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ESMO IMMUNO-ONCOLOGY AND TECHNOLOGY

REVIEW

A GD (Gamma-Delta) type of cancel culture

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γδ T cells represent an 'unconventional' class of CD3+ lymphocytes with unique phenotypical and functional attributes that distinguishes them from their αβ T-cell receptor-expressing counterparts. Studies investigating the roles of γδ T cells in cancer have shown that these cells are indispensable for effective tumor control and their presence within the tumor may be of prognostic significance. Currently, there is significant interest in harnessing γδ T cells for cancer treatment, and research efforts have focused on the development of γδ T-cell-based strategies that are efficacious against cancer. Several therapeutic approaches using γδ T cells have been described, premised on the expansion of γδ T cells or γδ chimeric antigen receptor T therapy. The potential for broad, unbiased and 'off-the-shelf' applicability in cancer treatment, drives ongoing and future research and methodologies by which γδ T cells can be exploited for therapeutic use. In this review, we will briefly outline the characteristics of γδ T cells and describe how these work within and promote proper functioning of the cancer-immunity cycle. Additionally, we will introduce strategies that are less commonly described and may potentially be more efficacious than other types of therapy. Our discussion will expand upon presently known applications and even highlight the versatility of this immune subset as cancer therapeutics. γδ T-cell-based treatment is an emerging strategy and should be considered for cancelling cancer.

Key words: tumor-infiltrating lymphocytes (TIL), T-cell product manufacturing/scalability, T-cell therapy for patients with solid tumors

INTRODUCTION

The discovery of the immune system's involvement in cancer surveillance decades ago sparked a new direction for cancer therapeutics. Since then, much research efforts in cancer have focused on dissecting the mechanisms of tumor immunological surveillance and developing strategies to restore or promote immune cell function against malignant cells. Immune-based therapies, or immunotherapy, has emerged as the fourth pillar of cancer treatment (after surgery, chemotherapy and radiotherapy), with an increasing number of patients receiving immunotherapeutic agents as part of their treatment regimen for both early- and late-stage disease. Cellular therapy is a category under the immunotherapy umbrella and typically involves the use and tweaking of patient (or healthy individuals', in some cases) immune cells to eliminate cancer cells. To date, cellular therapies based on different types of immune cells including dendritic cells (DCs), natural killer cells (NKs) and T cells have been reported.[1] Notably, among T cells, the γδ T-cell receptor (TCR)-expressing subclass of lymphocytes (γδ T cells) have garnered considerable attention in recent years due to their roles in tumor inhibition and the unique attributes possessed by these cells that not only distinguishes them from αβ T cells but may also confer superiority for current and future immunotherapy approaches. Several γδ T-cell-based treatment strategies have been described. These include methods for subset-specific expansion of γδ T cells from different sources, genetic engineering or even harnessing the by-products of γδ T-cell culture for therapeutic use.[2-9] Importantly, expanded γδ T cells and cell-derived molecules demonstrate antitumor activity across different cancers in preclinical tests, and showed considerable efficacy and tolerance in clinical studies.[2-6,10,11] With the plethora of information surrounding γδ T cells and growing interest in exploiting this immune subset for cancer treatment, it is necessary to reassess current understanding of γδ T cells and progress in therapeutic advancements as this will guide future research for the development of γδ T-cell-based therapies with enhanced potency and efficacy. This review aims to provide a concise summary of the therapeutic applications of γδ T cells in cancer. In particular,

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the antitumor role of γδ T cells will be described with reference to the cancer-immunity cycle to illustrate how these cells are suited for use in immunotherapy. Further, current advances in γδ T-cell-based treatment and future prospects of this therapeutic strategy will be discussed.

THE MULTIFACETED ROLE OF γδ T CELLS IN THE CANCER-IMMUNITY CYCLE

Tumor control and eradication is not elicited by a single class of immune cells.[12] Rather, an intricate interplay between different cell types of the immune system is necessary for effective antitumor responses to be induced—a concept illustrated by the cancer-immunity cycle.[12] This cycle describes a series of sequential and highly regulated steps required for optimal immune responses.[12] Briefly, cancer antigens released by dead or dying cancer cells are captured by antigen-presenting cells (APCs) such as DCs and presented to naïve T cells for priming and activation.[12] Activated T cells then traffic to and infiltrate the tumor bed where they recognize tumor cells and elicit cytotoxic responses for tumor control or eradication.[12] A noteworthy aspect of the cancer-immunity cycle is the indispensable roles played by both innate and adaptive cells for effective tumor clearance.[12] Interestingly, γδ T cells possess phenotypic and functional attributes of both innate and adaptive immunity.[5,10] This feature uniquely positions γδ T cells at the crossroads of both arms of immunity and even inspired the representation of these cells as 'adaptate' immune cells; 'adaptate' describes the integration of innate and adaptive traits in γδ T-cell biology.[13] It may be within reason to suggest that γδ T cells can fulfil the requirements of the cancer-immunity cycle at several steps and allow proper functioning of the cycle for potent tumor killing to be effected. In the following sections, we will describe the 'adaptate' nature of γδ T cells and their multifaceted roles in the context of the cancer-immunity cycle.

Cancer-immunity cycle: recognition of cancer cells

The TCR represents a natural and major mechanism for tumor cell recognition by T cells. In αβ T cells, antigen recognition by the TCR is dependent on prior processing of protein antigens into short, linear peptides and presentation on major histocompatibility complex (MHC) molecules.[5,14,15] γδ T cells, by contrast, adopt different mechanisms for ligand recognition: these cells are capable of detecting cognate antigens in their native forms without the need for antigen processing.[5,13,14] Ligands are also recognized independently of MHC molecules, and γδ T cells are hence not restricted by these.[5,14] The antigenic repertoire of γδ T cells even differs from their αβ TCR-expressing counterparts; instead of neoantigens, γδ T cells can be stimulated by a diverse range of molecules such as phosphoantigens, MHC-like proteins of the CD1 family including CD1d and intracellular proteins like Annexin A2 that are translocated to the cell surface upon cellular transformation.[5,13,14,16-18] Generally, the ligands for γδ TCRs are believed to be signals of cellular stress.[5,14,16-18] The ability of γδ T cells to recognize antigens without the requirement for antigen processing and/or MHC molecules and the unique antigenic repertoire of these cells are noteworthy as such features may confer major advantages for cancer immunosurveillance. Studies have shown that tumor cells can avoid immune detection by downregulating MHC molecules or fail to process antigens due to impaired intracellular antigen-processing machinery.[19-23] Similarly, cancer cells can be programmed to express poorly immunogenic antigens and reduce overall tumor neoantigen burden through the process of immunoediting.[24,25] Immune-evading strategies such as these may result in lowered expression of antigen-loaded recognition complexes on the surface of cancer cells such that αβ T cells will no longer be able to target these tumor cells, causing cytotoxic effector responses driven by this immune subset to be severely dampened. In comparison, by virtue of their unique modes of antigen recognition, γδ T cells may be less affected by defects in ligand presentation and maintain the ability to identify and kill cancer cells.

Apart from the TCR, γδ T-cell recognition of tumor cells can also be mediated by NK receptors (NKR).[5,14,26,27] γδ T cells have been shown to express various types of NKRs including, but not limited to, NKG2D and DNAM-1, and this feature allows γδ T cells to recognize ligands that are typically sensed by NK cells.[5,14,26,27] For example, γδ T cells can be stimulated by MHC class I chain-related proteins A and B (MICA and MICB), which are known ligands of the NKG2D receptor.[5,14,26] Importantly, MICA/B proteins, along with other NKR ligands, are commonly up-regulated on the surface of cancer cells[28-30] and these molecules may be sensed by γδ T cells through their NKRs and trigger γδ T-cell-mediated antitumor responses. It may be worth noting that the ligand specificities of NKRs largely differ from those of the TCR, and hence γδ T cells—by virtue of their expression of both the TCR and NKRs—are likely able to recognize a broad range of antigens on cancer cells.[5,13,14] The wide repertoire of cell surface receptors possibly also permit simultaneous detection of different ligands on cancer cells, thereby allowing γδ T cells to recognize tumor cells efficiently. Of note, αβ T cells have also been reported to express NKG2D receptors but the role of this molecule in αβ T-cell activation may be contentious as some studies showed that ligation of NKG2D receptor induced αβ T-cell-directed target cell lysis while others instead described a lack of response when CD8+ T cells were treated with NKG2D ligands.[26,31] NK cells, on the other hand, may share the repertoire of NKR ligands typically detected by γδ T cells, but recognition of TCR antigens by these innate immune cells is unlikely. It may also be reasonable to suggest that the presence of two distinct mechanisms for tumor sensing allows γδ T cells to prevent tumor escape caused by pathways such as lowered antigen expression or antigen loss. Taken together, the TCR and NKRs represent different 'layers' of the tumor recognition machinery in γδ T cells, and of which may synergize to form a sophisticated, 'two-pronged' strategy for effective tumor detection and subsequent clearance.

Cancer-immunity cycle: killing of cancer cells

Upon antigen encounter, γδ T cells can elicit a myriad of responses for overall clearance of tumor cells.[5,14] The effector functions of γδ T cells can be broadly categorized into 'direct' mechanisms consisting of cytokine and/or effector molecule secretion, death receptor engagement and antibody-dependent cellular cytotoxicity (ADCC), and 'indirect' effects where γδ T cells modulate the functions of other immune subsets.[5,14] *In vitro* studies in cancer have shown that upon stimulation, γδ T cells up-regulate the expression of type 1 (T_H1) and type 2 (T_H2) cytokines such as interferon (IFN)-γ and interleukin (IL)-10, respectively, death receptor ligands such as FasL and TRAIL, and granzyme B.[3,4,11,26,27,32-37] Importantly, these changes are coupled with cancer cell lysis, and are observed in different types of cancer.[3,4,11,27,32-37] Further investigation into the killing mechanisms revealed that γδ T-cell-mediated eradication of tumor cells may be mainly driven by the perforin—granzyme axis as the inhibition of this pathway abrogated tumor-killing effects more significantly than the blockade of other pathways.[38-40]

Given the roles of both the TCR and NKRs in cancer cell recognition (as described above), it is only to be expected that these receptors can also trigger the tumor-killing mechanisms of γδ T cells. Indeed, the significance of the TCR and NKRs in tumor eradication was demonstrated in receptor-blocking assays, in which inhibition of both receptors was found to completely abrogate tumor cell lysis.[38] Notably, single blockade of specific NKRs affected tumor cell kill to a limited extent whereas γδ TCR inhibition resulted in near-complete loss of target cell lysis.[39-41] Such variation in impact size suggests that the γδ TCR likely functions as the major determinant of γδ T-cell activation while NKRs act as costimulatory molecules.[5,38,40,41] Despite the differences, there is undeniable involvement of different receptors in γδ T-cell-mediated tumor killing.

It may be worth noting that the expression and role of NKRs in γδ T-cell physiology is one of the innate-like characteristics exhibited by these cells.[5] The presence and use of innate receptors for tumor sensing possibly allows γδ T cells to elicit rapid responses against a wide spectrum of ligands without the need for prior antigen exposure.[14] Perhaps the faster response rate is additionally supported by the unique modes of γδ TCR antigen recognition (i.e. lack of neoantigen and MHC restriction), and the broad antigenic repertoire conferred by the two types of receptors. The ligand diversity may even endow γδ T cells with the ability to target tumors in an antigen-agnostic fashion. In contrast, the initial response time of αβ T cells may be slower given that these cells require antigen presensitization in order to be activated.[14]

Cancer-immunity cycle: antigen presentation and immunomodulatory roles of γδ T cells

In addition to direct killing of tumor cells, γδ T cells can induce antitumor activity through indirect mechanisms. This involves regulating the function of other immune cells, including CD8+ T cells, DCs and even B cells.[5,11,42-44] In the context of CD8+ T cells, studies by Xu et al. and Capsomidis et al. showed that the expression of immunostimulatory molecules such as CD86, CD69 and HLA class II DR molecule/MHC-II were up-regulated in γδ T cells following stimulation, suggesting a possible role for these immune effectors as APCs.[11,45] Indeed, activated γδ T cells demonstrated the ability to process and present antigens to CD8+ αβ T cells, and also induced the proliferation of both CD4+ and CD8+ T cells.[42,45,46] Additionally, γδ T cells triggered the differentiation and proliferation of naïve αβ T cells.[42]

γδ T cells can also stimulate DCs.[43,47] Following culture with γδ T cells, DCs were found to express increased levels of costimulatory molecules CD86 and MHC-I.[43,47] These DCs were able to take up antigens, and could induce the proliferation of immune cells including αβ T cells.[43] Further investigation into the mechanisms of γδ T-cell-mediated activation of DCs revealed that IFN-γ and tumor necrosis factor-α secreted by stimulated γδ T cells play crucial roles in inducing DCs, as the blockade of either molecule suppressed the expression of costimulatory factors in DCs.[43] Strikingly, γδ T cells and DCs also demonstrate reciprocal activation and this likely occurs in a cell-to-cell contact-dependent fashion.[43,47]

In B cells, Bansal et al. and Rampoldi et al. reported that the levels of activation markers such as CD25 and CD69 and costimulatory markers such as CD40 and CD86 were up-regulated in B cells that were co-cultured with γδ T cells.[44,48] Under the same co-culture settings, γδ T cells also showed increased expression of inducible T-Cell costimulator—a marker of follicular T helper cells (T_{FH})—along with OX40, CD25, CD86 and HLA class II DR molecule, indicating that γδ T cells likely function as T_{FH} cells to induce B cells.[44] Such potential T_{FH} role of γδ T cells is in fact corroborated by the finding of significantly enhanced levels of immunoglobulin (Ig)G, IgM and IgA antibodies in γδ T-cell/B-cell co-cultures.[44] Taken together, these findings collectively suggest cross-talk between the two types of immune cells, and γδ T cells possess the ability to activate and promote antibody production in B cells.

The ability of γδ T cells to modulate the functions of other immune cells may have significant implications in cancer immunity. It is perhaps within reason to suggest that αβ T cells activated by γδ-APCs are potentially able to carry out antitumor activities and this, together with the known tumor-killing functions of γδ T cells, can result in better tumor control. On a similar note, DCs stimulated by γδ T cells possibly also induce cytotoxic αβ T cells that target cancer cells. However, in the case of γδ T-cell-activated B cells, antibodies produced by these B cells may exert antitumor effects through ADCC and perhaps other mechanisms.[49] It is likely conceivable that γδ T cells function as a 'signaling hub' wherein γδ T cells receive and integrate signals from the microenvironment, and 'transmit' signals to other innate and/or adaptive immune cells. In essence, it is undoubtable that γδ T cells play multiple roles in cancer immunity, inducing widespread effects that promote immunosurveillance and enhance tumor eradication.

Cancer-immunity cycle: trafficking to tumors

γδ T cells can be classified based on the TCR δ chain, and four main structural subsets—Vδ1, Vδ2, Vδ3 and Vδ5—have been reported to date.[5,13,14] Notably, different γδ T-cell subsets localize in distinct regions of the body.[5,14] In humans, Vδ1 cells are commonly found in the epithelia, spleen and liver while majority of γδ T cells in the peripheral blood (PB) is represented by Vδ2 cells (in particular, Vδ2Vγ9).[5,14] Vδ3 cells, on the other hand, reside in the gut epithelium and liver.[5,14] Such natural tissue tropism of γδ T cells is often thought to be essential for expanding immune responses to tissues that are not well served by B or αβ T cells.[5,13,14] The ability of γδ T cells to home to specific tissues may be attributed to the expression of chemokine receptor(s) and 'homing' factors unique to a tissue, on the surface of these cells.[50,51] For instance, intrahepatic Vδ1 cells express liver-associated receptors CXCR3 and CXCR6 as well as the tissue tropism molecule CD69.[50] In contrast, γδ T cells residing in the skin do not express these molecules but have high levels of CCR8 and the skin-homing marker cutaneous lymphocyte-associated antigen.[51] The homing behavior of γδ T cells suggests an intrinsic ability to traffic to tissues, and this is important in the context of adoptive T-cell transfer therapy as the success of such treatments is, at least in part, dependent on the ability of effector cells to traffic to the tumor compartment.[52] Additionally, the tissue specificity of γδ T cells suggests that effector responses can be limited to the target tissue such that off-target events may be minimized.

Overall, γδ T cells play diverse and intricate roles in cancer immunity and may even harbor the ability to potentiate the cancer-immunity cycle. It may be worth noting that the (i) tumor neoantigen- and MHC-independent patterns of antigen recognition, (ii) 'additional' immunomodulatory functions and (iii) natural tissue tropism are likely features unique to γδ T cells, given that the same characteristics have not been described in other types of cytotoxic immune cells including CD8+ αβ T cells. These attributes may confer γδ T cells the capacity to bypass the mechanisms commonly used by tumor cells to sabotage αβ T-cell-driven immunosurveillance, such that γδ T cells remain functional and promote optimal functioning of the cancer-immunity cycle for potent tumor killing to be effected. Given these, it is hence without doubt that γδ T cells are attractive candidates for cancer immunotherapy and should be considered as current and future therapeutic agents for the disease. A schematic overview of the contribution of γδ T cells in the cancer-immunity cycle is shown in Figure 1.

γδ T-CELL-BASED THERAPY FOR CANCER

The multiple roles played by γδ T cells in antitumor immunity motivated the development of γδ T-cell-based strategies as part of the armamentarium for cancer treatment. Currently, several approaches for harnessing these immune cells for therapeutic use have been proposed. These include expanding PB γδ T cells, genetic modification, engineering γδ T-cell-specific engagers and combinatorial use of γδ T cells and immune checkpoint inhibitors (ICIs)—all of which have been described in detail elsewhere.[5,14] This review will briefly outline the aforementioned strategies while focusing on other therapeutic applications of γδ T cells that are currently underexplored but hold potential for development into effective and efficacious treatments for cancer. More specifically, the use of non-PB sources of γδ T cells and cell-independent therapeutic agents will be discussed as novel γδ T-cell-based tools for cancer therapy.

Alternative sources of γδ T cells: umbilical cord blood-derived γδ T cells

Acquiring sufficient tumor-targeting effector cells is central to adoptive cell therapy. A common theme in γδ T-cell expansion is the large-scale culture of γδ T cells originating from PB.[5,11,33-36,38-41,53] Perhaps the ease of access of PB as compared to other anatomical sites, and the availability of established protocols for expanding PB γδ T cells, can explain the appeal of immune cells from this compartment for cancer treatment. Notably, γδ T cells can also be found in epithelial sites including tumor tissues and umbilical cord blood (UCB).[3,4,6,32] Studies investigating UCB γδ T cells have shown that these cells are mostly naïve—a feature worth highlighting as it suggests that UCB γδ T cells may be less prone to exhaustion during the expansion process where activating cytokines is often used.[4,54] PB γδ T cells, in contrast, carry a memory phenotype.[6] There are also structural differences between UCB and PB γδ T cells: Vδ1 cells dominate the UCB population whereas Vδ2 (in particular, Vδ2Vγ9 cells) form the majority of PB γδ T cells.[4-6,14] In addition, UCB γδ T cells are more clonally diverse as compared to those of PB.[4] Successful attempts at expanding UCB γδ T cells have been reported, and some protocols even achieved 100-fold change expansions.[3,4,6] UCB γδ T cells can be expanded using anti-CD3 or anti-γδTCR antibodies, as described by Hur et al. and Ng et al. respectively, and in the presence of cytokines and feeder cells.[3,4] These culture techniques resulted in the expansion of non-Vδ2 cells (comprising both Vδ1+ and Vδ1− cells) while the effect on Vδ2 cells was almost negligible.[3,4] Interestingly, Berglund et al. reported Vδ2 expansion from UCB γδ T cells but not non-Vδ2 cells in a zoledronate and interleukin (IL)-2-based culture.[6] These observations collectively suggest that (i) different structural subsets can be expanded from UCB γδ T cells and (ii) subset-specific amplification can also be achieved. It should be noted that culture of selected subpopulations in the context of UCB γδ T cells has only been described for Vδ2 cells. On a side note, the aforementioned selectivity of zoledronate for Vδ2 cells has also been indicated for expansions using PB γδ T cells—this may be important as it suggests that protocols used for expanding PB γδ T cells may apply to UCB cells of the same.[33] Different protocols for amplifying PB-derived Vδ1 cells are currently available, such as a report by Wu et al. where phytohemagglutinin (PHA) + IL-7 treatment was shown to promote the proliferation of PB Vδ1 cells but not those expressing the Vδ2 TCR, or another study by Almeida et al. which

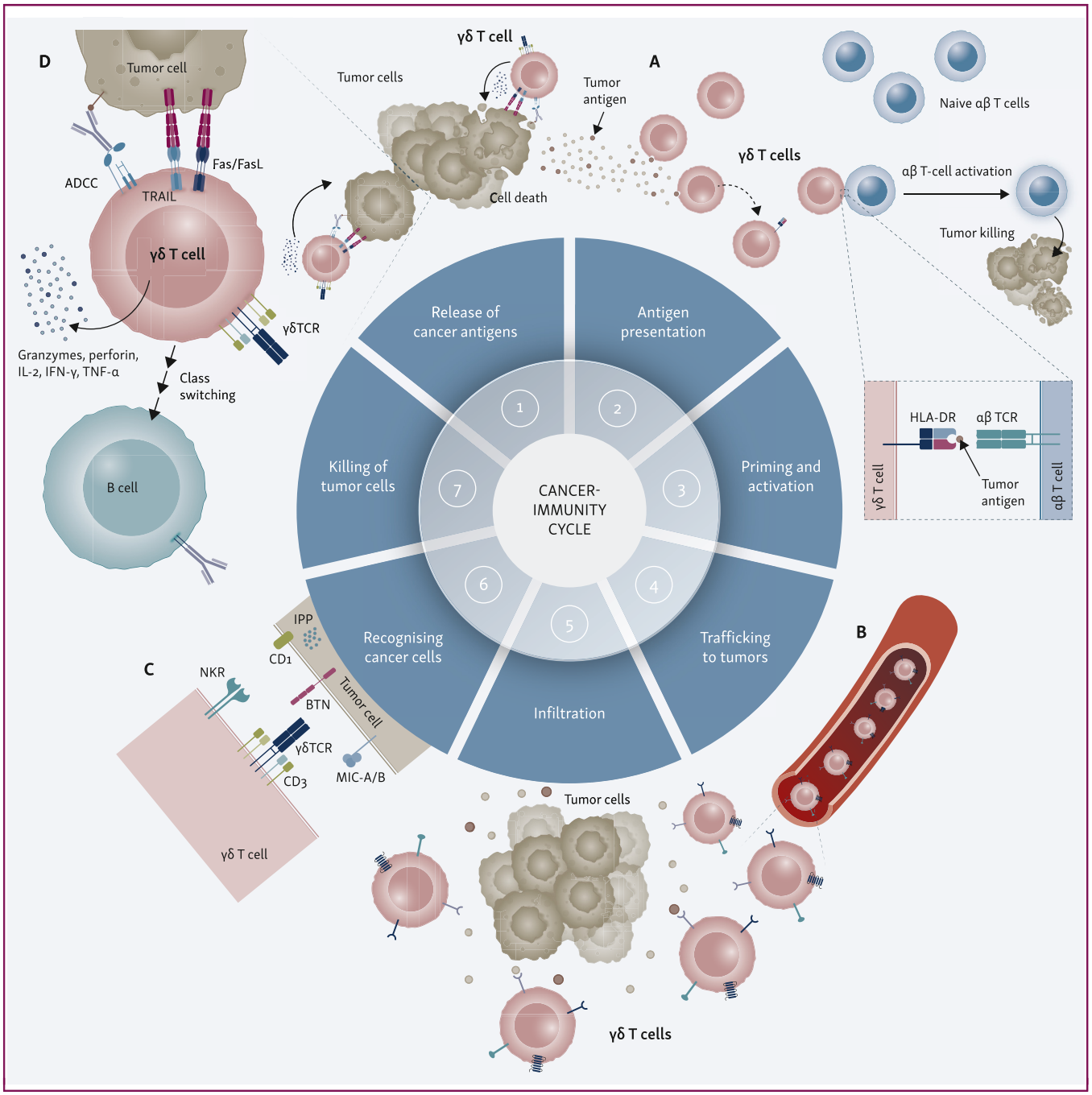

Figure 1. The multifaceted functions of γδ T cells compliment the cancer-immunity cycle. (A) γδ T lymphocytes function as APCs. Tumor-associated antigens that are released by apoptotic cancer cells are taken up by γδ T cells, processed and cross-presented to αβ T cells. These αβ T cells become activated and induce antitumor responses. (B) γδ T cells naturally home to tissues. γδ T cells express various chemokine receptors and tissue-associated molecules on their cell surface that underlies their tropism for specific anatomical regions. Such tissue-homing abilities may circumvent complications related to T_{eff} cell 'homing deficit' during immunotherapy wherein the expanded γδ T population can naturally traffic to the tumor site for efficient tumor eradication. (C) Unique mode of antigen recognition by γδ T cells. γδ cells interact with non-peptide ligands such as lipid or other stress-related molecules bound to non-classical, MHC-like molecules such as CD1 family proteins. (D) Direct and indirect mechanisms of γδ T-cell-mediated tumor killing. These cells can directly kill cancer cells via ADCC, death receptor engagement or secretion of cytotoxic molecules, or carry out T 'helper' functions and induce other immune cell types.
ADCC, antibody-dependent cellular cytotoxicity; APC, antigen-presenting cells; IFN, interferon; IL, interleukin; MHC, major histocompatibility complex; MICA/B, MHC class I chain-related proteins A and B; NKR, natural killer receptor; TCR, T-cell receptor; TNF, tumor necrosis factor.

described a two-step process for generating cytotoxic Delta One T cells (DOT) from PB γδ T cells.[34,35] Modified protocols for expanding DOT cells have also been reported, as described by Harmon et al.[55] Selective expansion of UCB Vδ1 cells is currently understudied; it may be worth attempting the amplification of this subset using strategies that were previously verified with PB cells, or perhaps new methods can be explored.

Apart from selectivity, another notable aspect of Berglund et al.'s study is expansion of the less-abundant Vδ2 subpopulation.[6] It is likely that other small subsets of UCB γδ T cells can also be amplified, although the success of

which may be dependent on the identification and use of stimulatory compounds that are both robust and subset specific.

Alternative sources of γδ T cells: intratumoral γδ T cells

The ability of some γδ T-cell subsets to naturally home to and reside in tissues suggests that these immune cells may also be found in solid tumors. Indeed, intratumoral γδ T cells have been identified in various cancer types like colon and gastric cancers, among others.[26,55-60] In gastric, kidney and breast cancers, studies have shown that Vδ1 cells form the majority of γδ T cells in the tumor.[26,55,58,59] While the origin of such intratumoral Vδ1 cells remains to be elucidated, it may be reasonable to speculate that these cells are derived from the immune cell population residing in tissues pre-tumorigenesis, especially since Vδ1 cells are commonly reported to localize in tissues.[5] Interestingly, there are also cases in which intratumoral γδ T cells are not dominated by Vδ1 cells: in glioblastoma, Juran et al. reported higher levels of Vδ2 cells in the tumor instead of Vδ1.[57] It is known that the diversity and abundance of immune cells, including γδ T cells, in the brain is limited.[61,62] Under homeostatic conditions, γδ T cells do not reside in the brain tissue but in the meninges where blood vessels also run through.[61] Perhaps PB Vδ2 cells circulated to the meninges and infiltrated the brain into the tumor site to elicit immune responses against glioblastoma cells. On a similar note, Lee et al. described preferential infiltration of Vδ2 cells in glioblastoma, although this study highlighted the presence of disease-specific Vδ2 repertoire in the tumor.[62] Intratumoral γδ T cells have also been described to carry prognostic significance: a study by Wang et al. found that gastric cancer patients with higher levels of γδ T cells within the tumor exhibited high overall survival (OS) and clinical benefit following adjuvant chemotherapy as compared with patients with low intratumoral γδ T cells.[59] In head and neck squamous cell cancer, enrichment of γδ T cells in the tumor is associated with improved survival and positively correlated with CD8+ T-cell abundance.[60] Similar findings were also observed in colorectal cancer and triple-negative breast cancer, in which Vδ1 cells is predictive of lower disease stage and increased OS, respectively.[26,27,55] It may be worth leveraging on the potential prognostic value of intratumoral γδ T cells to develop more robust methods for patient stratification. For instance, Wang et al. proposed the integration of intratumoral γδ T cells with TNM (tumor—node—metastasis) staging to create a 'TNM immune score' that may allow patients to be better classified for treatment.[59]

The presence of γδ T cells in solid tumors and clinical benefit conferred by this immune subset suggests that tumors may serve as a source of anticancer γδ T cells that can be exploited for cell therapy. As compared to PB γδ T cells, the expansion of solid tumor-derived γδ T cells is generally less studied, but attempts at amplifying intratumoral γδ T cells in colon and kidney cancers have been made.[32,58] In their colon cancer study, de Vries et al. cultured γδ T cells *ex vivo* with PHA, IL-2, IL-15, and in the presence of feeder cells. While the expansion efficiency was not reported, the expanded cells expressed the activation marker CD137 and effector molecule IFN-γ upon co-culture with patient-derived tumor cells, and also induced cancer cell apoptosis *in vitro*.[32] Rancan et al. described a different method for amplifying intratumoral γδ T cells in kidney cancer: instead of using PHA and a feeder cell layer, isolated γδ T cells were cultured with irradiated autologous tumor cells and a cytokine cocktail containing IL-2, IL-7, IL-15 and IL-21.[58] These expanded cells demonstrated killing of autologous cancer cells almost as efficiently as CD8+ αβ T cells.[58] Importantly, the same study also showed that sorted but unexpanded kidney tumor-derived γδ T cells secreted IFN-γ in the presence of tumor cells, suggesting that the reactivity of intratumoral γδ T cells against cancer cells is not an acquired function resulting from *ex vivo* expansion but an intrinsic feature of the cells.[58]

Noteworthy from both studies is the expansion of tumor-targeting γδ T cells from intratumoral cells expressing immune markers of exhaustion.[32,58] Rancan et al. additionally reported that programmed cell death protein 1 (PD-1) and TIM3 (and CD137) expression was retained in the expanded cells, and these cells even showed antitumor activity.[58] Perhaps phenotype(s) conventionally used to describe T-cell exhaustion instead indicates antitumor reactivity in γδ T cells and not functional inhibition, at least in these cases. Corroborating with this, Davies et al. recently showed that PD-1-expressing γδ T cells exhibited a molecular phenotype distinct from that of PD-1+ CD8+ T cells.[63] These PD-1+ γδ T cells were also functionally competent but not PD-1+ CD8+ T cells.[63] However, it should be noted that T-cell exhaustion encompasses a range of cellular dysfunction states,[64] and it may be necessary to investigate the proliferative ability of early-, mid- or late-exhausted intratumoral γδ T cells and tumor-targeting functions of the expanded population so that methods in which tumors are used as a source of immune cells can be better defined. Nevertheless, the findings of the studies by de Vries et al. and Rancan et al. suggest potential in harnessing tumor-derived γδ T cells for cell therapy.

The numerous studies focused on exploiting γδ T cells for the purpose of cell therapy in cancer have thus far demonstrated success in γδ T-cell expansion using different sources of cells and also verified the tumor-killing properties of the expanded population.[3-5,6,11,26,32-36,38-41,56,58] In spite of this, it may be worth mentioning that cell expansion is a complicated procedure in which successful generation of antitumor effectors in sufficient quantities is not always guaranteed, and the process is generally costly and time-consuming.[65] These challenges, however, can possibly be mitigated by modifying the cell expansion process. One may consider introducing quality control practices at various steps of the cell expansion cycle in order to ensure the quality of the expanded cell population. For example, the fitness of γδ T cells can be assessed before expansion and be used as a metric to predict the outcome of cell expansion. This may be important when patient-derived γδ T cells are used as the starting material, given that immune cells

obtained from these individuals may be of poorer quality as a result of the disease in some cases. Alternatively, healthy donor γδ T cells may be used. After expansion, the phenotypic and functional profile of the cell product should also be determined in order to ascertain the anti-targeting abilities of the resultant cells. With these measures, the cell expansion process can be streamlined and standardized such that consistency in the cell product can be attained. Furthermore, ongoing research will continue to improve upon current expansion protocols or develop new strategies for expanding γδ T cells with enhanced effectiveness, time and cost efficiency.

γδ T-cell-derived extracellular vesicles as anticancer therapeutic agents

Extracellular vesicles (EVs) are secreted by all living cells and play multiple physiological roles.[66] EVs carry various types of cargo such as protein and mRNA, and these are derived from the cytoplasm or cell membrane of the originating cell.[66] In the context of γδ T cells, studies have shown that EVs isolated from these cells (γδ-EVs) contain cytotoxic molecules such as IFN-γ and perforin, along with proteins known to be involved in γδ T-cell-mediated killing such as NKG2D and FasL.[67,68] Strikingly, γδ-EVs were found to induce tumor cell apoptosis *in vitro* and inhibited tumor progression *in vivo* in nasopharyngeal carcinoma, oral squamous cell cancer and Epstein−Barr virus-associated gastric cancer.[2,67-69] Superior tumor cell killing and tumor control were also observed with γδ-EVs as compared to EVs derived from other immune cells such as NK cells.[69] These observations collectively suggest a tumor-killing mechanism by γδ T cells that is independent of cell−cell contact, and even highlights the possibility of exploiting γδ-EVs for cancer therapy.

Interestingly, the antitumor functions of γδ-EVs are not limited to direct tumor killing. In two separate investigations, Wang et al. reported that γδ-EVs promoted proliferation and IFN-γ production in CD4+ and CD8+ T cells across different cancer types.[68,69] γδ-EVs also induced the cell surface expression of CCR5, a receptor involved in leukocyte recruitment, in CD4+ and CD8+ T cells.[69] Indeed, γδ-EV pretreatment enhanced the migratory activity of T cells in transwell assays and increased T-cell infiltration into tumors in mouse models.[68,69] T-cell migration was additionally proven to be mediated by CCR5 in both cases.[68,69] Another study by Li et al. reported the expression of antigen presentation-related proteins such as MHC-II, CD80 and CD86 in γδ-EVs, suggesting that γδ-EVs may even be involved in antigen presentation.[67] Taken together, these findings indicate possible immunomodulatory roles for γδ-EVs in addition to tumor cell killing. Such direct and indirect antitumor functions of γδ-EVs can perhaps be considered reminiscent of the functions of the parental cells.

A notable characteristic of EVs is the cell-independent nature of such molecules and this may serve as an advantage for cancer therapy. In adoptive T-cell transfer where viable T cells are the principal ingredient of the treatment, transferred T cells can be modulated by an immunosuppressive tumor microenvironment (TME) or even undergo malignant transformation to result in a secondary primary cancer.[70,71] Unlike cells, EVs do not contain dynamic and malleable cellular machinery that can respond to environmental cues, such that external influence conferred by the TME or other currently unidentified factors may only have limited effect on EV function. In fact, Wang et al. reported that the tumor cell-killing function of γδ-EVs was maintained even in co-cultures involving tumor cell-derived supernatant that contained immunosuppressive factors such as transforming growth factor-β.[68] It is also within reason to postulate that the risk of lymphomagenesis arising from γδ-EV treatment may be low given the lack of live cells in EVs for transformation to occur. Therefore, cell-independent treatments including γδ-EVs likely provide superior safety advantage over other types of therapy.

Therapeutic applications for γδ-EVs can also be extended to drug delivery carriers. In a study by Li et al., transduced γδ T cells were reported to express the exogenous gene and the gene product could be packaged into EVs.[67] These EVs were internalized by recipient CD8+ T cells, resulting in the transfer of the gene product into receiver cells.[67] Importantly, the gene product remained functional after delivery.[67] The authors also showed that gene product delivery to T cells was more efficient when γδ-EVs were used as compared to liposomes.[67] Taken together, these observations suggest that γδ-EVs can (i) function as nanocarriers for drug delivery and (ii) be used for targeted delivery to T cells.[67] The latter is noteworthy as T cells have been shown to demonstrate selectivity toward EVs for internalization, wherein these immune cells do not readily take up EVs produced by some cell types.[72]

Wang et al. widened the scope of applicability for γδ-EVs by investigating the use of these molecules as tumor vaccines.[2] When pulsed with tumor-associated antigens (TAAs), γδ T cells produced TAA-loaded γδ-EVs that served as immune adjuvants for DCs and induced antigen-specific T cells *in vitro*.[2] Similar observations were made *in vivo*, where TAA-loaded γδ-EV treatment suppressed tumor progression and improved survival in a humanized mouse model in comparison with mice treated with unpackaged TAAs or TAA-free γδ-EVs.[2] The authors also noted enhanced stimulation of tumor-specific T cells in mice which received TAA-loaded γδ-EVs, although T-cell induction is possibly mediated by B cells.[2] Notably, TAA-loaded γδ-EVs induced stronger adjuvant effects and better tumor control than TAA-rich EVs derived from DCs.[2]

An interesting aspect of the studies by both Li et al. and Wang et al. is the direct antitumor activities that γδ-EVs were reported to perform while simultaneously functioning as nanocarriers for drug/vaccine delivery.[2,67] Such functional duality may be important as it suggests that broad targeting of different cells in the TME or tumor macroenvironment can be achieved using a single 'drug' (i.e. γδ-EV nanocarrier). For example, γδ-EVs loaded with T-cell agonists may be able to target tumor cells in their own right, and concurrently activate T cells *in situ* which then

recognize and kill cancer cells, thereby providing a two-pronged approach for effective tumor control. In addition, given that γδ-EVs targeted different immune cell types in these studies, it is likely that γδ-EVs can transport molecules to various other types of immune or even non-immune cells.[2,67] Perhaps γδ-EV-based nanocarriers can even carry out APC functions especially since intrinsic antigen presentation roles for γδ-EVs have been suggested.[67]

Overall, current research suggests potential for γδ-EVs as therapeutic agents and there is increasing interest in harnessing these molecules for cancer treatment. However, one should note that γδ-EVs is an emerging application and much research is still required to better elucidate the features of γδ-EVs. It may be important to assess the phenotype and functions of γδ-EVs, their persistence *in vivo* and the cellular targets and specificity of these molecules. Furthermore, EVs produced using different subsets of γδ T cells should be examined and compared to determine whether γδ-EVs derived from various sources are functionally similar. For γδ-EV delivery systems, there is a need to optimize protocols for exogenous gene expression and loading of the gene product into γδ-EVs, as well as identifying the types of exogenously expressed cargo that can be loaded into γδ-EVs. Deep understanding of γδ-EVs will guide the improvement of current methods for γδ-EV manufacturing and isolation, and also drive the development of efficacious γδ-EV-based treatments. Finally, it may be worth considering the use of γδ-EVs for combinatorial treatment as γδ-EVs has been reported to synergize with standard therapies and result in enhanced tumor control.[68] This is illustrated in the study by Wang et al. wherein γδ-EVs in combination with radiotherapy induced higher tumor cell apoptosis as compared with single treatment with either agent.[68]

Other γδ T-cell-based therapeutics: genetic engineering

Chimeric antigen receptor (CAR)-T-cell-based therapy is one of the most remarkable advances in cancer treatment.[45,73] Since its introduction in 1993, studies on CAR-T cells were mostly focused on αβ T cells.[74] The unique biological properties of γδ T cells (as described in the earlier sections) render these immune cells good candidates for CAR-T therapies, and engineering of γδ CAR-T cells should be attempted. Indeed, recent studies reported successful transduction of healthy donor-derived γδ T cells with CAR constructs and expansion of γδ CAR-T cells.[45,75-81] These γδ CAR-T cells were cytotoxic against tumor cells *in vitro* and *in vivo*, and even demonstrated stronger antitumor activity as compared with αβ CAR-T cells in some cases.[75-81] γδ CAR-T cells were also found to accumulate in metastatic sites and induce cancer cell regression, suggesting the potential of γδ CAR-T cells for treatment of metastatic disease.[77] Interestingly, the function of γδ CAR-T cells was not limited to cytotoxic killing as these cells were capable of migrating toward tumor cells and even carried out APC functions to induce αβ T-cell proliferation in *in vitro* settings—this finding is noteworthy as it indicates that the endogenous features and functions of γδ T cells are preserved in CAR-transduced cells.[45] Taken together, the observations from these studies collectively demonstrate (i) feasibility in employing γδ T cells as vehicles for CAR-T expression and (ii) potential of γδ CAR-T cells as cancer therapeutics. It may be reasonable to consider that at least three different types of receptors—TCR, NKRs and CARs—constitute the tumor recognition machinery in γδ CAR-T cells. This likely endows γδ CAR-T cells with multi-antigen-targeting abilities that permit cancer cell detection more effectively as compared with primary γδ T cells or even other types of immune cells, and thus potentially minimize tumor antigen escape or off-target effects. Reduced effects of graft versus host disease with γδ CAR-T cells may also be conceivable, given the intrinsic MHC-independent antigen recognition patterns in γδ T cells.

The unique antigen recognition patterns of the γδ TCR make it an attractive option for T-cell engineering. In a report by Straetemans et al., T cells were engineered to express a specific, high-affinity γδ TCR clone and the resultant cells [T cells engineered to express γδ TCR (TEG)] also demonstrated antitumor activity.[82] Notably, this study described a good manufacturing practice-grade protocol for TEG manufacture, with the end product exhibiting high γδ T-cell purity and is of sufficient quantity for clinical testing.[82] Strategies for enhancing the therapeutic efficacy of TEGs have also been suggested, such as those illustrated in the study by Hernández-López et al., where TEGs were designed to co-express an NKG2D receptor chimera or CD277-recognizing receptor for sensing of stress-related ligands (i.e. NKG2D ligands and CD277, respectively) in addition to γδ TCR antigens.[83] Such dual mode of antigen targeting was found to enhance TEG activation, tumor-specific activity and serial killing of target cells *in vitro*, and promote tumor control and survival in mouse models.[83]

Genetic engineering-based strategies have broad applicability and may not be limited to the use of membrane-bound molecules to enhance effector cell activity. Instead of transducing γδ T cells with receptors such as CARs, Fowler et al. recently described the generation of γδ T cells that express and secrete tumor antigen-targeting opsonins and stable IL-15.[79] These engineered cells exhibited increased antigen-specific cytotoxicity against tumor cells *in vitro* and *in vivo* as compared with unmodified γδ T cells, and enhanced survival *in vivo*.[79] Additionally, the *in vivo* studies showed that opsonin/IL-15-engineered γδ T cells, but not unmodified ones, could be detected in the blood 7 days after administration; despite the short time point, this finding is still important as it hints at the potential of these engineered γδ T cells to persist.[79] Most notably, the authors of this study also reported the ability of engineered γδ T cells to induce antitumor activity in other types of immune cells.[79] This is observed with macrophages, in which macrophages cultured with opsonin/IL-15-engineered γδ T-cell-derived conditioned media demonstrated enhanced target cell lysis in an antigen-dependent fashion.[79] Perhaps such cell-to-cell contact-independent (engineered) γδ T-cell-induced triggering of other immune cells is akin to a

'bystander effect', and of which is possible likely due to the presence of 'free' molecules that have the propensity to induce immune responses.[79]

Evidently, the possibilities surrounding genetic engineering for γδ T-cell-based therapeutics are limitless. There is currently ongoing interest in engineering γδ T cells, and present and future research will improve upon current genetic engineering methods and/or develop new techniques for generating engineered γδ T cells with potent tumor-targeting abilities for cancer therapy.

Other γδ T-cell-based therapeutics: γδ T-cell engager molecules

Bispecific T-cell engager molecules (BiTE) are a novel subclass of bispecific antibodies that has gained much popularity in recent times as therapeutic agents for cancer.[7] These molecules are composed of two antibody fragments, each with different antigen-binding specificities.[7] In the context of cancer, BiTEs are typically designed to simultaneously bind a CD3 molecule on T cells and a tumor antigen expressed on the surface of cancer cells, and this consequently brings tumor and T cells in close proximity for cytotoxic killing by T cells.[7] Unsurprisingly, BiTE-based cancer cell-targeting strategies have also been applied to γδ T cells, although it should be noted that the immune cell-binding fragment of these engagers interact with the γδ TCR instead of CD3 molecules.[8,84-86] Several studies reported the design of γδ T-cell-specific BiTEs (γδ-BiTEs) and demonstrated the ability of these molecules to activate γδ T cells and trigger γδ T-cell-mediated antitumor activity *in vitro* and *in vivo*.[8,84-86] γδ-BiTEs were even found to selectively recruit γδ T cells of interest into the tumor bed in mouse models, suggesting specificity in γδ T-cell-based engagers.[86] Where investigated, T-cell activation in the presence of γδ-BiTEs was shown to be restricted to γδ T cells—this indicates that any off-target effects potentially induced by γδ-BiTEs will likely be minimal, thereby demonstrating the safety value of these molecules.[85] Interestingly, γδ-BiTEs used in these studies were described to target different tumor antigens and across different types of cancer.[8,84-86] Based on this, it may be within reason to suggest that γδ-BiTE is a versatile therapeutic modality that can possibly be engineered to target a broad spectrum of cancers. γδ-BiTEs can also be designed to be trispecific and stimulate other immune cells with tumor-killing properties in addition to γδ T cells; this is demonstrated in the study by Lameris et al. wherein γδ-BiTEs triggered both type I invariant NKT cells and γδ T cells, and resulted in robust antitumor activity.[84] This dual activation was also well tolerated in a non-human primate model.[84] On a side note, research involving γδ-BiTEs thus far have mainly focused on the Vδ2 γδ T-cell subset; other subpopulations of γδ T cells such as Vδ1 cells should also be considered for γδ-BiTE design, especially since this subgroup has been shown to target tumors.[3,34] Nevertheless, BiTEs represents a promising therapeutic avenue for γδ T cells in cancer and current and future investigations will delineate how γδ-BiTEs can be incorporated into the treatment regimen for this disease.

BiTE molecules and genetic engineering are both known to be promising approaches for cancer therapy. Becker et al. took it up a notch by combining the two techniques- here, a CD3 and CD19-targeting soluble BiTE construct (sBiTE) was introduced into γδ T cells, and these engineered cells demonstrated higher cytotoxicity against CD19-expressed tumor cells as compared to unmodified γδ T cells in *in vitro* and *in vivo* models.[81] Interestingly, sBiTE molecules produced by the engineered γδ T cells could potentially induce unmodified cells of the same, as unmodified γδ T cells showed improved tumor killing when treated with conditioned media obtained from sBiTE-expressing γδ T cells.[55] These findings collectively illustrate the possibility of amalgamating different approaches into one therapeutic agent for enhanced efficacy; further investigation is warranted to uncover the feasibility and full potential of such approaches.

Other γδ T-cell-based therapeutics: immune checkpoint inhibitors

ICIs have become part of the current standard of care for treatment of several types of cancer, including solid tumors, advanced cancers and metastatic diseases.[87] Although the significance of conventional T-cell exhaustion markers in γδ T cells remains to be elucidated, some studies have shown that immune checkpoint molecules such as PD-1 and CTLA-4 are up-regulated in γδ T cells upon exposure to tumor cells.[24,32,88] In these cases, the checkpoint proteins were found to diminish γδ T-cell cytotoxic responses, suggesting a possible role for immune checkpoint molecules in modulating γδ T-cell function.[88] Such antagonizing effects of checkpoint molecules on γδ T cells could be reversed in the presence of ICIs, and this indicates the potential of ICIs in reinvigorating or promoting γδ T-cell activity.[88] Indeed, reports by Simone et al. and de Vries et al. described increased infiltration of γδ T cells into tumors and overall γδ T-cell count in patients treated with ICIs.[32,88] Simone et al. further outlined an association between low immune checkpoint molecule expression in γδ T cells and improved patient survival.[88] Taken together, these observations highlight possible applications of ICIs for γδ T-cell-based treatments. Where applicable, combinatorial use of ICIs and adoptive γδ T-cell transfer should also be considered, for enhanced treatment effect.

CONCLUDING REMARKS

γδ T cells represent a paradigm shift in cell-based immunotherapies for cancer. By virtue of their unique functional and phenotypical features, these immune cells may be less susceptible to tumor-induced resistance and can promote complete functioning of the cancer-immunity cycle for effective anticancer responses to be elicited. The role(s) of γδ T cells in cancer immunity render them as attractive candidates for cancer immunotherapy, and special attributes further support the suitability of these cells as

therapeutic agents. For instance, the intrinsic, MHC-unrestricted modes of antigen recognition in γδ T cells likely minimize the risk of graft-versus-host diseases following adoptive transfer, and this may allow γδ T cells to be developed as off-the-shelf treatments. Currently, several methods for harnessing γδ T cells for therapeutic use have been reported and ongoing research will uncover other novel γδ T-cell-based treatment strategies. In this review, we highlight novel therapeutic avenues for γδ T cells, particularly (i) utilizing non-conventional sources of γδ T cells and (ii) cell-independent approaches. It may be worth noting that γδ T cells are less studied as compared to αβ T cells, and thus much about γδ T cells remains unexplored. Nevertheless, ongoing and future research will uncover the full potential of these immune cells in the context of cancer and improve upon current γδ T-cell-based immunotherapies or develop new treatment strategies. The cancel culture, while not socially accepted, is warranted for cancer diseases, and γδ T cells may be the physical tool for executing this ambition.

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