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EDITED BY

Paolo Dellabona,
San Raffaele Scientific Institute (IRCCS),
Italy

REVIEWED BY

Lauren J. Howson,
The University of Melbourne, Australia
Adriana Malheiro,
Federal University of Amazonas, Brazil

*CORRESPONDENCE

Serena Meraviglia
✉ serena.meraviglia@unipa.it

†These authors share first authorship

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$\gamma\delta$ T cells and their clinical application in colon cancer

Anna Maria Corsale^{1,2,3†}, Marta Di Simone^{1,2,3†}, Elena Lo Presti⁴,
Francesco Dieli^{1,3} and Serena Meraviglia^{1,3*}

¹Central Laboratory of Advanced Diagnosis and Biomedical Research (CLADIBIOR), University of Palermo, Palermo, Italy, ²Department of Health Promotion, Mother and Child Care, Internal Medicine and Medical Specialties (ProMISE), University of Palermo, Palermo, Italy, ³Department of Biomedicine, Neuroscience and Advanced Diagnosis (Bi.N.D.) University of Palermo, Palermo, Italy, ⁴Institute for Biomedical Research and Innovation (IRIB), National Research Council (CNR)I, Palermo, Italy

In recent years, research has focused on colorectal cancer to implement modern treatment approaches to improve patient survival. In this new era, $\gamma\delta$ T cells constitute a new and promising candidate to treat many types of cancer because of their potent killing activity and their ability to recognize tumor antigens independently of HLA molecules. Here, we focus on the roles that $\gamma\delta$ T cells play in antitumor immunity, especially in colorectal cancer. Furthermore, we provide an overview of small-scale clinical trials in patients with colorectal cancer employing either *in vivo* activation or adoptive transfer of *ex vivo* expanded $\gamma\delta$ T cells and suggest possible combinatorial approaches to treat colon cancer.

KEYWORDS

gamma delta T cells, immunotherapy, colon rectal cancer, MHC- unrestricted activation, tumor

Introduction

Colorectal cancer (CRC) is the third most frequent cancer, with about 1.8 million new cases worldwide in 2018, and the second leading cause of cancer-related deaths worldwide (1). At the onset of CRC, there is a combination of various intrinsic and extrinsic factors, such as genetic factors, gut dysbiosis, dysregulation of the immune system, and environmental factors (2). Although family history is involved in 10%–30% of cases in the pathogenesis of CRC, the majority are sporadic, and the most commonly associated mutations accumulate stepwise, following the so-called adenoma-carcinoma sequence (3). Although the 5-year survival rates for localized tumors are over 90% and 71% for regional tumors, 86% of patients with distant metastases die within 5 years (4). This indicates that, despite the adoption of several screening protocols and currently available standard treatments (surgical resection, radiotherapy, and chemotherapy), there is an urgent need to develop more effective treatments to reduce mortality and tumor relapse.

In this context, immunotherapy appears to be of considerable importance thanks to the unprecedented success obtained in clinical practice. Indeed, in recent years, various studies have been developed on CRC immunotherapy, showing very promising results, especially regarding the application of immune checkpoint inhibitors (ICIs). Pembrolizumab and nivolumab have been authorized for the treatment of advanced and metastatic CRC patients

with microsatellite instability-high/DNA mismatch repair deficiency (MSI-H/dMMR) in a chemotherapy-refractory scenario (5). However, ICIs are unsatisfactory in treating microsatellite stable/DNA mismatch repair proficient (MSS/pMMR) CRC because of the low tumor mutation burden and the lack of immune cell infiltration, which are regarded as immune resistance mechanisms (6). Furthermore, along with chemotherapy, targeted therapies such as monoclonal antibodies targeting epidermal growth factor receptor (EGFR) (e.g., cetuximab) and vascular endothelial growth factor (VEGF) (e.g., bevacizumab) are the gold standard treatments for advanced or metastatic colorectal cancer. At present, all of these treatment options are hampered by the possibility of primary or acquired resistance (7). Therefore, new approaches must be developed to enable more CRC patients to benefit from immunotherapy.

The presence of immune cells in the tumor microenvironment (TME) is known to be a strong predictor of clinical outcome (8). The number of tumor-infiltrating immune cells is referred to as the immunoscore, which is used in conjunction with other characteristics to categorize colorectal cancer. To demonstrate this, Pagès et al. observed a significant link between a 5-year survival rate and a high immunoscore in patients with microsatellite instability or microsatellite-stable CRC; this benefit was not identified when immunoscore was low (9). Among the immune cells that make up TME, a noteworthy population is $\gamma\delta$ T cells, an innate-like subset of T lymphocytes (10). As well as being located in peripheral blood, they are located in the intestinal epithelium, representing the major population of intraepithelial lymphocytes (IELs). Here, they carry out powerful anticancer and anti-inflammatory actions and are involved in maintaining homeostasis and regulating the microbiome (11, 12). $\gamma\delta$ T cells are a valid therapeutic option in the field of anticancer immunotherapy, ensuring efficacy and safety in side effects thanks to their unique recognition capabilities, their remarkable immunosurveillance properties, and their easy handling *in vitro* (13).

This review aims to highlight the role of $\gamma\delta$ T cells in colorectal cancer and to summarize the different approaches that allow their use as a therapeutic tool in the treatment of colorectal cancer.

Human $\gamma\delta$ T cells: Characteristics and general functions

$\gamma\delta$ T cells are a subset of CD3⁺ lymphocytes that, differently than $\alpha\beta$ T cells, express a T-cell receptor (TCR) composed of γ and δ chains. They bridge innate and adaptive immunity, generating quick immune responses with macrophages and neutrophils to several pathogens and helping adaptive immune cells such as T cells and B cells to exert their effector functions. Moreover, thanks to the expression of TCR and typical natural killer (NK) receptors, such as NKG2D and CD94, they can kill target cells and activate other immune cells (14). The vast majority of $\gamma\delta$ T cells (~70%) are CD4⁺CD8⁻, ~30% are CD8⁺CD4⁻, and only <1% are CD4⁺CD8⁻ (15). Based on differences in TCR δ -chain usage, human $\gamma\delta$ T cells are classified into three cellular subsets with specific tropisms: V δ 1⁺, V δ 2⁺, and V δ 1⁻V δ 2⁻ (16). V δ 1⁺ T cells are predominantly expressed in mucosal and epithelial tissues and represent a large portion of

human intestinal IELs (17). They show cytotoxic activities thanks to their ability to recognize stress antigens expressed by transformed cells, such as MHC class I-related molecules A and B (MICA/MICB) and UL16-binding proteins *via* the NKG2D receptor (18) (19). Also, they recognize self-glycolipids presented by CD1c and CD1d (20) (21) and are expanded during *Cytomegalovirus* (CMV) infection (22). The V δ 1 chain can be paired with several V γ chains (V γ 2, V γ 3, V γ 4, V γ 5, V γ 8, and V γ 10) (23), but in the gut, it is paired with the V γ 4 chain (12).

The most representative $\gamma\delta$ T-cell subset expresses the V δ 2 chain typically associated with V γ 9 chain. These cells are generally referred to as V γ 9V δ 2 T cells (24), and they account for 1%–10% of circulating T cells and 50%–95% of the total $\gamma\delta$ T-cell compartment (25). The distinctive feature of V γ 9V δ 2 T cells is to recognize and respond to phosphorylated metabolites called phosphoantigens (P-Ags) (14). These are intermediates of the endogenous mevalonate (MVA) pathway (e.g., isopentenyl pyrophosphate (IPP)), upregulated during tumor transformation, or the exogenous 1-deoxy-D-xylulose-5-phosphate (MEP) pathway (e.g., (E)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate (HMBPP)), upregulated during infections (26, 27). As a rule, P-Ags recognition is not MHC-restricted but requires butyrophilin (BTN)2A1 and BTN3A1, two type-I glycoproteins of the B7 family. The BTN2A1–BTN3A1 complex contacts with the $\gamma\delta$ TCR in two sites: BTN2A1 binds to the V γ 9 domain, whereas BTN3A1 may bind to the V δ 2 and γ -chain regions on the opposite side of the TCR (28). Once activated, V γ 9V δ 2 T cells kill infected, stressed, and tumor cells using the same mechanisms as NK and CD8⁺ T cells. Moreover, after activation, they interact with B cells, promoting CD4⁺ and CD8⁺ T-cell priming and inducing maturation of dendritic cells (DC) and survival of monocytes and neutrophils (14, 29, 30). These properties let V γ 9V δ 2 T cells orchestrate an innate-like response against pathogens and tumor cells. Recently, Davey et al. have described a population of V γ 9⁻V δ 2⁺ T cells that do not respond to P-Ags and have adaptive-like functions (31).

The V δ 1⁻V δ 2⁻ compartment consists of distinct minor subsets, such as V δ 3⁺ and V δ 5⁺ T cells. V δ 3⁺ T cells amount to 0.2% of T lymphocytes in the blood and are also located in the liver and gut. Currently, its paired γ chain is unknown. Due to a lack of specific antibodies for their identification, generally, they are characterized as V δ 1⁻V δ 2⁻-cells (32). V δ 3⁺ T cells recognize CD1d molecules expressed on target cells, have cytotoxic activity, and release Th1-, Th2-, and Th17-like proinflammatory cytokines (32). A recent study by Petrasca et al. described a role for this subset in the maturation of B cells and the production of IgM antibodies, without promoting class-switching (33).

V δ 5⁺ $\gamma\delta$ T cells are the rarest subset; in particular, Willcox et al. described a clone of these V δ 5⁺V γ 4⁺ T cells that recognize the endothelial protein C receptor expressed by stressed human cells (34).

Human $\gamma\delta$ T cells and their responses to tumors

$\gamma\delta$ T cells are one of the components of the tumor immune milieu and are well-known for influencing the antitumor immune response to a broad range of tumors (35). Indeed, $\gamma\delta$ T cells are recruited into

various types of malignancies, including breast cancer (36), melanoma (37), and lung cancer (38). In particular, with regard to colon cancer, we have previously shown that $\gamma\delta$ T lymphocytes, of which V δ 1 was the most represented subset, amounted to 4.5% of the total leukocyte population in CRC (39). In addition, Corvaisier et al. have described a V γ 9V δ 2 TCR clone isolated from colon cancer ascites (40). Recently, a TCR $\gamma\delta^+$ CD103 $^+$ PD-1 $^+$ subset was exclusively found by high-dimensional single-cell mass cytometry in MMR-deficient CRC, accounting for 8.4% of CD45 $^+$ cells (41, 42).

Despite some studies that have identified the presence of $\gamma\delta$ T cells among tumor-infiltrating lymphocytes (TILs) as a positive prognostic factor, it is not yet fully known whether they can promote or hamper tumor growth (43). Particularly, Gentles et al., analyzing the gene expression profile of 18,000 tumor fragments from 39 types of malignancies on the prognostic role of the individual infiltrating cell populations, showed that the neutrophils and plasma cells present in tumor infiltrate have a significant but opposite role in different tumors, while the presence of $\gamma\delta$ T cells was found to be the most favorable prognostic factor in analyzed tumors, both hematological and solid (44), including CRC. However, Tosolini et al., applying the CIBERSORT algorithm, showed that in Gentle's study, the presence of V γ 9V δ 2 was wrongly identified as CD4 $^+$ CD8 $^+$ T cells or NK and *vice versa* (45). Therefore, using an improved deconvoluted CIBERSORT matrix to investigate the abundance of TCR V γ 9V δ 2, they found that CRC patients with a high TCR V γ 9V δ 2 infiltration had a better survival rate than those with a low infiltration (45). Similarly, we have shown by transcriptomic analysis of a cohort of 557 CRC patients that 5-year disease-free survival was significantly higher in CRC patients with higher $\gamma\delta$ TILs (39). In addition, the decrease of NKp46 $^+$ /V δ 1 IELs in tumor-free specimens from CRC patients correlated with a higher risk of metastasis (12).

In contrast to these findings, a previous study highlighted the role of IL-17A-producing $\gamma\delta$ TILs in the promotion of tumor progression in patients with CRC (46), but these results were not confirmed in subsequent studies (39).

This twofold role can be linked to the plasticity of $\gamma\delta$ T cells, which includes the capacity to differentiate toward different functional cell subsets based on the microenvironment, which can direct their polarization. This implies that the positive or negative correlation with the tumor prognosis can be attributed to the specific $\gamma\delta$ T cell subsets recruited or resident at the tumor site (10).

Protumor activities of $\gamma\delta$ T cells

Notwithstanding that $\gamma\delta$ T cells exert antitumor effects, it is not surprising that they also have protumor activity due to their plasticity. Indeed, after recruitment to the tumor site, they can acquire a phenotype that mirrors T_{fh}, T_{reg}, Th1, Th2, and Th17, influenced by a plethora of cytokines present in the tumor microenvironment (47). Particularly, the polarized $\gamma\delta$ T17 and $\gamma\delta$ T_{reg} cells, which produce IL-17A or TGF- β , respectively, have been reported to support cancer progression and be associated with poor survival in the gallbladder (48), ovarian (49), and colorectal cancer (39, 46).

In a study by Wu et al., $\gamma\delta$ T cells were the major cellular source of IL-17A in human CRC. Activated inflammatory DC polarized V γ 9V δ 2 T cells into IL-17A-producing $\gamma\delta$ T cells. Together with the

secretion of IL-8, TNF- α , and granulocyte-macrophage colony-stimulating factor (GM-CSF), they induced an immunosuppressive microenvironment in human CRC, thus promoting tumor progression. The tumor infiltration of IL-17A-producing $\gamma\delta$ T cells was positively associated with advanced tumor stages of human CRC and with other clinicopathological features, including tumor size, lymphatic and vascular invasion, and lymph node metastasis (46). However, in a subsequent study (39) with two very large cohorts of CRC patients, we failed to confirm the results of Wu and colleagues. In ovarian cancer, $\gamma\delta$ T cells and their V δ 1 subset produced IL-17A, impaired antitumor responses, and enhanced immunosuppressive activities, as documented by their ability to inhibit the proliferation of naive CD4 $^+$ T cells upon co-culture with ovarian cancer tissue supernatants (49). Rong et al. described an imbalance in V δ 1 and V δ 2 subset distribution that may contribute to the growth of rectal cancer. Unlike V δ 2 T cells, V δ 1 $^+$ TILs were significantly increased, when compared with para-carcinoma tissues, and had strong inhibitory effects (50). Moreover, a CD39 $^+$ $\gamma\delta$ T_{reg} subset was identified in the human CRC, showing a superior immunosuppressive activity than CD4 $^+$ or CD8 $^+$ T_{regs} *via* the adenosine pathway. CD39 $^+$ $\gamma\delta$ T_{reg} cells also established an immunosuppressive milieu due to the chemoattraction of myeloid-derived suppressor cells (MDSCs) in an IL-17A and GM-CSF-dependent manner. In addition, their infiltration was correlated with unfavorable clinicopathological features in CRC (51). Recently, in the microenvironment of human breast cancer, a regulatory subset of CD73 $^+$ $\gamma\delta$ T cells was identified, which exerted immunosuppressive functions through the production of IL-10, IL-8, and adenosine (52).

Consequently, this evidence, although still very preliminary, suggests that $\gamma\delta$ T cells may establish an immunosuppressive microenvironment to inhibit antitumor immune responses, evade immune surveillance, and promote the progression of cancer.

Antitumor activities of $\gamma\delta$ T cells

$\gamma\delta$ T cells, particularly V δ 1 and V δ 2 subsets, have a significant antitumor activity that is exerted by direct and indirect mechanisms and through the involvement of several pathways. Cancer cells are recognized not only *via* TCR-dependent antigen recognition but also through NK receptors and or antibody-dependent cell-mediated cytotoxicity (ADCC) (Figures 1, 2).

V γ 9V δ 2 T cells generally recognize tumor target cells either through the TCR or through stimulatory NK receptors, such as NKG2D and DNAX accessory molecule 1 (DNAM-1), that recognize their specific ligands overexpressed on tumor cells. In addition to NKG2D, V δ 1 $^+$ T cells used natural cytotoxicity receptors (NKp30, NKp44, NKp46) for tumor cell recognition, although their expression is linked to specific conditions, such as the presence of IL-15 or IL-2 (12, 53). A recent study shows the strong antitumor activity of NKp46 $^+$ $\gamma\delta$ IELs against human colon adenocarcinoma cell lines (12).

Similar to NK cells, human $\gamma\delta$ T cells, once activated, upregulate on their surface type-III Fc γ receptor (CD16) and promote ADCC on opsonized tumor cells. Relevant to cancer immunotherapy, CD16-expressing $\gamma\delta$ T cells have been shown to improve the efficacy of therapeutic antibodies such as rituximab and trastuzumab for the

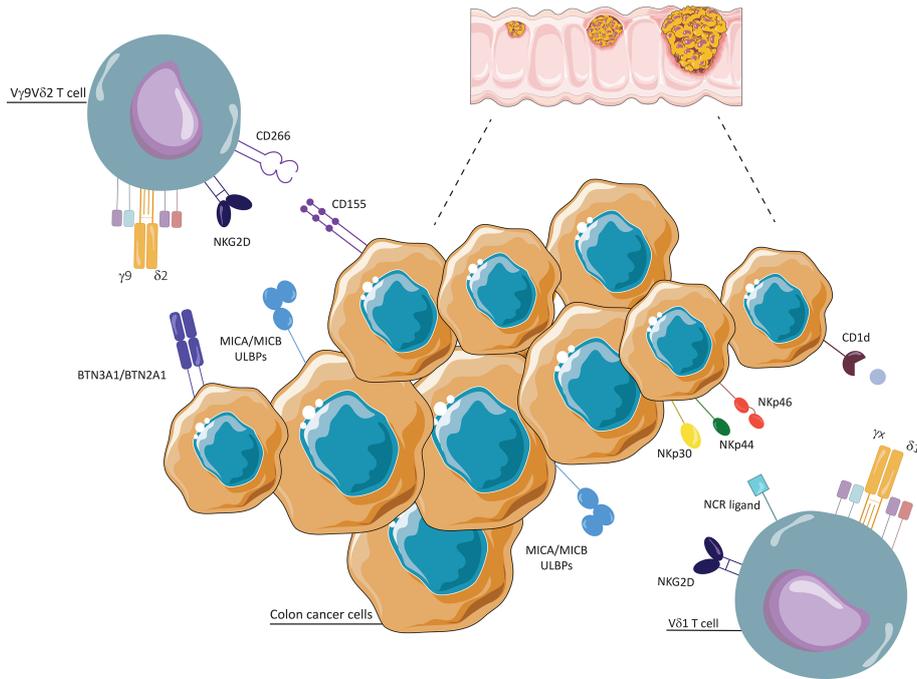


FIGURE 1

Interaction between $\gamma\delta$ T cells and colon cancer cells. $V\delta 1$ and $V\delta 2$ T cells conduct cytotoxic activities thanks to their ability to recognize stress antigens expressed by transformed cells, such as MHC class I-related molecules A and B (MICA/MICB) and UL16-binding proteins (ULBPs), via NKG2D receptor. $V\delta 1$ T cells recognize self-glycolipids presented by CD1c and CD1d, and tumor cells through natural cytotoxicity receptors (Nkp30, Nkp44, Nkp46). $V\delta 2$ T cells recognize and respond to phosphoantigens (P-Ags), through unrestricted MHC manner, with two types I glycoproteins of the B7 family, butyrophilin (BTN)2A1 and BTN3A1. They interact with tumor cells through CD226 (DNAM-1)/CD155 (Nectin-2).

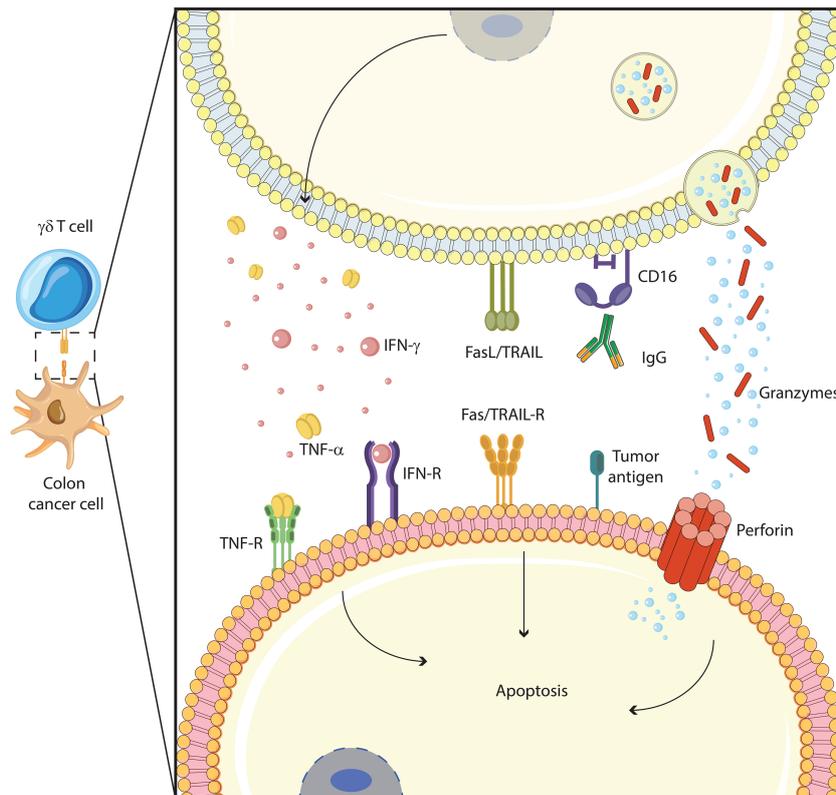


FIGURE 2

$\gamma\delta$ T cells function against colon cancer cells. $\gamma\delta$ T cells exert their cytotoxic functions through several mechanisms: induction of apoptotic cell death with Fas/TRAIL pathway, antibody-dependent cellular cytotoxicity (ADCC) with IgG/CD16 interaction, cytolytic granule (granzyme, perforin) polarization and degranulation, and proinflammatory cytokines (TNF- α , IFN- γ) production.

treatment of lymphoma, chronic lymphocytic leukemia, and human epidermal growth factor receptor 2 (HER2)-positive breast cancer cells (54, 55). Recent studies have shown that bispecific antibodies binding TCR-HER2 and CD19-specific triple bodies increase $\gamma\delta$ T cells cytotoxicity, improving ADCC (56) (57). Finally, $V\gamma9V\delta2$ T lymphocytes with high CD16 expression exert ADCC in the 3D cultures of colon cancer cells, inducing a decrease in size and cell number in spheroids (58). Whatever the mechanism of target cell recognition, killing by $\gamma\delta$ T cells involves the perforin/granzyme pathways (59, 60) and Fas/FasL, TRAIL/TRAIL-R, and TNF/TNF-RII interactions (61–63), depending on the nature of the target cells.

Instead, indirect antitumor mechanisms occur *via* interaction between $\gamma\delta$ T cells and other immune cells, such as B cells, DC, $\alpha\beta$ T cells, and NK cells. Acting similarly to Tfh cells, $\gamma\delta$ T cells can secrete cytokines that promote B cells to secrete specific antibodies (29). Furthermore, they take part in the maturation of DC, thus inducing helper and cytotoxic functions of $CD4^+$ and $CD8^+$ T cells (64, 65). Likewise, they improve NK cell-mediated cytotoxicity through CD137/CD137L engagement and upregulating NKG2D (66).

How to use $\gamma\delta$ T cells for immunotherapeutic protocols

In the last few years, immunotherapy has had great prominence in cancer treatment. It is based on enhancing the immune system to kill tumor cells and block or limit their growth. Most immunotherapeutic approaches make use of $\alpha\beta$ T cells, but efficacy is limited due to the characteristics of this subset, namely, their activation and functional

activities rely on MHC restriction, costimulatory signal, and specific tumor-associated antigen (TAA) recognition (67).

$\gamma\delta$ T cells provide themselves with a valid alternative to overcome these limits thanks to their unique features (68). Indeed, they recognize conserved antigens widely expressed on stressed and tumor cells without the need for MHC restriction and costimulatory molecules. Both of these aspects allow $\gamma\delta$ T cells to avoid immune escape due to the downregulation of MHC molecules and loss of antigen expression by tumor cells. In addition, they perform effector activity relevant to antitumor immune responses, such as cytotoxicity and cytokine (IFN- γ and TNF- α) production. Finally, their inbred tissue tropism, particularly for $V\delta1^+$ T cells, makes them more suitable than $\alpha\beta$ T cells for performing rapid antitumor responses. These features, together with their easy activation and expansion, make $\gamma\delta$ T cells attractive candidates for tumor immunotherapy (69).

Most $\gamma\delta$ T-cell-based immunotherapy clinical trials have used the $V\gamma9V\delta2$ subset (Figure 3). According to the modality of their expansion and activation, two types of $V\gamma9V\delta2$ T-cell immunotherapy have been considered: *in vivo* activation and adoptive transfer of *ex vivo* expanded $V\gamma9V\delta2$ T cells (17). The *ex vivo* approach requires the isolation of $\gamma\delta$ T cells from peripheral blood mononuclear cells (PBMCs), their expansion *in vitro*, and then their adoptive transfer into recipient patients. Specific stimulants for $V\gamma9V\delta2$ T cells include natural (IPP and HMBPP) or synthetic (bromohydrin pyrophosphate (BrHPP)) P-Ags. Furthermore, different drugs can be used to enhance the accumulation of endogenous P-Ags, among which are nitrogenous bisphosphonates (N-BPs), a class of drugs generally used in the treatment of osteoporosis and bone disorders. They act by inhibiting the farnesyl pyrophosphate synthase (FPPS) enzyme of the mevalonate

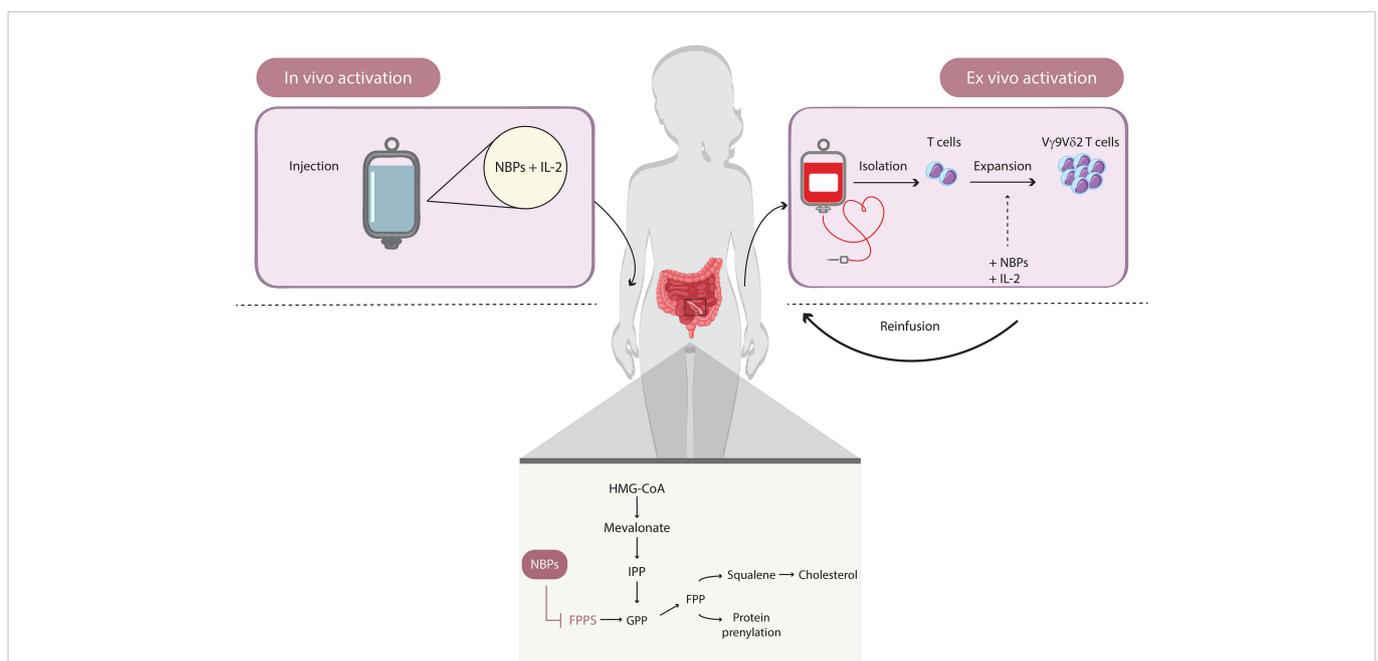


FIGURE 3

How to use $\gamma\delta$ T cells in immunotherapy. Immunotherapeutic approaches based on $V\gamma9V\delta2$ T cells involve two types of mechanisms: *in vivo* activation and adoptive transfer of *ex vivo*-expanded $V\gamma9V\delta2$ T cells. The *in vivo* activation requires the administration of amino bisphosphonates (e.g., zoledronate), which induce the accumulation of endogenous phosphoantigens (P-Ags; such as IPP) by inhibiting the farnesyl pyrophosphate synthase of the mevalonate pathway and upregulating isoprenoid biosynthesis within cells. The *ex vivo* approach is based on the isolation of $\gamma\delta$ T cells from peripheral blood mononuclear cells and the activation of $V\gamma9V\delta2$ T cell, induced directly by natural (e.g., IPP and HMBPP) or synthetic (e.g., BrHPP) P-Ags plus IL-2 and the adoptive transfer.

pathway, thus upregulating isoprenoid biosynthesis within cells (70). Zoledronic acid (zoledronate, Zometa (ZA)) is the most common *in vivo* and *in vitro* using N-BP and it also shows antitumor effects through induction of apoptosis and autophagy in cancer cell lines (71).

Another alternative approach is the *in vivo* activation of V γ 9V δ 2 T cells by the administration of P-Ags or N-BPs in combination with IL-2, which is necessary for V γ 9V δ 2 expansion (72). This strategy increases with success the number of circulating V γ 9V δ 2, but it does not ensure their recruitment to the target tissue, where they can exert antitumor functions.

However, it has been shown both in humans and in nonhuman primate models that, upon repeated injections of P-Ags, V γ 9V δ 2 do not respond to P-Ags, probably because of activation-induced energy or exhaustion of their functions, promoting terminal differentiation (73).

In light of these limits, V δ 1⁺ T cells apply for a possible alternative to or support to V γ 9V δ 2-based immunotherapy. Indeed, two studies conducted on primary malignant melanoma and colorectal cancer pinpointed V δ 1⁺ T cells as the major $\gamma\delta$ T-cell subset among TILs (37) (39). Indeed, as well as showing a higher cytotoxic capacity compared to V δ 2, V δ 1⁺ T cells have the advantage of tissue residency, so the adoptive transfer of V δ 1 may be efficient in reaching the tumor site. Once activated by a broad variety of ligands, V δ 1⁺ T cells are also able to circulate and persist for a long time in the body, promoting long-term therapeutic effects. This is probably due to the fact that they are resistant to activation-induced cell death (74). Nevertheless, their clinical application is difficult due to the lack of a well-established expansion protocol. Recently, one of the protocols developed that has given promising results is established by Almeida et al., in which they expanded V δ 1⁺ from the peripheral blood of healthy donors and patients with chronic lymphocytic leukemia (CLL). These cells differentiate into a highly cytotoxic subset and inhibit tumor growth and metastasis in xenograft models of CLL *in vivo* (53). Based on this expansion procedure, GammaDelta Therapeutics has developed an allogeneic, nonengineered V δ 1 T-cell therapy (GDX012) for the treatment of patients with acute myeloid leukemia with minimal residual disease (NCT05001451) (75).

The newest $\gamma\delta$ T-cell-based therapeutic strategies include the use of bispecific $\gamma\delta$ T-cell engagers and $\gamma\delta$ CAR-T cells (76). V γ 9-TCR-specific engagers binding to either CD123 (77) or HER2 (56) mediate cytotoxicity against tumor cells in acute myeloid leukemia and pancreatic adenocarcinomas, respectively. Moreover, they have the potential to enhance the efficacy of adoptively transferred $\gamma\delta$ T cells (78). Lava Therapeutics is currently conducting two open-label phase I/IIa studies to assess the efficacy and safety of LAVA-051 (79), a humanized bispecific single-domain antibody that directly engages CD1d and the V δ 2 chain of V γ 9V δ 2 T cells in patients with relapsed/refractory chronic lymphocytic leukemia, multiple myeloma, and acute myeloid leukemia (NCT04887259) and LAVA-1207, which activates V γ 9V δ 2 T cells upon crosslinking to prostate-specific membrane antigen (PSMA) in patients with metastatic castration-resistant prostate cancer (NCT05369000).

In addition to V γ 9V δ 2 T cells, recently $\gamma\delta$ CAR-T-cell-based therapy is also based on genetically modified V δ 1 T cells (80, 81). Adicet Bio is currently conducting a phase I clinical trial on anti-CD20 allogeneic $\gamma\delta$ CAR-T cells for patients with B-cell malignancies (NCT04735471) (81). Compared to $\alpha\beta$ CAR-T, the autologous or allogeneic V δ 1/V δ 2 T-cell-expressing CAR ensures a reduced risk of a

cytokine-related syndrome and a lower risk of off-target toxicity, as well as a long lifespan and preserved ability to act as antigen-presenting cells (82).

$\gamma\delta$ T-cell-based CRC immunotherapy

A growing body of evidence has allowed us to understand the role of $\gamma\delta$ T cells in the tumor milieu and pave the way for their potential use in new anti-CRC immunotherapy approaches. CRC immunotherapy based on $\gamma\delta$ T cells has only produced a small number of clinical results so far, although it has proven to be safe in terms of tolerability and side effects.

In a clinical trial with six CRC patients who had undergone lung metastasectomy, Izumi and colleagues used an injection of autologous V γ 9V δ 2 T cells, expanded with 5 μ M of ZA and 1,000 IU/ml of IL-2, for 8 weeks. During treatment, the number of V γ 9V δ 2 T cells increased and remained stable long after the last infusions in terms of percentage and absolute number. Moreover, V γ 9V δ 2 T cells expressed more IFN- γ and CD107a *ex vivo* than other V γ 9⁻ CD3⁺ T cells (83). In a phase I trial, Bennouna et al. tested *in vivo* the pharmacodynamic profile of $\gamma\delta$ -agonist, BrHPP (IPH1101), in 28 patients with solid tumors, among which there were three CRC patients. They first evaluated the maximum-tolerated dose of BrHPP administered alone during the first cycle. Because the expansion and activation of V γ 9V δ 2 T lymphocytes required IL-2, they combined BrHPP with a low dose of IL-2. This approach proved to be safe, generally well-tolerated, and induced a strong $\gamma\delta$ T lymphocyte expansion in these patients (84). Noguchi et al. performed a clinical trial enrolling 25 patients with advanced solid tumors, one of them with colon cancer. Once stimulated and expanded *ex vivo* with ZA and IL-2, $\gamma\delta$ T cells were reinfused into patients. This approach did not cause severe toxicity, but rather restored the number of effector $\gamma\delta$ T cells (85). Another phase I clinical study, including three patients with CRC, tested the adoptive transfer of *ex vivo* expanded autologous V γ 9V δ 2 T cells to evaluate whether the approach was doable and safe. No dose-dependent toxicity was found, but for some patients, the therapy was ineffective (86).

It is known that colon cancer is generated by a small population of cancer stem cells, which are relevant players in tumor persistence and drug resistance. Colon cancer stem cells are resistant to V γ 9V δ 2 T-cell cytotoxicity, but when sensitized with ZA (or even chemotherapeutic agents), they are efficiently killed by V γ 9V δ 2 T cells (60, 87). However, due to the biodistribution of ZA, including its bone tropism, its use *in vivo* can often be ineffective in properly reaching and stimulating $\gamma\delta$ T lymphocytes in tissues. In this regard, recent work has shown that it is possible to encapsulate ZA in spherical polymeric nanoparticles (SPNs), which, thanks to the enhanced permeability and retention effect, can easily reach the tumor site. ZA-SPNs are engulfed by CRC cells, and V δ 2⁺ T cells interact with ZA-SPN-treated CRC cells, promoting their killing. In addition, V δ 2⁺ T cells also kill ZA-SPN-treated CRC tumor spheroids and autologous tumor organoids (88).

Ang et al. expanded V γ 9V δ 2 T cells with ZA/IL2 and constructed NKG2D ligand-specific chimeric antigen receptors (NKG2DL) using mRNA electroporation. Upon interaction with NKG2DL⁺ cancer cells, the CAR- $\gamma\delta$ T cells enhanced their cytotoxic activity against a variety of cultured solid tumor cell lines, including those resistant to ZA treatment. They also evaluated the *in vivo* tumor-killing effect of the NKG2Dz RNA

CAR-modified V γ 9V δ 2 T cells in a colorectal cancer mouse model using repeated intraperitoneal injections of aggressive human colorectal cancer cell lines. NKG2Dz RNA CAR-modified V γ 9V δ 2 T cells significantly slowed tumor progression, resulting in a median survival time of 57 days (89). Gadeta's second cell therapy product, GDT201, which combines HLA-independent tumor recognition capabilities of $\gamma\delta$ TCRs with proliferative capacity and robust tumor killing of $\alpha\beta$ T cells, demonstrated long-lasting cytotoxicity effect on CRC cell lines, patient-derived tumor cells, and in a xenograft tumor model, suggesting the potential to address well-known clinical hurdles such as lack of tumor restricted antigens and safety (90).

As the functions of $\gamma\delta$ T lymphocytes are often switched off by the microenvironment, a further useful approach would be to combine their expansion with the use of inhibitors of their negative regulators, thus restoring their ability to mediate protective immunity against cancer cells. Lu et al. have shown a high expression of B7-H3, an important member of the B7 superfamily, on CRC-infiltrating V δ 2⁺ T cells. B7-H3 appears to be an important negative regulator of their V δ 2⁺ T-cell antitumor functions, specifically *via* the downregulation of IFN- γ and perforin/granzyme B expression. Indeed, by inhibiting B7-H3 with a specific blocking antibody (MIH35) or B7-H3 siRNA, they witness the reactivation of effector functions, a decrease of apoptosis rates, and an upregulation of the activation markers CD25 and CD69. These treatments promoted the cytotoxic activity of V δ 2⁺ T cells on colon cancer cell lines. Hereupon, this suggests that it would be useful to combine *in vivo* activation of V δ 2⁺ T cells with the inhibition or blockade of B7-H3 as another immunotherapeutic approach for colon cancer (91).

Tim-3 is another inhibitory receptor that can influence the activity of $\gamma\delta$ T lymphocytes. Its signaling significantly hindered the killing activity of V γ 9V δ 2 T cells against colon cancer cells and reduced the expression of perforin and granzyme B cells in an ERK1/2-dependent manner (92); because $\gamma\delta$ T cells express high levels of Tim-3 in peripheral blood and colon cancer tissue, Tim-3 blockade by neutralizing antibody restores their cytotoxicity towards colon cancer cells. Consistent with this strategy, the inhibition of glycogen synthase kinase-3 β with TWS119 inhibitor, boost proliferation, differentiation, and cytolytic activity of $\gamma\delta$ T cells against colon cancer cells, activating mammalian target rapamycin (mTOR) pathway and upregulating the antiapoptotic protein Bcl-2 as well as the expression of perforin and granzyme B *in vitro* and *in vivo* (59).

Exploiting $\gamma\delta$ T cells as a therapeutic tool can also be beneficial to overcome the failure of immunotherapies caused by the mutational state of the tumor. In this regard, de Bruin et al. generated and applied *in vitro* and *in vivo* a bispecific nanobody-based construct that, on the one hand, inhibited the signaling of EGFR and, on the other, induced target-dependent activation of V γ 9V δ 2 T cells. When co-cultured with two different EGFR⁺ human colon cancer cell lines, each with KRAS and BRAF mutations, and in the presence of the bispecific construct, V γ 9V δ 2 T cells produced IFN- γ and TNF- α and induced lysis of both cancer cell lines, irrespective of their mutational state. The same effects were found *in vivo*, combining the administration of V γ 9V δ 2 T cells with the bispecific construct (93).

Although most immunotherapy protocols are based on V γ 9V δ 2 T cells, promising results have been achieved using V δ 1⁺ T cells. After their isolation from PBMC and expansion with PHA and IL-7, V δ 1⁺ T cells were performed *in vitro* with strong cytotoxic and cytolytic

effects against adherent and sphere-forming human colon cancer cells in a contact-dependent manner. *Ex vivo* expanded V δ 1⁺ T cells were able to block tumor growth in a CRC xenograft model (94). Furthermore, in another murine model of metastatic colon cancer obtained from orthotopic implantation of human HT29 cells, the administration of CMV-induced V δ 1⁺ T cells suppressed the progression of the primary tumor and the onset of metastasis (95).

Conclusions

CRC is a very complex and multifactorial tumor, characterized by a high mutation burden. Due to these features, standard treatment regimens have been proven to be ineffective in many patients. Here comes immunotherapy into play, especially targeting the checkpoint blockade pathway to enhance antitumor responses, although so far in CRC it does not seem to be always applicable compared to other solid tumors that respond well. Indeed, in MSI-H/dMMR CRC, this therapeutic approach is useful, whereas the majority of MSS/pMMR CRC patients do not benefit from immunotherapy (2). Hence, new immunotherapeutic approaches are needed, taking advantage of the interactions between tumors and the immune system. In recent years, the role of $\gamma\delta$ T lymphocytes has emerged as an excellent therapeutic platform in hematological and solid tumors, including CRC. This is made possible by their hallmarks, which make them easily available, manipulable, and safe. Furthermore, to improve their immunogenicity and infiltration at the tumor sites in CRC, a combination of different strategies has been recently suggested, although their clinical relevance has yet to be evaluated. Little is known about the nature of the antigens and contacts that activate V δ 1 T cells, and a deeper understanding of their biology is required to maximize their therapeutic potential. Despite the beneficial aspects of $\gamma\delta$ -based immunotherapy, clinical trials for CRC are still in their early stages. Therefore, future colon cancer immunotherapy could benefit from new engineering technologies, such as CAR- $\gamma\delta$ T cells (96) and $\gamma\delta$ T-cell-based bispecific constructs (97), to improve their antitumor efficacy.

Author contributions

AC, MDS, and SM designed the entire review. AC and MDS wrote the paper. ELP, FD, and SM reviewed and edited the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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