Current status of chimeric antigen receptor therapy for haematological malignancies

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Summary

The field of adoptive cell transfer includes chimeric antigen receptor (CAR) engineered T cells, constructs that emerged from basic research into principles of immunology and have transformed into clinically effective therapies for haematological malignancies. T cells engineered to express these artificial receptors hold great promise, but also carry significant risk. While permanent genetic modification of mature T cells appears safe, modulating their *in vivo* function is difficult, partly because the robust response can trigger other arms of the immune system. Suicide systems and toxicity management with cytokine blockade or signal transduction modulators have emerged as a new frontier in this field, a far cry from early problems getting CAR T cells to work at all. Currently, clinical trials in patients with relapsed or refractory B cell malignancies treated with CD19-specific CAR T cells have induced durable remissions in adults and children. Results from these trials indicate that more work needs to be done to understand biomarkers of efficacy, the role of T cell persistence and how to integrate this care into standard practice. Cell therapy will not be a 'one size fits all' class of medicine, and here we will discuss the development of this therapy and important questions for its future.

Keywords: immunotherapy, chimeric antigen receptor, acute leukaemia, T cells.

A variety of cellular therapies have been implemented for the treatment of cancer. This includes many immune cell subtypes, from polyclonal T cells to dendritic cells and natural killer cells. These cells may also be modified *ex vivo* with DNA or peptide vaccines or further manipulated by culture conditions including cytokines or mitogens. In this review we focus on the background, rationale and current clinical use of chimeric antigen receptor (CAR) T cells targeting CD19 in paediatric B cell malignancies.

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Foundations of adoptive T cell transfer

History

As is often the case, animals research models provided the foundation of the concept of adoptive cellular therapy for tumour allografts (Mitchison, 1955). In the earliest work, researchers demonstrated that an allogeneic haematopoietic graft was the key to eradicating leukaemia after transplantation in mice (Barnes & Loutit, 1957; Barnes *et al*, 1957). The hypothesis that the graft itself had anti-leukaemia properties underlies the clinical strategy of allogeneic bone marrow transplant (Mathe *et al*, 1965; Rosenberg & Terry, 1977). This hypothesis was further supported by the seminal finding of Weiden *et al* (1979) that haematopoietic stem cell transplantation (HSCT) using allogeneic donors was more effective at preventing relapse of leukaemia than use of syngeneic donors. Allogeneic T cells can therefore probably recognize targets on leukaemia cells that syngeneic T cells cannot. Researchers built on this foundation, searching for ways to get autologous lymphocytes to recognize leukaemia cells to fulfill the promise of efficacy without the toxicity of graft-versus-host disease (GvHD). Many lessons were learned in early trials of adoptive cell therapy, most prominently that the key variables were target choice of the antibody fragment, the differentiation state of the cells to be transferred and the host environment.

Target antigens

Choosing a target antigen requires a delicate balance of risk and benefit. The ideal antigen is expressed only on the cancer, but not on normal tissue or only on an expendable cell type, is not shed into circulation and is essential to the growth or survival of the cancer cell (oncogene addiction) and thus not easily lost under selective pressure. Other than normal B cells, CD19 is not present on normal tissues, is not shed as a soluble form and appears to be relatively essential in the early stages of B cell development from which many

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malignancies tend to arise. This has made it an ideal target during the developmental stages of CAR research and the most successful early clinical trial target (Barrett *et al*, 2013a). In contrast to the experience with CD19, poor target choice or unexpected expression can lead to unwanted outcomes. The choice of carbonic anhydrase IX (CAIX), an antigen present on the surface of clear cell renal carcinoma, illustrates the problem of essential tissue expression (Lamers *et al*, 2006). Within a week of infusion of CAR T cells specific for CAIX, patients experienced hyperbilirubinaemia due to target expression in the biliary tract. At issue is how much expression on a normal tissue is too much? Does low-level expression make a tissue vulnerable? Is there a threshold of surface antigen expression above zero at which CAR T cells will not cause damage to normal tissues with a target antigen? Because the answers to these questions remain unknown, additional safety features, such as suicide systems and transient or tunable expression systems, are favoured during early phase trials to protect against unwanted off-tumour but on-target adverse effects (Di Stasi *et al*, 2011).

Host lymphodepletion

Adoptively transferred T cells engraft and expand more efficiently in a lymphopenic host, as lymphopenia favours homeostatic expansion of T cells. Host lymphodepletion is typically accomplished via chemotherapy or targeted monoclonal antibodies, and expansion can be further enhanced by application of supportive cytokines, such as interleukin (IL) 7 and IL15 (Klebanoff *et al*, 2005). After transfer, cells may gain enhanced effector function or proceed further down a differentiation path as a result of the homeostatic expansion (Dummer *et al*, 2002). The seminal work in this field was performed at the National Institutes of Health, where Dudley *et al* (2008) demonstrated that progressively more intense lymphodepletion was associated with better proliferation and efficacy of adoptively transferred tumour infiltrating lymphocytes in melanoma. Complementing these efforts was the work of Guimond *et al* (2009), who demonstrated the importance of homeostatic cytokines, such as IL7, on supporting the expansion of T cells through multiple mechanisms. Alternatively, researchers are pursuing methods to promote T cell proliferation in lymphoreplete hosts to minimize exposure to chemotherapy and the risks of immune suppression in patients with malignancies (Pegram *et al*, 2012).

T cell proliferative capacity and differentiation state

Originally, effector T cells (effector memory or terminal effector cells) that secreted high levels of cytotoxic cytokines and were proficient killers of tumour targets *in vitro* were thought to be the ideal components for clinical adoptive cell therapy. This was based on the concept that only $CD8^+$ cells were necessary (the killer T cells) and that $CD4^+$ cells [the helper T cells (Th)] were probably not necessary. The relative lack of clinical efficacy of largely terminally differentiated $CD8^+$ T cells, the type used most often in early T cell transfer trials, has provided several important lessons in the appropriate composition of an effective T cell therapy product. First, despite apparent optimal *in vitro* cytotoxicity and high cytokine secretion, the replicative capacity of these terminally differentiated cells was poor and consequently persistence of transferred cells in patients was very poor. Decades of research into these problems provides evidence indicating that infusion of naïve T cells (Hinrichs *et al*, 2009), central memory T cells (Berger *et al*, 2008), Th17 cells (Paulos *et al*, 2010), and T stem memory cells (Gattinoni *et al*, 2011) may all have certain advantages related to their high replicative capacity or previously unrecognized anti-tumour activity. Data also indicates that $CD4^+$ cells are probably required for efficacy of an adoptively transferred T cell product, and can have cytotoxic function on their own when armed with a CAR (Liadi *et al*, 2015). $CD4^+$ T cells also probably support a more robust physiological expansion of the T cell population after transfer, though whether this is from supportive cytokines or other factors remain unknown. This is one area where studies in mice were potentially misleading. Human T cells have limitations on replicative capacity by virtue of telomere degradation, whereas mouse T cells have extraordinarily long telomeres compared to the human T cells and do not face this limitation (Mestas & Hughes, 2004; Straetemans *et al*, 2011). The optimal state of differentiation of the infused T cells remains an important question, though researchers generally agree that less differentiated (i.e. 'younger') is better. Whether murine or human, naïve (T_N) or central memory (T_{CM}) cells result in higher efficacy in preclinical models and thus if given the choice clinicians would prefer to use T cell products enriched for these subtypes (Maus *et al*, 2004; Berger *et al*, 2008). This is often difficult in patients with cancer, especially elderly patients who have fewer naïve T cells and in general for all patients after lymphotoxic chemotherapy. T cells with high replicative capacity appear to be the most important biomarker of a successful cell therapy product when treating patients with CD19 CAR T cells (Kalos *et al*, 2011; Kalos & June, 2013). This appears translatable to other cancers, as replicative capacity of adoptively transferred T cells correlates with the engraftment and antitumour efficacy in patients with melanoma (Zhou *et al*, 2005).

There are two approaches to enriching for T cells with high replicative capacity: isolate them from bulk cells before proceeding with CAR modification or culture bulk cells in conditions that favour those subtypes. Cell sorting is expensive and time consuming, especially at Good Medical Practice (GMP) scale. During culture, activating specific costimulatory signals via receptor engagement promotes selected fate and differentiation. CD28 stimulation can program CD4 cells to maintain central memory states and maintain telomere length (Weng *et al*, 1996; Thomas *et al*, 2002; Kaneko *et al*,

2009), and stimulation of 4-1BB (also termed TNFRSF9, CD137) promotes the growth of CD8 central memory cells (Maus *et al*, 2002). Specifically for Th17 cells, ICOS (inducible costimulator) activation can promote outgrowth and stability of this rare subset (Paulos *et al*, 2010). The stem central memory T cell (T_{SCM}) has self-renewal and stem cell-like properties, and thus may have the potential for high replicative capacity and long persistence (Gattinoni *et al*, 2011; Graef *et al*, 2014). *In vitro*, activating *Wnt* signalling via inhibition of Glycogen Synthase Kinase 3 Beta (GSK3B) can promote the propagation of memory stem cells during culture (Gattinoni *et al*, 2009).

Conversely, regulatory T cells (T regs) are undesirable in cell therapy products for the treatment of malignancies and must be minimized both in the patient and in the transferred product based on pre-clinical models (Lee *et al*, 2011). Chimeric receptors in T regs, however, do have other promise as a therapy in autoimmunity. This concept relies on directing T regs to a vulnerable tissue via the CAR, where the goal is to activate the immunosuppressive effects of the T reg via CAR signalling. This was demonstrated in mouse T regs with a CAR targeting myelin basic protein that protected against autoimmune encephalitis, and other models are being pursued for colitis and diabetes (Esensten *et al*, 2009; Riley *et al*, 2009; Jethwa *et al*, 2013).

Based on these principles, the final clinical application of adoptive T cell transfer will probably employ a combinatorial approach mixing T cell subsets and preparatory regimens dependent on the goal at hand. This will be guided by the tumour type, prior chemotherapy regimens (subsets available for collection), host conditioning and the tumour microenvironment.

CAR design choices

The design of the CAR structure is historically broken into 3 generations. The first generation consisted solely of an antibody-based external receptor structure and a cytosolic domain including the immunoreceptor tyrosine-based activation motif (ITAM) from TCRζ or FcRγ (Eshhar *et al*, 2001). Second generation CARs added costimulatory signalling domains, such as CD28 or 4-1BB (Krause *et al*, 1998; Milone *et al*, 2009). Under the 'more is better' philosophy, third generation CARs include three or more cytosolic domains usually with multiple costimulatory sections (Till *et al*, 2012).

First generation CARs

The first CAR used in patients was a first generation structure combining the CD4 ectodomain with the CD3ζ endodomain that demonstrated modest antiviral efficacy in human immunodeficiency virus (HIV) but also long term persistence (Deeks *et al*, 2002). Long term follow up of these patients was reassuring in that no transformation events or oncogene insertions were noted with the modified lentiviral vector used to modify the T cells (Scholler *et al*, 2012).

This persistence was encouraging also because other trials of early CARs were not observing efficacy or persistence, reiterating a fundamental question in adoptive cell therapy: do cells need to persist to work or do they persist because they work? In the first generation CAR trials, T cells often failed to engraft at all, becoming undetectable after days or weeks. A pilot trial in neuroblastoma using a CAR targeting a tumour-associated adhesion molecule (CD171) had no toxicity but also poor persistence of the T cells (Park *et al*, 2007). Such results make it hard to say with confidence whether toxicity can be observed if efficacy is so poor. The best persistence of CARs reported in a cancer trial was in a paediatric neuroblastoma trial (clinicaltrials.gov NCT00085930), with low level polymerase chain reaction detection of CARs (0·0001–0·001%) up to 4 years after infusion (Louis *et al*, 2011). This is in contrast to the HIV CD4ζ CAR trial (clinicaltrials.gov NCT01013415), with much higher levels of persistence (0·6–6%) 5 years after infusion (Scholler *et al*, 2012). These trials reported some infusion toxicities, such as fever or chills, but no other adverse effects that could be attributed to the infused cells.

First generation CAR trials were informative as it was discovered that the infused product could be immunogenic. This includes responses from both B cells (Kershaw *et al*, 2006; Lamers *et al*, 2006) and T cells (Jensen *et al*, 2010; Lamers *et al*, 2011).

A final lesson from the first generation CAR trials in cancer patients was that efficacy was disappointing. The best clinical results were reported in patients after infusion of a GD2 (a disialoganglioside) specific CAR, with 2 of 11 patients having long term remissions (Louis *et al*, 2011). These surviving patients had the long term low level persistence cited above; patients with no persistence experienced disease relapse.

Second and third generation CARs

While first generation CARs were valuable for the establishment of chimeric receptor function in the laboratory setting, immunological principles would predict that a structure that lacks T cell costimulation would favour the development of anergy, unless costimulation was provided either from the target or exogenously (Bretscher & Cohn, 1970; Loskog *et al*, 2006; Brocker, 2000). The CD28 signalling domain, the key to T cell costimulation, provided this essential missing ingredient when engineered in *cis* with the T cell receptor zeta (TCRζ) domain in the CAR structure (Finney *et al*, 1998; Krause *et al*, 1998). Other members of the tumour necrosis factor receptor family, such as CD27, 4-1BB and CD134 (TNFRSF4), followed suit and also demonstrated the ability to provide costimulation (Finney *et al*, 2004; Imai *et al*, 2004; Song *et al*, 2012).

The comparison of CAR structure is complicated by the dense grid of variables that go into a functional CAR product (Fig 1). The most common comparison is the 4-1BB and CD28 endodomains, which have been extensively studied in pre-clinical models (Carpenito *et al*, 2009; Milone *et al*, 2009). Both CAR models showed preclinical efficacy, and both have demonstrated clinical efficacy (Grupp *et al*, 2013; Davila *et al*, 2014; Maude *et al*, 2014; Long *et al*, 2015) investigated the cause of early exhaustion in their CAR models, and found that CD28-based endodomains accelerate T cell exhaustion, whereas 4-1BB-based CARs did not. CD28-based endodomains in a CD28-based expansion system can mediate constitutive signalling, which seems to favour terminal differentiation of effector T cells (Frigault *et al*, 2015). These studies require careful consideration, as we also demonstrated that a 4-1BB based CAR cannot rescue more terminally differentiated cells generated by high-dose interleukin 2 (IL2) exposure (Barrett *et al*, 2014). As cell culture systems become more sophisticated, the influence and interaction of the T cell culture with the CAR signalling domains will be critical to the ultimate clinical success of these therapies.

Approaches for T cell culture

Early studies of T cell immunotherapy treated T cells as a traditional drug, where a large dose was desirable. In hindsight, this meant manufacturing large numbers of T cells with exhausted or terminally differentiated phenotypes, often the result of forced cell division from high dose IL2, which had little chance of resulting in the kind of lasting efficacy and persistence needed to clear refractory malignancies. T cells are not a traditional drug, however, as highly replicative early memory T cells can proliferate in the patient and result in clinical efficacy (Kalos *et al*, 2011; Porter *et al*, 2011). There is probably some lower threshold of CAR expressing T cells that will result in efficacy, but this has yet to be deter-

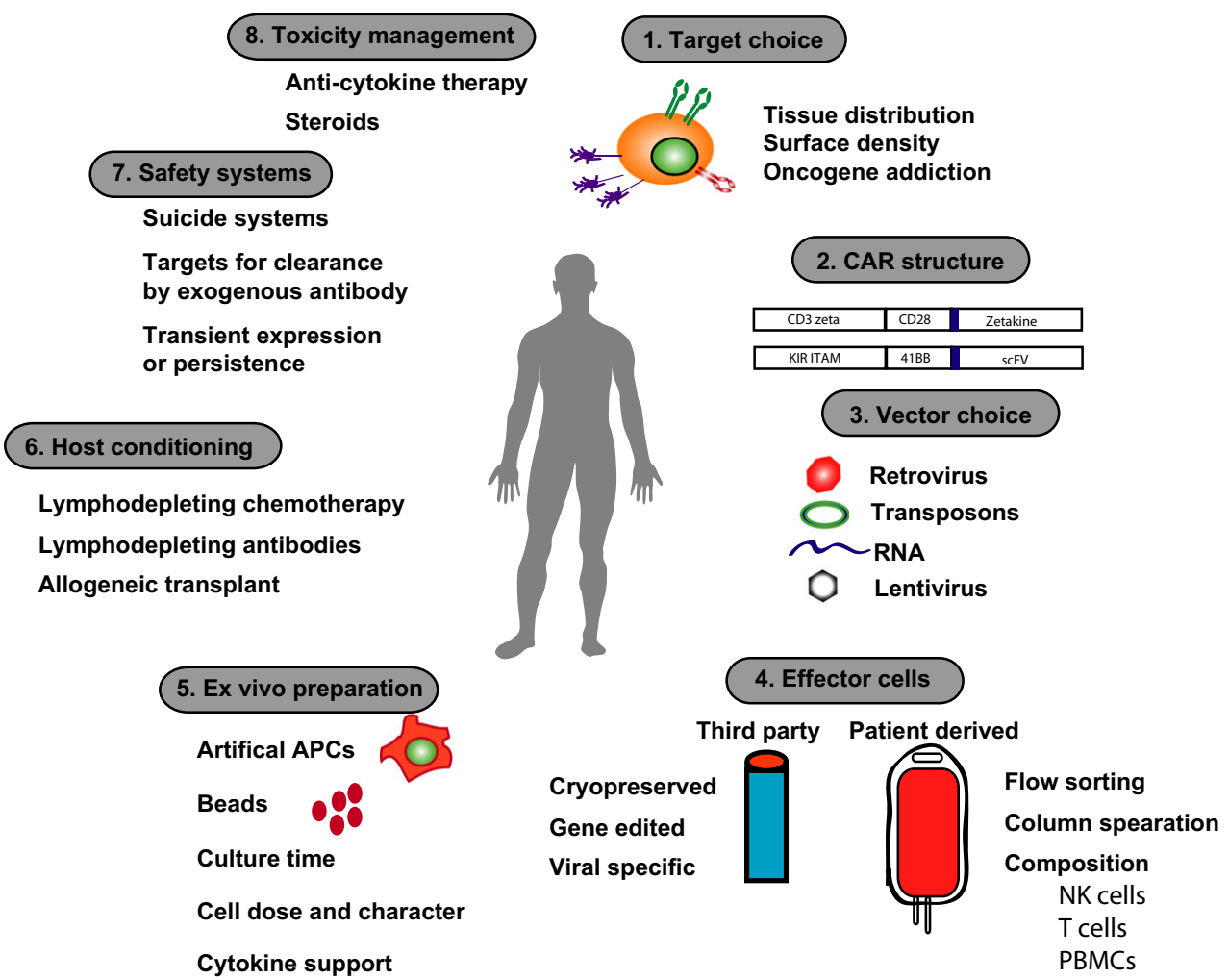

Fig 1. Variables that contribute to CAR design and efficacy. Change in any variable group can dramatically alter the function and efficacy of the overall product, making iterative comparisons in preclinical modelling extremely challenging. CAR, chimeric antigen receptor; APCs, antigen presenting cells; NK cells, Natural Killer cells; PBMCs, peripheral blood mononuclear cells.

mined. Alternative approaches using temporary expression systems, such as mRNA transfection, may actually benefit from large numbers of more effector-class cells or from multiple infusions similar to traditional 'loading and maintenance' dosing strategies(Zhao *et al*, 2010; Barrett *et al*, 2011).

The paradigm for assessing effective T cell therapy products has changed with the realization that, despite the high *in vitro* cytotoxicity and cytokine secretion of terminal effector cells, T cells with high replicative capacity are more effective (on a per cell basis) and persist longer after adoptive transfer(Zhou *et al*, 2005). With a better understanding of the differentiation of T cell memory states, this makes sense as T_{CM} cells can self renew and differentiate into effector T cells *in vivo*, while effector memory T cells (T_{EM}) can only progress to exhaustion and death (Seder *et al*, 2008). There are two approaches to *ex vivo* cell culture to try to select for cells that will be effective after transfer (i.e. have a high self-renewal and proliferative capacity): isolate the early memory T cells first or culture bulk cells in conditions that favour early memory states. In the first case, one could isolate naive (T_N) or T_{CM} cells by flow cytometric sorting or column separation, transduce with the desired CAR, expand and then infuse the resulting early memory cells (Berger *et al*, 2008). Manipulation of bulk T cell culture conditions via cytokines or artificial presenting cells with specific costimulatory ligands may also enrich and maintain T_{CM} cells, and thereby bypass the need for cell sorting procedures and the incumbent risk of cell loss (Levine *et al*, 1997; Maus *et al*, 2004). The use of stem central memory T cells (T_{SCM}), which may have the most capacity for self-renewal, also has significant potential (Turtle *et al*, 2009; Gattinoni *et al*, 2011).

Approaches for T cell engineering

Vectors for permanent genetic modification of T cells, such as the insertion of a CAR, include those derived from gamma retroviruses or lentiviruses with T cell trophy, integration into the host genome with low oncogenic potential and low immunogenicity (Miller & Rosman, 1989; Naldini *et al*, 1996). These vectors form the foundation of most on-going clinical trials of CAR therapies. A serious safety concern when infusing patients with engineered T cells is viral insertional mutagenesis, such as those that cause cellular transformation, seen with genetically engineered haematopoetic stem cells (HSCs) (Hacein-Bey-Abina *et al*, 2008). However, genetically modified T cells can persist in patients after adoptive transfer for more than a decade without transformation to a clonal or malignant process, highlighting that mature T cells are probably fundamentally safer for genetic modification that stem cells (Muul *et al*, 2003; Scholler *et al*, 2012). The nonviral vector *sleeping beauty* transposon system has also been described for permanent genetic modification with CARs, and is being tested in a clinical trial (clinicaltrials.gov NCT00968760) (Kebriaei *et al*, 2012). The *sleeping beauty* system is an elegant alternative to viral vectors, in which cells are transduced temporarily with a transposase and a DNA plasmid with a gene of interest flanked by the target sequences of the transposase enzyme. In this fashion, the gene sequence is randomly inserted into the genome by the transposase, which then degrades, limiting any further potential for genotoxicity. Advantages cited include the relatively lower cost and complexity of the transposase system compared to viral vectors and the potential to transduce non-dividing cells. RNA-based electroporation of lymphocytes using *in vitro*-transcribed mRNA mediates transient expression in situations where permanent expression is undesirable. This technique has been used with chimeric antigen receptors for CD19, mesothelin and GD2; with target specific activation and *in vivo* activity in pre-clinical models (Zhao *et al*, 2010; Barrett *et al*, 2013b; Singh *et al*, 2014). RNA electroporation and transient expression serves as another possible level of safety, as the degradation is always complete in all cells over time and does not require exposure to an activating agent.

Support and control of CAR T cells

By their nature, CARs bypass the normal checks and balances of immune recognition, overcoming the constraints of major histocompatibility complex (MHC)-restricted TCR recognition by targeting native cell surface molecules. Genetic modification of T cells is not limited to the CAR, but can also be used to insert genes that improve the efficacy or safety of the T cells that are transduced. Such payloads include more costimulation molecules (Krause *et al*, 1998), genes to prevent apoptosis (Charo *et al*, 2005), agents to remodel the tumour microenvironment (Kerkar *et al*, 2011), support homeostatic proliferation (Cheng *et al*, 2002) and chemokine receptors that promote directed T cell homing (Moon *et al*, 2011). Permanent and transient modification systems can be combined, such as having a permanently expressed CAR supported by RNA transfection of support molecules like interleukin-12, where permanent constitutive expression is probably undesirable. Permanent genetic modification remains a focus of significant regulatory oversight, due in no small part to concerns over clonal transformation or uncontrolled T cell proliferation. Therefore several approaches to incorporating suicide systems into the T-cell engineering process remain attractive, typified by the expression of a pro-apoptotic gene under the control of an inducible promoter responsive to a systemically delivered drug (Di Stasi *et al*, 2011). This does not guarantee elimination of all modified T-cells, however, though it is probably one of the best methods for halting an acute problem related to uncontrolled T cell proliferation.

The ability to introduce or delete genes in infused T cells has additional potential to provide 'off the shelf' cell products. This refers to T cells with no native TCR or human leucocyte antigen (HLA) genes, which would result in the prevention of rejection and a reduction in alloreactivity (Torikai *et al*, 2013; Tebas *et al*, 2014). While combining

CAR therapy with exogenous checkpoint blockade is being pursued, this concept can also be combined into switch receptors, such as one that has programmed death-1 (PD-1, also termed PDCD1) on the membrane and CD28 as an endodomain (Prosser *et al*, 2012).

Cytokines given to the host can have a major impact on the persistence and performance of adoptively transferred T cells. Coadministration of systemic IL2 has been reported to enhance the persistence of adoptively transferred human $CD8^+$ T cells(Yee *et al*, 2002). However, others have found that using autologous human $CD4^+$ T and $CD8^+$ T cells in combination eliminates the benefit of concomitant IL2 therapy (Mitsuyasu *et al*, 2000). Recent studies also seem to indicate that IL2 might actually reduce memory T cells and increase the number of T regs (Zhang *et al*, 2005). Conversely, IL15 and IL7 seem to select for the persistence of memory $CD8^+$ T cells, especially the T_{SCM} subset, and serve to decrease the number of T regs in experimental animal models (Ku *et al*, 2000) (Berger *et al*, 2009).

Clinical activity of CD19 CAR T cells

Adoptive transfer of T cells engineered to express a CD19-targeted CAR is the most advanced CAR-modified T cell technology currently being tested in clinical trials. As CD19 is expressed throughout B cell lineage development, it is found on the surface of nearly all B cell malignancies. Clinical trials of CAR-modified T cells directed against CD19 are enrolling patients with acute lymphoblastic leukaemia (ALL), chronic lymphocytic leukaemia (CLL) and non-Hodgkin lymphomas (NHL). Robust activity with striking clinical responses in patients with refractory, bulky CLL and relapsed, highly refractory ALL has been shown (Brentjens *et al*, 2011, 2013; Porter *et al*, 2011; Grupp *et al*, 2013; Kochenderfer *et al*, 2013; Davila *et al*, 2014; Lee *et al*, 2014; Maude *et al*, 2014). While the first reports included small numbers of patients, these have been validated and extended in larger studies with longer follow-up showing durable remissions.

Acute lymphoblastic leukaemia

Several clinical trials of CAR-modified T cells directed against CD19 have created immense optimism for the therapeutic potential of this technology in ALL. Early studies showed striking responses in patients who were not only considered to be incurable but also no longer responsive to chemotherapy (Brentjens *et al*, 2013; Grupp *et al*, 2013). However, these studies were quite small and needed to be expanded to determine the true potential. That potential may be even better than expected with complete remission (CR) rates of 70–90%. Importantly, these results have been replicated with three groups publishing larger studies using distinct CD19 designs (Davila *et al*, 2014; Lee *et al*, 2014; Maude *et al*, 2014). Our group reported a CR rate of 90% in 30 paediatric and adult patients with relapsed/refractory ALL treated with CAR T cells targeting CD19, which express a CAR composed of anti-CD19 single chain variable fragment (scFv), CD3ζ, and 4-1BB domains, on Children's Hospital of Philadelphia (CHOP) and University of Pennsylvania (Penn) phase I trials (Maude *et al*, 2014). In a cohort of 16 adults with relapsed B-ALL treated at Memorial Sloan Kettering Cancer Center (MSKCC), Davila *et al* (2014) reported a similar CR rate of 88% with 19-28z CAR T cells. In the third study, Lee *et al* (2014) reported a CR rate of 70% in a National Cancer Institute (NCI) intent-to-treat analysis of 20 children and young adults with ALL.

Toxicity associated with cellular therapies has several potential sources including those due to extrinsic factors present in the culture process, those due to co-infused cytokines and those intrinsic to the cells themselves. Respiratory obstruction following cytotoxic T lymphocyte (CTL) infusion for Epstein-Barr virus-related lymphomas has been reported in patients with pulmonary disease (Heslop & Rooney, 1997). This was hypothesized to be from a T cell–induced inflammatory response resulting in tumour oedema and necrosis. Effector functions of infused T cells also need to be considered, and may result in tissue damage similar to that encountered in T cell–mediated autoimmune diseases. In the case of allogeneic lymphocyte infusions, toxicities such as GvHD and bone marrow aplasia can occur (Kernan *et al*, 1986).

Similar toxicities were observed across all clinical studies of CAR T cells against CD19: cytokine release syndrome (CRS), B cell aplasia and neurotoxicity. CRS, the most notable and serious toxicity, is an inflammatory process associated with supraphysiological T cell proliferation and significant cytokine elevations, as its name suggests. Only seen with these third generation CAR products and with the infusion of highly proliferative T cells, CRS represents the toxicity not observed with first generation products and can be correlated with that first generation product's lack of efficacy. Observed in nearly all patients with ALL treated with highly active CAR-modified T cell therapies, CRS is a constellation of symptoms ranging from mild flu-like symptoms, with fevers, myalgias and nausea/vomiting being prominent features, to life-threatening, with hypotension and multi-organ system failure. Severe CRS has been shown by several groups to correlate with high disease burden and can be effectively reversed with IL6R blockade by tocilizumab (Grupp *et al*, 2013; Davila *et al*, 2014; Lee *et al*, 2014; Maude *et al*, 2014). B cell aplasia related to depletion of all cells of the B lineage, which express CD19, leads to hypogammaglobulinaemia requiring immunoglobulin replacement. Neurotoxicity, including confusion, aphasia, global encephalopathy and seizure, has been reported in several clinical trials of T cell engaging therapies, both with CAR T cells with distinct designs and with blinatumomab, a bispecific anti-CD3/CD19 antibody (Topp *et al*, 2011; Davila *et al*, 2014; Lee *et al*, 2014; Maude *et al*, 2014; Schlegel *et al*, 2014). In most cases

and in our experience, neurotoxicity is self-limited and resolves without apparent long-term sequelae. GvHD, a potential concern when infusing activated T cells, has not been observed to date in several studies that included patients with a prior history of allogeneic stem cell transplant (SCT) (Davila *et al*, 2014; Lee *et al*, 2014; Maude *et al*, 2014).

The enthusiasm surrounding CAR T cell therapies centres on not only high CR rates in a refractory population but also the potential for long-term remissions. For engineered T cells to provide continued protection from relapse, they need to persist, but the minimum length of time needed is unknown. While initial CR rates are comparable across distinct studies, institutions, and CAR designs, persistence of CAR-modified T cells can vary and may distinguish CAR designs. The costimulatory domain may be an important factor in determining persistence. Shorter persistence has been reported with the CD28 costimulatory domain, with loss of CAR T cells and recovery of normal B cells observed by 1–3 months in both the NCI and MSKCC studies using CD28 (Davila *et al*, 2014; Lee *et al*, 2014). With the 4-1BB costimulatory domain, we have observed longer persistence (up to 2 years) in our ALL cohort with the probability of CAR T cell persistence at 6 months being 68% [95% confidence interval (CI): 50–92%] (Maude *et al*, 2014). The duration of B cell aplasia, also longer at up to 3 years, suggests continued effector function of CAR cells. However, expanded cohorts and longer follow-up is needed to better elucidate these differences.

Durable remissions have been reported across studies of CD19-directed CAR-modified T cells. We reported sustained remissions of 2–24 months in 19/27 responding patients in the CHOP/Penn cohort, 15 of whom received no further therapy, with a 6-month event-free survival of 67% (95% CI: 51–88%) and 6-month overall survival of 78% (95% CI: 65–95%) (Maude *et al*, 2014). In an expanded cohort of 48 paediatric patients with ALL reported at the European Haematology Association meeting in 2015, we observed a 94% CR rate and a 6-month disease-free survival of 72% (95% CI: 60–87%) (Grupp *et al*, 2014; Maude *et al*, 2015). Five of the patients in this group went on to SCT. Relapse, occurring in 15 patients, resulted from short persistence or, more commonly, loss of CD19 antigen expression ($n = 10$). In the MSKCC and NCI studies, sustained remissions were described in the approximately 50% of patients who proceeded to allogeneic SCT (Davila *et al*, 2014; Lee *et al*, 2014). More mature follow-up will be needed across studies and CAR designs to determine the full potential of engineered T cell therapy as a bridge to SCT or as definitive therapy.

Non-hodgkins lymphoma

Pilot studies of CD19-directed CAR-modified T cell therapies have also shown promise in NHL. The NCI first reported partial responses (PR) in a small number of patients with follicular lymphoma (FL) treated with CD19 CAR T cells (Kochenderfer *et al*, 2010, 2012). They later extended these findings and recently reported remissions in diffuse large B cell lymphoma (DLBCL) (Kochenderfer *et al*, 2015). In heavily pretreated patients, CR was achieved in 4/7 patients and PR in 2/7. On-going remissions of 9–22 months were observed in 3 of the 4 patients who achieved CR. Similar findings were replicated in initial reports of a University of Pennsylvania phase IIa clinical trial of CD19 CAR T cells in relapsed/refractory NHL, in which the overall response rate was 67% (Schuster *et al*, 2015). The response rate was 50% in 12 patients with DLBCL and 100% in 6 patients with FL. Progression-free survival was 59% at a median follow-up of 6 months. Similar to CAR T cell trials in other disease types, CRS was common but fewer severe manifestations were seen in the initial reports (Kochenderfer *et al*, 2015; Schuster *et al*, 2015). Neurological toxicities appear to be prominent features of this therapy in NHL. These included confusion, encephalopathy, aphasia, facial paresis, myoclonus, and one death (Kochenderfer *et al*, 2015; Schuster *et al*, 2015). The mechanism responsible for neurotoxicity remains poorly defined, and further elucidation will be needed to aid toxicity management. Larger studies and more mature follow-up are needed to determine if long-term remissions can be achieved in NHL, but initial results are encouraging.

Chronic lymphocytic leukaemia

While not a paediatric disease, chronic lymphocytic leukaemia (CLL) offers important insights in to CAR therapy. Initial studies of CD19-targeted CAR T cells at the University of Pennsylvania demonstrated dramatic anti-tumour responses in patients with advanced CLL who had been highly refractory to standard therapy. Responses were observed in the first 3 patients treated, with 2 patients achieving long-term complete remissions (CRs) (Kalos *et al*, 2011; Porter *et al*, 2011). CD19 CAR T cells proliferated exponentially *in vivo*, eliminated bulky disease, and have now been shown to persist longer than 3 years (Kalos *et al*, 2013). Similar responses have been demonstrated by other groups using a different CD19-targeted CAR with a CD28 costimulatory domain, even in patients who have relapsed after prior allogeneic stem cell transplant (Kochenderfer & Rosenberg, 2013; Kochenderfer *et al*, 2013; Park & Brentjens, 2013).

In larger studies, the overall response rate of CD19 CAR T cells in CLL ranges from 35% to 57% (Porter *et al*, 2014a). All responding patients demonstrated CAR T cell proliferation *in vivo* and durable responses up to 4 years have been observed. In general, CAR T cell persistence appears to be longer in responding CLL patients compared to some patients with ALL, even though CR rates are lower than in ALL (Brown *et al*, 2014). The on-going antigen reservoir provided by the bulky disease characteristic of CLL along with the discrepant kinetics of tumour growth and elimination may account for this difference. Toxicities include CRS and B cell aplasia with associated chronic hypogammaglobu-

linaemia. The latter, B cell aplasia, is an 'on-target' toxicity as well as a surrogate marker of functional CAR T cell persistence. The former, CRS, tends to be delayed and less severe in CLL patients than in patients with ALL, for unclear reasons but possibly related to disease kinetics. Nonetheless, CRS is common and appears to correlate with response in CLL (Porter *et al*, 2014b).

CAR T cells and allogeneic stem cell transplant

Leukaemia relapse remains a major cause of failure after allogeneic HSCT, and evidence is emerging that this risk is higher in patients with notable minimal residual disease going into transplant (Alyea *et al*, 2010; Pulsipher *et al*, 2015). Unmodified donor lymphocyte infusions are commonly given to treat relapse and are often complicated by GvHD despite limited activity for patients with ALL. Infusion of co-stimulated but non-gene modified allogeneic T cells was safe in a phase I trial, giving credence to the idea that CAR-modified but donor-derived T cells could be used in the transplant setting, especially in the context of minimal residual disease positivity (Porter *et al*, 2006).

New targets for acute myeloid leukaemia

Given the success in ALL, there is tremendous desire to find a CAR target to treat AML. This is much more difficult, however, as many AML blasts share antigens with either HSCs or early progenitor cells (Rambaldi *et al*, 2015). This includes the IL13 receptor alpha (CD123), CD34, CD38 and CD33 (Kenderian *et al*, 2015). Unlike ALL, where elimination of normal B cells is manageable with immunoglobulin replacement, long-term suppression of early progenitor cells is likely to result in bone marrow aplasia unless the CAR T cells can be eliminated. This was seen in preclinical models of a CD123 CAR, where expression of the CAR was toxic to early progenitor cells in xenograft mouse models (Gill *et al*, 2014). AML is clearly a target wherein either CAR therapy will involve transient expression (i.e. RNA), suicide genes or combination with myeloablative transplant. As CAR therapy becomes more sophisticated, the idea of using multiple CARs in the same cell with Boolean properties ('AND' 'NOT' and so forth) is being considered. As other kinds of signalling domains can be linked to CARs, combinations of inhibitory and activating CARs might be able to be melded to yield better specificity (Wang *et al*, 2015).

Conclusions and future directions

The central questions in the field today revolve around the management of toxicity, where to integrate cellular therapies into the routine practice and standard of care as well as how to extend beyond CD19-positive malignancies. This is not to discount on-going iterative improvements in understanding T cell function, where basic science research into T cell exhaustion, checkpoint inhibition and the tumour microenvironment (especially in lymphoma) will be crucial to expanding the efficacy of this therapy. Gene transfer technologies, including genome editing with nuclease technologies, also continue to improve. A major challenge will be to identify unique tumour antigens that can be targeted with selective T cell therapy, as we revisit CD20 as a CAR target for mature B cell malignancies. Many investigators envision engineered autologous T cells to replace the need for allogeneic HSCT, though the clinical setting in which this could be tested in a randomized fashion is difficult to conceive. Demonstrating sufficient clinical benefit to justify the logistics and expense of customized cellular therapies is driving the investment of pharmaceutical companies, whose considerable financial might could be key to the global expansion of these kinds of therapies.

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Author contributions

SM and DMB conceived, wrote and reviewed the paper in equal contributions.

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