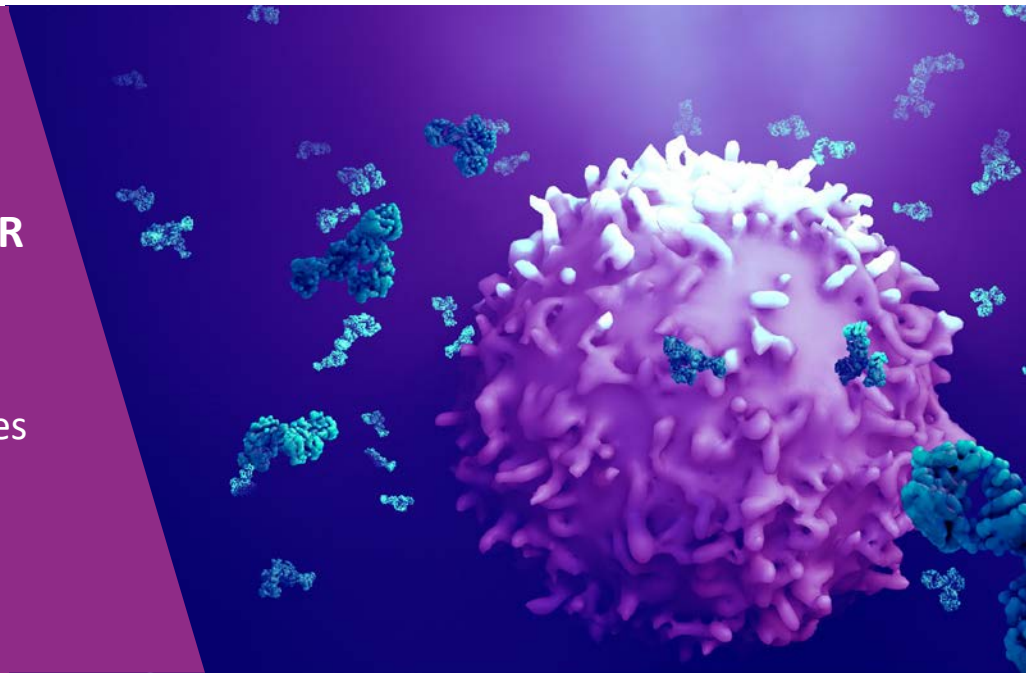
imSAVAR

**IMMUNE SAFETY AVATAR**

Nonclinical mimicking of the immune system effects of immunomodulatory therapies



# Deliverable 2.11
# 3rd iteration in vitro & MPS models

## DELIVERABLE REPORT

This project has received funding from the Innovative Medicines Initiative 2 Joint Undertaking (JU) under grant agreement No 853988.
The JU receives support from the European Union's Horizon 2020 research and innovation programme and EFPIA and JDRF INTERNATIONAL.

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## Abstract

A key objective in imSAVAR is to create a platform with novel tools, models and resources for early nonclinical prediction of possible immune-related adverse events (irAEs) of immunomodulatory therapies.

This deliverable report summarizes the progress that has been made since the last report (D2.8, 2nd iteration) regarding the refinement and development of different *in vitro* and organ-on-chip models to be used for chimeric antigen receptor (CAR) T cells and bispecific T cell engaging antibodies (BiTEs) as exemplary mode of actions (MoAs) and cytokine release syndrome (CRS) as their most frequent adverse outcome.

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# 1. Methods

*irAOP Development*

A team of different partners (UKW, Fraunhofer IZI/ITEM, NVS, BI, Labcorp, UniLu, PEI, Lund) that attended the CRS irAOP harmonization study-a-thon (see also D2.8) jointly collated the results in a manuscript. Here, already published data sets from recent CD19 CAR-T trials were used for visualisation in the CRS irAOP map hosted on the server of UniLu (see also D4.7).

A perspective manuscript on the current use of cytokine release assays (CRA) for immunotherapeutics in the context of our CRS irAOP was jointly prepared by partners from Labcorp, BI, NVS, Merck, UKW, Fraunhofer ITEM and UniLu.

*Experimental Campaign with bispecific T cell engagers*

As outlined in D2.8 a detailed study plan was established for testing bispecific T cell engagers in different cytokine release assay (CRA) platforms with the aim to identify differences between donors who are high or low responders following treatment. In addition, partner UKT will also test these tool bispecific T cell engagers in a newly developed lymphoid-tissue-on-chip.

*Experimental Campaign with CAR-Ts*

CAR-T cells generated from n=3 healthy donors (see also D2.3, D2.5 and D2.8) were tested in different 2D/3D *in vitro* models across the consortium. Models were further refined, e.g. by incorporation of primary tumor samples from cancer patients.

*Correlation to "real world" data sets*

Besides identifying improved molecular biomarkers for CRS in CAR-T patients, we are also interested to identify improved biomarkers for cytopenias in this patient cohort. Cytopenias, namely prolonged neutropenia, lymphopenia, anemia and thrombocytopenia, emerged as one of the most frequent causes for non-relapse mortality over the last few years as they can lead to life-threating infectious complications in patients (see e.g. doi: 10.1007/s40264-025-01538-5). To this end, we conducted multiplex cytokine analysis in patient sera and multi-color flow cytometric analysis of peripheral blood at timepoints prior and after CAR-T infusion in patients treated with FDA/EMA approved CAR-T products (see also D2.5, D2.8 and D4.8). Further, clinical data as well as flow cytometric analysis of immune cell subsets was correlated to e.g. best response after therapy, the occurrence, duration and grade of CRS, neurotoxicity, cytopenias, infectious complications, progression-free and overall survival. In addition, clinical parameters were also used as a guide for sample selection for subsequent single cell RNA-seq analysis.

# 2. Results

*irAOP Development*

The manuscript describing the results of our CRS irAOP harmonization / CRS irAOP Map was finalized and is currently under revision at Frontiers in Immunology. In addition, partners Labcorp, BI, NVS, Merck, UKW, Fraunhofer ITEM and UniLu prepared a manuscript on the current use of CRA in the field of immunotherapeutics. This manuscript will be submitted to ALTEX.

*Experimental Campaign with bispecific T cell engagers*

A final study with the CRS harmonized AOP using one CD3-binding T cell engager targeting CD123 was initiated, with the intention to cover all key events with the previously applied CRA platforms. To estimate effects of T cell engagers in lymphoid tissue, a newly developed lymphoid-tissue-on-chip (LToC) platform (UKT) was added to the previous CRA platforms. In a pilot trial, the selected T cell engager induced a dose-dependent activation of T cells within the LToC. Data for a total of 6 donors treated at a range of 0.01 ng/mL to 10 µg/mL are currently collected and will be used with readouts on molecular initiating events (lactate dehydrogenase assay), key event 1 (T cell activation and proliferation) and key event 2 (cytokine release) (results to be part of deliverable 4.10-4.13).

*Experimental Campaign with CAR-Ts*

1) Tumor-on-Chip

A tumor-on-chip model was developed to recapitulate the efficacy aspect of CAR T cell therapy against solid tumor as well as safety, namely by mimicking part of the resulting CRS on the chip systems over one week period of perfused culture. Specifically, the model is able to incorporate ROR1 expressing tumor spheroids/organoids and a perfusable endothelium that allows CAR T cell perfusion and extravasation. To capture patient-specific effects from the patient-derived cancer organoids, the model was refined by integrating human induced pluripotent stem cell-derived endothelial cells that are sourced from the same donor as CAR T cells, thereby excluding alloreaction between T cells and endothelial cells in the system.

Following the tumor-on-chip establishment, an equal ratio of $CD4^+$ and $CD8^+$ ROR1-specific CAR T cells (or control untransduced T cells from the same donor) was labelled and perfused through the chips. Over a one week period, (CAR) T cell migration, infiltration, and cytokine release (IL-2, IL-6, IL-10, TNFα, IFN-γ, granzyme B) were monitored. The model has been utilized to test CRS intervention using dasatinib at different time points and durations during the CAR T cell treatment period. As dasatinib enabled a transient “off” state of CAR T cells during their interaction with the tumor cells, a suitable time point and duration of its application enabled the management of cytokine release to ensure safety while keeping the efficacy of CAR T cells minimally affected. Further, we found that the relative ROR1 expression on the patient-derived organoids (5 donors) positively correlates with the persistence of CAR T cells within the tumor bulk and cytokine secretion. The integration of patient-derived organoids may therefore recapitulate target-antigen heterogeneity and patient-specific response. The breast-cancer-on-chip model is now accepted as an advanced model for CAR T cell efficacy and safety testing, as exemplified by target-expression specific modelling of efficacy of ROR1-CAR-T cells provided by partner UKW, and has been recently published in Cell Stem Cell (doi: 10.1016/j.stem.2024.04.018).

Further, an enhanced breast-cancer-on-chip model incorporating CAR T cell-autologous macrophages in the tumor microenvironment was used to study macrophage contribution to the amplification and perpetuation of the inflammatory cycle that would lead to CRS. The enhanced model was further used to test impact of macrophage blockade (anti-CSF1R) in the tumor microenvironment on CAR T cell recruitment and CRS. Effluent samples from CAR T cell-treated tumor-on-chips with or without CRS intervention strategies were shipped to partner UT and used to assess the downstream effect on endothelial damage using the vessel on-chip system (see below), and to partner UKW to assess the presence of clinically-relevant soluble markers of endothelial cell activation.

In addition, new renal-cancer-on-chip entities were generated using the tumor-chip and the 786-O, CAKI-2 or RCC-4 cell line. The renal-cancer-on-chips were used to test directed CAR T cell recruitment, efficacy and safety of adapter CAR T cells (adCAR-T) as well as conventional ROR2- or CD70-specific CAR T cell formats provided by partner UKW. Data from these experiments confirmed that adCAR-T cell presence in

the tumor was highly dependent on continuous adapter-compound application and suggests improved safety using this CAR-T format. For ROR2- and CD70-specific CAR-T cells, cell line-specific recruitment and cytokine release levels were observed. Findings on adapter compound-dependent recruitment of adCAR-T cells and application of another tumor entity demonstrate the versatility and adaptiveness of the model to address efficacy and safety concerns for various immunotherapeutic approaches.

Current results were presented at national and international conferences, including the 7th European CAR T cell Meeting (February 2025), Cancer Immunotherapy (CIMT) meeting (May 2024), EUROoCS (July 2024), Leipzig Immune ONcology (LION) conference (November 2024) and the annual meeting of DGHO (Deutsche Gesellschaft für Hämatologie und Medizinische Onkologie e.V.; October 2024).

2) Vessel-on-Chip

A previously established 3D vessel-on-chip model was used to model endothelial dysfunction, after exposure to CAR T cell derived culture media obtained from the University of Tübingen. The IPSC derived endothelial cells missed crucial growth factors in this medium, causing incompatibility that could be solved by addition of these growth factors. Experiments were conducted with the optimized medium, with readouts of markers for barrier permeability and endothelial dysfunction. In addition, a multiplexed chip with 16 channels and automated medium refreshment was developed to systematically study different cytokine mixtures. The results were collated in a manuscript that will be submitted to Cell Stem Cell.

3) MIMIC® CRA platform

Test runs with n=3 CAR-T donors on the MIMIC® CRA platform were completed incl. multiplex cytokine and flow cytometric analysis as already described (see also D2.5 and D2.8). Results of this study were published as part of the imSAVAR Special Issue in the Journal of Immunotoxicology (doi: 10.1080/1547691X.2024.2378729).

4) Gut-on-Chip

The final experimental setup included short-chain fatty acids (SCFA) in high (luminal) and low (serum) level concentrations to pretreat anti-ROR1 CAR-T cells and control anti-CD19 CAR-T cells in a ROR1-positive intestinal adenocarcinoma-on-chip model. The findings demonstrate that propionate and butyrate inhibit anti ROR1 CAR-T cell function by reducing infiltration, cytotoxicity, and cytokine release while preserving junctional integrity within the tumor model. Mechanistically, these SCFAs inhibit histone deacetylase activity and promote a phenotype switch toward regulatory T cells, as indicated by increased FoxP3 and ROR$\gamma$t expression. Additionally, propionate and butyrate upregulate PD-1 and TIM-3, markers of T-cell exhaustion and immune tolerance, and induce a dose- and time-dependent reduction in proinflammatory cytokines. In contrast, acetate and pentanoate promote a proinflammatory T helper 17 phenotype. These results highlight the immunomodulatory effects of SCFAs on CAR T-cell function, emphasizing the need to consider microbiota-derived metabolites in CAR-T cell therapies.

The described results were published in Advanced Healthcare Materials (doi: 10.1002/adhm.202405003).

5) Characterization of Immune Cell Subsets / soluble factors in patients undergoing CART treatment *(Biomarker Development for CAR-Ts)*

Besides CRS and neurotoxicity cytopenias, namely neutropenia, lymphopenia, anemia and thrombocytopenia, emerged as the most common adverse effects of CAR T cell therapy that are related to non-relapse mortality. As there are currently no specific molecular biomarkers available, we conducted a multiplex cytokine analysis in n =78 patients at the UKW partner site that were treated with either an approved CD19-specific CAR-T product (axicabtagene ciloleucel) to treat diffuse large B-cell lymphoma or an approved BCMA-specific CAR-T product (decabtagene vicleucel or ciltacabtagene autoleucel) to treat multiple myeloma. As we aim at identifying predictive biomarkers, we collected sera and EDTA blood samples prior and after CAR T cell therapy. So far, we identified two biomarkers in our training cohort that are linked to worse overall survival. Validation of these biomarkers in an independent cohort as well as the results of our in-depth molecular characterization of PBMC using scRNA-seq at the Fraunhofer IZI partner site are still pending.

Current results of our biomarker analysis were presented at the annual meeting of DGHO (*Deutsche Gesellschaft für Hämatologie und Medizinische Onkologie e.V.*; October 2024), Leipzig Immune ONcology (LION) conference (November 2024), 7th European CAR T-cell meeting (Strasbourg, February 2025) and the 5th international conference on lymphocyte engineering (ICLE; Munich, February 2025).

6) BOEC Autologous Assay

$CD4^+$ and $CD8^+$ CAR-T cells were generated targeting CD19 from two donors, which were cryopreserved and shipped to Labcorp along with untransduced (UTD) and peripheral blood mononuclear cells (PBMCs) from the same donors. PBMCs were then used to generate blood outgrowth endothelial cells (BOECs), serving as an autologous endothelial cell line for use in assays. Initial development runs of a CAR-T cytokine release assay focused on cellular response to endothelial, PBMC and CAR-Ts to an anti-CD3 stimulant in the absence of target cells, to establish assay performance in comparison to using in-house, fresh donors. A heterologous system using Human Umbilical Vein Endothelial Cells (HUVECs) in place of BOECs was also run to assess any difference in cytokine response in the two approaches. This assay demonstrated higher raw cytokine responses in the heterologous system compared to the autologous system (and thus a higher assay sensitivity in the BOEC assay; see also D2.8), and recapitulated the expected levels of cytokine response to in-house assays. Subsequent runs added in $CD19^+$ Raji target cells at an effector to target ratio of 2.5:1 and 1:1, demonstrating higher cytokine response in conditions of higher target concentration in line with higher clinical cytokine response to increased tumour burden. CAR-Ts used in these assays focused on $CD8^+$ CAR-T cells, but subsequent runs added a 1:1 of $CD4^+$:$CD8^+$ CAR-Ts, running 4:1, 2:1 and 1:1 with a Raji-GFP line to visualise target killing. The assay was also transferred into a 3D model to further recapitulate the in vivo setting, utilising the MIMETAS® 2-lane OrganoPlate® vessel-on-a-chip platform to generate autologous vessels from BOECs. This work demonstrated a proportional cytokine release response to target cell concentration, comparable data between 2D and 3D models and minimal cytokine release when using untransduced T cells. Cell killing functionality was confirmed through live cell imaging of GFP-labelled target cells within the assay, where CD19-targeting CAR-Ts maintained low GFP levels in comparison to untransduced T cell conditions where Raji-GFP signal increased with time. Future work could expand that dataset to include the BOECs generated from two additional donors with available CAR-T and untransduced T cells, and including anti-IL-6 treatment tocilizumab could assess the ability to reverse cytokine release in the model, to reflect the clinical treatment of cytokine release syndrome. Including an additional vascular leakage endpoint would

also enable the assay to assess any impact to barrier integrity, a known clinical effect seen with higher levels of cytokine release.

## 3. Discussion

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

## 4. Conclusion

imSAVAR established an irAOP enabling the development and validation of improved nonclinical models that aim to enhance the characterization of CAR-T and T-cell engager associated CRS. This effort is ultimately anticipated to enhance the safety assessment of these immunotherapeutics, thus potentially accelerating patient access to immunotherapies with an enhanced therapeutic index. This irAOP strategy forms part of imSAVAR's broader efforts to enhanced nonclinical safety assessment algorithms for additional immunomodulatory therapeutic modalities (e.g. immune checkpoint inhibitors), and a range of immune-related toxicities (e.g. ICANS, cytopenias and infections).

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