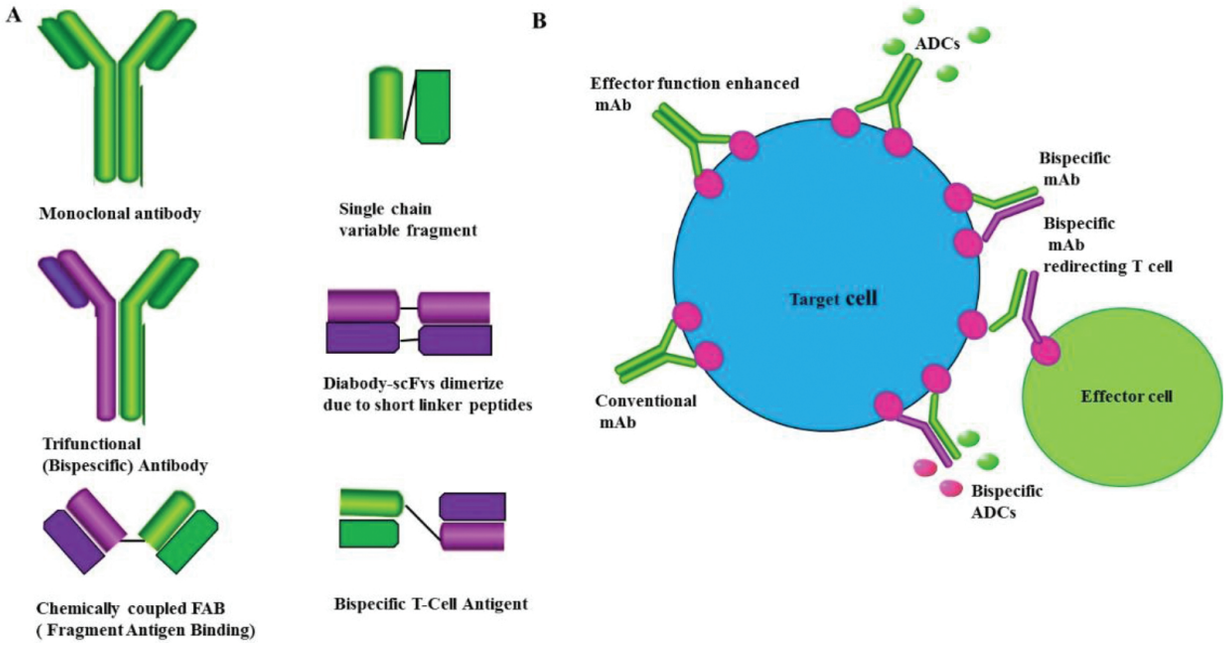

Figure 5. (A) Different forms of bispecific antibody. Trifunctional antibodies consist of two heavy and two light chains from two different antibodies. This results in an antibody with binding sites for two different antigens as well as an Fc region made up of two heavy chains forming a third binding site. A diabody consists of scFvs with very short linker peptides that force the closely positioned variable regions to fold together, forcing the scFvs to dimerize. Chemically coupled Fabs consist of the antigen-binding regions of two different monoclonal antibodies linked by a chemical means. Bispecific T-cell antigens are fusion proteins of two scFvs from four separate genes. (B) Development of bispecific compounds. Bispecific compounds enable simultaneous inhibition of two cell surface receptors, simultaneous blocking of two ligands, cross-linking of two receptors, and/or the recruitment of T cells to the proximity of tumor cells (redirected immune cell killing).

BsAb represent a class of antibodies that are yet to be fully explored in the treatment of cancer and other diseases. BsAb have a greater potential therapeutic efficiency than mono-targeted therapy, since they allow simultaneous engagements of two targets and limit potential escape pathways [84–86]. Numerous studies have shown that there is evidence of cross talk between receptor tyrosine kinases such as MET, VEGFR, and IGFR-IR which are known to promote cancer progression and drug resistance. And patients with colon cancer are known not to respond to anti-EGFR drugs with resistance emerging after initial usage [87, 88]. Engelman et al. showed that MET amplification leads to gefitinib resistance by activating the ERBB3 pathway, showing the complexity of tumor signaling pathways and a need to treat patients with drugs that target multiple targets [89].

In the use of BsAb, T cells are targeted because of their high cytotoxic retention, abundance in bloodstream, surveillance function, and proven ability to control malignant diseases [90, 91]. During cancer progression, cancer cells escape immune recognition by interfering with antigen presentation or T-cell activation or differentiation. In using the bispecific antibody, most

targeted antigens for tumor therapy are differentiation antigens such as CD19, CD33, CEA, EpCAM Epithelial cell adhesion molecule, PMSA Prostate-specific membrane antigen , and EGF receptors. In most cases these antigens are overexpressed in cancer cells compared to the normal cells.

Blinatumomab is an example of a bispecific antibody that has shown great promise clinically in cancer patients. Blinatumomab is a 55 kDa-fusion protein comprised of two single-chain antibodies to CD19 and CD3, recombinantly joined by a flexible, non-glycosylated five-amino acid non-immunogenic linker that affords a very short distance between arms [92, 93]. Blinatumomab has high affinity for CD19 which is important in sustaining the malignant B-cell phenotype via mechanisms of proliferation, cell survival, and self-renewal [94, 95]. It draws malignant B cells in close proximity to CD3-positive T cells without regard to TCR specificity or reliance on MHC class I molecules on the surface of APCs for activation. The nonspecific binding of the polyclonal T-cell population prevents resistances to T-cell-based therapies as a result of downregulation of MHC molecules. CD19 and CD3 binding results in T-cell activation, marked by upregulation of T-cell activation markers CD25, CD69, CD2, IFN-γ, TNFα, and IL-2, IL-6, and IL-10 [96]. Cell lysis is mediated by secretion of perforin and various granzymes stored in the secretory vesicles of cytotoxic T cells [97]. In vitro data suggest that efficacy of blinatumomab is not compromised or dependent upon T cells, which may be limited in number in heavily pretreated patients [98]. Also blinatumomab-activated T cells appear to effectively induce serial target cell killing [92, 93].

8. Adoptive immunotherapy

In the wake of cancer treatment challenges or therapies, adoptive immunotherapy is one of the novel strategies being researched for cancer treatment. This concept was presented five decades ago [99–101] and is based on the transfer of ex vivo expanded antitumor CD8 T cells into affected patients (**Figure 6**). Delorme and Alexander [101] showed that the transfer of immune lymphocytes could inhibit the growth rate of carcinogen-induced sarcoma.

The immune system is responsible for the prevention of tumors or elimination of pathogens that can cause inflammation or an inflammatory environment for tumorigenesis or destroy tumor cells expressing TSAs or molecules induced by stress [102, 103]. Therefore, tumor development and progression are largely dependent on the patient's immune system to effectively inhibit cancer growth using its network of immune cell types. Each and every cell type has a specific function in inhibiting tumor growth (**Figure 7**). Consequently, the success of adoptive immunotheraphy depends on approaches which will target different immune subsets.

Adoptive immunotherapies have explored the use of infiltrating T cells (CD8+ effector T cells and CD8+ effector memory cells), NK cells, and IL-2 for cancer-targeted therapies. The T cells are able to destroy tumor cells using cytotoxic granules containing perforin and granzymes and by using cell surface receptor such as TNF-related apoptosis-inducing ligand [104, 105]. Studies using mice have shown that adoptive transfer of T cells successfully induces antitumor

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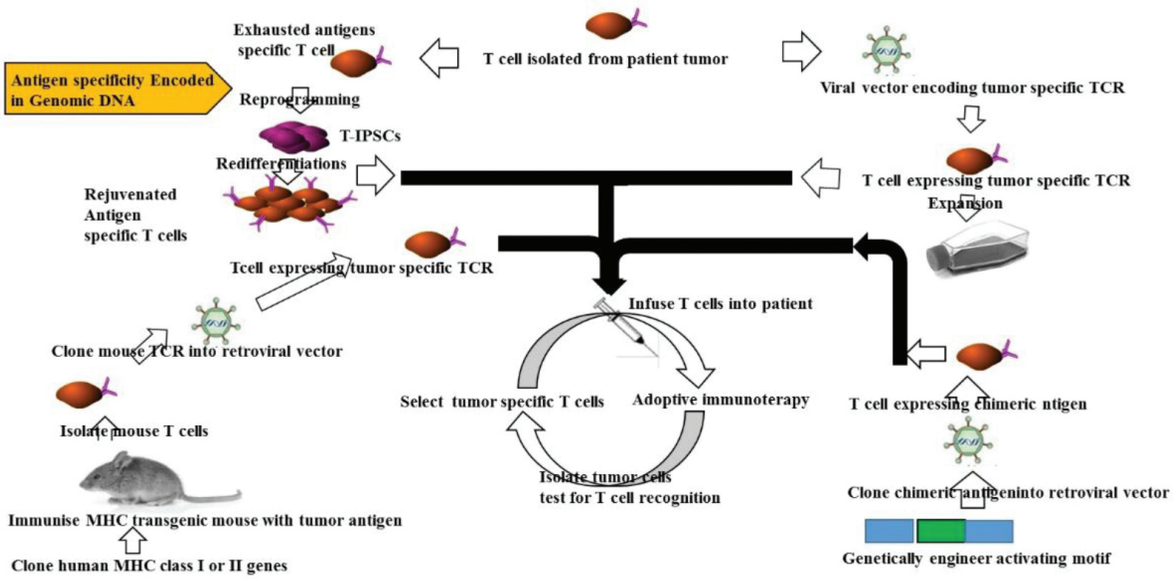

Figure 6. Different techniques used in adoptive immunotherapy. Adoptive immunotherapy with functional T cells can be performed in numerous ways. Exhaustion of antigen-specific T cells which can be a major problem in the practical application of this therapy can be solved through reprogramming clonally expanded antigen-specific $CD8^+$ T cells and then redirecting their redifferentiation into $CD8^+$ T cells possessing antigen-specific killing activity. T cells can be isolated from the patient. Isolated peptide antigens could be used to stimulate T cells that are already present in the patient's tumor or be used to prime tumor-specific T cells. If the T-cell populations generated are specific for the patient's tumor, they could be expanded and adoptively transferred if they are of human origin. T cells can be genetically engineered to recognize TAAs. TCRs from T cells that show a good antitumor response can be cloned and inserted into retroviruses, which are used to infect autologous T cells from the patient. Chimeric antigen receptors (CARs) can be generated through genetic engineering and then cloned into a retroviral vector and used to infect T cells from the patient. TCRs can also be isolated from humanized mice that express human MHC molecules and can be immunized with the tumor antigen of interest. Mouse T cells can then be isolated, and their TCR genes are cloned into recombinant vectors that can be used to genetically engineer autologous T cells from the patient.

response. Also, only a small number is required to mediate effective regression of tumor and survival [106–108]. Genetic modification of T cells has been successfully used to broaden their effective application by pairing with antigen receptors that recognize a range of different TAAs. Genetic engineering has also been employed to alter T cells so that they are able to avoid or be resistant to immune invasion strategies used by tumors such as the production of cytokines. Another modification of T cells involves attaching stimulatory signals for their activation [109, 110].

NK cells target and kill diseased cells using various mechanisms such as perforins and granzyme. The use of NK cells was first explored by Rosenberg et al. [111]. Lymphokine-activated killer cells were co-administered with IL-2 and resulted in a positive response in people with metastatic cancer [111]. Another combination of chemotherapy with transfer of allogeneic NK cells resulted in disease remission [112]. It is anticipated that a hybrid of T and NK cell will have great potential in the treatment of cancer using adoptive immunotherapy. However we are still a long way from the development of such a model treatment that can be developed for clinical trials and introduced into clinical practice.

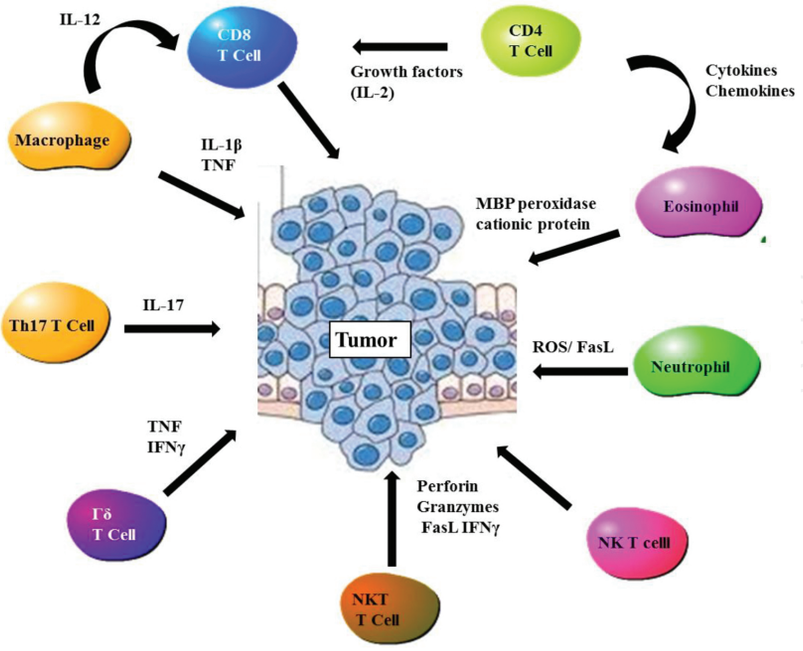

Figure 7. White blood cell types and mechanisms of response against tumors. The diagram illustrates all of the different immune-related cell types including T cells, NK, macrophages, eosinophils, and neutrophils that are able to respond against cancer cells. The method of response against the tumor cells is also indicated. In some cases these cell types can cooperate to produce additional responses.

9. Conclusion

The final goal of immunotherapeutic strategies to treat colon cancer would be the development of tumor-specific therapies that can be used in conjunction with standard chemotherapies with little side effects. The use of various combinations of different antibodies and OVs with synergistic antitumor activity and reduced toxicity will aim to achieve durable tumor eradication. One of the main obstacles is the identification of tumor-specific and essential tumor antigens. These antigens may differ with different tumors. In terms of checkpoint inhibition, it is important to establish the correct level of inhibition each patient requires to minimize toxicity. Another important goal is the identification and development of biomarkers to serve as prognostic markers for the monitoring of the individual patients response to immunotherapy, allowing for the identification of those patients who are most likely to benefit from these treatments. These goals require extensive further studies to refine immunotherapeutic strategies and combinatorial approaches.

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