



Deliverable 2.13

Evaluation of CPI combination therapy in Humanized Immune System (huNOG-EXL) Mice & 3rd iteration of strategies and experimental in vivo and in vitro immune-competent target organ models to study adverse events of CPI

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.











Abstract

Checkpoint inhibitor and small molecule combination therapies pose a particular challenge when serious adverse events (SAE) occur at higher incidence or in unexpected target organs compared to monotherapy (NEJMc1302338.pdf). The aim of this study was to compare the response of humanized immune system mice, huNOG-EXL, (NOD.Cg-Prkdcscid Il2rgtm1Sug Tg (SV40/HTLV-IL3, CSF2)10-7Jic/JicTac) reconstituted with hematopoietic stem cells (HSC) from three different donors to monotherapy with ipilimumab, palivizumab or combination therapy of ipilimumab with vemurafenib. Study endpoints consisted of recording of observations, body weight loss, survival rates and activity scores. Additionally, blood and tissue samples were collected for histology and clinical chemistry.

In addition, a series of organ-specific co-culture and triple-culture models were established to study hepatotoxicity and cytokine release syndrome (CRS), incorporating primary immune cells, hepatic cell lines, and endothelial components. Additionally, a precision-cut tissue slices (PCTS) model was developed using gingival tumor tissue to closely replicate the tumor microenvironment. The PCTS platform integrates autologous immune cells and enables multiomics analyses to assess immunotherapy-induced molecular and cellular changes. A range of CPIs, including anti-PD-1, anti-PD-L1, anti-CTLA4, and anti-GD2 antibodies, were evaluated for their effects under both physiological and disease-mimicking conditions. The models provide a robust, human-relevant system for characterizing immunotherapy responses, predicting adverse effects and reducing dependence on animal models in early-stage drug development.



Document Information

Deliverable Report	D2.13: Evaluation of CPI combination therapy in Humanized Immune System (huNOG-EXL) Mice & 3rd iteration of strategies and experimental in vivo and in vitro immune-competent target organ models to study adverse events of CPI
Date	12.06.2025
Report prepared by	MSD Fraunhofer ITMP Lund University
Project	imSAVAR - Immune Safety Avatar: nonclinical mimicking of the immune system effects of immunomodulatory therapies Grant Agreement No.: 853988 (IMI2-2018-15-04)
Project Coordinator	Fraunhofer-Gesellschaft zur Foerderung der angewandten Forschung e.V. Prof. Dr. Dr. Ulrike Köhl Dr. Kristin Reiche Novartis Pharma AG Dr. Jonathan Moggs
	Hannah Morgan, PhD
Туре	Deliverable Report Public

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.





Table of Contents

Al	ostract .						
Al	obrevia	tions	5				
1.	Met	Aethods6					
	1.1	CPI	combination therapy in huNOG-EXL mice6				
	1.2 events	Expo of C	erimental in vivo and in vitro immune-competent target organ models to study adverse Pl				
2.	Resu	ılts					
	2.1	CPI	combination therapy in huNOG-EXL mice10				
	2.1.1	1	Overall Survival				
	2.1.2	2	Body Weights				
	2.1.3	3	Observations and Activity Scores11				
	2.1.4	1	Histomorphologic Assessment				
	2.2 events	Expo of C	erimental in vivo and in vitro immune-competent target organ models to study adverse PI13				
3.	Disc	ussic	on14				
	3.1	CPI	combination therapy in huNOG-EXL mice14				
	3.2 events	Expo of C	erimental in vivo and in vitro immune-competent target organ models to study adverse PI14				
4.	Cond	clusio	on15				
	4.1	CPI	combination therapy in huNOG-EXL mice15				
	4.2 events	Expo of C	erimental in vivo and in vitro immune-competent target organ models to study adverse PI15				
Re	eferenc	es					
A	cknowle	edgei	ment17				



Abbreviations

BOECs	blood outgrowth endothelial cells
CD	cluster of differentiation
CRS	cytokine release syndrome
CPI	immune checkpoint inhibitor
CTLA4	cytotoxic T-lymphocyte-associated Protein 4
GD2	ganglioside 2
GM-CSF	granulocyte macrophage colony stimulating factor
lgG	immunoglobulin G
IL	interleukin
irAOP	immune-related adverse outcome pathway
MoA	mode of action
NK cells	natural killer cells
PD-1	programmed cell death protein 1
PD-L1	programmed death-ligand 1
OoC	organ-on-a-chip
PBMCs	peripheral blood mononuclear cells
PCTS	precision cut tissue slides



1. Methods

1.1 CPI combination therapy in huNOG-EXL mice

Prior to study start mice were engrafted with human CD4⁺ HSC from Donor 1, 2 and 3 at the breeder site (Taconic, Denmark). On Day 0 of the experiment six huNOG-EXL mice each were randomized into 9 treatment groups based on their body weights resulting in 3 different therapeutic groups per Donor.

Treatment was initiated on Day 1 and administered as follows: palivizumab in Groups 1-3, ipilimumab in Groups 4-7 and combination therapy of ipilimumab and vemurafenib in Groups 8-9 (Table 1). All antibody therapies were administered intraperitoneally (i.p.) at a dose of 7.5 mg/kg on Days 1 and 8 of the study. While therapies with vemurafenib were supplied twice daily via oral gavage (p.o.) with a 5-minute interval between dose administrations at a total daily dose of 200 mg/kg. Control and vehicle for vemurafenib was 2% hydroxypropyl cellulose (pH 4) and vehicle for palivizumab and ipilimumab was saline.

Group ID	Test Article	Donor	Dose Level [mg/kg/day]	Schedule* [Dosing days]	Route	No. of Animals
1	Palivizumab// Control Vehicle	Donor 1	7.5// 10	1,8// 1-9	ip// po	6
2	Palivizumab// Control Vehicle	Donor 2	7.5// 10	1,8// 1-9	ip// po	6
3	Palivizumab// Control Vehicle	Donor 3	7.5// 10	1,8// 1-9	ip// po	6
4	Ipilimumab// Control Vehicle	Donor 1	7.5// 10	1,8// 1-9	ip// po	6
5	Ipilimumab// Control Vehicle	Donor 2	7.5// 10	1,8// 1-9	ip// po	6
6	Ipilimumab// Control Vehicle	Donor 3	7.5// 10	1,8// 1-9	ip// po	6
7	Ipilimumab// Vemurafenib MRK- PA	Donor 1	7.5// 2×100	1,8// 1-9 (h:0+0:05)	ip// po	6
8	Ipilimumab// Vemurafenib MRK - PA	Donor 2	7.5// 2×100	1,8// 1-9 (h:0+0:05)	ip// po	6
9	Ipilimumab// Vemurafenib MRK- PA	Donor 3	7.5// 2×100	1,8// 1-9 (h:0+0:05)	ip// po	6

Table 1: Design of Experiment

To monitor animal wellbeing, mice were weighed daily and scored for activity. Animals were weighed daily, and relative body weights of individual animals were calculated by dividing the individual body weight on Day x (BW_x) by the individual body weight on the day of randomization (BW₀) multiplied by 100.

According to animal welfare regulations and the relevant SOP of Charles River Laboratories Germany, the following humane endpoints apply to individual animals, irrespective of the experimental status:

- Body weight loss > 30% on any measuring day
- Continued body weight loss > 20% for more than two days
- Rapid recorded decrease in body weight > 20% within two days



• Severe impairment of general condition (apathy, pain, markedly reduced feed and water intake, dyspnea, abnormal habitus or behavior)

Animal activity was examined once daily and categorized according to the scoring system below (used in terms of euthanasia criteria and for evaluation purposes). Animals with a score =3 for more than two days (i.e. three consecutive examinations) or mice with a score <3 for one day were euthanized. Only scores <5 are documented. In addition, animals showing an AS = 3 on a Friday were sacrificed to enable sample collection before the weekend.

Activity Score	Activity and Behavior	Measure(s)
5	Normal activity and behavior, normal movement in the cage including standing on hind legs, normal species-specific curiosity	Condition accepted
4	Less activity, less frequent standing on hind legs, stays mostly in the corners of the cage	Condition accepted but daily scoring documented from this point
3	Low activity level, discontinuous movements with pauses, mouse stays mostly in the corners of the cage	Repeated examination, if necessary, interruption of experimental therapy. Euthanasia if score 3 persists for more than two days.
2	Reluctance to move, short strides only upon touch, mouse stays mostly in the corners of the cage	Euthanasia if condition persists for one day
1	Moribund, apathy, neurological symptoms (e.g. unilateral paralysis)	Immediate euthanasia

On Day 9 of the study and upon animals reaching individual or experimental euthanasia criteria, samples were collected as laid out in Table 2.



Table 2: Sampling Schedule

Group ID	No. of Animals to be Sampled	Type of Sample, Fixation	Sampling Day	Sample Amount ²
1.0	2	Retrobulbar blood	Terminal Day 9	mou amount divided in 2 viola
1-9	4	$(100\mu l)^*$ and $-20^{\circ}C^{**}$	Terminal ¹	max amount divided in 2 viais
1-9	2	Colon	Terminal Day 9	Whole organ from caecum
	4	OCT/FFPE/ F	Terminal ¹	(divided in 3 sections)
1-9	2	Jejunum	Terminal Day 9	Whole organ
	4	OCT/FFPE/SF	Terminal ¹	(divided in 3 sections)
1-9	2	Liver OCT (×1)/SF (×1)	Terminal Day 9	Whole organ
	4	FFPE (×2) with and without gall bladder	Terminal ¹	(divided in four sections)
1-9	2	Spleen	Terminal Day 9	Whole organ
	4	OCT/FFPE/SF	Terminal ¹	(divided in three sections)
1-9	2	Lung FFPE (right lobe)/ SF (left lobe)	Terminal Day 9	Whole ergen (helved)
	4		Terminal ¹	whole organ (naived)

1.2 Experimental in vivo and in vitro immune-competent target organ models to study adverse events of CPI

Established *in vitro* models were used to analyze the mechanism of action (MoA) and toxicities of CPIs. These models consist of organ-specific immunologic cell cultures. For studying CPI-mediated hepatotoxicity, different types of models were generated and used, including mono-cultures, co-cultures, and triple-cultures of primary immune cells (e.g. CD8⁺ T cells, macrophages), hepatic cell lines (HepaRGs), or primary hepatocytes. Avelumab (anti-PD-L1), atezolizumab (anti-PD-L1), nivolumab (anti-PD1) and ipiliumab (anti-CTLA4) were used as test biologicals. As control, unspecific IgG antibody was applied. Primary human immune cells were screened for PD-1 and PD-L1 expression, and the effects of CPIs on various immune cells were investigated, including surface marker expression, cytotoxicity, cytokine and cytotoxic marker (e.g. granzyme B, perforin) release. CD8⁺ T cells were selected as the most interesting immune cell type for studying the immune modulatory effects of anti-PD-L1, anti-PD1 and anti-CTLA4 biologicals. Additionally, the effects of CPIs are being explored in a triple-culture model consisting of CD8⁺ T cells, macrophages, and hepatocytes. Moreover, a multicellular-culture model consisting of PBMCs and hepatocytes was established. Furthermore, to study long-term effects, we initiated the establishment of hepatic spheroids. These spheroids can be cultured for extended periods, allowing us to test long-term treatments with CPIs.

The significance of co-medication was assessed by pre-treating hepatocytes with drugs known to induce hepatotoxicity, such as acetaminophen and statins. To mimic a diseased state, hepatocytes were treated with an inflammatory cytokine, and CD8⁺ T cells were pre-activated. Notably, the cytokine treatment of hepatocytes resulted in an increased expression of PD-L1. These *in vitro* methods will be supplemented with microphysiological Organ-on-Chip (OoC) systems. For the analysis of hepatotoxicity, we will collaborate with Dynamic42 for complementary assessments using a Liver-on-Chip model.



In D2.10 we reported the use of an autologous co-culture system, comprised of PBMCs, serum and blood outgrowth endothelial cells (BOECs) isolated from tonsillar cancer donors' blood before and after they were treated in the clinic. This co-culture system aims to mimic CRS pathology, following stimulation with nivolumab *ex vivo*. To date we have continued to collect supernatants from these co-cultures of patients' cells (n=6).

A novel ex vivo model utilizing precision-cut tissue slices (PCTS) has been established, offering a promising approach for replicating the tumor microenvironment in three dimensions. In this method, freshly resected tumor specimens are finely sectioned (typically 100–400 µm thick) and cultured under ex vivo conditions. This advanced platform preserves the native tissue architecture and cellular diversity, enabling precise investigations of immune cell behavior and interactions within a physiologically relevant context. PCTS is especially valuable for studying immune-tumor dynamics using limited biopsy material, providing high-resolution insights into the immune landscape. By incorporating autologous immune elements, the model facilitates exploration of mechanisms driving immunotherapy response and toxicity and helps identify predictive immune signatures. Moreover, PCTS serves as a useful tool for early-stage evaluation of novel immunomodulatory agents, reducing reliance on animal testing. This model has been set up using gingival cancer tissue; gingival cancer is a relatively rare subtype of head and neck cancer originating in the gum. However, this model will also be established using tonsil tissue, in order to expand its applicability to more prevalent forms of head and neck cancer, enhance comparative analysis across tumor subtypes, and further investigate site-specific immune interactions within the tumor microenvironment. Using these models, the effects of different immunotherapies, including CPIs will be assessed. In parallel, multiomics approaches, particularly transcriptomic profiling, will be employed to elucidate molecular changes induced by CPI treatment, shedding light on the pathways and immune signatures associated with therapeutic response or resistance.

The experimental *in vitro* and *ex vivo* models used to analyse CPI-mediated toxicities are summarized in Table 3.

Partner	Pathology	Methods/Model
Fraunhofer ITMP	Hepatotoxicity	Co-cultures of CD8 ⁺ T cells and hepatic cell line (HepaRG) to determine hepatotoxicity, surface marker expression, cytokine release, cytotoxic marker release and function of hepatocytes.
		Triple-co-cultures of hepatic cell line (HepaRG), primary
		Human CD8 ⁺ T cells and macrophages to determine hepatotoxicity, surface marker expression on hepatocytes, cytokine release, cytotoxic marker release and function of hepatocytes. The optimal time point for culturing CD8 ⁺ T cells was determined to prevent T cell exhaustion.

Table 3: Experimental in vitro and ex vivo methods and models conducted by the different partners.



		 Starting to establish a model consisting of primary human immune cells and HepaRG spheroids
Lund University	CRS	Co-cultures of autologous PBMCs, serum and blood outgrowth endothelial cells (BOECs), stimulated with nivolumab and control biologics, known to induce CRS. Collection of supernatants after different timepoints and multiplex analysis for a panel of cytokines.
		Ex vivo 3D PCTS model of gingival and tonsillar cancers incorporating autologous immune cells to study immune- tumor interactions, CPI efficacy/toxicity, and treatment- induced molecular changes via multiomics. Enables early screening of immunomodulatory agents.

2. Results

2.1 CPI combination therapy in huNOG-EXL mice

2.1.1 Overall Survival

Overall, the survival rate was reduced in groups with Donor 3 humanized animals in comparison to Donor 1 and 2 humanized animals receiving same treatments. This finding highlights a potential dominance of a donor effect over effects due to group specific treatments (Figure 1).



Figure 1: Overall Survival



2.1.2 Body Weights

On Day 4 of the study bodyweight loss was significantly higher in animals receiving combination treatment in comparison to monotherapies, when humanized with Donor 1 or Donor 2. (p<0.05) No significant body weight differences were recorded between different treatment groups in Donor 3 humanized mice (Figure 2).



Figure 2: Relative Body Weight Changes

2.1.3 Observations and Activity Scores

No further significant differences of activity scores were found neither for donor-wise nor treatment-wise comparisons (Figure 3). Taken together, none of the administered therapies could outweigh the donor effect observed for survival, body weight loss and activity scores in this study rendering an assessment of the treatment effect difficult.



Table 4: Antemortem and Postmortem Findings

Dose Groupsª	Donor	Treatment 1ª 20 mL/kg (2*10mL/kg)	Treatment 2ª 10mL/kg	Unscheduled Deaths/ Clinical Signs/ Body Weights*	Clinical Pathology: Serum Biochemistry (ALT, AST)
1	Donor 1 (lot 49)	Vehicle Control 0 mg/kg/day	Palivizum ab 7.5 mg/kg/week	No unscheduled deaths: IN(3); ET(3)/ ruffled fur, impaired general condition/ no bwc	None
2	Donor 2 (lot 50)	Vehicle Control 0 mg/kg/day	Palivizum ab 7.5 mg/kg/week	No unscheduled deaths: IN(3); ET(3)/ ruffled fur, impaired general condition/ no bwc	None
3	Donor 3 (lot 51)	Vehicle Control 0 mg/kg/day	Palivizumab 7.5 mg/kg/week	ES: SD2(1), SD4(3); IN(2)/ ruffled fur, pale skin, impaired general condition/ mean bwc at ET -17% (maximum bwc -20.1% on SD8)	None
4	Donor 1 (lot 49)	Vehicle Control 0 mg/kg/day	Ipilimumab 7.5mg/kg/week	No unscheduled deaths: $IN(3)$; $ET(3)$ / ruffled fur, pale skin, impaired general condition/ no bwc	None
5	Donor 2 (lot 50)	Vehicle Control 0 mg/kg/day	Ipilimumab 7.5 mg/kg/week	ES: SD4(2); IN(2); ET(2)/ ruffled fur, impaired general condition/ no bwc	None
6	Donor 3 (lot 51)	Vehicle Control 0 mg/kg/day	Ipilimumab 7.5 mg/kg/week	ES: SD4(4), SD8(1); IN(1)/ ruffled fur, pale skin, impaired general condition, circling behavior/ mean bwc at ET -12.0% (maximum bwc -19.4% on SD8)	None
7	Donor 1 (lot 49)	Vemurafenib 200 mg/kg/day	Ipilimumab 7.5 mg/kg/week	ES: SD4(2); IN(2); ET(2)/ ruffled fur, impaired general condition/ mean bwc at ET -7.5% (maximum bwc -11.8% on SD8)	None
8	Donor 2 (lot 50)	Vemurafenib 200 mg/kg/day	Ipilimumab 7.5 mg/kg/week	ES: SD4(1); IN(3); ET(2)/ ruffled fur, impaired general condition/ mean bwc at ET -11.3%	None
9	Donor 3 (lot 51)	Vemurafenib 200 mg/kg/day	Ipilimumab 7.5 mg/kg/week	ES: SD4(3); IN(2) ;ET(1)/ ruffled fur, pale skin, impaired general condition/ mean bwc at term -10.2% (maximum bwc -16.3% on SD8)	None
huNOG-EXLmice: NOD.CgPrkdcscid 112rgtm 1Sug Tg(SV40/HTLV-IL3,CSF2)10-7/ic/Jic/Tac (Taconic model #13395-F) engrafted with human umbilical cord blood-derived CD34+ hematopoietic stem cells HSCCB-13395					

Treatment 1 Vehicle: 2% (w/v) hydroxypropyl cellulose (HPC) in deionized water, pH4 Treatment 1 Vehicle: 0.9% (w/v) sodium chloride in water for injection Treatment 1 was administered as two consecutive daily on all does at 10mL/kg (20mL/kg total daily dose volume). Each of 6 animals per cage were administered 10mL/kg of Treatment 1 daily, followed by the subsequent 10mL/kg of Treatment 1 daily, followed by Treatment 2 as an intraperitoneal injection at 10mL/kg once per week, before moving to the next cage.

*Animals generally had transient and/or sustained body weight losses throughout the study. Diet gel was given to all groups at the start of the study. ES=Early Sacrifice; FD= Found Dead; SD=Study Day; bwe=body weight change; IN=Interim Necropsy on Study Day 9; ET=Early Termination on Study Day 10.



Figure 3: Activity Scores



2.1.4 Histomorphologic Assessment

Histomorphologic changes were present in the liver, lung, and spleen of mice from each of the three donors.

The liver of all animals had multifocal aggregates of mononuclear cells containing intracytoplasmic brown, finely granular pigment. Intracytoplasmic erythrocytes (erythrophagocytosis) were occasionally observed in these areas suggesting the pigment is likely hemosiderin. The most common cause of hemosiderin accumulation within phagocytic cells is heme (Fe) catabolism from erythrocyte destruction. The etiology of the hemosiderin-laden phagocytic cells is not known; however, in this model, it may represent graft-host intolerance. These aggregates of pigment laden cells were often associated with minimal to marked inflammation characterized by infiltrates of mononuclear cells and less frequently Langerhans-type multinucleated giant cells. Infiltrates of mononuclear cells were also often present around blood vessels and bile ductules but were not associated with any parenchymal changes. The incidence and severity of inflammation varied among donor and treatment groups. Overall, mice from donor 3 exhibited higher incidence and severity of inflammation in the liver compared to mice from donors 1 and 2, regardless of the treatment conditions.

The lung had minimal to moderate, multifocal infiltrates of mononuclear cells present within the connective tissue surrounding blood vessels and/or within the alveolar parenchyma. Minimal numbers of pigment laden cells were occasionally admixed within the infiltrate, as were rare multinucleated giant cells. Overall, mice from donor 1 exhibited higher incidence and severity of lung infiltrates compared with mice from donors 2 and 3.

The spleen had minimal to mild increased extramedullary hematopoiesis (EMH) observed across donors and treatment groups. There was also increased cellularity of the splenic white pulp which consisted of expansion of the latter by accumulations of mononuclear cells. Langerhans-type multinucleated giant cells were rarely present in the white and/or red pulp in individual mice from donor 2. Overall, mice from donor 3 exhibited higher incidence and severity of EMH than mice from donors 1 and 2 (as with the liver findings). The EMH in mice from this donor often correlated with a subgross organ enlargement in both control and treated animals suggesting that this change may be related to the biology of the graft.

Most of the histomorphologic changes observed in the liver and lungs of mice in this study (treated or untreated) fell within the spectrum of changes described in the literature as the result of graft-versushost-disease or the administration of immune checkpoint inhibitors in immune-humanized mouse models, which are inherently difficult from another to distinguish one (https://pubmed.ncbi.nlm.nih.gov/30850839/). It is worth noting that, except for donor 3, the extent of the changes was overall modest (focal or multifocal, affecting a small fraction of the tissue) and that the incidence and severity of the changes in relation to treatment were inconsistent across donors.

2.2 Experimental in vivo and in vitro immune-competent target organ models to study adverse events of CPI

We evaluated avelumab, atezolizumab, nivolumab, and ipilimumab in a co-culture model (CD8⁺ T cells/hepatocyte cell line), a triple co-culture model (CD8⁺ T cells, macrophages, hepatocyte cell line), and a multi-cellular culture model (PBMCs, hepatocyte cell line). The concentrations used were comparable to those found in the plasma of patients. We also tested premedication with acetaminophen and statins while simulating an inflammatory state. However, none of these conditions resulted in CPI-



mediated hepatotoxicity. Consequently, we increased the concentration up to three times that observed in patients and combined the CPIs (as done in clinical settings), but again, no hepatotoxicity was observed. In patients, CPI-induced hepatotoxicity does not appear immediately; it takes weeks or months. Therefore, we decided to extend the culture time from 5 days to 10 days. While 2D cultured HepaRG cells showed reduced viability after 5 days, 3D HepaRG spheroids survived up to 10 days in culture. As a result, we initiated the establishment of a model combining primary immune cells and HepaRG spheroids.

A further CPI anti-GD2 used for the treatment of neuroblastoma was started to be investigated. Ganglioside 2 (GD2) is expressed on neuronal cells and hepatocytes. GD2 was identified as a ligand for the immune checkpoint receptor siglec-7, expressed on monocytes, NK cells and T cells [1,2]. The cytotoxicity of anti-GD2 in respect to cancer cells is enhanced by co-administration with granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin-2 (IL-2), due to their effects on neutrophils, macrophages, and immune effector cells; this is why anti-GD2 is commonly combined with these cytokines. A combination therapy induces a slightly higher increase in liver enzymes in comparison to the monotherapy with anti-GD2 [3]. We started to test an anti-GD2 antibody in combination with IL-2 and/or GM-CSF in our established triple-culture model (CD8⁺ T cells, macrophages, hepatocyte cell line).

The PCTS model has been established and optimized using gingival cancer tissue (n=2). Key parameters, including media composition, tissue slice thickness, viability, and nivolumab concentrations, have been tested and evaluated using flow cytometry. Sera from both nivolumab-treated and control samples have also been collected and will be analyzed for cytokine changes. The model will next be extended to include healthy tonsil and tonsillar cancer tissues to broaden its applicability.

3. Discussion

3.1 CPI combination therapy in huNOG-EXL mice

Even though some trends towards a test article effect were observed between the treatment (Ipilimumab alone or in combination with Vemurafenib) and control groups (e.g., inflammation in the liver of mice from donor 2 or perivascular infiltrates in the lung of mice from donor 1), these results were considered inconclusive due to the minimal nature of the changes, the intra- and inter-donor variability, the small number of mice per group, and the short treatment duration due to the early termination of all dose groups.

3.2 Experimental in vivo and in vitro immune-competent target organ models to study adverse events of CPI

We used the established irAOP (described in deliverable D2.10) to identify relevant *in vitro* models and read-outs. We concentrated on improving our models by transitioning from 2D to 3D formats. Additionally, we optimized our co-culture conditions by incorporating co-medication and simulating an inflammatory disease state. We also broadened the range of tested CPIs to include nivolumab (anti-PD1) and ipilimumab (anti-CTLA4), and we introduced a completely new CPI targeting anti-GD2.

Initial results on anti-GD2 therapy in combination with IL-2 and GM-CSF indicate the feasibility of using our models to explore emerging CPI strategies and combination therapies, including their potential off-target effects.



The PCTS model represents a major advance in modelling tumour-immune dynamics *ex vivo*. Its capacity to incorporate autologous immune components and support transcriptomic analyses enables precise dissection of immune-related responses and identification of predictive biomarkers. Expanding the model to include tonsillar tissues addresses both tissue accessibility and disease relevance, particularly for head and neck cancer subtypes.

4. Conclusion

4.1 CPI combination therapy in huNOG-EXL mice

The humanized immune system mice used in this study did not recapitulate the expected clinical observations of liver injury.

4.2 Experimental in vivo and in vitro immune-competent target organ models to study adverse events of CPI

We successfully established and optimized a suite of complementary in vitro and ex vivo models to evaluate CPI-mediated toxicity and immune modulation. These include hepatic co-culture systems, CRS-relevant endothelial co-cultures, and a novel tumour PCTS platform. Collectively, these models offer an integrated pipeline to study both efficacy and toxicity of immunotherapies in a human-relevant context. Ongoing work will focus on expanding the models' applicability across tissue types, refining long-term culture systems, and leveraging multiomics analyses to map immune signatures and mechanisms of action. These efforts will contribute to safer and more effective immunotherapy development and support a reduction in animal testing through the use of robust alternative models.



References

1. A, Handa K, Withers DA, Satoh M, Hakomori S. Binding specificity of siglec7 to disialogangliosides of renal cell carcinoma: possible role of disialogangliosides in tumor progression. FEBS Lett. 2001;498(1):116-120.

2. Haas Q, Markov N, Muerner L, et al. Siglec-7 represents a glyco-immune checkpoint for nonexhausted effector memory CD8+ T cells with high functional and metabolic capacities. Frontiers in immunology. 2022;13:996746.

3. Ladenstein R, Potschger U, Valteau-Couanet D, et al. Interleukin 2 with anti-GD2 antibody ch14.18/CHO (dinutuximab beta) in patients with high-risk neuroblastoma (HR-NBL1/SIOPEN): a multicentre, randomised, phase 3 trial. Lancet Oncol. 2018;19(12):1617-1629.

4. Majorova, D., et al., Use of Precision-Cut Tissue Slices as a Translational Model to Study Host-Pathogen Interaction. Front Vet Sci. 2021; 8:686088.

5. Siwczak, F., et al. Culture of vibrating microtome tissue slices as a 3D model in biomedical research. J Biol Eng. 2023. 17(1):36.



Acknowledgement

The authors would like to express their gratitude to the Innovative Medicines Initiative 2 Joint Undertaking (JU) for the financial support of this research 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.







