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Double-negative T cells: a promising avenue of adoptive cell therapy in transplant oncology

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Abstract: Tumor recurrence is one of the major life-threatening complications after liver transplantation for liver cancer. In addition to the common mechanisms underlying tumor recurrence, another unavoidable problem is that the immunosuppressive therapeutic regimen after transplantation could promote tumor recurrence and metastasis. Transplant oncology is an emerging field that addresses oncological challenges in transplantation. In this context, a comprehensive therapeutic management approach is required to balance the anti-tumor treatment and immunosuppressive status of recipients. Double-negative T cells (DNTs) are a cluster of heterogeneous cells mainly consisting of two subsets stratified by T cell receptor (TCR) type. Among them, TCR$\alpha\beta^+$ DNTs are considered to induce immune suppression in immune-mediated diseases, while TCR$\gamma\delta^+$ DNTs are widely recognized as tumor killers. As a composite cell therapy, healthy donor-derived DNTs can be propagated to therapeutic numbers in vitro and applied for the treatment of several malignancies without impairing normal tissues or being rejected by the host. In this work, we summarized the biological characteristics and functions of DNTs in oncology, immunology, and transplantation. Based on the multiple roles of DNTs, we propose that a new balance could be achieved in liver transplant oncology using them as an off-the-shelf adoptive cell therapy (ACT).

Key words: Double-negative T cell (DNT); Adoptive cell therapy (ACT); Liver cancer; Liver transplantation; Oncology

1 Introduction

Liver transplantation, a lifesaving intervention for patients with end-stage liver disease (ESLD), has grown by leaps and bounds in recent years. Since the transplantability of unresectable hepatocellular carcinoma (HCC) was discussed in the Milan criteria (Mazzaferro et al., 1996), a series of studies have been conducted to identify the suitable transplantation criteria for HCC patients (Takada et al., 2007; Xu X et al., 2016; Yao et al., 2001). In this context, a new concept named "transplant oncology" has entered the scene. Far beyond the scope of conventional surgery for hepatobiliary malignancies, it is a synthetic concept consisting of multiple disciplines. Generally, four pillars have been promoted to further elucidate the research priorities of transplant oncology: (1) promoting the development of multidisciplinary cancer care; (2) extending the limit of cancer surgery; (3) elucidation of a recognition system by combining tumor and transplant immunology; (4) exploration of the disease mechanism through genomic approaches (Hibi et al., 2017; Hibi and Sapisochin, 2019). Designed to extend the bridge between tumor biology and transplantation medicine, transplant oncology aims to create a new era of precise treatment for liver cancer.

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Currently, with the rapidly growing number of patients who receive transplantation to treat liver cancer, the risk of post-transplant tumor recurrence has attracted increasing attention. Circulating tumor cells (CTCs), released from the primary lesion before transplantation, are considered one of the causes of recurrence (Ye et al., 2019). Being hard to detect through conventional means, such escaped tumor cells in the peripheral blood provide opportunities for subsequent recurrence or even metastasis. Besides, research showed that an immunosuppressive regimen involving steroids, calcineurin inhibitors, and induction agents could contribute to the recurrence of liver cancer (Verna et al., 2020). Recently, immune checkpoint inhibitors (ICIs) are becoming a novel strategy to treat liver cancer, but an appropriate degree of immunologic activation must be taken into account, since hyperactivation of the immune system may bring damage to the graft (Nordness et al., 2020; Khan et al., 2021). Therefore, it is advisable to derive an integrated therapy that could induce the anti-tumor effect without increasing the immune burden of recipients.

2 Double-negative T cells (DNTs): fighters, peacemakers, or both?

2.1 An overview of DNTs

DNTs are a cluster of unconventional and less known cells expressing specific surface markers T cell receptor-positive (TCR^+), cluster of differentiation 3-positive ($CD3^+$), $CD4^-$, $CD8^-$, and $CD56^-$, which account for 1%–3% of peripheral blood mononuclear cells (PBMCs) and 3%–5% of the peripheral blood mature T lymphocyte pool (Lee et al., 2018; Yao et al., 2019). To begin with, it is necessary to differentiate DNTs from other $CD4^-CD8^-$ populations (Table 1). According to the surface TCRs, DNTs can be divided into two groups: $TCR\alpha\beta^+$ cells and $TCR\gamma\delta^+$ cells. $TCR\alpha\beta^+$ DNTs are referred to as "DN Tregs" for inducing the potent functions of immune regulation and tolerance (Velikkakam et al., 2022), while $TCR\gamma\delta^+$ DNTs tend to harbor tumor cytotoxicity and tumor infiltrating ability (Hoeres et al., 2018; Liu and Zhang, 2020; de Gassart et al., 2021). Despite these discrepancies, some researchers took the two subsets as a single category in expanded cell products, since comparable anti-tumor functions and cell surface marker expression were detected (Lee et al., 2018, 2019; Yao et al., 2019). They also proved that allogeneic human DNTs isolated from healthy donors were able to induce potent anti-tumor function in several hematologic malignancies and solid tumors. More importantly, little rejection or damage to the normal tissues was observed in the related experiments. In practice, a clinical-grade protocol has been developed to isolate and expand healthy donor-derived DNTs in therapeutic numbers (Lee et al., 2019). To sum up, there is available evidence that DNTs are able to function as both immune regulators and tumor killers, with such integrated characteristics mediated by two different subsets. This group of novel cells is likely to play dual roles of both "fighters" and "peacemakers" in terms of anti-tumor activity.

Table 1 Properties of the various $CD3^+CD4^-CD8^-$ T cell populations

Cell type	TCR	Restriction	Antigen recognition	Repertoire
DNT				
$TCR\alpha\beta^+$	αβ	MHC I/MHC II/CD1 (Chowdhary et al., 2017)	Peptide (Chowdhary et al., 2017)	Polyclonal (Fischer et al., 2005)
$TCR\gamma\delta^+$	γδ	MHC I/MHC II/CD1 (Kabelitz et al., 2020)	Soluble protein and non-protein antigens (Kabelitz et al., 2020)	Oligoclonal/polyclonal (Velikkakam et al., 2022)
MAIT	Semi-invariant αβ (Zhang et al., 2020)	MR1 (Legoux et al., 2017)	Microbial-derived vitamin B metabolites (Kjer-Nielsen et al., 2018)	Oligoclonal (Kjer-Nielsen et al., 2018)
NKT				
I	Semi-invariant αβ (Crosby and Kronenberg, 2018)	CD1d (Crosby and Kronenberg, 2018)	α-GalCer (Gálvez et al., 2021)	Oligoclonal (Gálvez et al., 2021)
II	αβ	CD1d (Almeida et al., 2021)	Lipid antigens (Pellicci and Uldrich, 2018)	Polyclonal (Pellicci and Uldrich, 2018)

TCR: T cell receptor; DNT: double-negative T cell; MAIT: mucosal-associated invariant T; NKT: natural killer T; MHC: major histocompatibility complex; MR1: MHC class I-related molecule; CD: cluster of differentiation; α-GalCer: α-galactosylceramide.

2.2 DNTs as novel adoptive cell therapies (ACTs) for malignancies

As early as 2003, the anti-tumor effect of DNTs was first reported in the treatment of lymphoma (Young et al., 2003). Thereafter, numerous successive studies of other hematologic or solid tumors came along, including those on melanoma (Strippoli et al., 2021), acute myeloid leukemia (Lee et al., 2018), pancreatic cancer (Chen et al., 2019), and lung cancer (Yao et al., 2019), in which the anti-tumor mechanisms of DNTs were investigated to varying degrees. In summary, the listed mechanisms can be concluded into two major types: receptor-ligand interaction and secretory factors (Fig. 1). Of note, previous research showed that the tumor-killing effect of DNTs is independent of TCR with a major histocompatibility complex (MHC)-unrestricted pattern (Lee et al., 2018), indicating that the two diverse DNT subsets are likely to display an innate-like anti-tumor function. For conventional ACTs, the high heterogeneity of cancer cells and their lack of tumor-specific antigen seriously hamper the recognition and elimination of the target by effector cells, which greatly impairs the therapeutic effect. Given the innate recognition mechanisms, DNTs as a cell therapy may be less affected by known primary or acquired drug resistance.

For clarity, we summarized the existing research of DNTs in oncology (Table 2). Despite all the above findings that DNTs exert their antitumor effects in a variety of ways, the current research progress in solid tumors is far from unimpeded. Firstly, the existence of tumor microenvironment in solid tumors could greatly impair the tumor-killing effect of ACTs (Mizukoshi and Kaneko, 2019). Besides, the localization of immune infiltration may further affect the therapeutic effect. A tumor model called immune-excluded phenotype showed that malignant tumors with poor prognosis may exclude effector cells from the lesion (Galluzzi et al., 2018), which may explain the reduced frequency of DNTs in tumor tissue compared with adjacent or normal lung tissue (Fang et al., 2019). To address this issue, Vasic et al. (2022) have developed a new strategy to use DNTs as chimeric antigen receptor (CAR) T cell therapy platforms, of which effective infiltration and anti-tumor function in lung cancer were observed. Nonetheless, more research is needed to improve the efficacy of DNTs against solid tumors.

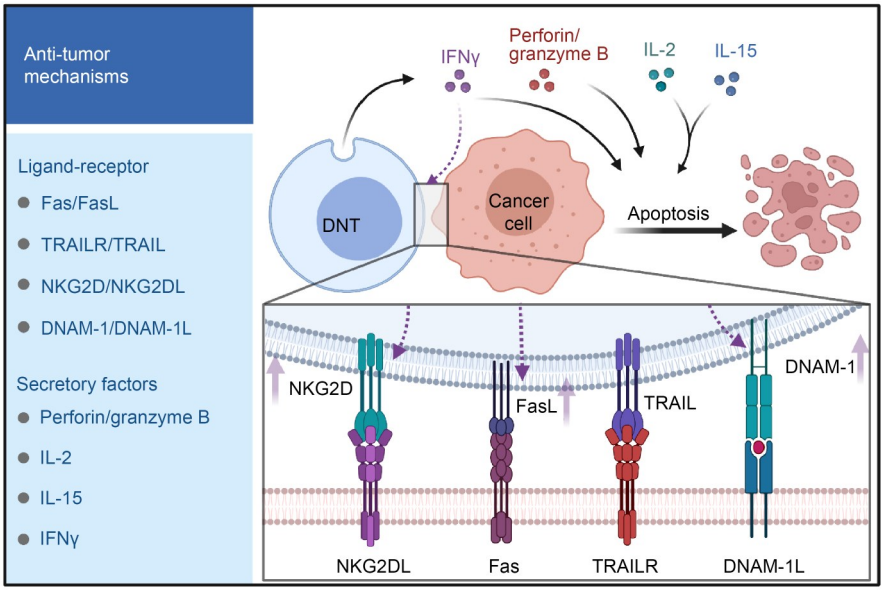

Fig. 1 Innate-like anti-tumor mechanisms of DNTs. The anti-tumor activity of DNTs is achieved through several ligand-receptor combination patterns. The typical surface receptors and ligands including NKG2D, DNAM-1, FasL, and TRAIL bind with their counterparts mostly expressed on cancer cells to induce tumor killing effects. Secretory factors such as IL-2, IL-15, IFNγ, and perforin/granzyme B play important roles in the above processes, further promoting the apoptosis of tumor cells. DNTs: double-negative T cells; Fas: factor-associated suicide; FasL: Fas ligand; TRAIL: tumor necrosis factor (TNF)-related apoptosis-inducing ligand; TRAILR: TRAIL receptor; NKG2D: natural-killer group 2, member D; NKG2DL: NKG2D ligand; DNAM-1: DNAX accessory molecule-1; DNAM-1L: DNAM-1 ligand; IL: interleukin; IFNγ: interferon-γ. Created with BioRender.com.

Table 2 Current research regarding DNTs in oncology

Tumor type	Subset of DNTs	Source of DNTs	Experiment*		Mechanism involved	Reference
			In vitro	In vivo		
Lymphoma	TCRαβ$^+$	Spleens of mice	Yes	Yes	Fas/FasL	Young et al., 2003
Melanoma	TCRαβ$^+$	PBMCs of a melanoma patient	Yes	No	Perforin/granzyme B	Voelkl et al., 2009
AML	TCRαβ$^+$ TCRγδ$^+$	PBMCs of AML patients	Yes	No	Perforin	Merims et al., 2011
Pancreatic carcinoma	TCRαβ$^+$ TCRγδ$^+$	PBMCs of healthy donors	No	Yes	NKG2D/MICA	Xu H et al., 2016
AML	TCRαβ$^+$ TCRγδ$^+$	PBMCs of AML patients and healthy donors	Yes	Yes	NKG2D/NKG2DL, DNAM-1/DNAM-1L	Chen et al., 2018
AML	TCRαβ$^+$ TCRγδ$^+$	PBMCs of healthy donors	Yes	Yes	NKG2D/NKG2DL, DNAM-1/DNAM-1L, IFNγ	Lee et al., 2018
Pancreatic carcinoma	TCRαβ$^+$ TCRγδ$^+$	PBMCs of healthy donors	Yes	Yes	Fas/FasL	Chen et al., 2019
Non-small cell lung cancer	TCRαβ$^+$ TCRγδ$^+$	PBMCs of healthy donors	Yes	Yes	IFNγ, TNFα, perforin/ granzyme B, CD107a	Fang et al., 2019
Myeloma, Burkitt lymphoma, AML, EBV-LCL, large cell lung cancer, and adenocarcinoma	TCRαβ$^+$ TCRγδ$^+$	PBMCs of healthy donors	Yes	Yes		Lee et al., 2019
Pancreatic carcinoma	TCRαβ$^+$	PBMCs of healthy donors	Yes	No	Fas/FasL, Nrf2, IFNγ	Lu et al., 2019
Sarcoma	TCRαβ$^+$	Spleens of mice	Yes	Yes	IFNγ	Ponzetta et al., 2019
Non-small cell lung cancer	TCRαβ$^+$ TCRγδ$^+$	PBMCs of healthy donors	Yes	Yes	NKG2D/NKG2DL, DNAM-1/DNAM-1L, TRAILR/TRAIL, NCR3, IFNγ, perforin/granzyme B	Yao et al., 2019
AML	TCRαβ$^+$ TCRγδ$^+$	PBMCs of healthy donors	Yes	Yes	CD64	Soares et al., 2021
B-ALL and lung cancer	TCRαβ$^+$ TCRγδ$^+$	PBMCs of healthy donors	Yes	Yes	Anti-CD19-CAR, LFA-1, perforin/ granzyme B	Vasic et al., 2022

* Refers to the in vitro cell experiments or in vivo animal experiments conducted in the corresponding article. DNTs: double-negative T cells; TCR: T cell receptor; Fas: factor-associated suicide; FasL: Fas ligand; PBMCs: peripheral blood mononuclear cells; AML: acute myeloid leukemia; NKG2D: natural-killer group 2, member D; MICA: major histocompatibility complex class I-related antigens A; NKG2DL: NKG2D ligand; DNAM-1: DNAX accessory molecule-1; DNAM-1L: DNAM-1 ligand; IFNγ: interferon-γ; TNFα: tumor necrosis factor α; CD: cluster of differentiation; EBV-LCL: Epstein-Barr virus lymphoblastoid cell line; Nrf2: nuclear factor erythroid 2-related factor 2; TRAIL: TNF-related apoptosis-inducing ligand; TRAILR: TRAIL receptor; NCR3: natural cytotoxicity triggering receptor 3; B-ALL: B-cell acute lymphoblastic leukemia; CAR: chimeric antigen receptor; LFA-1: lymphocyte function-associated antigen-1.

2.3 Immune tolerance induced by TCRαβ$^+$ DNTs

At present, in terms of immune tolerance and suppression, TCRαβ$^+$ DNTs comprise the main subset of DNTs to be studied. During the process of liver allograft rejection, activated CD8$^+$ cytotoxic T cells are known as the main effector lymphocytes to induce graft injury (Ronca et al., 2020). For CD8$^+$ T cells, the immunoregulation of TCRαβ$^+$ DNTs can be summarized into three approaches (Ford et al., 2002) (Fig. 2). Importantly, the above processes are conducted through an antigen-specific manner, which means that only those immune cells of the host that threaten the allograft would be attacked by TCRαβ$^+$ DNTs, and vice versa.

Based on their potent function of immune regulation, TCRαβ$^+$ DNTs have been promising therapeutic

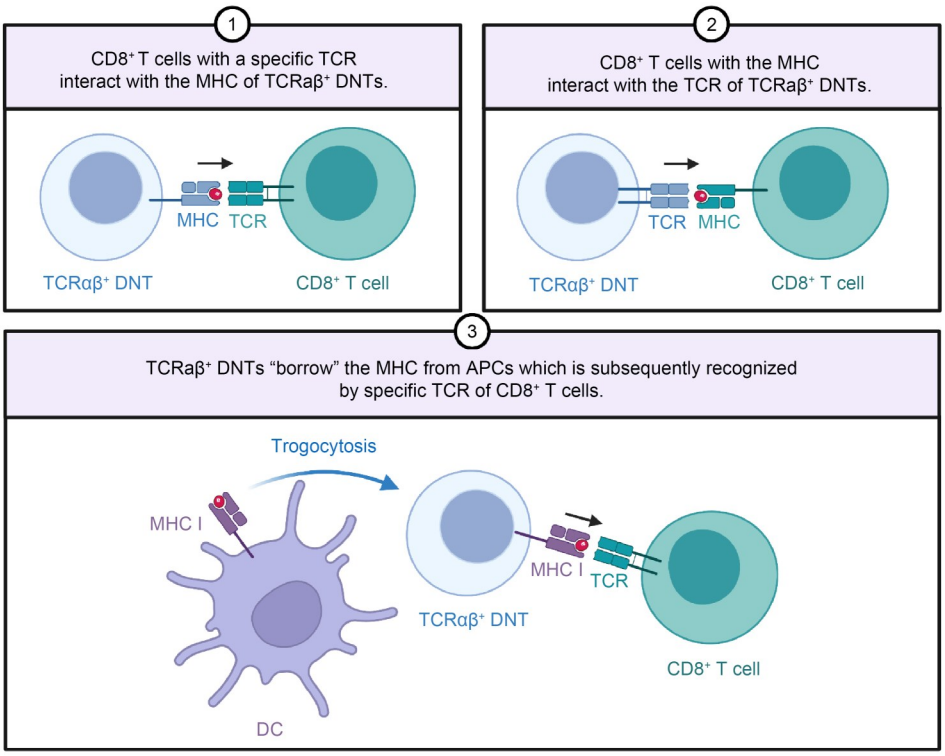

Fig. 2 Antigen-specific immune tolerance induced by TCRαβ⁺ DNTs, as typified by CD8⁺ T cells. Activated DNTs kill CD8⁺ T cells in an antigen-specific manner. The exact regulatory activity can be initiated according to three disparate situations (as shown by 1, 2, and 3). DNTs: double-negative T cells; CD: cluster of differentiation; TCR: T cell receptor; MHC: major histocompatibility complex; DC: dendritic cell; APCs: antigen-presenting cells. Created with BioRender.com.

agents in transplantation. Graft versus host disease (GVHD), a serious post-transplant complication, occurs when donor T cells are activated by the recipient alloantigen, which tends to cause multisystem organ failure (Chakraverty and Teshima, 2021). Achita et al. (2018) have confirmed that the infusion of allogeneic or xenogeneic TCRαβ⁺ DNTs alone does not cause GVHD due to the MHC-unrestricted characteristics, while adoptive CD4⁺ or CD8⁺ T cells are associated with a significantly high risk. In their study, DNTs were also observed to alleviate GVHD caused by other grafts. In addition to GVHD, host versus graft (HVG) rejection is another issue of major concern. Research showed that allogeneic DNTs are able to evade rejection by host immune cells (Lee et al., 2019), though the detailed mechanism remains elusive. For the allograft, studies have proved that a direct infusion of TCRαβ⁺ DNTs could inhibit the rejection and induce the long-term survival of cardiac, skin, and islet grafts (Young et al., 2002; Chen et al., 2005; Juvet and Zhang, 2012). As an important immunosuppressant, rapamycin is recommended in patients with HCC after liver transplantation (Ling et al., 2022). Interestingly, rapamycin enhanced the immunosuppressive function of TCRαβ⁺ DNTs in vitro and in vivo (Achita et al., 2018). The above-mentioned properties, albeit preliminary, could be indeed considered as a positive sign of future development and the potential application of DNTs to alleviate the immune burden of recipients in transplant oncology.

3 Future directions in the use of DNTs in transplant oncology

In practice, the infusion of allogeneic DNTs hardly brings about GVHD or damage to normal cells and tissues (Achita et al., 2018), thus ensuring their therapeutic safety as an ACT. Given the success of application in hematological malignancies (Lee et al., 2018, 2019), DNTs are likely to become an effective ACT in tumor immunotherapy. Despite the lack of

extensive data on the application of DNTs in patients after liver transplantation, the remarkable correlation of DNT infusion with an increased survival potential of organs in model animals still brings us great encouragement (Hu et al., 2021; Bafor et al., 2022; Newman-Rivera et al., 2022). We have divided the potential applications of DNTs in liver transplant oncology into two main fields: prevention and treatment (Fig. 3).

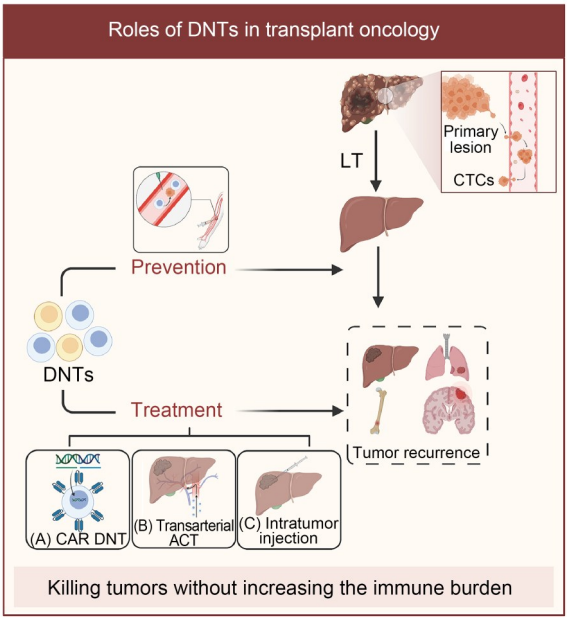

Fig. 3 Roles of DNTs in transplant oncology. DNTs are mainly composed of two cell populations, namely TCRαβ$^+$ and TCRγδ$^+$ DNTs, which show varying degrees of antitumor and immunosuppressive effects. In liver transplantation (LT) for liver cancer, DNTs may act as both preventive and treatment agents through alternative routes of administration. Importantly, the notable characteristic of DNTs to kill tumor cells without increasing the immune burden of recipients makes them potent ACT agents in transplant oncology. DNTs: double-negative T cells; TCR: T cell receptor; ACT: adoptive cell therapy; CTCs: circulating tumor cells; CAR: chimeric antigen receptor. Created with BioRender.com.

3.1 Prophylactic administration of DNTs

Based on the characteristics of the two cell subsets, DNTs are likely to become novel cell prophylactics that may prevent tumor recurrence without disturbing the immunosuppressive environment. Usually, liver cancer recurrences emerge in the liver and other sites with abundant vascularity such as lung, bone, adrenal glands, and even soft tissue (Verna et al., 2020), suggesting that CTCs left in the blood play a pivotal role after the complete removal of the primary lesion. Therefore, TCRγδ$^+$ DNTs may work even better against this part of tumor cells circulating in the blood, exactly as they would behave in the treatment of hematologic malignancies, contributing to an overall decreased recurrence rate.

Another inevitable issue lies in how to protect the allograft function. As an indispensable component, TCRαβ$^+$ DNTs tend to maintain an immune microenvironment conducive to subsequent graft survival with relatively low levels of CD4$^+$ and CD8$^+$ T cells (Juvet and Zhang, 2012) that play a key role in the HVG reaction. Early allograft dysfunction (EAD), as a life-threatening postoperative complication, is deemed to be largely the result of ischemia/reperfusion injury (IRI) caused by the transplant operation (Yang et al., 2021; Lu et al., 2022). In the lung and kidney, TCRαβ$^+$ DNTs are corroborated to function as important participants and play a protective role in response to IRI (Hsu et al., 2021), which makes the prophylactic use of DNTs more practical in transplant surgery. The above two aspects may be simply achieved through intravenous injection before and after the reception of transplant.

3.2 Therapeutic administration of DNTs

Other than acting as a form of prophylaxis in the peripheral blood, DNTs are expected to induce a direct killing effect inside the tumor. In acute myeloid leukemia, DNTs have been approved as an ACT for a human clinical trial (NCT03027102). Given the cytotoxicity of TCRγδ$^+$ DNTs, other clinical trials have also been conducted in lung, breast, and renal cancers (Hoeres et al., 2018; Pauza et al., 2018; Yao et al., 2019). Yet due to the general weakness of ACTs to treat solid tumors, most trials have shown limited efficacy. Intratumoral immunotherapy may help solve this problem (Marabelle et al., 2017). By applying direct injections, DNTs can be easily enriched in situ. Besides, transarterial chemoembolization (TACE) has become the standard-of-care for the treatment of intermediate-stage HCC (Raoul et al., 2019), the mechanisms of which involve drug local delivery and tumor embolization. Thus, it might also be effective to transfer our drugs into tumors by replacing the cytotoxic agents with DNTs. Moreover, among all sorts of cell therapies, autologous CAR T cells have great significance; such therapies are associated with little allogeneic

reaction but usually stymied by T cell dysfunction (Thommen and Schumacher, 2018), while allogeneic CAR T cells possess stable function but are likely to cause rejection (Depil et al., 2020). By contrast, CAR DNT therapy as an emerging ACT has shown great superiority, of which the cytotoxicity against solid tumors has been partly confirmed in previous research (Capsomidis et al., 2018; Vasic et al., 2022). However, for the lack of direct evidence, the above attempts require further exploration before being expanded to clinical treatment alternatives of liver cancer. Patient-derived xenograft (PDX) models may provide ideal ways for such preclinical research in oncology (Zhuo et al., 2020; Sun et al., 2021). To shorten the modeling time, patient-derived organoid (PDO) is another reliable model for validation (Broutier et al., 2017; Tuveson and Clevers, 2019). The above modern tools offer great convenience for the future applications of DNTs.

To sum up, by virtue of the unique characteristics of DNTs, they could play important roles in the treatment of liver cancer and prevention of its recurrence, especially under the background of transplantation. By not increasing the immune burden, DNTs can also make up for the shortcomings of traditional immunotherapies typified by ICI in transplantation. Notably, the administration of DNTs before liver transplantation may act as both a downstaging and a prophylactic strategy, which makes DNTs potential sequential therapeutics in transplant oncology.

4 Conclusions

Transplant oncology is a wide-ranging field, where anti-tumor effects and the maintenance of a suitable immune status are crucial issues to be investigated for a better prognosis of transplant recipients. DNTs are a cluster of immune cells mainly composed of two cell populations, including TCR$\alpha\beta^+$ and TCR$\gamma\delta^+$ DNTs, with great potential in terms of varying degrees of anti-tumor and immunosuppressive effects. Herein, we propose that DNTs could act as both "fighters" against liver cancer and "peacemakers" to maintain a suitable immune status of patients. Hopefully, further research will highlight the potential of this group of cells to act as a new ACT and provide an immunological equilibrium in transplant oncology.

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Author contributions

Zhihang HU: writing – original draft. Modan YANG: data curation and visualization. Xiao XU and Di LU: conceptualization and writing – review & editing. Hao CHEN, Chiyu HE, Zuyuan LIN, Xinyu YANG, Huigang LI, and Wei SHEN: supervision and writing – review & editing. All authors have read and approved the final manuscript, and therefore, have full access to all the data in the study and take responsibility for the integrity and security of the data.

Compliance with ethics guidelines

Zhihang HU, Modan YANG, Hao CHEN, Chiyu HE, Zuyuan LIN, Xinyu YANG, Huigang LI, Wei SHEN, Di LU, and Xiao XU declare that they have no conflict of interest.

This article does not contain any studies with human or animal subjects performed by any of the authors.

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