Application of a Performance Characterization Protocol for the Assessment of Normal and Diseased Cryopreserved Human Precision-cut Lung Slices.

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ABSTRACT

Human precision-cut lung slices (hPCLS) are a highly relevant 3-dimensional model of the lung, offer native architecture of the respiratory parenchyma and small airways, and contain immune competent cells involved in inflammatory and sensitization processes. We recently reported the proof of concept, "H. Behrsing, M. Marimoutou, K. Amin, et al., Cryopreservation of Human Precision-cut Lung Slices Provides an Immune Competent, Pulmonary Test System for 'On-demand Use, The Toxicologist: Supplement to Toxicological Sciences, 186 (1), Society of Toxicology, 2022. Abstract no. 4193. " in a 4-week culture using one donor's hPCLS. Adopting key elements from this study, we have cryopreserved, thawed, and assessed additional donors using a 2-week study intended for routine use in banking cryopreserved hPCLS.

The performance of fresh or cryopreserved (up to 6 weeks) hPCLS in a 2-week culture at the air-liquid interface was assessed from the same donor lungs. hPCLS from both normal tissues, tissues from donors with chronic obstructive pulmonary disease (COPD), or fibrosis were treated with either 5 µg/mL lipopolysaccharide (LPS) or vehicle (VC) for 24 hours at days 6 or 13 post culture initiation. The tissue biomass (BCA protein content), viability (metabolic activity via WST-8 assay), and LPS-induced immune responsiveness were determined at days 7 and 14. Some variability was observed at different time points but hPCLS (normal and COPD donors) in both fresh and cryopreserved groups demonstrated 2-week viability (4.18 and 3.90 OD450/mg protein in fresh and cryopreserved, respectively). The protein content in the fresh hPCLS from some donors was higher than that in the cryopreserved hPCLS (0.4 vs 0.25 mg/mL, respectively), but immune-responsiveness was not compromised. While finite cytokine pg/mL values varied between the fresh and cryopreserved slices, induced values (up to 139-fold increases in IL-6, TNF-α, and MMP-3) relative to the respective VC was maintained, suggesting biomass-equivalent functionality. Although cryopreserved IPF donor hPCLS showed a reduction in viability (relative to fresh) over 2-weeks, immune responsiveness was maintained. With improvements in slice creation, storage, and culture conditions, the hPCLS can be applied for larger scale testing, tissue banking, and repeat donor experimentation.

Performance testing of these hPCLS batches provides an understanding of banked tissues (normal and diseased) prior to selection and use for studies that have specific research questions. These improvements position hPCLS as an accessible, human-relevant, pulmonary test system suitable for testing inhaled materials, evaluating key events in AOPs, and the evaluation of therapeutics "on demand".

INTRODUCTION

hPCLS offer native lung architecture including small airways and respiratory parenchyma, making it one of the most physiologically-relevant non-animal models of the lower lung. These substructures within the hPCLS reflect the presence of the many cell types in the human lung, not present in other non-animal models used for toxicity studies. The complement of these cell types in hPCLS allows for a more realistic interpretation of complex pulmonary responses to various exposures. Reports of multi-week culture longevity and complex responses has positioned hPCLS as a candidate model to evaluate key events associated with severe lung disease.

However, with infrequent donor tissue availability and lack of reliable hPCLS preservation techniques, hPCLS are difficult to employ in research studies that require repetition. Moreover, excess hPCLS generated must be utilized immediately or discarded. Clearly, reliable preservation techniques coupled with optimized long-term culture method would position hPCLS as a pulmonary test system with increased utility and accessibility for researchers.

Here, we evaluated the performance of short- and long-term cryopreserved hPCLS for a period of up to 4 weeks in culture. The responses of the cryopreserved hPCLS to cytotoxic and inflammatory stimuli were evaluated in comparison to that of the fresh hPCLS from the same donor lung. The initial results suggest that researchers may be able to bank frozen hPCLS and has allowed us to generate a performance characterization process to assess all hPCLS stored in backs. Further, the principles of Good In Vitro Methods Practices (GIVIMP) are being applied to the workflow of obtaining and processing human lung tissue for hPCLS banking to enhance their credibility and optimize the utility of hPCLS for use in a regulatory setting.

METHODS

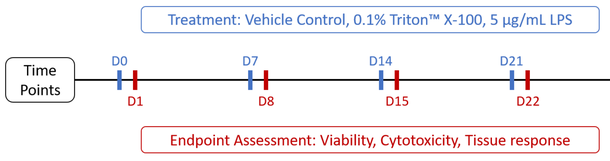


Figure 1. Treatment and endpoint assessment. After hPCLS acclimation, slices were exposed to 0.1% TX100 or 5 mg/ml LPS challenge at various time points during culture, for 24 h prior to endpoint assessments

PC Protocol: After demonstrating proof of principle with the 1st donor, 3 additional donor lungs (2 normal and one COPD) were processed and subjected to a more limited 2-week performance characterization protocol (evaluating protein content, viability, and inflammatory response of 3 cytokines at days 7 and 14) that compared fresh and cryopreserved hPCLS of the same donors, respectively.

Generation of hPCLS and Cryopreservation: Upon receipt, the non-transplantable human lungs (all ethically sourced from reputable procurement agencies with the consent of donor or next of kin) were inflated using 0.8% agarose solution. Upon gelling, the lung was cut into ~1.5 cm thick sections and cylindrical cores were generated using 8 mm circular knife. The cores were sliced using a Krumkieck slicer with approximate slice thickness of 500 μm. Some of the fresh hPCLS were used for the experiments and remaining were cryopreserved using a cryo buffer developed by IIVS for storage of hPCLS.

Viability assessment: The viability of hPCLS was assessed using WST-8 [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt] assay.

Histological assessment: The formalin-fixed hPCLS sections were stained using H&E solutions and assessed for cytomorphology. The slices were scored for viability and overall health compared to the negative control tissues (N.C.; never-frozen hPCLS).

Multiplex analyte analysis: The levels of various analytes (IL-6, CXCL-8/IL-8, CCL2/MCP-1, TIMP-1, CXCL-1/GRO-α) in the culture medium were determined using a Luminex assay kit (run on Luminex MAGPIX analyzer).

hPCLS Biochemical Characterization

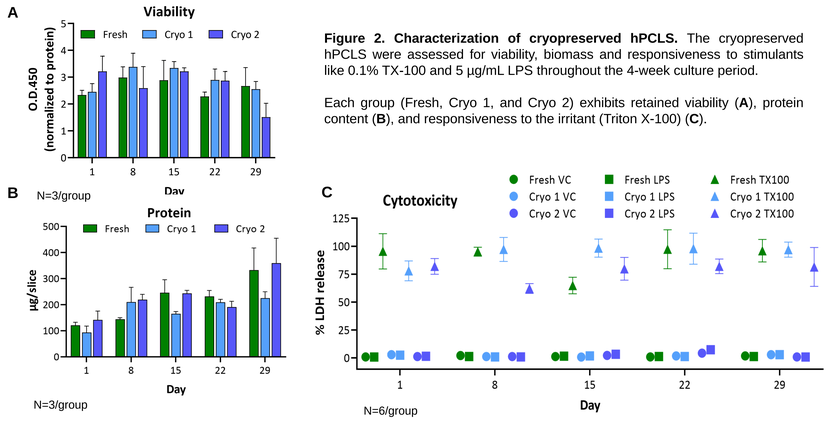


Figure 2. Characterization of cryopreserved hPCLS. The cryopreserved hPCLS were assessed for viability, biomass and responsiveness to stimulants like 0.1% TX-100 and 5 µg/mL LPS throughout the 4-week culture period.

Each group (Fresh, Cryo 1, and Cryo 2) exhibits retained viability (**A**), protein content (**B**), and responsiveness to the irritant (Triton X-100) (**C**).

hPCLS Immune Response

Table 1. Lipopolysaccharide (LPS)-induced responses in cryopreserved hPCLS. Following cryopreservation, the hPCLS were thawed and cultured at ALI. During the culture period a subset of hPCLS were treated with 0.1% TX-100, 5 µg/mL LPS or VC (culture medium) at days 0, 7, 14, 21 and 28. After 24hr exposure media samples were collected, assessed for cytokines and calculated as fold-change vs vehicle control

		IL-6					IL-8					MCP-1					IL-1β					TIMP-1					Gro-α				
	Days	1	8	15	22	29	1	8	15	22	29	1	8	15	22	29	1	8	15	22	29	1	8	15	22	29	1	8	15	22	29
Fresh	Mean	29.8	8.4	7.8	25.2	1.2	1.8	8.1	3.9	7.0	2.2	2.2	2.3	2.9	4.1	1.5	3.8	3.5	3.4	4.0	1.4	3.3	1.4	0.6	2.1	0.6	1.3	3.0	3.3	2.6	0.8
	SD	8.6	2.8	3.7	29.5	1.2	0.7	4.8	2.9	5.9	1.2	0.6	2.0	1.3	1.4	0.4	0.3	0.5	0.3	0.5	0.4	1.3	0.8	0.2	1.5	0.4	0.3	0.8	2.7	1.0	0.4
Cryo 1	Mean	9.1	3.9	26.1	4.4	7.9	2.4	3.9	23.6	2.4	13.6	1.7	1.3	5.2	2.3	5.3	5.2	na	na	na	7.4	1.4	0.9	2.0	1.0	2.4	1.3	1.9	13.4	1.1	9.5
	SD	4.8	1.9	9.8	2.1	3.0	1.2	1.9	9.1	1.3	2.5	0.9	0.2	1.6	0.6	1.2	2.1	na	na	na	2.1	1.5	0.3	0.5	0.3	0.4	1.1	0.5	6.5	0.5	3.0
Cryo 2	Mean	6.1	5.9	4.4	15.8	11.7	8.9	11.6	16.7	12.1	14.6	2.3	2.4	3.5	2.3	4.3	3.6	1.7	0.6	2.9	6.7	1.2	0.5	0.5	1.0	1.1	2.8	3.8	4.9	8.8	14.2
	SD	3.7	2.9	1.9	6.0	10.1	11.0	6.0	5.3	3.8	8.4	1.5	1.3	0.9	0.8	2.0	1.1	1.4	0.5	1.6	2.1	1.2	0.6	0.1	0.2	0.6	1.7	1.2	1.5	4.6	6.5

hPCLS Histological Evaluation

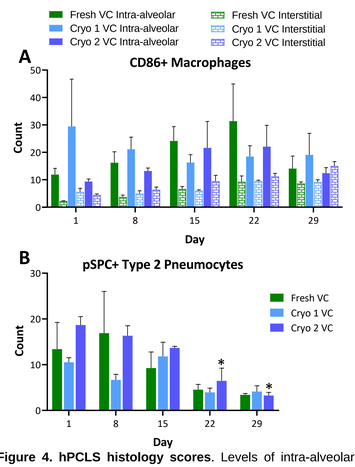


Figure 4. hPCLS histology scores. Levels of intra-alveolar and interstitial CD86þ macrophages (A) and type 2 pneumocytes (B, pSPC) are shown (AVE ± SEM). N=3/group

Changes in the levels of interstitial macrophages and type 2 pneumocytes were observed.

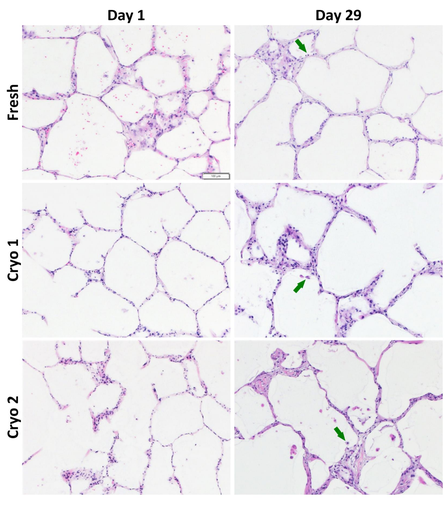


Figure 5. Histological evaluation of cryopreserved hPCLS. The optimized culture methodology applied results in excellent retention of lung architecture and normal cytological features after 29 days culture in fresh hPCLS. N=3/group. (green arrows = macrophages)

Application of hPCLS PC Protocol

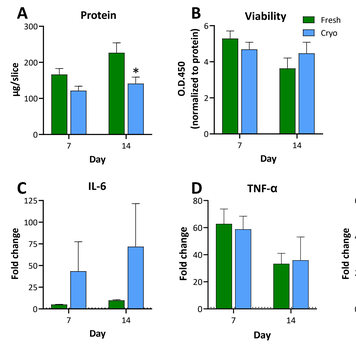


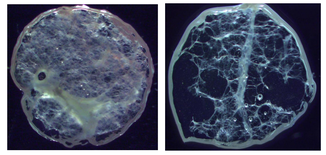
Figure 6. Retained viability and immune responsiveness in cryopreserved hPCLS from additional donor lungs. hPCLS were treated with 5 µg/mL LPS or VC (culture medium) at days 6 and 13.

After 24 hr of treatment assessments were made for:

- protein content (**A**)
- tissue viability (**B**)
- IL-6 (**C**)
- TNF-α (**D**)
- MMP-3 (**E**)

The data are presented as AVE ± SEM, or fold change vs vehicle control; N=3/group

Banking of Normal and Diseased hPCLS



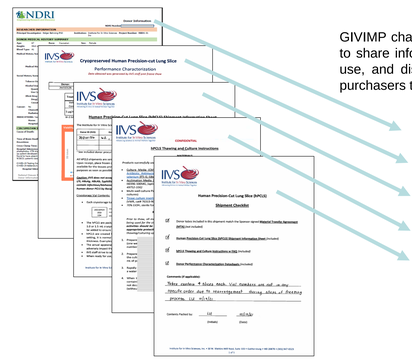
Normal tissue

Emphysemic tissue

IIVS Inventory				Donor Demographics					Lung Type	Known Behavior		
Donor ID (IIVS)	Total PCLS available	PCLS (reserved)	Mean Thickness (µm)	Age	Race	Height	Weight	Sex	Lung Status	Tobacco	Alcohol	Drugs
20210330	16		548 ± 65	66	Caucasian	173 cm	77.6 kg	M	COPD Dx 2010	Y	Y	Y
20210720	1878		493 ± 58	64	White	163 cm	62.6 kg	F	COPD	Y	N	N
20210923	396	396	520 ± 67	67	Caucasian	150 cm	54.4 kg	F	Normal	N	Y	Y
20211112	702		516 ± 53	59	Hispanic/Latina	145 cm	62 kg	F	Normal	N	N	N
20220209	984	24	524 ± 101	66	Caucasian	NA	NA	F	Fibrosis	N	Y	N
220628	1410	48	424 ± 131	66	Hispanic/Latino	157 cm	61.7 kg	M	Normal	N	Y	Y
220730	1020	48	437 ± 50	59	Hispanic/Latino	163 cm	88.6 Kg	M	Normal	N	N	N
220915	2470		535 ± 59	67	White	163 cm	117 kg	F	COPD (Asthma + Emphesema)	Y	Y	Y

indicates donor has been banked but PC data is incomplete

Application of GIVIMP Principles



Information Provided to Recipients

GIVIMP chapter 1 discusses the responsibility *in vitro* test system providers have to share information on test system characterization, authenticity, safe transport, use, and disposal. IIVS provides a package of this information to test system purchasers to satisfy the GIVIMP requirements of test system providers.

- Donor information excluding all personal identifiable information
- Performance characterization results of the batch of hPCLS
- A shipment inventory sheet traceable to the IIVS processing batch records
- Thawing and general culture instructions for the hPCLS
- A shipping checklist to assure all required information is included and serving as a chain of custody document

CONCLUSIONS

- With greatly increased production rates, the ability to bank cryopreserved hPCLS provides a means to maximally utilize the valuable donated human lung tissue (of all disease states), retest the same tissue, and make donor-donor comparisons that were not possible before.
- Long term hPCLS cultures (now also possible with cryopreserved hPCLS) can allow detection of Key Events involved in Adverse Outcome Pathways of pulmonary disease progression (e.g. fibrosis, respiratory sensitization) that require multiple cell types.
- The application of a performance characterization protocol for stored banks provides for a consistent method by which to evaluate frozen banks upon thaw that can be shared and screened with recipients to determine the most useful donor(s) for intended studies.
- Applying the principles of GIVIMP, the process of obtaining non-transplantable human lung tissues, creating and banking hPCLS establishes process credibility, provides transparency of source materials, and better positions cryopreserved hPCLS as an option for regulatory applications

ACKNOWLEDGMENT

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