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Ex vivo lung perfusion moderates gene expression differences between cardiac death and brain death donor lungs

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Donation after cardiac death (DCD) donor lungs have been shown to express less proinflammatory genes than donation after brain death (DBD) lungs, likely due to the absence of brain-death related inflammatory physiology. However, it is unclear whether this difference is clinically significant following reperfusion. To avoid confounding by the recipient immune system and activation state, we utilized ex vivo lung perfusion (EVLP) as a reperfusion-like event and examined the effect of EVLP on the transcriptome of DCD (n = 39) and DBD (n = 49) lungs. To validate our RNA results, banked EVLP perfusates from a separate cohort of DCD (n = 24) and DBD (n = 24) cases were assayed for IL-6, IL-8, IL-10, IL-1β, soluble TNFα receptor-1 (sTNFR1), and soluble triggering receptor expressed on myeloid cells-1 (sTREM1) protein levels at 15 minutes intervals for 3 hours. While DCD lungs demonstrated lower levels of proinflammatory transcripts and perfusate cytokine protein levels than DBD lungs prior to EVLP, after EVLP, there were no significant gene expression differences or cytokine protein levels between groups. Therefore, while DCD and DBD lungs differ by the amounts of proinflammatory cytokines following procurement, the propagation of inflammation becomes limited during EVLP, and DBD and DCD lungs reach a similar plateau of transcript expression, including proinflammatory cytokines at the end of perfusion. EVLP may therefore play a preconditioning role by dampening the proinflammatory state prior to transplant reperfusion.

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Background

Recently, lung transplantation activity has been boosted by the utilization of donation after cardiac death (DCD) lungs, reaching 10.1% of lungs transplanted in 22 transplant centers.[1]

While there were initial concerns about the suitability of these lungs for transplant, we and others have shown that DCD lungs have a reduced activation of inflammatory pathways and reduced levels of proinflammatory cytokines, such as interleukin (IL)-6, when compared to donation after brain death (DBD) lungs.[2,3] This is likely due to the lack of the "cytokine storm" physiology observed at the time of brain death. Propagation of the inflammatory state in the donor lung at the time of post-transplant reperfusion leads to ischemia-reperfusion injury and primary graft dysfunction (PGD).[4] Consequently,

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one hypothesis has been that DCD lungs would have superior outcomes to DBD lungs. Interestingly, despite differences in the pretransplant inflammatory state, the International Society for Heart and Lung Transplantation DCD registry demonstrated that DBD and DCD lungs have comparable recipient outcomes.[1,5] Therefore, it may be that these pretransplant differences are minor in comparison to the effect of post-transplant reperfusion.

Ex vivo lung perfusion (EVLP) is a well-established platform for the assessment and treatment of donor lungs in order to expand the donor pool and improve recipient prognosis.[6-8] The Toronto strategy of EVLP utilizes an acellular perfusate and therefore further isolates the lung from peripheral blood mononuclear cells found in blood, allowing for investigation of the donor lung itself in isolation.[6] As EVLP perfuses lungs at normothermia, it can also act as a platform for investigating the donor lung throughout reperfusion, allowing for sampling of lungs pre, post, and during reperfusion.[9,10] For this study, it provides a unique insight into reperfusion biology without interference and propagation of inflammation by the recipient immune system. This avoids confounding of donor-origin inflammation with the inflammatory milieu of the recipient.

In this study, we aimed to investigate how the transcriptome differs between DCD and DBD lungs before and after EVLP and validated these findings in another cohort by measuring cytokine protein levels taken from the EVLP perfusate.

Materials and methods

Patient cohort

This was a single-center retrospective cohort study of donor lungs undergoing EVLP followed by lung transplant for end-stage lung disease at Toronto General Hospital between October 2009 and December 2015. This study was approved by the University Health Network research ethics board (REB No. 5488) and the ethics board of Trillium Gift of Life Network.

The transcriptomes of DBD (n = 49) and DCD (n = 39) lungs matched before and after undergoing EVLP were analyzed. Perfusates from a separate cohort of DBD (n = 23) and DCD (n = 23) EVLP lungs were used for cytokine measurements. Clinical data were obtained from the Toronto Lung Transplant Program database.

RNA extraction and microarray

Lung tissues obtained at the end of the first cold ischemic time just before EVLP (CIT1) and after EVLP (CIT2) were used in this study. Around 1 cm^3 biopsy samples were collected from donor peripheral lung tissue and snap frozen in liquid nitrogen.

Total RNA was extracted and RNeasy Mini Kit (QIAGEN Canada, Toronto, ON, Canada) was used to extract RNA. Quality of the RNA extraction was assayed by Nanodrop spectrophotometer (Thermo Fisher Scientific Canada, Ottawa, ON, Canada), and Agilent Bioanalyzer (Agilent, Santa Clara, CA). Samples were hybridized to Affymetrix Clariom D microarrays, resulting in raw CEL files.

Microarray CEL files were normalized and background corrected using robust multiarray analysis (RMA affy 1.62.0), before being randomly split into 60 to 40 discovery and validation groups.[11] Limma 3.40.6 was used to generate a linear model to fit each of the discovery and validation groups, correcting for potential batch effect.[12] A contrast matrix of pre-EVLP DBD, pre-EVLP DCD, post-EVLP DBD, and post-EVLP DCD was generated creating contrasts of DBD vs DCD in CIT1, DBD vs DCD in CIT2, and CIT1 vs CIT2. For each contrast, empirical Bayes was applied to generate t-statistics, moderated F-statistics, and log-odds of differential expression between each contrast. Significantly differentially expressed genes were identified using a p-value cut-off of 0.05. Genes that were significantly differentially expressed and were of the same directionality of expression in both discovery and validation set were considered validated.

Cytokine measurement and analysis

To validate microarray findings, we used EVLP perfusate from a separate cohort of DBD and DCD lungs for cytokine protein analysis. Approximately 1 ml of perfusate from 24 DCD and 24 DBD lungs was collected from perfusate isolated from the left atrial side of the lung during EVLP over 15 minutes intervals for 3 hours. At each time point, the concentrations of 6 cytokines were recorded: IL-6, IL-8, IL-10, IL-1β, sTNFR1, and sTREM1. ELLA (Protein Simple, Santa Clara, CA) was used to assay cytokine concentrations.

Results

Demographic characteristics of donors

Table 1 summarizes the donor characteristics and demonstrates no significant differences between groups with the exception of last donor PO_2. A total of 49 DBD and 39 DCD lung tissues were sampled prior to EVLP at the end of cold ischemic time and then sampled once again post-EVLP during the second cold ischemic time creating paired pre- and post- EVLP samples.

Table 2 summarizes the donor characteristics of the validation cytokine cohort showing no significant differences except in age and smoking history between DCD and DBD donors.

Differences in inflammation between DCD and DBD lungs are lost after EVLP

Two hundred ninety-three differentially expressed genes were observed between DCD and DBD prior to EVLP (i.e., in CIT1 tissue). Of these 293 genes, 195 were upregulated in DBD and 98 in DCD ($p < 0.05$; Figure 1A; Supplementary Table 1). Pathway analysis by gene ontology enrichment analysis indicates that "cellular response to cytokine stimulus" was the major biologic process involved in the DBD upregulated genes, indicating that downstream genes from proinflammatory stimuli were the major differences between DCD and DBD. In contrast, genes upregulated in DCD lungs showed the nonspecific biologic process of "regulation of transcription."

Following EVLP, there were no differentially expressed genes observed between DCD and DBD lungs (Figure 1B). Principal component analysis of all transcripts shows a

Table 1 Donor Demographics

Demographic		DCD N = 39	DBD N = 49	*p*-value
Age, years, median (range)		*46 (12-72)*	*44(13-65)*	0.14
Sex				0.47
	Female	*19(48.7%)*	*19(38.8%)*	
	Male	*20(51.3%)*	*30(61.2%)*	
Body mass index, median (range)		*26.04 (16.22-53.15*	*26.58 (17.10-53.05)*	0.83
Cause of death				0.47
	Anoxia/cardiac arrest	*11(28.2%)*	*9(18.4%)*	
	Cerebrovascular/stroke	*15(38.5%)*	*27(55.1%)*	
	Head trauma	*10(25.6%)*	*10(20.4%)*	
	Other/unknown	*3(7.7%)*	*3(6.1%)*	
Smoking history, yes		*22(56.4%)*	*26(53.1%)*	0.99
Donor PaO_2/FiO_2, mm Hg, median (interquartile range)		406.0 (303.4-450.0)	318.0 (239.0-379.4)	0.01

Table 2 Donor Demographics of Validation Cohort

Demographic		DCD N = 24	DBD N = 24	*p*-value
Age, years, median (range)		*55 (22-74)*	*33 (23-71)*	0.002
Sex				0.49
	Female	*7(29.2%)*	*4(16.6%)*	
	Male	*17(70.8%)*	*20(83.4%)*	
Body mass index, median (range)		*26.67 (18.91-44.06)*	*27.02 (20.94-32.07)*	0.43
Cause of death				0.29
	Anoxia/cardiac arrest	*11(45.8%)*	*15(62.5%)*	
	Cerebrovascular/stroke	*3(12.5%)*	*5(20.8%)*	
	Head trauma	*4(16.7%)*	*2(8.3%)*	
	Other/unknown	*6(25.0%)*	*2(8.3%)*	
Smoking history, yes		*14(58.3%)*	*20(83.3%)*	0.05
Last Donor PaO_2/FiO_2, mm Hg, median (interquartile range)		389.0 (359.5-444.5)	419.0 (353.0-451.0)	0.57

separation of DBD and DCD pre-EVLP, but no separation of DBD and DCD post-EVLP (Figure 2A). The 195 upregulated genes in DBD seen pre-EVLP were all elevated to the same degree post-EVLP in the DBD and DCD groups, demonstrating increased transcriptional activation following EVLP reperfusion (Figure 2B, Supplementary Figure 1).

Cytokine protein analysis demonstrates similar differences in inflammation between DCD and DBD lungs pre-EVLP

As inflammation-related transcripts were significantly different between DCD and DBD pre-EVLP, we chose to validate these at the protein level in the EVLP perfusate. Significantly higher levels of IL-6, IL-8, and IL-10 were seen in EVLP perfusate from DBD lungs compared to DCD at 15 minutes of perfusion ($p = 0.002$, 0.004, 0.01, respectively). Soluble TNFR1 and soluble TREM1 had similar trends at 15 minutes of EVLP but were not statistically significant (Supplementary Figure 2). IL-1β was equal between the 2 groups (Supplementary Figure 2). By 3 hours of EVLP, there were no cytokines with concentrations significantly different between DBD and DCD (Figure 3A). Delta PO_2 at start of EVLP was not significantly different between DBD and DCD.

Time-series analysis demonstrates an initial large increase in cytokine concentrations at the beginning of reperfusion, followed by a drop in the rate of change as EVLP progresses in both DBD and DCD groups (Figure 3B).

Discussion

Though lung transplantation using DCD lungs has been shown to have similar 1-year mortality, PGD, and acute rejection after transplantation when compared to DBD, DCD organ donation remains limited in many jurisdictions.[13] In addition to logistical concerns, the reason for this may involve concerns about warm ischemic injury and the differences in biology between DCD and DBD lungs. We undertook this study to evaluate whether differences identified in the transcriptome between DCD and DBD lungs influence post-reperfusion outcomes.

When examining transcriptome differences between DCD and DBD lungs prior to EVLP, pathway analysis revealed that the major differences were in inflammation-related genes, confirming the known differences in inflammation between DCD and DBD lungs.[2] We further validated this at the protein level in EVLP perfusate. Despite this difference in proinflammatory signaling pre-EVLP, both types of lungs undergo a

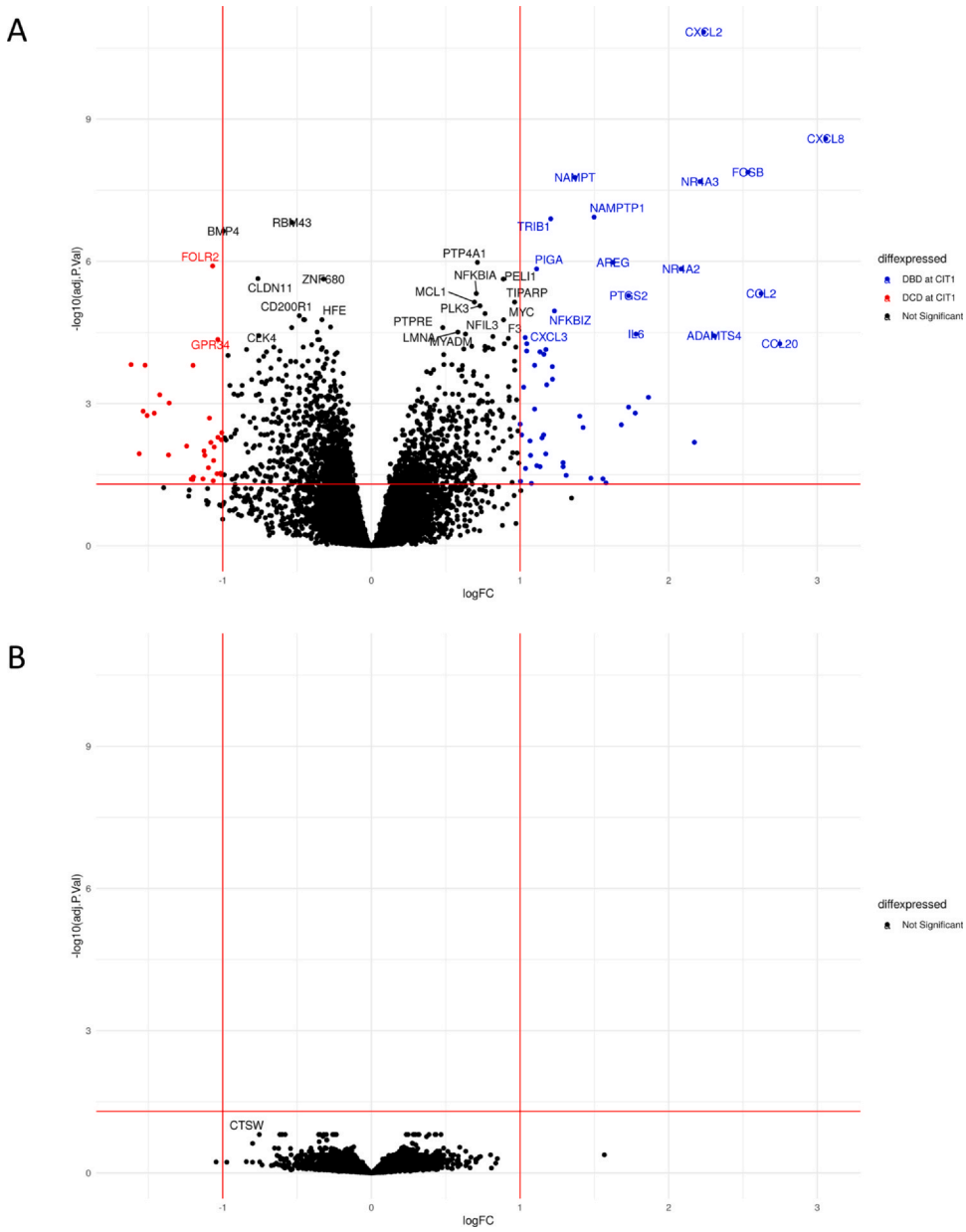

Figure 1 **(A)** Volcano plot of differentially expressed genes between DCD and DBD in biopsies taken prior to EVLP. Vertical red lines indicate log fold change of −1 and 1. Horizontal red line indicates significance at $p = 0.05$. **(B)** Volcano plot of differentially expressed genes between DCD and DBD in biopsies taken after EVLP. Vertical red lines indicate log fold change of −1 and 1. Horizontal red line indicates significance at $p = 0.05$.

rapid increase in transcript expression following the start of EVLP reperfusion, and differences seen pre-EVLP are lost post-EVLP. This suggests that the effect of ischemia-reperfusion overrides any initial differences in gene expression between DCD and DBD lungs.

As ischemia-reperfusion injury is largely mediated by inflammation, cytokines have been an important focus of lung transplantation research. We previously reported that in human lung transplantation using qRT-PCR, increased levels of IL-6, IL-8, TNF-α, and IL-1β in the accepted donor lung were risk factors for PGD, while IL-10 and IFN-γ were protective.[14] When we focused on proinflammatory cytokine differences at the protein level between DCD and DBD lungs in this study, these similarly equalized during EVLP. More interestingly, however, proinflammatory cytokine *production* levels eventually plateaued during EVLP at both the transcript and protein level, indicating that cytokine production in the lung ultimately slowed during EVLP. In earlier studies, circulating leukocyte cell-specific gene expression has been shown to be decreased during EVLP and thought to represent the washing out of leukocytes during perfusion.[10,15] In this study, we confirm that this also occurs at the protein level in the perfusate. This suggests that EVLP may dampen proinflammatory signaling prior to transplantation into the recipient and thereby play a preconditioning role by interrupting the propagation of the inflammatory response by the recipient's immune system.

Indeed, this study has focused on reperfusion during acellular EVLP. This has allowed for a highly controlled

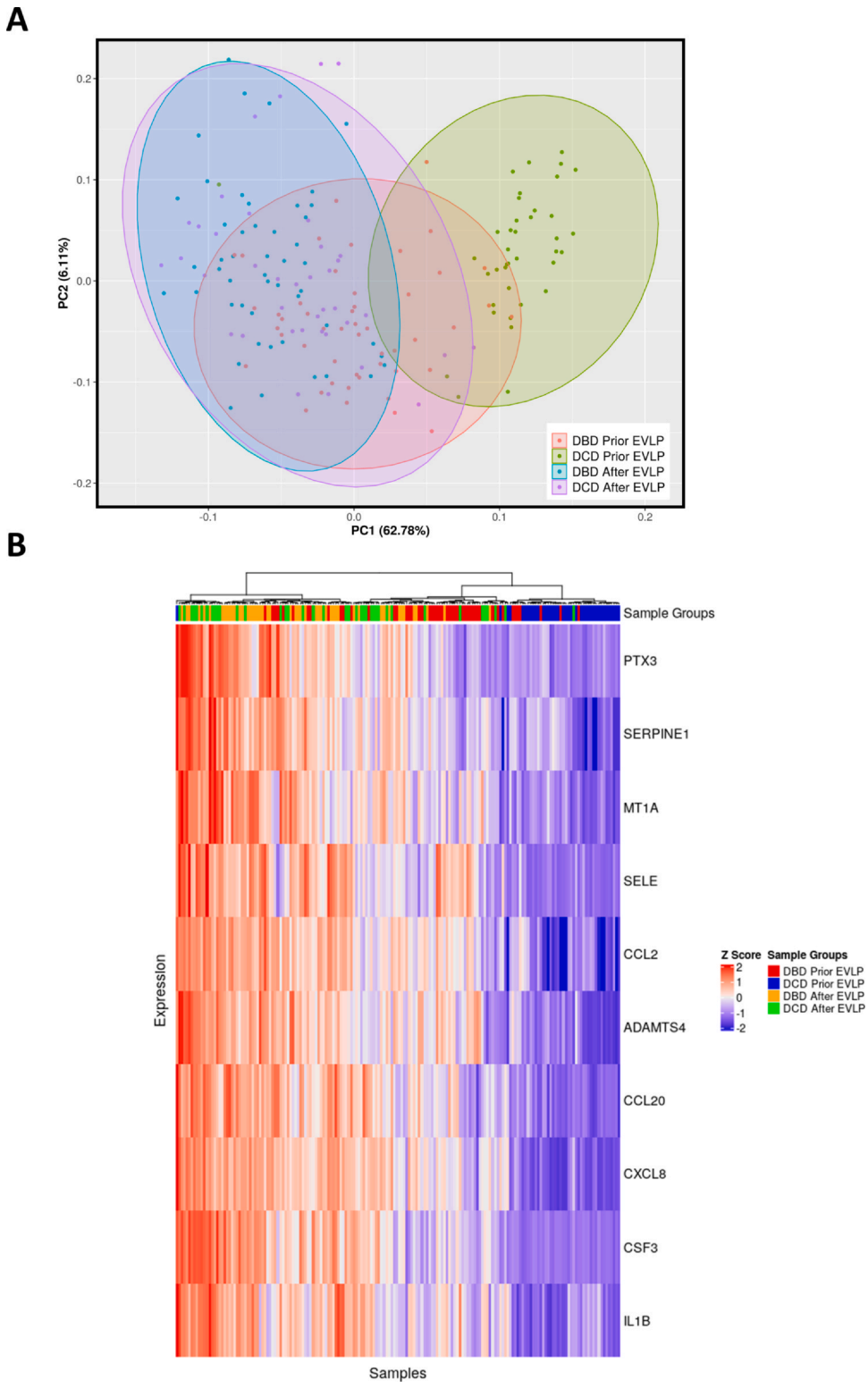

Figure 2 **(A)** Principal component plot using the 293 differentially expressed genes between DBD and DCD pre-EVLP and post-EVLP. **(B)** Heatmap of top 10 differentially expressed genes between DBD and DCD, pre- and post- EVLP.

look at the effect of reperfusion specifically on DBD and DCD donor lung biology without interference by the recipient immune system; however, the corollary is that the effect of the recipient inflammatory milieu is not examined, but this is being studied by others.[16] EVLP has been used to evaluate lungs prior to transplantation by us and other groups and thus does recapitulate transplant reperfusion to a large extent.[17] Another limitation includes the short observation period during EVLP; these changes may not reflect longer timepoints that occur in vivo after implantation, though the plateau in cytokine expression did occur during the study timeframe. Finally, these are findings from

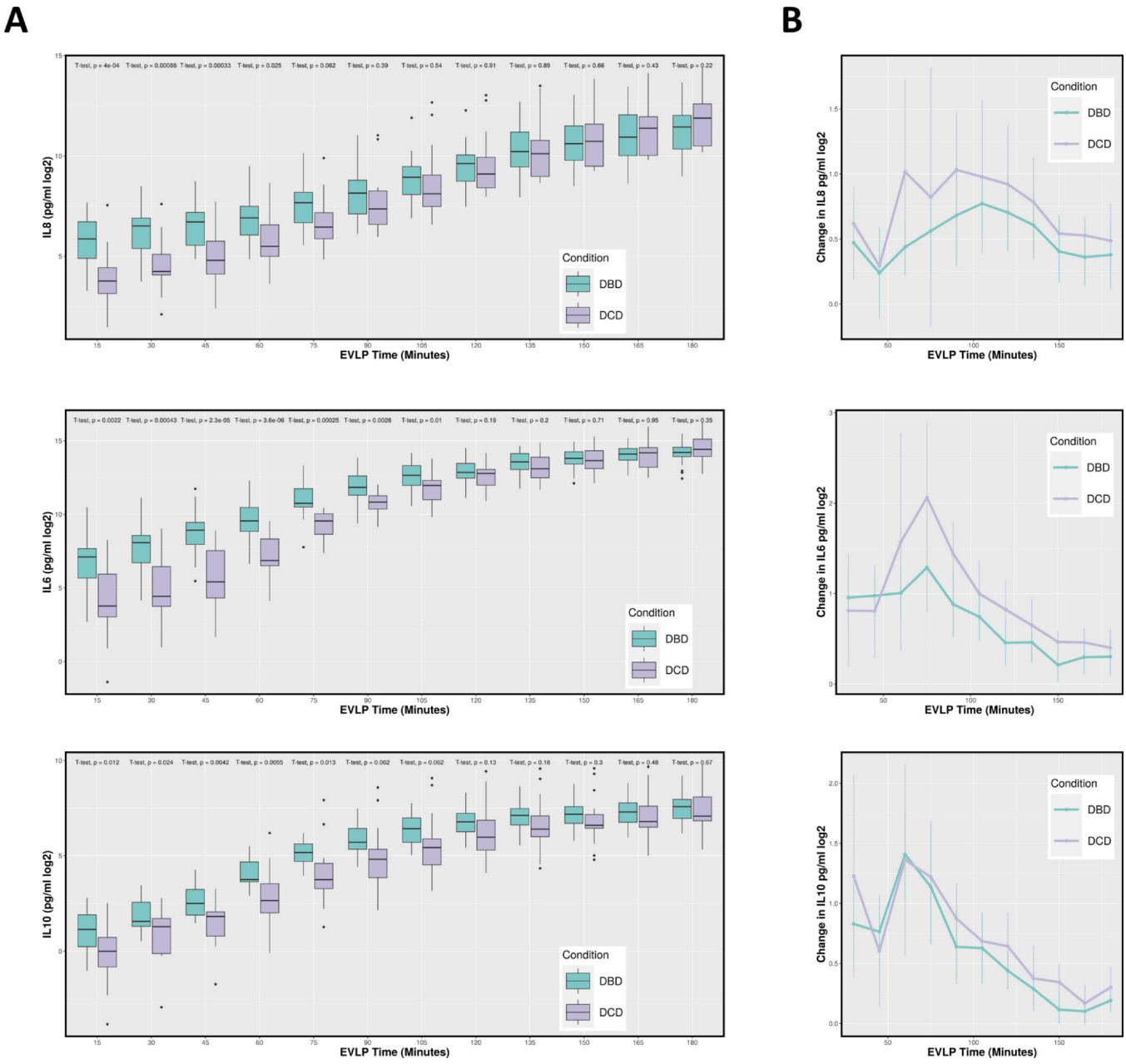

Figure 3 **(A)** Absolute values of IL-8, IL-6, and IL-10 protein levels during EVLP separated by DBD or DCD. **(B)** Change in levels in IL-8, IL-6, and IL10 protein levels during EVLP separated by DBD or DCD.

accepted and transplanted donor lungs. A future direction would be to study the perfusate of damaged organs that were not used clinically and investigate the potential of EVLP's mitigating influence on higher base levels of inflammation.[14] Another future study could compare the effect of EVLP and in vivo reperfusion on inflammation, which could assist in ascertaining if the effect of inflammation in this study is specific to EVLP or instead a time-effect of reperfusion.

In conclusion, we demonstrate that transcriptome differences between DCD and DBD donor lungs disappear following EVLP with a stabilization of the proinflammatory milieu during acellular EVLP. This will hopefully further spur the use of DCD lungs in lung transplantation to reduce waitlist mortality.

Disclosure statement

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Shaf Keshavjee reports financial support was provided by Genome Canada. Shaf Keshavjee reports a relationship with Traferox that includes: board membership. Marcelo Cypel reports a relationship with Traferox that includes: board membership. Shaf Keshavjee and Marcelo Cypel are co-founders and shareholders in Traferox Technologies, SK serves as Chief Medical Officer of Traferox technologies. Traferox devices were not used in the cases described in this study. SK and MC are consultants to Lung Bioengineering.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jhlto.2023.100027.

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