# Review Article Controversial role of γδ T cells in colorectal cancer

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Abstract: Colorectal cancer (CRC) is the third most frequent type of cancer, and the second leading cause of cancerrelated deaths worldwide. Current treatments for patients with CRC do not substantially improve the survival and quality of life of patients with advanced CRC, thus necessitating the development of new treatment strategies. The emergence of immunotherapy has revitalized the field, showing great potential in advanced CRC treatment. Owing to the ability of tumor cells to evade the immune system through major histocompatibility complex shedding and heterogeneous and low antigen spreading, only a few patients respond to immunotherapy.  $\gamma\delta$  T cells have heterogeneous structures and functions, and their key roles in immune regulation, tumor immunosurveillance, and specific primary immune responses have increasingly been recognized.  $\gamma\delta$  T cells recognize and kill CRC cells efficiently, thus inhibiting tumor progress through various mechanisms. However,  $\gamma\delta$  T cells can potentially promote tumor development and metastasis. Thus, given this dual role in prognosis, these cells can act as either a "friend" or "foe" of CRC. In this review, we explore the characteristics of  $\gamma\delta$  T cells and their functions in CRC, highlighting their application in immunotherapy.

Keywords: Colorectal cancer, γδ T cells, tumor microenvironment, immunotherapy, T-cell receptors

#### Introduction

Colorectal cancer (CRC) is one of the most common malignant tumors, with an estimated 1.9 million new CRC cases and 935,000 CRCrelated deaths in 2020, the third and second highest figures worldwide, respectively [1]. The 5-year survival rate for patients with stage I CRC is >90%, whereas that for patients with stage IV CRC is only 10% [2]. The main treatments for CRC are endoscopic resection, surgery, radiation therapy, and systemic therapy [3]. However, the efficacy of current treatment strategies for patients with intermediate to advanced CRC remains unsatisfactory. Therefore, more effective treatments are urgently needed for patients with advanced CRC. The emergence of immunotherapy has revitalized the field, showing great potential in advanced CRC treatment.

Unlike the effects of conventional cancer therapies that influence the proliferation, survival, and metabolic activities of tumor cells, the anti-tumor effects of immunotherapy are mainly manifested through the modulation of the tumor microenvironment (TME), restoration of anti-cancer immunity, and stimulation or suppression of the immune system [4]. The major factors affecting immunotherapy encompass the TME, tumor immunogenicity, major histocompatibility complex (MHC) dysfunction, irreversible T-cell failure, and tumor mutations [5]. In addition to malignant cells, the TME of CRC contains endothelial cells, stromal fibroblasts, and host-derived interacting cells, and the presence of immune cells is considered to be a strong predictor of clinical prognosis [6, 7].

Approximately, only 14% of patients with CRC who have mismatch repair defects or high microsatellite instability respond to immunothera-

py [8]. However, current common immune checkpoint inhibitors are nonfunctional in tumors with high mismatch repair capacity, microsatellite stability, or low microsatellite instability [9]. In the TME with low microsatellite instability, there are only a few functional tumorinfiltrating lymphocytes (TILs) and numerous immunosuppressive cells, which include T regulatory cells (Tregs), myeloid-derived suppressor cells (MDSCs), and tumor-associated macrophages [10]. Therefore, searching for new targets is imperative, and  $\gamma\delta$  T cells are attracting attention owing to their potent antitumor activity and unique role in immune surveillance, thereby making them an attractive tool for cancer immunotherapy.

Within the immune cells that comprise the TME, yo T cells are an innate subpopulation of T lymphocytes that are of interest owing to their ability to mediate natural immunity as the first line of defense [11]. Based on function, γδ T cells are classified into different subpopulations, such as yo T1, yo T17, follicular helper yo T (γδTfh), γδ Treg, and memory γδ T cells [12]. In oncology, γδ T cells constitute a strong positive prognostic indicator in most malignancies [13] and can infiltrate a variety of tumor tissues such as rectal, pancreatic, or breast cancer tissues [14]. While yo T cells can directly kill tumor cells, several of these cells have been used clinically for treating certain malignancies; however, further research is needed to apply these cells against CRC.

# $\gamma\delta$ T cells in the intestine

γδ T cells constitute a subpopulation of peripheral blood T lymphocytes. The two polypeptide chains that make up the yo T-cell receptors (TCRs): the  $\gamma$  and  $\delta$  chains, both of which are non-covalently linked to CD3 molecules and influence the TME [15]. According to their TCR-δ chains, human  $v\delta$  T cells can be divided into two major subpopulations. By fighting against invading intestinal pathogens, epithelial stress, and malignant transformation, yo T cells perform key roles in mucosal tissue homeostasis and immune surveillance. yo T cells act as a bridge between innate immunity and adaptive immunity, which are the first lines of defense against infection and malignancy [16]. In barrier sites including the intestine,  $\gamma\delta$  T cells are particularly abundant, with as much as 20% of

intraepithelial CD3+ T cells in the human colon expressing γδ TCRs [17]. γδ T cells are primarily found in the intestinal epithelial mucosa and represent the first line of resistance towards pathogens. yo T cells may play an important role in the pathogenesis of inflammatory bowel disease and colitis-associated cancer [18]. Normal colonic tissues are dominated by V $\delta$ 1 T cells, while V $\delta$ 2 T cells are less expressed. In patients with chronic inflammatory bowel disease, intestinal Vδ1 T cell frequency is lower, while that of V $\delta$ 2 T cells is high and correlates with the duration and severity of the inflammatory response [19]. Vδ1+ cells in colonic epithelial tissue can be powerful inflammatory cells, by producing IL-17 and other related cytokines. and suppressor cells that reduce the ability of the immune system to kill cancer cells [20]. Human yδ-intestinal epithelial lymphocytes (IELs) scan epithelial cells for the expression of MHC I-related genes, which are inducers of toxicity in γδ T cells, and account for approximately 40% of IELs [21]. Moreover, vo IELs mediate the intestinal-resident bacteria, which can penetrate the intestinal mucosal epithelial cells, to stimulate early protective immunity [22]. Vδ1 T cells are mainly expressed in mucosal and epithelial tissues and account for the majority of human intestinal IELs, which proliferate mainly in peripheral tissues, including solid tumors [23]. Furthermore, human intestinal epithelial Vo1T cells respond to tumor cells overexpressing MICA/B and ULBP through the synergistic action of TCR and NKG2D [24]. yo IELs stimulate mucosal healing and recruit macrophages to the area of injury, preventing symbiotic cell infiltration after injury, limiting excessive inflammatory responses, and maintaining homeostasis by removing damaged epithelial cells [25]. A recent study demonstrated that a subpopulation of human intestinal intraepithelial Vδ1 T cells constitutively expressing NKp46 exerted higher anti-tumor activity, and NKp46 expression on intestinal intraepithelial lymphocytes correlated with high cytolytic capacity. In tumor-free tissues of patients with CRC, a lower frequency of NKp46pos/Võ1 IELs increased the risk of metastasis [23]. yo T cells possess a range of biological effects, which include killing tumor cells, engaging in immunomodulation, presenting antigens, and assisting in dendritic cell (DC) maturation [26]. As vo T cells are not MHCrestricted, their activation does not require

antigen-presenting cells (APCs) for antigen processing and presentation; they are an antitumor immune complement to  $\alpha\beta$  T cells and can be activated rapidly in the early stages of immune response. Thus, these cells as a highly promising immune population to develop novel cancer immunotherapies [27]. Better control of tumor growth by  $\gamma\delta$  T cells than by conventional  $\alpha\beta$  T cells at the same quantity suggests that  $v\delta$  T cells have a better ability to suppress tumor growth and target tumors for killing [28]. During TCR vδ rearrangement. Vδ2 and Vy9 are nearly completely co-expressed to create Vy9Vo2 T cells, which are the predominant γδ T subtype in peripheral blood, accounting for approximately 90% of the total [29]. Brandes et al. [30] demonstrated that Vy9Vδ2 T cells present antigens 100-fold more efficiently than  $\alpha\beta$  T lymphocytes or monocytes. These can recognize phosphoantigens and exhibit powerful anti-tumor effects on, among others, cholangiocellular carcinoma, primary glioblastoma, and breast cancer [31].

In an azoxymethane-induced CRC model, mice deficient in yo T cells were more tumorigenic than mice deficient in  $\alpha\beta$  T cells, which suggests that yo T cells can effectively function as primary tumor suppressors [28]. γδ T cells killed cancer cells through the production of chemokines and cytokines or direct engagement with cancer cells via death receptor signaling, and their greater ability to survive in hypoxic environments suggests that they may be favorable target cells for immunotherapy, particularly, against CRC [32]. Flow cytometry and transcriptome analysis have shown that most of the  $\gamma\delta$  T cells in CRC tissues constitute a higher number of cytotoxic Vo1 cells [33]. Vδ1+ yδ T cells exhibit high anti-tumor activity against various cancers, such as chronic lymphocytic leukemia, multiple myeloma, breast cancer, and CRC [34]. In a study of yo T cells in the CRC TME, γδ T cells were detected predominantly in paracancerous tissues and rarely in intratumoral tissues, and there was no significant increase in the number of T-cell subpopulations of V $\delta$ 1 and V $\delta$ 2 in CRC-infiltrating  $\gamma\delta$  T cells; however, the predominant subpopulation comprised Vo1 T cells [35]. Vo3+ T cells recognize CD1d molecules expressed on target cells with cytotoxic activity and release Th1-, Th2-, and Th17-like pro-inflammatory cytokines [36].

The plasticity or polarization is another crucial characteristic of  $\gamma\delta$  T cells, and the TME provides abundant cytokines and hypoxic conditions that contribute to the polarization of  $v\delta T$ cells towards distinct effector subtypes, resulting in antitumor or tumor-promoting functions [37]. The antitumor function of  $y\delta$  T cells is often inhibited by the TME, which contributes to their transformation into a subpopulation that promotes tumor progression [38]. Owing to their plasticity,  $\gamma\delta$  T cells could be regarded as "friends" or "foes" of CRC. We discuss this dual role in CRC immunotherapy, shifting the perspective of  $\gamma\delta$  T cell-based immunity toward their newly discovered immunosuppressive regulatory function.

# Tumor cell recognition

γδ T cells recognize a variety of antigens in an MHC-dependent or -independent manner; they can be effective against tumors with low mutational load and MHC downregulation [39]. Unlike conventional  $\alpha\beta$  T cells, recognition of ligands by most yo T cells does not require antigen presentation by human leukocyte antigen molecules [40]. The vo TCR-recognition ligand is typically required to express stimulatory stress molecules on T lymphocytes to prevent self-reactions with negative effects [41]. BTN3A1 and BTN2A1 are immunoglobulin-like molecules with immunomodulatory functions that mediate the interaction of  $y\delta$  T cells with phosphorus antigen (PAG) or can be directly recognized by the Vy9Vo2 TCR [42]. Furthermore, Vδ1TCR can recognize tumor cells by MICA [43]. In addition to TCR, γδ T cells express multiple NK cell receptors, such as NKp30, NKG2D, NKp44, tumor necrosis factor (TNF), and DNAM-1, which recognize tumor cells, although their expression is associated with certain conditions [44]. Masking of DNAM-1 on the surface of yo T cells substantially inhibits their capacity to kill tumor cells [45]. When  $\gamma\delta$  T cells come in contact with tumor cells, the Vy9+ subpopulation proliferates rapidly, and NKG2D expression by yo T cells is upregulated [46]. Human vδ T cells exhibit direct cytotoxicity against different types of cancer cells, modulate anti-tumor cytokines, and interact with other immune cells to eliminate tumors [47].

Vy9Vo2 T cells recognize microbes and lipids conjugated to the non-classical MHC protein CD1d [48]. In the absence of MHC restriction, Vy9Vδ2 T cells can recognize non-peptide phosphorylation end-products of isoprene biosynthesis with phosphorylated antigens [49]. The isoprene biosynthetic pathway is upregulated in tumor cells to ensure availability of energy and accumulation of PAG. leading to its recognition by Vy9Vo2 T cells [50]. Additionally, Vy9Vo2 T cells recognize specific ligands overexpressed by tumor cells through the interaction of NKG2D and DNAM-1 with their ligands [51]. These receptors fine-tune the activation threshold of  $\gamma\delta$  T cells and enhance recognition of tumor targets on  $v\delta$  T cells, which induce immediate immune responses to tumor targets [52]. Vγ9Vδ2 T cells also recognize ApoA1 ectopically expressed on the tumor surface and Toll-like receptors, which together are involved in and contribute to the antitumor effects of Vy9Vδ2 T cells [53]. Corvaisier et al. [54] found that colon tumors were frequently infiltrated by Vy9Vo2 T lymphocytes. Expansion of yo T cells may be directly driven by intestinal bacterial products through an array of toll-like receptors that recognize pathogen-associated molecular patterns [55].

 $\gamma\delta$  T cells that infiltrate human CRC recognize stress-associated antigens expressed in transformed cells and react by immediate release of cytotoxic cytokines [56]. Moreover, in addition to the expressed tumor-associated antigens,  $\gamma\delta$  T cells are recognized by various intrinsic cytotoxic receptors expressed on the cell membrane [57].

# Antitumor functions of γδ T cells

Activated  $\gamma\delta$  T cells display potent cytotoxic activity by releasing granzyme B and perforin, amplifying the immune response through membrane-bound TNF-related apoptosis-inducing ligand (TRAIL) and Fas ligands, or producing TNF and interferon (IFN)- $\gamma$ , thereby inhibiting tumor progression [58]. Activated NKp30, NKp44, and NKp46 were also expressed on human V $\delta$ 1+ T cells followed by cytokine activation and co-stimulation to enhance IFN- $\gamma$  production [59].  $\gamma\delta$  T cells exposed to IL-2/IL-12/ IL-18 produce IFN- $\gamma$  and express cytotoxins and FasL, resulting in potent anti-tumor activity [60]. Release of cytokines, such as TNF and IFN- $\gamma$ , by  $\gamma\delta$  T cells activates T cells in the tumor site by acting as APCs [61]. These cytokines inhibit tumor growth through multiple mechanisms, such as downregulating the exposure of cyclin and cyclin-dependent protein kinase, thereby affecting the tumor cell cycle and inhibiting tumor cell proliferation and tumor angiogenesis, ultimately inhibiting the proliferation of tumor cells [62, 63].

Not limited to cytotoxicity, the activation of NKp30 on V $\delta$ 1+ T cells induces the production of CC chemokine ligands CCL3, CCL4, and CCL5, linking target recognition to the attraction of antigen-presenting cells, such as monocytes and conventional  $\alpha\beta$  T cells [64].

γδ T cells can also kill  $\alpha\beta$ -coated cancer cells via the cell surface CD16-like  $\alpha\beta$ -dependent cytotoxicity of NK cells [65]. Additionally, the accumulation of isopentenyl pyrophosphate (IPP) and dimethylallylpyrophosphate in cancer cells leads to aberrant expression of MICA/B and UL16-binding proteins recognizing the tumor cells, and this activates  $\gamma\delta$  T cells and enhances the antitumor efficacy [66]. MICA/B and ULBP are highly expressed in mismatchrepair-deficient CRC cell lines and tumor organs, and removal of these ligands reduced yo T cell activation and cytotoxicity. This suggests a role for the activation receptor, NKG2D, in γδ T cell immunity to mismatch-repair-deficient tumors [67]. During tumorigenesis, Vy9Vδ2 T cells in circulation are inactivated by excessive production of IPP in cancer cells, leading to the expression of inflammatory homing chemokine receptors and mediating infiltration toward tumor tissues and organs [68]. Vy9Vδ2 cell activation with phosphoantigens is currently the most common approach in γδ T cell-based antitumor research and is a key approach in the development of antitumor immunotherapy drugs [69]. Zoledronate (ZOL) enhances IPPinduced  $\gamma\delta$  T cell activation by inhibiting the activity of the IPP-metabolizing enzyme farnesyl diphosphate synthase that increases IPP levels [70]. In vitro, Vγ9Vδ2 T also overregulates both MHC and costimulatory molecules in response to IPP stimulation. Such an APC-like phenotype permits  $V\gamma 9V\delta 2$  T cells to initiate CD4+ T cells, which are polarized in a Th1 antitumor direction [71]. CD3 monoclonal antibodystimulated tumor-derived yo T cells produce large amounts of IFN-y and show strong cytotoxic activity against autologous and allogeneic gastrointestinal tumor cells [72]. In contrast,



Figure 1.  $\gamma\delta$  T cells function against CRC cells.

the use of nitrogen-containing bisphosphonates increase IPP incubation in CRC stem cells and cancer cells sensitized to Vy9Vo2-induced cytotoxicity [73]. yδ T cells further facilitate the phagocytosis of tumor antigens through perforin and granzyme production and CD36 expression. These mechanisms directly induce toxicity in CRC cells, which subsequently lead to the induction of tumor antigen-specific CD8+ T cell responses [74]. Phosphoantigen expression enhances the toxicity of yo T cells against tumor cells, which is partially mediated by the increased expression of hydroxymethylglutaryl CoA reductase [75]. γδ T cells recognize tumor cells and release perforin and granzyme B into the synaptic gap, which further activates caspases and destroys the DNA of tumor cells, leading to tumor cell mortality [76]. Antibodydependent cytotoxicity is also an important mechanism of death induction that allows FcγRIIIA (CD16)-expressing Vδ2 T cells to kill colon cancer cells through antibody-dependent cytotoxicity [77]. Meraviglia et al. [78] showed that most  $\gamma\delta$  T cells in colorectal tumor and adjacent normal tissues comprised the Vo1 T cell subtype and exhibited an effector phenotype. The production of IFN- $\gamma$  is significantly regulated by V $\delta$ 1 T cells in tumor tissues of patients with CRC when compared with normal colonic tissues adjacent to the cancer, possibly due to the presence of certain identified inhibitory molecules in colonic tumor stem cells. The NKp46+ V $\delta$ 1 IEL subpopulation is rare in the intestinal tissue surrounding tumors in patients with CRC and the low frequency of NKp46+ V $\delta$ 1 IELs is associated with rapid progression of tumors to metastatic disease [23].

γδ T cells interact with B helper cells to produce immunoglobulin (Ig)A, IgM, and IgG antibodies while interacting with other immune cells to regulate the immune response [79]. Additionally, granulocyte-macrophage colony-stimulating factor (GM-CSF) production is associated with vδ T cells that maintain potent tumor-killing activity and produce immunomodulatory cytokines [80]. Upon binding to NKG2D, γδ T lymphocyte signals are activated, rapidly exerting their effector functions to destroy bacteria or infected cells by proliferating and producing pro-inflammatory and antibacterial cytokines, and releasing lytic enzymes in response to injury signals [81, 82]. At the level of PAG synthesis, valproic acid synergizes with ZOL to enhance γδ T cytotoxicity. It further affects the association between  $v\delta$  T cells and tumor cells at the site of the NKG2D receptor/ligand axis [83].

 $\gamma\delta$  T cell-mediated TRAIL and Fas ligands form another death receptor signaling pathway. The combination of death effector domains with Fas-associated death domain-containing proteins leads to the activation of caspases and induction of target cell apoptosis [58] (Figure 1). In vitro-expanded V $\delta$ 1 T cells expressing Fas ligands are highly toxic to colon cancer cells [84]. In a subpopulation of Vδ1 γδ T cells, NKp30 interacts with B7-H6 expressed on tumor cells for precise antitumor activity [85]. Additionally, IL-22 produced by vo T cells acts as a regulator of the DNA damage response of enterocyte stem cells against environmental genotoxic factors, preventing malignant transformation and cancer development [86]. In a recent study, the expression of NKp46 on γδ IELs was associated with high antitumor activity against CRC [23]. Li et al. [87] found that the NKp46+ Vδ1 T cell subpopulation was able to restrict the metastatic spread of primary CRC to the liver and confirmed similar results in mice. Meraviglia et al. [78] showed that patients with CRC containing large numbers of yo T cells had a remarkably higher 5-year diseasefree survival rate, and  $\gamma\delta$  T cells were effective in controlling early-stage tumor growth. Vδ1 and V $\delta$ 2 TILs produced low levels of IFN- $\delta$  in colorectal tissues when compared with those in neighboring tissues; however, IL-17 was barely secreted [78]. In particular, an analysis of 585 patients with CRC showed that a high number of yo T cells in the TIL and a reduced production of IFN-y were associated with the likelihood of 5-year disease-free survival [78]. The unique mechanism underlying γδ T cell recognition of CRC cells, tumor invasion, and potent cytotoxicity suggests that vo T cells are promising candidates for antitumor therapy.

# Protumor activities of γδ T cells

Tumor-infiltrating  $\gamma\delta$  T cells promote tumor progression and metastasis by recruiting suppressor cells and inducing the apoptosis of antitumor immune cells [20]. IL-17 produced by  $\gamma\delta$ T cells promotes tumorigenesis and development through multiple downstream effects on tumor, endothelial, and other immune cells [88]. In a preclinical mouse model of CRC, Reis et al. [89] demonstrated that cytotoxic IFN- $\gamma$ producing V $\gamma$ 7- and V $\gamma$ 1-positive cells are essential for tumor surveillance and that IL-17producing V $\gamma$ 6+ T cells promote tumor proliferation.

In colon cancer, IL-17 is mainly produced by  $\gamma\delta$  T cells, in particular, 83% by V $\delta$ 1 T cells [90]. Inflammatory DCs in the tissues of patients with CRC produce IL-23 when irritated by bacterial products leaking from the colonic epithelial

barrier, which drives the V $\delta$ 1+ y $\delta$ Th17 response and results in IL-17 production [91]. Even tumorfree intestinal tissue from patients with CRC contains IL-17-producing y $\delta$  T cells [20]. IL-17 can directly stimulate CRC cells and increase their migratory behavior via the PI3K/AKT signaling pathway [92, 93], which can directly stimulate the proliferation of intestinal epithelial cells by activating the MAPK signal transduction pathway and promote early CRC [94].

In CRC, due to the disruption of the tumor epithelial barrier and inflammatory DCs in the TME, the presence of microorganisms polarizes  $\gamma\delta$  T cells to produce cytokines, such as TNF-a, granulocyte colony-stimulating factor (GM-CSF), IL-17, and IL-8. These cytokines recruit MDSC into the TME, which regulates tumor cell development and induces Treg differentiation, underlining their functional plasticity shaped by the TME [20, 89].

Particularly, in human CRC, because IL-17producing  $\gamma\delta$  T cells can act as pro-carcinogens, they are linked to worse survival and increased metastasis risk; more advanced the disease, the larger the tumor and the higher the risk of metastasis [95]. Additional evidence indicates that the toll-like receptor pathway plays an essential role upstream of IL-17-producing  $\gamma\delta$  T cells in inducing IL-1 $\beta$  and IL-23 production by cancer-related myeloid cells, as this process is initiated by colonic bacteria during carcinogeninduced CRC development [96]. Moreover, IL-17 induces M2 macrophage polarization [97].

γδ T cells can contribute to tumor growth through tumor cell-induced programmed death-1 (PD-1)/PD-L1 interactions and the inhibition of neutrophil and  $\alpha\beta$  Treg production. The TME regulation induced by hypoxia induces tumor cells to shed MICA/B to block the NKG2Dmediated cytotoxicity of yo T cells [98]. Regulatory Vδ1+ T cells recruited by CXCL10 inhibit the activation of  $\alpha\beta$  T cells and DCs and trigger immune senescence, increasing the probability of tumor recurrence and metastasis [99]. Chen et al. [100] discovered a γδ T subpopulation showing high PD-1 expression that was considerably increased in CRC tissues. PD-1+  $v\delta$  T cells infiltrating these tumors expressed tissue-resident, activation, and depletion markers and maintained some level of granzyme B (GzmB)- and perforin-secretion capacity.



Figure 2. γδ T cells promote CRC cells.

γδ Treg lymphocytes induce cell cycle arrest in cytotoxic T lymphocytes and inhibit proliferation by inducing T cell senescence [101]. The polarized vo T17 or vo Treg cells generate high amounts of IL-17 and transforming growth factor- $\beta$  (TGF- $\beta$ ), which trigger an inhibitory immune response and promote CRC advancement [102]. Wakita et al. [103] reported that yo T17 cells induced angiogenesis and promoted tumor growth in CRC through the secretion of vascular endothelial growth factor. yo T17 invasion is positively correlated with clinicopathological features, such as CRC stage, and tumorinduced disruption of the epithelial barrier may lead to invasion by commensal and bacterial products. Thus, dendritic cells activated by microbial products produce vo T17 via de novo polarization [104]. Strong signaling on the CD27TCR along with yo co-stimulation and lymphotoxin signaling directs progenitor cells into interferon γ-producing γδ T-cells, downregulating CD24 and CD25 [105].

Meanwhile, IL-10 and TGF- $\beta$ , secreted by regulatory T cells or myeloid cells, induce the transformation of epithelial cells to mesenchymal cells, thereby increasing cancer aggressiveness [106]. Tumor-derived TGF- $\beta$  induces differentiation of immunosuppressive CD39+ $\gamma\delta$  Treg cells in CRC [107]. Rong et al. [14] described the disproportionate distribution of

 $V\delta1+$  and  $V\delta2+$  T cells in rectal cancer tissues, with a higher proportion of  $V\delta1+$  T cells and a lower proportion of  $V\delta2+$  T cells in tumor tissues than adjacent normal tissues. Tumorinfiltrating  $V\delta1+$  T cells had a strong suppressive function and positively correlated with the patient's tumor stage.

Although intestinal inflammatory responses persist, the Vo1+ T cells underlie angiogenesis, the activation of survival signaling pathways, and the recruitment of MDSC, resulting in a chronic precancerous inflammatory environment [108]. A study of patients with CRC showed that tumor-infiltrating yo T cell-produced IL-17A polarizes inflammatory macrophages, induces MDSC recruitment, and recruits neutrophils to the tumor location, thus promoting cancer metastasis [109]. CRC cell secretions also inhibit the growth of yo T cells, which produce IFN-y, and inhibit the production of CD4+ and CD8+ T cells [78]. Additionally, pro-inflammatory Vo2 T cells may be involved in the pathogenesis of CRC through chronic inflammation [108] (Figure 2).

CD39+ inhibitory  $\gamma\delta$  T cells from human CRC increase GM-CSF and IL-17 levels in tumor tissues [107]. In CRC, CD39+  $\gamma\delta$  T cells inhibit the proliferation of peripheral blood mononuclear cell (PBMC)-derived T cells by expressing FOXP3

while additionally inhibiting the immune response and recruiting monocyte-derived DCs through the adenosine pathway [107], suggesting that they are potential prognostic factors. The right CRC had significantly higher proportions of CD39+  $\gamma\delta$  Tregs, and showed increased expression of IL-17A and Tim-3 and decreased expression of IFN- $\gamma$ ; whereas, the opposite was observed for the left CRC [110].

Treg-like Vδ2 T cells expressing FOXP3 alter the structure of the TME and inhibit the effector T cell activities by inducing the interaction of CD86/cytotoxic T lymphocyte-associated protein 4 (CTLA-4) and PD-L1/PD-1 [111]. Due to the multifaceted and immunosuppressive nature of TME, the balance between pro- and anti-tumor effects of TILs often skews toward a tumor-supporting environment. Specifically, γδ T cells are often exhausted or impaired [112]. Activated V $\delta$ 1+ and V $\delta$ 2+ y $\delta$  T cells upregulate various C-C chemokine receptors (CCRs), such as CCR1 and CCR8, CCR2, CCR5, and CX-C chemokine receptor 3 (CXCR3), to mediate infiltration into the TME [113]. Notably, unlike Vo2 T cells, Vo1 T cells express CCR2 and exhibit a migratory response to CCL2. The expression of this chemokine is significantly altered in several human tumors, such as lung, prostate, liver, and breast cancer [114]. Due to the complex interactions between TME and tumor-infiltrating γδ T cells, activated γδ T cells can be functionally polarized to become antitumor Th1 and follicular Th (Tfh) cells or protumor Th17 and T regulatory (Treg) cells [115].

# Expansion of γδ T cells

γδ T cells can recognize malignant cells, infiltrate tumors, and exert potent cytotoxicity, thereby making them ideal candidates for CRC treatment. However, owing to the low numbers of vo T cells in human peripheral blood, using them in cancer immunotherapy requires substantial in vitro expansion. Vδ2T cells represent the major mutant cells in the circulation, whereas Vo1T cells are enriched in tissues. These cells exhibit potent anti-tumor cytotoxicity and are easily amplified from peripheral blood and ascites [116]. Moreover, Vo1 T cells separated from the blood of patients with CRC and amplified ex vivo showed stronger cytotoxic activity than Vo2 T cells [84]. Santos et al. [117] demonstrated that the amplified V $\delta$ 1 T cells effec-

tively inhibited tumor proliferation and prevented tumor spread in xenograft models, thereby offering a preclinical rationale for their use in tumor-relay immunotherapy. In addition to being isolated from tissues and amplified in vitro, Vo1 cells can also be separated from peripheral blood and amplified using the Vo1+ T cell generation regimen that includes a 3-week clinical-level regimen of TCR and cytokine stimulation [118]. Human peripheral blood Vδ1 yδ T cells amplified in vitro can kill more CRC cells through the cytolytic pathway and receptor-ligand interactions [119]. Ang et al. [120] expanded Vy9V $\delta$ 2 T cells with Zometa/ IL-2 and implanted extracellular domain of NKG2D (NKG2DL)-based CARs in the cells using mRNA electroporation. The authors conducted a preclinical study in mice injected intraperitoneally with colon tumors and demonstrated that the NKG2DL CAR-yo T cells led to tumor regression and prolonged the survival of mice.

Currently, there are two strategies for expanding  $\gamma\delta$  T cells. The first approach is to separate γδ T lymphocytes from PBMCs and stimulate them in vitro with synthetic PAGs or BPs. The second approach stimulates and amplifies vo T cells in vivo through the systemic administration of PAG or nitrogen-containing bisphosphonates [83]. The clinical applications of yo T cells started with the attempt to directly amplify in vivo autologous Vδ2 T cells and were based on their ability to penetrate solid tumors and readily expand [121]. Most early studies attempted to activate Vo2T cells by direct application of nitrogen-containing bisphosphonates or synthetic PAG and low doses of IL-2. Several clinical studies have used this approach in solid tumors and hematological malignancies [122]. The addition of IL-15 to IL-2/ZOL also promotes γδ T cell expansion from PBMCs [123]. Xiao et al. [124] developed a method to increase vo T cell proportion by more than 500,000-fold in just 17 days using K562 cells as artificial APCs, anti-CD3, ZOL, and IL-2. Bennouna et al. [125] treated patients with solid tumors via subcutaneous injection of BrHPP and low-dose IL-2. The outcomes showed that 1,500 mg BrHPP effectively increased the number of human V $\delta$ 2+ T cells in the peripheral blood of patients. Moreover, ZOL remarkably altered the phenotype of Vδ2T cells, transforming them into effector memory cells [126]. BPs stimulate the proliferation of V $\delta$ 2+ T cells *in vitro* and sensitize tumor cells *in vivo*, thus exerting antitumor effects [127]. V $\gamma$ 9V $\delta$ 2 T cells also recognize colon cancer stem cells that are sensitized by ZOL through TCR-PAG interactions and enhance the lethality of  $\gamma\delta$  T cells against these target cells using TRAIL and granzymes [128].

Furthermore, Zocchi et al. [129] found that zoledronate-stimulated local TME of CRC expresses BTN3A1/CD277, which stimulates and expands effector  $V\gamma 9V\delta 2$  T cells with antitumor activity. Therefore, systemic or topical ZOL may be envisaged to improve the therapeutic efficacy of metastatic V $\delta 2$  T cells.

Following zoledronic acid stimulation in vitro, Vγ9Vδ2 T cells transmit co-stimulatory signals to DCs, thereby increasing the number of antigen-specific CD8+  $\alpha\beta$  T cells while inhibiting the amplification of IL-2-dependent Tregs [130]. Additionally, Vy9Vδ2 T cells reportedly expanded and survived well in patients with CRC in the absence of co-cytokines, with a promoting effect on the secretion of either endogenous IL-2 or IL-15 [131]. Cytomegalovirus-induced Vδ1 T cells inhibit not only the growth of primary colon cancer, but also the appearance of metastases [132]. Wu et al. [84] found that freshly isolated human peripheral blood Vδ1 T cells, especially ex vivo and ex vivo-expanded Vδ1 T cells, showed better cytotoxicity against adherent and spherical colon tumor cells when compared to V $\delta$ 2 T cells.

# Preclinical research and clinical efficacy

The ability of yδ T cells to directly recognize and kill tumor cells makes the adoption and in vivo induction of yo T cell expansion therapy a promising direction to explore in anti-cancer immunotherapy. Similar protective effects of yo T cells have been validated in models of bladder cancer [133], malignant melanoma [134], B-cell lymphoma [135], and prostate cancer [136]. These important contributions to tissue homeostasis and cancer immunosurveillance have stimulated scientific interest in further exploring the biology of  $v\delta$  T cells and their clinical translational potential. Generally, γδ T cells correlate positively with a better outcome in CRC [137]. Immunotherapy based on yo T cells has demonstrated promising antitumor effects in vitro in transplantation tumor models and in phase I clinical trials [138]. Safety and tolerability of γδ T cell-based immunotherapies clinically approved for the treatment of non-small cell lung cancer and breast cancer have been reported [139].  $V\delta 2$  T cells are highly appealing candidates for over-the-counter cellular immunotherapy, and the innate-like feature of MHCindependent activation readily permits the transfer of Vδ2 T cells between individuals in a syngeneic manner without incurring the risk of graft-versus-host disease [140]. Clinical studies have utilized aminobisphosphonates for inhibiting farnesyl pyrophosphate synthase activity in the mevalonate pathway, thereby promoting IPP buildup in cells, or synthetic phosphoantigen analogs for activating Vy9Vδ2+ T cells in patients with malignancies [113]. Repeated infusions of allogeneic Vy9Vδ2 T cells are safe and show favorable results, especially in treating liver and lung cancers [141]. Such therapies have been proven to be capable of killing cancer stem cells in colon cancer [141]. Moreover, tumor-infiltrating PD-1+ cells preferentially expressed IL-17, whereas PD-1preferentially secreted IFN-y. There was no overlap between IL-17 and IFN-γ-producing γδ cells [89].

Ang et al. [120] amplified  $V\gamma 9V\delta 2$  T cells with ZOL/IL2, and evaluated the in vivo tumor-killing effect of Vγ9Vδ2 T cells modified with NKG2D RNA chimeric antigen receptor (CAR) in a mouse model of CRC through multiple intraperitoneal injections of an invasive human CRC cell line. The modified  $V\gamma 9V\delta 2$  T cells substantially retarded tumor advancement, leading to a median survival time of 57 days in mice. Devaud et al. [132] reported that in a CRC model, treatment with human V $\delta$ 1 v $\delta$ T cells not only controlled the progression of the primary tumor, but also inhibited the migration of tumor cells. In a previous study, tumor-infiltrated V $\delta$ 1 γδ T cells from patients with CRC can lyse not only autologous tumor cells but also tumor cells, demonstrating the important role of V $\delta$ 1  $v\delta$  T cells in tumor monitoring [142]. Toussaint et al. [143] collected peripheral blood samples from 11 patients (6 CRC, 4 hepatocellular carcinomas (HCCs), 1 sarcoma) and 16 healthy donors for experiments. Treatment of tumor cells with the mevalonate pathway inhibitor ZOL enhanced the killing of HCC and CRC. The expansion index of Vy9Vδ2 T cells was at similar levels in healthy donors or patients with cancer, and the total expansion was suitable for adoptive immunotherapy. The number of tumor

cells in ascites was significantly reduced and the level of IFN-y increased after the transfer of activated Vy9Vo2 T cells. No major negative reactions were observed during the treatment period, and the proportion of ascites was markedly reduced [144]. ZOL can be used for the selective expansion of Vy9Vo2 T cells in blood specimens and for sensitizing tumor cells to kill Vy9Vo2 T cells. Thus, the addition of ZOL and IL-2 to PBMC cultures resulted in the selective expansion of  $V\gamma 9V\delta 2$  T cells, which in turn were highly efficient cancer cell killers after ZOL sensitization of cancer cells [145]. Izumi et al. [131] conducted a clinical trial using Vy9Vδ2 T cell transfer therapy in six patients with CRC undergoing resection of lung metastases. Expansion with zoledronate and IL-2 increased the number of Vy9V $\delta$ 2 T cells during treatment and remained stable long after the last infusion, expressing higher INF-y and CD107a.

Nicol et al. [146] reported the results of a clinical study of autologous γδ T-cell immunotherapy for various metastatic solid tumors, including CRC. A total of 18 patients were recruited for the study. Among the 14 evaluable patients, 3 had stable disease and 11 had progressive disease. A phase I trial explored the treatment of 10 patients with metastatic CRC with adenosine polyphosphate-expanded  $y9\delta2$  T cells. The most common adverse events included cold, runny nose, hypotension, and tachycardia. One patient developed disseminated intravascular coagulation, which lasted 25.7 weeks [147]. Immunotherapy with yδ T cells is a clinically safe method, and adverse reactions of severity greater than grade 2 are generally not associated with  $y\delta$  T-cell therapy and can be managed properly [148].

# Optimization of γδ T-cell immunotherapy regimens

Several novel tactics have been developed to increase the antitumor potency and reduce the adverse effects of  $\gamma\delta$  T cell-based immunotherapies. Additionally, aberrant effects of the combination of radiation and photodynamic or thermotherapy regimens may increase the expression of  $\gamma\delta$  T-cell ligands, leading to substantial clinical efficacy [83]. Previous studies have confirmed that CAR- $\gamma\delta$  T cells targeting CD19 considerably increase the cytotoxicity of CD19+ tumor cells, suggesting that CAR- $\gamma\delta$  T

cells hold great promise in tumor immunotherapy [149].

The T cell activities of  $\gamma\delta$  are also modulated by immune checkpoint receptors, including PD-1, CTLA-4, lymphocyte activation gene 3, T cell immunoglobulin structural domain, and mucin structural domain 3 [113]. Integrating approaches aimed at pre-tumor vo T cells with immune checkpoint blockade therapies considerably increase the response rate and long-term survival of patients with cancer [150]. Li et al. [151] found that there was a remarkable increase in the ratio of Tim-3+  $y\delta$  T cells in the peripheral blood and colon cancer tissues of the patients, and the stimulation of the Tim-3 signaling pathway remarkably suppressed the killing efficiency of Vy9Vo2 T cells against co-Ion cancer cells. Additionally, activation of the Tim-3 signaling pathway decreased perforin and GZMB expression in Vy9Vo2 T cells and reduced the toxicity of Vy9V $\delta$ 2 T cells against colon cancer cells, suggesting Tim-3 as a promising therapeutic target for improving the efficacy of the adoptive immunotherapy of Vγ9Vδ2 T cells for colon cancer. It was recently reported that PD-1+ γδ T cells aggregate in CRC tumor tissues with defective mismatch repair genes, and the PD-1+ γδ T cells were stimulated by PMA to produce IFN-y, GZMB, and perforin, indicating the potential cytotoxic activity of completely activated PD-1+ yo T cells in the antitumor immune response [100]. A research trial using the CTLA-4-targeting monoclonal antibody ipilimumab showed a significant correlation between the proportion of V $\delta$ 2 T cells and survival outcomes in patients who received the treatment [152].

To ameliorate the low persistence of V $\gamma$ 9V $\delta$ 2 T cells in patients with advanced cancer, one strategy under clinical development is to transduce selected high-affinity V $\gamma$ 9V $\delta$ 2 TCRs into  $\alpha\beta$  T cells, and this strategy is expected to produce durable memory responses [153]. In a colon cancer model, a nanoantibody was constructed to induce V $\gamma$ 9V $\delta$ 2 T cell activation and tumor cell killing by targeting V $\gamma$ 9V $\delta$ 2 T cells and epidermal growth factor receptors *in vitro* and *in vivo* [154]. Bispecific antibodies have been employed to promote  $\gamma\delta$  T cell activity and enhance tumor targeting. Transfer to V $\gamma$ 9V $\delta$ 2 T cells with HER2/V $\gamma$ 9 bispecific antibodies con-

siderably reduced colon cancer growth in a mouse xenograft model [155].

Simultaneous chemotherapy and radiotherapy, conventional treatments, enhance the toxicity of yo T lymphocytes against malignant cells [156, 157]. Low doses of 5-fluorouracil, doxorubicin, and cisplatin sensitize colon cancer cells to Vy9Vδ2 T cell killing [158]. NKG2D-targeted antibody inhibits Vy9Vo2 yo T cell lysis in chemosensitive colon cancer-induced cells, whereas chemotherapeutic agents enhance TRAIL expression in colon cancer-induced cells [159]. Topical low-dose y irradiation results in the normalization of abnormal blood vessels and effective recruitment of tumor-specific T cells, which could also be utilized to potentiate the translation of  $y\delta$  T cells to the tumor [160]. While vitamin C is known to reduce the rate of apoptosis of  $\gamma\delta$  T cells during stimulation, the proliferation of  $v\delta$  T cells is promoted by L ascorbic acid 2-phosphate. Thus, it has been used to increase the effectiveness of  $\gamma\delta$  T cells in antitumor immunotherapy [161].

# Conclusions

As a common cancer and a serious human health problem, the incidence of CRC is progressively increasing among younger individuals; however, very few patients with CRC have benefited from conventional immunotherapy. In recent years, the potential of  $y\delta$  T cells in anticancer immunotherapy has attracted considerable attention. An increasing number of studies have demonstrated that yo T cells have antitumor activity against a variety of malignant tumors using their innate and adaptive immunity, and this has shown for the development of yo T cell therapy for CRC. yo T cells have received attention for their antitumor potential and MHC-independent recognition of antigens. yδ T cells help to regulate the balance between immune and immunosuppressive responses. γδ T cells may alternatively support tumorigenesis. Therefore, strategies must be developed to evade the suppressive activity of  $\gamma\delta$  T cells while augmenting their antitumor effects. Despite their promising applications, research on γδ T cells remains in its infancy, with unresolved challenges and obstacles. The main challenge in this area is the small number of yδ T cells and the absence of homology among animal models and humans, rendering the study of the biological features and functions of these cells

challenging. Further evaluation of the clinical efficacy of  $\gamma\delta$  T-cell transfer therapy in prospective clinical trials is warranted, and the combination of this novel therapy with existing therapies is promising to enhance the survival of patients with CRC.

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#### Disclosure of conflict of interest

#### None.

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