CAR-T cells for the treatment of cancer and the future of preclinical models for predicting their toxicities

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Abstract

CAR-T cell therapy has achieved highly promising results in clinical trials, particulary in B-cell malignancies. However, reports of Serious adverse events (SAE) including a number of patient deaths has raised concerns about safety of this treatment. Presently available pre-clinical models are not designed for predicting toxicities seen in human patients. Besides choosing the right animal model, careful considerations must be taken in CAR T-cell design and the amount of T-cells infused. The development of more sophisticated *in vitro* models and humanized mouse models for preclinical modeling and toxicity tests will help us to improve the design of clinical trials in cancer immunotherapy.

Introduction

The conventional treatment of cancer relies mostly on radiation, chemotherapy and surgery, which is successful in some patients but not all. Therefore, there is an unmet need to develop new approaches to treat cancer, such as immunotherapy. One exciting immunotherapeutic strategy entails the use of chimeric antigen receptor (CAR)-engineered T-cells. In this approach, T-cells isolated from patients are engineered to express an activating fusion receptor that binds directly to a cell surface target that is found on tumour cells alone, or also on healthy cell types that are dispensable or whose function can be replaced [1, 2].

Chimeric antigen receptors are classified based upon their signalling capabilities. First generation CARs contain a source of signal 1, most commonly TCRζ. While these have achieved some success pre-clinically [3], they have had limited impact in the clinical arena. Second generation CARs contain a single co-stimulatory module, most commonly derived from CD28 [4, 5] or 4-1BB [6] and which is placed upstream of TCRζ. Provision of co-stimulation enhances the target-dependent proliferation of CAR T-cells, potentiating their anti-tumour effect and prolonging their persistence *in vivo* [5, 7]. Second generation CARs directed against CD19 have achieved striking efficacy in the treatment of B-cell malignancy - most notably acute lymphoblastic leukaemia in which complete remission rates of 80-90% have been reported by independent centres using distinct CAR configurations [8-13]. Since CD19 antigen is also expressed on normal B-cells, it was predicted and subsequently confirmed that CD19-targeted CAR T-cells would commonly induce B-cell aplasia and hypogammaglobulinaemia – an on-target toxicity that can be managed effectively with the use of immunoglobulin replacement therapy. However, severe and

sometimes unanticipated toxicities have also been reported, most notably in the form of Cytokine release syndrome (CRS) and neurotoxicity.

The first detailed case report of CAR T-cell induced CRS involved a patient with metastatic ErbB2-expressing colon cancer. She received an intravenous infusion of 10 billion 3rd generation CAR-T cells targeted against this naturally occurring receptor [14]. Within 15 minutes, the patient developed respiratory distress and progressed to cytokine release syndrome, multi-organ failure, repeated cardiac arrests and death 4 days later. Post mortem examination revealed that the toxicity may have resulted from binding to low levels of ErbB2 expressed on healthy lung parenchyma or endothelium [15].

A similar SAE was reported at the same time in a patient with chronic lymphocytic leukaemia (CLL) who received 3x10^{7} 2nd generation CAR-T cells targeting CD19 [16]. In this case, it is possible that occult infection was a cofactor in the development of this event. In both of these patients, CAR T-cells were infused after lymphodepleting chemotherapy.

Clinical experience of toxicity induced by CD19-targeted CAR T-cells

More recently, as clinical experience with CD19-targeted CAR T-cells has grown, significant issues have been observed with toxicity due to CRS and neurotoxicities. CRS is a result of an overshooting and highly activated immune system [17]. The severity of CRS and the symptoms displayed can vary greatly [18]. The symptoms can include fever, vomiting, tachypnoea and tachycardia. Neurotoxicities can include headache, delirium, hallucinations and seizures. In general CRS and neurotoxicities are broadly defined as mild (grade 1), moderate (grade 2), severe (grade 3), life-threatening (grade 4) and grade 5 which corresponds to the death of the patient [18]. Most clinical trials of CD19-targeting CAR T-cells running currently reported CRS and neurotoxicities (Table 1). Juno Therapeutics developed their anti-CD19 CAR T-cell product to treat B-ALL and decided to halt their clinical trial due to the development of cerebral edema which led to the death of patients (Clinicaltrials.gov identifier: NCT02535364) (Crow, David. *Juno shares tumble as cancer drug trial halted.* The Financial Times Limited, 2017. Web. 23.11.2016).

Affinity of CAR T-cells

The CD19-targeting CAR T-cells used to treat the patient with CLL were designed with the scFv derived from the SJ25C1 hybridoma [3]. The anti-CD19 scFv from SJ25C1 does recognise the CD19-antigen, as assessed in a FACS competition

assay [19]. In order to evaluate the affinity of the scFv from SJ25C1 the protein was iodinated and Scatchard analysis performed to evaluate binding parameters with its receptor. However, the specific affinity of scFv from SJ25C1 was too low to be determined, thus indicating that we are dealing with a low-affinity CAR-T cell construct.

The role of affinity in CAR-mediated T-cell activation in response to their cognate antigen remains controversial. When the antigen was immobilised on a solid surface CAR-mediated T-cell activation was reported to be dependent on the strength of the interaction, because of the correlation between the affinity of different CAR T-cell-constructs and IFN-γ release by the CAR T-cells [20] .

In contrast, the co-culture of those different affinity CAR T-cell-constructs with tumour cell lines expressing the cognate antigen in different quantities revealed that effective lysis of the target cell was dependent on the amount of antigen expressed on the surface rather than the binding affinity of the scFv domain [20]. Furthermore, high affinity CAR T-cell constructs were less able to discriminate between tumour cells that express either high or low levels of antigen (Ag) on their surface [20]. The loss of selectivity can have fatal consequences when infused into patients, as healthy cells with a basal level of Ag-expression will be also eliminated along the cancerous cells with a high Ag-expression profile. However, not only the affinity of the scFv domain is important, the position of the targeted epitope within the antigen plays an important role in the activation of the CAR T-cell. This was shown with engineered immunoreceptors targeting the carcinoembryonic antigen (CEA) expressed on cancerous gastrointestinal cells which either targeted a domain distal or proximal from the cell membrane [21]. The proximally located scFv receptor

elicited increased cytolysis and IFN-γ release in comparison to the distal location upon binding to its epitope, although the distal epitope binds with higher affinity. This suggests that there is for CAR T- cells an optimal distance for their interaction with their target, irrespective of their affinity.

The structure of the CD19 antigen is currently unclear and, consequently, the binding of the CD19 scFv derived from the SJ25C1 hybridoma to its target is unknown and its interaction can merely be modeled [22].

CART-cell dose

Another important aspect of CAR T-cell therapy is the quantity of T-cells infused into the patient. Some researchers are of the opinion that the infused dose does not correlate with SAEs or severe neurotoxicity although it has been reported that toxicities develop more often after the infusion of a high CAR T-cell dose [12, 23]. These neurotoxicities may be caused by CAR T-cells migrating into the brain because a higher CAR T-cell number was found in the Cerebrospinal fluid (CSF) of patients with severe signs of neurotoxicity [12]. This migration of CAR T-cells into the brain is not a surprise as the CSF of healthy individuals can contain up to 3000 leukocytes per ml and drains into cervical lymphatics allowing the CAR T-cells to enter the brain and cause potential neurotoxicities [24]. In both reports of SAEs the patients received a high dose of CAR T-cells with 10^{10} and $3x10^{7}$/kg respectively. Coming from the field of peptide immunotherapy the administered dose plays a huge role and as Paracelsus stated correctly: “The dose makes the poison”. A dose escalation is always recommended to identify the maximum tolerated dose and a successful dose escalation is able to prevent the development of adverse side

effects in peptide immunotherapy [25]. The same principle was seen as the company Kite performed a dose escalation trial with their KTE-C19 product targeting haematological malignancies and observed dose-limiting toxicity with grade 3-4 CRS predominantly at a dose of $3x10^6$/kg anti-CD19 CAR T-cells but not at the lower dose of $1x10^6$/kg CAR T-cells [12].

However, even in a clinical trial conducted by Kite which is considered successful, evident by high overall response rates and high complete remission rates, patients developed serious side effects [26].

Kite target with their KTE-C19 product in the ZUMA-1 trial refractory aggressive non-Hodgkin lymphoma (NHL) (trial number: NCT02348216). Recently published results reported safety, *in vivo* effects and efficacy of the ZUMA-1 trial [26]. Kite reported the development of adverse events after a CAR T-cell dose of $1\text{-}2x10^6$ anti-CD19 CAR^+ T-cells/kg. The majority of the patients experienced CRS and neurological toxicities of grade 3 or less but all treatment-related AEs were successfully managed within 30 days.

This trial used the monovalent scFv derived from the mouse hybridoma cell line FMC63 which binds the $CD19^+$-target cell with an affinity constant of 2.3×10^{-9} M, which corresponds to a high-affinity interaction [27]. One could speculate that although a scFv domain with a high affinity was used, the lower dose of infused T-cells prevented the death of patients in contrast to the trial initiated by the Memorial Sloan Kettering Cancer Center where $3x10^7$ CAR-T cells were infused targeting CD19 using a scFv domain with low affinity. Differences in toxicities could be potentially induced by the usage of different co-stimulatory domains, although both studies used the signalling domain of the CD28 receptor, or the different retroviral vectors used for delivery. The safety of CD19 targeting CAR T-cells can also be

influenced by a number of factors other than CAR T-cell design including the heterogeneity of the targeted disease and the age differences of the patient population.

The balance between a successful anticancer response and the danger of eliciting serious toxicities seems precarious. Both studies tested their CAR T-cell constructs in mouse models beforehand, but these were xenograft models established in immunocompromised mice [28, 29].

Preclinical modeling

Would it be possible to prevent the death of patients resulting from adverse effects of CAR T-cell therapy by using more sophisticated *in vitro* models and humanized mouse models for preclinical modeling and toxicity tests?

Rather than requesting unreliable xenograft mouse model experiments, effort should be made to to develop animal models that reflect a human immune system more accurately. Mouse models can also help us to develop treatments for very rare diseases where the number of patients would otherwise be too low to initiate a clinical trial.

The specificity of CAR T-cells and the different functional response is generally tested *in vitro* in cultures of monolayers of cells expressing the target antigen in comparison to cells that do not express the antigen. The development of organoids provides an important bridge between the traditional *in vitro* monolayer cell culture and the *in vivo* mouse/human models. Organoids are 3D organotypic cultures and represent 'mini-organs' in a dish [30]. Organoids can be derived from stem cells and exploit their intrinsic biological capability of self-organisation [31]. The cells are able to organise themselves between each other and also within the tissue which allows the development of a mini-organ reflecting the structure and functional properties of organs such as brain, lung or intestine [32]. In addition, organoids can also be generated from human cancer specimens like glioblastomas, colorectal tumours and pancreatic cancer and can recreate the histopathology seen in the primary tumour [33-36]. Of course, there are still limitations to the use of cancer organoids, such as the absence of cell interactions between the epithelia or stroma and the lack of an intact human immune system, which greatly affect disease progression. One study successfully grew cancer cells together with endothelial and stromal cells in a 2D culture [37]. It would be possible to overcome the potential limitations, such as the lack of stroma and the missing immune system, by culturing cancer organoids together with endothelial and stromal cells as a 3D model and then infuse those

mini-organs into mice engrafted with a human immune system. This humanisied, patient-derived organoid model system would allow the investigation of the efficiacy of CAR T-cell therapy and also determine the immune responses elicited.

Using mice as a model organism in research has helped us to develop treatments of severe diseases. The successful treatment of acute promyelocytic leukemia (APL) using a combination of retinoic acid and arsenic, which was mainly untreatable previously, was a direct result from pioneering work in mice [38]. This cancer, caused by gene fusions that affect the bone marrow, can now be treated after extensive research using genetically engineered mice (GEMMs) resembling different types of APL. In a similar vein, GEMMs bearing the human breast cancer gene BRCA1 mimicked features of the human breast cancer better than xenograft models and helped in defining a treatment using chemical inhibitors of poly-(ADP-ribose)-polymerase-1, which delays the onset of resistense to the treatment [39].

Some researchers see mouse models just as a passport to be allowed to perform a clinical trial or are wary of possible adverse effects they might see in a mouse model, which could delay initiation of a clinical trial, even though these adverse effects may not necessarily represent the human situation. However, even though animal models are by no means perfect, sometimes seeing adverse side effects of the treatment in a mouse model can help in improving the design of a future human clinical trial. Work by our group showed that the treatment of SCID/Beige mouse with $T4^+$ T cells a T1E28z second-generation CAR targeting ErbB-1 homodimers and ErbB2/3 heterodimers resulted in severe toxicities dependent on the route of administration [40]. Tumour-bearing animals who received $T4^+$ T cells either intravenously (i.v.) or

intratumoural (i.t.) showed tumour regression with no sign of severe toxicities. $T4^+$ T cells, which have been administered intraperitoneal (i.p.) have led to the rapid death of the animals caused by the induction of a CRS indicated by the upregulation of human IFN-γ, human IL-2 and mouse IL-6 in the serum of the animals. The severe on-target off-tumour toxicity seen may have been caused by ErbB1 expression by healthy mesodermal cells in the peritoneal cavity. This study demonstrated the importance of the route of administration and led to the initiation of a clinical trial treating patients with head and neck cancer by the infusion of T1E28z CAR T-cells i.t. [41].

Certainly, current mouse models have not always proven reliable as preclinical models in particular in CAR T-cell therapy of cancer. But this is mainly because the current available mouse models are imperfect and the improvement of mouse models would help us to generate knowledge that translates more directly to the human situation. The most common model used in studying cancer therapy is the xenograft model, established by either transferring cancer cell lines or patient-derived tumours (PDX) into immune-deficient mice. It is clear that the assessment of CAR T-cell therapy using xenograft models without immune sytem components does not help us in gaining any further knowledge about how this T-cell construct might act in a human being.

The efficacy of CAR T-cell treatment can also be better examined by the addition of other cell types into xenograft models, which are known to diminish the effect of CAR T-cells, such as regulatory T-cells (Tregs) or myeloid-derived suppressor cells (MDSC). In one study, carcinoembryonic antigen$^+$ (CEA) C15A3 tumor cells were

transferred into immunodeficient mice together with human Tregs, to investigate the efficiacy of tumour cell lysis by CEA-specific CAR T-cells [42]. CEA-specific CD3ζ CAR T-cells were able to prevent tumour formation even in the presence of Tregs. However, the infusion of CD28-CD3ζ CAR T-cells led to a progressive tumour development in the presence of Tregs, even though the cytolytic efficiency assessed *in vitro* was the same for both T-cell constructs. Further analysis clarified that in this case the production of IL-2 by the CD28-TCRζ CAR T-cells after target engagement improved the survival of Treg cells. The subsequent introduction of a mutation in the lck binding domain of the CAR CD28 moiety to prevent IL-2 secretion led to a reduction of tumour-infiltrating Tregs and improved the anti-tumour response. The normally considered improved design of addition of the CD28-domain supported Treg development and abolished CAR T-cell efficiency in the model of CAR T/Treg-cell co-transfer.

In another immunocompetent murine model of CEA^+ liver metastases (LM) anti-CEA CAR T-cell therapy was investigated under the influence of MDSCs [43]. Hepatic MDSC expanded more than 3-fold in response to LM. Mice with established LM received CEA CAR T-cells in combination with a MDSC depletion strategy, which led to a prolonged survival of the mice compared to CEA CAR T-cell treatment only. MDSC suppressed CAR T-cells via the expression of PD-L1 on their surface. When tumour-bearing animals received CEA CAR T-cell in combination with a PD-L1 blocking antibody and a reduction in tumour burden could be detected compared to the treatment with the CAR T-cells only.

A very good example of the need for better animal models to test T cell-mediated cancer immunotherapy is the use of the anti-cytotoxic T lymphocyte-associated molecule-4 (CTLA-4) antibody Ipilimumab for the treatment of melanoma. Before a clinical trial was initiated, Ipilimumab was used in studies to treat tumour models of fibrosarcoma and ovarian carcinoma in mice [44]. Blocking CTLA-4 in tumour-bearing mice led to the development of anti-tumour T-cell responses and subsequently tumour regression. There were no adverse toxic effects nor any signs of the development of autoimmunity reported, which led to the initiation of a clinical trial. Patients with metastatic melanoma were treated with anti-CTLA-4, which resulted not only in tumour regression but also led concurrently to the development of severe autoimmune toxicities including colitis and dermatitis [45]. A later study used a humanized mouse model reconstituted with human HSCs, and anti-CTLA-4 treatment could mirror the side effects seen in the clinical trial [46]. Ipilimumab-treated humanized mice developed autoimmune hepatitis. Taken together it is important to test therapies in an appropriate mouse model to rule out unwanted severe side effects before initiating a clinical trial.

It is beyond doubt that the mouse models come with many challenges:

- Animals used in experiments tend to be rather young while many human cancers develop later in life.
- maintenance of the animals happens in a clean and controlled environment and does not take environmental factors into account.
- Animals are mostly inbred which does not take genetic variability of diseases into account.

- Animal models do not reflect the patient situation with relapsed, refractory or resistant disease.

Development of humanized mouse models

One approach to model human cancer or to “humanize” mouse models is to genetically modify mice. In this way, human oncogenes are introduced into the mouse genome and tumour development can be observed as already mentioned. Another approach is to introduce human hematopoietic stem cells (HSCs) or peripheral blood mononuclear cells (PBMCs) into immunocompromised mice to create mice with a humanized immune system. Immunodeficient mice like those with severe combined immunodeficiency (SCID) support the engraftment of human peripheral blood leukocytes (PBLs) (Hu-PBL-SCID) and HSCs (Hu-SCR-SCID) cells [47-49] at a low level. SCID mice have the disadvantage that their condition is “leaky” and can still generate a few functional T and B cell clones in addition to having no impairment in myeloid cell differentiation and natural killer cell (NK) activity [50]. The level of human cell engraftment was efficiently increased by crossing the

SCID mouse with the nonobese diabetic mouse (NOD-SCID mice) because this mouse strain showed reduced levels of innate immune cells [51].

The breakthrough in the field of human cell engraftment came with the creation of the NOD-SCID mouse with either a deletion of the IL-2R common γ–chain (NSG mouse) or a truncated mutation of the IL-2R (NOG mouse), which both allow the engraftment of human cells to a previously unachievable level [52]. The NSG mouse was found to support greater engraftment of human cells than any of the other available strains [53]. Engraftment of HSCs allows the development of a multilineage immune system while the engraftment of peripheral blood mononuclear cells (PBMCs) represents an already matured immune system. The approach of transferring PBMCs into NSG mice only allows short-term experiments because the animals develop graft-versus-host disease (GvHD) within 4-5 weeks [54]. Furthermore, this NOD-scid IL2Rγnull mice engrafted with $CD34^+$ HSCs develop a diverse TCR repertoire, have a prolonged life span and develop no graft-versus-host (GvH) disease [55]. The downside of this model is that the developing human T-cells in the thymus lack the ability of recognising antigens in an HLA-restricted manner because the murine thymic epithelium lacks the expression of HLA molecules. This limitation was overcome by the development of the NSG-HLA-A2/HHD strain expressing HLA class I heavy and light chains which led to differentiation of functional $CD4^+$ and $CD8^+$ T-cells able to secrete cytokines *in vivo* [56]. A third method for the generation of humanized mice is the bone-liver-thymus (BLT) model. In this model, foetal thymus and liver tissue are implanted beneath the renal capsule of the kidney along with the co-transfer of autologous $CD34^+$ cells into an immunodeficient murine host [57, 58].

The co-engraftment of thymic tissue and human hematopoietic stem cells in immunocompromised mice allows the development of human T-cells, which interact with human MHC molecules, able to mount an antigen-specific immune response. One of the main disadvantages of the BLT model is the development of GvHD affecting skin, lungs and the gastrointestinal tract, which limits the experimental window[59].

The further engraftment of humanized mice with human tumour cell lines allowed the investigation of the interaction of the tumour with the immune system [60]. In the humanized tumour mouse model (HTM), human $CD34^+$ HSCs were co-transfered with human breast cancer cells into irradiated newborn NSG mice [60]. The mice developed a human immune system characterized by the presence of T-, B-, NK-cells and macrophages besides the development of solid human breast cancer without eliciting any rejection caused by MHC-mismatched cells.

The disadvantage of using cancer cell lines for engraftment is the loss of tumour heterogeneity and the molecular make-up that is clinically relevant.
To further enhance the use of humanized mice in cancer immunotherapy the generation of humanized patient-derived xenograft tumour models (PDX) would be essential. This was achieved by the engraftment of irradiated NSG mice with *in vitro* expanded HSCs and the subsequently transplantation with a patient's head and neck carcinoma sample, called xenochimeric mice (Xact mice) [61]. The tumour cell stroma of Xact mice contained human $CD45^+$ $CD151^+$ cells originating from the bone marrow of engrafted mice. Furthermore, the intra-tumoural lymphatic vasculature in

Xact mice showed an increase in vessel density, suggesting that the Xact model is able to recreate the native tumour environment.

Humanized mice cannot develop all characteristics of a human immune system. In particular, they lack innate immune cells due to the absence of human cytokine signaling and exhibit poor lymph node development [62]. Several strategies have been developed to overcome those limitations. Human cytokines were either delivered exogenously to humanized mice or knock-in models were developed where single human cytokine genes were introduced into the mouse genome, replacing the mouse cytokine gene [63-66]. All of those modifications improved the development of individual cell populations like human NK cells [63], human alveolar macrophages [65] or human monocytes [66] but did not result in a complete reconstitution of a human innate immune system.

An attempt to express a range of human cytokine genes in order to enable complete innate immune cell development led to the generation of MITRG mice [67]. These mice harbor a knock-in of human M-CSF, IL-3/GM-CSF and human thrombopoietin (TPO) in their respective gene loci. Humanized MITRG mice develop functional myeloid cells, monocytes and NK cells. However, these mice allow human HSC engraftment to a great extent, which in turn leads to massive deficits in mouse red blood cells resulting in severe anemia and the death of the animals [67]. This just leaves a very short experimental window of approximately 2 to 3 weeks, which makes this model not ideal.

As mentioned earlier humanized mice exhibit poor lymph node development. This obstacle could be overcome by a tissue engineering approach where artificial lymph nodes (LNs) could be generated in humanized mice. Lymph nodes as a secondary lymphoid organ (SLO) are important organs for T cell activation and the initiation of adaptive immunity [68]. Live *in vivo* two-photon imaging revealed that lymphocytes migrate into LNs and interact with antigen-presenting cells [69]. Tertiary lymphoid organs (TLOs), which can develop at sites of inflammation anywhere in the body, share the same function as SLOs [70] and are very similar in their organizational structure [71]. The successful engineering of artificial lymph node-like tertiary lymphoid organs (artTLOs) showed that these artificial structures are transplantable and retain their immunological function [72-74]. The newest approach from the Watanabe lab is a stromal cell-free model where slow-releasing gel-beads containing a chemokine cocktail, lymphotoxin-$\alpha 1\beta 2$ and soluble RANK ligand (sRANKL) are embedded in a collagen sponge [75]. This collagen sponge was then transplanted into the subcapsular space of the kidney of mice. After 3 weeks, immunological active artTLOs have been formed. This tissue engineering approach could be used to create human artTLOs by transplanting those collagen sponges into humanized mice.

Another approach to test CAR T-cell therapy is by using humanized mouse models where human tumours are generated *de novo* alongside the development of a matched human immune system. A human mixed-lineage-leukemia (MLL) model was developed by transduction of $CD34^+$ HSC with the oncogene MLL-AF10 along with the gene for K-ras and transfer into NOG mice which led to the development of acute monoblastic leukemia [76]. The strength of this approach is the ability to study

the steps of human tumourigenesis including initiation, development and dissemination of cancer alongside the development of a matched human immune system.

The use of a humanized mouse model of treatment refractory B-cell leukemia showed that tumour cells in the bone marrow were resistant to treatment with the anti-CD52 antibody alemtuzumab [77, 78]. Combination therapy using alemtuzumab and the chemotherapeutic agent cyclophosphamide led to a near complete elimination of the disease in the bone marrow. Furthermore, the initial dose of the toxic cyclophosphamide could be reduced by a third and still resulted in disease elimination to the same extent. Very recently, a humanized mouse model of spontaneous B-cell acute lymphoblastic leukemia (B-ALL) was developed [79]. Human foetal thymic tissue (FTHY) and liver plus $CD34^+$ HSCs transduced with the oncogene MLL-AF9 were transplanted into irradiated NSG mice, which led to the development of human T -, B and myeloid cells as well as leukemic cells which makes this model system superior to other available models evaluating cancer immunotherapy using anti-CD19 CAR T-cells.

Conclusion

CAR T-cell therapy, especially in B-cell malignancies, has delivered impressive and promising results in the treatment of haemotological cancer. It is a balancing act to design improved CAR T-cells for successful tumour elimination while also limiting the development of serious adverse effects. In CAR T-cell design, there does not seem to be a consensus or rule that defines the best scFv targeting domain or signaling domain, resulting in diverse clinical trials targeting the same antigen. Predicting CAR

T-cell toxicities that result from their activation, using existing preclinical models, is still in its infancy. Current preclinical models are mostly unable to recapitulate the severe toxicities seen in human clinical trials after CAR T-cell infusion. Testing CAR T-cells for their suitability in the clinic implies the use of immuno-compromised mouse models. Mostly, immuno-compromised animals are challenged with human tumours where the antigen targeted by the CAR T-cells is exclusively expressed on the tumour cell preventing the development of potential on-target, off-tumour toxicities.

In reality, clinical trials have been initiated without the input from valuable preclinical mouse models that could have aided in improving their design. But, following reports of deaths in clinical trials, the scientific community tries to improve their understanding of mechanism leading to toxicities by developing new murine humanized cancer models (summarized in Table 2).

Future perspective

Several new strategies are in place and will be exploited to create better preclinical murine models to hopefully further reduce treatment-related toxicities in the future (Figure 1). Validated humanized mouse models hold great promise for improving safety of CAR T-cell therapy. The challenge to develop these chimeras, which are viable enough to provide a healthy environment for the transplanted human cells to thrive in while at the same time limiting the development of GvHD, is also strongly dependent on future developments in stem cell research and tissue engineering. However, recent successes in developing improved preclinical humanized tumour mouse models represent a step in the right direction. Further developments over the years to come are likely to provide new models that will allow for accurate modelling

of CAR T cell therapy in vivo, thus accelerating clinical translation of new strategies, while limiting devastating adverse effects.

Executive summary

- Despite promising results in CAR-T cell therapy, reports of SAEs in 2010 raised concerns about safety.
- The majority of anti-CD19 CAR T-cell clinical trials reported CRS and neurotoxicities.
- The choice of the scFv for the CAR-T cell defines the affinity of the CAR-T cell construct and can affect their efficacy.
- Infusion of high CAR-T cell doses correlates with incidence of severe toxicities.
- Current preclinical models are unable recapitulate toxicities seen in patients.
- Cancer organoids could provide a bridge between traditional monolayer cell culture and *in vivo* models.
- Humanized mouse models were developed by engraftment of $CD34^+$ HSCs into immunodeficient mice.
- Humanized mouse models are further advanced by co-engraftment of human tumour cell lines or patient-derived tumour samples.
- Humanized mice still exhibit deficits in the development of a complete human immune system.
- Expression of various human cytokines in humanized mice enables development of innate immune cells.
- Poor lymph node development in humanized mice could be overcome by a tissue engineering approach.

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Tables

Table 1: Anti-CD19 CAR T-cell clinical trials and their reported toxicities.

Institution	Ref.	Disease	No. patients	Toxicities	Clinicaltrials.gov identifier
Memorial Sloan Kettering Cancer Center	[80]	B-ALL	46	n=11 with severe CRS; n=13 with neurotoxicity grade 3-4	NCT01044069
	[81]	CLL	8	most patients with mild CRS	NCT00466531
	[82]	CLL	8	n=4 with mild CRS	NCT01416974
	[83]	B-NHL	8	n=4 with CRS	NCT01840566
University of Pennsylvania	[84]	$CD19^+$ ALL	59	n=52 with CRS; n=16 with severe CRS	NCT01626495
	[85]	$CD19^+$ ALL	n=27	n=21 with CRS grade 3 or greater; 3 deaths due to CRS	NCT02030847
	[86]	CLL	n=35	n=19 with CRS; n=7 with CRS grade 3 or greater	NCT01747486
	[87]	B-NHL	n=29	Not reported	NCT02030834
	[88]	multiple myeloma	n=10	n=1 with CRS grade 1	NCT02135406
Fred Hutchinson Cancer Research Center	[89]	$CD19^+$ ALL	n=36	Not reported	NCT02028455
	[90]	B-ALL B-NHL CLL	n=90	n=14 with severe CRS; n=3 with CRS grade 4; n=27 with neurotoxicities grade 3 or greater, 4 deaths due to CRS	NCT01865617
National Cancer Institute	[12]	B-ALL B-NHL	n=21	n=3 with CRS grade 4	NCT01593696
	[91]	B-NHL	n=22	Not reported	NCT00924326
City of Hope Duarte	[92]	B-NHL	n=8	No toxicties of grade 2 or greater	NCT01318317
Comprehensive Cancer Center	[92]	B-NHL	n=8	no tocixities of grade 2 or greater	NCT01815749
Kite Pharma	[26]	B-NHL	n=7	n=3 with CRS grade 3; n=3 with CRS grade 4 and neurotoxicity; n=1 with CRS grade 4 and neurotoxicity	NCT02348216

ALL = acute lymphoblastic leukemia; CLL = chronic lymphocytic leukemia; B-NHL = B-cell non-Hodgkin lymphoma; CRS = cytokine release syndrome

Table 2: Examples of preclinical humanized tumour models.

Category	Model	Characteristics	Ref
Humanized tumour mouse model	Cotransfer of human HSC and human Her2 overexpressing breast cancer cells into NSG mice	Presence of human T-, B-, NK-cells and macrophages and solid breast cancer	60
Humanized PDX tumour model	Xact mice Transfer of *ex vivo* expanded human HSC and transplantation with human PDX from head and neck carcinoma	Tumour stroma is infiltrated with human B- and T-cells and increase in intra-tumoural vessel density	61
Humanized *de novo* tumour mouse models	MLL model Transfer of human HSC transduced with oncogene MLL-AF10 and K-ras into NOG mice	Development of acute monoblastic leukemia	76
	MYC/BCL2 (hMB)-model Transfer of human HSC transduced with oncogene Bcl-2 and c-Myc into NSG mice	Development of refractory B-cell leukemia	78
	BLT B-ALL model Cotransfer of foetal thymic and liver tissue and human HSC transduced with oncogene MLL-AF10 into NSG mice	Presence of human T-, B and myeloid cells and leukemic cells	79

HSC = Hematopoetic Stem Cells; ALL = Acute Lymphoblastic Leukemia; PDX = Patient-Derived Xenograft; MLL = Mixed Lineage Leukemia; BLT = bone marrow-liver-thymus

Figure 1: Future of preclinical models testing CD19-targeting CAR T-cells. A Humanized mouse models with components of the human immune sytem were developed by the transfer of human stem cells (HSCs) into immunocompromised mice. The further subsequent engraftment with human tumour cell lines (**C**), tumours from human patients (**D**) or cancer organoids (**E**) will improve pre-clinical modelling and predicting CAR T-cell toxicities. Humanized mouse models lack the development of lymph nodes, which could be overcome by implanting artificial lymph nodes (**F**). **B** The co-transfer of HSCs and tumour cell lines into immune-compromised mice allows the simultaneously development of a human immune sytem and solid cancer.