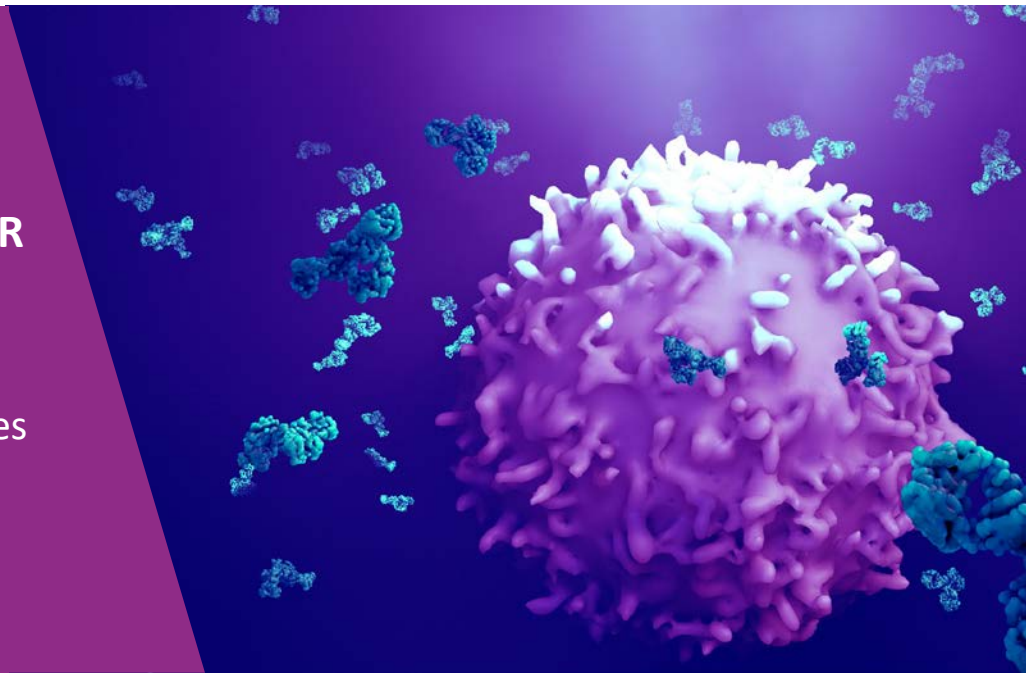
imSAVAR

IMMUNE SAFETY AVATAR

Nonclinical mimicking of the immune system effects of immunomodulatory therapies



Deliverable 2.3

CAR-T & BiTE immune safety assessment research roadmap

DELIVERABLE REPORT

This project has received funding from the Innovative Medicines Initiative 2 Joint Undertaking (JU) under grant agreement No 853988.
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Abstract

A key objective in imSAVAR is to create a platform with novel tools, models and resources for early preclinical prediction of possible immune-related adverse events (irAEs) of immunomodulatory therapies. WP2 focuses on refining/adjusting/developing models for three different immune-oncology modes of action (MoA) with a high medical need for improved preclinical safety assessment: chimeric antigen receptor (CAR) T cells, bispecific T cell engagers (BiTEs) and immune checkpoint inhibitors (ICIs). The following immune related adverse outcomes are in the focus of the work program: cytokine release syndrome (CRS), neurotoxicity (CAR T cells, BiTEs) and hepatotoxicity (ICIs). This deliverable summarizes the progress that has been made and the accomplishments in establishing the research roadmap for CAR T cells and BiTEs as exemplary MoAs and CRS as their most frequent adverse outcome.

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1. Methods

1.1 Literature Review / Development of immune-related adverse outcome pathways (irAOPs)

As a first step for the development of irAOPs for CAR T cells and BiTEs, a scientific literature review on the current state-of-knowledge on their pathophysiology and the current state-of-the-art of preclinical assessment was conducted. This effort was closely aligned with the systematic literature review conducted in WP4, which focuses on the biomarkers related to the prediction of CRS in CAR T cell therapy. This effort identified a substantial amount of peer-reviewed publications (173) that were evaluated in a first iteration based on the PRISMA statement[1]. After exclusion of articles that did not meet the ranking criteria (no correlation with biomarkers, no data sets available), 50 publications remained for full text evaluation. In five publications, data were accessible for re-analysis and one particular publication was deemed to be of particular interest[2]. The WP2 leads reached out to the senior author of this publication (Prof. Cameron J. Turtle, MD/PhD, Fred Hutch, Seattle, WA) and were able to hold a consultation where they introduced the imSAVAR project and objectives of WP2. At present, efforts are ongoing to engage Prof. Turtle as an advisor for the imSAVAR consortium and to re-analyze the available (public) data sets from Fred Hutch using the algorithms established in imSAVAR (joint activity with WP4).

To guide the refinement and development of improved preclinical test systems, the current knowledge on the different biological key events and the current or new test systems and related molecules which could serve as predictive biomarkers were combined in immune-related adverse outcome pathways (irAOPs) for the most relevant irAEs mediated by CAR T cells and BiTEs, namely CRS and neurotoxicity, in accordance with the OECD guidelines on AOP development[3].

These irAOPs enable the definition of biological key events and key event relationships mediated by the molecular initiating event mediated by the MoA (here: CAR T cells or BiTEs) and facilitate the identification of gaps in current model systems. Subsequently, the irAOPs guide the refinement and development of assays/models for improved preclinical safety assessment and will also be instructive for the development of suitable predictive biomarkers (joint activity with WP4).

1.2 Workshops

The WP2 leads organized a series of workshops and congress presentations to advance irAOP development, increase visibility of the imSAVAR project, engage stakeholders and collect valuable feedback and input from the field.

The first event was the 1st imSAVAR stakeholder workshop (June 29, 2020; virtual event with 87 attendees) that introduced the general concept of AOPs (speaker: Katherina Sewald, Fraunhofer ITEM, WP3) and the working model for CAR T cell and BiTE-mediated CRS (speakers: Michael Hudecek, Universitätsklinikum Würzburg, WP2 lead; Birgit Fogal, Boehringer Ingelheim). This event provided feedback for a first iteration and revision cycle of the irAOP. The updated CAR T cell mediated CRS irAOP was then presented as an ePoster at the 46th annual meeting of the European Society for Bone Marrow Transplantation (EBMT) that attracts clinicians and scientists from the field of hematology (i.e. the key 'users' of CAR-T cell therapy). The ePoster was well received and triggered feedback and input from multiple academic and industry stakeholders and patients.

The second key event in this project period was a 2nd workshop, organized jointly between imSAVAR and the T2EVOLVE IMI projects, at the 3rd EBMT/EHA European CAR-T Meeting (February 5, 2021; virtual event

with 136 attendees). The workshop was integrated into the meeting program and garnered widespread attention and visibility for the two IMI projects. The workshop comprised a panel discussion with panelists from the two IMI2 projects imSAVAR and T2EVOLVE, a moderator, and a key opinion leader in the field of adoptive T-cell immunotherapy – Prof. Stanley R. Riddell, MD, Fred Hutch, Seattle, WA. The panel discussion and lively interaction with the audience yielded valuable feedback and input that is currently integrated into the next updated version of the irAOP. The imSAVAR coordinator and WP2 lead have taken measures to keep the workshop participants engaged through a follow-up note and invitation to the stakeholder community. The series of workshops will be continued at the annual EBMT and EHA meetings in 2021 and 2022, and the joint EBMT/EHA CAR-T Meeting in 2022.

1.3 Experimental campaign with CAR T cells

As simplistic immune cell / tumor cell co-culture *in vitro* tests "ignore" the role of innate immune cells and endothelial cells in the pathophysiology of CRS, cytokine release test platforms incorporating these cell types shall be explored for their suitability in modelling for example the activation of endothelial cells (defined as key event 3 in the irAOP for CAR T cells, see **Figure 3**). Furthermore, test systems shall also be ranked according to their suitability to model e.g. release of cytokines that are associated with CRS in patients treated with CAR T cells.

To minimize donor variability and hence increase comparability of results obtained on different test platforms, partner UKW obtained leukapheresis collected from n=2 healthy donors from the German Red Cross (DRK) center in Frankfurt. From these, peripheral blood mononuclear cells (PBMCs) were isolated, followed by isolation of CD4 and CD8 T cells and subsequent production of CD4 and CD8 CD19- and ROR1-specific CAR T cells (see **Figure 1**; also reported as part of deliverable report D5.3). These set of matched PBMCs, control T cells as well as CAR T cells of each donor were distributed among partners (see **Table 1** in Results section). In total, approx. 35 vials of each cell type (PBMC, CD4 and CD8 control T cells, CD4 and CD8 CD19- and ROR1-specific CAR T cells) were cryopreserved per donor.

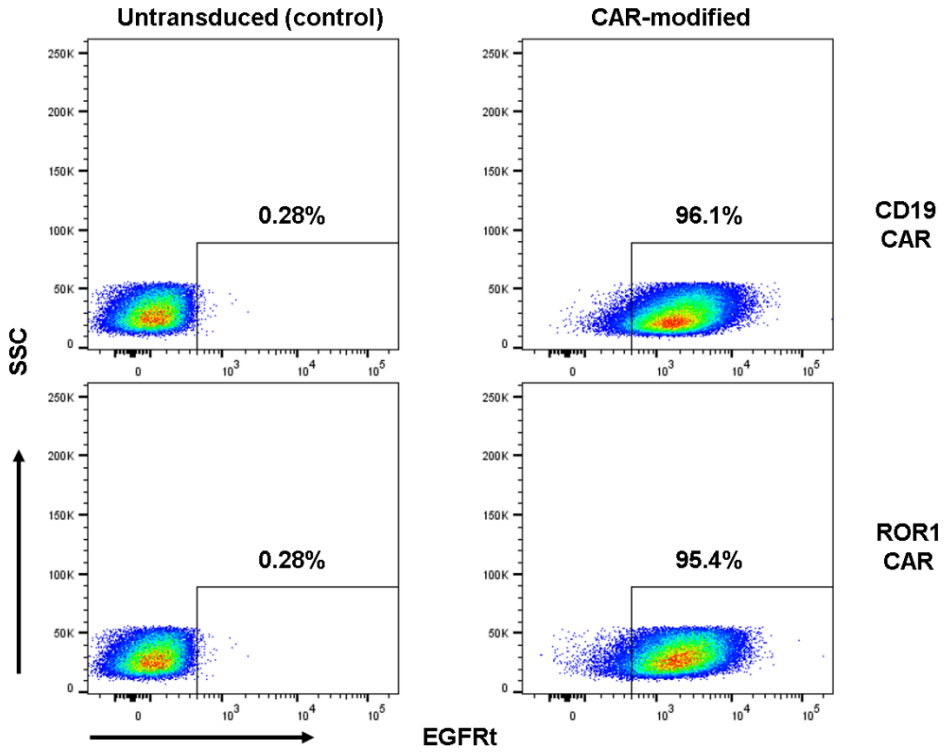

Figure 1: Phenotype of CD19 and ROR1 specific CAR T cells after expansion. *CD4 and CD8 T cells were isolated from PBMCs, stimulated with CD3/CD28 beads and transduced with lentiviral particles encoding the CAR of choice. CAR T cells were purified using a biotinylated antibody targeting the transduction marker (EGFRt) and expanded using an irradiated feeder cell line and third party PBMCs according to an UKW internal SOP. Dot plots show the expression of the CAR (visualized via staining of the transduction marker) after expansion of control T cells (untransduced; left panel) and CD19- or ROR1-specific CAR T cells (CAR-modified; right panel). Representative dot plots from donor 1 gated on live CD4⁺ T cells. EGFRt: truncated epidermal growth factor receptor; SSC: side scatter*

Furthermore, the CD19 and ROR1 expressing tumor cell line Raji (Burkitt lymphoma; obtained from Leibniz Institute DSMZ, Braunschweig, Germany) was made available to all partners. Additionally, the breast cancer cell line MDA-MB-231 (obtained from ATCC, Manassas, VA, USA) was shared with partner Fraunhofer IGB for tumor-on-chip test runs.

2. Results

2.1 Literature Review

One of the most common and potentially fatal immune-related adverse events of both CD19 CAR T cell therapy (e.g. with the FDA/EMA approved CD19 CAR-T products Kymriah®, tisagenleucel or Yescarta®, axicabtagene ciloleucel) and CD19 targeting BiTE therapy (e.g. with the FDA/EMA approved BiTE Blincyto®, blinatumomab) is cytokine release syndrome [4-7]. Patients receiving such immunotherapies are closely monitored within the first ten days after infusion of the cell product/the BiTE for any sign of CRS (e.g. fever > 38°C). Patients who develop CRS of grades 3 to 4 require intensive care due to serious side effects such as capillary leakage, hypotension and end organ failure [8-10].

Although it is now well understood, that particularly in CAR T cell therapy the release of interleukin (IL)-6 by macrophages and/or monocytes is a key factor of CRS pathophysiology [10,11] (see **Figure 2**), it is currently hard to predict the occurrence of such a safety issue with "conventional" *in vitro* tumor cell/immune cell co-culture assays and NSG mouse models lacking a human immune cell compartment [12].

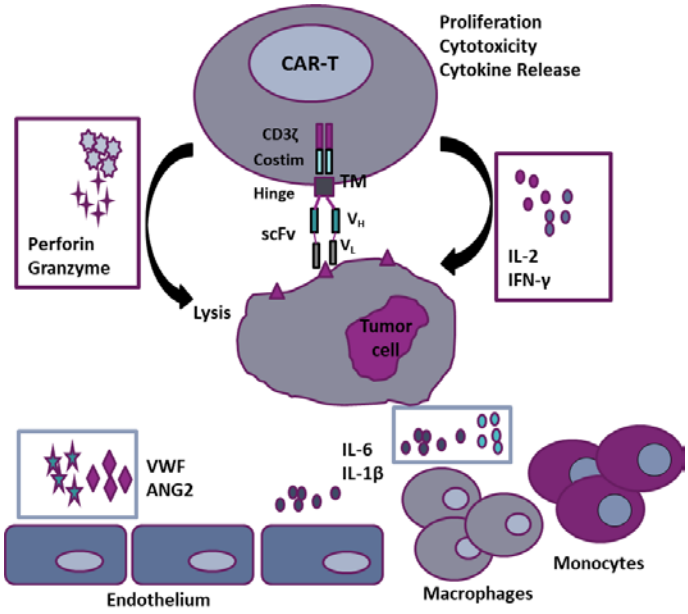

Figure 2: Illustration of some key aspects of CRS pathophysiology mediated by CAR T cells. *T cells equipped with a CAR of choice are able to target tumor cells expressing this antigen in a non-MHC restricted manner. Upon binding to the antigen, CAR T cells are activated and will therefore start to proliferate and release cytokines like IL-2 or interferon (IFN)-γ or in case of CD8 CAR T cells*

release additionally cytolytic molecules such as perforin and granzyme B. Furthermore, innate immune cells will be recruited/will migrate in response to the released cytokines, will be activated and in turn release additional pro-inflammatory mediators such as IL-1β and IL-6. The release of these molecules also activates endothelial cells which will release additional molecules like angiopoietin-2 (ANG2[13]) and von Willebrand factor (VWF). This abundant release of different pro-inflammatory mediators can lead to systemic inflammation and organ dysfunction with a rapid onset of fever, capillary leakage, hypotension, hypoxia, and organ failure. Presented at 1st imSAVAR stakeholder workshop.

The key aspects of this literature review were then translated into an irAOP for CAR T cell mediated CRS (see **Figure 3**). The framework of such AOPs can be instructive to find the gaps in current test systems and can guide the refinement and development of test systems towards an improved preclinical hazard assessment. AOPs can be seen as "living" documents, which means that the results of different test campaigns in imSAVAR along with the review of current literature will be also incorporated into the irAOPs later on.

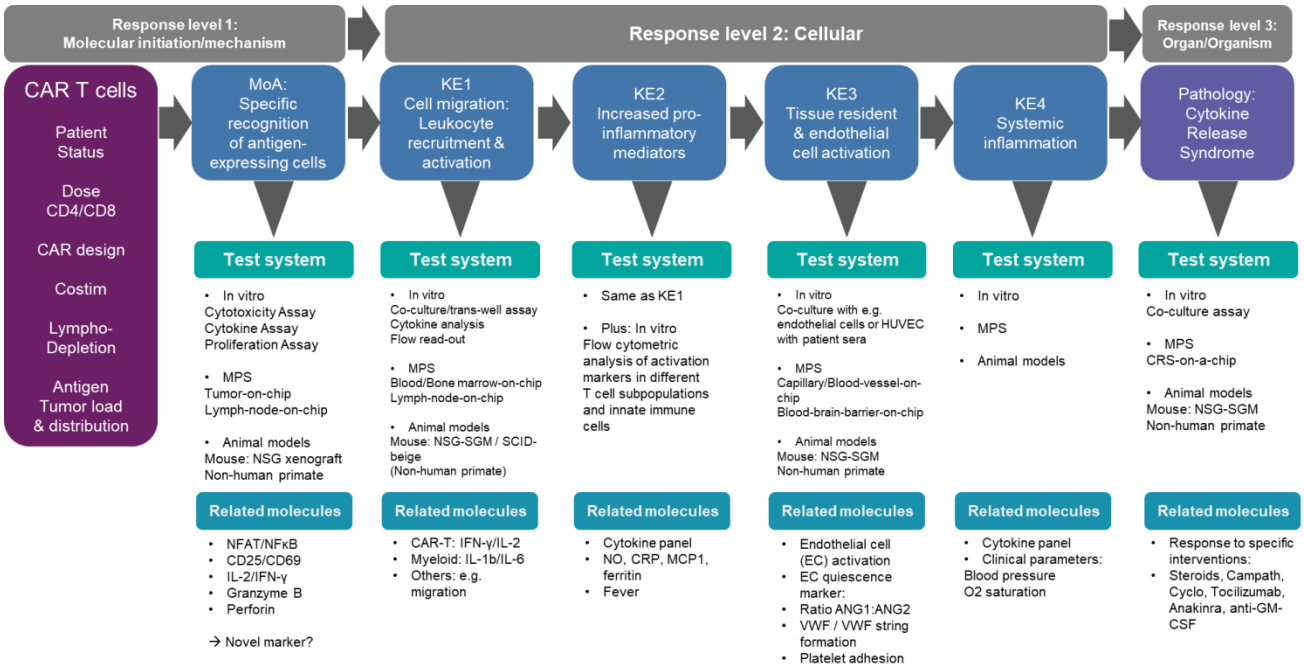

Figure 3: irAOP framework for CAR-T mediated CRS. *The framework of the irAOP not only lists the biological key events (KE) that finally lead to the adverse outcome on an organ/organism level (here: CRS) but also gives an overview of current and/or new test systems to model these key events and molecules that can be measured in these test systems and which are linked to the respective KE. Presented at 1st imSAVAR stakeholder workshop and as an ePoster at the 46th annual EBMT meeting. KE: key event; MPS: micro-physiological system (organ-on-chip models).*

2.2 Feedback on CAR-T and BiTE irAOPs presented at workshops

One main aspect that was discussed at the 1st imSAVAR stakeholder workshop and the panel discussion at the 3rd European CAR T cell meeting was that current test systems, which are broadly used for efficacy testing of novel CAR T cell constructs or BiTEs, mostly "ignore" the contribution of innate immune cells and endothelial cells to the adverse outcomes CRS and neurotoxicity. Therefore, models that incorporate the relevant cell types for hazard assessment are needed. Such models are available in the imSAVAR consortium and have been integrated (and prioritized) in the research roadmap during the first part of the project period (**Figure 4**).

The advantage of fully autologous over allogeneic test systems was also discussed as autologous test systems could help to minimize confounding results due to MHC/HLA mismatch. It was agreed that the refinement and/or development of test systems should be conducted with cells from healthy donors with minimal HLA mismatch within one test system (e.g. by using endothelial cells and T cells from only one donor). As patient samples are mostly restricted to small sample sizes and cell numbers it was agreed that patient samples shall be used to validate the test systems at a later stage.

It was also discussed that refined/adjusted models and new models (e.g. organ-on-chip models) should be adapted to the "real world", i.e. to the cytokines and other parameters which are assessed in CAR T cell or BiTE therapy in the clinic/in clinical trials. The systematic literature review conducted in WP4 focuses already on this key aspect, e.g. if these molecules could be suitable predictive biomarkers for CRS. Therefore, models should be benchmarked against already available data sets of e.g. clinical trials with CAR T cells. This aspect has been considered in the research roadmap during the first part of the project period (**Figure 4**). Furthermore, within WP4 of imSAVAR new data sets shall be analyzed to gain further insight on the cellular composition in patients prior and after immunotherapies.

2.3 Experimental campaign for CAR T cells and BiTEs

In the first part of the imSAVAR project, experiments will focus on key events 1-2 (activation and migration of immune cells and increased release of pro-inflammatory mediators) and key events 2-3 (pro-inflammatory mediators and activation of endothelial cells). Data sets will then be reviewed, the roadmap re-assessed / re-adjusted as necessary and more complex models tackled in the second part of the project, including key event 4 (systematic inflammation) and assessments at the organ/organism level (see **Figure 4**).

Here, test systems incorporating the relevant cell types, which are associated with CAR T cell mediated CRS, such as endothelial cells, will be refined or adjusted as needed or new models as tumor-on-chip models will be explored for their suitability to predict the adverse outcome. As mentioned in the Methods section, partner UKW distributed a set of matched PBMCs, control T cells as well as CAR T cells of two healthy donors among partners with relevant test systems available at their sites (see **Table 1** below).

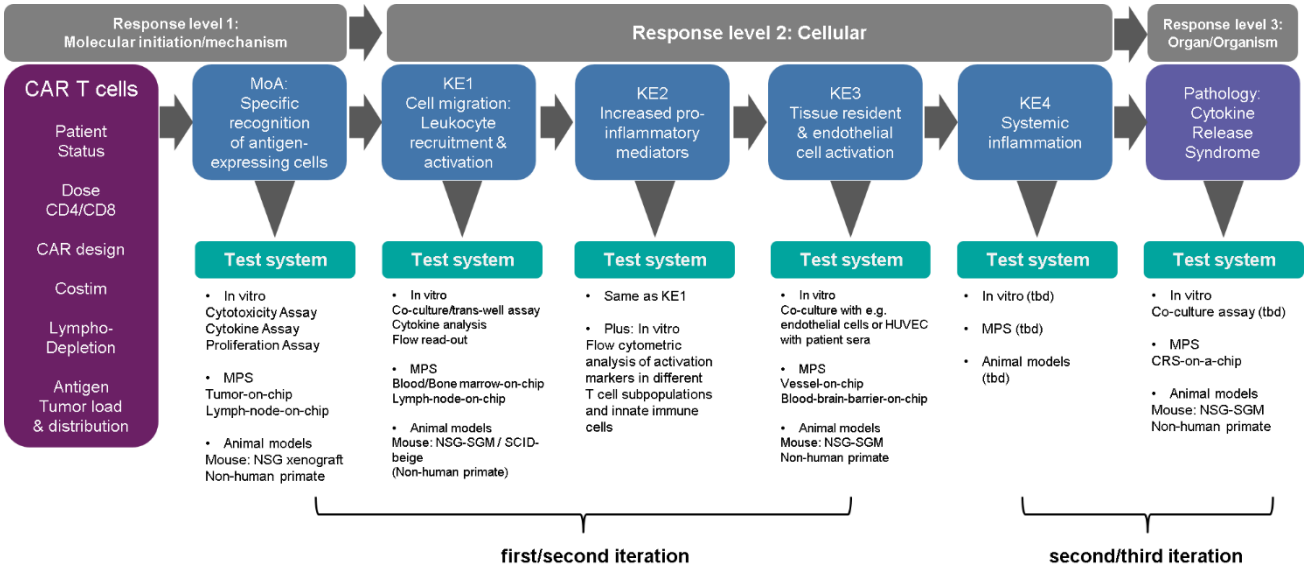

Figure 4: Research Roadmap for CAR T cells. *The first and second iteration will focus on test systems that model the recruitment and activation of endogenous and infused immune cells as well as the increased release of pro-inflammatory mediators (key event 1 and 2) and the activation of endothelial cells (key event 3). Later on, complex models shall be explored for their suitability to assess systemic inflammation (key event 4) and assessments on the organ/organism level. KE: key event; MPS: micro-physiological system (organ-on-chip models).*

To further minimize the variability of cell types used on different platforms (e.g. usage of human umbilical vein endothelial cells (HUVECs) from a third party donor in co-culture experiments) PBMCs from the UKW healthy donors shall be reprogrammed to obtain induced pluripotent stem (iPS) cell lines which can be further differentiated to e.g. endothelial cells. By this, it is possible to obtain a fully autologous set of different cell lines from one or more donors, which will minimize donor variability across different test systems.

Table 1: *Test systems available at different partner sites to assess cytokine release by CAR T cells*

Assay Format	Partner	Key events of irAOP	Possible Biomarker
BOEC Assay	Covance	KE 1 to KE 3	IL-2, IFN-γ, TNF-α, Granzyme B, Perforin, IL-6, VWF
MIMIC (Whole-blood)	Sanofi	KE 1 to KE 3	IL-2, IL-6, IL-10, IFN-γ
Co-culture with tumor cells and HUVECs	Boehringer Ingelheim	KE 1 to KE 3	IL-2, IL-6, IL-10, IFN-γ, Granzyme B
Tumor-on-chip	Fraunhofer IGB	KE 1 to KE 3	IL-2, IFN-γ, TNF-α, Granzyme B, Perforin, IL-6
Vessel-on-chip	University of Twente	KE 3	VWF
Intestine-on-chip	JUH	To be determined	To be determined

BOEC: blood-outgrowth endothelial cells; HUVECs: human umbilical vein endothelial cells; KE: key event; MIMIC: Modular IMmune In vitro Construct; TNF-α: tumor necrosis factor alpha; VWF: von Willebrand factor

Likewise, model bispecific molecules (T-cell engagers) that partners Boehringer Ingelheim, Novartis and Sanofi can share with the imSAVAR consortium shall be tested in a similar manner using the *in vitro* test platforms (established *in vitro* test systems as well as newly developed organ-on-chip models) available within the imSAVAR consortium. Here, WP2 will also closely collaborate with WP4 to identify and incorporate suitable predictive biomarkers from existing data sets as well as from data sets that will be generated within imSAVAR.

2.4 Outlook

To gain further insight into the cellular composition of the CAR T cell products used in the different test systems, CAR T cells as well as matched control T cells and PBMCs from the same donor shall be analyzed by mass cytometry (cytometry by time of flight [CyTOF]) at the Roche partner site as well as by single cell RNA-seq analyses at the Fraunhofer IZI partner site.

In addition, clinical samples from patients treated at UKW with FDA/EMA approved CD19 CAR T cell products (Kymriah®, tisagenleucel or Yescarta®, axicabtagene ciloleucel) will be collected for a in-depth characterization of these cell products before and after infusion (“real world” datasets) to facilitate the identification of suitable predictive biomarkers which can be incorporated in the above mentioned test systems.

Analyses of clinical samples will also enable insight into the cellular composition of endogenous innate immune cells prior and after infusion of the CAR T cell product and can therefore give additional guidance

for the optimization/mimicking of the microenvironment seen in patients in established and newly developed CRS test systems.

Additionally, experiments in relevant CRS mouse models shall also be conducted[14]. Samples obtained from such models can also be analyzed by OMICS techniques (e.g. single cell RNA-seq) and help to elucidate the contribution of the infused CAR T cell product and other immune cells to the pathophysiology of CRS which might also be instructive for an optimized CRS management e.g. with IL-6 receptor antagonists.

3. Discussion

No relevant deviations from the Description of Action and contingency plans identified.

4. Conclusion

The fruitful discussions and feedback during and after the 1st imSAVAR stakeholder workshop and the panel discussion at the 3rd European CAR T cell meeting concerning the irAOPs that were developed for CAR T cells as well as BiTEs fostered the refinement of the research roadmap for improved preclinical safety assessment for these immunotherapeutic modalities. By adjusting and refining as well as incorporating new test systems to model cytokine release syndrome mediated by CAR T cells or BiTEs the consortium aims at a more precise prediction of adverse events and intends to incorporate the results of retrospective analyses of existing data sets from clinical trials as well as data sets that will be generated within imSAVAR as suitable predictive biomarkers.

References

1 Moher, D., Liberati, A., Tetzlaff, J., Altman, D. G. & Group, P. Preferred reporting items for systematic reviews and meta-analyses: the PRISMA statement. *J Clin Epidemiol* **62**, 1006-1012, doi:10.1016/j.jclinepi.2009.06.005 (2009).

2 Sheih, A. *et al.* Clonal kinetics and single-cell transcriptional profiling of CAR-T cells in patients undergoing CD19 CAR-T immunotherapy. *Nat Commun* **11**, 219, doi:10.1038/s41467-019-13880-1 (2020).

3 Sakuratani, Y., Horie, M. & Leinala, E. Integrated Approaches to Testing and Assessment: OECD Activities on the Development and Use of Adverse Outcome Pathways and Case Studies. *Basic Clin Pharmacol Toxicol* **123 Suppl 5**, 20-28, doi:10.1111/bcpt.12955 (2018).

4 Teachey, D. T. *et al.* Cytokine release syndrome after blinatumomab treatment related to abnormal macrophage activation and ameliorated with cytokine-directed therapy. *Blood* **121**, 5154-5157, doi:10.1182/blood-2013-02-485623 (2013).

5 Brentjens, R., Yeh, R., Bernal, Y., Riviere, I. & Sadelain, M. Treatment of chronic lymphocytic leukemia with genetically targeted autologous T cells: case report of an unforeseen adverse event in a phase I clinical trial. *Mol Ther* **18**, 666-668, doi:10.1038/mt.2010.31 (2010).

6 Locke, F. L. *et al.* Long-term safety and activity of axicabtagene ciloleucel in refractory large B-cell lymphoma (ZUMA-1): a single-arm, multicentre, phase 1-2 trial. *Lancet Oncol* **20**, 31-42, doi:10.1016/S1470-2045(18)30864-7 (2019).

7 Curran, K. J. *et al.* Toxicity and response after CD19-specific CAR T-cell therapy in pediatric/young adult relapsed/refractory B-ALL. *Blood* **134**, 2361-2368, doi:10.1182/blood.2019001641 (2019).

8 Dholaria, B. R., Bachmeier, C. A. & Locke, F. Mechanisms and Management of Chimeric Antigen Receptor T-Cell Therapy-Related Toxicities. *BioDrugs* **33**, 45-60, doi:10.1007/s40259-018-0324-z (2019).

9 Lee, D. W. *et al.* ASTCT Consensus Grading for Cytokine Release Syndrome and Neurologic Toxicity Associated with Immune Effector Cells. *Biol Blood Marrow Transplant* **25**, 625-638, doi:10.1016/j.bbmt.2018.12.758 (2019).

10 Shimabukuro-Vornhagen, A. *et al.* Cytokine release syndrome. *J Immunother Cancer* **6**, 56, doi:10.1186/s40425-018-0343-9 (2018).

11 Garcia Borrega, J. *et al.* In the Eye of the Storm: Immune-mediated Toxicities Associated With CAR-T Cell Therapy. *Hemasphere* **3**, e191, doi:10.1097/HS9.0000000000000191 (2019).

12 Chulpanova, D. S., Kitaeva, K. V., Rutland, C. S., Rizvanov, A. A. & Solovyeva, V. V. Mouse Tumor Models for Advanced Cancer Immunotherapy. *Int J Mol Sci* **21**, doi:10.3390/ijms21114118 (2020).

13 Scholz, A., Plate, K. H. & Reiss, Y. Angiopoietin-2: a multifaceted cytokine that functions in both angiogenesis and inflammation. *Ann N Y Acad Sci* **1347**, 45-51, doi:10.1111/nyas.12726 (2015).

14 Norelli, M. *et al.* Monocyte-derived IL-1 and IL-6 are differentially required for cytokine-release syndrome and neurotoxicity due to CAR T cells. *Nat Med* **24**, 739-748, doi:10.1038/s41591-018-0036-4 (2018).

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