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Prospects for chimeric antigen receptor (CAR) γδ T cells: a potential game changer for adoptive T cell cancer immunotherapy

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Abstract

Excitement is growing for therapies that harness the power of patients' immune systems to combat their diseases. One approach to immunotherapy involves engineering patients' own T cells to express a chimeric antigen receptor (CAR), to treat advanced cancers, particularly those refractory to conventional therapeutic agents. Although these engineered immune cells have made remarkable strides in the treatment of patients with certain hematologic malignancies, success with solid tumors has been limited, probably due to immunosuppressive mechanisms in the tumor niche. In nearly all studies to date, T cells bearing αβ receptors have been used to generate CAR T cells. In this review, we highlight biological characteristics of γδ T cells that are distinct from those of αβ T cells, including homing to epithelial and mucosal tissues and unique functions such as direct antigen recognition, lack of alloreactivity, and ability to present antigens. We offer our perspective that these features make γδ T cells promising for use in cellular therapy against several types of solid tumors, including melanoma and gastrointestinal cancers. Engineered γδ T cells should be considered as a new platform for adoptive T cell cancer therapy for mucosal tumors.

Keywords

γδ T cells; chimeric antigen receptor; cancer therapy

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Introduction

Harnessing the immune system to recognize and destroy tumor cells is quickly becoming a cornerstone of cancer treatment. One of the principal treatment modalities within the field of cancer immunotherapy has been adoptive T cell therapy (ACT). In this strategy, patient-derived T cells specific for tumor-associated antigens (TAA) are expanded outside the patient's body and re-infused into the bloodstream to target and destroy cancer cells. These tumor-specific cells may be derived in a number of ways, including expansion of antigen-specific T cell clones, genetic modification of polyclonal T cells to express either a T cell receptor or CAR targeting TAAs, or expansion of tumor-infiltrating lymphocytes (TILs)(1–7). The most widely employed strategy has been TIL infusion, for which a robust body of evidence exists indicating that this treatment can induce durable complete responses, even in patients in whom other immunotherapies have failed (8, 9). Advances in genetic engineering have made it possible to confer tumor specificity to T cells, thus circumventing the need to isolate tumor-infiltrating T cells, an obstacle that has restricted broad application of TIL therapy beyond a narrow subset of tumors characterized by extensive T cell infiltrates. Using viral and non-viral integration approaches, antigen-specific receptors can be introduced into T cells (10–12). One such example of an antigen-specific receptor is a CAR, a fusion protein in which a TAA-binding moiety (usually a single chain variable fragment [scFv] derived from a monoclonal antibody) is linked to an intracellular immunoreceptor signaling domain, typically the CD3 ζ chain. CAR T cells can potentially redirect the effector functions of a T cell towards any protein or non-protein target expressed on the cell surface. Therefore, CAR T cells can recognize a various range of protein and non-protein antigens without requirement of antigen processing and presentation by the target cell (6, 13–15). Bypassing the requirement for major histocompatibility complex (MHC)-restricted targets also means that the CAR T-cell approach can be used as a universal treatment, broadening the potential of applicability of adoptive T-cell therapy. In the vast majority of CAR T cell studies, the source of T cells used to generate the therapeutic cell product has been the peripheral blood, and the T cells expressed αβ receptors rather than γδ receptors (10–13, 16). Moreover, as we progress toward better understanding of different aspects of immune system and how immune responses generated and regulated in situ, it is becoming clear that the characteristics of the tissue microenvironment is as decisive as immune cells in determining the initiation, polarization and effector function of immune responses. This therefore highlights how local tissue microenvironment in different organs can shape and influence the outcome of immune responses (17–20). In this regard, we offer an appraisal of how adoptive therapy using CAR T cells bearing γδ receptors may be a promising therapeutic strategy for cancers particularly mucosal (epithelial) cancers.

γδ T cells: development, tissue distribution, and function

Mucosal (epithelial) tissues act as physical barriers and contain a wide range of cell populations including non-lymphoid and lymphoid immune cells, notably T cells. It has been shown that T cells, particularly those bearing γδ receptors, play a pivotal role in mucosal tissue homeostasis and immunosurveillance against invading pathogens and epithelial stresses such as malignant transformations (21–25). γδ T cells develop mainly in the thymus and generate their γδ T cell receptor through V(D)J recombination (26–29).

After characteristic gene rearrangements two T cell lineages expressing γδ and αβ receptors diverge from a common lymphoid precursor (CLP) (30–32). T cells bearing γδ receptors transduce a TCR signal through associated CD3 complexes. In contrast to γδ T cells, which comprise 1–10% of circulating T cells in the peripheral blood of healthy adults, T cells expressing αβ receptors comprise about 90% of circulating T cells and direct intracellular signaling through associated CD3 complexes (33). In contrast to an αβ TCR, a γδ TCR directly binds to an antigen without requiring antigen presentation by MHC molecules and, as a result, CD4 and CD8 are uncommon on γδ T cells. One of the distinct features of T cells bearing γδ receptors is that the majority of these cells are found primarily in epithelial and mucosal sites (34, 35). Relatively little is known about the ligands recognized by γδ T cells.

Several studies have demonstrated that γδ TCRs can recognize and be activated by a wide range of structurally different ligands with various sizes, compositions, and molecular structures (36–38). Table 1 summarizes potential ligands of human γδ T cells.

During embryonic development, γδT cells encoding specific Vγ gene segments exit from thymus at defined periods during fetal and neonatal development, and then migrate to and populate different epithelial tissues in adult animals (39). The first T cells appear to express γδ T cell receptors. In the mouse, γδ T cells are developed in distinct waves in the fetus, and each wave homes to specific sites in the adult animal. At about two weeks of gestation, the first wave of γδ T cells, expressing Vγ5, populates the epidermis. After a few more days, Vγ5 bearing T cells decline and are replaced by a second wave of T cells expressing Vγ6, which homes to the epithelia of the reproductive and airway tracts. The third wave represents Vγ4 bearing T cells, which become established in the spleen and epithelium of the lung. After birth, although γδ T cells expressing Vγ1, 2, and 7 are still produced and migrate to lymph nodes, the αβ T cell lineage becomes dominant and comprises the majority of thymocytes (~95%). The γδ T cells produced at this stage (expressing Vγ1, 2, and 7) are different from those produced earlier. They have much more diverse receptors, and most of these γδ T cells migrate to peripheral lymphoid tissues rather than to epithelia (Figure 1). Their functional significance is unclear; however, it seems that all these changes are related to the pattern of receptors expressed by γδ T cells in humans. It should be noted that, however, the thymus is not necessarily required for complete development of some γδ T cells, so that many γδ T cells, after exiting from bone marrow, can directly migrate to peripheral tissues, such as vagina, intestine, lung, and skin, where they can employ their effector functions. Such thymus-independent γδ T cells comprise about 50% of the T cell subsets in intestinal epithelial tissues (27, 29). Extensive investigations have demonstrated that human γδ T cells consist of three main populations based on δ chain expression. γδ T cells expressing Vδ1 chains are the dominant population in the intraepithelial layer of mucosal surfaces and comprise a minor population in the peripheral blood. They have a central role in maintenance of epithelial integrity with respect to damage, infection, or transformation (33, 40–42). Another major subset of γδ T lymphocytes expresses a Vδ2 chain, which is almost exclusively paired with one particular Vγ chain (Vγ9, also known as Vγ2), and comprises the majority of circulating γδ T cells in healthy human adults, populating up to 50%-90% of the peripheral γδ T cell (33). Intriguingly, upon activation, Vδ2 T cells acquire features of professional antigen presenting cells (APCs) including the

expression of costimulatory, adhesion, and antigen presenting molecules such as CD86, CD80, CD11b, CD18, CD54 and MHC-II (43–45). A third population of γδ T cells express Vδ3 and account for approximately 0.2% of circulating T cells, comprising $CD4^+$, $CD8^+$, and $CD4^-$ $CD8^-$ subsets. They variably express CD56, CD161, HLA-DR, and NKG2D. Vδ3 T cells are a minor population in the blood but are more dominant in the liver and in leukemic patients. Upon activation with IL-2, Vδ3 T cells are expanded and recognize CD1d and, thereby, can lyse $CD1d^+$ target cells and release cytokines such as IL-17 and IFN-γ (46). A substantial body of evidence now demonstrates that γδ T cells play a central role in defending the host against a wide range of infections as well as sterile stresses such as malignant transformation. γδ T cells accomplish this through multiple mechanisms including regulation of stromal cell function by production of growth factors, granzyme-mediated lysis of infected or stressed cells, production of a range of cytokine and chemokines to regulate both immune and non-immune cells (Table 2), antigen presentation leading to αβ T cell priming, and induction of dendritic cell (DC) maturation (43, 47–50).

Adoptive T cell Therapy for Cancer

Adoptive T cell therapy involves the isolation and ex vivo expansion of tumor-specific T cells, often isolated from tumor-infiltrating lymphocytes (TILs), and then re-infusion of these lymphocytes either in a modified or unmodified state into the patient's body. Adoptive transfer of tumor-specific T cells has demonstrated robust antitumor immune responses in some cancers such as melanoma and virus-associated malignancies (8, 9). For instance, it has been shown that TIL infusion can induce complete remissions even in patients with metastatic melanoma who have not responded to other immunotherapy options (8). However, the generation of TILs has generally not been feasible for most cancers and even in melanoma is not successful for all patients. Moreover, in the majority of cancers, tumor cells evolve and deploy multiple mechanisms to escape immunity through either evading antigen recognition or subverting normal antitumor immune responses. For example, tumors downregulate MHC expression, express inhibitory ligands such as PD-L1, and produce or induce immunosuppressive cytokines or tumor-favoring growth factors such as TGF-β (84, 85). In this regard, efforts to stimulate endogenous T cells against cancer are often futile.

One strategy to overcome the paucity of TILs available in most tumor types is to reprogram T cells to recognize tumor-associated antigens using genetic engineering approaches. The most common strategies have been to introduce genes encoding either 1) high-affinity αβ TCRs that were previously cloned from tumor-reactive T cells, or 2) chimeric antigen receptors, usually comprising an antigen-specific single-chain antibody variable fragment (scFv) linked, via hinge and transmembrane domains, to one or more of the intracellular domains of T cells such as CD3ζ, CD28, or 4-1BB. In these treatment modalities, T cells are isolated from the blood of patients, genetically modified in vitro, expanded, and re-infused back into the bloodstream (86, 87). T cells expressing αβ TCRs can target intracellular antigens but are restricted to a specific HLA type, require costimulatory signals, are susceptible to antigen presentation defects such as MHC loss, and, as explained above, have the potential to pair with endogenous TCR αβ chains to create new TCRs with unknown and potentially self-reactive specificities. Chimeric antigen receptor (CAR) T cells have several potential advantages, including the ability to provide a costimulatory signal through the

CAR, lack of requirement for a MHC-restricted peptide complex, and ability to recognize a wide range of antigens including carbohydrates and lipids without the need for antigen presentation (6, 13–15). One limitation is that CARs require cell surface antigen targets.

Clinical trials testing adoptive transfer of CAR T cells have shown remarkable responses in patients with B lymphoid malignancies, notably relapsed or refractory acute lymphoblastic leukemia (ALL) (11–13, 88–90). However, adoptive CAR T cell therapy for solid tumors has shown limited success so far, likely due to immunosuppressive tumor microenvironments, lack of tumor-specific antigens, and insufficient trafficking of CAR T cells to tumor sites (91, 92). To overcome these barriers, several ingenious strategies have been deployed, including design of inhibitory CARs (iCARs), logic-gated CARs, introduction of chemokine receptor genes that match the chemokines produced either by tumor or tumor associated cells (e.g. CCR2b which binds to CCL2-derived neuroblastoma cells), or endowing CAR T cells with immunostimulatory ligands (e.g. CD40L), immunostimulatory cytokines (e.g. IL-12, IL-15, and IL-7), chimeric inhibitory receptors (e.g. PD-1/CD28), or basement membrane-degrading enzyme (e.g. heparanase) (93–104). However, these interventions have yet to be proven in clinical trials, and it remains to be seen whether effective responses against solid tumors can be achieved with these measures.

Why adoptive CAR γδ T cell cancer therapy?

Most current immunotherapeutic approaches aim at inducing antitumor responses via stimulation of the adaptive immune system, which is dependent on MHC–restricted αβ T cells. Most current adoptive T cell therapies for cancer have employed αβ T cells with MHC-restricted TCRs or MHC-independent CARs (8, 9, 13, 105). Despite remarkable progress in our understanding of adaptive immunity toward tumors, durable responses are rare. Adoptive T cell therapy using αβ TCRs has several disadvantages: αβ T cells require specific tumor-associated antigens (TAAs) and appropriate costimulatory molecules for activation. Loss of TAA expression, development of defects in antigen presentation, loss of MHC molecules, and/or absence of costimulatory molecules renders tumor cells resistant to αβ T-cell–mediated cytotoxicity or induces anergy of specific T cells (106). We postulate that several characteristics of γδ T cells make them an attractive T cell subset in which to apply CAR T cell therapy for solid tumors, including their inherent anti-tumor activity and ability to home to epithelial tissues.

Anti-tumor activity of γδ T cells

In contrast to αβ T-cells, γδ T cells are not susceptible to antigen processing and presentation defects (although tumors could still potentially lose expression of the TAA γδ TCR ligand), and are thus an appealing T cell subset for clinical cancer immunotherapy. Growing evidence indicates that γδ T cells play a critical role in tumor immunosurveillance and anti-tumor immune responses. Girardi et al. showed that epithelial localization of γδ T cells may contribute to prevention of tumor formation in mice prone to develop epithelial malignancies. They demonstrated that mice lacking γδ cells are highly susceptible to cutaneous carcinogenesis (107). Liu and colleagues also showed that prostate tumor-bearing mice treated intravenously (i.v.) with syngeneic γδ T cells developed measurably less disease compared with control mice. Tumor-bearing mice treated i.v. with γδ T cells also

showed superior survival compared with untreated mice (108). An interesting study on human dysgerminoma and seminoma conducted by Zhao and colleagues showed that γδ TILs accumulate within the granulomatous inflammation of tumor tissues. Such infiltrating γδ T cells showed autologous tumor killing activity, which could be inhibited by monoclonal antibodies against Vδ. These cells also produced proinflammatory cytokines such as TNF-α and IFN-γ. The authors concluded that γδ T cells accumulating in dysgerminoma and seminoma exhibit anti-tumor activity through TCRs and these γδ T cells also play a role in the formation of granulomatous inflammation (109). Todaro et al. showed that γδ T cells can kill colon cancer stem cells, a subpopulation demonstrated to be responsible for tumor initiation, growth, metastasis, resistance to conventional cancer therapies, and thereby, cancer relapse (110). A separate study showed that Vγ9Vδ2 T lymphocytes recognize, trogocytose, and efficiently kill imatinib-resistant CML cell lines pretreated with zoledronate (111). Liu and colleagues demonstrated that ex vivo expanded apoptosis-resistant human Vγ9Vδ2 T cells are able innately to recognize and kill human prostate tumor cell lines in vitro (112). γδ T cells have been consistently identified and isolated from TIL in various types of cancer, including colorectal, breast, prostate, ovarian, and renal cell carcinoma (25, 113–116). γδ T cell lines and clones established from TIL recognize and destroy autologous tumor cell lines and a wide range of related tumors probably due to the recognition of shared activating ligands (See Table 1). γδ T cells show potent MHC-unrestricted cytotoxicity, a high potential for cytokine secretion, inherent potential for antitumor effects, apparent lack of alloreactivity, broad-spectrum recognition of cancer cells through direct recognition of TAAs (e.g. heat shock proteins, major histocompatibility complex class I chain-related gene A/B, F1-ATPase and phosphoantigens), and ability to present antigens to αβ T cells professionally. These features not only lead to direct recognition of tumor cells but also enhance their antitumor activity through recruitment of other immune cells (Figure 2) (37, 42, 44).

Several studies have demonstrated a role for human γδ T cells in recognition of transformed cells. γδ T cells have been found with increased frequency in disease-free survivors of acute leukemia following allogeneic bone marrow transplantation (117, 118). In addition, adoptive transfer of ex vivo-expanded human γδ T cells in a mouse tumor model further supports the in vivo antitumor effects of γδ T cells. For example, Devaud and colleagues demonstrated that concomitant injections of Vδ2-(negative) clones could prevent the development of HT29 tumors (119). Moreover, they showed that a systemic i.p. treatment with Vδ2-(negative) clones delayed the growth of HT29 s.c. tumors. Various clinical trials have demonstrated that γδ T cells-based immunotherapy is a promising approach for fighting many cancers (Table 3). Intriguingly, γδ T cells preferentially destroy cancer cells and show low, if any, reactivity towards healthy cells, a characteristic that has inspired considerable interest in exploring their therapeutic potential. Xu and colleagues showed that synthesized TCR Vδ2 CDR3 peptides derived from tumor infiltrating lymphocytes (TILs) in ovarian epithelial carcinoma (OEC) could bind specifically to tumor cell lines and tissues but not normal tissues (116). In another study, Corvaisier et al isolated a Vγ9Vδ2 T cell clone from the ascites of a colon cancer patient. This isolated clone showed robust activity against a large fraction of colon carcinoma and melanoma cell lines, but did not affect a normal colon cell line, colon fibroblasts, or melanocytes. Similar reactivity patterns against colon

carcinoma cell lines were also observed using polyclonal Vγ9Vδ2 T cells of various origins (120). Viey and colleagues also shown that phosphostim-expanded peripheral Vγ9Vδ2 T cells have a selective lytic potential toward autologous primary renal tumor cells but not renal normal cells. The lytic activity involved the perforin-granzyme pathway and was mainly TCR and NKG2D receptor-dependent (114). The impact of γδ TCR expression intensity in natural γδ T cells on anti-tumor activity is not known yet and should be investigated.

Genetically engineered γδ T cells

Many studies have shown that TCR αβ gene transfer might lead to generation of neoreactive TCR heterodimers resulting from pairing with the endogenous α and β chains. The possible formation of such mixed TCRs, which are not subject to thymic selection and thus might have harmful autoreactive specificities, is an inherent disadvantage of αβ TCR transfer to αβ T cells. For instance, Bendle et al demonstrated that mice adoptively transferred with TCR gene-modified polyclonal T cells developed a lethal autoimmune disease (121). One potential solution to this problem is transfer of αβ TCRs into γδ T cells to eliminate the possibility of mispairing. Van der Veken et al. investigated the function of γδ T cells engineered to express human αβ TCRs and reported that these cells exhibited high levels of cytotoxic activity and cytokine release. They also confirmed the absence of mixed TCR heterodimer formation (122). In another study, the same team also demonstrated that TCR-transduced γδ T cells have potent antileukemic activity and produce IFN-γ and IL-4, particularly in the presence of transferred CD4 or CD8 molecules (123). Hiasa and colleagues showed that γδ T cells co-transduced with TCR αβ and CD8 αβ genes acquire antitumor activity and secrete cytokines in both αβ- and γδ-TCR-dependent manners. Furthermore, αβ TCR and CD8-transduced γδ T cells rapidly respond to target cells compared with conventional αβ T cells (124).

Rischer et al. demonstrated that peripheral blood-derived Vγ9Vδ2 T cells transduced with retroviral vectors encoding either GD2 or CD19-specific CARs had high CAR expression, could be readily expanded, and demonstrated antigen-specific IFN-γ secretion and cytotoxicity against tumor cell targets. These in vitro tests suggested that CAR-expressing γδ T cells might serve as potent and specific antitumor effector cells (125). More recently, a study conducted by Deniger et al. also showed that in vitro aminobisphosphonate-propagated Vγ9Vδ2 CAR T cells could secrete proinflammatory cytokines and kill $CD19^+$ tumor cell lines in vitro, but that they could also inhibit tumor growth in a mouse xenograft model (16). In addition to CAR-mediated stimulation, direct tumor antigen recognition by the γδ TCR and its consequent signaling cascade might have an additive stimulating effect on CAR γδ T cells.

Regulatory functions of γδ T cells

While γδ T cells clearly show potent antitumor activity, there are some reports that describe regulatory function of these cells in the tumor microenvironment. Peng et al. reported a dominant γδ T cell population among lymphocytes infiltrating breast tumors that exhibited a potent immunosuppressive activity on naive and effector T cell responses and also blocked the maturation and function of DCs. These regulatory γδ T cells did not express FoxP3 or

CD25 (classical markers of conventional Tregs) and did not exert their immunosuppressive activities by IL-10 or TGF-β. The authors showed that these immunosuppressive activities could be reversed by human TLR-8 ligands (126). However, Hua and colleagues demonstrated that blood-derived γδ T cells can acquire a classical regulatory phenotype (i.e. expression of FoxP3, CD25, and CTLA-4) following stimulation with plate-bound anti-Vδ antibody. These cells could also secrete IL-10 and TGF-β and, as a consequence, suppress CD4+ T cell proliferation (127). It is important to note that the suppressive γδ T cells in these studies were of a distinct subtype expressing Vδ1. However, Traxlmayr and colleagues showed that peripheral blood Vγ9Vδ2 T cells can acquire inhibitory function in response to IL-12 secreted by DCs. Thus, it appears that while Vγ9Vδ2 have inherent anti-tumor activity, they are subject to IL-12 mediated negative feedback (128).

Clinical-scale expansion of γδ T cells for therapeutic application

One important consideration in adoptive T cell therapy is the ability to generate sufficient numbers of cells to conduct human clinical trials. The conventional approaches used to expand αβ T cells such as antiCD3 antibodies and IL-2 usually do not result in efficient expansion of γδ T cells. Two strategies using γδ T cells for cancer immunotherapy have so far been explored: i) the adoptive transfer of ex vivo-expanded γδ T cells and ii) in vivo therapeutic application of γδ-stimulating phosphoantigens or aminobisphosphonates together with low-dose IL-2. Several investigators developed protocols for culturing and expansion of γδ T cells based on their reactivity to bisphosphonate drugs. These drugs, however, expand Vγ9Vδ2 cells but do not stimulate Vδ1 T cells. Lopez and colleagues developed a pan-γδ T cell expansion protocol in which anti-CD2 monoclonal antibody can generate IL-12-dependent signals that not only protect human γδ T cells from mitogen-induced apoptosis (i.e. activation induced cell death) but also lead to production of large numbers of viable and functional γδ T cells. They showed that these expanded γδ T cells retain their anti-tumor activity against a wide range of hematologic and solid primary tumors and cell lines. (129) In another study, Siegers et al. enhanced expansion capacity of γδ T cells (up to 24,000-fold) via stimulation of peripheral blood mononuclear cells (PBMC) by Concanavalin A (ConA) without requirement for feeder cells (130). Lamb et al, using irradiated leukemic feeder cells and low-dose IL-2, were able to enhance expansion of γδ T cells up to 1200-fold (131). Finally, Deniger and colleagues, using γ-irradiated K562-derived artificial antigen presenting cells (aAPCs) plus soluble IL-2 and IL-21, could generate up to 10^9 CAR γδ T cells start in with fewer than 10^5 total cells (16). In most clinical trials, 1–5 × 10^6 CAR+ αβ T cells/kg (~ 10^8 total) are infused (11, 13). It seems likely that clinical-scale generation of CAR γδ T cells will be possible using these optimized expansion protocols.

Migration pattern of γδ T cells

Another favorable characteristic of γδ T cells is the localization of specific subsets to mucosal epithelial surfaces. This could be a decisive factor for successful immune or tumor-surveillance function. Until recently, the nature of the molecular interactions between epithelial cells and epithelia-associated T cells was elusive, particularly how the inherent cytotoxic activity of such T cells is regulated and targeted properly to stressed or transformed, but not healthy, epithelial cells. Two different forms of co-receptor molecules

have been identified that enable epithelial cells to interact with and to regulate the activity of dETCs and intestinal intraepithelial T lymphocytes (iIELs) independent of antigen recognition and TCR specificity. Mouse dETCs and the Vγ2Vδ2 population present in human peripheral blood express NK cell receptors such as NKG2D, which deliver an activating stimulus when ligated. The ligands for NKG2D are MICA and MICB, which are expressed on human intestinal epithelial cells, and Rae1 (retinoic acid early inducible 1) and the minor histocompatibility antigen H60 which are expressed on mouse skin epithelial cells (132–134). A second γδ TCR co-receptor is the non-classical MHC class I molecule, thymus leukemia antigen (TL), which is expressed solely by intestinal epithelial cells that preferentially bind the homotypic form of CD8 (CD8αα) that is uniformly expressed by γδ iIELs (135, 136). Such co-receptor interactions might inhibit iIEL proliferation and cytotoxicity and stimulate cytokine release instead which might have an important role in homeostatic regulation of epithelial lining and activation and survival of iIELs (137, 138). Various studies showed that MICA/B and Rae1 are expressed by tumor cells (139, 140). Since MICA/B, and Rae1 are expressed on epithelial tumor cells, these proteins provide a means by which γδ T cells might function in antitumor immunity, as a consequence of signals derived from both the γδ T cell receptor and NKG2D.

Tissue-specific homing of γδ T cells to mucosal epithelial tissues such as skin, reproductive, and gastrointestinal tracts, as well as to tumors originating from these tissues, has important implications for the design of novel immunotherapeutic approaches. As mentioned above, one of the potential problems of adoptive T cell therapy is insufficient trafficking of effector T cells to tumor sites. The efficiency of adoptively transferred T cells infiltrating the tumor site has been found to correlate well with clinical responses in patients (141–144). Of the large number T cells expanded ex vivo and infused, only a small fraction eventually reaches the tumor site. Because γδ T cells inherently express different adhesion molecules and chemokine receptors that facilitate their migration to mucosal or epithelial tissues, these cells may penetrate mucosal-derived tumors much more efficiently than αβ T cells. For example, γδ T cells express CCR6, which is required for epidermal trafficking, and thus these cells are a logical choice for introducing CARs targeting malignant skin lesions(145). Adhesion molecule αEβ7 (CD103) is also found on 95% of iIELs and on other mucosal T cells but on only 2% of peripheral blood lymphocytes (146, 147). Nicol and colleagues have also reported that ex vivo aminobisphosphonate-activated autologous Vγ9Vδ2 T cells have an activated effector memory phenotype and express chemokine receptors predictive of homing to peripheral tissues. As a result of these phenotypic traits, adoptively transferred Vγ9Vδ2 T cells predominantly traffic to the lungs, liver, and spleen and, in some patients, to metastatic tumor sites outside these organs (148). In another study, using radioisotope-labeled human and mice γδ T cells, Beck and colleagues reported that adoptively-transferred γδ T cells localize to breast tumors in a mouse model of human breast cancer. Furthermore, their biodistribution studies showed that adoptively transferred γδ T cells traffic differently in tumor-bearing mice compared to healthy with fewer γδ T cells localizing into the spleens of tumor-bearing mice. They concluded that their findings provide a robust preclinical evidence for using ex vivo expanded adoptively transferred γδ-T cells as a form of cell-based immunotherapy for the treatment of breast cancer (149). Ali et al demonstrated that the microbial phosphoantigen (*E*)-4-hydroxy-3-methyl-but-2-enyl

pyrophosphate (HMBPP) plus IL-2 treatment of macaques induced a prolonged major expansion of circulating Vγ2Vδ2 T cells that expressed CD8 and produced cytotoxic perforin. Interestingly, HMBPP-expanded Vγ2Vδ2 T cells accumulated in the lung and lasted for 3–4 months. Lung- accumulated Vγ2Vδ2 T cells are also had an effector memory phenotype and produced considerable amounts of IFN-γ up to 15 weeks post treatment (54). Brandes et al. also showed that peripheral blood Vγ9Vδ2 T cells express CXCR4 and transiently increase its expression following phosphoantigen stimulation (150). Consequently, high production of CXCL12 by breast cancer associated fibroblasts (CAFs) or any tumor microenvironment containing CXCL12 could recruit Vγ9Vδ2 cells to the tumor site (151, 152). It should be noted that γδ T cells cannot be considered as a single group of cells; rather, the functions they carry out differ according to the tissue distribution of the cells, the structure of their antigen receptors and the local microenvironment.

Conventional therapies and γδ T cells

Interestingly, it has been shown that dermal γδ T cells are radioresistant, a quality that could permit the infusion of cells concomitantly with radiotherapy; however, this requires further study (153, 154). Ma and colleagues have reported that chemotherapy induces a rapid and prominent infiltration of IL-17–producing γδ (Vγ4 and Vγ6) T lymphocytes (γδ T17 cells) that precedes the accumulation of Tc1 CTLs within the tumor site (155). They concluded that γδ T17 cells contribute to chemotherapy-induced anticancer immune responses. Contrary to naive αβ T cells or stem central memory αβ T cells, this chemoresistant feature of γδ T could also be strategically incorporated into clinical trials in which CAR γδ T cell therapy is given in combination with chemotherapy regimens (156). Another potential advantage of γδ T cells is that unlike αβ T cells, they are not restricted to MHC, and thus utilizing engineered allogeneic donor-derived γδ T cells expressing CAR transgene could theoretically be used as an off-the-shelf universal product, though this application would be limited to very immunocompromised patients (e.g. after allogeneic stem cell transplantation) or following intensive lymphodepletion to avoid host immune rejection.

CAR γδ T optimization and manipulation

One question that has not yet been addressed is whether CAR design for γδ T cells might require optimization in light of the γδ TCR molecular structure and costimulation. It has been shown that γδ T cells express a series of costimulatory molecules such as CD28, CD27, and 4-1BB (CD137). Ribot et al showed that CD28 is constitutively expressed on γδ T cells and promotes survival and proliferation via IL-2 production (157). In another study, DeBarros and colleagues addressed the impact of CD27 costimulation on activation of human γδ T cells. They found that administration of soluble recombinant CD70 (CD27 ligand) enhanced Vγ9Vδ2 T cell expansion in vitro. Moreover, CD27 signals not only promote upregulation of Cyclin D2 and anti-apoptotic gene regulator Bcl2a1 but also induce production of high levels of IFN-γ (158). Thus, the synergy between TCR γδ and CD27 signals should be explored for clinical expansion of Vγ9Vδ2 T cells. Upon activation, γδ T cells also express CD137 (4-1BB). Intriguingly, Maniar et al. demonstrated that activated Vγ9Vδ2 T cells express high levels of CD137L, which can act as a ligand for CD137 on T and NK cells and may also have a role in Vγ9Vδ2 T cell activation, likely by reversing signal transduction (159). A similar possibility may apply for CD70, which is highly up-

regulated in Vγ9Vδ2 T cells following stimulation by phosphoantigens, but this requires further investigation. Song et al. reported that αβ T cells expressing CARs with CD27 signaling domains exhibited increased proliferation, Bcl-XL up-regulation, and resistance to apoptosis. They also showed that tumor regression effected by these cells was similar to that of CD28- or CD137-costimulated CARs, and in vivo persistence was superior to CD28 and similar to 4-1BB (160). Given the role of CD27 in γδ T cell physiology, transducing these cells with CD27-containing CARs would be an appealing strategy.

Concluding remarks

γδ T cells are a unique and conserved population of lymphocytes. The identification of tumor-expressed ligands that are recognized by these cells (but not by αβ T cells), together with their potent cytotoxic antitumor activity, have recently stimulated considerable interest in the development of γδ T cell-based immunotherapies for several types of cancers, including renal cell carcinoma, colorectal cancer, multiple myeloma, and certain leukemias. In this review, we offer the hypothesis that utilizing a γδ-derived CAR T cell product to target mucosal epithelial cancers will improve antitumor immune responses. This is because γδ T cells not only have inherent migration tropism to mucosal sites but also because NKG2D ligands expressed on tumor cells derived from these tissues can enhance the antitumor activity of the adoptively transferred T cells, potentially acting in synergy with CAR stimulation and reducing the likelihood of immune escape through antigen loss. It is worthy of note that Deniger and colleagues have observed decent antitumor immune responses with anti-CD19 CAR γδ T cells, and thus similar antitumor responses might be expected against mucosal epithelial cancers, but this remains to be investigated. As we described above, some γδ T cells have immunosuppressive function, and it may be important to eliminate some subsets such as Vδ1 from infusion products prior to administration. Recent data also suggest that the in vitro activation of human γδ T cells in response to phosphoantigens is enhanced in the absence of $CD4^{+}CD25^{high}$ regulatory T cells (Tregs), supporting the idea that diminishing Treg activity might be beneficial in CAR γδ T cell-based immunotherapy of cancers (164). Combining CAR αβ T cells with CAR γδ T cells might also enhance therapeutic efficacy due to concomitant targeting of both circulating and tissue-resident tumor cells. Additionally, because CAR γδ T cells can act as a professional antigen presenting cells, combination therapies with other modalities of immunotherapy such as checkpoint inhibitors, oncolytic viruses, vaccines, or cytokines could synergistically amplify recruitment and function of tumor-infiltrating lymphoid and non-lymphoid cells. However, little is known about the effect of tumor-infiltrating immune inhibitory cells, cytokines, and checkpoint ligands on γδ T cell antitumor activity and more investigation will be important to understand the function of γδ T and/or CAR γδ T cells in context of the tumor immunosuppressive microenvironment. Conceptualizing which tumor types are most likely to respond to CAR αβ and/or γδ T cell therapy by categorizing those tumors according to their origin and their microenvironment will help investigators choose the appropriate combinations of immunotherapy for each particular cancer. Finally, although γδ T cells are an appealing T cell subset for adoptive T cell therapy, protocols for their therapeutic use, particularly in the case of expansion in vitro to obtain sufficient cell

numbers for adoptive cell transfer, need to be optimized. We look forward to the results of future studies unlocking the promise of γδ T cells for adoptive cellular cancer therapy

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Highlights

- γδ T cells are unique and crucial cell population in mucosal epithelial microenvironment.
- Utilizing CAR γδ T would be a promising immunotherapeutic strategy at least for mucosal-derived malignant lesions.
- Engineered γδT cells would be as a new platform for adoptive T cell cancer therapy

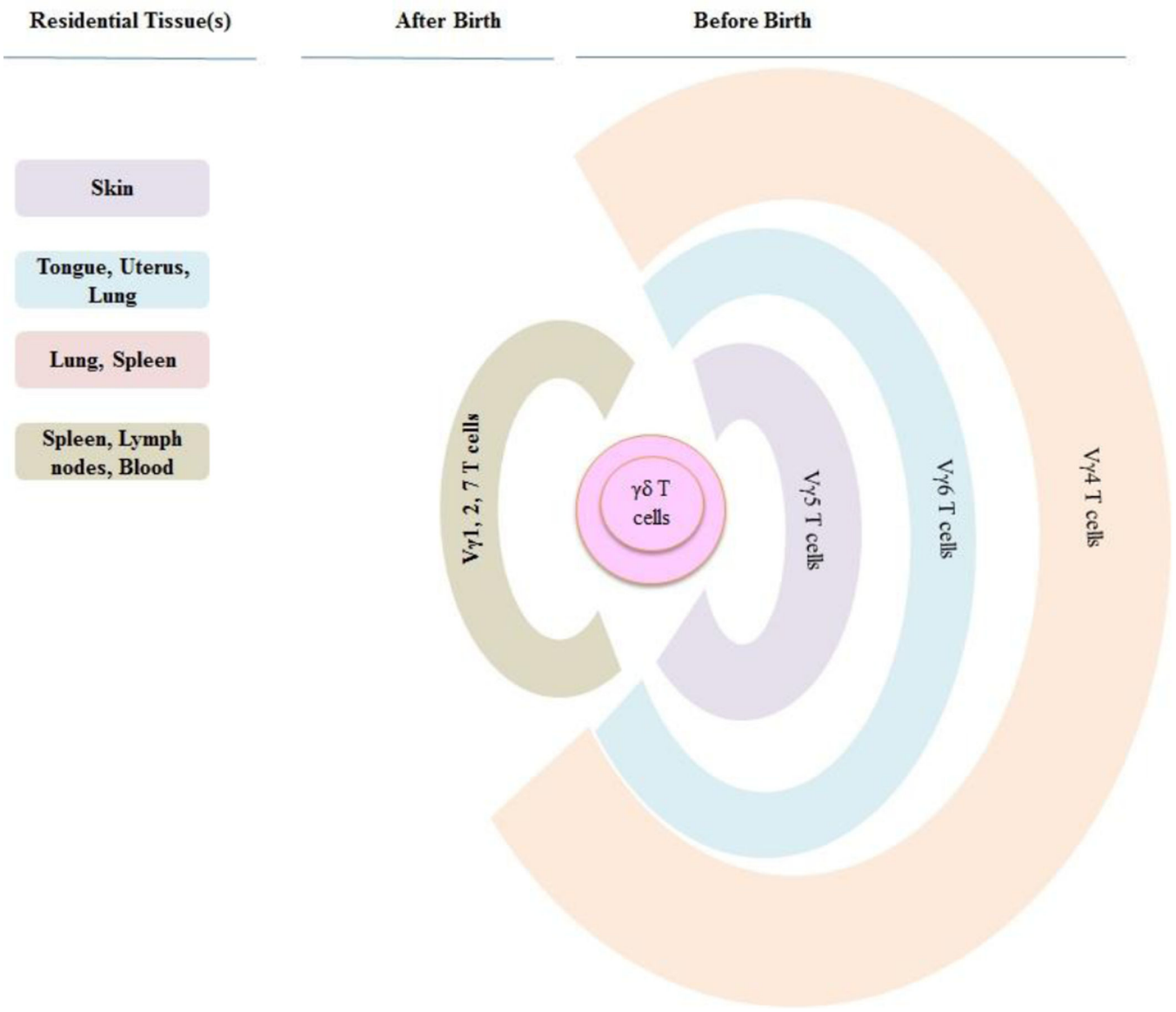

Figure 1.
Developmental waves of mouse γδ T cell generation

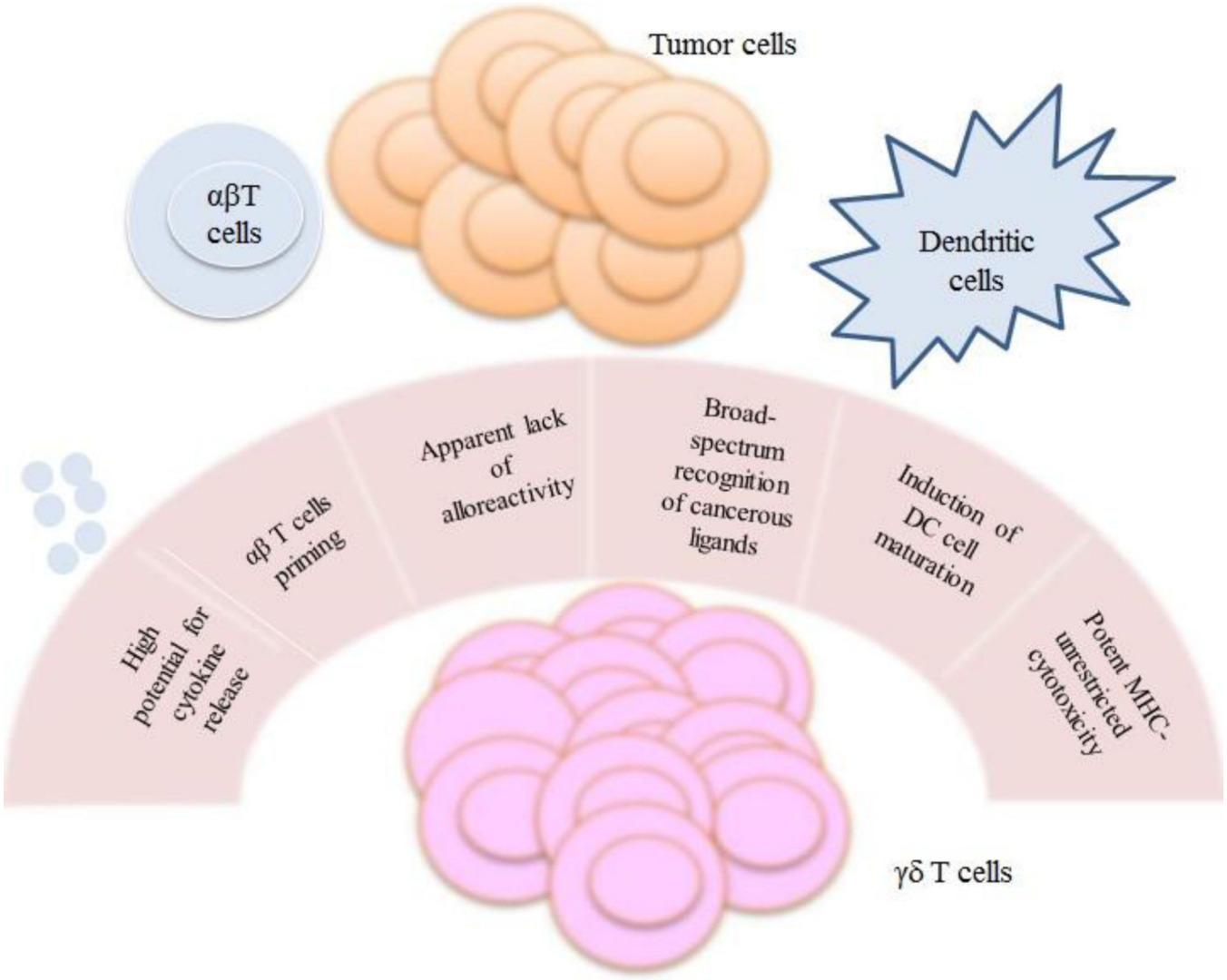

Figure 2.
Functional advantages of γδ T cells for CAR T cell cancer therapy

Table 1

human γδ T cell ligands

Subset	Antigen		Reference(s)
Vγ1.3Vδ2	Histidyl-tRNA synthetase		(51)
Vγ4Vδ5	Endothelial protein C receptor (EPCR)		(52)
Vγ9Vδ2	Alkylamines		(53)
	Phosphoantigens (pAgs)	Butyrophilin 3A1 (BTN3A1)+ Pyrophosphate molecules	(38)
		(E)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate (HMBPP)	(54)
		Isopentenyl pyrophosphate (IPP)	(55)
		Bromohydrin pyrophosphate (BrHPP)	(56)
		Endogenous mevalonate metabolites	(57)
	Agonistic monoclonal antibody (called 20.1)		(58)
	Surface mitochondrial F1-ATPase-related structure/Apolipoprotein A-I		(59, 60)
	ULBP4(a soluble isoform of RAET1E)		(61)
	Ligands on Daudi & Molt-4 cell lines		(62, 63)
	Human MutS homolog		(64)
Vδ1	Lipohexapeptides		(65)
	CD1c–expressing cells		(66)
Vγ2Vδ8(clone)	HSV glycoprotein I		(67)
Vδ1 (IELs)	MICA & MICB		(25, 68)
Vδ1 (blood γδ T cells)	CD1d–sulphatide		(69)
	CD1d-α-GalCer		(70)
Various	Phycoerythrin		(71)

HSV, herpes simplex virus; MICA, MHC class I polypeptide-related sequence A; MICB, MHC class I polypeptide-related sequence B; RAET1E, Retinoic acid early transcript 1E, ULBP4, UL16-binding protein4

Table 2

Cytokines produced by human and murine γδ T cells

Subset (Origin)	Cytokines	Reference(s)
Murine		
DETCs	IL-1α, IL-2, IL-3, IL-4, IL-6, IL-7, GM-CSF, TNFα, IFNγ, CCL3, CCL4, CCL5, XCL1	(72–74)
Lung-resident γδ T cells	IL-2, IL-4, IFNγ, IL-17, CXCL1, CXCL10	(75–77)
Peritoneal Vδ1 cells	IL-17, CCL3, CCL5	(78, 79)
Human		
Vδ1 cells	CCL3, CCL4, CCL5	(80)
Vδ2 cells	IL-2, TNFα, IL5, IL-13, IL-4, GM-CSF, CCL3, CCL4, CXCL10, CXCl13	(48, 81)
Skin Vδ2 cells	TNFα, IFNγ, IL-17, IL-8, CCL1, CCL-3, CCL-4, CCL-5	(82, 83)

CCL, CC-chemokine ligand; CXCL, CXC-chemokine ligand; DETC, dendritic epidermal γδ T cell; XCL, XC-chemokine ligand

Table 3

Summary of selected published reports of adoptive γδ T cell therapy in clinical trials

Cancers	Treatment regimen	Year reported	No. of patients	Responses	Reference
T-NHL, AML, SPL and MM	Haploidentical γδ T lymphocytes, Zoledronate, and IL-2	2014	T-NHL(1 pt), AML (1 pt), SPL(1), MM(1 pt)	CR expect for MM pt which was not evaluable	(161)
Colorectal Cancer	Zoledronate-activated, ex vivo-expanded Vγ9Vδ2 T cells	2013	6 pts	CR was observed in one pt	(162)
RCC, Melanoma and AML	Bisphosphonate-reactive γδ T lymphocytes, Low-dose IL-2 and Zoledronate	2012	RCC(7 pts), Melanoma(6 pts), AML(8 pts)	No objective response for RCC and Melanoma pts, PD for 2 AML pts	(163)
	IL-2 and Zoledronate - activated Vγ9Vδ2 T celle and	2011	Melanoma (pts), Ovarian cancer (2 pts), Cervical cancer(1 pt), Colon cancer (3 pts), Breast cancer (2 pts), Duodenal cancer (1 pt), Cholangiocarcinoma (1), Adenocarcinoma (1 pt),	Melanoma: PD (4 pts), SD (2 pts), NE(1 pt); Ovarian cancer: PD (1 pt), SD (1 pt); Cervical cancer: PD; Colon cancer: PD (3 pts); Breast Cancer: PD(1 pt), CR (1 pt); Duodenal cancer: PD; Cholangiocarcinoma: PD; Adenocarcinoma: PD	(148)
ALL and AML	Allogeneic HSCT depleted of αβT cells	2007	ALL(77 pts), AML(76 pts)	CR in 36 patients	(118)

T-NHL, T cell non-Hodgkin lymphoma; AML, acute myeloid leukemia; SPL, secondary plasma cell leukemia; MM, multiple myeloma; RCC, Renal cell carcinoma; ALL, acute lymphoblastic leukemia; HSCT, hematopoietic stem cell transplant; CR, complete response; PD, progressive disease; SD, stable disease; Pt(s), patient(s).