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1 **CAR T-cells for T-cell malignancies: challenges in distinguishing**  
2 **between therapeutic, normal, and neoplastic T-cells**

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40

1 **Abstract**

2  
3 Chimeric antigen receptor (CAR) T-cells targeting CD19 demonstrated remarkable efficacy  
4 for the treatment of B-cell malignancies. The development of CAR T-cells against T-cell  
5 malignancies appears more challenging due to the similarities between the therapeutic,  
6 normal and malignant T-cells. The obstacles include CAR T-cell fratricide, T-cell aplasia, and  
7 contamination of CAR T-cell products with malignant T-cells. Here, we review these  
8 challenges and the solutions proposed to overcome these limitations.

9  
10  
11 **Introduction**

12  
13 Chimeric antigen receptor (CAR) T-cells demonstrated remarkable efficacy for the treatment  
14 of B-cell malignancies and have been approved by the US Food and Drug Administration  
15 (FDA) for the treatment of relapsed/refractory B-cell acute lymphoblastic leukemia (B-ALL)  
16 and diffuse large B-cell lymphoma (DLBCL).(1–5) This proof of concept generated great  
17 enthusiasm for the development of CAR T-cells directed against other types of cancer,  
18 including T-cell malignancies.(6,7)

19  
20 T-cell malignancies encompass immature (i.e. T-cell acute lymphoblastic leukemias (T-ALL))  
21 and mature (i.e. T-cell lymphomas (TCL)) lymphoid neoplasms and are often associated with  
22 a dismal prognosis.(8–10)

23  
24 Despite great interest, the development of CAR T-cells against T-cell malignancies has been  
25 limited so far due to the difficulties to distinguish between therapeutic, normal and  
26 malignant T-cells. Here, we review the challenges raised by such development and describe  
27 the solutions that have been proposed to address these limitations.

## 1 **Challenges**

2  
3 CAR T-cells, directed against antigens shared with normal T-cells, may recognize and kill 3  
4 types of cells: tumor T-cells, normal T-cells and CAR T-cells (Figure 1). Mutual killing of CAR T-  
5 cells, also called fratricide, may prevent the generation, expansion and persistence of CAR T-  
6 cells. Prolonged and profound T-cell aplasia induced by the destruction of normal T-cells  
7 exposes patients to severe opportunistic infections.(11,12) Thus, developing CAR T-cells for  
8 T-cell malignancies requires targeting malignant T-cells while sparing normal and CAR T-cells,  
9 or at least some subsets of them.

10  
11 Furthermore, CAR T-cell products may be contaminated with malignant T-cells. Indeed,  
12 circulating tumor T-cells are often found in the peripheral blood of patients with T-ALL(9,13–  
13 15) and, although less frequently, with TCL.(8,16) Because tumor T-cells may harbor the  
14 same phenotypic and functional properties as normal T-cells(17), they may be harvested,  
15 transduced, expanded and infused concomitantly with normal T-cells. This process may lead  
16 to the generation of “CAR tumor T-cells”. Ruella et al. recently described accidental  
17 transduction of CAR construct in leukemic B-ALL cells leading to CAR expressing blasts (so  
18 called “CARB”).(18) In these patients, CD19 CAR at the blast surface bound to CD19, thus  
19 preventing their recognition by CAR T-cells. A similar mechanism may be anticipated with  
20 malignant T-cells if transduced with the CAR construct. Furthermore, contaminating tumor  
21 T-cells may also be genetically edited to prevent the expression of a T-cell target along with  
22 normal T-cells and thereby escape CAR T-cells recognition and eradication. Thus, developing  
23 CAR T-cells for T-cell malignancies requires avoiding contamination of the CAR T-cell product  
24 with malignant transduced T-cells.

25

1 **Proposed solutions**

2

3 ***Fratricide***

4

5 To prevent fratricide, CAR cells should be directed against a tumor antigen that is not shared  
6 (or not completely) between malignant and therapeutic T-cells. This can be achieved in 2  
7 ways: i) either by targeting a tumor antigen that is not naturally expressed by the CAR T-  
8 cells, ii) or by using CAR cells that do not express the T-cell target which can be achieved by  
9 using CAR T-cells that have been genetically edited *ex vivo* to prevent expression of the T-cell  
10 target or by using non-T CAR cells such as NK-cells.

11

12 *CAR T-cells directed against antigens that spare CAR T-cells (Table 1)*

13

14 Most target antigens are shared between normal and malignant T-cells(8,19,20) rendering  
15 specific targeting of tumor T-cells challenging. Strategies that target tumor antigens while  
16 sparing CAR T-cells include targeting of pan-T antigens which are down-regulated during CAR  
17 T-cell expansion (e.g. CD5) thereby preventing or minimizing CAR T-cell fratricide(21), or  
18 targeting of antigens which are expressed only by a subset of normal T-cells (e.g. CD4 or  
19 CD30) thereby sparing subsets of CAR T-cells (Table 1).(22–24)

20

21 CD5 CAR T-cells

22 CD5 is expressed by normal T-cells and by most T-ALL and TCL.(8,25) In preclinical models,  
23 CD5 is downregulated through internalization following CAR T-cell expansion and activation,  
24 thus preventing fratricide.(21,26) However, this is not observed when using CAR T-cells  
25 containing a TNFR superfamily co-stimulatory domain such as 4-1BB.(27,28)

26

1 CD4 CAR T-cells

2 CD4 is expressed by two thirds of normal T-cells, by most TCL and a subset of T-ALL.(8,17,25)  
3 Preclinical data testing CD4 CAR T-cells have shown a highly enriched CD8+ CAR T-cells  
4 product which efficiently killed lymphoma cells *in vitro* and *in vivo*.(22) However, such CD4  
5 CAR T-cells will induce a CD4 T-cell aplasia, and this would result in a syndrome similar to  
6 HIV/AIDS. Thus, we view this approach as a temporary or bridging strategy.

7

8 CD30 CAR T-cells

9 CD30 is expressed by a subset of activated B and T-cells, virtually all Hodgkin lymphomas  
10 (HL) and anaplastic large cell lymphomas (ALCL), subsets of peripheral TCL, and about one  
11 third of T-ALL.(29,30) Following demonstration of a preclinical activity(31), CD30 CAR T-cells  
12 have been evaluated in two phase I clinical trials, mostly in patients with HL.(23,24) No  
13 decrease in B or T-cell counts were described in these studies. Furthermore, T-cell immunity  
14 to common viral pathogens did not seem to be impaired.(23) This is in line with what has  
15 been observed in patients treated with brentuximab-vedotin, an antibody-drug conjugate  
16 directed against CD30 where no unexpected opportunistic infections have been  
17 observed.(32) In the study by Ramos et al., two patients with ALCL (one systemic ALK+ and  
18 one cutaneous ALK-) were infused with CD30 CAR T-cells.(23,31) One of the two patients  
19 achieved a complete remission which lasted for 9 months following 4 infusions of CD30 CAR  
20 T-cells at the highest dose ( $2 \cdot 10^8$  cells/m<sup>2</sup>). (23) Wang and colleagues observed a partial  
21 response following infusion without conditioning regimen in one (and only) ALCL  
22 (cutaneous) patient.(24) These encouraging results may be further improved by adding a  
23 lymphodepleting conditioning regimen prior to the CD30 CAR T-cell infusion.(33,34)

24

1 *Genetically-edited CAR T-cells to prevent target expression*

2

3 Fratricide may also be avoided by knocking-out the target gene using gene editing (such as  
4 TALEN or CRISPR system). This approach has been evaluated preclinically with CD7 CAR T-  
5 cells. CD7 is expressed by normal T and NK-cells, by most T-ALL and a subset of TCL.(8,25)  
6 Unlike CD5, CD7 is poorly downregulated upon CAR T-cell expansion/activation. Thus,  
7 prevention of fratricide requires genomic disruption of CD7 prior to CAR transduction. In  
8 preclinical models, CRISPR/Cas9-mediated editing of CD7 abrogates fratricide and enables  
9 the expansion of CAR T-cells.(35,36) Similar results have been achieved using PEBL  
10 technology(37), a method that prevents CD7 surface expression by anchoring newly  
11 synthesized CD7 in the endoplasmic reticulum and/or golgi.(38–40) Gomes-Silva et al.  
12 suggested that the infused CD7 CAR T-cells may retain antiviral activity through their native  
13 receptor and therefore counteract the profound immunodeficiency induced by on-  
14 target/off-tumor effects of CD7 CAR T-cells.(35)

15

16 *CAR NK-cells*

17

18 CARs are commonly transduced into T-cells but the use of NK-cells is emerging.(41) Using  
19 NK-cells is a promising strategy to avoid fratricide. The typical cell surface phenotype of NK-  
20 cells shows lack of TCR, CD3 and CD5 expression.(42,43) Conversely, NK-cells are  
21 characterized by CD56 and CD7 expression.(44) NK-cells are part of the innate immune  
22 system and have natural cytotoxic properties against tumors which can be further improved  
23 by CAR engineering.(41,45,46) NK-cells present several advantages for CAR engineering: i)  
24 their phenotype (different from T-cells) can be used to prevent fratricide and avoid

1 contamination, ii) due to their lack of a TCR receptor, they do not naturally induce graft-  
2 versus-host disease (GVHD)(47) and thus can be used in allogeneic conditions, iii) their short  
3 lifespan may prevent prolonged T-cell aplasia.(41) However, using blood NK-cells to  
4 manufacture CAR cells is challenging because the collection, expansion and transduction of  
5 these cells is difficult.(48) For these reasons, CAR NK-cells directed against CD3, CD4 and CD5  
6 have been engineered using the NK-92 cell line, a human cell line derived from a patient  
7 with a NK-cell lymphoma, rather than natural NK-cells.(43,48–52) No fratricide is expected  
8 since NK-cells do not express these targets. In preclinical models however, CAR T-cells seem  
9 to outperform CAR NK-92 cells. Although CAR NK-92 cells can induce significant reduction of  
10 tumor burden, they lack persistence in xenograft mouse models, consistent with the short  
11 lifespan of NK-cells. Moreover, some concerns may be raised regarding the potential  
12 tumorigenicity of CAR NK-92 cells since they originate from a transformed cell line. To  
13 prevent this risk, NK-92 cells are irradiated before injection to patients. NK-92 cells (not  
14 genetically engineered) have been evaluated in phase I clinical trials in patients with  
15 metastatic solid tumors.(53,54) Another safety concern is the advent of neurotoxicity  
16 (strokes) after infusion of CD3 and CD5 CAR NK-92 cells in mice.(43,49)

17  
18 ***T-cell aplasia***

19  
20 Unlike B-cell aplasia which is usually well tolerated and can be compensated with continuous  
21 infusion of immunoglobulins for the lack of humoral adaptive immunity(55,56), prolonged T-  
22 cell aplasia exposes patients to opportunistic infections.(11,12) Prevention of prolonged T-  
23 cell aplasia may be achieved in 3 ways: i) either by targeting a tumor antigen that is not  
24 expressed by all or a subset of normal T-cells, ii) by using short-lived CAR cells and iii) by



1 myeloablation and subsequent bridging to allogeneic hematopoietic stem cell  
2 transplantation (HSCT).

3

4 *CAR T-cells directed against antigens that spare all or subsets of normal T-cells (Table 1)*

5

6 Such strategies have been previously described (paragraph “Fratricide”). However, depletion  
7 of certain T-cell subsets which are quantitatively (CD5+ or CD7+) or qualitatively (CD4+)  
8 important may still induce profound immune suppression. Depletion of other T-cell subsets  
9 such as CD30 may be better tolerated.(23,24) Another promising approach is the targeting of  
10 the T-cell receptor beta constant 1 (TRBC1) or TRBC2. Physiologically, the TCR  $\beta$  chain  
11 expresses either TRBC1 or the TRBC2 constant region.(57) Maciocia *et al.* have shown that  
12 the proportion of TRBC1+ T-cells varies between 25% to 47% in healthy donors, regardless of  
13 the T-cell subset.(58) T-cell leukemias and lymphomas, instead, are clonally TRBC1 positive  
14 or negative.(58) Therefore, TRBC1 CAR T-cells kill specifically TRBC1 malignancies while  
15 sparing TRBC2+ normal T-cells.(58) A clinical trial testing TRBC1 CAR T-cells in T-cell  
16 lymphomas is about to start (AUTO4).

17

18 *Short-lived CAR cells*

19

20 Another way to prevent prolonged T-cell aplasia is to use CAR cells with limited lifespan. This  
21 can be achieved by using i) allogeneic CAR T-cells, ii) CAR NK-cells, iii) non-viral mRNA  
22 transfection with electroporation(59), or iv) a safety switch (such as suicide gene or a  
23 targetable surface marker).(60–64) However, these strategies do not allow prolonged

1 persistence of CAR T-cells meant to prevent disease recurrence. Thus, they may rather be  
2 used as a bridge to transplant.

3

#### 4 ***Contamination of CAR T-cells product with malignant T-cells***

5

6 Purifying the apheresis product from circulating tumor T-cells to produce CAR T-cells is  
7 challenging since it is often difficult to distinguish between normal and neoplastic T-cells.  
8 Thus, avoiding contamination can be achieved in 2 ways: i) either by purifying and  
9 transfecting non-T cells, such as NK-cells (described previously), or ii) by producing CAR T-  
10 cells from an allogeneic healthy donor.

11

#### 12 *Allogeneic CAR T-cells*

13

14 CAR T-cells can be generated from allogeneic donors.(2) Nevertheless, infusion of allogeneic  
15 CAR T-cells may cause life-threatening GVHD, even after HLA matching.(65,66) To overcome  
16 this issue, Cooper et al. developed “off-the-shelf”, universal CD7 CAR T-cells (UCART7).(36)  
17 Using multiplex CRISPR/Cas9 gene editing of T-cells before CAR transduction, they deleted  
18 both CD7 and T-cell receptor alpha chain (TRAC). In preclinical models, their CD7 CAR  
19 efficiently killed T-ALL without inducing xenogeneic GVHD in a patient-derived xenograft  
20 (PDX) mouse model.(36) These allogeneic CAR T-cells are expected to have a short lifespan  
21 because they will be eliminated upon immune reconstitution of the host. This short  
22 persistence may be seen as an advantage to prevent T-cell aplasia but as a disadvantage to  
23 prevent cancer recurrence.

24

25

26

1 **Conclusion**

2

3 The development of CAR T-cells for T-cell malignancies faces unique challenges due to the  
4 similarities between therapeutic, normal, and malignant T-cells. Many of the solutions that  
5 have been proposed do not seem optimal, either because they lack specificity (risk of  
6 fratricide, immune suppression and/or contamination) or persistence (risk of tumor  
7 recurrence). Targeting of certain subsets (e.g. CD30 or TRBC1 CAR T-cells) seems promising  
8 but is restricted to subtypes of T-cell malignancies. It is unlikely that one type of CAR T-cells  
9 will be used for all T-cell malignancies (unlike CD19 CAR T-cells for B-cell malignancies). To  
10 date, few studies evaluated CAR T-cells in patients with T-cell malignancies(23,24) but  
11 several trials are underway or about to be launched (Table 1). Results from these clinical  
12 trials are eagerly awaited.

13

14

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16 manuscript, and created the table and figure.

17

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19 declares no competing financial interests. C.H.J. reports sponsored research from Novartis,  
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21 inTmunity. R.H. received consulting fees/honoraria from Novartis and Kite/Gilead.

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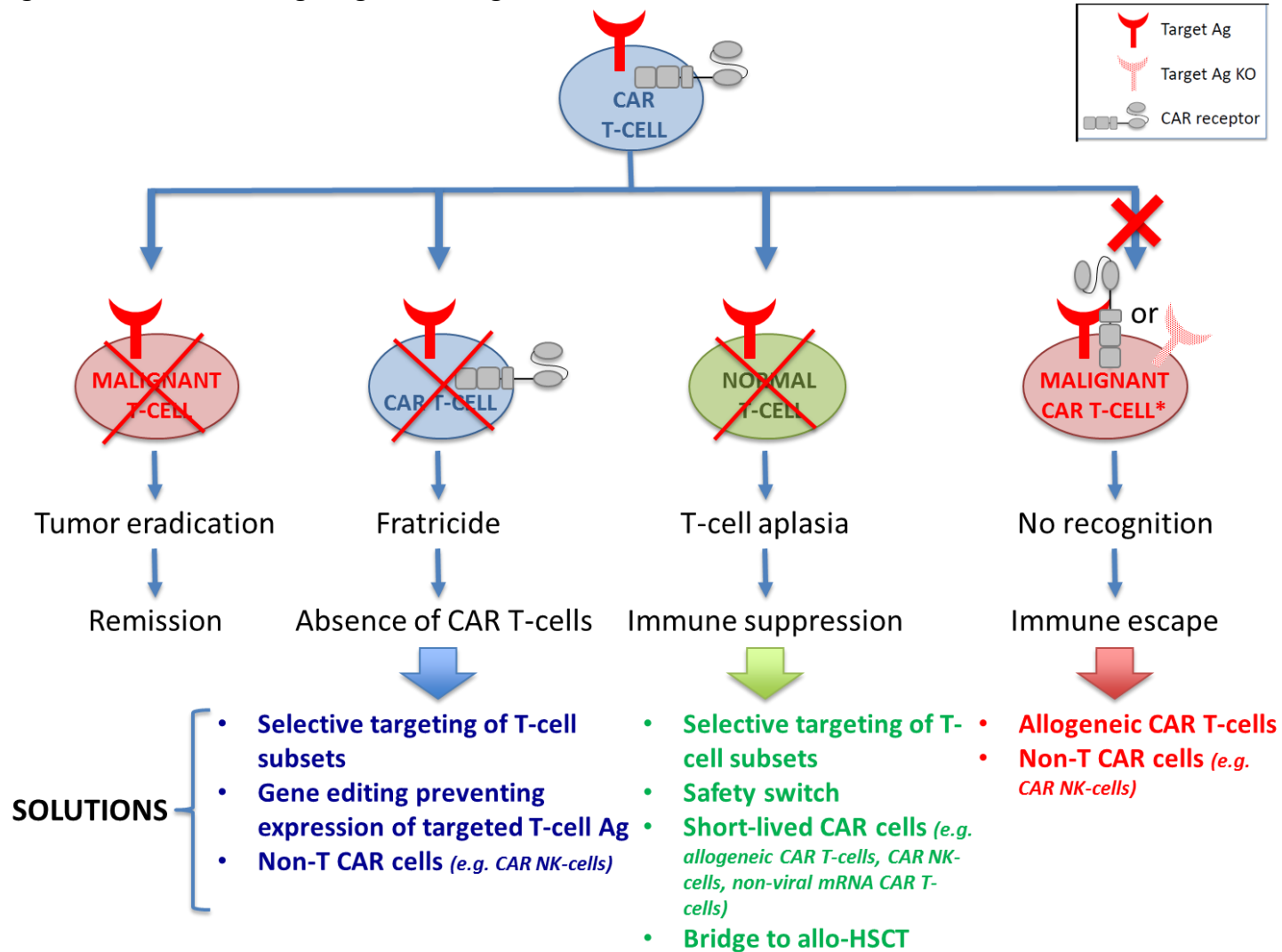
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3

Figure 1. Challenges and solutions in targeting T-cell antigens with CAR T-cells



\*Contaminating malignant T-cells transduced with CAR may escape recognition by normal CAR T-cells if the chimeric receptor binds to its target at the cell surface or if it has been genetically edited to prevent target expression.

**Table 1. Effects of different CAR cells constructs on the three T-cell compartments (therapeutic, normal and malignant T-cells)**

CAR cells	Target	CAR cells	Normal T-cells	Malignant T-cells*	Preclinical studies	Clinical studies
CAR T-cells	CD4	Partially depleted ( <i>CD4 expressed by two-thirds of T-cells</i> )	Partially depleted ( <i>CD4 expressed by two-thirds of T-cells</i> )	CD4 expressed in the majority of TCL and in a subset of T-ALL	Specific killing of ALCL cell line and human primary samples <i>in vitro</i> ; prolonged survival in ALCL xenograft (cell line) mouse model(22)	No clinical studies
	CD5	Transiently depleted ( <i>CD5 expressed by all T-cells but downregulated in CAR T-cells expressing CD28 costimulatory domain</i> )	Depleted ( <i>CD5 expressed by all T-cells</i> )	CD5 expressed in most T-ALL and TCL	<ul style="list-style-type: none"> <li>• 28.z CD5 CAR : Transient depletion (CD5 is lost following CAR expression)(21)</li> <li>• 4-1BB.z CD5 + conditional CAR expression system (4-1 BB.z Tet OFF CD5 CAR cells): CAR cells preserved(28)</li> <li>• Both constructs showed cytotoxicity against T-ALL cell lines <i>in vitro</i>. <i>In vivo</i>, survival of T-ALL xenograft (cell line) mice models enhanced with BB.z Tet OFF CD5 CAR.</li> </ul>	Ongoing trial : NCT03081910
	CD7	Depleted ( <i>CD7 expressed by all T-cells and poorly downregulated in CAR T-cells</i> )	Depleted ( <i>CD7 expressed by all T-cells</i> )	CD7 expressed by most T-ALL and a subset of TCL	<ul style="list-style-type: none"> <li>• CD7 downregulation before CAR expression (CD7 PEBL construct, whereby CD7 scFv is linked to ER retention domains)(37)</li> <li>• CRISPR-mediated deletion of CD7 prior to CAR transduction(35)</li> <li>• CRISPR-mediated deletion of CD7 and TCR alpha chain (UCART7)(36)</li> <li>• All three constructs showed <i>in vitro</i> lysis of T-ALL cell lines and primary T-ALL cells. In</li> </ul>	Ongoing trial : NCT02742727

					vivo, anti-leukemic effects were observed in T-ALL xenograft (cell lines) and PDX models.	
	<b>CD30</b>	Partially depleted ( <i>CD30 expressed by a small subset of activated T-cells</i> )	Partially depleted ( <i>CD30 expressed by a small subset of activated T-cells</i> ) No significant changes in T-cell counts observed in clinical trials.(23,24)	CD30 expressed by virtually all HL and ALCL, a subset of other TCL, and about one third of T-ALL(30)	<i>In vitro</i> , cytotoxic activity of CD30 CAR EBV-CTL against an ALCL cell line. <i>In vivo</i> , tumor growth control in HL xenograft (cell line) model.(31)	<ul style="list-style-type: none"> <li>Phase I study which included 2 ALCL patients. One patient experiences a 9 month-long complete remission after 4 CAR T-cells infusion. No response observed in the second patient.(23)</li> <li>Phase I study which included 1 ALCL patient who showed a partial remission after the first CAR T-cells infusion.(24)</li> <li>Ongoing trials : NCT03049449 , NCT02917083, NCT02663297</li> </ul>
	<b>TRBC1</b>	Partially depleted	Partially depleted ( <i>TRBC1 expressed by ≈1/3 of normal T-cells</i> )(58)	Around 40% of T-cell malignancies were found to be TRBC1+(58)	Persistence of normal T-cells in T-ALL xenografts injected with human peripheral blood mononuclear cells.(58)	Pending trial : AUTO4
<b>CAR NK-cells</b>	<b>CD3</b>	Preserved ( <i>CD3 not expressed by NK-cells</i> )	Transiently depleted ( <i>CD3 expressed by all T-cells but CAR NK short-lived</i> )	CD3 expressed by the majority of TCL and a subset of T-ALL	<i>In vitro</i> , lysis T-ALL cell line and primary TCL. <i>In vivo</i> , anti-leukemic effects in xenograft models (cell lines) but relapses observed.(49)	No clinical studies
	<b>CD4</b>	Preserved ( <i>CD4 not expressed by NK-cells</i> )	Partially and transiently depleted ( <i>CD4 expressed by two-thirds of T-</i>	CD4 expressed in the majority of TCL and in a subset of T-ALL	<i>In vitro</i> , lysis of T-ALL and lymphoma lines, primary Sezary syndrome and primary T-ALL cells. <i>In vivo</i> , anti-leukemic effects in a xenograft lymphoma model	No clinical studies

			<i>cells and CAR NK short-lived)</i>		(ALCL cell line).(50)	
	<b>CD5</b>	Preserved <i>(CD5 not expressed by NK-cells)</i>	Transiently depleted <i>(CD5 expressed by all T-cells but CAR NK short-lived)</i>	CD5 expressed in most T-ALL and TCL	<i>In vitro</i> , lysis T-ALL and lymphoma cell lines, primary T-ALL, TCL and Sezary syndrome cells. <i>In vivo</i> , anti-leukemic effects in a xenograft T-ALL model (cell line) when injected in multiple cell doses.(43)	No clinical studies

*\*Based on Ref 8, 19 and 20*