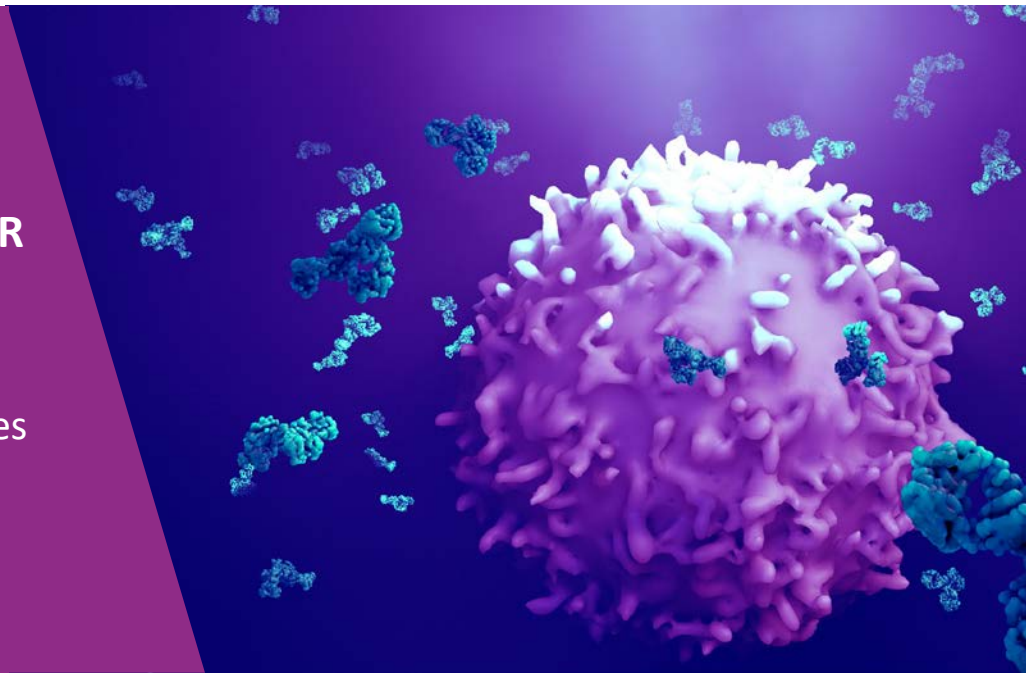
imSAVAR

IMMUNE SAFETY AVATAR

Nonclinical mimicking of the immune system effects of immunomodulatory therapies



Deliverable 4.3
2nd iteration of advanced immune cell profiling

DELIVERABLE REPORT

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Abstract

Work package 4 (WP4) focuses on the development of biomarkers for predicting the risk of observing harmful adverse outcomes in first-in-human (FIH) studies of immunomodulatory therapeutics. Current preclinical models to assess safety of immunotherapies are often species-dependent and incomplete, since they reflect only limited areas of the human immune system, which often leads to wrong predictions of human immune-related adverse events (irAEs). Hence, WP4 aims at establishing biological characteristics (biomarkers) that are measurable and evaluable and can be integrated into safety models in order to (i) assess if the model mimics the underlying human biological processes leading to an immune-related adverse outcome as closely as possible, to (ii) assess if the biomarker is reliably predicting the risk of harmful adverse outcomes in FIH studies, and to (iii) support safe starting dose selection for FIH studies.

In imSAVAR four firstly defined mode of actions (MoAs) of immunomodulatory therapeutics will be addressed and require development and confirmation of biomarkers: (i) CAR (chimeric antigen receptor) T-cells, (ii) BiTEs (bispecific T-cell engagers), (iii) CPI (checkpoint inhibitors) and (iv) IL-2. We align biomarker development with immune-related AOPs (irAOPs) to foster a common understanding of the processes triggered through a molecular initiating event and eventually leading to adverse outcomes.

With Deliverable D4.3, we describe a second version of datasets that have undergone bioinformatic processings and are useful for identifying biomarkers as well as mechanistic insights from the models being refined and developed in imSAVAR.

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1. Background

The project imSAVAR (Immune Safety Avatar: nonclinical mimicking of the immune system effects of immunomodulatory therapies) aims at creating a platform that provides novel tools, models and resources for early pre-clinical prediction of adverse events of immunomodulatory therapeutics.

Within imSAVAR this is implemented by defining immune-related adverse outcome pathways (irAOPs) to guide the development of novel test systems. Adverse Outcome Pathways describe the interconnection of a molecular initiating event (MIE) with a series of key events eventually leading to an adverse outcome. The interconnection between two subsequent key events (KEs) is defined by key event relationships (KERs).

Biomarkers as measurable and evaluable biological characteristics evaluating the MIE, the KEs or the adverse event of an irAOP. The first step towards biomarker development were systematic reviews of pre-existing data sets on human and non-human immune cell subsets to identify knowledge gaps and to guide future research directions (see Deliverable Report D4.1). In a second step specific research questions were formulated and appropriate data sets identified. Here we report the second round of datasets available and provide details on their processing state.

2. Data

The following datasets are included in the second round of biomarker development. They have been identified by systematic reviews (as described in D4.2) or have been newly generated in close cooperation with WPs 2 and 3.

Table 1: Ongoing biomarker studies in WP4.

No.	Study	Aim of study	Study design	Status of data (raw / (pre)processed)	MoA
1	Single cell RNA and bulk sequencing of in vitro stimulated PBMCs (CD3/CD28) in healthy humans and non-human primates (NHPs)	Determining baseline characteristics of CD3/CD28 engagement of T cells, cross-species comparison	Isolation of PBMCs of healthy donors (human, NHP), incubation with CD3/CD28 and subsequent single cell sequencing of RNA	Single cell RNA sequencing: preprocessed Bulk RNA sequencing: preprocessed	Baseline Immune Cell Profiles
2	ROCHE Immune Cell Atlas	Baseline characteristics of immune cells (bulk RNA sequencing)	Characterize immune cells of healthy H. sapiens, M. fascicularis, and M. musculus	Processed according to FAIR principles	Baseline Immune Cell Profiles
3	Patient Research Study	Identify biomarkers for CRS	Collect PBMCs of patients receiving FDA/EMA-approved	Flow cytometry data: raw/processed	CAR T cells

		development in CAR T cell patients	CAR T cell products, characterize cytokine/chemokine profile, immune cell composition, transcriptome on single cell level	Cytokine data: raw/to be generated (in part already processed) scRNA seq data: to be generated	
4	Single cell CAR T Cell atlas	Develop a specialized single-cell atlas for CAR T-cells with a large number of samples to distinguish the spectrum of transcriptional states that may explain the irAOPs.	Re-analysis of publicly available single-cell data including infusion products as well as pre- and post-infusional samples	Gene expression and clinical metadata were processed and harmonized across studies	CAR T cells
5	In vitro assay to study vascular leakage	Identification of potential novel biomarkers to better predict/manage vascular leakage e.g. after IL-2 therapy.	HUVECs are treated with 18 leakage-associated stimuli; Regulation of 5 surface markers, cellular vitality, and 11 cytokines as well as permeability assessed	Time resolved raw data on surface markers, cell viability, cytokine levels and leakage for multiple stimuli and unstimulated controls	IL-2
6	Re-analysis of Deng et al., 2020	Adaptation and optimization of the oposSOM data portraying analysis workflow for single-cell data	Modularization of gene expression patterns in infusion product T cells and characterization of cellular subpopulations using single-cell oposSOM	Original data publication by Deng et al. 2020 (PMID: 33020644). Processed meta-cell data and analysis results were integrated into the oposSOM-browser and published in PMID: 36248848	CAR T cells
7	TCE CRA Assessment	Identification of potential differences in healthy donor baseline phenotypes that correlate with high	Baseline phenotype characterization will include hematology, clinical chemistry, flow cytometry assessment of immune cell subsets	Flow cytometry data: raw/processed	BiTEs

		or low responses in four different in vitro cytokine release assays	and scRNA-Seq of high and low responding donors		

3. Results

In the following we provide an overview of the current results for each of the individual studies listed in Table 1.

3.1 Study 1 – Single cell RNA and bulk sequencing of in vitro stimulated PBMCs (CD3/CD28) in healthy humans and non-human primates (NHPs)

Within the NHP working group we used this study to search for specific gene signatures for each cell types, primarily immune cell types, for the purpose of development of a validated reliable cell-decomposition method for non-human primates models, which would fill the currently existing niche. Deconvolution methods for NHP models will support the assessment of cell type subset changes based of different conditions. The single-cell data for non-human primates were preprocessed and clustered using the Seurat R package. Based on the highly expressed marker genes for each of the identified cell clusters, the clusters were annotated with a particular celltype. The identity of cells was also confirmed using the cell signatures for NHP data generated from the Roche Immune Cell Atlas and from the publicly available Non-Human Primate Cell Atlas, NHPCA (https://db.cngb.org/nhpca/). The identified phenotypes serve to the improvement of the reference cell-type signature, which is essentially needed for the development of the deconvolution methods.

3.2 Study 2 – Roche Immune Cell Atlas

The NHP working group used the RNAseq data for non-human primates from the Roche Immune Cell Atlas to create preliminary gene signatures for available immune cell types and evaluated its enrichment in the bulk RNAseq datasets (of the Roche Immune Cell Atlas and a the publicly available NHPCA atlas) in order to validate their specificity and accuracy. We also used the Roche Immune Cell Atlas to compare the cell type signatures of the human (14 different immune cell types) and NHP (9 different immune cell types) data for the intersecting cell types to highlight the differences in the number and type of the marker genes.

The dataset has been processed according to FAIR principles in collaboration with FAIRPlus. It is now available from https://datacatalog.elixir-luxembourg.org/develop/e/study/f85998b0-7479-11ed-a0d3-acde48001122 .

3.3 Study 3 – Patient Research Study

With this study we aim to identify biomarkers that discriminate patients that will develop high grade CRS (grade 3-4) after CAR T cell infusion from those that will present with lower grades of CRS (grade 1-2). These biomarkers will eventually be transferred to the in vitro models developed in imSAVAR and will allow for a more reliable prediction of CRS. To this end, we collect peripheral blood samples of patients receiving FDA/EMA-approved CAR T cell therapies at the University Hospitals Würzburg and Leipzig, twice before and twice after CAR T cell infusion. Additionally, extensive clinical data sets of these patients are collected. Immune cell composition of the fresh sample as well as CAR positive T cells are analyzed using

flow cytometry. The patients' sera and their peripheral blood mononuclear cells (PBMCs) are retrieved to determine cytokine levels and conduct single cell transcriptome sequencing using next generation sequencing (NGS), respectively.

The flow cytometry data and patient clinical data from the two centers have been processed and correlated. The data show a high inter-patient variability in immune cell composition and CAR T cell expansion after infusion. As patients that present with CRS grade 1 or 2 are treated with dexamethasone and/or tocilizumab (anti-Il-6 antibody) and thus rarely develop higher grades of CRS, we are analysing the clinical data for surrogate parameters, that indicate the patients that would have developed a higher grade CRS (3 or 4) if not treated with the above mentioned medication. The data analyses done so far will be the basis to determine the patients to include in the NGS measurements.

3.4 Study 4 – CAR T cell atlas

The rationale of this study is to elucidate the functional fidelity and activity of CAR T-cells with the aim of defining therapeutically relevant T-cells for safety assessment. By developing a specialized single-cell atlas for CAR T-cells, a level of resolution can be achieved that is required to distinguish the spectrum of transcriptional states that may explain the irAOPs. Together with other modalities such as single-cell TCR sequencing to characterize the fate of T-cells in response to therapy, we aim to develop a unified and reliable CAR T cell reference atlas.

Publicly available single-cell data from five studies (PMID: 33020644, PMID: 36097223, PMID: 36097221, PMID: 31924795, PMID: 35792801) were downloaded. After quality control, we re-analyzed single-cell sequencing data from approximately 200 samples of CAR T-cells, including infusion products as well as pre- and post-infusional samples, obtained from PBMCs of 90 patients with Non-Hodgkin's lymphoma or acute lymphocytic leukemia. After filtering for CAR+ T-Cells approximately 400,000 cells were used for downstream analysis. T-Cell subtypes were annotated using CD4 and CD8 single-cell atlases using transfer learning approaches. Clinicopathological metadata were harmonized, and a first version of an integrated transcriptomic single-cell reference atlas was developed. In addition, a first version of a web application was developed, that allows the user to analyze the CAR atlas by visualizing cell information and gene expression in reduced dimensions. In addition, gene co-expression can be analyzed, and expression patterns of multiple genes can be visualized by grouped categorical cell annotations (e.g., cell type, response to treatment, time point after treatment). For functional interpretation, the user can analyse gene signatures that describe coordinated variation between cells and capture transcriptional differences between different conditions of interest.

3.5 Study 5 – In vitro assay to study vascular leakage

Rationale of this study is the identification of potential biomarkers or biomarker combinations, which can be statistically correlated to increased endothelial permeability and hence vascular leakage, a severe adverse outcome during e.g. immunotherapy. In this study, HUVECs are treated with 18 leakage-associated stimuli. We analyzed regulation of 5 endothelial-characteristic surface markers (ICAM-1, VCAM-1, E-selectin, JAM-3, and VE-cadherin), cellular vitality, and 11 different cytokines. Furthermore, changes in HUVEC-permeability upon treatment with these stimuli were analyzed using an in vitro vascular leakage assay, we established in the course of imSAVAR. The data was shared with WP4 and processed as follows: Datasets were analyzed for quality and outliers were identified and reviewed. Surface markers, cytokines, cell survival and leakage were compared between stimulated cells and unstimulated controls at different time-points. Measured levels were compared descriptively and by U-tests. In addition, association of surface markers, cytokines and endpoints were analyzed by Spearman correlation and respective correlation tests. With the current results, we could identify/confirm potent

HUVEC-activating compounds (e.g. IL-1β, TNF-α, thrombin, and LPS). This activation is characterized by upregulation of ICAM-1, VCAM-1, and E-selectin and is associated with decreased vitality. Certain stimuli were able to induce vascular leakage (e.g. thrombin and VEGF).

3.6 Study 6 – Re-analysis of Deng et al.

Rationale of this study was to extend the bulk data-focused analysis framework 'oposSOM' (PMID: 26063839) with focus on challenges of single-cell analyses and on specifics of immune-related liquid biopsies, and to apply this novel methodology to single-cell transcriptome data of CAR T cell therapy infusion products to extract patterns of transcriptional activity and cellular subpopulations. Therefore, we adapted already implemented routines for processing the large-scale input of single-cell data sets, and extended functionalities especially by down- and upscaling procedures and visualization options.

The method was applied to data previously published by Deng et al. in 2020 (PMID: 33020644), comprising single-cell RNA sequencing data of 24 patients comprising more than 130,000 cells. Pre-processed data was obtained from Gene Expression Omnibus and processed using the novel single-cell oposSOM software. We demonstrated capabilities of our approach to disentangle transcriptional states using intuitive visualization, functional mining, molecular cell stratification, and variability analyses. Our analysis revealed that the T cell composition of the patient's infusion product as well as the spectrum of their transcriptional states of cells derived from patients with low ICANS grade do not markedly differ from those of cells from high ICANS patients, while the relative abundancies, particularly that of cycling cells, of LAG3-mediated exhaustion and of CAR positive cells, vary.

The results were published in Frontier Immunology Loeffler-Wirth et al. in 2020 (PMID: 36248848), and meta-cell data and analysis results uploaded to oposSOM-Browser, an interactive data and analysis browsing tool (PMID: 33076824).

3.7 Study 7 – TCE CRA Assessment

Rationale of this study is to conduct a cross-consortium study using four different in vitro cytokine release assay formats to identify potential differences in donor phenotypes that correlate with high or low responses in the in vitro assays.

Cell assay systems involve: WB, endothelial cell co-cultured with PBMCs or RBC depleted WB consisting of heterologous (HUVEC/EA.hy926) and autologous (Blood Outgrowth Endothelial Cell) platforms.

Generation of Blood Outgrowth Endothelial Cells (BOEC) involves culturing of donor PMBCs for several weeks until BOEC colonies start to form. Colonies are expanded before cryopreservation. The BOEC assay then uses these cryopreserved cells and recalls the same donor in order to remove patient mismatch by using the same donor cells and PBMCs during stimulations. This has shown increased assay sensitivity with regards to cytokine release serum.

A pilot study has been completed to assess and optimise the methods that will be used to generate data in the main study that will undergo biomarker detection.

4. Summary

In the second iteration of advanced immune cell profiling, seven datasets, either generated by imSAVAR partners or publicly available, have been (re-)analysed or prepared for later analysis. These comprise either baseline immune cell data that allow insights in the mechanisms of the immune system, also comparing human and NHPs, or data for the MoAs BiTEs, IL-2 or CAR T cells.

5. Abbreviations

BiTE – bispecific T-cell engagers

BOEC – blood outgrowth endothelial cell

CAR – chimeric antigen receptor

CLL – chronic lymphocytic leukemia

CPIs – checkpoint inhibitors

CRA – cytokine release assay

CRS – cytokine release syndrome

HUVEC – Human Umbilical Vein Endothelial Cells

ICANS – immune effector cell associated neurotoxicity,

irAOPs – immune-related adverse outcome pathways

KE – key event

KER – key event relationships

LBCL – large B-cell lymphoma

MIE – molecular initiating event

MoA – mode of action

NGS – next generation sequencing

NHL -- non-hodgkin lymphomas

NHP – non-human primates

PBMCs – peripheral blood mononuclear cells

PCL – plasma cell leukemia

RBC – red blood cell

RNA-seq – RNA sequencing

scRNA-Seq -- single-cell RNA sequencing

TCE – Bispecific T cell engager

6. References

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