Trends in Immunology

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Review

Targeting γδ T cells for immunotherapies against colorectal cancer

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The advancement of immunotherapy faces significant challenges, including extending its benefits to a growing number of patients and enhancing its efficacy across different tumor types. In this context, γδ T cells emerge as particularly promising candidates owing to their distinctive biological features such as MHC-independent activation, potent cytotoxicity, and capacity to bridge innate and adaptive immunity. Recently, advanced single-cell techniques have allowed detailed γδ T cell characterization in the tumor microenvironment (TME) and have emphasized their heterogeneity, mechanisms of activation, and response to immune checkpoint blockade (ICB). This review provides a comprehensive summary of recent advances in understanding γδ T cells in colorectal cancer (CRC), with a particular emphasis on their prognostic and therapeutic relevance in both primary tumors and metastatic disease.

Highlights

Colorectal cancer (CRC) represents a major clinical issue, with growing rates in younger patients and few effective therapies.

γδ T cells in primary and metastatic CRC are highly heterogeneous and comprise functionally distinct subpopulations.

Some γδ T cell phenotypes protect against tumor progression and correlate with improved survival, suggesting that they have potential prognostic value.

γδ T cells respond to PD-1/PD-L1 axis inhibitors, particularly in HLA class I-negative CRC tumors.

PD-1$^+$ γδ T cells show a profile of tumor-reactive cells that can be reinvigorated via immune checkpoint blockade (ICB).

Combination strategies such as *in vivo/ex vivo* activation and expansion, adoptive transfer, genetic engineering, and ICB are now being investigated to enhance γδ T cell specificity, persistence, and antitumor efficacy.

Significance

Growing evidence highlights the key role of the immune response in controlling colorectal cancer (CRC) and the need to better understand its immune evasion mechanisms to identify new therapeutic targets. γδ T cells are emerging as promising candidates owing to their ability to eliminate tumor cells in an MHC-independent manner while bridging innate and adaptive immunity. Strategies such as adoptive transfer, engineered γδ T cell products, and their engager-based or pharmacological activation and expansion make them particularly appealing for CRC therapy.

Therapeutic potential of γδ T cells in cancer immunotherapy

Immunotherapy has become a cornerstone of modern oncology owing to its ability to enhance the immune responses of patients against malignant cells. Strategies such as ICB, multispecific **antibody engagers** (see Glossary), adoptive cell therapies, and chimeric antigen receptor (CAR)- or T cell receptor (TCR)-engineered T cells have gained increasing clinical interest. Within this landscape, γδ T cells are emerging as particularly promising targets [1,2]. Their therapeutic potential stems from their unique biological features, including a highly efficient cytotoxicity machinery and the production of proinflammatory cytokines (IFN-γ, TNF) and chemokines (CCL3/5, XCL1/2) that can modulate the TME, as well as a relatively low risk of cytokine release syndrome (CRS). These cells circulate in the peripheral blood and populate multiple tissues, such as the colon mucosa, which supports their potential for therapeutic targeting in both hematological and solid malignancies. Activation of γδ T cells occurs predominantly in an MHC-independent manner [3–5], which makes them ideal candidates for allogeneic strategies with a reduced risk of graft-versus-host disease (GVHD), a common limitation of αβ T cell-based strategies. Importantly, this MHC-independent activation also enables γδ T cells to recognize and eliminate tumor cells that have downregulated MHC, a prevalent mechanism of αβ T cell-related tumor evasion. Recent evidence suggests that some γδ T cells can recognize classical MHC molecules expressed by tumor cells; however, this interaction appears to be peptide-independent and occurs even in the presence of peptide-loading defects [6]. γδTCRs recognize **phosphoantigens (pAgs)** presented by **butyrophilin** molecules (BTN), the MHC-like molecule MR1, lipid-presenting CD1 family members, and the endothelial protein C receptor (EPCR) [7]. In addition, γδTCRs can bind to non-MHC-related and stress-induced molecules such as annexin A2 and the ephrin type A receptor 2 (EPHA2) [7] which are overexpressed following metabolic reprogramming of cancer cells.

A distinctive feature of γδ T cells is their dual innate and adaptive immune functions. In addition to TCR-mediated recognition, they express activating receptors that are typically found on natural

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killer (NK) cells (e.g., NKG2D, DNAM-1, and NKp30/44/46) [8,9], which allows them to detect stress-induced ligands on tumor cells. Their cytotoxic activity also involves FAS/FASL and TRAIL/TRAIL-R pathways, as well as the CD16 (FcγRIII) receptor, which recognizes the Fc portion of IgG and plays a crucial role in triggering antibody-dependent cellular cytotoxicity (ADCC) [10,11]. However, the full spectrum of ligands for γδ T cell activation, across both adaptive and innate pathways, remains incompletely characterized, and the crosstalk between these signaling pathways is not yet fully understood. For instance, the stress-inducible MHC class I-related molecule ULBP4 can act as a ligand for both γδTCR and NKG2D [12], revealing the multifaceted integration of innate and adaptive functions in γδ T cells. Furthermore, γδ T cells can prime αβ T cell responses and interact with other immune cells, thereby orchestrating a cascade of antitumor responses [13].

The clinical exploitation of γδ T cells requires careful consideration of their heterogeneity and functional plasticity. The main human subsets, Vδ1, Vδ2, and Vδ3, display different tissue distributions, antigen recognition patterns, and activation mechanisms (Box 1). These differences shape their interactions within the TME and influence the recognition and killing of tumor cells, and thus impact on their potential use in immunotherapy. High-resolution profiling technologies such as single-cell RNA sequencing (scRNA-seq) have substantially expanded our understanding of γδ T cell biology in recent years (Box 2). In CRC, such analyses have uncovered previously unappreciated aspects of γδ T cell functional states, heterogeneity, and therapeutic relevance.

This review summarizes current knowledge about γδ T cells in CRC, and outlines their distribution and phenotypic and functional diversity across primary and liver metastases (LMs), alongside their prognostic significance. We then discuss γδ T cell-based immunotherapeutic strategies in CRC, including ICB, modulators of γδ T cell activity, and adoptive γδ T cell therapies, and provide a concise overview of their therapeutic potential in this disease.

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Box 1. Human γδ T cell subsets

The classification of γδ T cells is based on their expression of the T cell receptor δ (TCRδ) locus (*TRD*) which undergoes recombination of variable (V), diversity (D), and joining (J) segments. In humans, Vδ1, Vδ2, and Vδ3 are most commonly utilized among the eight known Vδ gene segments, and these are rearranged in one of the four *TRD* J segments (J1–4). The TCRγ locus (*TRG*) also undergoes VJ recombination, but only six of the 14 *TRG* V segments are functional (Vγ2–5, Vγ8, and Vγ9) and can be recombined with five *TRG* J segments (JP1, JP, J1, JP2, and J2) to generate a diverse TCR repertoire. The Vδ2 chain preferentially pairs with the Vγ9 chain, giving rise to a semi-invariant Vγ9Vδ2 TCR repertoire which may be shared across individuals and constitutes ~90% of the total γδ T cells in adult peripheral blood**.** Owing to their high relative abundance and accessibility, blood Vγ9Vδ2 cells have been the most extensively characterized and were the first to be translated into clinical applications [1]. Vγ9Vδ2 cells rapidly respond to phosphoantigens (pAgs) such as hydroxymethy-but-2-enyl pyrophosphate (HMBPP) and isopentenyl pyrophosphate (IPP) that increase during microbial infection or in malignant cells triggered by metabolic stress signals. The presentation of pAgs depends on butyrophilin (BTN) family members, specifically BTN3A1 and BTN2A1, that enable their binding to the Vγ9 chain [93,94] (Figure 1). In addition, the NKG2D ligand ULBP4 was reported to bind to both Vγ9Vδ2 TCR and NKG2D [12]. Although Vγ9Vδ2 cells are the most common subset among Vδ2 T cells, the rarer Vγ9^{neg}Vδ2 T cells have been also described as a more adaptive-like cell type with a more diverse TCR [95].

Non-Vδ2 γδ T cells are typically enriched in tissues [96,97]. Among these, Vδ1 cells are the most abundant and, as such, have been the most extensively studied, particularly in the context of solid tumors [2]. Vδ1 cells exhibit highly individual TCR repertoires and are often marked by clonally expanded cells. In healthy human intestine and metastatic CRC (mCRC), the main Vγ chains pairing with Vδ1 are Vγ4, followed by Vγ3. Although the full spectrum of human Vδ1 ligands remains poorly defined, their TCRs recognize lipid antigens presented by CD1a–d [98–102], MR1 [103], stress-induced annexin A2 [104] and EPHA [105,106], and BTNL3/8 via the Vγ4 chain [48] (Figure 1). Human Vδ3 chains are CD1d-restricted [107] and can also bind to annexin A2 [104] and MR1 [108], whereas EPCR, an MHC-like phospholipid-binding molecule, is the only known human Vδ5 TCR ligand [109].

Box 2. Exploring γδ T cells in cancer: opportunities from single-cell RNA-sequencing (scRNA-seq) technology

Despite their potential, the study and clinical applications of γδ T cells in immunotherapy still face numerous obstacles. One of the main challenges concerns the limited understanding of the mechanisms that regulate their activation and crosstalk between signals originating from the γδTCR and innate receptors, an aspect that remains a significant gap in our current knowledge. This difficulty is largely due to their high variability and low abundance in peripheral tissues, which tends to further decrease under pathological conditions [35]. In clinical settings where access to samples and cells is limited, high-resolution single-cell analyses represent a valuable tool to investigate the heterogeneity of γδ T cells and to gain deeper insights into their functional states. Among these technologies, scRNA-seq has emerged as a key resource in cancer research because it can unravel the complexity of the TME and thus facilitate the identification of new therapeutic targets and mechanisms of immune evasion [110–112]. In recent years, several studies have used scRNA-seq to characterize γδ T cells across different tumor types and have highlighted the great potential of this approach in clarifying their role in cancer biology. Nonetheless, important challenges remain in the single-cell transcriptional analysis of these cells, including accurate cell clustering and annotation [113]. Indeed, the rarity of γδ T cells can result in their transcriptional profiles being obscured by those of more abundant and phenotypically similar populations such as CD8 T cells and natural killer (NK) cells. In this context, γδ T tumor-infiltrating lymphocytes (TILs) and their heterogeneous profile pose technical challenges for scRNA-seq analysis. In addition, a major limitation of scRNA-seq is that it only provides a snapshot of the transcriptomic cell state and does not capture the corresponding protein expression levels. As a result, important activation and memory markers that are tightly regulated post-transcriptionally or at the protein level may not be accurately predicted from transcriptomic data alone. This disconnection between mRNA and protein expression can limit the ability to infer the true functional state of γδ T cells. To address this issue, integrating scRNA-seq with complementary multimodal approaches, such as cellular indexing of transcriptomes and epitopes by sequencing (CITE-seq), combining transcriptomic and surface protein profiling, or validating key findings through flow cytometry, can provide a more comprehensive characterization of γδ T cell activation and differentiation states.

γδ T cells in CRC: modulators and prognostic indicators of disease progression

CRC is the third most commonly diagnosed malignancy and the second leading cause of cancer-related death worldwide, and its incidence is rising both among individuals under 50 years of age and in patients with advanced disease [14]. Prognosis varies widely: 5 year survival is ~90% for localized tumors but drops to 15% in metastatic CRC (mCRC) [15]. Over 25% of patients with early-stage disease progress to mCRC, and the liver represents the most frequent metastatic site owing to portal venous drainage. About 25% develop **synchronous metastatic liver disease** and an additional 20–25% develop **metachronous metastatic liver disease**. As result, hepatic disease occurs in roughly half of all CRC patients, who also face a recurrence risk of up to 60% [16,17]. The TME in CRC is highly complex, and is shaped by the composition and functional state of immune cells which can establish both antitumor and immunosuppressive niches in primary tumors and LMs [18–20]. A deeper understanding of this TME will be crucial to accurately delineate immune dynamics and their clinical implications, and thus enable improved patient stratification and the development of effective therapies.

γδ T cell heterogeneity across primary and metastatic CRC

γδ T cells in CRC display a heterogeneous landscape (Figure 1). In healthy intestinal tissue, their frequency is highly variable and they account for a mean of 20–25% of intraepithelial lymphocytes [21,22]. Similar percentages are observed in peritumoral areas; however, within the tumor core, where the anatomical architecture of the intraepithelial and lamina propria compartments is disrupted, the frequency of γδ T cells is markedly reduced and represent 10% of total T cells [21,22]. In both tumors and peritumoral specimens, the predominant subset is Vδ1, followed by Vδ2 and Vδ3 [21,23–26]. Single-cell analysis of primary CRC revealed an enrichment of effector γδ T cells that express the tissue-retention markers CD103 and CD69 [21,27], as well as activating receptors including the natural cytotoxicity receptors (NCRs) NKp30 and NKp46, NKG2D, NKG7, FASL, CD16, and DNAM-1 [24–28]. Among the subsets, Vδ1 cells mainly express NKp46, TRAIL, and CD1; notably, NKp46^{+} Vδ1 cells exhibit enhanced cytotoxic potential and greater IFN-γ-responsiveness [21].

Glossary

Antibody engagers: engineered proteins that recognize specific antigens and trigger immune responses against target cells. Bispecific antibodies bind to two targets simultaneously, usually a tumor antigen and an immune effector receptor, and actively redirect immune cells to kill cancer cells. Tribodies extend this approach by engaging three targets, often one on the tumor and two on immune cells, to enhance the potency and flexibility of immune-mediated tumor elimination.

Butyrophilins (BTNs): a family of molecules involved in the regulation of γδ T cell activation through γδ T cell receptor (TCR) signaling. Specifically, BTN2A1 and BTN3A1 mediate recognition of phosphoantigens by Vγ9Vδ2 TCR cells. By contrast, BTNL3 and BTNL8 (butyrophilin-like 3/8) modulate the activation of tissue-resident Vδ1 T cells.

Consensus molecular subtypes (CMS1–4): a classification system for colorectal cancer (CRC) based on gene expression profiles that define four subtypes, CMS1–4, according to their distinct biological features, prognostic implications, and therapeutic relevance. CMS1 is characterized by high microsatellite instability (MSI-H) and strong immune activation, CMS2 by WNT/MYC-driven epithelial tumors, CMS3 by metabolic dysregulation, and CMS4 by mesenchymal features, stromal infiltration, and poor prognosis.

Delta One T (DOT) cells: a clinical grade Vδ1 T cell product based on peripheral blood-derived Vδ1 T cells that are expanded *ex vivo* over 2–3 weeks through anti-CD3 and cytokine stimulation to induce natural cytotoxicity receptor (NCR) expression and enhance cytotoxicity against tumors.

Metachronous CRC: CRC that develops liver metastases >6 months after the initial cancer diagnosis, which reflects differences in tumor biology and treatment strategy.

Phosphoantigens (pAgs): small metabolites that specifically activate Vγ9Vδ2 T cells They are naturally produced in cells via the mevalonate pathway by tumor cells or are derived from bacterial metabolism. Key examples include isopentenyl pyrophosphate (IPP), which activates Vγ9Vδ2 T cells via BTN3A1, and bromohydrin pyrophosphate (BrHPP), a synthetic phosphoantigen that is

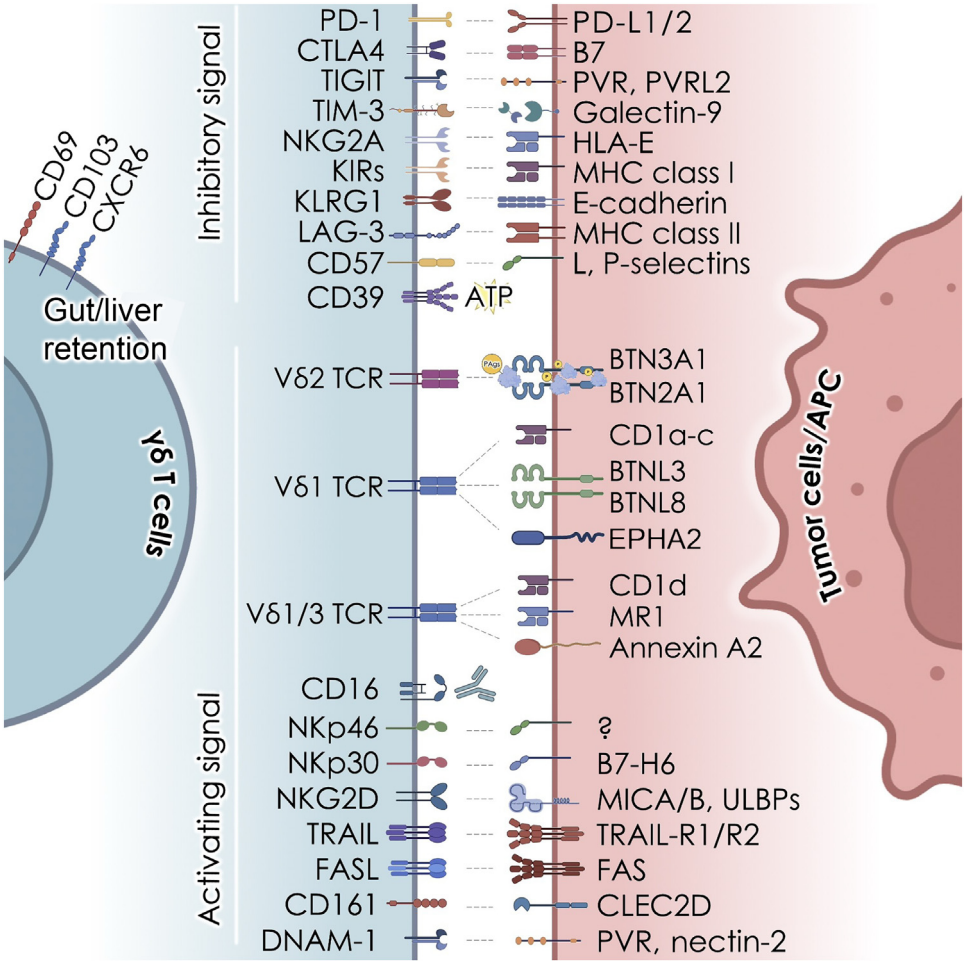

Figure 1. Ligand–receptor landscape shapes γδ T cell activity in colorectal cancer (CRC). Schematic representation of the key activating and inhibitory interactions that shape γδ T cell function within the tumor microenvironment (TME). This overview illustrates how ligand–receptor networks in CRC may influence γδ T cell activation, inhibition, and tissue retention. Markers expressed by γδ T cells (shown on the left) are organized into activating (bottom: γδTCRs, CD16, NKp30/46, NKG2D, TRAIL, FASL, CD161, DNAM-1) and inhibitory (top: PD-1, CTLA-4, TIGIT, TIM-3, NKG2A, KIRs, KLRG1, LAG-3, CD57, CD39) groups, together with tissue-retention markers (CD69, CD103, CXCR6) that are characteristic of gut/liver tissue tumor-associated γδ T cells. On the right, ligands expressed by tumor cells or antigen-presenting cells (APCs) are depicted, including inhibitory ligands (PD-L1/L2, B7 family members, PVR/PVRL2, galectin-9, HLA-E, MHC class I/II, E-cadherin, L/P-selectins, soluble ATP), activating/costimulatory ligands engaging both adaptive or innate receptors (BTN/BTNL, CD1 isoforms, MR1, annexin A2, EPHA2, MICA/B, ULBPs, TRAIL receptors, FAS, CLEC2D), and adhesion molecules (nectin-2 and the PVR family). Abbreviations: BTN/BTNL, butyrophilin/butyrophilin-like molecules; HLA-E, human leukocyte antigen E; KIRs, killer immunoglobulin-like receptors; MHC class I/II, major histocompatibility complex class I/II; TCR, T cell receptor. Figure generated with BioRender.

The inhibitory receptor pattern also varies between subsets: Vδ1 cells express PD-1, TIGIT, TIM-3, CD39, CTLA-4, and killer cell immunoglobulin-like receptors (KIRs), whereas Vδ2 cells are enriched in KLRG1 and NKG2A [24–26,28]. KIRs are mainly inhibitory receptors that recognize specific HLA class I alleles and mediate the so-called 'missing-self recognition' mechanism that regulates the effector response of NK cells [29]. Although KIRs have long been known to be exclusively expressed on human NK cells, their regulatory role in γδ T cells has more recently been recognized [30]. Moreover, PD-1 expression in Vδ1 cells has been linked to tumor-reactive cells

designed to selectively stimulate these cells.

Synchronous CRC: refers to CRC diagnosed together with liver metastases, or when liver metastases appear within 6 months of the initial cancer diagnosis.

Tumor mutational burden (TMB): the total number of somatic mutations present in the genome of a tumor. A higher TMB is often associated with increased production of tumor-associated antigens (TAAs), which can enhance recognition by the immune system, and is correlated with a better response to immunotherapy, particularly to immune checkpoint inhibitors.

Zoledronate: a drug that inhibits farnesyl pyrophosphate synthase in the mevalonate pathway of target cells (e.g., myeloid or tumor cells), leading to the accumulation of PAgs that trigger Vγ9Vδ2 TCR activation via BTN family members. Zoledronate is used to expand and activate Vγ9Vδ2 T cells *in vitro* and *in vivo* for cancer immunotherapy.

[31] and correlates with clinical response to ICB in CRC patients [24]. NKG2A, on the other hand, identifies a subset of 'educated' Vδ2 cells that are endowed with superior antitumor function, in terms of cytokine production and cytotoxicity, compared to their NKG2A-negative counterparts [32,33]. This increased effector potential of NKG2A$^+$ γδ T cells is tempered by inhibitory signaling upon NKG2A binding to its ligand HLA-E, which is expressed in malignant cells, and can be restored upon NKG2A blockade [32]. These findings indicate that, in CRC, distinct γδ T cell subsets are constrained by specific checkpoint ligands which limit their function. This inhibitory circuitry is intertwined with the emergence of protumorigenic γδ T cell populations such as Vδ1 cells that produce amphiregulin (AREG), an epidermal growth factor (EGF)-like ligand involved in epithelial cell proliferation. In pediatric tissues, Vδ1 cells that produce AREG are linked to tissue repair functions during early life [32]. In CRC, dysregulated AREG production by Vδ1 cells may contribute to tumor progression [33]. Indeed, binding of AREG to EGFR-expressing tumor cells can activate downstream pathways (e.g., PI3K/AKT and IKK/NF-κB) that promote tumor cell survival and migration, as well as immune evasion through enhanced regulatory T cell (Treg) suppressive function and upregulation of PD-L1 [34,35]. Finally, although IL-17 production has long been associated with the protumor activity of γδ T cells in CRC, recent scRNA-seq data have challenged this view (Box 3).

Similarly to the primary tumor, in LM lesions the mean percentage of γδ T cells is lower than in the peritumoral area (5% vs 15%), and Vδ1 cells represent the most abundant subset in both compartments [34,35]. scRNA-seq analysis of LMs identified Vδ1, Vδ2, and Vδ3 cells with a proliferative and cytotoxic profile, shaped by subset-specific activation programs. In fact, Vδ1 and Vδ3 cells displayed strong IFN-γ-driven activation, whereas Vδ2 cells adopted a type 3 profile (expression of *CCR6*, *IL23R*, and *RORC*) [36] that is linked in LMs to TNF-responsiveness [34]. Moreover, different cell subsets express the tissue-retention markers CD69 and CXCR6 which favor their persistence within the tumor, and exhibit distinct receptor signatures. Indeed, Vδ1 cells are enriched in CD16, KIRs, PD-1, and TIGIT, whereas Vδ2 cells express high levels of NKG2A and KLRG1 [34].

Prognostic value of γδ T cells in CRC progression

Tumor-infiltrating γδ T cells have been consistently associated with favorable outcomes across multiple solid tumors [37–40], and both the Vδ1 and Vδ2 subsets demonstrate clear prognostic significance [41–45]. In primary CRC, enrichment of Vδ1 cells correlates with longer 5 year disease-free survival [46], and a high frequency of NKp46$^+$Vδ1 cells in adjacent healthy tissue is linked to reduced disease progression [21]. BTN-like (BTNL) molecules shape and maintain human gut-resident γδ T cells through direct γδTCR interactions [47–50]. Reduced BTNL expression, which has been reported in CRC and inflammatory bowel disease (IBD), is associated with a decreased frequency of gut γδ T cells [49,51]. Among these molecules, BTNL3/8

Box 3. Controversial protumoral role of Th17-polarized γδ T cells in CRC

Mouse γδ T cells are important source of the proinflammatory IL-17, as shown in multiple disease models including cancer [114]. However, the proposed protumoral role of human γδ T cells in CRC, that is attributed to their production of IL-17, remains highly debated [26]. This hypothesis initially gained traction from flow cytometry studies suggesting that γδ T cells were the predominant source of IL-17 within CRC tumor tissues [115,116]. However, subsequent investigations using similar approaches have yielded conflicting results, indicating that the majority of IL-17-producing cells in CRC tissues are αβ T cells [46], whereas γδ T cells predominantly produce IFN-γ [25,46]. Further insights from scRNA-seq analyses on sorted γδ T cells from CRC lesions failed to detect IL-17A transcription and showed minimal expression of canonical Th17-associated genes such as *RORC*, *IL23R*, and *CCR6* [22,26]. In addition, an integrated analysis of whole-tissue scRNA-seq datasets from 187 CRC patients across nine studies confirmed that IL-17-producing cells in both tumor and adjacent normal tissues are predominantly CD4 T cells [22]. Similarly, scRNA-seq analysis of liver metastases (LMs) from mCRC patients identified a minor subset of Vδ2 T cells with Th17 profile [34]; however, these cells lacked detectable IL-17A expression and mainly produced TNF.

heterodimers sustain the intrinsic intraepithelial CD103^{+}NKp46^{+}Vδ1Vγ4 subset [52]. Loss of the BTNL3/8–Vδ1 axis in CRC may weaken epithelial integrity and increase susceptibility to tumor development, consistent with findings in IBD where BTNL3/8 deficiency correlates with more severe disease [52]. Therefore, this specific axis could represent a valuable prognostic biomarker.

In LMs, elevated levels of CD69^{+}Vδ1 cells predict fewer metastases and improved survival [34]. In particular, a subset of terminally differentiated effector memory (T_{EMRA}) CD69^{+}Vδ1 cells can recirculate from the liver into the bloodstream while retaining transcriptional and clonal features characteristic of the LM site. Importantly, their number in blood (as detected via liquid biopsy), similarly to tumor-associated CD69^{+}Vδ1 cells, is associated with a favorable prognosis. Human Vδ3 cells, although typically rare in healthy blood, represent a substantial liver population [3,53]. Similarly to Vδ1 cells, paired blood–LM scRNA-seq analyses confirm Vδ3 cell presence in both compartments, suggesting that they recirculate via the blood [34]. Vδ3 cell frequencies increase in liver inflammatory conditions [54]; although their clinical relevance in hepatic mCRC remains to be defined, their cytotoxic potential and detectability in patient blood support their potential as a prognostic marker.

Chemotherapy, that is commonly administered to mCRC patients, affects both the circulating γδ T cell compartment and LMs [35]. It depletes naïve and central memory (T_{CM}) Vδ2 cells while increasing senescent CD57^{+}Vδ2 T_{EMRA} cells with reduced effector function, whereas Vδ1 cells appear more resistant. These different effects reflect intrinsic variations in differentiation and effector status across γδ T cell subsets, referred as 'effectorness' [55], and suggest that preservation and recovery of these subsets after treatment may correlate with improved prognosis.

γδ T cell-based immunotherapeutic approaches in CRC

In non-metastatic CRC, treatment is primarily curative, and surgery is followed by chemotherapy [folinic acid, 5-fluorouracil, and oxaliplatin (FOLFOX), or capecitabine and oxaliplatin (CAPOX)] in high-risk patients. In mCRC, systemic chemotherapy (FOLFOX, folinic acid, 5-fluorouracil, and irinotecan (FOLFIRI), or FOLFOX plus irinotecan (FOLFOXIRI)] with bevacizumab (anti-VEGF) or cetuximab/panitumumab (anti-EGFR) antibodies is the standard of care, and surgery is feasible in only 20% of cases [56]. The marked heterogeneity of CRC complicates the selection of optimal treatment regimens. Molecular markers such as mismatch repair/microsatellite-instability (MMR/MSI) status and *RAS*/*BRAF* mutations are routinely used to guide therapy; however, a significant proportion of patients within these subgroups do not benefit from available treatments. Recently, the classification of CRC into four **consensus molecular subtypes (CMS1–4)**, based on intrinsic tumor biology rather than clinical endpoints, has offered the potential to better predict both prognosis and response to systemic therapy [57]; however, further studies will be necessary to support its clinical implementation.

New therapeutic strategies that have emerged over the past decade for CRC treatment include ICB, CAR-T cells, vaccines, and multimodal regimens [58]. ICB is effective in tumors with MMR-deficiency (MMR-d), MSI-high (MSI-H), and *POLE* mutations, collectively referred to here as MSI-H. These tumors exhibit a strong immunogenic profile driven by their high **tumor mutational burden (TMB)** and abundant tumor-associated antigens (TAAs), features that supported FDA approval of anti-PD-1 for first-line therapy in 2020 [59]. By contrast, limited TAA expression contributes to resistance in MMR-proficient (MMR-p) and microsatellite-stable (MSS) CRC, hereafter termed MSS tumors, which account for 95% of all mCRC cases. Nevertheless, accumulating evidence suggests that ICB and CAR-T therapies may also induce responses in MSS tumors (Box 4).

In parallel with these established approaches, γδ T cells have emerged as a novel immunotherapeutic target in CRC. Their MHC-independent recognition and innate-like cytotoxicity provide a

Box 4. Emerging immunotherapeutic approaches in advanced mCRC

Evidence for immune checkpoint blockade (ICB) therapy

Randomized controlled clinical trials in microsatellite-stable (MSS) mCRC patients are limited, and the only international randomized Phase 3 trial, LEAP-0176, revealed a less favorable outcome with anti-PD-1 versus standard of care [117]. On the other hand, recently potential clinical predictors of ICB response in MSS tumors were observed [118]. Analyses of the Phase 2 AtezoTRIBE randomized trial, which evaluated FOLFOXIRI/bevacizumab alone or in combination with anti-PD-L1, provided compelling evidence that the novel 'Immunoscore-IC' may predict the efficacy of ICB in MSS mCRC [119]. This assay, that measures the densities and spatial organization of PD-L1$^+$ CD8 cells, identified ~30% of MSS tumors that were responsive to ICB, and also retained its predictive impact in patients with LMs [119,120]. In accordance, the CheckMate 9×8 Phase 2 randomized trial, that compared upfront FOLFOX/bevacizumab alone or in combination with anti-PD-1, was able to use the CD8 T cell level in MSS tumors to identify patients who derive benefit from anti-PD-1 treatment [121]. Overall, these findings highlight the need for deeper investigation of the mCRC TME to identify immune-competent niches in MSS tumors and uncover potential immunotherapeutic targets. Indeed, the AtezoTRIBE study revealed poor concordance between the Immunoscore-IC and tumor-infiltrating lymphocyte (TIL)-based tests, and provided evidence that rough evaluation of immune cells in MSS is insufficient to predict the benefit of ICB [122]. Moreover, there was poor agreement between PD-L1 expression and the response to ICB in MSS tumors [121,122]. Another aspect that requires further investigation regards the combination of ICB treatment with chemotherapy that could potentially overcome immune refractoriness by increasing the release of tumor-associated antigens (TAAs) [119,120,123].

Evidence for CAR-T cell therapy

Current preclinical evidence supports CAR-T cell therapy as a viable and potentially effective treatment for patients with CRC, also in the metastatic setting. One of the major challenges in applying CAR-T therapy to solid tumors is the identification of TAAs that allow selective targeting of cancer cells. Carcinoembryonic antigen (CEA) is one of the most clinically advanced targets in CRC that show therapeutic activity [124]. Other significant targets are guanylyl cyclase C (GUCY2C) and CD18 that have demonstrated antitumor efficacy and relevance to mCRC onset in preclinical models [125–127]. Lastly, the full-length ectodomain sequence of CD6, which binds to CD166 and CD318, was used to build CARs for CRC [128].

complementary mechanism to conventional αβ T cell-based therapies. Several innovative strategies are under development to harness γδ T cells in the clinic, including ICB therapies, *in vivo* and *ex vivo* activation and expansion, and CAR/γδTCR engineering, that have potential applications in mCRC (Figure 2).

Harnessing ICB to activate γδ T cells in CRC

Based on scRNA-seq analysis, γδ T cells have been identified as key effectors that sustain the response to ICB in patients with MSI-H CRC and HLA class I deficiencies [24]. Responses to PD-1 therapy in these patients showed that mutations in *B2M*, that encodes an essential component of HLA class I, are associated with significant clinical benefit from PD-1 blockade. This finding suggests that immune cells beyond HLA class I-restricted cells contribute to tumor control. Further analyses indicate that PD-1 is primarily expressed on γδ T cells, and the Vδ1 and Vδ3 subsets represent the predominant tumor-infiltrating γδ T cell populations in these patients [24]. PD-1-expressing γδ T cells also exhibit proliferation and an activated phenotype characterized by the expression of NKp46 and NKG2D. This activated profile of Vδ1 cells in MSI-H CRC is consistent with previous observations that PD-1$^+$ γδ T cells in MSI-H tumors coexpress activation markers such as CD103, CD38, and HLA-DR, along with effector and cytotoxic mediators (IFN-γ, granzymes, perforin) [60]. PD-1$^+$ γδ T cells isolated from MSI-H lesions and expanded *in vitro* demonstrate strong reactivity against CRC cells and tumor-derived organoids, highlighting their functional relevance [24]. Interestingly, their reactivity against B2M-deficient organoids, compared to wild-type organoids, suggests that loss of HLA class I may release γδ T cells from inhibitory control. This is particularly relevant in the light of evidence that γδ T cell activity can be negatively regulated by KIRs upon HLA class I binding [30]. On the other hand, in MSS tumors, a dysfunctional transcriptional profile of tumor-infiltrating Vδ1 cells has been associated with the expression of TIGIT – which can interact with NECTIN expressed on fibroblasts to suppress their activity [28]. Importantly, blocking this axis with an anti-TIGIT antibody partially restored the cytotoxicity of the dysfunctional Vδ1 cells.

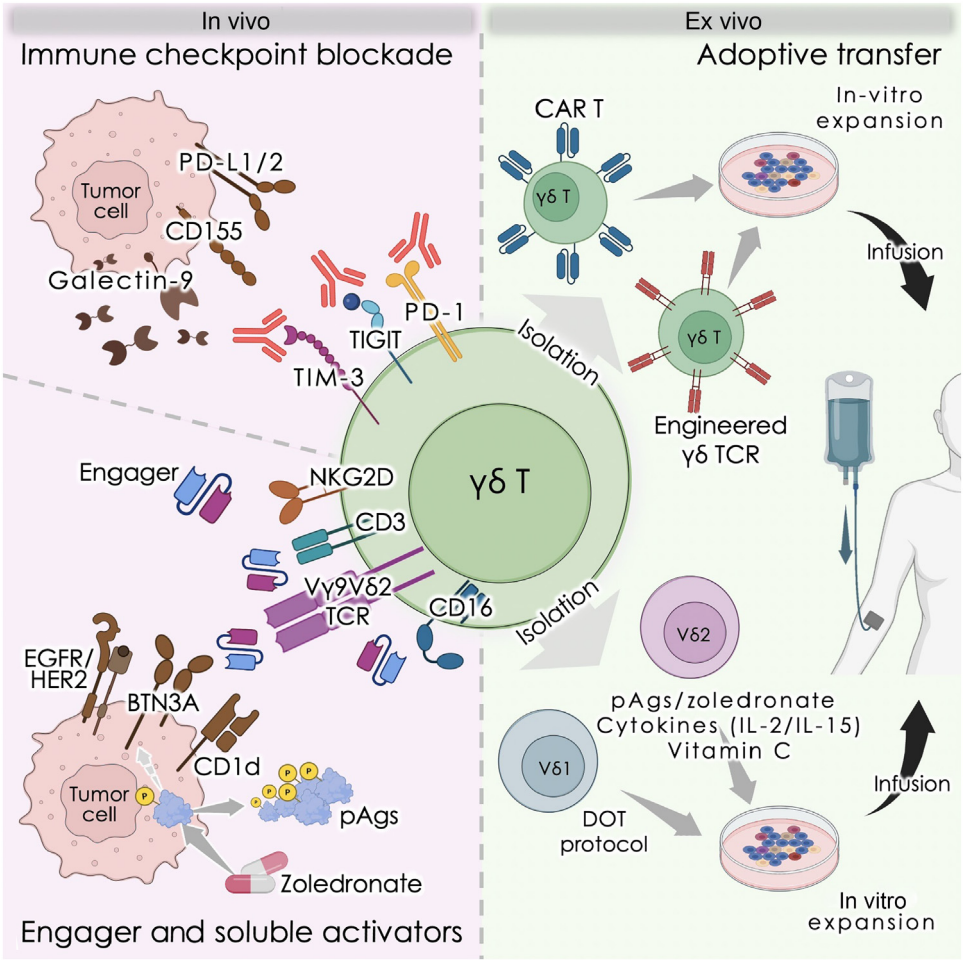

Figure 2. Immunotherapeutic strategies to exploit γδ T cells in colorectal cancer (CRC). Schematic representation of key immunotherapeutic targets and strategies to exploit γδ T cells in CRC. The figure is divided into two main panels, illustrating the *in vivo* (left panel) and *ex vivo* (right panel) approaches that represent alternative strategies to exploit the intrinsic antitumor activity of γδ T cells. *In vivo* strategies include γδ T cell activation mediated by immune checkpoint blockade (ICB) that targets major immune checkpoints relevant to CRC, such as PD-1, CTLA4, TIGIT, and TIM-3. In addition, *in vivo* approaches include the use of bispecific antibodies designed to engage γδTCRs, with most studies focused on the Vγ9Vδ2 subset. These antibodies can simultaneously target activating receptors, including CD3, NKG2D, and CD16, as well as tumor-associated molecules such as EGFR, HER2, CD1d, and B7M3A, and thereby enhance the specificity and efficacy of γδ T cell-mediated cytotoxicity. Furthermore, *in vivo* stimulation can involve phosphoantigen (pAg)-dependent activation of Vγ9Vδ2 cells, where pAgs can be produced by tumor cells or induced pharmacologically using drugs such as zoledronate. *Ex vivo* strategies focus on the isolation, activation, and expansion of γδ T cells outside the patient. Cells obtained from autologous or allogeneic sources are then expanded *in vitro* using protocols tailored to specific subsets. For Vδ1 cells, DOT-based protocols are used whereas Vδ2 cells are expanded using pAgs or zoledronate. These expanded cells are then reinfused into patients, either directly or following genetic modifications such as CAR engineering or γδTCR modifications. Abbreviations: CAR, chimeric antigen receptor; DOT, Delta One T cells; EGFR, epidermal growth factor receptor; HER2, human epidermal growth factor receptor 2; TCR, T cell receptor. Figure generated with BioRender.

Recent findings showed that MSI-H and MSS CRC cells can coexist in the same TME [61,62], a phenomenon with important biological and therapeutic implications. In particular, preclinical studies demonstrated that immune responses directed against the MSI-H component can extend to neighboring MSS cells within the same tumor niche, and both γδ T cells and CD8 T cells

contribute to this antitumor activity [63]. These observations strengthen the notion that the mixed TME may provide an exploitable therapeutic opportunity by leveraging the higher immunogenicity of the MSI-H component to also sensitize surrounding MSS cells.

It is now widely accepted that, in MSI-H tumors, a high TMB represents the main driver of immune activation rather than direct upregulation of immune checkpoint expression. Building on this concept, several studies are exploring strategies to convert MSS tumors, that are typically refractory to immune responses, into immunologically 'hot' tumors by inducing hypermutation and enhancing responsiveness to ICB-based therapies [64]. Along these lines, treatment with the alkylating agent temozolomide (TMZ) has been investigated in several independent Phase 2 trials in patients with MSS CRC [65,66]. These studies demonstrated that TMZ can induce tumor hypermutation and potentially sensitize the cells to ICB; however, this approach requires further validation. The involvement of γδ T cells in this context warrants particular attention because drug-induced TMB does not necessarily correlate with antigen presentation via MHC class I. Therefore, γδ T cells, through their MHC-independent recognition mechanisms, may play a central role in mediating antitumor immune responses under these conditions. Moreover, comparative bulk and single-cell RNA-seq analyses between MSI-H and MSS tumors revealed increased infiltration of Vδ1 T cells in MSI-H, along with higher expression of effector mediators such as IFN-γ, granulysin, and PD-1 [28]. Moreover, CMS1 tumors, which are enriched for MSI-H status, exhibit higher γδ T cell infiltration compared to the CMS2–4 types [67]. Thus, such treatment may not only enhance γδ T cell activation but also promote their increased infiltration into the tumor. It is also important to note that TMZ-resistant γδ T cells have been developed for the adoptive cell therapy of high-grade gliomas [68].

Among the inhibitory receptors not yet established as therapeutic targets in the clinic, TIM-3 may play a relevant role in regulating γδ T cell function against CRC cells. High TIM-3 expression on γδ T cells has been observed both in primary CRC lesions and in peripheral blood, and was significantly associated with TNM (tumor size, lymph node involvement, metastasis) stage and tumor volume [69,70]. Moreover, TIM-3 has been shown to significantly impair the cytotoxic activity of Vγ9Vδ2 T cells against CRC cells through an ERK1/2-dependent mechanism [69]. As mentioned earlier, NKG2A, that is constitutively expressed on Vδ2 T cells, also warrants further investigation, and several studies are underway to evaluate its potential clinical use [33]. To maximize ICB efficacy in γδ T cells, combined checkpoint targeting may represent a more effective strategy. **Delta One T (DOT)** cells are a clinical grade Vδ1 cell product generated through a 3 week TCR- and cytokine-based expansion protocol which induces *de novo* NCR expression and enhances cytotoxic activity [71,72]. This product demonstrated synergistic inhibitory interactions of TIGIT and PD-1 when tested against both MSI-H and MSS CRC lines [73,74]. Regarding Vδ2 cells, combined ICB treatment has not been sufficiently explored. However, in patients with leukemia, Vδ2 cells coexpressing TIM-3 and PD-1 exhibited significantly reduced effector functions, which were restored by TIM-3 blockade alone or in combination with PD-1, whereas anti-PD-1 treatment alone had no significant effect [75].

Engagers and other *in vivo* modulators of γδ T cell activity

Antibody engagers, which combine a tumor-recognition domain with a T cell engagement domain, are designed to recruit and activate cytotoxic T cells. Building on substantial progress with conventional T cells, recent efforts have focused on developing engagers for γδ T cell activation, although most studies so far have primarily involved Vδ2 cells. A bispecific construct targeting both Vγ9 and EGFR [76] – which is overexpressed in ~60–80% of CRC cases – was shown to engage Vγ9Vδ2 T cells *in vitro* and triggered IFN-γ and TNF production as well as induced the lysis of $EGFR^+$ CRC lines carrying *KRAS* or *BRAF* mutations. This activation was

independent of the cell mutational status or variations in the Vγ9Vδ2 cell receptor sequence. Comparable effects were observed *in vivo* [76]. Furthermore, the companies Acepodia and LAVA Therapeutics have developed engagers that link Vγ9 to EGFR [1,71]. Other engagers include HER2-targeting tribodies [$(HER2)_2$ × Vγ9, or $(HER2)_2$ × CD16] as well as a bispecific Vγ9/CD3 molecule (GAB) that have been tested in solid tumors [77–79]. In prostate cancer, bispecific constructs are under evaluation that simultaneously target Vγ9 and prostate-specific membrane antigen (PMSA) that is highly expressed on tumor cells [1]. Additional targets such as CD1d, CD40, and CD123, as well as anti-Vγ9 bispecific approaches, have also been tested [1,80,81], confirming the potential of γδ T cell engagers [1]. Another area of investigation involves the development of NKG2D-targeted antibodies. For instance, bispecific engagers using NKG2D binders to retarget immune cells toward HER2-positive malignant cells showed enhanced cytotoxicity [82]. Given the role of BTN3A molecules in Vγ9Vδ2 T cell activation, ImCheck Therapeutics has developed a specific anti-BTN3A-agonist antibody that is capable of driving Vγ9Vδ2 T cell activation, and this is currently being tested in Phase 1/2 dose-escalation trails in advanced solid tumors (NCT05307874, NCT05307874).

Administration of natural or synthetic pAgs, such as the bromohydrin pyrophosphate (BrHPP), has also been tested to promote Vγ9Vδ2 T cell expansion *in vivo* in solid tumors, including CRC [83]. Although this approach proved to be safe and well-tolerated, it showed poor pharmacokinetics *in vivo* [1]. Likewise, drugs such as **zoledronate**, which causes intracellular pAg accumulation in tumor cells, have shown limited efficacy despite favorable safety profile, likely due to Vγ9Vδ2 cell exhaustion from chronic stimulation. In this context, recent studies have demonstrated that encapsulation of zoledronate in spherical polymeric nanoparticles can enhance its permeability and retention at the tumor site [84]. These nano-formulated zoledronate particles can be taken up by CRC cells, tumor spheroids, and autologous tumor organoids, and subsequently promote Vδ2 cell-mediated cytotoxicity. If proven effective, γδ T cell engagers could offer a potent and cost-effective immunotherapy compared to more complex and expensive approaches such as CAR-γδ T cell therapies. However, to overcome TME immunosuppression, combining them with ICB may be necessary to maximize their therapeutic potential.

Adaptive γδ T cell-based therapy in CRC

γδ T cell-based immunotherapies have largely focused on the adoptive transfer of *ex vivo* expanded cells in both autologous and allogeneic settings. In particular, the allogeneic setting is especially interesting because γδ T cells mediate independent of MHC-antigen presentation tumor killing. This provide an alternative to αβ T cell-mediated recognition of tumors that evade αβ T cell cytotoxicity while maintaining a low risk of GVHD and CRS. Most clinical efforts have concentrated on Vγ9Vδ2 cells, which can be easily isolated from peripheral blood and efficiently expanded *in vitro* using pAgs. Over the past two decades, several clinical studies have evaluated the safety and efficacy of infusing activated and expanded *in vitro* Vγ9Vδ2 cells into cancer patients. In a small non-randomized exploratory adoptive-cell therapy trial involving six patients with mCRC, autologous Vγ9Vδ2 cells were expanded with zoledronate and IL-2 over an 8 week period [85]. During treatment, both the percentage and absolute number of Vγ9Vδ2 cells increased and remained stable long after the final infusion. Notably, these cells displayed higher *ex vivo* expression of IFN-γ and CD107a compared to their $Vγ9^-$ cell counterparts. Similarly, a proof-of-concept study in 25 patients with advanced solid tumors, including one CRC patient [86], showed that γδ T cells expanded with zoledronate and IL-2 could be safely reinfused and restored effector γδ T cell numbers without causing severe toxicity. Moreover, a Phase 1 single-arm study, including three patients with CRC, demonstrated the safety and feasibility of adoptive transfer of *ex vivo* expanded autologous Vγ9Vδ2 cells [87]. Interestingly, a novel method for expanding Vγ9Vδ2 cells was developed that used stimulation with zoledronate, IL-2, IL-15,

and vitamin C to generate cells with improved proliferation capacity and cytotoxicity [88]. When tested in a Phase 1 single-arm clinical trial in 132 patients with late-stage solid tumors in an allogeneic setting, these expanded Vγ9Vδ2 cells prolonged survival in 18 patients with advanced lung or liver cancer who received five or more infusions.

Although most γδ T cell-based strategies have focused on Vγ9Vδ2 cells, Vδ1 cells have also produced encouraging results. Following isolation from peripheral blood and expansion using phytohemagglutinin (PHA) and IL-7, Vδ1 cells exhibited potent *in vitro* cytolytic activity against both adherent and sphere-forming human CRC cells, and effectively suppressed tumor growth in a CRC xenograft model [89]. Supporting these findings, in a murine model of mCRC generated by orthotopic implantation of human HT29 cells, cytomegalovirus (CMV)-induced Vδ1 cells inhibited both primary tumor growth and metastatic spread [90]. Moreover, DOT cells showed efficacy against both MSI-H and MSS CRC lines, as well as against patient-derived organoids [73,74].

Beyond adoptive transfer, engineered γδ T cells are a promising complementary approach, as described in several recent reviews [1,71,91,92]. Multiple studies have reported the feasibility of transducing γδ T cells with different CAR constructs to generate CAR-γδ T cells with enhanced cytotoxicity. Additional strategies exploit tumor-reactive αβ T cells engineered to express γδTCR. These CAR-γδ T cells and γδTCR-modified cells are currently being tested in Phase 1/2, single-arm, dose-escalation clinical trials for refractory solid tumors (NCT06150885, NCT05302037, NCT04864054, NCT04502082, NCT04634357). None of these studies specifically target CRC, except for an allogeneic CAR-γδ T cell product directed against NKG2D ligand that was evaluated in patients with various solid tumors including CRC (NCT04107142). Nevertheless, advances in expansion and engineering protocols for Vδ1 and Vδ2 cells, together with growing evidence of safety and potent antitumor efficacy, encourage future trials in CRC patients.

Concluding remarks

Immunotherapy has improved outcomes for some CRC patients but its efficacy remains limited in advanced disease and LM. γδ T cells are emerging as promising effectors owing to their MHC-independent CRC recognition and ability to enhance the response to ICB in αβ T cell-resistant settings. Innovative strategies to exploit their antitumor potential include engager molecules, *ex vivo* expansion and infusion of autologous or allogeneic γδ T cells, and CAR-γδ/γδTCR-engineered cells to boost their specificity, persistence, and cytotoxicity. Although many of these approaches have not yet been tested in CRC, preclinical and clinical data from other tumors suggest that γδ T cell-based therapies could improve outcomes in CRC. However, to fully exploit this potential, several issues must be clarified (see Outstanding questions). A more comprehensive understanding is needed of how innate and adaptive γδ T cell programs contribute to antitumor immunity in the CRC TME, which specific ligands are recognized, particularly by Vδ1 cells, and how tumor-reactive γδ T cells can be phenotypically defined, clonally tracked, and selectively expanded for therapy. It also remains unclear how these therapies can be integrated with conventional treatments to maximize efficacy. Finally, robust biomarkers will be necessary to guide patient selection and predict response or resistance to γδ T cell-based interventions.

Outstanding questions

How do innate and adaptive γδ T cell programs mediate antitumor activity in CRC, and how do they interact?

Which ligands are specifically recognized by CRC-infiltrating γδ T cells, particularly Vδ1 subsets, and how does the TME influence this recognition?

What are the key phenotypic, transcriptional, and clonal features of tumor-reactive γδ T cells in CRC, and how can these subsets be isolated and expanded for therapy?

Which strategies best integrate γδ T cell-based therapies with standard treatments to maximize clinical benefit?

Which biomarkers can guide patient selection and predict response or resistance to γδ T cell-directed immunotherapies in CRC?

Author contributions

J.M. developed and wrote the review. P.M. and V.C. contributed to manuscript preparation. D.M. critically reviewed and revised the manuscript. All authors contributed to the article and approved the final version.

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Declaration of interests

The authors declare no competing interests.

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