Review

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CAR-T Therapy for the Treatment of Colorectal Cancer

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Colorectal cancer (CRC) is one of the most common malignancies worldwide. Advanced CRC has a poor prognosis, with treatment primarily relying on chemotherapy combined with targeted therapies. Currently, immunotherapy based on immune checkpoint inhibitors is reserved exclusively for mismatch repair-deficient (dMMR) or microsatellite instability-high (MSI-H) tumors, which represent less than 10% of advanced CRC cases. Chimeric antigen receptor (CAR)-T cell therapy is a type of adoptive cell therapy involving modified T-lymphocytes engineered to express chimeric antigen receptors, enabling them to recognize surface antigens expressed by tumor cells. CAR-T cell therapy has demonstrated efficacy in treating hematological malignancies such as lymphoma, myeloma, and leukemia. However, its efficacy in solid tumors remains limited due to several limitations such as antigen heterogeneity, restricted CAR-T cell trafficking into the tumor area, and the presence of an immunosuppressive tumor microenvironment. Developing novel CAR-T cell therapies for solid tumors represents an unmet need, particularly for cases where immune checkpoint blockade is ineffective, such as CRC. Preclinical studies have shown the efficacy of various CAR-T cell models targeting a wide range of tumor-associated antigens in CRC, both *in vitro* and *in vivo*. Despite these promising results, the clinical efficacy of CAR-T cell therapy for CRC has been limited in early-phase clinical trials. Factors such as trial design or tumor characteristics, including antigen heterogeneity and the immunosuppressive microenvironment, should be considered. The development of innovative CAR-T cell models and the identification of novel antigens may improve the effectiveness of CAR-T cell therapy for CRC patients.

Keywords: colorectal cancer; tumor-associated antigen; chimeric antigen receptor; immunotherapy; CAR-T cell therapy

Introduction

Colorectal cancer (CRC) is one of the most common solid neoplasms worldwide. In 2022, CRC ranked as the third most incident malignant tumor and was the second leading cause of cancer-related deaths [1]. When diagnosed in its early stages, CRC is amenable to curative therapeutic strategies and has a favorable prognosis, with 5-year survival rates ranging from 45 to 99%, depending on the stage at diagnosis [2,3]. Conversely, patients with advanced CRC are rarely candidates for curative therapies, resulting in a poor prognosis with 5-year survival rates below 20%.

The treatment of advanced CRC primarily relies on systemic therapies combining chemotherapy with targeted therapies, such as antiangiogenics or anti-epidermal growth factor receptor (EGFR) monoclonal antibodies, depending on the primary tumor location and molecular biomarkers [4,5]. While immunotherapy with checkpoint inhibitors has demonstrated remarkable results and is widely used for various solid tumors [6], its administration in advanced CRC is exclusively reserved for mismatch repair-deficient (dMMR) or microsatellite instability-high (MSI-H) tumors, which represent less than 10% of all advanced CRC cases [7–9].

Previous clinical trials investigating the role of checkpoint blockade in refractory mismatch repair-proficient (pMMR)/microsatellite stable (MSS) advanced CRC failed to demonstrate an improvement in overall survival for this population [10–12]. Recently, a combination of cytotoxic T-lymphocyte antigen 4 (CTLA4)/programmed cell death protein 1 (PD1) blockade exhibited anti-tumor activity in pMMR/MSS advanced CRC; however, the presence of liver metastases negatively impacted its efficacy [13]. Additionally, pMMR/MSS advanced CRC patients treated with an anti-programmed death ligand 1 (PDL1) antibody (atezolizumab) in combination with chemotherapy and antiangiogenic therapy showed better outcomes when the Immunoscore Immune-Checkpoint status was high [14].

Given the limited efficacy of immune checkpoint blockade for treating this population, improved patient selection based on innovative biomarkers and the development of novel alternatives, such as adoptive cell therapy,

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may help extend immunotherapy options to pMMR/MSS advanced CRC patients.

Overview of CAR-T Cell Therapy

Chimeric antigen receptor (CAR)-T cell therapy is a type of adoptive cell therapy based on engineered T cells expressing a CAR construct that enables them to recognize tumor-associated antigens (TAAs) and acquire anti-tumor activity [15]. CARs are synthetic receptors composed of four different domains: an antigen-binding domain, a spacer domain or hinge region, a transmembrane domain, and an intracellular signaling domain (Fig. 1) [16].

The antigen-binding domain is typically derived from the variable heavy and light chains of monoclonal antibodies, forming a single-chain variable fragment (scFv). The scFv allows for the recognition of surface antigens in a major histocompatibility complex (MHC)-independent manner [17]. In order to enhance CAR-T cell efficacy, novel antigen-binding domains different from scFvs are emerging, such as nanobodies, natural ligands, designed ankyrin repeat proteins (DARPins), and *de novo*-designed proteins [18].

The complexity of the intracellular domain defines the generation of the CAR (Fig. 2). First-generation CARs harbor a single intracellular signaling domain, usually CD3ζ. Second-generation CARs include two intracellular domains: a signaling domain and a costimulatory domain, such as CD28 or 4-1BB/CD137. The choice of the costimulatory domain impacts CAR-T cell activation, expansion, and persistence. Third-generation CARs harbor an additional costimulatory domain, typically combining both CD28 and 4-1BB [16,19].

Moreover, new CAR constructs are being developed, such as fourth-generation CARs, also known as armored CAR-T cells or T-cells redirected for universal cytokine-mediated killing (TRUCK-T). Fourth-generation CAR-T cells are armed with cytokine signals to enhance anti-tumor activity [20].

The overall process of CAR-T cell manufacturing encompasses the following stepwise phases: patient leukapheresis, T cell activation and expansion, genetic modification of T cells, and further expansion [21]. *Ex vivo* processing conditions of T cells, such as culture media selection or cytokines involved in T-cell expansion, can impact the functionality of CAR-T cells [22].

Different approaches can be employed for T cell engineering; however, viral vectors (lentivirus or γ-retrovirus) have been the most widely used for CAR transgene transduction. Novel non-viral transfection methods, such as membrane permeabilization-based or carrier-based methods, are being developed for CAR-T cell manufacturing. The application of these novel transfection methods can lead to cost-effective T-cell engineering of CAR-T cells with a better safety profile [23].

CAR-T cell delivery methods include intravenous administration and locoregional strategies, such as intratumoral, intrapleural, or intraperitoneal infusion [24]. Before CAR-T cell infusion, administration of lymphodepleting chemotherapy is advised. The aims of lymphodepleting chemotherapy are to reduce endogenous lymphocytes, downsize the tumor, and reprogram the tumor microenvironment, therefore improving CAR-T cell engraftment, survival, and efficacy [25].

Challenges and Recent Advances of CAR-T Cell Therapy for Solid Tumors

CAR-T cell therapy has achieved groundbreaking results in the treatment of hematological neoplasms such as lymphoma, leukemia, and myeloma [26]. In contrast, the efficacy of CAR-T cell therapy for the treatment of solid tumors has been hindered by multiple tumor-related factors.

For solid tumors, adequate target selection is particularly challenging. CAR-T cell efficacy is affected by tumor heterogeneity, as some tumor cells may not express the target antigen, and by common on-target, off-tumor effects, which limit treatment intensity [27]. The immunosuppressive tumor microenvironment and limited CAR-T cell penetration into the tumor also affect CAR-T therapy. Novel CAR-T cell designs, such as armored CAR-T cells, may improve CAR-T cell penetration and persistence and help overcome immunosuppression driven by the tumor microenvironment [28].

Despite these limitations, recent clinical trials have shown promising results. Administration of CAR-T cells targeting claudin18.2 (CLDN18.2) in patients diagnosed with advanced gastrointestinal malignancies, mainly gastric cancer, demonstrated high overall response (38.8%) and disease control (91.8%) rates in a phase 1 trial [29]. Additionally, another phase I trial reported tumor regression and clinical benefit following sequential intravenous and intracerebroventricular administration of CAR-T cells targeting disialoganglioside GD2 in patients with histone 3 lysine27-to-methionine (H3K27M)-mutant diffuse midline glioma [30].

These results highlight the therapeutic potential of adoptive cell therapies for treating solid tumors. Further development of novel CAR-T cell therapies against these malignancies is needed and offers an opportunity to expand the use of immunotherapy for cases in which immune checkpoint blockade is ineffective, such as pMMR/MSS CRC. In this review, we provide a broad summary of recent advances in CAR-T cell therapy for CRC treatment in both preclinical and clinical settings.

CAR-T Cell Therapy for CRC in the Preclinical Setting

Several preclinical studies have developed novel CAR-T cell therapies using different tumor antigens as tar-

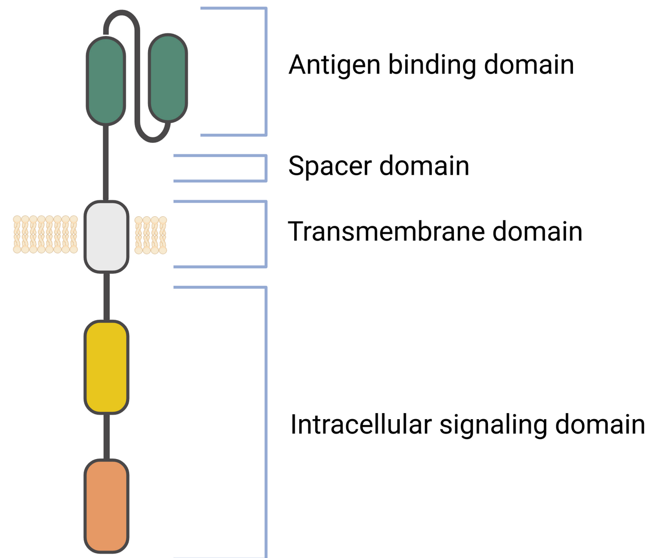

Fig. 1. Chimeric antigen receptor (CAR) structure. CARs are composed of four different domains. The antigen-binding domain confers specificity to the CAR and typically is a single-chain variable fragment (scFv). The spacer domain connects the antigen-binding domain to the transmembrane region. The intracellular signaling domain transduces the activating signal and may contain costimulatory domains. Created in BioRender. Labiano, I. (2025), https://BioRender.com/h85w923.

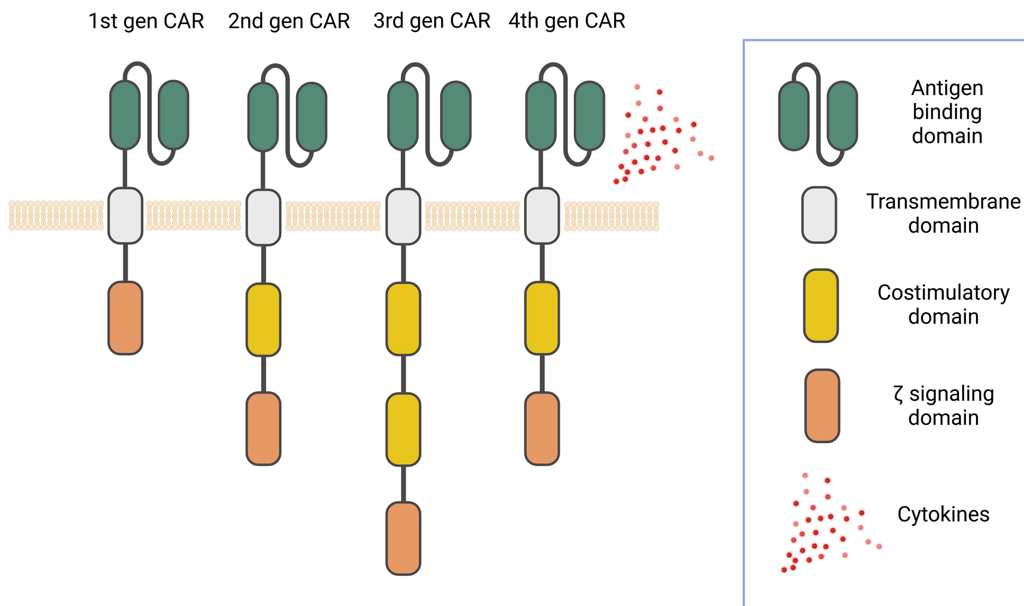

Fig. 2. Evolution of CAR-T cell generations. First-generation CARs are composed of an antigen-binding domain, a spacer domain, a transmembrane region, and an intracellular signaling domain. Second-generation CARs comprise two intracellular domains, the signaling domain and a costimulatory domain (usually CD28 or 4-1BB). Third-generation CARs have three intracellular domains, as they incorporate an additional costimulatory domain. Fourth-generation CARs are engineered to induce cytokine secretion. Created in BioRender. Labiano, I. (2025), https://BioRender.com/w27a681.

gets for CRC (Table 1, Ref. [31–59]). These studies mainly generated second or third-generation CAR constructs and scFvs were the most frequently used antigen-binding domain. The efficacy evaluation of the CAR-T cells generally implied the use of *in vitro* cytotoxicity assays and evaluation of tumor growth and/or survival in *in vivo* models.

Carcinoembryonic Antigen (CEA)

CEA is a membrane-bound glycoprotein strongly positive in CRC and differentially expressed compared to healthy tissue [60,61]. Thus, it has been considered an adequate target antigen for CAR-T cell therapy. Second-generation CEA-CAR-T cells designed by Chi *et al*. [31]

Table 1. Overview of preclinical studies evaluating CAR-T cell therapy in CRC.

Target	CAR Generation	Publication	Antigen-binding domain	Efficacy experiments in CRC
CEA	2nd	Chi, 2019 [31]	scFv	*In vitro*, CDX
	2nd	Hombach, 2020 [32]	scFv	*In vitro*, CDX
	2nd	Chai, 2022 [33]	scFv	*In vitro*, CDX
	2nd	Qian, 2024 [34]	scFv	*In vitro*, CDX
	3rd	Zhang, 2023 [35]	scFv	*In vitro*, CDX
EpCAM	2nd	Zhou, 2019 [36]	scFv	*In vitro*
	2nd	Li, 2023 [37]	Nanobody	*In vitro*, CDX
	3rd	Ang, 2017 [38]	scFv	CDX
	3rd	Zhang, 2019 [39]	scFv	*In vitro*, CDX
	3rd	Zeng, 2024 [40]	scFv	*In vitro*, CDX
HER2	2nd	Teng, 2019 [41]	scFv	*In vitro*, PDX
	2nd	Xu, 2021 [42]	scFv	*In vitro*, CDX, PDX
NKG2DL	1st Dual (1st/2nd)	Jiang, 2023 [43]	NKG2D ED, NKG2D ED/PD1 ED, NKG2D ED/antiPDL1 scFv	*In vitro*, CDX
	3rd	Deng, 2019 [44]	NKG2D ED	*In vitro*, CDX
	1st, 2nd, 3rd	Li, 2020 [45]	NKG2D ED	*In vitro*, CDX
MSLN	2nd	Zhou, 2024 [46]	scFv	*In vitro*, CDX, PDX
	3rd	Zhang, 2021 [47]	scFv	*In vitro*, CDX, PDX
GUCY2C	3rd	Magee, 2016 [48]	scFv	*In vitro*, CDX
	3rd	Magee, 2018 [49]	scFv	*In vitro*, CDX
CD166	2nd	He, 2023 [50]	CD6 ED	*In vitro*
CD318	3rd	Li, 2023 [51]	scFv	*In vitro*, CDX
PLAP	2nd	Li, 2020 [52]	scFv	*In vitro*, CDX
ROR1	3rd	Meng, 2023 [53]	scFv	*In vitro*, CDX
DCLK1	2nd	Sureban, 2019 [54]	scFv	*In vitro*, CDX
Hsp70	2nd	Bashiri Dezfouli, 2022 [55]	scFv	*In vitro*
Gb3	2nd	Meléndez, 2022 [56]	Gb3-binding lectin	*In vitro*
CDH17	2nd, 3rd	Feng, 2022 [57]	Nanobody	Murine pCRC model
nfP2X7	2nd	Bandara, 2023 [58]	scFv	*In vitro*
CD133	2nd	Kieliszek, 2024 [59]	scFv	*In vitro*, PDX

CRC, colorectal cancer; CEA, carcinoembryonic antigen; EpCAM, Epithelial Cell Adhesion Molecule; HER2, Human Epidermal Growth Factor Receptor 2; NKG2DL, Natural Killer Group 2, member D ligands; PD1, programmed cell death protein 1; PDL1, programmed death ligand 1; MSLN, mesothelin; GUCY2C, Guanylate Cyclase C; PLAP, Placental Alkaline Phosphatase; ROR1, Receptor tyrosine kinase-like orphan receptor 1; DCLK1, Doublecortin-like kinase 1; Hsp70, Heat shock protein 70; Gb3, globotriaosylceramide; CDH17, cadherin-17; nfP2X7, non-functional version of P2X purinoceptor 7; scFv, single-chain variable fragment; ED, extracellular domain; CDX, cell line-derived xenograft; PDX, patient-derived xenograft; pCRC, primary colorectal cancer.

induced *in vitro* cytotoxicity when cocultured with CRC cells. The addition of Interleukin 12 (IL12) led to higher CAR-T cell proliferation and enhanced cytotoxicity *in vitro*. Consequently, intravenous administration of CEA-CAR-T cells and IL12 in CRC mouse xenografts produced stronger anti-tumor activity than CEA-CAR-T cells alone [31].

Hombach *et al*. [32] engineered mesenchymal stem cells (MSCs) to release Interleukin 7 (IL7) and IL12. Second-generation CAR-T cells displayed increased killing activity when incubated with CEA+ CRC cells in the presence of IL7/12-MSCs. Co-injection of CEA+ CRC cells and CEA-CAR-T cells suppressed tumor growth in mice and led to a survival advantage compared to controls. Co-injection with IL7/12-MSCs proved superior to CAR-T cell injection alone [32].

Chai *et al*. [33] developed second-generation CEA-CAR-T cells and explored various delivery methods in CRC liver metastasis murine models. Regional delivery via portal vein increased CAR-T penetration into the tumor compared to systemic delivery. Additionally, regional delivery

of CAR-T cells resulted in tumor regression, whereas systemic delivery led to tumor progression [33].

Similarly, second-generation CEA-CAR-T cells developed by Qian *et al*. [34] demonstrated efficacy when delivered regionally. Intraperitoneal administration was more effective than systemic administration, exerting antitumor activity in CRC xenograft models of peritoneal carcinomatosis and extraperitoneal disease [34]. Furthermore, third-generation CEA-CAR-T cells developed by Zhang *et al*. [35] were capable of suppressing tumors when administered intravenously in CRC xenograft models.

Epithelial Cell Adhesion Molecule (EpCAM)

EpCAM is a transmembrane glycoprotein found on the basolateral surface of normal epithelia and is highly expressed in the majority of gastrointestinal tumors, including CRC [62,63]. CAR-T cells targeting EpCAM have shown efficacy against CRC in several preclinical studies.

Zhou *et al*. [36] developed second-generation EpCAM-CAR-T cells. *In vitro* experiments with different CRC cancer cell lines showed a cytotoxic effect that was dependent on the effector-to-target ratio and the levels of EpCAM surface expression. Li *et al*. [37] designed second-generation CARs targeting EpCAM comprising nanobodies as the antigen-binding domain and different costimulatory domains (CD28, 4-1BB, or dectin-1). Dectin-1-costimulated EpCAM-CAR-T cells displayed similar efficacy to 4-1BB- or CD28-costimulated cells but showed lower exhaustion-associated gene transcription. Administration of dectin-1-costimulated EpCAM-CAR-T cells successfully eradicated CRC in cell line-derived mouse xenografts. Both intravenous and intraperitoneal administration were effective; however, peritoneal infusion resulted in faster tumor eradication. Regrettably, tumors relapsed in both groups by the end of the experiment [37].

Further studies evaluated the efficacy of third-generation EpCAM-CAR-T cells. Ang *et al*. [38] generated third-generation EpCAM-CAR-T cells using lentiviral transduction and mRNA electroporation. Repeated intraperitoneal administration of electroporation-generated EpCAM-CAR-T cells in CRC models led to a modest survival advantage and mice eventually died of tumor progression. Both lentivirally transduced and mRNA electroporated CAR-T cells showed better efficacy in ovarian cancer cell line-derived murine carcinomatosis models [38].

Additionally, third-generation EpCAM CAR-T cells developed by Zhang *et al*. [39] elicited *in vitro* cytotoxicity against EpCAM-expressing cells and delayed tumor growth after subcutaneous co-inoculation with CRC cells in mice. Intravenous administration of third-generation EpCAM-CAR-T cells, as developed by Zeng *et al*. [40], resulted in tumor volume reduction and improved survival in CRC cell line-derived xenografts.

Human Epidermal Growth Factor Receptor 2 (HER2; ERBB2)

HER2 is a receptor tyrosine kinase implicated in cell growth, survival, and differentiation. Dysregulation of its signaling contributes to various malignancies, including breast and gastric cancer [64]. Anti-HER2 therapy has been shown to be effective in treating HER2-positive breast and gastric cancer, and HER2-mutant lung cancer [65–68]. Although HER2-positive tumors account for less than 5% of all CRC cases [69], this small subset of CRC seems to benefit from HER2-targeted treatments.

Teng *et al*. [41] established a patient-derived xenograft (PDX) from HER2-positive colon carcinoma. In their study, second-generation HER2-specific CAR-T cells were able to infiltrate into the xenograft and suppress tumor growth. Complete tumor elimination was achieved in mice treated with HER2-CAR-T cells, and no new tumors developed after additional viable tumor injections [41].

Similarly, Xu *et al*. [42] developed second-generation HER2-CAR-T cells that induced *in vitro* cytotoxic activity against HER2-expressing CRC cells. Administration of these cells suppressed tumor growth in cell line-derived xenografts, and complete tumor elimination was achieved in some mice. In PDX models, HER2-CAR-T cells reduced tumor size only when HER2 expression was high, suggesting selective killing activity [42].

Natural Killer Group 2, Member D Ligands (NKG2DL)

NKG2DL refers to a family of stress-induced molecules that are expressed at low levels in healthy tissue but are frequently found in various tumor types. NKG2D is expressed in natural killer cells and other immune cells, and its ligation to NKG2DL mediates lytic synapse formation and degranulation [70]. Several studies have designed CAR-T cell therapies targeting NKG2DL for the treatment of CRC.

Jiang *et al*. [43] analyzed the co-expression of NKG2DL and PD1 in ovarian cancer specimens, observing co-expression in 45% of cases, as well as in ovarian and CRC cell lines. Consequently, they developed two different dual CAR-T cell models expressing a first-generation NKG2D-CAR and a second-generation CAR comprising a PDL1 targeting domain, either PD1 ectodomain or PDL1-specific scFv respectively. First-generation NKG2D-CAR-T cells were also generated. Both NKG2D-CAR-T and NKG2D/PDL1 dual CAR-T cells showed cytotoxic activity; however, dual CAR-T cells harboring the PDL1-specific scFv-CAR demonstrated superior activity and expansion. Intraperitoneal administration of dual CAR-T cells in CRC peritoneal carcinomatosis models resulted in complete tumor elimination, although some tumors eventually relapsed. Despite this, dual CAR-T administration significantly improved mice survival [43].

Deng *et al.* [44] designed a third-generation CAR targeting NKG2DL. These NKG2D-CAR-T cells exhibited *in vitro* cytotoxicity against CRC cell lines in an effector-to-target-dependent manner, and effectively reduced tumor growth while prolonging survival in mouse xenografts [44].

Li *et al.* [45] constructed first-, second-, and third-generation NKG2D-CAR-T cells using mRNA electroporation. All NKG2D-CAR-T cells demonstrated *in vitro* cytotoxicity against CRC cell lines, with the second-generation CAR-T cells incorporating CD28 as a costimulatory molecule showing enhanced activity. However, the first-generation CAR-T cells exhibited marginally superior efficacy. Intraperitoneal administration of first-generation NKG2D-CAR-T cells significantly reduced tumor number and weight in CRC peritoneal carcinomatosis mouse models [45].

Mesothelin (MSLN)

MSLN is a membrane protein with limited expression in healthy tissues but is frequently expressed in ovarian and pancreatic cancers. MSLN expression can be found at lower levels in CRC [71–73]. Zhou *et al.* [46] examined the effects of second-generation MSLN-CAR-T cells in orthotopic CRC liver metastasis models. Both caudal vein and portal vein injections of CAR-T cells reduced tumor volume; however, portal vein injection was more effective. Administration of CAR-T cells via portal vein increased T cell infiltration into the tumor. Intratumoral administration of MSLN-CAR-T cells in a PDX model led to increased tumor T cell infiltration and elevated expression of PD1, Lymphocyte activation gene-3 (LAG3), and T-cell immunoglobulin and mucin domain 3 (TIM3) compared to caudal vein administration [46].

Zhang *et al.* [47] developed third-generation MSLN-CAR-T cells, which displayed *in vitro* cytotoxicity against various MSLN-positive cancer cell lines. Injection of MSLN-CAR-T cells into cell line-derived xenografts resulted in complete tumor elimination in ovarian cancer models and tumor growth reduction in CRC models. Similarly, anti-tumor effects of MSLN-CAR-T cells were observed in CRC PDX models and were found to be independent of tumor burden [47].

Guanylate Cyclase C (GUCY2C)

GUCY2C is a transmembrane receptor found almost exclusively in healthy intestinal epithelium and is widely expressed by CRC [74]. Magee *et al.* [48] proved that third-generation CAR-T cells could recognize and lyse CRC cells expressing murine GUCY2C. Administration of GUCY2C-CAR-T cells in CRC lung metastasis mouse models reduced the number of lung metastases, decreased morbidity, and prolonged survival. Intestinal toxicity was not observed in this experiment [48]. Subsequently, they generated third-generation CAR-T cells targeting human GUCY2C. Intravenous administration of GUCY2C-CAR-T cells in GUCY2C-expressing CRC mouse xenografts eliminated lung metastases, prolonged survival, and prevented new tumor formation after further inoculation of GUCY2C-expressing CRC cells [49].

CD166 & CD318

CD166 and CD318 are ligands for CD6, a member of the scavenger receptor cysteine-rich superfamily primarily expressed in lymphocytes. Overexpression of CD166 and CD318 has been observed in various malignancies [75]. He *et al.* [50] assessed CD166 expression in multiple CRC cell lines, finding high levels in all of them. In their study, they designed a CAR comprising an extracellular domain of CD6 as the antigen recognition domain, instead of a scFv. Subsequently, second-generation CD6-CAR-T cells were generated using lentiviral vectors. CD6-CAR-T cells targeted CD166-positive CRC cells and exhibited *in vitro* anti-tumor activity. Interestingly, the CD6-CAR-T cells anti-tumor activity was independent of CD318 expression [50].

Li *et al.* [51] identified CD318 expression in CRC tissue and cell lines. To target CD318, they developed a third-generation CAR that harbored a CD318-specific scFv. Co-culture of CD318 CAR-T cells with CD318-positive cells elicited cytotoxic activity. Intratumoral injection of CD318-CAR-T cells also demonstrated anti-tumor activity in CRC cell line-derived mouse xenografts [51].

Placental Alkaline Phosphatase (PLAP)

PLAP expression in adult healthy tissues is mainly restricted to the placenta. PLAP positivity has been observed across various tumor types, especially in germ-cell tumors. A small percentage of CRC cases exhibit PLAP expression, which is typically weak [76]. Li *et al.* [52] examined *PLAP* mRNA expression levels across various cell lines, finding high levels in gastrointestinal cancers, including CRC. Positive PLAP expression was detected by immunohistochemistry staining in 23.6% of all CRC samples tested. Consequently, second-generation PLAP-CAR-T cells were manufactured. PLAP-CAR-T cells selectively killed PLAP-positive CRC cells without affecting PLAP-negative cells. Administration of PLAP-CAR-T cells in CRC xenograft models reduced tumor growth and did not cause significant toxicity. Combining PLAP-CAR-T cells with checkpoint inhibitors enhanced CAR-T cell efficacy *in vitro* [52].

Receptor Tyrosine Kinase-Like Orphan Receptor 1 (ROR1)

ROR1 is a receptor expressed during embryonic development, in some adult healthy tissues such as the gastrointestinal tract, and in human epithelial cancers [77]. Meng *et al.* [53] analyzed RNA expression data from public databases and found high expression of *ROR1* in CRC, cholangiocarcinoma, clear cell renal carcinoma, and prostate cancer. ROR1 expression was also con-

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firmed by flow cytometry in CRC cell lines. Third-generation ROR1-CAR-T cells demonstrated anti-tumor activity against CRC cells *in vitro* and reduced tumor volume in mouse xenografts [53].

Doublecortin-Like Kinase 1 (DCLK1)

DCLK1 is a CRC stem cell marker, and its expression is associated with a worse prognosis [78]. Sureban *et al.* [54] designed second-generation DCLK1-CAR-T cells that could successfully kill CRC cells at high effector-to-target ratios. Intravenous administration of DCLK1-CAR-T cells in xenograft models decreased tumor volume without relevant toxicities [54].

Heat Shock Protein 70 (Hsp70) and Globotriaosylceramide (Gb3)

In contrast to healthy tissue, malignant tumors can present Hsp70, a stress-inducible member of the HSP70 family, on their cell surface. The glycosphingolipid Gb3 is a partner of Hsp70 and enables its presence on the cell surface [79]. Bashiri Dezfouli *et al.* [55] targeted Hsp70 with second-generation CAR-T cells. Hsp70-CAR-T cells displayed cytolytic activity when cultured with CRC cells expressing Hsp70. This cytolytic activity was comparable to that of stimulated NK cells, which are known to effectively kill Hsp70-expressing cells. *In vivo* experiments were not conducted in this study [55]. Meléndez *et al.* [56] designed a CAR targeting Gb3. They used lectins, which are glycan-binding proteins, as the antigen-binding domain of the CAR. Lectin-based CARs were expressed by T lymphocytes following lentiviral transfection. Lectin-based CAR-T cells elicited potent anti-tumor activity against Gb3-expressing CRC cell lines [56].

Other Targets

Other studies evaluated the efficacy of CAR-T cells targeting additional antigens in various neoplasms, including CRC. Feng *et al.* [57] designed a CAR targeting cadherin-17 (CDH17), which utilized a nanobody as the antigen-binding domain instead of a scFv. Third-generation CDH17-CAR-T cells potently killed CDH17-positive cells when cocultured and eliminated neuroendocrine tumors in cell line-derived mouse xenografts. Additionally, CDH17-CAR-T cells were able to kill CDH17-positive gastric and pancreatic cancer cells in both *in vitro* and *in vivo* experiments. Furthermore, CAR-T cells targeting CDH17 reduced tumor volume in a primary murine CRC model [57]. Bandara *et al.* [58] designed CAR-T cells targeting a non-functional version of P2X purinoceptor 7 (nfP2X7), which is overexpressed in solid tumors. Third-generation nfP2X7-CAR-T cells showed *in vitro* cytotoxicity against various solid tumor cell lines, including CRC. Anti-tumor activity was also observed in breast and prostate cancer xenograft models. However, CRC xenograft models were not tested [58]. Kieliszek *et al.* [59] designed CAR-T cells targeting CD133, a stem cell marker, for the treatment of brain metastases. Brain metastasis cell lines were derived from patient samples. CD133-CAR-T cells exhibited *in vitro* cytotoxicity against CD133-positive CRC, breast, and lung cancer cells in a dose-dependent manner. Intratumoral delivery of CD133-CAR-T cells reduced tumor volume and increased survival in CRC and lung cancer brain metastasis PDXs [59].

CAR-T Cell Therapy for CRC in the Clinical Setting

Despite the efficacy of CAR-T cell therapy for the treatment of CRC in a preclinical setting, showing acceptable outcomes in both *in vitro* and *in vivo* models, its implementation in clinical practice remains distant. To date, few clinical trials have reported results regarding the efficacy of CAR-T cell therapy for CRC (Table 2, Ref. [29,37,80–86]). The available data came from early-phase clinical trials, and only a minority exclusively focus on CRC patients. Most trials enrolled cancer patients based on tumor expression of the target antigen, resulting in a low proportion of CRC patients.

Carcinoembryonic Antigen (CEA)

First-generation CEA-CAR-T cells were administered in a dose-escalation, phase I trial conducted by Thistlethwaite *et al.* [80]. Fourteen patients diagnosed with advanced CEA+ malignancies, including six with CRC, received CEA-CAR-T cells and Interleukin 2 (IL2) infusion after pre-conditioning chemotherapy. No objective responses were reported; nevertheless, disease stabilization was observed in seven patients, including four with CRC. Patients who received the highest doses of CAR-T cells and increased-intensity pre-conditioning chemotherapy experienced acute respiratory toxicity, which led to premature trial closure [80].

Zhang *et al.* [81] conducted a phase I trial with second-generation CEA-CAR-T cells. Ten patients with CEA-positive CRC were enrolled and treated with CEA-CAR-T cells at different dose levels following lymphodepletion. Two out of ten patients experienced grade 2 fever after CAR-T administration. Hematological toxicity was attributed to lymphodepletion, and one case of duodenum perforation was reported. In terms of efficacy, seven out of ten patients experienced disease stabilization, with durable stabilization (>30 weeks) observed in two patients. Objective responses were not reported [81].

Katz *et al.* [82] carried out a phase I trial to assess the efficacy of hepatic artery infusion of second-generation CEA-CAR-T cells for treating CEA-positive liver metastases. Patients with unresectable CEA-positive liver metastases who had progressed after one or more lines of therapy were included, with minimal extrahepatic disease permitted. Patients received increasing doses of CEA-CAR-T

Table 2. Overview of CAR-T cell therapy published clinical trials including CRC.

Target	Phase	Registration Number	Tumor Types	CRC patients/ patients treated	Treatment	Best response in CRC
CEA	Phase I	NCT01212887 [80]	CEA+ tumors	6/14	PCh + 1st gen CEA-CAR-T cells (IV) + IL2	SD
CEA	Phase I	NCT02349724 [81]	CEA+ CRC	10/10	PCh + 2nd gen CEA-CAR-T cells (IV)	SD
CEA	Phase I	NCT01373047 [82]	Unresectable CEA+ LM	5/6	2nd gen CEA-CAR-T cells (HAI) ± IL2	SD
CEA	Phase Ib	NCT02416466 [83]	Unresectable CEA+ LM	4/6	2nd gen CEA-CAR-T cells (HAI) + IL2+ SIRT	SD
CD133	Phase I	NCT02541370 [84]	CD133+ tumors	2/23	PCh (non HCC) + 2nd gen CD133-CAR-T cells (IV)	SD
EpCAM	Phase I	NCT02915445 [37]	EpCAM+ tumors	4/12	PCh + 2nd gen EpCAM-CAR-T cells (IV, IP)	SD
GD2	Phase I	ACTRN 12613000198729 [85]	GD2+ tumors	4/12	3rd gen GD2-CAR-T cells (IV) ± BRAF/MEKi	PD
GUCY2C	Phase I	ChiCTR2000040645 [86]	GUCY2C+ CRC	15/15	PCh + GCC19-CAR-T cells	PR/mCR
CLDN18.2	Phase I	NCT03874897 [29]	CLDN18.2+ GI cancer	8*/98	PCh + CLDN18.2-CAR-T cells (IV) ± antiPD1 therapy	Not specified

CEA, carcinoembryonic antigen; EpCAM, Epithelial Cell Adhesion Molecule; GD2, disialoganglioside GD2; GUCY2C, Guanylate Cyclase C; CLDN18.2, claudin18.2; CRC, colorectal cancer; LM, liver metastases; GI, gastrointestinal; PCh, pre-conditioning chemotherapy; IV, intravenous; HAI, hepatic artery infusion; SIRT, selective internal radiotherapy; IP, intraperitoneal; SD, stable disease; PR, partial response; PD, progressive disease; mCR, metabolic complete response; * reported as intestinal cancer.

cells (cohort 1) or CEA-CAR-T cells combined with systemic infusion of IL2 (cohort 2). Eight heavily pretreated patients were enrolled, and six completed the treatment protocol, of whom five had been diagnosed with CRC. Four out of five patients who underwent radiological assessment showed progression at the first evaluation. Grade 4–5 adverse events were not reported. Fever was frequent (4/6), and all patients experienced transient elevations in phosphatase alkaline, bilirubin, and aspartate aminotransferase, with grade 3 elevations in one case [82]. Subsequently, Katz *et al.* [83] conducted a phase Ib trial combining hepatic artery infusion of second-generation CEA-CAR-T cells and selective internal radiation therapy (SIRT). The treatment protocol consisted of three hepatic artery infusions of CEA-CAR-T cells and continuous systemic IL2 infusion followed by SIRT. Six patients completed the planned treatment, including four with CRC. After CEA-CAR-T cell administration, five out of six patients showed progression at the first CT/MRI evaluation, while liver disease stabilization was achieved in two cases. The safety profile was similar to that reported in the previous trial [83].

CD133

Second-generation CD133-CAR-T cells were investigated in a phase I trial conducted by Wang *et al.* [84]. Twenty-three patients diagnosed with CD133-positive hepatocellular carcinoma (HCC) or solid malignant tumors that had progressed after two previous lines were enrolled, including two patients with CRC. In non-HCC patients, CD133-CAR-T cell infusion was preceded by a lymphodepleting regime. Disease control for ≥3 months was achieved in 65% of the patients. Both enrolled CRC patients experienced disease stabilization. Regarding the safety profile, hematological toxicity and hyperbilirubinemia were the most frequent adverse events [84].

Epithelial Cell Adhesion Molecule (EpCAM)

Li *et al.* [37] developed a dectin-1-costimulated EpCAM-CAR-T cell and conducted a phase I trial. Twelve patients with EpCAM-positive tumors were treated, including four with gastric cancer and four with CRC. EpCAM-CAR-T cells were administered intravenously, while patients with gastric cancer and peritoneal spread received intraperitoneal administration. A partial response was achieved in two patients, and disease stabilization was observed in five. Fever and hematological toxicity were the most frequent adverse events. Cytokine release syndrome was not reported, nor was grade ≥3 EpCAM targeting-related toxicity [37].

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Disialoganglioside GD2

Gargett *et al*. [85] conducted a phase I trial of GD2-CAR-T cells for the treatment of GD2-positive metastatic melanoma and other solid tumors. Fourteen patients were enrolled, including four with advanced GD2-positive CRC. Third-generation GD2-CAR-T cells were administered intravenously at three different dose levels. All four CRC patients experienced disease progression, and three of them died within three months of CAR-T cell administration. The most common adverse events were rash, fever, and diarrhea. No grade $\geq$3 adverse events or dose-limiting toxicity were reported [85].

Guanylate Cyclase C (GUCY2C)

Chen *et al*. [86] evaluated the safety and efficacy of CAR-T cells targeting GUCY2C in a phase I trial. GCC19-CAR-T cells were generated by simultaneously transducing T cells with lentiviral vectors encoding one GUCY2C-CAR and three different CD19-CARs. Fifteen CRC patients received GCC19-CAR-T cell infusions following lymphodepleting chemotherapy. The overall response rate, as assessed by CT, was 40%, and one case of metabolic complete response was observed. Eight patients (53%) experienced grade 3 diarrhea. Diarrhea of any grade and cytokine release syndrome were common, affecting 93% of the patients [86].

Claudin18.2 (CLDN18.2)

A phase I trial conducted by Qi *et al*. [29] evaluated the role of CLDN18.2-CAR-T cells in the treatment of CLDN18.2-positive gastrointestinal malignancies. This trial enrolled 98 patients, of whom 73 (74.5%) had gastric/gastroesophageal junction cancer, and eight had been diagnosed with intestinal cancer. Patients received CLDN18.2-CAR-T cells alone or in combination with PD1 blockade (cohort 2; n = 15). The disease control rate among intestinal cancer patients was 87.5%, with only one patient (diagnosed with duodenal adenocarcinoma) achieving a partial response [29].

Ongoing Clinical Trials

CAR-T cell therapy for the treatment of CRC is still under evaluation in various active clinical trials. CEA-CAR-T cells for the treatment of advanced CEA-positive malignancies, including CRC, are being investigated in several phase I trials (NCT05415475, NCT05396300, and NCT06043466). The administration of CEA-CAR-T cells following CRC primary and liver metastases surgery is being studied in another early-phase trial (NCT05240950). Additional antigens such as CDH17 (NCT06055439), EpCAM (NCT05028933), guanylate cyclase (NCT06653010, NCT06675513, NCT05319314), LGR5 (NCT05759728) or NKG2DL (NCT03692429, NCT04991948) are being targeted in early-phase trials for the treatment of gastrointestinal malignancies and CRC.

Conclusion

A growing interest in developing CAR-T cell therapy for the treatment of CRC has emerged over the last decade. A wide variety of tumor antigens have been explored as potential targets for CAR-T cells, including well-known molecules overexpressed in CRC, such as CEA or EpCAM, as well as novel targets like NKG2DL. Comparing the effectiveness of targeting different antigens remains challenging, as preclinical studies have primarily focused on single targets, with nearly all showing anti-tumor activity. Moreover, these studies seldom develop different generations of CAR-T cell models. Regional delivery of CAR-T cells demonstrated better results in animal models with controlled disease location. However, these delivery methods could be limited in patients with disease spread patterns involving multiple organs.

Some of the targets evaluated in preclinical research have also been assessed in clinical trials. Despite promising results in the preclinical setting, most early-phase clinical trials with CAR-T cell therapies focusing on CRC have shown limited or nonexistent efficacy. The lack of objective responses in the majority of these trials does not support their development in later phases. The trial design, including enrollment criteria, heterogeneity of the patients, and CAR-T cell delivery methods, may have influenced these outcomes. Additionally, the inherent challenges of CAR-T cell therapy for solid tumors - such as antigen heterogeneity, CAR-T cell trafficking into the tumor, and the immunosuppressive tumor microenvironment - likely contributed to these discouraging results, particularly as most trials used second-generation CAR-T cells. These obstacles could be addressed through improved trial design, the exploration of novel target antigens, and the generation of armored CAR-T cells capable of better tumor penetration and resistance to the immunosuppressive milieu. The development of these innovative CAR-T cell products may enhance the efficacy of CAR-T cell therapies for CRC and facilitate their incorporation into future clinical practice.

Availability of Data and Materials

Not applicable.

Author Contributions

AL, MA, NR and RV conceived the review. AL, HA and IC performed the literature search and drafted the original manuscript. AL, HA, IC, AEH, NC and IL were involved in data curation and visualization. AL, HA, IC, AEH, NC, IL, MA, NR and RV critically revised the final draft. All authors have read and approved the final manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

Not applicable.

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Conflict of Interest

AL has received speaker honoraria from Pierre Fabre and support for attending meetings and/or travel from PharmaMar, Lilly, Merck, Seagen, Novartis and MSD. HA has been involved as a consultant for advisory roles from Astra Zeneca, received speaker honoraria from Takeda and for trial coordination from Ferrer Farma. NC has received speaker honoraria from Pierre Fabre and Roche and support for attending meetings and/or travel from BMS, Lilly, Merck, MSD, Pfizer and Roche. MA has been involved as a consultant for advisory roles with Amgen, AstraZeneca, BeiGene, Dragonfly Therapeutics, Jazz Pharmaceuticals, BMS, Novartis and MSD and received speaker honoraria from Beigene, Jazz Pharmaceuticals and MSD. RV has been involved as a consultant for advisory roles with Servier, Roche and Merck Sharp and has received speaker honoraria from Roche, Amgen, MSD and Astra Zeneca. IC, AEH, IL and NR declare no conflict of interest.

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