Tumor resistance mechanisms and their consequences on γδ T cell activation

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Abstract
Human γδ T lymphocytes are predominated by two major subsets, defined by the variable domain of the δ chain. Both, Vδ1 and Vδ2 T cells infiltrate in tumors and have been implicated in cancer immunosurveillance. Since the localization and distribution of tumor-infiltrating γδ T cell subsets and their impact on survival of cancer patients are not completely defined, this review summarizes the current knowledge about this issue. Different intrinsic tumor resistance mechanisms and immunosuppressive molecules of immune cells in the tumor microenvironment have been reported to negatively influence functional properties of γδ T cell subsets. Here, we focus on selected tumor resistance mechanisms including overexpression of cyclooxygenase (COX)-2 and indolamine-2,3-dioxygenase (IDO)-1/2, regulation by tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)/TRAIL-R4 pathway and the release of galectins. These inhibitory mechanisms play important roles in the cross-talk of γδ T cell subsets and tumor cells, thereby influencing cytotoxicity or proliferation of γδ T cells and limiting a successful γδ T cell-based immunotherapy. Possible future directions of a combined therapy of adoptively transferred γδ T cells together with γδ-targeting bispecific T cell engagers and COX-2 or IDO-1/2 inhibitors or targeting sialoglycan-Siglec pathways will be discussed and considered as attractive therapeutic options to overcome the immunosuppressive tumor microenvironment.

KEYWORDS
bispecific T cell engager, galectin, kynurenine, prostaglandin, TRAIL, γδ T cells

1 | INTRODUCTION

The immune system has successfully developed various mechanisms to recognize and respond to foreign antigens including tumor antigens. The different mechanisms include recognition of microbe (pathogen) and damage-associated molecular patterns by cells of the innate and adaptive immune systems including γδ T cells, missing-self and induced-self recognition by Natural Killer (NK) cells, antigen-specific recognition by αβ T cells and B cells, and selective recognition of non-proteinaceous antigens by γδ T cells.

Cancer is a devastating disease and a leading cause of death worldwide. The difficulties of effective tumor-specific T cell responses are summarized as follows (a) low frequency of tumor antigen-specific T cells, (b) weak immunogenicity of tumor antigens, (c) downregulation of antigen-presenting human leukocyte antigen

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(HLA) molecules and adhesion molecules as well as shedding of natural killer group 2 member D (NKG2D ligands), (d) clonal deletion of high affinity T cells, and (e) existence of tumor-derived immunosuppressive molecules and immune suppressor cells. Based on diverse intrinsic and acquired tumor resistance mechanisms, complexity of tumor microenvironment (TME), and heterogeneity of tumor cells, only certain tumor entities can be treated by novel biological therapies including targeted therapies and immune checkpoint blockade. Although significant improvement has been obtained such novel biological therapies, many patients do not experience clinical benefit due to other tumor resistance mechanisms. These mechanisms negatively influence T cell effector function including the anti-tumor response of γδ T cells.

γδ T cells are a heterogeneous subpopulation of lymphocytes that combine innate and adaptive properties. A prognostic significance of γδ T cells in a broad range of human tumor entities and a correlation with patient outcome have been revealed by recent bioinformatic analyses of a meta-genomic datasets. A further improved deconvolution of human cancer microarrays revealed a considerable inter-individual variation of tumor-infiltrating Vγ9Vδ2 γδ T cell abundance across the 50 analyzed types of hematological and solid malignancies. Although the correlative data of γδ T cell infiltration in tumors are encouraging, an immunosuppressive TME can hinder γδ T cells from exerting an effective anti-tumor response and can actually promote γδ T cell differentiation into an immunosuppressive phenotype based on their high plasticity. Otherwise, the high functional plasticity of γδ T cells offers interesting perspectives for their application as effector cell population for T cell-based immunotherapy.

2 | γδ T LYMPHOCYTES

2.1 | Ligand recognition of γδ T cells

Apart from the difference in their mode of antigen recognition, γδ T cells differ from αβ T cells in T cell receptor (TCR) diversity, the structure of TCR-CD3 complex, and their frequency in tissue and blood distribution. γδ T cells are suggested to play an important role in immunity to infection, stress surveillance, and carcinogenesis. Human γδ T cells can be classified in at least three major subsets. Human Vδ1 T cells constitute a main population in the mucosa and skin. Several Vδ1 T cell clones or lines have been shown to recognize microbial and self-lipids bound to non-classical CD1c and CD1d molecules with high affinity, but also CD1d molecules without presented antigens with low affinity. Similar to unloaded CD1d molecules, several cancer patients-derived Vδ1 T cell lines directly bind via NKG2D to stress-induced MHC class I-related chain (MIC) A and UL16-binding protein (ULBP)-3 expressed on tumor cells. The current knowledge about recognition of MHC-like molecules as well as other recognition concepts for Vδ1 and Vδ1-negative T cells is extensively summarized in recent reviews. Vδ1 T cells express a TCR composed of any of the six different variable (V) genes (Vγ2, 3, 4, 5, 8, or 9) paired with Vδ1. Human Vδ2 T cells which co-express Vγ9 are mainly found in the peripheral blood. Vγ9Vδ2 T cells recognize pyrophosphate intermediates of a dysregulated mevalonate pathway in tumor cells and of the prokaryotic non-mevalonate pathway by a butyrophilin (BTN) molecule-dependent mechanism. A blockade of the isoprenoid pathway with aminobisphosphonates (n-BP) including zoledronic acid, which is used for the treatment of bone-related diseases, inhibits the pathway and induces an Vγ9Vδ2 proliferation via accumulation of pyrophosphate intermediates. In addition, TCR ligand F1-ATPase in a complex with apolipoprotein A-I and ectopically expressed DNA mismatch repair protein human MutS homologue (hMSH2) are recognized by several Vγ9Vδ2 T cell clones or lines, respectively. Cytotoxic activity of Vγ9Vδ2 T cells can be also induced TCR-independently via NKG2D binding to NKG2D ligands expressed on tumor cells. Human Vδ3 T cells are present in skin and liver, and their antigens are so far not defined in detail. Recently, ligands for peripheral blood-derived Vδ3 T cell lines such as glycolipids binding to CD1d molecules and for a Vδ8Vδ3 T cell clone as well as Vδ2-negative T cell subsets within PBMC binding to stress-induced Annexin A2 expressed on the tumor cell surface have been described. Additionally, human intestinal Vγ4 T cells paired with Vδ1 or Vδ3 recognize BTN-like proteins BTN-L3 and BTNL-B on intestinal epithelial cells. Moreover, a human Vγ4Vδ5 T cell clone directly bound the endothelial protein C receptor expressed on CMV-infected endothelial cells and also aberrantly on epithelial tumor cells independently of antigens.

2.2 | γδ T cells in cancer

Peripheral γδ T cell lymphomas (γδ TCLs) are very rare with <1% of lymphoid neoplasms but aggressive and accompanied with a dismal prognosis. While hepatosplenic γδ TCLs are usually associated with Vδ1 T cells, cutaneous γδ TCLs are associated with Vδ2 T cells. An abundance of Vγ9Vδ2 γδ T cells has been shown in B cell-acute lymphoblastic leukemia, chronic myeloid leukemia, and promyelocytic leukemia by transcriptome analysis. Circulating γδ T cells were increased in chronic lymphocytic leukemia (CLL), and dysfunctional Vγ9Vδ2 γδ T cells were reported to be a negative prognostic factor in CLL. In addition, analysis of paraffin-embedded sections from patients with Epstein-Barr virus (EBV)-associated Hodgkin’s lymphoma demonstrated that most of the γδ T cells from these patients expressed Vγ9, whereas γδ T cells expressing Vγ2, 3, or 4 were nearly absent. γδ T cell infiltration and correlation with patient outcome have been also demonstrated in epithelial and solid tumor cells including melanoma, colorectal, breast, pancreatic, and ovarian cancer. Immunohistochemistry (IHC)-based analysis allows analysis of tumor-infiltrating γδ T lymphocytes (γδ TIL) within the tumor as well as in the context of the TME or the surrounding tissue. γδ T cells moderately infiltrate in superficial intraepithelial and superficial spreading melanoma, whereas a higher amount of γδ TIL has been demonstrated in ulcerated nodular melanoma. γδ TILs are localized in the
periphery of the tumor in all analyzed melanomas. A localization of γδ TILs rather than in the tumor stroma than in the intratumoral tissue has also been shown in patients with colorectal cancer, whereas γδ TIL in patients with hepatocellular carcinoma localized in the peritumoral liver tissue. Regarding pancreatic ductal adenocarcinoma (PDAC), our studies initially demonstrated that γδ T cells were mainly localized in the stroma adjacent to the tumor cells or within malignant ductal epithelium in 44% of a cohort of 41 patients. Our further studies revealed a significant correlation of TCRγδ expression with CD3 and CD8. Different stromal localization of γδ T cells in PDAC patients compared with chronic pancreatitis patients may be explained by changes of stromal composition during progression to invasive PDAC characterized by a pronounced immunosuppressive TME. Although the frequency of γδ T cells in PDAC tissue is low, γδ T cells accumulate in malignant ductal epithelium in close interaction with regulatory T cells (Treg) and immunosuppressive fibroblasts. By analyzing the distribution of the PDAC γδ TIL subsets, Daley et al demonstrated that non-Vγ9 γδ T cells infiltrate in human PDAC. A more detailed analysis of γδ TIL subsets by flow cytometry demonstrated a low frequency of unresponsive non-Vγ9 γδ TIL and an increased number of Vδ1 T cells. Moreover, Daley and collaborators delineated a molecular mechanism through which γδ TIL within pancreatic cancer promote oncocenic progression by impairing anti-tumor response of αβ T cells as shown by multiple murine pancreatic tumor models.

Intratumoral γδ T cells in breast cancer patients are reported to serve as a prognostic marker for an advanced tumor stage and poor clinical outcome, probably due to the enrichment of immunosuppressive CD73-expressing Vδ1 T cells in immunoregulatory γδ T cells. In contrast, several other studies with triple negative breast cancer patients revealed that the infiltration of γδ T cells is accompanied with good prognosis which is discussed in more detail in the recent publication of Chabab and colleagues. In addition, adjuvant chemotherapy and physical activity of breast cancer patients did not significantly alter absolute cell number of peripheral blood γδ T cells.

2.3 Distribution of tumor-infiltrating γδ T cell subsets

The immunohistological approaches are often combined with flow cytometry analysis to determine the different γδ T cells by antibodies which are not suitable for IHC studies. An important issue for cancer immunotherapy is the immune-profiling of the different tumor-derived γδ T cell subsets. Analysis of γδ TIL by flow cytometry revealed an inversion of the Vδ1/Vδ2 ratio in tumors including melanoma, colorectal cancer, squamous cell carcinoma, esophageal tumors, non–small-cell lung cancer, PDAC, and ovarian cancer. The inversion of the Vδ1/Vδ2 ratio is demonstrated by a high percentage of Vδ1 γδ T cells and a reduced percentage of Vδ2 γδ T cells within TIL compared to peripheral blood lymphocytes (PBL) of the same donors. An enriched percentage of Vδ1 and Vδ1/Vδ2-negative γδ TIL co-expressing Vγ2, 3, or 4 is also reported for ovarian cancer patients. Similar to the results in ovarian cancer measured by flow cytometry, a co-expression of Vγ2, 3, or 4 on Vδ1 TIL is also detected by IHC in frozen sections of colon adenocarcinoma patients. In contrast, an enhanced number of Vδ2 was found in glioblastoma multiforme, whereas no significant difference in the percentage of Vδ1 and Vδ2 T cells was observed in prostate cancer tissue in comparison with PBL.

In the majority of the above described tumor entities, γδ TIL are described to be in an activated stage accompanied by an increased number of Vδ1 TIL with a TCM or TEM phenotype and Vδ2 TIL with an TEM or TEMRA phenotype. Although a clear correlation with γδ T cell infiltration and patients’ prognosis is missing, there is a tendency that a reduction of Vδ2 γδ T cells correlates with an advanced stage of the disease. An enhanced Vδ2 T cell percentage has been shown to correlate with an early stage of development of melanoma and the absence of metastasis. The abundance of γδ T cells (mainly Vδ2 T cells) with a T helper (TH) 1 phenotype and cytotoxic capacity revealed a significantly longer 5-year disease free survival rate of colorectal cancer patients determined by transcriptome analysis. In contrast to an initial study demonstrating a positive correlation of interleukin (IL)-17 producing γδ TIL with tumor stages and other clinicopathologic features in colorectal cancer patients, Meraviglia and colleagues reported that interferon (IFN)-γ production of Vδ1 and Vδ2 TIL was reduced in colorectal tissue compared to adjacent tissue; however, IL-17 secretion was nearly absent. In this context, it is very interesting that freshly isolated Vδ1 and Vδ2 derived from PBL and TIL of ovarian cancer patients highly express intracellular granzyme A and B without additional phorbol ester and ionomycin (PMA/Iono) stimulation suggesting a pre-activated state of both γδ TIL subsets. In striking contrast, intracellular IFN-γ production was nearly absent but was inducible donor-dependently after PMA/Iono stimulation. Conversely, intracellular IL-17 was detected after PMA/Iono in ovarian γδ TIL in the publication of Chen et al., but not in our own studies. This may suggest a donor-dependent influence by the TME or an inclusion of IL-17-producing non-γδ TIL in the flow cytometric gating-strategy. Interestingly, CD4-expressing γδ T cells were identified in ovarian tumor to be beneficial for the patients’ survival.

Although the number of Vδ2 TILs is lower than the number of Vδ1 TILs in some tumor entities, Vδ2 TILs are a very interesting effector cell population for T cell-based immunotherapy as extensively summarized in recent reviews. The interest is based on the capacity of Vδ2 T cells to present soluble proteins and cell debris to CD8 αβ T cells by a proteasome-dependent cross-presentation pathway, to recognize phosphorylated antigen (PAG) released by tumor cells in a HLA-unrestricted manner and their reduced potential to cause graft versus host disease.

The frequency, phenotype and functions of γδ TILs are presumably influenced by genetic differences between tumor cells, polymorphisms of regulatory genes, exposure to certain pathogens (eg intestinal microbiome) and TME. The TME comprises a cellular compartment composed of a variety of immunosuppressive cells
including mesenchymal cells (eg, fibroblasts), myeloid-derived suppressor cells (MDSC), tumor-associated macrophages (TAM), and Treg, which release immunosuppressive molecules or express immunosuppressive molecules and thereby prevent the penetration or activation of cytotoxic γδ T cells. This review presents a crucial update of potential molecular mechanisms partially described in recent publications.11,12,60,81,88 More importantly, it focuses on some novel aspects of selected tumor resistance mechanisms influencing the effector function of γδ TILs.

3 | TUMOR RESISTANCE MECHANISMS

3.1 | Cyclooxygenase enzymes

Cyclooxygenase (COX) enzymes convert arachidonic acid, which is released from plasma membrane phospholipids to prostacyclins, thromboxanes, and prostaglandins (PG)89,90 [Figure 1(A)]. While COX-1 is constitutively expressed in the majority of mammalian tissues, COX-2 is induced by a variety of pro-inflammatory stimuli including tumor necrosis factor (TNF)-α.89,91 Both COX enzymes can promote tumorigenesis, tumor progression, and metastasis in a variety of human tumor entities including pancreatic ductal and colorectal adenocarcinoma as well as pulmonary, mammary, prostate, kidney, ovary, and liver tumors.92-96 However, this is not a general tumor resistance mechanism of these tumor entities, since COX overexpression is also variable in any tumor entity due to the heterogeneity of tumor cells in different patients. In addition, COX-2 is also overexpressed in cells of the TME including cancer-associated fibroblasts in nasopharyngeal and colorectal cancer as well as mesenchymal stem cells (MSC) in colon-, breast-, and ovarian cancer, glioma, and melanoma.97-100

An enhanced COX expression of tumor cells results in an increased release of its metabolite PGE2 which recruits Treg to the tumor site and suppresses the effector function of cells of the innate and adaptive immunity including γδ T cells.91,98,101-103

3.2 | Cross-talk between COX-2-expressing cells and γδ T cells

Inflammatory cytokines such as IFN-γ and TNF-α strongly released by tumor-surrounding cells and activated immune cells at the tumor-site including macrophages, αβ and γδ T cells enhance COX-2 expression in MSC and in PDAC98,99,103 [Figure 1(B)]. Subsequently, cytotoxic activity and cytokine release of γδ T cells are inhibited by PGE2 released by COX-2 expressing MSC and PDAC [Figure (1C)]. Activated γδ T cells (as they occur at the

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**FIGURE 1** Tumor resistance mechanisms (COX, TRAIL, IDO). A, Cyclooxygenase (COX) enzymes convert arachidonic acid to prostaglandins (PG) like PGE2. B, Inflammatory cytokines such as IFN-γ and TNF-α released by γδ T cells and other tumor-surrounding cells enhance COX-2 expression in mesenchymal stem cells (MSC) and tumor cells. MSC-mediated immunosuppression in turn exerts a negative feedback on the proliferation and cytokine production of γδ T cells. C, PGE2 binds to G-protein-coupled PGE2 and E4 receptors (EP2/4) and inhibits cytotoxic activity and cytokine release of γδ T cells. D, Cytokines, growth factors, and tumor promoters enhance COX-2 expression in tumor cells via mitogen-activated protein kinases (MAPK). E, TRAIL-R4 knockdown (ko) upregulates COX-1/2 expression and thereby PGE2 secretion in tumor cells, which is accompanied by an increased ERK activity. F, Indoleamine-2,3-dioxygenase (IDO) −1 and −2 convert the essential amino acid tryptophan in N-formyl-kynurenine and picolinic acid. G, Kynurenine, released by tumor and stromal cells in the tumor microenvironment (TME), suppresses degranulation of granzyme/perforin (Grz/Prf) of γδ T cells.
tumor-site) bind PGE2 via G-protein-coupled PGE2 and E4 receptors [Figure 1(C)], and as a consequence increase intracellular second messenger cyclic adenosine monophosphate (cAMP) via adenylyl cyclase activation.\textsuperscript{11,104} cAMP activates the transcription factor cAMP responsive element binding (CREB) via protein kinase A and the raft-associated enzyme c-src tyrosine kinase (CSK). While CREB proteins are essential regulators for T cell function and cytokine production, CSK negatively regulates phosphorylating activities of the TCR signaling kinase LCK, the \( \zeta \) chain TCR-associated kinase ZAP-70, and AKT in T cells including \( \gamma \delta \) T cells.\textsuperscript{98,105-107} Because PGE2 inhibits TCR-activated \( \gamma \delta \) T cell cytotoxicity, high PGE2 and COX-2 levels determined in many cancer patients can negatively influence the benefit of \( \gamma \delta \) T cells.\textsuperscript{98,105-107} Because PGE2 inhibits TCR-activated \( \gamma \delta \) T cell cytotoxicity, high PGE2 and COX-2 levels determined in many cancer patients can negatively influence the benefit of \( \gamma \delta \) T cell-based immunotherapy.\textsuperscript{98,99,103,108,109}

An enhanced COX-2 expression has also been shown to alter the cytokine profile of T cells by switching from TH1 to TH2-type cytokines. This could be converted by inhibition of COX-2 activity after in vivo application of selective COX-2 inhibitors or silencing with anti-sense oligonucleotides in an experimental colitis mouse model.\textsuperscript{110}

### 3.3 | Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)

Ligation of tumor necrosis factor-related apoptosis-inducing ligand (TRAIL or Apo2L) with cognate receptors such as TRAIL-R1 (death receptor 4, DR4) and TRAIL-R2 (death receptor 5, DR5) induces apoptosis in different tumor entities by activation of the caspase cascade, and thereby regulating the immune surveillance of tumors.\textsuperscript{111,112} Downregulation or shedding of TRAIL-R1 and -R2 on tumor cells results in tumor escape and acquired resistance of tumor cells toward TRAIL-induced apoptosis.\textsuperscript{113-115} Conversely to the function of plasma membrane-expressed TRAIL-R1/-R2, intracellular localization of TRAIL-R1/-R2 can influence malignant progression of tumor cells which is accompanied with patients' poor prognosis.\textsuperscript{113,116-119} Two additional TRAIL receptors, TRAIL-R3 (decoy receptor 1, DcR1) and TRAIL-R4 (decoy receptor 5, DcR5), which lack the pro-apoptotic death domain, are unable to induce apoptosis and negatively regulate TRAIL-induced apoptosis by binding TRAIL or by forming hetero-complexes with TRAIL-R1/-R2.\textsuperscript{120-124} Although the function of TRAIL-R4 is largely unexplored, several recent reports demonstrated a possible promutual function.\textsuperscript{120,125}

### 3.4 | Interaction of \( \gamma \delta \) T cells and tumor cells—Role of TRAIL/TRAIL receptor pathways

\( \gamma \delta \) T cells have a potent anti-tumor activity mediated by the cytotoxic effector molecules including perforin, granzymes, granulysin, TNF-\( \alpha \), soluble CD95-L, and soluble TRAIL.\textsuperscript{126,127} Initially, high concentrations of TRAIL were determined in the serum of hormone-refractory prostate cancer patients treated with \( \gamma \delta \) T cell agonist zoledronic acid and interleukin (IL)-2. Simultaneously performed microarray analysis revealed TRAIL production by \( \gamma \delta \) T cells, and a correlation of enhanced TRAIL serum level and increased number of \( \gamma \delta \) T cells with declining prostate-specific antigen levels and improvement of patients' clinical outcome upon zoledronic acid/IL-2 treatment.\textsuperscript{126} TRAIL and granzymes were also produced after exposure of V\( \gamma \)9V\( \delta \)2 \( \gamma \delta \) T cells to zoledronic acid-sensitized colon cancer-derived cancer stem cells (CSC) which results in an enhanced \( \gamma \delta \) T cell cytotoxicity against these target cells. The recognition and lysis of CSC, which are responsible for tumor maintenance and resistance to conventional cancer therapies, was TCR-dependent but only NKG2D-dependent when tumor targets expressed NKG2D ligands.\textsuperscript{128} In this context, it is very interesting that the lysis of chemotherapy-sensitized colon cancer-initiating cells by V\( \gamma \)9V\( \delta \)2 \( \gamma \delta \) T cells is inhibited by NKG2D-targeting antibodies but not by anti-CD3 antibodies. Moreover, chemotherapeutic agents enhance the TRAIL-R2 expression on colon cancer-initiating cells.\textsuperscript{129} In addition, the triggerring of NKG2D expressed on \( \gamma \delta \) T cells by agonistic NKG2D-antibodies induces the release of soluble TRAIL by \( \gamma \delta \) T cells and their cytotoxic activity against ULBP2 and TRAIL-R2-expressing H460 lung cancer cells in vitro and in vivo. This TRAIL-mediated lysis of H460 lung cancer cells was abrogated by blocking antibodies against NKG2D or ULBP2.\textsuperscript{130,131} These studies revealed that \( \gamma \delta \) T cell cytotoxicity can be mediated via TCR or NKG2D-dependent release of soluble TRAIL. Recently, Yang and colleagues demonstrated that a long noncoding RNA of TRAIL, called TANCR, regulates TRAIL expression by \( \gamma \delta \) T cells.\textsuperscript{132}

Regarding tumor resistance mechanisms mediated by down-regulation of TRAIL-R1 or TRAIL-2, it is very interesting to note that TRAIL-mediated lysis of osteosarcoma cells by \( \gamma \delta \) T cells was improved by treatment with celestrol, which enhanced TRAIL-R1 or TRAIL-2 expression.\textsuperscript{133} Additionally, resistance of TRAIL can be overcome by IFN-\( \gamma \) which is produced in high amounts by activated T cells including TH1-\( \gamma \delta \) T-cells as well as by NK cells.\textsuperscript{134,135}

While the majority of the studies analyzed so far focus on V62 T cells, the effects of the TRAIL/TRAIL-R pathway is less well examined for V61 T cells, which are enriched in most tumor entities. Our recent publication demonstrated that activated V61 T cells have a superior cytotoxic activity against PGE2-secreting PDAC cells compared to V62 T cells from the same donors.\textsuperscript{136} Since the knockdown of TRAIL-R1 or R2 in PDAC cells as well as the neutralization of TRAIL did not significantly impaired the V61 T cell cytotoxicity against PDAC cells, a minor role of the TRAIL/TRAIL-R pathway is suggested for V61 T cells. However, TRAIL-R4 expressed by PDAC cell can regulate the cytotoxicity of V61 and V62 \( \gamma \delta \) T cells. Knockdown of anti-apoptotic TRAIL-R4 in PDAC and breast cancer cells increase PGE2 release of these tumor cells and rendered them even more resistant to \( \gamma \delta \) T cell-mediated cytotoxicity by reducing granzyme production of \( \gamma \delta \) T cells.\textsuperscript{136}

### 3.5 | Liaison between TRAIL-R4 and COX

TRAIL, which can be produced in small amounts by tumor cells, tumor-surrounding cells or V62 T cells, has been shown to upregulate...
COX-1 by caspase and/or nuclear factor of κB (NFκB) activation. Cytokines, growth factors, and tumor promoters have been shown to enhance COX-2 expression in tumor cells via signal transduction pathways including mitogen-activated protein (MAP)-kinases p38, extracellular signal-regulated kinases (ERK) 1/2, and Jun N-terminal kinases (JNK). An accumulation of IDO-1/-2 enzymes in PDAC cells with metastatic origin and an upregulation in primary PDAC cells after co-culture with IFN-γ-producing γδ T cells has been recently shown to inhibit degranulation and cytotoxicity of γδ T cells. Additionally, the IDO-down-stream molecule kynurenine, which is released by tumor and stromal cells in the TME suppresses anti-tumor response of γδ T cells.

An involvement of IDO in immune modulatory capacities is not limited to tumor cells but has been also reported in the cross-talk of γδ T cells with human MSC or mesenchymal stroma cells, respectively. Activated γδ T cells can induce a MSC-mediated immunosuppression by their release of IFN-γ which in turn exerted a negative feedback mechanism on proliferation and cytokine production of γδ T cells. A similar, an interaction of γδ T cells with human mesenchymal stroma cells propagate an immunosuppressive milieu in the TME.

A liaison between COX-2 and IDO has been reported in a mammary as well as in lung carcinoma mouse models by an anti-tumor vaccination strategy which prevents IDO activation after application of the selective COX-2 inhibitor Celecoxib.

3.8 | Glycan-lectin interactions—An important role of galectins

Several technology advances including genomic, proteomics, glycomics, and glycoproteomics have developed to optimize personalized medicine. Aberrations in protein glycosylation have been implicated as a hallmark of cellular oncogenesis, tumor progression, and poor patients’ prognosis, although the complexity of cancer-associated glycosylation mechanisms is not completely understood. Several efforts have been made to influence glycan-lectin interactions by pharmacological anti-tumor agents that target lectins such as sialic-acid-binding immunoglobulin-like lectins (siglecs) and C-type lectin receptors such as selectins or galectins.

Galectins are a family of soluble proteins that bind β-galactoside-containing glycans. Here, a focus is set on galectins including galectin-1, galectin-3, and galectin-9, which so far are described to play a role in the interaction of tumor cells and γδ T cells.

Mammalian galectins are defined by a conserved carbohydrate recognition domain (CRD) and common structural fold. Galectin-1 belongs to the prototype galectin subgroup, galectin-3 is the only chimera-type member of the galectin family and galectin-9 belongs to the tandem-repeat-type galectins. As multifunctional proteins, all three galectins are overexpressed by tumor cells and secreted in the serum of cancer patients. However, an enhanced galectin expression correlates with poor patients’ survival in only in a few tumor entities, but not in others. Galectin-1, 3 and 9 are expressed also by immune cells and by tumors. All three galectins have multiple binding partners and can contribute to immune evasion by regulating tumorigenesis and metastasis.

For instance, galectin-1 is described to serve as negative immune regulator of immune responses by inducing tumor-mediated TH1-cytokine suppression, IL-10 release and apoptosis in TH1 and TH17 cells but not in TH2 cells due to differential sialylation of cell surface glycoproteins. Targeting galectin-1 might promote anti-tumor response by reduction of T-cell apoptosis, abrogation of tumor and
Galectin-3 mediates both pro- and anti-apoptotic activity depending on the subcellular localization. Galectin-3 mediates malignant tumor transformation and is involved in different aspects of cancer biology including angiogenesis, cell adhesion, and migration. Galectin-3 released by tumor cells induces immunosuppression and regulates immune cell activities. Extracellular galectin-3 binds glycosylated T cell and NK cell surface receptors thereby inducing anergy of CD8 TIL and impairment of NK cell activity in bladder cancer. Several clinical trials combining galectin-3 inhibitor GR-MD-02 and DG-MD-02 together with immune checkpoint inhibitors have started to enhance therapeutic effects of immunotherapy.

Galectin-9 modulates also multiple biological functions including regulation of cell adhesion, chemo-attraction, lattice formation of glycoconjugates and cell death depending on their subcellular localization. Galectin-9 inhibits proliferation of cancer cells and treatment with recombinant galectin-9 prevents metastatic spread in multiple preclinical cancer models, which support the discussion about the therapeutic potential of galectin-9 in cancer. In addition, galectin-9 induces tolerogenic macrophages via binding of decitin-1, exhaustion of cytotoxic CD8 cells, and cell death in TH1 and TH17 T via binding of T cell immunoglobulin mucin-3 (TIM-3) as well as the polarization of induced Treg cells.

Galectin-1 is expressed by cervical cancer tumors. Targeting galectin-1 by appropriate antibody or lactose enhanced cytotoxicity of ex vivo-expanded γδ TILs derived from cervical cancer patients against cervical tumor cells in vitro and in vivo in a xenograft severe combined immunodeficiency (SCID) mouse model [Figure 2(A)]. Similar to galectin-1, galectin-3 is regarded as an intrinsic tumor escape mechanism. Galectin-3 is expressed in PDAC cells and γδ T cells, but is released only in small amounts by either cell population. In addition, the results of Gonnermann and colleagues demonstrated that PDAC cells transport galectin-3 in vesicles to the cell surface after having direct cell contact with γδ T cells. Consequently, galectin-3 is released in higher amounts by PDAC cells and, similar to recombinant galectin-3, inhibits proliferation of circulating and tumor-infiltrating γδ T cells of healthy and cancer patients by interacting with glycosylated Vδ2 γδ T cells.
cell surface receptor α3β1 integrin [Figure 2(B)]. Knockdown of galectin-3 in PDAC cells restored the γδ T cell proliferation when co-culturing both cell populations. While galectin-3 has an immunosuppressive function by inducing anti-proliferative signaling, it did not influence IFN-γ production, degranulation or cytotoxic activity of γδ T cells co-cultured with PDAC cells. Interestingly, in contrast to galectin-9, physiological concentrations of galectin-3 did not induce cell death in γδ T cells.Galectin-9 has been reported to be strongly expressed in peripheral blood γδ T cells and tumor tissue of PDAC patients in comparison with healthy controls. Galectin-9 can polarize macrophages to a protumoral M2-phenotype and thereby inhibit the IFN-γ and TNF-α secretion of γδ T cells.[71] In accordance, Daley et al demonstrated that the targeting of galectin-9 in γδ T cell–competent hosts restored the activity of γδ TILs suggesting that release of galectin-9 by γδ T cells might play a role. However, an effect of galectin-9 on cytokine secretion of γδ T cells is not examined. In contrast to galectin-3, galectin-9 was enhanced in the serum of PDAC patients compared with other diseases and healthy individuals suggesting galectin-9 could serve as a biomarker for the determination of PDAC.[63,71]

In sum, galectin-1, 3, and 9 play an important role in the interaction of cervical cancer or PDAC cells and γδ T cells by differentially modulating anti-tumor responses of γδ T cells. Since γδ T cells infiltrate in PDAC tissue[57,60-62] which overexpress galectin-1, 3, and 9,[198] the different impact of the three galectins may be one important aspect for the impaired responsiveness of Vδ2 γδ TILs against PDAC. Accordingly, the low absolute cell number of Vδ2 γδ TILs within PDAC tissue is further reduced after their stimulation with zoledronic acid. In consequence, zoledronic acid–stimulated PDAC cells co-cultured with autologous γδ TILs released an enhanced amount of galectin-3.[62] Certainly, further studies are required in an autologous assay of PDAC cells and γδ TILs to investigate in detail the different impact of the described galectins.

4 | TUMOR-TARGETING BY γδ T CELLS

4.1 | Common strategy to enhance γδ T-cell cytotoxicity against tumor cells

A series of clinical trials have examined the anti-tumor response of Vγ9Vδ2 T cells in different types of cancer after the application of n-BP or Pag plus IL-2 in vivo or after repetitive adoptive transfer of in vitro expanded Vδ2-expressing γδ T cells. An overview on current γδ T cell–based immunotherapies is summarized extensively in different reviews.[80,82,199,200] Briefly, although the application of n-BP or Pag ± γδ T cells is well tolerated in these clinical trials, limited promising results have been delivered. Strikingly, a complete remission has been shown in 4 patients with hematological malignancies after adoptive transfer of haploidentical γδ T cells, and in one patient with renal-carcinoma–derived lung metastases after adoptive transfer of autologous γδ T cells.[201,202] In contrast to other clinical trials, adoptively transferred γδ T cells were additional stimulated by additional intravenous administration of n-BP together with subcutaneous application of IL-2 in both trials, which enabled a necessary restimulation of γδ T cells initially expanded for 11 to 14 days under GMP-conditions in vitro before they were adoptively transferred.

A restimulation of adoptively transferred γδ T cells with n-BP and IL-2 was initially demonstrated in a preclinical study.[203] The repetitive combination of n-BP activated γδ T cells together with n-BP and IL-2 significantly prolonged the survival of SCID mice inoculated with tumor cells. Interestingly, our further preclinical studies demonstrated that activation of ex vivo–expanded γδ T cells with γδ-targeting bsTCE further enhanced their cytotoxic capacity and prevented cell death of these adoptively transferred γδ T cell. Adoptively transferred γδ T cells together with γδ-targeting bsTCE more significantly reduced growth of pancreatic tumor engrafted in SCID-Beige mice than n-BP.[57,204]

4.2 | Bispecific antibodies

Bispecific antibodies (bsAb) are a family of biological molecules designed in their simplest format with two different antigen-bind sites which are lack a constant immunoglobulin domain.[205,206] Improvement of the design of bsAb has been generated by different means in various formats.[206,207] BsTCE targeting CD3ε are the most advanced. BsTCE directly targets CD3 T cells and tumor cell via binding of different tumor-associated antigens, and thereby inducing TCR activation, release of cytotoxic mediators and target cell lysis. Blinatumomab [CD19xCD3] was approved by FDA for the clinical use in patients with B-cell lymphoma or leukemia.[208,209] A great number of further clinical studies (phase I or I/II) has been started with bsTCE equipped with a specificity for CD3 and another one for antigens expressed by tumor cells to treat mainly hematological malignancies but also several solid tumors.[206] Although the CD3-targeting bsTCE revealed promising results, some disadvantages including the activation of Treg or potential high toxicity for commonly expressed tumor-associated antigen such as Epithelial cell adhesion molecule (EpCAM) or Epidermal growth factor receptor (EGFR) have been reported.[210,211] Under these circumstances, it would be an advantage to activate solely γδ T cells rather than all CD3 T cells.

The first γδ-based bsTCE was a tribody comprising bivalent targeting of human epidermal growth factor receptor (HER)-2 overexpressed on PDAC cells and monovalent binding of Vγ9TCR. Our studies demonstrated that tribody [HER2]2 × Vγ9] selectively targets Vγ9 γδ T cells to tumor-associated antigen HER-2, thereby enhancing the Vγ9 T cell cytotoxicity in vitro as well as in vivo in a PDAC grafted SCID-beige mouse model.[57] Similar to the enhanced reactivity against PDAC, tribody [[HER2]2 × Vγ9] efficiently enhanced the lysis against other HER2-expressing tumor cells including ovarian and breast cancer cells.[58,136] HER-2 is often selected as tumor-associated target antigen, since anti-HER-2 therapies are successful for the treatment of HER-2-expressing tumor cells.[212]

Of interest is also a new bspecific nanobody in a different format, which targets Vγ9Vδ2 T cells and has specificity for EGFR
expressed on tumor cells. This Vγ9Vδ2 T cell-specific nanobody induced lysis of patient-derived colorectal carcinoma cells in vitro as well as in vivo in a xenograft mouse model and has minimal reactivity to other EGFR-expressing cells such as keratinocytes.213,214

An enhanced cytotoxic activity of Vδ1 and Vδ2 T cells toward CD20-expressing lymphoma cells and, more importantly, against patient-derived chronic lymphocytic leukemia cells were induced by two immunoligands, designated [MICA × 7D8] and [ULBP2 × 7D8].215 The two immunoligands have specificity for CD20 (7D8) and NKG2D ligand MICA or ULBP-2, respectively, expressed on tumor cells including lymphoma and leukemia cells.215,216 Beside Vδ1 and Vδ2 T cells, these immunoligands also attract NK cells and synergistically enhanced antibody-dependent cell-mediated cytotoxicity. An additional approach focuses on re-directing CD16-expressing CD16-expressing innate cells against HER-2-expressing cancer cells.217 Targeting of different innate cells can be promising with respect to their differential infiltration in different tumor entities.

Overall, these γδ-targeting bsTCE approaches offer new possibilities for γδ T cell-based immunotherapy.

4.3 | Effects of γδ T-cell engager on tumor resistance mechanisms

Since the interaction of tumor cells and tumor-surrounding cells with γδ T cells can increase inhibitory mechanisms including overexpression of COX-2 or IDO, downregulation of TRAIL-R4, or release of galectins, the anti-tumor response of γδ T cells is impaired. The application of γδ-targeting bsTCE can drastically enhance impaired cytotoxic activity by targeting γδ T cells to tumor cells and increasing the release of cytotoxic mediators. Since γδ-targeting bsTCE are not designed to reduce immunosuppressive molecules such as PGE2 and kynurenine, the application of appropriate inhibitors has to be considered. A combination of γδ-targeting bsTCE with COX-2 or IDO inhibitors completely restored the γδ T cell cytotoxicity against tumor cells resistant against γδ T cell-mediated lysis in vitro.103,218 Interestingly, in a lung and mammary carcinoma mouse model, COX-2 inhibitor Celecoxib prevents IDO activation.164-166 In addition, COX inhibitors improved the anti-tumoral γδ T cell cytotoxicity regulated by TRAIL-R4.136 So far, it is uncertain whether an in vivo administration of γδ-targeting bsTCE and COX-2 or IDO inhibitors can overcome an immunosuppressive TME. Of course, other inhibitory mechanisms including oxidative and metabolic stress, expression of inhibitory receptors and galectins can inhibit γδ T cell proliferation,11,73,88,171,218,219 which is only marginally affected by γδ-targeting bsTCE. In this context, an activation of γδ T cells by γδ-targeting bsTCE in vivo may not abolish a suppressed proliferation or activate accumulating exhausted Vδ2 TIL. For instance, co-culture of freshly isolated PDAC tissue containing tumor-associated immune cells together with TIL in the presence of n-BP revealed an unresponsiveness of Vδ2 TILs and an enhanced secretion of galectin-3.218 As a consequence to overcome exhaustion of Vδ2 TIL, an adoptive transfer of Vδ2 γδ T cells together with proliferation-inducing n-BP and enhanced cytotoxicity-inducing γδ-targeting bsTCE can be a therapeutic option. If a possible suppressive activity of galectin-9, produced by adoptively transferred Vδ2 γδ T cells, inhibits αβ T cell activation or can be abolished by targeting sialoglycan-Siglec pathway has to be elucidated.

Surely, the data summarized in this review support the idea of an (personalized) assessment of phenotypic and functional characterization of the different T cell subsets of cancer patients. This analysis is important to get insights in the anti-tumor response of circulating and tumor-infiltrating γδ T cells. In addition, γδ-targeting bsTCE provide a valuable tool to analyze the functional capacity of γδ T cell subsets within peripheral blood and tumor-infiltrating cells co-cultured with autologous tumor cells.58,82,220

5 | FUTURE PERSPECTIVES

To understand the functional properties of γδ T cell subsets and to improve γδ T cell-based immunotherapy, additional studies are required to further determine their frequency and distribution of γδ TIL in human tumor tissues, natural ligands, homing, survival, cytotoxicity, cytokines/chemokine production, requirement of costimulatory molecules, pro-and anti-tumoral activities, and regulatory functions. Additionally, different factors influencing the interaction of γδ TIL and tumor cells have also to be considered in more detail. These factors include genetic differences between tumor cells, oncogenic signaling pathways and inflammatory conditions at the tumor-site, intrinsic tumor escape mechanisms, influence of TME-associated cells, and cancer-associated glycosylation.

A new wave of innovative strategies, including new design of γδ T cell engagers such as bsAb targeting different tumor-associated tumor antigens, CAR γδ T cell therapy, targeting of tissue-resident Vδ1 T cells, agonistic anti-BTN3A Ab, allogeneic γδ T cell adoptive cell therapy, and combination therapies will certainly help to improve the success of γδ T cell-based immunotherapy in the future.81,221,222

A further understanding of functional properties of γδ T cell subsets, development of three-dimensional spheroid culture systems, use of non-human primate models, and further clinical studies will hopefully pave the way for the future success.81,223

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