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Nonclinical safety assessment of engineered T cell therapies

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ABSTRACT

Over the last decade, immunotherapy has established itself as an important novel approach in the treatment of cancer, resulting in a growing importance in oncology. Engineered T cell therapies, namely chimeric antigen receptor (CAR) T cells and T cell receptor (TCR) T cell therapies, are platform technologies that have enabled the development of products with remarkable efficacy in several hematological malignancies and are thus the focus of intense research and development activity. While engineered T cell therapies offer promise in addressing currently intractable cancers, they also present unique challenges, including their nonclinical safety assessment. A workshop organized by HESI and the US Food and Drug Administration (FDA) was held to provide an interdisciplinary forum for representatives of industry, academia and regulatory authorities to share information and debate on current practices for the nonclinical safety evaluation of engineered T cell therapies. This manuscript leverages what was discussed at this workshop to provide an overview of the current important nonclinical safety assessment considerations for the development of these therapeutic modalities (cytokine release syndrome, neurotoxicity, on-target/off-tumor toxicities, off-target effects, gene editing or vector integration-associated genomic injury). The manuscript also discusses approaches used for hazard identification or risk assessment and provides a regulatory perspective on such aspects.

1. Introduction

Anti-tumor immunity is an intense field of research and many biological processes contribute to what has been described as the cancer-immunity cycle (Chen and Mellman, 2013). Among cellular functions in the cancer-immunity cycle contributing to inhibition of tumor growth, T cell function (priming and activation, trafficking to and infiltration into tumors, killing of cancer cells) is recognized as paramount (Taube et al., 2018; Galon et al., 2006). Considering this, cancer immunotherapy occupies a growing treatment option with approved and marketed biotechnology products targeting enhancement or restoration of individual anti-tumor immunity: immune check point inhibitors (CTLA-4: Ipilimumab; PD-1: Nivolumab, Pembrolizumab, Dostarlimab, Cemiplimab; PD-L1: Avelumab, Durvalumab, Atezolizumab), a bi-specific T cell engager (Blinatumomab, a BiTE® molecule) antibody construct, an oncolytic virus (Talimogene laherparepvec) and engineered T cell therapies, namely chimeric antigen receptor (CAR) T cells (Axicabtagene ciloleucel, Brexucabtagene autoleucel, Tisagenlecleucel).

Remarkable clinical responses have been obtained with CAR-T cell therapies in patients with otherwise intractable hematological malignancies (Holstein and Lunning, 2020; Majzner and Mackall, 2019). The therapeutic success of these CAR-T cell strategies has spurred tremendous interest in developing improved CAR-T therapies for use in both hematological malignancies and solid tumors, as well as alternative T cell engineering strategies, including T cell receptor (TCR) T cell therapies.

Engineered T cell therapies provide huge opportunities to address current unmet medical needs but present also unique challenges in terms of safety assessment during nonclinical and clinical development. The

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main safety risks for this class of products include cytokine release syndrome, neurotoxicity, on-target/off-tumor effects or off-target toxicities. The current use of integrating viruses to manufacture the T cell product also give rise to a theoretical concern about integration-associated genomic injury, including mutagenic transformation. Regulatory agencies generally discourage conducting in vivo toxicity studies with non-relevant animal models and recognize that standard safety assessments in animals are largely not appropriate given the nature of this class as a human cellular product. Instead, nonclinical development efforts should mainly focus on target expression analysis to evaluate potential on-target/off-tumor activity and specificity assessments to rule out off-target activity. Those assessments heavily rely on in silico and in vitro tools and assays. Viral vector integration site assessments provide a profile of genomic integration patterns for these vectors and can inform the potential for insertional mutagenicity following viral transduction.

The continued development of engineered T cells presents on-going challenges for nonclinical safety assessment. Beyond considering the engineering of novel cell types (for use in cancer and in other diseases), current approaches for engineering T cells include enabling manufacture of allogeneic T cells or suppressing/altering response to T cell regulatory factors (via gene editing), expressing cytokines or chemokines to increase efficacy (also referred to as armoring T cells), controlling/regulating T cell expansion/deletion (e.g., via introducing gates or switches), alternative manufacturing approaches and others. These strategies will continue to challenge the nonclinical safety evaluation of these products. In particular, consideration is needed to address the risks associated with off-target genomic risk associated with gene editing including genomic deletions, translocations, and missense/nonsense mutations that could lead to malignant transformation.

In summary, the current tools and in vivo models to address the nonclinical development of engineered T cell products are constrained, and thus nonclinical safety assessment strategies require a thoughtful and tailored approach based on both the nature of the product and the intended application. Moreover, nonclinical safety scientists will need to closely follow the clinical safety profile of these cell products to understand the emergent safety profile and identify opportunities to apply specialized methods to understand the mechanisms underlying emergent toxicities, patients who may be at increased risk, and therapeutic or engineering strategies to mitigate these toxicities. Thus, current tools and models will need to evolve to address risks associated with the growing complexity of these products, and to establish credible means for identifying and managing potential safety liabilities.

A workshop organized by HESI and the Food and Drug Administration (FDA) was held to provide an interdisciplinary forum for representatives of industry, academia and regulatory authorities to share information and debate on the safety assessment engineered T cells (September 24–25, 2019, US FDA, Silver Spring, MD). The following topics were discussed current practices for the nonclinical safety evaluation of engineered T cell therapies, including in vitro and in vivo methods and studies applicable to such products; how the clinical experience to date with such products informs on potential safety assessment strategies; what are the global regulatory expectations and considerations for the successful safety assessment of such therapies. This manuscript leverages what was discussed at this workshop to provide an overview of the current important nonclinical safety assessment considerations for the development of engineered T cells. Specifically, it reviews safety issues encountered with JCAR015 as a case example, summarizes current trends in designing engineered T cells for optimal activity and safety, discusses the importance of target expression analysis and associated liability assessment, discusses in vivo models informing safety and activity, it links nonclinical development with the clinical experience, and provides regulatory perspectives and considerations.

2. Review of safety issues related to engineered T cell therapies – experience with JCAR015 as a case study

The example of the experience with JCAR015, as detailed below, illustrates some safety-related challenges associated with T cell therapies and the need for proper models to understand and potentially predict the severe side effects that can be associated with such modalities. JCAR015 is a second-generation (Abate-Daga and Davila, 2016) CD19-directed autologous chimeric antigen receptor (CAR) T cell product with a CD28 co-stimulatory domain. Development of this product was terminated by Juno Therapeutics (Seattle, WA) after fatal neurotoxicity was observed in a Phase 2 clinical study (called the ROCKET trial) in adult patients with relapsed or refractory acute lymphoblastic leukemia (ALL). As a result of these neurotoxicities, a root cause assessment was conducted by the sponsor to evaluate drug product, treatment, and baseline patient characteristics in an effort to identify possible factors contributing to the patient deaths (Research, 2018).

Chemotherapeutic lymphodepletion prior to CAR-T cell infusion is designed to reduce competition of the engrafted T cells with endogenous T cells for critical homeostatic cytokines such as IL-7 and IL-15, while also potentially removing some burden of tumor and reducing potentially inhibitory Treg cells (Klebanoff et al., 2005). The Phase 2 ROCKET trial originally used cyclophosphamide preconditioning prior to T cell infusion in the patient. Because a combination of fludarabine and cyclophosphamide was reported to deepen the patient response compared to cyclophosphamide alone (Turtle et al., 2015), a protocol amendment was instituted to allow preconditioning with both fludarabine and cyclophosphamide. Analysis of this trial showed that dual agent preconditioning was superior to cyclophosphamide alone with regards to improving patient anti-tumor outcomes, however, it also appeared to increase the adverse event rate and severity, with the observation of cerebral edema and patient death in three patients. After a trial halt and review of the available data, preconditioning was returned to cyclophosphamide alone and the trial was reopened. Whereas the adverse event rate improved overall, two additional patient deaths were reported, and the trial was ultimately terminated.

Post-hoc analyses of available clinical data demonstrated that patients with higher baseline IL-15 level, who were younger than 30 years old, and who had fewer prior lines of therapy were at increased risk for a severe adverse response. Characterization of drug product indicated that batches that were enriched for T cells producing IL-2 and TNF-$\propto$ were also associated with increased risk of fatal cerebral edema/neurotoxicity. Taken together, these data suggest that patient and product factors contributing to more potent T cells increased patient risk.

There is underlying biology supporting differential metabolic programming for CD28− vs 4-1BB-costimulated T cells (Kawalekar et al., 2016). In this model, CD28-costimulation promotes a glycolytic metabolism yielding an effector memory T cell phenotype with rapid expansion, and short persistence. In contrast, 4-1BB-costimulation promotes oxidative metabolism yielding a central memory T cell phenotype with slower expansion, and longer persistence. Thus, in the context of the JCAR015 program, this combination of both a CD28-costimuated CAR, which is designed to expand rapidly upon activation, the availability of high levels of IL-15 (and likely IL-7) following preconditioning, and the ready availability of antigen in the blood/bone marrow, appear to have been sufficient to drive a rapid T cell expansion and cytokine release. In addition, the composition of T cells in the delivered product may also underlie patient risk, with increased risk of adverse events among patients receiving a greater proportion of $CD8^{+}$ CAR + T cells. Among the patients that died from cerebral edema, the T cell expansion was much more rapid, and peaking within a week of dosing compared to the >10 days found in patients that didn't experience the neurotoxicity. Whether this early rapid expansion is attributed to relative patient and T cell health, CD28 co-stimulation, chemotherapeutic preconditioning regimen (or a combination of these factors) is unclear. However, the early, rapid T cell expansion and concomitant cytokinemia are strongly

implicated as underlying the observed neurotoxicity risk in these patients.

How the T cell activation and cytokine release promoted cerebral edema remains to be established, although evaluation of available samples indicated a vasogenic edema involving injury to vascular endothelia and astrocytes, along with microglial activation. Additional work, not detailed here, has been reported by Gust et al., 2017, 2018 and others.

3. Current trends in designing engineered T cells for optimal activity and safety

Many advances in improving safety and efficacy of CAR T cells have focused on optimizing CAR T cell biology, overcoming tumor antigen loss, and mitigating risk of off-tumor activity. A primary goal for effective CAR T functionality is engineering CARs that overcome their inherent deficiencies in forming physiologically relevant immunological synapses that are necessary for effective granzyme B and perforin-mediated killing and production of cytokines that promote T cell effector activity and drive T cell differentiation (Benmebarek et al., 2019). Engineering modifications to improve CAR functionality have targeted antigen affinity, intracellular signaling domains, and extracellular structural features (Benmebarek et al., 2019). CAR antigen affinity must be high enough to engage the tumor antigen and activate T cells, but not so high that it leads to activation induced cell death (AICD). The spacer length for both the hinge region between the cell membrane and single chain variable fragment (scFv) and the linker connecting the V_H and V_L of the scFv can affect CAR functionality. Different spacer lengths can modulate antigen interaction and may attenuate tonic signaling that, in some instance, leads to T cell exhaustion (Watanabe et al., 2016). The co-stimulatory domains engineered in these products, which currently consist of CD3-zeta plus CD28 and/or 4-1BB intracellular signaling domains in second and third generation CARs (Abate-Daga and Davila, 2016), provide co-stimulation for promoting both optimized antigen-mediated T cell activation, proliferation, and persistence as well as shaping the differentiation fate of the engrafted T cells (Kawalekar et al., 2016; Brentjens et al., 2007). T stem cell and central memory T cell (Tcm) phenotypes have been shown to improve anti-tumor T cell functionality, durable expansion and long-term memory in vivo (Gattinoni et al., 2011; Biasco et al., 2015; Wang et al., 2016).

To overcome antigenic escape and disease recurrence in B cell malignancy patients treated with current generation CD19 CAR T cells, multi-targeted CAR T cells are undergoing clinical evaluation. However, such strategies may give rise to new safety concerns. For example, CD22xCD19 bivalent CARs have reported promising results in overcoming relapse associated with downregulation of CD19 (Gardner et al., 2018). Whether bi-specific CAR T cells targeting these antigens would have increased toxicity is not known.

Successful approaches to address severe adverse events associated with on-target/off-tumor activity observed with HER2 CAR T cells have included switching to less potent signaling domains, local delivery (e.g., intra-ventricular for brain metastases), and fine-tuning CAR affinity. For example, HER2 CAR T cells with nanomolar affinity had activity against all cells tested expressing HER2, but HER2 CAR T cells with micromolar affinity no longer killed cells with normal antigen density (Liu et al., 2015). Novel approaches are also being used to overcome the suppressive tumor micro-environment (e.g. dominant-negative TGF-βRII) and off-switches for over-exuberant responses (e.g., armored CARs co-expressing EGFR that can be targeted by cetuximab) (Kloss et al., 2018).

As compared to CAR T cells, which can only recognize extracellular antigens, engineered TCR cell therapies have the potential therapeutic advantage of being able to recognize peptides presented from intracellular tumor antigens. However, engineered TCR T cells present new development and nonclinical safety challenges because these cell products are designed to recognize peptides presented in the context of major histocompatibility complex restriction (Ping et al., 2018). While the ability to test the off-tumor activity these cell products in vivo is constrained by MHC restriction, novel in vitro strategies have developed to de-risk the TCRs proposed for use in peptide targeting, including deep peptide scanning and screening for alloreactivity, described below (Sharpe, 2018). Overall, these innovative approaches are promising solutions to more effective and safe engineered T cells therapies.

4. Target expression and associated liability assessment

Engineered T cell therapies have demonstrated remarkable potency in the clinic where other treatments have failed. This is attributed to the unique properties of T cells, which can kill cells that express a target antigen even at low levels and mount an effective immune response that eradicates them. Due to this highly effective and sensitive immune response, a suitable target for an engineered T cell therapy must be selected to maximize efficacy but also minimize the safety risks from any off-tumor targeting. The appropriate target and the scope of any off-target assessment plan will vary by product and target type and require case-by-case evaluation of the risk/benefit ratio.

Off target assessments for engineered T cells generally consider two aspects:

1) On-target/off-tumor. This category provides the field with one of its major challenges, to find tumor-specific antigens that have minimal expression in healthy cells and tissues. It is addressed with analysis of target expression in cells and tissues in order to identify potential risk. Due to the potential trafficking of CAR-T cells to the brain and the known risk of neurotoxicity, potential expression of the tumor antigen in the brain should be considered in the risk assessment. Although immune effector cell associated neurotoxicity syndrome (ICANS) is not thought to be an on-target effect at this time (Gust et al., 2017), on-target cytotoxicity in the CNS could also result in neurotoxicity.
2) Off-target. This category refers to cross-reactivity of a binder or TCR to unknown targets. It is addressed with cross-reactivity studies using protein, peptide-MHC, cellular or tissue panels. Consideration of cross-reactivity to closely related proteins can be built into CAR design and selection.

The process of on-target/off tumor assessment begins at target selection; the ideal target for an engineered T cell therapy is highly and uniformly expressed on all tumor cells while completely absent from healthy cells and tissues. Since such “ideal” targets are rare to non-existent, the risk/benefit ratio for any target must be considered for a particular indication. In addition, the potential for up-regulation of target in vital cells and tissues in circumstances relevant to the patient population should be considered. Typically, bioinformatics/in silico searches are conducted to gather expression data for the intended target in healthy and disease tissue in order to make this assessment. Several cancer and healthy tissues gene expression data resources exist that can facilitate this process, such as mRNA and/or protein expression in primary cells, cell lines, and tumor samples (e.g. proteinatlas.org, gtexportal.org, the Cancer Genome Atlas [TCGA]).

Next is an effort to investigate the findings regarding target expression/distribution in actual tissue samples from healthy and diseased individuals using in situ hybridization (ISH) or immunohistochemistry (IHC), flow cytometry or other methods. To obtain meaningful results from these screening efforts, it is essential to qualify both the antibodies/probes and the cells and tissues being used. For IHC for example, this can be done using a set of positive and negative cell pellets and control tissues to confirm the specificity of detection reagents. Reference genes with known expression patterns can be used to confirm the quality of tissues from a given source. These steps are critical to obtain meaningful results but are often not performed in a rigorous manner. The specificity of antibodies for a given target cannot be assumed without proper

testing, and the results obtained from tissue screens largely depend on the quality of tissue. Although tumor tissue can generally be readily sourced, truly healthy human tissues, labeled and preserved appropriately, can be difficult to acquire and quality control checks are important. Additional caveats with these approaches include the limited diversity of tissues typically screened (e.g. tissues from sick patients or individuals with other disorders/co-morbidities are often excluded) and the lack of availability of pediatric tissues, both of which can have very different expression levels/patterns.

For TCR programs, initial target selection is similar on the level of the protein being pursued, with additional consideration given to whether a given target also has a dominant and potentially immunogenic peptide presented by MHC I. Once target-specific TCRs have been identified along with the peptide they recognize, the peptide sequence might be blasted against the genome to identify potential on-target/off-tumor interactions. In order to test/confirm target peptide-MHC expression in a given tissue mass spectrometry might be performed on membrane preparations from relevant cells/tissues.

Most developers of engineered T cell therapies perform an off-target screen against tissues, primary cells, cell lines, differentiated iPSCs, and/or protein expression array systems. Most screening efforts utilize binding as a readout (fluorescence microscopy or flow cytometry) due to the high throughput nature of these assays, but it has been observed that the most sensitive readout is functional, such as direct killing of cells which can be measured using T-cell activation readouts (degranulation or cytokine release) or target cell focused readouts (apoptosis markers such as caspase activation, cell lysis markers or morphological measurements). These screens can provide information on potential off-target interactions as well as on-target/off tumor activities. Such assays are not easily scalable for large screening purposes and are best applied to select tissue/cell types identified to be at risk.

For TCR programs, the assessment of off-target binding includes an analysis of the "sensitive" residues in the targeted peptide which are implicated in the TCR-target recognition binding (typically obtained using an alanine scan or x-scan (Docta et al., 2019)), followed by a bioinformatics approach to identify potential peptide homologs in proteins other than the intended target that might also cross react with the same TCR. In addition, alloreactivity also must be considered where unrelated peptides presented by a different HLA type than the intended target might cross-react with the TCR being developed. This can be done for most common HLA types by screening TCRs against panels of EBV-transformed B cells expressing a wide range of HLA types. As mentioned above, a functional readout such as cytotoxicity is the most sensitive readout of cross-reactivity. Since TCRs have been shown to require binding to single digit targets in order to mount an immune response (Fooksman et al., 2010), extensive testing of primary tissues in 2D and 3D cultures is sometimes performed. As with CAR programs, no screening effort can be fully predictive of off target binding and the toxicity risk of a TCR product must be weighed against the potential benefit. The value of animal models to identify off-target activity is discussed in the next section of the manuscript.

In summary, workshop presentations and the following discussions revealed a common approach to assessing the risk of off-tumor effects from engineered T cell therapies but highlighted that the methods and approaches used for such assessments should be carefully selected and qualified to provide meaningful results. Even with carefully executed studies, the full risk of off-target activity cannot be measured for most products due to limitations of current models, and therefore the risk/benefit ratio must be evaluated for each product on a case-by-case basis.

5. In vivo models informing safety and activity

While animal toxicology studies are generally a key component of the overall nonclinical safety assessment of a variety of types of new pharmaceutical products (e.g., small molecules, biologics, oligonucleotides, oncolytic viruses, vaccines), such studies might offer unique challenges when applied to cellular therapies and engineered T cells specifically. These challenges can be divided into several categories: (a) the frequent lack of recognition of the target of interest in animal species (e.g., chimeric antigen receptors may not be cross-reactive in animal species as is sometimes the case for other products; human TCRs are never cross-reactive to the intended peptide/MHC in animal species due to HLA restriction), (b) graft versus host and host versus graft reaction if human cells are administered to animals, (c) technical feasibility of generating surrogate autologous cells in animals.

Mouse models have been used, notably for the evaluation of activity/efficacy of human CAR-T therapies. In particular, human tumor models have been developed in immunocompromised animals such as severe combined immunodeficiency (SCID) mice to enable administration of xenografts and human engineered T cells while minimizing host versus graft responses. In such models, biodistribution and persistence of engineered T cells can be measured as well but the distribution is likely to be affected by target distribution and cross-reactivity of binding to mouse antigens. A limiting factor of in vivo models is the occurrence of graft versus host disease (GvHD) which is associated with decreased health of the animals as manifested by hair loss, decreased body weight and moribundity. In addition, alloreactivity of engineered T cells towards xenografts may contribute to the anti-tumor activity measured. Despite such limitations it is worth noting that some have leveraged such models for safety assessments including in vivo product characterization for process change control (Fraietta et al., 2018).

The utilization of such models for safety assessment purposes is limited. Such models have not been well predictive of cytokine release (notably due to the absence of human myeloid cells) and/or neurotoxicity even though these models can be further improved by administration of human PBMCs or further humanizing mice via implantation of $CD34^+$ hematopoietic stem cells. The possibility of using such mouse models to identify on-target/off-tumor or off-target activity must be closely evaluated on a case-by-case basis. Differences between humans and animal models in homology of protein targets, levels of expression, distribution of the target within tissues and cells, and variable expression of checkpoint proteins/receptors and other immune modulators can all contribute to underpredicted or overpredicted toxicity. GvHD resulting from allogeneic activation of human TCRs against mouse antigens, can overpredict/exacerbate off-target toxicity or mask it, leading to misleading conclusions.

To remedy the limitations associated with the rodent models described above, various groups have explored the utilization of nonhuman primate models, notably for the development of CAR-T cells (such models cannot be used for TCR-T therapies, as mentioned above, due to HLA restrictions) (Berger et al., 2015; Karbowski et al., 2020). In a case study recently published, authors have evaluated the possibility to evaluate the safety profile of an investigational chimeric antigen receptor (CAR) T-cell immunotherapy for the treatment of AML (AMG 553) by targeting Feline McDonough Sarcoma (FMS)-like tyrosine kinase 3 (FLT3). For that purpose, they established a tolerated regimen of the lymphodepleting and preconditioning agents cyclophosphamide and fludarabine in cynomolgus monkeys and optimized the transduction of the CAR construct in cynomolgus monkey T cells using a retroviral vector pseudotyped with a Gibbon-Ape leukemia virus (GALV) envelope protein. Indeed, nonhuman primate T-cells are not readily transduced by lentiviral vectors (He et al., 2017). The administration of autologous anti-FLT3 CAR T cells to the animals, with or without preceding administration of a cyclophosphamide and fludarabine regimen demonstrated no evidence of CAR T-cell-mediated toxicity, expansion, or persistence, likely due to limited cell surface FLT3 protein expression in healthy animals. This demonstrated the challenges associated with such in vivo studies for safety assessment of the CAR T-cell modality, when directed against a target with restricted expression. It should be noted that the manufacture of enough properly characterized autologous macaque T cells offers significant challenges. Ultimately, the nonclinical safety assessment of this investigational therapy relied also

on other approaches (Karbowski et al., 2020). Specifically, the nonclinical safety assessment of AMG 553 included a thorough assessment of FLT3 expression in normal human tissues and the in vitro assessment of AMG 553-mediated cytotoxicity against cells from a variety of tissues, expressing or not the tumor associated antigen. In addition and fortuitously, the availability of in vivo toxicology studies with 2 distinct FLT3-targeting BiTE® molecules in cynomolgus monkeys complemented the above mentioned data sets and confirmed that while AMG 553 targeted FLT3 protein on AML cells, effects in normal cynomolgus monkeys were limited to a small percentage of normal hematopoietic stem and progenitor cells.

Overall, it appears that animal models are not best suited and sometimes not relevant for these cellular therapies. This emphasizes the importance of alternative methods for hazard identifications and/or risk assessment.

6. Safety of viral and non-viral gene delivery systems – utility and risk of genomic injury

Viral vectors are used to introduce genetic material into human cells because, by converting their RNA genome into DNA and integrating this DNA into the chromosomes of target cells, stable integration and expression of novel elements can be achieved. Lentiviral vectors offer efficient transfer and stable integration of the transgene of interest in the host genome. There are many clinical studies involving CAR-T cell immunotherapies with gene transfer being performed with lentivirus transduction (Holzinger et al., 2016). Concerns regarding lentiviral vector manufacture and clinical use include the potential generation of replication-competent lentivirus (RCL) during lentiviral production or in the patient post-dosing and insertional mutagenesis caused by integration of the proviral DNA and viral promotor within or in close proximity to active genes and especially proto-oncogenes. Attributes of the vectors used for the manufacture of engineered T cells and how these attributes and manufacturing controls mitigate risks of generation of RCL and insertional oncogenesis (Zhao et al., 2017) are points to consider when conducting a vector-specific risk assessment. Lentiviral vectors have not been considered to be associated with a significant risk of oncogenic mutagenesis based on clinical studies (Milone and O'Doherty, 2018; Dropulić, 2011) and mature T-cells have a low propensity for oncogenic transformation after retroviral transduction in comparison to hematopoietic stem cells (Cattoglio et al., 2010; Newrzela et al., 2008). In addition, third-generation lentiviral vectors including a self-inactivating 3′ long-term-repeat (LTR) instead of an active LTR pose a reduced risk of oncogenic activation when compared to vectors with active LTRs (Cesana et al., 2012).

A current trend in the field of engineered T cell therapies is increased complexity of design to make next generation products that are more potent, persistent, more effective against solid tumors, and allogeneic to overcome the limitations associated with autologous manufacturing. Some of the common tools for T cell enhancement involve the use of nucleases to knock-out genes that negatively regulate potency/activity or to enable targeted integration via homology-directed repair of the CAR/TCR into specific loci that are intended to provide superior expression/function (Rafiq et al., 2020; Hong et al., 2020; Eyquem et al., 2017). The most common gene editing platforms being used include Zinc Finger Nucleases (ZFNs), CRISPR, TALENs, and MegaTALS (Maeder and Gersbach, 2016; Ashmore-Harris and Fruhwirth, 2020). ZFNs and TALENs work similarly in that they require dimerization of two domains of the FOKI nuclease for activity, and the specificity of the double-stranded break (DSB) site is provided by two separate ZFP arrays or TALE arrays that recognize particular DNA sequences with DNA-protein binding interactions. MegaTALs use a meganuclease that recognizes a specific sequence and specificity is increased by fusing it to TALE DNA-binding domains. CRISPR is a protein-RNA enzyme that uses the RNA-DNA interaction for specificity. Despite these differences, all nucleases present similar safety challenges when used for engineered T cell therapies and many drug developers are using similar tools for assessing their safety. The main risks associated with gene editing are derived from off target cutting of the genome (double stranded breaks, or DSBs) that can result in novel functions, deletions, translocations, genotoxicity, and ultimately tumorigenicity.

The general approaches to assessing and mitigating these risks include:

- Identification/quantification and characterization of off-target DSBs by employing in silico predictions based on sequence homology, unbiased in vitro assays such as guide-seq (Tsai et al., 2015) or site-seq (Cameron et al., 2017) to identify DSBs associated with a particular dose of a particular nuclease, and amplicon sequencing to validate DSBs identified by other means. Follow up may include ranking of identified DSBs in terms of potential risk and assessing the biological consequences of specific gene disruptions.
- Optimization of nuclease design, dose, and delivery to minimize off-target DSBs.
- Limiting the number of edits performed in the same product or temporally separating edits to prevent translocations between editing sites or off-target DSBs.
- Monitoring translocations, deletions, and genotoxicity through molecular/in vitro assays and karyotyping.
- Assaying for tumorigenicity as a comprehensive functional readout for off-target effects that might lead to growth advantages, clonal enrichments, and potentially cancer.

Assessing the tumorigenesis potential of engineered T cells in vivo is extremely limited by development GvHD and is therefore of questionable relevance. An in vitro method currently being explored and used to evaluate the likelihood of uncontrolled T cell growth in engineered T cell products relies on cytokine independent growth (IL-2). The origins of this assay can be traced to publications regarding HTLV-1 infection in T cells resulting in malignant transformation that was associated with cytokine independent growth after many weeks in culture (Nagarkatti et al., 1994; Futsch et al., 2018; Matsuoka and Jeang, 2011). Borrowing from the observation that malignant T cells could grow in the absence of IL-2 in culture, an ~18- to 60-day assay was developed to compare growth of an engineered T cell product in culture with and without IL-2 support. Whether one should rely on results from this assay, however, is open to questions. The methodology/assay lacks a positive control that would validate its ability to detect a transformation event. Furthermore, there are products types, such as enhanced T cell products that are engineered to be more proliferative and even cytokine-independent, for which this assay would not be relevant. At best, this assay measures the propensity of a T cell product to develop the ability to proliferate without IL-2, which says nothing about the tumorigenic potential of the product, and in fact might be desirable for the therapeutic effects of the product. There is a need to develop more informative and relevant models to assess T cell tumorigenicity, and the IL-2 independent growth assay should be used and interpreted with caution.

Gene editing plays a major role in the development of the next generation of engineered cell therapies that are potentially safer and more effective. Mature T cells appear to be quite resistant to transformation from lentivirus integration (the main tool used in the field for gene transfer), as supported by the history of HIV infection and engineered T cell therapies in which no T cell malignancy has been recorded to date (Milone and O'Doherty, 2018). Despite the added risk of inducing DSBs, often in combination with lentiviral-mediated gene transfer, developing cell therapy products from differentiated autologous or allogeneic T cells raises fewer concerns for transformation/tumorigenicity than using less differentiated cell types or pluripotent cells. As this field advances, increased expertise and knowledge over time will enhance our understanding and mitigation approaches to reduce the potential risks of gene editing.

7. Linking nonclinical development with the clinical experience

Although mouse models have been used to predict efficacy (e.g., select the CAR-T cell product to advance to the clinic) their utility to predict human safety remains uncertain. Since CAR-T cells are a human cell-based therapy, immunodeficient mouse models bearing human tumor xenografts are generally used which can pose challenges for human translation. Toxicity has been observed in mouse efficacy models in cases where the CAR cross-reacts to the mouse ortholog, and the mouse ortholog is expressed on normal cells/tissues, suggesting that in these circumstances the model could inform on the potential for a therapeutic index. However, as demonstrated by GD2-targeting CAR-T cells, differentiating between on-target/off-tumor toxicity and effects secondary to cytokine release/immune activation may prove challenging (Richman et al., 2018; Majzner et al., 2018; Richman and Milone, 2018). Mouse models of cytokine release and neurotoxicity are in development but require additional investigation and optimization to determine if the mechanisms involved reflect the CRS and ICANS observed in patients. In addition, no animal model exists for rapid-onset edema, which has been a severe adverse event observed clinically with some CD19 CAR T-cell therapies.

Unlike traditional small molecule or protein-based therapeutics, CAR-T cells are a living drug and proliferate (expand) in the patient after dosing. Thus, selection of a first-in-human dose for CAR-T cell therapies does not generally follow traditional paradigms based on allometric scaling of doses between nonclinical species and humans or traditional pharmacokinetic and pharmacodynamic correlations. For example, persistence of a human CAR-T cell product in an immunodeficient mouse may not reflect persistence in patients. Instead, starting dose is generally based on clinical experiences with other CAR-T cell therapies especially those against the same or a similar target. Nonclinical data may provide information to determine if a specific starting dose has an acceptable level of risk.

Despite leveraging external experience with later stage CAR-T cell therapies to help select first-in-human doses for CAR-T cell therapies entering the clinic, direct comparisons of CAR-T cell safety profiles have been hampered until recently by a lack of standardized grading system for CRS and ICANS. In 2018, a manuscript was published by Lee et al. (2019) that described a consensus grading system agreed by a group of experts in a meeting supported by the American Society for Transplantation and Cellular Therapy (ASTCT; formerly American Society for Blood and Marrow Transplantation, ASBMT). The consensus grading system is applicable to both clinical trials and commercial use. The application of this consensus grading system is enabling more direct comparisons of CAR-T safety profiles.

Finally, investigations on bio-banked patient samples and drug product characteristics are an area for further investment. Such investigations may inform on mechanisms of response and resistance, identify screening biomarkers, and support development of novel manufacturing procedures. A resource that provides extensive data on a large number of patients, even with the limits of retrospective analyses, could enable the identification of candidate causes for toxicities observed with CAR-T cells.

8. Regulatory perspectives and considerations

The global regulatory environment is evolving rapidly to keep up with new developments in the engineered T-cell therapy field. There is regional diversity in how engineered T cells are classified (e.g., as Advanced Therapy Medicinal Products in the European Union, Regenerative Medicine Advanced Therapies in the United States, and Regenerative Medicine Products in Japan; however nonclinical, clinical and manufacturing guidance documents for cellular and tissue-based products are applicable. Both EMA and FDA have recently released a number of guidance documents that pertain to the development of engineered T cell products including: "Guideline on quality, non-clinical and clinical aspects of medicinal products containing genetically modified cells," "Guideline on quality, non-clinical and clinical requirements for investigational advanced therapy medicinal products in clinical trials," and "Preclinical Assessment of Investigational Cellular and Gene Therapy Products" (EMA, 2020; EMA, 2019; FDA, 2019). To this end, there are several regional guidance documents, new guidance documents emerging (e.g., Brazil, China, India, S. Korea), but no harmonized guidance for nonclinical safety assessment of engineered T-cell products. However, there is harmonized guidance relevant to aspects of gene therapy in development, specifically focused on nonclinical biodistribution studies (ICH, 2021).

Many rely on the publicly available reviews conducted by various health authorities for the approved CAR-T cell products – Yescarta® and Kymriah® – for an initial view on what assessments were conducted and may be required. For example, the nonclinical safety assessment for Yescarta® leveraged an analogous murine CAR construct recognizing the murine CD19 molecule in a lymphodepleted syngeneic mouse model of $CD19^+$ B cell lymphoma whilst recognizing the limitations of the model, demonstrated specificity to $CD19^+$ tumor cell in vitro and provided a weight-of-evidence risk assessment relevant to oncogenic potential (FDA, 2017). Nonhuman primate in vivo toxicology studies were not conducted for either approved product. To this end, despite the lack of harmonized guidance, there is a general recognition that traditional in vivo toxicology studies may not be possible or appropriate based on the views expressed by participants from various Regulatory Agencies. The speaker from PMDA reported that due to the high frequency of GvHD in animals, in vivo toxicity testing is thought to be inappropriate in most cases, and clinical observation in xenograft models to demonstrate proof-of-concept is often sufficient. In vitro assessments were considered valuable to address on-target/off-tumor toxicity, off-target/off-tumor toxicity and potential for uncontrolled growth (e.g., proliferation assays) or oncogenesis (e.g., karyotyping) as described earlier in this document.

PMDA specifically addressed tumorigenicity assessment for inducible pluripotent stem cell (iPSC)–derived T-cell products and manufacturing impurities. To evaluate the tumorigenic risk of residual iPSCs in iPSC–derived T-cell products, it was recommended that in vitro/in vivo tumorigenicity testing be conducted according to the Japanese technical guidance for the cellular and tissue-based products (referenced in Preclinical Toxicity Studies for Regenerative Medicine in Japan (Shigeto et al., 2018)). In regard to manufacturing impurities the recommendation was for complete elimination, if possible, and the potential risk of remaining impurities should be assessed based on relevant data from a variety of sources: toxicity profiles of chemical or biological products, evidence of use in humans, serum levels in humans, and reports of acceptable daily intake.

Health authorities have a unique opportunity to evaluate data from multiple sponsors. To leverage this, the FDA is building a database – the FDA CAR-T cell safety database – to capture the impact of manufacturing changes on clinical outcomes across multiple products. The intent is to use the accumulated data to provide advice for sponsor-proposed risk-mitigation strategies and to mine the data for correlates between adverse events and specific patient population or product attributes. However, the data set is still immature at this stage.

Given the pace of innovation in the engineered cell therapy space, there are mechanisms in place to consult regional health authorities on nonclinical and/or clinical aspects for a specific product. CBER can be engaged via the Initial Targeted Engagement for Regulatory Advice on CBER Products (INTERACT) program, as well as via pre-IND meeting(s). It was noted that INTERACT meetings are a new opportunity and that they are not always granted depending on the novelty of the product, stage of development, and scope of the request. The MHRA can be consulted on clinical trial designs for engineered T-cells via the Clinical Trials, Biologicals and Vaccines Expert Advisory Group (CTBVEAG).

9. Conclusions

Engineered T cells constitute novel therapeutic strategies that combine elements of both cell therapies and gene therapies and therefore requires novel approaches to safety assessments. Following the approval of the first CAR-Ts, next generation T cell therapies are more complex and may present new risks for which appropriate safety assessment tools are needed. It should be noted that progress has been made in engineering safer plasmids, virus particles, and cell products. Currently, the main risks from a safety perspective for this class of products arise from on-target/off-tumor effects, CRS, and neurotoxicity. The risk of insertional mutagenesis remains something to be measured and monitored but no cases of insertional mutagenesis from lentiviral integration into mature T cells resulting in tumorigenicity products have ever been recorded. The increased likelihood of mutagenesis from gene editing in advanced T cell products is challenging to predict and also requires appropriate nonclinical assessments as well as close monitoring in humans to inform the field of the risks. There is still no harmonized regulatory guidance for engineered T cell therapies. Academic groups, industry groups, and consortia that recognize the need for filling these gaps are taking steps to develop and evolve methods to address them.

It should be noted that while this manuscript summarized approaches available to address several aspects on the nonclinical safety assessment of such therapies, it is recognized that each product has to be evaluated as an individual case and that generally regulatory agencies discourage conducting studies with non-relevant models. Instead, nonclinical development efforts should be scientifically sound and based on relevant data, and safety assessments in animals should be considered only if the model is appropriate. Since current tools and models for the safety assessment of T cell therapies are limited, mitigation for unknown and uncharacterized safety risks should be incorporated into clinical trial design. Furthermore, traditional allometric methods for translating dose from animals to humans cannot be used in the case of cell therapies and this represents another challenge in the field when trying to translate nonclinical data into a safe FIH dose.

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CRediT authorship contribution statement

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