Potential therapeutic targets for lung repair during human *ex vivo* lung perfusion

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ABSTRACT

Introduction: The *ex vivo* lung perfusion (EVLP) technique has been developed to assess the function of marginal donor lungs and has significantly increased donor lung utilisation. EVLP has also been explored as a platform for donor lung repair through injury-specific treatments such as antibiotics or fibrinolytics. We hypothesised that actively expressed pathways shared between transplantation and EVLP may reveal common mechanisms of injury and potential therapeutic targets for lung repair prior to transplantation.

Materials and methods: Retrospective transcriptomics analyses were performed with peripheral tissue biopsies from "donation after brain death" lungs, with 46 pre-/post-transplant pairs and 49 pre-/post-EVLP pairs. Pathway analysis was used to identify and compare the responses of donor lungs to transplantation and to EVLP.

Results: 22 pathways were enriched predominantly in transplantation, including upregulation of lymphocyte activation and cell death and downregulation of metabolism. Eight pathways were enriched predominantly in EVLP, including downregulation of leukocyte functions and upregulation of vascular processes. 27 pathways were commonly enriched, including activation of innate inflammation, cell death, heat stress and downregulation of metabolism and protein synthesis. Of the inflammatory clusters, Toll-like receptor/innate immune signal transduction adaptor signalling had the greatest number of nodes and was central to inflammation. These mechanisms have been previously speculated as major mechanisms of acute lung injury in animal models.

Conclusion: EVLP and transplantation share common molecular features of injury including innate inflammation and cell death. Blocking these pathways during EVLP may allow for lung repair prior to transplantation.

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Introduction

Lung transplantation is the only effective treatment for patients with end-stage lung disease. In 2018, the International Society for Heart and Lung Transplantation reported that over 64 000 lung transplants had been performed worldwide, with over 4500 being conducted annually [1]. Despite improvements in the transplant procedure, ischaemia–reperfusion-induced injury remains the leading cause of primary graft dysfunction (PGD) and early recipient mortality, and contributes to the development of chronic lung allograft dysfunction (CLAD). Fear of these complications leads to a low utilisation rate (~20%) of donated lungs [2].

The *ex vivo* lung perfusion (EVLP) technique has been developed over the past 10 years. This technique restores normothermic temperature, ventilation and circulation to cold preserved donor lungs. This allows for lung function to be evaluated under physiological conditions prior to transplantation. We have demonstrated that the application of the Toronto EVLP protocol has significantly increased the utilisation of marginal donor lungs with promising clinical outcomes [3, 4].

EVLP has been further explored as a platform for donor lung repair. Clinically, high-dose antibiotics have been used to treat donor lung infection and fibrinolytic agents have been used to treat pulmonary embolism [5, 6]. These successful clinical case reports suggest that injury-specific repair of donor lungs is possible. This would be able to improve the quality of donor lungs, reduce ischaemia–reperfusion-induced lung injury, and prevent the incidence of PGD and CLAD.

During transplantation, the donor lung graft undergoes reperfusion with recipient blood which induces significant biological processes that affect reactive oxygen species production, inflammation and cell death [7]. In contrast to reperfusion, the Toronto EVLP protocol uses an acellular perfusate that contains electrolytes, buffers, albumin, dextran 40 and glucose, without blood cells, antibodies and most serum proteins found in whole blood [8]. EVLP then may conceptually be thought of as a "partial" reperfusion that essentially only restores normothermia, ventilation and circulation to donor lungs without introducing recipient blood cells or serum proteins (supplementary figure S1). Common molecular mechanisms shared by EVLP and lung transplantation may represent a subset of ischaemia–reperfusion injury that is caused by physical stress. Molecular mechanisms predominant to transplant and to EVLP may represent the effect of recipient–donor interactions during reperfusion and the biological effects of EVLP, respectively.

Injury caused by recipient–donor interactions during transplant reperfusion may vary greatly based on several factors, such as cause of donor death, donor lifestyle and condition of the recipient. In contrast, damage caused by the physical stress of ischaemia–reperfusion is far more consistent and predictable due to the use of standardised cold ischaemic preservation protocols. Therefore, targeting mechanisms of lung injury induced by the physical stress of ischaemia–reperfusion may be ideal for lung repair. We hypothesised that the shared molecular mechanisms between EVLP and transplantation may be targets for therapeutic intervention during EVLP. In this retrospective study, we conducted pathway analyses on two gene expression datasets: one containing paired pre-/post-transplant samples (direct to transplant) and another with paired pre-/post-EVLP samples from clinical cases. This allowed us to identify and compare biological pathways regulated by ischaemia–reperfusion in lung transplant or by ischaemia–perfusion in EVLP.

Methods

Study design and lung tissue

This study was approved by the Research Ethics Board of the University Health Network (Toronto, ON, Canada) (REB12-5488 and REB08-0114) and the Ethics Review Board of the Trillium Gift of Life Network (Toronto, ON, Canada). Peripheral lung tissues biopsies were collected from transplant cases. All donor lungs in this study were from "donation after brain death" (DBD) and eventually transplanted bilaterally. For the transplant group, lung samples were collected during a period from 2007 to 2012, at the end of cold ischaemic time (CIT) and after 2 h of reperfusion in the recipient (n=46 paired samples). In the EVLP group, samples were collected during a period from 2011 to 2015. Indications for EVLP are outlined in Cypel *et al.* [9]. The first sample was collected at the end of CIT1 (pre-EVLP CIT). Lungs in this group were then placed on the EVLP circuit, which was followed by a second round of cold preservation (CIT2 (post-EVLP CIT)) prior to transplantation. Post-EVLP samples were then collected at the end of CIT2 (n=49 paired samples). The transplant and EVLP datasets are available at Gene Expression Omnibus (GEO): GSE127003 and GSE127055. For validation, we used gene expression microarray data from DBD donor lungs from Kang *et al.*'s [10] study, which had samples collected at the end of CIT (pre-transplant) and after reperfusion (post-transplant) (n=12 paired samples). From Yeung *et al.*'s [11] study we used data from human lungs declined for transplant which had samples collected at the end of CIT (pre-EVLP) and at 6 h of EVLP (post-EVLP) (n=17 paired samples). Validation datasets are available at GEO: GSE127242 and GSE127057. Further details on the validation analysis can be found in the supplementary material.

Gene expression

Peripheral lung tissue biopsies were collected and snap frozen in liquid nitrogen. Gene expression profiles were measured with microarrays by the Princess Margret Genomics Center (Toronto, ON, Canada) according to the manufacturer's protocol. Transplant samples were run on Human Genome U133 Plus 2.0 arrays (Affymetrix, Santa Clara, CA, USA) and EVLP samples were run on Clariom D arrays (Affymetrix).

Pathway analysis and network generation

To enable us to perform a comparison of cross-model transcriptomic changes, we followed an analysis pipeline as described in figure 1a [12]. Differential gene expression was calculated using the Bayes moderated paired t-test comparing the "post" *versus* "pre" time-points for the transplant and EVLP groups. To create ranked lists for pathway analysis, genes were ranked based on a gene score (supplementary material). A pre-ranked gene set enrichment analysis (GSEA) was conducted on each ranked list [13].

Enriched pathways from the transplant and EVLP samples which met the cut-off of false discovery rate (FDR) <0.05 were plotted together and clustered to group highly similar pathways using the EnrichmentMap and AutoAnnotate Cytoscape apps [14, 15]. Pathway clusters were manually annotated. Only clusters with four or more pathways were included in this analysis. Each cluster was then categorised based on a predominance score calculated as: (number of transplant enriched gene sets−number of EVLP enriched gene sets)/total number of nodes in cluster. Clusters with a predominance score $\geqslant 0.8$ were classified as predominant in transplant while clusters with a predominance score $\leqslant -0.8$ were classified as predominant in EVLP. Clusters with a predominance score between −0.8 and 0.8 were classified as common to both transplant and EVLP.

Detailed information on sample processing, microarray pre-processing, GSEA parameters, network construction and principal component analysis (PCA) can be found in the supplementary material.

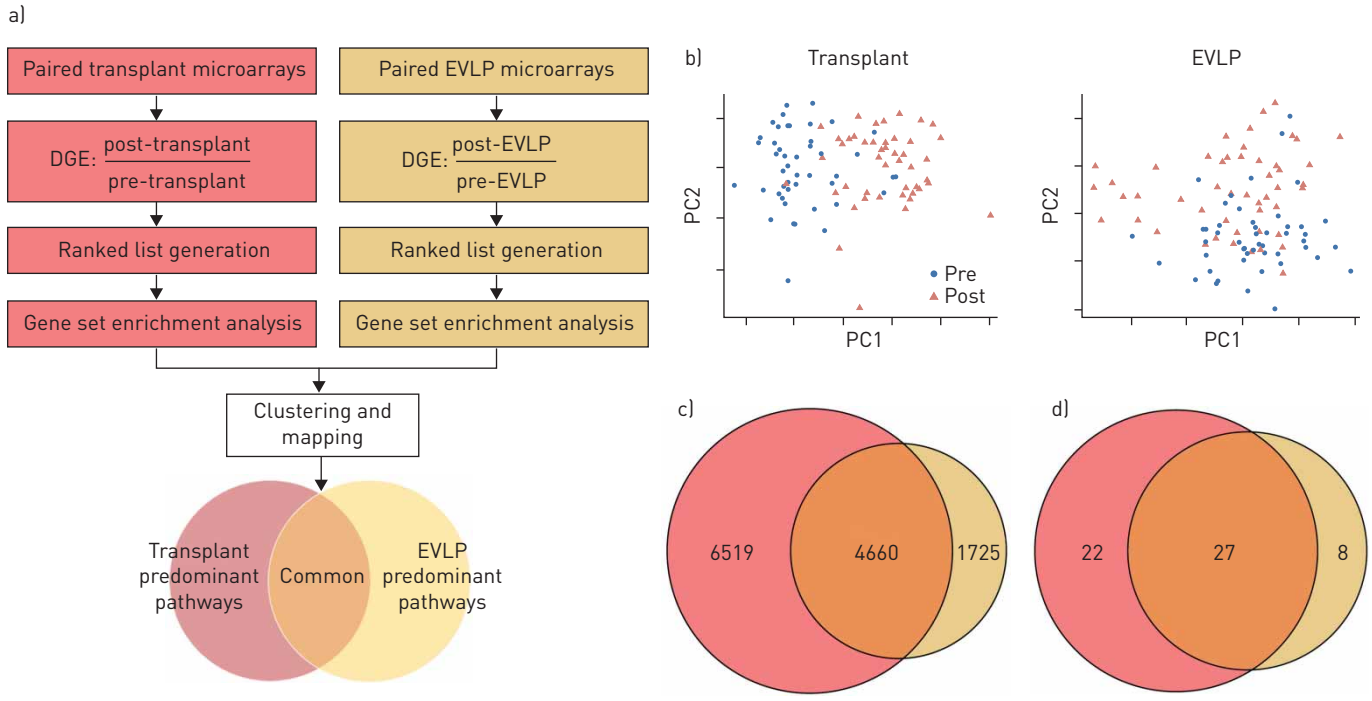

FIGURE 1 Gene expression profiling from human lung transplant and *ex vivo* lung perfusion (EVLP) samples. DGE: differential gene expression; PCA: principal component analysis; FDR: false discovery rate. a) Study design. Transplant and EVLP gene expression profiles were independently pre-processed. DGE between "post" and "pre" time-points was calculated. p-values from DGE were used to score genes and create a ranked list. Pathway enrichment analysis was conducted. Pathways from both datasets were visualised using EnrichmentMap and AutoAnnotate. Pathways were sorted into one of three categories based on their enrichment pattern among datasets. b) PCA of all genes from each microarray which reveal distinct clusters between "pre" and "post" time-points in the transplant and EVLP datasets. c) Comparison of the differentially expressed genes in the transplant (red) and EVLP (yellow) groups using the 20 297 genes which were commonly detectable on both microarray platforms at FDR <0.05. d) Comparison of the number of enriched pathways in transplantation (red) and EVLP (yellow) at FDR <0.05.

Results

Donor lung demographics are shown in table 1. The mean donor age in the transplantation dataset (49.0±16.1 years) was higher than in the EVLP dataset (40.5±14.4 years) (p=0.008).

CIT, male:female ratio and transplant outcomes were not significantly different between datasets (table 1).

Reperfusion of lung allografts or EVLP induces significant gene expression changes

PCA analysis revealed that in both transplant and EVLP datasets, "pre" and "post" time-points resulted in distinct clusters indicating that transplantation and EVLP (figure 1b) induced major changes in gene expression.

Of the 20 297 genes analysed on both microarray platforms, 11 179 genes were differentially expressed in the transplant group and 6385 genes were differentially expressed in the EVLP group at FDR <0.05. When compared, 4660 differentially expressed genes were found to be in common (figure 1c).

GSEA identified 822 enriched pathways induced by transplantation and 271 enriched pathways induced by EVLP (FDR <0.05). Enriched pathways from both groups were plotted and clustered in Cytoscape, forming 101 clusters. We removed any clusters with fewer than four pathways from the analysis, leaving 57 pathways. After sorting, 22 were classified as predominant in transplant, eight predominant in EVLP and 27 in common (figure 1d and supplementary table S1).

Reperfusion of lung allografts is associated with the upregulation of inflammatory pathways and downregulation of metabolic pathways

During reperfusion, recipient leukocytes enter donor lung tissues. As a result, transplant enriched pathways contain both the response of the donor lung tissue to the reperfusion of recipient blood and signals from the recipient leukocytes. Three major pathway themes were enriched predominantly in transplant: inflammation, cell death and metabolism. Inflammation was upregulated and regulation of cell death was increased. The most prominent pathways in the inflammation theme were HIV-negative regulatory factor (NEF) and tumour necrosis factor (TNF) signalling and regulation of mitogen-activated protein kinases signalling, followed by leukocyte chemotaxis and T-cell receptor (TCR)/B-cell receptor (BCR) signal transduction. In contrast, genes related to metabolism (oxidative phosphorylation) were downregulated (figure 2).

EVLP is associated with downregulation of pathways relating to leukocyte function

In the predominantly EVLP enriched pathways, leukocyte-associated processes were downregulated, especially phosphatidylinositol biosynthesis, as well as phospholipase C (PLC) signalling, Golgi vesicle trafficking, protein targeting to vacuole and cholesterol biosynthesis. Pathways relating to vascular processes such as adherens junction organisation and regulation of vasodilation were upregulated (figure 3). A single cell death pathway relating to the negative regulation of cell death in epithelial and endothelial cells was upregulated.

TABLE 1 Donor lung characteristics for transplant and *ex vivo* lung perfusion (EVLP) datasets

	Transplant	EVLP	p-value
Period	Feb 2007–Jan 2012	Feb 2011–Dec 2015	
Mean donor age years	48.98±16.07	40.49±14.39	0.008
Donor male:female ratio	21:25	31:18	0.10
CIT[#] h	4.48±1.57	4.54±1.81	0.7034943
Mean EVLP time h		5.02±0.84	
PGD grade[¶]			
0/1	19	29	
2	14	8	
3	13	8	0.0863
ICU length of stay days	18.37±38.72	9.82±14.49	0.1645

Data are presented as mean±SD or n, unless otherwise stated. CIT: cold ischaemic time; PGD: primary graft dysfunction. PGD was graded over the first 72 h post-transplant according to International Society for Heart and Lung Transplantation guidelines. The Chi-squared test was used to compare PGD grade between the transplant and EVLP groups. [#]: CIT was only available for 35 lungs in the EVLP dataset; [¶]: PGD grade was only available for 45 lungs in the EVLP dataset.

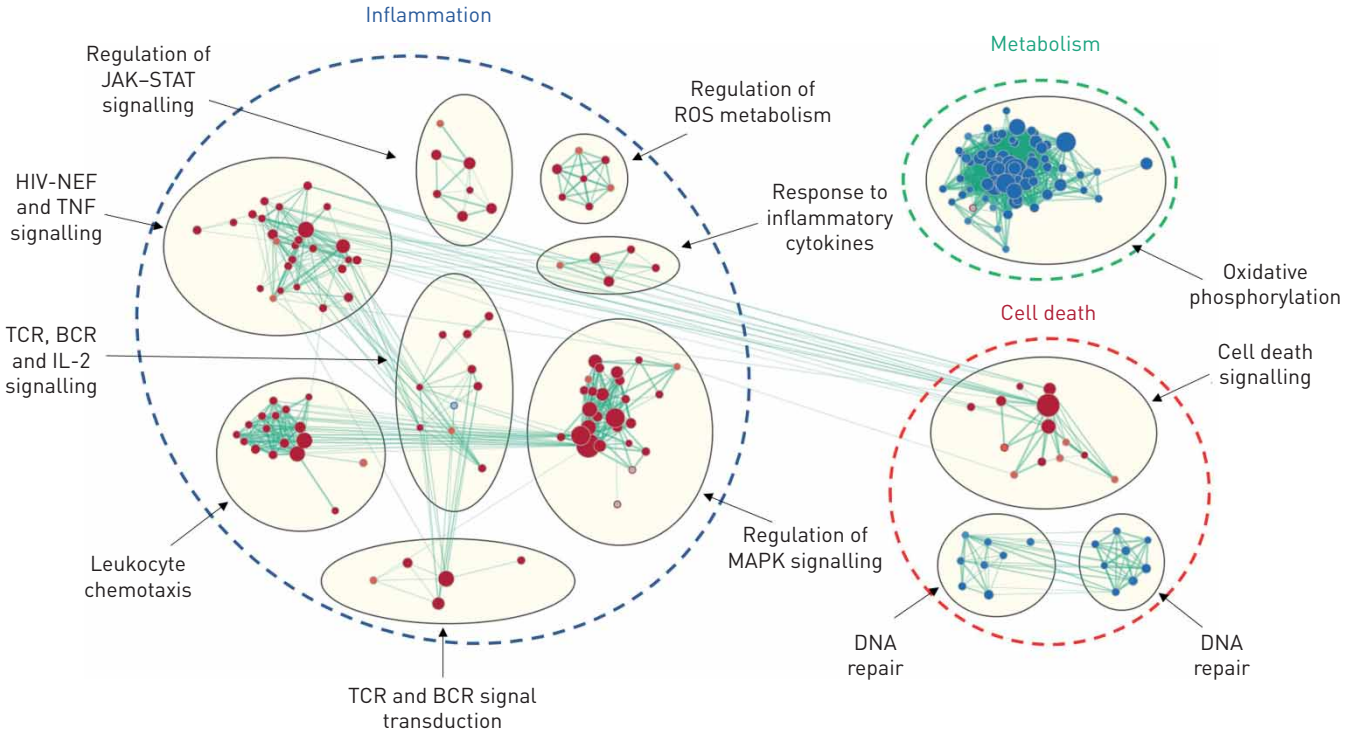

FIGURE 2 Pathways enriched predominantly in lung transplant. JAK: Janus kinase; STAT: signal transducer and activator of transcription; ROS: reactive oxygen species; NEF: negative regulatory factor; TCR: T-cell receptor; BCR: B-cell receptor; IL: interleukin; MAPK: mitogen-activated protein kinase. Pathways fell into three major themes, with inflammation and cell death generally upregulated (red nodes) and metabolism downregulated (blue nodes).

Pathways related to inflammation and cell death are significantly upregulated during both transplantation and EVLP

Inflammation and apoptosis are upregulated in commonly enriched pathways (figure 4). These include Toll-like receptor (TLR)/innate immune signal transduction adaptor (MYD88) signalling, response to TNF and interleukin (IL)-1, a response to bacteria, regulation of leukocyte chemotaxis, and regulation of

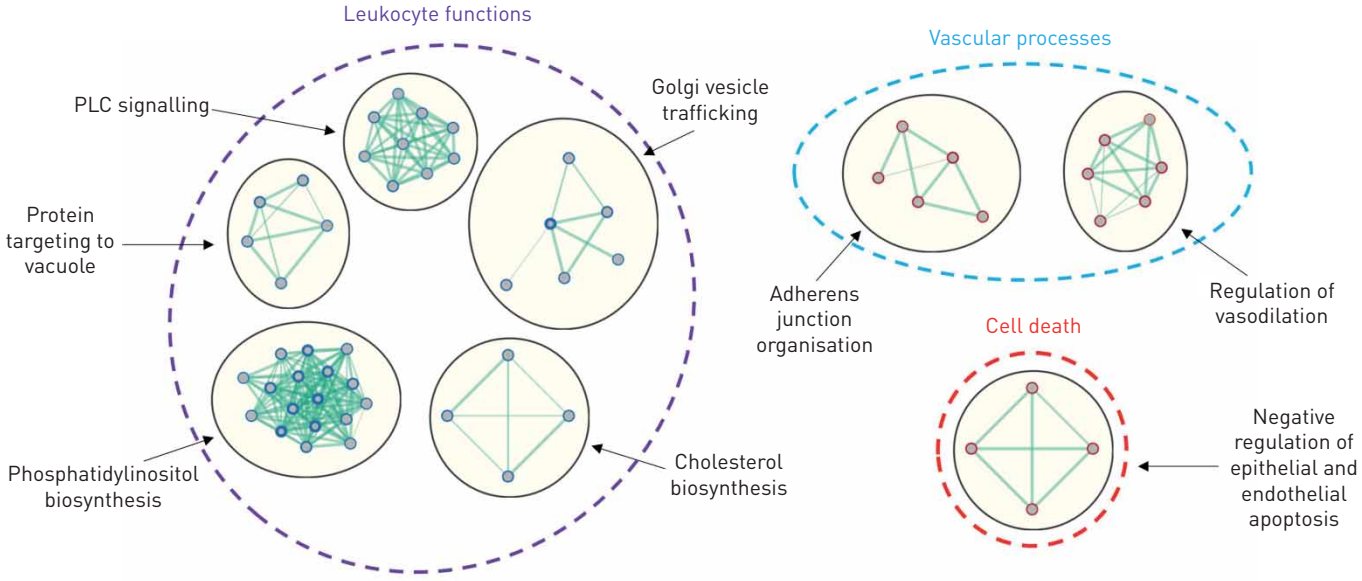

FIGURE 3 Pathways enriched predominantly in *ex vivo* lung perfusion. PLC: phospholipase C. Pathways fell into three major themes, with vascular processes and cell death generally upregulated (red nodes) and leukocyte functions downregulated (blue nodes).

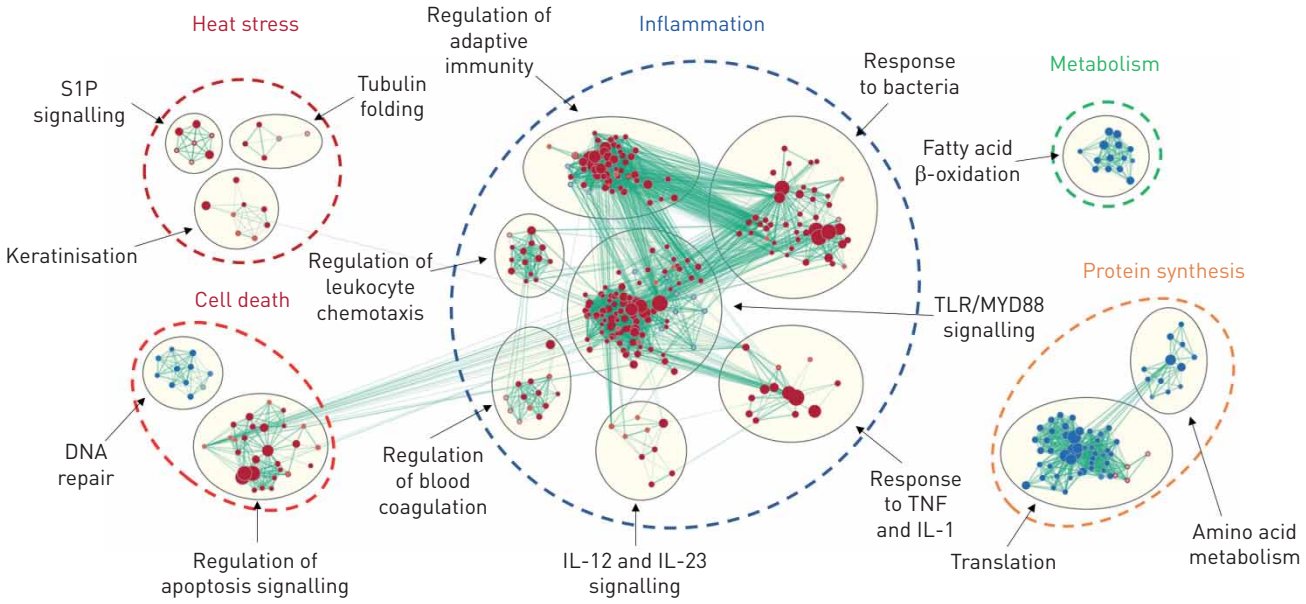

FIGURE 4 Pathways enriched in both transplant and *ex vivo* lung perfusion. S1P: sphingosine 1-phosphate; TLR: Toll-like receptor; MYD88: innate immune signal transduction adaptor; TNF: tumour necrosis factor; IL: interleukin. Pathways fell into five major themes, with inflammation, cell death and heat stress generally upregulated (red nodes) and metabolism and protein synthesis downregulated (blue nodes).

adaptive immunity. Indeed, these have been speculated and empirically explored as major mechanisms of acute lung injury [16]. Activation of cell death pathways is found commonly in both transplant and EVLP. Regulation of blood coagulation was upregulated. In addition, heat stress (keratinisation, sphingosine 1-phosphate signalling and tubulin folding) pathways were upregulated, while metabolism (fatty acid β-oxidation) and protein synthesis (translation and amino acid metabolism) were downregulated.

Validation

To confirm the robustness of our newly identified associations, we used gene expression data from two independent studies including donor lungs pre-/post-transplant and pre-/post-EVLP [10, 11]. In Kang *et al.*'s [10] pre-/post-transplant dataset, five themes were present in the enriched pathways: inflammation, heat stress and cell death were generally upregulated, and metabolism and protein synthesis downregulated (supplementary figure S2). From Yeung *et al.*'s [11] pre-/post-EVLP dataset, six major themes were observed: inflammation, cell death and vascular processes were generally upregulated, and metabolism, protein synthesis and leukocyte processes downregulated (supplementary figure S3).

Discussion

When donor lungs undergo hypothermic preservation followed by normothermic EVLP or reperfusion, there are major changes in temperature, ventilation and perfusion, which have a significant impact on lung cell biology. This may, in part, explain why EVLP or transplantation induces major changes in gene expression. In lung transplants there are additional interactions between donor lung and recipient cells, cytokines and proteins in the blood, which complicates the comparison with the EVLP group. However, the present experiments were designed based on these two clinical situations, and our objective was to determine the differences and similarities of the gene profiles between them. Our results implicate acute inflammation and cell death as major molecular events in both EVLP and transplantation. These may be the most important mechanisms in ischaemia–reperfusion injury in lung transplants and EVLP may provide opportunities for donor lung repair through targeting these pathways.

Lymphocyte activation and inhibition of metabolism in lung allograft reperfusion

Leukocyte recruitment and activation have been considered to be major inflammatory events in lung allografts during reperfusion [17]. Indeed, pathways associated with regulation of leukocyte chemotaxis and response to inflammatory cytokines were upregulated in the lung after reperfusion. Importantly, previous studies which measured gene expression in bronchoalveolar lavage fluid of lung transplant recipients found that innate inflammation was upregulated in those that developed PGD grade 3 *versus* those that did not [18].

We were surprised to find a pathway associated with HIV-NEF and TNF signalling predominant to transplant because HIV-positive lungs are not used for transplantation. Curiously, previous studies have found that the HIV-NEF protein is involved in modulating T-cell activation which is thought to aid the virus in infecting T-cells [19]. Enrichment of HIV-NEF pathways in the context of transplantation may indicate that T-cells are being activated during reperfusion. Pathways associated with TCR/BCR signal transduction and Janus kinase/signal transducer and activator of transcription (JAK–STAT) signalling were also upregulated. IL-2 is considered a robust marker of $CD4^+$ T-cell activation [19], while the JAK–STAT signalling pathway is known to be involved in $CD4^+$ T-cell differentiation [20]. Taken together, these pathways plausibly suggest that lymphocytes from the recipient blood migrate into the allograft and become activated during reperfusion. This idea is further supported by previous work showing that lymphocytes both accumulate in lung grafts and mediate ischaemia–reperfusion injury in rat lung transplant models [21, 22]. Lymphocyte activation and cytokine production pathways could be important therapeutic targets for ischaemia–reperfusion injury during lung allograft reperfusion. Early accumulation of lymphocytes may contribute to allograft-induced acute and chronic rejection after transplantation.

EVLP associated depletion or inhibition of passenger leukocytes

Marginal (injured) donor lungs transplanted after EVLP showed similar outcomes to regular lung transplants, which has led to the clinical observation that EVLP may benefit donor lungs. In the present study, pathways associated with leukocyte function such as phosphatidylinositol biosynthesis, PLC signalling, cholesterol biosynthesis, protein targeting to vacuole and Golgi vesicle trafficking were all downregulated in EVLP samples. These results support previous studies which have inferred that passenger leukocytes are depleted during EVLP [11]. The phosphatidylinositol biosynthesis and PLC signalling pathways are essential in neutrophil degranulation [23, 24]. Cholesterol biosynthesis has also been shown as a mechanism by which neutrophils modulate adherence to activated endothelium [25]. Protein targeting to vacuoles and Golgi vesicle trafficking may represent the biosynthesis and packaging of inflammatory cytokines by neutrophils and macrophages.

We noted that pathways related to cell adherens junction organisation were upregulated, which is consistent with our previous report that EVLP protects the alveolar epithelial junctions in porcine lungs [26]. A pathway relating to the negative regulation of epithelial and endothelial cell death was also upregulated. These results suggest that short periods of EVLP may protect lungs by depleting/inhibiting passenger leukocytes and providing an opportunity for lungs to recover and heal.

Inflammatory responses and apoptotic cell death as therapeutic targets during EVLP

Knowing inflammation and cell death are major mechanisms of ischaemia–reperfusion injury (figure 2) and the partial beneficial effects of EVLP (figure 3), we are surprised to find that inflammation and apoptosis signalling are also shared between transplant and EVLP gene clusters (figure 4). Of the five major pathway themes common to transplant and EVLP, inflammation had the greatest number of pathways, including upregulation of TLR/MYD88 signalling (figure 4). Activation of TLRs is consistent with previous studies which have found that TLR expression is upregulated in the peripheral blood of transplant recipients within 2 h of reperfusion [27]. Studies in mouse lung transplant models have also shown that TLRs are activated during pulmonary ischaemia–reperfusion and mediate injury [28]. Activation of TLR/MYD88 pathways triggers the release of inflammatory cytokines, TNF and IL-12, from neutrophils and macrophages [29]. Consistent with these events, we observed upregulation of pathways associated with the cellular response to TNF and IL-12. We also observed upregulation of pathways relating to a response to bacteria, which is likely attributed to TLR4 activation. TLR4, the most studied TLR activated during lung transplant, is a sensor for lipopolysaccharides (LPS) and may be responsible for inducing a bacterial response when activated [30]. Human lung epithelial cells have been shown to express TLR4, respond to LPS and secrete inflammatory cytokines when stressed by conditions simulating lung preservation and reperfusion [31–33]. Intriguingly, we also found that during EVLP an upregulated pathway associated with regulation of adaptive immunity was highly connected to TLR/MYD88 signalling in terms of shared genes. This suggests that activation of TLR signalling in the lung allograft may promote lymphocyte activation independent of the recipient. Taken together, these data suggest that there is activation of innate immunity during both reperfusion and EVLP.

Metabolism (β-oxidation) and protein synthesis (translation and amino acid metabolism) were downregulated. This may represent mitochondrial dysfunction and an effort by cells to downregulate energy-intensive processes to conserve cytosolic ATP [34]. Mitochondrial dysfunction has been well studied in cardiac ischaemia–reperfusion and mitochondria have been shown to be damaged in rat lung ischaemia–reperfusion models [35, 36]. These novel findings should also be considered when developing strategies to ameliorate reperfusion-induced lung injury.

On the theme of cell death, the apoptosis signalling pathway was upregulated, while the DNA repair pathway was downregulated. Cell death is a major contributing factor in acute lung injury [37]. Apoptosis of tissue cells has been noted in human donor lungs [38]. In a rat lung transplant model, apoptosis was shown to be the major mode of cell death in hypothermic preserved lungs, while necrosis was predominantly observed after reperfusion [38]. Blocking apoptosis pathways in donor lungs has partially protected lung function after reperfusion [39].

EVLP has been developed as a platform for donor lung repair and regeneration. The discovery of activation of inflammation and cell death pathways in the present study indicates that these could be further developed as therapeutic targets during EVLP. In our recent studies we found that α_1-antitrypsin could inhibit inflammation and cell death [40]. When used in a cell culture model that simulates ischaemia–reperfusion conditions seen in lung transplantation, it reduced inflammatory cytokines and cell death [41]. It prevented ischaemia–reperfusion injury in pig lung transplants and improved pig lung function during EVLP [42, 43]. Thus, targeting inflammation and cell death during EVLP could improve donor lung quality and improve outcome after lung transplantation. To identify potential therapeutics we used the enrichment map post-analysis and a database of US Food and Drug Administration-approved drugs to identify potential therapeutics targeting the commonly activated pathways, and a number of potential drugs were identified (supplementary table S2). These could be further tested and validated through experimentation.

Limitations and resolutions

In this retrospective study, the lung transplant and EVLP datasets were from two separate cohorts. The mean age of donor lungs in the transplant dataset was significantly higher than in the EVLP dataset. As a proxy comparison for a relationship between age and gene expression, we compared the 10 oldest and youngest lungs from each dataset. This analysis did not identify any differential gene expression between young and old lungs at FDR <0.05 (supplementary material).

EVLP is predominantly used to evaluate marginal donor lungs for transplantation. Therefore, the quality of lungs in the EVLP group may have been lower than those in the direct-to-transplant group. Our experimental design utilised a paired analysis strategy, which allowed each lung to act as its own baseline control. This allowed us to minimise the possible effects of differences in the mean donor age and collection periods between transplant and EVLP datasets.

We wanted to identify potential therapeutic targets for further study during EVLP; we selected a threshold of |0.8| to capture common pathways between lung transplant and EVLP. We compared trends on categorisation across a range of thresholds between |0.6| and |0.9| (supplementary figure S4). As expected, the number of common pathways decreased with a lower threshold and increased with a higher threshold. The threshold of |0.8| represents a sharp transition from less to more stringent selection.

Moreover, we validated our results through analysis of independent data. Results from our validation sets recapitulated similar pathways that we identified as being predominant in transplantation, predominant in EVLP and common to both (supplementary figures S2 and S3).

Results from the present study will trigger more mechanistic research in lung transplantation. For example, we could compare cases which did or did not develop PGD, compare lungs treated with or without EVLP and compare donor lungs declined after EVLP with those successfully used for transplantation. These studies are currently being addressed in our own group and others. With the decreasing cost of bulk and single-cell RNA sequencing, these new technologies will allow us to identify case-specific factors which affect clinical outcome.

In conclusion, we compared the effects of EVLP and transplantation in human lungs using a transcriptome-wide approach. We found that both forms of reperfusion enrich for gene set clusters associated with inflammation and apoptosis. Therapeutic targeting of these pathways during EVLP may allow for lung repair prior to implantation and improve transplant outcomes.

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