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Potential Therapeutic Targets for Lung Repair During Human Ex Vivo Lung Perfusion

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Take home message:

Inflammation and cell death pathways are common molecular features of ischemia-reperfusion and ischemia-ex vivo lung perfusion. These may represent therapeutic targets for lung repair prior to transplantation.

Abstract

Introduction: The ex vivo lung perfusion (EVLP) technique has been developed to assess the function of marginal donor lungs which has significantly increased donor lung utilization. EVLP has also been explored as a platform for donor lung repair through injury specific treatments such as antibiotics or fibrinolytics. We hypothesized 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: A 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, TLR/MYD88 signaling 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.

Keywords: ischemia reperfusion injury, lung transplantation, EVLP, bioinformatics

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 have been performed worldwide, with over 4,500 being conducted annually (1). Despite improvements in the transplant procedure, ischemia-reperfusion (IR) induced injury remains the leading cause of primary graft dysfunction (PGD), early recipient mortality, and contributes to the development of chronic lung allograft dysfunction (CLAD). Fear of these complications leads to low utilization rate (approximately 20%) of donated lungs (2).

The ex vivo lung perfusion (EVLP) technique has been developed over the past ten 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 utilization 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 (5), and fibrinolytic agents have been used to treat pulmonary embolism (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 IR 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 1). Common molecular mechanisms shared by EVLP and lung transplantation may represent a subset of IR 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 biological effects of EVLP respectively.

Injury caused by recipient-donor interactions during transplant reperfusion may vary greatly based on several factors such as the cause of donor death, donor lifestyle, and condition of the recipient. In contrast, damage caused by the physical stress of IR is far more consistent and predictable due to the use of standardized cold ischemic preservation protocols. Therefore, targeting mechanisms of lung injury induced by the physical stress of IR may be ideal for lung repair. We hypothesized 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 IR in lung transplant, or by ischemia-perfusion in EVLP.

Methods

Study Design and Lung Tissue

This study was approved by the University Health Network research ethics board (REB #12-5488, REB #08-0114) and ethics review board of the Trillium Gift of Life Network. 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 ischemic time (CIT) and after 2-hour reperfusion (R) 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 GEO: GSE127003 & GSE127055 (Reviewer Access Token: “wpevuemixtuvxmr”). For validation, we used gene expression microarray data from DBD donor lungs from Kang et al.’s study (10) 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 study (11) we used data from human lungs declined for transplant which had samples collected at the end of CIT (pre-EVLP) and at 6h EVLP (post-EVLP) (n=17 paired samples). Validation datasets available at GEO: GSE127242 & GSE127057. Further details on the validation analysis can be found in the Supplementary Methods.

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, Canada) according to the manufacturer protocol. Transplant samples were run on Human Genome U133 Plus 2.0 arrays (Affymetrix; Santa Clara, CA) and EVLP samples 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 (12) as described in Figure 1A. Differential gene expression was calculated using Bayes moderated paired t-test comparing the post versus pre timepoints for the transplant and EVLP groups. To create ranked lists for pathway analysis, genes were ranked based on a gene score (Supplementary Methods). A pre-ranked gene set enrichment analysis (GSEA) (13) was conducted on each ranked list.

Enriched pathways from the transplant and EVLP samples which met the cutoff of FDR < 0.05 were plotted together and clustered to group highly similar pathways using the Enrichment Map 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 categorized based on a predominance score calculated by the formula:

$$\frac{number\ of\ transplant\ enriched\ gene\ sets\ -\ number\ of\ EVLP\ enriched\ gene\ sets}{total\ number\ of\ nodes\ in\ cluster}$$

Clusters with a predominance score of ≥ 0.8 were classified as predominant in transplant while cluster with a score of ≤ -0.8 were classified as predominant in EVLP. Clusters with a score between -0.8 and 0.8 were classified as common to both transplant and EVLP.

Detailed information on sample processing, microarray preprocessing, GSEA parameters, network construction, and principal component analysis (PCA) can be found in the Supplementary Appendix.

Results

Donor lung demographics can be found in Table 1. The mean donor age in the transplantation dataset (49.0 ± 16.1) was higher than those in the EVLP dataset (40.5 ± 14.4) (p=0.008).

Cold ischemic time, male to 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 analyzed on both microarray platforms, 11,179 genes were differentially expressed in the transplant group and 6,385 in EVLP at FDR<0.05. When compared, 4,660 differentially expressed genes were found to be in common (Figure 1C).

GSEA identified 822 enriched pathways induced by transplantation, and 271 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 4 pathways from the analysis, leaving

57 pathways. After sorting, 22 were classified as predominant in transplant, 8 predominant in EVLP and 27 in common (Figure 1D, 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 up-regulated and regulation of cell death was increased. The most prominent pathways in the inflammation theme was human immunodeficiency virus (HIV) Negative Regulatory Factor (NEF) and Tumor Necrosis Factor (TNF) signaling, Regulation of mitogen-activated protein kinases (MAPK) signaling, followed by leukocyte chemotaxis and T-cell receptor (TCR) / B-cell receptor (BCR) signal transduction. In contrast, genes related to metabolism (oxidative phosphorylation) were down-regulated (Figure 2).

EVLP is associated with downregulation of pathways relating to leukocyte function

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

Pathways related to inflammation and cell death are significantly up-regulated during both transplantation and EVLP

Inflammation and apoptosis are up-regulated in commonly enriched pathways (Figure 4). This includes Toll like receptor (TLR) / innate immune signal transduction adaptor (MYD88) signaling, response to TNF and interleukin 1 (IL-1), a response to bacteria, regulation of leukocyte chemotaxis, and regulation of 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 up-regulated. In addition, heat stress (keratinization, S1P signaling, and tubulin folding) pathways were up-regulated, while metabolism (fatty acid β-oxidation), and protein synthesis (translation, amino acid metabolism) were down-regulated.

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 (10) and pre-/post-EVLP (11). In Kang's pre-/post-transplant dataset, 5 themes were present in the enriched pathways with inflammation, heat stress and cell death were generally up-regulated, and metabolism, and protein synthesis down-regulated (Supplementary Figure 2). From Yeung's pre-/post-EVLP dataset, 6 major themes were observed, with inflammation, cell death and vascular processes generally up-regulated. Metabolism, protein synthesis and leukocyte processes down-regulated (Supplementary Figure 3).

Discussion

When donor lungs undergo hypothermic preservation followed by normothermic EVLP or reperfusion, there are major changes in temperature, ventilation, perfusion, which have significant impact on lung cell biology. This may in part, explain why EVLP or transplantation induce 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 are designed based on these two clinical situations, and our objective is to determine the differences and similarities of the gene profiles between them. Our results implicate that acute inflammation and cell death are major molecular events in both EVLP and transplantation. This may be the most important mechanisms in IR 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 a major inflammatory event in lung allografts during reperfusion (17). Indeed, pathways associated with regulation of leukocyte chemotaxis and response to inflammatory cytokines were up-regulated in the lung after reperfusion. Importantly, previous studies which measured gene expression in the bronchoalveolar lavage fluid of lung transplant recipients found that innate inflammation was upregulated in those that developed PGD 3, versus those that did not (18).

We were surprised to find a pathway associated with HIV-NEF and TNF signaling 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 transducers and activators of transcription (JAK-STAT) signaling were also up-regulated. IL-2 is considered a robust marker of CD4+ T cell activation (19), while the JAK-STAT signaling 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 that has shown that lymphocytes both accumulate in lung graft and mediate IR injury in rat lung transplant models (21, 22). Lymphocyte activation and cytokine production pathways could be important therapeutic targets for IR 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 with regular lung transplants, which has led to the clinical observation that EVLP may benefit donor lungs. In the present study, pathways associated with leukocyte functions such as phosphatidylinositol biosynthesis, PLC signaling, cholesterol biosynthesis, protein targeting to vacuole and Golgi vesicle trafficking were all down-regulated in EVLP samples. These results support previous studies which have inferred that passenger leukocytes are depleted during EVLP (11). Phosphatidylinositol biosynthesis and the PLC signaling pathway 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 organization were up-regulated, 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 IR injury (Figure 2) and partial beneficial effects of EVLP (Figure 3), we are surprised to find that inflammation and apoptosis signaling are also shared between transplant and EVLP gene clusters (Figure 4). Of the 5 major pathway themes common to transplant and EVLP, inflammation had the greatest number of pathways, including upregulation of TLR/MYD88 signaling (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 hours of reperfusion (27). Studies in mouse lung transplant models have also shown that TLRs are activated during pulmonary IR and mediate injury (28). Activation of TLR/MYD88 pathways triggers the release of inflammatory cytokines, TNF and interleukin-12 (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 (31), and secrete inflammatory cytokines when stressed by conditions simulating lung preservation and reperfusion (32, 33). Intriguingly, we also found that during EVLP an upregulated pathway associated with regulation of adaptive immunity was highly connected to TLR/MYD88 signaling in terms of shared genes. This suggests that activation of TLR signaling 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 IR (35), and mitochondria have been shown to be damaged in rat lung IR models (36). These novel findings should also be considered when developing strategies to ameliorate reperfusion-induced lung injury.

In the theme of cell death, the apoptosis signaling pathway was up-regulated while the DNA repair pathway was down-regulated. Cell death is a major contributing factor in acute lung injury (37). Apoptosis of tissue cells have 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 indicate that these could be further developed as therapeutic targets during EVLP. In our recent studies we found that, alpha 1-antitrypsin (A1-AT) can inhibit inflammation and cell death (40). When used in a cell culture model that simulates IR conditions seen in lung transplantation, it reduced inflammatory cytokines and cell death (41). It prevented IR injury in pig lung transplants (42) , and improved pig lung function during EVLP (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 FDA approved drugs to identify potential therapeutics targeting the commonly activated pathways, a number of potential drugs was identified (Supplementary Table 2). This 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 the EVLP group. 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 Data 1).

EVLP is predominantly used to evaluate marginal donor lungs for transplantation. Therefore, the quality of lungs in the EVLP group may be lower in quality than those in the direct to transplant group. Our experimental design utilized a paired analysis strategy, which allowed

each lung to act as its own baseline control. This allowed us to minimize the possible effects of differences in the mean donor age and collection periods between transplant and EVLP datasets.

We want 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 categorization across a range of threshold between 0.6-0.9 (Supplementary Figure 4). 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 Figure 2 and 3).

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 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 ex vivo lung perfusion 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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Declaration of Interest

The authors have no conflicts of interests to report.

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Figure Legends

Figure 1. **Gene Expression Profiling from Human Lung Transplant and Ex Vivo Lung Perfusion Samples (EVLP)**. **A.** Study design**.** Transplant and EVLP gene expression profiles were independently preprocessed. Differential gene expression (DGE) between post and pre time points is calculated. P-values from DGE are used to score genes and create a ranked list. Pathway enrichment analysis is conducted. Pathways from both datasets were visualized using Enrichment Map and AutoAnnotate. Pathways were sorted into one of three categories based on their enrichment pattern among datasets. **B** principal component analysis (PCA) of all genes from each microarray which reveal distinct clusters between pre (blue circles) and post (red triangles) time points in transplant and EVLP datasets. **C.** Comparison of the differentially expressed genes in transplant (red) and EVLP (beige) groups using the 20,297genes which were commonly detectable on both microarray platforms at an FDR < 0.05. **D.** Comparison of the number of enriched pathways in transplantation (red) and EVLP (beige) at FDR < 0.05.

Figure 2. Pathways Enriched Predominantly in Lung Transplant. Pathways fell into three major themes with inflammation and cell death generally up-regulated (red nodes) and metabolism down-regulated (blue nodes).

Figure 3. Pathways Enriched Predominantly in EVLP. Pathways which fell into three major themes with vascular processes and cell death generally up-regulated (red nodes) and leukocyte functions down-regulated (blue nodes).

Figure 4. Pathways Enriched in both Transplant and EVLP. Pathways fell into five major themes with inflammation, cell death and heat stress generally up-regulated (red nodes) and metabolism and protein synthesis down-regulated (blue nodes).

Table 1: Donor Lung Characteristics for Transplant and EVLP Datasets. Primary graft dysfunction was graded over the first 72 hours post transplant according to ISHLT guidelines. The Chi-square test was used to compare PGD grade between the transplant and EVLP groups.

		Transplant	EVLP	P Values
Period		Feb 2007-Jan 2012	Feb 2011-Dec 2015	—
Mean donor age		48.98 ± 16.07	40.49 ± 14.39	0.008
Donor M:F Ratio		21:25	31:18	0.10
Cold Ischemic Time		4.48 ± 1.57	4.54 ± 1.81**	0.7034943
Mean EVLP time (h)		—	5.02 ± 0.84	—
PGD#	**Grade 0/1**	19	29	
	Grade 2	14	8	
	Grade 3	13	8	0.0863
ICU Length of Stay (days)		18.37 ± 38.72	9.82 ± 14.49	0.1645

*Data above presented as mean ± standard deviation.

**Cold ischemic time was only available for 35 lungs in the EVLP dataset.

PGD grade was only available for 45 lungs in the EVLP dataset.

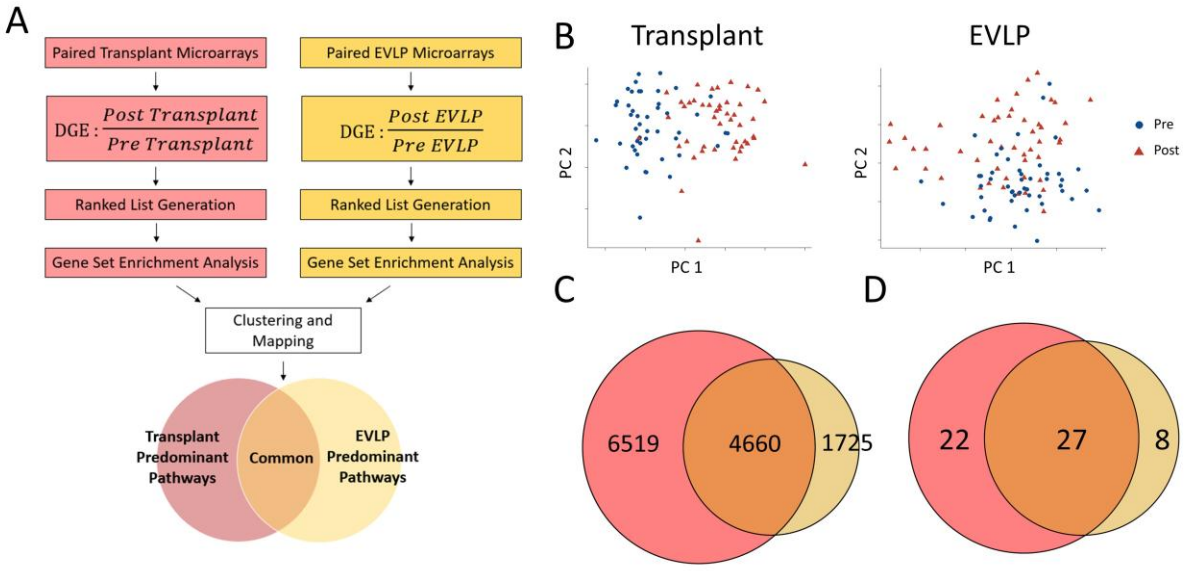
A
Paired Transplant Microarrays
Paired EVLP Microarrays
DGE : Post Transplant / Pre Transplant
DGE : Post EVLP / Pre EVLP
Ranked List Generation
Ranked List Generation
Gene Set Enrichment Analysis
Gene Set Enrichment Analysis
Clustering and Mapping
Transplant Predominant Pathways
Common
EVLP Predominant Pathways
B
Transplant
EVLP
PC 2
PC 1
PC 2
PC 1
Pre
Post
C
6519
4660
1725
D
22
27
8

Figure 1

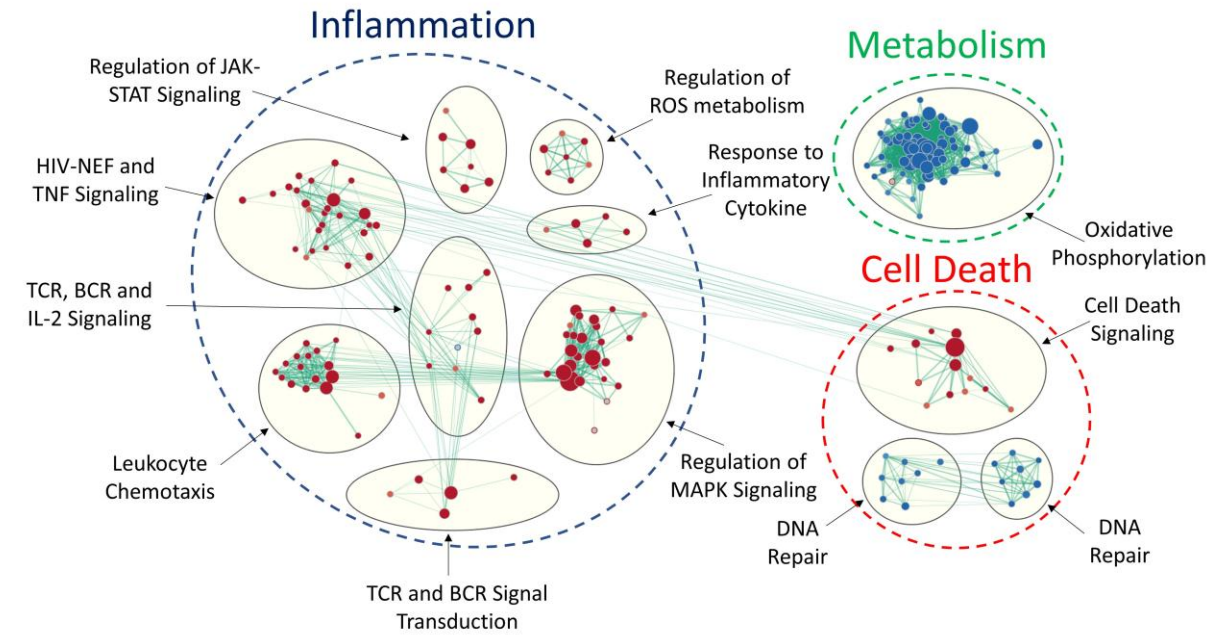

Figure 2: Transplant Predominant

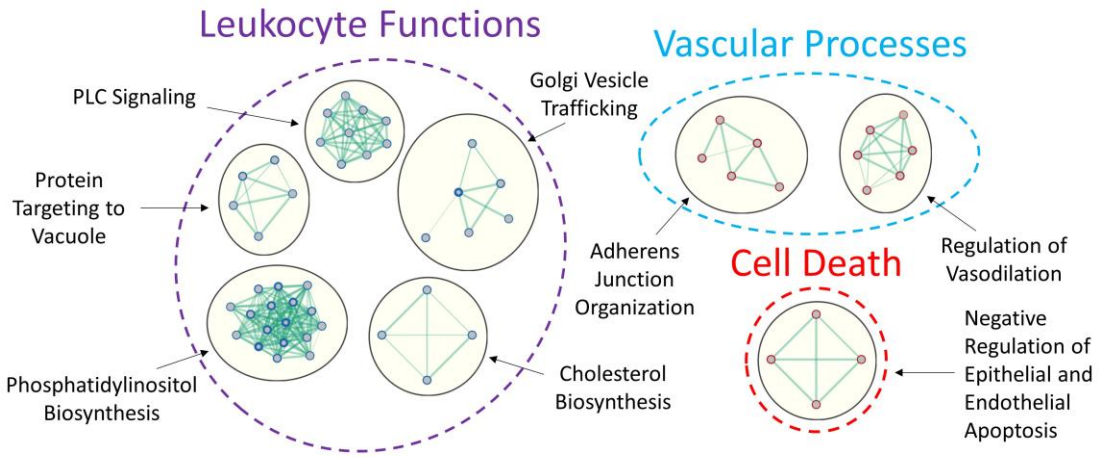

Figure 3: EVLP Predominant

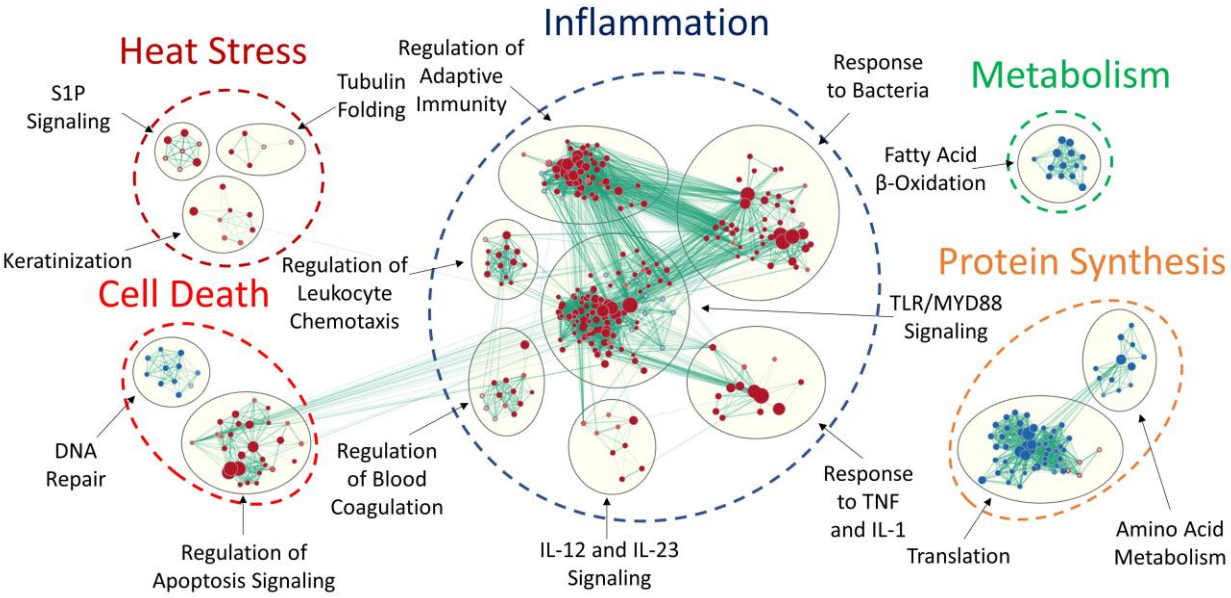

Figure 4: Common

Supplementary Appendix

Potential Therapeutic Targets for Lung Repair During Human Ex Vivo Lung Perfusion

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Supplementary Methods:

RNA Extraction and Microarrays. Peripheral lung tissue biopsies were collected and snap frozen in liquid nitrogen in the operating room. Total RNA was purified using RNeasy Mini Kit (Qiagen; Hilden, Germany). RNA quality was assessed using Nanodrop spectrophotometer (VWR; Radnor, PA) and Bioanalyzer (Agilent; Santa Clara, CA). Samples with concentration above 100ng/ul and RNA Intergaty Number (RIN) above 7.0 were used for microarray analysis. Purified RNA was stored at -80°C. Microarrays were processed by Princess Margret Genomics Center (Toronto, Canada). Transplant samples had gene expression measured on Human Genome U133 Plus 2.0 Array (Affymetrix; Santa Clara, CA) and EVLP samples on Clariom D Assay (Affymetrix). Analysis was conducted in R version 3.5.1 (1).

Microarray Preprocessing. Microarrays were normalized using Robust Multi-array Average (RMA) (2) in the oligo package (3). Genes were annotated using Brainarray version 22 custom annotation files (4).

Limma Analysis. Differential gene expression (DGE) was calculated using Bayes moderated paired t-test in the Limma package (5). The transplant group had differential gene expression calculated between paired post and pre transplant samples, while the EVLP group had differential expression calculated between paired post and pre EVLP samples. Differentially expressed genes were defined as having an FDR<0.05 using the Benjamin-Hochberg correction procedure (6).

Generation of Ranked list for Gene Set Enrichment Analysis. Only the genes detectable on both microarray platforms were used in the generation of ranked lists. For each dataset, genes were ranked based on their score according to the formula:

$$\text{Gene score} = -\ln(\text{p value from DGE})(\text{Sign of gene fold change})$$

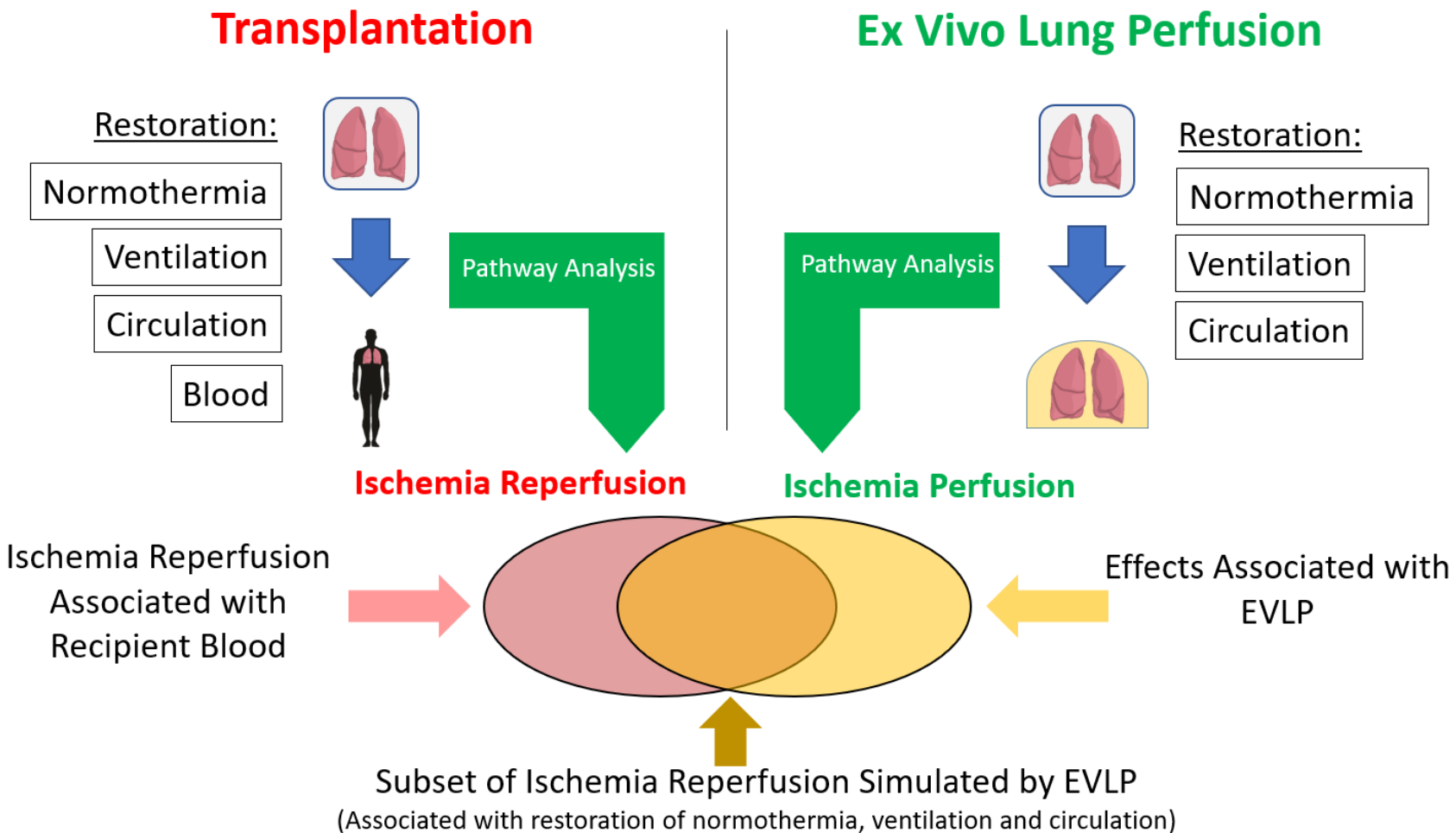
Ranked lists were then passed to GSEA.

Gene Set Enrichment Analysis 3.0 (GSEA) (7)**.** A PreRanked analysis was run with the following parameters: gene set min=15, gene set max=500, permutations=1000, scoring scheme = weighted, normalization mode = mean div. The gene set database used is from the Bader lab: Human_GOBP_AllPathways_no_GO_iea_June_01_2017_entrezgene.gmt from the Bader Lab. More information on the database can be found at: http://baderlab.org/GeneSets.

Cytoscape Visualization. Gene set reports were filtered for gene sets which met the FDR < 0.05. Cytoscape version 3.5.0 was used (8). Pathway network was generated using EnrichmentMap version 2.2.1 (9) using the follow parameters: similarity overlap = overlap coefficient, cutoff=0.5. Clusters were annotated with AutoAnnotate 1.1.0 (10) using the parameters: clustering algorithm = MCL, edge weight column = similarity coefficient. Each cluster with more than 4 nodes was reviewed and had a name assigned.

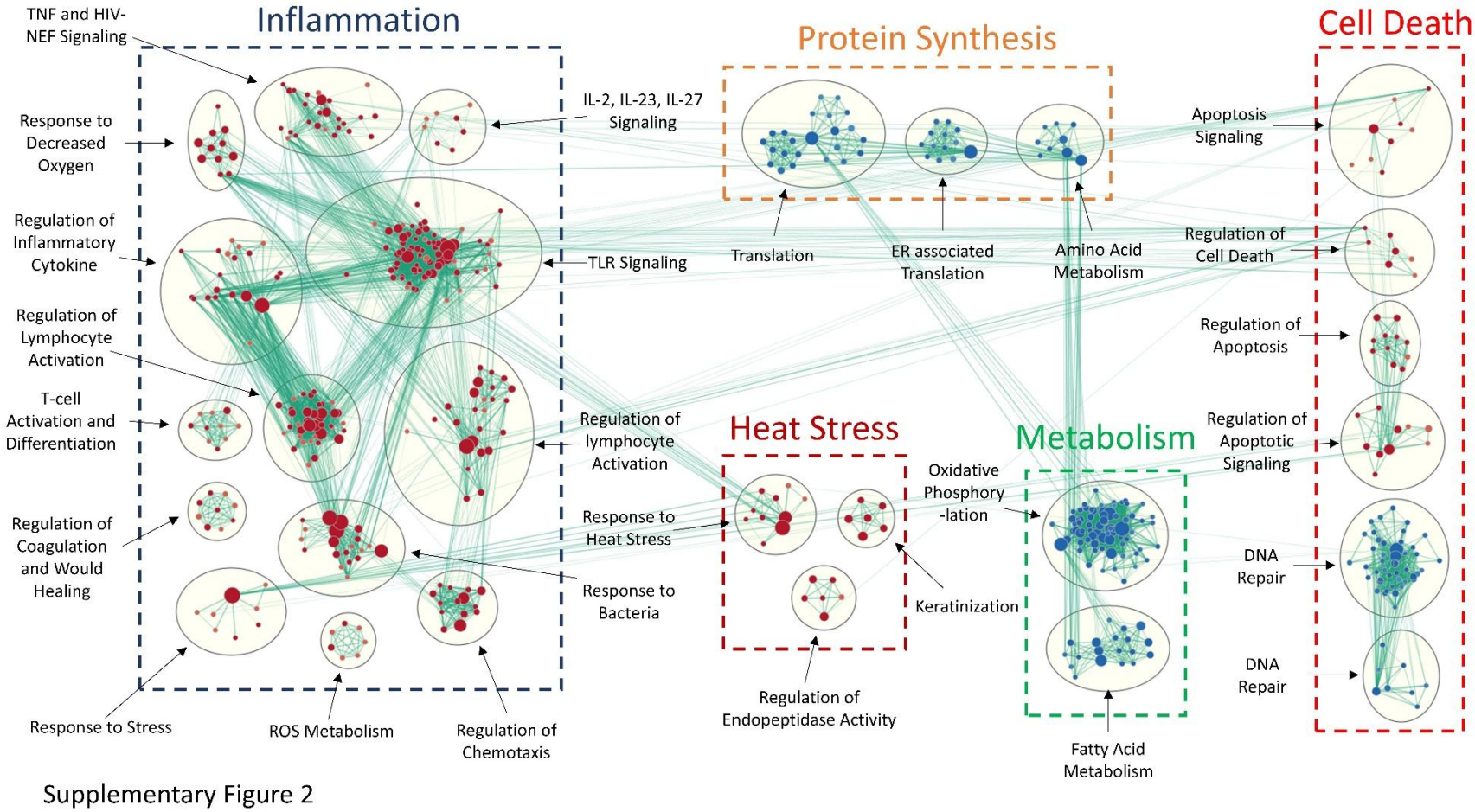
PCA Visualization. PCA visualization were made using the R package pca3d (11). All genes from each microarray platform were used to create plots.

Validation Analysis. Validation datasets were analyzed using the same pipeline as the study dataset. Due to the small sample size of both datasets, we increased the cutoffs for gene sets to FDR<0.10.



Supplementary Figure 1. Comparison of Transplantation and EVLP as Models of Reperfusion.

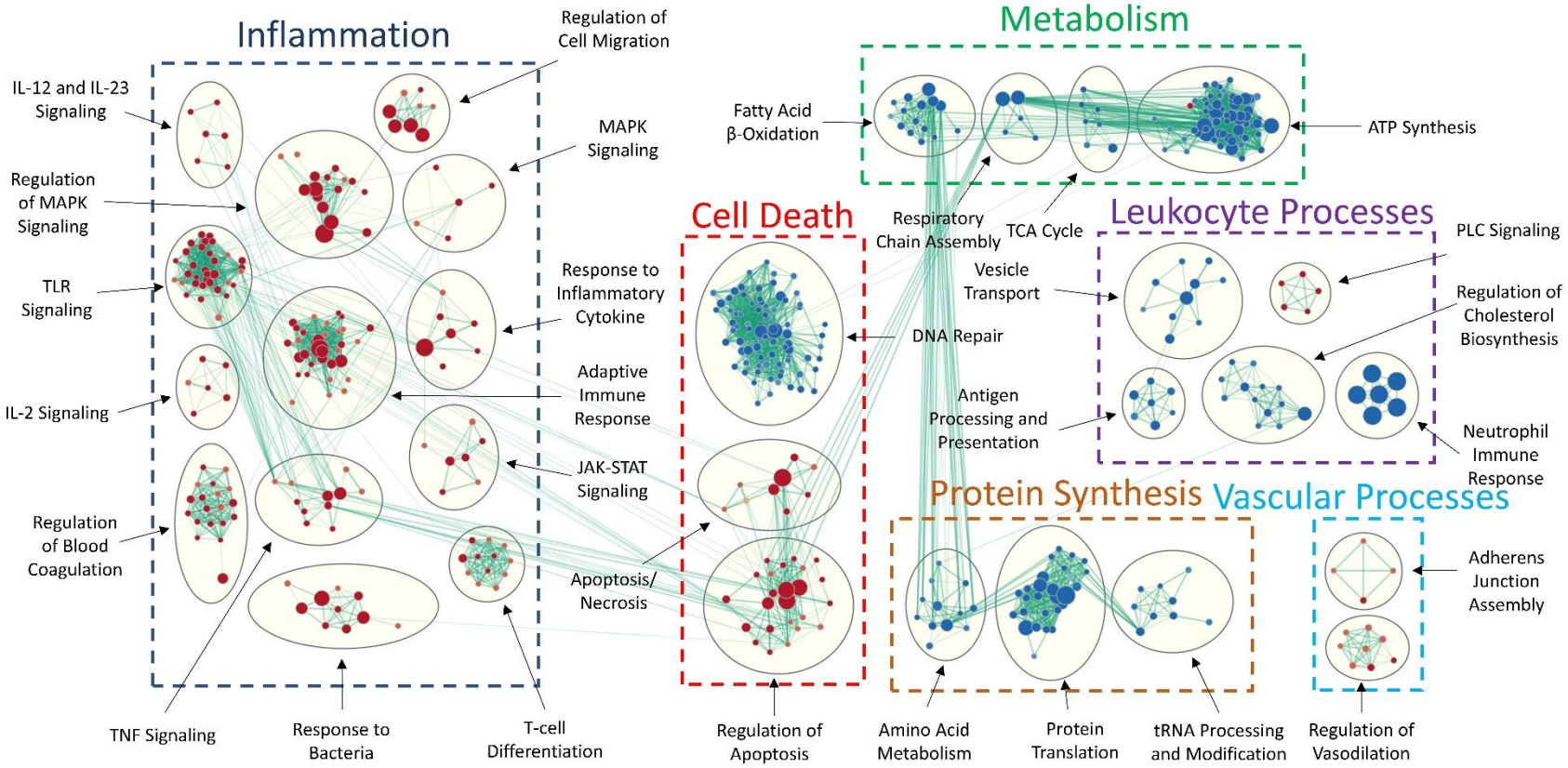
The left side of the panel represents the biological response induced by recipient reperfusion, while the right side represents the response induced by EVLP. The Venn diagram represents a comparison of the enriched biological pathways in transplantation and EVLP, which can be divided into three categories.



Supplementary Figure 2

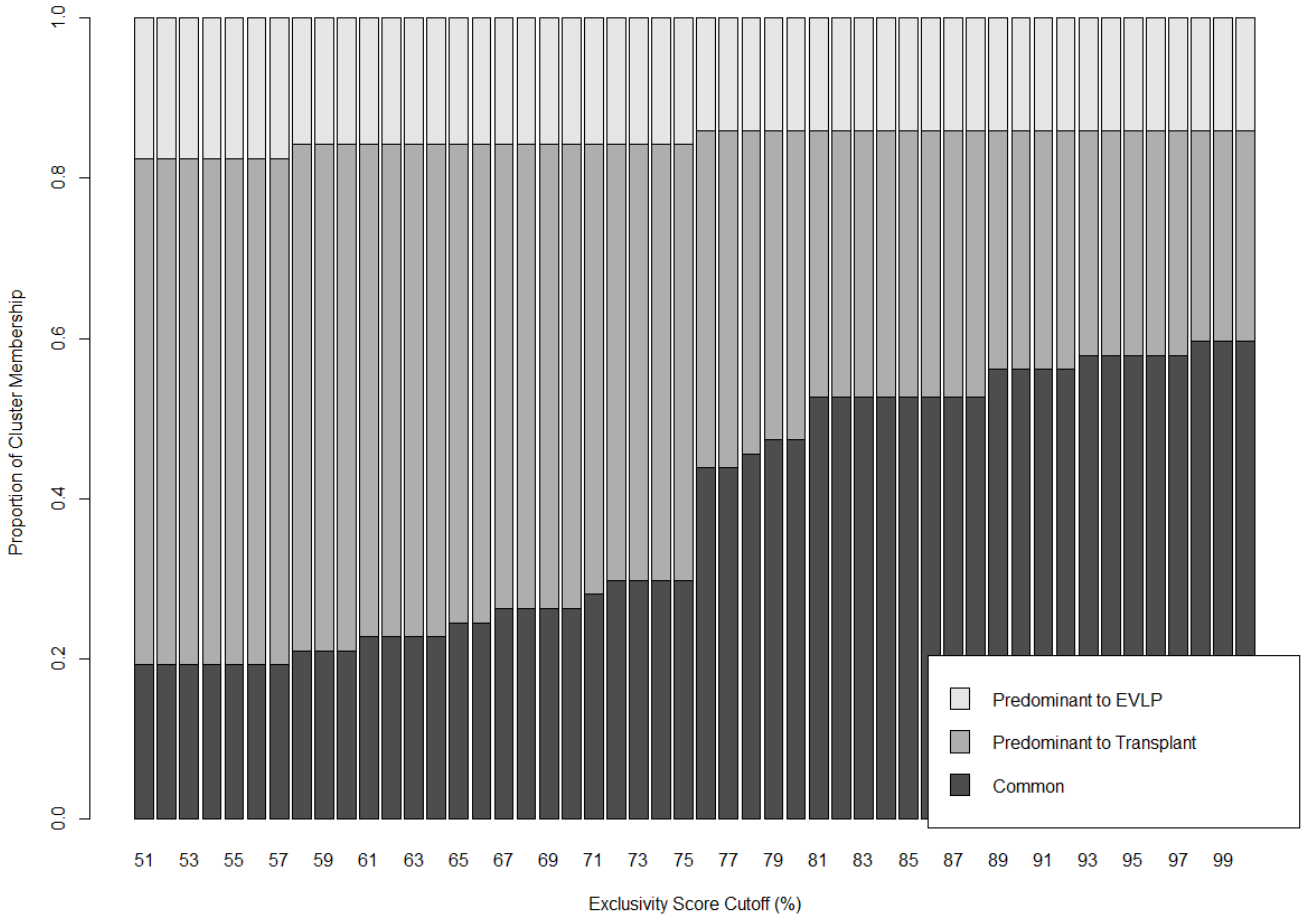
Supplementary Figure 2. Transplant Validation - Gene Set Clusters Enriched from the Kang et al. Dataset (12)**.**

Gene set clusters which met cutoffs (FDR<0.10) fell into five major categories with inflammation, cell death and heat stress were generally up-regulated (red nodes) and metabolism and protein synthesis down-regulated (blue nodes). The gene set clusters observed here recapitulate the same themes classified as being predominant to transplant and common from the study dataset.



Supplementary Figure 3. EVLP Validation - Gene Set Clusters Enriched from the Yeung et al. Dataset(13)**.**

Gene set clusters which met cutoffs (FDR<0.10) fell into six major categories with inflammation, cell death and vascular processes generally up-regulated (red nodes) and metabolism, protein synthesis and leukocyte processes down-regulated (blue nodes). The gene set clusters observed here recapitulate the same themes classified as being predominant to EVLP and common from the study dataset.



Supplementary Figure 4. Proportion of cluster membership across a range of thresholds between 0.5-1.0.

Supplementary Table S1. Enriched Gene Set Clusters from Transplant and EVLP Comparison |0.8| threshold.

Cluster Name	Number of Gene Sets	Predominance Score	Classification
TLR/MYD88 Signaling	99	0.71	common
Regulation of Adaptive Immunity	58	0.76	common
Translation	49	0.78	common
Response to Bacteria	46	0.72	common
Regulation of Apoptic Signaling	28	0.64	common
Cillium Organization	14	0.29	common
Regulation of Leukocyte Chemotaxis	14	0.79	common
Fatty Acid Beta Oxidation	14	0.43	common
Regulation of Blood Coagulation	13	0.23	common
Response to TNF and IL-1	13	0.23	common
Amino Acid Metabolism	12	0.75	common
DNA Repair	11	0.18	common
Epigenetic Regulation of Expression	11	0.09	common
IL-12 and IL-23 Signaling	9	0.67	common
Regulation of Alternative Splicing	8	0.75	common
S1P Signaling	8	0.75	common
Keratinization	7	-0.57	common
unknown	5	0.20	common
Tubulin Folding	5	-0.40	common
Response to Hormone	5	0.40	common
Cofactor Metabolic Process	5	0.60	common
Amino Acid Metabolism	4	0.25	common
Intracellular Transport	4	-0.75	common
AP1 and Fra Pathway	4	0.00	common
unknown	4	0.75	common
Regulation of Endocrine Process	4	0.75	common
NFAT and TCR Pathway	4	0.75	common
Oxidative Phosphorylation	74	0.97	transplant predominant
Regulation of MAPK Signaling	26	0.81	transplant predominant
HIV-NEF Signaling and TNF Signaling	26	0.81	transplant predominant
Leukocyte Chemotaxis	17	0.88	transplant predominant
Cell Death Signaling	13	0.92	transplant predominant
IL-2 and GMCSF Signaling	12	1.00	transplant predominant
TCR, BCR, IL-2 Signaling	10	0.80	transplant predominant
Regulation of Protein Import to Nucleus	9	0.89	transplant predominant
Regulation of FGFR Signaling	9	1.00	transplant predominant
DNA Repair-1	8	1.00	transplant predominant
DNA Repair-2	8	1.00	transplant predominant
Regulation of ROS Metabolism/Biosynthesis	7	1.00	transplant predominant
Regulation of JAK-STAT Signaling	7	1.00	transplant predominant
MAPK Signaling	6	1.00	transplant predominant

Symbiont Host Modulation	6	1.00	transplant predominant
ERBB and TRK Receptor Signaling	5	1.00	transplant predominant
Response to Inflammatory Cytokine	5	1.00	transplant predominant
TCR and BCR Signal Transduction	5	1.00	transplant predominant
Leukocyte Mediated Cytotoxicity	5	1.00	transplant predominant
Leukocyte Homeostasis	4	1.00	transplant predominant
Thromboxane and IL-8 Signaling	4	1.00	transplant predominant
Heart Morphogenesis	4	1.00	transplant predominant
Phosphatidylinositol Biosynthesis	17	-1.00	EVLP predominant
PLC Signaling	9	-1.00	EVLP predominant
Vesicle Transport	6	-1.00	EVLP predominant
Regulation of Vasodilation	6	-1.00	EVLP predominant
Adherens Junctions	5	-1.00	EVLP predominant
Protein Localization to Vacuole	5	-1.00	EVLP predominant
Cholesterol Biosynthesis	4	-1.00	EVLP predominant
Regulation of Epithelial and Endothelial Apoptosis	4	-1.00	EVLP predominant

*Red cluster names indicate upregulation while blue names indicate downregulation of the pathway. Only clusters which contained at least 4 nodes were included in this table.

Supplementary Table S2. Number of FDA approved therapeutics predicted to target pathways. Enrichment Map post analysis was used to identify drugs from the Drug Bank database: October_1_2019_Human_DrugBank_approved_entrezgene.gmt (available at http://download.baderlab.org/EM_Genesets/October_01_2019/Human/Entrezgene/). Potential therapeutics are defined as having an overlap of at least 3 genes between an enriched geneset and drugset.

Cluster Number	Cluster Name	Number of Drug Hits
1	TLR/MYD88 Signaling	37
2	Oxidative Phosphorylation	30
3	Regulation of Adaptive Immunity	15
4	Translation	17
5	Response to Bacteria	25
6	Regulation of Apoptic Signaling	11
7	Regulation of MAPK Signaling	88
8	HIV-NEF Signaling and TNF Signaling	14
9	Phosphatidylinositol Biosynthesis	20
10	Leukocyte Chemotaxis	0
11	Cillium Organization	3
12	Regulation of Leukocyte Chemotaxis	10
13	Fatty Acid Beta Oxidation	19
14	Regulation of Blood Coagulation	7
15	Response to TNF and IL-1	12
16	Cell Death Signaling	34
17	IL-2 and GMCSF Signaling	13
18	Amino Acid Metabolism	28
19	DNA Repair	1
20	Epigenetic Regulation of Expression	1
21	TCR,BCR,IL-2 Signaling	8
22	PLC Signaling	0
23	Regulation of Protein Import to Nucleus	8
24	Regulation of FGFR Signaling	20
25	IL-12 and IL-23 Signaling	0
26	Regulation of Alternative Splicing	1
27	S1P Signaling	0
28	DNA Repair-1	1
29	DNA Repair-2	11
30	Keratinization	0
31	Regulation of ROS Metabolism/Biosynthesis	1
32	Regulation of JAK-STAT Signaling	42
33	Vesicle Transport	0
34	Regulation of Vasodilation	5
35	MAPK Signaling	0
36	Symbiont Host Modulation	0
37	unknown	11
38	Adherens Junctions	8

39	Protein Localization to Vacuole	10
40	Tubulin Folding	2
41	Response to Hormone	20
42	ERBB and TRK Receptor Signaling	5
43	Response to Inflammatory Cytokine	15
44	Cofactor Metabolic Process	0
45	TCR and BCR Signal Transduction	0
46	Leukocyte Mediated Cytotoxicity	0
47	Cholesterol Biosynthesis	0
48	Regulation of Epithelial and Endothelial Apoptosis	15
49	Amino Acid Metabolism	3
50	Intracellular Transport	6
51	Leukocyte Homeostasis	2
52	AP1 and Fra Pathway	47
53	unknown	1
54	Regulation of Endocrine Process	17
55	Thromboxane and IL-8 Signaling	0
56	NFAT and TCR Pathway	0
57	Heart Morphogenesis	0

Supplementary Data. 1

Donor lungs in the EVLP group were significantly older than lungs in the transplant dataset (Table 1). As a proxy, we analyzed differential gene expression between the 10 youngest and 10 oldest donor lungs in the transplant and evlp datasets at both pre and post timepoints (4 comparisons total) using limma (5). In the transplant dataset the mean age of young lung group was 24 (±8.26) and the mean age of old lung group was 67.18 (±5.016). In the EVLP dataset the mean age of young lung group was 19.1 (±3.84), and the mean age of old lung group was 58.55 (±4.344). No differential gene expression was found at FDR<0.05, and a few genes were found at FDR<0.10 (see below).

	Transplant	**EVLP**
Pre	**0 DGE @FDR<0.05, 0 DGE @FDR<0.10**	**0 DGE @FDR<0.05, 5 DGE @FDR<0.10**
Post	**0 DGE @FDR<0.05, 6 DGE @FDR<0.10**	**0 DGE @FDR<0.05, 0 DGE @FDR<0.10**

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