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# Arming V $\delta$ 2 T cells with chimeric antigen receptors to combat cancer

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## Abstract:

Immunotherapy has emerged as a promising approach in the field of cancer treatment, with chimeric antigen receptor (CAR) T cell therapy demonstrating remarkable success. However, challenges such as tumor antigen heterogeneity, immune evasion, and limited persistence of CAR-T cells have prompted the exploration of alternative cell types for CAR-based strategies. Gamma delta T cells, a unique subset of lymphocytes with inherent tumor recognition capabilities and versatile immune functions, have garnered increasing attention in recent years. In this review, we will present how arming V $\delta$ 2-T cells might be the basis for next-generation immunotherapies against solid tumors. Following a comprehensive overview of  $\gamma\delta$  T cell biology and innovative CAR engineering strategies, we will discuss the clinical potential of V $\delta$ 2 CAR-T cells to overcome the current limitations of immunotherapy in solid tumors. Indeed, while applications of V $\delta$ 2 CAR-T cells in cancer research are relatively in their infancy and many challenges are yet to be identified, V $\delta$ 2 CAR-T cells represent a promising breakthrough in cancer immunotherapy.

## Introduction

Immunotherapy has emerged as a promising approach in cancer treatment, with chimeric antigen receptor (CAR) T-cell therapy showing remarkable success in treating aggressive B-cell lymphomas, leading to durable remissions. However, these positive results are counterbalanced by serious life-threatening side effects of CAR-T cell therapies, including cytokine release syndrome (CRS) and ON-Target/OFF-Tumor toxicity. Indeed, most tumor-associated antigens (TAAs) are shared with normal cells. OFF-Tumor toxicity can have severe consequences, particularly when vital

tissues are affected. Additionally, CAR-T cells have not succeeded similarly in treating solid tumors due to antigenic heterogeneity, the immunosuppressive tumor microenvironment, and challenges related to CAR-T cell homing.

While CAR engineering has primarily focused on conventional  $\alpha\beta$  T cells, V $\delta$ 2-T cells hold promise as immune effectors for cancer immunotherapy. They are easy to manufacture and engineer and may be used in allogeneic settings. However, the applications of V $\delta$ 2-T cells in cancer research are still in the early stages, and many challenges need to be addressed. This review provides a comprehensive overview of V $\delta$ 2-T cell biology and CAR engineering, to finally highlight the potential of V $\delta$ 2 CAR-T cells as the basis for next-generation immunotherapies.

## **I- V $\delta$ 2 T cells, an ideal immune effector for cancer immunotherapy**

### **A- A bridge between innate and adaptative immunity**

#### **1- $\gamma\delta$ T cells, an unconventional population of T cell**

To defend the organism against pathogens, the immune system includes the innate and the adaptative immunity. In contrast to conventional  $\alpha\beta$  T cells which are commonly discussed when referring to T cells,  $\gamma\delta$  T cells break the dichotomy between innate and adaptative immunity. Indeed, they can rapidly initiate an immune response, without going through the expansion and differentiation phases required for the conventional T cells, to secrete a large panel of cytokines and pro-inflammatory chemokines and to collaborate and/or activate other cells of the immune system to increase the immune response against a pathogen (for review (1)).

In humans,  $\gamma\delta$  T cells represent 3-5% of the T cell population in peripheral blood and can quickly expand upon stimulation. They are the first lymphocytes to develop, even before other immune cells become functional. Their primary role is to monitor the environment and initiate immune responses against infections, cellular transformations, and tissue damage. Their abundance varies based on factors such as age, organ, and the specific  $\gamma\delta$  paired chains that form their TCR. Interestingly, their TCR is not MHC-restricted, enabling their rapid activation. They also express various activating and inhibitory NK receptors, along with different cytokine receptors like IL-2R and IL-15R, which finely regulate their immune activation. Once activated,  $\gamma\delta$  T cells exhibit similar cytotoxic activity to  $\alpha\beta$  T cells, releasing soluble mediators such as perforin and granzymes, as well as pro-inflammatory cytokines. They can also display a regulatory phenotype when exposed to immunosuppressive cytokines like IL-10 and TGF- $\beta$ , and perform immunosuppressive functions by directly secreting these cytokines. Moreover,  $\gamma\delta$  T cells can function as antigen-presenting cells (APC) and prime immature dendritic cells and naive  $\alpha\beta$  T cells following the phagocytosis of apoptotic or live infected or cancer cells (2). They also express classic APC markers including MHC, CD80, CD86, or CD11a.

## 2- V $\delta$ 2-T cells, the standard-bearer of $\gamma\delta$ T cells

More than 90% of  $\gamma\delta$  T cells in peripheral blood express the V $\delta$ 2 chain, almost exclusively paired with the V $\gamma$ 9 chain. These cells can be found at various stages of differentiation, including naive and central memory T<sub>CM</sub> states when in circulation. Once at the tumor or infection site, they transition into effector memory T<sub>EM</sub> and resident memory T<sub>RM</sub> population states.

V $\delta$ 2-T cells are activated by non-peptidic phosphorylated molecules called phosphoantigens (pAg), produced by bacteria and eukaryotic cells (Figure 1). Among these pAgs, isopentenyl pyrophosphate (IPP) is generated from the endogenous mevalonate pathway of cholesterol synthesis. Synthetic or bacterial IPP, such as BrHPP or HMBPP respectively, are more efficient at stimulating V $\delta$ 2-T cells compared to endogenous IPP. Aminobisphosphonates (ABPs), which disrupt the mevalonate pathway, lead to IPP accumulation and subsequent activation of V $\delta$ 2-T cells. The discovery of the pivotal role played by butyrophilin BTN3A1 has significantly advanced our understanding of pAg-mediated activation. BTN3A1, a type-1 glycoprotein structurally similar to molecules of the B7 receptor family, is expressed on the surface of target cells. Its intracellular domain is necessary for sensing pAg and forms a complex with butyrophilin BTN2A1, which results in conformational changes that enable direct interaction with the V $\delta$ 2-TCR (3–5). However, it remains unclear whether pAg recognition follows a classic antigenic presentation or an allosteric model, and whether the BTN3A1/BTN2A1 complex is sufficient to activate the V $\delta$ 2-TCR or if it requires another unidentified ligand (3).

In addition to the TCR, V $\delta$ 2-T cells express secondary receptors that modulate their activation, including NK receptors like NKG2D and DNAM-1. Upon recognizing their respective ligands, these receptors enhance V $\delta$ 2-T cell activation by providing a co-stimulatory signal. Whether these receptors can directly activate  $\gamma\delta$  T cells or if TCR engagement is required is still debated. V $\delta$ 2-T cells also express inhibitory receptors like NKG2A and immune checkpoint proteins, which play a role in immune tolerance to spare normal cells.

## **B- V $\delta$ 2-T cells, a key player in cancer immunosurveillance**

### 1- Lessons from murine models

Despite the inability of murine  $\gamma\delta$  T cells to recognize pAgs, several preclinical studies have highlighted the protective role of  $\gamma\delta$  T cells in tumor development. Initial studies revealed that mice are prone to develop tumors following the genetic deletion of  $\gamma\delta$  T cells (6). Further studies demonstrate a protective effect of  $\gamma\delta$  T cells against spontaneous B cell lymphomas (7), prostate cancer (8), and in the widely used B16-F0 melanoma model (9). A therapeutic effect was even demonstrated following the adoptive transfer of human  $\gamma\delta$  T cells into mice bearing established adenocarcinomas of the prostate (8), B16-F0 melanomas (10), and glioblastoma (11). In most studies, the protective role of  $\gamma\delta$  T cells against tumor progression involved the TCR engagement and/or the NKG2D pathway (6,11). Accordingly, NKG2D-deficient mice exhibit a higher susceptibility to cancer (12).

### 2- V $\delta$ 2-T cells, a first-line defense in cancer immune response

The innate ability of  $\gamma\delta$  T cells to recognize tumor cells has garnered significant attention as a potential approach to harness their potency in cancer immunotherapy. Furthermore,  $\gamma\delta$  T cells have a natural tendency to infiltrate tumor sites and are frequently observed among tumor-infiltrating lymphocytes (TILs). Intriguingly, an extensive pan-cancer study analysis using CIBERSORT revealed that  $\gamma\delta$  T cells are the immune subset most significantly associated with favorable overall survival across 25 different cancer types (13). However, this study also highlighted significant variability in tumoral  $\gamma\delta$  T cell infiltration and some challenges in distinguishing between  $\gamma\delta$  T cells, natural killer (NK) cells, and other T cell subsets. Further computational analysis revealed that the abundance of V $\delta$ 2-TILs is associated with a favorable outcome in various hematological and solid tumors (14). Additionally, high circulating levels of V $\delta$ 2-T cells have been linked to reduced cancer risks and improved survival in certain patients with acute leukemia (15).

### 3- Current evaluation of V $\delta$ 2-T cells in cancer immunotherapy

Given their broad and potent recognition of tumor cells and their potential for allogeneic use,  $\gamma\delta$  T cells are highly attractive immune effector candidates across a wide range of tumors and patients with diverse MHC alleles. Furthermore, abnormal activity of HMG-CoA reductase, the rate-limiting enzyme in the mevalonate pathway, has been observed in various tumors, leading to the accumulation of IPP involved in V $\delta$ 2-T cell activation (16). Consequently, several clinical trials have sought to activate V $\delta$ 2-T cells *in vivo* by either systemically delivering ABP or *ex-vivo* expanding autologous V $\delta$ 2-T cells from patient peripheral blood followed by adoptive cell transfer (ACT) (Table 1) (17). These strategies have been explored against multiple cancers including multiple myeloma, non-Hodgkin lymphoma, acute myeloid leukemia, prostate cancer, renal cell carcinoma, colorectal cancer, breast cancer, melanoma, or neuroblastoma (17). ABP administration is generally well-tolerated, increases the number of circulating V $\delta$ 2-T cells, and triggers various clinical responses among patients. In some cases, autologous V $\delta$ 2-T cell ACT following *ex-vivo* expansion, has effectively controlled or reduced tumor progression. However, ABPs are rapidly eliminated from circulation through renal excretion and bone absorption. Additionally, the activation of V $\delta$ 2-T cells by ABP appears to induce exhaustion in preclinical primate models and clinical trials (18). Furthermore, little is known about the ability of systemic ABP to activate V $\delta$ 2-T cells within the tumor microenvironment. Finally, few clinical trials have been initiated involving an allogeneic ACT of  $\gamma\delta$  T cells. Only two studies have been published, reporting the clinical safety and feasibility of this approach in patients with refractory/relapsed acute myeloid leukemia (19) and hepatocarcinoma (20).

## **II- Manufacturing and engineering of V $\delta$ 2 T cells**

The allogenic potential of V $\delta$ 2 T cells allows their expansion from peripheral blood cells from healthy donors rather than cancer patients, facilitating their manufacturing, engineering, and administration strategy, as well as the establishment of a bank that would be readily available anytime for cancer patient infusion.

### **A- Manufacturing of V $\delta$ 2 CAR-T cells**

Given their broad and potent recognition of tumor cells and their potential for allogeneic use, V $\delta$ 2 T cells are highly attractive immune effector candidates across a wide range of tumors and patients with diverse MHC alleles. Indeed, one of their main advantages is the establishment of a bank from healthy donors that could be readily available anytime for cancer patient infusion. V $\delta$ 2 T cells are commonly expanded from freshly isolated PBMC from healthy donors in the presence of a combination of IL-2 with natural or synthetic pAg agonists such as HMBPP or BrHPP. Globally, within 2 weeks, at least  $10^8$  V $\delta$ 2-T cells can be generated from whole blood-derived PBMC (<10 ml) (21). These cells showed potent cytotoxic and anti-tumor activity *in vitro* against numerous tumor cell types. Further studies reported that the combination of pAg agonists with other cytokines such as IL-15 or IL-21 enhances anti-tumor cytotoxicity of V $\delta$ 2 T cells (22,23). Other protocols expanded V $\delta$ 2 T cells using zoledronate and IL-2 (24). More recently, Xu *et al.* developed a new V $\delta$ 2 T cell expansion protocol involving incubation with zoledronate, IL-2, IL-15, and vitamin C, which resulted in improved cell proliferation, differentiation, cytotoxicity, and persistence when injected in murine xenograft models (25). In addition, the feeder cell-based expansion method for V $\delta$ 2 T cells has also been investigated. In particular, the presence of MHC-II+ cells or the depletion of  $\alpha\beta$  T cells seems to improve *in vitro* expansion of V $\delta$ 2 T cells (26,27).

However, the success of an expansion cannot be predicted based on initial  $\gamma\delta$  T cell percentages (28), highlighting the complexity of finding optimal donors for expansion. Several studies revealed an interindividual V $\delta$ 2 subpopulation heterogeneity among healthy individuals, including variable frequency of proliferative versus cytotoxic V $\delta$ 2 functional subpopulations (29). In line with these results, we reported that *in vitro* expansion of V $\delta$ 2 T cells resulted in highly cytotoxic or poorly cytotoxic V $\delta$ 2 T cells against glioblastoma cells, depending on the donor (11). Long-term persistence of adoptively transferred V $\delta$ 2 T cells could also be a problem, as reported for the transfer of NK cells which are under the regulatory control of comparable receptors (30). Finally, while the absence of MHC restriction prevents GVHD,  $\gamma\delta$  T cells express HLA molecules that can be recognized by the host immune system, which may result in a host vs graft effect reducing  $\gamma\delta$  T cells persistence and efficacy *in situ*.

## B- CAR engineering of T cells

Most authorized CAR-T cell therapies are developed from autologous  $\alpha\beta$  T cells obtained from patients through leukapheresis. Nevertheless, *in vitro* studies have demonstrated the feasibility of CAR transduction in V $\delta$ 2-T cells and their ability to exhibit TAA-targeted cytotoxicity similar to conventional T cells (31–34). Here, we will report the main developments in CAR-T cell engineering.

### 1- Conventional CAR-T cell backbone

CARs are synthetic receptors that redirect antigen recognition and trigger T cell activation by merging an extracellular antigen-binding component with an intracellular signaling domain. While CAR generation is empiric, its design is key in balancing immunotherapy efficacy and toxicity (Figure 2). Commonly, CARs are composed of an antigen-binding domain commonly derived from the single-chain fragment variable (scFv) of an antibody, a costimulatory domain and an intracellular domain typically deriving from the CD3 $\zeta$  chain of the TCR. All domains impact CAR efficacy. For example, the scFv can induce intrinsic CAR-T cell tonic signaling and exhaustion while other studies demonstrate a close relationship between CAR affinity, safety, and efficacy (35–

38). Replacing CD3 $\zeta$  with other proximal signaling molecules or altering the number of ITAM present in the CD3 $\zeta$  intracellular domain improves conventional CAR-T cell cytotoxicity and persistence (39,40). The costimulatory domains also play a crucial role with the CD28 costimulatory domain conferring potent and immediate antitumoral cytotoxicity, while the 4-1BB domain enhances CAR-T cell persistence (37,41,42). Whether these costimulatory domains are the best ones for V $\delta$ 2 T cell engineering has to be investigated. Finally, CARs can also be engineered to secrete various cytokines at the tumor site, to improve their persistence and antitumor effects. These TRUCK-CARs are being evaluated in clinical trials (NCT03721068)(43).

## 2- Enhancing conventional CAR-T cell potential by Boolean logic-gating CAR

In solid tumors, CARs targeting a single antigen often prove ineffective. The main current limitations of CAR immunotherapies include the heterogeneity and loss of the target antigen as well as ON-Target/OFF-Tumor toxicity. Therefore, various systems have been developed to enhance tumor targeting while minimizing normal tissue toxicity.

### a. OR-gated strategy to widen tumor targeting

Targeting simultaneously multiple antigens can be achieved through (1) the co-administration of several CAR-T cell products, (2) the co-transduction of T cells with vectors encoding different CARs, (3) the transduction of T cells with a bicistronic vector encoding two CARs, or the use of a bispecific CAR where several scFvs are incorporated into one CAR (Figure 2). Co-administration or co-transduction allows the combination of several vectors, requires minimal optimization but results in a heterogeneous mixed product with a more complex and costly manufacturing procedure. In contrast, bicistronic and bispecific CARs are cheaper to manufacture, and generate more uniform products but their design makes it more challenging to maintain their multiple functionalities (44,45). Indeed, each scFv must not interfere with others, and the risk of cumulative side-effects minimized. To encompass constant re-engineering, SUPRA-CAR has been developed offering enhanced flexibility, specificity, and controllability (46). SUPRA-CAR features an RR-leucine-zipper in the CAR (zipCAR) and a scFv-adaptor linked to an EE-leucine-zipper (zipFv). The ZipFv recognizes its specific TAA and dimerizes with the zipCAR to activate the CAR. This simplified approach of multiple targeting only requires the generation of various zipFvs.

Several preclinical studies are actively exploring multiple targeting strategies. A recent study conducted a head-to-head investigation of mono- and dual-targeting conventional CAR-T cells against BCMA and GPRC5D in a preclinical model of multiple myeloma where they assessed the morbidity of tumor-bearing mice following (1) administration of either BCMA-CAR and/or GPRC5D-CAR-T cells, (2) bicistronic BCMA/GPRC5D CAR-T cells, and (3) bispecific CAR-T cells expressing both scFvs on a single CAR (47). While all strategies effectively eliminated tumor cells and resulted in long-term survival, the bicistronic CARs and co-administration approaches exhibited superior efficacy. Similar preclinical results have been observed in solid tumors with dual targeting of various antigen pairs, some of them demonstrating limited immune evasion and persistent T cells with limited exhaustion (45,48–50). Finally, efficient targeting of three different antigens was demonstrated in several preclinical models (46,51,52).

The safety of multiple targeting has also been demonstrated in several clinical studies including patients with B-cell malignancies with an initial remission rate of approximately 80% within the first 6 months. However, all patients experienced tumor relapse, despite a reduction in antigen-negative relapse (53). A phase 1 study is underway, evaluating a tri-specific approach involving the co-administration of bispecific CD19/CD20 CAR-T cells with CD22-CAR-T cells.

*b. AND-gated strategies to limit ON-target/OFF-tumor toxicity*

To enhance tumor specificity, a Boolean AND-gated strategy can be achieved where the presence of all targeted TAAs is required. Roybal *et al.* developed a synthetic Notch receptor (synNotch), which, upon engagement of a "gate" TAA, drives the transcription of a CAR targeting another TAA (54). The Split-CAR also restricts T cell activity to tumor cells through combinatorial antigens recognition since the activation and the costimulatory domains are provided by two distinct structures, each targeting a different TAA (55–57). Split-CARs have been further refined into co-LOCKR-CARs, by exploiting the reversible interaction between two colocalized proteins (58). Lastly, exploiting the fine dissection of intracellular proximal TCR signaling molecules, a LINK-CAR has been developed, incorporating LAT and SLP76 fused to CD28 and CD8 respectively. While all CAR-T cells efficiently eradicate tumor cells without toxicity to normal tissues, the LINK-CAR outperforms all other Boolean-gated CARs in ROR1<sup>+</sup>-tumor models, one of the best preclinical models to investigate ON-Target/OFF-Tumor toxicity (40,59).

An alternative approach to enhance tumor specificity is to use an AND-NOT-gated strategy, often referred to as NOT-gated, in which a CAR is expressed with an inhibitory CAR targeting an antigen present on healthy tissues but not on the tumor (Figure 2). While this strategy is barely investigated, a proof-of-concept has been recently provided (60).

3- Current V $\delta$ 2 CAR-T cell approaches

CAR engineering in V $\delta$ 2-T cells was reported two decades ago (61). V $\delta$ 2-T cells were expanded from the peripheral blood of healthy donors and efficiently transduced with recombinant retrovirus encoding GD2- or CD19-CARs. These V $\delta$ 2 CAR-T cells efficiently recognized and killed antigen-expressing tumor cells (61). Interestingly, and unlike CD19-CAR conventional T cells, CD19-CAR V $\delta$ 2 T cells were also able to kill CD19-negative leukemia cells when the endogenous production of pAg was enhanced by ABP, or tumor cells lacking MHC-I expression (32,62). More recently, V $\delta$ 2 CAR-T cells generated from iPSC demonstrated sustained tumor cytotoxicity in the presence of IL-15 (63). Finally, we recently demonstrated that V $\delta$ 2 CAR-T cells exhibited similar cytotoxicity to conventional CAR-T cells against pediatric high-grade glioma (BioRxiv 2023.11.17. 567375v1). Importantly, we also highlighted their promising allogeneic settings using 3D tumor models.



Two clinical approaches of ACT with allogeneic V $\delta$ 2 CAR-T cells have recently been initiated (Table 1). Most studies include patients with B-cell malignancies and investigate ACT of allogeneic V $\delta$ 2-T cells expressing anti-CD20-CAR. Initial results reported that these V $\delta$ 2 CAR-T cells were well tolerated, with a favorable safety profile and encouraging preliminary efficacy (64). The other approach, conducted in Singapore, investigates the safety and tolerability of NKG2L-CAR V $\delta$ 2-T cells in patients with relapsed or refractory tumors of different types. No published results are currently available.

Collectively, these data have created considerable enthusiasm regarding the clinical application of V $\delta$ 2 CAR-T cells, particularly in an allogeneic setting. In particular, the application of conventional CAR engineering to these cells should improve immunotherapy efficacy. While further development is required for Boolean logic-gating CAR with conventional T cells, such strategies should also be applied to V $\delta$ 2-T cell immunotherapy. Recently, V $\delta$ 1-T cells have also been explored for therapeutic purposes owing to the recent development of a safe clinical-grade protocol to expand them (65). These DOT cells (Delta One T cells) showed potent and specific cytotoxicity against AML and Glioblastoma cells *in vitro*. They recently entered clinical testing in patients with AML (NCT05001451; Table 1).

### **III- V $\delta$ 2 CAR-T cells as the basis for next-generation immunotherapies against solid tumors**

Most current immunotherapies utilize the patient immune system to eradicate cancer and involve *ex vivo* expansion of patient lymphocytes (TIL) before ACT. Given the ability to engineer V $\delta$ 2 T cells, using V $\delta$ 2 CAR-T cells rather than conventional  $\alpha\beta$  CAR-T cells brings numerous advantages, in particular (1) an innate OR-gated strategy through their TCR, NKG2D diversity, and/or CAR engineering, and (2) an allogeneic setting of ACT and off-the-shelf CAR-T cells (Table 2).

#### **A- Multiple immunoreactivities of V $\delta$ 2 CAR-T cells to overcome tumor heterogeneity**

Solid tumors are characterized by strong inter- and intra-patient heterogeneities that definitively limit actual CAR-T cell therapies, which is even reinforced by the loss of the targeted TAA expression following CAR-T cell therapy. In this context, using V $\delta$ 2 CAR-T cells rather than conventional  $\alpha\beta$  CAR-T cells may prevent tumor escape caused by antigen loss or downregulation observed with conventional CAR-T cell therapy. Indeed, V $\delta$ 2 CAR-T cells can recognize a myriad of TAAs through their TCR, and NKG2D receptors. This diversity of ligands combined with their differential regulation makes V $\delta$ 2 T cells sensitive to a wider range of tumor signals and more alert to tumor immune evasion (66). As stated before, tumor cells exhibit a deregulated mevalonate pathway leading to V $\delta$ 2 T cell activation (16). Numerous studies have reported that BTN3A1, along with various NKG2D ligands, are commonly expressed in cancer (11,34,67,68). The importance of the NKG2D pathway in tumor immunosurveillance has been demonstrated through both preclinical and clinical studies involving conventional NKG2D-CAR-T cells (50,69,70). These cells can migrate to tumor sites, extending the survival of tumor-bearing mice and providing protection against tumor rechallenge (71). Notably, some tumor cells shed NKG2D ligands from their surface to evade immunosurveillance (67,72). Accordingly, elevated serum concentrations of shed MICA

have been associated with disease progression in numerous human cancers (73). Interestingly, inhibition of this shedding with specific antibodies has shown a significant survival benefit in humanized metastasis models (73).

This innate immunoreactivity against a wide range of tumor cells from patients with diverse MHC alleles may be further complemented with CAR engineering, as with conventional CAR-T cells. As stated above, preclinical studies have reported a dual reactivity of V $\delta$ 2 CD19-CAR-T cells, engaging both their CAR and TCR, against leukemia cells in the presence of ABP (32). This dual reactivity was confirmed with the demonstration that CAR and TCR form distinct signaling synapses, and even collaborate in CAR-T cell activation (74). Interestingly, V $\delta$ 2 CD19-CAR-T cells were also able to kill CD19-negative leukemia cells when the endogenous production of pAg was enhanced by ABP (32). Consequently, multiple groups are currently working to improve the biodistribution of ABP to enhance V $\delta$ 2-CAR-T cell efficacy. The current urgent need is the identification of relevant TAAs. (for review see (75)). TRUCK-CAR engineering might also be important to consider to improve V $\delta$ 2 T cell amplification and/or immunoreactivity.

## **B- Limiting the immunosuppressive tumor microenvironment with V $\delta$ 2 CAR-T cells**

V $\delta$ 2 T cells are actively recruited within the tumor microenvironment (TME) and have the potential to kill tumor cells. However, the TME also attenuates V $\delta$ 2 T cell responses in multiple ways. A major challenge is thus to limit this TME-induced immunosuppression.

### **1- Cytokinic immunosuppressive microenvironment**

The TME of solid tumors is mainly dominated by pro-inflammatory cytokines such as TNF- $\alpha$ , IL-1, and IL-6, which promote tumor cell proliferation and recruit immunosuppressive cells including regulatory T cells (Treg), M2-polarized tumor-associated macrophages (M2-TAM), and immature myeloid cells (MDSC). These cells produced various immunosuppressive cytokines, including TGF- $\beta$ , IL-4, or IL-10 involved in tumor escape. Accumulation of Treg and M2-TAM also correlates with a poor prognosis. Accordingly, the depletion of endogenous lymphocytes through chemotherapy improves ACT clinical responses (76,77). Thus, chemotherapy or radiotherapy might be required for optimal clinical responses of ACT in patients, for those who can support such conditioning regimens.

TRUCK-CAR engineering should also be considered to improve V $\delta$ 2-T cell amplification and/or immunoreactivity. Indeed, several studies revealed that infusion of conventional CAR-T cells secreting IL-12 or IL-18 prevents Treg- and M2-TAM immunosuppression while promoting their activation and recruitment (77–79). CAR-T cells secreting both IL-7 and CCL19 also showed superior tumor suppression to conventional CAR-T cells in several murine models (80,81). Based on these results, a phase 1 clinical trial has been initiated on patients with advanced carcinoma (NCT03198546). All these strategies currently developed using conventional T cells can be applied to V $\delta$ 2 T cells. Finally, as potent activators of V $\delta$ 2-T cells, both IL-15 and IL-21 could be considered to be secreted by V $\delta$ 2 CAR-T cells (22,23). A major concern with IL-15 or IL-21 is an enhanced proliferation of V $\delta$ 2 CAR-T cells leading to the production of toxic levels of IFN $\gamma$  and TNF $\alpha$  which might favor the incidence of Cytokine Release Syndrome (CRS), a potential fatal

adverse effect of CAR-T cell immunotherapy. In this context, the generation of V $\delta$ 2 CAR-T cells secreting CK in an inducible manner may guarantee the safety of this strategy.

## 2- Metabolic immunosuppressive microenvironment

The TME exhibits abnormal metabolic characteristics, including hypoxia, acidic conditions, and limited glucose availability, due to dysfunctional blood vessels and high nutrient demand of tumor cells. This metabolic environment hampers T cell function while promoting Treg differentiation and M2-TAM polarization (82–85). Hypoxia further reduces V $\delta$ 2-T cell cytotoxicity and persistence while enhancing Treg activity (86–88). L-arginine and lipid metabolism also play vital roles in modulating V $\delta$ 2-T cell activity (89).

In this context, metabolic inhibitors may improve V $\delta$ 2-T cell function. The hyperactive mevalonate metabolism in tumor cells may counterbalance the reduction in V $\delta$ 2-T cell cytotoxicity due to LDL binding on their receptors upon activation (90). Metformin, which preferentially inhibits mitochondrial oxidative phosphorylation in tumor cells, effectively reduces tumor hypoxia and specifically restores V $\delta$ 2-T cell cytotoxicity against tumor cells in preclinical models (87). Additionally, PI3K inhibitors reduce Treg and M2-TAM polarization while improving CAR-T cell efficacy and persistence in preclinical studies leading to its clinical evaluation (91,92).

To optimize V $\delta$ 2 CAR-T cell functionality, CAR design is crucial for achieving rapid tumor regression and sustained immunosurveillance. A dual CAR design incorporating both CD28 and 4-1BB costimulatory domains holds promise, especially in cases of heterogeneous tumor antigen expression (93). Finally, the genetic engineering of CAR-T cells can be harnessed to confer metabolic advantages, as demonstrated with enzymes involved in arginine metabolism (94).

## 3- Recruitment of V $\delta$ 2 CAR-T cells

Patient data and *in vivo* imaging revealed that CAR-T cells primarily circulate in the bloodstream but struggle to infiltrate solid tumors due to their dense extracellular matrix (ECM) and cancer-associated fibroblasts (CAFs)(95). Through the production of the ECM, CAFs are a shell that traps T cells and inhibits their homing to and infiltration within the tumor (95) Depletion of CAF enhances anti-tumor immunity in murine models but was not successful in clinics (96). Re-expression of heparanase in engineered T cells enhances CAR-T cell infiltration, improving survival in murine models (97). Heparanase may also improve tumor homing and infiltration by facilitating chemokine release. Excluded cytotoxic T cells also upregulate CTLA-4, which modifies integrin expression reducing their migration. Accordingly, anti-CTLA-4 antibodies boost T-cell motility (98) but also increase V $\delta$ 2-T cell frequency in some patients, adding another benefit of V $\delta$ 2 CAR-T cell immunotherapy (99).

Chemokines and cytokines, such as TGF- $\beta$  and soluble NKG2D ligands secreted by CAFs, are also interfering with V $\delta$ 2 T cell proliferation, function, and recruitment (100). Mismatched chemokines and TCR also hinder CAR-T cell homing. Tumor-produced CCL22 or CCL28 favor Treg recruitment and M2-TAM polarization, leading to TGF- $\beta$  secretion, CAF differentiation, and T cell exclusion. Interestingly, blocking NOX4, a TGF- $\beta$  downstream target, reverses CAF differentiation and promotes T cell infiltration (95) while reducing TGF- $\beta$  interference with V $\delta$ 2-T

cell function (100). Expression of specific receptors (CXCR2, CCR2, CCR4) and chemokines (IL-7, CCL19) by CAR-T cells also enhances tumor homing and infiltration without compromising cytotoxicity (80,101). It is also noteworthy that IPP released by tumor cells treated with ABP can act as a chemotactic molecule on V $\delta$ 2 T cells, a function that could help their tumor homing, even though this has yet to be confirmed *in vivo*.

Considering the challenging weak immune cell infiltration into tumors, the mode of CAR-T cell administration is crucial. Clinical trials have shown that locoregional delivery is a safe and effective approach (NCT00730613, NCT01082926). Additionally, engineered biomaterial delivery systems for CAR-T cells are under investigation. For example, bioactive polymer implants acting as reservoirs, releasing CAR-T cells as the material biodegrades or bioactive carriers delivering, expanding, and dispersing tumor-reactive T cells have been developed (102,103). Both approaches have shown better tumor regression in preclinical models compared to systemic lymphocyte injections. These implants can also release vaccine adjuvants to activate robust T cell responses. For example, combining CAR-T cells with STING agonists in biomaterial implants triggers a synergistic activation of host APC and CAR-T cells, leading to local tumor elimination, systemic antitumor immunity, and protection against tumor rechallenge (103). This approach could potentially be employed to deliver additional therapies, like ABP, to reinvigorate V $\delta$ 2 CAR-T cells, enhance their infiltration, and boost their immunoreactivity.

## **C- Combining V $\delta$ 2 CAR-T cells with conventional treatments**

### **1- Combination with standard-of-care therapies**

Radiotherapy remains a standard cancer treatment, including against solid tumors. This treatment can trigger various potent antitumor immune responses, including unmasking hidden tumor antigens, generating neoantigens, and inducing the expression of NKG2D ligands (104). A recent study highlighted the synergistic effect of combining radiotherapy with conventional NKG2D-CAR-T cells in a preclinical model (71). This combination enhanced CAR-T cell homing to the tumor site and increased their cytotoxicity, leading to long-term protection against tumor rechallenge. Retrospective analyses have also indicated that radiotherapy can improve local tumor control (105). Similar benefits have been observed with chemotherapy, which not only induces the rapid infiltration of  $\gamma\delta$  T cells into the tumor but also results in a significant increase in tumor cell death in various preclinical models (106–108). Engineered drug-resistant V $\delta$ 2-T cells have even been designed to prevent their elimination by chemotherapy (106). The feasibility of combining this approach with V $\delta$ 2 CAR-T cells is currently under investigation in a clinical setting, and the initial results have yet to be reported (NCT04165941).

### **2- Combination with Immune Checkpoint Inhibitors**

The discovery of immune checkpoint proteins and their role in tumor tolerance and T cell exhaustion has led to significant advancements in cancer immunotherapy. Clinical trials have been exploring the use of immune checkpoint inhibitors (ICI) to reinvigorate preexisting antitumor T-cell responses (109–111). Treatments with ICIs, such as PD-1, PD-L1, and CTLA-4 inhibitors, have been approved for various cancer types and have shown promise in reactivating the immune

response against tumors (111). Combining ICI with CAR-T cell therapies is under active clinical evaluation with promising results (NCT02706405, NCT03310619, NCT02926833, NCT03287817). However, many patients do not respond to ICI treatment and experience tumor relapse within two years. In this context, V $\delta$ 2 CAR-T cells could be designed to secrete antibodies against immune checkpoints like PD-L1 or PD-1, a strategy that has shown promise in preclinical studies with conventional CAR-T cells. A recent clinical study, involving CD19/PD-1-CAR-T cells in 17 patients with PD-L1+ B lymphoma, has demonstrated the feasibility and potential efficacy of this approach (111). As a result, numerous clinical trials are currently recruiting to explore the potential of autologous CAR-T cells that express immune checkpoint antibodies (Table 3). Interestingly, in a model of EBV-driven B cell lymphoma, while conventional T cell responses were held in check by inhibitory ligands and required co-administration of anti-PD-1 and anti-CTLA4 antibodies to reduce tumor burden, a single dose of V $\delta$ 2 T cells almost completely prevented the outgrowth of tumors (112).

### Conclusions

Recent advances in cancer immunotherapy have revolutionized the treatment of several cancers. However, further strategies are definitively required to fully realize the potential of cancer immunotherapy. V $\delta$ 2-T cells, with their unique characteristics, present promising opportunities for cancer immunotherapy. The combination of the anti-tumor-directed V $\delta$ 2 TCR with the combination of intrinsic NK cell properties and CAR engineering make them attractive candidates for fighting solid tumors. These features are reinforced with their potential for allogeneic use, and their banking feasibility. Clinical trials in the past have shown that ACT of autologous V $\delta$ 2-T cells is well-tolerated and has the potential to trigger antitumor immune responses. Ongoing clinical trials will provide crucial results to determine the benefits of CAR-engineered V $\delta$ 2-T cells. These results will be instrumental in shaping the future of cancer immunotherapy and may open new avenues for the treatment of solid tumors.

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## Figures legends

### Figure 1 Exploiting V $\delta$ 2 T cells particularities to their fullest.

(A) V $\delta$ 2 T cells present interesting innate and HLA-independent tumor recognition, sensitive to the intracellular concentration of phosphoantigen (pAg), that can be upregulated with zoledronate or IPP analogs. Several strategies can also be used to increase their efficacy, including (B) the increase of an NK-like response with the use of different cytokines (IL-2, IL-15, IL-21, or IFN- $\gamma$ ) or (C) engineering to increase their recognition against other antigens through the use of CAR. (D) On top of these reactivities, V $\delta$ 2 T cells exhibit CPA-like features that allow them to mature other immune cells like immature dendritic cells or naïve  $\alpha\beta$  T cells. (Adapted from an image created with BioRender.com.)

### Figure 2 Innovant strategies enhancing CAR T cell efficiency

Chimeric antigen receptors (CARs) endow T cells with customizable antigen recognition depending on scFv specificity and antigen-binding properties. While the generation of optimized CAR T therapies is empiric, its design is key in balancing immunotherapy efficacy and toxicity (A). It relies on fine-tuning CAR affinity through scFv design, enhancing T-cell survival and persistence through the costimulatory domains and the transducing domains. Current CAR T cell improvements include multiple targeting (B) and reducing ON-Target/OFF-Tumor toxicity (C) using various boolean-gated strategies. Multiple targeting may be achieved by an OR-gated strategy through the co-administration of several CAR T cell products, the transduction of T cells with a bicistronic vector encoding two CARs, or the use of a bispecific CAR where several scFvs are incorporated into one CAR. To reduce ON-Target/OFF-Tumor toxicity, CAR specificity may be strengthened through AND-gated CAR strategies (SynNotch-CAR, co-LOCKR-CAR, Split-CAR, or LINK-CAR) or AND-NOT-gated strategies. Finally, the SUPRA-CAR features an RR-leucine-zipper in the CAR (zipCAR) and a tumor-targeting scFv-adaptor linked to an EE-leucine-zipper (zipFv). This approach only requires the generation of various zipFvs and might be applied to various boolean-gated strategies (OR-, NOT-, and AND-gated strategies).

SUPRA-CAR: Split, universal, and programmable CAR system; TRUCKs: T-cell redirected for antigen-unrestricted cytokine-initiated killing CARs

(Adapted from an image created with BioRender.com.)

Table 1 Immunotherapy with  $\gamma\delta$  T cells

Study design	Patients	Reference	Outcomes
<b>Clinical trials with autologous <math>\gamma\delta</math> T cells</b>			
Phase I, single arm, to test the safety of intra-arterial injection of $\gamma\delta$ T cells	Patients with HCC in a palliative setting	NCT00562666	Completed, no published results
Observational study of ex vivo expansion of $\gamma\delta$ T cells in combination with chemotherapy	Patients with ovarian cancer	NCT01606358	Completed, no published results
Exploratory study to assess the feasibility of expansion of circulating $\gamma\delta$ T cells	Patients with newly diagnosed or relapsed/refractory AML	NCT03885076	Unknown status
Phase I, single arm, to test the safety and tolerability of $\gamma\delta$ T cells in combination with chemotherapy	Patients with newly diagnosed glioblastoma receiving maintenance chemotherapy	NCT04165941	Not yet recruiting
<b>Clinical trials with allogeneic <math>\gamma\delta</math> T cells</b>			
Phase I, single arm, to test the safety and efficacy of ex-vivo expanded allogeneic $\gamma\delta$ T-lymphocytes	Patients with active relapsed/refractory AML	NCT03790072	Completed Vydra et al 2023 showed the feasibility, safety, and tolerability of allogeneic haploidentical $\gamma\delta$ T cells, now progressing in a phase II clinical trial using an off-the-shelf, unrelated allogeneic donor source of $\gamma\delta$ T-cells (1)
Phase I/II, single arm, to test the efficacy of allogeneic $\gamma\delta$ T-lymphocytes	Patients with advanced HCC hepatocyte carcinoma	NCT05628545	Zhang et al 2021 demonstrated the clinical safety of adoptive transfer of allogeneic $\gamma\delta$ cells in combination with locoregional ablation, with encouraging clinical efficacy against HCC (2)
Phase I/II, single arm, to assess the safety, tolerability, and efficacy of allogeneic $\gamma\delta$ T cell therapy	Patients with AML patients who are MRD positive	NCT05001451	Completed, no published results
Phase I, single arm, to test the maximum tolerated dose and effectiveness of donor $\gamma\delta$ T cells	Patients with AML at high risk of relapse after allogeneic hematopoietic stem cell transplantation	NCT05886491	Active, recruiting
Phase I/II, single arm, to test the Safety and impact and/or the rate of GVHD	Patients with hematological malignancies with a partially mismatched bone marrow transplant.	NCT03533816	Active, recruiting
Phase I, single arm, to test the efficacy and safety of ex-vivo expanded allogeneic $\gamma\delta$ T lymphocytes	Patients with refractory/relapsed AML	NCT04008381	Unknown status
Phase I, single arm, to test the safety, therapy dose, and efficacy of ex-vivo expanded $\gamma\delta$ T cells in combination with chemotherapy	Children with refractory, relapsed, or progressive neuroblastoma	NCT05400603	Active, recruiting
Phase I/II, single arm, to test the safety, efficacy and tolerability of allogeneic $\gamma\delta$ T therapy	Patients with relapsed/refractory AML	NCT05886491	Not yet recruiting
<b>Clinical trials with allogeneic CAR <math>\gamma\delta</math> T cells</b>			
Phase I, single arm, to test the safety, activity and safe dose of haploidentical or allogeneic CAR-grafted $\gamma\delta$ T cells	Patients with relapsed/refractory Solid Tumor.	NCT04107142	Completed, no published results
Phase I, single arm, to evaluate the safety, tolerability, pharmacokinetics, pharmacodynamics, and efficacy of allogeneic CAR $\gamma\delta$ T-cell Therapy,	Patients with relapsed/refractory B-cell Malignancies	NCT05653271	Active, recruiting
Phase I, to test the safety and efficacy of allogeneic CAR $\gamma\delta$ T therapy	Patients with relapsed/refractory B-cell malignancies	NCT04735471	Active, recruiting
Observational study to assess long-term side effects of allogeneic $\gamma\delta$ CAR T cell infusion (safety, efficacy, pharmacokinetics and immunogenicity)	Patients with B-Cell lymphoma previously enrolled in Adicet Bio study	NCT04911478	Prospective
Phase I, single arm, to evaluate the safety, tolerability and optimal dose of allogeneic $\gamma\delta$ CAR T cell infusion	Patients with advanced cancers	NCT05302037	Not yet recruiting

AML : Acute Myeloid Leukemia ; HCC : hepatocyte carcinoma; MRD (Minimal Residual Disease)

Table 2 Comparison of V $\delta$ 2 CAR-T cell strategies with conventional CAR-T cells

Immune features	$\alpha\beta$ T	V $\delta$ 2 T
Innate immunity		
Immunosurveillance	no	yes
Early cytotoxicity	no	yes
APC	no	yes
Adaptative immunity		
Delayed cytotoxicity	yes	no
Memory phenotype	yes	yes
Antigenic diversity	high	low
Tumor targeting properties		
Innate recognition	no	yes
Exhaustion	medium	high
Regulatory phenotype	yes	yes
Tumor homing	low/ medium	low/ medium
ACT manufacturing & settings		
Autologous	yes	yes
Allogeneic	no	yes
Amplification	yes	yes
CAR engineering	yes	yes

Table 3 Clinical trials with autologous CAR T cells expressing ICIs in solid tumors

Study design	Sponsors (Reference)
CTLA-4 and PD-1 Antibodies Expressing EGFR-CAR-T Cells for EGFR Positive Advanced Solid Tumor	Shanghai Cell Therapy Research Institute, China (NCT03182816, NCT03182803), Shanghai International Medical Center, China (NCT02862028)
PD-1 Antibody Expressing CAR T Cells for Mesothelin Positive Advanced Malignancies	Ningbo Cancer Hospital, China (NCT03030001, NCT02873390)
Anti-MUC1 CAR T Cells and PD-1 Knockout Engineered T Cells for NSCLC	The First Affiliated Hospital of Guangdong Pharmaceutical University, China (NCT03525782)
CAR T and PD-1 Knockout Engineered T Cells for Esophageal Cancer	The First Affiliated Hospital of Guangdong Pharmaceutical University, China (NCT03706326)
CTLA-4 and PD-1 Antibodies Expressing MUC1-CAR-T Cells for MUC1 Positive Advanced Solid Tumor	Ningbo Cancer Hospital, China (NCT03179007)
PD-1 Knockout Anti-MUC1 CAR-T Cells in the Treatment of Advanced Breast Cancer	Sun Yat-Sen Memorial Hospital of Sun Yat-Sen University, China (NCT05812326)
PD-1 Antibody Expressing mesoCAR-T Cells for Mesothelin Positive Advanced Solid Tumor	Shanghai Cell Therapy Research Institute, China (NCT03615313)
$\alpha$ PD1-MSLN-CAR T Cells for the Treatment of MSLN-positive Advanced Solid Tumors	Wuhan Union Hospital, China (NCT04489862), Shanghai Mengchao Cancer Hospital, China (NCT05373147), Shanghai Cell Therapy Group Co.,Ltd, China (NCT04503980, NCT05089266)
Study of CRISPR-Cas9 Mediated PD-1 and TCR Gene-knocked Out Mesothelin-directed CAR-T Cells in Patients With Mesothelin Positive Multiple Solid Tumors	Chinese PLA General Hospital, China (NCT03545815)
Study of PD-1 Gene-knocked Out Mesothelin-directed CAR-T Cells With the Conditioning of PC in Mesothelin Positive Multiple Solid Tumors	Chinese PLA General Hospital, China (NCT03747965)
PD-1 Silent PSMA/PSCA Targeted CAR-T for the Treatment of Prostate Cancer	Shanghai Unicar-Therapy Bio-medicine Technology Co.,Ltd, China (NCT05732948)
Clinical Study on the Efficacy and Safety of c-Met/PD-L1 CAR-T Cell Injection in the Treatment of HCC	The Second Hospital of Nanjing Medical University, China (NCT03672305)
Dual-targeting CLDN18.2 and PD-L1 CAR-T for Patients With CLDN18.2-positive Advanced Solid Tumors	Sichuan University, China (NCT06084286)
Anti-PD-L1 Armored Anti-CD22 CAR-T/CAR-TILs Targeting Patients With Solid Tumors	Hebei Senlang Biotechnology Inc., Ltd., China (NCT04556669)
Dual-targeting HER2 and PD-L1 CAR-T for Cancers With Pleural or Peritoneal Metastasis	Sichuan University, China (NCT04684459)
Dual-targeting VEGFR1 and PD-L1 CAR-T for Cancers Patients With Pleural or Peritoneal Metastases	Sun Yat-Sen Memorial Hospital of Sun Yat-Sen University, China (NCT05812326)

Figure 1

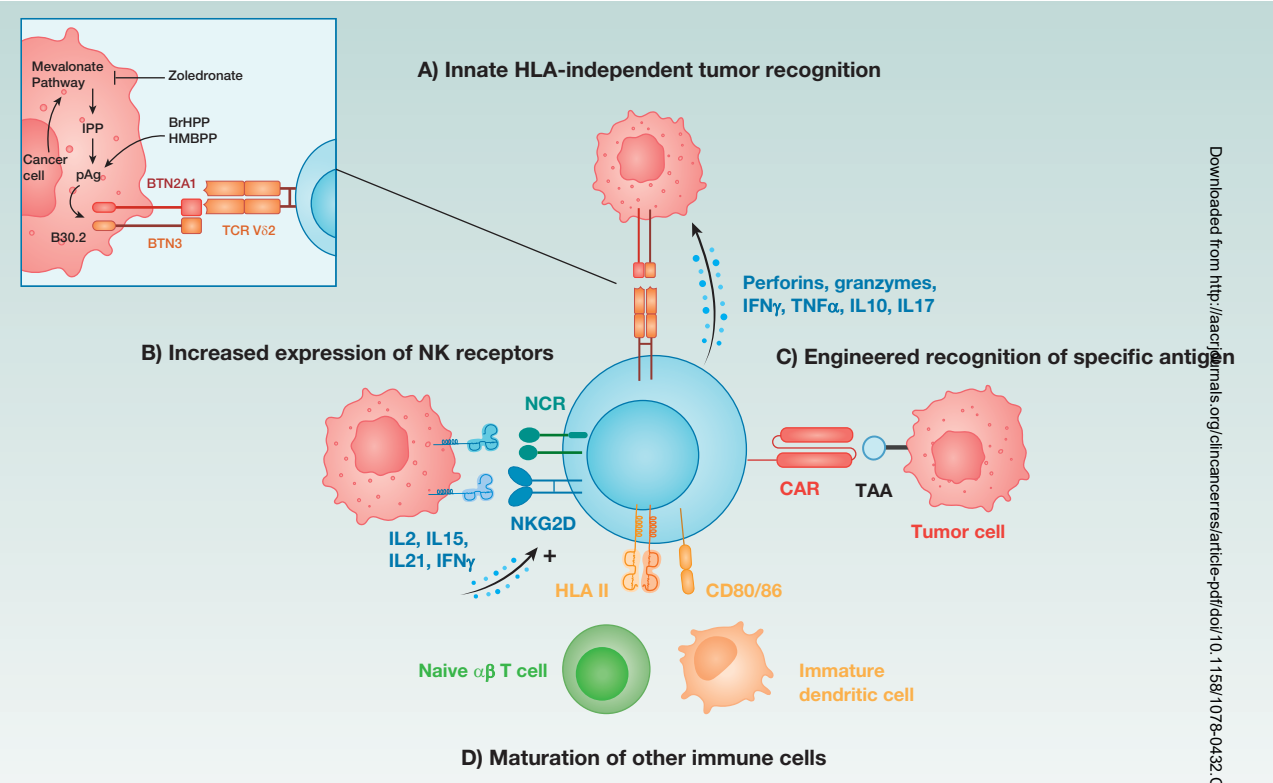




Figure 2

