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Normothermic Ex Vivo Lung Perfusion in Brain-dead Donors Reduces Inflammatory Cytokines and Toll-like Receptor Expression

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ABSTRACT

Inflammatory responses and innate immunologic reactions play an important role in the respiratory system. Ex vivo lung perfusion (EVLP) is considered a novel method in the evaluation and reconditioning of donor lungs prior to transplantation. However, EVLP's effect on inflammatory and metabolic markers of human lung tissue is unknown. This study investigated how the performance of EVLP on brain-dead (BD) donor lungs affects the production and release of inflammatory cytokines (IL-6, IL-8, and TNF-α), inflammatory cells and toll-like receptors (TLR) -2, 4.

This study was conducted with an animal subject for qualification of EVLP team and then EVLP was performed on 4 human cases referred to Masih Daneshvari Hospital (Tehran,Iran), from May 2013 to July 2015. Two of these cases, who had acceptable lung function parameters, were enrolled in this study for immunologic investigations. Bronchoalveolar lavages (BAL) were taken before and after EVLP. Cytokines were quantitatively measured before lung retrieval, at the end of the lung removal, at the start of EVLP, and at the end of the each hour of EVLP. TLR expression was measured on the cells obtained by flow cytometry.

TNF-α, IL-6 and IL-8 decreased in each stage of washing perfusate in both cases, and the level of cytokines in serum was in the normal range. Flow cytometry analysis revealed a decreasing expression of CD3, CD4/8, CD19, and CD16+56, as well as TLR-2 and TLR-4 in both cases.

Intra-capillary pools of pro-inflammatory cytokines (IL-6, IL-8, and TNF-α) were determined to contribute to the lung injury during prolonged lung perfusion. This raises the possibility that EVLP donor lungs could be less immunogenic than standard lungs. However, to assess EVLP's effects on lung grafts and optimize recipient outcomes, further studies with a sufficient number of lungs are required.

Keywords: Cytokines; Ex vivo lung perfusion (EVLP); Lung transplantation; TNF-α; TLR

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INTRODUCTION

Lung transplantation is a well-established treatment in patients with end-stage lung diseases. Although the number of lung transplant candidates has grown significantly, only 15-20 percent of brain-dead (BD) donor lungs are usable for transplant, which results in a high mortality rate among lung transplant patients on waiting lists.[1,2]

Greater susceptibility of the lungs to the deleterious effects of brain death and to ICU complications contribute to the low rate of available lungs.[3,4]

Recently, various strategies have been proposed to increase the number of transplanted lungs using marginal-lung and non-heart-beating donors. However, an increased incidence of some complications such as primary graft dysfunction (PGD) in recipients receiving marginal organs was reported.[5] PGD is a form of acute lung injury that develops in recipients early after lung transplantation[6] and is the leading cause of 30-day mortality accounting for 29 percent of early deaths.[7] It was originally thought that PGD was principally a manifestation of ischemia reperfusion injury.[8] However, it is now accepted that PGD is the result of a series of injuries that start in the donor during the events leading up to brain death and culminating in the reperfusion of the organ in the recipient.[9] Although human leukocyte antigen (HLA) typing is not significant in lung transplantation, other unknown factors play an important role in the rejection of this organ. It is well-established that BD influences the outcome of lung transplantation[10,11] and the magnitude of the pulmonary inflammatory response in the donor predicts PGD in the recipient.[12]

To overcome both the scarcity of donor lungs and post-operative complications, scientists have introduced ex vivo lung perfusion (EVLP), a novel approach that reverses lung injury.[13] In this method, poorly oxygenated lungs with pulmonary edema or atelectasis without established pneumonia, severe contusions or gross gastric aspiration are washed and perfused ex vivo with a high oncotic pressure solution called Steen solution (XVIVO, Vitrolife, Sweden).[5]

The BD process can activate an inflammatory cascade in the airway resulting in the production of a number of potent cytokines and chemokines that are important mediators in lung defense and inflammation.[14] The airway epithelium is the first barrier for pathogens and particles and is also a target for factors released by infiltrating inflammatory cells.[15] Furthermore, airway epithelial cells are themselves potent producers of inflammatory mediators by which the inflammation can be inhibited and sustained within the airways. In addition, the effect of BD on inflammation in the lungs has been described by Fisher et al.[16] They found a higher level of IL-8, GRO-a (CXCL1) and neutrophils in bronchoalveolar lavage (BAL) fluid and a higher expression of IL-8 mRNA in biopsies from BD donors than from controls.[16]

External stimuli, such as major surgery, infections, burns and trauma elicit an inflammatory response. This response is mediated by the immune system, the coagulation system, the fibrinolytic system and the complement system. The cellular response is organized by a complex interaction of cytokines produced locally by many different cells, particularly leukocytes.[17]

In many studies, the primary outcome of inflammation after BD is the production of cytokines. Important pro-inflammatory cytokines include TNF-α , IL-1b, and IL-6, whereas IL-10 has anti-inflammatory effects. TLRs are also pathogen-associated molecular pattern receptors for diverse microbial-derived molecules that are expressed and activated predominantly on innate immune cells.[18] TLR activation is ligand binding to receptor and causing activation of receptor. To date, eleven TLR family members have been identified in the human genome.[19] TLR4 and TLR9 play critical roles in lung and respiratory tract diseases,[20-23] and TLRs 2 and 4 are trans membranes receptors for innate immunity which recognizing the gram negative and positive bacteria, respectively. It has been shown that with the onset of reperfusion, the production of TNF-α increases and together with IL-1β, they up-regulate Toll-like Receptor (TLR) 2 on pulmonary endothelial cells. Versican which is induced by fibroblasts,binds TLR2, resulting in a significant increase in IL-8 production.[24-26] It has been suggested that versican and hyaluronan synthesis may play an important role in the innate immune response to gram-negative lung infection.[24]

The expression of other receptors is not considered to be important in brain dead lung inflammation response.[20] Considering that the role of TLRs and their downstream signaling pathways are poorly understood, the main aim of this study is to assess the effect of normothermic EVLP on the production and release of inflammatory cytokines (IL-6, IL-8, and TNF-α), inflammatory cells (CD3, CD4/8, CD19, CD16+56),

TLR-2, and TLR-4 receptors in BD donor lungs.

MATERIALS AND METHODS

Donor Selection

The Iranian EVLP team's qualification course was conducted using an animal subject. After this course, four human lungs were selected for EVLP at Masih Daneshvari Hospital, Tehran, Iran, two of which could be considered for immunologic investigations in this study according to acceptable lung functionparameters. These parameters are described in Ex Vivo Functional Assessment section.

Between May 2013 and May 2014, all BD multi-organ donors were evaluated according to lung transplant or EVLP criteria. If a donor did not have any sign of severe chest trauma or pneumonia, but showed poor oxygenation due to possible atelectasis or neurogenic pulmonary edema, the lungs would be candidated for EVLP. Lung functions such as PaO2/FIO2 and lung compliance were evaluated every hour during the operation.

This study was approved as a PhD dissertation by the Ethics Committee of Shahid Beheshti University of Medical Sciences (No.1392019). Written informed consent was obtained from the donors' families. Before the animal study, all the related protocols were approved by the institution's animal welfare regulatory committee.

Sample Collection

Bronchoalveolar lavage (BAL) were taken before (BAL 1) and after EVLP (BAL 2).

Blood samples from BD cases were collected via arterial needle aspiration before lung retrieval for the measurement of serum cytokines. Inflammatory cytokines were quantitatively measured several times using Perfadex (Perfadex, Vitrolife, Sweden) preservative solution (PBS +0.1FBS, Merck, Germany), firstly before the lung retrieval or at the end of the lung removal (sample 1) and then at the start of EVLP from Perfadex (sample 2). Finally, using a human cytokine enzyme-linked immunosorbent assay (ELISA) kit, inflammatory cytokines were measured at the end of the each hour of EVLP from the Steen solution (XVIVO, Vitrolife, Sweden) circulating in the lung (samples 3 to 6). Two BAL samples were taken for each case. In addition, samples were taken and stored at -20 ºC; three samples for case one and six samples for the second case from the preservative solutions including Perfadex (Perfadex, Vitrolife, Sweden) or Steen (XVIVO, Vitrolife, Sweden). TLRs expression was measured via BAL cells of BAL obtained by flow cytometry.

Lung Retrieval Technique

It is essential to follow standard lung harvesting for EVLP and the following points should be taken precisely into account. First, 500 international unit (IU)/kg of heparin (about 30000 IU) was injected before the harvest to prevent blood clots in the system, and surface (with ice) and core (with 8ºC Perfadex) lung cooling were initiated afterwards to immediately decrease cell metabolism.In the second and third cases, surface lung-cooling was imperfect, but in the fourth and fifth, it was achieved completely. Intravenous prostacyclin (500 mg) was administered before aortic clamping to prevent vasoconstriction and pulmonary edema during EVLP. However; prostacyclin was not available in the hospital, and it was not used in the third case of this study.Then, lung ante-grade flushing was completed by 60 mL/kg of buffered Perfadex (Perfadex, Vitrolife, Sweden) plus one vial of isotonic tromometrol solution. After the antegrade dose, 200 ml Perfadex was given down each pulmonary vein as a final retrograde flush. The trachea and pleura was dissected perfectly to prevent lung damage. An adequate portion of the main pulmonary artery (PA) and the left atrial cuff was dissected for good cannula anastomosis. A long trachea (at least 4 cm) was stapled in a moderately inflated state, considering the point that, a short trachea may lead to repeated disconnection from the ventilator during the process.

The Ex Vivo Lung Perfusion Technique

The Toronto technique was used in this study which was developed by Cypel, et al.[27] This method involves prolonged perfusion, organ recovery as well as active treatment for organ repair along with a lung assessment.

The circuit was briefly primed with two liters of Steen Solution (XVIVO, Vitrolife, Sweden). In addition, imipenem/cilastatin (500 mg, Jaberebne Hayan Pharmaceutical Company, Iran), heparin (3000 IU, Caspian Tamin Pharmaceutical Company, Iran), and methylprednisolone (500 mg, Solu-Medrol; Sandoz Canada, Boucherville, Canada) were added to the perfusate solution.

After the first hour of EVLP, 500 mL of circulated perfusate were removed and replenished with 500 mL of fresh solution. Subsequently, 250 mL were exchanged every hour until the end of the procedure. After transferring the lungs to the XVIVO chamber, the pulmonary artery and left atrium cannulas were connected to the circuit and anterograde flow with the perfusate was started at 150 mL/min at room temperature. The temperature of the perfusate was then gradually increased to 37°C. When 32°C was reached (usually >30 minutes), ventilation was started and then the perfusate flow rate was increased gradually. To deoxygenate and provide carbon dioxide to the inflow perfusate, a mixture of N_2, O_2, and CO_2 gasses (Roham Gas, Iran) was introduced via a gas exchange membrane, initiated at 0.5 L/min and adjusted according to ABG analysis. In this method, mechanical ventilation is based on a protective mode with a tidal volume of 6-8 ml/kg, a positive end expiratory pressure of 5 cm H_2O, a respiratory rate of 7 breaths/ min, and a FIO_2 of 21 percent. Perfusion flow was limited to 40 percent of cardiac output, pulmonary arterial pressure of 10–15 mmHg, and a left atrial pressure of 3-5 mmHg in a closed circuit. In the final step, the lung block was cooled down in the circuit to 10°C for 10 minutes. Thereafter, perfusion and ventilation were stopped (FIO_2 was increased to 50 percent for lung storage), and the trachea was clamped to maintain the lungs in an inflated state. The lungs were then statically preserved at 4°C in Perfadex (Perfadex, Vitrolife,Sweden) plus one vial of isotonic tromometol (Addex-Tham, Kabi, Sweden) solution until transplantation. Figure 1 and 2 depict a lung on the EVLP system and an ex vivo lung perfusion circuit diagram, respectively.

Ex Vivo Functional Assessment

This method was conducted as described by earlier studies.[28] The PH, PCO_2, electrolytes, and glucose were maintained at physiologic levels in the perfusate. For the functional ex vivo assessment, the tidal volume was set at 10 ml per kilogram of donor body weight and 10 breaths per minute for 5 minutes, with FIO_2 at 1.0. Lung function was evaluated hourly during EVLP according to the following calculations: PO_2= [left atrial PO_2 – pulmonary artery PO_2 (in mmHg)], and pulmonary vascular resistance (PVR)=[(pulmonary artery pressure – left atrial pressure) × 80] ÷ pulmonary artery flow (in dynes.seconds.cm -5), dynamic compliance (in ml/cmH_2O), and peak inspiratory pressure (in cmH_2O). Radiography and flexible bronchoscopy of the ex vivo lung were performed at 1 hour and 3 hours of EVLP.

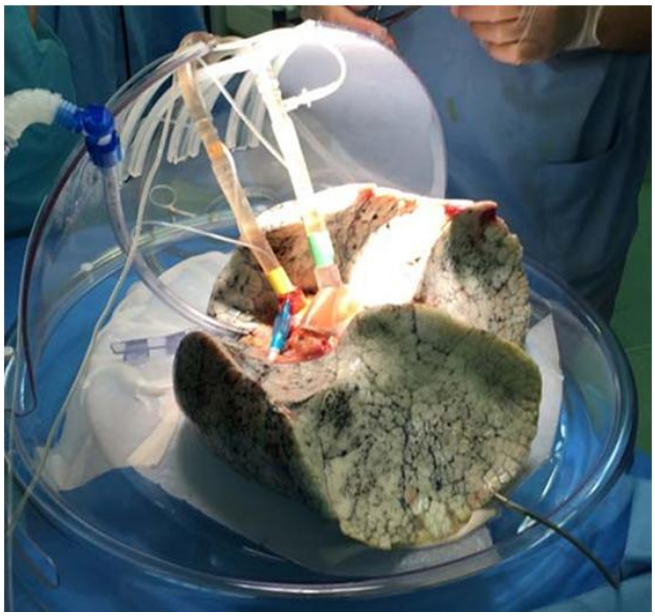

Figure 1. Lung on the EVLP system; Brain-dead donor lungs have been placed in the chamber. Pulmonary artery (green cannula) and left atrium (yellow cannula) were cannulated. Trachea (blue tube) is intubated and connected to the ventilator.

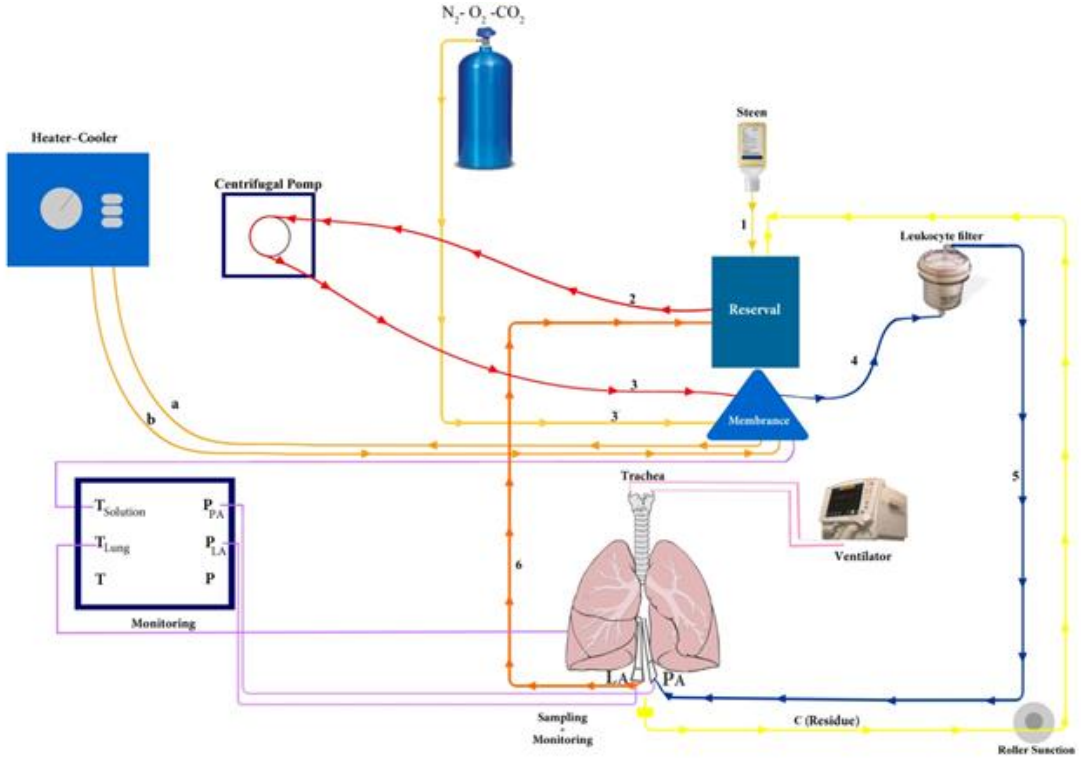

Figure 2. Ex vivo lung perfusion circuit. Circuit 1: Steen solution poured from bottle into reservoir of cardio pulmonary bypass pump, circuit 2: Steen circulated from reservoir to centrifugal pump, circuit 3: circulation of Steen from centrifugal pump to membrane, circuit 3`: circulation of mixed gas from gas cylinder to membrane, circuit a: solution moved from membrane to heater-cooler, circuit b: solution passes from heater-cooler to membrane, circuit 4: solution flows from membrane to leukocyte filter, circuit 5: solution flows from leukocyte filter to pulmonary artery, circuit 6: solution returns from left atrium to reservoir, circuit c: solution residue returns from chamber to reservoir through roller suction, purple circuit: solution and lung temperature monitoring, left atrial (LA) and pulmonary artery (PA) pressure monitoring.

Ex Vivo Functional Assessment

This method was conducted as described by earlier studies.[28] The PH, PCO_2, electrolytes, and glucose were maintained at physiologic levels in the perfusate. For the functional ex vivo assessment, the tidal volume was set at 10 ml per kilogram of donor body weight and 10 breaths per minute for 5 minutes, with FIO_2 at 1.0. Lung function was evaluated hourly during EVLP according to the following calculations: PO_2= [left atrial PO_2 – pulmonary artery PO_2 (in mmHg)], and pulmonary vascular resistance (PVR) = [(pulmonary artery pressure – left atrial pressure) × 80] ÷ pulmonary artery flow (in dynes.seconds.cm -5), dynamic compliance (in ml/cmH_2O), and peak inspiratory pressure (in cmH_2O). Radiography and flexible bronchoscopy of the ex vivo lung were performed at 1 hour and 3 hours of EVLP.

Measurement of Cytokine Concentration

IL-8, IL-6, and TNF-α from supernatants of serum and BAL of harvested samples, which were stored at -80°C, were analyzed using the ELISA method. All analyses were applied based on manufacturer instructions. Assays were performed in duplicate.

Flow Cytometry

IL-8, IL-6, and TNF-α from harvested samples were analyzed using the ELISA method provided by BD Biosciences and eBioscience (San Diego, USA). FITC anti-TLR2, APC anti-TLR4, APC anti-CD3, FITC/PE anti-CD4/8, PE-CD19, PE-CD16+56 antibodies, and isotype controls were provided by eBioscience (San Diego, CA).

The BAL fluid was centrifuged at 800 ×g for 10 minutes. The supernatant was then aliquoted and frozen at −80°C until the cytokines were measured.

The flow cytometric determination of blood immune phenotyping was done using CD3, CD4/8, and CD19, and CD16 + 56 markers.

To investigate the immune phenotyping and expression of TLR in BAL fluid, 5 x 10^6 cells/ml were re-suspended in a FACS buffer, and then 50-µl aliquots were stained with appropriate Abs and were matched to isotype controls in ice for 30 minutes, then washed in an ice-cold FACS buffer (Becton Dickinson, USA), pelleted by centrifugation (1000 × g for 2 minutes at 4°C), and re-suspended in PBS. Flow cytometric analysis was performed using a FACS Calibour (BD, USA), and the data were analyzed using Cell Quest software program (Becton Dickinson, USA). An increase in expression was expressed as the median fluorescence relative to baseline expression.

RESULTS

Lung Functional Assessment

An increasing trend in ΔPO_2 was observed in all cases in EVLP study. In addition, there was an increasing trend in other functional parameters including dynamic lung compliance and a decreasing trend in the pulmonary vascular resistance. The first case considered for immunologic investigation was a non-smoker, 63 year-old donor, brain-dead due to a benign brain tumor. The patient had been intubated for 9 days with a PaO_2/ FIO_2 equal to 125 mmHg, pulmonary edema in both lungs, and a normal bronchoscopy finding. EVLP was started 130 minutes after the application of the aortic clamp. Anastomosis was satisfactory, but there were problems with air leak in the circuit and liquid leak from the site of lung biopsy that was performed for research study. In the end, foamy yellowish secretions, which were suggestive of pulmonary edema, were removed from the lungs. ΔPO_2 increased from 19 to 110 mmHg. The PVR at first decreased from 780 to 360, but increased to 800 dynes×sec×cm^{-5}. The second immunologic case was a 43-year-old brain-dead donor due to a hypertension crisis, with PaO_2/ FIO_2 equal to 220 mmHg and obvious atelectasis in the right lung and normal bronchoscopy findings. The lungs had been harvested in another center, kept in an icebox for one hour, and transferred to Masih Daneshvari Hospital. It took 180 minutes from the application of the aortic clamp to the onset of EVLP. Pressures were kept at an overall acceptable level; however problems with temperature monitoring occurred, especially during the first thirty minutes. ΔPO_2 increased from 62 to 223 mmHg. In addition, the PVR decreased from 350 to 130 at the beginning and then increased to 444 dynes ×sec ×cm^{-5}. Lung dynamic compliance improved from 44 to 69 ml/cm H_2O and stabilized around 45.

Cytokine Assessment

Table 1 shows the degranulation changes in the constitution of the leukocyte cells trapped inside the filters. TNF-α, IL-6 and IL-8 decreased with each circulation of the perfusate and the level of cytokines in serum was in the normal range for both cases (Table 1).

Table 1. Cytokine levels of brain dead cases in serum, circulating solutions, and bronchoalveolar lavages

	Cytokines	Serum	Circulating solution						BAL	
			1	2	3	4	5	6	1	2
Case 1	IL-8 pg/ml	68	57.2	53.2	45.4	-	-	-	60	38.7
	IL-6 pg/ml	39	85.5	8.3	7	-	-	-	9	6.98
	TNF-α pg/ml	7	28.6	22	20	-	-	-	30	10
Case 2	IL-8 pg/ml	129	65.6	56.4	50.0	43.5	41.2	31.1	70	23.3
	IL-6 pg/ml	42	9.7	9	7.5	7	6.99	5	10	4.76
	TNF-α pg/ml	1.12	2.99	2.87	2.5	1.98	1.7	1.45	3	1

EVLP: Ex vivo lung perfusion

Bronchoalveolar lavage (BAL) were taken before (BAL 1) and after EVLP (BAL 2).

Blood samples from brain dead cases were collected before the lung retrieval for the measurement of serum cytokines. Inflammatory cytokines were quantitatively measured from a Perfadex preservative solution before the lung retrieval or at the end of the lung removal (sample 1), at the start of EVLP (sample 2) and circulating in the lung at the end of the each hour of EVLP (samples 3 to 6). Limitation of IL-8 kit was 15.6–1,000 pg/ml and for TNF-αwas 1-1200 pg/ml and for IL-6: 1-10000 pg/ml.

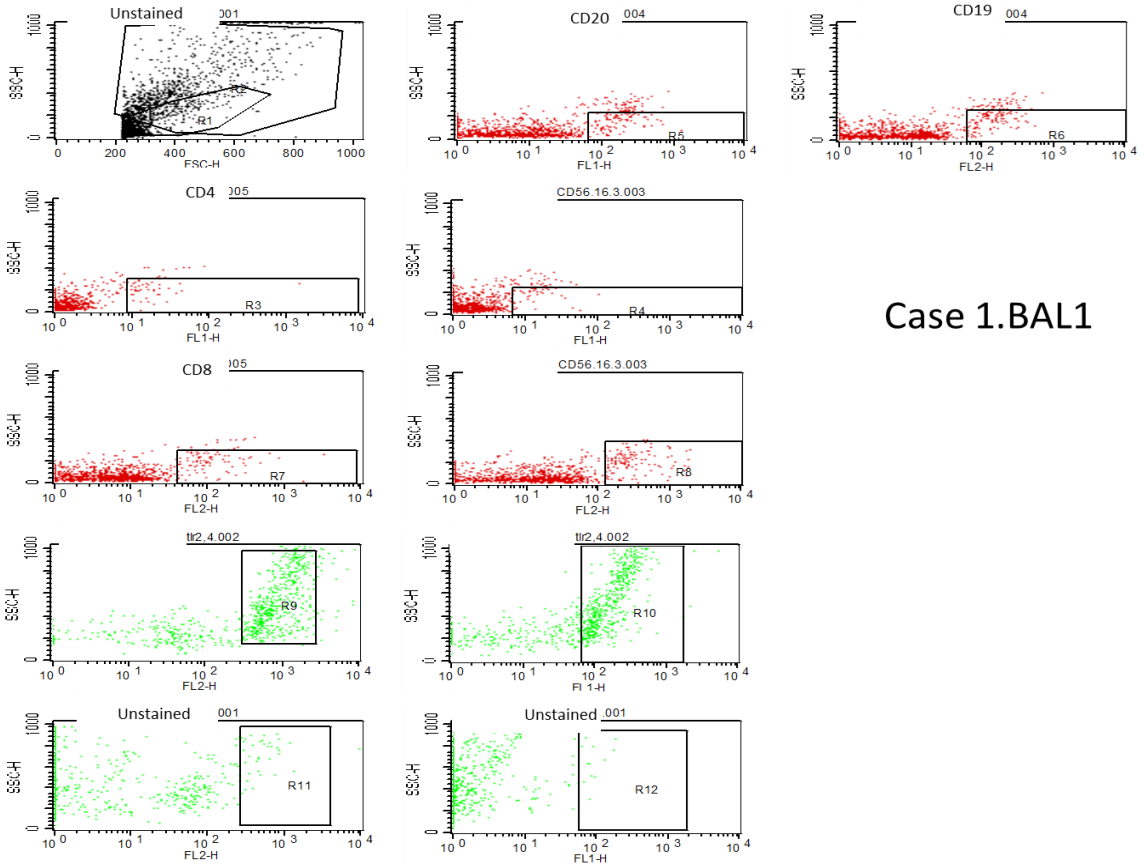

Figure 3. Flowcytometry dotblots of cell markers and toll-like receptors in bronchoalveolar lavage (BAL) of the brain dead case 1 .

BAL1: before lung retrieval.

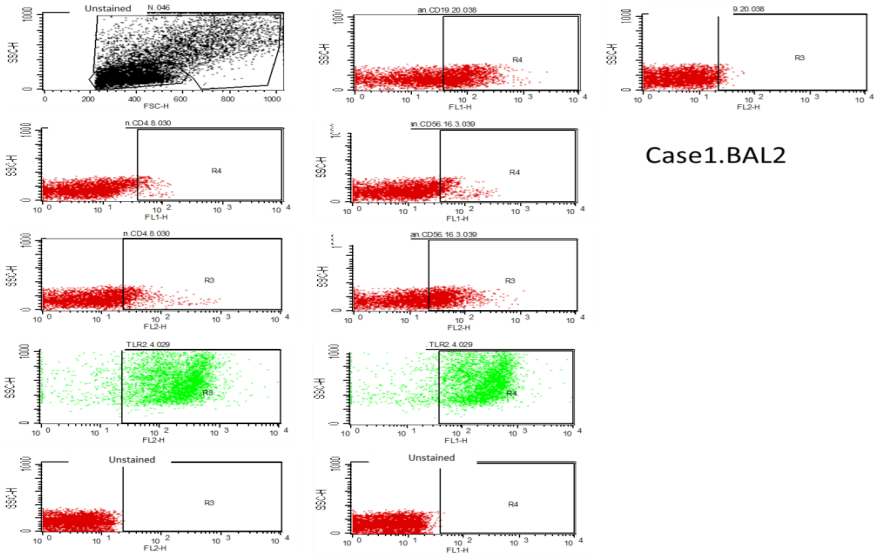

Figure 4. Flowcytometry dotblots of cell markers and toll-like receptors in bronchoalveolar lavage (BAL) of the brain dead case 1.

BAL2: after ex vivo lung perfusion (EVLP).

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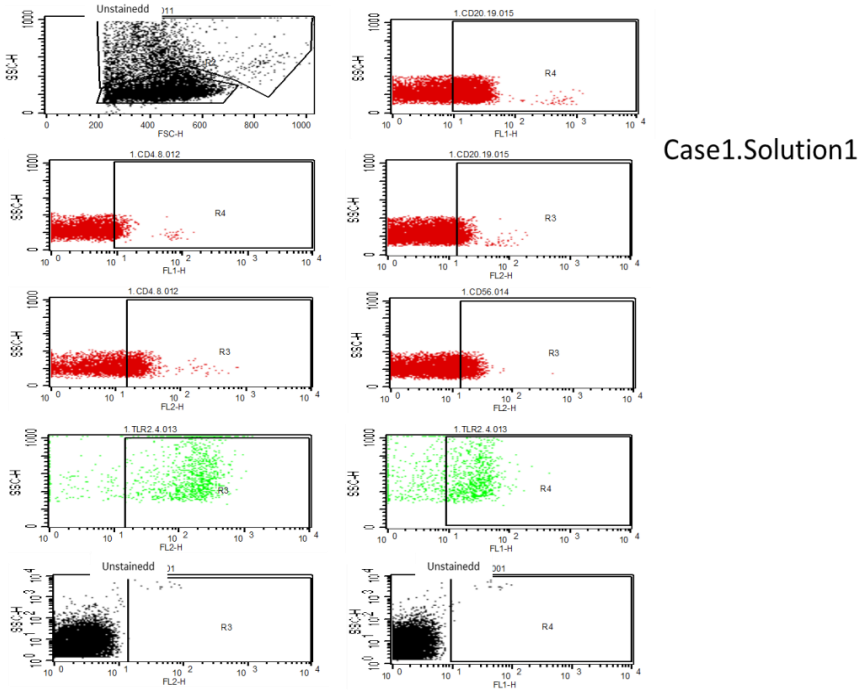

Figure 5. Flowcytometry dotblots of cell markers and toll-like receptors in circulating solution1 of the brain dead case 1. Solution 1: before lung retrival.

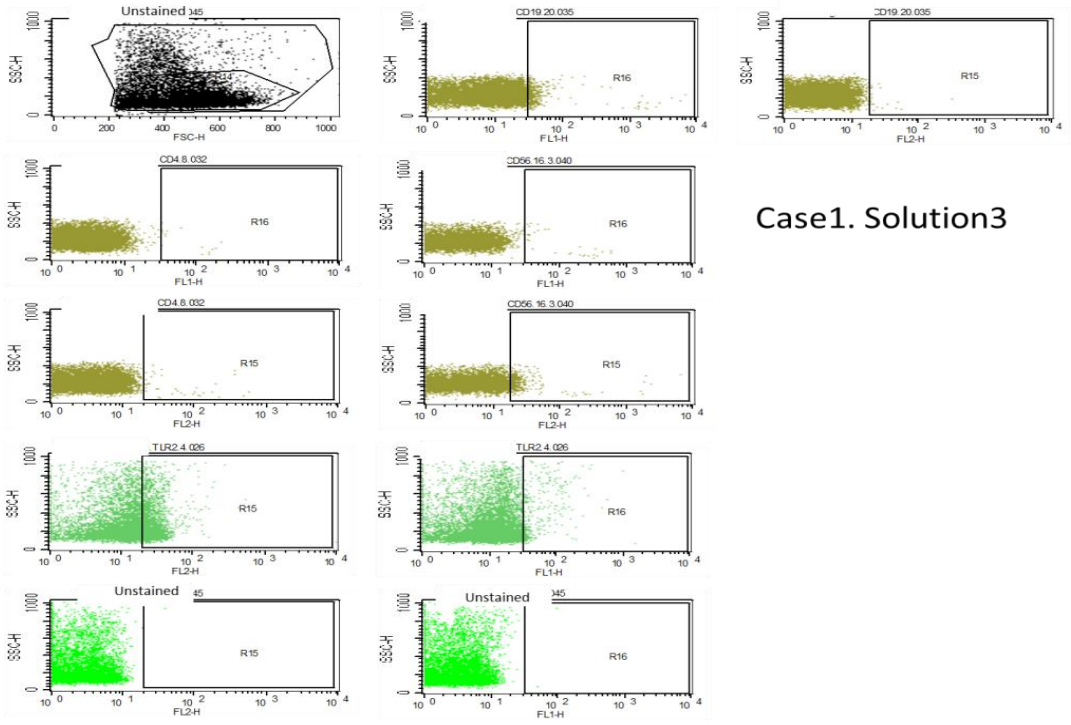

Figure 6. Flowcytometry dotblots of cell markers and toll-like receptors in circulating solution 3 of the brain dead case 1. Solution 3: after ex vivo lung perfusion (EVLP).

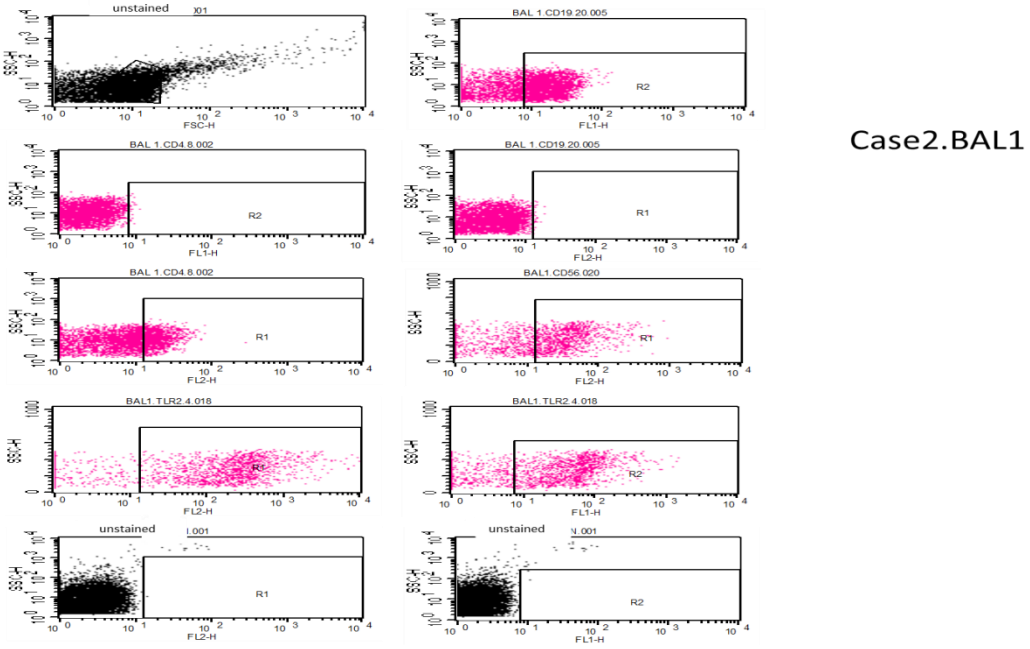

Figure 7. Flowcytometry dotblots of cell markers and toll-like receptors in bronchoalveolar lavage (BAL) of the brain dead case 2.
BAL1: before lung retrieval.

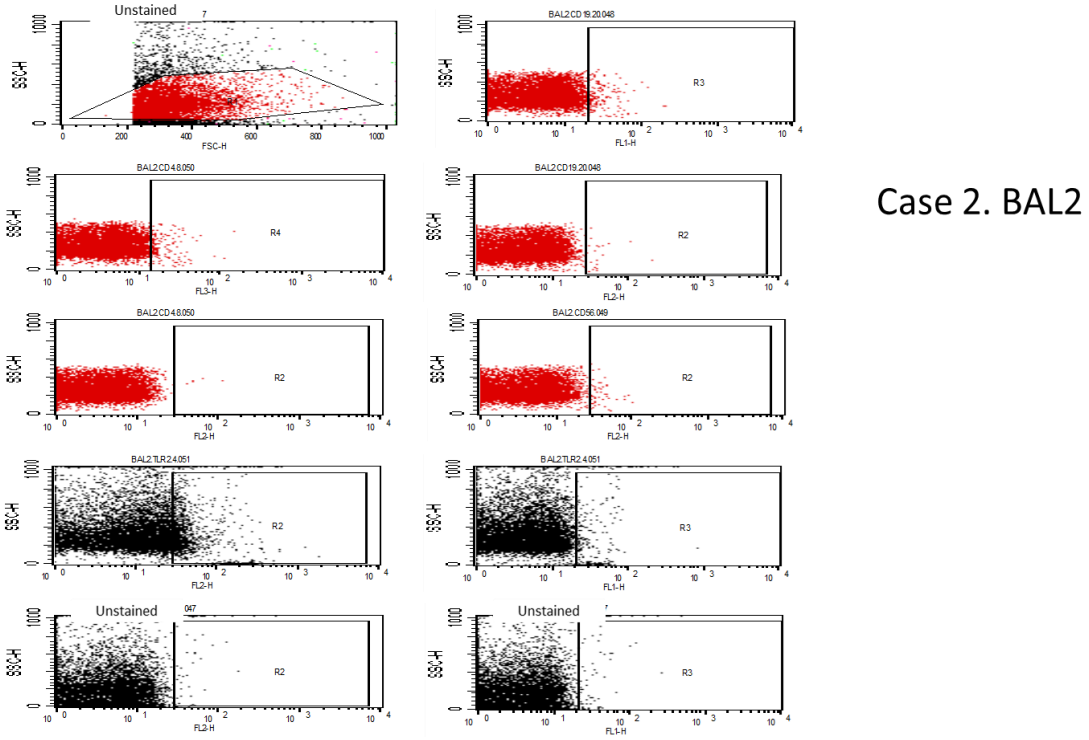

Figure 8. Flowcytometry dotblots of cell markers and toll-like receptors in bronchoalveolar lavage of the brain dead case 2.
BAL2: after ex vivo lung perfusion (EVLP).

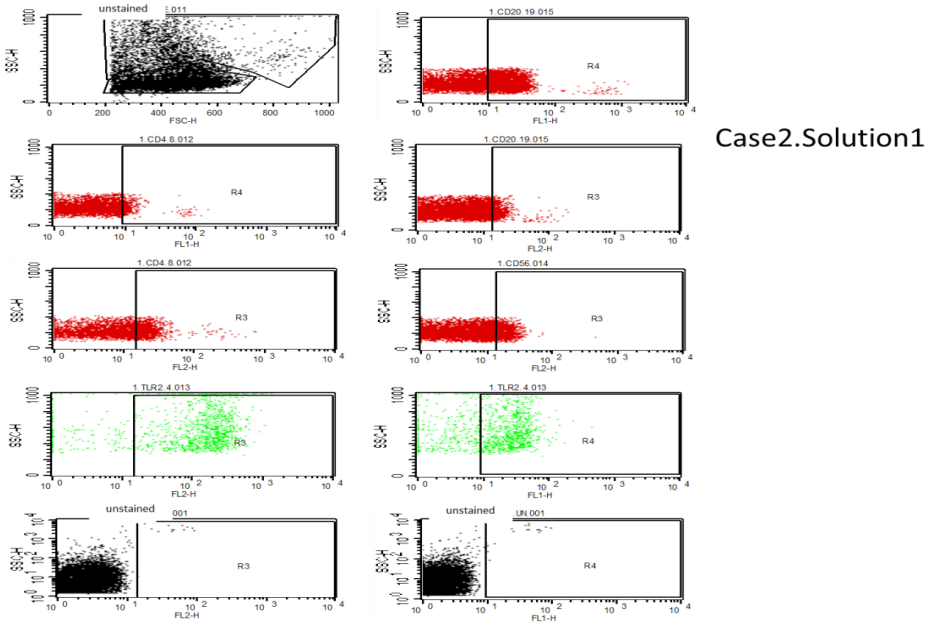

Figure 9. Flowcytometry dotblots of cell markers and toll-like receptors in circulating solution1 of the brain dead case 2. Solution 1: before lung retrival.

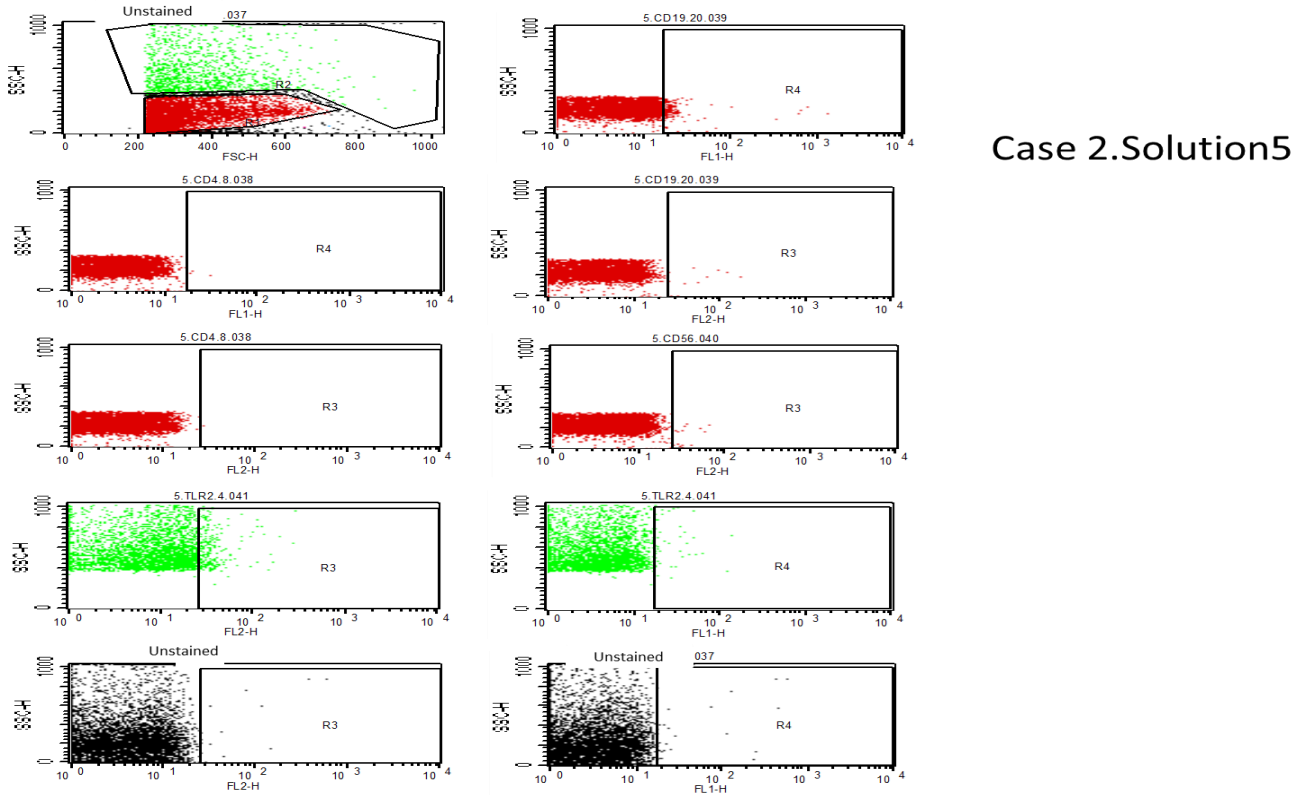

Figure 10. Flowcytometry dotblots of cell markers and toll-like receptors in circulating solution 5 of the brain dead case 2. Solution 5: after ex vivo lung perfusion (EVLP)

Table 2. Cell markers and toll-like receptors in circulating solutions, and bronchoalveolar lavages (BAL) of brain dead cases 1, 2

		CD4/CD8	CD3 (%)	CD19 (%)	CD16+56 (%)	TLR2 (%)	TLR4 (%)
Case 1	Solution1	0.8	70	5	42	0.9	0.63
	Solution2	0.74	72	4	35	0.7	0.54
	Solution3	0.62	58	3.7	32	0.3	0.45
	BAL1	0.9	84	5	42	1	0.7
	BAL2	0.6	40	2.1	28	0.3	0.3
Case 2	Solution1	1.2	90	5.9	50	0.9	0.7
	Solution2	1	77	5.7	46	0.8	0.68
	Solution3	0.9	66	5.8	38	0.6	0.59
	Solution4	0.8	57	5	37	0.4	0.5
	Solution5	0.82	52	3.2	28	0.4	0.3
	Solution6	0.8	51	3.2	29	0.5	0.25
	BAL1	1.3	90	6	50	1	0.8
	BAL2	0.7	90	3.2	18	0.2	0.1

Circulating Cells and TLRs analysis

The results showed that the numbers of leukocytes were decreased within the EVLP procedure (Table 2). The flow cytometry findings revealed a decreasing expression of CD3, CD19, CD4/8, and CD16+56 as well as TLR-2 and TLR-4 on the obtained cells from the Steen solution in both cases (Table 2). Decreasing pattern of cell markers and toll-like receptors in flow cytometry of BALs and circulating solutions of both BD cases are demonstrated in Figures 3-10.

DISCUSSION

The aim of this investigation was to determine the effect of a normothermic EVLP of BD donor lungs on the production and release of inflammatory cytokines, the type of inflammatory cells, the expression of TLR-2, and TLR -4 receptors on BAL cells before and after the EVLP procedures.

This study revealed that TNF-α, IL-6, and IL-8 decreased in both cases with each circulation of washing perfusate. Recently it has been shown that a granulocyte macrophage colony-stimulating factor was down-regulated during EVLP ($p<0.05$). IL-1 β, IL-4, IL-7, IL-12, interferon-γ, macrophage inflammatory protein-1 β, and tumor necrosis factor-α were detectable and unchanged.[29]

The role of leukocyte filters in the EVLP circuit is to remove circulating inflammatory cells from the perfusate. Leukocyte filters are the main component of the EVLP circuitry, and their value has been confirmed in animal studies.[12,30] Current models use short-period filters (<30 minutes)[31]. Intra-capillary pools of pro-inflammatory cytokines (IL-1β, IL-6, IL-8, and TNF-α) have been identified to contribute to lung injury during prolonged lung perfusion.[31]

Kakishita et al. demonstrated a significant reduction of IL-8 and TNF-α levels in the perfusate and lung tissue by using an adsorbent membrane in a porcine model. However, this failed to lead to any improvement in lung function after EVLP.[30,32]

Fildes et al. reported an increased percentage of non-classic monocyte cells (NCM, $CD14^+$, and $CD163^{-/+}$) in the leukocyte filters in a porcine model by using flow cytometry.[33] Donor-derived monocytes contribute significantly to the onset and progression of immune reactions resulting in rejection episodes after transplantation.[34] Fildes et al. showed that leukocyte filters remove lung-derived non-classic monocytes, representing 80 percent of the circulating cell population in a porcine EVLP model.[33] This raises the possibility that EVLP donor lungs could be less immunogenic than standard lungs.[34]

Cytokine profiling studies of EVLP perfusate indicated an increase in the levels of IL-6, IL-8, granulocyte colony-stimulating factor, and monocyte

chemotactic protein-1. IL-1-β, IL-4, IL-7, IL-12, interferon-γ, and TNF-α remained unchanged during EVLP.[29] In a recent study, this variation in cytokine levels in the EVLP perfusate did not lead to pulmonary edema or acute lung injury.[29] The rise in EVLP perfusate levels of IL-6, IL-8, and IL-10 was observed mainly during the first 6 hours of EVLP and this was associated with a perfusate washout effect.[35]

Further studies on both animal and human cases should be carried out to confirm, or refute similar cell populations in EVLP models.

Studies suggest that stimulation of both TLR2 and TLR4 can contribute to the inflammatory sequela of brain death which may prime the lung for subsequent ischemia reperfusion ischemia (IRI) and primary graft dysfunction (PGD).[35] Furthermore, other investigations showed that inflammatory lung injuries, which were assumed to be triggered by hypertensive crises,[11,36] can be separated from the hemodynamic response to brain death by specific desensitization of TLR signaling.[37]

Hemodynamic injury and the subsequent inflammatory response that accompanies brain death in the donor may therefore be linked to the generation of endogenous TLR ligands. Further research will identify the TLR ligands released during brain death, potentially allowing beneficial modulation of their downstream effects and mitigation of lung dysfunction after transplantation.

It has been shown that activating TLRs, especially TLR-2 and TLR -4 can lead to the maturation and activation of inflammatory cells such as DCs. This group of cells secretes pro-inflammatory cytokines such as TNF-α via a MYD88-dependent pathway.[38] They also carry antigen- derived peptides and lipids via MHC class II and CD1 molecules to T cells, natural killer cells (NK cells). Furthermore, they secrete IL-12, which stimulates native T cells to differentiate into Th1 cells.[39] Th1 cells in turn secrete IFN-γ, which stimulates monocytes to differentiate into macrophages, causing the inflammatory responses and the secretion of cytokines, particularly (IL-6, IL-1β, TNF-α, IL-1α, IL-10 and IFN-γ), chemokines (CXCL-10, CCL-2) and colony-stimulating factors.[37]

Transplantation is often the only treatment option in end-stage organ failures and BD donors are often considered as candidates in many transplant cases.[40,41]

Brain death itself impairs organ function in the potential donor, which can limit the number of suitable organs for transplantation. In fact, management of the BD donor has been found to be the most neglected area of transplantation medicine.[42]

The mechanism causing the deteriorating effect of brain death on the organs has not yet been fully understood, but it is obvious that brain death triggers massive circulatory, hormonal and metabolic changes in donors. Moreover, there is much evidence indicating that brain death is associated with systemic inflammatory responses.[43]

Experimental studies confirmed lower graft survival for kidneys from BD compared with living donors.[44-46] Thus, brain death is known to be an important antigen-independent risk factor which harms organs before transplantation to the recipient.[47] It can be concluded that after brain death, due to the release of unknown mediators, the inflammatory response is triggered, which induces the inflammation via up-regulation of cytokine release and cell- population modification.

TLRs are key sensors of the innate immune system and are pattern recognition receptors that sense pathogens associated molecular patterns (PAMPs) through leucine-rich repeats in their extracellular domain. Even when there is no infection, injured cells can release damage associated molecular patterns (DAMPs), which activate TLRs.[48,49]

There is increasing evidence that damaged tissues may release endogenous ligands called alarmins, including heparan sulphate,[50] hyaluronan[51] and high mobility group box 1 (HMGB1)[52] that can activate TLRs in the absence of infection. There is clear potential for these endogenous ligands to develop during the process of brain death in organ donors.[36,53]

In many studies of inflammation after BD, cytokines have been evaluated. . Important pro-inflammatory cytokines include TNF-α and IL-1 β, and TLR-6, whereas IL-10 has anti-inflammatory effects. It has been shown that with the onset of reperfusion, the production of TNF-α increases. Together with IL-1β, cytokines up-regulate TLR2 on pulmonary endothelial cells. TLR-4 induced by fibroblasts, and TLR-2 which binds to Versican , lead to significant increase in IL-8 production.[24-26]

Another study demonstrated that within the graft, there was an increased release of IL-1β and IL-18, which could have lead to the induction of IL-6 release (IL-6 correlates with the 30-day post-transplant mortality)[54] and an increased amount of TNF-α and IL-8.[55] The origin of these cytokines is not only the graft macrophages, but also other cell types within the graft.

These cytokines are pro-inflammatory proteins that induce other cytokines, inflammatory cell infiltration and additional inflammatory reactions.[56]

In conclusion, understanding the molecular basis of IRI in the lung and how it might be linked to cytokine production and inflammation could improve the EVLP protocol and the lung transplantation practice. Additionally, further studies including more cases are needed to assess EVLP's effects on lung graft, as this could optimize recipient outcomes.

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