T-Cells targeting CD19 have shown significant antitumour efficacy and clinical success from 57 up to 90% overall complete remission rate when treating relapsed/refractory B cell acute lymphoblastic leukemia (B-ALL) in children and young adults[28–31], relapsed/refractory chronic lymphocytic leukemia (CLL)[32] and B-cell non-Hodgkin lymphoma (NHL)[33]. Recently, two anti-CD19 CAR T-Cell therapies were approved by the FDA. Gilead's Yescarta (Axicabtagene ciloleucel, October 2017) with CD3ζ/CD28 co-stimulatory domains treats large B-cell lymphoma and Novartis's Kymriah (Tisagenlecleucel, August 2017) with CD3ζ/4-1BB co-stimulatory domains treats B-ALL. In 2018, NHS UK struck a deal with Novartis and Gilead in September, providing Kymriah and Yescarta to 30 and 200 patients per year respectively. This is a very small number of patients that can be benefited from CAR T-Cell therapy. Although proven effective against B lymphocyte malignancies, there are still potential toxicities that can affect the survival rate of patients after the therapy.

Cytokine Release Ryndrome (CRS)

Although second generation constructs of CAR T-cells have increased cytokine production and a higher cytotoxicity, this a double-edged sword as the immune activation induces adverse events (AE) such as hypotension, febrile neutropenia, acute vascular leak syndrome[34], fevers. Some of these events were reported to evolve into symptoms similar to macrophage activation syndrome[29]. The incorportation of CD28 or 4-1BB expresses difference in cytokines release, 4-1BB induced signalling shows less potency in secreting IFN-α and Il-2 compared with CD28[35]. It seems that the concentration of cytokine released is correlated to the tumour burden and CAR T cells expansion of the patient[36,31], indicating that the more effective the therapy is, the higher there is in developing more severe CRS.

Table 1: Cytokines related to CRS and their effects on CAR T-Cell therapy clinical trials

Cytokines	Sources	Principle Targets and Functions	Symptoms in CRS
IFN-γ	NK cells, Th1 cells and Cytotoxic T lymphocytes (CTLs)	Macrophages: classical activation T cells: Th1 differentiation B cells: isotype switching to opsonizing Various cells: increases MHC expression and antigen processing to T cells	Fever, chills, dizziness and headache, fatigue
TNF-α	Macrophages, NK cells and T cells	Endothelial cells: activation (inflammation) Neutrophils and macrophages: stimulates microbicidal activity Liver: synthesis of acute phase proteins	Flu-like syndrome, fever, general malaise, rigor, and watery diarrhea, vascular leak, inhibits myocardial contractility and vascular muscle tone, lung injury, synthesis of acute phase proteins
Il-2/sIL2Ra	T cells	T cells: proliferation and differentiation into effector and memory T cells NK cells: proliferation and differentiation B cells: proliferation and antibody synthesis Augment immune response	Flu-like syndrome, vascular leak, inhibitory actions on myocyte contractility
Il-6	T cells, monocytes, macrophages, fibroblasts and endothelial cells	B cells: proliferation of antibody-producing cells Neutrophils: stimulates production from the bone marrow Liver: synthesis of acute phase proteins	Fever, chills, nausea, vomiting and fatigue, vascular leak, lung injury
Il-10	Th2 cells and macrophages	Macrophages and DCs: inhibition of the expression of IL-12, costimulators and class II MHC	Fever, headache, back pain, dizziness
Il-12	Macrophages and DCs	T cells: Th1 differentiation NK cells and T cells: IFN-c synthesis, increasing cytotoxicity	Fever, chills, nausea/vomiting, hypotension, anorexia, myalgia, fatigue, liver toxicity

In various studies there were around 10-45% of patients developed a severe CRS (grade $\geq$3)[37–42]. The most frequently observed cytokines related to CRS are interferon (IFN)-γ, tumour necrosis factor (TNF)-α, Il-6, Il-10 and interleukin-2 receptor α (sIL2Ra). Currently, CRSs are usually controlled by treating with

high-dose steroids such as corticosteroids or monoclonal antibody (mAb) such as tozilizumab that blocks interleukin receptor, in this case Il-6R[28]. However, using steroids in CRS management results in depletion of CAR T cells by five-folds when compared with using tozilizumab alone with similar results in reversing CRS symptoms[28]. It is a tricky situation to balance between the efficacy of the therapy and CRS management, early immunosuppression may result in severe decrease in efficacy but late CRS management can become fatal. Patients treated with tozilizumab alone showed persisting T cells culture, indicating that tozilizumab did not affect long term T cells survival[32] and this suggests that using tozilizumab as the first line of immunosuppressive therapy might be a better choice. However it is still unclear if targeting Il-6 directly would be more effective as Il-6 inhibitors are not extensively tested in treating CRS.

A study suggested that more investigations should be done regarding CRS to isolate cytokines that are required for increasing efficacy of the CAR T-Cell therapy and those that induced adverse events[42]. This allows us to target CRS-indusing cytokines and apply mAb to reduce toxicity and increase safety. Recently, a new approach in managing CRS has been explored. Macrophages are observed to release catecholamines upon activation by IFN-γ released by CAR T cells in a mouse model and this catecholamine production is essential in cytokine release[43]. Application of atrial natriuretic peptide (ANP) or metyrosine (MTR) significantly lowered the levels of catecholamines and certain cytokines (human IFN-γ, TNF and mouse Il-6, KC(CXCL1), MIP-2 (CXCL2)) when treating mice with high tumour burdens. However, they died prematurely as a result of progressive disease. Treatment of mice with low tumour burdens with ANP and MTR also significantly lowered levels of catecholamines, human TNF, mouse Il-6, KC but not so much in human IFN-γ and Il-2. The antitumour effects and expansion of CAR T cells didn't seem to be affected by ANP and MTR but but cytokines associated to CRS decreased in concentration, showing the effectiveness of the treatment in managing CRS without affecting the efficacy of CAR T cells.

As the experiment was based on mouse model, clinical trials are required to confirm that ANP and MTR have similar effects in human. The potential of CRS management by ANP, MTR and other molecules that can inhibit the production of catecholamines might provide more options in cytokine toxicity.

Neurotoxicity

There are evidences that early CRS developed after CAR T-Cell infusion leads to a higher risk of developing reversible neurological toxicities[44,28,45] including confusion, seizure, headache, decreased level of consciousness, language disturbance, delirium and in extreme cases lethal. 133 adults with refractory B-ALL, NHL, or CLL after receiving lymphodepletion chemotherapy prior to anti-CD19 CAR T-Cell therapy with 4-1BB as the co-stimulatory domain were studied and 40% (53/133) of them developed one or more grade $\geq$1 neurological AEs[45] within 28 days after the infusion. 48 out of those 53 patients with AEs also had CRS. Patients showed symptoms of CRS like fever earlier developed higher grade of neurotoxicity ($\geq$3) compared to those developed grade 1-2 neurotoxicity, show-

ing the correlation between CRS severity and neurotoxicity severity. Neurotoxicity is usually treated with tozilizumab as well given its relationship with CRS but once it is developed it is less responsive to treatment compared to those patients that only have CRS[45].

The severity of neurotoxicity correlates to the level of C-reactive protein (CRP, acute-phase protein released by the liver responding to inflmmation), ferritin and other cytokines (Il-6, IFN-γ and TNF-α) that lead to endothelial activation[45,28]. Since real-time monitor of serum cytokines before onset of toxicities is difficult so CRP is chosen and test as a potential target. It is proven successfully that CRP is an excellent indication for CRS as they show very strong correlation[28]. The study suggested that patients with early fevers and a CRP level of $\geq$20 mg/dl are advised to be managed as if they have CRS as these indications suggest a very high risk in developing neurotoxicity as well.

The causes of neurotoxicity is still unclear but clinical evidences such as endothelial dysfunction, including vascular instability, capillary leak, disseminated intravascular coagulation (DIC) are accompanied with elevation of serum cytokines especially Il-6, IFN-γ and TNF-α in patients with severe neurotoxicity[45]. The paper proposed that one of the potential causes of neurotoxicity is the vascular leak of high concentration of cytokines into the cerebrospinal fluid (CSF) due to disruption of the blood brain barrier (BBB). The permeability of the BBB increased after lymphodepletion chemotherapy, showing higher concentration of protein and leukocyte detected in CSF compared to before lymphodepletion. This disruption of BBB is considered to be induced by the affects of cytokines[46]. Although there were some arguments regarding the effects of TNF-α on the permeability of BBB in the earlier days[47], it is hypothesised to increase the BBB permeability in different ways such as affecting the architecture, increase the expression of adhesion molecules for immune cells and synthesis/release of cyclooxygenase products[48]. BBB-endothelial cells (BBB-ECs) express tight junction (TJ) and adherens junction (AJ) proteins that limit cellular permeability[46]. TNF-α downregulates occludin expression in BBB-ECs in a mouse model, which is one of the major components of TJ proteins[49]. This effect was not observed in human model but in vitro TNFα and IFNγ stimulation is shown to change the architecture of junction proteins without affecting their expression[49,50]. Adhesion molecules such as CXCL10, CXCL9, CX3CL1, CCL3, CCL4 and CCL5 are shown to be upregulated by the combination of IFN-γ and TNF-α[46] and they induce the attraction, activation and migration of various immune cells towards BBB.

These findings confirm that the patients with more severe CRS have a higher risk in developing neurotoxicity. The increase in BBB permeability leads to cytokines diffuse into CSF and possible increase in local cytokines production due to migration of immune cells across the BBB. Although there are risks in neurotoxicity, this side effect may be utilised to direct CAR T cells to the brain and improve the penetration of BBB when treating glioma.

Anaphylaxis

The construct of CAR usually includes a single-chain variable fragment (scFv) derived from murine origin. It is expected to show some degree of graft-versus-host disease (GVHD) which is the immune cells in the donor (infused CAR T cells in this case) attack the patient's body's cells. In some cases antibodies targeting the CAR were detected[51,52] and reversible GVHD (aggravated hyperbilirubinemia, elevated amino-transferases and chronically aggravated skin damage was also observed[53] but no large number of adverse effects had been recorded. There was a clinical trial where 1 patient out of 4 developed acute anaphylaxis which resulted in a lethal cardiac arrest event after the third infusion of anti-mesothelin CAR T cells with 4-1BB stimulatory domain[54]. The same batch of cryopreserved CAR T cells were used in those 3 infusions. The patient also underwent cardiac arrest within 1 minute after the second infusion and was treated with aggressive volume of resuscitation and high-dose steroids. Given the same CAR T cells were used, it was proposed that the serious adverse event was caused by an accumulated immune response. Further investigation was conducted based on the suspicion that this adverse event is caused by Ig-E mediated degranulation of mast cells leading to anaphylaxis. Tryptase, which is the marker for mast cells degranulation was recorded to have an elevated level, confirming the adverse event was caused by Ig-E mediated anaphylaxis. Although this is a very small sample size with 4 patients enrolled in the clinical trial, the research had been conducting trials on over 400 patients before infusing them with T cells cultured with immobilised mouse anitbodies to CD3 and CD28 on beads[54] and no anaphylaxis was observed so alloimmune response might be a very rare incident. Due to this event that occurred within a 49 days period, future trail that requires multiple infusions will be designed such that infusions must not be separated for more than 10 days and all infusions should be completed within 21 days to minimise the chance of class switching from IgG to IgE.

Interestingly, the use of CD28 as the co-stimulatory domain seems to reduce the probability of developing GVHD and increase survival rate when compared to using 4-1BB or not having a co-stimulatory domain[55]. The research group hypothesised the repetitive stimulation signals from CAR and alloreactive TCR lead to T cells exhaustion and eventually being deleted, reducing the number of alloreactive CAR T cells. They found out that the expansion of CD8+ effector-memory T cells and cytokines production (Il-6, IFN-γ and TNF-α) was significantly lower when using CD28. This confirmed their hypothesis that T cell exhaustion protects patients from GVHD but this finding contradicts with previous studies which showed that adding in co-stimulatory domain improves T cell proliferation and cytokines production[4,5]. This difference might be due to the different in CAR constructs design and remain unanswered. However, this finding agrees with previous researches that the use of CD28 instead of 4-1BB induces T cell exhaustion and affects the persistent of CAR T cells[8,9,56], and shows the importance in CAR construct design to achieve a minimal toxicity.

On-Target/Off-Tumour

The major on-target/off-tumour toxicity observed when applying CAR T cells is B cell aplasia, the depletion of nonmalignant B cells. This is an expected side effect as CD19 is expresses on both B cell malignancies and also normal B cells so the CAR T cells targeting CD19 will also attack normal B cells. This suggests that B cell aplasia can also be served as an indicator for the effectiveness of the therapy as it correlates with the persistence of CAR T cells in the patient. The severity of B cell aplasia varies from months[29,57] to over 15 months[58] and CAR T cells using 4-1BB showed a prolonged B cell aplasia[59,58], agreeing with the findings that 4-1BB improves CAR T cells persistence. This can be easily managed with infusion of γ-immunoglobulin (IgG) as replacement therapy[29,60,61] to reduce risk of infection but the cost of the therapy will be further increased.

3 Discussion: Future CARs development

Although we have seen great success in applying CAR T Cell therapy in B cell malignancies, there are way more cancer incidences in solid tumour than leukaemia[62] and the next step is to explore the therapeutic potential of CAR T Cell therapy in solid tumours. To translate this technology from liquid to solid tumours, balance between therapeutic effectiveness and potential toxicities must be evaluated carefully. Targeting solid tumours is more challenging than leukaemia as one of the key factors to an effective and safe therapy is unique targetable antigens. CD19 is an excellent target in leukaemia due to its exclusive expression on B cells, even though nonmalignant B cells will also be deprived during the therapy, aplasia can be easily managed. On the other hand, a lot of antigens expressed on cancer cells in solid tumours are also expressed on normal cell and the on-target/off-tumour effect may results in organ damages. There are also some other factors that we have to overcome in order for CAR T Cell therapy be viable in treating solid tumour.

3.1 Challenges in Designing CAR T-Cell for Solid Tumours and New Construct Designs

Trafficking and Infiltration

CAR T cells localisation to the target tumour site is essential in targeting solid tumours. The expression of E-, P-selectin ligands and L-selectin on the CAR T cells and cell adhesion molecules (CAM) on the endothelial cells is needed for T cells rolling on the tumour cells[63]. Matching between chemokine receptors on CAR T cells and the chemokines released by the tumour cells is also vital in T cells trafficking[64].

Glioblastoma is the most aggressive cancer that begins in the brain in human so we will use it as an example in the following discussion about the importance of adhesion molecules. Activated leukocyte cell adhesion molecule (ALCAM) has a major role in T cells recruitment associated with inflammatory brain diseases[65].

It is shown that brain cancer endothelial cells overexpress ALCAM but they down-regulate the expression of intercellular adhesion molecule-1 (ICAM1) and clear out vascular cell adhesion molecule-1 (VCAM1)[65]. Similar changes are observed in multiple sclerosis which ICAM1 and VCAM1 act as the secondary signal required to reach the threshold needed to recruit T cells from the bloodstream[66]. This alteration allows the tumour cells to evade the immune system. It is still unclear the effects of the overexpression of ALCAM apart from tumour invasion but ALCAM alone is not sufficient for T cells homing to the tumour cells. Due to this similarity to multiple sclerosis, the research group hypothesised that they can apply what they learnt from multiple sclerosis and overcome this immune-evasion mechanism of glioblastoma. They created an in vitro BBB model by sandwiching a polycarbonate membrane between primary tumour endothelial cells (pTECs) and pericytes.

ALCAM usually binds to CD6, which is expressed on activated T cells but despite the overexpression of ALCAM on tumour endothelial cells, poor transendothelial migration of T cells was observed. Therefore they developed a homing system (HS) by re-engineering CD6 on T cells which the prototype HS molecule included a D3 (extracellular domain 3 of CD6) exodomain, an IgG hinge and a CD6 transmembrane and signalling domain. They also created variants such as multimerising of the D3 domain (denoted as 3HS and 5HS, figure 2) and some without the signalling domain (ΔHS) to study the effect of crosslinking avidity to ALCAM and importance of CD6 signalling respectively.

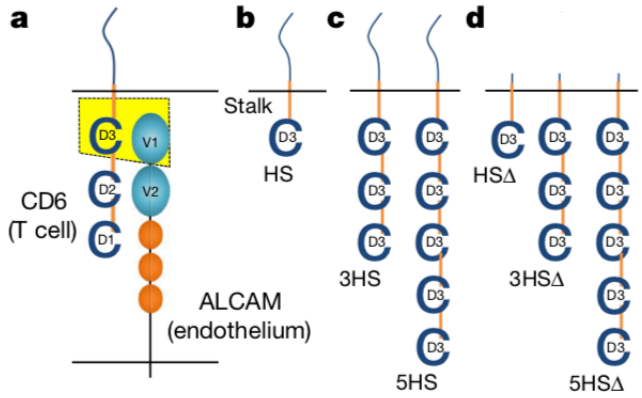

Figure 2: Schematic representation of the homing system (a)ALCAM binding to CD6 (b)prototype HS (c)multimerisation of D3 domain (d)without signalling domain

They demonstrated that the T cells with HS showed a more frequent capture, rolled more slowly and stopped more quickly when they were in contact with ALCAM+ cells compared to normal T cells. They were also more resistant to mechanical detachment which allows them to be less affected by the fluctuated blood flow in the tumour neovasculature which is more chaotically arranged with blind ends.

Study had shown that the matching of expressed chemokine receptors and the chemokines released by the tumour cells are essential in T cells trafficking. Nor-

mal T cells activated by anti-CD3/anti-CD28 beads via T cell receptor upregulate the expression of CCR5, CCR7 and CXCR3[67] and they are likely to be attracted to tumour cells releasing CCL5 (binding to CCR5) or CXCL9, CXCL10, or CXCL11 (binding to CXCR3). They found very little concentration of these chemokines in mesothelioma cell lines and patients with malignant mesothelioma but instead CCL2 was highly secreted. However, the chemokine receptor CCR2b was poorly expressed on the anti-mesothelin CAR T cells they were using so the potential of directing CAR T cells to the tumour site was tested by transducing CCR2b into the CAR construct using a lentiviral vector. Dual expression of the CAR and the CCR2b was observed in almost one quarter of the cells indicating some technical limitations when performing two transduction events with less than 100% efficiency. There was a significant increase in infiltrated T cells ($>$12.5 fold) with CAR$+$CCR2b T cells and surprisingly augmented antitumour activity as well when compared to CAR T cells without the CCR2b (killing 20% more tumour cells). Similar results were obtained by another research group when transducing CCR2b to anti-GD2 CAR T cells targeting neuroblastoma[68] showing $>$10 fold increase in trafficking and enhanced killing capacity. Despite different CAR constructs were used (second generation 4-1BB in anti-mesothelin CAR and third generation CD28/OX40 in anti-GD2), the CAR T cells with coexpression of CCR2b showed enhanced killing. This suggests that the incorporation of chemokine receptor matching the chemokine released by the tumour might be a way to further improve antitumour efficiency.

Immunosuppressive Tumour Microenvironment

Hinge domain

Homing System

3.2 Complementation with other molecules and therapies

(hypoxia switch from oxidative to glycolytic, talk about VHL-HIF?)

PD1

3.3 Safety monitoring

C-reactive protein[28]

organoid

4 Conclusion