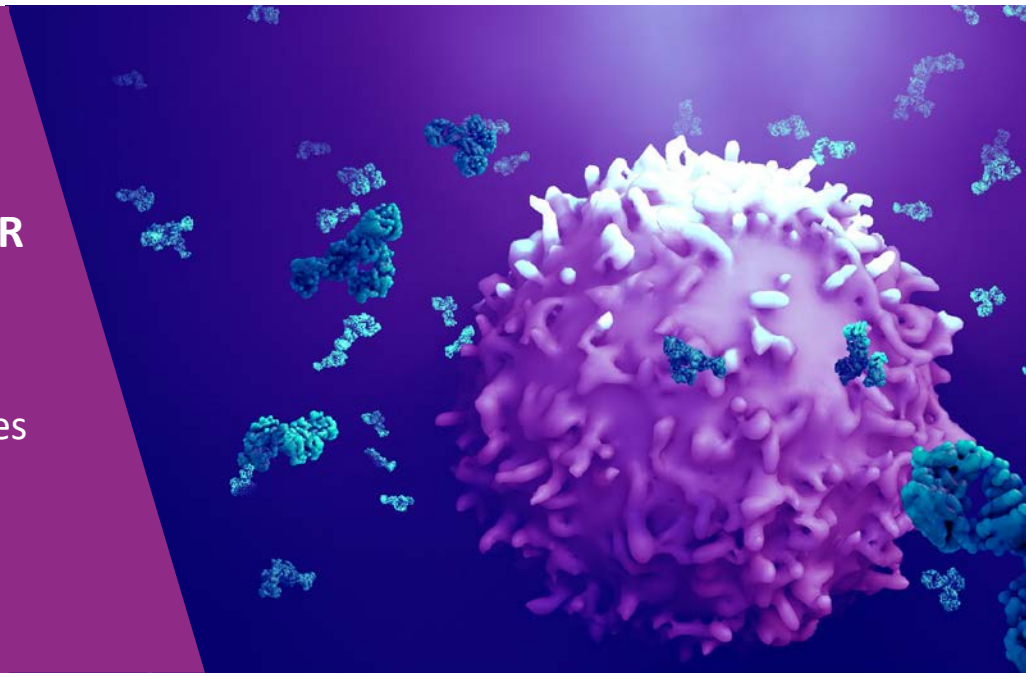
imSAVAR

IMMUNE SAFETY AVATAR

Nonclinical mimicking of the immune system effects of immunomodulatory therapies



Deliverable 3.3

1st iteration in vivo and in vitro model readouts IL-2 MoA

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

Work package (WP) 3 of imSAVAR focusses on innovative models for safety assessment of immuno-inflammatory disease therapeutics. Immunotherapy with interleukin-2 (IL-2) was chosen as a first concrete use case for the work in WP3. In a first step, a preliminary immune-related outcome pathway (irAOP) has been generated and published in deliverable D3.1. This irAOP of IL-2-mediated toxicities was used as a tool for the progress of the WP on a theoretical and practical level. The information gathered by intensive literature research was structured with the help of the irAOP. Its refinement resulted in the identification of irAOP-branches leading to organ-specific toxicities mediated by IL-2 immunotherapy. According “sub-AOPs” were used as central orientation for the development of experimental models. This provided the basis for the planned lab work analysing IL-2-mediated immunological effects on different levels of complexity and within different organs. Accordingly, the readouts chosen for the different assays were tested and evaluated for their usefulness and capability of reflecting the desired parameters in a required sensitivity and reproducibility. The models and the cooperation of imSAVAR partners initiated to work on the identified critical topics are described in the deliverable report 3.4, as the current report focuses on the corresponding readouts.

Document Information

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Report prepared by	**Fraunhofer ITEM** Paul-Ehrlich-Institute, Fraunhofer IZI, Fraunhofer ITMP, Lund University
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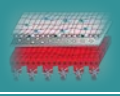
1. Methods

irAOPs as a theoretical structuring tool are the central method for the progress of the imSAVAR project. Preliminary irAOPs for IL-2-mediated toxicities were generated based on the OECD Guidance Document and published in the imSAVAR deliverable D3.1. Over the last months, we continued to structure the information gained for IL-2 immunotherapy. Intensive literature research and cooperative work of all WP3 subtask-groups (table 1) resulted in the refinement of these irAOPs (figure 1). These irAOPs served as valuable orientation for the design of experimental assays, as described in the deliverable report 3.4. The experimental assays selected, necessitate certain appropriate readouts reflecting key events identified within the irAOPs as well as biomarkers for pathological outcomes (figure 1).

Table 1: Partners of WP3 working on IL-2

WP subgroup	irAOP and team	Cellular complexity
WP 3.1/3.2	Hepatotoxicity (ITMP)	
WP 3.1/3.2	moDCs (University Lund)	
WP 3.1/3.2	Vascular leakage (PEI)	
WP 3.1/3.2	Pulmonary toxicity/Dermatotoxicity (ITEM)	
WP 3.3	Gut on a chip (Uniklinikum Jena)	
WP 3.4	*In vivo* SLE-Model (Fraunhofer IZI)	

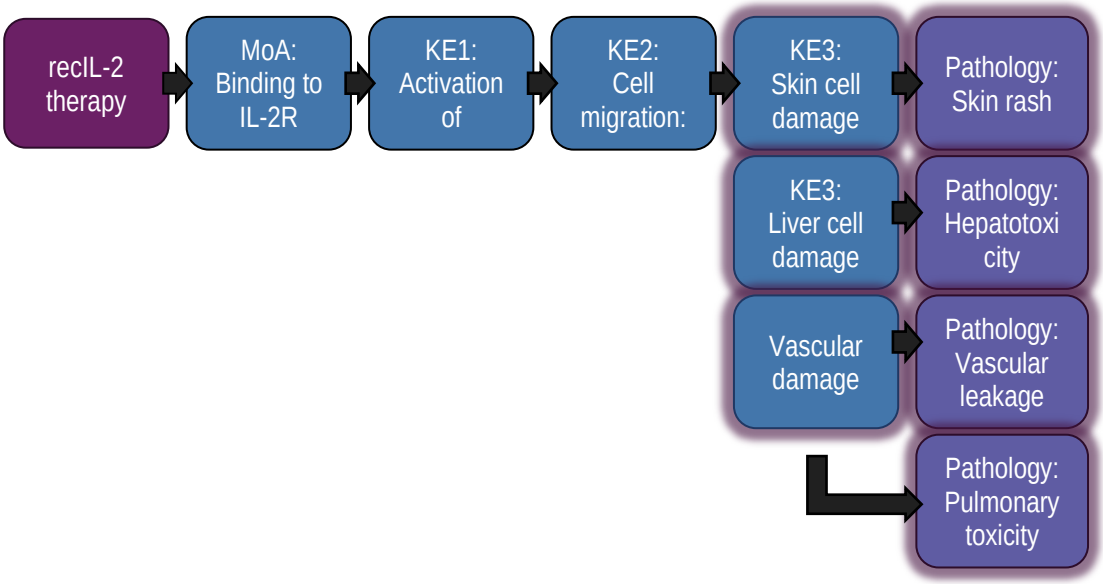


*Figure 1: **The irAOP of IL-2-mediated toxicities diverges into different branches**. The mode of action (MoA) and key events (KE) 1 and 2 are similar to initial irAOP while in KE3, branches of organ-specific damage are defined. Accordingly, different organ-specific damage are defined. Accordingly, different organ-specific model systems were chosen to approach experimental analyses of these toxicities.*

This deliverable report focuses on the developed readouts of the assay systems deviated from the irAOPs and are described as results in deliverable D3.4.

2. Results

Using the irAOPs as a tool gave rise to results on both theoretical and practical level. The theoretical structuring of the information around IL-2-mediated pathologies resulted in the identification of different organ-specific toxicological effects (figure 1). Integration of the different pathology branches will generate a complete picture regarding adverse outcomes induced by IL-2 immunotherapy. To that point, refined irAOPs for IL-2-induced skin rash, hepatotoxicity, and vascular leakage were developed, as described in D3.4.

Practically, the refined irAOPs were used for the identification of potential assay systems (see imSAVAR deliverable D3.1). *In vitro* models to analyse the mode of action (MoA) and toxicities of low and high dose recombinant IL-2 comprise organ-specific immunologic cell cultures (see D3.4). The experimental models and readouts used to analyse IL-2-mediated toxicities are summarized in table 2.

For the *in vitro* analysis of IL-2-mediated dermal toxicities as described in D3.4, the readouts chosen in the first phase of assay development included flow cytometric analysis (FACS) for the detection of specific cell surface marker. In addition, histological assessment of changes in skin structure will be performed. Furthermore, cytokine levels and beta-hexaminidase assay (especially for mast cell degranulation) were selected. As a quality criterion to ensure and provide a stable model system, cell vitality is measured routinely for each experimental run.

IL-2-mediated lung-toxicities were investigated using cultivated human precision-cut lung slices (PCLS) as an *ex vivo* lung-model. As for the readout parameters in the assay development, a flow cytometric analysis of cell surface markers was used to verify the presence of the irAOP identified target cells within the

primary tissue. Quality was checked by different established vitality assays. These vitality assays were also used for the analysis of drug-induced cytotoxic effects. To assess immunotoxicity, cytokine levels, changes in cell populations within the PCLS, and surface markers were determined by multiplex Enzyme-linked Immunosorbent Assay (ELISA), FACS, and microscopy.

The IL-2-induced hepatotoxic effects are analysed within single and co-cultures. FACS was also used to verify the presence of IL-2 receptors on target cells identified in the irAOP. Various vitality assays were used to assess drug-induced cytotoxic effects. Drug-induced effects on hepatocyte function was addressed by functional assays. Moreover, changes of the cellular composition and cytokine patterns were determined by FACS and ELISA.

IL-2-induced vascular leakage is measured by flux of the tracer molecule FITC-albumin across an endothelial cell layer in a trans-well based *in vitro* assay. For the analysis of IL-2-mediated activation of cells used in the vascular leakage assay, FACS is also used as a central read-out endpoint. So far, IL-2 receptor expression, cell surface marker expression, and vitality were analysed by FACS. Cytokine levels will be measured by using bead arrays and/or ELISA.

As illustrated in the preliminary irAOP (figure 1), a connection of IL-2-induced vascular leakage and pulmonary toxicity has been identified. Concerted cross-cutting experiments combining PCLS and vascular leakage assay are currently discussed and the above mentioned established readouts for each separate assays will be transferred to this combined approach.

Additionally, the role of distinct immune cells (i.e. dendritic cells, monocyte-derived dendritic cells, or *in vitro*-differentiated Langerhans cells) during IL-2 therapy will be analysed (LUND).

Currently, planned *in vivo* studies will analyse IL-2-mediated skin toxicities on the one side. On the other side, the therapeutic efficacy and safety of long-term low-dose IL-2 will be investigated within subtask 3.4 with a murine model of induced systemic lupus erythematosus (SLE). Besides optimal IL-2 dose, parameters such as (auto-) antibodies, tumor necrosis factor (TNF), immune cell analysis (ratio of regulatory and effector T cells) and viral load in lungs will be determined.

Table 2: Methods conducted by the different partners

Partner	Pathology	Read outs
Fraunhofer ITEM	Skin rash/pulmonary toxicity	• Extrinsic cytokine levels/patterns (multiplex ELISA) • Surface marker expression (flow cytometry/Microscopy) • Vitality (LDH) • Degranulation (Beta-Hexaminidase assay/flow cytometry)
Fraunhofer ITMP	Hepatotoxicity	• Surface marker expression (flow cytometry) • Cytokine secretion (flow cytometry) • Liver inflammation and functional marker (ELISA) • Vitality (LDH) • Functional assay (Bilirubin glucoronidation/LC MS/MS analysis; MRP2 assay)

Paul-Ehrlich-Institut	Vascular leakage	• Activation marker-expression and vitality of HUVECs and PBMCs (flow cytometry) • Cytokine secretion (Bead arrays, ELISA) • Vascular leakage (FITC-albumin flux across a HUVEC-monolayer)
University Lund	Diverse	• Flow cytometry (FACS) • Intrinsic and Extrinsic cytokine levels (ELISA) • Cellular composition (FACS/Microscopy) • Vitality
University Jena*	Gut toxicity	• Flow cytometry (FACS) • Cellular patterns (FACS/Microscopy) • Vitality • Transepithelial resistance (TEER)
Fraunhofer IZI	Lupus and infections	• (auto-) antibodies (via ELISA) • Tumor necrosis factor (TNF) • Immune cell analysis (ratio of regulatory and effector T cells, via FACS) • Viral load in lungs (via RT qPCR)

3. Discussion

The different assay systems and their corresponding readouts described briefly in this deliverable and in deliverable report D3.4 are currently under further establishment. Furthermore, they are being used to generate original data, which are intended to be published in peer review journals. After publication, these data will be incorporated in the following iteration deliverables. Additionally, these data will be the basis for further refinement of the generated irAOPs.

The used readouts for the developed assays are well-established in the field of cell biology and standardly applied for in vitro experiments. Also within our WP, they reliably showed the expression of the targeted surface receptors as in our first case study with IL-2: the different subsets of the IL-2 receptor. Moreover, the readouts were sensitive enough to distinguish between different levels of expression as well as between the different receptor subtypes. Additionally, other surface markers to identify and characterize variations in the composition of cellular populations due to IL-2 treatment were established. All in all, the first readouts were established and used in a – for assay development – robust manner. For further assay development, the readouts might need to be adapted, optimized or complemented by additional readouts, which will then be discussed in the second iteration of "in vivo and in vitro model readouts IL-2 MoA".

Experimental setups were designed to fill the "black boxes" identified using the irAOP for knowledge structuring. Resulting data will be applied to fill these knowledge gaps, the development of suitable pre-clinical safety assessment models, and/or biomarker identification. In addition, the gained insights will be used for the optimization of existing assay systems. Taken together, the usage of irAOPs is a suitable tool

in the scope of imSAVAR. Structuring the theoretical background helped to focus the available expertise of the different partners on the relevant knowledge gaps. The data from experimental work as well as the theoretical background from the irAOPs within the reviews will be an important orientation for further concrete subprojects, such as the development of the irAOP disease maps (D4.5).

4. Abbreviations

HUVEC	Human umbilical vein endothelial cells
IL-2	Interleukin-2
irAOP	Immune related adverse outcome pathways
MoA	Mode of action
NK cells	Natural killer cells
OECD	Organisation for Economic Co-operation and Development
PBMC	Peripheral blood mononuclear cell
SLE	Systemic lupus erythematosus
Tregs	Regulatory T cells
FACS	Flow cytometry analysis
ELISA	Enzyme-linked Immunosorbent Assay
LDH	Lactate dehydrogenase assay
WST-1	WST-1 Cell Proliferation band viability assay

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