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γδ T cells as critical anti-tumor immune effectors

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While the effector cells that mediate anti-tumor immunity have historically been attributed to αβ T cells and natural killer cells, γδ T cells are now being recognized as a complementary mechanism mediating tumor rejection. γδ T cells possess a host of functions ranging from antigen presentation to regulatory function and, importantly, have critical roles in eliciting anti-tumor responses where other immune effectors may be rendered ineffective. Recent discoveries have elucidated how these differing functions are mediated by γδ T cells with specific T cell receptors and spatial distribution. Their relative resistance to mechanisms of dysfunction like T cell exhaustion has spurred the development of therapeutic approaches exploiting γδ T cells, and an improved understanding of these cells should enable more effective immunotherapies.

The majority of T cells express T cell receptors (TCRs) composed of alpha and beta (αβ) chains, which are the dominant mediators of adaptive immunity. T cells expressing TCRs composed of gamma and delta (γδ) chains represent a small fraction of lymphocytes but contribute to both adaptive and innate immunity. Since their discovery in the 1980s[1,2], their role in contributing to anti-tumor immunity has slowly been acknowledged. In contrast to αβ T cells, the study of γδ T cells presents some challenges. These include their low abundance in blood (representing 5–10% of the total circulating lymphocytes), tissue-specific distribution of specific populations and the discrepancy between mouse and human γδ subsets[3]. Moreover, the ligand specificity for each of the different γδ TCRs was mostly unknown until the early 2000s, when γδ T cells were found to elicit tumor regression in a preclinical skin tumor model through engagement of the NKG2D receptor[4]. They were also reported as a main source for interferon-γ (IFNγ) in the tumor microenvironment (TME)[5]. Preclinical studies in a prostate cancer mouse model demonstrated that γδ T cells were mediators of tumor cytotoxicity in autologous cell-transfer experiments[6]. In recent years, γδ T cell infiltration has been shown to be the most favorable prognostic factor among different cancer types, with a stronger association than αβ T cells[7]. In this Review, we will examine the most recent advances in understanding the roles of γδ T cells in tumor immunity as well as efforts to leverage these cells for cancer immunotherapy.

γδ T cell biology

γδ T cell ontogeny and biology have been extensively reviewed[8–14]. Similar to the αβ lineage, the γδ TCR is a heterodimer composed of a γ chain paired to a δ chain. Unlike the adaptive response for αβ T cells, γδ T cells can elicit antigen-driven cytotoxicity in a major histocompatibility complex (MHC)-independent fashion, behaving as innate effectors, and can recognize a broad range of known antigens as well as antigens yet to be identified. Upon activation, γδ T cells can clonally expand[15,16] and can induce type 1 helper T cell responses in vivo in mice and humans in response to diverse disease scenarios, such as cytomegalovirus infection[17–19] or cancer[4–6,20].

The classification of γδ T cells is based on receptor gene usage, which is species specific. The principal murine γδ T cell subsets are classified by γ-chain usage (Vγ1–Vγ7), whereas the main human γδ T cell subsets are classified by their δ-chain usage[21]. While the most-studied human γδ T cells, using the Vδ2 chain, mostly coupled with the Vγ9 chain and usually termed Vγ9Vδ2 cells (Vδ2 onward), are predominant in the periphery (up to 95% of circulating γδ T cells), other subsets (mainly Vδ1, Vδ3, Vδ5) are primarily tissue resident. The Vδ2 TCR repertoire, the first γδ T cell repertoire generated in the human body, appears to have low variability and is even shared between individuals[22]. Vδ2 T cells express multiple natural killer (NK) cell receptors that contribute to their cytotoxic activity upon ligand recognition[23,24]. Because they express CD16 or FcγRIII, Vδ2 T cells are also able to

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induce antibody-dependent cellular toxicity[25]. Their unique bridging of innate and adaptive immune responses is evidenced by their ability to cross-present antigens to induce αβ $CD8^+$ T cell responses[26,27]. Vδ1 T cells, the most promiscuous γδ subset, pairing with γ-chains Vγ2–Vγ5, Vγ8 and Vγ9, are enriched in the skin, spleen, liver and gut, where they facilitate tissue homeostasis[10]. They can represent up to 30% of circulating γδ T cells and present a private (nonshared) and more variable adult TCR repertoire than Vδ2 T cells[28,29]. Subset characterization in healthy donors has shown that most circulating Vδ1 T cells could be categorized as primarily $CD27^-$ naive or $CD27^+$ effector memory cells[30], while Vδ2 cells presented a more terminal effector phenotype in circulation, with no significant differences in early or late activation marker expression[30]. Vδ1 T cells express NK cell receptors like NKG2D as well as other innate receptors, such as NKp46, NKp30 or NKp44, that enable cytotoxicity[31–34]. They are major mediators of cytomegalovirus and clearance of Epstein–Barr virus infection[17,19,35–37]. Vδ3 T cells, paired with either Vγ2 or Vγ3 cells, are a minor circulating (<1%) γδ T cell subset that is also enriched in the intestines and the liver, showing functional overlap with Vδ1 T cells[38–40]. Finally, Vδ5 cells, paired with the Vγ4 chain, tend to be found mainly in peripheral blood, although at much lower frequencies than Vγ9Vδ2 cells, and in the skin[21,41].

Continued efforts have been made for comprehensive identification of γδ TCR ligands, thoroughly reviewed by others[42]. Vδ2 cells recognize phosphoantigens, small nonpeptidic byproducts from bacteria and tumor cell metabolism, through a mechanism regulated by the endogenous butyrophilin (BTN) protein family. Since the first studies in the 1990s, activation of Vδ2 cells by phosphoantigens or phosphoantigen analogs, such as zoledronate, has been largely reported to induce activation and cytotoxicity[43–46]. However, the specific molecular hierarchies orchestrating γδ ligand sensing are still not fully understood. It is generally accepted that the participation of the protein complex composed of up to three members of the BTN protein family, BTN3A1, BTN3A2 and BTN2A1, is critical for effective signaling. The necessary binding of the Vγ9 domain of the γδ TCR to BTN2A1 and the potential binding of BTN3A1 to the Vδ2 chain for effective activation upon phosphoantigen recognition have been previously demonstrated[47]. Recent work has hugely contributed to the elucidation of such mechanisms by identifying the critical role for the BTN2A1 V domain in the recruitment of the γδ TCR as well as the improved characterization of efficient signal-transducing BTN3A heteromers[48]. Further studies of ligand-mediated activation of γδ T cells may enable more effective γδ-based technologies.

γδ T cells in cancer immunity

The field of cancer immunology does not cease to expand. The study of tumor-infiltrating γδ T cells has spurred huge interest in the past decades, which has led to important yearly milestones. However, γδ T cell research often faces challenges in resolving ambiguous observations, and there are still many underexplored or not yet fully disclosed features of γδ T cell biology impacting the fate of tumors. In the following section, we will discuss the current knowledge of protumoral and anti-tumoral functions of γδ T cells as well as their ligand specificity and functional status.

Protumoral and anti-tumoral roles for infiltrating γδ T cells

γδ cells can possess both innate and adaptive pleiotropic functions that either enhance tumor progression or help to mediate tumor rejection, summarized in Fig. 1 and previously reviewed by others[11,49–51] (Fig. 1). Tumor-infiltrating γδ populations have been known to contribute to anti-tumor immunity[52–56] and can mediate strong anti-tumor responses via IFNγ and tumor necrosis factor (TNF) by cytolytic mechanisms[57]. On the other hand, they can also possess immunosuppressive function. For example, interleukin-17 (IL-17)-expressing γδ T cells, enacting the main γδ-mediated immunosuppressive pathway identified to date, can lead to the accumulation of myeloid-derived suppressor cells[58,59], among many other functions. Tumor-infiltrating γδ T cells have also been described to suppress αβ T cell and dendritic cell function in a fashion reversible by engaging Toll-like receptor (TLR) signaling[60]. Conversely, they have also been shown to function as professional antigen-presenting cells to αβ T cells in lymph nodes[26,61] as well as to enable antibody production by B cells[62] or to contribute to anti-tumor neutrophil infiltration[63].

Beyond $IL\text{-}17^+$ γδ subsets, recent work has uncovered other markers for γδ T cell immunosuppression in $IL\text{-}17^-$ cells, such as promyelocytic leukemia zinc finger (PLZF) or amphiregulin (AREG) in tumor-infiltrating Vδ1 T cells in colorectal cancer[64]. Tumor-infiltrating γδ T cells possessing a regulatory T (T_{reg}) cell-like phenotype have also been associated with impaired dendritic cell and $CD8^+$ T cell responses[60,65] as well as with poor survival in breast[66], pancreatic[67] and colorectal cancers[68] and multiple myeloma[69]. T_{reg} cell-like γδ T cells expressing high levels of IL-10 and IL-17 were found to hamper immune surveillance in pancreatic ductal adenocarcinoma[67]. An immunosuppressive role for IL-4-secreting γδ T cells has also been described in vitro[70].

The contribution of different γδ populations in anti-tumor immunity varies greatly between subsets and even tumor types. $V\delta2^+$ T cells have been most studied in the context of antigen reactivity and have been shown to kill tumor cells upon antigen encounter[46,71]. In addition, other subsets expressing alternative Vδ genes ($V\delta2^-$) such as those encoding Vδ1, Vδ3 or Vδ5 have also emerged as mediators of cytotoxic function in tumors[52–54]. However, others questioned a therapeutic role for γδ cells in renal cancer by showing reduced tumor-infiltrating γδ T cell frequencies and lack of correlation with any of the clinicopathological features included in the study[72]. In the case of liver cancer, both $V\delta2^+$ and $V\delta1^+$ cells showing a $CD69^+CD49a^+CD103^+$ tissue-resident memory phenotype have been recently associated with anti-tumoral activity[56]. Overall, protumoral or anti-tumoral roles in γδ T cells cannot solely be predicted by subset or discrete marker expression. Ligand specificity and immune functional status, reviewed in the following sections, are therefore pivotal determinants of tumor-infiltrating γδ T cell fate.

γδ ligands in cancer

In contrast to αβ T cells, which rely on the recognition of processed peptides presented on MHCs, identified γδ ligands span a wide range of disparate molecules presented in different contexts, ranging from MHC class I-like proteins like CD1c or CD1d[73,74] to co-stimulatory-like proteins like murine SKINT1 (ref. 75) or the BTN protein family[76]. Vδ1 γδ T cells recognize multiple MHC-like proteins but also 'unexpected' self-ligands such as members of the heat shock protein family as well as other proteins such as MutS homolog 2 (MSH2) or other stress-related candidates[8,41,76–80]. Vδ3 T cells can recognize CD1d-presented glycolipids and translocated annexin A2 in transformed cells[39,77] as well as the MHC-like protein MR1 in an antibody-like recognition fashion[81,82], even though the latter has not been linked yet to tumoral tissue. Vδ5 cells have been reported to recognize the endothelial protein C receptor (EPCR), also not yet linked to cancerous transformation[41]. γδ T cells that are self-reactive toward specific MHC class I molecules have also been suggested[83] (Fig. 2).

Malignant transformation can upregulate stress-associated molecules, such as MHC class I chain-related protein A (MICA), MICB, ULBP1–ULBP6, NECTIN2 and NECTIN5 as well as integrins such as ICAM-1 (refs. 41,78,84–86). γδ T cells, like NK cells, express a wide range of innate surveillance receptors, including NKG2D, DNAM-1, NKp30, NKp44 or LFA-1, to target cells expressing these stress ligands and act as co-stimulators of the γδ TCR[23,87–89]. Vδ2 ligands characterized so far are mostly phosphoantigens like isopentenyl pyrophosphate, which accumulates when the mevalonate metabolic pathway is altered in tumor cells, or the microbial metabolite methyl-but-2-enyl-pyrophosphate, produced by the non-mevalonate isoprenoid synthesis pathway in

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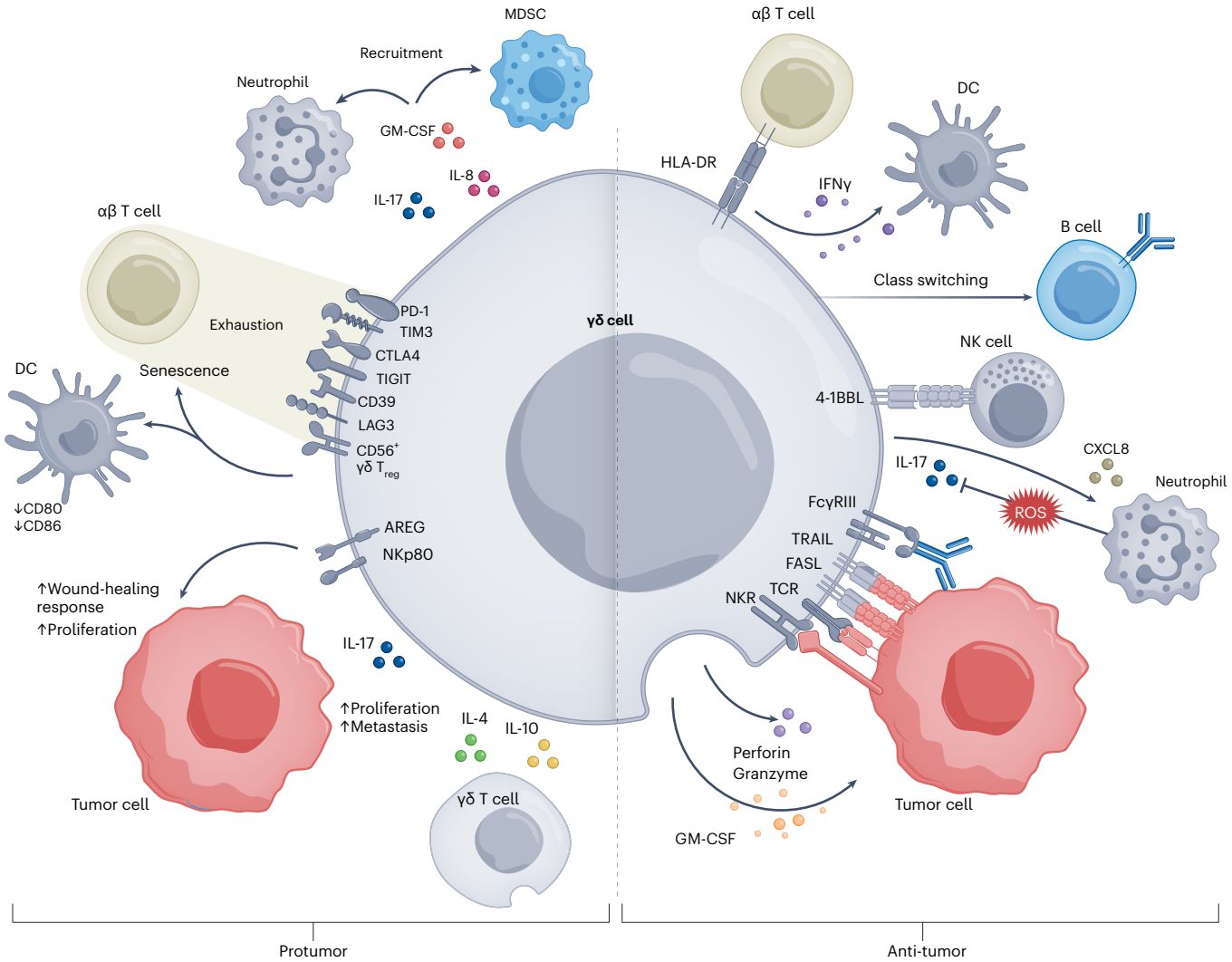

Fig. 1 | Protumoral and anti-tumoral roles of γδ T cells. Tumor-infiltrating γδ T cells present a wide and often opposing functional spectrum. They can contribute to anti-tumor immunity by acting as antigen presenters to αβ T cells, by activating dendritic cells (DCs), by promoting antibody production by B cells, by co-stimulating innate cells, such as NK cells, by promoting reactive oxygen species (ROS) production by neutrophils, thereby inhibiting immunosuppressive IL-17 signaling, and by mediating direct or indirect cytotoxicity on target tumor cells (right). On the other hand, they can contribute to tumor immune escape by recruiting immunosuppressive myeloid-derived suppressor cells (MDSCs) and neutrophils by means of IL-17 secretion, by inducing senescence in tumor-reactive αβ T cells and neighboring dendritic cells, by upregulating immune checkpoints leading to exhausted dysfunctional states, by promoting wound healing and survival pathways in tumor cells via cytokine signaling and by inhibiting bystander γδ T cell responses by IL-4 and IL-10 release (left). CXCL8, C–X–C motif chemokine ligand 8; FASL, Fas ligand; GM-CSF, granulocyte–macrophage colony-stimulating factor; HLA, human leukocyte antigen; NKR, NK cell receptor; TRAIL, TNF-related apoptosis-inducing ligand.

non-eukaryotic organisms, in a mechanism governed by the initial interaction between phosphoantigens and members of the BTN protein family[90,91], although Vδ2 recognition of alternative ligands, such as apolipoprotein A1 (ApoA1) or ectopically expressed MSH2, has been reported[80,85,92]. Phosphoantigen analogs, such as the aminobiphosphonate zoledronic acid (zoledronate), can also activate Vδ2 cells in the cancer setting[93]. Non-Vδ2 subsets, collectively termed 'Vδ2^{-}', recognize a spectrum of tumor antigens that have not been fully determined. They can bind MICs, lipid-loaded CD1 molecules, among others mentioned above. Studies in lung, breast, kidney or colorectal cancer have also underscored the potential of exploiting tumor-infiltrating Vδ2^{-} γδ T cells in cancer immunotherapy[52–54,56].

While the thresholds enabling γδ T cells to recognize stress conditions are still largely unknown, the interaction between the γδ TCR and members of the BTN family on target cells, particularly on Vδ2 T cells, can serve as triggering events[94]. Recently, functional CRISPR screens of tumor cells have elucidated how such stress-induced ligands, such as members of the BTN2A1–BTN3A protein complex, become upregulated in target tumor cells and influence Vδ2-mediated target cell killing. These studies demonstrate the importance of metabolic pathways in response to alterations of the cellular energetic state, such as the AMP-activated protein kinase (AMPK) pathway, in driving γδ T cell responses. γδ T cells were shown to simultaneously detect these increased stress-induced pathways along with reduced oxidative phosphorylation, one of the key features of transformed malignant cells. These findings, which were validated in patients with cancer, highlight opportunities for exploiting γδ T cell tumor immune surveillance and could guide parallel studies for elucidating the specific stress-induced pathways that engage Vδ2^{-} γδ T cell responses[95].

γδ T cell exhaustion in tumors

Mixed immunosuppressive and anti-tumoral γδ T cell phenotypes are commonly found in human tumors, which is prompting researchers to refine their analysis tools to minimize ambiguity[96]. While T cell

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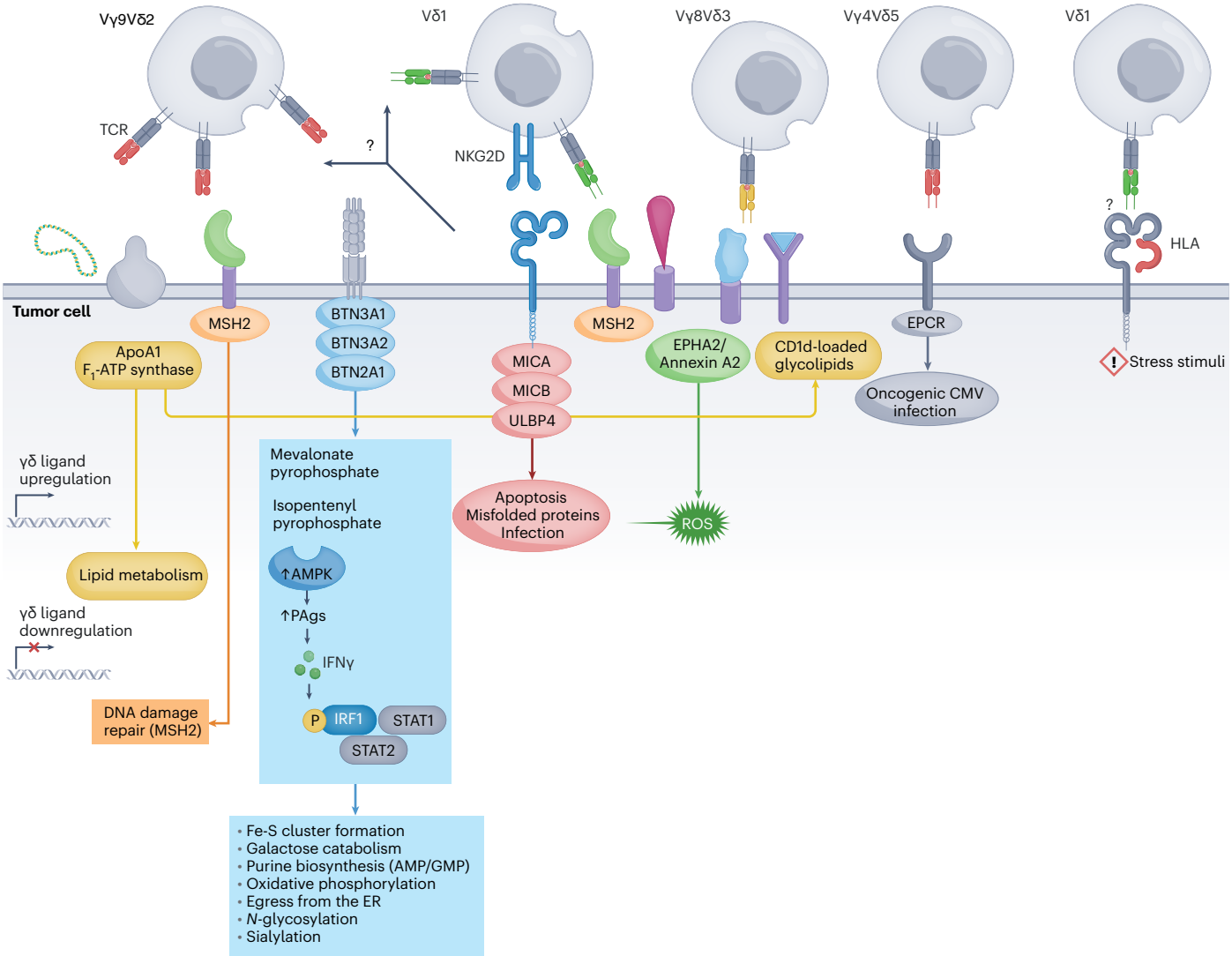

Fig. 2 | Pathways regulating γδ T cell ligand expression. Several biological pathways regulate the expression of a diverse range of γδ ligands during cancer. Stress-induced accumulation of intermediate metabolites of the mevalonate pathway such as isopentenyl pyrophosphate, also known as phosphoantigens (PAgs), drives the upregulation of members of the BTN protein family, the major Vδ2 activating ligands. However, highly active Fe-S cluster formation, galactose catabolism, purine biosynthesis, oxidative phosphorylation and the balance in post-translational modification, among others, lead to BTN downregulation. Other known ligands with their respective activating γδ subset and the underlying gene expression regulating pathways are shown. The full list of γδ ligands is still unknown. CMV, cytomegalovirus; EPHA2, EPH receptor A2; ER, endoplasmic reticulum; IRF1, IFN regulatory factor 1; STAT, signal transducer and activator of transcription.

exhaustion as a dysfunctional tumor-infiltrating T cell state resulting from chronic antigen stimulation has been clearly established in αβ T cells[97–99], the capacity of γδ T cells to become exhausted is less clear. Tumor-infiltrating γδ T cells can express immune checkpoints representing putative exhaustion markers[100], generally higher in Vδ1 cells than in Vδ2 cells[30]. The sensitivity of human Vδ2 T cells to inhibition by the CD86–cytotoxic T lymphocyte-associated protein 4 (CTLA4) axis has been described[101]. Exclusively immunosuppressive γδ T cell function by immune checkpoints has indeed been reported in multiple malignancies. PD-1 ligand-positive (PD-L1$^+$) tumor-infiltrating γδ T cells in response to IFNγ, similar to αβ T cells, have been identified in neuroblastoma[102]. CD39$^+$CTLA4$^+$PD-1$^+$ T_{reg} cell-like γδ T cells were found to hamper αβ CD8$^+$ responses via the adenosine receptor pathway in colorectal cancer[68]. Similarly, irreversibly dysfunctional Vδ2 T cells have been identified in the bone marrow of patients with multiple myeloma[69], and PD-1$^+$ γδ T cells from patients with leukemia were found to secrete significantly reduced levels of IFNγ in vitro, in an apparently immune checkpoint inhibition-resistant fashion[103]. In this latter and other studies, Vδ2$^-$ T cells, particularly from the Vδ1 subset, appear to be the predominant PD-1-expressing γδ T cell population, but more extensive research is needed to fully elucidate their role in tumors and the contribution of the relevant subsets.

Despite the expression of canonical exhaustion markers including the combination of PD-1, TIM3 and TIGIT, γδ T cells were still able to expand and kill autologous tumor cells in vitro and were associated with favorable clinical responses in patients with urothelial cancer treated with immune checkpoint inhibition[53]. Similarly, PD-1$^+$ Vδ1 and Vδ3 tumor-infiltrating γδ T cells were key contributors to autologous tumor killing in patients with mismatch repair-deficient colon cancer[52]. PD-1$^+$ Vδ2 cells were reported to induce IFNγ–TNF-driven anti-tumoral responses in hematological malignancies[104]. TIGIT$^+$TIM3$^+$PD-1$^+$CD39$^+$ Vδ1 and Vδ2 γδ T cells were found in tissues and the circulation in patients with ovarian cancer, with an observed high frequency of tissue-resident effector memory traits in the Vδ1 compartment[105]. Another study in patients with colorectal cancer showed significantly increased cytotoxic molecule expression (GZMB, PRF1), moderate

exhaustion (TIGIT, lymphocyte-activating protein 3 (LAG3), TIM3, CTLA4) and glycolytic metabolic advantage in tumor-infiltrating γδ T cells compared to $CD8^+$ TILs[64]. In the context of non-small cell lung cancer (NSCLC), effector-like and tissue-resident memory-like subsets of Vδ1 infiltrating γδ T cells with varying expression of the exhaustion markers PD-1, PD-L1 or TIGIT were associated with increased relapse-free survival[55]. In the context of immune checkpoint inhibition with blocking antibodies, it has been shown that increased Vδ2 cells, but not Vδ1 cells, correlated with clinical benefit in patients with melanoma treated with anti-CTLA4 therapy[106]. Similar findings involving exhausted $PD\text{-}1^+TIM3^+CD39^+$ γδ T cells associated with clinical benefit were reported in ovarian cancer[107]. In a recent side-by-side comparison of melanoma- and lung-infiltrating αβ and γδ T cells by means of ex vivo tissue explants, $PD\text{-}1^+$ Vδ1 T cells showed diminished transcriptional evidence of terminal exhaustion compared to $PD\text{-}1^+CD8^+$ T cells, were more able to maintain effector function and were valid predictors of response to checkpoint inhibition in patients with melanoma[108]. A similar observation was also recently reported showing that clonally expanded $PD\text{-}1^+TIGIT^+$ Vδ1 T cells were correlated with clinical benefit in a patient with Merkel cell carcinoma who had a complete response after treatment with anti-PD-1 therapy[109]. Thus, although γδ T cells can express markers of exhaustion, it seems that they are able to maintain their anti-tumor effector function.

The potential resistance to TME-mediated exhaustion in comparison to αβ T cells in certain circumstances, summarized with reported T cell functional states in Fig. 3, showcases γδ T cell biology as a very appealing platform to develop or improve current cancer immunotherapies that are not able to provide sustained tumor-eliminating capabilities (Fig. 3). Unambiguous success using γδ T cell-based immunotherapies has been infrequent. To date, most efforts have revolved around Vδ2 γδ T cells due to their known cytotoxic features. With the emerging data in which anti-tumoral $V\delta2^-$ subsets have been identified in different cancer types[52,53,55,108], expanding therapeutic approaches that tap the potential of $V\delta2^-$ T cells represents an opportunity for the future.

Adoptive γδ T cell therapies

The initial clinical trials to leverage γδ T cells focused on adoptive transfer of Vδ2 cells, specifically Vγ9Vδ2 T cells[110], given their high prevalence in the blood. Overall, this approach has been safe but also showed modest clinical efficacy. These studies included a range of different cell-expansion protocols and delivery methods (with or without cytokines or other stimulating drugs), summarized in Table 1.

One of the earliest demonstrations of using adoptively transferred Vγ9Vδ2 T cells for the treatment of renal cell carcinoma was reported in 2007 (refs. 111,112). In this first open-label pilot study, peripheral blood nuclear cells were isolated from patients, treated with 2-methyl -3-butenyl-1-pyrophosphate antigen and recombinant human IL-2 to selectively expand Vγ9Vδ2 T cells and subsequently adoptively transferred into seven patients with cytokine-refractory metastatic renal cell carcinoma. Following adoptive transfer of Vγ9Vδ2 T cells, these cells were detected in systemic circulation, although at low levels. The treatment was tolerated without notable toxicity but also without clear efficacy.

In 2008, an autologous Vγ9Vδ2 T cell product (Innacell γδ) was studied in combination with a low dose of IL-2 in patients with metastatic renal cell carcinoma[113]. Ten patients were treated, and immunophenotyping data demonstrated that IL-2 rescues Vγ9Vδ2 T cells following initial clearance. Disseminated intravascular coagulation was detected as a dose-limiting toxicity in one patient who received a dose of 8 billion cells. Other toxicities included gastrointestinal side effects, fatigue, fevers, rigors, hypotension and tachycardia. There were no responders, and the best outcome was stable disease in six patients.

In another phase 1 study, autologous zoledronate and IL-2-expanded γδ T cells were studied in patients with recurrent non-small cell lung cancer[114]. Ten patients were treated with adoptively transferred autologous γδ T cells 2 to 12 times every other week. No treatment-related adverse events or objective responses were seen. Plasma IFNγ was used as a biomarker, and there was measurable elevation of IFNγ in five of ten treated patients, who happened to be those who had stable disease as the best response.

Similar efforts were made in breast cancer in 2010. In a phase 1 study, zoledronate was used as a Vγ9Vδ2 T cell agonist and combined with low-dose IL-2 for treating advanced metastatic breast cancer[115]. The study demonstrated good tolerability and showed that the intervention promoted effector maturation of Vγ9Vδ2 T cells. Cancer antigen (CA)15-3 levels were reduced in patients who showed sustained levels of peripheral Vγ9Vδ2 T cells. The remainder of the patients who did not have detectable sustained Vγ9Vδ2 T cells in the periphery showed disease progression. In 2011, another trial of autologous Vγ9Vδ2 T cells in patients with metastatic solid tumors was performed[116]. The cell therapy was well tolerated, with only 7 out of 18 patients experiencing fevers that were manageable and resolved within 24 h. Of the three patients who continued their anti-cancer therapy that was not working before cell therapy, the authors saw two partial responses and one CR. While this experience was quite limited, these results provided the rationale for subsequent studies combining γδ T cells with chemotherapy. The authors also demonstrated using ^{111}In radiolabeling that adoptively transferred Vγ9Vδ2 T cells localize to lungs, liver and spleen within the first 24 h.

A more recent open-label single-arm phase 2 trial investigated zoledronate-expanded autologous Vγ9Vδ2 T cells for the treatment of refractory NSCLC. Of 25 treated patients, 16 patients completed the planned six infusions of γδ T cells[117]. Median progression-free survival (PFS) was 3.4 months, and median overall survival was 15.5 months, and there was only one objective partial response (4%). Severe adverse events developed in nine patients, with one patient developing pneumonitis related to Vγ9Vδ2 T cell infusion. One patient displayed reduced lesions in the lung, and lymphadenopathy was reduced substantially following the second infusion, but, unfortunately, the patient had disease progression due to new liver metastasis. Immunophenotyping revealed that Vγ9Vδ2 T cell number and percentage in peripheral blood nuclear cells gradually increased with each injection over the course of the treatment, and adoptively transferred Vγ9Vδ2 T cells displayed a $CD45RA^-CD27^-$ effector memory phenotype in the blood.

Combinations of γδ T cells with systemic therapy other than IL-2 in earlier disease settings have also been reported[118], such as adjuvant gemcitabine with autologous γδ T cell transfer in patients with curatively resected pancreatic cancer. Safety signals were consistent with expected toxicity arising from gemcitabine chemotherapy without severe adverse events related to the added γδ T cell therapy. However, relapse-free survival and overall survival of patients treated with the γδ–gemcitabine combination treatment were no different than in those treated with gemcitabine only. Serial infusions were correlated with

Fig. 3 | Immune checkpoint expression related to the function of tumor-infiltrating γδ T cells. a, Summary of immune checkpoint expression on tumor-infiltrating αβ T cells (left) and γδ T cells (right) with associated function and clinical relevance across different cancer types. Correlation with clinical benefit has been reported for both Vδ1 and Vδ2 T cells in patients with malignancies treated with immune checkpoint inhibition, such as the anti-PD-L1 antibody atezolizumab for urothelial tumors[53] or the anti-CTLA4 antibody ipilimumab in melanoma[106], respectively. The following references are cited in panel **a**: refs. 52,53,55,64,68,69,102–109. **b**, Vδ1 T cells in the TME generally express higher levels of PD-1 than Vδ2 T cells but can retain equal or even higher levels of effector and memory function. CRC, colorectal cancer; FOXP3, forkhead box P3; freq, frequency; MM, multiple myeloma; MMRd, mismatch repair-deficient tumors; RCC, renal cell carcinoma; TRM, tissue-resident memory.

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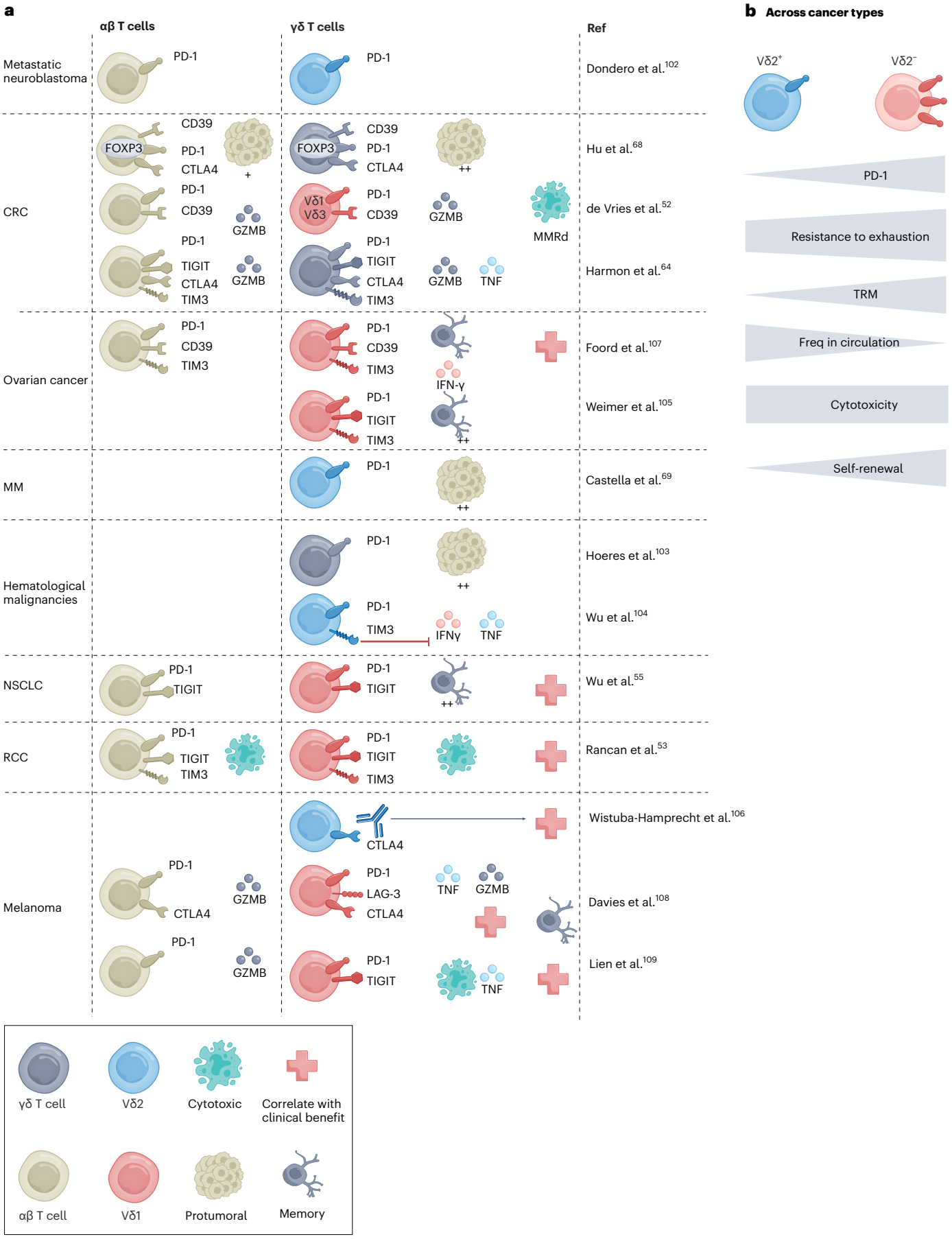

Table 1 | γδ T cell products under development

Drug name	Allogeneic vs autologous	Company	Indication	Current phase	Trial number
ADI-001	Allogeneic	Adicet Bio	Diffuse large B cell lymphoma, NHL	1	NCT04735471
INB-100	Allogeneic	IN8bio	AML	1	NCT03533816
INB-200	Autologous	IN8bio	Brain cancer (malignant glioma; anaplastic astrocytoma and glioblastoma)	1/2	NCT05664243
OmnImmune	Allogeneic	TC BioPharm	AML	1/2	NCT05358808
ICT01	N/A	ImCheck	Multiple solid tumors	1/2	NCT05307874 NCT04243499
GDX012	Allogeneic	Takeda Pharmaceutical	AML	1/2	NCT05886491
INB-400	Autologous	IN8bio	Brain cancer (malignant glioma; anaplastic astrocytoma and glioblastoma)	IND	NCT04165941
ADI-270	Allogeneic	Adicet Bio	Solid tumors	Preclinical	N/A
GDT201	Undisclosed	Gadeta	Colorectal cancer	Preclinical	N/A
INB-300	Undisclosed	IN8bio	Solid tumors	Preclinical	N/A

IND, Investigational New Drug application; N/A, not available.

greater levels of γδ T cells detected in the circulation, and those who had higher amounts of detected peripheral γδ T cells experienced a trend toward more favorable relapse-free survival and overall survival.

Genetically modified γδ T cells

γδ T cells have also been modified in an effort to enhance their anti-tumor efficacy. An autologous γδ T cell product has been genetically modified with *an MGMT* chemotherapy-resistance gene (auto-drug resistant immunotherapy)[119] and was studied in a phase 1 trial in patients with glioblastoma after surgical recession and 6 weeks of induction with concurrent temozolomide and radiation. During the subsequent six cycles of maintenance temozolomide, patients also received INB-200, a γδ T cell therapy.

Of the 18 patients that enrolled, 4 autologous γδ T cell products failed manufacturing. There were three deaths related to disease progression and three deaths that were unrelated to treatment. There were no serious toxicities attributable to the cell therapy; however, there were also no responders, and the PFS ranged between 7.1 and 22.8 months and ongoing. Temozolomide is known to have lymphosuppressive effects, and, indeed, immunophenotyping studies showed that there was significant reduction of T cells, B cells and NK cells to low normal or below-normal levels. Tumor biopsies comparing baseline and 148 d after infusion of INB-200 with concurrent temozolomide demonstrated the presence of tumor-infiltrating γδ T cells, which indicated that the engineered *MGMT* resistance gene was functional. A phase 1b–2 study (NCT05664243) of allogeneic or autologous γδ T cells in combination with maintenance temozolomide is ongoing for patients with recurrent or newly diagnosed glioblastoma.

Allogeneic γδ T cell therapies

Beyond autologous γδ T cells, there have also been efforts to engineer off-the-shelf allogeneic γδ T cells for more acute conditions that require prompt therapeutic interventions, previously reviewed by others[120]. OmnImmune (TC BioPharm) is an unmodified allogeneic Vγ9Vδ2 γδ T cell product being studied in patients with AML. In a phase 1 trial (NCT03790072), adoptive transfer of γδ T cells from haploidentical donors was used to treat patients with refractory or relapsed AML.

Seven patients with bone marrow blasts >5% received donor-derived T cell products[121]. There were in total four serious treatment-emergent adverse events including febrile neutropenia, *Clostridium difficile* colitis, gastrointestinal hemorrhage and one incident of grade 5 pneumonia. Of the seven treated patients, only four lived long enough to have a 28-day bone marrow evaluation, of which one achieved a complete response and one achieved a morphological leukemia-free state, one had stable disease and one showed no response. Redosing was performed for the three patients who experienced a complete response, a morphological leukemia-free state and stable disease. Despite redosing, all the patients had disease progression in the marrow by day 100. This outcome again highlights the lack of persistence that has yet to be overcome.

An allogeneic γδ T cell (DeltaEx Allo) is being tested in patients with acute leukemia after haploidentical hematopoietic stem cell transplant. This is a single ascending-dose 3 + 3 design, with two dose cohorts at 10^6 cells per kg and 3×10^6 cells per kg as the two treatment arms (NCT03533816).

There were no dose-limiting toxicities, cytokine-release syndrome, immune effector cell-associated neurotoxicity syndrome or graft-versus-host disease grade 3 or higher. Of the four patients in cohort 1, there were two of three patients who are still on study over 2 years who remain in morphological complete remission, with another third patient approaching the 2-year follow-up including one patient surpassing 3 years without leukemic relapse. The fourth study patient also has a complete remission and is ongoing at month 7. Three patients have been treated with dose level 2, and all are in remission with ongoing follow-up. In vivo persistence and expansion of γδ T cells were measured, and there was a dose-dependent increase of circulating γδ T cells at days 60 through 180 for DeltaEx Allo γδ T cell-treated patients, which is promising. At the 2023 American Society of Hematology conference, updated data showed that 100% of patients (ten out of ten) remained in morphological complete remission and are still on study, including six patients that have persistence of more than a year[122,123]. Impressive in vivo persistence and expansion of γδ T cells were detected for up to 1 year of follow-up. A phase 1b expansion of ten patients at dose level 2 is underway to validate this result.

Chimeric antigen receptor-modified γδ T cells

An allogeneic γδ chimeric antigen receptor (CAR) T cell therapy (ADI-001) is being developed as a potential treatment for relapsed or refractory B cell non-Hodgkin's lymphoma (NHL) (B-NHL)[124]. ADI-001 targets lymphoma cells via an anti-CD20 CAR and γδ innate and T cell endogenous cytotoxic receptors. Vδ1 cells were expanded from peripheral blood of normal healthy donors and engineered with a second-generation CAR construct (4-1BBz) to create an allogeneic CAR T cell product. ADI-001 was studied in the first-in-human study GLEAN in a 3 + 3 dose-escalation design[125]. Four dose cohorts were studied, from 30 million cells up to 1 billion cells. Patients underwent

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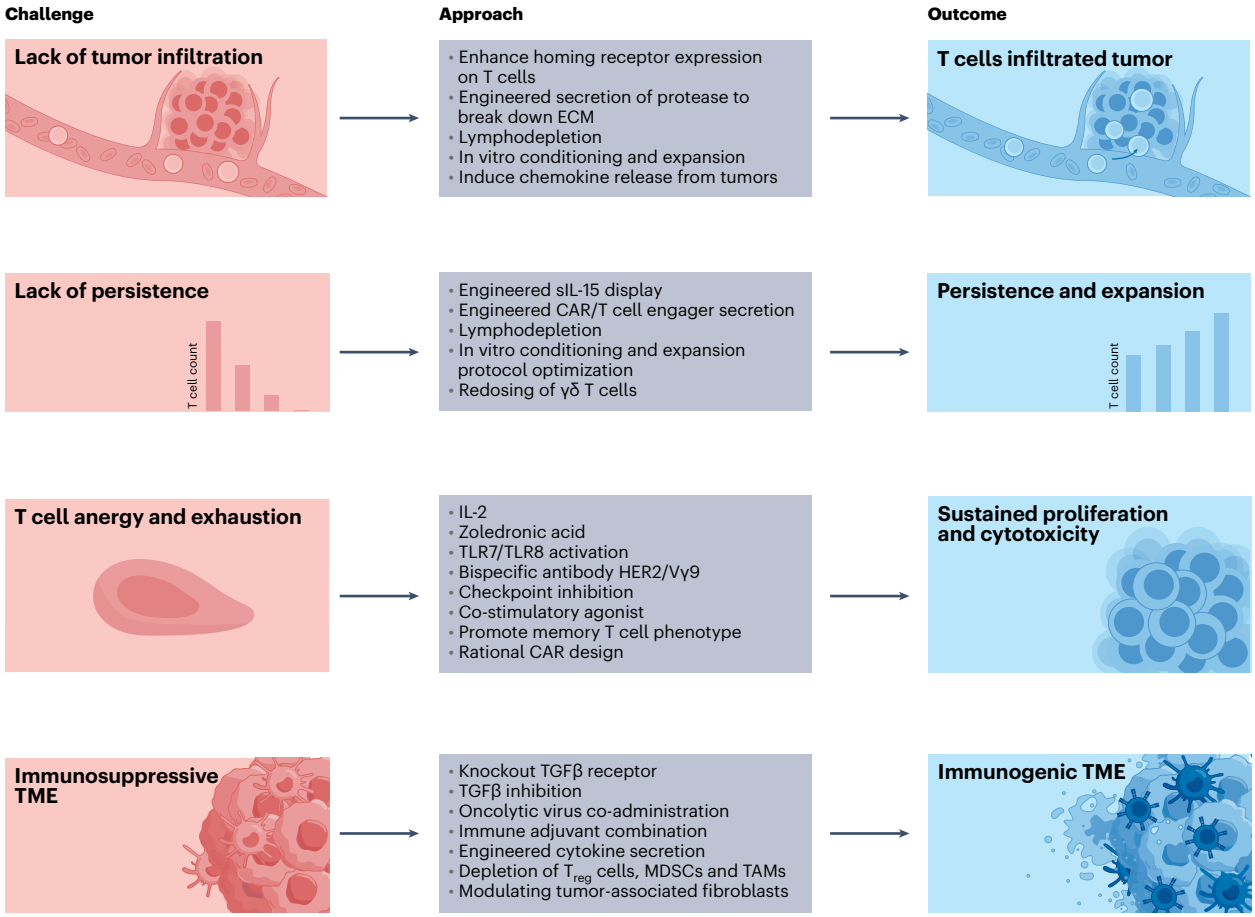

Fig. 4 | Approaches to current challenges and desired outcomes. γδ T cell studies have yielded mixed results and may be explained by lack of tumor infiltration, lack of persistence, anergy and exhaustion of cells and an immunosuppressive TME (left). There are numerous approaches being explored to overcome these limitations, with the goal of having increased T cell-infiltrated tumor tissue, improved cell persistence and in situ expansion, sustained proliferation and cytotoxicity and a more immunogenic TME (middle and right). ECM, extracellular matrix; HER2, human epidermal growth factor receptor 2; sIL-15, soluble IL-15; TAMs, tumor-associated macrophages; TGFβ, transforming growth factor-β.

lymphodepletion under a standard regimen or an enhanced regimen before the infusion of ADI-001. Thus far, 24 heavily pretreated patients have been enrolled, with 12 of these patients having received prior anti-CD19 CAR T therapies. Notably, 8 of these 12 patients had progressed within 6 months from the date of autologous CD19 CAR T cell therapy.

ADI-001 was reasonably tolerated without any serious incidence of cytokine-release syndrome or immune effector cell-associated neurotoxicity syndrome, which are toxicities often seen with αβ T cell-based CAR T therapy. Starting at dose level 3, there was one patient with cytokine-release syndrome and two patients with infection. However, no dose-limiting toxicities have been observed. In the overall population, ADI-001 achieved a 71% objective response rate (ORR) and a 63% complete response rate across all dose levels in patients with a median of four prior lines of therapy. At the highest dose level 4, which is also the recommended phase 2 dose, of eight patients, there was a 75% ORR, including a 62.5% complete response rate. However, the durability of response is inadequate, as only 25% of patients remain in complete response at 6 months. In three patients after CAR T therapy, ADI-001 achieved 100% ORR and 67% complete response, with just one of three patients still in complete response at 6 months. The persistency of γδ CAR T cells after infusion was measured by flow cytometry, and there was a dose-responsive relationship of higher levels of detected CAR^+ Vδ1 cells per microliter with each subsequent increased dose level. ADI-001 CAR transgene copies were also measured, and, at dose level 4, which was 1 billion cells infused, the transgene copy number was maintained throughout day 28. Longer-term persistence has yet to be established.

One approach to overcome this persistence obstacle is the addition of cytokine production into the CAR T cells. Second-generation CD20 γδ T cells with the 4-1BB co-stimulatory domain with third-generation CD20 γδ T cells engineered with the 4-1BB co-stimulatory domain and IL-15 secretion drastically improved anti-tumor activity in preclinical models[126]. This will be tested in a phase 1 trial that will explore the maximum tolerated dose of ADI-001 alone or in combination with IL-2, which may lead to better persistence. The durability of efficacy still falls below the autologous CD19 CAR T standard of care.

Bispecific antibodies

A bispecific antibody that binds to CD16A on NK cells and CD30 on lymphoma cells (AFM13) can activate both NK cells and γδ T cells to target CD30-positive lymphomas[127]. Treatment with AFM13 was well tolerated: only two treatment-associated serious adverse events were reported, and all serious adverse events resolved completely. With an ORR of 16.7% and a 12-month PFS of 12.6% (95% confidence interval, 3.2–28.9), treatment efficacy of AFM13 monotherapy in all evaluable patients was modest.

Activation of endogenous γδ T cells

An anti-BTN3A monoclonal antibody (ICT01) that was designed to induce specific activation of Vγ9δ2 T cells[128] has been studied in combination with IL-2 in a phase 1 study in patients with colorectal, ovarian,

prostate and pancreatic cancer[129]. The combination of ICT01 with low-dose subcutaneous IL-2 has been safe thus far. Of 18 patients, the best response was stable disease up to 24 weeks. The combination of ICT01 with low-dose subcutaneous IL-2 induced robust and selective expansion of γ9δ2 T cells above baseline across all cohorts, peaking at days 8–15 of expansion, recurring in subsequent cycles. Expansion of γ9δ2 T cells was also associated with increases in the Ki67 proliferation marker and increased immune checkpoint expression.

Translational challenges

Expansion and manufacturing

γδ T cells occur at low physiological frequency in the periphery and are difficult to expand ex vivo. Phosphoantigen-mediated activation of γδ T cells hinges on their intracellular binding to members of the BTN protein family, which are then able to activate the γδ TCR[47]. Within the γδ T cell, phosphoantigen recognition leads to phosphorylation events, calcium influx and the activation of various kinases (for example, MAPK, PI3K–AKT), which collectively orchestrate cellular responses. Phosphoantigen–BTN activation of γδ T cells initiates signaling cascades, including the activation of protein kinases and phosphatases, ultimately leading to T cell activation.

The Vγ9Vδ2 subset of γδ T cells, found predominantly in human peripheral blood, is highly responsive to phosphoantigens. While MHC-unrestricted BTN–phosphoantigen recognition is a pivotal trigger, optimal activation and expansion of γδ T cells necessitate co-stimulation through co-receptors, such as NKG2D, and cytokine support. IL-2 and IL-15, for instance, provide essential signals for γδ T cell proliferation, survival and effector functions[11]. Challenges in the field, summarized in Fig. 4, include optimizing culture conditions, minimizing exhaustion of expanded cells and enhancing homing to target tissues (Fig. 4).

Persistence

The allogeneic ADI-001 therapy showed initial complete response and ORR against NHL, but with most patients relapsing in less than 6 months, which makes it less attractive than approved CD19 CAR T products. Persistence represents another major challenge for innate-like immune cellular therapies like NK and γδ T cells; further cellular engineering and innovative combinations would be needed to overcome this barrier. For example, off-the-shelf Vδ1 γδ T cells engineered with a glypican 3 (GPC-3)-specific CAR and soluble IL-15 display robust anti-tumor efficacy against hepatocellular carcinoma[130]. Tumor control is dependent on the long-term ability of Vδ2 T cells to effectively discern and target malignant cells, orchestrate robust immune responses against neoplastic tissues and sustain the production of cytotoxic effector molecules and immunomodulatory cytokines. This would also involve mechanisms to uphold cellular activation, preserve effector functions and sustain effector cytotoxicity when confronted with tumor-associated antigens.

Extended and repeated stimulation can drive functional exhaustion of Vδ2 T cells. Strategies aimed at mitigating or forestalling this exhaustion are paramount for the preservation of therapeutic efficacy. Genetic modifications to counteract exhaustion-associated signaling pathways or the integration of combination therapies are strategies being employed to address this challenge.

Protection from immunoregulatory mechanisms

Tumor cells adeptly construct an immunosuppressive microenvironment that may neutralize the activity of therapeutic immune cells such as Vδ2 T cells (Fig. 4). Long-term persistence of γδ T cell function necessitates the implementation of strategies to shield these cells from immunoregulatory elements, either by combination with immune checkpoint inhibitors or by genetically engineering γδ T cells to augment cellular activity and resilience to these inhibitory influences. The overarching goal is to conceive therapeutic regimens that not only elicit initial tumor control but also provide patients with durable, sustained and protracted therapeutic responses to effectively combat malignancies.

Conclusions

While immunotherapy approaches including immune checkpoint blockade and CAR T cells have historically focused on αβ T cells for effector function, it is now clear that γδ T cells can also be leveraged for cancer rejection. Indeed, γδ T cells have advantages over αβ T cells in a growing number of settings. In many cases, αβ T cell responses are susceptible to tumor escape by MHC loss in tumor cells, which renders the treatments futile[131]. Alternative immune checkpoint upregulation can also hamper anti-tumor αβ responses. After prolonged exposure to tumor antigens, lack of persistence and metabolic burden can also limit their function. On the other side of the balance, overwhelming αβ T cell activation and amplification can also lead to severe adverse effects.

γδ T cell antigen recognition is MHC independent, which not only overcomes MHC loss but also has huge implications for the feasibility of γδ T cells as off-the-shelf therapeutic products for allogeneic treatment of cancer. They seem more resistant to immune checkpoint-mediated dysfunction than their αβ counterparts as well as less prone to excessive activation, as shown in the currently available clinical data. Their broad, and still in ongoing discovery, tumor ligand reactivity and innate receptor spectrum might render them prepared for more sensitive sensing of emerging malignancy. They are also able to sustain and amplify anti-tumor responses by cytokine secretion and interaction with other tumor-infiltrating immune cells like neutrophils, dendritic cells, B cells and even αβ T cells.

After years of great focus on the Vδ2 subset, recent evidence in multiple tumors showed associations of other γδ subsets with increased patient survival or clinical benefit, posing the consideration of the more diverse, yet unexplored, Vδ2$^-$ subsets as serious complementary candidates for bringing qualitative improvement to current γδ-based immunotherapies[52,53,55,64]. Their advantageous tissue homing facilitates their enrichment upon oncogenic insults, and their accentuated resistance to exhaustion renders them more responsive to immunostimulatory signals. The metabolic homeostasis of tumor-infiltrating cytotoxic γδ T cells seems to be less prone to abrupt alterations, which likely translates into more sustained and long-term surveillance, reducing the theoretical effector/target ratio needed for effective tumor clearance. Importantly, they have been shown to significantly contribute to clearance of highly mutated tumors, which decouples their efficacy from the need for the presence of immunogenic tumor neoantigens found in αβ T cell tumor recognition[52,64,108]. All these are unique γδ features that seem very desirable for any cell therapy-based strategy.

The nature of γδ T cells, however, also poses challenges. They are largely outnumbered by αβ T cells in the body, which means subset-specific optimized ex vivo expansion protocols need to be tailored, and their ability to naturally generate effective memory is still in debate. A deeper understanding of tumor-induced stress surveillance and unambiguously anti-tumoral phenotypes is needed. This entails not only the discovery of yet-unknown ligands and engaging mechanisms but also additional studies in patients and relevant comparisons with healthy donors, owing to the suboptimal translatability of γδ research in non-human models. Dissecting the clinical importance of the γδ innate receptor repertoire could benefit not only cell therapies but also boost current antibody or small-molecule discovery processes by providing new and relevant targets. More high-dimensional studies that include relevant clinical information and refined γδ T cell annotation will enrich our knowledge on how γδ T cells impact the TME[96]. More intelligent and integrative approaches, such as the thriving field of spatial transcriptomics, constitute invaluable tools to overcome these difficulties and improve our knowledge on γδ T cells and will likely translate into important advances toward the establishment and recognition of the γδ T cell therapy field.

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