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Novel therapies for indolent lymphomas in the cellular therapy era

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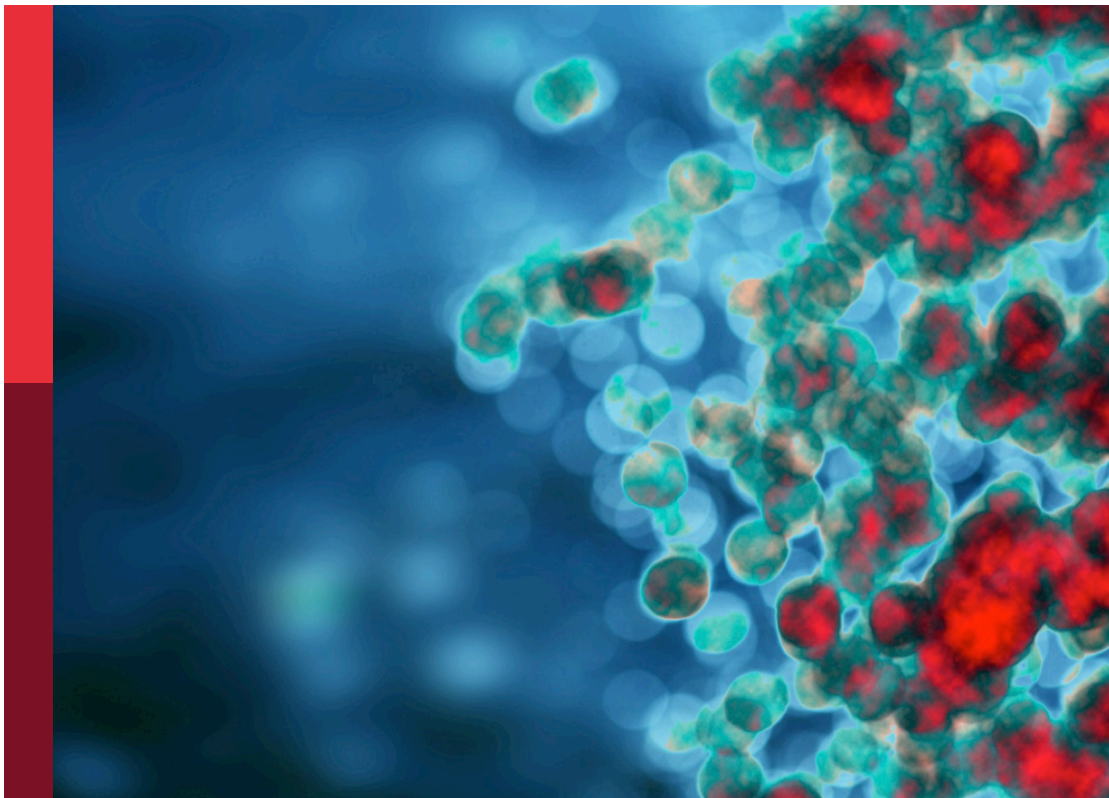

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Novel therapies for indolent lymphomas in the cellular therapy era

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Table of contents

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CASE REPORT
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Case Report: Clinical Responses to Tislelizumab as a First-Line Therapy for Primary Hepatocellular Carcinoma With B-Cell Indolent Lymphoma

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Background: As an emerging therapy with a promising efficacy, immunotherapy has been widely used in the treatment of solid tumors and hematologic malignancies. This clinical study compares the efficacy of tislelizumab, a domestic immune checkpoint inhibitor (ICI), to that of sorafenib when used as a first-line therapeutic option in hepatocellular carcinoma (HCC), and the concurrence of HCC and non-Hodgkin's lymphoma (NHL) is rare, especially in the treatment of ICIs.

Case presentation: A 61-year-old patient presenting with primary HCC and indolent B-cell lymphoma had a partial clinical response to tislelizumab for his primary HCC. Besides, we described a phenomenon of pseudo-progression and delayed diagnosis of his lymphoma during a long course of treatment.

Conclusion: Tislelizumab, an immunotherapeutic option with a favorable efficacy and toxicity, can be used to manage double primary tumors. However, studies should aim to elucidate the probable mechanisms of this therapy. Pseudo-progression and separation remission make the treatment of double primary tumors even more challenging, which calls for additional caution in patients undergoing immunotherapy to avoid misdiagnosis and, therefore, begin early appropriate interventions.

Keywords: immune checkpoint inhibitors, double primary tumors, hepatocellular carcinoma, B-cell indolent lymphoma, tislelizumab, misdiagnosis, case report

INTRODUCTION

Globally, liver cancer is the sixth most prevalent malignant tumor, however, it is the second most common cause of tumor associated mortalities (1, 2). Due to its aggressive behavior and limited therapeutic options, hepatocellular carcinoma (HCC), a type of primary liver cancer, has a poor prognosis. Conversely, the nature of indolent non-Hodgkin's lymphoma (NHL) is relatively mild, despite there being no effectively radical treatment during its long chronic process. Therapeutic resistance, multiple relapses, and biological characteristic transformations lead to poor clinical outcomes (3). Occurrence of double tumors, comprising HCC and NHL, is fairly rare, and

treatment is based on individual experience rather than standard protocols. Immunotherapy has rapidly developed and become an efficient therapy for non-small cell lung cancer, malignant melanoma, and other diseases (4, 5). It is a promising option for treating drug-resistant HCC (6). Tislelizumab, a newly humanized IgG4 antibody against programmed cell death-1 (PD-1), has been approved for the treatment of Hodgkin's lymphoma (HL), and a large number of clinical studies, such as the Phase III clinical trial of sorafenib as a first-line treatment for HCC (NCT03412773), have been performed. We report a case of indolent B-cell lymphoma-complicated HCC, which was effectively controlled by tislelizumab as the first line treatment. Authors also reviewed current literature and discussed the possible interaction between HCC and B-cell lymphoma during the long course of treatment.

CASE PRESENTATION

In April 2013, a 61-year-old man was found to have a liver mass by abdominal ultrasonography during regular physical examination. The patient did not exhibit gastrointestinal reactions or abdominal pain symptoms, and he was therefore referred to the Dongyang People's Hospital for further examination. There was no significant personal or family history that could have aided the diagnosis. During hospitalization, he was diagnosed with chronic hepatitis B (no hepatitis C) with liver cirrhosis, and the level of HBV-DNA was $3.33x10^{\wedge 5}$ IU/ml. The levels of serum tumor markers such as AFP and CEA were found to be 48.32 ng/ml and 5.71 U/ml, respectively. Abdominal computed tomography (CT) revealed a left lateral segment lesion of the liver with multiple enlarged lymph nodes around the lesser peritoneal sac, porta hepatis, retroperitoneum, and right paracardiac regions, which was radiologically suspected for small HCC and lymphadenopathy of infectious etiology. He was subjected to the left lateral lobe hepatectomy and celiac lymphadenectomy for the clinical diagnosis of HCC. Intraoperative findings revealed that the diameter of the lump was 0.9 x 0.8 cm. Pathology indicated hepatocellular carcinoma (**Figure S1**), while the two lymph nodes were negative. Therefore, his final diagnosis was HCC (pT1N0M0 stage I, BCLC 0). The patient was administered with entecavir (0.5 mg) once a day for antiviral treatment. Regular follow-up showed no tumor recurrence or active hepatitis for more than three years (**Table S1**).

Unfortunately, in July 2016, a new lesion in the right lobe segment was discovered by abdominal CT, without abnormal levels of serum liver function and tumor markers. However, the patient declined treatment with western medicines. In December 2016, the CT scan showed enlarged multiple liver nodules with vascular involvement, and he was hospitalized with HCC (cT3bN0M0 stage IIIB, BCLC B) in our hospital. Transarterial chemoembolization (TACE) was successively performed twice to downstage the tumor. After neoadjuvant therapy, he was histologically reassessed as HCC (ypT3N0M0 stage IIIB, BCLC B). Then, laparoscopic R0 resection of the right posterior liver lobe as well as the gall bladder was performed in April 2017 to eliminate residue lesions. Intraoperative findings revealed that lesion diameters were 9x8.5x10 cm with negative surgical margins. After partial liver resection, the patient's HBV-DNA copies increased to $2.35x10^{\wedge 4}$ IU/ml, with mildly abnormal AST, ALT, and bilirubin levels. He was continuously administered with antiviral therapies and other medicines to protect liver functions. After successful management of the disease, he was discharged and regular follow-ups were performed thereafter. In December 2018, the patient was found to have hepatopulmonary metastases. CT scans showed multiple small nodules, the largest of which (in the lung) was about 8.6x6 mm, most possibly a result of HCC metastases, and about 18 mm in the liver. The patient was clinically diagnosed with HCC (cT3N0M1 stage IV, BCLC C). Notably, these enlarged lymph nodes in the peritoneum remained the same as before. Systemic therapies are available to treat patients with unresectable HCC. Multitargeted tyrosine kinase inhibitors (TKIs), such as sorafenib or lenvatinib, are first-line systemic therapies. The patient had not had a previous prescription of systemic treatments, and therefore, he met the inclusion criteria for BGB-A317-301(NCT03412773), a global study designed to compare the clinical efficacy as well as safety of tislelizumab and sorafenib (1:1 randomized) as a first-line systemic treatment for unresectable HCC, which was considered beneficially to the patient (clinical trial protocols are provided in the supplementary materials). After signing the informed consent, the patient was serially screened for confounding factors (exclusion criteria). On January 18th 2019, he was administered with tislelizumab 200 mg igtt q3w. To accurately evaluate immunotherapeutic efficacy, we used iRECIST to determine disease progression (7). Reductions in pseudo-progressions (PsPDs) during immunotherapy were recorded. During two cycles of tislelizumab, the patient developed multiple rashes with pruritus (grade 2, CTCAE 5.0), and on February 1st 2019, he was treated with loratadine, ebastin, and mometasone ointment. Since the rashes got worse (grade 3, CTCAE 5.0), the third cycle of therapy was suspended. From February 25th 2019, the patient was orally administered with 35 mg prednisolone tablet once a day, and the rash was gradually alleviated. Dosage of prednisolone was reduced stepwise by the order of 25 mg, 15 mg, and 10 mg, with the last dose on March 21st, 2019. Before the third immunotherapeutic cycle, CT scans indicated that pulmonary lesions almost disappeared while the liver lesions remained stable (**Figure 1B**). Moreover, before the seventh cycle, the liver lesions were significantly enlarged, without a corresponding enlargement of lymph nodes, evaluated as iuPD (immunity unconfirmed progressive disease). In the ninth cycle, lesions were similar to the seventh cycle and evaluated as icPD (immunity confirmed progressive disease). During the treatment, AFP reached the highest level (696.18 ng/ml) before the eighth treatment (**Figure 1A**). Given that the patient did not meet the exclusion criteria, it was considered that he could still benefit from tislelizumab, and therefore, his participation in the clinical trial was continued after deep consideration. CT scan before the tenth cycle showed that the lesion size was smaller than the last assessment, and

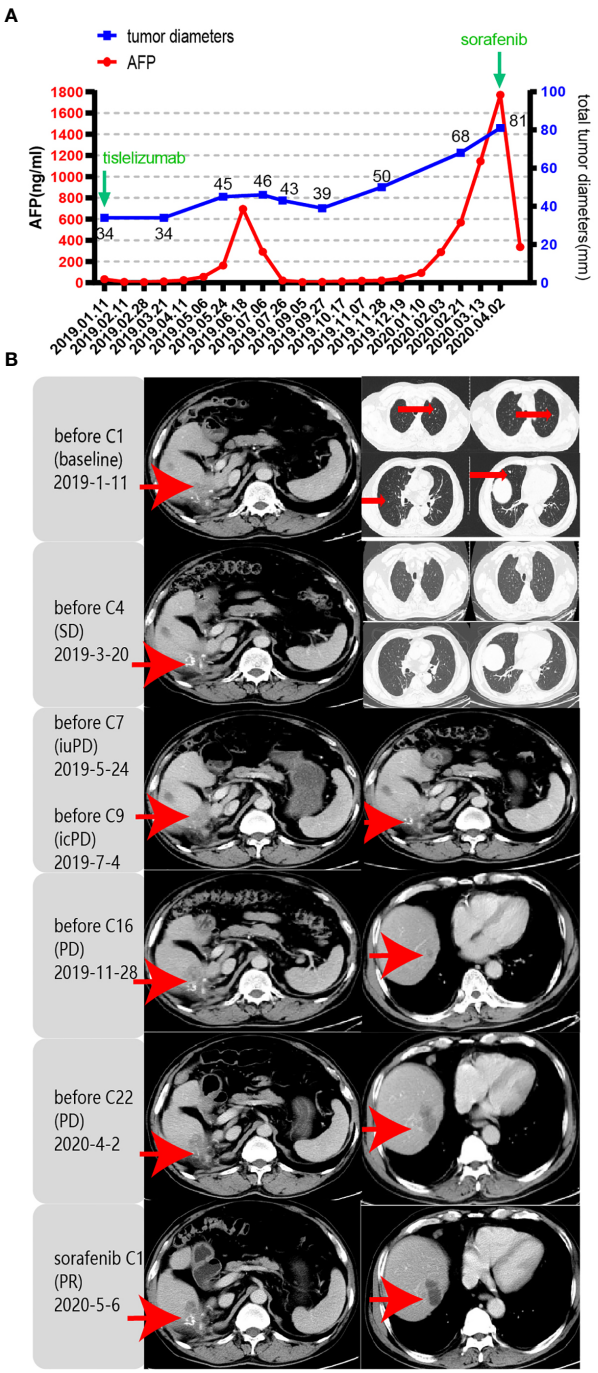

FIGURE 1 | Response evaluation during the clinical course including changes in imaging and quantitative data. iuPD (immunity unconfirmed progressive disease); icPD (immunity confirmed progressive disease). **(A)** Trends in the levels of tumor monitoring indicators, including AFP (left Y-axis) and tumor diameters (right Y-axis) corresponding to the treatment timeline. X-axis showing the date of the disease course. The frequency of imaging evaluations is less than that of AFP. **(B)** Representative images of the CT scan revealed the increasing and decreasing process of both primary and metastatic lesions in the liver and lung after PD-1 antibody (tislelizumab) and sorafenib treatment. Red arrows indicate tumor lesions.

simultaneously, AFP decreased to normal (21.14 ng/mL). Therefore, the researchers considered that the patient exhibited "false progression" in the previous treatment. Liver lesions remained stable until the thirteenth cycle. Before the sixteenth cycle, there was slow disease progression, while before the 22nd cycle (2020–4–2), liver lesions exhibited significant progression. Therefore, it was considered that tislelizumab was no longer beneficial to the patient, and he was withdrawn from the clinical trial. Disease progression is shown in **Figure 2**, and except for the maculo-papule, all adverse events are described in **Table 1**.

OUTCOMES AND FOLLOW-UP

After being discontinued from the clinical trial, the patient was treated with sorafenib. One month later, the liver tumor was found to be smaller and AFP decreased significantly. Meanwhile, the care team noticed that during treatment with tislelizumab, his lymph nodes did not exhibit any change, regardless the lesions were either enlarged or shrank, HBV was either well or poorly controlled. For further evaluation, we performed a lymph node biopsy. Histological examinations of axillary and inguinal lymph nodes revealed indolent B-cell lymphoma (**Figure 3**). Retrospectively analyzed, his peritoneal sac, paracardiac regions, and superficial lymph nodes remained the same in size and unparallel to tumor progression, which supported that the patient had lymphoma seven years ago. It is unfortunate that a lymph node biopsy was not performed at that time, therefore, there is no definite pathological evidence to support our hypothesis. Currently, clinical follow-ups are still being performed and to date, the patient continues receiving sorafenib treatment and survives his double tumors. Under the treatment of ICIs, despite signs of "false progression", his disease course showed his liver lesions were responsive to tislelizumab overall, but not his lymphoma. Recently, the patient was discovered to have an elevated lymphocyte count but without clinical symptoms, which may be correlated with the indolent lymphoma. Hematological assessments are shown in the Supplementary material (**Figure S2**).

DISCUSSION

Warren's definition of multiple primary tumors refers to the simultaneous or successive occurrence of two or more unrelated primary malignant tumors (8, 9). Patients with malignant tumors are more likely to develop a second malignant tumor, which may be due to the persistent effects of risk factors, radiotherapy, and chemotherapy (10). Currently, incidences of synchronous multiple primary cancer are increasing. Carson H. J. documented the reported cases of synchronous NHL with other cancers. The most common are colon cancer, prostate cancer, and lung cancer respectively, while the HCC, is penultimate (11). In case reports of HCC with lymphoma (**Table 2**), the most common is HCC with invasive diffuse large B-cell lymphoma (DLBCL), while follicular lymphoma

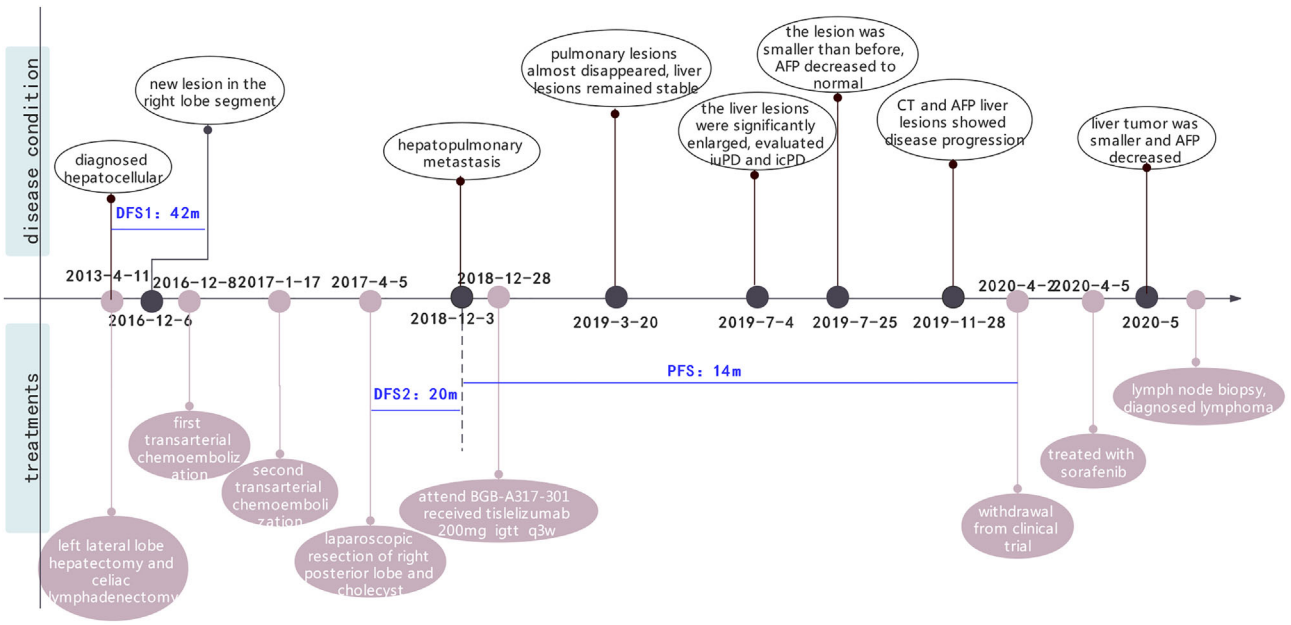

FIGURE 2 | The whole clinical timeline of the patient, with major treatment and disease status. DFS, disease-free survival; PFS, progression-free survival.

TABLE 1 | Adverse events in the tislelizumab therapeutic course in the patient (graded by CTCAE 5.0).

Adverse events	Baseline	Maximum grade	Duration	irAE	Treatment
Pruritus	0	II	C2-C3	Yes	Glucocorticoid
Rash maculopapule	0	III	C2-C3	Yes	Glucocorticoid
Leukocytosis	0	III	C2-C5, C18-C21	No, may be unrelated	No
Lymphocyte count increased	I	III	C2-C5, C18-C21	No, may be related	No
Alanine transaminase (ALT) levels increased	I	I	C8	May be related	Medicine
Aspartate aminotransferase (AST) levels increased	0	I	C8	May be related	Medicine
Blood bilirubin levels increased	0	I	C14-C15	May be related	Medicine

The first screening indicators after the clinical trial as the baseline; CTCAE, Common Terminology Criteria for Adverse Events; C, cycle; irAE, immune-related adverse events.

(FL) represents the majority of indolent NHL, which is consistent with the incidence of NHL (33). The overall survival (OS) of indolent NHL is high, with 70% of patients having more than 10 years of survival. However, in multiple malignant tumors, interactions between tumors may affect the OS. Retrospective studies have shown that the prognosis of gastric cancer patients complicated with lymphoma may depend more on gastric cancer (34), but Lee SI et al. documented that in patients with both HCC and hepatitis, delayed diagnosis of NHL, especially DLBCL, is associated with a poor prognosis (29). Therefore, it is crucial to identify multiple primary tumors early and precisely.

In our case, the patient with HCC was not diagnosed with B-cell indolent lymphoma until the lymph node biopsy was implemented at the later stage, so we made a retrospective analysis. Occurrence of both HCC and chronic hepatitis affects the diagnosis of multiple-lymphadenopathy, which is easily misdiagnosed as "reactive hyperplasia" or "lymph node metastasis of HCC". Misdiagnoses resulted in delayed treatments. Therefore, NHL should be regarded as a differential diagnosis for HCC and chronic hepatitis patients (29). Compared to metachronous neoplasms, synchronous multiple neoplasms are perhaps tougher to identify. Next-generation sequencing (NGS) is effective in the identification of tumor-specific genes, which is important in the diagnosis of multifocal tumors and in informing clinical treatments (35). In our case, swollen lymph nodes were found in the initial treatment of HCC and were not parallel to the changes in HBV-DNA or tumor development. Therefore, if there is "separation remission" during the clinical treatment process, the possibility of double primary tumors should be ruled out. In our case, lymph node biopsy was negative for lymph node metastasis of liver cancer, so in the status of liver tumor progression with lung metastasis disappearance, local treatments of the liver can also be considered in the following treatments.

As mentioned in the treatment strategies, HCC is often diagnosed in the advanced stage, therefore, systemic treatment plays an essential role in its control. However, as the first-line treatment for advanced HCC, sorafenib does not show a

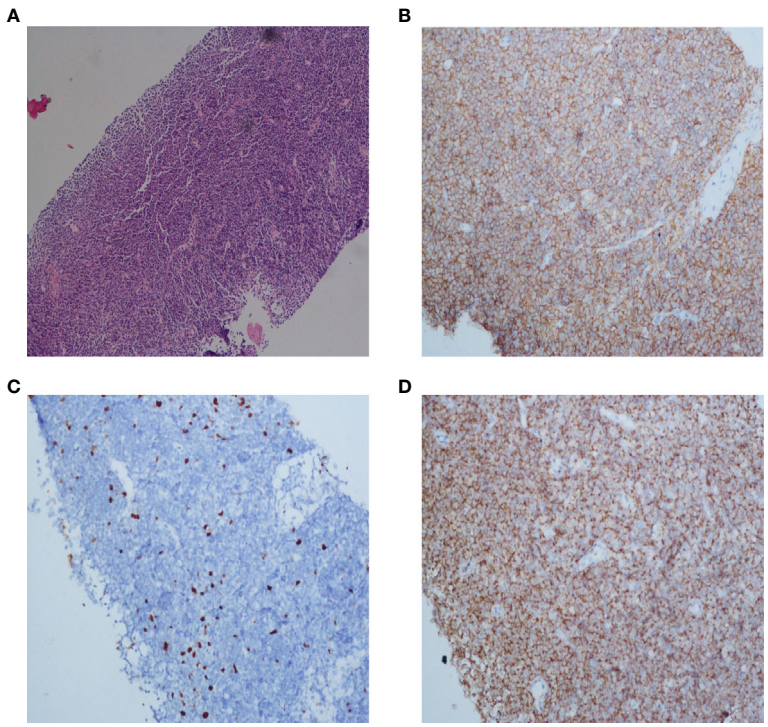

FIGURE 3 | Histopathology and immunohistochemistry (IHC) of the lymph node of this patient. Microscopic observation (10×) of H&E staining showed a dense diffuse lymphoid cells infiltration **(A)**. Immunohistochemical staining of CD20 and Bcl-2 expression (20×) showed that tumor cells were positive for CD20 and Bcl-2, respectively **(B, D)**. The Ki-67 proliferative index (20×) was low **(C)**.

dramatic benefit. The median survival time of sorafenib was only three months longer than placebo (36). Advances in immunotherapy have enhanced tumor treatment (5, 37), and some have shown good therapeutic effects in HCC. A phase III trial involving a combination of atezolizumab and bevacizumab as first-line treatment for unresectable HCC revealed a significant improvement, with a 12-month prolonged OS compared to sorafenib (38). Phase III randomized controlled trials comparing nivolumab (39) and tislelizumab (NCT03412773) with sorafenib as first-line therapeutic options for advanced HCC have been launched in succession. Unlike solid tumors, the decisive prognostic factor for patients with lymphoma is the pathological type rather than clinical stage. Even though the clinical course of indolent lymphoma is always stable or spontaneously relieved before progression (40), it is mostly incurable and has the probability of transforming to invasive lymphoma such as DLBCL (41), especially under the circumstance of immune disorders in patients with active tumor. Therefore, indolent lymphoma might require the same aggressive treatments under those scenarios. R-CHOP (a scheme including rituximab, which is an anti-CD20 monoclonal antibody, mAbs) is the first-line recommended treatment for indolent lymphoma at stages III and IV. In refractory HL, ICIs have shown good clinical outcomes (42). There are no clinical trials of tislelizumab for NHL, however, ICIs such as nivolumab (NCT02038946) have been used in the treatment of NHL, some case reports and small sample research studies of certain NHLs have displayed durable response under the treatment of ICIs (43, 44). At molecular and cytological levels, PD-1 positive expression was detected in DLBCL, FL and marginal zone lymphoma (MZL) (45). The density of PD-1 positive cells in FL is associated with the prognosis and possibility of transformation to DLBCL. However, expression levels of PD-1 vary from different studies (45–47). Most FLs have been shown to have a stronger immune escape (48), which may be due to the rich $PD1^+$ γδ T lymphocytes. PD-1 regulates the immune components of γδ T cytotoxic cells, resulting in the hypofunction of γδ T lymphocytes which reduces antibody-dependent cell-mediated cytotoxicity (ADCC) (49). Therefore, ICIs have the ability to slow down FL development. Anti-CD20 mAbs can enhance intratumoral infiltration of γδ T cells (50), which provides the possibility to improve the efficacy of ICIs against immune desert tumors by rituximab. It is theoretically proven that a combination or bispecific antibodies of anti-CD20 mAbs and ICIs in the treatment of NHL complicated HCC can enhance the therapeutic effect.

Moreover, both NHL and HCC are associated with hepatitis B or C viruses (51, 52), and NHL patients have higher odds for HCC development (53, 54). Activated NF-κB pathways have been simultaneously reported in a mantle cell lymphoma (MCL), renal cell carcinoma and stromal tumor tissue (55), suggesting that there may be a common pathway between lymphoma and solid tumors. Similarly, through different mechanisms such as cell proliferation and oxidative stress, Bccip, miR-29, and PI3K

TABLE 2 | Clinical information of hepatocellular carcinoma with lymphoma patients.

Case	Age (year)	Gender	HBV/ HCV	Lymphoma		Hepatocellular adenocarcinoma		OS (months)
				Diagnosis	Treatment	Staging	Treatment	
Talamo T (12)	67	male	HBV	malignant lymphoma	palliative	IV stage	palliative	<1
Cavanna L (13)	50	male	Neither	NHL	chemotherapy	pT1NxM0	operation	30
Ono T (14)	59	female	HCV	DLBCL	None	T1NxM0	TACE	18
Shikuwa S (15)	64	male	HBV	B cell	None	TxNxM1	chemotherapy and radiotherapy	11
Monarca R (16)	66	male	HBV	chronic and indolent B-cell	Not mentioned	T1N0M0	Not mentioned	Not mentioned
Suriawinata A (17)	55	male	HCV	DLBCL	None	T2NxM0	liver transplantation	>15
Shapira M (18)	70	male	HCV	DLBCL	/	T2NxM0	/	/
Takeshima F (19)	65	female	HBV	MALT	hepatic segmentectomy	pT1N0M0	hepatic segmentectomy	>10
Kataoka T (20)	64	male	Neither	DLBCL	conservative therapy	T1NxM0	conservative therapy	1.5
Othsubo K (21)	66	male	HCV	DLBCL	R-CHOP	T2NxM0	RFA	Not mentioned
Himoto T (22)	63	male	HCV	DLBCL	CHOP	T1N0M0	PEIT and RFA	Not mentioned
Nonami A (23)	73	male	HBV	DLBCL	R-CHOP	T2N0M0	hepatectomy	>22
Lin A (24)	70	male	HCV	DLBCL	R-CHOP	Not mentioned	Not mentioned	>5
Lin A (24)	65	female	HCV	MZL	R-CHOP and radiotherapy	T2NxMx	None	14
Utsunomiya T (25)	70	female	HCV	DLBCL	Not mentioned	pT2N0M0	partial hepatectomy	4
Becker D (26)	68	male	HCV	SLL/CLL	untreated	pT1N0M0	RFA	Not mentioned
Heidecke S (27)	70	male	Neither	CLL	untreated	pT2NxMx	operation	>17
Tajiri H (28)	75	male	HCV	DLBCL	R-THP-COP	pT1N0M0	hepatectomy and chemotherapy	>12
Lee S (29)	60	male	HCV	FL	R-CVP	T2NxM0	TACE and RFA	>20
Chan R (30)	59	male	HBV	MALT	Right hepatectomy	pT1N0M0	Right hepatectomy	>48
Lee M (31)	52	male	HBV	MCL	CHOP	T3N0M0	RFA	>12
Meng J (32)	58	male	HBV	DLBCL	CHOP	I stage	operation	>62

HBV, chronic hepatitis B; HCV, chronic hepatitis B; NHL, non-Hodgkin's lymphoma; DLBCL, diffuse large B-cell lymphoma; MALT, mantle cell lymphoma; SLL, small lymphocyte lymphoma; CLL, chronic lymphocytic leukemia; FL, follicular lymphoma; MZL, marginal zone lymphoma; MCL, mantle cell lymphoma; R-CHOP, a combination of rituximab, cyclophosphamide, adriamycin, vincristine and prednisone; R-THP-COP, a combination of rituximab, pirarubicin, cyclophosphamide, vincristine, and prednisolone; R-CVP, a combination of rituximab, cyclophosphamide, vincristine and prednisone; TACE, transarterial chemoembolization; RFA, radiofrequency ablation; PEIT, percutaneous ethanol injection therapy.

pathways can simultaneously induce NHL and HCC (56–59). Therefore, the correlation between NHL and HCC theoretically implies that multiple primary tumors of NHL and HCC have a higher incidence rate. Moreover, immunotherapy makes it possible to manage related multiple primary tumors at the same time.

Even though we adopted tislelizumab instead of the standard first-line treatment of sorafenib when we treated the disease as primary HCC initially, treatment indications and the patient's views were fully considered. The clinical trial provided the patient more treatment options, and the patient truly had clinical benefits from tislelizumab. Immunotherapy for HCC is promising and may also benefit indolent NHL patients that are resistant to chemotherapy. In our case, while lymphoma did not respond to tislelizumab, the influence of tislelizumab in NHL progression and histological transformation cannot be negated. The patient had no significant progression in lymph nodes and hematological indicators during treatment of tislelizumab, however, outcomes were different during sorafenib treatment. The natural course of NHL could not be excluded. Hematological tumors are often caused by multiple co-inhibitory signaling pathways (60), which suppress immune activation. Compared to chemotherapy, rituximab has been shown to reduce the risk of histological transformation in indolent lymphoma (2, 61). Treatments for asymptomatic indolent lymphoma are unnecessary, however reducing incidences of histological transformation in double or multiple primary tumors with indolent lymphoma may be a consideration for treatment options.

One of drawbacks of this case is the failure to identify lymphoma at the early stage, which made accurate parallel analysis along the treatment course impossible. In addition, detection of immune treatment resistance of HCC, such as the accumulation of β-catenin, was not completed in the later stage, and lacked the underlying relevant mechanism.

CONCLUSION

There are no standard therapeutic options for lymphoma and HCC, as well as for double primary tumors. Individualized

treatments should be decided based on the behavior of tumor and patients' overall conditions. Clinical management of HCC with indolent B-cell lymphoma has rarely been reported in previous studies. Among the described cases, most are early HCC with local therapy, while standard therapy is used to treat lymphoma. To our knowledge, there are no reports of advanced HCC complicated with lymphoma using systemic therapy and achieves a long survival in literature.

For double primary tumors, latent cancer may be misdiagnosed as the progression of the first primary tumor due to primary drug resistance of ICIs (62). Therefore, it is necessary to determine the pathology of abnormal lymph nodes through biopsy. NHL can infiltrate into other tumors, such as HCC and renal cell carcinoma, so when atypical lymphoid cells are detected in tumor tissues, attention should be paid to mixed pathological features (25) since diagnosis of a double tumor will substantially affect clinical management. Moreover, NHL invasion may lead to the occurrence of precancerous lesions and promote the development of HCC (12, 20). Therefore, early diagnosis of lymphoma and simultaneous treatment of lymphoma and HCC can theoretically prolong survival time. Pathological characteristics for HCC and NHL are different, therefore, when standard systemic therapy for both tumors is applied synchronously, there may be great toxicity and poor tolerability. Treatment of HCC in the remission stage of NHL may improve the cure rate. This study elucidates the correlation between NHL and HCC, and the necessity for treating both diseases at the same time. The synergistic effects of CD20 mAbs and ICIs might provide a potential therapeutic option for double primary tumors with NHL. However, this conclusion should be verified further.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Sir Run Run Shaw Hospital, Zhejiang University School of Medicine. The patients/participants provided their written informed consent to participate in this study. Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

All authors have contributed to the preparation of this manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.634559/full#supplementary-material

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CAR T-Cell-Based gene therapy for cancers: new perspectives, challenges, and clinical developments

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Chimeric antigen receptor (CAR)-T cell therapy is a progressive new pillar in immune cell therapy for cancer. It has yielded remarkable clinical responses in patients with B-cell leukemia or lymphoma. Unfortunately, many challenges remain to be addressed to overcome its ineffectiveness in the treatment of other hematological and solidtumor malignancies. The major hurdles of CAR T-cell therapy are the associated severe life-threatening toxicities such as cytokine release syndrome and limited anti-tumor efficacy. In this review, we briefly discuss cancer immunotherapy and the genetic engineering of T cells and, In detail, the current innovations in CAR T-cell strategies to improve efficacy in treating solid tumors and hematologic malignancies. Furthermore, we also discuss the current challenges in CAR T-cell therapy and new CAR T-cell-derived nanovesicle therapy. Finally, strategies to overcome the current clinical challenges associated with CAR T-cell therapy are included as well.

KEYWORDS

immunotherapy, gene therapy, CAR T-cell therapy, solid cancers, hematologic malignancies

1 Cancer immunotherapy

The immune component plays a critical role in maintaining a balance between recognizing cancer cells as foreign bodies and showing tolerance towards self-antigens. The cancer immunity cycle depends on the ability of T-cells to attack and eliminate cancer cells. Antibodies against PD-1 and PD-L1 have significantly improved the outcomes of patients with melanoma and lung cancer (1, 2).

Cancer immunotherapy relies on the immune system of patients to recognize and attack cancer cells. Cancer immunotherapies potentiate immune cells by relieving their suppression or directly activating them to perform their immune function more effectively. There are different cancer immunotherapies based on the targeted immune components.

1.1 Cytokines

In the 1970s, tumor necrosis factor (TNF) was systemically injected into patients with cancer as a cancer immunotherapy modality. However, toxicities due to TNF infusion, such as fever, rigors, and pulmonary edema, limited its use in cancer treatment (3). Interleukin 2 (IL-2) is another cytokine that demonstrated efficacy and was approved by the Food and Drug Administration (FDA) for metastatic renal cell cancer in 1992 and metastatic melanoma in 1998. However, similar to TNF, the use of IL-2 was limited due to the severe toxicities it induced in the patients, which outweighed the benefits of the treatment (4).

1.2 Vaccines

The Bacillus Calmette-Guerin (BCG) vaccine was the first vaccine approved by the FDA in 1990 for the treatment of superficial bladder cancer. In 2010, the FDA approved a sipuleucel-T vaccine for castrate-resistant prostate cancer to extend the overall survival of patients. However, these vaccines failed to confer durable responses (5). This was perhaps due to the limited knowledge on dosing, vaccine availability in the tumor microenvironment, and engagement of T cells.

1.3 Checkpoint inhibitors

The discovery of immune checkpoint inhibitors was a breakthrough in cancer research. Allison showed that blocking cytotoxic T lymphocyte antigen 4 (CTLA-4) releases the brake on the immune system and boosts the immune response against cancer cells (6). Ipilimumab, a CTLA-4 checkpoint inhibitor, significantly improves survival in patients with metastatic melanoma (7). The CTLA-4 receptor is induced on T cells 48-72 h after T-cell receptors are engaged with antigen-presenting cells. The CTLA-4 receptor is also expressed on FOXP3 positive regulatory T cells (8). Mechanistically, CTLA-4 is known to have a PI3K-like motif, implying that it may interact with the PI3K, MAPK, and NF-kB pathways (9). Following CTLA-4 treatment, the FDA approved the inhibition of programmed death-1 (PD-1) and its ligand PD-L1 as immune checkpoint inhibitors for metastatic melanoma and lung cancers (10). PD-1 and PD-L1 interactions regulate immune escape in the tumor and tumor microenvironment. PD-1 expression on T-cells is a marker of antigen-experienced exhausted T-cells (11). Mechanistically, ligation of TCR and PD-1 leads to phosphorylation of a tyrosine residue located within the immunoreceptor tyrosin-based switch motifs (ITSM) of the PD-1 cytoplasmic tail. These events, including binding of phosphatases and augmentation of PTEN, expression contribute to decreased T-cell proliferation, survival, protein synthesis, and IL-2 production (12). An increasing number of clinical trials are being launched every year using these checkpoint inhibitors as monotherapies or in combination with standard of care or targeted therapies for various malignancies.

1.4 Adoptive cell therapy

CAR T-cell therapy is an adoptive cell-transfer-based immunotherapy developed by genetically modifying T cells. CAR T-cell therapy is directed against tumor-associated antigen and is independent of MHC-receptor presentation by the. This therapy has revolutionized the treatment of patients with B-cell lymphomas by conferring durable clinical responses. Several ongoing clinical trials have tested the efficacy of CAR T-cell therapy for different malignancies (13).

2 Genetic engineering of T-Cells

The source of T cells for CAR T-cell production can be either the patient (autologous) or a donor (allogenic). Blood is collected by venipuncture or apheresis from the patient and donor. The T cells undergo purification and are subjected to genetic engineering (14). CARs are artificially generated receptors that have been built to specifically target antigens expressed on the cell surface (15). T cells are typically engineered to express CARs by transducing patient T cells with a virus that encodes aDNA construct. The resulting CAR T cells are then expanded ex vivo and infused back into the patient (**Figure 1A**). Genetic engineering is performed using viral or non-viral methods to eliminate the expression of proteins such as HLA class I and II, in allogeneic T cells (16). This helps mitigate rejection by the hosts' immune system. These vectors are also co-delivered with transposase to enable the integration of transgenes into the genome in a random fashion (17). Transgenes are typically introduced under the control of endogenous promoters. A typical CAR consists of a single-chain variable fragment (scFv) with a flexible hinge domain, transmembrane domain, and CD3ζ activation domain (14) (**Figure 1A**) and several CAR T-cell generations have been engineered (18) (**Figure 1B**). The key raw material for CAR T-cell products is the viral vector. The viral vector is stored in large quantities at −80°C for up to 9 years (19). Safety, sterility, titer, purity, and potency of the vector are crucial for infusion into patients (20). Lentiviral and retroviral vectors are potentially oncogenic however, vectors are associated

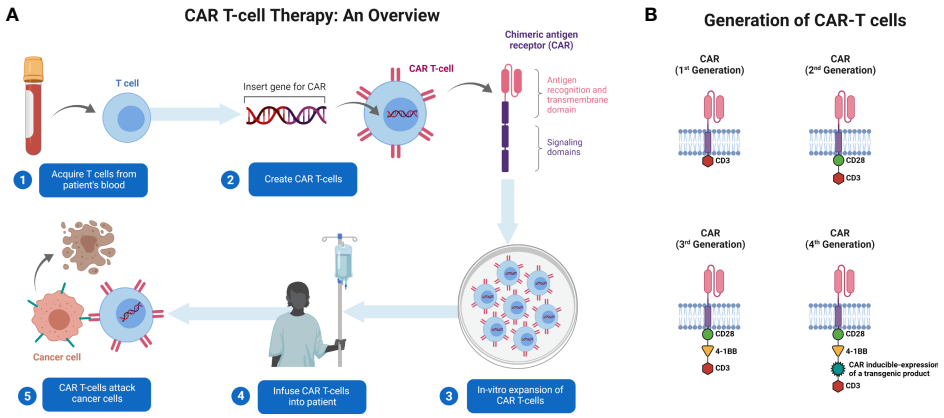

FIGURE 1
Generation and administration of CAR T-cells in patients with cancer. **(A)** T cells are collected from patients' blood *via* apheresis. They are genetically engineered to express CAR and cultured *ex vivo* for expansion. CAR T-cells are then administered to patients. The cells identify their target and kill the tumor cells expressing that target. **(B)** Illustration of basic structure of four generations of CAR T-cells. Created with BioRender.com.

with a lower risk of mutagenesis (21). It is also important to increase the safety of CAR T-cell therapy to improve the specificity of modified T cells.

3 CAR T-Cell therapy

3.1 Solid tumors

Tumors can suppress T-cells activity through various methods, and several studies have examined engineering cells to overcome this suppression. We evaluated clinical trials for the adequacy of CAR T-cell therapies in solid tumors (**Table 1**) and important targeted surface markers (**Figure 2**).

CAR T, chimeric antigen receptor-T; CAE, carcinoembryonic antigen; CD276, cluster of differentiation 276; CT 041, claudin 18.2; BPX-601, PSCA-Targeted CAR T-Cells; hCD70, human cluster of differentiation 70; 4S CAR T, fourth-generation safety-designed CAR; GFRA4, GDNF Family Receptor Alpha 4; EGFR, epidermal growth factor receptor and CD133, cluster of differentiation 133.

3.1.1 Pancreatic tumor

CAR T-cells have demonstrated therapeutic efficacy both *in vitro* and in orthotopic or metastatic xenograft mouse models. Studies have hypothesized that chemokine receptors CXCR2-expressing CAR T-cells could traffic towards IL-8 more efficiently. In xenograft animal models, CAR T-cells expressing CXCR2 showed significant antitumor activity against αvβ6-expressing pancreatic tumors (23). Interestingly, 4-1BB co-stimulation can lower PD-1 expression in the generated T cells, showing more potent antitumor activity against PD-L1-expressing tumor cells (24, 25). Additionally, clinical trials for pancreatic, colorectal, and hepatocellular carcinomas demonstrated the inhibitory effect of CD133-CAR T-cells on the metastatic potential of the cancers (26). In addition, other varieties of antigen targets for pancreatic cancer CART-cell therapy, such as CD24 (27), MUC-1 (28), PSCA (29), mesothelin (30), and FAP (31), have been investigated in preclinical studies and clinical trials.

3.1.2 Breast cancer

Several studies have shown that, CAR T-cells are very potent at killing triple-negative breast cancer (TNBC) tumor cells in an exceedingly tMUC1-highly specific manner. MUC28z CAR T-cells, a specifically contain CAR with both CD3ζ and CD28 signaling domains, which increases the synthesis of cytokine IFN-γ, granzyme B, and other kinds of cytokines or chemokines produced by Th1 cells. In addition, a single dose of MUC28z CAR T-cells could significantly abolish TNBC cell proliferation and increase survival benefits in xenograft models (32). Another study revealed that 4-1BB or CD27 co-stimulation enhanced NKG2D CAR T-cells involved in anticancer function in TNBC tumor models (33). Another study showed that CAR T-cells support HRG1β to successfully abolish breast cancer cell proliferation through HER family receptors and deliver a practical therapeutic approach to overcome cancer resistance, specifically against HER2-based targeted therapy (34). Human anti-HER2 CAR T-cells also exhibit desirable targeting, triggering cell death in HER2 overexpressing breast cancer cells (35). Furthermore, another biomarker, mesothelin, identified by special CAR T-cells, has been reportedly as promising in immunotherapy for breast cancers (36).

TABLE 1 Ongoing and currently recruiting clinical trials involving CAR T-cell therapies for solid tumors (22).

Intervention	Condition	Location	ClinicalTrials.gov Identifier
CEA CAR T-cells	Pancreatic Cancer	Chongqing University Cancer Hospital Chongqing, Chongqing, China	NCT04348643
CD276 CAR T-cells	Advanced Pancreatic Cancer	Li Yu Shenzhen, Guangdong, China	NCT05143151
CT041 autologous CAR T-cell	Pancreatic Cancer	Anhui Provincial Cancer Hospital Hefei, Anhui, China	NCT04581473
BPX-601 CAR T-cells	Metastatic Castration-resistant Prostate Cancer, Metastatic Prostate Cancer, Metastatic Pancreatic Ductal Adenocarcinoma, Metastatic Pancreatic Cancer and Metastatic Pancreatic Adenocarcinoma	Moffitt Cancer Center Tampa, FL, USA	NCT02744287
Anti-hCD70 CAR transduced PBL	Pancreatic Cancer	National Institutes of Health Clinical Center Bethesda, MD, USA	NCT02830724
CEA CAR T-cells	Breast Cancer	Chongqing University Cancer Hospital Chongqing, Chongqing, China	NCT04348643
4S CAR T-cells	Breast Cancer	The Seventh Affiliated Hospital, Sun Yat-Sen University Shenzhen, Guangdong, China	NCT04430595
CD44v6-specific CAR T-cells	Cancers Which Are CD44v6 Positive	Shenzhen Children's Hospital, Shenzhen, Guangdong, China	NCT04427449
Anti-hCD70 CAR transduced PBL	Breast Cancer	National Institutes of Health Clinical Center, Bethesda, MD, USA	NCT02830724
AIC100 CAR T-cells	Anaplastic Thyroid Cancer and Relapsed/Refractory Poorly Differentiated Thyroid Cancer	Weill Cornell Medical College New York, NY, USA	NCT04420754
single dose of CAR T-GFRa4 cells	Metastatic Medullary Thyroid Cancer	University of Pennsylvania Philadelphia, PA, USA	NCT04877613
EGFRv III -CAR transduced PBL	Malignant Glioma	National Institutes of Health Clinical Center, 9000 Rockville Pike Bethesda, MD, USA	NCT01454596
anti-CD133-CAR vector-transduced T cells	Brain Tumor	Biotherapeutic, Department and Pediatrics Department of Chinese PLA General Hospital Beijing, Beijing, China	NCT02541370

3.1.3 Thyroid cancer

The first study on CAR T-cell therapy for advanced thyroid cancer revealed the development of an intercellular adhesion molecule 1 (ICAM 1)- specific CAR T-cell and its preclinical efficacy (37). However, various factors may impede clinical translation of anti-ICAM 1-CAR T-cells. While T cells upregulate ICAM 1 expression and are followed by activation (38), it is possible that anti-ICAM 1-CAR T-cells might target each other, resulting in poor *in vitro* proliferation and persistence in patients with thyroidcancer. Another condition reported was elevated soluble ICAM 1 found in the serum of patients with thyroid cancer (39), which might neutralize anti-ICAM 1-CAR T-cells in the periphery before recognizing ICAM 1^+ tumor cells. In the absence of a tumor-associated antigen target (TAA), alternative technologies using antibody-based CARs to mimic T cell receptor (TCR) recognition of specific tumor-neoantigens, such as the complex of $BRAF^{V600E}$ oncoprotein with MHC, could be further investigated (40). The transgenic TCR tumor-infiltrating lymphocyte approach requires tumor cells to maintain the ability to process and present antigens at the cell surface. Medullary thyroid cancer (MTC) may be an excellent target for CAR T-cells therapies, given that these tumors commonly express carcinoembryonic antigen (CEA) and GDNF family receptor α4 (GFRA4). Indeed, GFRA4-specific CAR T-cell strategies are currently under preclinical development (41).

3.1.4 Brain cancer

Various clinical studies have been completed and are ongoing using CAR T-cells in glioblastoma (GBM). The first clinical trial on humans involving 10 patients with recurrent GBM evaluated the effect of intravenously injected EGFRvIII-CAR T-cells; while CAR T-cells expanded within the blood and were trafficked to the tumor region, they found antigen loss in five out of seven patients, and therefore, the tumor microenvironment indicated higher expression of inhibitory molecules, and the rate of occurrence of Treg cells was higher, as indicated (42). Improve the CAR T-cell therapy requires identifying TAA expressed with stability and specificity with definite heterogeneity throughout the tumor region. An

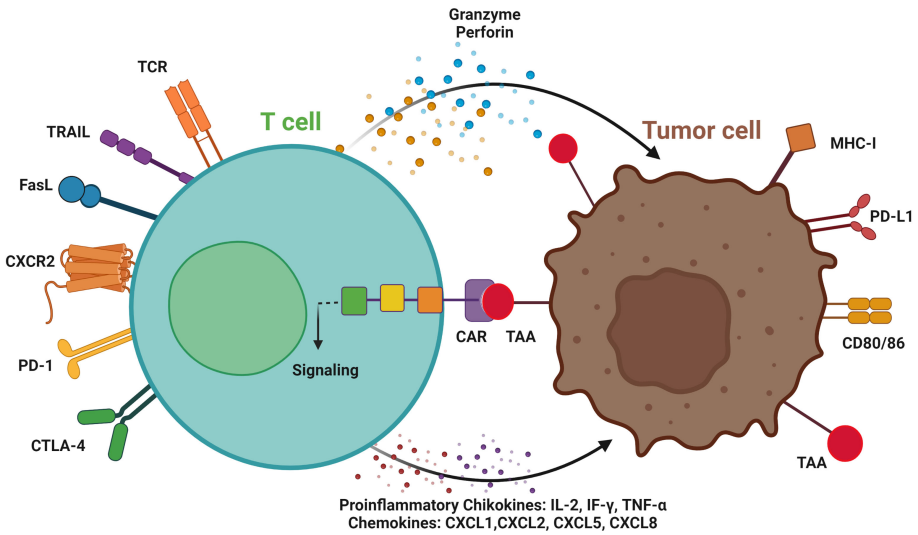

FIGURE 2
T cell-mediated antitumor effects by chimeric antigen receptors (CAR). Engineered CAR T-cells can recognize tumor cells by CAR binding to tumor-associated antigen (TAA), signaling activation and targeting the tumor cells by secreting granzymes, and perforins, and inducing TRAIL and FasL expression. CAR T-cells can be used as an ideal platform to deliver immune checkpoint therapeutic antibodies, such as anti-PD1 and CTLA-4 antibodies. CC-chemokine receptor 2; CD, cluster of differentiation; CTLA-4, cytotoxic T-lymphocyte associated protein 4; MHC, major histocompatibility complex; PD-1, programmed cell death protein-1; PD-L1, programmed death-ligand 1; and TCR, T cell receptor. Immune cells invade the tumor by activating proinflammatory cytokines and chemokines. Created with BioRender.com.

appropriate target was identified for these criteria. A study demonstrated *in vivo* therapeutic effects of intracranial delivery of chondroitin sulfate proteoglycan 4 (CSPG4)-CAR T-cells in nude mice transplanted with CSPG4-expressing glioma cells or GBM neurospheres models (43). As the endmost CAR T-cell product mixes with $CD4^+$ and $CD8^+$ CAR T-cells, this approach was refined to distinguish the T cell subsets that arbitrate antitumor activity. Another study revealed that the $CD4^+$ CAR T-cell subset, was more effective than $CD8^+$ CAR T-cells in orthotopic GBM mouse models and IL-13Rα2-CAR T-cells, which possibly indicated that $CD8^+$ CAR T-cells were rapidly exhausted (44). Co-expression of the IL-8 receptor, CXCR1, and CXCR2, enhanced CAR T-cell trafficking and was stably retained at in the glioma tumor site in a mouse model (45). Genetically engineered EGFRvIII-CAR T-cells co-expressing a bispecific T-cell engager (BiTE) directed against EGFR (wild-type) were established in GBM tumor models (46). Additionally, various CAR target antigens in GBM tumors, including B7-H3 (47, 48), HER2 (49–51), and EphA2 (52), have been demonstrated in advanced phase I clinical trials using HER2-CAR T cells and in other preclinical studies (50, 53).

The development of a universal CAR T (UCAR T) cell, which allows a tri-cistronic transgene to encode three CAR molecules against HER2, IL-13Rα2, and EphA2, overcame the interpatient variability and targeted 100% of GBM tumor cells (54). In a different way to overcome antigen escape problems and tumor heterogeneity, a new CAR approach was designed that employs a toxin as the targeting entity, which was developed and tested in a murine glioma model. Chlorotoxin (CLTX) directed CAR T-cellsshowed GBM cell binding affinity by matrix metalloproteinase-2 and CLTX- CAR T-cells efficiently limited tumor growth in mouse model, which addressed the off-target effects (55) The ongoing and currently recruiting phase II clinical trials (thyroid tumor: I clinical trials) involving CAR T-cell therapies for solid tumors are listed in **Table 1**.

3.2 Hematologic malignancies

Hematologic malignancies, also known as blood cancers, arise from the uninhibited proliferation of abnormal blood cells and made up approximately 10% of all cancers in 2019 in the United States (56). CAR T-cell therapies have shown significant promise in the treatment of hematologic malignancies in recent years (57–61), although the first insight into their efficacy of CAR T-cell therapy was obtained from the clinical trials involving solid tumors (62, 63). The response time for CAR T-cell therapy is lower than that for other therapeutic strategies, such as tumor vaccines and immune checkpoint blockade, although this is not always true since, some of the CAR T-cells persist with a memory phenotype and respond more quickly (64, 65). These efforts have resulted in three FDA-approved first-of-

their-kind therapies for treating refractory diffuse large B-cell lymphoma (DLBCL) and acute lymphoblastic leukemia (ALL) (66).

3.2.1 Hodgkin's lymphoma

Hodgkin's lymphoma (HL) is less common than other hematologic malignancies, accounting for <1% of all cancers in the United States (56). In 2020, 83,087 new HL cases and 23,376 HL-related deaths were estimated worldwide (67). HL is characterized by Hodgkin Reed-Sternberg (HRS) cells belonging to the B-cell lineage. HRS and anaplastic large cell lymphoma (ALCL) cells highly express the cell surface marker CD30 (68). While the FDA-approved antibody-drug conjugate brentuximab vedotin is clinically effective in treating these tumors by targeting CD30 (68, 69), the progression-free survival (PFS) rate remains low at 5 years, suggesting that improved targeted therapies could cure the disease by driving tumor cells in long-term remission (70). CAR T-cell therapies directed towards CD30 have shown durable antitumor response in HL cell lines and mouse models (71, 72). Inducing expression of CCR4 in anti-CD30 CAR T-cells promotes their migration towards tumors in HL mouse xenografts (73). In phase I clinical trials, antitumor responses have been observed in the presence or absence of conditioning chemotherapy when patients with brentuximab-refractory HL and ALCL patients were treated with anti-CD30 CAR T-cells containing a CD28 (74) or 4-1BB costimulatory domain (75).

3.2.2 Non-hodgkin lymphoma

Non-Hodgkin lymphoma (NHL) is more common than HL and constitutes approximately ~4% of all cancers in the United States (56). In 2020, 544,352 new NHL cases and 259,793 NHL-related deaths were estimated worldwide (67). NHL can be categorized as B-cell lymphoma (BCL) and T-cell lymphoma (TCL). Most BCL cells express the B-cell differentiation markers - CD19 and CD20, whereas some TCLs express the CD30 marker (76).

3.2.2.1 B-Cell lymphoma

BCL constitutes the majority (~85%) of NHLs (77). DLBCL (26%), follicular lymphoma (FL; 13%), marginal zone lymphoma (MZL; 7%) and mantle cell lymphoma (MCL; 3%) are the main subtypes of NHL (76). CAR T-cell therapies targeting these antigens have shown a high overall response rate (ORR) and complete response rate (CRR) in NHL in clinical trials (60).

In a clinical trial involving seven patients, the City of Hope National Medical Center and Fred Hutchinson Cancer Research Center researchers used electroporation to introduce the CD20-specific CAR transgene into the T cells of patients with MCL and refractory BCL (78). This resulted in either stable disease (n=4) or partial response (n=1) or complete responses (n=2) with minimal toxicities (78). In another clinical trial published by the City of Hope, patients with recurrent DLBCL and refractory FL were treated with CD20- and CD19-specific CAR T-cells. Although minimal toxicity was observed, the persistence of infused cells remained low (79). The National Cancer Institute (NCI) first reported the efficacy of CD19-specific CAR T-cells incorporated with a CD28 costimulatory domain (FMC63-28Z) in combination with chemotherapy and IL-2 administration in the treatment of treating refractory FL and splenic MZL in a clinical setting (58, 80). While patients did not suffer from evident chronic toxicities, cytokine release syndrome (CRS) was observed (58). In a pilot study conducted by Till et al. (2012), patients with FL and MCL received CD20-specific CAR T-cells with costimulatory domains *via* electroporation followed by conditioning chemotherapy (81). Notably, patients showed partial or complete response and the persistence of T cells in the blood lasted for 9-12 months, which may be attributed to multiple IL-2 treatments (81). Another clinical trial involving the administration of anti-CD19 CAR T-cells in two children with relapsed and refractory (R/R) pre-B-cell ALL resulted in complete remission (82). Interestingly, one of the patients relapsed due to the emergence of CD19-negative cells, demonstrating a classic immune escape mechanism, indicating that and other B-cell markers are needed to improve the efficacy of treatment (82).

The NCI first reported successful administration of anti-CD19 CAR with a CD28 costimulatory domain in patients with DLBCL (83). Cyclophosphamide and fludarabine was included in their chemotherapy regimen prior to CAR T-cell infusion. The combination therapy worked well, driving refractory BCLs, including DLBCL, into complete remission (83). Another clinical trial demonstrated the efficacy of anti-CD19 CAR T cells containing CD28 and TCR zeta domains with reversible toxicities, when administered to children and young adults with relapsed or refractory B-cell ALL (B-ALL) following the aforementioned chemotherapy regimen (83, 84). Antitumor responses have also been observed when anti-CD19 CAR T-cells with a 4-1BB costimulatory domain were administered to patients with NHL or B-ALL (85, 86). Fludarabine conditioning chemotherapy proved effective in improving ORR (86). Clinical trials involving anti-CD19-CAR T-cells have shown better clinical responses in patients with ALL and chronic lymphocytic leukemia (CLL) when combined with cyclophosphamide conditioning (57, 87). Relapses were observed due to the low *in vivo* persistence of CAR T-cells and the emergence of CD19-negative cells as a mechanism of immune escape (57, 87). Interestingly, reports also showed the efficacy of anti-CD19 FMC63-28Z CAR T-cells alone in treating patients with ALL, CLL, DLBCL, and MCL, in the absence of prior chemotherapy (88). Graft-versus-host disease (GVHD) was observed in one patient (64, 88). Anti-CD19 CAR T-cells

therapies have shown promising results when used as adjuvant treatments following autologous or allogeneic hematopoietic cell transplantation (HCT) in patients with ALL or B-cell NHL, with the former resulting in a higher ORR and 30-month PFS rate than allogeneic HCT (89). Phase I and II trials of axicabtagene ciloleucel, anti-CD19 CAR T-cells with CD28 costimulatory domain, have demonstrated anticancer response in refractory NHL when combined with cyclophosphamide and fludarabine chemotherapy, with an ORR of 82% and complete response rate of 54% in more than 100 treated patients (90). Similarly, anti-CD19 CAR T-cells with a 4-1BB costimulatory domain in combination with the aforementioned chemotherapy (90), resulted in an impressive ORR of 80% and a complete response rate of 60% in patients with lymphoma (91). Clinical trials using this combination therapy in patients with DLBCL are underway (92). While CD20-specific second-generation CAR T-cells containing a 4-1BB costimulatory domain were able to drive refractory DLBCL into partial remission when administered with prior conditioning chemotherapy (93), a phase II trial using the same CAR T-cells resulted in complete remission in six out of 11 patients with NHL (FL, MCL, DLBCL) patients (94).

Recent efforts in CAR T-cell development have targeted the identification of novel B-cell surface markers to improve selectivity of the therapy toward tumor cells, thereby sparing normal cells and reducing the side effects of CART-cell therapy. Three attractive targets, CD23 (present on CLL cells) (95), ROR1 (present on CLL and MCL) (96), and immunoglobulin kappa (κ) light chain (present on MCL, DLBCL, and some other NHLs) (97) are being evaluated for their anticancer activity in preclinical models since they are either not expressed or present at low levels in normal cells. CD22 is another potential target antigen expressed on B-ALL and other B-cell lymphomas (98). Preclinical results have demonstrated potent antitumor activity when at monoclonal antibody targeting a proximal epitope on CD22 is used for CAR T-cell production (98).

3.2.2.2 T-Cell lymphoma

While TCL accounts for only a small proportion (~15%) of all NHL cases, they are associated with a worse prognosis compared to B-cell NHL (77, 99). Currently, therapeutic options for the treatment of TCL are limited to allogeneic HCT (100). Developing CAR T-cell therapies can be a breakthrough; however, it is imperative to do so by identifying antigen markers that are exclusively present on malignant T cells. One potential target antigen could be CD30 since some TCLs such as ALCL express it on their cell surfaces (68). Although high cytotoxicity was observed, natural killer cells have shown antitumor activity in preclinical T-cell ALL-derived cell lines (101). This study suggests that CAR T-cell therapies have the potential to treat complex, difficult-to-treat diseases. However, a better understanding of cytotoxicity management is required to improve the effectiveness of these therapies.

3.2.3 Acute myeloid leukemia

In 2019, acute myeloid leukemia (AML) accounted for <2% of all cancers in the United States (56). The disease is associated with a poor prognosis owing to the limitation in finding a suitable target that is only present in AML cells and absent in normal hematopoietic stem cells (102). CD123, a hematopoietic cell marker, has shown efficacy in preclinical models (102, 103). A phase I clinical trial is currently ongoing to determine the safety and efficacy of second-generation autologous or allogeneic anti-CD123 CAR T-cells (with a CD28 costimulatory domain) in combination with cyclophosphamide and fludarabine chemotherapy (104). In addition to CD123, CAR-T cells specific for CD33, another myeloid antigen, have also shown promise *in vivo* for refractory AML (105). Higher expression of CD33 on normal cells makes them a less attractive target for treatment than CD123 (105). A phase I clinical trial, involving anti-Lewis Y (LeY) CAR T-cells with a CD28 costimulatory domain, demonstrated modest responses in two patients who had received prior fludarabine chemotherapy (106). CAR T-cells show durable persistence in patients, leading to mild toxicity (106). Other potential CAR T-cell therapy targets, including CD47, CD96, and CD44v6, are currently being investigated in preclinical models (100).

3.2.4 Multiple myeloma

In 2019, 176,404 new multiple myeloma (MM) cases and 117,077 MM-related deaths are estimated worldwide (67). In the United States, in 2019, MM accounted for <2% of all cancers (56). MM cells express plasma cell surface antigens CD138 and CD38 (107). A phase I clinical trial involving CD138-specific CAR T-cells demonstrated efficacy with tolerable toxicities in five patients with refractory MM, with 4 patients reaching a stable disease state and one demonstrating a marked reduction of MM cells in the peripheral blood (108). Another phase I trial is ongoing to determine the dose-limiting toxicities associated with anti-CD138 CAR T-cell therapy in relapsed or refractory MM (NCT03672318).

B-cell maturation antigen (BCMA) is another surface marker present in B, plasma and MM cells (109). A clinical trial of anti-BCMA CAR T-cells with CD28 costimulatory domain conducted at NCI demonstrated partial responses in two patients and stable disease in 10 patients when treated with low doses of cells in combination with chemotherapy (110). High doses of CAR T-cells resulted in complete response in one patient and partial response in the other (110). Patients also experienced a higher degree of toxicity with increasing CAR T-cell doses (110). Anti-BCMA CAR T-cells alone have also shown efficacy in the absence of chemotherapy, leading to partial

response in one patient and complete response in another, with toxicity levels similar to those observed in the NCI trial (111). MM cells demonstrate a classic immune escape strategy through the emergence of BCMA-negative cells (111). The infusion of low doses of anti-BCMA CAR T-cells with the 4-1BB costimulatory domain after chemotherapy resulted in partial response and mild toxicities in one patient, while high doses resulted in partial or complete responses in 11 out of 15 patients (112). A phase III trial is currently ongoing to determine the safety and efficacy of bb2121 in combination with standard MM treatment regimens and chemotherapy (**Table 2**). Another phase I trial with anti-BCMA CAR T-cells called LCAR-B38M has resulted in partial or complete responses with mild toxicities in 18 of the 19 treated patients (113). Anti-CD19 CAR T-cells administered to a patents with refractory MM following melphalan chemotherapy and autologous stem cell transplantation resulted in a complete response (114).

Preclinical evaluation of other potential antigen targets for CAR T-cell therapy such as CD38, CD44 isoform variant 6 (CD44v6), CD70, CD56, immunoglobulin κ light chain and signaling lymphocyte–activating molecule F7 (SLAMF7) is underway (115).

Currently, several phase III clinical trials are ongoing to determine the efficacy of CAR T-cells therapies targeting various antigens in combination with chemotherapy in patients with ALL, MM, AML and BCL (**Table 2**).

CAR T, chimeric antigen receptor-T; R/R, relapsed or refractory; B-ALL, B-cell acute lymphoblastic leukemia; B-LLy, B-cell lymphoblastic lymphoma; BCL, B-cell lymphoma; DLBCL, Diffuse Large B Cell lymphoma; MRD, minimal residual disease; CLL-1, C-type lectin-like molecule-1; AML, acute myeloid leukemia; MM, multiple myeloma; NHL, non-Hodgkin lymphoma; BCMA, B-cell maturation antigen.

4 Side effects of CAR T-Cell therapy

CAR T-cell therapies are known to cause severe side effects in various malignancies including CRS, GVHD, tumor lysis syndrome (TLS) and immune effector cell associated neurotoxicity syndrome (ICANS) (82, 116–119). CRS is activated by a massive increase in serum cytokine levels followed by T-cell activation (58, 65, 120) and is accompanied by nausea, vomiting, headaches, fever, myalgia, anorexia, coagulopathy, hypotension, renal dysfunction, and pulmonary edema (118). Severe CRS has been reported following by the administration of anti-CD19 CAR T-cell therapies in patients with NHL (86). A study conducted by Grupp et al. demonstrated the potential of tocilizumab, an anti-IL6 receptor antibody, in rapidly eliminating CRS (82).

Neurological toxicities may lead to B-cell aplasia, confusion, unresponsiveness, and seizures (118, 121), especially when anti-CD19 CAR T-cell therapies are administered in patients with lymphoma (86, 89). However, the mechanisms underlying these toxicities remain unknown (119). Notably, CRS and NS rates

TABLE 2 Ongoing and currently recruiting phase III clinical trials involving CAR T-cell therapies for hematologic malignancies (22).

Intervention	Condition	Location	ClinicalTrials.gov Identifier
Anti-CD19 CAR T-cells with concurrent BTK inhibitor for BCL	BCL	Union Hospital, Wuhan, Hubei, China	NCT05020392
CAR-transduced autologous T cell intravenous infusion in subjects with R/R DLBCL with chemotherapy	R/R DLBCL	Multi-center study	NCT03391466
Anti-CD19 CAR T-cells with chemotherapy or blinatumomab in adults with B-ALL	B-ALL	Multi-center study	NCT04530565
BiRd regimen combined with BCMA CAR T-cell therapy in patients with MM	MM	The First Affiliated Hospital of Soochow University Suzhou, Jiangsu, China	NCT04287660
VRd regimen combined with autologous BCMA CAR T-cell therapy in patients with MM	MM	Multi-center study	NCT04923893
Autologous CAR T cell therapy targeting BCMA	MM	Multi-center study	NCT04181827
Efficacy and Safety Study of bb2121 Versus Standard Triplet Regimens in Subjects with R/R Multiple Myeloma (RRMM)	MM	Siteman Cancer Center, Saint Louis, MO, USA Hackensack University Medical Center, NJ, USA Sarah Cannon Research Institute Center for Blood, TN, USA	NCT03651128
Intravenous autologous CD19 CAR T-Cells for R/R B-ALL	R/R B-ALL	UKM Medical Centre Bandar Tun Razak, Kuala Lumpur, Malaysia	NCT03937544
Tisagenlecleucel in adult patients with aggressive B-cell NHL	B-cell NHL	University of Chicago Medical Center, Hematology & Oncology, IL, USA Sarah Cannon, Research Institute, TN, USA	NCT03570892

were higher in patients with hematologic malignancies than in those with solid tumors (60).

GVHD is often experienced by patients following the infusion of allogeneic lymphocytes from HTC donors, because of the response elicited by non-cancerous cells (122). Allogeneic anti-19 CAR T-cells cause chronic GVHD but no acute GVHD in patients with various B-cell lymphomas (118). The lack of GVHD may be attributed to the low persistence of CAR T-cells (76).

TLS is characterized by hyperkalemia, hyperuricemia, hypocalcemia, and hyperphosphatemia (118). Severe TLS has been observed in patients following infusion of anti-CD19 CAR T-cell therapies in various studies (59, 64).

Other less common side effects of CAR T-cell therapies include hypotension (87), pulmonary toxicity (123), hemorrhagic events (86, 93), and even death in rare cases (124). Strategies to eliminate CAR T-cells once the desirable response is achieved, are urgently required. Several studies have reported the use of biodegradable CAR T-cells, addition of an EGFR on the T-cell surface to be targeted by anti-EGFR antibodies, RNA electroporation (125, 126) or suicide gene incorporation (using target epitopes from CD34/CD20/caspase 9) (102, 103, 127–131). Although these approaches may work well, they should be used with caution since, the antitumor response achieved in patients may be affected in the absence of CAR T-cells (76).

5 Current challenges in CAR T-Cell therapy

The major challenges in the field of CAR T-cell therapy are to improve the *in vivo* persistence of CAR T-cells and identify ways to mitigate therapeutic toxicity. In addition, many unknowns in the field remain to be investigated, such as the mechanism of target-cell death, optimal dose needed for maximum efficacy, duration of *ex vivo* T-cells expansion, and efficacy of single vs multiple infusions of CAR T-cells.

CAR T-cells must persist and remain functional for a long time to prevent relapse. Long-term persistence of anti-CD19 CAR T-cells has been demonstrated in patients for many years after infusion (65, 82, 132). The limiting factors for *in vivo* CAR T-cell persistence may include *ex vivo* conditions in which T cell expansion occurs, stability of transgene expression, and immune responses developed against the transgene (133). Similarly, severe toxicities associated with CAR T-cell therapy may be due to the disease burden (84), high-dose chemotherapy regimen (87), high-dose CAR T-cell infusion (86), and as peak levels of serum cytokines and C-reactive protein (83, 86).

Determining the mechanism underlying target cell death, which may be caused by signaling domains associated with antigens or TCR complex chain, is crucials (134). The fate of the residual natural TCR remains unclear. T cells can also mediate target-cell death *via* granzyme release, cytokine release, and other immune effectors.

Responses to different doses of CAR T-cell therapy vary on a patient-by-patient basis. Some patients can greatly benefit from small doses, while others may not show any effect after infusion of a large dose. Therefore, it is challenging to determine the optimal T-cells dose for individual patients. Other important factors that may modulate this response are disease burden and toxicity levels (65, 82). A few studies recommend infusion of less than 10^8 CAR T-cells following lymphodepletion in clinical trials to achieve a higher complete response rate (60, 135). Although infusion of multiple small doses of CAR T-cells has not shown any toxicity, it is still unknown whether single or multiple infusions lead to optimal efficacy remains unknown (59, 136).

The duration for which T cells need to be expanded in culture before infusion remains unclear. Since a less differentiated and more proliferative phenotype (such as T memory stem cells) is associated with better responses in preclinical models (137, 138), long-term *ex vivo* T cell expansion may not yield optimal results. Several crucial details regarding T-cell trafficking after infusion are currently unknown. Homing and trafficking of molecules on tumor vessels play a key role in modulating T-cell recruitment into the tumor microenvironment (139), thereby influencing the response in patients (140).

6 CAR T-Cell-derived nanovesicle therapy

Extracellular vesicles (EVs) are nano-sized membrane based-vesicles secreted by almost all cells and consistof exosomes (small EVs), microvesicles, apoptotic bodies and larger vesicles. EVs are capable of carrying various biological cargoes such as lipids, proteins and nucleic acids and resembles of their origin cells compositions (141–145). They are involved in local or distal intercellular communication by interacting with or delivering biologically active cargoes to recipient/target cells (146, 147). Immune cells such as dendritic cells, natural killer cells, macrophages, B- cells, and T-cellshave been shown to release EVs and are capable of modulating immunoregulation, tumor microenvironment and EV-based immunotherapy for cancers (148–152).

As EVs are mirror images of their parent cells in terms of their composition, CAR T-cell-derived EVs may substitute CAR T-cells and overcome some limitations. For example, CAR T-cells can proliferate in an uncontrolled manner thus inducing cytokine release syndrome (58, 123), which can lead to complications and even death (153),whereas EVs are non-proliferative biological nano-materials. Unlike cell therapies,

EVs may not cause immune rejection (154). Immunotherapies can be hampered by tumor microenvironments; however, EVs are not influenced by the tumor microenvironments (155, 156).

Recent studies have reported the use of CAR T-cell derived EVs (exosomes or EVs) in cancer therapies (157–159). Exosomes derived from CAR T-cells (CAR-T exosomes) have shown high levels of cytotoxic molecules, such as perforin and granzyme B. CAR-T exosomes inhibit the growth of human breast tumors. Moreover, an *in vivo* preclinical model showed that the administration of CAR-T exosomes is safer than CAR-T cell therapy (157). Another study compared the penetration and cytotoxic activities of stimulated Anti-HER-2+ CAR T-cells and their CAR-T EVs. CAR-T EVs contain lower interferon gamma levels than CAR T-cells. Granzyme B levels were approximately 20-fold higher in CAR-T EVs than in EVs from unstimulated CAR T-cells. Anti-HER-2+ CAR-T EVs targeted HER-2 expressing cells. CAR T-cells showed more rapid cytotoxicity than their EVs (159). HEK293T cells were transduced with CD19 CAR plasmids, and their exosomes (Exo-CD19 CAR) were used to treat CD19 B-lineage leukemia. The results showed that Exo-CD19 CAR treatment induced cytotoxicity in CD19-positive leukemia B-cells but not in CD19-negative cells (158). These studies support the therapeutic use of EVs derived from CAR T-cells as a cell-derived nanovesicle-based therapeutic approach against tumors (**Figure 3**).

7 Strategies to overcome current clinical challenges associated with CAR T-Cell therapies

CAR T-cell persistence is major challenge faced by the CAR T-cell research community. Some of the strategies that can improve T cell persistence include administration of cytokines such as IL2, IL7, and IL15, and upregulation of proliferative or anti-apoptotic signals (87, 160, 161). In contrast, some studies have found that skipping IL-2 during CAR T-cell production resulted in higher ORR in patients with solid tumors and hematologic malignancies (60, 135).

Optimization of the CAR design is equally important for better persistence and overall treatment efficacy. Second-generation CARs have been shown to improve persistence compared to first-generation CARS;however, it remains unclear whether third-generation CARs are better at improving persistence than those in the second-generation CARs (81, 162). Among the different costimulatory molecules, CD137 and 4-1BB seem to work better than CD28 molecules in enhancing persistence and tumor trafficking, thereby improving the antitumor response in preclinical models (163, 164). Changes in the hinge and transmembrane regions of CAR regulate cell death and cytokine production (98, 165). A fully human CAR construct

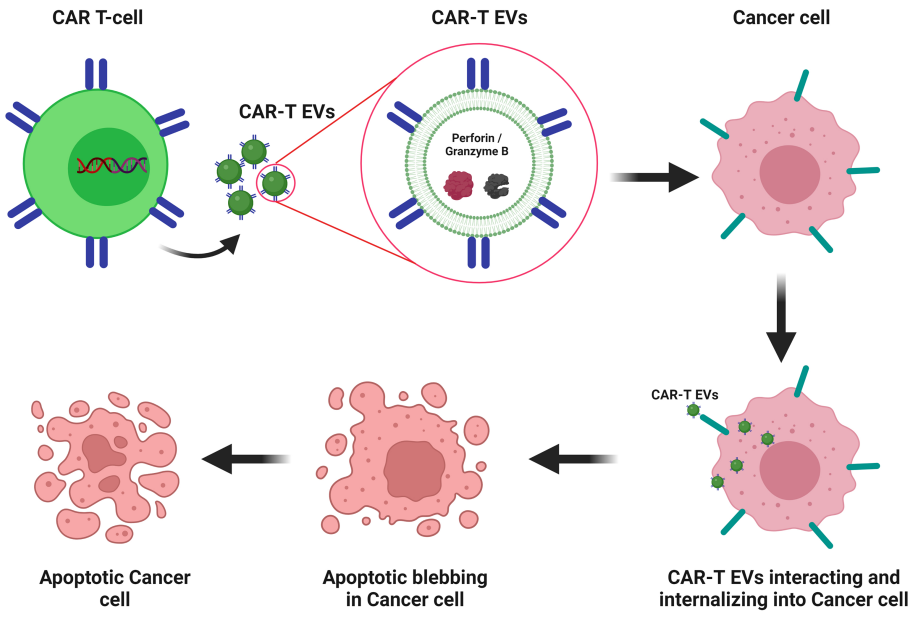

FIGURE 3
CAR-T EV-based therapy for cancer CAR-T EVs containing catalytic proteins (perforin and granzyme B). CAR-T EVs' interacting and internalizing into cancer cells and leading to apoptotic blebbing and apoptosis. Created with BioRender.com.

(HuCAR-19), designed to reduce immunogenicity and improve persistence (76), has shown an 86% ORR in patients with NHL in a first-of-its-kind clinical trial (166, 167). Clinical trials using two fully humanized CAR constructs are currently underway in patients with CD30+ NHL and HL as well as in those with CD19+ ALL and NHL. Preclinical studies have suggested an improved antitumor response when pharmaceutical agents and conditioning chemotherapy are administered in combination with CAR T-cell therapy (86, 168).

Tumor cells modulate the antigen expression on their cell surface to facilitate immune escape (57, 82, 87, 111). Therefore, CAR T-cells can no longer recognize and kill these cells. The efficacy of CAR T-cell therapy can be enhanced, and toxicity can be minimized by incorporating molecules specific for two or more target antigens, as demonstrated by some preclinical studies (169, 170). CAR T-cell therapies in conjunction with immune-checkpoint blockade are currently being investigated in patients with refractory or relapsed NHL (171).

Therefore, safer and cheaper gene transfer approaches are needed to reduce the overall cost of CAR T-cell therapy. While non-viral approaches, such as Sleeping Beauty, are inexpensive compared to lentiviral/retroviral vector-mediated gene transfer, there is a growing body of clinical evidence using the latter approach (172, 173).

Finally, CAR T-cell therapies have also been applied much later during the course of disease progression usually following chemotherapy, hematopoietic stem cell transplantation, or other treatments. The tremendous potential of applying CAR T-cell therapy at the beginning or earlier during the treatment course was unraveled and the strategy revealed higher success rates and reduced toxicity associated with anticancer treatments (174). Early administration of the therapy earlier may also give us access to a higher proportion of naïve, unexposed T-cell populations to facilitate the production of CAR T-cells.

Author contributions

MJ, PG, and B-CA contributed to the conception, writing, and discussion of this manuscript. MJ, FK, CD, RR, and PG wrote the initial draft of the manuscript. B-CA Contributed to the critical conclusion of the manuscript. The final version of the manuscript has been approved by all authors.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Cytopenia after chimeric antigen receptor T cell immunotherapy in relapsed or refractory lymphoma

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Background: Patients with relapsed or refractory (R/R) lymphomas have benefited from chimeric antigen receptor (CAR)-T-cell therapy. However, this treatment is linked to a high frequency of adverse events (AEs), such as cytokine release syndrome (CRS), immune effector cell-associated neurotoxicity syndrome (ICANS), and hematologic toxicity. There has been increasing interest in hematological toxicity in recent years, as it can result in additional complications, such as infection or hemorrhage, which remain intractable.

Methods: We conducted a retrospective, single-institution study to evaluate the patterns and outcomes of cytopenia following CAR-T-cell infusion and potential associated factors.

Results: Overall, 133 patients with R/R lymphoma who received CAR-T-cell therapy from June, 2017 to April, 2022 were included in this analysis. Severe neutropenia, anemia and thrombocytopenia occurred frequently (71, 30 and 41%, respectively) after CAR-T-cell infusion. A total of 98% of severe neutropenia and all severe thrombocytopenia cases occurred in the early phase. Early severe cytopenia was associated with CRS incidence and severity, as well as peak inflammatory factor (IL-6, C-reactive protein (CRP), and ferritin) levels. In multivariate analysis, prior hematopoietic stem cell transplantation (HSCT), baseline hemoglobin (HB), and lymphodepleting chemotherapy were independent adverse factors associated with early severe cytopenia. In addition, 18% and 35% of patients had late neutrophil- and platelet (PLT)-related toxicity, respectively. In multivariate analysis, lower baseline PLT count was an independent factor associated with late

thrombocytopenia. More severe cytopenia was associated with higher infection rates and poorer survival.

Conclusions: This research indicates that improved selection of patients and management of CRS may help to decrease the severity of cytopenias and associated AEs and improve survival following CAR-T-cell therapy.

Clinical Trial Registration: https://www.clinicaltrials.gov/ct2/show/NCT03196830, identifier NCT03196830.

KEYWORDS

Lymphoma, chimeric antigen receptor, hematological toxicity, cytopenia, cytokine release syndrome

Introduction

Although chemoimmunotherapy regimens and novel targeted drugs have substantially improved lymphoma care (1), patients with treatment failure after chemoimmunotherapy often have a poor outcome, especially those with disease that is refractory to frontline or subsequent therapies (2). Chimeric antigen receptor (CAR)-T-cell therapy is a novel type of tumor immunotherapy that has improved outcomes for many patients with relapsed or refractory (R/R) lymphomas, showing varied efficacy in diffuse large B-cell lymphoma (DLBCL) (objective response rate (ORR) 52-88%; complete response (CR) 40-59% (3), mantle cell lymphoma (MCL) (CR 59%) (4), and chronic lymphocytic leukemia (CLL) (ORR 57-74%; CR 21%) (5). To date, several CAR-T-cell therapies have received FDA approval in the USA, including axicabtagene ciloleucel (axi-cel) (6) and lisocabtagene maraleucel (liso-cel) (7) for R/R DLBCL, axi-cel for R/R follicular lymphoma (FL) (8), and brexucabtagene autoleucel (brexu-cel) for R/R MCL and B-cell acute lymphoblastic leukemia (ALL) in adults (4). CAR-T-cell therapy has had a substantial impact on overall survival (OS) and progression-free survival (PFS), but it also comes with a host of adverse events (AEs), including cytokine release syndrome (CRS) and immune effector cell-associated neurotoxicity syndrome (ICANS), which can lead to treatment failure (9). Increasing CAR-T-cell therapy experience suggests that these complications can be effectively controlled in a timely manner through early monitoring and intervention, which makes this treatment safer than ever (7).

However, with long-term patient follow-up, cytopenia has been increasingly reported after CAR-T-cell therapy. Cytopenia after infusion of CAR-T cells is extremely common. In the ZUMA-1 study, 78% of patients had neutropenia, 43% of patients had anemia and 38% of patients had thrombocytopenia (10). Similarly, any-grade cytopenia occurred in 44% of patients in the JULIET study (11). Severe cytopenia may result in higher risks of infection (12) and hemorrhage, requiring more blood transfusion support (13). Transfusion-related responses, iron overload, and circulatory overload are relevant to continuous transfusion needs. As a result, there might be a decline in quality of life, and an increase in treatment-related morbidity and mortality (14). Prior studies have mainly considered the following potential risk factors: patient median age, number of prior lines of therapy, baseline blood counts, baseline LDH levels, CRS grade, and CRS-related inflammatory factor levels (14–16). However, these factors have shown contradictory utility in identifying and predicting the risk of this complication. Here, we retrospectively characterized the hematological toxicities of patients treated in our center who participated in a phase I/II clinical trial of CAR-T-cell therapy for R/R lymphoma and explored potential causes of hematologic toxicities, specifically cytopenias, with CAR-T-cell therapy.

Methods

Patients

In this single institution retrospective observational study, 171 patients with R/R lymphoma, including DLBCL, transformed lymphoma (transformed FL (tFL), transformed mucosa-associated lymphoid tissue (tMALT) lymphoma, or transformed CLL (tCLL)), indolent lymphoma (marginal zone lymphoma (MZL), CLL, or FL) and other diseases (Table 1), were treated with CAR-T cells targeting CD19/CD22/CD20/CD30 between June, 2017 and April, 2022

TABLE 1 Demographic and clinical characteristics of patients.

Total	N=133
Age, year	44 (range, 18-71)
Sex, male n (%)	75 (56.39%)
Bone marrow involvement n (%)	45 (33.83%)
Prior HSCT n (%)	24 (18.05%)
Median lines of prior therapy (range)	2 (range, 1-7)
CAR Product	
CD19 n (%)	29 (21.80%)
CD19/CD22 n (%)	84 (63.16%)
CD19, CD20 n (%)	12 (9.02%)
CD30 n (%)	8 (6.01%)
Disease Entity	
DLBCL n (%)	87 (65.41%)
PMBCL n (%)	5 (3.76%)
GZL n (%)	2 (1.50%)
HL n (%)	3 (2.26%)
Transformed Lymphoma (trFL, trMCL, trMZL, trCLL) n (%)	17 (12.78%)
Indolent lymphoma (MZL, CLL, FL) n (%)	6 (4.51%)
AITL n (%)	3 (2.26%)
MCL n (%)	5 (3.76%)
BL n (%)	5 (3.76%)
Pre-lymphodepletion	
Median NE, $\times10^9$/L	1.91 (0.56-28.77)
Median PLT, $\times10^9$/L	104 (20-776)
Median HB, g/L	89.5 (61-179)
Median LDH, U/L	237.15 (115.6-11637)
Median CRP, mg/L	36.73 (1.04-182.02)
Median Ferritin, ng/mL	520.10 (17.56-8969.29)
Lymphodepleting preparative regimen	
FC n (%)	120 (90.22%)
SEAM n (%)	13 (9.77%)
CRS, n (%)	
Grade 0	46 (33.08%)
Grade 1-2	67 (50.38%)
Grade 3-5	20 (15.03)
Neurotoxicity, n (%)	9 (6.43%)

Data were described as n (%) or median [range].
HSCT, hematopoietic stem cell transplantation, DLBLC, Diffuse Large B-Cell Lymphoma, PMBCL, Primary Mediastinal B-Cell Lymphoma, GZL, Gray zone lymphoma, HL, Hodgkin lymphoma, FL, Follicular lymphoma, MCL, Mantle cell lymphoma, MZL, Marginal zone lymphoma, CLL, Chronic lymphocytic leukemia, AITL, Angioimmunoblastic lymphoma, BL, Burkitt lymphoma, NE, neutrophil, PLT, Platelet, HB, Hemoglobin, LDH, lactate dehydrogenase, CRP, C reactive protein, FC, fludarabine 30 mg/m2 d-5 to -3, cyclophosphamide 300 mg/m2 d-5 to -3, SEAM: 250 mg/m2 Me-CCNU d-10, etoposide 100 mg/m2 every 12h d−9 to−6, cytarabine every 12 h d−9 to-6, hematopoietic stem cell infusion d-2.

(NCT03196830) at our center. We analyzed 133 patients without severe cytopenia before reinfusion, death prior to d14 or incomplete data for early hematologic toxicity within one month after infusion. 113 patients without disease progression, additional cytotoxic therapy or incomplete data were further analyzed for late hematologic toxicity at one month after infusion (Figure 1). This study was approved by the Institutional Ethics Committee of the First Affiliated Hospital of Suzhou University and was in line with the guidelines of the Declaration of Helsinki.

Study design

Patients were assessed for eligibility and their peripheral blood mononuclear cells (PBMCs) were obtained for CAR-T-cell production. The costimulatory domain of all CARs was 4-1BB. Prior to this report, our center reported the protocol for manufacturing CAR-T cells and the construct designs for the CARs (17). Patients received lymphodepleting chemotherapy with FC (fludarabine 30 mg/m^2 d-5 to d-3, cyclophosphamide 300 mg/m^2 d-5 to d-3) or SEAM (250 mg/m^2 Me-CCNU d-10, etoposide 100 mg/m^2 every 12 h d-9 to d-6, cytarabine every 12 h d-9 to d-6, hematopoietic stem cell infusion d-2). A total of 1×10^7 CAR-T-cells/kg were infused over 3 consecutive days (d0, 10%; d1, 30%; d2, 60%). Subsequently, the hospital observation period was at least two weeks, and the period was extended according to whether the patient developed AEs. Regular outpatient follow-up monitoring was performed after the patients were discharged from the hospital for one month. In addition to routine blood tests, the patients' cytokine, serum ferritin, and C-reactive protein (CRP) levels were also regularly monitored.

Definitions of hematologic toxicity

We defined neutropenia, anemia, and thrombocytopenia using guidelines provided by the Center for International Blood and Marrow Transplant Research (CIBMTR). The definitions of neutropenia and severe neutropenia were an absolute neutrophil count below 1.5×10^9/L and 0.5×10^9/L peripheral blood, respectively. Anemia was defined as a hemoglobin (HB) concentration less than 120 g/L in men and less than 110 g/L in women; severe anemia was defined as an HB concentration lower than 60 g/L. Platelet (PLT) counts below 100×10^9/L and below 20×10^9/L were used to characterize thrombocytopenia and severe thrombocytopenia, respectively. Severe cytopenia was defined as any occurrence of severe neutropenia, anemia or thrombocytopenia. Early hematologic toxicity was defined as cytopenia that occurred within one month, and late hematologic toxicity was defined as neutrophil count lower than 1.0×10^9/L or PLT count lower than 80×10^9/L at one month after infusion. The primary observational endpoint of the study was the severity of hematological toxicity up to 30 days after CAR-T-cell infusion,

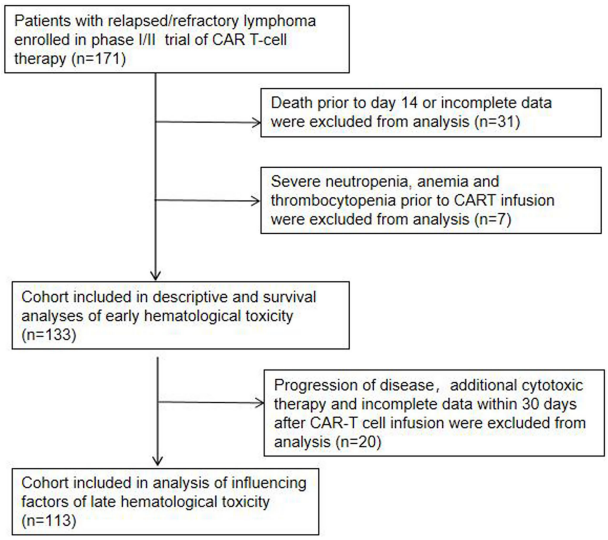

FIGURE 1
Patient selection.

and the secondary observational endpoint was neutrophil- and PLT-related toxicity 30 days after CAR-T-cell infusion.

CRS, ICANS, serum biomarkers, cytokines and additional clinical characteristics

Experienced doctors evaluated CRS and ICANS in accordance with the criteria agreed upon by the American Society for Transplantation and Cellular Therapy (ASTCT) (18). Patient serum was examined for the levels of IL-2, IL-4, IL-6, IL-10, IL-17, IFNγ, TNF,CRP, and ferritin. We also closely monitored patient vital signs and complications, such as infection and hemorrhage. In addition, patient clinical outcomes were determined.

Statistics

The baseline features and cytopenia statuses of the patients were described using descriptive statistics. For the purpose of determining statistical significance ($p<0.05$) between groups, a nonparametric Mann-Whitney test was performed. Categorical variables were analyzed with the chi-square test. Spearman correlation coefficients were used to evaluate associations between continuous variables. OS data were displayed using Kaplan-Meier curves. Independent predictors of cytopenia were assessed by univariate and multivariate logistic regression analyses. IBM SPSS Statistics version 26 and GraphPad Prism v9.0 were used to conduct the statistical analysis.

Results

Patient characteristics

Between June, 2017 and April, 2022, 171 patients with R/R lymphoma were treated with CAR-T-cells. A total of 133 patients who survived for more than 14 days and had complete data were included in this analysis. The median age was 44 years (range, 18-71). Forty-five (34%) patients had bone marrow involvement at diagnosis. Patients received a median of 2 prior therapies (range, 1-7), and 24 (18%) had HSCT before the lymphodepletion regimen. Twenty-nine (21%) patients were infused with CD19 CAR-T-cells, 84 (63%) patients were treated with CD19/CD22 bispecific CAR-T-cells, 12 (9%) patients were sequentially infused with CD19 and CD20 CAR-T-cells, and eight (6%) were treated with CD30 CAR-T-cells. The main indications for CAR-T-cell therapy were R/R DLBCL (n=87), transformed B-cell lymphoma (n=17), indolent lymphoma (n=6), and primary mediastinal large B-cell lymphoma (PMBCL) (n=5). Eighty-seven (65%) patients presented with CRS after CAR-T-cell infusion, and ICANS occurred in 9 (6%) patients. A total of 31 (22%) patients received steroids or tocilizumab to cure severe (grade ≥2) CRS and ICANS. The patient characteristics are depicted in Table 1.

Hematologic toxicity after CAR-T-cell Infusion

We observed that prior to lymphodepleting chemotherapy, the median neutrophil count was 1.96 (range, 0.56-28.77) $\times 10^9$/L, the median HB concentration was 89.5 (range, 61-179) g/L, and the median PLT count was 104 (range, 20-776) $\times 10^9$/L (Table 1). After CAR-T-cell infusion, 130 (98%) patients developed neutropenia, 71% of them developed severe neutropenia, and 30% and 41% of patients developed severe anemia and thrombocytopenia, respectively (Figure 2A).

Next, we observed three lineages kinetics after CAR-T-cell infusion. The median time to severe neutropenia was 4 days from cell infusion (range, 1-58), and the cumulative incidence of severe neutropenia on d30 was 69.45% (95% CI, 64.32%-75.34%). The median time to severe anemia and thrombocytopenia was not reached, and the cumulative incidence of severe anemia and thrombocytopenia on d30 after CAR-T-cell infusion was 39.88% (95% CI, 29.92%-50.04%) and 28.82% (95% CI, 16.88%-39.81%), respectively. Compared to severe neutropenia, severe anemia and thrombocytopenia occurred more slowly (Figure 2B).

Early hematological toxicity

We considered that lymphodepleting chemotherapy may induce cytopenia, which generally occurs within 3-4 weeks after chemotherapy. Apart from this, we explored other factors that may be associated with the onset of early severe cytopenias. Based on the data of 133 patients without severe cytopenia before reinfusion, death prior to d14 and incomplete data within one month after infusion, 98% of severe neutropenia cases and all severe thrombocytopenia cases occurred in the early phase.

CRS and inflammatory cytokine levels correlate with the occurrence of early severe hematologic toxicity

CRS was the most frequent AE recorded in patients receiving CAR-T-cell treatment. We found that as the CRS developed more severe, the incidence of each degree of cytopenia tended to increase accordingly (Figure 3A). We further analyzed the correlation between the minimum blood cell count and maximum inflammatory factor levels related to CRS in the early phase. The peak IL-6, CRP, and ferritin levels were substantially inversely related to the neutrophil, HB, and PLT counts. Furthermore, the peak value of IFNγ was negatively correlated with the minimum HB value, and the peak IL-4 level was negatively correlated with the minimum PLT count (Figures 3B–L).

Factors associated with the occurrence of early severe cytopenia

According to previous studies (16, 19), 14 baseline clinical characteristics considered to have potential prognostic value for predicting severe cytopenia are age, sex, disease type, tumor stage (≤II/>II), bone marrow involvement (yes/no), lines of prior therapy (≤2/>2), response to pre-CAR-T-cell treatment (partial response (PR)/stable disease (SD), progressive disease (PD)), prior HSCT (yes/no), LDH, lymphodepleting chemotherapy (FC/SEAM), CAR product, baseline blood parameters, baseline CRP and baseline ferritin. In multivariate analysis, prior HSCT and baseline HB were independent adverse factors associated with the occurrence of early severe neutropenia and thrombocytopenia. In addition, lymphodepleting chemotherapy with SEAM was another independent adverse factor associated with the occurrence of early severe thrombocytopenia (Figure 4) (Supplemental Tables 1–3).

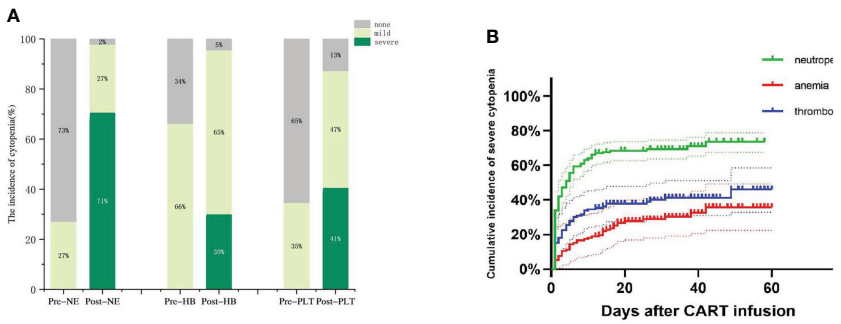

FIGURE 2
Hematologic toxicity after CAR-T cells. **(A)**The incidence of cytopenia before and after CAR-T cell infusion. **(B)** Cumulative incidence of severe cytopenia.

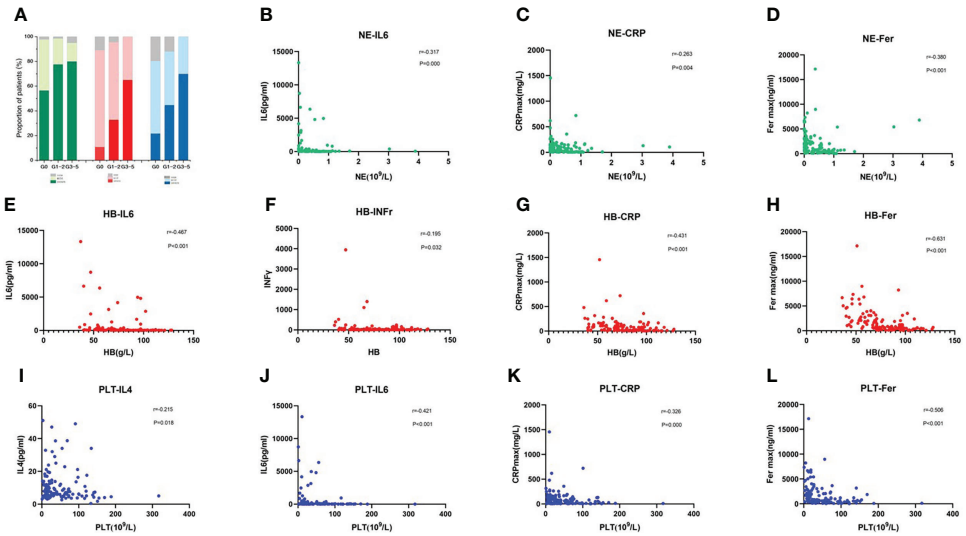

FIGURE 3
CRS and Inflammatory Cytokine Levels Correlate with the Occurrence of Early Severe Haematologic Toxicity **(A)** Incidence of each cytopenia is shown in patients without CRS or mild or severe CRS. **(B–L)** Correlation of inflammatory factors and blood cell parameters, including neutrophil count **(B–D)**, hemoglobin concentration **(E–H)**, and platelet count **(I–L)**. P values and r values were determined by Spearman correlation analysis.

Complications, treatments and prognoses associated with early severe cytopenia

We analyzed the correlation between severe cytopenia and early CAR-T-cell infusion outcomes. Patients with severe cytopenia had a significantly increased infection rate. (P=0.001). In the subgroup that received broad-spectrum carbapenem and tigecycline antibiotics, the number of patients with severe cytopenia was significantly higher than the number of patients with non-severe cytopenia (P=0.000). Although there were no significant differences in hemorrhage rate (P=0.328), patients with severe cytopenia received significantly more blood transfusions than patients without severe cytopenia (P<0.001) (Table 2).

In addition, 133 patients were followed up until April 1, 2022, and the survival curves are shown in Figure 5A. The median follow-up time was 10.2 months (range, 0.3 to 60.3 months), and compared with severe cytopenia patients, patients with non-severe cytopenia experienced significantly prolonged survival (P=0.031). We further analyzed the association of early severe neutropenia and thrombocytopenia with the occurrence of mortality 6 months post CAR-T-cell therapy. The 6-month survival rate of patients in the early severe neutropenia and thrombocytopenia group was significantly lower than that in the other group (P=0.049, P=0.000) (Figures 5B, C).

Late hematological toxicity

Cytopenia after 30 days is considered as late hematologic toxicity. We further analyzed the 113 patients without progression of disease, additional cytotoxic therapy and incomplete data for late hematologic toxicity at one month after infusion. Thirty days after CAR-T-cell infusion, 20 (18%) patients had neutrophil count lower than 1.0×10^9/L, and 39 (35%) patients had PLT count lower than 80×10^9/L. Similarly, we analyzed the association between late cytopenia and survival. There was no statistically significant difference in 6-month survival between patients who had late neutropenia and no neutropenia (P=0.291), but compared with the late thrombocytopenia group, patients without late thrombocytopenia had a significantly prolonged 6-month survival (P=0.017) (Figures 5D, E).

Factors associated with late hematological toxicity

We also included early CRS and inflammatory factor variables as well as clinical baseline characteristics to explore potential factors associated with late hematologic toxicity. In univariate analysis, prior HSCT, lower baseline HB/PLT, higher baseline ferritin and ferritin peak after infusion were associated with late thrombocytopenia, while no factor was found to be significantly associated with late neutropenia (Supplement Table 4). In multivariate analysis, lower baseline PLT was an independent adverse factor associated with late thrombocytopenia (Table 3).

Discussion

CAR-T-cell therapy has been shown to provide long-term remission or cure for patients with R/R lymphoma. However, there is a significant incidence of AEs with this medication, including CRS, ICANS, and hematologic toxicity. CAR-T-cell

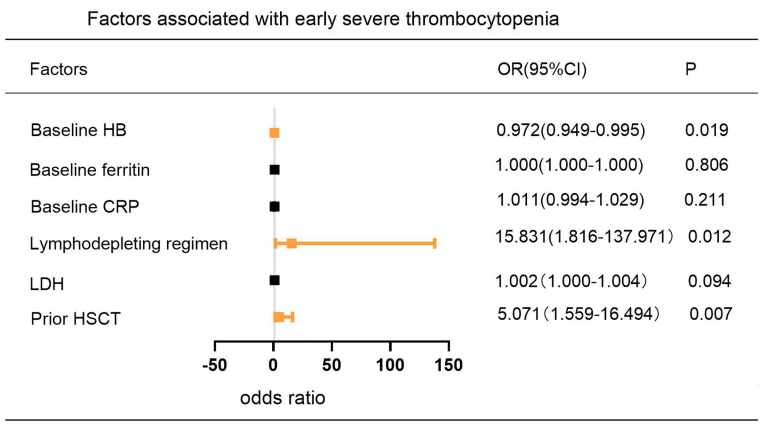

FIGURE 4
Multivariable Analysis of Factors Associated with the Incidence of Early Severe Thrombocytopenia. P values were tested by logistic regression model.

related CRS and neurotoxicity have been widely reported, and grading systems and care guidelines developed. However, hematologic toxicity following CAR-T-cell treatment for lymphoma remains poorly characterized. This retrospective review details the early and late hematologic toxicity patterns seen at our institution in a sizable cohort of R/R lymphoma patients who received CAR-T-cell treatment.

After CAR-T-cell infusion, we observed a high incidence of cytopenia, almost all of our patients had at least one type of cytopenia, and a majority of them developed a severe cytopenia, consistent with the results reported in previous studies (10, 11). From the perspective of three-lineage kinetics, the occurrence of severe neutropenia peaked earlier than those of severe anemia and severe thrombocytopenia, emphasizing the importance of early infection prevention. Since the immediate effects of lymphodepleting or bridging chemotherapy regimens on the hematopoietic system, previous studies have divided cytopenias after CAR-T-cell infusion into two stages (14). Accordingly, we explored risk factors that may lead to early and late cytopenias through univariate and multivariate analyses.

Our results showed that prior HSCT, and baseline HB were significantly associated with the occurrence of early severe cytopenia. Simultaneously, baseline PLT was an independent adverse factor associated with late thrombocytopenia. This finding indicates the possibility of direct toxicity to the hematopoietic system as a result of earlier treatment (including prior HSCT and numerous cycles of cytotoxic chemotherapy) (20), which may have resulted in inadequate bone marrow function in the presence of hematopoietic stress. Prior research has shown that myeloablative conditioning regimen combined with autologous hematopoietic stem cell transplantation might provide better disease debulking than conventional bridging regimens and thereby lead to greater efficacy of subsequent CAR-T-cell therapy in patients with high-risk LBCL (21). Therefore, for some young patients with better physical fitness, we adopted the myeloablative lymphocyte depletion regimen. Although this regimen could inevitably lead to severe early cytopenia, it had no effect on late cytopenia.

Correlation analysis showed that there was a certain correlation between the grade of CRS and the severity of cytopenia, which highlighted CRS as a common risk factor for

TABLE 2 Early severe hematological toxicities with cost of treatment and other adverse events after CAR-T-cell infusion.

Characteristic	Severe cytopenia	Non-Severe cytopenia	p
	n=99	n=34	
Infection, n (%)	32 (94.11%)	2 (5.88%)	0.001
Number of patients using broader-spectrum antibiotic, n (%)	73 (87.95%)	10 (12.05%)	0.000
Hemorrhage, n (%)	5 (100%)	0 (0.00%)	0.328
Number of blood transfusions, n (%)	7.05 (0-52)	0.76 (0-17)	<0.001

Data were described as n (%).
P value were tested by Chi-Square test.

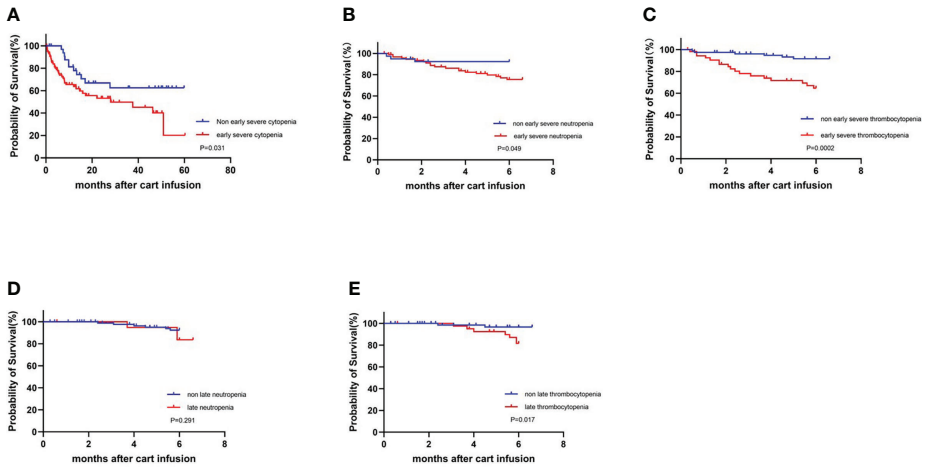

FIGURE 5
Survival curves for all patients who underwent infusion of CART cell with or without severe cytopenia. KM estimates of OS **(A)** in patients with non-severe cytopenia (blue) and with severe cytopenia (red). **(B, C)** Early severe neutropenia and thrombocytopenia compared with non-occurring group at 6-month survival.**(D, E)** Late neutropenia and thrombocytopenia compared with non-occurring group at 6-month survival.

early severe cytopenia. This conclusion is consistent with previously reported findings (13, 14). In addition, in our study, inflammatory indicators such as CRP and ferritin were substantially related to early hepatotoxicity, and spikes in cytokines, especially IL-6, were also significantly associated with early severe trilineage cytopenia, the rates of which were significantly elevated in groups with severe CRS in previous reports (22). Likewise, we noted that baseline ferritin, and peak ferritin were significantly associated with late thrombocytopenia in the univariate analysis. Consistent with a recent report that Rejeski and colleagues developed a predictive model to identify biomarkers, including baseline inflammatory markers (CRP and ferritin), to predict hepatotoxicity after CAR-T-cell infusion. According to their findings, high CAR-HEMATOTOX scores were associated with higher incidences of severe thrombocytopenia (16). Notably, although severity of CRS was associated with the early severity of hematologic toxicity, it did not affect hematologic recovery. In addition to the potential predictive value of the above serum markers for cytopenia, previous studies have reported that in lymphoma patients, late cytopenia may be related to the tumor microenvironment. Fried (14) argues that a chemokine critical for B-cell maturation and hematopoietic stem cell migration, stromal derived factor-1 (SDF-1), may be related to late cytopenia. Furthermore, in the ZUMA-1 study, four patients were diagnosed with myelodysplastic syndrome (MDS) after a median of 13.5 months (range 4-26), attributed to previous systemic therapies (23). Therefore, we further assessed bone marrow morphology in five patients with severe hematologic toxicity over 3 months and used an MDS FISH panel to determine whether the patients had cytogenetic abnormalities, and there was no evidence of clonal hematopoiesis of indeterminate potential (CHIP) or early MDS among them. However, this conclusion needs to be confirmed in studies with larger sample sizes and more precise detection methods, such as next-generation sequencing (NGS).

Previous report has shown that CD19/CD22 bispecific CAR-T product had equivalent potency and lower CRS response versus CD19 (24). To reduce the risk of relapse mediated by antigen negative clonal escape, in our institution a considerable proportion of patients were treated with CD19/CD22 bispecific CAR-T cells. Encouragingly, our observations demonstrated that modifications to the CAR-T target did not raise the risk of hematologic toxicity. In addition, according to previous reports (25), CRS cases are more severe with the usage of CARs that employ CD28 as a costimulatory domain compared with the usage of CARs that employ 4-1BB constructs, and Tania Jain's research showed (26) that the CAR construct was significantly associated with differences in the peak level, expansion, and persistence of various CAR-T-cells, factors that contribute to the development of cytopenias. In our study, the patients all received CAR-T-cells with 4-1BB as the costimulatory domain, and we could include commercial CAR-T-cells with the CD28 construct as a control for further comparative analysis in the future.

Overall, the infection rates after CAR-T-cell therapy appeared to be equivalent to those seen in similarly intensively pretreated patients. Many studies have been conducted to assess infection rates in individuals receiving CAR-T-cell treatment. The incidence of early infection (<30 d) ranges from 18% to 60%

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TABLE 3 Univariate and Multivariable Analysis of Factors Associated with the late neutropenia.

Variable	Univariable		Multivariable	
	Hazard ratio (95%CI)	P	Hazard ratio (95%CI)	P
Age	0.993 (0.962-1.024)	0.650		
Sex (M/F)	0.481 (0.218-1.059)	0.069		
Disease Type				
Aggressive B-cell lymphoma[a]	1[c]			
Indolent B-cell lymphoma[b]	8.567E+8 (0.000-)	0.999		
AITL	8.077E+8 (0.000-)	0.999		
HL	2.610E+18 (0.000-)	0.999		
Ann Arbor Stage (≤II/>II)	1.230 (0.353-4.285)	0.745		
Bone marrow involvement (yes/no)	0.852 (0.376-1.930)	0.701		
lines of prior therapy (≤2/>2)	1.561 (0.711-3.425)	0.267		
Prior HSCT (yes/no)	2.818 (1.118-7.102)	0.028	2.300 (0.742-7.126)	0.149
Response before treatment (PR/SD, PD)	1.318 (0.591-2.940)	0.500		
Pre-LD NE	0.985 (0.879-1.105)	0.801		
Pre-LD HB	0.960 (0.938-0.982)	0.000	0.980 (0.955-1.006)	0.140
Pre-LD PLT	0.985 (0.977-0.992)	0.000	0.989 (0.980-0.997)	0.006
LDH	1.001 (0.999-1.003)	0.245		
Lymphodepleting chemotherapy (FC/SEAM)	1.195 (0.363-3.935)	0.770		
CAR Product				
CD19	1[d]			
CD19/CD22	1.385 (0.207-9.241)	0.737		
CD19, CD20	0.920 (0.157-5.389)	0.926		
CD30	1.667 (0.210-13.223)	0.629		
Baseline CRP	1.013 (0.998-1.029)	0.093		
Baseline ferritin	1.001 (1.000-1.001)	0.024	1.000 (0.999-1.001)	0.834
CRS grade (<2/≥2)	0.850 (0.329-2.194)	0.737		
CRP max	0.999 (0.997-1.002)	0.613		
Ferritin max	1.000 (1.000-1.001)	0.015	1.000 (1.000-1.001)	0.294
IL2 max	0.974 (0.933-1.016)	0.219		
IL4 max	0.952 (0.906-1.000)	0.050		
IL6 max	1.000 (1.000-1.000)	0.974		
IL10 max	0.999 (0.993-1.004)	0.603		
TNFmax	0.996 (0.976-1.017)	0.707		
IFNγ max	0.997 (0.991-1.003)	0.339		
IL17 max	0.991 (0.977-1.006)	0.232		

Aggressive B-cell lymphoma[a] include DLBCL, PMBCL, GZL, Transformed Lymphoma, MCL, BL.
Indolent B-cell lymphoma[b] include MZL, CLL, FL.
Aggressive B-cell lymphoma[c] group was defined as the control group.
CD19[d] group was defined as the control group.

(12, 27–29) in prospective clinical trials and retrospective analyses. For example, Hill (12) described 23% of patients with different B lymphoid malignancies had infection during the first 28 days following CAR-T-cell infusion. Park (27)reported that adult B-ALL patients who underwent CD19-28z CAR-T-cell treatment experienced a 40% frequency of infections. The causes of infection in patients who have received CAR-T-cell treatment are multifactorial, including cumulative immunodeficiency brought on by prior therapies and the inherent susceptibility of patients with hematological malignancies to infection (12). The use of lymphodepleting chemotherapy to eliminate immune cells (30) as well as the use of immune-suppressing medicines such as high-dose corticosteroids and tocilizumab to alleviate the side effects of CAR-T-cell therapy may significantly increase the risk of infection (31). Furthermore, CD19 CAR-T-cell products that target B cells may cause B-cell aplasia, as a result, hypoimmunoglobulinemia, which increases the risk of infection. Post-CAR-T-cell cytopenia is also gradually being

recognized as an important factor, and this cytopenia is more severe than predicted following chemotherapy and lasts long after CRS has resolved. Our study also suggests that severe cytopenia is associated with an increased incidence of infection and significantly higher rates of using broad-spectrum antibiotics. Consistent with its association with infection, severe cytopenia is also associated with an increased risk of hemorrhage and a significantly increased number of blood transfusions, which indirectly suggests the need of more treatment related cost. Furthermore, cytopenia can also negatively impact CAR-T-cell therapy outcomes. According to Rejeski and colleagues, poorer clinical outcomes were associated with higher CAR-HEMATOTOX scores. In that analysis, the CAR-HEMATOTOX score was found to have a relationship with OS (P=0.09) and PFS (P=0.07) (16). This conclusion is consistent with the results of our survival analysis. In fact, most patients eventually develop disease relapse, and cytopenia jeopardizes the capacity to provide continued treatment after relapse and may limit participation in clinical studies. This factor further contributes to the poorer prognosis of patients.

In view of the above possible causes of severe cytopenia and adverse outcomes, the predictors we explored have instructive significance for the clinical management of cytopenia. These mainly included the following points: 1. Early supportive intervention is required for patients with high-risk baseline characteristics, 2. Potential modifications to lymphodepleting protocols. For example, cyclophosphamide can be replaced by bendamustine (32), And 3. Reasonable application of corticosteroids and tocilizumab for CRS. For the management of late hematologic toxicity, in addition to supportive care, Naman et al. (33) suggest that hematopoietic stem cell rescue is also an appropriate option.

In general, this study has the following limitations. First, this was a single-institution study with a high ratio of CD19/CD22 CARs, limiting its overall applicability. Next, this was a retrospective study with a relatively short follow-up time, and time of blood cells recovery was not traced in all patients. Again, we lacked peripheral blood samples from patients with persistent cytopenia, precluding us from studying other mechanistic reasons for late hematological toxicity. Furthermore, in clinical practice, the use of granulocyte-colony stimulating factor (GCSF), blood transfusions or corticosteroids will inevitably interfere with blood count results. But we can't get the complete data. This problem will need to be further studied in a prospective manner.

In summary, our study provides clinical evidence for the hypothesis that CRS and CRS-related cytokines (eg. IL-6, CRP and ferritin) are associated with the severity of early cytopenia. Myelotoxic conditioning regimen, prior HSCT, and baseline HB were independent adverse factors associated with the occurrence of early severe cytopenia. Furthermore, baseline PLT count was an independent factor associated with late thrombocytopenia after CAR-T-cell therapy. Our data suggest that clinicians should focus on cytopenia and guide the accurate prediction and appropriate management of post-CAR-T-cell therapy cytopenia.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The studies involving human participants were reviewed and approved by the Institutional Ethics Committee of the First Affiliated Hospital of Suzhou University. The patients/participants provided their written informed consent to participate in this study. Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

Author contributions

DW, CL, and YX contributed to the conception of the study and manuscript revision. HG, JL, and GC contributed to data collection. LY designed and manufactured the CAR-T-cells; JZ, YZ, MS, and XZ analyzed data and wrote the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.997589/full#supplementary-material

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Efficacy and safety of copanlisib in relapsed/refractory B-cell non-Hodgkin lymphoma: A meta-analysis of prospective clinical trials

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Background: Copanlisib is an intravenously administered pan-class I PI3K inhibitor that has been demonstrated to have appreciable effects in the treatment of patients with lymphoma. The purpose of this meta-analysis was to evaluate the efficacy and safety of copanlisib for treating patients with relapsed/refractory (R/R) B-cell non-Hodgkin lymphoma (B-NHL).

Methods: PubMed, Web of Science, EMBASE, and the Cochrane Central Register of Controlled Trials were searched for relevant studies published prior to July 2022. The efficacy evaluation included complete response rate (CR), partial response rate (PR), rate of stable disease (SDR), overall response rate (ORR), disease control rate (DCR), rate of progressive disease (PDR), median progression-free survival (PFS), and median overall survival (OS). Any grade adverse events (AEs) and grade ≥3 AEs were synthesized to assess its safety.

Results: Eight studies with a total of 652 patients with R/R B-NHL were identified. The pooled CR, PR, ORR, SDR, DCR, and PDR from all 8 articles were 13%, 40%, 57%, 19%, 86%, and 9%, respectively. The CR and ORR of combination therapy with rituximab were higher than those with copanlisib monotherapy for R/R B-NHL (34% vs. 6%, p<0.01; 89% vs. 42%, p<0.01). For patients with R/R indolent B-NHL, CR and ORR were lower with copanlisib monotherapy than with combination therapy with rituximab (7% vs. 34%, p<0.01; 58% vs. 92%, p<0.01). In R/R B-NHL patients receiving copanlisib monotherapy and combination therapy with rituximab, the risk of any grade AEs was 99% and 96%, respectively, and the risk of grade ≥3 AEs was 84% and 91%, respectively. The common any grade AEs included hyperglycemia (66.75%), hypertension (48.57%), diarrhea (35.06%), nausea (34.98%) and fatigue (30.33%). The common grade ≥3 AEs included hyperglycemia (45.14%), hypertension (35.07%), and neutropenia (14.75%). The comparison of AEs between the copanlisib monotherapy and the combination therapy with rituximab showed that hyperglycemia of any grade (p<0.0001), hypertension of any grade (p=0.0368), fatigue of any grade (p<0.0001), grade ≥3 hypertension

(p<0.0001) and grade ≥3 hyperglycemia (p=0.0074) were significantly different between the two groups.

Conclusion: Our meta-analysis demonstrated that the efficacy of both copanlisib monotherapy and combination therapy with rituximab in patients with R/R B-NHL was satisfactory, while treatment-related AEs were tolerable. Compared with copanlisib monotherapy, combination therapy with rituximab showed superior efficacy for treating R/R B-NHL, and its safety was manageable.

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KEYWORDS

copanlisib, rituximab, R/R B-NHL, efficacy, safety, meta-analysis

Introduction

B-cell non-Hodgkin lymphoma (B-NHL) is a large group of lymphomas that can be divided into indolent B-NHL and aggressive B-NHL. A survey found that the incidence of both indolent and aggressive B-NHLs has increased in recent years (1). Patients with indolent B-NHL are generally considered incurable, often recur repeatedly, receive multiple lines of antitumor therapy and are prone to drug resistance (2). Approximately 25%-30% of patients with aggressive B-NHL have a poor reaction to first-line therapy or relapse (3). Diffuse large B-cell lymphoma is a common aggressive B-NHL, accounting for approximately 30%-58% of NHL cases (4). A large-scale cohort study showed that 2778 patients with refractory diffuse large B-cell lymphoma had a median overall survival (OS) of 5.9 months and a 2-year OS rate of 16% (5).

Treatment of B-NHL mainly includes alkylating agents, combination chemotherapy, chemoimmunotherapy, high-dose chemotherapy + autologous/allogeneic stem cell transplantation, radiotherapy, chimeric antigen receptor T-cell therapy, and so on (6, 7). However, less than half of patients with relapsed/refractory (R/R) indolent B-NHL were responsive to subsequent treatment (8, 9). No standard treatment has been developed for patients with R/R aggressive B-NHL. Currently, high-dose salvage chemotherapy or bone marrow transplantation are often used, but the overall effect is not satisfactory (10). The management of patients with R/R B-NHL has become a major difficulty for hematologists. Therefore, it is necessary to develop more effective drugs for treating patients with R/R B-NHL.

The B-cell receptor (BCR) signaling pathway accounts for much of the development of B-cell lymphoma. In the BCR pathway, phosphatidylinositol 3-kinase (PI3K) and Bruton tyrosine kinase play significant roles (11). PI3K is a key downstream effector of the BCR (12). Since PI3K is important in carcinogenesis, it has become one of the potential targets for lymphoma treatment. PI3K is classified into three types (I, II, III) based on their distinctive substrates and structures. Class I PI3K is a heterodimer formed from class IA and class IB PI3K, both of which consist of catalytic subunits (p110 or p110γ) and regulatory subunits (p85 or p101) (13). Class II PI3K comprises only one catalytic subunit, including three isoforms: PI3K–C2α, PI3K–C2β, and PI3K–C2γ (14). Class III PI3K is only formed from Vps34p. Of the three classes of PI3Ks, class I is most closely associated with tumorigenesis and progression (12, 15).

Class I PI3K includes four isoforms, namely, PI3Kα, PI3Kβ, PI3Kγ, and PI3Kδ. PI3kα and PI3Kβ generally exist in various kinds of cells, whereas PI3Kγ and PI3Kδ are mainly expressed in the hemopoietic system (16). PI3K inhibitors can be classified into pan-PI3K inhibitors, isoform-specific inhibitors, and dual PI3K/mTOR inhibitors according to their different selectivity. Pan-PI3K inhibitors are effective against all four isoforms of class I PI3K, such as buparlisib, which has not been approved for treating lymphoma by the Food and Drug Administration (FDA) (17). Isoform-specific inhibitors are selective for a specific isoform of PI3K, and they can be separated into selective PI3Kα inhibitors, selective PI3Kβ inhibitors, selective PI3Kγ inhibitors, and selective PI3Kδ inhibitors. Dual PI3K/mTOR inhibitors can also specifically bind to a domain of mTOR, so they can simultaneously inhibit mTOR. To date, dual PI3K/mTOR inhibitors have not been approved for cancer treatment, but clinical trials are underway (18). Currently, the only oral PI3K inhibitors approved by the FDA for the treatment of lymphoma are idelalisib (PI3Kδ inhibitor) and duvelisib (PI3Kδ and PI3Kγ inhibitor) (19, 20). However, these two oral drugs have serious safety problems in clinical application, such

as severe intestinal adverse events (AEs) and infections (21). Therefore, the FDA also gives a corresponding warning statement in the drug label.

Copanlisib is a pan-class I PI3K inhibitor that is highly selective for PI3Kα and PI3Kδ isoforms (22). It is administered intravenously. The CHRONOS-1 (a large multicenter phase 2 clinical trial) study showed a considerable overall response rate (ORR, 60.6%), progression-free survival (PFS), and OS with copanlisib for R/R indolent lymphoma, as well as satisfactory safety (23). Based on the results of CHRONOS-1, the FDA rapidly approved copanlisib for treating relapsed follicular lymphoma in 2017. Most B-NHLs express the CD20 antigen, so rituximab is one of the standard options for treatment. However, due to the drug resistance of patients with R/R B-NHL and the poor efficacy of monotherapy, researchers have been exploring combination regimens. A randomized double-blind phase 3 trial (CHRONOS-3) indicated that the ORR (81% vs. 48%) and median PFS (21.5 months vs. 13.8 months) in the copanlisib plus rituximab group were significantly higher than those in the placebo plus rituximab group (24).

At present, various researchers have been exploring the curative effect of copanlisib-containing regimens in patients with B-NHL. Therefore, this meta-analysis was performed to comprehensively evaluate the efficacy and safety of monotherapy or combination therapy with rituximab for patients with R/R B-NHL to provide a basis for clinical practice.

Methods

Search strategy

Original studies that described the efficacy or safety of copanlisib monotherapy or combination therapy, including copanlisib plus rituximab, for treating B-NHL were systematically searched for in the PubMed, Web of Science, EMBASE, and Cochrane Central Register of Controlled Trials. The search terms were combined as follows: "Copanlisib OR Aliqopa OR BAY80-6946" AND "lymphoma". The search included only articles published before July 2022 and had no language restrictions. This meta-analysis followed the Preferred Reporting Items for Systematic reviews and Meta-Analyses (PRISMA) guidelines.

Inclusion and exclusion criteria

We followed the following inclusion criteria to screen the literature: 1) prospective clinical trials at any stage; 2) studies including patients diagnosed with R/R B-NHL; 3) articles studying copanlisib monotherapy or combination therapy with rituximab; and 4) clinical trials reporting any data involving their efficacy or safety.

The exclusion criteria were as follows: 1) no available data of efficacy or safety; 2) reviews, case reports, news, editorials, meta-analyses, and meeting/conference abstracts.

Data extraction and quality assessment

Two authors (JW and HZ) independently screened the literature and collected the data, and any difference was settled by the third author. The extracted data were sorted into a designed spreadsheet that mainly included the first author, ClinicalTrials.gov number, phase, study design, number of patients, disease, ages, treatment, prior lines of anticancer therapy, any grade AEs, grade ≥3 AEs, complete response rate (CR), partial response rate (PR), rate of stable disease (SDR), ORR, disease control rate (DCR), rate of progressive disease (PDR), median PFS, and median OS. These terms are defined in the Supplementary material. For all enrolled studies, we only extracted information about copanlisib monotherapy or combination therapy with rituximab, for treating B-NHL. For the included randomized controlled trials (RCTs), the quality was estimated by the Cochrane Collaboration Risk of Bias Tool (25). The methodological index for nonrandomized studies (MINORS) was utilized to assess the quality of the non-RCTs (26).

Statistical analysis

Statistical analysis of the data was performed using R 4.1.1 software. The I^2 statistic test was applied to appraise the heterogeneity among studies. The value of the I^2 statistic is 0 to 100%. I^2<25% indicates mild heterogeneity, I^2 25–50% means moderate heterogeneity, and I2>50% manifests obvious heterogeneity. A fixed-effects model was employed if the I^2 statistic was low ($I^2 \leq 50\%$), while a random-effects model was utilized with I^2>50%. Subgroup analysis (copanlisib vs. including copanlisib plus rituximab; R/R indolent B-NHL vs. R/R aggressive B-NHL) was employed to address any heterogeneity.

Results

Study characteristics

A total of 741 records were retrieved from PubMed (n=68), Web of Science (n=125), EMBASE (n=463), and the Cochrane Central Register of Controlled Trials (n=85). After removing 242 duplicate studies and 486 articles for various reasons, we read the full text of 13 articles. Finally, eight qualified studies were included in the meta-analysis (23, 24, 27–32). Figure 1 shows the complete screening process. All eight articles were prospective clinical trials, including three phase I trials, three

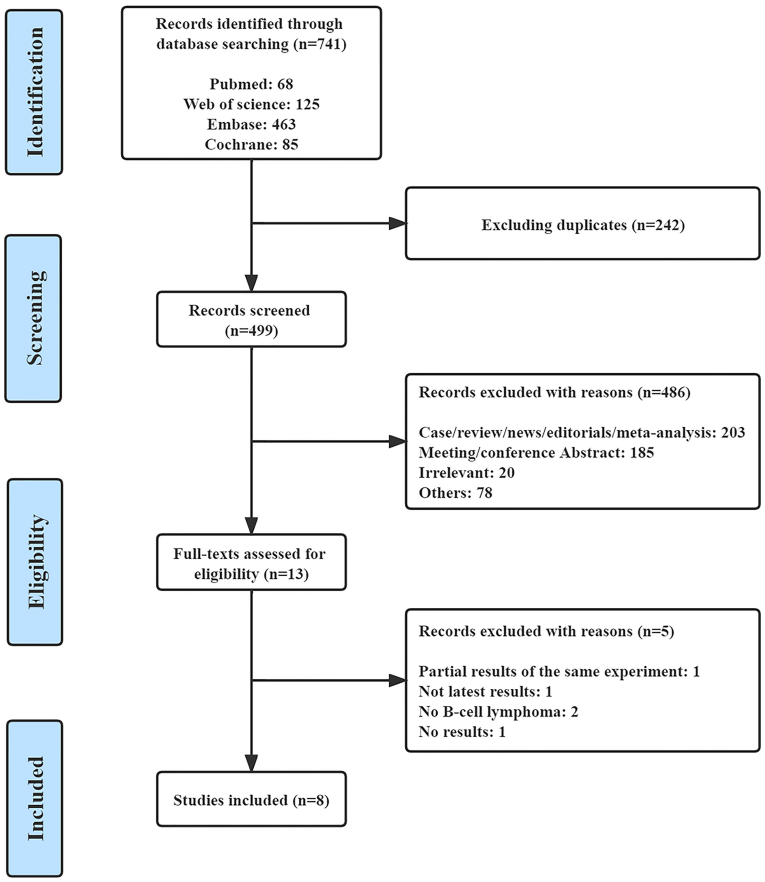

FIGURE 1
The flow chart.

phase II trials, and two phase III trials. The included studies were published from 2016 to 2022. The features of all of the eligible studies are shown in Table 1. Altogether, 652 patients with R/R B-NHL were included, of whom 516 had R/R indolent B-NHL, 127 had R/R aggressive B-NHL, and the remaining nine were unable to distinguish between indolent and aggressive B-NHL. The median age of all patients ranged from 60 to 72 years.

In the eight articles, patients received copanlisib monotherapy in six trials and combination therapy including copanlisib plus rituximab in the remaining 2 trials (copanlisib + rituximab, copanlisib + rituximab + bendamustine, copanlisib + rituximab + cyclophosphamide/doxorubicin/vincristine/prednisone). The participants had received 1 to 13 prior lines of anticancer therapy. Seven of the included studies reported complete information on efficacy (CR, PR, SDR, ORR, DCR, and PDR), and six trials showed full information on safety. Supplementary Table S1 shows information on the dose of copanlisib, frequency of administration, median duration of treatment, follow-up time, and modification of doses (reduction or interruption, or delay) or discontinuation due to AEs in the included studies. The doses of copanlisib in all studies included 45 mg, 60 mg, 0.4 mg/kg, and 0.8 mg/kg, and the frequency of copanlisib intravenous infusion was Days 1, 8, and 15 (28 days per cycle). The median duration of treatment ranged from 6 to 33.2 weeks. The rates of discontinuation of treatment due to AEs ranged from 15.4% to 31.3%.

Efficacy

We synthesized CR, PR, ORR, SDR, DCR, and PDR to assess the efficacy of copanlisib monotherapy or its combination with rituximab for patients with all R/R B-NHL. All enrolled studies reported CR, PR, and ORR for patients treated with copanlisib-containing regimens. The pooled CR, PR and ORR were 13% (95% CI: 4%-23%), 40% (95% CI: 32%-50%), and 57% (95% CI: 46%-71%), respectively. For copanlisib monotherapy, the pooled CR and ORR were 6% (95% CI: 1%-12%) and 42% (95% CI: 30%-59%), respectively. For combination therapy, including copanlisib plus rituximab, the pooled CR and ORR were 34%

TABLE 1 The characteristics of included studies.

N0.	Study	Clinical Trials. gov number	Phase	Study design	Number of patients	Disease	Ages (years), medium (range)	Treatment	Prior lines of therapy	Any grade AEs (n)	grade ≥3 AEs (n)	CR (%)	PR (%)	ORR (%)	SDR (%)	DCR (%)	PDR (%)	Median PFS (m)	Median OS (m)
1	Liu et al., 2022 (27)	NCT03498430	I	single-arm	13	R/R indolent B-NHL	40 (30–64)	copanlisib	1-8	13	10	0	58.3%	58.30%	41.6%	100%	0	—	—
2	Lenz et al., 2020 (28)	NCT02391116	II	single-arm	67	R/R aggressive B-NHL	69(25–93)	copanlisib	1-13	65	58	7.5%	11.9%	19.40%	20.9%	40.30%	44.80%	1.8	7.4
3	Dreyling et al., 2017 (1) (29)	NCT01660451 -part A	II	single-arm	33	R/R indolent B-NHL	66.5 (22–90)	copanlisib	1-10	33		6.3%	34.4%	43.80%	45.5%	90.60%	3%	9.8	21.9
	Dreyling et al., 2017 (2) (29)				34	R/R aggressive B-NHL				—	—	0	23.5%	29.40%	17.6%	47.10%	32.40%	—	—
4	Dreyling et al., 2020 (23)	NCT01660451 -part B	II	single-arm	142	R/R indolent B-NHL	63 (25–82)	copanlisib	2-9	140	118	16.9%	43.7%	60.60%	28.9%	89.40%	2.10%	12.5	42.6
5	Patnaik et al., 2016 (30)	NCT00962611	I	dose-escalation	9	R/R B-NHL	72 (40–84)	copanlisib	1-8	8	6	11.1%	66.7%	77.80%	0	77.80%	22.20%	—	—
6	Morschhauser et al., 2020 (31)	NCT02155582	I	single-arm	26	R/R aggressive B-NHL	61 (38–80)	copanlisib	—	—	—	3.8%	19.2%	23.10%	—	—	—	—	—
7	Matasar et al., 2021a (24)	NCT02367040	III	double-blind, randomised	307	R/R indolent B-NHL	63 (54–70)	copanlisib +rituximab	—	293	280	33.9%	44.6%	81%	11.7%	89%	2%	21.5	—
8	Matasar et al., 2021b (1) (32)	NCT02626455	III	double-blind, randomised	10	R/R indolent B-NHL	62 (41-82)	copanlisib+ +R-B	1-3	10	7	50%	40%	90%	10%	100%	0	—	—
	Matasar et al., 2021b (2) (32)				11	R/R indolent B-NHL	64 (46-78)	copanlisib+ +R-CHOP		11	10	30%	70%	100%	0	100%	0	—	—

R/R, relapsed or refractory; B-NHL, B-cell non-Hodgkin lymphoma; R-B, rituximab + bendamustine; R-CHOP, rituximab + cyclophosphamide, doxorubicin, vincristine, and prednisone.

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(95% CI: 29%-39%) and 89% (95% CI: 77%-100%), respectively. The above subgroup analysis suggested that the CR and ORR of combination therapy, including copanlisib plus rituximab, were higher than those of copanlisib monotherapy for R/R B-NHL (34% vs. 6%, p<0.01; 89% vs. 42%, p<0.01) (Figure 2). There was no significant difference in PR between copanlisib monotherapy and combination therapy, including copanlisib plus rituximab (Supplementary Figure S1). The SDR, DCR and PDR were shown in seven articles, which were 19% (95% CI: 10%-29%), 86% (95% CI: 78%-94%), and 9% (95% CI: 3%-14%), respectively. However, the copanlisib monotherapy subgroup displayed a higher SDR and PDR than the combination therapy subgroup, including copanlisib plus rituximab (25% vs. 9%, p<0.01; 16% vs. 2%, p=0.02) (Figure 2). No significant difference occurred for DCR between copanlisib monotherapy and combination therapy, including copanlisib plus rituximab (Supplementary Figure S1).

For patients with R/R indolent B-NHL, six studies reported all efficacy data (CR, PR, ORR, SDR, DCR, and PDR) with copanlisib monotherapy or copanlisib plus rituximab. The pooled CR, PR, ORR, SDR, DCR and PDR were 18% (95% CI: 7%-33%), 44% (95% CI: 40%-49%), 74% (95% CI: 58%-88%), 21% (95% CI: 9%-33%), 91% (95% CI: 88%-93%), and 2% (95% CI: 1%-3%), respectively. Subgroup analysis of CR and ORR showed that both CR and ORR were lower in patients with R/R indolent B-NHL receiving copanlisib monotherapy than in those receiving combination therapy, including copanlisib plus rituximab (7% vs. 34%, p<0.01; 58% vs. 92%, p<0.01) (Figure 3). The SDR in the copanlisib monotherapy subgroup was higher than that in the combination therapy subgroup, including copanlisib plus rituximab (32% vs. 11%, p<0.01) (Figure 3). The difference in PR, DCR, and PDR between copanlisib monotherapy and combination therapy, including copanlisib plus rituximab, was not statistically significant (Supplementary Figure S2).

For patients receiving copanlisib monotherapy, six trials included patients with R/R indolent B-NHL or R/R aggressive B-NHL, and all reported CR, PR, and ORR. The pooled CR, PR, and ORR were 6% (95% CI: 0-12%), 30% (95% CI: 16%-44%), and 38% (95% CI: 21%-56%), respectively. The subgroup analysis showed that patients with R/R indolent B-NHL treated with copanlisib monotherapy had higher PR and ORR than patients with R/R aggressive B-NHL (43% vs. 15%, p<0.01; 58% vs. 22%, p<0.01) (Figure 4). No significant difference existed in CR between the R/R indolent B-NHL subgroup and the R/R aggressive B-NHL subgroup (Supplementary Figure S3). Five studies displayed SDR, DCR, and PDR. The pooled SDR, DCR and PDR were 29% (95% CI: 20%-38%), 79% (95% CI: 51%-97%), and 15% (95% CI: 3%-28%), respectively. In the R/R indolent B-NHL subgroup, the SDR and DCR were higher than those in the R/R aggressive B-NHL subgroup (36% vs. 20%, p=0.03; 93% vs. 43%, p<0.01) (Figure 4), while the PDR was lower than that in the R/R aggressive B-NHL subgroup (2% vs. 40%, p<0.01) (Figure 4).

Of all included studies, only four described the survival outcomes, of which three reported median PFS and OS with copanlisib monotherapy and one showed median PFS with combination therapy, including copanlisib plus rituximab. The

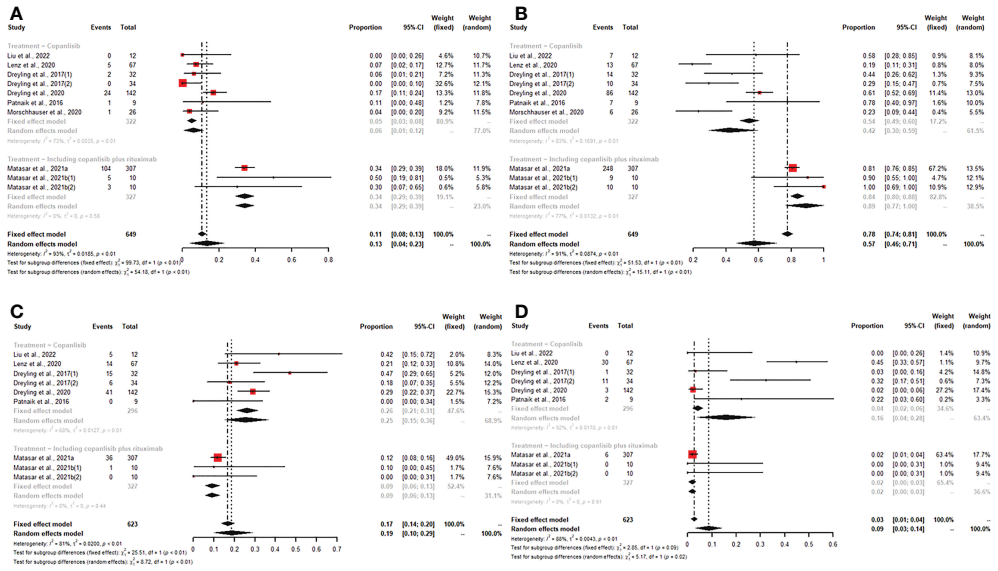

FIGURE 2
The pooled CR **(A)** and ORR **(B)** in patients with R/R B-NHL receiving copanlisib monotherapy were significantly higher than those receiving combination therapy, including copanlisib plus rituximab, while results for pooled SDR **(C)** and PDR **(D)** were reversed between the two groups.

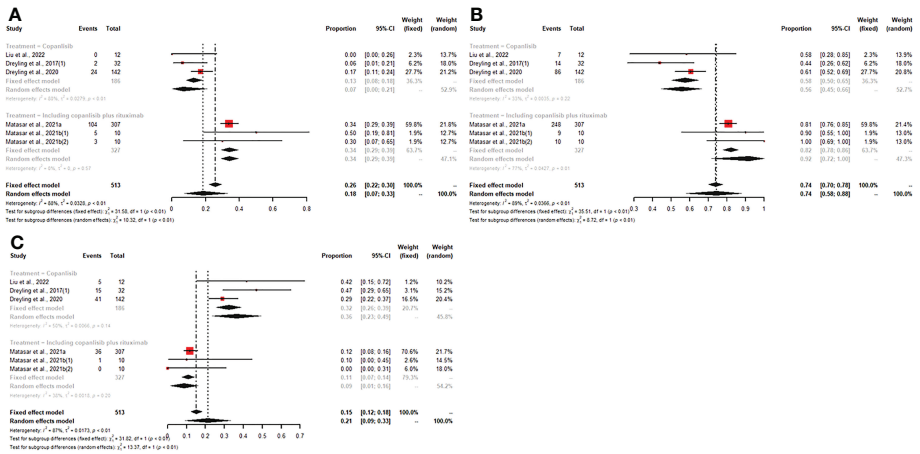

FIGURE 3
The pooled CR **(A)** and ORR **(B)** in patients with R/R indolent B-NHL receiving copanlisib monotherapy were significantly lower than those receiving combination therapy, including copanlisib plus rituximab, while result for pooled SDR **(C)** was reversed between the two groups.

best survival outcomes with copanlisib monotherapy for patients with R/R B-NHL were a median PFS of 12.5 months and a median OS of 42.6 months (Table 1). Due to incomplete data, the survival outcomes were not further synthesized.

Safety

Of all studies, seven studies reported any grade AEs, and six articles described grade ≥3 AEs. In patients with R/R B-NHL

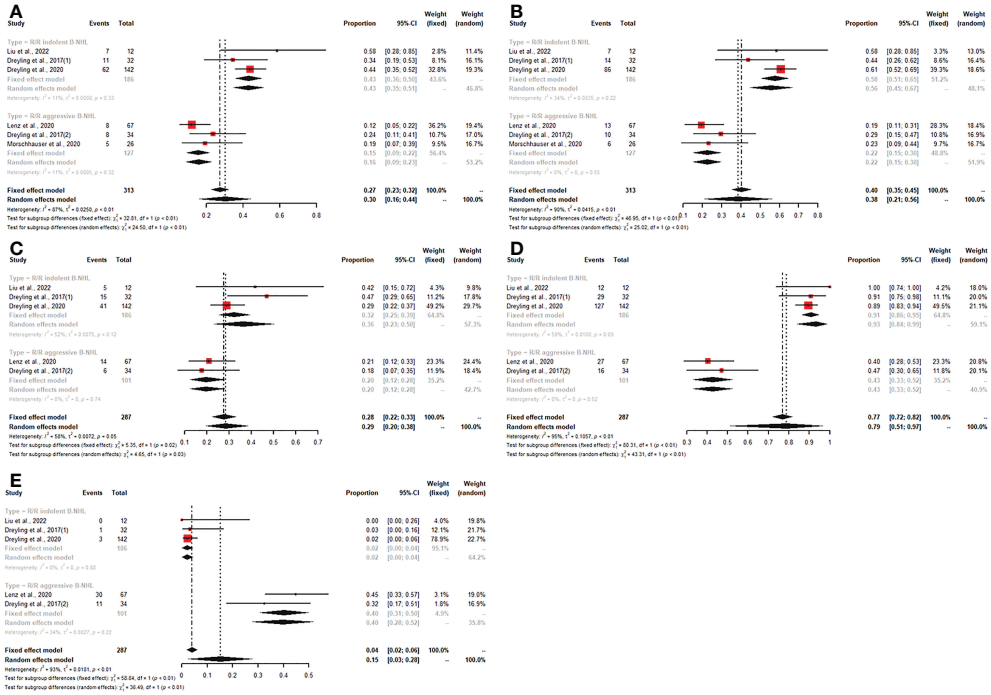

FIGURE 4
The pooled PR **(A)**, ORR **(B)**, SDR **(C)**, and DCR **(D)** in patients with R/R indolent B-NHL receiving copanlisib monotherapy were significantly higher than those in patients with R/R aggressive B-NHL receiving copanlisib monotherapy, while result for pooled PDR **(E)** was reversed between the two groups.

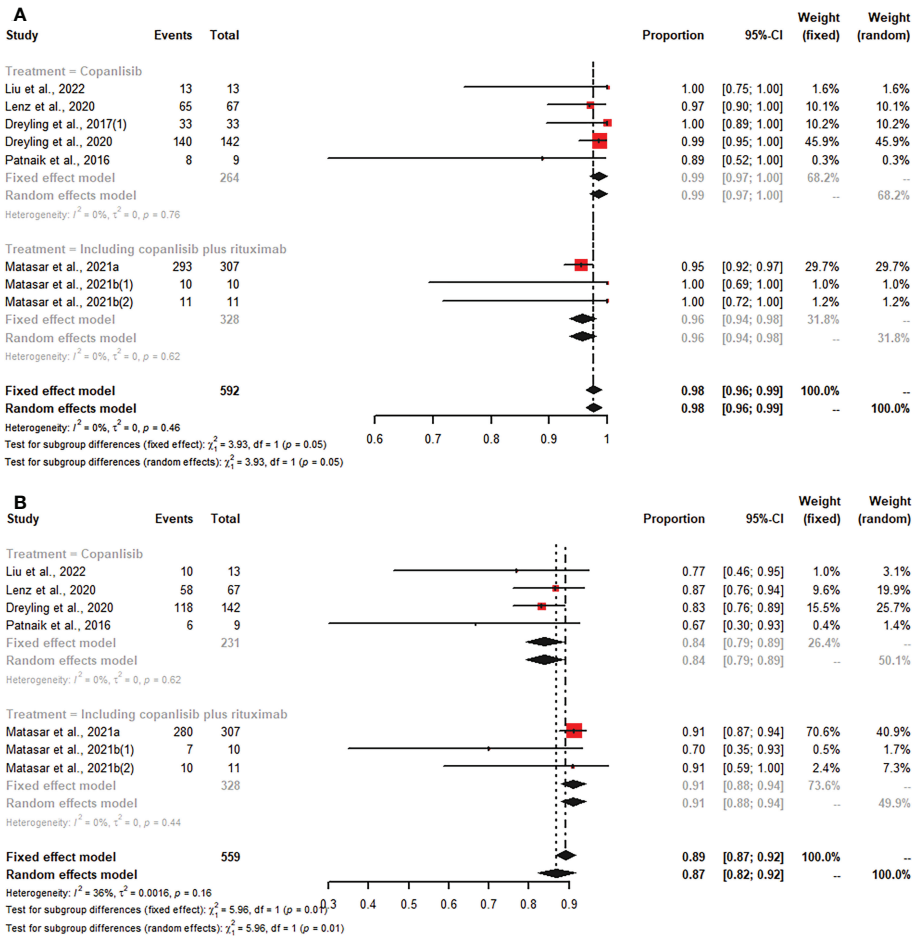

FIGURE 5
The pooled risk of any grade **(A)** and grade ≥3 **(B)** AEs in patients with R/R B-NHL receiving copanlisib monotherapy or combination therapy with rituximab.

who were treated with copanlisib monotherapy, the pooled risks of any grade and grade ≥3 AEs were 99% (95% CI: 97%-100%) and 84% (95% CI: 79%-89%), respectively (Figure 5). Patients with R/R B-NHL receiving combination therapy, including copanlisib plus rituximab, had a 96% (95% CI: 94%-98%) risk of any grade AEs and a 91% (95% CI: 88%-94%) risk of grade ≥3 AEs (Figure 5). For all patients with R/R B-NHL, the difference in any grade AEs was not statistically significant between copanlisib monotherapy and combination therapy, including copanlisib plus rituximab (99% vs. 96%, p=0.05; Figure 5). However, the pooled risk of grade ≥3 AEs for the combination therapy, including copanlisib plus rituximab, was significantly higher than that for copanlisib monotherapy (91% vs. 84%, p=0.01; Figure 5).

For all R/R B-NHL patients treated with copanlisib monotherapy or in combination with rituximab, the common any grade AEs included hyperglycemia (66.75%), hypertension (48.57%), diarrhea (35.06%), nausea (34.98%) and fatigue (30.33%) (Table 2). The common grade ≥3 AEs included hyperglycemia (45.14%), hypertension (35.07%), neutropenia (14.75%), pneumonia (7.03%), and diarrhea (5.09%) (Table 2). For all R/R B-NHL patients treated with copanlisib monotherapy, the common any grade toxicities were hyperglycemia (63.69%), hypertension (49.69%), diarrhea (35.94%), nausea (35.8%) and fatigue (32.76%). Hyperglycemia (37.61%), hypertension (27.57%), and neutropenia (18.01%) were the common grade ≥3 AEs in copanlisib monotherapy. For all R/R B-NHL patients treated with copanlisib plus rituximab, the common any grade toxicities included hyperglycemia (69.93%), hypertension (47.89%), nausea (40.53%), and decreased platelet count (38.87%), and the common grade ≥3 AEs included hyperglycemia (56.41%), hypertension (39.34%), and neutropenia (9.36%). Other AEs in patients with R/R B-NHL receiving copanlisib monotherapy or combination therapy with rituximab are listed in Supplementary Table S2.

TABLE 2 The incidence of adverse events in any grade or grade ≥3 for patients with R/R B-NHL.

AEs	Treatment	Any grade					Grade ≥3				
		Included study	Event	Total patients	Pooled rate (95% CI)	*p-value*	Included study	Event	Total patients	Pooled rate (95% CI)	*p-value*
Hematological											
Neutropenia	Copanlisib	3	59	242	0.2278 [0.1297;0.3440]	P=0.2661	2	42	209	0.1801 [0.0782;0.3124]	P=0.1811
	Including copanlisib plus rituximab	2	66	328	0.1067 [0.0043;0.3190]		2	50	328	0.0936 [0.0067;0.2645]	
	Overall	5	125	570	0.1899 [0.1202;0.2711]		4	92	537	0.1475 [0.0843;0.2246]	
Decreased platelet count	Copanlisib	3	27	188	0.1436 [0.0973;0.1972]	P=0.7445	2	7	155	0.0435 [0.0100;0.0769]	P=0.5806
	Including copanlisib plus rituximab	2	52	328	0.3887 [0.0770;0.7655]		2	10	328	0.0440 [0.0000;0.1109]	
	Overall	5	79	516	0.2101 [0.1249;0.3105]		4	17	483	0.0281 [0.0132;0.0429]	
Non-hematological											
Fatigue	Copanlisib	4	75	251	0.3276 [0.2354;0.4557]	P<0.0001	2	4	209	0.0190 [0.0050;0.0419]	P=0.2762
	Including copanlisib plus rituximab	2	52	328	0.2828 [0.1143;0.6994]		2	5	328	0.0143 [0.0043;0.0299]	
	Overall	6	127	579	0.3033 [0.2038;0.4513]		4	9	537	0.0160 [0.0072;0.0284]	
Diarrhea	Copanlisib	5	95	264	0.3594 [0.3015;0.4172]	P=0.7627	3	13	222	0.0289 [0.0001;0.1058]	P=0.8811
	Including copanlisib plus rituximab	2	113	328	0.3437 [0.2925;0.3949]		2	17	328	0.0514 [0.0302;0.0779]	
	Overall	7	208	592	0.3506 [0.3122;0.3889]		5	30	550	0.0509 [0.0341;0.0708]	
Nausea	Copanlisib	4	71	251	0.3580 [0.2117;0.5042]	P=0.3357	2	2	209	0.0086 [0.0000;0.0284]	P=0.6445
	Including copanlisib plus rituximab	2	80	328	0.4053 [0.1214;0.6892]		2	2	328	0.0000 [0.0000;0.0019]	
	Overall	6	151	579	0.3498 [0.2505;0.4491]		4	4	537	0.0000 [0.0000;0.0053]	
Pneumonia	Copanlisib	2	25	175	0.1403 [0.0912;0.1972]	P=0.9685	1	12	142	0.1056	_
	Including copanlisib plus rituximab	2	45	328	0.1175 [0.0806;0.1590]		2	21	328	0.0610 [0.0352;0.0869]	
	Overall	4	70	503	0.1261 [0.0959;0.1591]		3	33	470	0.0703 [0.0472;0.0933]	
Hyperglycemia	Copanlisib	5	136	264	0.6369 [0.4379;0.9264]	P<0.0001	4	87	231	0.3761 [0.3148;0.4394]	P<0.0001
	Including copanlisib plus rituximab	2	228	328	0.6993 [0.6513;0.7508]		2	185	328	0.5641 [0.5101;0.6173]	
	Overall	7	364	592	0.6675 [0.5399;0.8254]		6	272	559	0.4514 [0.5101;0.6173]	

(Continued)

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TABLE 2 Continued

AEs	Treatment	Any grade					Grade ≥3				
		Included study	Event	Total patients	Pooled rate (95% CI)	*p-value*	Included study	Event	Total patients	Pooled rate (95% CI)	*p-value*
Hypertension	Copanlisib	5	106	264	0.4969 [0.3045;0.6898]	P=0.0368	4	66	231	0.2757 [0.2168;0.3384]	P=0.0074
	Including copanlisib plus rituximab	2	161	328	0.4789 [0.2664;0.6950]		2	131	328	0.3934 [0.3380;0.4502]	
	Overall	7	267	592	0.4857 [0.3604;0.6118]		6	197	559	0.3507 [0.2576;0.4492]	

For all R/R B-NHL patients, the comparison of AEs between the copanlisib monotherapy group and the combination therapy, including copanlisib plus rituximab group, suggested that significant differences existed in any grade of hyperglycemia (63.69% vs. 69.93%, p<0.0001), hypertension (49.69% vs. 47.89%, p=0.0368) and fatigue (32.76% vs. 28.28%, p<0.0001) between the two groups, and significant differences were shown in grade ≥3 hypertension (37.61% vs. 56.41%, p<0.0001) and hyperglycemia (27.57% vs. 39.34%, p=0.0074) between the two groups. The above results are shown in Table 2.

Study quality

Six of the studies were open-label, and two were double-blind. MINORS was applied to evaluate the quality of the six nonrandomized trials. The scores for each article ranged from 11 to 13 (Table 3). Meanwhile, the quality of the two RCTs was estimated by the Cochrane Collaboration's Risk of Bias Tool. The quality assessment of the included RCTs was good (Figure 6). Therefore, the quality of the enrolled studies was not poor.

Discussion

R/R B-NHL indicates a poor prognosis. Patients with R/R B-NHL have often received multiple lines of treatment and have poor responses to various treatment regimens. Therefore, there is an urgent need to find effective treatments for patients with R/R lymphoma. Copanlisib is active against all four isoforms; however, it has higher targeting of PI3Kα and PI3Kδ. We conducted a meta-analysis to evaluate the efficacy and safety of copanlisib monotherapy or combination therapy, including copanlisib plus rituximab, for patients with R/R B-NHL.

Our analysis showed that among patients receiving monotherapy, the pooled ORRs of patients with R/R B-NHL,

TABLE 3 Quality assessment of the non-randomized studies.

Reference	Study aims	Consecutive patient inclusion criteria	Prospective collection of data	Endpoint consistent with the study aim	Unbiased evaluation of endpoints	Follow-up period	Loss to follow-up less than 5%	Prospective calculation of the sample size	Total
Liu et al., 2022 (27)	2	2	2	2	0	2	2	0	12
Lenz et al., 2020 (28)	2	2	2	2	0	2	2	0	12
Dreyling et al., 2017 (29)	2	2	2	2	1	2	2	0	13
Dreyling et al., 2020 (23)	2	2	2	2	1	2	2	0	13
Patnaik et al., 2016 (30)	2	2	2	1	0	2	2	0	11
Morschhauser et al., 2020 (31)	2	2	2	1	0	2	2	0	11

0. not reported.
1. reported but inadequate.
2. reported and adequate.

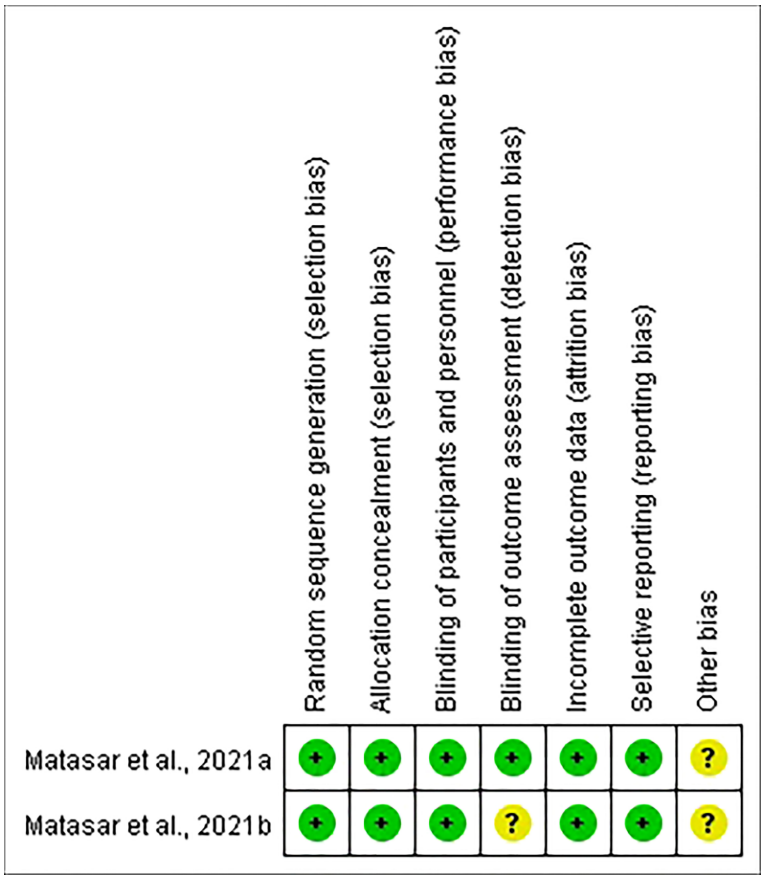

FIGURE 6
Quality assessment of the randomized studies.

R/R indolent B-NHL and R/R aggressive B-NHL were 42%, 58%, and 22%, respectively. The results indicated that copanlisib had promising efficacy in patients with R/R B-NHL who failed to respond to previous antitumor therapy. The number of lines of previous anticancer therapy the participant had received was from 1 to 13, mainly including rituximab, alkylating agents, high-dose chemotherapy/autologous stem cell transplant, radioimmunotherapy, and so on. The efficacy results of the patients with R/R B-NHL receiving copanlisib monotherapy were similar to the efficacy results of a meta-analysis that enrolled five clinical trials involving a total of 331 NHL patients receiving copanlisib, 174 of whom were indolent and 115 of whom were aggressive (33). In addition, similar ORRs have been reported for other PI3K inhibitors (duvelisib and idelalisib) approved for the treatment of lymphoma. Previous studies showed that the ORRs of patients with lymphoma receiving duvelisib were 47.3%-58.1% (34–36), and the ORRs of patients with lymphoma receiving idelalisib were 40%-57% (37–39).

PI3K is essential in the BCR signaling pathway. Dysregulation of PI3K is directly associated with the development of cancer, while abnormal activation of class I PI3K is related to acquired drug resistance (40). PI3Kδ is only expressed in hematopoietic cells and is usually expressed in B-cell malignancies (41). Copanlisib is highly selective for PI3Kα and PI3Kδ, so it can play a good role in treating B-NHL. In addition, the subgroup analysis revealed that copanlisib monotherapy had better efficacy in patients with R/R indolent B-NHL than in those with R/R aggressive B-NHL. Aggressive NHL is difficult to control once relapsed or refractory (10).

The results of subgroup analysis showed that combination therapy, including copanlisib plus rituximab, had a higher effect (CR and ORR) than copanlisib monotherapy for R/R B-NHL and R/R indolent B-NHL. At the same time, patients with R/R B-NHL receiving combination therapy had lower PDR. The above results suggest that combination therapy of copanlisib plus rituximab is a promising regimen for patients with R/R B-NHL. Rituximab, a CD20 monoclonal antibody, is often the

first-choice treatment for patients with B-NHL and is one of the standard options for patients with R/R B-NHL (42). PI3K plays a part in the development of B-cell lymphoma. A few previous studies have investigated the efficacy of other PI3K inhibitors in combination with rituximab for NHL. The results of a phase I study showed that the ORR of idelalisib plus rituximab in patients with R/R indolent NHL was 75% (43). Flinn IW et al. revealed that duvelisib plus rituximab had an ORR of 71.4% in patients with NHL (44). The ORR of the above studies seemed to be lower than that of our meta-analysis (89%). However, there are few relevant trials of copanlisib/idelalisib/duvelisib in combination with rituximab for patients with lymphoma. Meanwhile, there were only 2 studies on combination therapy included in our analysis, so more prospective clinical trials about combination therapy, including copanlisib plus rituximab, for lymphoma are needed.

Our meta-analysis suggested that the common any grade AEs included hyperglycemia, hypertension, diarrhea, nausea, and fatigue. The common grade ≥3 AEs included hyperglycemia, hypertension, neutropenia, pneumonia, and diarrhea. It is worth noting that the most common AEs, whether of any grade or grade ≥3, were hyperglycemia and hypertension. Hyperglycemia was also found to be a common AE in clinical trials of other PI3K inhibitors (45, 46). Alterations in PI3K signaling play a role in the development of noninsulin-dependent diabetes (47). Hyperglycemia induced by copanlisib may be related to the targeting of PI3Kα inhibition (23). Hyperglycemia often occurs during intravenous infusion of copanlisib and is often transient and controllable. In most patients, blood sugar levels can be normalized with fluid replacement (48). Before using copanlisib, it is recommended to screen the patient for diabetes, and if the patient is diagnosed with diabetes, they can receive copanlisib until their blood glucose is adequately controlled (49). If the blood sugar level of the patient is not effectively controlled, it is best to switch to other drugs that do not affect blood sugar.

In studies of other PI3K inhibitors, hypertension has been less frequently reported as an AE (48). The mechanism by which copanlisib causes hypertension is unclear, but it may be related to the interaction of PI3Kγ and angiotensin II (47). Hypertension often occurs during intravenous infusion and is usually transient and manageable. During an infusion of copanlisib, the patient's blood pressure should be closely monitored. If the patient's blood pressure continues to rise, antihypertensive drugs can be given appropriately, and the dose of copanlisib should be reduced or discontinued if necessary (50).

Diarrhea is also a common AE that is usually less than grade 3 and can be relieved by dietary or drug therapy (47). Nausea and fatigue were also mostly mild and could be alleviated with medication or rest. Compared with idelalisib and duvelisib, copanlisib exhibited less gastrointestinal toxicity, possibly related to its intermittent intravenous infusion (51). Hematological toxicities caused by copanlisib, including neutropenia, decreased platelet count, anemia, etc., may be related to the suppression of the bone marrow by copanlisib (50). Patients should have their blood monitored during the use of copanlisib, and severe hematological toxicities can be managed by reducing or discontinuing copanlisib. Pneumonia is a common infection induced by copanlisib. Patients using copanlisib should be closely monitored for symptoms and signs related to infection. For infections of grade 3 or higher, it is recommended to discontinue copanlisib treatment and actively take anti-infective treatment (50).

The risk of other AEs of special interest, including increased ALT/AST and rash, in patients with lymphoma using copanlisib was lower than that in patients with lymphoma using idelalisib or duvelisib (34, 35, 37, 39). This suggested that copanlisib may have superior safety for the liver and skin. With copanlisib, ALT and AST levels should be monitored closely. If ALT/AST exceeds 5 times the upper limit, copanlisib should be stopped temporarily, and a reduced dose of copanlisib should be restarted after the ALT/AST returns to normal. When severe liver toxicity occurs, copanlisib should be permanently discontinued (52). Patients with severe or grade ≥3 cutaneous reactions during the use of copanlisib should consult a dermatologist to evaluate their need for medication (47). The comparison of the incidence of AEs between copanlisib and other PI3K inhibitors (idelalisib and duvelisib) was shown in Supplementary Table S3.

Previous studies have revealed that patients with lymphoma receiving duvelisib have a 99%-100% risk of any grade AEs and an 88.4%-87% risk of grade ≥3 AEs (34, 53, 54). This was similar to our analysis of the risk of any grade (99%) and grade ≥3 (84%) AEs in patients with R/R B-NHL receiving copanlisib monotherapy. However, compared with our results, patients with lymphoma using idelalisib had a lower risk of any grade (82%-98.6%) and grade ≥3 (54%-65.3%) AEs (37, 38, 55). PI3kα and PI3Kβ are expressed in various kinds of cells, while PI3Kγ and PI3Kδ are mainly expressed in the hemopoietic system (16). The incidence of AEs with idelalisib was lower than that with copanlisib and duvelisib, which may be because idelalisib is an isoform-specific inhibitor and only has targeting activity for PI3Kδ.

In recent years, serious safety concerns about idelalisib and duvelisib have attracted significant attention. The FDA gave a black box warning for the AEs caused by these two drugs (56, 57). Idelalisib mainly leads to serious or fatal hepatotoxicity, diarrhea/colitis, pneumonitis, infections, and intestinal perforation, and duvelisib mostly causes serious or fatal diarrhea/colitis, cutaneous reactions, infections, and pneumonitis (49). Compared to the serious toxicities caused by idelalisib and duvelisib (21), copanlisib seems to have manageable safety.

This meta-analysis revealed that the risk of grade ≥3 AEs in combination therapy, including copanlisib plus rituximab, was higher than that in copanlisib monotherapy. The difference in grade ≥3 AEs between the two groups was mainly reflected in hyperglycemia and hypertension. However, these AEs, which were significantly different between the two groups, were

manageable. Therefore, the AEs in patients with R/R B-NHL receiving either copanlisib monotherapy or combination therapy, including copanlisib plus rituximab, were tolerable.

Our study has several limitations. First, the number of articles included in our analysis was limited. Second, most of the involved clinical trials were single-arm trials. Third, the dose of copanlisib varied among the studies. All of the above may cause bias. Similarly, due to the inconsistent follow-up times and incomplete data among the articles, our meta-analysis did not conduct a synthetic analysis of the survival outcomes.

In conclusion, our meta-analysis demonstrated that the efficacy of both copanlisib monotherapy and combination therapy, including copanlisib plus rituximab, in patients with R/R B-NHL was satisfactory, while treatment-related AEs were tolerable. Compared with copanlisib monotherapy, combination therapy of copanlisib plus rituximab showed superior efficacy for treating R/R B-NHL, and its safety was manageable. Furthermore, this research revealed that copanlisib monotherapy had better efficacy for patients with R/R indolent B-NHL than for patients with R/R aggressive B-NHL. The efficacy and safety of copanlisib needs to be compared with other drugs for treating lymphoma and there is a need to explore the efficacy and safety of copanlisib-based combination therapy for patients with lymphoma further.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding author.

Author contributions

JW collected, analyzed the data and wrote the article. JW, HZ and MM performed the statistical analysis. AZ and ZC analyzed and interpreted the data. LL and MW prepared the pictures and tables. TN and HZ provided the idea and modified the article. All authors read and approved the final manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.1034253/full#supplementary-material

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Targeting 4-1BB and PD-L1 induces potent and durable antitumor immunity in B-cell lymphoma

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Introduction: Although PD-1/L1 mAb has demonstrated clinical benefits in certain cancer types, low response rate and resistance remain the main challenges for the application of these immune checkpoint inhibitors (ICIs). 4-1BB is a co-stimulator molecule expressed in T cells, which could enhance T cell proliferation and activation. Herein, the synergetic antitumor effect and underlying mechanism of 4-1BB agonist combined with PD-1/PD-L1 blockade were determined in B-cell lymphoma (BCL).

Methods: Subcutaneous transplantation BCL tumor models and metastasis models were established to evaluate the therapeutic effect of PD-L1 antibody and/or 4-1BB agonist in vivo. For the mechanistic study, RNA-seq was applied to analyze the tumor microenvironment and immune-related signal pathway after combination treatment. The level of IFN-γ, perforin, and granzyme B were determined by ELISA and Real-time PCR assays, while tumor-infiltrating T cells were measured by flow cytometry and immunohistochemical analysis. CD4/CD8 specific antibodies were employed to deplete the related T cells to investigate the role CD4+ and CD8+ T cells played in combination treatment.

Results: Our results showed that combining anti-PD-L1 ICI and 4-1BB agonists elicited regression of BCL and significantly extended the survival of mice compared to either monotherapy. Co-targeting PD-L1 and 4-1BB preferentially promoted intratumoral cytotoxic lymphocyte infiltration and remodeled their function. RNA-sequence analysis uncovered a series of up-regulated genes related to the activation and proliferation of cytotoxic T lymphocytes, further characterized by increased cytokines including IFN-γ, granzyme B, and perforin. Furthermore, depleting CD8+ T cells not CD4+ T cells totally abrogated the antitumor efficacy, indicating the crucial function of the CD8+ T cell subset in the combination therapy.

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Discussion: In summary, our findings demonstrated that 4-1BB agonistic antibody intensified the antitumor immunity of anti-PD-1/PD-L1 ICI via promoting CD8+ T cell infiltration and activation, providing a novel therapeutic strategy to BCL.

KEYWORDS

B-cell lymphoma, anti-PD-L1 ICI, 4-1BB agonist, CD8+ T cells, combination therapy

Introduction

Although PD-1/PD-L1 axis-targeting immune checkpoint inhibitor (ICI) has revolutionized the therapeutic modality of several types of cancers, an obvious fraction of cancer patients showed no responses to ICI monotherapy (1, 2). Multiple factors such as immune cell infiltration, expression of immune checkpoint and cytokine signaling could monitor the response to ICIs and influence their efficacy (3, 4). Elucidation of the underlying immunologic characteristic of the tumor microenvironment (TME) associated with resistance will benefit the patients treated with ICI monotherapy and reveal the determinants for ICIs combination therapy.

As one prominent co-stimulator, 4-1BB is mainly expressed on natural killer T and $CD4^+$/$CD8^+$ T cells (5–7). Upon conjunction with soluble 4-1BBL or agonistic monoclonal antibody (mAb), 4-1BB forms a heterotrimer and induces T-cell proliferation, cytokine release, and upregulation of antiapoptotic molecules (8). In 4-1BB-deficient models, the function of cytotoxic T lymphocytes is mostly diminished, and 4-1BB on infiltrated T cells in TME could serve as a marker to predict the antitumor effect of immunotherapy (9). A comprehensive study revealed for the first time that targeting 4-1BB has strong antitumor effects *via* injecting mice bearing Ag104A sarcoma and P815 mastocytoma with anti-4-1BB mAbs (10). Kinetic studies of 4-1BB expression on T cells indicate that although mechanisms for the differential costimulatory ability are not completely elucidated in T cell subsets, 4-1BB is an ideal target on $CD8^+$ T cells for immunotherapy. Indeed, while agonic mAb of 4-1BB could reduce the tumor size and increase survival in multiple preclinical studies (11–13), only limited clinical benefit has been observed due to dose-dependent hepatic toxicity (14–16).

Anti-4-1BB mAb has been proved as an enhancer in the antitumor immunity (17). The bispecific Ab targeting 4-1BB and HER2 showed potent therapeutic effects in HER2 positive cancer (18). IL-12 and 4-1BB were found to possess synergistic antitumor effect *via* activating $CD8^+$ T cell response in mouse models (19). As to the impact of 4-1BB on the therapeutic effect of ICIs, researches demonstrated that costimulatory pathways and immune checkpoint pathways were interdependent (20, 21). Lack of the costimulatory signals led to increased PD-1 expression, which further decreased IL-2 receptors that were necessary for T cell proliferation, whereas 4-1BB co-stimulation potently enhanced reinvigoration of infiltrated T cells in TME (22). Although these findings imply a potential synergy between PD-1 blockade and 4-1BB agonist, the synergistic antitumor effect between PD-L1 blockade and 4-1BB agonist remains unclear (23).

In this context, antitumor immunity of PD-1/PD-L1 blockade and 4-1BB agonist was evaluated in immunocompetent B-cell lymphoma (BCL) models. The transcriptional profile was further analyzed to uncover the underlying mechanisms. Our results highlighted PD-L1 blockade combined with 4-1BB agonist as a potential therapeutic strategy for BCL.

Results

Anti-PD-L1 mAb and 4-1BB agonist elicited synergistic antitumor activity

To investigate the feasibility of dual-targeting PD-L1 and 4-1BB in BCL tumors, mice implanted with A20 or WEHI-231 tumors were treated with 4-1BB agonist, anti-PD-L1 mAb, 4-1BB agonist combined with anti-PD-L1 mAb, respectively. As shown in Figures 1A, B, compared with an equivalent dose of ICI alone, the combined therapy showed a more potent anti-tumor effect in both well-established A20 and WEHI-231 models. A20 tumor-bearing mice were sacrificed on day 18. All mice in the combined group were tumor-free, and the relative tumor weight compared to control in the groups of anti-PD-L1 and 4-1BB agonist antibody were 20.7 ± 4.1% and 33.9 ± 27.5% respectively (Figure 1C). Similar results were observed in WEHI-231 model. Compared with the group under monotherapy with anti-PD-L1 or 4-1BB agonist, tumor volume in the combined group began to decrease on day 8 and continued until the end of treatment (Figure 1B). Relative tumor weight in the groups of anti-PD-L1, 4-1BB agonist, anti-PD-L1 plus 4-1BB agonist were 66.8 ± 7.5%, 52.2 ± 7.0% and 5.0 ± 4.6% respectively (Figure 1D). To further

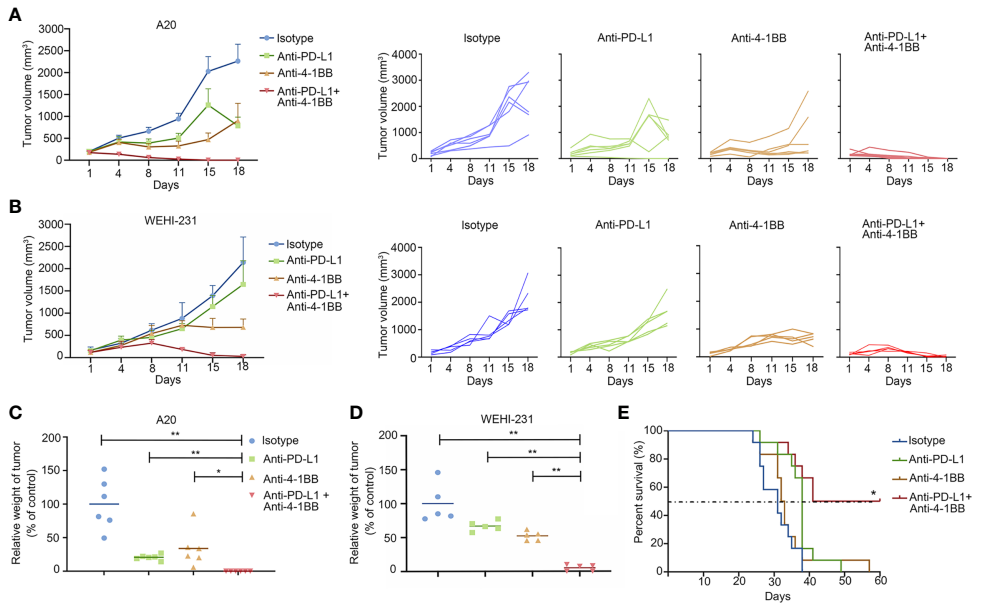

FIGURE 1
4-1BB agonism and PD-L1 blockade elicited synergistic antitumor effect in B-cell lymphoma. **(A, B)** The subcutaneous A20 tumor model (n = 6) or WEHI-231 tumor model (n = 5) were well-established in BALB/c mice. Tumor volume was measured twice a week. Treatment was initialized when the tumor volume reached 100 mm^3. **(C, D)** Tumor weight was measured at the end of the treatment. **(E)** The metastatic A20 model was established to evaluate the antitumor effect on survival (n = 10). * means p-value < 0.05, and ** means p-value < 0.01.

explore whether the combined therapy could extend the survival, A20 metastatic model was established. Compared with the 4-1BB agonist or anti-PD-L1 monotherapy, combined therapy significantly extended the median survival. Importantly, half of the mice were still vigorous by the end of this experiment (Figure 1E). These data showed that anti-PD-L1 therapy combined with 4-1BB agonist elicited potent and durable antitumor effect in subcutaneous and metastatic BCL models.

Transcriptional profile involved in the synergistic antitumor effect

To reveal mechanisms underlying the synergistic antitumor effect, we performed RNA-sequencing on mouse A20 tumor tissue at 10 days post-treatment. In brief, Six tumor-bearing mice were divided equally into two groups and treated with PD-L1 antibody or a combination of 4-1bb agonist and PD-L1 antibody, and their tumor tissues were examined ten days later to analyze the transcriptional profile in the TME. Compared to anti-PD-L1 monotherapy, anti-PD-L1 therapy combined with 4-1BB agonist significantly altered the expression profiles of 538 genes (defined as $P<0.05$, fold change>2) in TME (Figure 2A). Furthermore, the biological functions of the modules were analyzed by KEGG (Figure 2B). Interestingly, most of the increased genes were associated with classical antitumor immunity signal pathways, including cytokine and chemokine pathways (1^{st} and 2^{nd}), and differentiation of Th1, Th2, and Th17 cells (3^{rd}) (adjusted $P<0.0005$). In addition, there were enrichments in Protein digestion and absorption (4^{th}), ECM-receptor interaction (5^{th}), NF-kappa B signaling pathway (7^{th}), PI3K-Akt signaling pathway (8^{th}), and Cell adhesion molecules (9^{th}), demonstrating that the combination therapy had a widespread influence on the metabolism and metastasis of tumor cells (Figure 2B). Integrally, GSEA analysis showed that combined therapy increased T cell activation and IFN-γ signal pathway (Figures 2C, D). These results uncovered the transcriptional landscape in BCL tumors under the cotreatment with anti-PD-L1 ICI and 4-1BB agonist.

4-1BB agonist potentiated anti-PD-L1 mAb-induced T cell immunity

Next, we sought to detect T cells infiltration in TME *via* flow cytometry. Compared to anti-PD-L1 monotherapy, a significant increase of tumor-infiltrating lymphocyte (TIL) was observed in the group of combinatorial therapy (Figure 3A). Furthermore, we determined T cell subsets in the indicated cohorts and found that anti-PD-L1 monotherapy presented modest impact on the infiltration of T cells, whereas there were obvious changes in the combinatorial group with a two-fold increase of $CD8^+$ T cells

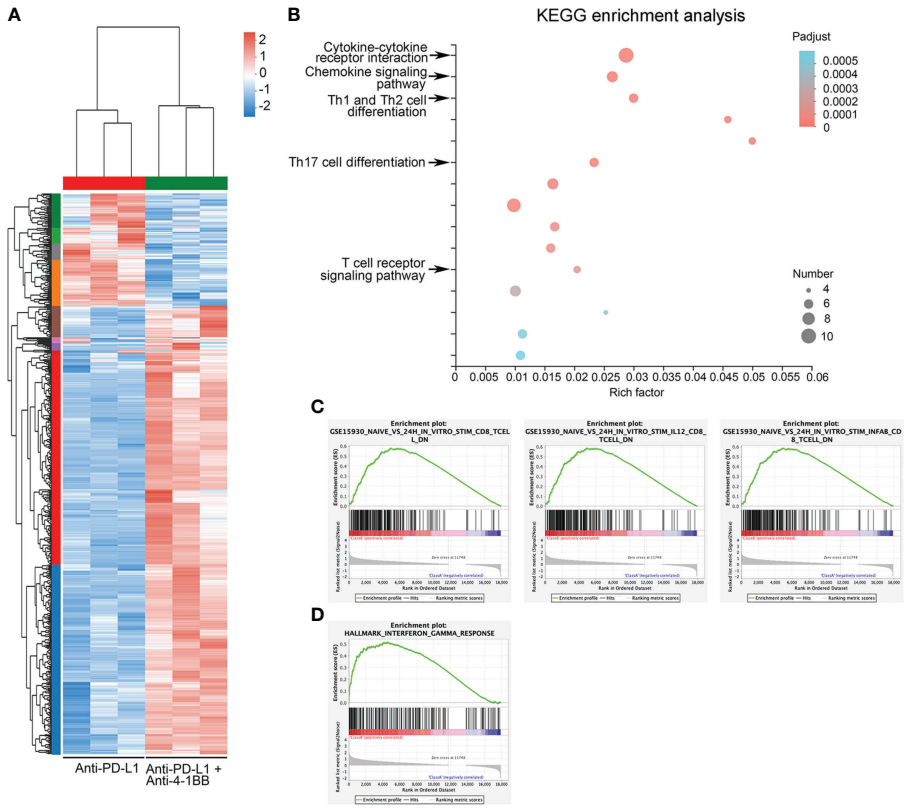

FIGURE 2
Mechanisms underlying the synergistic antitumor efficacy of the combination therapy. **(A)** Heatmap showed the differentially expressed genes between the anti-PD-L1 group and the combined therapy group. **(B)** Enrichment of KEGG pathway of differentially expressed genes. **(C)** GSEA showed an enrichment of the gene set related to $CD8^+$ T cell activation. **(D)** GSEA showed enrichment of activation of IFN-γ response gene sets.

(Figures 3B, C). Immunohistochemistry staining was also performed to confirm $CD8^+$ T cell infiltration. As shown in Figure 3D, compared to anti-PD-L1 alone, PD-L1 blockade plus 4-1BB agonism indeed promoted the infiltration of $CD8^+$ T cells. Summary, these data indicated that 4-1BB agonist and PD-L1 blockade resulted in synergistic antitumor efficacy *via* reinforcing T-cell immunity and PD-L1 expression.

4-1BB agonist potentiated cytolytic capacity of infiltrated T cells

Infiltrated T cells in an established TME are mostly dysfunctional. Thus, gene signature and function of the infiltrated T cells were further evaluated after anti-PD-L1 and 4-1BB agonist combined therapy. We examined the genes related to T cell function in TME, and found that the upregulated genes were significantly enriched in T cell chemotaxis, differentiation, proliferation and activation (Figures 4A–C). Importantly, compared to anti-PD-L1 monotherapy, transcriptional levels of *ifng*, *gzma*, *prf1* and *gzmb* were also intensified by the combined therapy. To confirm immune-related gene expression signatures associated with response to T cell cytotoxicity, mRNA expression of key factors related to T cell cytotoxicity were also measured *via* RT-PCR and ELISA assay. As shown in Figure 4D, 4-1BB signaling significantly potent the release of IFN-γ, perforin and granzyme B from T cells induced by anti-PD-L1 mAb. In summary, these results indicated that 4-1BB agonist in combination with anti-PD-L1 ICI potentiated cytolytic capacity of infiltrating T cells.

$CD8^+$ T cell was essential to the antitumor efficacy elicited by the combined therapy

To illuminate which T cell subsets were indispensable to the combined therapy-mediated tumor regression, we depleted $CD4^+/CD8^+$ T cells in mice using CD4/8 antibodies, respectively. We then constructed the A20 subcutaneous

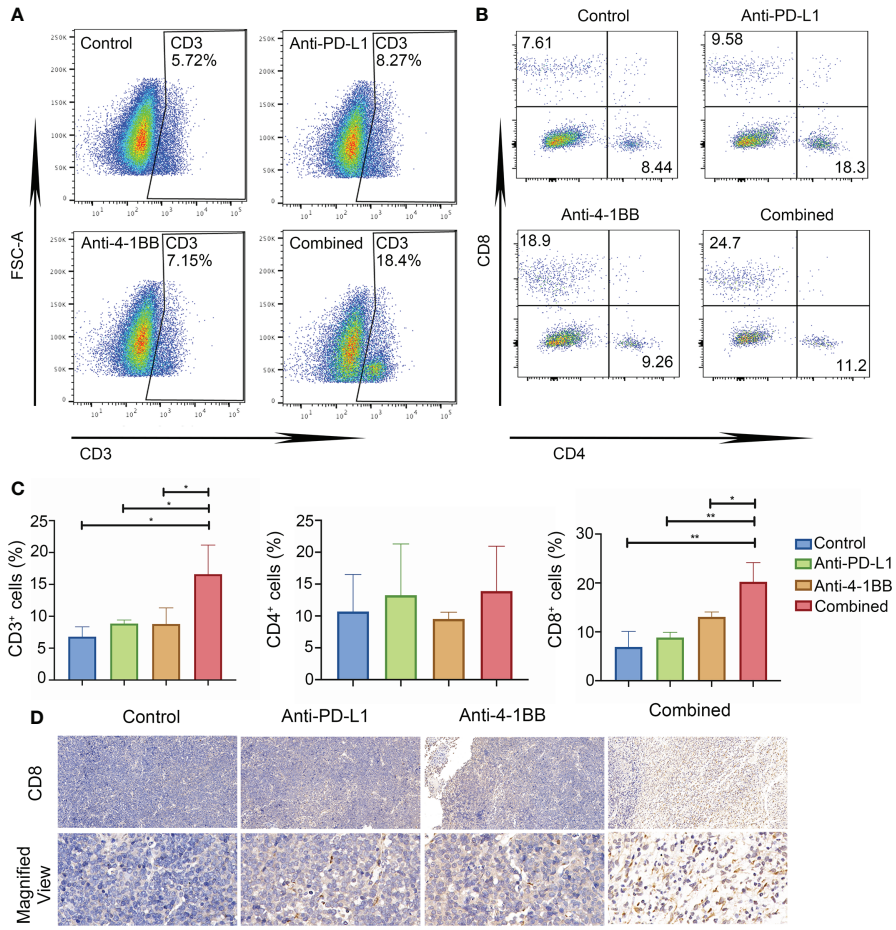

FIGURE 3
The combined therapy increased cytotoxic T-cell infiltration and expression of PD-L1. **(A–C)** The proportion of CD4/CD8 positive cells in the tumor tissue in each group (n = 3) after one week of treatment. **(D)** The infiltration of $CD8^+$ T cell in tumor tissue detected by Immunohistochemistry (IHC) (Magnification × 20 (up), Magnification × 90 (below)). * means p-value < 0.05, and ** means p-value < 0.01.

tumor model and treated the mice with a combination of anti-PD-L1 antibody and 4-1BB agonist. Depleting $CD8^+$ T cell completely abolished the therapeutic effect, while $CD4^+$ T cell depletion had no obvious effect on the combined therapy (Figure 5). The above results confirmed that $CD8^+$ T cells played an essential role in the combined therapy.

Discussion

Considering the fact that PD-1/PD-L1 axis confers cancer cells evasion from hosts' immune system, digging excellent anti-PD-1/PD-L1 ICIs will inspire patients against malignancies (24). However, only a small subset of patients received benefits from these ICIs, of which the absence and exhaustion of tumor-infiltrated cytotoxic T lymphocytes are the main symptoms (25, 26). As one of the prominent co-stimulators (19, 27), 4-1BB signaling could stimulate T cell proliferation and activation, and enhance the cytotoxicity of adaptive T therapy (28–30). Herein, we determined the synergistic effect of anti-PD-L1 therapy in combination with 4-1BB agonists in BCL.

TIL in tumor environment is considered to be the crucial factor determining the antitumor immunity of PD-1/PD-L1 blockade (31, 32). Various efforts have been made to enhance antitumor immunity of tumor infiltrating lymphocytes. Fusion protein consisting of chemokine CCL4 and collagen-binding domain was applied to recruit TILs to improve the antitumor immunity of anti-PD-L1 monotherapy in multiple tumor models (33). Positive correlation has been uncovered in elevated collagen and exhausted T cells in lung cancers. Reducing tumor collagen deposition could increase infiltration of $CD8^+$ T cells and overcome resistance to anti-PD-L1 therapy (34). Physical therapies including thermotherapy and radiation therapy were also reported to promote T cell infiltration and

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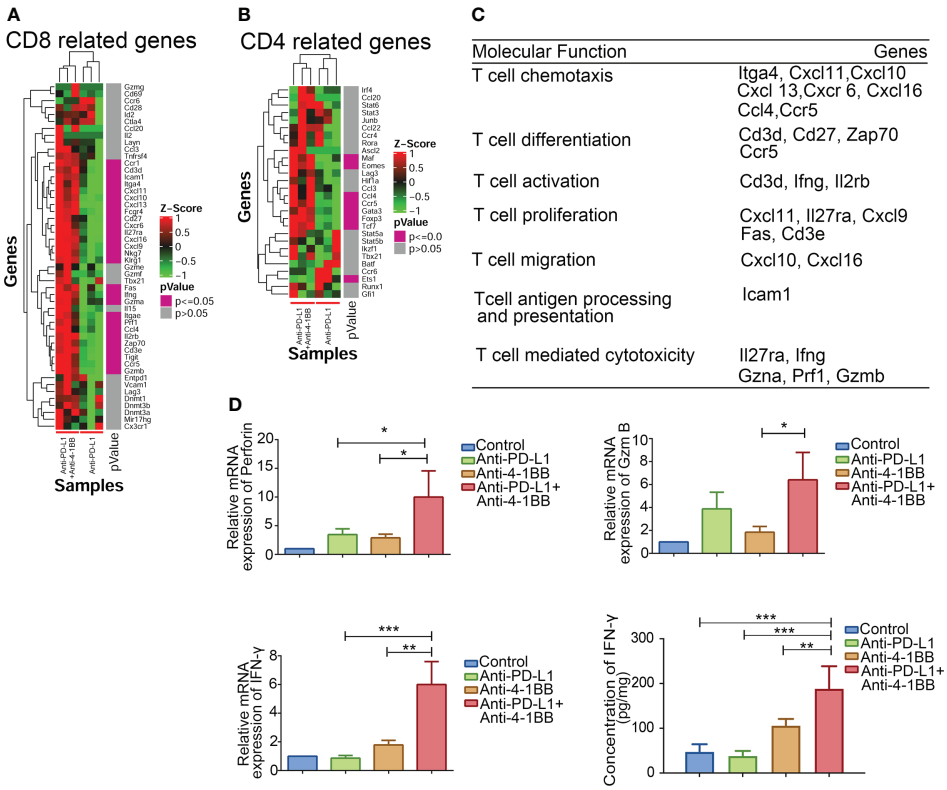

FIGURE 4
Functions of $CD8^+$ T cells in the combination therapy. **(A, B)** Combinational therapy significantly upregulated genes related to $CD8^+$ and $CD4^+$ T cells. **(C)** Genes were classified and grouped based on their molecular function. **(D)** Perforin, IFN-γ, and granzyme B in tumors were measured by RT-PCR or ELISA after combinational treatment (n = 3, mean ± SD). * means p-value < 0.05, ** means p-value < 0.01, and *** means p-value < 0.001.

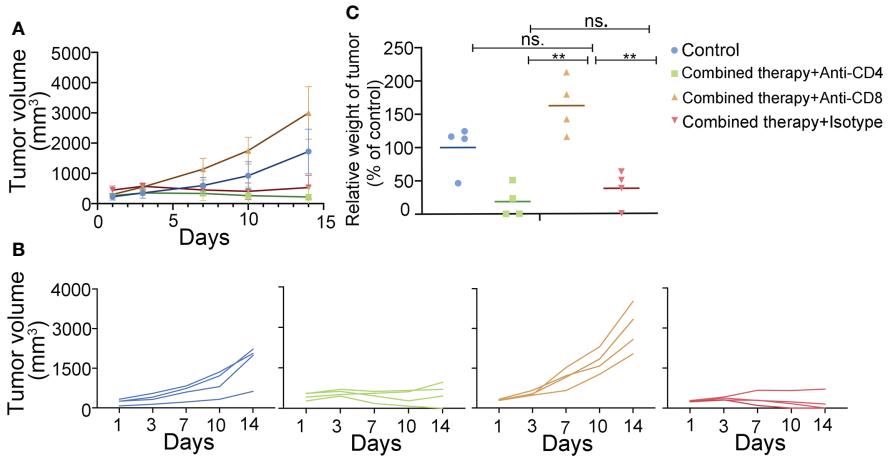

FIGURE 5
T cell subsets in the antitumor efficacy of anti-PD-L1 mAb combined with 4-1BB agonist. The antitumor effect of the combined therapy was abolished by $CD8^+$ T cell depletion. **(A, B)** Tumor volume was measured twice a week. **(C)** Tumor weight was measured at the end of the treatment. (n = 4, mean ± SD). ** means p-value < 0.01. ns means no significance.

synergize with anti-PD-L1 treatment (35). In addition, activating 4-1BB mAb was found to increase the ratio of tissue-resident T cells in pulmonary and hepatocellular carcinoma (36, 37). Bispecific antibodies MCLA-145 and ABL503 were generated to evaluate the antitumor effect and liver toxicity of co-targeting 4-1BB and PD-L1 (21). Results indicated that high dose of bispecific antibody (10 mg/kg) could induce strong antitumor efficacy with low liver toxicity in MC38 tumor model (38). Our research showed that anti-PD-L1 mAb in combination with low dose of 4-1BB agonistic Ab (1 mg/kg) elicited synergistic antitumor activity with no obvious toxicity, whereas PD-L1 blockade or 4-1BB agonism alone only had modest antitumor effects. Considering the significant discrepancy in dosage, combination therapy targeting 4-1BB and PD-L1 is still a potential option in the clinic.

Analyzing tumor-infiltrating T cells would not only contribute to investigating the T cell subsets, but also provide insights into the function of tumor-specific cytotoxic T lymphocytes. Generally, T cells are essential to eliminate tumor cells and higher cytotoxic T lymphocytes infiltration in tumor correlates with better prognosis (39–42). While characteristics underlying the infiltrated T lymphocytes in TME are still unclear, and functions of TILs in the context of anti-PD-L1 ICI and 4-1BB agonist have not been fully elucidated. Here, we observed that targeting 4-1BB and PD-L1 not only activated T cell immunity, but also promoted recruitment of effector T lymphocytes into TME. We also analyzed the transcriptional profile of TME, and data indicated that anti-PD-L1 mAb in combination with 4-1BB agonist resulted in reinforcing $CD8^+$ T-cell immunity *via* cytokine and chemokine signaling pathway. Furthermore, T cell depletion confirmed that the effect of combinational therapy was dependent on $CD8^+$ T cells in BCL.

Currently, ICI has elicited promising antitumor effects in several clinical trials. PD-1 monoclonal antibody nivolumab achieved an objective remission rate of 87% in a phase I clinical trial against refractory recurrent classic Hodgkin's lymphoma (43). In a phase II clinical trial, the objective remission rate reached 68% with overall survival of 6 months (44). However, the efficacy of PD-1 monoclonal antibodies in the treatment of non-Hodgkin's lymphoma is not satisfactory, with objective remission rates of only 40% and 36% for nivolumab alone in clinical trials for refractory relapsed follicular lymphoma and refractory relapsed diffuse large B-cell tumor (45, 46). Studies have shown that PD-L1 ligands are associated with 9p23-24 gene amplification (47) and that the 9p24 gene has a higher probability of mutation in Hodgkin's lymphoma cells (48), whereas the gene is rarely altered in non-Hodgkin's lymphoma cells (47), thus PD-1/PD-L1 blockade has shown better efficacy in the treatment of Hodgkin's lymphoma.

To further enhance the efficacy of PD-1/PD-L1 monoclonal antibodies in the treatment of B-cell lymphoma, various combination regimens have been proposed, including other immune checkpoint inhibitors, co-stimulatory molecular agonists, and other types of therapeutic antibodies. An objective remission rate of 80% was achieved in clinical trials combining the PD-1 monoclonal antibody pembrolizumab and the CD20 monoclonal antibody rituximab for the treatment of relapsed follicular lymphoma (45). Complete remission rates of 50%-65% were achieved using PD-1 monoclonal antibody nivolumab in combination with brentuximab vedotin, an ADC drug targeting CD30, for refractory relapsed Hodgkin's lymphoma (49).

In conclusion, this paper demonstrated that the combination of 4-1BB agonist and anti-PD-L1 antibody can activate T cell function and increase the expression of cytokines such as IFN-γ. On the other hand, it can release the inhibition of T cells by tumor cells by blocking the PD-1-PD-L1 signaling axis. Thus, eliciting a more significant anti-tumor effect (Figure 6). Our results confirmed the synergetic antitumor effect of anti-PD-L1 mAb and 4-1BB agonist, providing an effective approach to treating BCL.

Materials and methods

Antibodies

Anti-PD-L1 mAb was supplied by Shanghai Hankon Biosciences Co. Ltd (Shanghai, China). 4-1BB agonistic antibody was provided by Eutilex Co., Ltd (Korea). APC anti-CD3 antibody, PE anti-CD8a antibody, and PerCP/Cyanine5.5 anti-CD4 antibody were purchased from BioLegend, Inc.

Cell culture

A20 and WEHI-231 were purchased from Nanjing Cobioer biotechnology company and cultured in the indicated medium with 10% FBS, and 50 μM B-mercaptoethanol. The cells were incubated at 37 °C and 5% CO_2.

In vivo therapeutic effect

A20 and WEHI-231 subcutaneous graft tumor models were constructed. The cells were collected by low-speed centrifugation (1200 rpm, 5 min), resuspended in sterile phosphate buffer, and the cell concentration was adjusted to a final concentration of 2.5×10^7 cells/mL. BALB/c mice were ready for tumor

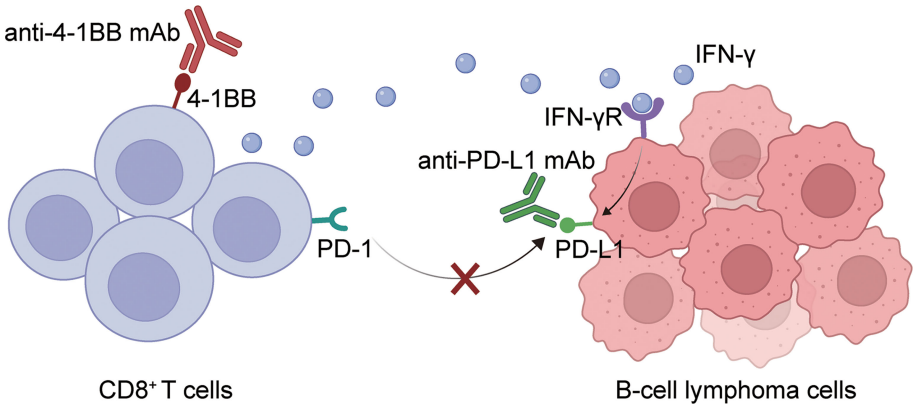

FIGURE 6
A graphical description of how PD-L1 blockade and 4-1BB agonism elicited enhanced antitumor effect in BCL. The combination of 4-1BB agonist and anti-PD-L1 antibody can activate T cell function and increase the expression of cytokines such as IFN-γ. On the other hand, it can release the inhibition of T cells by tumor cells by blocking the PD-1-PD-L1 signaling axis. Thus, eliciting a more significant anti-tumor effect.

inoculation at six weeks of age, weighing about 20 g. Each mouse was injected with 5×10^6 cells, as soon as the tumor volume reached 100 mm^3, the mice were randomly divided into groups and treated. Body weight and tumor diameter (a=long diameter, b=short diameter) were measured and recorded twice a week.
Tumor volume = $\frac{a\times b^2}{2}$.
Relative tumor weight = $100\,\% \times \frac{\text{control group}-\text{treatment group}}{\text{control group}}$.

To construct the A20 metastatic tumor model, the cell concentration was adjusted to 1×10^7 cells/mL using sterile PBS. 100 µL of the cell suspension was injected into the mice through the tail vein, i.e., 1×10^6 cells/mouse. Treatment was started the day after injection, Mice were treated for one week and administered twice a week (Anti-PD-L1 10mg/kg, 4-1BB agonist 1mg/kg). The survival of mice was monitored.

RNA-seq

RNA extraction

TRIzol® Reagent (Invitrogen) and DNAase I (TaKara) were utilized for total RNA extraction and genomic DNA removal respectively. The quantity and quality of RNA were detected by the ND-2000 (NanoDrop Technologies) and 2100 Bioanalyse (Agilent). The next experiment can only be performed if the RNA meets the following requirements. OD260/280 = 1.8~ 2.2, OD260/230≥2.0, RIN≥6.5, 28S:18S≥1.0, >1µg

Library preparation, and Illumina Hiseq xten/ Nova seq 6000 sequencing

Firstly, mRNA was isolated from 1 µg of total RNA and fragmented. Then double-stranded cDNA was synthesized and modified with end-repair, 'A' base addition and phosphorylation. The 300bp size cDNA was isolated and amplified using PCR. After quantification, sequencing was performed on Illumina Hiseq xten/Nova seq 6000 with read length = 2 × 150bp.

Read mapping

SeqPrep and Sickle for quality control. HISAT2 for clean reads aligning to reference genome. StringTie for the assembly of mapped reads.

Differential expression analysis and functional enrichment

The expression level was determined by the transcripts per million reads. Q value ≤ 0.05 (DESeq2) was considered to be significantly different. KEGG pathway analysis was carried out by KOBAS.

GSEA analysis

GSEA was performed with GSEA v3.0 (http://www.broadinstitute.org/gsea/). Gen sets were obtained from MSigDB (http://www.broadinstitute.org/gsea/msigdb).

Flow cytometry

4.5.1 Preparation of single cells suspensions of tumor tissues

Mice were executed to obtain tumor tissue. The tumors were cut into small pieces and placed on a cell sieve with PBS for grinding. The collected grinds were centrifuged to remove the supernatant. After 5 min, the supernatant was discarded by

centrifugation, washed once with PBS, and PBS was added again to obtain a single-cell suspension.

Antibody staining

The supernatant was removed by centrifugation according to the instructions, and the blocking antibody was added and blocked for 30 min at 4°C. The blocking antibody was then removed by centrifugation and the flow-labeled antibody (Anti-mouse CD3 (APC), anti-mouse CD4 (PerCP/Cy5.5), anti-mouse CD8a (PE) (Biolegend)) was added and incubated for a half-hour at 4°C in dark. Centrifuge to remove supernatant, wash twice with PBS, and resuspend in 300μl PBS.

Immunohistochemistry

Mouse tumors were fixed in 4% paraformaldehyde. After the fixed tissues were embedded in wax blocks and sectioned, the tissue sections were stained using CD 8 antibody. The infiltration of CD8-positive cells in the tissues was examined and analyzed by light microscopy.

T cells depletion

All antibodies used for T-cell depletion were purchased from bioxcell. Mice were injected intraperitoneally with an anti-CD4 antibody (200μg, colone GK1.5), anti-CD8 antibody (200μg, colone 2.43), and Isotype control (IgG2b, 200μg, colone LTF-2) on days 1, 3, 5, and 7, respectively, and then the antibodies were injected once a week until the end of the experiment.

Statistical analysis

Data in this study were analyzed by GraphPad Prism 9. Comparison was determined by Student's *t*-test and One-Way ANOVA analysis, and P value< 0.05 was considered as statistical significance.

Data availability statement

The original contributions presented in the study are publicly available. The data is deposited in the SRA database, accession number: PRJNA904505.

Ethics statement

The animal study was reviewed and approved by Animal Ethical Committee of School of Pharmacy at Fudan University.

Author contributions

DJ and XZh designed the study. YW, XZh, CX and YN performed the experiments and wrote the paper. YW, XZh, JF, XZe, and BK analyzed the data. All authors contributed to the article and approved the submitted version.

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Conflict of interest

BK is the founder and Chief Executive Officer of Eutilex Co., Ltd.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.1004475/full#supplementary-material

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Biology and clinical relevance of follicular cytotoxic T cells

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Follicular cytotoxic T (Tfc) cells are a newly identified subset of $CD8^+$ T cells enriched in B cell follicles and their surroundings, which integrate multiple functions such as killing, memory, supporting and regulation. Tfc cells share similarities with follicular helper T (Tfh) cells, conventional cytotoxic $CD8^+$ T (Tc cells)cells and follicular regulatory T (Tfr) cells, while they express distinct transcription factors, phenotype, and perform different functions. With the participation of cytokines and cell-cell interactions, Tfc cells modulate Tfh cells and B cells and play an essential role in regulating the humoral immunity. Furthermore, Tfc cells have been found to change in their frequencies and functions during the occurrence and progression of chronic infections, immune-mediated diseases and cancers. Strategies targeting Tfc cells are under investigations, bringing novel insights into control of these diseases. We summarize the characteristics of Tfc cells, and introduce the roles and potential targeting modalities of Tfc cells in different diseases.

KEYWORDS

follicular cytotoxic T cell, phenotype, cellular crosstalk, disease relevance, transcription factor

1 Overview of Tfc cells

Follicular cytotoxic T (Tfc) cells are a $CD8^+$ T cell subset initially discovered by Quigley et al. (1) in 2007. Tfc cells were found primarily inside and around the B cell follicles (2–4), while a small subset of Tfc cells localized in peripheral blood (1, 2). In addition, they were found in a variety of species, including mice, rhesus monkeys, and humans (5–7). Tfc cells are typically identified as $CXCR5^+$ $Tcf1^+$ $Tim3^-$ $CD8^+$ T cells, and their development is regulated by core transcription factors Tcf1, Bcl6, Blimp-1, E2a and Runx3. Meanwhile, Tfc cell differentiation is positively regulated by cytokines IL-21, IL-6, IL-23 and TGF-β. Since they maintain stemness and undergo a follicular development

pathway, they share similarities with follicular helper T (Tfh) cells, follicular regulatory T (Tfr) cells, and newly identified "stem-like" $CD8^+$ T cells. Furthermore, Tfc cells secrete cytokines IL-2, IL-4, IL-21, IFN-γ, TNF-α, granzyme B (Gzmb) and perforin under different conditions, and perform multiple functions such as killing, supporting and regulation. Participation of Tfc cells in chronic infections, immune-mediated diseases and tumors have been revealed. Changes in the frequency, phenotype and functions of Tfc cells affect the local immune homeostasis, which may mediate the pathophysiology and affect the severity of these diseases. In recent decade, great progress has been made in our understandings of Tfc cells. Deeper understandings on Tfc cell biology and their roles in diseases have shed light on potential therapeutic modalities targeting Tfc cells.

2 The characteristic surface markers of Tfc cells

Tfc cells derive from $CD8^+$ T cells which migrate towards GCs, and express signature markers of both $CD8^+$ cytotoxic T cells and follicular T cells. $CXCR5^+$ $Tcf1^+$ $Tim3^-$ $CD8^+$ are classical surface markers to identify Tfc cells (8). Stromal cells and follicular dendritic cells (FDCs) secrete large amount of CXCL13, and construct an environment with both soluble and immobilized CXCL13 gradients (9, 10). CXCR5 is the corresponding receptor of CXCL13, which induces T cells to migrate toward B-cell follicles. Naïve $CD8^+$ T cells are activated and differentiated into activated $CD8^+$ T cells. A part of them express CXCR5 and migrate to B cell follicles and their surroundings along the gradient of CXCL13 concentration (Figure 1). In contrast to CXCR5, CCR7 is an important factor facilitating the migration of Tfc cells towards the T cell zone in response to CCL21, which is highly expressed in the T cell area. Se Jin Im et al. (5) observed that $CXCR5^+$ $CD8^+$ Tfc cells with high level of CCR7 mRNA expression resided in the T cell zone of mice spleen. Down-regulation of CCR7 led to the migration of Tfc cells from the T cell zone or T-B borders to the B cell zone (7). In addition, CXCR3, CD62L and CD69 are related to Tfc cell chemotaxis toward lymphoid tissue. CXCR3 is found on activated T cells and assists in their recruitment (11, 12). CXCR3 expression on Tfc cells is higher than that on naïve $CD8^+$ T cells and Tc cells, which may facilitates their migration from peripheral blood toward infected B lymphocyte follicles (4, 8). Tfc cells express higher level of Sell and its encoded protein CD62L than Tc cells, indicating a memory phenotype and mediating lymphocyte adhesion and lymph node (LN)-homing of Tfc cells (4, 13). CD69 has been linked to the rapid activation of T cells during acute inflammation, and it has been shown to interfere with the function of S1P receptors, limiting S1P-mediated egress of immune cells from lymphoid organs into

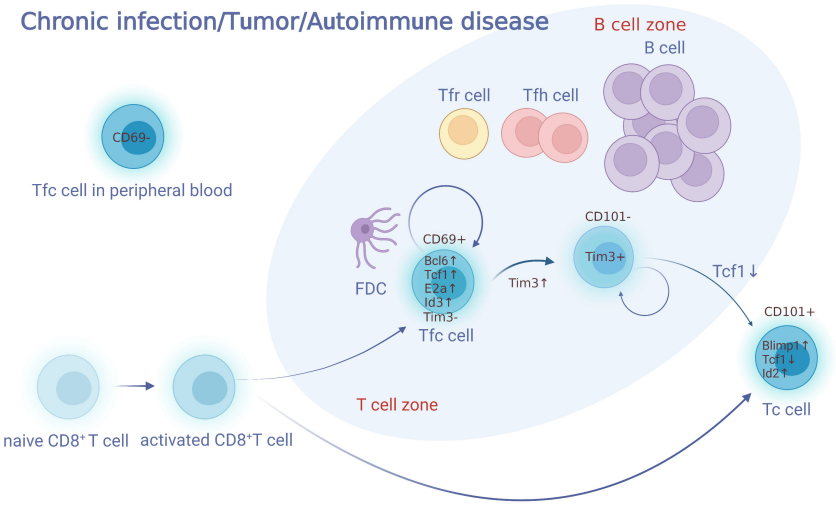

FIGURE 1
Development of Tfc cells. Naïve $CD8^+$ T cells are activated and differentiated into activated $CD8^+$T cells. A part of activated $CD8^+$ T cells express CXCR5 and migrate to B cell follicles and their surroundings along the gradient of CXCL13 concentration. These cell subsets, defined as Tfc cells, perform various biological functions such as killing, memory, supporting, and regulation. Tfc cells do not express Tim3, and have the ability to self-renew. Tfc cells upregulate Tim3 and differentiate into two groups of $Tim3^+$ $CD8^+$ T cells ($CD101^-Tim3^+$ $CD8^+$T and $CD101^+Tim3^+CD8^+$T). $CD101^-Tim3^+CD8^+$ T cells is a transitory cell population, which preserves proliferative and effector activity before transform into effector $CD101^+Tim3^+CD8^+$ T cells irreversibly. Furthermore, Tfc cells present in both of the lymphoid organs and the peripheral blood, with those in the lymphoid organ expressing CD69 while those in the peripheral do not express. FDC, follicular dendritic cell; Tfh, follicular helper; Tc, cytotoxic T; Tfc, follicular cytotoxic T.

lymphatic vessels (8). Therefore, CD69$^+$ Tfc cells reside in the lymphoid organs quiescently, whereas CD69$^-$ Tfc cells remain in the peripheral blood.

45RO is highly expressed on Tfc cells, indicating that they may be derived from naive CD8$^+$ T cells after contact with antigens (1). CD27, CD28 and CD69 are specific biomarkers on the early stage of T cell differentiation, which are also highly expressed on Tfc cells, suggesting Tfc cells as an early effector memory T cell subset. After antigen stimulation, Tfc cells elevate the expression of CD40L and ICOSL, which activates GC B cells *via* the CD40L/CD40 and ICOSL/ICOS, respectively (14).

Compared with Tc cells, Tfc cells express lower level of inhibitory molecules such as PD-1, GITR, CD244 and CD160 (3–6, 15–19). Tfc cells do not express Tim3, and have the ability to self-renew. Tfc cells upregulate Tim3 and differentiate into two groups of Tim3$^+$ CD8$^+$ T cells (CD101$^-$ Tim3$^+$ CD8$^+$ T and CD101$^+$ Tim3$^+$ CD8$^+$ T). In addition, CD8$^+$ T cells up-regulate the PD-1 expression and develop status of cell exhaustion under the long and high-load antigen exposure, so the lower expression of PD-1 on Tfc cells might indicate a lower degree of cell exhaustion (3–5, 20). Notably, the expression level of PD-1 on Tfc cells varies among diseases and different levels of antigen stimulation. Compared with healthy individuals or NHL patients, PD-1 expression on Tfc cells is lower among CLL patients (5). Moreover, PD-1 expression on Tfc cells infiltrated in the tumor samples of follicular lymphoma patients is higher than that expressed on Tfc cells sorted from tonsil samples of healthy individual (15). Interestingly, upon specific condition such as allograft transplant, a subset of PD-1 negative Tfc cells have been observed to inhibit allo-antibody secretion from alloreactive B cells (21). Finally, given that PD-1 controls the localization of Tfh cells in both co-stimulus-independent and co-stimulus-dependent manners, it is also speculated that PD-1 may be also related to the localization of Tfc cells (15, 22–24).

3 Transcription factors involved in Tfc cells differentiation

Bcl6, Tcf1, Eomes and E2a inhibitors Id2 and Id3 form a transcriptional control loop that together guide the development of Tfc cells (Figure 2). The differentiation of Tfc cells can be promoted by the TFs Bcl6, E2a, and Tcf1, and inhibited by Blimp1 and Id2 (4, 15). Bcl6 and Blimp1 are antagonistic TFs. Compared with CXCR5$^-$ non-Tfc cells, Tfc cells express higher levels of Bcl6, while Blimp1 expression is significantly reduced. Cluster analysis has shown that a large number of differentials expressed genes which drive Tfc cell differentiation are Bcl6-bounded. The proportion of CXCR5$^+$ CD8$^+$ T cells among CD8$^+$ T cells in Bcl6 overexpressed mice infected with LCMV increased significantly from 20% to 60%. After knocking down the *Bcl6*, CD8$^+$ T cells failed to differentiate into Tfc cells *in vivo*

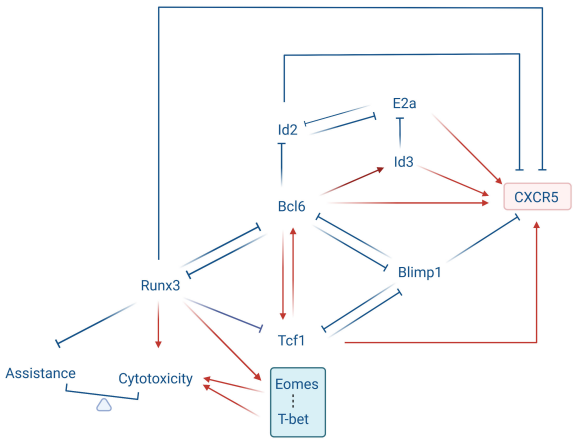

FIGURE 2
Transcriptional loop governing Tfc cell development. Bcl6, Tcf1, Eomes, and the E2a inhibitors Id2 and Id3 form a transcriptional loop that determines Tfc cell development. Tcf1 is highly expressed in Tfc cells and acts as an upstream regulator of the Bcl6-Blimp1 crosstalk, promoting Bcl6 expression while suppressing Blimp1 expression. Tcf1, Bcl6 and Blimp1 are mutually antagonistic or promoting. Bcl6 is highly expressed in Tfc cells and promotes CXCR5 protein expression while decreasing Runx3 and Id2 expression. Blimp1 is downregulated in Tfc cells, and it suppresses Cxcr5 gene transcription by binding to its 5′ upstream and intronic regions. E2a activation enhances Cxcr5 transcription and protein production through binding to its intron region. Id2, which antagonizes E2a transcriptional activity, suppresses Cxcr5 expression. Id3, however, a transcriptional antagonist of E2a, promotes the Cxcr5 expression. Runx3 is down-regulated in Tfc cells, it represses the Bcl6 expression and the Tfh program, and activates the cytotoxic program directly. In addition, Runx3 promotes the expression of T-box family transcription factors (T-bet and Eomes), which promotes the cytotoxicity of Tfc cells, while limiting the assistant capacity.

on the 8th day after infection with LCMV. In the transcriptional regulation aspects, up-regulation of Bcl6 enhanced the expression of *Tcf7* (encoding Tcf1) and *Id3*, while inhibited the expressions of *Prdm1* (encoding Blimp1) and *Id2*. Meanwhile, over-expression of Bcl6 resulted in most phenotypic changes on Tfc cells, including up-regulation of CD127, CD62L and ICOS, and down-regulation of Tim-3 (4). In contrary to Bcl6, Blimp1 inhibits Tfc cells differentiation. It was observed that in Blimp1 deficient CD8$^+$ T cells the characteristic TFs of Tfc cells were upregulated, and the CXCR5 expression was significantly increased (4, 25). Finally, up-regression of Blimp1 in activated CD8$^+$ T cells using retroviral vectors containing Blimp1-binding motifs has shown that Blimp1 suppresses CXCR5 promoter activition (4).

Tcf1 is a key transcription factor regulating the Bcl6-Blimp1 axis during Tfc cell development. Tcf1 induced Bcl6 expression and repressed several pro-exhaustion factors including Blimp1, Tim3 and Cish, which could repress T cell exhaustion and maintain T cell stemness (26). During chronic infection,

Virus-specific Tcf1^{high} CD8$^+$ Tfc cells differentiate into a less exhausted Tcf1^{high} CD8$^+$ T cell subset and a more exhausted Tcf1^{low} CD8$^+$ Tc cell subset, respectively. Here Tfc cells serve as progenitor-like subsets supplementing Tcf1^{low} CD8$^+$ Tc cells, which is critical for persistent antiviral CD8$^+$ T cell responses in chronic infection (27).

Compared to CXCR5$^-$ CD8$^+$ T cells, Tfc cells express lower level of runt-related transcription factor 3 (Runx3) (6). Runx3 modulates a broad transcriptional network. Owing to the down-regulation of Runx3, the major function of CD8$^+$ T cells changes from cytotoxicity to B cell assistance (28). It is revealed that Runx3 enhances the cytotoxic function of Tc cells through *Prf1* and *Gzmb* binding, and its knockout leads Tc cells to differentiate into Tfc-like cells, which migrate into the B follicles and assist antibody production (28–30). In Runx3-difficient Tc cells, characteristic genes associated with follicular T cell linage including *Bcl6, Tcf7, cxcr5, Icos, il6ra and il21* are up-regulated. Meanwhile, GSEA plot has also shown gene expression similarities between Runx3$^{-/-}$ Tc cells and follicular T cells (28). Finally, during acute infection Runx3 promotes Tc cell clonal expansion, and prevents activation of the Tfh program in CD8$^+$ T cells through Tcf1 repression.

During Tfc cell development, During Tfc cell development, Runx3 induces the expression of T-box family TFs (30). Tfc cells only express two T-box family TFs: T-bet and Eomes, which antagonize with each other during the linage differentiation of CD8$^+$ T cells (31–33). T-bet regulates cytotoxicity of CD8$^+$ T cells by increasing perforin and IFN-γ secretion and promoting IL-2 and PD-1 expression (34–36). It is observed that Tfc cells express lower level of T-bet and higher level of Eomes as compared to Tc cells, and this Eomes^{hi} T-bet^{low} feature confers Tfc cells an early memory phenotype with lower cytotoxicity than their CXCR5$^-$ counterparts (15).

Id2 and E2a antagonize each other not only in the CXCR5 expression but also the development of Tfc cells. Id2 inhibits the Cxcr5 expression and the Tfc cells differentiation. He et al. observed that on the 21st day after LCMV infection, the number and frequency of Tfc cells in Id2$^{-/-}$ mice were higher than those in wildtype group, and the virus titer in Id2$^{-/-}$ mice was significantly lower. In Tc cells, E2a promotes CXCR5 expression *via* binding with a conserved E2a-binding sequence in *Cxcr5* intron region. Over-expressing E2a in LCMV-specific P14 CD8$^+$ T cells remarkably upregulated CXCR5 expression and the frequency of CXCR5$^+$ cells in P14 CD8$^+$ T cells, whereas co-overexpressing Id2 compromised such effect. In addition, E2a overexpression is related to increased CD107 expression and cytokine secretions, and decreased PD-1 expression on P14 CD8$^+$ T cells. Importantly, during chronic viral infection, the Id2/E2a axis plays a key role in driving the differentiation from virus-specific CD8$^+$ T cells to CXCR5$^+$ and CXCR5$^-$ subgroups, performing anti-viral effect both inside and outside the follicles. It was observed that Tfc cells appear in chronic infected organisms and supplement CXCR5$^-$ CD8$^+$ T cells outside the follicles. Abundance of Id2 in Tfc cells help them to transform into CXCR5$^-$ CD8$^+$ T cells, which secrete more IFN-γ and TNF- compared to Tc cells originally reside outside the GCs (3). By contrast, down-regulation of Id2 has been found to transfer virus-specific exhausted CD8$^+$ T cells towards Tfc cells and migrates into follicles (28). Interestingly, another E2a antagonize TF Id3 is highly-expressed in Tfc cells, indicating a potential self-limiting regulation of CXCR5 expression in Tfc cells through the Id3-E2a crosstalk. When *Id3* expression is disrupted, the CXCR5 expression on LCMV-specific Tfc cells was significantly increased. Meanwhile, over-expression of Id3 prevents CD8$^+$ T cell transformation towards Tfc cells due to down-regulation of several genes relevant to Tfc development (4).

4 Cytokines related to Tfc cell development and functions

4.1 IL-21 and IL-6

IL-21 receptor (IL-21R) is expressed on Tfc cells, while the effects of IL-21 on Tfc development and functions remain elusive. It is observed that during chronic inflammation, activated CD4$^+$ Tfh-like cells enter tissues, produce IL-21 and activate CD8$^+$ T cells through JAK/STAT protein phosphorylation (37). Afterwards, CD8$^+$ T cells release IFN-γ and change their metabolic profile. Target genes of the IL-21/IL-21R signaling include *Batf*、*Bcl6*、*Eomes*、*Gzma*、*Gzmb*、*Il10*、*Maf*、*Prdm1* (38, 39). In addition, IL-21 promotes the transcription of Id2, which down-regulates CXCR5 expression on CD8$^+$ T cells (40, 41). It is possible that Tfc cells are also affected in a similar way, while more experimental evidence should be provided.

Tfc cells secrete IL-21, which promotes B cell maturation, antibody secretion and class-switch recombination *in vitro* (42, 43). Circulating Tfc cells in chronic HBV infection express IL-10 and IL-21, which enhance Tfh cell function and induce B cell antibody production synergistically (42). In addition, IL-21 secreted by Tfc cells may promote the development of HL by stimulating the IL-21R on the surface of R-S cells (44). Deficiency in Runx3 or STAT5 elevates the IL-21 secretion from Tfc cells (28). Down-regulation of STAT5 impairs the Blimp1 expression, and increases the expressions of Bcl6, Batf and IL-21. In some certain conditions, Tfc cells do not secrete IL-21. For example, Tfc cells in LCMV infected mice do not express ICOSL and IL-21, while they perform more potent cytotoxicity (4).

The IL-6 signaling is essential for Tfc cell differentiation at the early stage, which promotes IL-21 secretion through Stat3 up-regulation. Yang et al. (43) have found that IL-6R is an important marker to identify Tfc cell subsets producing IL-21, and an IL-6-rich microenvironment is necessary for naïve CD8$^+$

T cells to develop into Tfc cells highly secrete IL-21. Notably, the effect of IL-6 is timely-dependent, since late stimulation of activated $CD8^+$ T cells with IL-6 can no longer induce their IL-21 production (43).

4.2 TGF-β

When PBMCs are cultured with anti-CD3/CD28 *in vitro* together with TGF-β, the expression of CXCR5 is up-regulated. When TGF-β is added to the $CD8^+$ T_{RM} cell culture system, CXCR5 expression of $CD8^+$ T_{RM} cells was higher than those stimulated by IL-12 and IL-23 (6). In addition, IL-23 and TGF-β induce a higher expression of CXCR5 in naïve $CD8^+$ T cells (15, 45). TGF-β and IL-23 down-regulate the expression of Prdm1 and Id2 and increase the expressions of Bcl6 and Id3 by Tfc cells. It is also found that TGF-β activates E2a and promotes $CD8^+$ T cells to go through the follicular differentiation pathway. In the meantime, TGF-β induces the Foxp3 expression of $CD8^+$ T cells by promoting the E2a and Foxp3 promoter binding (46–48). Finally, non-canonical pathway of TGF-β and interactions with other signals may be also involved in Tfc cell follicular differentiation (45).

5 Comparison of Tfc cells and relevant T cells

There are similarities and disparities between Tfc cells and other cell types (summarized in Table 1). Firstly, for Tfc and Tfh cells, both of them undergo similar follicular development pathways regulated by core transcription factors Bcl6, Blimp1 and Tcf1. Meanwhile, their developments are regulated by cytokines IL-21 and IL-6, and their surface markers (CXCR5, ICOS, CD40L, etc.) are similar. However, some differences have made Tfc cells distinct from Tfh cells. Tfc and Tfh cells belong to $CD8^+$ and $CD4^+$ T cell lineages, respectively. Tfc cells may stay at an earlier stage of differentiation than Tfh cells. According to Yu Di et al. (8), the stage of differentiation of Tfc cells are between Tscm and Tcm cells, which may explain that stemness exist in Tfc cells but not in Tfh cells. Moreover, not like Tfh cells, Tfc cells are capable of self-renew, and have the ability to develop into cells with a killing function before entering an exhausted state. Tfc cells secrete cytokines IL-2, IL-4, IL-21, IFN-γ, TNF-α, Gzmb and perforin under different conditionsm, while Tfh cells secrete IL-6, IL-10, IL-21 to help them serve as B cell helpers. Tfc and Tfh cells can help B cells through CD40L/CD40, ICOSL/ICOS, TCR/MHC, as well as the cytokine IL-21 (2).

Recent studies have described a specific subset of $CD8^+$ T cells namely "stem-like" $CD8^+$ T cells, which significantly overlap with Tfc cells in transcription factors, phenotypes, and functions (49–53). "Stem-like" $CD8^+$ T cells express CXCR5 throughout their development but not when they develop into mature couterparts. Therefore, CXCR5 was the key surface marker for distinguishing this subset of "stem-like" $CD8^+$ T. However, recent studies have identified Tcf1 as a more prominent marker than CXCR5 for Tfc cell identification. Tcf1-mediated Bcl6 induction and Blimp1 repression constitute crucial regulatory circuits in promoting "stem-like" $CD8^+$ T cell fate, as well as regulated Cxcr5 expressions (4). Notably, "stem-like" $CD8^+$ T cells and Tfc cells share many similarities, including the high expressions of Tcf1, CXCR5, ICOS, CD28, and weak expression of surface markers related to the cell depletion. In addition, both of them have the ability to self-renew and transform into more cytotoxic and exhausted T cells. However, Tfc cells not only exhibit "stem-like" features and serve as a $CD8^+$ T storage pool, but they also eliminate Tfh cells and B cells, execute B cell helper functions, regulate B cells, and play a role in anti-tumor, antiviral, and autoimmune diseases. Collectively, Tfc cells seem to perform broader range of functions than "stem-like" $CD8^+$ T cells, but it remains unclear whether "stem-like" $CD8^+$ T cells represent a subset of Tfc cells, or "stem-like" $CD8^+$ T cells are indeed Tfc cells with function partially revealed.

Tfc and Tfr cells are both important members of the humoral immunity. There are similarities and differences between Tfc and Tfr cells on transcription factors, surface markers, cytokines and functions. Firstly, Tfc cell development is dependent on Tcf1, Bcl6, Id2, and Runx3, and the expression of Blimp1 is down-regulated. However, Tfr cell development is dependent on Bcl6, Foxp3, and the expression of Blimp1 is up-regulated. Tfc cell differentiation is positively regulated by cytokines IL-21, IL-6, IL-23, and TGF-β, whereas Tfr cell differentiation is negatively regulated by cytokines IL-2, IL-6 and IL-21 (54–56). Secondly, even though Tfc and Tfr cells are $CD8^+$ and $CD4^+$ T cell lineages respectively, they both express CXCR5, Bcl6, Tcf1, PD1, and ICOS. However, Tfc cells expressed memory T cell markers CD28, CD27, and CD62L, while Tfr cells expressed Treg cell markers CTLA4, GITR, and Foxp3. Thirdly, different from Tfc cells, Tfr cells release IL-10, barely express IL-4 and IL-21 and cytokines related to cytotoxicity such as IFN-γ, TNF-α, Gzmb and perforin. Finally, both Tfc and Tfr cells play a vital role in regulating Tfh cells and GC-B cells, but the mechanisms are different. Tfc cells exert its regulatory role through not only Tfh and B cell elimination, but also cytokine secretion (described in the next chapter). Tfr cells produce IL-10, TGF-β to suppress Tfh and GC-B cells directly (15). Meanwhile, Tfr cells produce IL-1R2 and IL-1Rα to inhibit Tfh cell activation, and suppress the expression of B7-1 and B7-2 on GC-B cells *via* CTLA4, which finally down-regulate GC-B cell stimulation (57).

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TABLE 1 Comparison among Tfc cell and other T cell subsets.

Cell subsets			Naïve $CD8^+$ T	Stem-like CD 8^+T	Tfc	Tc	Tfh	Treg	Tfr
Lineage		CD4	–	–	–	–	+	+	+
		CD8	+	+	+	+	–	–	–
Surface marker	**Migration**	CCR5			+	+	–		
		CCR7	+		+/-	–	–		–
		CXCR5	–	+	+	–	+	–	+
		CXCR3	+	+	+	+	+	+/-	
		CD62L	+	+	+/-	–	–	–	
	Activation	CD69	–	+/-	+/-	+	+	+	–
		CD27		+	+	+			
		CD45RA	+	–	–	–	–	–	–
		CD45RO	–	+	+	+	+	+	+
	TNF superfamily	CD40L			+	+			–
		FasL			+	+			
	Co-stimulating molecules	CTLA-4		–	+	+		+	+
		PD-1	–	+	+	+	+	+	+
		CD28	+	+	+	+			–
		ICOS	–	+	+	+	+	+	+
	Others	CD107	–		+	–			
		CD127	+		+	–	–		–
Transcription regulator		Bcl6	–	+	+	+	+	+	+
		Blimp1	–	+	+	+	+	+	+
		Tcf1	–	+	+	–	+		+
		Runx3			+		+		
		Tim3		–	–	+			
		T-bet	–	+	+	+	+	+	
		Eomes	–		+	–	+/-	+	
		Id2	–		+/-	+	–		+
		Id3	+/-		+		+		+
		E2a			+	+	+		+
		Stat3			+	+/-	+	+	+
		Maf			+		+	+	
		Batf			+		+	+	
		Foxp3	–	–	–	–	–	+	+
Cytokines required for differentiation *in vitro*			–	–	IL-23, TGF-β, IL-6, IL-21	IL-2, IL-4, IL-6, IL-12, IL-21, TGF-β	IL-6, IL-12, TGF-β, IL-21, IL-23	IL-2, TGF-β	
Cytokines secretion			–	–	IL-21, IL-10, IFN-γ, perforin	IL-4, IL-5, IL-9, IL-10, IL-13, IL-17, IL-21, granzyme, perforin, TNF-α, TGF-β, IFN-γ, GM-CSF,	IL-4, IL-10, IL-17A, IL-17F, IL-21, CXCL13, IFN-γ, TNF-α	IL-9, IL-10, IL-35, TGF-β, CLL3, CLL4	IL-10, TGF-β, granzyme

+, positive; -, negative; +/-, either positive or negative, context-dependent.

6 Crosstalk among Tfc, Tfh and B cells

Functions of Tfc cells can be divided into four categories: cytotoxicity, memory, B cell antibody class-switch facilitation and B cell function enhancement (literatures summarized in Table 2). The common anatomical location provides a good communication space for Tfc, Tfh and B cells (crosstalk among these three types of T cell is illustrated in Figure 3). Cytotoxicity of Tfc cells manifests as removal of the infected/cancerous Tfh cells or B cells. Tfc cells secrete IFN-γ, TNF-α and granzymes to eliminate infected Tfh and B cells, and the number of Tfc cells was inversely correlated with viral load. In patients with chronic hepatitis B virus (HBV) infection and HBV-infected hepatocellular carcinoma, frequency of Tfc cells in the peripheral blood is significantly up-regulated, which is negatively correlated with frequency of Tfh cells (2). Besides, Tfc cells are less exhausted than $CXCR5^{-}$ $CD8^{+}$ T cells during chronic infection, and they serve as a $CD8^{+}$ T storage pool and differentiate into terminally exhausted $CD8^{+}$ T cells.

Tfc cells are early memory-like cells. Genes related to Tfc cell differentiation are enriched in mitochondrial fatty acid β-oxidation, mTOR signaling and Wnt signaling, which are all related to cell maintenance and self-renewing. Moreover, it is also observed that Tfc cells are similar to $CD8^{+}$ memory precursor cells in gene set enrichment analysis (5). Expression of Tcf1 is essential for Tfc cells maintenance and longevity. Meanwhile, over-expressions of CD62L, CD127, KLRG1 and low expressions of molecules associated to effector T cells make Tfc cells less differentiated and gain memory-like phenotype (7, 10). In addition, Tfc cells in both PB and LN express high levels of co-stimulatory molecules such as CD27 and CD28, which is compatible with their early effector memory phenotype (7).

Under specific pathogenic conditions, Tfc cells support B cell functions or assist antibody class switch through direct or indirect patterns. Tfc cells play a B cell helper function to promote virus-specific IgG production during influenza infection (43). In autoimmune diseases, increased frequency of Tfc cells leads to the breakdown of B cells tolerance and antibody over-production (2). *In vitro* experiments have shown that Tfc cells promote naïve B cells to transform into mature plasma cells secreting IgG1 antibodies. Interestingly, Tfc cells and Tfh cells are equally potent during this process, which is twice as efficient as Tc cells (44, 62). Concerning mechanism, Tfc cells facilitate antibody production from B cells *via* IL-21 and CD40L secretions (62). Moreover, *in vivo* experiment showed that after using anti-CD8 antibodies in IL-2 deficient mice, Tfc cells were eliminated, leading to a dramatic reduction of IgG1 production from GC B cells. Interestingly, Tfc cells have the potential to provide a helper-like function alone, and they also act synergistically with Tfh cells to enhance B cells differentiation, immunoglobulins production and specific class switching (42, 62). Compared to the Tfh and B cell co-culture alone, IgG1 and IgG2b secretions from B cells increase significantly when Tfc cells are added (42). Moreover, in humoral immunity, IFN-γ secreted by Tfc cells is delivered locally to B cells that localize in the T-B border and promote B cell antibody class-switching to IgG2c, and finally participate in antiviral responses (65). TCR, CD40L and ICOS are expressed on Tfc cells, and MHCI, CD40 and ICOSL are present on B cells (62, 66). It is therefore hypothesized that Tfc cells interact with B cells through the CD40L-CD40 and ICOSL-ICOS crosstalk (42). Additionally, it has been observed that in autoimmune diseases, Tfc cells assist B cells to produce IgG and IgE *via* TCR-MHCI and CD40L-CD40 interactions (2, 67).

Tfc cells perform their regulate function use either cytokines or cell-cell contact manner. Tfc cells suppress Tfh cell helper function and antibody response through ICOSL/ICOS, CD122/TIGHT and TCR/Qa-1, and inhibit antibody responses to sustain self-maintenance and suppress immunity (63). Moreover, Tfc cells selectively inhibit Tfh cells in a perforin-dependent and IL-10-independent manner, and IL-21 secreted by Tfc cells can also promote the perforin-dependent Tfh cell killing (63). In addition, Tfc cells also secrete IFN-γ, TNF-α, and granzymes A/B/K to inhibit Tfh-dependent plasma blast cell differentiation (15).

7 Clinical relevance of Tfc cells

7.1 Secondary immunodeficiency diseases

Simian immunodeficiency virus (SIV) and human acquired immunodeficiency virus (HIV) are pathogens for secondary immunodeficiency diseases in primates. Tfc cells are only observed in monkeys infected with acquired SIV, but not in the individuals that infected with SIV naturally (61). During pathogenic SIV infection, inflammatory cells infiltration and immune activation in and outside follicles are the main reasons for Tfc cell mobilization and accumulation (61). Compared to the rhesus monkeys which are under progressive stage of SIV infection, the SIV-specific Tfc cells accumulate in "SIV elite controller" monkeys, and they eliminate the infected Tfh cells in GCs efficiently. Meanwhile, in "elite controllers" the frequency of Tfc cells is negatively correlated with the peripheral viremia titer (6). The cytotoxicity of Tfc cells is mild, and their granzymes A, B, and K secretion levels are lower than those of Tc cells. In addition, Tfc cells up-regulate the expression of anti-apoptotic gene Bcl-2, which helps them to survive for a long term during SIV infection (6).

Increasing number of Tfc cells are also observed in the LNs from untreated HIV-infected patients (4, 59), and the frequency of HIV-specific Tfc cells is negatively correlated with peripheral viremia load (68). Sustained immune activation mediated by local inflammation is the main reason for HIV-specific Tfc cells expansion, while the number of Tfc cells is not related to local

TABLE 2 Tfc cell functions and their settings.

Reference	Tfc definition	Function	WT/KO	Disease	Setting *in vitro*	Setting *in vivo*	Function
(58)	$CD3^+CXCR5^+CD45RA^-CD8^+$ T	Tfc cells secrete higher levels of IFN-g, IL-2, TNF and IL-10 than non-Tfc $CD8^+$ T cells.	–	non-small cell lung cancer	Human tumor Infiltrating Tfc cells	–	cytotoxic
(59)	$CCR7^{lo}CXCR5^{hi}CD27^{hi/lo}CD45RO^{hi}$ $CD8^+$ T	Tfc cells show good cytolytic potential characterized by high expression of granzyme B and perforin. Tfc cells with potent cytolytic activity are recruited to GCs during HIV infection and kill HIV infected cells.	-	HIV	Tfc cells from LN of HIV+ humans	-	cytotoxic
(60)	SIV-specific $CD8^+$ T cell	SIV-specific $CD8^+$ T cells restrict productive SIV infection to Tfh cells in elite controller monkeys.	–	SIV	–	SIV elite controllers/ SIV typical progressors in rhesus monkey model	cytotoxic
(4)	$CXCR5^+CD200^+ICOS^+PD\text{-}1^+Tcf1^+Tim3^-CD8^+$ T	Tfc cells control viral infection in Tfh cells; the frequency of LCMV-infected Tfh cells in mice that have received $Cxcr5^{-/-}$ P14 cells is about twofold higher than that in mice receiving $Cxcr5^{+/+}$ P14 cells. Tfc cells control viral infection in B cells; the frequency of MuHV-4-infected B cells is about 4.5-fold higher in mice that have received Tc cells than in mice that have received Tfc cells.	-	HIV, LCMV, murid herpesvirus 4	-	LCMV-infected mice; MuHV-4 infected mice	cytotoxic
(3)	$CXCR5^+CD44^{hi}ICOSL^-CD8^+$ T	Tfc cells are less exhausted than $CXCR5^-CD8^+$ T cells and control viral load during chronic	$Cd4^{Cre}$ transgenic, μMT and C57BL/6J	Acute and chronic LCMV infection	–	$CXCR5^+CD44^{hi}CD8^+$ T cells were adoptively transferred into Cl13-infected $CD4^+$ T-cell-depleted	cytotoxic

(Continued)

TABLE 2 Continued

Reference	Tfc definition	Function	WT/KO	Disease	Setting *in vitro*	Setting *in vivo*	Function
		infection. Upon stimulation with the indicated peptides, the IFN-γand TNF-αproductions from Tfc cells are higher than $CXCR5^{-}CD8^{+}$ T cells.				recipients after LCMV infection.	
(61)	$CCR7^{lo}CD95^{hi}CXCR5^{hi}CXCR3^{hi}CD8^{+}T$	Tfc cells have cytolytic potential and can be redirected to target and kill HIV-infected cells.	-	chronic SIV	Tfc cells from chronically SIV-infected rhesus macaques	chronically SIV-infected rhesus macaques	cytotoxic
(62)	$CXCR5^{+}PD\text{-}1^{+}ICOS^{+}CD40L^{+}CD45RO^{+}CD27^{+}CCR7^{-}CD62L^{-}CD8^{+}$ T	Tfc cells express ICOS and CD40L, which interact with their corresponding ligands on B cells, and secrete IL-21, which could help B cells in the GC for Ig production. Most of Tfc cells in tonsils are effector or central memory cells. Tfc cells expressed higher level of granzyme B than $CXCR5^{-}$ $CD8^{+}$ T cells in tonsils and lymph nodes but not in PBMCs.	–	colorectal cancer, HIV	Human tonsils and lymph nodes tumor infiltrating Tfc cells		cytotoxic, memory
(6)	$CXCR5^{+}$ SIV-specific $CD8^{+}$ T	Tfc cells contribute to control of chronic SIV replication; Rapid expansion of $CXCR5^{+}$ SIV-specific CD8 T cells is associated with enhanced control of chronic SIV infection.	-	chronic SIV infection	Tfc cells from LNs and blood of DNA/MVA vaccinated SIV-infected rhesus monkeys	-	cytotoxic, memory
(7)	$CXCR5^{+}PD\text{-}1^{+}Tcf1^{+}CD8^{+}$ T	Tfc cells are memory-like T cells with low expression of	–	MM, CLL, DLBCL, FL	Human Tfc cells from PB and LN		cytotoxic, memory

(Continued)

TABLE 2 Continued

Reference	Tfc definition	Function	WT/KO	Disease	Setting *in vitro*	Setting *in vivo*	Function
		effector molecules, that adequately produce effector cytokines and differentiate into effector cells upon stimulation.					
(5)	$CXCR5^+Tim\text{-}3^-PD\text{-}1^+ICOS^+CD28^+OX40^+CD8^+T$	Tfc cells resemble stem cells during chronic LCMV infection, undergoing self-renewal and also differentiating into the terminally exhausted $CD8^+$ T cells. Tfc cells selectively proliferate after PD-1 blockade.	-	LCMV	Tfc cells from spleens of LCMV infected C57BL/6 mice	Tfc cells from $CD45.2^+$ LCMV chronically infected mice are adoptively transferred into naive $CD45.1^+$ recipient mice	memory
(27)	$TCF1^{high}Tim3^{low}Blimp1^{high}CD8^+$ T	Tfc cell is a less exhausted T cell population in chronic viral infection and cancer. Like stem cells, they can either maintain their phenotype or differentiate into terminally differentiated $Tim3^{high}$ $TCF1^{low}$ cells to maintain persistence of T cell responses.	$Tcf7^{loxP/loxP}$; CD4-Cre (cKO), Blimp1-YFP, P14 and Ifnar1 KO P14			TCR transgenic mice recognizing LCMV	memory
(1)	$CXCR5^+CD27^+CD28^+CD45RO^+CD69^+CD7^{low}CD8^+$ T	Tfc cells express CD27, CD28, CD45RO, CD69, and are $CD7^{low}$, and produce IFN-γ and granzyme A but lack perforin, suggesting that these cells are early effector memory T cells. CD70, OX40 and ICOS are induced upon activation, and Tfc cells could secrete IFN-γ, TNF-α and IL-2.	-	-	Peripheral blood and tonsil Tfc cells from healthy human		memory, assistance

(Continued)

TABLE 2 Continued

Reference	Tfc definition	Function	WT/KO	Disease	Setting *in vitro*	Setting *in vivo*	Function
		Tfc cells support survival and IgG production in tonsil B cells.					
(21)	$CXCR5^{+}IFN\gamma^{+}PD\text{-}1^{-}CXCR3^{-}CD8^{+}T_{ab\text{-}supp}$ cells	Tfc cells play an antibody-suppressor role. Tfc cells inhibit IL-4 expression by allo-specific $CD4^{+}$ T cells, and kill allo-primed IgG^{+} B cells directly. As a result, this process improves survival of transplanted hepatocytes after transplantation.	–			C57BL/6 mice transplanted with FVB/N hepatocytes	regulation
(2)	$CXCR5^{+}$ PD-1^{+} $CD40L^{+}$ $CD8^{+}$ T	Tfc cell require CD40L/CD40 and TCR/MHCI interactions to deliver help to B cells. Tfc cells contribute to the breakdown of B-cell tolerance. Tfc cells regulate the GC-B cell response and control autoantibody production.				$Stat5^{fl/-}$ $CD8^{Cre/YFP}$ mice	regulation
(63)	$CD44^{+}ICOSL^{+}CXCR5^{+}GITR^{+}Foxp3^{-}CD8^{+}$ Treg cells	Tfc cells reduce the numbers of Qa-1 WT donor $BTLA^{+}$ OT-II cells to control the adoptive response. Interaction between Tfc cells and Qa-1^{+} Tfh cells inhibits production of both high affinity antibody and autoantibody.	B6. Qa-1 (WT) or B6.Qa-1 (D227K) mice infected with LCMV. naïve WT B6 mice, $Rag2^{-/-}$ mice	SLE-like autoimmune diseases	Tfc cells collected from WT OT-II or B6. Qa-1 OT II mice	B6.Qa-1(WT) and B6.Qa-1(D227K) mice; naïve WT B6 mice, $Rag2^{-/-}$ mice;	regulation
(15)	$CD3^{+}CXCR5^{+}CD45RA^{-}CD8^{+}T$	Tfc cells exhibit high cytotoxic activity, increased expression of IFN-γ, TNF-α, and granzymes A/B/K, and displayed	-	follicular lymphoma	Human Tfc cells	EG7-OVA lymphoma mouse model	regulation, cytotoxic

(Continued)

TABLE 2 Continued

Reference	Tfc definition	Function	WT/KO	Disease	Setting *in vitro*	Setting *in vivo*	Function
		antitumor efficacy *in vitro* against human follicular lymphoma cells. Tfc cells inhibit Tfh-dependent plasma blast cell differentiation.					
(42)	$CXCR5^+$ $PD\text{-}1^{high}$ $CD40L^+$ $CD8^+$ T	Tfc cells promote B cell antibody class-switch in autoimmune disease. Allo-primed Tfc cells kill self $IgG1^+$ B cells. Tfc cells express B cell costimulatory proteins, and promote B cell differentiation and Ab isotype class switching. CD8 T cells facilitate enhanced B cell expansion and Ab production in $IL\text{-}2^-$ KO mice.	–	autoimmune disease		BALB/c IL-2–KO mice, scurfy and MRL/MpJ-FASlpr mice	regulation, cytotoxic
(64)	IFN-$\gamma^+$$CD40L^+$$perforin^-$$CD8^+$ T	Tfc cells regulate the structural integrity and functional activity of GCs in ectopic lymphoid follicles. In the absence of CD8 T cells, follicular dendritic cells disappear, production of lymphotoxin-α1β2 markedly decrease, and immunoglobulin secretion cease.	NOD.CB17-Prkdc scid/J mice (NOD-SCID) treated with anti-CD8 mAb	rheumatoid arthritis	Human Tfc cells from synovial tissue samples of rheumatoid arthritis	human synovium-SCID mouse chimeras	assistance
(65)	$CXCR5^+CD8^+$ T	$CXCR5^+CD8^+$ T cells arise in response to protein immunization and peripheral viral infection, displaying a follicular-homing phenotype, expression of cell		Influenza A			assistance

(Continued)

TABLE 2 Continued

Reference	Tfc definition	Function	WT/KO	Disease	Setting *in vitro*	Setting *in vivo*	Function
		surface molecules associated with Tfh cells and limited cytotoxic potential. $CXCR5^+$ $CD8^+$ T cells shape the antibody response to protein immunization and peripheral viral infection, promoting class switching to IgG2c in responding B cells.					
(44)	$CXCR5^+ICOS^+$ $CD8^+$ T	Tfc cells have deficient cytotoxicity, low IFN-γ secretion, and produce IL-4, IL-21, CXCL13. Tfc cells are capable of supporting B cell responses *in vitro*. Coculture of B cells with Tfc cells induced a twofold increase in IgG production when compared with $CXCR5^-ICOS^-$ $CD8^+$ T cells. ICOS is a surface marker for Tfc cell interaction with B cell. Tfc cells may be related to an unusual, CD8-mediated, antitumor reaction, mainly acting in particular cHL.		classic HL, CLL, DLBCL, FL, marginal zone lymphoma, mantle cell lymphoma,	Tfc cells from human classic HL, CLL, DLBCL, FL, marginal zone lymphoma, mantle cell lymphoma samples		assistance

Tfc: follicular cytotoxic T; SIV, simian immunodeficiency virus; HIV, human immunodeficiency virus; LCMV, lymphocytic choriomeningitis virus; MuHV, B cell-tropic herpesvirus; SLE, systemic lupus erythematosus; MM, multiple myeloma; HL, hodgkin lymphomas; CLL, chronic lymphocytic leukemia; DLBCL, diffuse large B-cell lymphoma; FL, follicular lymphoma; Gzmb, granzyme B, GC, germinal center.

viral replication directly (59, 61). Cytokine productions of Tfc cells are severe impaired in HIV-infected patients, even if they highly express Gzmb and perforin, and have good cytolytic potential (3, 4, 15, 59, 61). In addition, Reuter et al. observed that in HIV patients, $CD8^+$ Tc cells lose their cytotoxic capacity, while non-cytolytic Tfc cells may be responsible for the control of HIV replication. Therefore, the failure of HIV clearance may be related to the weak non-cytolytic response of Tfc cells (68).

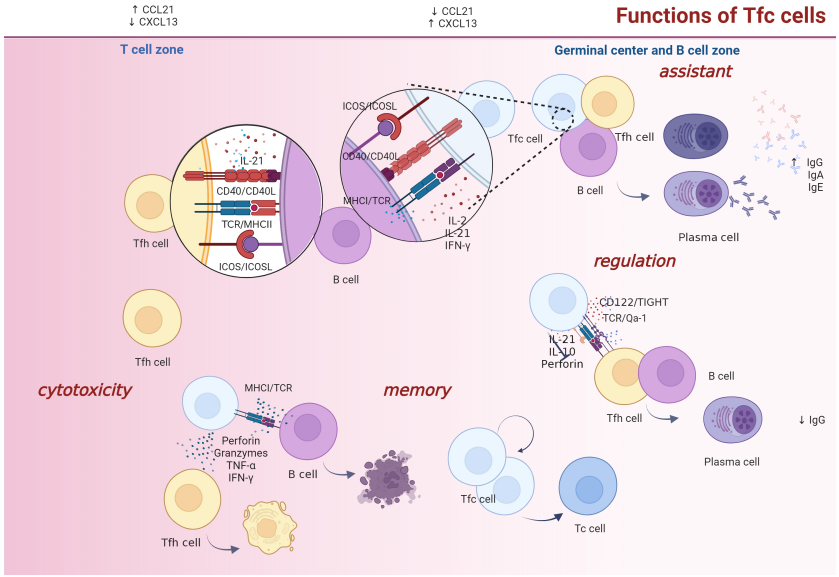

FIGURE 3
Tfc/Tfh/B cell interactions. Tfc cell functions are categorized as cytotoxic, memory, assistance, and regulatory. Firstly, Tfc cells perform their killing functions mainly through their cytokine secretion. They secrete IFN-γ, TNF-α, granzymes and perforin to eliminate infected Tfh and B cells. Secondly, under chronic infection settings, Tfc cells serve as a CD8+ T cell storage pool, which maintain stemness and differentiate into effector CD8+ T cells. Thirdly, Tfc cells help B cells produce antibodies and antibody isotype class switching *via* cytokines IL-2, IL-21, IFN-γ or with the help of CD40L/CD40, ICOSL/ICOS, and TCR/MHC I interactions. Fourthly, under a perforin-dependent and IL-10-independent condition, Tfc cells suppress Tfh cell helper function and antibody response through ICOSL/ICOS, CD122/TIGHT and TCR/Qa-1, and inhibit antibody responses to sustain self-maintenance and suppress immunity. Moreover, the IFN-γ, TNF-α, and granzymes A/B/K expression of Tfc cells increase, inhibit Tfh cell function in a cell-cell contact independent manner, and inhibit Tfh-dependent plasma blast cell differentiation indirectly. FDC, follicular dendritic cell; Tfh, follicular helper; Tc, cytotoxic T; Tfc, follicular cytotoxic T.

7.2 B cell infection and malignant B lymphoproliferative diseases

EBV activation often occurs in immunodeficiency patients, and may leads to a variety of diseases ranging from non-malignant diseases such as infectious mononucleosis to malignancies such as lymphomas. EBV-specific Tfc cells can be detected in tonsils from patients who are currently or previously infected with EBV to control the EBV-infected B cells (7). Similar to the response in humans, on the 15th day after B-cell herpesvirus MuHV-4 (murine herpesvirus 4) infection, a significant accumulation of Tfc cells was observed in the mediastinal LNs. Comparatively, in infected mice infused with Tc cells, the frequency of MuHV-4 infected B cells was 4.5 times higher than that in mice infused with Tfc cells (4).

Interactions between immune cells potentially regulate the occurrence and development of B cell malignancies. Hofland et al. (7) observed that Tfc cells are enriched in B cell follicles of HL, non-Hodgkin's lymphoma (NHL), follicular lymphoma (FL), multiple myeloma and chronic lymphocytic leukemia patients. They may exert antitumor activity through Tfh cell suppression in a cell-cell contact manner and dose-dependent inhibition of plasma blast cell differentiation. In addition, in Tfc cell-FL cell line co-culture, CD107a expression was found to be associated with cell degranulation and tumor killing ability of Tfc cells (15).

Interestingly, Tfc cells might assist tumor proliferation in certain conditions. The ICOS-ICOSL and IL-21-IL-21R crosstalk between Tfc and R-S cells are suspected to be promotive for malignancy development. In addition, Tfc cells can be observed in tissue samples with more activation-induced cytidine deaminase(AID)$^+$ B cells, which are prone to transform into spontaneous B-cell lymphoma cells (44, 69).

7.3 Other tumors

Tfc cells have been studied in various solid tumors such as melanoma, non-small cell lung cancer (NSCLC), pancreatic cancer, colorectal cancer and hepatocellular carcinoma (7, 58, 70, 71). Tfc cells are enriched in the TLS of tumors, but it is unknown whether they are involved in the TLS formation (59, 72, 73) In NSCLC patients Tfc cells are found both in the tumor and in the peripheral blood. When Tfc cells sorted from NSCLC patients' tumor and blood were activated with anti-CD3/CD28, their CD107a expressions were higher than those expressed by their CXCR5$^-$ counterparts (58). It is therefore suspected that Tfc cells may play a tumor-suppressive role. In addition, Tfc cells

have been observed to infiltrate the tumor microenvironment of pancreatic cancer, and the frequencies of tumor-infiltrated and peripheral blood Tfc cells are positively correlated to the disease-free survival of pancreatic cancer patients. Similarly in colorectal cancer, higher frequency of Tfc cells in tumor-draining lymph nodes is related to a better prognosis of patients (74). Meanwhile, these Tfc cells express higher levels of effector genes and lower levels of genes related to cell exhaustion. Besides, the CD40L expressions on Tfc cells are positively related to the disease stage, and the levels of gzmb and perforin productions by Tfc cells decrease with disease progression (71). The frequency of Tfc cells in the peripheral blood of HBV-related hepatocellular carcinoma patients are obviously higher than that in healthy controls. Meanwhile, the Tfc cell frequency is negatively related to the HBV load and alanine aminotransferase level in these patients (75). From studies mentioned above, Tfc cells have potential anti-tumor capacity, while this effect might be affected largely by the tumor microenvironment and attenuated during disease progression.

7.4 Autoimmune diseases

In autoimmune diseases, Tfc cells promote B cell antibody class switching and antibody production directly, or through Tfh cells enhancement *via* cytokines indirectly (42, 62). Tfc cells assist GC-B cell tolerance and autoantibody production through CD40L/CD40 and TCR/MHCI interactions (2). Deficiency of Stat5 leads to an increase of Tfc cells, resulting in the breakdown of B cell tolerance and concomitant autoantibody production (2). In IL-2 knockout mouse model which manifests as autoimmune hemolytic anemia, Tfc cells were found to cooperate with Tfh cells to promote B cell proliferation and antibody production. When Tfc cells were depleted, the autoimmune feature of these mice mitigated and the survival prolonged due to B cell frequency reduction and decreased anti-RBC antibody production (42). Notably, affected by a variety of cytokines released during autoimmune responses including IL-21, IL-4 and IFN-γ, Tfc cells produce IL-21, which furtherly induces plasma cell differentiation and antibody class switching, constructing a positive feedback loop in autoimmune diseases (42).

7.5 Rejection after transplantation

The donor-specific antibodies (DSA) generation and DSA-mediated organ rejection are big challenges for organ transplantation (76, 77). Donor MHC molecules present on extracellular vesicles are recognized by alloreactive B cells, which differentiate under the help of Tfh cells to generate DSA (78). In the past decades, rejection after transplantation was regulated by T cell "depletion" or Tfh cells inhibition, however the side effects of these treatments could be severe (57, 79, 80). Zimmerer et al. (21) found that Tfc cells in hepatocyte transplant mice were located in GCs, which down-regulated the frequency of B and Tfh cells, and inhibited Tfh cell auxiliary function, reduced the generation of DSA, and finally improved the long-term survival of the graft. These Ag-specific, IFN-γ-dependent and self-MHC class I-restricted Tfc cells in the post-rejection condition up-regulated CXCR5 and down-regulated Foxp3. In addition, they may eliminate the B cells through perforin- and FasL-dependent manner.

8 Treatment prospects

8.1 Anti PD-1 therapy

Immune checkpoint blockade (ICB) is an effective treating method to maintain effective anti-tumor response by blocking inhibitory receptors on effector T cells. Thus, the emerging therapy targeting on PD-1 may restore the Tfc cell function (81). After PD-1 inhibitory pathway blockade in chronic LCMV infection mice, Tfc cells significantly proliferate and differentiate towards CXCR5$^-$ CD8$^+$ T cells (5). Interestingly, during chronic HIV infection, the PD-1 expression level may represent the anti-viral capacity of Tfc cells, and PD-1 inhibition decreases IFN-γ and TNF-α productions from HIV-specific Tfc cells (18). Finally, PD-1 ICB might exert anti-tumor efficacy through Tfc-modulation since PD-1 is related to Tfc cell function and localization, while the exact effects and mechanism remain to be elucidated.

8.2 Genetic engineering and adoptive therapy

To control viral infection effectively, researchers have tried to use Tfc cell adoptive therapy and T cell genetic engineering to increase their abundance in follicles. In Leong et al. study, Tc cells and Tfc cells were selected from chronic LCMV-infected mice, and infused into chronic LCMV-infected mice respectively. Tfc cells were found to amplify in B cell follicles efficiently, and exert stronger cytotoxic effect than Tc cells (4, 44). Ayala et al. (82) expressed CCR7 and CD62L on the surface of SIV-specific T cells through genetic engineering and infused then into rhesus monkeys. These modified T cells preferentially located in the LNs. In addition, they amplified CD8$^+$ T cells with CXCR5 expression *in vitro* and infused them into rhesus monkey, and these cells were observed to enter B cell follicles effectively. They also used human CXCR5 murine leukemia virus (MuLV)-based retroviral expression vector to insert *CXCR5* gene to CD8$^+$ T cells, and the expression of PD-1 was downregulated, that could be associated with the homing of remodeled-Tfc cells into B cell follicles (22). As for the potential

therapeutic modalities in antibody mediated disease, Zimmerer et al. (83) found that after receiving adoptive therapy with alloprimed CXCR5$^+$ CD8$^+$ T cells, the alloantibody titer in kidney transplant mice was reduced, which ameliorated antibody-mediated rejection and prolonged allograft survival.

8.3 The application of IL-15 super agonist

IL-15 is a regulator of T cell homeostasis. Topical IL-15 super agonist ALT-803 increase the Tfc cells abundance in B cell follicles to eliminate chronic viral pathogens. ALT-803 up-regulates CXCR5 expression on Tfc cells precisely, and finally help them to localized in secondary lymphoid tissues (84). Apart from IL-15 super agonist, more stimulators are expected to enhance the function of Tfc cells.

9 Conclusion and perspectives

Tfc cells are an important immune cell subset with multifaceted functions in humoral immunity. There are similarities among Tfc cells and other cell types such as Tfh, Tfr or the newly identified 'stem-like' CD8$^+$ T cells, bringing inconsistencies in identification of these cells among literatures. Deeper understanding on disparities of phenotype, transcription factors, secreted cytokines and functions between Tfc cells and other relevant T cells is needed to alleviate confusion in the field. Tfc cells eliminate infected Tfh/B cells, promote Ig secretion and regulate the B cell antibody class switch. In addition, Tfc cells have self-renewal ability and can convert to CXCR5$^-$ CD8$^+$ T cells in specific conditions such as chronic infection. Moreover, Tfc cells' interactions with Tfh and B cells are complex and context-dependent. Further in-depth studies on Tfc cell transcriptome and metabolome, as well as spatial transcriptome studies on GCs/TLSs may help us further understand the whole picture of Tfc cell intrinsic signaling and their interconnections with other cells. With our deeper understanding of Tfc cell biology and more pre-clinical studies conducted in typical disease mouse models, innovative targeted therapies using or killing Tfc cells may further enlarge our arsenal towards cancer or immune-mediated diseases.

Author contributions

YY and HH contributed to the conception of this review. YL, LR and YY prepared the manuscript. HH and BG provided expert comments. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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CLIC-01: Manufacture and distribution of non-cryopreserved CAR-T cells for patients with CD19 positive hematologic malignancies

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Access to commercial CD19 CAR-T cells remains limited even in wealthy countries like Canada due to clinical, logistical, and financial barriers related to centrally manufactured products. We created a non-commercial academic platform for end-to-end manufacturing of CAR-T cells within Canada's

publicly funded healthcare system. We report initial results from a single-arm, open-label study to determine the safety and efficacy of in-house manufactured CD19 CAR-T cells (entitled CLIC-1901) in participants with relapsed/refractory CD19 positive hematologic malignancies. Using a GMP compliant semi-automated, closed process on the Miltenyi Prodigy, T cells were transduced with lentiviral vector bearing a 4-1BB anti-CD19 CAR transgene and expanded. Participants underwent lymphodepletion with fludarabine and cyclophosphamide, followed by infusion of non-cryopreserved CAR-T cells. Thirty participants with non-Hodgkin's lymphoma (n=25) or acute lymphoblastic leukemia (n=5) were infused with CLIC-1901: 21 males (70%), median age 66 (range 18-75). Time from enrollment to CLIC-1901 infusion was a median of 20 days (range 15-48). The median CLIC-1901 dose infused was 2.3 × 10^6 CAR-T cells/kg (range 0.13-3.6 × 10^6/kg). Toxicity included ≥ grade 3 cytokine release syndrome (n=2) and neurotoxicity (n=1). Median follow-up was 6.5 months. Overall response rate at day 28 was 76.7%. Median progression-free and overall survival was 6 months (95%CI 3-not estimable) and 11 months (95% 6.6-not estimable), respectively. This is the first trial of in-house manufactured CAR-T cells in Canada and demonstrates that administering fresh CLIC-1901 product is fast, safe, and efficacious. Our experience may provide helpful guidance for other jurisdictions seeking to create feasible and sustainable CAR-T cell programs in research-oriented yet resource-constrained settings.

Clinical trial registration: https://clinicaltrials.gov/ct2/show/NCT03765177, identifier NCT03765177.

KEYWORDS

CAR-T cells, hematologic malignancies, immune effector cells, point-of-care manufacturing, prodigy, in-house manufacturing, lymphoma, leukaemia

Introduction

Chimeric Antigen Receptor (CAR)-T cells are a powerful tool for treating cancer. CAR-T cells are genetically modified T cells that are programmed to target a specific cell surface antigen. The use of CAR-T cells has been explored in clinical trials for various cancers, mainly hematologic malignancies such as B-cell acute lymphoblastic leukemia (1) and non-Hodgkin's lymphoma (2–4), where the pooled complete response rate is 56% (95% CI: 44%, 67%; I2 69%) (5). This degree of efficacy is remarkable given that most of these patients only had palliative options prior to CAR-T therapy and has prompted rapid regulatory approval of CD19 CAR-T cell interventions for these cancers worldwide (6–8).

Despite this promise, CAR-T cell production and distribution is complex and operationally challenging, which hampers access to these lifesaving therapies. At the time of writing, Canadian cancer patients have highly variable access to CAR-T cell therapy depending on jurisdiction. While most CAR-T products are currently manufactured in centralized facilities, academic CAR-T studies have explored various approaches to in-house manufacturing and delivery of CAR-T cells, and in clinical trials these efforts have yielded high quality products with safety and efficacy profiles that compare favourably to commercially available CAR-T cell products (9–14). This paradigm has been greatly facilitated by closed benchtop systems that make manufacturing within the hospital setting more feasible. Miltenyi's CliniMACS Prodigy is one such closed system in which the patient's T cells, CAR-encoding virus, cytokines and T-cell growth media are all manipulated on the instrument within a single-use closed tubing set. The Prodigy has a relatively small footprint, allowing multiple instruments to be operated in a single facility. Notable advantages to in-house CAR-T manufacturing include opportunities to actively manage production capacity, pursue continuous process improvement, minimize the cost of these expensive interventions (15), and ultimately improve their value for money.

Our research consortium was established to test the feasibility of providing high quality, cost effective, in-house manufactured CD19 CAR-T cells within Canada's financially strained healthcare system. We designed, manufactured, and functionally verified a "2nd generation" 4-1BB-containing CD19 CAR construct and lentiviral vector system to enable CAR-T manufacturing using the Miltenyi Prodigy platform. All aspects of vector and CAR-T manufacturing, clinical trial design, patient enrollment and treatment, clinical trial monitoring, and regulatory filings were undertaken by our academic team.

Here we report interim results from CLIC-01, a single-arm, open-label phase I/II study (NCT03765177) to evaluate the safety and efficacy of our CLIC-1901 non-cryopreserved CAR-T cell product in participants with CD19 positive hematologic malignancies. We successfully implemented this trial at two Canadian hospitals on opposite sides of the country, with cell manufacturing at a third site. We leveraged hematopoietic stem cell transplantation protocols already available in Canada to successfully transport fresh apheresis and final CAR-T products, with no delays or interruptions despite our large geography. In fact, the time sensitivity of transporting rapidly expiring cell products over large distances imparted a sense of urgency to this study, which benefitted patients by removing any possibility of delay when treating their rapidly progressing malignancies. This clinical trial represents an important step towards enhancing CAR-T cell innovation and equitable access for Canadian cancer patients and may offer helpful guidance to other jurisdictions seeking to incorporate this transformative form of cancer treatment in resource-constrained settings.

Methods

Study protocol

The protocol and amendments were reviewed by Health Canada prior to implementation and approved by the institutional review boards at both study sites, Vancouver General Hospital (VGH) and The Ottawa Hospital (TOH). Participants aged 18 years or older were eligible if they had relapsed or refractory CD19 positive disease including acute lymphoblastic leukemia (ALL), chronic lymphocytic leukemia (CLL) or histologically confirmed B-cell non-Hodgkin's lymphoma (NHL). Relapsed or refractory disease was defined by one of the following: a) second or greater relapse, b) any relapse after autologous or allogeneic stem cell transplantation, or c) chemorefractory as defined by not achieving complete remission after 2 cycles of a standard induction chemotherapy or 1 cycle of salvage therapy. All eligible participants had to have documentation of CD19 tumour expression demonstrated in tissue biopsy, bone marrow or peripheral blood within 6 months prior to study screening, as well as adequate organ function defined as: creatinine clearance > 30 mL/min, ALT/AST < 3X upper limit of normal (ULN), and bilirubin < 2X ULN. Exclusion criteria included: isolated extra-medullary disease, concomitant genetic syndrome (such as Fanconi anemia, or any other known familial bone marrow failure syndrome), malignancy in the last 5 years or concurrent active malignancy (with the exception of non-melanomatous skin cancer), prior treatment with any gene therapy product, PCR positive hepatitis B, C or HIV, any uncontrolled infection, active graft-versus-host disease requiring systemic therapy, allogeneic stem cell transplant less than 6 months prior to CLIC-1901 cell infusion or donor lymphocyte infusion less than 6 weeks prior to CLIC-1901 cell infusion, active Central Nervous System (CNS) involvement by malignancy, history of anaphylaxis to gentamicin or its derivatives, or participants receiving an investigational agent within the 30 days prior to enrolment.

Lentivector production

The Supplementary Material provides detailed methods for lentivector and CAR-T manufacturing (S1). The CLIC-1901 CAR construct is a second-generation CAR as described by Imai et al, 2004 (16), with modifications to the leader and linker sequences, as described by Kochenderfer et al. (17). In brief, the CAR consists of a GM-CSF receptor alpha (GM-CSFRα) signal peptide, the scFv fragment derived from the FMC63 mouse monoclonal antibody, the CD8α-derived hinge and transmembrane region, a 4-1BB co-stimulatory domain, and a CD3ζ signaling domain. We created a three plasmid self-inactivating lentivector system (18, 19) comprising a packaging plasmid encoding gag, pol and rev, a VSVG envelope protein expressing plasmid (VSV-G), and a transfer plasmid encoding the CAR transgene. The design incorporates standard biosafety features (20). Synthetic transgene sequences encoding the CLIC-1901 CAR were manufactured, subcloned into the transfer plasmid and verified by Sanger sequencing. Clinical grade plasmid production of all DNA was carried out at BC Cancer Research Institute (Vancouver, Canada) using endotoxin-free Purelink Expi Giga Plasmid Purification Kit (Invitrogen #A31232).

Plasmid DNA was used by the Biotherapeutics Manufacturing Centre (BMC) at Ottawa Hospital Research Institute (OHRI) to create replication-incompetent CD19-CAR lentivirus by transient transfection of a master cell bank of 293T/17 cells (ATCC CRL-11268) and expansion in 36-layer HYPERstacks (Corning). Crude supernatant was clarified, treated with benzonase to remove residual cellular DNA, and concentrated *via* tangential flow filtration (TFF). Diafiltration was performed to formulate the CD19 CAR lentivirus in TexMACS Medium (Miltenyi), further concentrated using high-speed centrifugation, resuspended in TexMACS buffer (Miltenyi) and then aliquoted into 2mL cryovials at 500 µL/vial and stored at -80°C. The following release tests were performed on the final *CD19CAR* lentiviral product: 1) host cell DNA detection by qPCR, 2) residual

benzonase quantitation, 3) residual VSV-G plasmid, E1A, or SV40 detection, 4) endotoxin level, 5) sterility, 6) pH & appearance, 7) identity, and 8) titer assay. Additionally, the replication-competent lentivirus release test was performed on the crude lentivirus harvest, and four release tests (for mycoplasma, 9 CFR Bovine Virus, adventitious viral contaminants, and replication competent lentivirus) were performed on the control cells in spent media. Empirical evaluation of the percentage of CAR-T cells generated using the Miltenyi Prodigy protocol per volume of lentivirus provided indicated that one vial per manufacturing run was favourable in terms of handling and transduction efficiency.

CAR-T cell production

Patients underwent apheresis using a standard MNC collection procedure. Apheresis volume ranged from 219 to 348 mL. Immediately following apheresis collection, the product was packaged in a standard transfer device (Credo cube) and shipped cold (1-10°C), but without cryopreservation, from the apheresis location (Ottawa, Ontario or Vancouver, British Columbia) to the cell processing facility in Victoria, British Columbia. CAR-T cells were manufactured using the Miltenyi CliniMACS Prodigy system installed in a classified Grade D manufacturing suite. The exception to this is that the first four manufacturing runs used apheresis that was shipped at ambient temperature (15-25° C). 'Functionally open' steps (i.e., media preparation and cell manipulation) were performed in a classified Grade A isolator (NuAire). We followed the 'enhanced feeding protocol' version of the pre-installed T cell transduction (TcT) protocol for serum-free cultivation. In-process control samples obtained on day 5 and final product samples obtained on day 12 were subjected to sterility testing using a BacT ALERT system (BioMerieux) and Mycoplasma testing using a MycoTool PCR test. Final samples taken on day 12 were additionally tested by Gram stain and Endotoxin (LAL) testing. Cell counts were obtained by conventional Trypan Blue stain and CAR-T cell content was assessed by staining cells with FITC-conjugated CD19 protein (Acro) followed by analysis on a CytoFlex cytometer (Beckman Coulter). The final product was shipped to the clinical site as a fresh (non-cryopreserved) product using the Credo cube at ambient temperature (15-25°C).

CAR-T cell administration

Participants received lymphodepletion with fludarabine (40 mg/m^2 by IV daily × 3 days on days -4, -3, and -2) and cyclophosphamide (500 mg/m^2 by IV daily × 2 days on day -4 and -3) prior to CLIC-1901 infusion. Patients were treated with a single intravenous infusion of autologous CLIC-1901 cells on day 0 at a minimum dose of 1 × 10^6 CAR positive CLIC-1901 cells per kilogram of body weight (to a maximum of 2 × 10^8 total CLIC-1901 cells). In participants with high disease burden, the minimum dose was reduced to 1 x 10^5 cells/kg (with the respective maximum dose reduced to 2 × 10^7 total CLIC-1901 cells) to reduce the risk of toxicity. High disease burden was defined as more than 20% bone marrow blasts in ALL; or a mediastinal mass greater than 1/3 of the intra-thoracic diameter on PA chest x-ray or any mass ≥ 10 cm on CT or PET scan at time of enrolment in NHL.

Safety and statistical analysis

An independent data safety monitoring board (DSMB) consisted of two physicians with expertise in CAR-T cells and stem cell transplantation, and one statistician. This independent DSMB met after the fourth participant received CLIC-1901 cells and had completed 28 days of follow-up, and every 6 months thereafter to review safety data including unexpected adverse events (AEs) and any deaths. Demographics and baseline characteristics are summarized for all participants. Proportions and 95% confidence intervals are summarized for dichotomous data and means, and standard deviations or medians and interquartile ranges are provided as appropriate for continuous data. Progression-free and overall survival were calculated using the Kaplan-Meier method.

Outcomes

The primary endpoint of the study was the proportion of participants experiencing either grade 3 or 4 cytokine release syndrome (CRS), grade 3 or 4 neurotoxicity, other grade 3 or 4 toxicity (by CTCAE 4.03) or non-relapse related death within the first 28 days after CAR-T infusion. Safety outcomes included the proportion of CRS and neurotoxicity at 28 days from CLIC-1901 infusion. Grading and management of CRS and neurotoxicity was suggested by guidelines available at the time of protocol development (21). Overall response was defined as the sum of complete and partial responses. In ALL, bone marrow biopsies were performed to determine disease response and was defined by NCCN guidelines version 1.2017.42 (22). In NHL, disease assessments were made by PET-CT scan using Lugano criteria (23).

Correlative analysis

Leukapheresis product, CAR-T cell product, and peripheral blood samples were collected, processed and biobanked for correlative analysis, with informed consent under the CLIC-01 clinical trial protocol as approved by local Research Ethics Boards. Samples were assessed for immunophenotype using multi-parameter spectral flow cytometry, for cytokine levels using the Mesoscale platform, and for CAR transgene levels

using quantitative real-time PCR analysis. Please see Supplemental Material (S2) for detailed biobanking and correlative analysis methods.

Results

Patient characteristics

From October 1, 2019, until July 1, 2021, 48 consecutive patients were screened for eligibility, with 35 (73%) enrolled onto the study (Figure 1). Out of 35 participants who underwent cell collection, 30 received lymphodepleting chemotherapy and CLIC-1901 cells. Reasons for not receiving chemotherapy and CLIC-1901 cells included manufacturing failure (n=2), rapid disease progression leading to death within 2 weeks of leukapheresis (myocarditis and multiorgan failure from ALL; airway obstruction from MCL; n=2), and infection/respiratory failure in DLBCL (n=1). Characteristics of the 30 participants who received CLIC-1901 are summarized in Table 1. The majority were male (n=21; 70%), with a median age of 66 (range 18-75). The median number of prior therapies was 3 (range 2-6), including 13 (43%) patients who had relapsed after hematopoietic stem cell transplant (allogeneic (n=5), autologous (n=6), both (n=2)). Most participants had lymphoma (n=25) (DLBCL (n=10), MCL (n=8), DLBCL transformed from indolent lymphoma (n=4), Richter's transformation (n=1), follicular lymphoma (n=1), plasmablastic lymphoma (n=1)), and 5 participants had B-ALL. Details regarding disease risk of the 30 participants who underwent CLIC-1901 infusion are presented in Supplemental Table 1. Eighteen participants had disease that was primary refractory to front-line therapy, while 2 had relapsed disease within 12 months of front-line therapy, and the remaining 8 had disease that relapsed more than 12 months after front-line therapy.

CAR-T cell product characteristics

The default parameters of the 12-day T cell transduction (TcT) enhanced feeding protocol that are pre-installed on the CliniMACS Prodigy by the manufacturer (Miltenyi) were used throughout, with the exception of one modification. In brief, during the pre-clinical development phase, we noted that when using the default CD4/CD8 enrichment protocol, the number of CD4/CD8 T cells that is obtained during enrichment is typically in vast excess of the required number. Thus, we calculated the volume of apheresis product containing 0.9×10^9 T cells and loaded the Prodigy with this volume rather than the full volume as recommended (the Prodigy can accommodate 50-280 mL). This approach allowed the CD4/CD8 cell enrichment phase to be reduced by 1 to 3 hours (only one magnetic column pass was required instead of up to 3). All runs yielded an enriched CD4/CD8 population of sufficient quantity for subsequent steps (range of 1.64 - 11 $\times 10^8$ enriched CD4/CD8 cells). Table 2 provides a summary of starting apheresis products, yield of CD4/CD8 T cells obtained from bulk apheresis during magnetic enrichment, the number of CD4/CD8 T cells used to initiate culture, and the final (day 12) product yields.

For the first 6 participants enrolled on the study, culture was initiated using 5×10^7 CD4/CD8 T cells, a starting number that was based upon the results of 14 pre-clinical development runs using healthy donor apheresis material. However, when this pre-clinical protocol was applied to patient apheresis materials in the

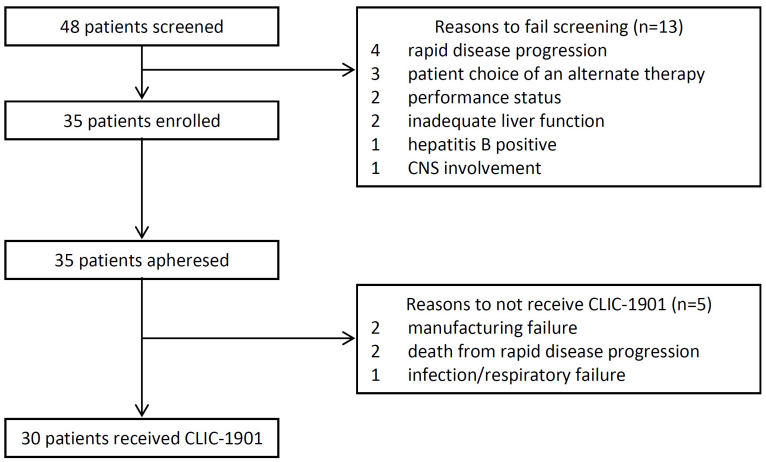

FIGURE 1
Patient Flow Diagram.

TABLE 1 Patient Characteristics for N=30 Infused.

Characteristic	N (%)
Age (median, range)	66 (range 18-75)
Male sex	21 (70)
BMI (median, range)	25.8 (19.7-37.3)
Race	
Caucasian	24 (80)
Asian	5 (16.7)
Did not disclose	1 (3.3)
ECOG	
0	8 (26.7)
1	19 (63.3)
2	3 (10)
Disease	
DLBCL	10 (33.3)
Transformed DLBCL	4 (13.3)
MCL	8 (26.7)
ALL	5 (16.7)
Other	3 (10)
LDH at enrollment (median, range)	245 (134-1145)
Disease Stage (excludes ALL)	
I	2 (6.7)
II	3 (10)
III	8 (26.7)
IV	12 (40)
Number of prior therapies	3 (range 2-6)
Response to front-line therapy	
Primary refractory	18 (60)
Relapse within 12 months	2 (6.7)
Relapse after 12 months	10 (33.3)
Prior transplant	
Allogeneic	5 (16.7)
Autologous	6 (20)
Both	2 (6.7)

trial, 3 of the first 6 runs (50%) had poor expansion, with 2 runs failing to yield sufficient numbers of CAR-T cells for infusion and one run barely meeting the minimum cell dose for high disease burden after 12 days of culture (1.1×10^5 CAR-T cells/kg). We adjusted the protocol to start with 1×10^8 enriched T cells, after which the manufacturing success rate was 100% (n=29), with all products demonstrating excellent *ex vivo* expansion characteristics (Figure 2 left). In addition, one of these failures occurred when the culture was initiated with cells that had been shipped to the manufacturing site at ambient temperature and exhibited low viability in comparison to other starting cells. This prompted a change in the apheresis shipping temperature to cold shipping (1-10°C). These failures, and the subsequent 100% success rate after adjusting these parameters, suggested that seeding density and apheresis shipping temperature may be critical variables in this process. In addition, clinical factors may play a role, although our analysis did not uncover any clear clinical explanations for the manufacturing failures. The mean transduction frequency (% of cells expressing the CD19 CAR) in the final products was 28.9% (range 11 - 65.3%). Interestingly, the product with a transduction frequency of 65.3% represented one of the early manufacturing failures, suggesting that excess virus multiplicity of infection (MOI) may have been a contributing factor when starting with only 5×10^7 enriched T cells. All 30 treated patients received their target dose of CLIC-1901, which was defined as a minimum of 1×10^6 CAR-T cells/kg (up to a maximum of 2×10^8 total CAR-T cells) for standard dose or a minimum of 1×10^5 CAR-T cells/kg (up to a maximum of 2×10^7 CAR-T cells) for patients with high disease burden. The actual yield of CAR-T cells on day 12 was typically in vast excess (Figure 2 right); consequently, the majority of patients (n=28, 93%) were infused with the maximum target dose. The ratio of CD4:CD8 T cells in the final infusion product spanned a broad range (1:10 to 7:1). When comparing CD4:CD8 ratios of cell subsets within each product, the CAR+ fraction consistently contained a higher frequency of CD4+ T cells (47%) compared to CD4+ frequency in the bulk CD3+ population (36%) (paired t-test, $p < 0.0001$). T cell phenotypes for the infusion products are shown in Figure 3. The predominant memory cell phenotype was either central memory (CCR7+ CD45RO+) or effector memory (CCR7- CD45RO+) in each product.

Clinical outcomes

Feasibility

Recruitment opened on Oct 1, 2019, in Ottawa and on Feb 1, 2020, in Vancouver. The first participant was screened for the trial on October 26, 2019. In the first and second year of the trial, 9 and 21 participants were infused with CLIC-1901 respectively. The beginning of the COVID-19 pandemic led to a 3-month pause in recruitment from March to May 2020. The median time from screening to enrolment was 17 days (range 0-98) which accounted for the time from signing consent to obtaining all needed testing for eligibility (bloodwork, PET scan or bone marrow biopsy, echocardiogram, and pulmonary function tests). The median time from screening to CLIC CAR-T infusion was 38 days (range 16-117). The median time from enrolment to apheresis was 4 days (range 0-33) and enrolment to CAR-T infusion was 19 days (range 15-48 days). The median time from leukapheresis to CAR-T infusion was 15 days (range 13-16 days for fresh product, one participant received frozen product at 28 days from leukapheresis). The median time from last disease progression to eligibility was 47 days (range 10-183), and was notably longer for MCL (median 88 days, range 26-183 days). Otherwise, there were no differences in median times described by disease group. In summary, accrual and time from progression to enrollment and CAR-T infusion was deemed feasible and appropriate for this patient population.

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TABLE 2 Apheresis material and CAR-T cell product characteristics.

ID	Total apher.volume	# of MNC in apher.(x10^9)	% CD3 in apher.	Vol. of apher. loaded onto Prodigy	CD4/ 8yield (x10^6)	# of CD4/ 8 used to seed culture (x10^6)	Total # of cells harvested on day 12 (x10^8)	% CAR positive on day 12	Total # of CAR T+ cells harvested on day 12 (x10^8)	Total # of CAR T+ cells infused (x10^8)	Dose of CAR T cells infused (x10^6/kg)
S001	348	10.9	13.1	219.3	660	49.5	47	36.9	17	2	3.5
S002	274	20.2	20.3	60.1	616	46.2	39	30.9	12	NI	NI
S003	291	20.0	60.3	21.7	500	50	–	–	–	MF	MF
S004	294	17.1	51.2	30.2	808	40.4	12	35.8	04	2	2.3
S005*	329	5.1	11.2	280	400	50	0.22	38.5	0.09	0.077	0.11
S006	213	7.8	34.8	70.5	716	53.7	0.38	65.3	0.25	MF	MF
S007	234	11.2	32.5	57.9	1100	113	55	28.9	16	2	2.8
S008	280	18.9	47.8	27.9	852	106.5	17	36.6	6	2	2.6
S009	288	23.9	50.9	21.3	652	97.8	35	28.3	10	2	2.7
S010	252	19	44	27.1	692	103.8	53	21.1	11	2	2.3
S011*	263	22.2	50.3	21.2	612	107.1	37	39.2	14	0.2	0.33
S012	314	17.8	15.9	99.9	564	98.7	53	33.3	18	2	2.5
S013	325	8.1	49	74.1	788	98.5	17	42.7	7	2	2.3
S014	306	29.2	56.1	16.8	536	107.2	52	29.4	15	2	2.4
S015	322	27	71.4	15.0	584	102.2	36	26.1	9	2	3.3
S016	341	13.3	81.4	28.3	820	102.5	41	20.0	8	NI	NI
S017	344	12.3	46.9	53.7	624	93.6	58	21.3	12	2	2.8
S018	278	160.9	10.1	15.4	620	93	58	21.9	13	2	2.2
S019	280	17.6	42.7	33.5	720	108	27	30.1	8	2	2.4
S020	219	9.1	43	50.6	648	97.2	49	20.2	10	2	3.6
S021*	307	20.1	33.1	41.6	628	94.2	25	18.6	5	0.2	0.32
S022	305	5.8	9.2	280.0	372	102.3	19	30.8	6	2	1.9
S023	280	34.4	3.3	220.7	624	93.6	53	24.9	13	NI	NI
S024	318	41.6	52	13.2	616	92.4	17	39.0	7	2	3.5
S025	295	19.6	66	20.5	526	105	41	16.2	7	2	2.1
S026	280	26.2	30.7	31.3	624	93.6	19	11.0	2	1.85	2.8
S027*	328	70.7	44.1	9.5	164	98.4	32	30.0	10	0.2	0.2
S028	280	30	59.4	14.1	508	101.6	33	23.2	8	2	1.7
S029	269	22	66.9	16.5	656	98.4	26	25.0	7	2	3.0
S030	280	23.7	57.1	18.6	696	104	55	32.4	18	2	2.9
S031	279	34.6	49.1	14.8	652	97.8	41	21.2	9	2	2.2
S032*	287	9.9	71.7	36.4	592	103.6	56	22.5	13	0.2	0.35
S033	280	92.5	8.5	32.1	400	100	49	23.3	11	2	1.7
S034	280	9.1	36.9	75.2	544	95.2	48	32.0	15	2	2.1
S035	330	46.5	59.8	10.7	756	94.5	55.4	26.4	15	2	2.6

*Targeted infusion dose reduced 10-fold due to high disease burden (in accordance with clinical protocol). Apher, apheresis.
The 5 gray rows represent participants not infused with CAR-T either for clinical reasons (NI) or for manufacturing failure (MF).

Safety

A total of 7 out of 30 participants (23.3%) had grade 3 or 4 CRS, grade 3 or 4 neurotoxicity, other grade 3 or 4 toxicity or non-relapse related death within the first 28 days after CAR-T infusion (the defined primary endpoint). CRS was observed in 18 participants (60%) at a median onset of 1.5 days after CLIC-1901 infusion (range 0-9 days). Two participants (7%) experienced grade ≥ 3 CRS; of note, these were the only 2 participants on the study to have any grade of neurotoxicity. One participant with primary refractory DLBCL developed CRS grade 3 on day one, and subsequently developed concomitant grade 2 neurotoxicity on day 4, both of which resolved on day 7. This participant had significant bowel involvement of lymphoma and ultimately died on day 28 from a bowel perforation. The other participant had B-ALL and developed grade 1 CRS on day 0 which progressed to grade 3 by day 5 with

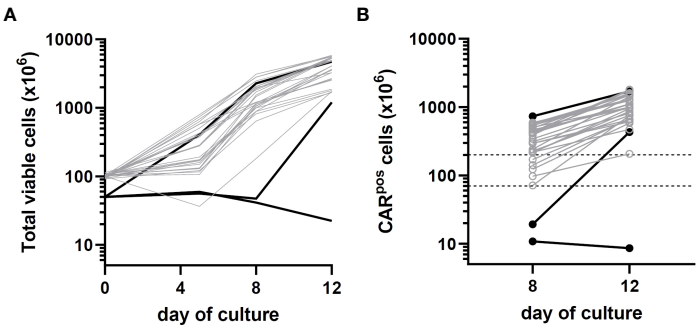

FIGURE 2
Growth kinetics of patient CLIC-1901 CAR-T cell products (n=30) manufactured using the CliniMACS Prodigy platform. CD4/CD8 T cells were enriched from bulk patient apheresis and either 50 million (black lines) or 100 million (grey lines) cells were used to initiate a 12-day culture using serum-free TexMACS medium and the 'enhanced feeding protocol' version of the TcT process. Cells were activated with TransAct immediately upon seeding and were transduced with lentivirus the following day. **(A)** shows the total number of viable cells present (measured on days 0, 5, 8 and 12) and **(B)** shows the number of CAR T-expressing cells present (measured on days 8 and 12). Dotted lines on **(B)** indicate the upper (200 × 10^6) and lower (1 × 10^6/kg; average 70 kg) numbers of CAR-T cells required for standard dosage according to clinical protocol (10-fold lower doses are required for patients with high disease burden).

onset of grade 4 neurotoxicity on day 5 as well. The patient ultimately died on day 9 from CRS and multiorgan failure, without resolution of neurotoxicity. Adverse events occurring in more than one participant per CTCAE version 4.03 are reported in Table 3. Two patients died from non-relapse causes within the first 28 days of receiving CLIC-1901 CAR-T cells. These are the same 2 participants who had high-grade CRS and neurotoxicity as described above.

Persistent cytopenias are a well-known side effect after CD19 CAR-T cell therapy (2, 24). Cytopenias were graded using CTCAE version 4.03. In brief, of the 28 participants who were evaluable at day 28, 2 (7%) had grade 3 anemia, 10 (36%) had severe neutropenia (n=5; grade 3, n=5; grade 4) and 10 (36%) had severe thrombocytopenia (n=2; grade 3, n=8; grade 4). By month 3, of the 20 evaluable patients, zero participants had severe anemia, 5 (25%) participants had severe neutropenia (all grade 3) and 3 (15%) had severe thrombocytopenia (all grade 4). At month 6, of 13 evaluable patients, 2 (15%) had grade 3 neutropenia, 1 (8%) had grade 3 thrombocytopenia and 1 (8%) had grade 4 thrombocytopenia. The participants with ongoing severe neutropenia were not the same as the participants who had severe thrombocytopenia. Two participants had bone marrow biopsies to assess for ongoing cytopenias (one with marrow-based lymphoma and one with B-ALL), one case showed a hypocellular marrow and the other showed a hypercellular marrow, but no evidence of disease or myelodysplasia in either biopsy.

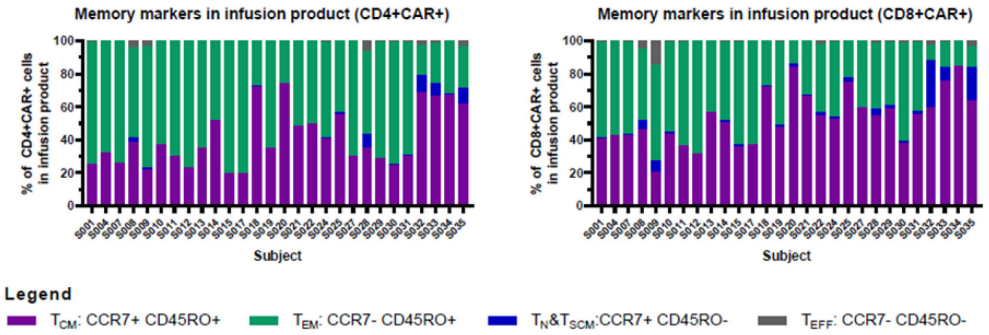

FIGURE 3
Memory markers in infusion product. CD4+ and CD8+ T cells in infusion products consisted mainly of cells with central memory or effector memory phenotypes. Cryopreserved cells were thawed, stained, and analyzed using an Aurora spectral cytometer as described in Materials and Methods. Graphs show the percentage of central memory (CCR7+ CD45RO+), effector memory (CCR7- CD45RO+), naïve/stem cell memory (CCR7+ CD45RO-), and effector (CCR7- CD45RO-) cells within the CAR+CD4+ (left), and CAR+CD8+ (right) T cell populations (gated on CD3+ CD45+ single viable cells).

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TABLE 3 Adverse Event Data to 30 days after CLIC-1901 Infusion.

Adverse Event	Any grade (N patients, %)	Grade ≥ 3 (N patients, %)
CRS	18 (60%)	2 (6.7%)
Neurotoxicity	2 (6.7%)	1 (3.3%)
Vomiting	11	0
Edema	10	2
Anorexia	8	0
Nausea	8	0
Diarrhea	7	0
Headache	6	0
Abdominal pain	5	1
Fatigue	5	0
Febrile Neutropenia	5	4
Pain	5	0
Rash maculo-papular	5	0
Tachycardia	5	0
Bruising	4	0
Constipation	4	0
Decreased fibrinogen	4	0
Dizziness	4	0
Infection	4	0
Chills	3	0
Hypokalemia	3	0
Hypotension (*not CRS)	3	0
Rigors	3	0
Tremors	3	0
Cough	2	0
Dehydration	2	0
Dyspepsia	2	0
Dyspnea	2	0
Increased INR	2	1
Memory impairment	2	0
Pruritus	2	0
Urticaria	2	0
Weight Gain	2	0

Efficacy

As of March 7, 2022, the median follow-up for the 30 participants was 6.5 months (205 days; range 9-601 days). In the 28 participants evaluable at day 28, the overall response rate was 76.7% (CR n=12, PR n=11, SD n=1 and PD n=4). The median progression-free was 6 months (95%CI 3-not estimable, NE; Figure 4A) and appeared to be best in ALL (Figure 4B). The median overall survival was 11 months (95% CI 6.6-NE), Figure 5. The median PFS and OS for DLBCL was 3 months (95%CI 1.9-5.9) and 10 months (95%CI 3.6-NE) respectively. The median PFS and OS for MCL was 6.3 months (95%CI 0.9-NE) and 9.8 months (95%CI 4.8-NE) respectively. The median PFS and OS was not estimable for ALL or other diagnosis. Patient level data is presented in Figure 6.

Correlative analysis

We performed multi-parameter flow cytometry analysis of patient PBMC isolated from peripheral blood samples drawn at the time of apheresis, and of the infusion products. There was a nominally significant association between CD28 expression on CD4+ T cells and response (CR or PR) at day 180 after CAR-T cell infusion (91.3% CD28+, range 56.9–99.9% for responders versus 79.4%, range 44.6–99.9% for non-responders, p=0.048, t-test), but no other statistically significant associations between clinical outcomes and the frequencies of immune cell subsets in PBMC samples or the infusion products. We then compared the total number of cells infused to overall response at day 180. This was of interest because dose is defined by the number of CAR positive T

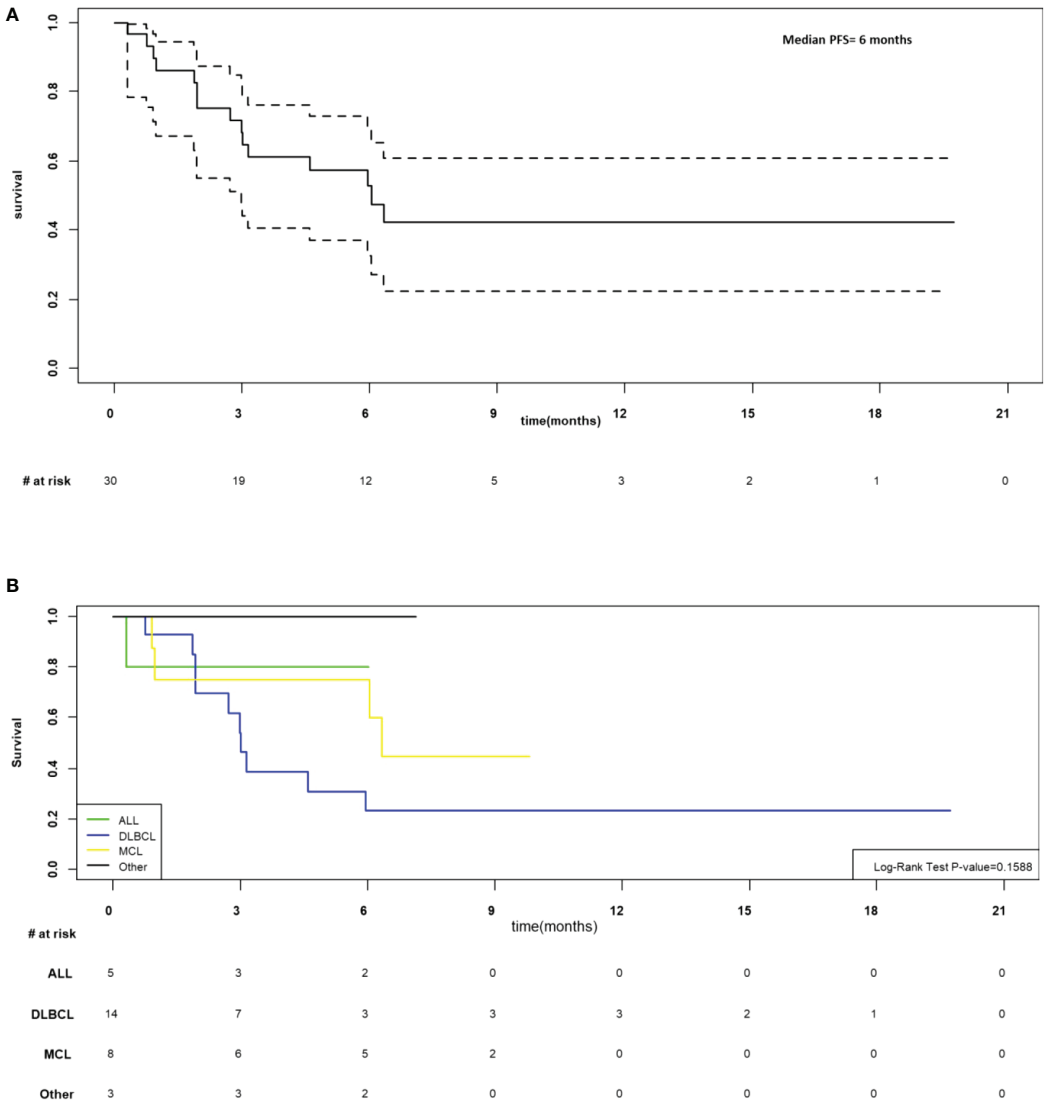

FIGURE 4
(A) Progression-free survival for N=30 infused. **(B)** Progression free survival by disease.

cells such that products with lower CAR-T content have greater numbers of total infused cells; however, no significant correlation was observed (p = 0.304, t-test). We then compared the phenotypes of CAR-T cells in the infusion product to overall response and noted that favourable response at day 180 was associated with increased frequency of naïve/SCM CD4+ CAR-T cells (ANOVA p = 0.010, BH-adjusted p = 0.077), increased frequency of CD27+ CD4+ CAR-T cells (ANOVA p = 0.019, BH-adjusted p = 0.107), decreased frequencies of PD1+ CD4+ CAR-T cells (ANOVA p = 0.00041, BH-adjusted p = 0.009) and decreased frequencies of TIGIT+ CD4+ CAR-T cells (ANOVA p = 0.0057, BH-adjusted p = 0.066) in the infusion product (Figure 7). These findings are consistent with previous reports of CAR-T cell phenotypes associated with favourable outcomes.

We then evaluated B cell persistence and CAR-T persistence in the blood at days 3, 7, 14, 28, month 2 and month 3. As measured by flow cytometry, we saw the level of CAR-T cells in the blood peak at day 7 or 14 and typically saw B cell levels drop at day 14 (Supplemental Figure 1). To give an independent and sensitive measure of CAR-T abundance in participant blood over time, and to provide data for time points where there were not enough cells to analyze by flow cytometry, we developed a qPCR assay to quantify the CAR-T transgene in total nucleic acid extracted from PBMC. Supplemental Figure 2 shows the results from this assay for the first 90 days, which was largely consistent with CAR-T cell frequencies obtained by flow cytometry. We did not observe any correlations between CAR-T cell expansion dynamics and participant outcomes. Finally, we

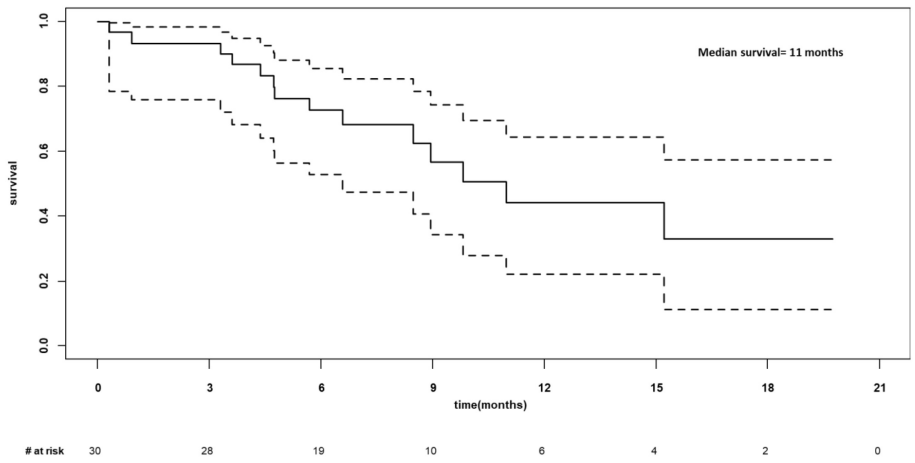

FIGURE 5
Overall survival for N=30 infused.

measured cytokine levels in patient serum during the first 28 days. In general, we saw trends towards increased cytokine levels at days 3 and 7 compared to pre-infusion time points, and in patients that experienced CRS grade 3 or greater. The largest increases were seen with GM-CSF, IFN-g, and IL-6, and to a lesser extent, IL-1b and TNF-b (Supplemental Figure 3). However, these trends in cytokine levels did not reach statistical significance. While the correlative data presented

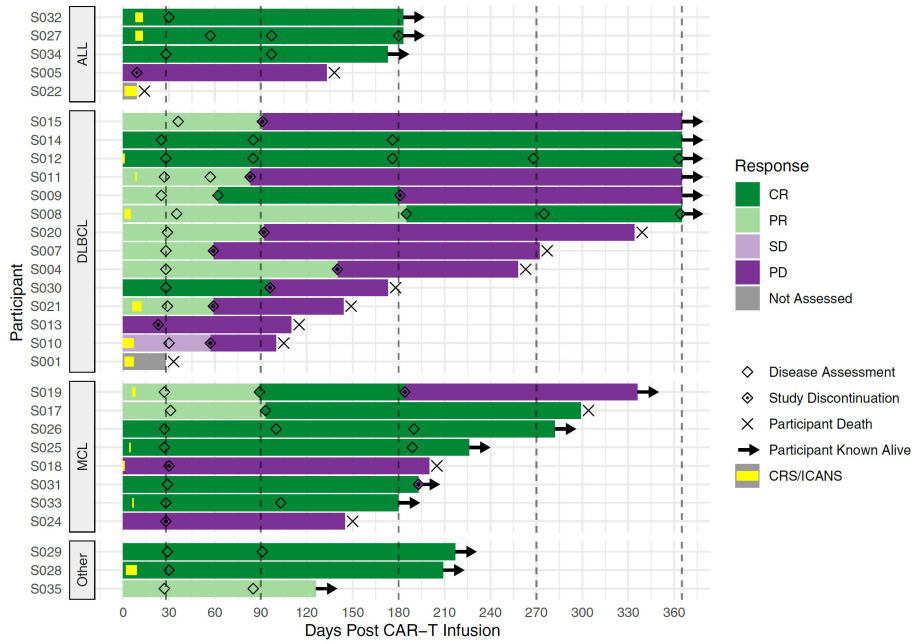

FIGURE 6
Swimmer's Plot of Patient-level Data. Each bar represents one CLIC-01 participant infused with CLIC-1901. Participants are grouped into disease type (ALL, DLBCL, MCL, and Other). X-axis denotes the days after CAR-T infusion. Vertical dashed lines mark expected disease assessment time points. Hollow diamonds indicate the days where a disease assessment was made for each participant. Bars are coloured according to the response at each disease assessment, with the time between 2 assessments being coloured by the earlier assessment time. For the time before the first assessment, bars are coloured by the response observed at the first assessment. Periods of CRS or neurotoxicity are denoted by yellow bars within the overall bar. An × marks the death of a participant, and → denotes the patient is still known to be alive at that time point. For this figure, follow up is truncated at 1 year.

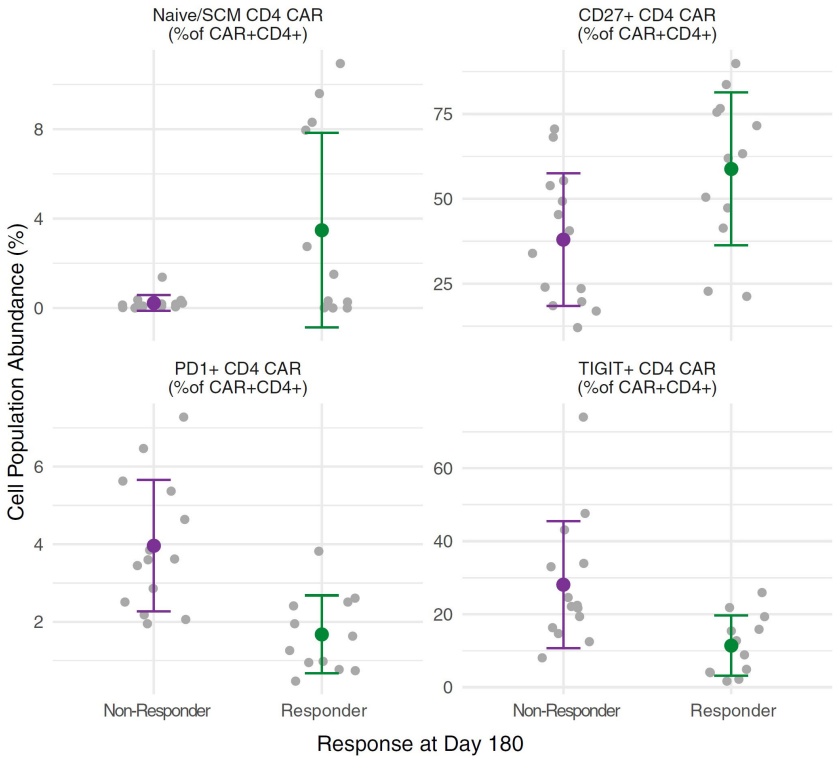

FIGURE 7
CAR cell phenotypes in the infusion product for responders versus non-responders. Independent ANOVAs were run to test for differences in CAR-T cell phenotypes between responders (n = 12) and non-responders (n = 14) at day 180, with Benjamini & Hochberg (BH) multiple test correction. Increased frequencies of Naïve/SCM CD4+ CAR-T cells (ANOVA p = 0.010, BH-adjusted p = 0.077) and CD27+ CD4+ CAR-T cells (ANOVA p = 0.019, BH-adjusted p = 0.107) in the infusion product correlated with favourable responses at day 180, as did decreased frequencies of PD1+ CD4+ CAR-T cells (ANOVA p = 0.00041, BH-adjusted p = 0.009) and TIGIT+ CD4+ CAR-T cells (ANOVA p = 0.0057, BH-adjusted p = 0.066). For each cell subset, grey points show individual measurements for each subject, while the green (responders) or purple (non-responders) points show the mean with whiskers extending to the mean ± standard deviation for each group.

here are consistent with favourable immune activation in study participants, there is insufficient power to link specific markers to outcomes. We will conduct a more extensive correlative analysis when accrual to the study is complete.

Discussion

Here we report our first experience with an in-house manufactured non-cryopreserved CAR-T cell product. Consistent with previous studies, we demonstrate that CAR-T cell manufacturing using the Miltenyi Prodigy platform is feasible in an academic setting. In this trial, we had two early manufacturing failures, both of which were due to a process design issue that was successfully addressed leading to zero further failures. We were able to provide fresh (i.e. non-cryopreserved) CAR-T product, with a 12-day manufacturing platform and a rapid 15-day turnaround from apheresis to CAR-T infusion. This is significantly shorter than the 4-to-6-week manufacturing and turnaround of commercial CAR-T products that we experience at our centers, and significantly shorter than other reported in-house manufacturing efforts (25). Our short manufacturing time allowed all patients to avoid the need for bridging therapy between apheresis and CAR-T infusion, which has been associated with poor clinical outcomes with CAR-T cell treatment (26).

Our trial showed that despite the COVID-19 pandemic and large geographical size of Canada, non-cryopreserved cells could be transported by volunteer couriers to and from our manufacturing facility (Victoria, British Columbia) and two clinical sites over 4,300 km apart (Vancouver, British Columbia and Ottawa, Ontario). Thus, we successfully leveraged the current standard of care for transportation of hematopoietic stem cell transplant products for Canada to achieve shorter vein-to-vein time than previous studies.

Lastly, in this early analysis of 30 patients treated on trial, safety and efficacy results were comparable to early phase trials performed for the other CD19 CAR-T products. In the ZUMA-1

trial of 111 patients with DLBCL who were treated with axicabtagene ciloleucel, grade 3 or higher CRS or neurotoxicity was 13% and 28% respectively (2). In the JULIET trial which reported the results of 167 patients and led to the approval of tisagenlecleucel for clinical use in DLBCL, the rate of grade 3 or higher CRS was 23% and the rate of severe neurotoxicity was 11% (3). In this CLIC-01 study, only 2 of the first 30 patients infused experienced grade 3 or higher CRS and neurotoxicity, giving this trial a low rate of 7% for these common CAR-T cell toxicities. We did not find a correlation between patient characteristics or infusion product characteristics in predicting toxicity, but with such a low incidence of CRS and neurotoxicity in this first group of patients, we are likely underpowered to report this at present. Our reported median progression-free survival is 6 months, which cannot at this time be compared to commercial products as this includes multiple disease types. In DLBCL, the median PFS in this trial was 3 months which does not differ significantly from the JULIET (2.9 months) and ZUMA-1 (5.9 months) trials (2, 3). Our experience in this trial is difficult to compare directly to real-world data with commercial products as we found that patients were often referred to the CLIC-01 trial because they were considered too sick (due to rapid disease progression or performance status) to wait for commercial CAR-T funding approvals and manufacturing times.

While this study provides promise for an in-house manufactured CD19 CAR-T product, there are limitations. With only 30 patients reported here and short follow-up, we cannot draw firm conclusions about safety and efficacy. In addition, the trial is currently underpowered to provide conclusive correlative results to better predict for toxicity, CAR-T persistence, and overall response. With multiple diseases included in this analysis, it is also not possible to conclude what the response rate is for each specific disease type. Nonetheless, this trial demonstrates that non-commercial organizations, working together, can effectively and efficiently manufacture in-house, high-quality CAR-T products for distribution and administration across a network of clinical sites. While this is a large undertaking for academic institutions, as has been outlined elsewhere (27), it also represents an opportunity to reduce costs and improve efficiency of manufacturing processes for future CAR-T therapies. While we must estimate the cost of our innovative platform and measure how this may change over time, there is compelling evidence (28–30) that our platform could fix the cost-prohibitive nature of commercial products that have placed immense pressure on publicly funded health care systems like Canada. In addition, our platform will not only provide a feasible approach with fast manufacturing for a conventional CAR-T, but it will also allow for flexibility of building the next CAR-T product, due to the rapid vector and CAR development which is not restricted by meeting world-wide supply and demand constraints of pharmaceutical companies, allowing for more rapid improvement in patient outcomes with CAR-T therapies.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The studies involving human participants were reviewed and approved by Ottawa Health Science Network Research Ethics Board (OHSN-REB) and The University of British Columbia - BC Cancer Research Ethics Board. The patients/participants provided their written informed consent to participate in this study.

Author contributions

NK, RAH, HA, JB, BN, ML, DF, JP, and KT designed the research. RM and SB analyzed data. JW, JN, EY, LD, NG, KC, TD, RH, BL, RF, VH, HL, LMC, JQ, PY, and DV were responsible for process development and manufacturing of the products. MB, LM, DC, MG, SB, CM, AS and EW provided quality and regulatory oversight. NK, KH, A-MC, AB, SM, MK, LH, KS, SN and JR conducted the clinical trial. JN, LD, DW and JS designed and executed immune monitoring assays. MS, DB and SY provided project management and administrative support. SD and TH provided patient perspective for the study design and consent. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.1074740/full#supplementary-material

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Safety and efficacy of dual PI3K-δ, γ inhibitor, duvelisib in patients with relapsed or refractory lymphoid neoplasms: A systematic review and meta-analysis of prospective clinical trials

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Background: Duvelisib is the first FDA-approved oral dual inhibitor of phosphatidylinositol-3-kinase PI3K-delta (PI3K-δ) and PI3K-gamma (PI3K-γ). Although many clinical studies support the efficacy of duvelisib, the safety of duvelisib remains with great attention. This systematic review and meta-analysis aimed to evaluate the safety and efficacy of duvelisib in treating different relapsed or refractory (RR) lymphoid neoplasm types.

Methods: We searched prospective clinical trials from PUBMED, EMBASE, Cochrane Library, and ClinicalTrials.gov. For efficacy analysis, Overall response rate (ORR), complete response rate (CR), partial response rate (PR), rate of stable disease (SDR), rate of progressive disease (PDR), median progression-free survival (mPFS), 12-/24-month PFS, and 12-month overall survival (OS) were assessed. For safety analysis, the incidences of any grade and grade ≥3 adverse events (AEs), serious AEs, and treatment-related discontinuation and death were evaluated. Subgroup analysis based on the disease type was performed.

Results: We included 11 studies and 683 patients, including 305 chronic lymphocytic leukemia/small lymphocytic lymphoma (CLL/SLL), 187 B-cell indolent non-Hodgkin lymphoma (iNHL), 39 B-cell aggressive non-Hodgkin lymphoma (aNHL), and 152 T-cell non-Hodgkin lymphoma (T-NHL) patients. The pooled ORR in CLL/SLL, iNHL, aNHL and T-NHL was 70%, 70%, 28% and 47%, respectively. Additionally, the pooled ORR in CLL/SLL patients with or without TP53 mutation/17p-deletion (62% vs. 74%, p=0.45) and in follicular lymphoma (FL) or other iNHL (69% vs. 57%, p=0.38) had no significant differences. Mantle cell lymphoma (MCL) patients had higher pooled ORR than other aNHL (68% vs. 17%, p=0.04). Angioimmunoblastic TCL (AITL) patients had higher pooled ORR than other PTCL patients (67% vs. 42%, p=0.01). The pooled incidence of any grade, grade ≥3, serious AEs,

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treatment-related discontinuation and death was 99%, 79%, 63%, 33% and 3%, respectively. The most frequent any-grade AEs were diarrhea (47%), ALT/AST increase (39%), and neutropenia (38%). The most frequent grade ≥3 AEs were neutropenia (25%), ALT/AST increased (16%), diarrhea (12%), and anemia (12%).

Conclusion: Generally, duvelisib could offer favorable efficacy in patients with RR CLL/SLL, iNHL, MCL, and AITL. Risk and severity in duvelisib treatment may be mitigated through proper identification and management.

KEYWORDS

dual PI3K-δ, γ inhibitor, duvelisib, lymphoid neoplasms, safety, efficacy, meta-analysis

Introduction

Lymphoid neoplasms comprise a heterogeneous group of lymphoproliferative malignancies with a variety of clinical, morphologic, and molecular features, for which about 150,000 new cases and 40,000 deaths expected to occur in 2022 in the United States alone (1). Mature B-cell neoplasms and mature T-cell neoplasms represent the most typical lymphocytic tumors originating from cells at stages of maturation after stem cell differentiation (2). Mature B-cell neoplasms account for nearly 65% of all lymphoid neoplasms, with both aggressive and indolent subtypes (2). The latter mainly including chronic lymphocytic leukemia/small lymphocytic lymphoma (CLL/SLL), Follicular lymphoma (FL), Marginal zone lymphoma (MZL), Lymphoplasmacytic lymphoma (3).Widespread use of chemoimmunotherapy has greatly improved the survival of patients with CLL/SLL and indolent non-Hodgkin lymphomas (iNHLs). However, these diseases are currently incurable (4–9). Patients with aggressive B-cell lymphoma(aNHLs), such as diffuse large B-cell lymphoma (DLBCL) and mantle cell lymphoma (MCL), are often diagnosed with advanced stage, and a substantial proportion of patients are refractory to initial chemotherapy or relapse in early years. High-dose chemotherapy with autologous stem cell transplantation may be the only curative choice for patients with relapsed/refractory (R/R) aNHLs. Radiotherapy and single-agent therapies, which play roles in the treatment of iNHL, have shown low expected response rates and duration of responses (10–16). Mature T-cell non-Hodgkin lymphomas (T-NHLs) are another heterogeneous group representing approximately 6% of all lymphoid neoplasms (2), usually including cutaneous T-cell lymphoma (CTCL) and peripheral T-cell lymphoma (PTCL). With a relatively poor prognosis, PTCL includes PTCL not otherwise specified (NOS), angioimmunoblastic T-cell lymphoma (AITL), anaplastic large-cell lymphoma (ALCL), and others (17). In contrast to mature B-cell neoplasms, T-cell lymphomas, especially PTCL, have a high failure rate of first-line chemotherapy, prone to relapse, and lacking an effective monoclonal antibody, like anti-CD20 in B-cell lymphoma (18, 19). Some agents have been approved by the FDA for relapsed PTCL, unfortunately with low response rates and short median progression-free survival (PFS) (20–22). New and more effective agents with distinct mechanisms are urgently needed for patients with relapsed or refractory mature lymphoid neoplasms, whether the tumor originates from B cells or T cells.

Duvelisib is the first FDA-approved oral dual inhibitor of phosphatidylinositol-3-kinase PI3K-delta (PI3K-δ) and PI3K-gamma (PI3K-γ) and shows great potential in many clinical trials for the treatment of relapsed or refractory lymphoid neoplasms, including CLL/SLL, iNHLs, and T-NHLs (23–33). Phosphatidylinositol 3-kinase (PI3K) is a lipid kinase involved in lots of signal transduction. Class I PI3K consists of four catalytic subunits (α, β, γ, δ) in human cells. PI3K-α and PI3K-β show a broad tissue distribution. In contrast, the PI3K-δ and PI3K-γ isoforms are primarily expressed in leukocytes, extensively regulating both innate and adaptive immune function in lymphocyte and myeloid cell function (34–39).PI3K-δ inhibition directly targets proliferation and survival of lymphoid neoplasm cells, while PI3K-γ inhibition reduces the differentiation and migration of crucial tumor support cells in the tumor microenvironment, such as Treg cells and M2 tumor-associated macrophages (33, 40–44). With dual inhibition of PI3K-δ and PIK3-g in preclinical models of CLL, B-cell lymphomas, and T-cell lymphomas, duvelisib showed more robust anti-tumor activity than inhibitors of PI3K-δ isoform alone (33, 44–46).

However, due to the heterogeneity of lymphoid neoplasms, the efficacy of PI3K inhibition in lymphoid neoplasms ranged widely. Meanwhile, the PI3K/AKT/mTOR regulates a range of cellular activities, whether in malignant or normal cells, so off-target effects and side effects are inevitable (47, 48). Some of the toxic effects reported in clinical trials of PI3K-δ inhibitors, incredibly immune dysregulation, and immune dysfunction, have raised concerns about safety (49–51). Compared with other approved PI3K-δ inhibitors, duvelisib has certain safety advantages. Compared with idelalisib, only inhibiting PI3K-δ,

and based on preclinical data, duvelisib may reduce autoimmune complications through the inhibition of PI3K-γ (52, 53). Additionally, duvelisib doesn't need infusion, without producing hyperglycemic effects mediated by PI3K-α isoform inhibition, which reduces the usage of copanlisib in older adults. A better understanding of the complexities of the adverse events and subgroup of lymphoid neoplasms patients benefitting most from duvelisib treatment could provide more precise treatment schedule. In this systematic review, we analyzed the efficacy and safety of duvelisib monotherapy in patients with relapsed or refractory lymphoid neoplasms. Besides, subgroup analysis was conducted to compare the efficacy and safety of duvelisib between different disease groups. These findings lead to offering evidence-based references for clinicians to optimize future clinical trials and treatment options.

Methods

Literature search

The study design and literature search strategy for this article followed Systematic Reviews and Meta-Analyses (PRISMA) guidelines. The relevant studies were identified by searching Medline (PubMed), Embase, Cochrane Library, and ClinicalTrials.gov. We used a combination of terms: "(Leukemia OR Lymphoma) AND ((Duvelisib) OR (COPIKTRA) OR (IPI-145))" to search for clinical studies evaluating the safety and efficacy of duvelisib in the treatment of relapsed or refractory lymphoid neoplasms, the data cut-off was May 20, 2022. There were no date or language restrictions.

Inclusion criteria and exclusion criteria

Studies must meet the following inclusion criteria: 1) clinical trials in any phase of Duvelisib therapy for patients with relapsed/refractory CLL/SLL or relapsed/refractory NHL; 2) analyzable data on safety or efficacy available in the study; 3) drugs used in humans; 4) the patients are over 18 years old. Exclusion criteria are: (1) Studies not related to our topic; (2) studies without usable results; and (3) reviews, letters, editorials, patents, news, case reports, and retrospective or observed studies. Two authors independently searched, screened, and determined study eligibility, and any disagreements were resolved by discussion.

Data extraction and quality control

Eligible studies were reviewed, and data were extracted independently by two authors. We identified the first author, publication year, ClinicalTrials.gov number, phase, study design and treatment, disease type, patients numbers, age, gender, previous systemic therapy, overall response rate (ORR), complete response rate (CR), partial response rate (PR), rate of stable disease (SDR), rate of progressive disease (PDR), median progression-free survival (mPFS), PFS, overall survival (OS), any grade AEs, grade ≥3 AEs, serious AEs, treatment-related discontinuation and death.

Statistical analysis

The package meta version 5.2-0 Index in the R-4.1.1 was used to evaluate the results of efficacy and safety. To analyze heterogeneity between studies we included, the I-squared test (I2 test) was used. A random effects model was used when I2 >50%, and a fixed effect model was conducted when I2 ≤50%. All analyses were based on the intention-to-treat population of the studies included. The subgroup analysis by disease type or treatment was applied. Sensitivity analysis was carried out by using different effect models. No dose effect was considered. $P < 0.05$ suggested statistical significance.

Study qualitative assessment

Methodological Indicators for Nonrandomized Studies (MINORS) were used to assess the methodological quality of non-randomized surgical studies included. MINORS contain 12 items; The first eight items are dedicated to non-comparative research. Items include the stated purpose of the study, The inclusion of consecutive patients, prospective collection of data, endpoints suitable for study purpose, unbiased endpoints evaluation, adequate follow-up time enough for the endpoint, loss to follow-up of patients not exceeding 5%, and sample size calculated prospectively. Each item is scored from 0 to 2; 0 is not reported, 1 is insufficiently reported, and 2 is adequately reported. The Cochrane Collaboration Risk of Bias Tool (Review Manager 5.4) is used to evaluate the bias of the RCT studies involved, including six criteria: random sequence generation, allocation concealment, blinding of participants and personnel, blinding of outcomes assessment, completeness of the outcome data, and selective reporting.

Results

Study selection

Through the above search strategy, we retrieved 494 studies. 205 were dropped after the duplication check, and 266 were excluded for the reasons we mentioned above. After Inclusion, 6 studies were removed with combined therapy, 3 studies were removed with first-line treatment, and 2 studies had no

analyzable results. Figure 1 showed the details of our study selection process. Ultimately, our meta-analysis included 11 studies (23–33).

Study characteristics

Table 1 presents the baseline characteristics of the studies included. These studies were published from 2018 to 2022. We included total of 11 studies, 683 patients, of which 305 were CLL/SLL included in 3 studies, 378 in other 8 studies were NHL [187 B-cell indolent non-Hodgkin lymphoma(iNHL), 39 B-cell aggressive non-Hodgkin lymphoma (aNHL), 152 T-cell lymphomas (T-NHL)]. There were 6 phase I studies,3 phase II studies, and 2 phase III studies. Patients in all 11 studies received duvelisib monotherapy. We assessed safety and efficacy only in patients with RR CLL/SLL or RR NHL. All studies presented information on efficacy or safety.

Assessment of study quality

The MINORS scores of 10 single arm studies ranged from 7 to 13. 3 studies without full context cannot be evaluated totally. The item of sample size calculated prospectively was not mentioned. The bias of the only 1 RCT study included was assessed by the Cochrane Collaboration Risk of Bias Tool with an acceptable quality result. Therefore, the overall quality of the 11 studies included was satisfactory. More details are shown in Supplementary Figure 1 and Supplementary Table 1.

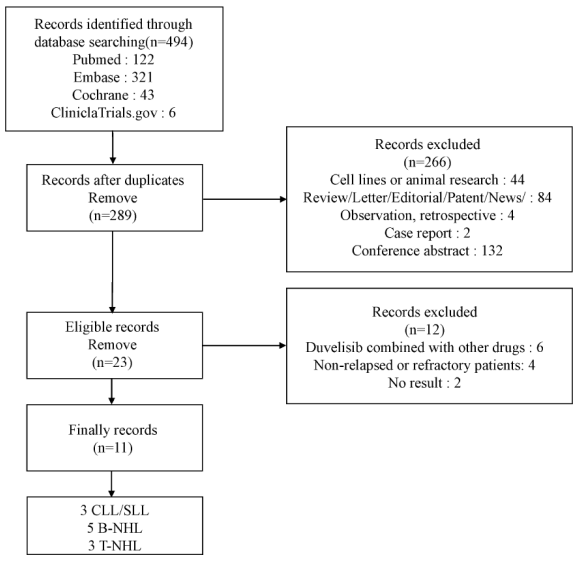

FIGURE 1
The flow chart.

Efficacy

All studies reported the efficacy outcomes such as overall response rate (ORR), complete response rate (CR), partial response rate (PR), stable disease rate (SDR), and progressive disease rate (PDR). The response was based on International Workshop on CLL or Revised International Working Group response criteria for CLL/SLL patients, and the International Working Group response criteria for NHL patients.

All studies focused on evaluating the ORR. The pooled ORR in CLL/SLL, iNHL, B-aNHL and T-NHL was 70% (59-81%), 70% (48-93%), 28% (14-42%) and 47% (39-55%), respectively (Figure 2). Additionally, the ORR in CLL/SLL patients with or without TP53 mutation/17p-deletion (62% vs. 74%, p=0.45) had no significant difference (Figure 3A). Follicular lymphoma (FL) had higher ORR than iNHL, yet without statistical significance (69% vs. 57%, p=0.38) (Figure 3B). Besides, Mantle cell lymphoma (MCL) patients had higher pooled ORR than other aNHL (68% vs. 17%, p=0.04) (Figure 3C). The ORR was 49% and 32% in PTCL and CTCL, respectively (Supplementary Figure 2A). In the subgroup of PTCL, Angioimmunoblastic T cell Lymphoma (AITL) patients had higher pooled ORR than other PTCL patients (67% vs. 42%, p=0.01) (Figure 3D).

The pooled CR in CLL/SLL, iNHL, B-aNHL and T-NHL was 2% (0–1%), 16% (1–31%), 8% (0–17%) and 22% (6–38%), respectively (Figure 4). In the subgroup analysis, the CR in CLL/SLL patients with or without TP53 mutation/17p-deletion (6% vs. 1%, p=0.21) had no significant difference (Figure 5A). FL had higher CR than other iNHL, yet without statistical significance (18% vs. 2%, p=0.08) (Figure 5B). Besides, the CR in MCL patients and CR in other aNHL are similar (9% vs. 7%, p=0.87) (Figure 5C). PTCL had a higher CR than CTCL (29% vs. 0%, p<0.01) (Figure 5D).

The pooled PR in CLL/SLL, iNHL, B-aNHL and T-NHL was 64% (53–74%), 46% (39–53%), 20% (8–33%) and 22% (10–33%), respectively (Figure 6). In the subgroup analysis, the PR in CLL/SLL patients with or without TP53 mutation/17p-deletion (54% vs. 73%, p=0.13) had no significant difference (Figure 7A). the PR in FL and that in other iNHL had no significant difference (43% vs. 53%, p=0.20) (Figure 7B). Besides, the PR in MCL had higher PR than other aNHL, yet without statistical significance (64% vs. 11%, p=0.08) (Figure 7C). There is no significant difference between the PR of PTCL and CTCL (19% vs. 32%, p=0.30) (Figure 7D).

The pooled SDR in CLL/SLL, iNHL, and T-NHL was 22% (12–33%), 25% (8–42%), and 10% (0–27%), respectively (Supplementary Figure 3A). The pooled PDR in CLL/SLL, iNHL, and T-NHL was 1% (0–3%), 11% (6–16%), 43% (29–57%), respectively (Supplementary Figure 3B). In the subgroup of T-NHL, the SDR was 2% (0–5%) and 32% (13–57%) in PTCL and CTCL, respectively (Supplementary Figure 2B). The PDR was 47% (38–56%) and 32% (13–57%) in PTCL and CTCL,

TABLE 1 The characteristic of include studies.

Study	ClinicalTrials.govidentifier	Phase	Design	Disease	Treatment	No.ofpatients	Agerange	Male/ female (n)	Median number ofprior regimen, (range)	ORR %	12-month PFS rate	12-month OS rate	mPFS (months)	Discontinued treatment due of Aes (n)	Any grade adverse events (n)	Grade≥3 adverse events (n)	Serious adverse events (n)
O'Brien-2018	NCT01476657	I	multicohort, single arm	relapsed or refractory CLL/SLL	25 or 75 mg BID in 28-day cycles	55	66(42–82)	42/13	4(1–11)	56.4%	57.1%	65.5%	ALL:15.7 TP53 mutation/ 17p-deletion:27.9	20	_	_	_
Flinn-2018	NCT02004522	III	multicenter, randomized	relapsed or refractory CLL/SLL	25 mg BID in 28-day cycles	160	69(39-90)	96/64	2(1-10)	73.8%	60.0%	86.3%	ALL:13.3 TP53 mutation/ 17p-deletion:12.7	55	156	138	115
Davids-2020	NCT02049515	III	two-arm, non-randomized	relapsed or refractory CLL/SLL	25 mg BID in 28-day cycles	90	68(39–90)	57/33	3(2-8)	76.7%	64.4%	82.2%	ALL:15.7 TP53 mutation/ 17p-deletion:14.7	47	90	80	67
Flinn-2018	NCT01476657	I	multicohort, single arm	relapsed or refractory idolent NHL	25 or 75 mg BID in 28-day cycles	31	64(37-78)	18/13	3 (1-8)	58.1%	58.1%	77.4%	9.5	6	_	_	_
Flinn-2019	NCT01882803	II	single arm	relapsed or refractory idolent NHL	25 mg BID in 28-day cycles	129	65(30-90)	88/41	3 (1-18)	47.3%	31.8%	76.7%	14.7	40	128	114	74
Zheng-2021	NCT04707079	II	multicenter, single arm	relapsed or refractory Follicular lymphoma	25 mg BID in 28-day cycles	23	49(31-70)	16/7	≥2	82.6%	_	_		_	21	12	_

(Continued)

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TABLE 1 Continued

Study	ClinicalTrials.govidentifier	Phase	Design	Disease	Treatment	No.ofpatients	Agerange	Male/ female (n)	Median number ofprior regimen, (range)	ORR %	12-month PFS rate	12-month OS rate	mPFS (months)	Discontinued treatment due of Aes (n)	Any grade adverse events (n)	Grade≥3 adverse events (n)	Serious adverse events (n)
Izutsu-2020	NCT02598570	I	multicenter, single arm	relapsed or refractory B cell NHL	25 mg BID in 28-day cycles	7	61(54–74)	4/3	3 (1–5)	71.4%	_	_		2	7	_	2
Flinn-2018	NCT01476657	I	multicohort, single arm	relapsed or refractory B cell NHL	25 or 75 mg BID in 28-day cycles	36	_	_	≥1	27.8%	_	_		_	_	_	_
Horwitz-2018	NCT01476657	I	multicohort, single arm	relapsed or refractory T cell NHL	25 or 75 mg BID in 28-day cycles	35	64 (34-86)	16/19	4 (1-10)	40.0%	31.4%	65.7%	PTCL:8.3 CTCL:4.5	13	34	24	20
Horwitz-2019	NCT02783625	I	single arm	relapsed or refractory T cell NHL	25 or 75 mg BID in 1 month lead-in	16	_	_	≥1	50.0%	_	_		_	_	_	_
Zinzani-2022	NCT03372057	II	multicenter, parallel cohort	relapsed or refractory T cell NHL	25 mg BID after 75 mg BID for 2 cycles	101	67(21-92)	_	3 (1–9)	48.5%	_	_	3.6	20	_	_	_

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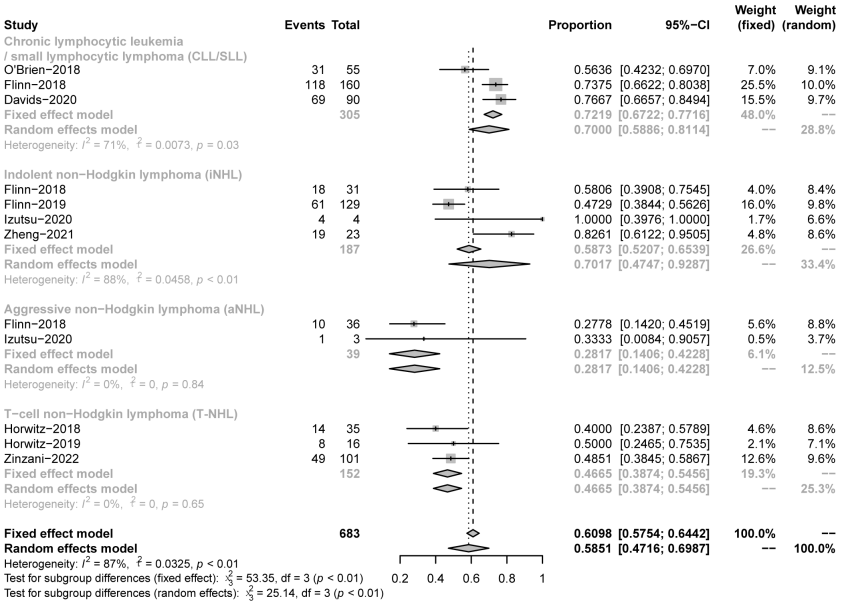

FIGURE 2
The forest plot of pooled ORR. Disease including chronic lymphocytic leukemia/small lymphocytic lymphoma (CLL/SLL), indolent non–Hodgkin lymphoma (iNHL), aggressive non–Hodgkin lymphoma (aNHL) and T–cell non–Hodgkin lymphoma (T-NHL).

respectively (Supplementary Figure 2C). No enough SDR or PDR data is available to analyze other subgroups.

Seven studies presented mPFS data. For all CLL/SLL patients, the mPFS reported in the three studies was 15.7, 13.3, and 15.7 months, respectively. For CLL/SLL patients with TP53 mutation/17p−deletion, the mPFS was 27.9, 12.7, and 14.7, respectively. For patients with iNHL, the mPFS reported in the two studies was 9.5 and 14.7 months, respectively. The mPFS for patients with PTCL in the reported 2 studies was 8.3, and 3.6 months, and CTCL in 1 study was 4.5 months. The mPFS data reported in studies were shown in Table1. The rate of PFS and OS was performed to assess the efficacy of duvelisib treatment in CLL/SLL and NHL. For all

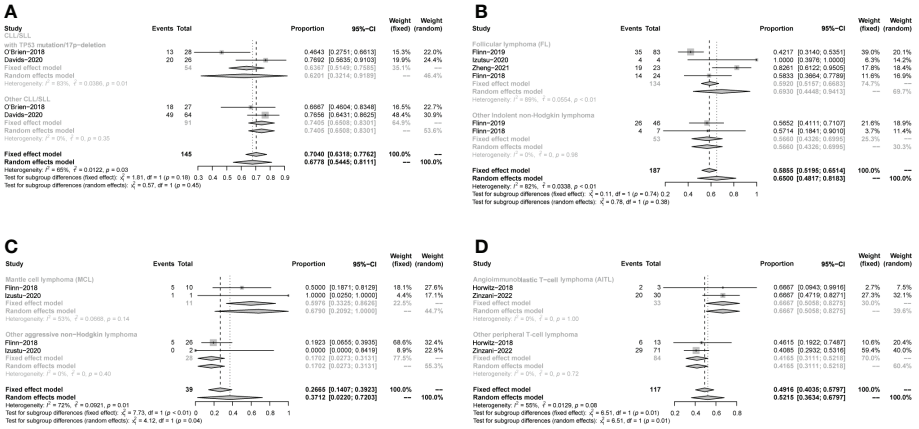

FIGURE 3
Subgroup analysis of the pooled ORR. **(A)**. Subgroup of CLL/SLL, the pooled ORR of CLL/SLL with TP53 mutation/17p−deletion and others. **(B)**. Subgroup of iNHL, the pooled ORR of Follicular lymphoma (FL) and others. **(C)**. Subgroup of aNHL, the pooled ORR of MCL and others. **(D)**. Subgroup of PTCL, the pooled ORR of AITL and others.

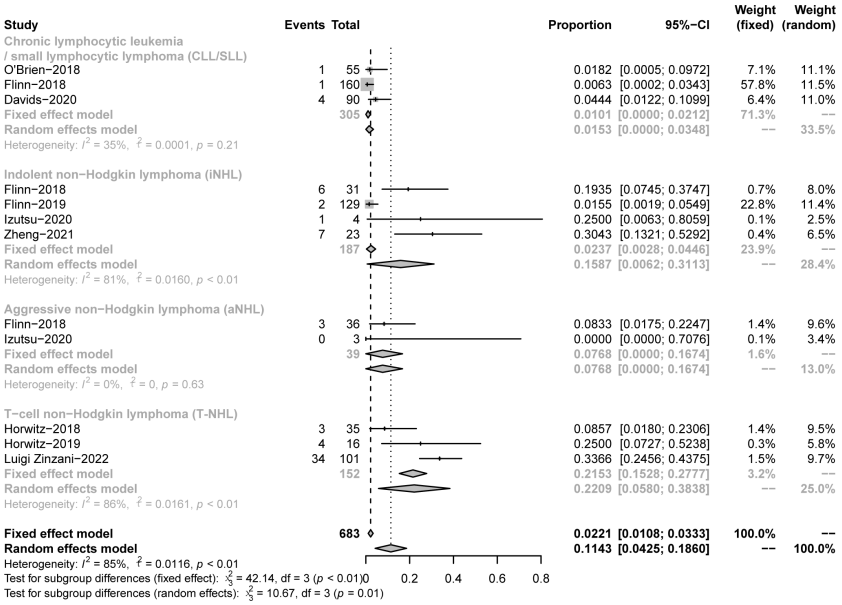

FIGURE 4
The forest plot of pooled CR. Disease includes chronic lymphocytic leukemia/small lymphocytic lymphoma (CLL/SLL), indolent non−Hodgkin lymphoma (iNHL), aggressive non−Hodgkin lymphoma (aNHL) and T−cell non−Hodgkin lymphoma (T-NHL).

patients, the pooled 12-month PFS rate was 49% (37%–60%). Besides, in CLL/SLL, iNHL, and CLL/SLL with TP53 mutation/17p−deletion, the pooled 12-month PFS rate was 61% (55–66%),44% (18–69%), and 62% (52–72%), respectively (Figure 8). Only 1 T-NHL study reported the 12-month PFS of PTCL and CTCL was 38% (15–65%), 26% (9–51%), respectively. Moreover, the 24-month PFS rate was also similar to the rate between all CLL/SLL patients (27%, 22–32%) and those with TP53 mutation/17p−deletion (27%, 12–42%) (Supplementary Figure 4). For all patients, the pooled 12-month OS rate was 76% (69–84%). Besides, in CLL/SLL and iNHL, the pooled 12-month OS rate was 79% (68–66%) and 77% (70–83%), respectively (Figure 9). Only 1 T-NHL study reported the 12-month OS of PTCL and CTCL was 44% (20–70%) and 79% (54–94%), respectively.

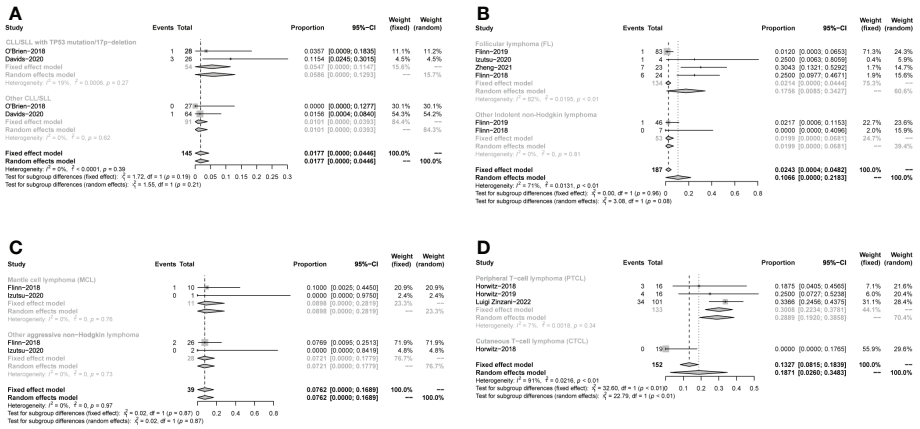

FIGURE 5
Subgroup analysis of the pooled CR. **(A)**. Subgroup of CLL/SLL, the pooled CR of CLL/SLL with TP53 mutation/17p−deletion and others. **(B)**. Subgroup of iNHL, the pooled CR of Follicular lymphoma (FL) and others. **(C)**. Subgroup of aNHL, the pooled CR of MCL and others. **(D)**. Subgroup of T-NHL, the pooled CR of PTCL and CTCL.

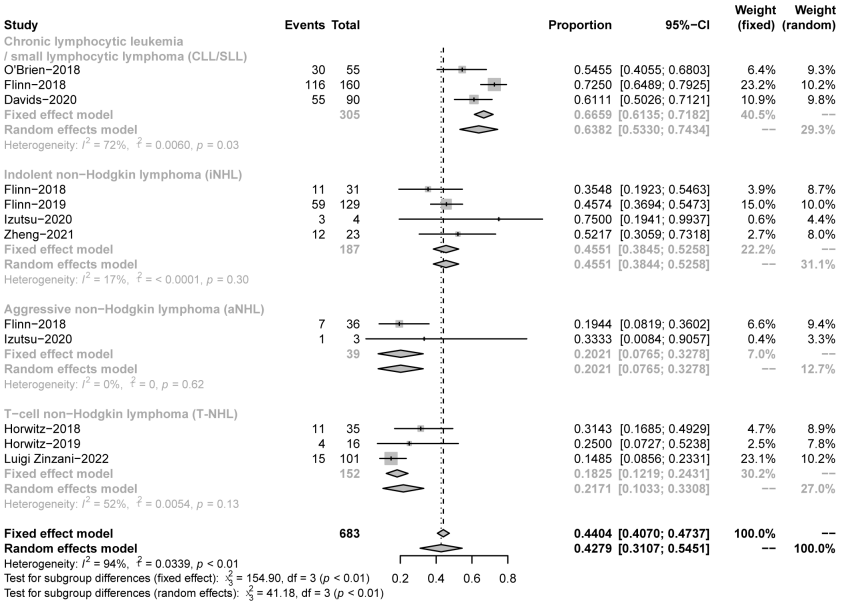

FIGURE 6
The forest plot of pooled PR, disease includes Chronic lymphocytic leukemia/small lymphocytic lymphoma (CLL/SLL), Indolent non−Hodgkin lymphoma (iNHL), Aggressive non−Hodgkin lymphoma (aNHL) and T−cell non−Hodgkin lymphoma (T-NHL).

Safety

The safety profiles in individual disease subgroups were generally consistent with those of the entire subjects. The safety assessments recorded in studies on patients were listed in Table 1. Six studies were included in the analysis of the pooled incidence of any grade AEs (99%, 98–100%), and five studies were included to evaluate the pooled incidence of grade ≥3 AEs (79%,67–91%). 5 studies were included in the analysis of pooled serious AEs (63%,53–74%). The pooled rate of treatment discontinuation due to AEs was 33% (25–41%) in 8 included studies (Figure 10). And the most frequent AEs leading to treatment discontinuation were elevated transaminases (6%, 0-11%), colitis (5%, 2-7%), diarrhea (4%, 2-6%) (Supplementary Figure 5).

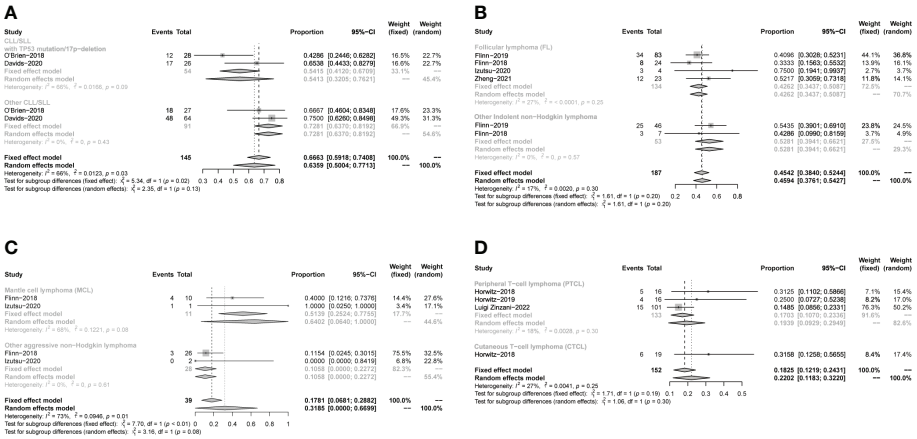

FIGURE 7
The forest plot of pooled PR of disease subgroup, **(A)**. Subgroup of CLL/SLL, the pooled PR of CLL/SLL with TP53 mutation/17p−deletion and others. **(B)**. Subgroup of iNHL, the pooled PR of Follicular lymphoma (FL) and others. **(C)**. Subgroup of aNHL, the pooled PR of MCL and others. **(D)**. Subgroup of T-NHL, the pooled PR of PTCL and CTCL.

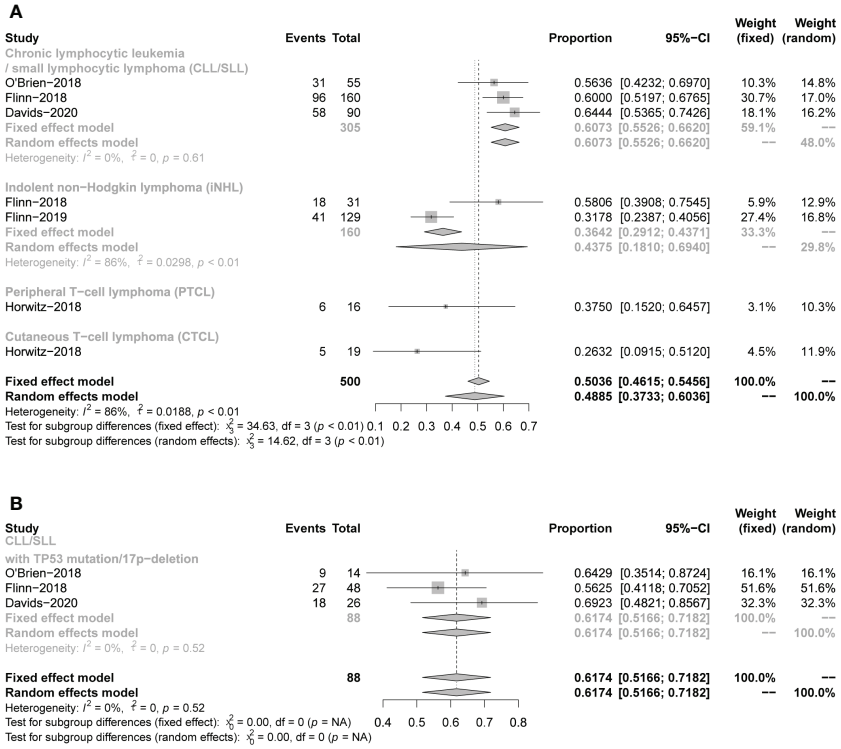

FIGURE 8
The forest plot of pooled 12-month PFS rate, **(A)**. pooled 12-month PFS rate of CLL/SLL, iNHL, PTCL and CTCL. **(B)**. Pooled 12-month PFS rate of CLL/SLL with TP53 mutation/17p-deletion.

The most frequent any-grade AEs were Diarrhea (47%, 43-52%), ALT/AST increased (39%, 23-55%), neutropenia (38%, 26-50%), fatigue (29%, 18-40%), fever (29%, 25-32%) and cough (26%, 18-34%). The most frequent grade 3 or greater AEs were neutropenia (25%, 19-31%), ALT/AST increased (16%, 5-26%), diarrhea (12%, 6-18%) and anemia (12%, 5 -18%) (Table 2).

The subgroup analysis of CLL/SLL, B-NHL, and T-NHL were shown in the Table 2. The incidence of grade≥3

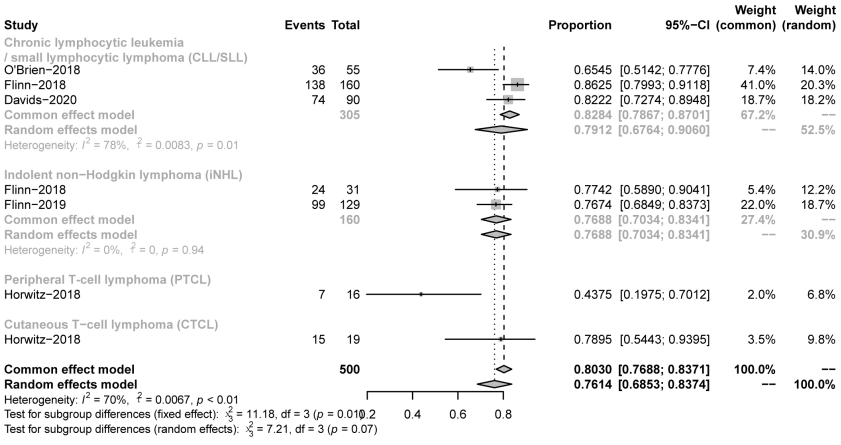

FIGURE 9
The forest plot of pooled 12-month OS rate of CLL/SLL, iNHL, PTCL and CTCL.

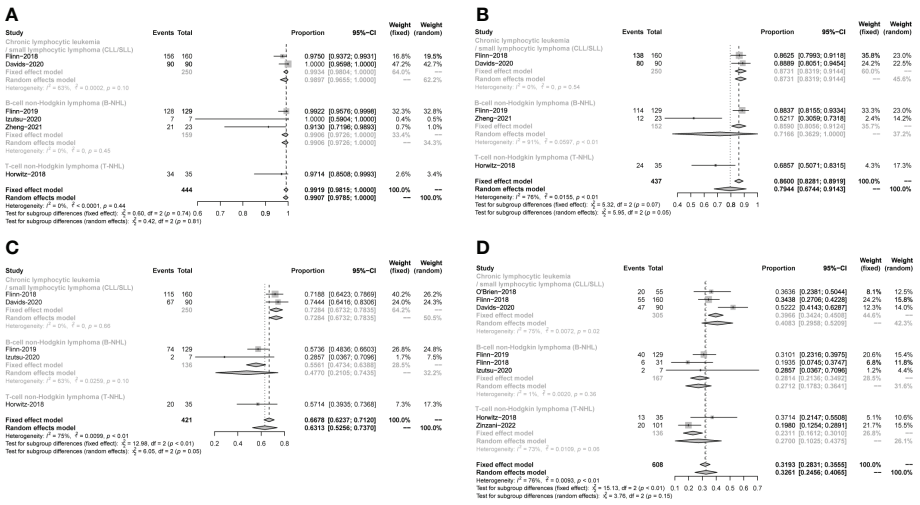

FIGURE 10
The forest plot of incidences of AEs. **(A)**. AEs in any grade. **(B)**. AEs in grade ≥3. **(C)**. Serious AEs. **(D)**. Treatment discontinuation due to AEs.

transaminase elevations in CLL/SLL (3%) is much lower than in B-NHL (19%, p=0.05) or T-NHL (30%, $p < 0.01$). And the incidence of grade≥3 diarrhea in T-NHL (3%) is much lower than in CLL/SLL (15%, p=0.02) and B-NHL (16%, p<0.01). Furthermore, the incidence of grade≥3 colitis in CLL/SLL(12%) is higher than in T-NHL(0%,$p < 0.01$) and B-NHL (6%, p=0.03) (Table 2). Consistent with this difference, elevated transaminases (CLL/SLL:1%, B-NHL:8%, T-NHL:15%) was a major factor for treatment discontinuation in NHL, while diarrhea (CLL/SLL:5%, B-NHL:2%) or colitis (CLL/SLL:6%, B-NHL:2%, T-NHL: 6%) were major factors for treatment discontinuation in CLL/SLL (Supplementary Figure 5). Furthermore, the CLL/SLL patients have shown higher incidence of any grade (22% vs. 8%, p=0.04) and grade≥3 pneumonia (14% vs. 6%, p<0.01) compared to B-NHL patients (Table 2).

A total of 17 fatal AEs related to duvelisib application by 577 patients were reported in the 7 studies we included (pooled rate of 3%). Infectious complications are the leading causes of duvelisib-related mortality (n=12, pooled rate of 2%). Details were shown in Table 3 and Figure 11.

Discussion

Duvelisib, the world's first approved oral dual inhibitor of PI3K-δ and PI3K-γ, has been approved for treating patients with R/R CLL/SLL after at least two other treatments in the US and Europe. In Europe and China, duvelisib is indicated for treating adult patients with R/R FL after at least two prior systemic therapies. Recent clinical data on duvelisib suggest a satisfactory efficiency and acceptable safety profile in patients with advanced lymphocyte neoplasms, including R/R CLL/SLL and R/R NHL (23–29). However, the toxicity problems of PI3K inhibitor application cannot be ignored, especially immune-related and infection-related adverse events (47, 54). Unfortunately, at this point the assessments recently reaffirmed by the Panel of experts of the Oncologic Drugs Advisory Committee (ODAC) cannot be omitted, which analyzing the follow-up data of the DUO trial, among others, believes that 'the risks associated with taking duvelisib by patients with certain blood cancers appear to outweigh the benefits ". So, it is essential to accurately identify the patients benefitting from PI3K inhibitor therapy, and to properly recognize and manage adverse events during treatment. We conducted this meta-analysis and literature review to evaluate the efficacy and safety of duvelisib in different lymphocyte neoplasms, and possible solutions to treatment-relevant toxicity were reviewed. We summarized the results of 11 prospective studies, including 683 patients, 305 patients with CLL/SLL and 378 patients with NHL [187 B-cell iNHL, 39 B-cell aNHL, 152 T-NHL], to assess the safety and efficacy.

This meta-analysis showed that duvelisib could provide satisfactory efficacy in patients with mature B-cell/T-cell Neoplasms. It is worth noting that response rates were particularly noteworthy in patients with RR CLL/SLL and RR iNHL, in whom ORR both were 70%,12-month PFS was 61% and 44%, and 12-month OS was 79% and 77%, respectively. ORR was 49% in RR PTCL patients and 67% in AITL, which are also compelling compared to the poor response rates of other approved single agents. But among the aNHL patients we included, the pooled ORR was only 17%. It's clear that with the heterogeneity of lymphoid neoplasms, different therapeutic impacts of PI3K-δ and PI3K-γ inhibition could be derived from following aspects.

The first is tumor intracellular impact itself. A key factor is that some lymphocyte neoplasms are highly dependent on

TABLE 2 The incidence of adverse events in all grade or grade equal or greater than 3.

AEs	Diease	Any grade						Grade≥3					
		Includestudy	Event	Total	Pooled rate (95%CI)	Model	P-value	Includestudy	Event	Total	Pooled rate (95%CI)	Model	P-value
Hematological													
Thrombocytopenia	CLL/SLL	3	45	305	0.1461 [0.0837; 0.2084]	Random		3	27	305	0.0881 [0.0322; 0.1440]	Random	
	B-NHL	4	39	190	0.2005 [0.1439; 0.2571]	Fixed		3	19	167	0.1048 [0.0586; 0.1509]	Fixed	
	T-NHL	–	–	–	–	–		–	–	–	–	–	
	ALL	7	84	495	0.1576 [0.1258; 0.1893]	Fixed		6	46	472	0.0847 [0.0598; 0.1096]	Fixed	
Anemia	CLL/SLL	3	62	305	0.2082 [0.0595; 0.3569]	Random		3	34	305	0.1124 [0.0048; 0.2199]	Random	
	B-NHL	2	40	160	0.2475 [0.1807; 0.3142]	Fixed		2	23	160	0.1434 [0.0891; 0.1977]	Fixed	
	T-NHL	–	–	–	–	–		–	–	–	–	–	
	ALL	5	102	465	0.2145 [0.1257; 0.3033]	Random		5	57	465	0.1186 [0.0533; 0.1840]	Random	
Neutropenia	CLL/SLL	3	106	305	0.3752 [0.1993; 0.5510]	Random		3	93	305	0.3133 [0.2081; 0.4186]	Random	
	B-NHL	4	68	190	0.4402 [0.2492; 0.6312]	Random		4	54	232	0.2350 [0.1382; 0.3319]	Fixed	
	T-NHL	1	7	35	0.2000 [0.0844; 0.3694]	–		2	25	136	0.1836 [0.1185; 0.2486]	Fixed	
	ALL	8	181	530	0.3795 [0.2643; 0.4947]	Random		9	172	673	0.2508 [0.1912; 0.3105]	Random	
Febrile neutropenia	CLL/SLL	1	9	55	0.1641 [0.0661; 0.2610]			1	9	55	0.1641 [0.0661; 0.2610]		
	B-NHL	1	12	129	0.0930 [0.0434; 0.1431]			1	12	129	0.0930 [0.0434; 0.1431]		

(Continued)

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TABLE 2 Continued

AEs	Diease	Any grade						Grade≥3					
		Includestudy	Event	Total	Pooled rate (95%CI)	Model	P-value	Includestudy	Event	Total	Pooled rate (95%CI)	Model	P-value
	ALL	2	21	184	0.1152 [0.0511; 0.1798]	Fixed		2	21	184	0.1152 [0.0511; 0.1798]	Fixed	
Non-Hematological													
ALL infections	CLL/ SLL	2	150	215	0.6983 [0.6370; 0.7596]	Fixed		1	29	90	0.3222 [0.2275; 0.4290]		
	B-NHL	1	19	31	0.6129 [0.4219; 0.7815]			2	12	96	0.1463 [0.0000; 0.3371]	Random	
	T-NHL	–	–	–	–	–		2	20	136	0.1791 [0.0000; 0.3602]	Random	
	ALL	3	169	246	0.6886 [0.6309; 0.7463]	Fixed		5	61	322	0.1941 [0.0852; 0.3030]	Random	
Fatigue	CLL/ SLL	2	41	215	0.2464 [0.0000; 0.4977]	Random		2	8	215	0.0520 [0.0000; 0.1451]	Random	
	B-NHL	2	49	160	0.3268 [0.1965; 0.4570]	Random		2	6	160	0.0245 [0.0000; 0.0700]	Random	
	T-NHL	1	11	35	0.3143 [0.1685; 0.4929]			1	3	35	0.0857 [0.0180; 0.2306]		
	ALL	5	101	410	0.2885 [0.1805; 0.3966]	Random		5	17	410	0.0348 [0.0024; 0.0672]	Random	
Dyspnoea	CLL/ SLL	2	29	215	0.1582 [0.0260; 0.2904]	Random		2	7	215	0.0291 [0.0067; 0.0516]	Fixed	
	B-NHL	1	7	31	0.2258 [0.0959; 0.4110]			1	0	31	0.0000 [0.0000; 0.1122]		
	T-NHL	1	6	35	0.1714 [0.0656; 0.3365]			1	4	35	0.1143 [0.0320; 0.2674]		
	ALL	4	42	281	0.1671 [0.0917; 0.2424]	Fixed		4	11	281	0.0256 [0.0052; 0.0461]	Fixed	
Cough	CLL/ SLL	3	64	305	0.2182 [0.1064; 0.3300]	Random		3	2	305	0.0039 [0.0000; 0.0146]	Fixed	
	B-NHL	2	47	160	0.2906 [0.2206; 0.3607]	Fixed		2	0	160	0.0000 [0.0000; 0.0103]	Fixed	

(Continued)

10.3389/fimmu.2022.1070660

TABLE 2 Continued

AEs	Diease	Any grade						Grade≥3					
		Includestudy	Event	Total	Pooled rate (95%CI)	Model	P-value	Includestudy	Event	Total	Pooled rate (95%CI)	Model	P-value
	T-NHL	1	12	35	0.3429 [0.1913; 0.5221]			1	0	35	0.0000 [0.0000; 0.1000]		
	ALL	6	123	500	0.2620 [0.1844; 0.3396]	Random		6	2	500	0.0018 [0.0000; 0.0091]	Fixed	
Fever	CLL/SLL	3	84	305	0.2742 [0.2242; 0.3242]	Fixed		3	10	305	0.0308 [0.0114; 0.0501]	Fixed	
	B-NHL	2	48	160	0.3698 [0.1082; 0.6314]	Random		2	1	160	0.0007 [0.0000; 0.0112]	Fixed	
	T-NHL	1	13	35	0.3714 [0.2147; 0.5508]			1	0	35	0.0000 [0.0000; 0.1000]		
	ALL	6	145	500	0.2848 [0.2456; 0.3240]	Fixed		6	11	500	0.0066 [0.0000; 0.0157]	Fixed	
Diarrhea	CLL/SLL	3	148	305	0.4852 [0.4292; 0.5413]	Fixed		3	49	305	0.1528 [0.0781; 0.2275]	Random	
	B-NHL	3	82	167	0.4892 [0.4138; 0.5646]	Fixed		3	28	167	0.1615 [0.1060; 0.2170]	Fixed	p<0.01, B-NHL vs T-NHL
	T-NHL	1	11	35	0.3143 [0.1685; 0.4929]			2	7	136	0.0326 [0.0000; 0.1004]	Random	p=0.02, CLL/SLL vs T-NHL
	ALL	7	241	507	0.4730 [0.4299; 0.5162]	Fixed		8	84	608	0.1232 [0.0623; 0.1841]	Fixed	
Nausea	CLL/SLL	3	60	305	0.1892 [0.0921; 0.2863]	Random		3	1	305	0.0006 [0.0000; 0.0079]	Fixed	
	B-NHL	3	51	167	0.2988 [0.2299; 0.3677]	Fixed		3	4	167	0.0134 [0.0000; 0.0329]	Fixed	
	T-NHL	1	9	35	0.2571 [0.1249; 0.4326]			1	0	35	0.0000 [0.0000; 0.1000]		
	ALL	7	120	507	0.2336 [0.1623; 0.3049]	Random		7	5	507	0.0022 [0.0000; 0.0090]	Fixed	
Vomiting	CLL/SLL	3	43	305	0.1377 [0.0991; 0.1763]	Fixed		3	1	305	0.0006 [0.0000; 0.0079]	Fixed	

(Continued)

10.3389/fimmu.2022.1070660

TABLE 2 Continued

AEs	Diease	Any grade						Grade≥3					
		Includestudy	Event	Total	Pooled rate (95%CI)	Model	P-value	Includestudy	Event	Total	Pooled rate (95%CI)	Model	P-value
	B-NHL	3	31	167	0.1852 [0.1263; 0.2441]	Fixed		3	6	167	0.0363 [0.0056; 0.0671]	Fixed	
	ALL	6	74	472	0.1519 [0.1197; 0.1842]	Fixed		6	7	472	0.0025 [0.0000; 0.0097]	Fixed	
Decreased appetite	CLL/SLL	3	43	305	0.1457 [0.0694; 0.2221]	Fixed		3	1	305	0.0006 [0.0000; 0.0079]	Fixed	
	B-NHL	2	26	160	0.1588 [0.1024; 0.2153]	Fixed		2	1	160	0.0066 [0.0000; 0.0234]	Fixed	
	ALL	5	69	465	0.1385 [0.1073; 0.1696]	Fixed		5	2	465	0.0015 [0.0000; 0.0083]	Fixed	
Hypokalemia	CLL/SLL	1	10	55	0.1821 [0.0801; 0.2840]			1	3	55	0.0551 [0.0008; 0.1151]		
	B-NHL	1	17	129	0.1320 [0.0734; 0.1901]			1	4	129	0.0314 [0.0001; 0.0608]		
	ALL	2	27	184	0.1442 [0.0931; 0.1948]	Fixed		2	7	184	0.0358 [0.0091; 0.0623]	Fixed	
URTI	CLL/SLL	2	40	215	0.2025 [0.0908; 0.3143]	Random		2	1	215	0.0007 [0.0000; 0.0091]	Fixed	
	B-NHL	1	6	31	0.1935 [0.0745; 0.3747]			1	1	31	0.0323 [0.0008; 0.1670]		
	ALL	3	46	246	0.1798 [0.1321; 0.2274]	Fixed		3	2	246	0.0011 [0.0000; 0.0095]	Fixed	
Arthralgia	CLL/SLL	1	14	55	0.2545 [0.1467; 0.3900]			1	0	55	0.0000 [0.0000; 0.0649]		
	B-NHL	2	20	136	0.1471 [0.0875; 0.2066]	Fixed		2	0	136	0.0000 [0.0000; 0.0268]	Fixed	
	ALL	3	34	191	0.1697 [0.1169; 0.2226]	Fixed		3	0	191	0.0000 [0.0000; 0.0191]	Fixed	
Edema	CLL/SLL	1	10	55	0.1818 [0.0908; 0.3090]			1	0	55	0.0000 [0.0000; 0.0649]		

(Continued)

10.3389/fimmu.2022.1070660

TABLE 2 Continued

AEs	Diease	Any grade						Grade≥3					
		Includestudy	Event	Total	Pooled rate (95%CI)	Model	P-value	Includestudy	Event	Total	Pooled rate (95%CI)	Model	P-value
	B-NHL	2	27	160	0.1687 [0.1107; 0.2267]	Fixed		2	4	160	0.0164 [0.0000; 0.0397]	Fixed	
	ALL	3	37	215	0.1719 [0.1215; 0.2223]	Fixed		3	3	215	0.0086 [0.0000; 0.0256]	Fixed	
Pneumonia	CLL/SLL	3	62	305	0.2226 [0.0838; 0.3614]	Random		3	45	305	0.1399 [0.1012; 0.1786]	Fixed	
	B-NHL	2	11	136	0.0795 [0.0341; 0.1250]	Fixed	p=0.04 (CLL/SLL vs B-NHL)	2	8	136	0.0562 [0.0176; 0.0949]	Fixed	p<0.01 (CLL/SLL vs B-NHL)
	T-NHL	1	8	35	0.2286 [0.1042; 0.4014]			2	8	136	0.0829 [0.0000; 0.2293]	Random	
	ALL	7	81	476	0.1839 [0.0970; 0.2708]	Random		8	64	608	0.1026 [0.0549; 0.1502]	Random	
Stomatitis	CLL/SLL	1	10	55	0.1818 [0.0908; 0.3090]			1	3	55	0.0545 [0.0114; 0.1512]		
	B-NHL	2	7	38	0.1775 [0.0567; 0.2982]	Fixed		2	1	38	0.0035 [0.0000; 0.0460]	Fixed	
	ALL	3	17	93	0.1800 [0.1021; 0.2579]	Fixed		3	4	93	0.0193 [0.0000; 0.0546]	Fixed	
Colitis	CLL/SLL	2	33	250	0.1320 [0.0900; 0.1740]	Fixed		2	29	250	0.1159 [0.0762; 0.1556]	Fixed	
	B-NHL	3	14	167	0.0777 [0.0373; 0.1181]	Fixed		2	8	136	0.0562 [0.0176; 0.0949]	Fixed	p=0.03, CLL/SLL vs B-NHL
	T-NHL	1	1	101	0.0099 [0.0003; 0.0539]		p<0.01, CLL/SLL vs T-NHL	1	0	101	0.0000 [0.0000; 0.0359]		p<0.01, CLL/SLL vs T-NHL
	ALL	6	48	512	0.0788 [0.0287; 0.1288]	Random		5	36	487	0.0660 [0.0091; 0.1229]	Random	
Constipation	CLL/SLL	1	26	160	0.1625 [0.1090; 0.2290]			1	1	160	0.0063 [0.0002; 0.0343]		
	B-NHL	2	16	136	0.1174 [0.0633; 0.1715]	Fixed		2	0	136	0.0000 [0.0000; 0.0106]	Fixed	

(Continued)

10.3389/fimmu.2022.1070660

TABLE 2 Continued

AEs	Diease	Any grade						Grade≥3					
		Includestudy	Event	Total	Pooled rate (95%CI)	Model	P-value	Includestudy	Event	Total	Pooled rate (95%CI)	Model	P-value
	T-NHL	1	6	35	0.1714 [0.0656; 0.3365]			1	0	35	0.0000 [0.0000; 0.1000]		
	ALL	4	48	331	0.1417 [0.1042; 0.1792]	Fixed		4	1	331	0.0020 [0.0000; 0.0104]	Fixed	
Asthenia	CLL/SLL	2	29	250	0.1158 [0.0762; 0.1555]	Fixed		2	3	250	0.0059 [0.0000; 0.0185]	Fixed	
	B-NHL	1	15	129	0.1163 [0.0666; 0.1845]			1	3	129	0.0088 [0.0000; 0.0203]		
	ALL	3	44	379	0.1160 [0.0838; 0.1482]	Fixed		3	6	379	0.0110 [0.0000; 0.0266]	Fixed	
Abdominal pain	CLL/SLL	2	26	250	0.1038 [0.0660; 0.1416]	Fixed		2	4	250	0.0150 [0.0000; 0.0301]	Fixed	
	B-NHL	1	19	129	0.1473 [0.0911; 0.2204]			1	2	129	0.0155 [0.0019; 0.0549]		
	ALL	3	45	379	0.1158 [0.0836; 0.1479]	Fixed		3	6	379	0.0152 [0.0029; 0.0275]	Fixed	
Bronchitis	CLL/SLL	1	21	160	0.1262 [0.0750; 0.1763]			1	5	160	0.0312 [0.0102; 0.0714]		
	B-NHL	1	1	7	0.1428 [0.0000; 0.4021]			1	0	7	0.0000 [0.0000; 0.4096]		
	ALL	2	22	167	0.1263 [0.0768; 0.1763]	Fixed		2	5	167	0.0304 [0.0027; 0.0581]	Fixed	
Headache	B-NHL	2	28	160	0.1696 [0.1117; 0.2275]	Fixed		2	1	160	0.0007 [0.0000; 0.0112]	Fixed	
	T-NHL	1	8	35	0.2286 [0.1042; 0.4014]			1	0	35	0.0000 [0.0000; 0.1000]		
	ALL	3	36	195	0.1783 [0.1249; 0.2317]			3	1	195	0.0006 [0.0000; 0.0108]	Fixed	
ALT/AST increased	CLL/SLL	1	17	55	0.3091 [0.1914; 0.4481]			3	13	305	0.0319 [0.0123; 0.0515]		

(Continued)

10.3389/fimmu.2022.1070660

TABLE 2 Continued

AEs	Diease	Any grade						Grade≥3					
		Includestudy	Event	Total	Pooled rate (95%CI)	Model	P-value	Includestudy	Event	Total	Pooled rate (95%CI)	Model	P-value
	B-NHL	4	49	190	0.3657 [0.1489; 0.5825]	Random		3	20	167	0.1863 [0.0000; 0.3960]	Random	
	T-NHL	1	20	35	0.5714 [0.3935; 0.7368]			2	37	136	0.2990 [0.1327; 0.4653]	Random	p<0.01 (CLL/SLL vs T-NHL)
	ALL	6	86	280	0.3890 [0.2331; 0.5449]	Random		8	70	608	0.1562 [0.0540; 0.2583]	Random	
Rash	CLL/SLL	2	37	250	0.1613 [0.0311; 0.2916]	Random		2	7	250	0.0238 [0.0049; 0.0426]	Fixed	
	B-NHL	4	44	190	0.1933 [0.1372; 0.2493]	Fixed		3	14	167	0.0257 [0.0000; 0.0535]	Fixed	
	ALL	7	91	523	0.1752 [0.1165; 0.2339]	Random		5	21	417	0.0246 [0.0083; 0.0409]	Fixed	
Rash maculo-papular	CLL/SLL	1	10	55	0.1818 [0.0908; 0.3090]			1	0	55	0.0000 [0.0000; 0.0649]		
	B-NHL	2	7	38	0.1822 [0.0597; 0.3048]	Fixed		2	2	38	0.0494 [0.0000; 0.1307]	Fixed	
	T-NHL	1	8	35	0.2286 [0.1042; 0.4014]			1	6	35	0.1714 [0.0656; 0.3365]		
	ALL	4	25	128	0.1932 [0.1249; 0.2615]	Fixed		4	8	128	0.0501 [0.0000; 0.1253]	Random	
Pneumonitis	CLL/SLL	1	5	55	0.0909 [0.0302; 0.1995]			2	23	250	0.0804 [0.0076; 0.1531]	Random	
	B-NHL	1	2	31	0.0645 [0.0079; 0.2142]			3	12	167	0.0705 [0.0299; 0.1111]	Fixed	
	T-NHL	–	–	–	–	–		1	1	101	0.0099 [0.0003; 0.0539]		
	ALL	2	7	86	0.0794 [0.0223; 0.1365]	Fixed		6	36	518	0.0565 [0.0179; 0.0951]	Random	

10.3389/fimmu.2022.1070660

TABLE 3 AEs leading to death related to treatment.

	Total patients	number	AEs leading to death related to treatment(n)
O'Brien-2018	55	2	respiratory syncytial viral pneumonia (1), metabolic acidosis in the setting of sepsis and renal failure (1)
Flinn-2018	160	4	staphylococcal pneumonia (2), sepsis (1), general health deterioration (1)
Davids-2020	90	2	general health deterioration (1), Pneumocystis jirovecii pneumonia (1)
Izutsu-2020	7	0	-
Flinn-2019	129	5	severe skin toxicity (2), suspected viral infection (1), fatal septic shock (1), pancolitis (1)
Horwits-2018	35	1	HSV pneumonia (1)
Zinzani-2022	101	3	pneumonitis (1), Epstein-Barr associated lymphoproliferative disorder (1), sepsis (1)

maintenance and survival signals from BCR/TCR, chemokine receptors, or co-stimulatory molecules, in which the PI3K-mTOR signaling plays an important role. It's well known that many mature B-cell malignancies show constitutive PI3K pathway activation because of chronic BCR activation, and are susceptible to kinase inhibitors that disrupt BCR signaling (55–57). In addition, deregulation of the PI3K-AKT pathway has been shown to have a role in the pathogenesis of TCL

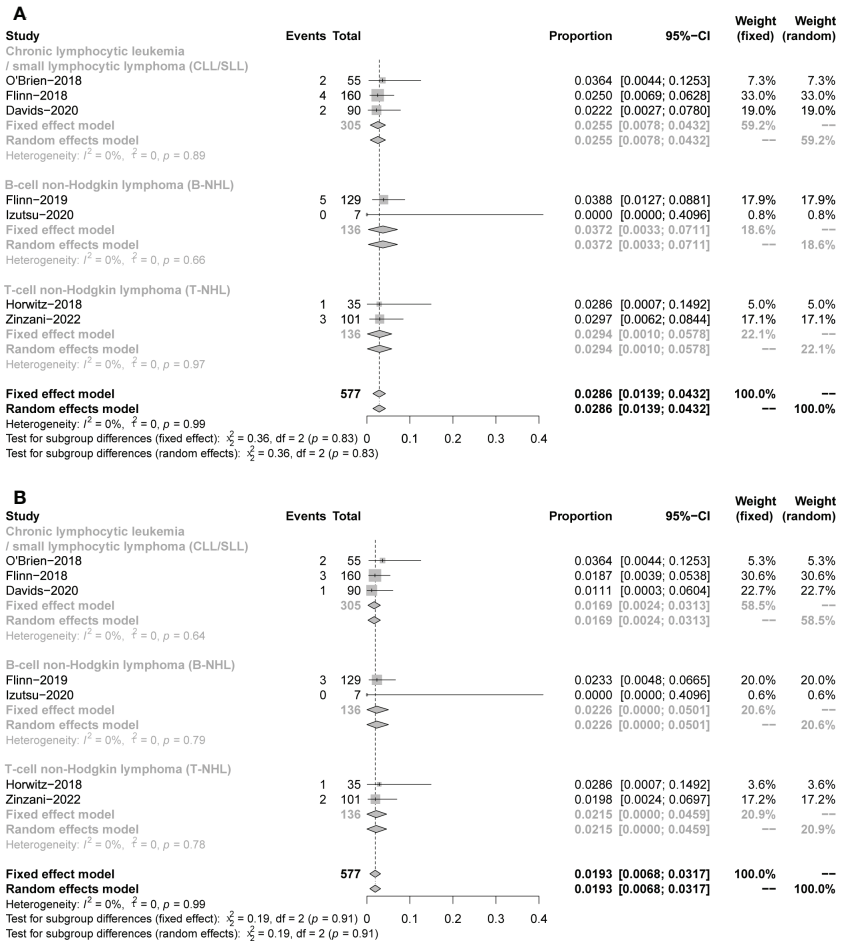

FIGURE 11
The forest plot of treatment-related mortality. **(A)**. The incidence of fatal AEs related to duvelisib. **(B)**. The incidence of fatal infectious AEs related to duvelisib.

(46, 58–61).Meanwhile, inhibitors of PI3K-mTOR signaling can effectively treat AITL in preclinical models (46, 58, 62). The second aspect of PI3Kδ inhibition is direct negative impact of the tumor microenvironment on the mitogenic and survival signaling of cells. Inhibition of PI3K-δ induces multiple effects on malignant B-cells, including the arrest of malignant B-cell proliferation and migration mediated by the tumor microenvironment, and inhibition of chemokine secretion derived from tumor cell (44, 56, 63, 64). In some PTCL subtypes, particularly AITL, molecular profiling has elucidated specific microenvironmental signatures associated with poor outcome (65). Of note, the study also shows that AITL presents significant enrichment of B-cell in the microenvironment. These mechanisms can explain why PI3Kδ inhibitors can be used to treat AITL. The third aspect focuses on the fact that inhibition of PI3K-δ, PI3K-γ, or both could activate antitumor immune responses. Indeed, PI3K-δ inhibition enhances antitumor immune response primarily due to a preferential inhibition of immunosuppressive Treg cells in the preclinical model (66–72). while PI3K-γ inhibition reduces the differentiation and migration of key Immunosuppressive cells in the tumor microenvironment, such as M2 tumor-associated macrophages, negatively regulating effector T and natural killer (NK) cells (33, 41, 42).

Despite therapy advances targeting CLL patients (73), CLL/SLL remains incurable (74). Thus, novel and effective agents for R/R CLL/SLL patients are needed. Duvelisib is currently the only FDA-approved PI3K inhibitor for the monotherapy of CLL/SLL. Our meta-analysis revealed that duvelisib could offer reasonable efficacy in patients with RR CLL/SLL without being negatively affected by del17P/TP53 mutation. The pooled ORR of duvelisib was 70%, 12- month PFS and OS were 61% and 79%, respectively. Another approved PI3K-δ inhibitor idelalisib demonstrated only 48% of ORR and 6.9-month median PFS (75). Our data show duvelisib provides improved efficacy over idelalisib. This conclusion is also consistent with previous preclinical CLL models. Dual PI3K-δ, g inhibition has shown stronger activity than blocking either isoform alone (44, 56, 76). Moreover, in a phase 3 randomized study in CLL/SLL, duvelisib demonstrated significantly improved ORR, PFS, and OS compared with ofatumumab (CD20 inhibitor) (24). In addition, duvelisib has also shown high response rates in patients with R/R CLL/SLL who progressed on ofatumumab (77). Many preclinical studies indicate that p53 could inhibit PI3K/AKT/mTOR pathway through multiple targets, mutation or deletion of p53 leading to abnormal activation of these pathways (78–83). Studies show that patients with del(17p) and/or TP53 mutations are more likely to relapse, even when treated with ibrutinib (BTK inhibitor) or venetoclax (BCL2 inhibitor) (84–86). However, duvelisib can prevent CLL/SLL tumor cell proliferation and metabolism by inhibiting abnormally activated PI3K/AKT/mTOR signaling in the context of del(17p) and/or TP53 mutations. Similarly, in our study, ORR,12-month PFS and 24-month PFS, in the subgroup of patients with del(17p) and/or TP53 mutations were similar to those of the whole population, which meant duvelisib could decrease the recurrence due to del(17p) and/or TP53 mutations. Considering the increasing use of ibrutinib and venetoclax in first-line therapy (87–90), duvelisib has been shown to effectively increase the sensitivity of CLL cells to BCL2i and BTKi (91, 92). For patients who are refractory to or intolerable to BTKi or BCL2i, duvelisib is an effective option for RR CLL/SLL with or without del(17p) and/or TP53 mutations.

Although most iNHL patients initially respond to standard chemoimmunotherapy with prolonged remission, eventually, all patients experience disease progression or relapse (93). There are currently several approved options for relapsed or refractory iNHL, but the multiple toxicities of therapies and resistance or transformation to advanced or aggressive lymphomas remain challenges. In our meta-analysis, Response rates of duvelisib were clinically meaningful in patients with RR iNHL across subtypes. In all patients, the pooled ORR was 70%, the 12-month PFS was 44%, and the 12-month OS was 77%. In FL patients, the pooled ORR was 69%. There are already several PI3K inhibitors in clinical trials for RR iNHL patients: copanlisib (intravenous inhibitor of PI3K-α, -β), idelalisib (oral inhibitor of PI3K-δ), umbralisib (oral inhibitor of PI3K-δ, CK-e). ORR in these trials ranged from 47% to 59% in all patients and 45% to 59% in FL patients (94–96). Despite heterogeneity in cross-trial patient selection and prior treatments, our data reveal that duvelisib had higher efficacy than other PI3Ki treatments. Repeating chemotherapy, even combined with different CD20 antibodies like Obinutuzumab, caused cumulative toxicities and decreased efficacy. In our study, almost all RR iNHL patients had been previously treated with rituximab (100%, 94%, 100%, and 100%, reported in 4 studies, respectively) or alkylating agent (98%, 81% reported in 2 studies, respectively). Given the increasing use of rituximab and alkylating agents for the untreated or RR iNHL, duvelisib monotherapy may provide an option for R/R patients. Additionally, PI3Kδ inhibition restores the sensitiveness of FL cells on the anti-apoptotic protein BCL-2 (63), showing a rationale potent for combined PI3Kδ and BCL-2 inhibition.

In the aggressive B-NHL, the pooled ORR was 68% in MCL and 17% in other aNHL (Mainly DLBCL), respectively, while in copanlisib, the ORR of the aggressive cohort ranged from 7% in DLBCL patients to 64% in MCL patients (97). The responses to ibrutinib have been reported at 37% in ABC DLBCL patients and 5% in GCB DLBCL patients. Although no data on DLBCL subtypes is available here, considering that ABC DLBCL often selectively acquires mutations targeting B-cell receptors (BCRs) that promote chronically active BCR signaling (98), duvelisib may be a rationale candidate for ABC DLBCL. Data from a phase 1 study have demonstrated that the combination of duvelisib with standard therapies, bendamustine, and rituximab, is well tolerated and presents a novel therapeutic option in B-NHL, including DLBCL and MCL (99).

Peripheral T cell lymphomas (PTCLs) are highly heterogenous diseases with a poor prognosis. Immunotherapy and novel chemotherapy protocols are active in B cell lymphomas, unluckily with a high failure rate and frequent relapses in T-NHL. Indeed, new treatments for peripheral T cell lymphomas (PTCLs) are developing, but patients with PTCLs still have poor survival. In our analysis, the pooled ORR was 49% in patients with RR PTCL, which is also satisfactory, for the response rates of other approved single agents for RR PTCL, like romidepsin (HDACi), belinostat (HDACi), and pralatrexate (antifolate), range from 25% to 29% (20, 100, 101). And, for another PI3K inhibitor copanlisib, the ORR of PTCL was only 21% (97). Additionally, a phase I study of duvelisib combined with romidepsin, has shown a better ORR(58%) than previous therapy using romidepsin alone (102). In the subgroup analysis of the PTCL subtype, we found that AITL patients appeared to have higher pooled ORR than other PTCL patients (67% vs. 42%, p=0.01). The similar results could be observed in phase I study of duvelisib combined with romidepsin, AITL has also shown a better ORR than other PTCL (68% vs. 53%). Meanwhile, we also noticed a high pooled PDR of 47%, also observed in copanlisib (36%) for all PTCL. However, in one of our studies included patients with AITL had a lower PDR (0 of 3) compared to other PTCLs (6 of 13) (33). These results suggested the existence of heterogeneity between diseases. Long-term outcomes of retrospective series, such as the International T-cell lymphoma project (ITCP), displayed that the 5-year failure-free survival (FFS) for the AITL patients receiving CHOP was only 18%. Therefore, more novel therapies should be explored for T-NHL. Duvelisib would be a good option for patients with RR PTCL, especially AITL. Given the phenomenon observed in our study, clinical trials with larger sample size were needed to characterize more details of differential efficacy of duvelisib in PTCL subtypes.

PI3K-δ, g dual inhibition significantly changes the cellular composition of the microenvironment by reducing Treg cell numbers and activating CD4+ and CD8+ cells, which clonally expand and display enhanced cytotoxic and cytolytic properties. Despite enhancing anti-tumor immunity, activated T cells invariably cause immune dysregulation in normal tissues. The safety profile in the individual disease subgroup was found to be generally consistent with the subjects. Nearly all patients in both subgroups experienced an AE. AEs were generally low grade and manageable, probably leading to dose reductions/interruptions, among which 33% of patients discontinued treatment, similar to observations with other PI3K-δ inhibitors (95, 103). Immune-related toxicities are common, leading cause of treatment discontinuation, including transaminase elevations, diarrhea or colitis, pneumonitis, and rash. In our meta-analysis, diarrhea and transaminase elevations were the most frequent non-hematologic AEs (47% and 39%, respectively) and the most common grade≥3 non-hematologic events (12% and 16%, respectively) in all diseases, which could be managed by adjusting dose, discontinuing and recovering treatment when monitoring hepatic function. Only 1 patient was reported to die of treatment-induced pancolitis. Similar results have been observed in other PI3K inhibitors. The incidence of diarrhea in any grade and grade ≥3 was 43% and 13% in idelalisib, and 34% and 5% in copanlisib, respectively. For transaminase elevations, the incidence in any grades and grade ≥3 were 47% and 13% in idelalisib, 28% and 2% in copanlisib, respectively (94–96). The subanalysis showed the incidence of grade≥3 spoke of potential differences among subgroup patients. This difference may be due to toxic modulation by disease-specific factors, or different prior treatments, or differences in immune cell populations. Consistent with this difference, elevated transaminases are a major factor for treatment discontinuation compared to diarrhea in NHL, whereas in CLL/SLL, the opposite could be witnessed.

Pneumonitis and rash, which are also common in the application of idelalisib and copanlisib, are the other leading cause of treatment discontinuation. Grade≥3 pneumonitis occurred in 6% of patients, including 1 fatal thought to be duvelisib relevant. Some patients experienced skin toxicity at any grade (rash 18%, rash maculo-papular 19%), and greater than grade 3 are uncommon (rash 2%, rash maculo-papular 5%) but still cause 2 casualties, which are duvelisib relevant. Duvelisib has a satisfactory hematological safety profile. The most frequent hematologic AEs were neutropenia (38%) and anemia (21%). Notably, neutropenia was the most frequent severe AEs (25%). The incidences of neutropenia and anemia in other PI3K inhibitors were similar to our results (94, 95). These events seldom require treatment modifications due to their reversibility, and rarely result in treatment discontinuations (neutropenia in 1 patient).

Grade≥3 serious infections occurred in 19% of patients treated with duvelisib. Infectious complications are major causes of mortality in duvelisib treatment, as a result of the humoral immunodepression inherent to the disease and therapy-induced immunosuppression (104, 105). A total of 17 treatment-related deaths in 577 patients (pooled rate of 3%) were reported in our included studies, 12 (pooled rate of 2%) were infection-related. These were similar in copanlisib (3/142,1 infection-related) and idelalisib (8/125, 4 infection-related). Severe pneumonia occur in 10% of patients, and few are fatal, 5 of which were assessed as related to duvelisib: staphylococcal pneumonia (n=2), pneumocystis jirovecii pneumonia (PJP), respiratory syncytial viral pneumonia, and HSV pneumonia (n=1 each). If pneumonias are suspected, appropriate and extensive evaluations should be performed for infectious etiologies of pneumonias. In clinical trials, many patients have been treated with antibiotics and corticosteroids and most recovered. Prophylaxis for PJP infections is required to mitigate the risk of these opportunistic infections, which have been reported in other B-cell receptor inhibitors (106–109). And antiviral prophylaxis also should be implemented at the consideration of the clinicians.

Generally, duvelisib is effective in treating mature lymphocyte neoplasms. Still there are significant patients requiring dose adjustments, and up to 33% of patients discontinue the treatment because they could not tolerate the AE (41% in CLL/SLL, 27% in B-NHL and T-NHL). A reasonably designed intermittent dosing regimen may help reduce the incidence of AEs without compromising efficacy. The TEMPO study (NCT04038359) evaluates the effects of duvelisib prespecified 2-week dose holidays on responses and safety/tolerability in patients with iNHL (110). Similarly, in mouse models, a modified treatment with intermittent dosing of PI3Kδi led to a decrease in tumor growth without inducing pathogenic T cells in colonic tissue, indicating that alternative dosing regimens might limit the toxicity of colits (111). What's more, extended survival is observed in patients who had treatment interruptions with the PI3Kδ inhibitor idelalisib in FL and CLL, indicating that discontinuous PI3Kδ therapy may also achieve clinical benefit (112).

Due to the differences in study design and patient enrollment, it is difficult to compare the risk of immunotoxicity directly. between different PI3K-δ inhibitors. However, compared with idelalisib, which only inhibits PI3K-δ, duvelisib may reduce autoimmune complications through the simultaneous inhibition of PI3K-γ based on preclinical data, preventing leukocyte recruitment and reducing dextran sulfate sodium-induced colitis in mice (53). Pharmacological blockade of PI3K-γ suppresses joint inflammation in mouse models of rheumatoid arthritis and eases inflammation in a model of colitis-associated cancer (113, 114). Further studies to elucidate the effect of PI3K-γ on the immune system during duvelisib treatment are of great interest. Another intravenous PI3K-δ/α inhibitor copanlisib, has a specific AE profile, including hypertension and hyperglycemic effects mediated by PI3K-α isoform inhibition, limiting its application in elderly patients with a high prevalence of these comorbidities. And hospital visit for infusional therapies represents an essential concern for some people, who are likely to benefit significantly from oral drug treatment.

There are still some limitations in our study. Firstly, most of the studies involved were single-armed studies without double-blinded randomized controlled trials, which may lead the potential performance bias. In addition, a small number of patients received different drug doses, and some AEs may be dose-dependent. Finally, although the diseases were all relapsed or refractory, the degree of lymphoma and leukemia was dispersive, which might have caused bias in the final analysis and made comparisons with other studies difficult. No results of survival benefit were defined in the study due to the varied length of follow-up time and survival data in some studies shown incompletely.

In conclusion, our analysis shows that duvelisib is an effective monotherapy for RR mature lymphocyte neoplasms. Duvelisib could offer favorable efficacy in patients with RR CLL/SLL and is not negatively affected by del17P/TP53 mutation. Besides, duvelisib has better efficacy than other approved PI3K inhibitors in iNHL treatment, including FL. And duvelisib monotherapy shows unexpectedly good efficacy in PTCL, especially in AITL. However, the efficacy of duvelisib in aNHL was limited. Although fatal and severe toxicity occasionally exists, risk and severity in duvelisib treatment have the potential to be mitigated through identification and management properly. Based on the limits of the single-arm studies, more randomized controlled studies are needed to explore the efficacy and safety of duvelisib with or without combination with other drugs for patients with RR lymphoma and leukemia.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding author.

Author contributions

ZW and HZ have contributed equally to this work and share first authorship. ZW and HZ participated in the study design. ZW and HZ acquired, analyzed and interpreted the data. ZW wrote the manuscript. HZ and TN revised the manuscript. JX proofread the language. JW provided methodological advice. All authors contributed to the manuscript and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.1070660/full#supplementary-material

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