T-ALERT: animal-free tumourigenicity assessment of CAR-T and other genetically modified T cells

Overall aim

1. The aim of this Challenge is to develop an *in vitro* assay(s) that can reliably evaluate tumourigenicity of human-engineered T cell therapies (primary and/or induced pluripotent stem cell (iPSC) -derived) with the potential to be applied to multiple immune cell types (e.g. NK or B cells).

Duration

2. Phase 1: up to six months, Phase 2: up to three years.

Budget

3. Phase 1: up to £100k, Phase 2: up to £1M.

Sponsors

4. Challenge Sponsors define the Challenges in collaboration with the NC3Rs to set out the business case and 3Rs benefits, with a view to using the product developed. Sponsors are required to provide in-kind contributions to help solve the Challenge.

5. The Sponsors for this Challenge are Novartis, Takeda, AstraZeneca, Sonoma Biotherapeutics and GentiBio.

Partners

6. Challenge Partners collaborate with the NC3Rs to provide additional resources to successful applicants to help deliver the Challenge.

7. The Challenge Partner is the Health and Environmental Sciences Institute (HESI) Cell Therapy - TRAcking, Circulation, and Safety (CT-TRACS) Committee

Background

8. Cell therapies are medical treatments where viable cells are grafted into a patient to achieve a therapeutic effect, such as replacing damaged tissue or regaining lost functionality. Engineered T cells expressing a

chimeric antigen receptor (CAR-T) are cell therapies capable of targeting cancer cells via cell-mediated immunity – they are proving to be effective therapeutic approaches for some cancers, such as haematologic cancers, in patients who have not responded to traditional therapies. There are currently six FDA approved CAR-T cell therapies, including two of the first anti-CD19 CAR-T therapies, Kymriah and Yescarta, that have a potential target population of 10,000 patients in the USA alone.

Types of T cell therapies

9. Genetically engineered T cells for therapeutic use include CAR-T cells and TCR-T cells. TCR-T cells are T cells engineered to express a T cell receptor (TCR) that recognises a peptide antigen only when loaded within a specific human leukocyte antigen protein. CAR-T cells or TCR-T cells can also be either effector T cells, in the case of oncology applications, or regulatory T cells (Tregs) developed to treat autoimmune or inflammatory conditions. Effector T cells generally serve to eliminate antigen-positive cells (such as tumour cells) and promote an inflammatory environment, whereas Tregs – used in autoimmune and inflammatory diseases – dampen inflammation through bystander suppression mechanisms. Antigens recognised by CAR- or TCR-T cells are typically over-expressed in diseases (e.g. tumour-associated antigens), making them robust targets for therapies.

Generation of T cell therapies

10. Engineered T cell products can be manufactured from the patient's own cells (autologous) or from healthy donors (allogeneic) generated either from primary T cells or derived from iPSCs. Generation of specific types of CAR- or TCR-T cells is achieved through expansion of the chosen cell type or through forced lineage commitment. The T cell products can then be further engineered to express cytokine receptors or to secrete cytokines or antibody-like proteins – known as armoured T cells to achieve their desired therapeutic effect. For allogenic products, further genetic manipulation (e.g. inactivation/elimination of donor TCR) is required to prevent graft versus host disease (GVHD).

Potential risks with T cell therapies

11. Although viral transduction of primary differentiated T cells has not been directly associated with leukaemia transformation to date, processes using genetic manipulation in the generation of T cell therapies can all present a risk of cell transformation and tumourigenicity which needs to be investigated [1]. There are a number of factors that could drive cell transformation, including:

    a) Random integration (and potential insertional mutagenesis) of the viral vector or transposon used for transgene delivery,

    b) Introduction of unintended alterations to the genome (e.g. point mutations, deletions, inversions and translocations) from further modification of the T cells performed with designer nucleases (e.g. zinc fingers, TALEN or CRISPR/Cas9).

12. Off-target genome editing through the introduction of mutations that may lead to tumourigenicity or uncontrolled cell growth presents a key safety concern. Recently, two clinical cases of CAR-T derived lymphoma have been reported, resulting in the death of one patient and the clinical trial withdrawal [2, 3].

13. The current approaches to evaluate T cell transformation and tumourigenicity concerns are limited. *In silico* and molecular biology assays can identify viral insertions, mutations, or translocations, but their interpretation needs to be linked to functional assays that directly assess cell transformation or tumourigenicity. The conventional approach for tumourigenicity testing is based on the *in vivo* tumourigenicity assay, where cells are implanted at an ectopic site (e.g. subcutaneously, under the kidney capsule or testis capsule) in immunodeficient mice and which are then monitored for the formation of tumour masses. These studies are limited in their value, primarily due to a lack of scientific consensus on the selection of the most relevant animal models to evaluate tumourigenic potential and how predictive these models are of clinical outcome.

14. The tumourigenic potential of cell therapy products manufactured from mature human T cells can be evaluated *in vitro* using the cytokine-independent growth assay. This assay is based on the principle that lymphocyte growth and survival are dependent on common gamma chain cytokines, which include Interleukin (IL)-2, IL-7, and IL-15 [4, 5, 6]. Lymphocyte growth in the absence of these cytokines is used as an indicator of cellular transformation and can help inform tumourigenicity risk assessment. While the cytokine-independent growth assay may be useful for the identification of potential hazards, it has a number of limitations. These include the absence of relevant positive controls that mimic cell transformation of the T cell product, undefined sensitivity (currently only technically assessed using cell lines), and lack of a standardised protocol. Mature T cell leukaemia and lymphomas are also highly heterogeneous, and not all these neoplasms do exhibit growth in the absence of cytokines [7, 8]. The cytokine-independent growth assay also failed to predict the recent documented CAR-T derived lymphoma cases [2, 3].

15. A robust, *in vitro* assay (or suite of assays) capturing key characteristics of transformed T cells is required to support and complement the interpretation of *in silico* and molecular assays for robust assessment of potential T cell transformation, with the aim of replacing *in vivo* studies and preventing T cell therapy-derived leukaemia in patients.

3Rs benefits

16. There are over a thousand (mainly CAR-T) T cell-based therapies currently in clinical trials [9] and many more are at the preclinical stage of development. While the relevance of the use of mice to assess modified T cell tumourigenicity potential is debatable, *in vivo* studies are sometimes requested by regulatory authorities. *In vivo* studies to assess tumourigenicity of modified T cells involve a minimum of 30 to 40 immunocompromised mice and can run from six to 12 months in duration. However, the standard *in vivo* experiment using NOD SCID gamma (NSG) mice is ill-suited to detect human tumourigenic T cells, as xenogeneic GVHD appears within a few weeks resulting in termination of the study before tumourigenic events, which may take months to develop, can be detected. Even in models where GVHD

is delayed or mitigated, such as the MHCI/II knockout mice or humanised mice [10], the GVHD will still occur during the six to 12 months required for a complete tumourigenicity study. As a result, large numbers of animals are needed to compensate for those euthanised due to the appearance of GVHD.

17. Specific constructs such as lentiviruses over-expressing NPM-ALK can potentially generate tumours within weeks [11]. However, animal models may not provide an appropriate physiological environment to allow most types of human-engineered T cells to persist, which may explain why no CAR-T cell transformation has been reported to date. Even if T cell transformation occurs, the translatability of a positive result to humans or comparison with a traditional two-year carcinogenicity study in mice is not robust.

18. The use of animals is predicted to keep increasing with the rapidly expanding research and development programmes on CAR-T therapies for solid tumours and additional indications such as the emerging field of regulatory T cell engineering for autoimmune diseases. For non-oncology indications, the limitations of current *in vivo* approaches may mean the tumourigenicity risk outweighs the benefit of treatment. An *in vitro* human based assay that can reliably assess the risk of T cell transformation has the potential to completely replace *in vivo* studies and improve the assessment of the tumourigenicity potential of modified T cell therapies.

Key deliverables

19. The key deliverable for this Challenge is to develop an *in vitro* assay(s) that can differentiate normal from aberrant tumourigenic behaviour of human T cells, using a multiparametric approach. The project will benefit from expertise in CAR-T call generation, *in vitro* leukemogenesis, genome editing, integration site analysis and tumourigenicity assessment.

20. The assay or model developed must:

    - Be suitable for human cell products (ideally primary cells, but also surrogate cell lines).
    - Include clinically relevant positive controls (e.g. piggyBac constructs, NPM-ALK, Myc-Ras).
    - Deliver quantitative endpoints. Exploration of additional endpoints beyond cell proliferation, such as, but not limited to genetic analysis, phenotypic and functional readouts linked to transformation events, ideally linking to the causal mechanism(s) for transformation are encouraged.
    - For each endpoint, include definition of its sensitivity and specificity using appropriate controls.
    - Be benchmarked against the cytokine-independent growth assay and *in vivo* tumourigenicity models.
    - Be amenable to low or medium throughput, though higher throughput is preferred where possible.
    - Deliver a reliable result within a reasonable assay timeframe (ideally a few weeks but up to six months).

Phase 1 Deliverables

- Establishment of the assay system (e.g. using 2D or 3D culture, microfluidic, cells grown in semi-solid media/3D printed structures, or other).
- Establishment of a human surrogate cell line (if an approach based on surrogate cell line is proposed).
- Establishment of clinically-relevant positive and negative controls to define assay sensitivity and specificity.
- Identification of the parameters needed to differentiate between normal and tumourigenic T cells.
- Proof-of-concept experiments with appropriate controls.

Phase 2 Deliverables

Essential

- Characterisation of the system (e.g. low limit of quantification or sensitivity, reproducibility, robustness) with multiple donors for each parameter/endpoint.
- Validation of the parameters needed to differentiate between normal and tumourigenic T cells.
- Evaluation of all positive and negative controls and definition of endpoint thresholds to enable assay interpretation.
- Identification of the minimum time required for a reliable discrimination between normal and transformed T cells.
- Benchmarking against the cytokine-independent growth assay and *in vivo* tumorigenicity models.
- Suitability of the assay(s) for implementation in standard laboratories.

Desirable

- Adaptation of the assay(s) to additional cells grown in suspension (e.g. B cells, NK cells).
- Scale-up of the assay for higher throughput.

21. It is important to note that the CRACK IT Challenges competition is designed to support the development of new 3Rs technologies and approaches, which will improve business processes and/or lead to new marketable products. The application must include a plan to commercialise the results into a product or service. This should be taken into consideration when completing your application.

Sponsor in-kind contributions

22. The Sponsors will provide:

- Scientific support.
- Data collected from *in vivo* experiments and/or clinical trials to be used for identification of appropriate biomarkers, where possible.
- Reagents to assist in the development of the assay, where possible.
- Bioinformatics analysis and/or support.
- Testing of the model system in Phase 2.
- Dissemination of the model and validation in multi-site studies.

Partner contributions

23. HESI CT-TRACS will provide a platform for exposure to and potential collaboration with an international network of experts from across multiple sectors (research science, regulatory, clinical, health foundations, and technology developers). The Tumourigenicity working group is specifically focused on addressing concerns regarding the potential for tumourigenicity of hiPSC-derived products by assessing and/or developing methodologies and approaches that could support tumourigenicity evaluation and has successful track record of multi-laboratory evaluation of relevant methods and assays.

References

1. Nahmad AD., *et al.,* (2022). Frequent aneuploidy in primary human T cells after CRISPR-Cas9 cleavage. Nat Biotechnol. https://doi.org/10.1101/2021.08.20.457092
2. Micklethwaite, K. *et al.*, (2021). Investigation of product-derived lymphoma following infusion of *piggyBac*-modified CD19 chimeric antigen receptor T cells. *Blood*, 138(16), 391-1405. doi: 10.1182/blood.2021010858.
3. Bishop, D., *et al*., (2021). Development of CAR T-cell lymphoma in 2 of 10 patients effectively treated with *piggyBac*-modified CD19 CAR T cells. *Blood*, 138(16), 1504-09. doi: 10.1182/blood.2021010813
4. Migone, T., *et al.,* (1995). Constitutively Activated Jak-STAT Pathway in T Cells Transformed with HTLV-I. *Science*, 269(5220), 79-81. doi: 10.1126/science.7604283
5. Akbar, A., *et al*., (1996). Interleukin-2 receptor common γ-chain signaling cytokines regulate activated T cell apoptosis in response to growth factor withdrawal: Selective induction of anti-apoptotic (bcl-2, bcl-xL) but not pro-apoptotic (bax, bcl-xS) gene expression. *European Journal of Immunology*, 26(2), 294-99. doi: 10.1002/eji.1830260204
6. D'Souza, W. and Lefrançois, L., (2003). IL-2 Is Not Required for the Initiation of CD8 T Cell Cycling but Sustains Expansion. *The Journal of Immunology*, 171(11), 5727-35. doi: 10.4049/jimmunol.171.11.5727

7. T Hori, *et al.*, (1987) Establishment of an interleukin 2-dependent human T cell line from a patient with T cell chronic lymphocytic leukemia who is not infected with human T cell leukemia/lymphoma virus. *Blood* ; 70 (4): 1069–1072. https://doi.org/10.1182/blood.V70.4.1069.1069

8. Kees UR, *et al.,* (1991) Intrathoracic carcinoma in an 11-year-old girl showing a translocation t(15;19). The American Journal of Pediatric Hematology/oncology. 13(4):459-64. 10.1097/00043426-199124000-00011. Doi: 10.1097/00043426-199124000-00011

9. Clinicaltrials.gov. 2022. *Home - ClinicalTrials.gov*. [online] Available at: <https://clinicaltrials.gov/> [Accessed July 2022].

10. Yin, L., *et al*., (2020). Humanized mouse model: a review on preclinical applications for cancer immunotherapy. American journal of cancer research, 10(12), 4568–84. PMID: 33415020

11. Congras A., *et al.,* (2020) ALK-transformed mature T lymphocytes restore early thymus progenitor features. J Clin Invest. Dec 1;130(12):6395-6408. doi: 10.1172/JCI134990