Transcriptomic investigation reveals donor-specific gene signatures in human lung transplants

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ABSTRACT

Introduction: Transplantation of lungs from donation after circulatory death (DCD) in addition to donation after brain death (DBD) became routine worldwide to address the global organ shortage. The development of *ex vivo* lung perfusion (EVLP) for donor lung assessment and repair contributed to the increased use of DCD lungs. We hypothesise that a better understanding of the differences between lungs from DBD and DCD donors, and between EVLP and directly transplanted (non-EVLP) lungs, will lead to the discovery of the injury-specific targets for donor lung repair and reconditioning.

Methods: Tissue biopsies from human DBD (n=177) and DCD (n=65) donor lungs, assessed with or without EVLP, were collected at the end of cold ischaemic time. All samples were processed with microarray assays. Gene expression, network and pathway analyses were performed using R, Ingenuity Pathway Analysis and STRING. Results were validated with protein assays, multiple logistic regression and 10-fold cross-validation.

Results: Our analyses showed that lungs from DBD donors have upregulation of inflammatory cytokines and pathways. In contrast, DCD lungs display a transcriptome signature of pathways associated with cell death, apoptosis and necrosis. Network centrality revealed specific drug targets to rehabilitate DBD lungs. Moreover, in DBD lungs, tumour necrosis factor receptor-1/2 signalling pathways and macrophage migration inhibitory factor-associated pathways were activated in the EVLP group. A panel of genes that differentiate the EVLP from the non-EVLP group in DBD lungs was identified.

Conclusion: The examination of gene expression profiling indicates that DBD and DCD lungs have distinguishable biological transcriptome signatures.

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Introduction

Lung transplantation is a life-saving therapy for patients with end-stage lung disease. However, there is a significant shortage of donor lungs to meet this therapeutic need. When compared with other organs, the utilisation rate of donated lungs is the lowest, which further exacerbates the organ shortage and leads to an increased mortality rate for patients on the transplant wait list [1]. This is partially caused by the lower availability of lungs from conventional donation after brain death (DBD) compared with other organs [2], as lungs are more vulnerable during retrieval and preservation.

Utilisation of lungs from donation after circulatory death (DCD), in addition to DBD lungs, has become worldwide practice to increase the number of donor organs for transplantation [3, 4]. According to the International Society for Heart and Lung Transplantation Registry report, the percentage of lung transplants from DCD donors was 20.9% in 2015 and the post-transplant survival for recipients receiving lungs from DCD *versus* DBD donors is comparable [5, 6].

The development of *ex vivo* lung perfusion (EVLP) technology has increased the utilisation of donor lungs, as it enables further organ assessment, treatment and rehabilitation of donor lungs at body temperature [7, 8]. By providing the donor lung with perfusion and ventilation in a normothermic environment, EVLP can help restore physiological metabolism so that more accurate assessments and more effective therapies are possible prior to transplantation. The use of EVLP for extended criteria donor lungs led to a 20% increase in available donor lungs for transplant as of 2015 and this number has surged even further over recent years [9]. Lungs assessed by EVLP that progress to transplant have similar post-transplant outcomes compared with standard donor lungs [10, 11]. Better understanding of the differences between DCD and DBD lungs, and identification of the indications for proceeding to EVLP, will be crucial to finding precise therapeutic targets and deciding which lungs would benefit from EVLP.

The present study aims to answer the following two questions: 1) are DBD and DCD donor lungs different at the transcriptional level? and 2) do EVLP and non-EVLP lungs have a different gene signature at cold ischaemic time (CIT)?

There is a lack of systematic investigation of transcriptional signatures specific to donor types. A pilot study using microarray data from 12 DBD and six DCD human lung samples, performed by our group, showed more inflammation-related genes in DBD lungs compared with lungs from DCD donors [12]. However, the sample size at the time was very small, lacking in statistical power, details on the inflammatory genes and pathways were very limited, and a comparison of EVLP *versus* non-EVLP samples was not performed. The objective of the current study was to examine a large dataset of human lungs to conduct comprehensive bioinformatics and systems biology analyses to answer the aforementioned questions, and to validate the findings of our previous work, giving that the validation using a different cohort of samples is extremely important.

Materials and methods

Donor lungs, RNA extraction and microarrays

Donor peripheral lung tissue biopsies were collected at the end of CIT before EVLP or transplant and snap frozen in liquid nitrogen by the Toronto Lung Transplant Program (University Health Network, Toronto, ON, Canada), from 2010 to 2015. Total RNA was extracted and then purified using a RNeasy Mini Kit (Qiagen, Hilden, Germany). RNA quality was verified by a Nanodrop spectrophotometer (VWR, Radnor, PA, USA) and a Bioanalyzer (Agilent, Santa Clara, CA, USA). All samples were processed with a Clariom D Assay (Thermo Fisher Scientific, Waltham, MA, USA). Raw data and processed files are accessible at Gene Expression Omnibus with series accession number GSE128204.

General clinical indications to select donor lungs for EVLP were defined by the following criteria: arterial oxygen tension (P_{aO_2})/inspiratory oxygen fraction (F_{IO_2}) <300 mmHg, presence of pulmonary oedema or infiltrates on chest imaging.

This study was approved by the University Health Network Research Ethics Board (REB11-0509 and REB12-5488) and the Ethics Review Board of the Trillium Gift of Life Network (Toronto, ON, Canada). All patients provided written consent for tissue biobanking.

Bioinformatics analyses

The entire flow of bioinformatics analyses and the corresponding methods are depicted in supplementary figure S1.

Gene expression and clustering analysis

We selected only those samples that were transplanted directly or following EVLP. Lungs rejected for transplant were not considered.

There are several group comparisons defined in this study (table 1): 1) all DBD (n=177) *versus* all DCD (n=65), 2) non-EVLP DBD (n=123) *versus* non-EVLP DCD (n=22), 3) EVLP DBD (n=54) *versus* EVLP DCD (n=43), 4) EVLP DCD (n=43) *versus* non-EVLP DCD (n=22) and 5) EVLP DBD (n=54) *versus* non-EVLP DBD (n=123) lung samples.

Differential gene expression, principal component analysis and hierarchical clustering were performed in R version 3.5.0 with various packages (affy, limma, annotate and pca3d) [13–17]. For heatmap visualisation, we employed MetaboAnalyst [18]. Microarray data were pre-processed by RMA utilising the affy package [14]. We used the Entrez Gene ID alternative annotation package from Brainarray [19]. p-values for differential gene expression were obtained using the limma package [15]. Batch effects were minimised by adjusting for microarray lot within the limma models. A gene was considered differentially expressed between two groups if a p-value corrected for false discovery rate (FDR) using the Benjamini–Hochberg method was <0.05 (FDR <0.05) [20]. In addition, stronger effects were defined for differentially expressed genes with fold change ⩾2 (upregulation) or ⩽0.5 (downregulation), or as otherwise indicated.

Pathway and network analysis

The lists of the differentially expressed genes and their statistical and experimental parameters (FDR-corrected p-value and $\log_2$ fold change) corresponding to each group comparison in this study were uploaded to Ingenuity Systems (www.ingenuity.com) to perform Ingenuity Pathway Analysis (IPA). For network analysis we employed STRING database version 10.5 and the igraph package [21, 22].

Multiple logistic regression and 10-fold cross-validation

We investigated the correlation between the seven highly differentially expressed genes in the EVLP DBD *versus* non-EVLP DBD comparison using the stepwise multiple logistic regression method. We validated the best model with the 10-fold cross-validation method. Area under the curve (AUC) was calculated with ROCR [23].

More details on bioinformatics methods are included in the supplementary material.

Protein assays

EVLP perfusate samples of the donor lungs were collected at 1 h into perfusion. Samples were taken directly from the pulmonary venous outflow and tested on an automated ELISA for interleukin (IL)-6, IL-8 and IL-1β, as per the manufacturer's instructions (Protein Simple, San Jose, CA, USA).

Results

Clinical data of donors are provided in table 2. The donors were similar in terms of age, sex, smoking status, chest radiograph infiltration and mechanism of injury leading to brain death (head trauma or anoxia/cardiac arrest) or the decision to withdraw life-sustaining therapies, in the case of DCD donors. 58.76% of donors had a cerebrovascular/stroke mechanism in the DBD group compared with 41.54% in the DCD group (p=0.02). The ratio of last P_{aO_2}/F_{IO_2} was significantly higher in the DCD group (p=0.019), with proportionally more DCD cases being assessed by EVLP (66.15%), as per institutional practice.

Transcriptional signatures show significant differences between DBD and DCD donor lungs

Differential gene expression analysis at FDR <0.05 revealed 5196 differentially expressed genes in the all DBD *versus* all DCD group comparison, 1972 differentially expressed genes in the non-EVLP DBD *versus* non-EVLP DCD group comparison and 2792 differentially expressed genes in the EVLP DBD *versus* EVLP DCD group comparison. These genes displayed a fair separation between DBD and DCD groups by principal component analysis (supplementary figure S2a–c). Genes with fold change ⩾2 or ⩽0.5 (supplementary table S1) showed a very good delineation between DBD and DCD samples, as presented by heatmaps with unsupervised hierarchical clustering (figure 1). The large numbers of differentially expressed genes and the distinct gene clustering between DBD and DCD lungs indicate that the pathophysiological conditions of these two types of organ donations are quite different.

TABLE 1 Human lung samples used in this study

	Donation after brain death	Donation after circulatory death
Non-EVLP	123	22
EVLP	54	43
Total	177	65

Data are presented as n. EVLP: *ex vivo* lung perfusion.

TABLE 2 Donation after brain death (DBD) and donation after circulatory death (DCD) donor characteristics

	DBD	DCD	p-value
Donors	177	65	
Age years	45.65±17.60	45.97±17.57	0.902
Sex			0.772
Male	87 (49.15)	34 (52.31)	
Female	90 (50.85)	31 (47.69)	
Donor smoking	89 (42.94)	37 (61.66)	0.545
Mechanism leading to brain death or irreversible brain injury			
Cerebrovascular/stroke	104 (58.76)	27 (41.54)	0.020
Head trauma	37 (20.90)	19 (29.23)	0.173
Anoxia/cardiac arrest	27 (15.25)	16 (24.61)	0.128
Donor last P_{aO_2}/F_{IO_2} mmHg	386.9±105.0	421.87±86.9	0.019
Chest radiograph infiltration	80 (45.20)	35 (53.85)	0.429
EVLP	54 (30.51)	43 (66.15)	7.69E–07

Data are presented as n, mean±SD or n (%), unless otherwise stated. P_{aO_2}: arterial oxygen tension; F_{IO_2}: inspiratory oxygen fraction; EVLP: ex vivo lung perfusion. Statistical p-values calculated with Fisher's exact test, except for numerical data (age and donor last P_{aO_2}/F_{IO_2}) where the t-test was applied.

Inflammation is dominant in DBD lungs and cell death is associated with DCD lungs

Within the list of highly differentially expressed genes in DBD *versus* DCD samples identified based on FDR and fold change (⩾2 or ⩽0.5), 18 are common to all three group comparisons and are highly upregulated (supplementary table S1). Among them are: 1) members of the chemokine family (*CCL2*, *CXCL2* and *CXCL8* (*i.e. IL8*)) involved in immunoregulatory and inflammatory processes [24]; 2) genes from nuclear receptor subfamily 4 (*NR4A1*, *NR4A2* and *NR4A3*), shown to regulate neutrophil lifespan and homeostasis [25]; 3) several metallothioneins (*MT1M*, *MT1G*, *MT1X*, *MT1A* and *MT1JP*) involved in cellular homeostasis, but also in differentiation and proliferation of normal and tumour cells, and tumour angiogenesis [26]; and 4) others, *e.g. AMADTS4* and *SELE* with established roles in fibrosis [27], and *FOSB*, a regulator of cell proliferation, differentiation and transformation [28].

There are nine differentially expressed genes shared between EVLP and all samples, of which chemokine *CCL20* and cytokine *IL6* are well known for their role in inflammatory responses. There are several differentially expressed genes exclusive to the EVLP group. Of these, activation of *IL1B* and *PTX3* has been linked to donor lung injury or poor outcome after lung transplantation [29, 30]. The upregulated *NFKBIZ* gene in the EVLP group, which encodes the transcription factor IκBζ, is associated with increased susceptibility to invasive pneumococcal disease [31]. *IER3*, found to be activated in the EVLP category of lungs, has roles in immune responses, inflammation, tumorigenesis and rheumatoid arthritis [32].

Following gene expression analysis, we investigated the pathways, diseases and functions associated with differentially expressed genes using IPA. When compared with DCD lungs, the majority of canonical pathways were upregulated and very few were downregulated in DBD samples (see table 3 for pathways and abbreviations). Among the upregulated pathways common to all three comparisons, IL-6 signalling, HMGB1 signalling, TREM1 signalling and p38 MAPK signalling are known to play roles in pulmonary inflammation, infections and immune responses. Of the pathways specific to EVLP samples, IL-1 signalling, LPS-stimulated MAPK signalling, NRF2-mediated oxidative stress response, role of IL-17F in allergic inflammatory airway diseases and iNOS signalling are activated in DBD donor lungs. These are pathways prominent in inflammation [7, 33–35]. Detailed information on the p-values and *z*-scores of these pathways is given in supplementary table S2.

IPA predicted a wide range of activated diseases and functions in DBD *versus* DCD samples. More specifically, cell viability and cell survival pathways frequently seen in tumor-related research are activated in DBD, whereas pathways related to cell death, apoptosis and necrosis are activated in DCD samples (figure 2).

Network centrality reveals specific drug targets to potentially rehabilitate DBD lungs

For network analysis, we performed a STRING analysis using the three short lists of highly differentially expressed genes (supplementary table S1), without protein–protein interactors from the database. The resulting networks have protein–protein interaction enrichment p<1.0E–16 (figure 3), suggesting that these

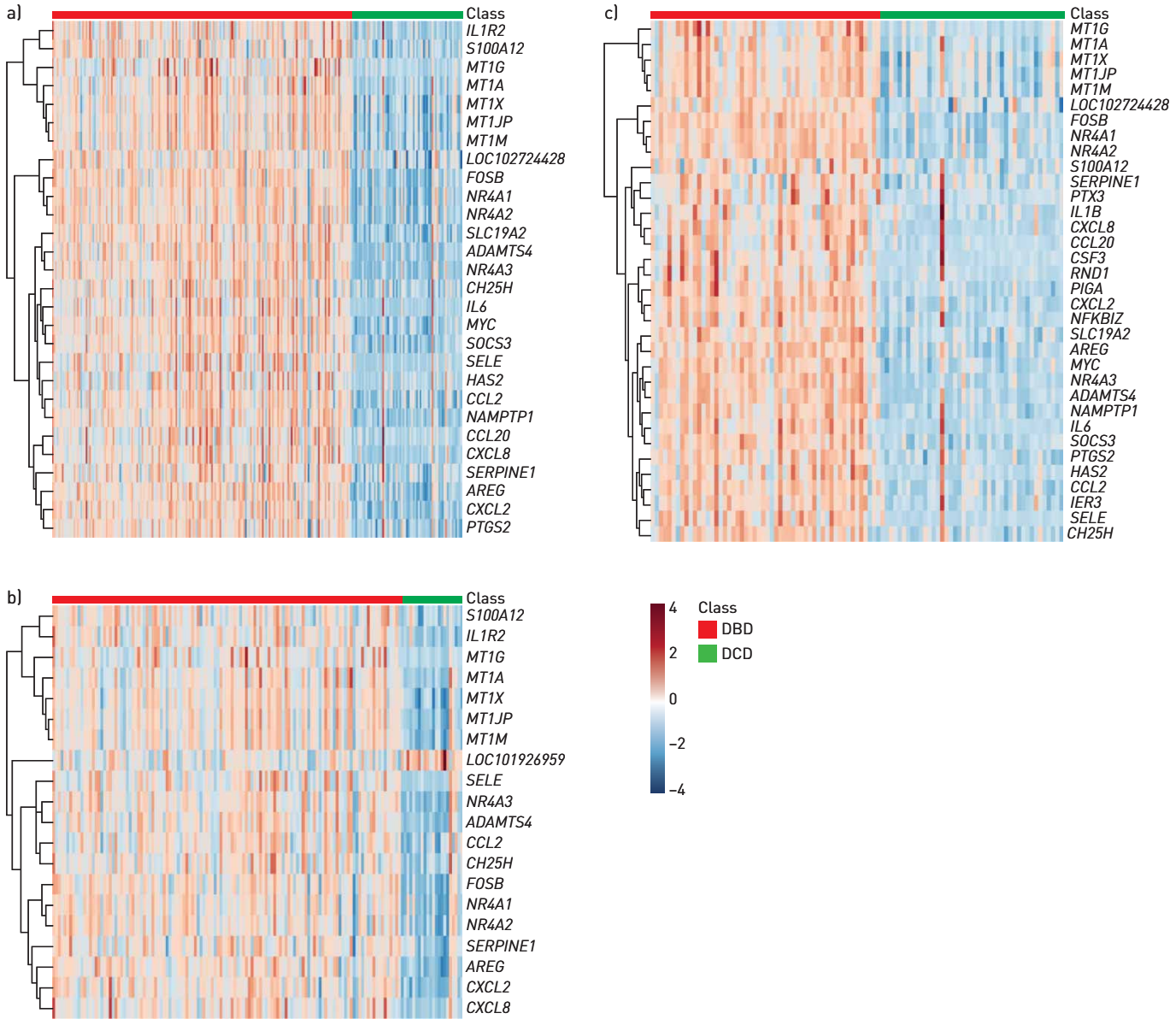

FIGURE 1 Differentially expressed genes between donation after brain death (DBD) and donation after circulatory death (DCD) lungs. EVLP: *ex vivo* lung perfusion. Heatmaps with differentially expressed genes by strict filtering (false discovery rate <0.05 and fold change ⩾2 or ⩽0.5) with unsupervised hierarchical clustering: a) all DBD *versus* all DCD, b) non-EVLP DBD *versus* non-EVLP DCD and c) EVLP DBD *versus* EVLP DCD.

are very strong biological networks solely based on differentially expressed genes between DBD and DCD lung samples.

The central node is IL-6 for biological networks derived from either all DBD *versus* DCD lungs (figure 3a; betweenness score 29.3) or using EVLP-only samples (figure 3c; betweenness score 41.8). These results also reflect the pathway analysis that lists IL-6 as the top altered pathway (table 3). For non-EVLP samples only (figure 3b), the central node was NR4A1 (betweenness score 18.5). The centrality analysis could be important for drug targeting. For instance, targeting IL-6 may result in the inhibition of the entire network, thus triggering inhibition of inflammation. More mechanistic and experimental studies are necessary to test this hypothesis.

EVLP lungs are different from non-EVLP lungs at the transcriptomic level

Comparisons of the EVLP DBD (n=54) and non-EVLP DBD (n=123) samples revealed 401 differentially expressed genes. Pathway analysis showed the involvement of genes in TNF family member receptors, TNF receptor (TNFR)-1/2 signalling pathways and macrophage migration inhibitory factor (MIF)-related pathways in the EVLP lungs (supplementary figure S3), which supports inflammatory responses as

TABLE 3 Summary of pathways activated or inhibited in donation after brain death (DBD) *versus* donation after circulatory death (DCD) samples

	All samples	Non-EVLP	EVLP
IL-6 signalling	Yes	Yes	Yes
HMGB1 signalling	Yes	Yes	Yes
TREM1 signalling	Yes	Yes	Yes
p38 MAPK signalling	Yes	Yes	Yes
ERK5 signalling	Yes	Yes	Yes
MIF-mediated glucocorticoid regulation	Yes	Yes	
Acute-phase response signalling	Yes		Yes
Hypoxia signalling in the cardiovascular system	Yes		Yes
LXR/RXR activation#	Yes		Yes
Pyridoxal 5′-phosphate salvage pathway	Yes		Yes
B-cell receptor signalling	Yes		
Complement system#	Yes		
MIF regulation of innate immunity	Yes		
Th1 pathway#	Yes		
iCOS–iCOSL signalling in T-helper cells#	Yes		
1D-myo-inositol hexakisphosphate biosynthesis II		Yes	
AMPK signalling		Yes	
Chondroitin sulfate biosynthesis		Yes	
Dermatan sulfate biosynthesis		Yes	
ERK/MAPK signalling		Yes	
Valine degradation I#		Yes	
IL-1 signalling			Yes
LPS-stimulated MAPK signalling			Yes
NRF2-mediated oxidative stress response			Yes
Role of IL-17F in allergic inflammatory airway diseases			Yes
iNOS signalling			Yes
IL-17A signalling in gastric cells			Yes
4-1BB signalling in T-lymphocytes			Yes
Aryl hydrocarbon receptor signalling			Yes
Lymphotoxin β receptor signalling			Yes
PI3K signalling in B-lymphocytes			Yes
Salvage pathways of pyrimidine ribonucleotides			Yes

EVLP: *ex vivo* lung perfusion; IL: interleukin; HMGB: high mobility group box; TREM: triggering receptor expressed on myeloid cells; MAPK: mitogen-activated protein kinase; ERK: extracellular signal-regulated kinase; MIF: macrophage migration inhibitory factor; LXR: liver X receptor; RXR: retinoid X receptor; Th1: T-helper type 1; iCOS(L): inducible T-cell costimulator (ligand); AMPK: AMP-activated protein kinase; LPS: lipopolysaccharide; NRF2: nuclear factor erythroid 2-related factor 2; iNOS: inducible nitric oxide synthase; PI3K: phosphatidylinositol 3-kinase. #: pathways inhibited in DBD lungs (or activated in DCD lungs); everything else is activated in DBD samples.

potential therapeutic targets for DBD lung repair during EVLP [36]. These pathways are known to exert potent pro-inflammatory effects and regulate immune responses [35, 37, 38]. Several genes are shared by these pathways, including *NFKB1* (NF-κB subunit 1), *NFKBIE* (NF-κB inhibitor ε), *NFKBIA* (NF-κB inhibitor α), *BIRC3* (also called inhibitor of apoptosis protein 1) and *PLA2G5* (phospholipase A2 group V, a secretory enzyme that can induce inflammatory responses in neighbouring cells) (supplementary table S3), indicating these pathways are highly related to each other.

Further filtering the differentially expressed genes by FDR <0.05 and fold change ⩾1.5 or ⩽0.7 identified eight genes (*SCGB1A1*, *C20orf85*, *CFAP126*, *SNTN*, *FAM216B*, *MS4A8*, *TSPAN1* and *LOC101928817*) downregulated in EVLP samples (figure 4a). From this list we excluded the noncharacterised gene (*LOC101928817*) with unknown function. To validate these results, we performed multiple logistic regression analysis, which showed high correlation among the other seven differentially expressed genes (figure 4b). This implies that each of these genes could be predictive of EVLP assessment on its own. The step procedure determined that the best model was with *CFAP126* (cilia and flagella associated protein 126); the 10-fold cross-validation showed 70% prediction accuracy, with an AUC of 0.70 (figure 4c).

Comparison of non-EVLP DCD lungs (n=22) *versus* EVLP DCD lungs (n=43) resulted in no significant differences at the transcriptional level. This may be due to the small number of DCD donor lungs in our

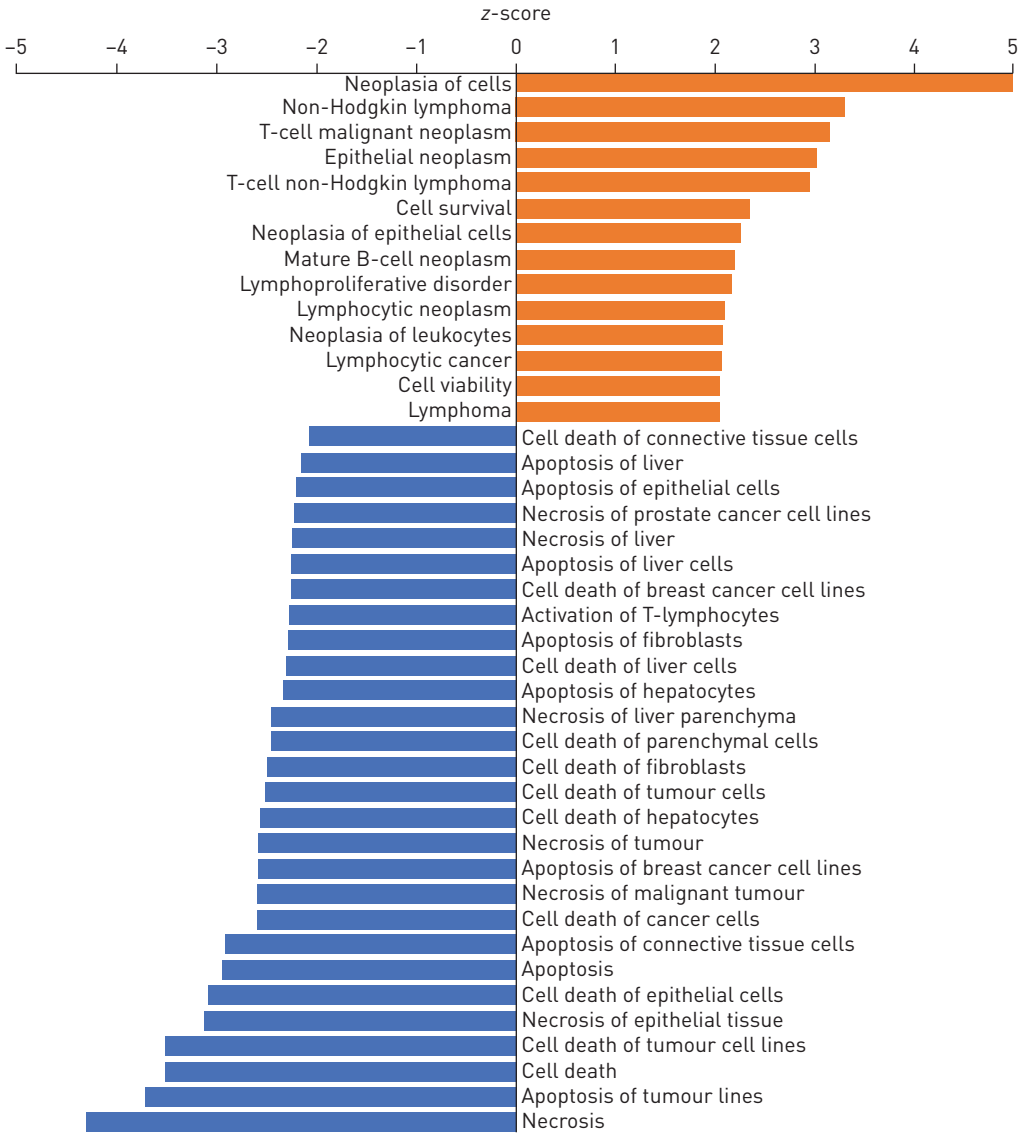

FIGURE 2 Ingenuity Pathway Analysis shows different diseases and functions for all donation after brain death (DBD) *versus* all donation after circulatory death (DCD) comparison. Orange: activation in DBD; blue: activation in DCD.

dataset and to the fact that EVLP has been more frequently used for DCD lungs at our centre (66.15%) (table 2).

Experimental validation

In an effort to recapitulate our findings at the transcript level, we performed parallel protein work using perfusate samples collected during EVLP after the first hour. Consistent with our transcript data, there was a significantly higher concentration of IL-6, IL-8 and IL-1β proteins from DBD donors compared with DCD donors (figure 5).

Discussion

Our study demonstrated that the lung injuries in DBD and DCD lungs arise from different biological mechanisms, as evidenced by their different respective transcriptomic signatures. With this work, we have validated the results from our previous pilot study showing that lungs from DBD donors present higher inflammation [12], and we further revealed details on activation of immunological diseases and immune responses. IL-6, HMGB1, TREM1 and p38 MAPK signalling pathways were found to be activated in DBD *versus* DCD comparisons. In addition, as a novelty of our study, we also found that the activation of cell death, apoptosis and necrosis pathways was associated with DCD donor lungs. We further identified IL-6

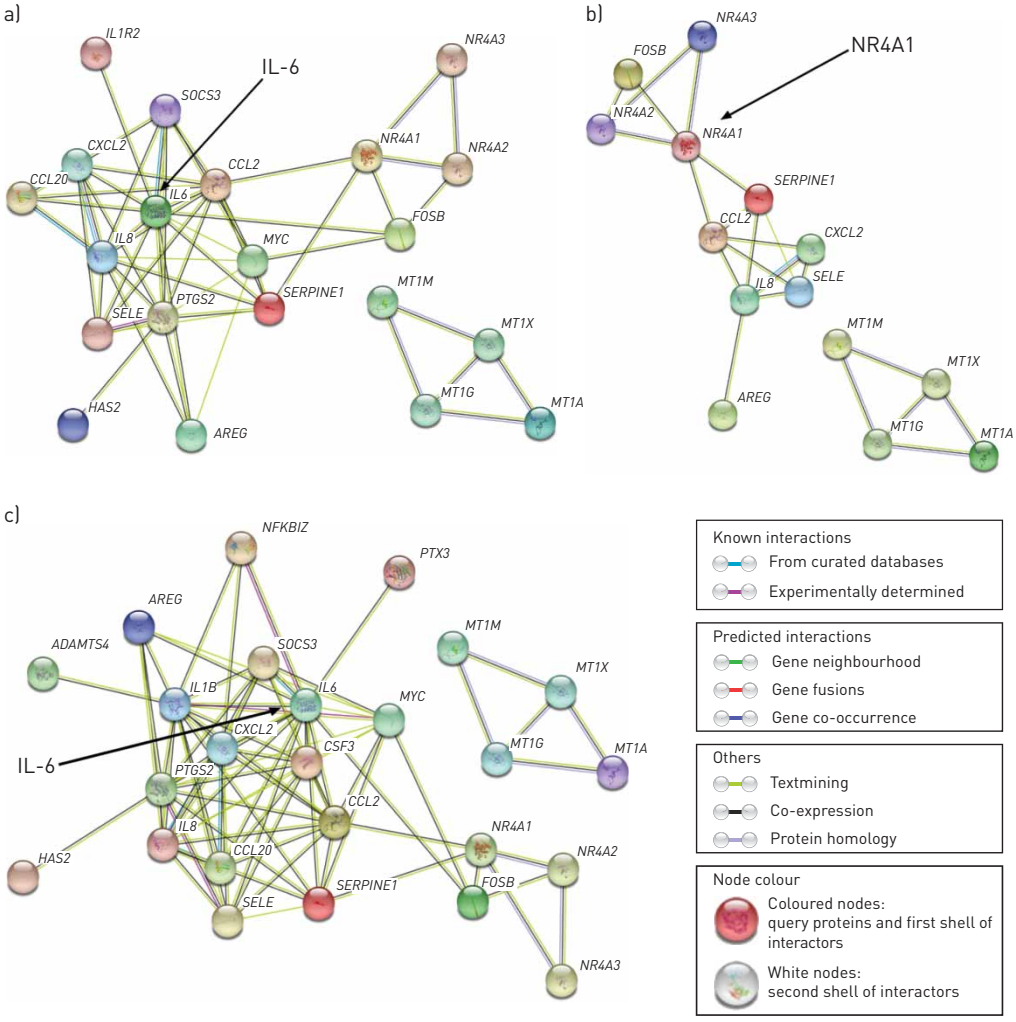

FIGURE 3 Highly differentially expressed genes are functionally connected as networks. DBD: donation after brain death; DCD: donation after circulatory death; EVLP: *ex vivo* lung perfusion. STRING networks with differentially expressed genes by strict filtering (false discovery rate <0.05 and fold change ⩾2 or ⩽0.5): a) all DBD *versus* all DCD, b) non-EVLP DBD *versus* non-EVLP DCD and c) EVLP DBD *versus* EVLP DCD. The black arrows with the corresponding text indicate the central nodes. Only connected nodes are shown.

as a central node for a network of highly differentially expressed genes in DBD lungs, especially in lungs assessed with EVLP. These results will be very valuable for further drug targeting studies, since they demonstrate that the two types of donor lungs may require different types of treatment.

To the best of our knowledge, this is the first large-scale study of human lungs aiming to identify the transcriptional differences, molecular pathways and networks between DBD and DCD donor lungs used for transplantation. The results at the transcript level between DBD and DCD lungs were also partially confirmed at the protein level, in perfusate samples taken 1 h after the start of EVLP. This confirms that the pathway activation reported here does lead to translational changes at the protein level in the lung, as demonstrated by the protein assays on the common molecules (IL-6, IL-8 and IL-1β) present in IL-6, HMGB1 and TREM1 signalling pathways.

EVLP provides the means to evaluate extended criteria donor lungs, thus increasing the number of utilised lungs for transplantation and reducing the risk of using poor quality donor lungs. EVLP also provides the opportunity to repair or to improve donor lung quality. Identification of possible drug targets for different types of lung injuries is critical, and our study revealed genes and pathways that mediate inflammation and/or cell death as potential targets for lung repair during EVLP. Recently, we have shown α_1-antitrypsin reduced porcine donor lung injury by inhibiting acute inflammatory responses and cell death during EVLP [39]. It is possible to use this or other anti-inflammatory and/or anti-cell death drugs to repair human donor lungs during EVLP. Hozain *et al.* [40] have developed xenogeneic cross-circulation for extracorporeal recovery of injured human lungs, which provides a new platform for donor lung repair.

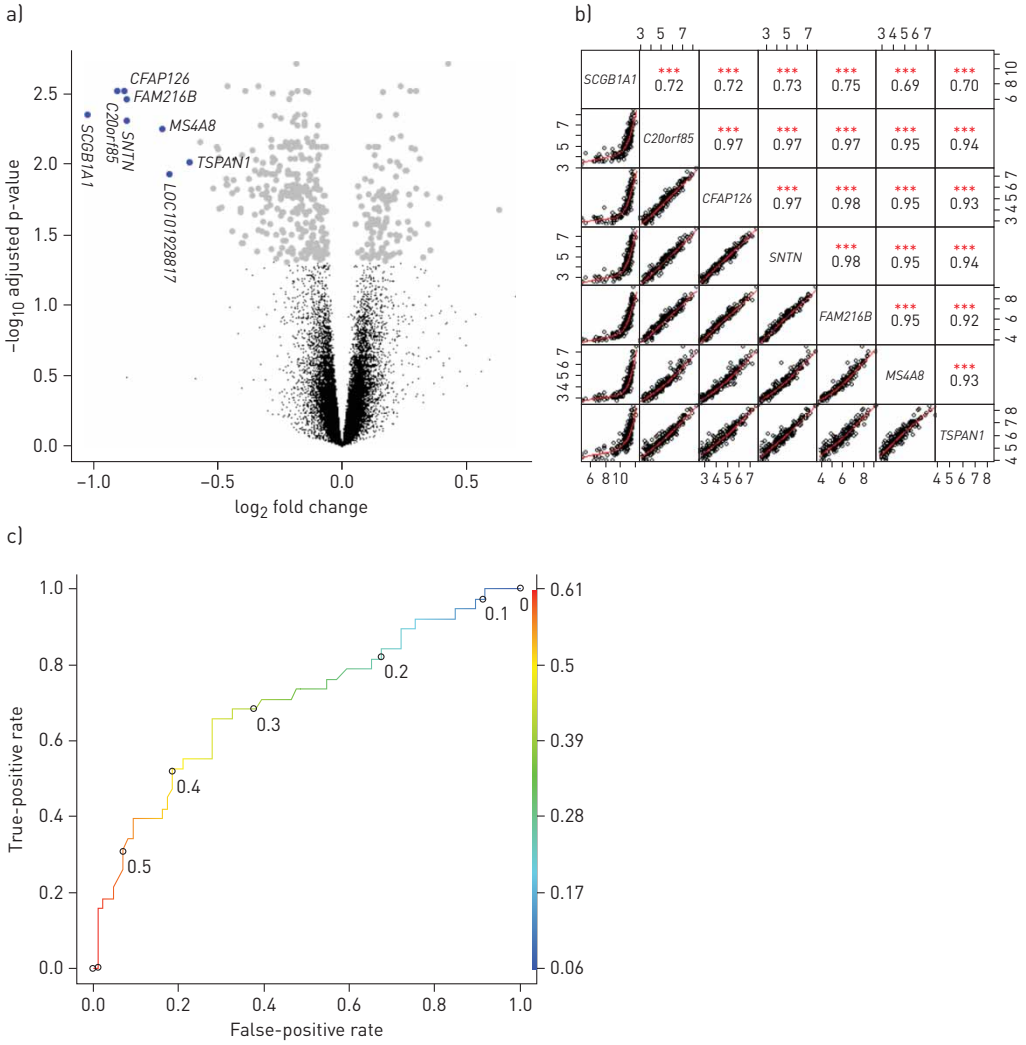

FIGURE 4 Differentially expressed genes in donation after brain death (DBD) lungs (*ex vivo* lung perfusion (EVLP) *versus* non-EVLP). a) Volcano plot showing highly differentially expressed genes (blue dots) by filtering criteria (false discovery rate <0.05 and fold change ⩾1.5 or ⩽0.7) in DBD samples (EVLP *versus* non-EVLP). b) Logistic regression model shows high correlation between the seven differentially expressed genes for DBD (EVLP *versus* non-EVLP) lungs at the end of cold ischaemic time (pre-transplant or pre-EVLP). Data values represent the correlation coefficients. ***: p<0.001. c) Receiver operating characteristic curve for the best model (*CFAP126*) determined by the stepwise procedure (area under the curve 0.70).

In current transplantation practice, clinical experience dictates which extended lungs are directed to EVLP. Our analyses revealed molecular differences between EVLP and non-EVLP samples. First, the heatmaps of differentially expressed genes of the DBD *versus* DCD lungs comparisons look more similar between all samples and EVLP-only samples than with non-EVLP lungs (figure 1). Second, significant pathways from all sample comparison shows more overlap with EVLP-only samples than with non-EVLP samples (table 3). Third, the biological networks derived from highly differentially expressed genes from all or from EVLP-only samples shared the same central node, *i.e.* IL-6 (figure 3).

We have further shown that activation of inflammatory pathways is characteristic of lungs selected for EVLP, as TNFR-1/2 signalling pathways and MIF-mediated pathways are activated in the EVLP group. A group of genes highly differentiate the EVLP group from the non-EVLP group in DBD samples. This panel of differentially expressed genes could be further tested and developed to facilitate clinical decision making on which DBD donor lungs should be subjected to EVLP assessment and repair. One of the limitations of this study is the smaller number of non-EVLP *versus* EVLP samples in the DCD group, as DCD was a relatively new practice during 2011–2015 when the samples were collected. Due to the uncertainty of using DCD lungs, the majority of DCD lungs proceeded to EVLP in our programme. The differences between the non-EVLP and EVLP groups in the DCD group are currently unknown.

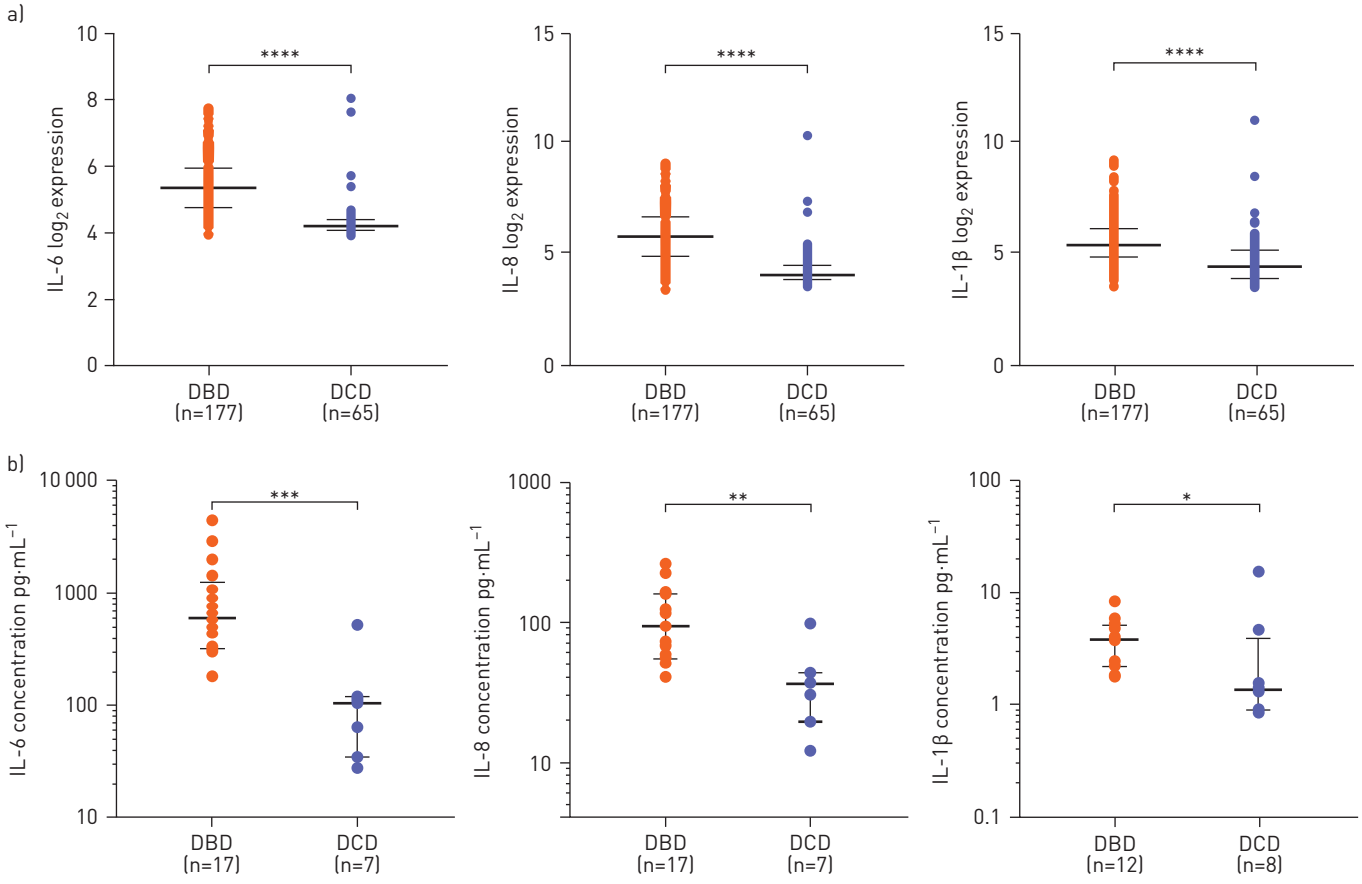

FIGURE 5 Interleukin (IL)-6, IL-8 and IL-1β levels in a) lung tissue (microarray data) and b) *ex vivo* lung perfusion perfusate (protein level). DBD: donation after brain death; DCD: donation after circulatory death. Data are presented as aligned dot plots with median and interquartile range. Statistical analyses were performed with the two-tailed nonparametric Mann–Whitney test. *: p<0.05; **: p<0.01; ***: p<0.001; ****: p<0.0001.

The second limitation is the relatively small number of EVLP perfusate samples tested for IL-6, IL-8 and IL-1β by protein assays. These cases were part of a prospective validation study for cytokine biomarkers to assess donor lung repair. As such, we used all samples so far collected. We also recognise the importance of investigating the outcome from lung transplant, *e.g.* using gene expression as biomarkers to predict donor lungs that may develop primary graft dysfunction after transplantation, which constitutes the objective of a future investigation and cannot be addressed in the present study.

Overall, our findings constitute important information for the clinical lung transplantation community, delineating different types of donor-specific lung injuries and providing important clues to potential actionable drug targets to facilitate donor lung recovery for subsequent transplantation. The EVLP signature reflects that based on current criteria on which EVLP was assigned; these lungs have distinct transcriptomic features that are different from those of directly transplanted lungs. These results provide a deeper understanding on donor lung biology, and although the immediate clinical implication of these findings remains unclear, it is hoped that they will ultimately help us to improve donor lung management, increase the number of lungs available for transplantation and potentially even improve lung allograft outcomes.

Author contributions: C. Baciu participated in the study design, performed all bioinformatics analyses, submitted the raw data to GEO and wrote the manuscript; A. Sage performed protein analysis; R. Zamel provided raw microarray data and revised the manuscript; J. Shin and X-H. Bai performed experimental validation; O. Hough prepared the figures; M. Bhat, M. Cypel and J.C. Yeung assisted with review and editing; S. Keshavjee led the biobanking and microarray studies; M. Liu and S. Keshavjee conceived the study design and supervised the project; all authors revised the final draft of the manuscript.

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