



Fludarabine and neurotoxicity in engineered T-cell therapy

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Abstract

Adoptive T-cell therapy, incorporating engineered T cell receptors (TCRs) or chimeric antigen receptors (CARs), target tumor antigens with high affinity and specificity. To increase the potency of adoptively transferred T cells, patients are conditioned with lymphodepleting chemotherapy regimens prior to adoptive T-cell transfer (ACT), and data suggest that fludarabine is an important component of an effective regimen. In a recent clinical trial using CAR-T cells engineered to target the CD19 B-cell antigen to treat acute lymphoblastic leukemia, JCAR-015 (NCT02535364), two patient deaths due to cerebral edema led to trial suspension. The lymphodepleting agent fludarabine was suggested as the causative agent, in part due to its known association with neurotoxicity and its ability to induce greater potency. In a similar CAR-T study also incorporating fludarabine in the preconditioning regimen, ZUMA-1 (NCT02348216), one patient died of cerebral edema. However, subsequent deaths in the JCAR-015 study after removal of fludarabine and improved understanding behind the mechanisms of CAR-T-related encephalopathy syndrome (CRES) indicate that fludarabine is not the primary causative agent of cerebral edema and that it can be safely incorporated into the preconditioning regimen for ACT. Since entering clinical use in the late 1980s as a chemotherapy agent, fludarabine and similar analogs have been associated with lethal neurological toxicity, yet the manifestation and timing of symptoms are distinct to those observed recently in ACT. Herein, we review the history of fludarabine development as a chemotherapeutic agent, and discuss the safety of its continued use in preconditioning regimens for ACT.

Introduction

The term “adoptive immunotherapy” was first used to explain the graft-vs.-tumor effect observed following allogeneic stem cell transplants for leukemia [1]. In these early studies, chemotherapy-induced immunosuppression was found to be necessary to enable successful stem cell grafts. Later studies demonstrated the importance of the T-cell component of the stem cell graft in preventing relapse [2]. Allogeneic stem cell transplantation (ASCT) signified the first potentially curative approach to improve cancer therapy

for patients with hematological malignancies, and it was also a first example of cell-based immune oncology.

Lymphodepleting agents were selected based on clinical experience from single agent and combination chemotherapies and include the alkylating agents chlorambucil and cyclophosphamide, which were among the first identified chemotherapeutics. The family of nucleoside analogs shortly followed, encompassing the pyrimidine nucleoside cytarabine and the purine nucleosides cladribine, pentostatin and fludarabine that demonstrate potent cytotoxic activity [3, 4]. Purine nucleosides were developed specifically to improve on the activity of cytarabine, and fludarabine’s relative resistance to adenosine deaminase improved its bioavailability. While not used in the original transplant regimens, fludarabine became an important part of pre-transplantation conditioning regimens due to its improved tolerability and outcomes [5–7]. Fludarabine induces cellular cytotoxicity via multiple pathways that ultimately lead to an inhibition of DNA synthesis. A rate-limiting step in this process is the activity of deoxycytidine kinase, which is abundant in lymphocytes, making them susceptible to accumulation of F-ara-ATP, the active metabolite of fludarabine, and hence

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Table 1 Details of neurotoxicity observed in clinical trials using fludarabine chemotherapy

Reference	Year	No. of patients	Disease	Fludarabine (mg/m ²)	Treatment regimen	Onset of symptoms (days)	Reported symptoms	Recovered (Y/N)	Autopsy (Y/N)
Spriggs et al. [22]	1986	2	AML	60	x d1-5; 15 d; 2 c	70	Dementia, incontinence, and quadriparesis	N	Y
Spriggs et al. [22]	1986	1	Acute leukemia	100	x d1-5; 15 d; 1 c	35	Dementia, incontinence, blindness, quadriparesis and coma	N	Y
Warrell et al. [21]	1986	5	Acute leukemia	150	x d1-5; 7 d; 1 c	21-43	Dementia, incontinence, blindness, seizure and coma	1Y 4N	1Y 3N
Chun et al. [23]	1986	13	Acute leukemia	96	x d1-5; x d; x c	NS	Dementia, hallucination, blindness and quadriparesis	N	N
Chun et al. [23]	1986	1	Acute leukemia	125	Single bolus	NS	Dementia, blindness, quadriparesis	N	N
Merkel et al. [24]	1986	1	T-ALL	77	x d1-5; 28 d; x c	60	Dementia, myoclonus and coma	N	N
Weiss et al. [25]	1986	1	NSLCC	22	x d1-5; 28 d; x c	41	Dementia, cerebral atrophy and coma	N	N
Rainey et al. [26]	1988	1	SLCC	1	x d1-5; 28 d; x c	NS	Ataxia and dizziness	NS	NS
Hochster et al. [27]	1992	6	NHL	18	x d1-5; 28 d; 1-25 c	NS	Vision loss, hearing loss and confusion	Y	N
Komblau et al. [28]	1993	2	Acute leukemia	30	x d1-5; x d; x c	NS	Severe, progressive cerebral dysfunction	N	N
Cohen et al. [29]	1993	1	CLL	20-25	x d1-5; 28 d; 6 c	>170	Blurred vision and gait disturbance	Y	N
Cohen et al. [29]	1993	1	Mycosis fungoides	20-25	x d1-5; 28 d; 8 c	>220	Seizure, loss of consciousness and hemiparesis	Y	N
Cheson et al. ^a [30]	1994	3	CLL	25	x d1-5; 28 d; x c	>120	Dementia, vision loss, seizure and paralysis	N	N
Cheson et al. ^a [30]	1994	1	FSCL	18,25	x d1-5; 28 d; x c	>220	Ataxia and dysphasia	N	N
Cheson et al. ^a [30]	1994	5	CLL	25-40	x d1-5; 28 d; 3-10 c	>90	Dementia, dizziness, seizures and quadriparesis	Y	N
Johnson et al. [31]	1994	5	CLL	25	x d1-5; 28 d; x c	20-Jan	Headaches, hemiparesis and quadriparesis	4 Y 1N	Y
Zabernigg et al. [32]	1994	1	CLL	20-25	x d1-5; 28 d; 6 c	360	Dementia, hemiparesis and coma	N	Y
Johnson et al. [33]	1996	3	CLL	25	x d1-5; x d; 6 c	NS	NS	N	N
Gonzalez et al. [34]	1999	3	CLL	25	x d1-5; x d; x c	120-250	Paralysis, quadriparesis and dementia	N	1Y 2N
Sorensen et al. [35]	1997	1	CLL	25	x d1-5; 21-28 d; 1-12 c	180	Visual and cerebral disturbances	Y	N
Cid et al. ^c [36]	2000	1	CLL	18-25	x d1-5; 21-28 d; x c	>220	Hemiparesis	NS	NS
Leonard et al. ^c [37]	2002	1	CLL	NS	NS	150	Aphasia and right hemiparesis	NS	NS
Saumoy et al. ^c [38]	2002	1	CLL	25	x d1-5; 21-28 d; x c	NS	Progressive neurological syndrome	N	N

Table 1 (continued)

Reference	Year	No. of patients	Disease	Fludarabine (mg/m ²)	Treatment regimen	Onset of symptoms (days)	Reported symptoms	Recovered (Y/N)	Autopsy (Y/N)
Kiewe et al. ^c [39]	2003	1	CLL	25	x d1-5; 21-28 d; x c	>180	Progressive neurological syndrome	N	N
Rossi et al. [40]	2004	1	CLL	40	Oral x d1-5; 21-28 d; ≤8 c	NS	Peripheral neuropathy	Y	N
Hagenbeek et al. [41]	2006	1	NHL	25	x d1-5; 21-28 d; x c	NS	Loss of consciousness and peripheral neuropathy	Y	N
Kalita et al. ^c [42]	2008	1	CLL	NS	x d1-5; 21-28 d; 2 c	180	Loss of consciousness, seizures and right hemiparesis	Y	N
Lee et al. ^c [43]	2010	1	MM	40	x d1-5; 21-28 d; x c	35	Dementia, vision loss and quadriparesis	N	N
Lejniece et al. ^c [44]	2011	1	CLL	NS	x d1-5; 21-28 d; 7 c	NS	Hemiparesis, tremors, speech loss and motor aphasia	N	N

NS not stated, ^c case studies, *d* days, *c* cycles, *x c* number of cycles not stated, *AML* acute myeloid leukemia, *T-ALL* T-cell acute lymphoblastic leukemia, *NSCLC* non-small cell lung carcinoma, *CLL* chronic lymphocytic leukemia, *Follicular* small-cleaved lymphoma, *NHL* non-Hodgkin's lymphoma, *MM* metastatic melanoma

^aIndependent studies reviewed by Cheson et al. Unless stated, infusions were administered intravenously

giving fludarabine particularly potent lymphodepleting properties [8, 9].

In the setting of adoptive T-cell transfer (ACT)-based immune oncology, use of lymphodepleting chemotherapy enhances potency, in addition to making “room” for the infused cells to engraft [10, 11]. Lymphodepletion was applied to the adoptive transfer of tumor-infiltrating lymphocytes (TILs) for the treatment of metastatic melanoma and led to enhanced TIL homing and anti-tumor efficacy [12]. Use of the non-myeloablative transplant regimen containing fludarabine and cyclophosphamide improved overall response rates compared to patients who did not receive preconditioning, and this regimen therefore was taken forward in a majority of subsequent engineered ACT studies [13]. ACT engineered with chimeric antigen receptors (CARs) and high affinity T-cell receptors (TCRs) targeted against tumor-specific antigens have shown promise in the clinic [14–16].

Recently there have been reports of lethal neurotoxicity due to cerebral edema in six patients treated with CD19 CAR-T cells, across two different CD19 CAR-T studies [17–19]. After the first two patients were reported, links between this neurotoxicity and fludarabine were suggested, as these patients had received fludarabine in addition to cyclophosphamide as part of their preconditioning regimen, and neurotoxicity is known to be associated with this drug. However, the neurological symptoms associated with fludarabine as a chemotherapeutic agent are distinct from CD19 CAR-T neurotoxicity in both symptoms and timing. Specifically, fludarabine-associated neurotoxicity has a later onset, and cerebral edema is not reported. Since initial reports, significant neurotoxicity, including cerebral edema, occurred in the absence of fludarabine-containing preconditioning regimens.

Although it is now broadly accepted that fludarabine is not a primary driver of neurotoxicity at the doses used in ACT studies, it is important to understand its role in the efficacy and toxicity of adoptive T-cell therapy, in order to develop products with the optimal balance of risk and benefit for patients. Here, we summarize the use of fludarabine from a historical perspective to the present day, reporting efficacy and toxicity of fludarabine compared to alternative agents and in different therapeutic regimens, and the rationale for its use in combination with ACT.

History of fludarabine safety and efficacy as a chemotherapeutic agent

Initial phase I clinical trials testing fludarabine for the treatment of solid tumors started intravenous (i.v.) dosing at 260 mg/m² for 5 days, which was soon reduced by 60% due to severe neutropenia [20]. Anti-tumor activity was most

potent against hematological malignancies resulting in dose escalating Phase II trials for treatment of acute leukemia ($\geq 100 \text{ mg/m}^2$) (Supplementary Tables 1 and 2). Although efficacious, high incidence of delayed, progressive central nervous system (CNS) toxicity was reported (described in more detail in the following section). This toxicity was associated with high dose of fludarabine and was addressed by reducing doses to $25\text{--}40 \text{ mg/m}^2$ for 5 days i.v. in monthly cycles [21–24]. Generally, $25\text{--}40 \text{ mg/m}^2$ dose regimens were well tolerated, although neurotoxic events continued to be reported [25–44] (Table 1, Supplementary Table 2). By the late 1990s, fludarabine-based chemotherapy regimens became the standard first- and second-line therapies for the most common adult leukemia, chronic lymphocytic leukemia (CLL), and a common therapeutic for many other leukemias and lymphomas.

Compared to alternative chemotherapy regimens, fludarabine as a monotherapy produced superior or statistically similar response rates to the chlorambucil, cyclophosphamide-doxorubicin hydrochloride-vincristine (CHOP) regimen, cyclophosphamide-adriamycin-cisplatin (CAP) regimen and fludarabine-prednisone, with no significant increase in toxic effects. However, no difference in survival was observed (Supplementary Table 3). Combining fludarabine and cyclophosphamide (FC) gave rise to both enhanced response rates and extended survival with no increase in toxicity (Supplementary Table 3). The addition of targeted monoclonal antibodies, such as the anti-CD20 antibody rituximab (FCR) or the anti-CD52 antibody alemtuzumab, further enhanced response and survival in CLL and B-cell non-Hodgkin's lymphoma (B-NHL), although increasing potency also led to increased incidence of toxicity in more vulnerable patients, such as the elderly (Supplementary Table 3). Lowering the dose of FC (FCR-Lite regimens) or replacing FC with alternate agents such as bendamustine often showed more favorable toxicity profiles, making them more suitable for vulnerable patient populations (Supplementary Table 3).

Fludarabine-induced neurotoxicity

Fludarabine is rapidly converted in plasma to F-araA, which accumulates in cells where it is phosphorylated to its active metabolite F-araATP by deoxycytidine kinase [9]. This metabolite has a half-life of approximately 20 h in vivo, and its clearance is dependent upon adequate renal function. The main dose-limiting toxicities associated with fludarabine that are common among chemotherapy agents were myelosuppression and risk of infection, which accounted for the majority of fatalities and failed outcomes. Neurological abnormalities continued to cause concern and were initially confounded by an absence of a mechanism to explain their

onset and occurrence. Somnolence and peripheral neuropathy during and immediately following fludarabine infusion were frequently observed but usually reversible, and these symptoms had been described previously with other anti-metabolite drugs [22] (Supplementary Table 2). Of greater concern were the late-onset neurological symptoms (20–250 days) that manifested in a pattern of progressive visual disturbances, peripheral neuropathy, dementia, ataxia, hemiparesis, quadriparesis and blindness, sometimes leading to coma and death (Table 1). Cheson et al. [30] described these late onset symptoms to be associated specifically with purine analogs. While the mechanisms of purine analog-mediated neurotoxicity are still not entirely clear, there is evidence to support the hypothesis that neurotoxicity results from disruption of normal synaptic function. Purine analogs are known to cross the blood brain barrier (BBB), and reduced uptake into the CNS protects against neurotoxicity [45–47]. Adenosine is a major regulator of neuromodulation, and purine analogs have been shown to bind to adenosine receptors [48]. Specifically, fludarabine is an A_1 receptor agonist [49]. The A_1 receptor is expressed primarily on neurons, and agonism causes somnolence and may induce coma, while antagonism can induce seizures. The concentration at which fludarabine is predicted to agonize the A_1 receptor is pharmacologically relevant for patients receiving higher doses of fludarabine [50].

Where autopsies or MRI could be performed, neurological abnormalities were characterized by demyelination of white matter, variable extents of necrosis, areas of enlarged astrocytes and oligodendrocytes, and multiple lesions in white matter identified by high signal by MRI [21–23, 29, 30, 34, 36, 38, 42, 43] (Table 1). Evidence of JC virus in cerebrospinal fluid or brain biopsies supported the diagnosis of the leukoencephalopathy (LE) or progressive multifocal leukoencephalopathy (PML) [32, 34, 36–39, 44] as a contributing factor to purine analog neurotoxicity. JC virus infects over 70% of the population and only becomes pathological in immunosuppressive environments, again increasing susceptibility to the elderly and patients with advanced disease. The virus infects oligodendrocytes and astrocytes leading to irreparable degeneration [34]. In the case of CLL, 90% of PML diagnoses since 1990 occurred in patients treated with purine analogs, such as fludarabine [51].

In summary, fludarabine demonstrated enhanced potency against hematological malignancies compared to alternative agents but was associated with a distinct set of late onset, progressive neurological symptoms in isolated cases. Neurological abnormalities were more prominent at high doses and in more vulnerable patients, such as those with advanced disease or elderly patients that often harbor renal insufficiency, thereby heightening fludarabine exposure.

Dose reduction of fludarabine is associated with a reduction of these toxicities in patients.

Development of lymphodepleting pre-conditioning regimens for ASCT

Despite increasing response rates with new combination-based chemotherapy regimens, patients with advanced hematological malignancies gained little survival advantage until the emergence of ASCT as the first potentially curative treatment strategy [1, 52, 53]. Successful engraftment and durability of cells relied heavily on conditioning the host immune system to “create room” by increasing the levels of homeostatic cytokines including IL-7 and IL-15 and diminishing regulatory mechanisms [54, 55].

Myeloablative pre-conditioning regimens were associated with high risk of toxicity and were considered unsuitable in vulnerable patients [56]. While non-myeloablative regimens reduced toxicity, durability of remission was compromised, leading to the development of reduced intensity conditioning (RIC) regimens that characteristically included a nucleoside analog, such as fludarabine, in combination with an alkylating agent, such as melphalan or busulfan [57–59] (Table 2).

In clinical trials, fludarabine-melphalan (Flu-M) RIC proved superior to FC in increasing engraftment of ASCT and prolonging disease-free survival [53, 59–62] (Table 2). However, non-relapse mortality (NRM) rates that largely resulted from acute graft-vs.-host disease (GVHD) remained high, often correlating with starting disease burden [58, 59, 61]. Efforts to enhance cytotoxic activity of pre-conditioning regimens led to trials replacing fludarabine for a more active nucleoside analog, clofarabine, increasing durability [63–68]. However, RIC regimens consisting of Flu-M, Flu-busulfan, clofarabine-M or clofarabine-busulfan resulted in similar progression-free survival (PFS) and NRM rates across malignancies, making it difficult to dissect optimal treatment strategies [58, 61, 65–77]. Irrespective of individual combinatorial approaches, pre-conditioning regimens containing nucleoside analogs were associated with increased engraftment and durability of transferred cells.

Fludarabine-associated neurotoxicity in ASCT

A retrospective analysis of patients receiving fludarabine-containing pre-conditioning for hematopoietic cell transplantation showed a 2–3% incidence of grade 3 or higher neurologic events, manifesting in a similar pattern of symptoms to those observed previously from fludarabine

alone, from somnolence during infusion, headaches, blurred vision to blindness, seizure, dementia, cognitive decline and paralysis [78]. These chemotherapy-induced LEs can be characterized by MRI as being posterior reversible encephalopathy syndrome (PRES), acute toxic leukoencephalopathy (ATL), or other leukoencephalopathy (OLE), which is similar to ATL but with lesser white matter changes. PRES is associated with better survival outcomes than ATL. The timing of events tended to plateau at one-month post treatment. A low incidence of neurological events were also recorded in studies reviewed here using either fludarabine or clofarabine preconditioning [43, 58, 63, 64, 71, 74, 78, 79] (Table 2). In a specific case study, neurological symptoms were described to be consistent with LE [43]. Beitinjaneh et al. [78] performed a comprehensive analysis of CNS toxicity associated with fludarabine preconditioning for ASCT. Distinct stages of CNS disease were categorized by MRI and correlated with different forms and severities of LE that influenced survival. Notably, cerebral edema was not described in the imaging findings. The exact occurrence of neurological toxicity following ASCT is likely masked by short follow-up times resulting from disease progression, incidence of GVHD or transition to other treatment regimens.

Lymphodepletion in adoptive T-cell therapy

TILs

Following the clinical success of using preconditioning regimens to support engraftment of ASCT for the treatment of both hematological and solid malignancies, the same principle was applied to ACT of TILs for the treatment of metastatic melanoma [80, 81]. Dudley et al. [13, 82] applied FC non-myeloablative conditioning to ACT of TILs cultured from patients with metastatic melanoma and demonstrated enhancement of anti-tumor activity (Table 3). A series of studies expanded on these observations, including combining FC with total body irradiation (supplemented with hematopoietic stem cells) to induce more potent lymphodepletion, resulting in higher objective responses [83–87]. The rationale for chemotherapy preconditioning and the mechanisms by which it enhances anti-tumor activity are multi-pronged, including the reduction of immunosuppressive cell populations (e.g. regulatory T cells and myeloid-derived suppressor cells), an increase in tumor antigen presentation by inducing cell death, a reduction of sinks for the T-cell proliferative cytokines IL-7 and IL-15 and an improvement in the reactivity of the adoptively transferred cells [10, 88, 89].

One major caveat of ACT using TILs is that many cancers are unsuitable for TIL extraction, and the process is

Table 2 Results from clinical trials using reduced intensity conditioning regimens prior to ASCT

References	Year	No. of patients	Disease	Pre-conditioning regimen	Engraftment success (%)	PFS (%); Years (Yr)	NRM (%); Years (Yr)/Days (d)
Giralt et al. [59]	2001	78	HMs	Flu-M	80–100	53; 1Yr	37; 100d
van Besien et al. [53]	2003	31	HMs	Flu-M	100	33; 1Yr	23; NS
Schetelig et al. [77]	2003	30	CLL	Flu-Busulfan	93	67; 2Yr	15; 2Yr
Morris et al. [58]	2004	88	NHL	Flu-M- Alemtuzumab	97	50; 3Yr	29; 100d
Anderlini et al. [60]	2005	14	NHL	FC	69	21; 1.5Yr	5; 1.5Yr
		26	NHL	Flu-M	100	37; 1.5Yr	22; 1.5Yr
Brown et al. [75]	2006	46	CLL	Flu-Busulfan	>75	34; 2Yr	15; 2Yr
Delgado et al. [76]	2006	24	CLL	Flu-M- Alemtuzumab	85	45; 2Yr	26; 2Yr
Oran et al. [61]	2007	112	AML & MDS	Flu-M	82	15; 2Yr	54;>2Yr
Shimoni et al. [74]	2007	72	HMs	Flu-Busulfan	97	72; 2Yr	16; 2Yr
		79	HMs	Flu-M	99	36; 2Yr	40; 2Yr
Anderlini et al. [62]	2008	58	NHL	Flu-M	NS	32; 2Yr	15; 2Yr
Valcarcel et al. [69]	2008	93	AML & MDS	Flu-Busulfan	NS	43; 4yr	16; 1Yr
Lee et al. ^C [43]	2010	2	AML	FC	–	–	2 ^a
Beitinjaneh et al. [78]	2011	1596	HMs	Flu (+various agents)	NS	NS	NS
Santarone et al. [70]	2011	44	ALL	Flu-Busulfan	100	63; 2Yr	18; 2Yr
Kebriaei et al. [71]	2012	51	ALL	Clo-Busulfan	94	54; 1Yr	6; 100d
van Besien et al. [64]	2012	72	HMs	Clo-M	NS	45; 1Yr	26; 1Yr
Kirschbaum et al. [63]	2012	14	HMs	Clo-M	100	61;>1Yr	21;<1Yr
Baron et al. [73]	2015	218	AML	Flu-Busulfan	99	53; 2Yr	18; 2Yr
		176		Flu-M	100	60; 2Yr	20; 2Yr
Annalaro et al. ^C [79]	2015	1	Myelofibrosis	Flu	CR	–	1 ^a
Rambaldi et al. [72]	2015	125	AML	Cyc-Busulfan	NS	NS	17.2; 1Yr
		127	AML	Flu-Busulfan	NS	NS	7.9; 1Yr
El-Jawahri et al. [66]	2016	33	AML, ALL and MDS	Clo-Busulfan	100	50; 2Yr	24; 1Yr
Alatrash et al. [67]	2016	70	AML, CML & MDS	Clo-Flu-Busulfan	100	0.9Yr ^b	4; 100d
Chevallier et al. [65]	2016	316	AML & MDS	Flu-Busulfan	NS	51.1; 2Yr	17.3; 2Yr
		39	AML & MDS	Clo-Busulfan	NS	61.5; 2Yr	10.3; 2Yr
Kebriaei et al. [68]	2017	107	ALL	Clo-Busulfan	NS	44; 2Yr	34; 2Yr

Incidence of NRM were primarily related to opportunistic infections and GVHD-related morbidities, including mucosal, hepatic, pulmonary, renal, cardiac, skin and gastrointestinal

PFS progression-free survival, *NRM* non-relapse mortality, *NS* not stated, *C* case study, *HMs* hematological malignancies, *CLL* chronic lymphocytic leukemia, *NHL* non-Hodgkin's lymphoma, *AML* acute myeloid leukemia, *MDS* myelodysplastic syndromes, *ALL* acute lymphoblastic leukemia, *CML* chronic myeloid leukemia, *Flu* fludarabine, *M* melphalan, *FC* fludarabine-cyclophosphamide, *Clo* clofarabine

^aIndividual instances of NRM

^bMedian PFS

both cost and labor intensive [90]. As a result, a search for a more widely applicable ACT methodology has continued, which led to the emergence of genetically modified lymphocytes expressing tumor-targeting receptors in the form of TCRs or CARs.

Engineered TCR-transduced T cells

TCR engineered T cells are attractive because the TCR recognizes the HLA-peptide complex, and the presented

peptides are derived from both membrane and intracellular proteins. This enables TCRs to recognize virtually any protein in the cell, which is useful when engineering specificity for tumor recognition. Affinity optimization of tumor antigen-specific TCR-transduced T cells offers significant advantages over natural affinity TCRs, since the majority of tumor antigens are self-antigens, thus rendering the naturally occurring repertoire of TCRs to be low affinity due to thymic selection mechanisms [91]. Affinity optimization has been shown to improve the potency and

Table 3 Results from clinical trials using ACT of TILs with or without pre-conditioning regimens

References	Year	No. of patients	Disease	Therapeutic regimen	CR/ORR (%)	PFS >1 year	Neurotoxicity ≤Grade 3	Neurotoxicity ≥Grade 4
Rosenberg et al. [80]	1988	20	MM	IL-2	–; 55	–	–	–
Rosenberg et al. [81]	1994	29	MM	IL-2	–; 31	–	–	–
Rosenberg et al. [81]	1994	57	MM	FC + IL-2	–; 35	–	–	–
Dudley et al. [13]	2002	13	MM	FC + IL-2	–; 46	–	–	–
Dudley et al. [82]	2005	35	MM	FC + IL-2	9; 51	6%	–	1
Dudley et al. [83]	2008	43	MM	FC + IL-2	9.3; 48	9%	–	–
Dudley et al. [83]	2008	25	MM	FC + TBI + IL-2	16; 72	12%	4	–
Rosenberg et al. [84]	2011	43	MM	FC + IL-2	12; 49	12%	–	–
Rosenberg et al. [84]	2011	25	MM	FC + TBI + IL-2	40; 72	40%	–	–
Pilon-Thomas et al. [86]	2012	13	MM	FC + IL-2	15; 26	NS	–	–
Besser et al. [87]	2013	54	MM	FC + IL-2	9; 40	9%	4	–
Goff et al. [85]	2016	50	MM	FC + IL-2	24; 45	24%	–	–
Goff et al. [85]	2016	51	MM	FC + TBI + IL-2	24; 54	24%	–	–

CR complete response, ORR overall response rate, PFS progression-free survival, MM metastatic melanoma, TIL tumor-infiltrating lymphocyte, IL-2 interleukin-2, FC fludarabine-cyclophosphamide, TBI today body irradiation

functionality of TCR engineered T cells, which is necessary to recognize immune-selected tumors [92]. TCR-transduced T cells utilize the natural T-cell signaling infrastructure and therefore maintain the inherent capability to induce physiologically appropriate levels of T-cell activation, costimulation, expansion, memory cell formation and APC interactions [93].

Several TCR engineered T-cell therapy studies have been carried out, including both natural and affinity-optimized TCRs [94]. The antigens MART-1 and gp100 were identified as melanoma-specific antigens during the course of ACT clinical trials with TILs and became the first targets for TCR-transduced T-cell therapy [95–97]. In the majority of these studies, an FC regimen was used for preconditioning. In the first application of ACT using TCR-transduced T cells for the treatment of metastatic melanoma, response rates were low despite preparative lymphodepletion [95] (Table 4). Efforts to enhance the affinity of TCRs to their target antigen, MART-1, reflected positively in response rates but simultaneously raised issues of enhanced on target, off tumor toxicity, with patients experiencing non-lethal adverse reactions in the skin, eyes and ears due to Mart-1 expression in the cells in those tissues [97]. Subsequent studies expanded into non-melanoma tumors, targeting the NY-ESO-1 cancer testis antigen, with promising results in melanoma and synovial sarcoma [15, 16, 98]. In a TCR-transduced T-cell therapy targeting the cancer testis antigen MAGE-A3, two patients experienced lethal neurological abnormalities said to result from cross-reactivity to other members of the MAGE family that were expressed in brain [99]. A different MAGE-A3 TCR also showed off tumor –off target toxicity in recognition of the cardiomyocyte

protein titin, resulting in two patient deaths [100]. In no cases were these adverse events related to use of fludarabine in the preconditioning regimen. Dose reduction of fludarabine is under investigation in a pilot study in synovial sarcoma, as this may reduce other fludarabine-related toxicities such as prolonged neutropenia and bone marrow failure [16]. In this same study, removal of fludarabine appeared to have a negative effect on efficacy.

In summary, the incidence of neurotoxicity appears to be lower with TCR engineered ACT studies, and fludarabine appears to play role in enhancing ACT potency.

CAR-T cells

CAR-T cells are engineered with synthetic receptors comprised of an antibody single chain variable fragment (scFv) specific for a cell surface protein, a transmembrane domain, and intracellular signaling domains [101]. Second-generation CARs incorporate various T-cell costimulatory activation domains such as CD137 (4-1BB) and CD28 to enhance expansion and persistence of the CAR-T cells [102, 103]. Signaling mechanisms are likely to be different with CARs than with TCRs, which harness the natural immunologic synapse, and whether this may lead to differences in the toxicity profiles of the two modalities is an area of active investigation.

As with TIL and TCR-transduced T-cell therapy, preparative lymphodepleting regimens using mainly cyclophosphamide and fludarabine were used to increase efficacy and durability of adoptively transferred CAR-T cells [104–124] (Table 5). In mouse tumor models and human studies, lymphodepletion by cyclophosphamide was

Table 4 Results from clinical trials using TCR-transduced T cells with or without pre-conditioning regimens

References	Year	No. of patients	Disease	Target	Pre-conditioning regimen	CR/ORR (%)	PFS >1 year	Neurotoxicity ≤Grade 3	Neurotoxicity ≥Grade 4
Morgan et al. [95]	2006	17	MM	MART-1	FC	–; 11	–	–	–
Burns et al. [96]	2009	40	MM	MART-1	FC	–; 13	–	–	–
Johnson et al. [97]	2009	20	MM	MART-1	FC	–; 30	–	–	–
Johnson et al. [97]	2009	16	MM	gp100	FC	6; 18	6	–	–
Robbins et al. [98]	2011	17	MM and SC	NY-ESO-1	FC	11; 52	11	–	–
Morgan et al. [99]	2013	9	MM, esophageal and SC	MAGE-A3	FC	11; 55	–	3	2
Linette et al. [100]	2013	2	Multiple myeloma	MAGE-A3	Cyclophosphamide	–; –	–	–	–
Rapoport et al. [15]	2015	20	Multiple myeloma	NY-ESO-1	Melphalan	NS; 80	50	–	–

CR complete response, ORR overall response rate, PFS progression-free survival, MM metastatic melanoma, SC synovial carcinoma, FC fludarabine-cyclophosphamide, NS not stated

critical for CD19 CAR-T-cell engraftment and persistence [106, 125]. Fludarabine enhanced in vivo persistence of adoptively transferred T cells 2.9 fold, in part by reducing starting tumor burden prior to ACT [106, 126]. Considered here are clinical trials using second-generation CAR-T cells engineered to target B-cell-related hematological malignancies through the CD19 or CD20 surface B-cell antigens. Overall, response rates were much higher than conventional chemotherapy, other ACT methods, and the bispecific anti-CD19 BiTE blinatumomab [127] (Table 5).

Few studies have directly compared pre-conditioning regimens; however, trials that did not use pre-conditioning gave rise to substantially lower response rates and PFS [106, 107, 110] (Table 5). Turtle et al. [120] used both cyclophosphamide and FC pre-conditioning regimens and showed that FC improved CAR-T-cell persistence and disease-free survival compared to cyclophosphamide alone. Clear differences in toxicities between lymphodepleting regimens were not described. While enhanced lymphodepletion correlated with increased efficacy and durability of CAR-T cells, deeper conditioning can promote enhanced initial expansion of CAR-T, which along with cell dose and tumor burden, is known to increase the frequency of severe cytokine release syndrome (CRS).

CRS is a systemic inflammatory response caused by high levels of inflammatory cytokines that potentiate T-cell activation and proliferation, and its occurrence in the context of engineered T-cell therapy has a unique algorithm for accurate diagnosis, classification and treatment [128, 129]. CRS can be associated with a broad range of symptoms, often mimicking infection or sepsis. Typical presenting symptoms include fever, anorexia, nausea, fatigue and myalgia/arthralgia, and symptoms can progress to more serious life-threatening complications, including hypotension, capillary leak, and hypoxia. More severe cases can be associated with organ toxicity such as cardiac dysfunction, adult respiratory distress syndrome, and neurologic involvement [118]. In most cases, symptoms of CRS can be effectively managed with supportive care and by blocking the trans IL-6 signaling mechanism through the IL-6 receptor blocking antibody tocilizumab or the IL-6 blocking antibody situximab, and earlier administration provides more effective management of symptoms. Tocilizumab is generally considered not to reduce initial anti-tumor activity, although more research is needed to determine what effect blocking IL-6 activity has on CAR-T cell durability [122]. CRS observed in TCR studies has been less frequent and less severe than in CAR-T studies, which may be due to differences in T-cell signaling, tumor target density, and tumor burden and accessibility [16].

We described earlier a distinct set of late-onset neurological symptoms associated with fludarabine therapy and other purine nucleoside analogs. Neurological events

Table 5 Results from clinical trials using CAR-T-cell therapy with or without pre-conditioning regimens

References	Year	No. of patients	Disease	CAR	Pre-conditioning regimen	ORR (%)	PFS >1 year	Neurotoxicity ≤Grade 3	Neurotoxicity ≥Grade 4
Till et al. [124]	2008	9	FL or MCL	CD20	Various	42	14	—	—
Kochenderfer et al. ^c [104]	2010	1	FL	CD19;CD28+IL-2	FC	PR	Y	—	—
Porter et al. ^c [105]	2011	1	CLL	CD19;CD137	Pentostatin-cyclophosphamide	CR	N	—	—
Brenijns et al. [106]	2011	3	CLL or B-ALL	CD19;CD28	—	—	—	—	—
Brenijns et al. [106]	2011	4	CLL or B-ALL	CD19;CD28	Cyclophosphamide	—	—	—	—
Savoldo et al. [107]	2011	6	NHL	CD19;CD28	—	—	—	NS	NS
Kalos et al. [108]	2011	3	CLL	CD19;CD137	Bendamustine, bendamustine & rituximab, pentostatin-cyclophosphamide	100	—	—	—
Kochenderfer et al. [109]	2012	8	B-cell malignancies	CD19;CD28+IL-2	FC	88	38	—	—
Cruz et al. [110]	2013	8	B-cell malignancies	CD19;CD28 (VST)	—	25	—	NS	NS
Brenijns et al. [111]	2013	5	B-ALL	CD19;CD28	Cyclophosphamide	100	NS	—	—
Grupp et al. [112]	2013	2	B-ALL	CD19;CD137	Cyclophosphamide	100	50	1	—
Davila et al. [113]	2014	16	B-ALL	CD19;CD28	Cyclophosphamide	88	NS	3	3
Maude et al. [114]	2014	30	B-ALL	CD19;CD137	FC	90	67	13	8
Park et al. [115]	2015	43	B-ALL	CD19;CD28	Cyclophosphamide	84	16	NS	NS
Kochenderfer et al. [116]	2015	15	B-cell malignancies	CD19;CD28	FC	80	46	3	1
Lee et al. [117]	2015	19	B-cell malignancies	CD19;CD28	FC	68	NS	7	—
Porter et al. [118]	2015	14	CLL	CD19;CD137	FC, cyclophosphamide-pentostatin or bendamustine	57	29	6	1
Dai et al. [119]	2015	9	B-ALL	CD19;CD137 (autologous)	C-MOAD/-	44	—	1	1
Turtle et al. [120]	2016	12	B-ALL	CD19;CD137	Cyclophosphamide	83	8	15 ^a	6 ^a
Turtle et al. [120]	2016	17	B-ALL	CD19;CD137	FC	95	65	—	—
Turtle et al. [17]	2017	24	CLL	CD19;CD28;4-1BB;CD3 ζ	Cyclophosphamide, fludarabine, or FC	71	—	7	1
Gardner et al. ^A [121]	2016	43	B-ALL (Pediatric)	CD19;CD28;4-1BB	Cyclophosphamide-FC	93	51	21	10
Brudno et al. [122]	2016	20	B-cell malignancies	CD19;CD28	—	40	NS	—	—
Maude et al. ^A [123]	2016	30	B-ALL	CD19;CD137	FC	87	NS	9	9

All patients had undergone previous treatment. All studies report varying degrees of infection and symptoms of cytokine release syndrome, including hepatic, pulmonary, renal, cardiac and skin abnormalities as well as fever, vascular leak, hypotension and hypoxia

^c case study, ^A abstract, CR complete response, ORR overall response rate, PFS progression-free survival, IL-2 interleukin-2, NHL non-Hodgkin's lymphoma, CLL chronic lymphocytic leukemia, HL Hodgkin's lymphoma, FL follicular lymphoma, MCL mantle cell lymphoma, FC fludarabine-cyclophosphamide, VST virus-specific T cell, C-MOAD methotrexate, rituximab, vincristine, pegylated L-asparaginase and dexamethasone

^aNot detailed whether patients treated with cyclophosphamide or FC

reported following CD19-targeted CAR-T-cell therapy, recently named CAR-T-cell-related encephalopathy syndrome (CRES), manifest in a pattern distinct from that previously described with fludarabine, occurring within the first week of therapy. Several groups reported diagnoses of encephalopathy associated with symptoms of delirium, confusion and hallucinations that occurred soon after CAR-T cell transfer. Symptoms in many cases were severe but reversible [113, 114, 116–118, 120] (Table 5). Factors speculated to contribute to the onset of neurological events include circulating CAR-T-cell concentration and the onset of CRS [117]. Studies identified a correlation between the severity of CRS, the intensity of lymphodepletion and starting tumor burden [111, 113]. In one study, a high incidence of grade 3 or above neurotoxicity (50%) was reported using CAR-T cells for treatment of B-ALL. Peak levels of IL-6, IFN- γ , ferritin, and CRP were significantly higher in patients who developed neurotoxicity. Furthermore, six of six patients with high tumor burden and also treated with higher cell doses developed severe neurotoxicity [120]. In an analysis of the incidence and grade of neurotoxicity, CRS, and correlative biomarkers in blood and CSF in adult patients with relapsed or refractory B-ALL, severe neurotoxicity correlated with pre-infusion disease burden and peak CAR-T-cell expansion in the blood. Neurotoxicity grade correlated with elevations of CSF protein level as well as elevated cytokines IL6, IL8, IL10, IFN γ and G-CSF in CSF over serum at the time of neurotoxicity [130]. These factors suggest a causal role of CRS in the development of neurotoxicity.

CRES is often biphasic, with an initial phase occurring during CRS, and a second phase occurring days later. This second phase may be why some neurological events do not correlate with cytokine levels or CRS. Treatment algorithms for CRES include blockade of IL-6 signaling as well as systemic steroids; however, neurotoxicity has not been consistently responsive to these therapies, making it difficult to elucidate whether early identification and treatment of CRS will be effective in treating neurological symptoms [116, 120, 123, 129]. The presentation, time course, proposed monitoring, and treatment algorithm for CRES was recently reported by Neelapu et al. [129].

In a recent clinical trial, JCAR-015 (NCT02535364), investigating the application of CD19 CAR-T cells for the treatment of B-ALL, two patient deaths due to cerebral edema resulted in the trial being suspended, pending investigation. This finding was not previously reported in CAR-T studies. The cerebral edema was initially thought to be associated with the addition of fludarabine to the treatment cohort. Fludarabine was subsequently removed from the preconditioning regimen in the trial; however, two further patient deaths reported shortly after the trial was re-initiated indicated that fludarabine was not the causal agent

for the fatal cerebral edema seen in the trial. The trial has since been suspended [131]. Another study, ZUMA-1 (NCT02348216), reported a similar incident, where a patient with advanced refractory NHL experienced cerebral edema following CD19 CAR-T-cell infusion after FC preconditioning, resulting in lethality [132]. This event was an isolated incident, and the trial continued (now complete). However, the CAR constructs engineered for both of these studies share the same CD28 signaling domain, which has been reported to result in greater T-cell expansion [133]. This may contribute to the CRS toxicity and thus neurotoxicity. Investigations into the pathophysiology of these events have recently been reported and include an early endothelial activation event mediated by the CAR-T cells. This initiates a cascade of coagulopathy concomitant with increased BBB permeability. Cytokines produced during CRS, as well as the CAR-T cells, then traffic into the CNS, inducing neurotoxicity [134].

The contribution of the target antigen to neurological abnormalities should be considered. We previously noted the incidence of neurological toxicity within days following adoptive transfer of T cells transduced with high affinity TCRs against the MAGE-A3 cancer testis antigen, which also cross-reacted with the MAGE-A9 and MAGE-A12 antigens that can be expressed in the CNS. Two patients exhibited lethal global encephalopathy characterized by white matter abnormalities, vacuolation and edema by MRI [99]. Although the cause of toxicity was attributed to cross-reactivity with another MAGE family member, the impact of lymphodepletion on the exposure of these potent molecules was not assessed. The contribution of a specific antigen is unique in each case and relates to the level of antigen expression, highlighting the need for thorough research into antigen expression profiles of antigen and related protein sequences.

The spotlight on CD19 CAR-T toxicity is driven by the success and potency of these molecules in the clinic. Interestingly, early results in a primate model suggest that similar neurotoxicity occurs following treatment with CD20 CAR-T cells [135]. In a small number of non-human primates, CRS and neurotoxicity were recapitulated following infusion of CD20 CAR-T cells in healthy animals following preconditioning with cyclophosphamide alone. The autopsy of the animals showed extensive infiltration into the brain of CD20⁺ CAR-T cells [135]. This increased CNS infiltration may be due to the CAR-T cells reacting to naturally trafficking B cells in the brain parenchyma [136]. Furthermore, CD19 CAR-T cells have been identified in the CSF of some subjects with neurotoxicity and the extent of infiltration correlates with severity in some but not all studies [117, 119, 130]. Neurotoxicity with encephalopathic symptoms has also been described with the bispecific anti-CD19 BiTE blinatumomab. Despite the recently described

pathophysiology of CD19 CAR-T-mediated neurotoxicity described earlier, the significance of CD19 or other antigens on the mechanism of neurological toxicity cannot be excluded and will only be further understood with continuing evaluation of affected subjects and as increasing numbers of CAR-T cells targeting different antigens are tested for a range of malignancies.

Conclusions

The early onset neurological symptoms observed following CD19 CAR-T-cell therapy that include encephalopathy and more rarely cerebral edema appear to be driven by a distinct set of mechanisms from those that drive late-onset neurological toxicity associated directly with fludarabine. To our knowledge, late-onset neurotoxicity associated with fludarabine has not been reported in ACT studies. There should be careful monitoring for this potential toxicity, particularly in patients with increased sensitivity to fludarabine, such as heavily pre-treated patients or patients with renal insufficiency.

The mechanisms that induce early onset cerebral edema are just beginning to be elucidated. CRES severity is highly correlated with CRS severity, which is known to be related to expansion of CAR-T cells *in vivo*. As suspected then, risk factors for CRES include tumor burden and the depth of preconditioning, as both of these factors affect the expansion of infused T cells. This may explain why the first cerebral edema events on the JCAR015 study were seen once fludarabine was added to the preconditioning regimen, as it leads to a more profound lymphopenia. Presence of the target in the CNS (e.g. on B cells circulating through the CNS) may also contribute to CRES. CRES is a common neurotoxic side effect in CAR-T-cell studies, and similar side effects appear to be less frequent and less severe in TCR and TIL studies. Although encephalopathy can be severe, complete recovery is typical. A second neurological toxicity, cerebral edema, has only rarely been observed in CAR-T-cell studies, has not been observed to date in TCR T cell studies, and is acutely life threatening. It now appears that cerebral edema is an extreme manifestation of CRES. The recent elucidation of the mechanism behind this toxicity and components of the etiology with other described medical events, such as thrombotic thrombocytopenic purpura and malaria-associated neurologic dysfunction, suggest approaches for therapeutic intervention [137]. Early identification of certain biomarkers and careful management of CRS may help reduce side effects of neurotoxicity [129]

Fludarabine is an important component of the preconditioning regimen for effective ACT. Consideration of the intensity of fludarabine preconditioning should include reduction of dose for patients with known risk factors for

fludarabine toxicity, as described in the product label. Next generation engineered T-cell therapies that include approaches to circumvent the requirement for preconditioning may lead to ACT regimens with improved tolerability. Until then, fludarabine continues to be an important component of the preconditioning regimen for supporting the potency of ACT. At doses used in this context, the evidence to date does not support a direct role for fludarabine in initiation or exacerbation of CRES.

Compliance with ethical standards

Conflict of interest KLL is an employee of Immunocore; EN, RA, BKJ and GB are employees of Adaptimmune. CLM is a member of the Parker Institute for Cancer Immunotherapy, which supports the Stanford University Cancer Immunotherapy Program.

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