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Endothelial cell activation mediated by cold ischemia-released mitochondria is partially inhibited by defibrotide and impacts on early allograft function following liver transplantation

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ABSTRACT

DAMPs (danger-associated molecular patterns) are self-molecules of the organism that appear after damage. The endothelium plays several roles in organ rejection, such as presenting alloantigens to T cells and contributing to the development of inflammation and thrombosis. This study aimed to assess whether DAMPs present in the organ preservation solution (OPS) after cold ischemic storage (CIS) contribute to exacerbating the endothelial response to an inflammatory challenge and whether defibrotide treatment could counteract this effect. The activation of cultured human umbilical vein endothelial cells (HUVECs) was analyzed after challenging with end-ischemic OPS (eiOPS) obtained after CIS. Additionally, transwell assays were performed to study the ability of eiOPS to attract lymphocytes across the endothelium. The study revealed that eiOPS upregulated the expression of MCP-1 and IL-6 in HUVECs. Moreover, eiOPS increased the membrane expression of ICAM-1and HLA-DR, which facilitated leukocyte migration toward a chemokine gradient. Furthermore, eiOPS demonstrated its chemoattractant ability. This activation was mediated by free mitochondria. Defibrotide was found to partially inhibit the eiOPS-mediated activation. Moreover, the eiOPS-mediated activation of endothelial cells (ECs) correlated with early allograft dysfunction in liver transplant patients. Our finding provide support for the hypothesis that mitochondria released during cold ischemia could trigger EC activation, leading to complications in graft outcomes. Therefore, the analysis and quantification of free mitochondria in the eiOPS samples obtained after CIS could provide a predictive value for monitoring the progression of transplantation. Moreover, defibrotide emerges as a promising therapeutic agent to mitigate the damage induced by ischemia in donated organs.

Abbreviations: CCL, C-C motif chemokine ligand; CIS, cold ischemic storage; CIT, cold ischemic time; CXCL, C-X-C motif chemokine ligand; DAMP, danger-associated molecular pattern; DBD, donation after brain death; DCD, donation after circulatory death; EC, endothelial cell; eiOPS, end-ischemic organ preservation solution; EVs, extracellular vesicles; HLA, human leukocyte antigen; HLA-DR, major histocompatibility complex, class II, DR; HUVEC, human umbilical vein endothelial cell; ICAM-1, intercellular cell adhesion molecule 1; IFN, interferon; IL, interleukin; LPS, lipopolysaccharide; MCP-1, monocyte chemoattractant protein-1; MEAF, model for early allograft function; MHC, major histocompatibility complex; mitDAMP, mitochondrial DAMP; mitDNA, mitochondrial DNA; NFP, N-formylated peptide; NRP, normothermic regional perfusion; OPS, organ preservation solution; SRR, super rapid recovery; TNF, tumor necrosis factor; VCAM-1, vascular cell adhesion molecule 1; VOD/SOS, veno-occlusive disease/sinusoidal obstruction syndrome.

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1. Introduction

The evolution and success of organ transplantation has been fueled by the advances in immunosuppressive therapy [1]. These drugs inhibit the function of the adaptive immune system T cells, which are the main cellular effectors of rejection. However, during graft ischemia, damaged tissues and cells release self-molecules that are not usually found extracellularly, known as danger signals or DAMPs (danger-associated molecular patterns) [2]. Likewise, organ donation after circulatory death (DCD), compared with the classical donation after brain death (DBD), influences the signature of the released DAMPs, which can have an impact on early liver allograft function [3,4]. Among others, apoptotic and necroptotic cells secrete both free or encapsulated mitochondria [5], which induce innate immune responses [6]. The bacterial origin of mitochondria could explain the similitude among several molecules (i.e., cytochrome C, N-formylated peptides (NFPs), or mitochondrial DNA (mitDNA)) and their ability to work as mitochondrial-derived DAMPs (mitDAMPs) [7]. In addition, other non-immune cells, including endothelial cells (ECs), are also activated by mitDAMPs. Moreover, freely circulating mitochondria are abundant in the circulation of deceased donors [8] and are able to activate ECs [9].

EC activation involves a sequence of processes, including cytokine secretion, changes from the anti-thrombotic to the pro-thrombotic phenotype, loss of vascular integrity, expression of leukocyte adhesion molecules, and upregulation of human leukocyte antigen (HLA) molecules [10]. The graft endothelium is the first wall between the self and the non-self that host lymphocytes encounter after the reperfusion of vascularized solid transplants. Graft vessels must be healthy to preserve homeostatic functions but can be activated by mediators of innate and adaptive immunity to mediate graft inflammation [11]. Moreover, the vascular endothelium also presents the ability of antigen presentation [12]. HLA class II molecules on the surface of ECs can be recognized by T-cell receptors on circulating T cells, triggering both transendothelial migration and activation; therefore, they represent a preferential target in organ rejection [13].

Defibrotide is an anti-thrombotic, pro-fibrinolytic, and anti-inflammatory drug formed by a polydisperse mixture of predominantly single-stranded polydeoxyribonucleotide sodium salts, currently derived via the controlled depolymerization of porcine intestinal mucosal DNA [14]. Defibrotide is used in bone marrow transplant-associated hepatic veno-occlusive disease/sinusoidal obstruction syndrome (VOD/SOS), a potentially fatal complication occurring in hematological patients receiving conditioning regimens for hematopoietic-stem-cell transplantation (HSCT) or chemotherapy [15]. Defibrotide has a particularly complex mechanism of action [16–19]. It has been described that this drug enters the interior of endothelial cells mainly by macropinocytosis, and remains in the cytoplasm without entering the nucleus, where it exerts anti-inflammatory effects, increases nitric oxide generation, induces PI3K/Akt and nitric oxide synthase activation, and attenuates adhesion molecule expression, heparanase activity and reactive oxygen species generation [20–22].

Therefore, the aim of the present study was to determine whether the DAMPs present in the organ preservation solution (OPS) collected from explanted livers after static cold ischemic storage contribute to activating ECs and influence the outcome of liver transplantation. We found a variety of chemokines in end-ischemic OPS (eiOPS) with the ability to attract leukocytes to the inflamed tissue and the presence of extracellular mitochondria, in addition to other mitDAMPs, that activated ECs. Moreover, this eiOPS-mediated EC activation was partially inhibited by defibrotide and correlated with poor early allograft function.

2. Methods

2.1. Cells and reagents

Gibco™ human umbilical vein endothelial cells (HUVECs), MitoTracker® Red FM and anti-human ICAM-1-APC antibody (clone HA58; #17–0549–42) were obtained from ThermoFisher Scientific (Waltham, USA), whereas Calcein AM, anti-human VCAM-1-BV605 (clone 51–10C9; #563307) and anti-human HLA-DR-APC-H7 (clone G46–6; # 561358) antibodies were obtained from BD Biosciences (Franklin Lakes, USA). Anti-translocase of outer mitochondrial membrane 20 (Tomm20) rabbit antibody was from Abcam (#ab232589; Cambridge, UK). Anti-mouse CD45-PerCP/Cy5.5 (clone 30-F11; #103131), anti-mouse F4/80-APC (clone BM8; #123115) and anti-mouse Ly6G-PE (clone 1A8; #127607) antibodies were obtained from Biolegend (San Diego, USA). Defibrotide was provided by Jazz Pharmaceuticals (Dublin, Ireland). *E. coli* lipopolysaccharide (LPS) 055:B5, recombinant human tumor necrosis factor alpha (TNF-α), and Corning® Transwell with 5-μm pore polycarbonate membrane cell culture inserts were obtained from Sigma-Aldrich (San Luis, USA). Recombinant human interferon gamma (IFN-γ), C-C motif chemokine ligand (CCL) 2, C-X-C motif chemokine ligand (CXCL) 10, and chemokine (C-X3-C motif) ligand 1 (CX3CL1) were obtained from Peprotech (London, UK).

2.2. Organ preservation solution collection

A volume of 50 mL of OPS from 79 explanted livers from deceased donors was recovered aseptically after cold ischemic storage and before implantation into the recipient as previously explained [3]. Briefly, the donor aorta and donor portal vein were each flushed with 3 L of Celsior® solution (IGL, Lissieu, France) after supraceliac aortic cross-clamping, and liver grafts were stored refrigerated at 4ºC in a Celsior® bath. After static cold storage and before implantation, the infrahepatic inferior vena cava was ligated, and liver grafts were flushed with 500 mL of 5% human albumin (Grifols, Barcelona, Spain) via the portal vein. At that moment, the first 50 mL of intrahepatic eiOPS was retrieved directly from the hepatic vein outflow into the suprahepatic inferior vena cava. eiOPS was stored refrigerated and promptly centrifuged at 400 x*g* for 10 min, and the supernatants were aliquoted and frozen at − 80 ºC until use.

2.3. Patients

Seventy-nine liver transplant patients who received one of the studied donated livers participated in the present study after providing their informed consent; the study was approved by the ethical committee of Hospital Clínico Universitario Virgen de la Arrixaca (2019–6–2-HCUVA) and conforms to the ethical guidelines of the 1975 Declaration of Helsinki. The demographic and clinical characteristics of the donors and recipients have been previously published [3]. In brief, 49 explanted livers came from DBD donors (62%). In DCD donors (n = 30; 38%), 73.4% were recovered with the super rapid recovery (SRR) technique, whereas the other 27.6% were recovered under normothermic regional perfusion (NRP). The mean age of donors was 60.6 ± 14.2 years-old, and male represented 53.2%. Cold ischemic time (CIT) reached a mean of 331.8 ± 151.4 min. No significant differences were found between the different types of donations in terms of age (p = 0.964), sex (p = 0.627), or CIT (p = 0.455). Likewise, functional warm ischemia was similar between SRR and NRP donors (p = 0.146). Recipients had a mean age of 57.0 ± 9.6 years-old, with 64.7% being males. The main etiology for transplantation was alcoholic cirrhosis (46.8%), followed by viral infection (13.9%). Nevertheless, 11 patients (13.9%) were treated with a re-transplant, and 2 (2.5%) died at surgery (See Table S1).

2.4. Cell culture and human peripheral blood mononuclear cell (PBMC) isolation

Gibco™ HUVECs from a single donor (#C00035C; ThermoFisher Scientific) were cultured in Gibco™ Medium 2000 supplemented with Low Serum Growth Supplement in the absence of antibiotics from

passage numbers 2–12. A total of 1×10^5 cells/well were cultured in 24-well plates to achieve confluence. They were then incubated for 16 h at 37 ºC in the presence of 500 ng/mL LPS, 10 ng/mL TNF-α, 10 ng/mL IFN-γ, all diluted in Celsior®, or non-diluted eiOPS recollected from explanted livers after cold ischemic storage.

Blood from healthy volunteers was collected after informed consent and PBMCs were isolated from whole blood via centrifugation in Ficoll-Paque gradient.

2.5. Determination of endothelial adhesion proteins and major histocompatibility complex (MHC) class II cell surface molecules in HUVECs

The membrane expression of those molecules was determined with flow cytometry. Briefly, cultured HUVECs were detached from wells using TrypLE™ Express (ThermoFisher Scientific) and were then stained with anti-ICAM-1-APC, anti-VCAM-1-BV605, and anti-HLA-DR-APC-H7 antibodies.

After staining, the cells were subjected to a flow cytometry analysis using a BD FACSCanto flow cytometer and FACSDiva software (BD Biosciences), gating for singlets based on the forward scatter (FSC-A, FSC-H) and side scatter (SSC-A) parameters. Data were analyzed using FCS Express 5 software (DeNovo Software, Pasadena, USA).

2.6. Determination of chemokines in eiOPS

A Luminex multiplex assay (R&D Systems, Minneapolis, USA) measured with MagPix System (Luminex, Austin, USA) was carried out to detect the presence of 17 chemokines (CCL2, CCL3, CCL4, CCL5, CCL8, CCL17, CCL20, CCL22, CCL28, CXCL1, CXCL2, CXCL5, CXCL6, CXCL9, CXCL10, CXCL16, and CX3CL1).

2.7. Quantification of extracellular mitochondria in eiOPS

For the determination of extracellular mitochondria, 500 μL of eiOPS were incubated with 10 μM Calcein AM and 100 nM MitoTracker® Red FM for 20 min at 37ºC. After that, eiOPS was centrifuged at 15,000 x*g* for 15 min at 4ºC and the resulting pellet was resuspended and subjected to flow cytometry analysis. Free mitochondria were identified as events exhibiting single positive fluorescence for MitoTracker® Red FM, while mitochondria packed within extracellular vesicles (EVs) also exhibited positivity for Calcein AM (Fig. S2b).

2.8. Determination of mitochondrial DAMPs

A volume of 600 μL of eiOPS was centrifuged at 15,000 x*g* for 15 min at 4 ºC, and the supernatant was used for free-mitDNA determination. mitDNA was extracted and quantified with quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) as previously indicated [23]. NFPs were quantified using a Human Formylmethionine (fMET) ELISA kit (SAB-Signalway antibody, Maryland, USA), whereas cytochrome C was measured using a Cytochrome C Human ELISA kit (Invitrogen) following the manufacturer's instructions.

2.9. Quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR)

Total RNA was extracted from HUVECs with RNAqueous Micro Kit (Invitrogen), followed by reverse transcription. qRT-PCR was performed using SYBR Premix ExTaq (Takara Bio Inc., Kusatsu, Japan). The samples were run in duplicate, and the relative gene expression levels were calculated using the 2-ΔCt method, normalizing to 18 S rRNA.

2.10. Transwell migration assays

For the determination of the chemoattractant ability of the OPS, 600 μL of eiOPS was added to the bottom chamber, whereas 3×10^5 human PBMCs resuspended in 100 μL of DMEM medium (Sigma-Aldrich) containing 0.3% BSA were added to the upper chamber of a Corning® Transwell polycarbonate membrane cell culture insert (5-μm pore size). Next, cells were allowed to migrate for 6 h at 37 ºC; then, the migrated cells present in the lower chamber were recovered and stained for the detection of $CD45^+$ cells by flow cytometry.

On the other hand, 7.5×10^4 HUVECs were cultured over a Transwell polycarbonate membrane and activated for 16 h with 500 ng/mL LPS, 10 ng/mL TNF-α, or eiOPS. After that, 100 ng/mL recombinant human CCL2, CXCL10, and CX3CL1 were added to the lower chamber, whereas 3×10^5 human PBMCs were added to the upper chamber. Then, cells were allowed to migrate for 16 h at 37 ºC; then, the migrated cells present in the lower chamber were recovered and stained for the detection of $CD45^+$ cells with flow cytometry and quantified using Trucount Absolute Counting tubes (BD Biosciences).

2.11. In vivo mouse model of peritoneal neutrophil infiltration

All experimental protocols for animal handling were refined and approved by the Animal Health Service of the General Directorate of Fishing and Farming of the Council of Murcia (Servicio de Sanidad Animal, Dirección General de Ganadería y Pesca, Consejería de Agricultura y Agua Región de Murcia, reference A13220602). Animal welfare-related assessments, measurements, and interventions were conducted before, during, and after the experiments. C57BL/6 (wild-type) mice were obtained from Charles River Laboratories (Saint-Germain-Nuelles, France). For all experiments, male mice between 8 and 10 weeks of age were used. Mice were randomly assigned to each group, and 3–5 mice were housed per cage with a 12:12 hr light-dark cycle, according the Animal Experimentation Guidelines of the Hospital Clínico Universitario Virgen de la Arrixaca, and in accordance with Spanish national (Royal Decree 1201/2005 and Law 32/2007) and EU (86/609/EEC and 2010/63/EU) legislation. A volume of 300 μL of Celsior® as negative control, LPS (diluted in Celsior®) or eiOPS were injected intraperitoneally. When indicated, mice were treated intraperitoneally with 25 mg/Kg/day defibrotide from day − 3 until the end of the experiment [20]. Mouse eating, drinking, movement, and the operated area were monitored daily. After 24 h, mice were euthanized by exposure to CO_2 followed by cervical dislocation, and a peritoneal lavage was performed with 3 mL of PBS to recover peritoneal cells. Cells were then counted and stained with anti-mouse CD45-PerCP-Cy5.5, anti-mouse F4/80-APC and anti-mouse Ly6G-PE antibodies, and subsequently analyzed by flow cytometry.

2.12. Model for early allograft function score

The model for early allograft function (MEAF) score was calculated as previously described [24] based on the maximum alanine aminotransferase (ALT) and international normalized ratio (INR) during the first 3 postoperative days and the serum bilirubin levels on day 3 after transplantation.

2.13. Statistical analysis

Statistics were calculated with GraphPad Prism 8.0.2 software (GraphPad Software Inc., San Diego, USA). The results are here represented as means ± SEMs. All group sizes were at least $n = 5$. The statistical calculations were performed with a one-way ANOVA followed by Bonferroni's post hoc tests for multiple comparisons if F reached $P < 0.05$ and there was no significant variance inhomogeneity. The logarithmic transformation of some results was performed to avoid unwanted sources of variation. Student's t-test was used for comparing the two groups. For all comparisons, $P < 0.05$ was accepted to indicate a statistically significant difference. Correlation analyses were evaluated using Spearman's rank correlation coefficient. The eiOPS used for each

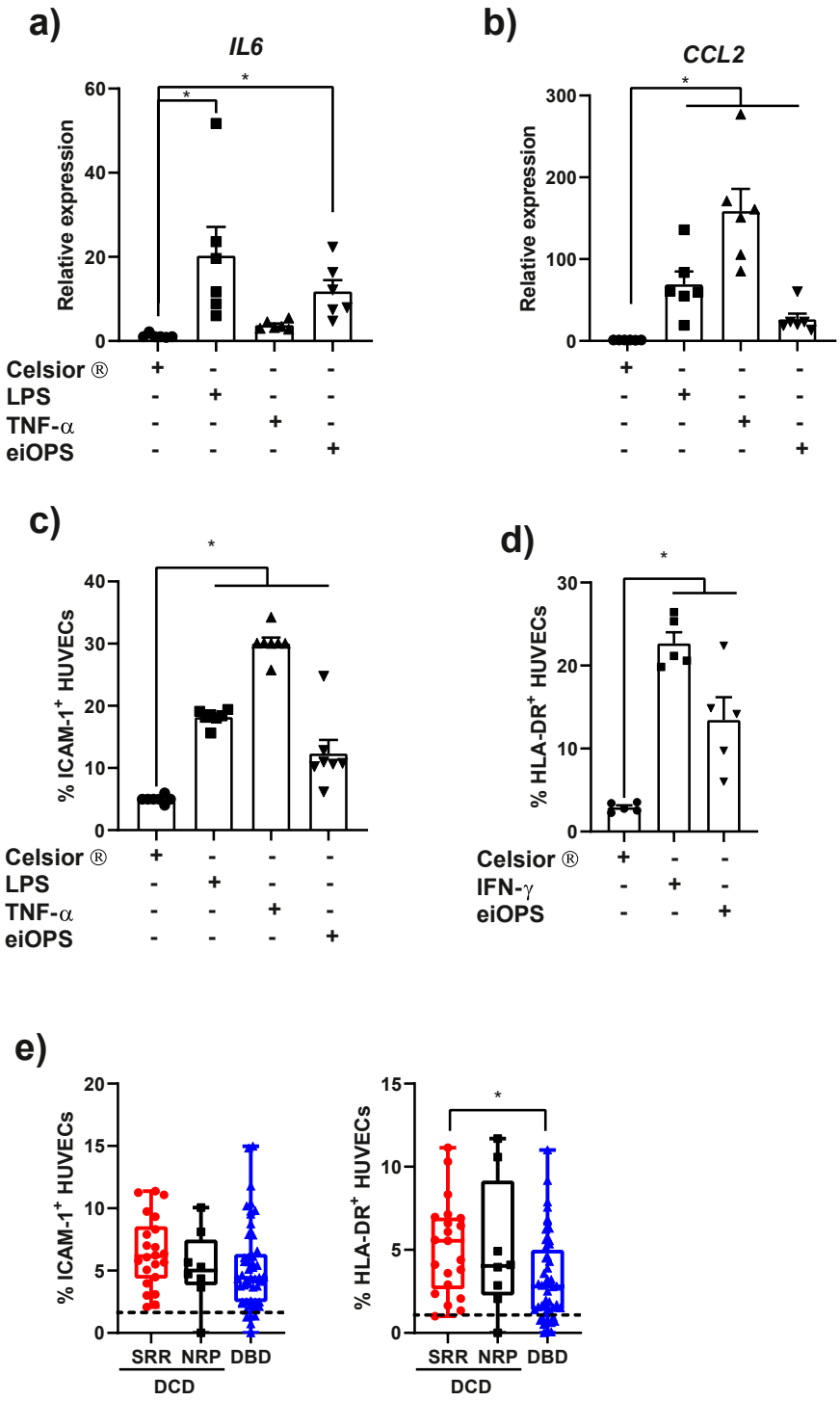


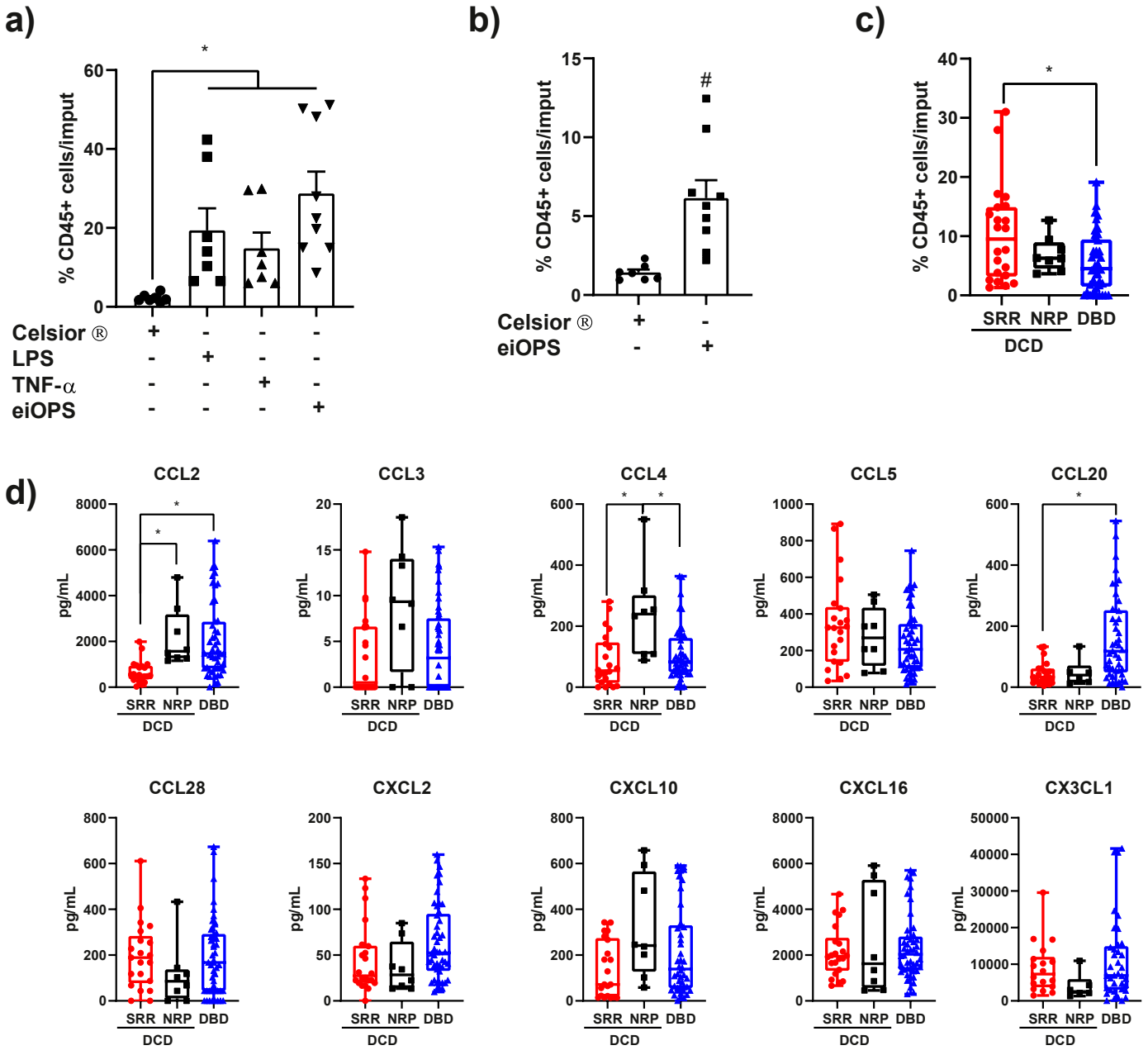
Fig. 1. End-ischemic OPS activates endothelial cells. (a, b) Relative expression of *IL6* (a) and *CCL2* (b) in HUVECs as analyzed with qRT-PCR; n = 6. (c-e) Expression of membrane ICAM-1 (c, e) and HLA-DR (d, e) in HUVECs as detected by flow cytometry. In total, 1×10^5 HUVECs were incubated for 16 h in the presence of Celsior® as the negative control or with 500 ng/mL LPS, 10 ng/mL TNF-α, 10 ng/mL IFN-γ (diluted in Celsior®) or non-diluted eiOPS coming from a different pool of donors. Results are presented as means ± SEMs; n = 6 (c); n = 5 (d). In (e), 1×10^5 HUVECs were incubated for 16 h only in the presence of eiOPS, and each dot represents a single donor. Mean membrane protein expression in resting cells is represented as a dashed line. Outliers from datasets were identified using the ROUT method, with Q= 1%, and were eliminated from analyses and representation. Results are presented as medians, interquartile ranges, and minimum and maximum values. * $p \leq 0.05$, one-way ANOVA followed by Bonferroni's test.

experiment came from a different pool of donors, and each experiment was conducted in duplicate.

3. Results

3.1. OPS collected after cold ischemia activates endothelial cells

As described in our previous work, several DAMPs released to OPS during static cold ischemic storage are able to activate NLRP3 inflammasome in monocytes/macrophages [25]. The activation of ECs in allotransplantation can induce inflammation and thrombosis, leading to graft rejection [11]. In this context, we wondered if eiOPS could also activate ECs. When HUVECs were cultured in the presence of eiOPS for 16 h, different markers of EC activation were increased with respect to cells cultured in the presence of Celsior® as negative control (Fig. 1). The gene expression of *IL6* (Fig. 1a) and *CCL2* (Fig. 1b), similar to the membrane expression of the adhesion molecules ICAM-1 (Fig. 1c; Fig. S1a) or to a lesser extent VCAM-1 (Fig. S1b), increased significantly in response to eiOPS, an effect which was similar to that found after exposure to LPS or TNF-α, used as positive controls. Likewise, we analyzed the expression of MHC class II molecules in ECs, as it allows ECs to act as antigen-presenting cells and to influence transplant



e)

	Spearman's rho	CCL3	CCL4	CXCL10
% CD45+ cells/imput	Correlation coeficient	0.316	0.316	0.234
	Sig. (2-tailed)	0.005	0.005	0.038
	N	79	79	79

(caption on next page)

Fig. 2. End-ischemic OPS induces leukocyte transendothelial migration. (a-c) Percentages of $CD45^+$ human leukocytes migrated to the bottom chamber of Corning® Transwell polycarbonate membrane cell culture inserts (5-μm pore size) with respect to total cells (input) added to the upper chamber are shown. In (a), $7.5x10^4$ HUVECs were cultured over a polycarbonate membrane and were activated for 16 h in the presence of Celsior® as the negative control or with 500 ng/mL LPS, 10 ng/mL TNF-α (diluted in Celsior®) or non-diluted eiOPS coming from a different pool of donors. After that, 100 ng/mL recombinant human CCL2, CXCL10, and CX3CL1 were added to the lower chamber, whereas $3x10^5$ human PBMCs were added to the upper chamber. After 16 h of incubation at 37 ºC, migrated cells in the lower chamber were recovered and stained for flow cytometry; $n \geq 7$. In (b, c), 600 μL of non-diluted eiOPS or Celsior® as the negative control (b; $n \geq 7$) was added to the bottom chamber, whereas $3x10^5$ human PBMCs were added to the upper chamber of Corning® Transwell polycarbonate membrane cell culture inserts (5-μm pore size) for 6 h at 37 ºC; then, migrated cells in the lower chamber were recovered and stained for flow cytometry. (d) Concentrations of several chemokines found in eiOPS are shown. In (a, b), results are presented as means ± SEMs. In c-d, each dot represents a single donor. Outliers from datasets were identified using the ROUT method, with Q= 1%, and were eliminated from analyses and representation. Results are presented as medians, interquartile ranges, and minimum and maximum values. * $p \leq 0.05$, one-way ANOVA followed by Bonferroni's test (a, c-d), or $^{\#}p \leq 0.05$, unpaired Student's t-test (b). (e) Correlation matrix between migrated leukocyte percentages in the transwell assays and several chemokines levels found in eiOPS are shown.

Table 1
Quantification of chemokines detected in eiOPS from 79 explanted livers.

Chemokines	Concentration (pg/mL)[a]
CCL2	1270 (0–8863)
CCL20	75.18 (0–5113)
CCL28	167.4 (0–1057)
CCL3	4 (0–19.31)
CCL4	87.45 (0–629.8)
CCL5	242.5 (16.61–875.8)
CX3CL1	11,298 (0–2366,039)
CXCL2	44.45 (0–233.16)
CXCL10	172.1 (0–994.5)
CXCL16	1981 (295.1–9057)

[a] Variables are expressed as median (range).

rejection [12]. Also, eiOPS successfully induced the membrane expression of HLA-DR in HUVEC cells (Fig. 1d; Fig. S1c), with IFN-γ serving as a positive control [26]. Moreover, eiOPS from DCD livers had a relatively greater ability to upregulate membrane cell adhesion molecules such as ICAM-1 (Fig. 1e) and VCAM-1 (Fig. S1d) and for activating HLA-DR expression in HUVECs, principally in the SRR group, compared with DBD livers (Fig. 1e).

3.2. eiOPS induces leukocyte extravasation and exhibits chemoattractant ability

Endothelial activation leads to leukocyte adherence and recruitment, with subsequent leukocyte extravasation to inflamed tissues [27]. Thus, we studied the ability of eiOPS to induce such action in ECs using HUVEC transwell assays. As shown in Fig. 2a, eiOPS mediated the migration of $CD45^+$ human leukocytes through a monolayer of HUVECs towards a gradient of recombinant human chemokines (CCL2, CXCL10, and CX3CL1), similar to when HUVECs were stimulated with LPS or TNF-α. Even more, eiOPS exhibited its own chemoattractant ability, as human leukocytes were able to migrate through the 5-μm pore size membrane towards eiOPS, but not towards Celsior® (Fig. 2b). Again, eiOPS from DCD livers displayed a significantly higher chemoattractant ability, principally in the SRR group, compared with DBD livers (Fig. 2c). These data suggested the presence of active chemokines in eiOPS. Thus, we analyzed the presence of 17 chemokines, of which only 10 were detected in eiOPS from most of the 79 explanted livers (Table 1 and Fig. 2d). Only CCL2, CCL4, and CCL20 showed significant differences among donors, finding a lower amount of these chemokines in SRR livers from the DCD group (Fig. 2c). Moreover, a significant but weak positive correlation was found between the concentration of CCL3, CCL4, or CXCL10 measured in eiOPS and its chemoattractant ability in the HUVEC transwell experiments (Fig. 2e). However, no significant correlation was found between CIT and chemokine levels (Table S2).

Table 2
Quantification of mitDAMPs detected in eiOPS coming from 79 explanted livers.

mitDAMPs	Concentration[a]
Free mitochondria (number/mL)	34,764 (3013–195,093)
Mito-EVs (number/mL)	70,110 (4532–518,900)
mitDNA (pg/mL)	5.27 (0.10–125.80)
Cytochrome C (ng/mL)	0.97 (0.31–15.72)
NFPs (pg/mL)	60.31 (12.76–147.80)

[a] Variables are expressed as median (range). Mito-EVs (Extracellular vesicles including mitocondria); mitDNA (mitochondrial DNA); NFPs (N-formylated peptides).

3.3. Extracellular mitochondria present in eiOPS mediates the activation of endothelial cells

Bacterial LPS and TNF-α are the principal endothelial activators, together with IL-1β [28], whereas IFN-γ induces the expression of HLA-DR [26]. We previously demonstrated the presence of low amounts of endotoxins in eiOPS from the 79 explanted livers and practically no detectable amounts of TNF-α, IL-1β, or IFN-γ [25]. In this regard, low amounts of LPS (0.01 ng/mL), similar to those found in eiOPS, were not able to activate HUVECs (Fig. S2a). Recently, it was demonstrated that extracellular mitochondria are abundant in the circulation of deceased donors [8] and that they are able to activate ECs [9]. Thus, we analyzed the presence of extracellular mitochondria in eiOPS and found a large amount of free, whole mitochondria in eiOPS, but also mitochondria packed within EVs (Fig. S2b, Table 2). Then, we found that the removal of total extracellular mitochondria from eiOPS using centrifugation (Figs. S2c) impaired the ability of eiOPS to induce the expression of ICAM-1 in HUVECs (Fig. 3a), as well as HLA-DR (Fig. 3b). When the pellet from centrifuged eiOPS was added to HUVECs, activation was recovered (Fig. 3a, b). Likewise, when only free mitochondria were removed from eiOPS (Fig. S2d), ECs activation was also impaired (Fig. 3a, b). Although a trend to present higher amounts of free mitochondria in eiOPS from DCD compared with DBD livers was observed, no statistical significance was achieved (Fig. 3c). Moreover, other mitDAMPs associated with mitochondrial damage, including mitDNA, NFPs, and cytochrome C, were found in eiOPS (Fig. 3d). NFPs, cytochrome C, and free mitochondria showed a positive correlation both among themselves and with the induction of HLA-DR membrane expression in HUVECs (Fig. 3e). Likewise, CIT showed a modest correlation solely with the amount of free mitochondria released into eiOPS during cold ischemic storage (Fig. 3e).

3.4. Defibrotide inhibits the activation of endothelial cells induced by eiOPS

Defibrotide is used for the treatment of bone marrow transplant-associated hepatic veno-occlusive disease/sinusoidal obstruction syndrome (VOD/SOS) [15], and its great potential to modulate endothelial activation and damage has been previously described [16,22]. Thus, we wanted to assess whether defibrotide was able to impair the ability of

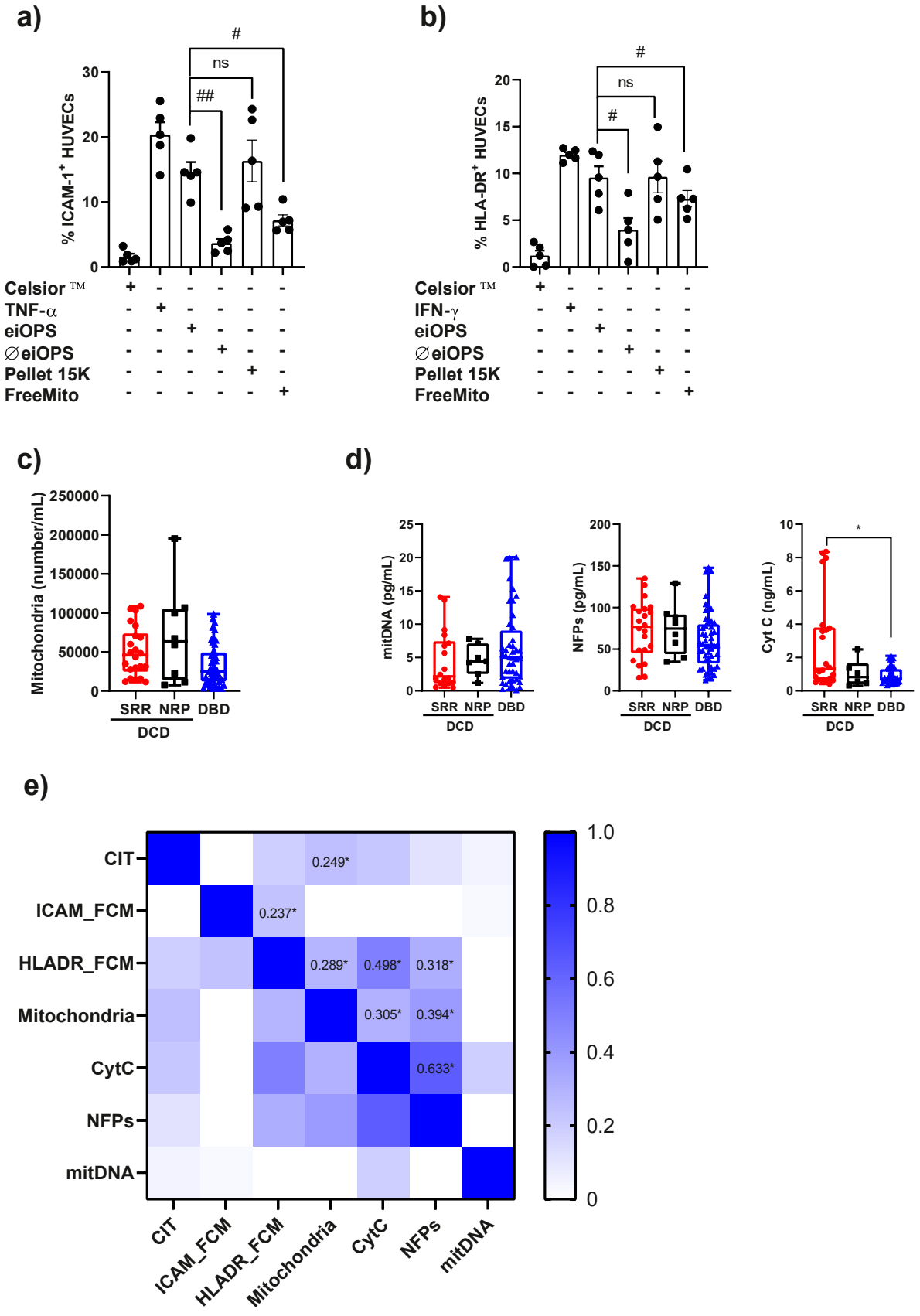


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Fig. 3. Mitochondria present in eiOPS activate endothelial cells. (a, b) Expression of membrane ICAM-1 (a) and HLA-DR (b) in HUVECs was detected by flow cytometry. In total, 1×10^5 HUVECs were incubated for 16 h in the presence of Celsior® as the negative control, or with 10 ng/mL TNF-α (a), IFN-γ (diluted in Celsior®) (b) or non-diluted eiOPS coming from a different pool of donors, before and after (ø) total mitochondria removal by means of centrifugation at 15,000 *xg* for 15 min. Pellet obtained after centrifugation, was resuspended in Celsior® and added to HUVECs (Pellet 15k). For free mitochondria removing, eiOPS pools were incubated with anti-Tomm20, followed by anti-rabbit magnetic beads incubation and magnetic separation. eiOPS depleted of free mitochondria (FreeMito) was also added to HUVECs in this experiment. Results are presented as means ± SEMs. $^{\#}p \leq 0.05$, unpaired Student's t-test; n = 6 (a); n = 5 (b). (c) The number of free mitochondria found in eiOPS was detected by flow cytometry. 500 μL of eiOPS were stained with 10 μM Calcein AM and 100 nM MitoTracker® Red FM during 20 min at 37ºC. Free mitochondria are detected as single positive MitoTracker® Red FM events, while mitochondria packed within extracellular vesicles appear as double positive MitoTracker® Red FM and Calcein AM events. Absolute mitochondria counts were obtained using Trucount Absolute Counting tubes (BD Biosciences). (d) The concentrations of mitDNA, NFPs, and cytochrome C in eiOPS were measured with qPCR (mitDNA) or ELISA (NFPs and cytochrome C). Each dot represents a single donor. Outliers from datasets were identified using the ROUT method, with $Q = 1\%$, and were eliminated from analyses and representation. Results are presented as medians, interquartile ranges, and minimum and maximum values. * $p \leq 0.05$, one-way ANOVA followed by Bonferroni's test. (e) Spearman's correlation matrix among cold ischemic time (CIT), membrane expression of ICAM-1 (ICAM_FCM) or HLA-DR (HLADR_FCM) in HUVECs, number of mitochondria, and concentration of cytochrome C (CytC), NFPs, or mitDNA found in eiOPS. Numbers indicate the correlation coefficient (Spearman's rho). * $p \leq 0.05$.

eiOPS to activate ECs. The pre-treatment of HUVECs with 100 μg/mL defibrotide [20] was enough to significantly prevent the upregulation of the expression of the membrane ICAM-1 molecule, such in the presence of TNF-α as eiOPS (Fig. 4a). Likewise, defibrotide significantly inhibited the transendothelial migration of leukocytes through a monolayer of activated HUVECs after treatment with both TNF-α and eiOPS (Fig. 4b). However, defibrotide did not significantly impair the upregulated expression of MHC class II molecules in HUVECs after IFN-γ or eiOPS treatment (Fig. 4c). Similar results were obtained from an in vivo mouse model of peritoneal neutrophil infiltration (Fig. 4d). Intraperitoneal injection of eiOPS significantly induced the infiltration of $F4/80^{-}Ly6G^{+}$ cells, although to a lesser extent than that induced by a high concentration of LPS. The elimination of mitochondria from eiOPS partially inhibited neutrophil infiltration, which was practically recovered when the mitochondria pellet was injected separately. Similarly, mice treated with defibrotide showed a partial inhibition of peritoneal neutrophil infiltration. The presence of very low concentrations of LPS, such as those observed in eiOPS [3] had no effect on the neutrophil infiltration.

3.5. eiOPS-mediated expression of membrane ICAM-1 and HLA-DR in HUVECs correlates with early allograft dysfunction after liver transplantation

To find out if the ability of eiOPS to activate ECs could influence liver transplantation outcomes, we analyzed early allograft dysfunction after liver transplantation using the MEAF score, which predicts recipient and graft survival [24]. In this regard, the eiOPS-mediated expression of membrane ICAM-1 and HLA-DR in HUVECs was found to be positively correlated with the MEAF score (Fig. 5), although no additional analyzed parameter appeared as correlated with early graft dysfunction (Table S3). Overall, these data suggest that the activation of the endothelium, probably mediated by mitochondria released in eiOPS, could compromise the early outcomes of transplanted livers.

4. Discussion

Human vascularized allografts are subject to host alloimmune responses. Graft vascular cells can be activated by mediators of innate and adaptive immunity to participate in graft inflammation, contributing to both ischemia–reperfusion injury and allograft rejection [11]. We previously demonstrated that eiOPS is rich in inflammatory and danger signals that directly activate the inflammasome of myeloid cells [25]. Now, we show how eiOPS is able to even activate ECs. ECs play a significant role in upregulating inflammation promptly after transplantation. The ECs of an allograft are subjected to constant pressure from inflammatory stimuli. They enable the recruitment of immune effector cells and regulate leukocyte extravasation at the specific sites of inflammation after the inducible expression of EC adhesion molecules such as ICAM-1 or VCAM-1. In such situations, the activation of ECs upregulates HLA antigens [29]. HLA class II molecules on the surface of ECs can mediate antigen presentation and initiate rapid and localized memory immune responses in peripheral tissues; therefore, they represent a preferential target in organ rejection [13]. In this work, we show that eiOPS-mediated EC activation is found at the level of the expression of HLA class II antigens and adhesion molecules that mediates the transmigration of leukocytes through ECs. Although LPS, TNF-α, IL-1β, and IFN-γ are well-known activators of ECs [26,28], we demonstrated that mitochondria released during cold ischemic storage are able to activate the expression of both adhesion molecules and HLA class II antigens. Freely circulating and membrane-bound mitochondria have been detected in plasma from organ donors [8], and it has previously been shown that EC activation, leading to an increase in membrane adhesion molecules and HLA class II antigens, could be induced by the uptake of isolated mitochondria [9]. Whilst it is striking that mitochondria activate such different pathways in ECs, it is worth noting that, for instance, TNF-α also increase membrane Class II molecules in combination with IFN-γ [30,31]. Although we cannot rule out a plausible effect of EV-embedded mitochondria in ECs activation, as seen for brain ECs [32], our data point to free mitochondria as the main activator. Puhmet et al. showed that depletion of free mitochondria significantly reduced the ability of microvesicles derived from activated monocytes to induce TNF responses in ECs [33]. The number of free mitochondria found in eiOPS correlated with the induction of the expression of HLA-DR antigens, and both were found to be higher in DCD livers. Although under continuous improvement, DCD involves warm ischemic time and more complications than DBD grafts [34], which is consistent with a higher presence of different DAMPs in eiOPS [25]. In this regard, we also found higher concentrations of mitDAMPs, mostly NFPs and cytochrome C, in eiOPS from DCD livers, with a high correlation with total free mitochondria, indicating that mitochondria can be damaged to some extent [35]. Zhu et al. demonstrated in a rat liver transplant model that mitochondria from apoptotic cells were highly inflammatory, principally due to the presence of cardiolipin [6], a mitochondrion-specific lipid located in the inner mitochondrial membrane [36] that translocates to the outer membrane after mitochondrial damage [37]. Likewise, only mitochondria isolated from LPS-treated, but not vehicle-treated, THP-1 human monocytic cells were able to activate HUVECs [33]. Stimulation with LPS disturbs the activity of mitochondria [38], which are significant intracellular mediators of the immune response [39]. It is worth noting that mitDNA, a potent mitDAMP [40], did not correlate with other mitDAMPs or EC activation. In contrast to cytochrome C, the release of mitDNA, similar to NFPs, requires the rupture of the mitochondrial inner membrane [41], a well-documented event that occurs in necroptotic cells [42]. Nevertheless, more recently, it has been shown how the permeabilization of the mitochondrial inner membrane enables mitDNA release during apoptosis [43] and pyroptosis, probably mediated by gasdermin pores [23]. However, it is still believed that NFPs are only secreted by necrotic cells [44]. This discrepancy could be due to a lower stability of mitDNA in the sample, a greater difficulty in permeating through the inner membrane, or even a greater complexity in the system of purification and analysis.

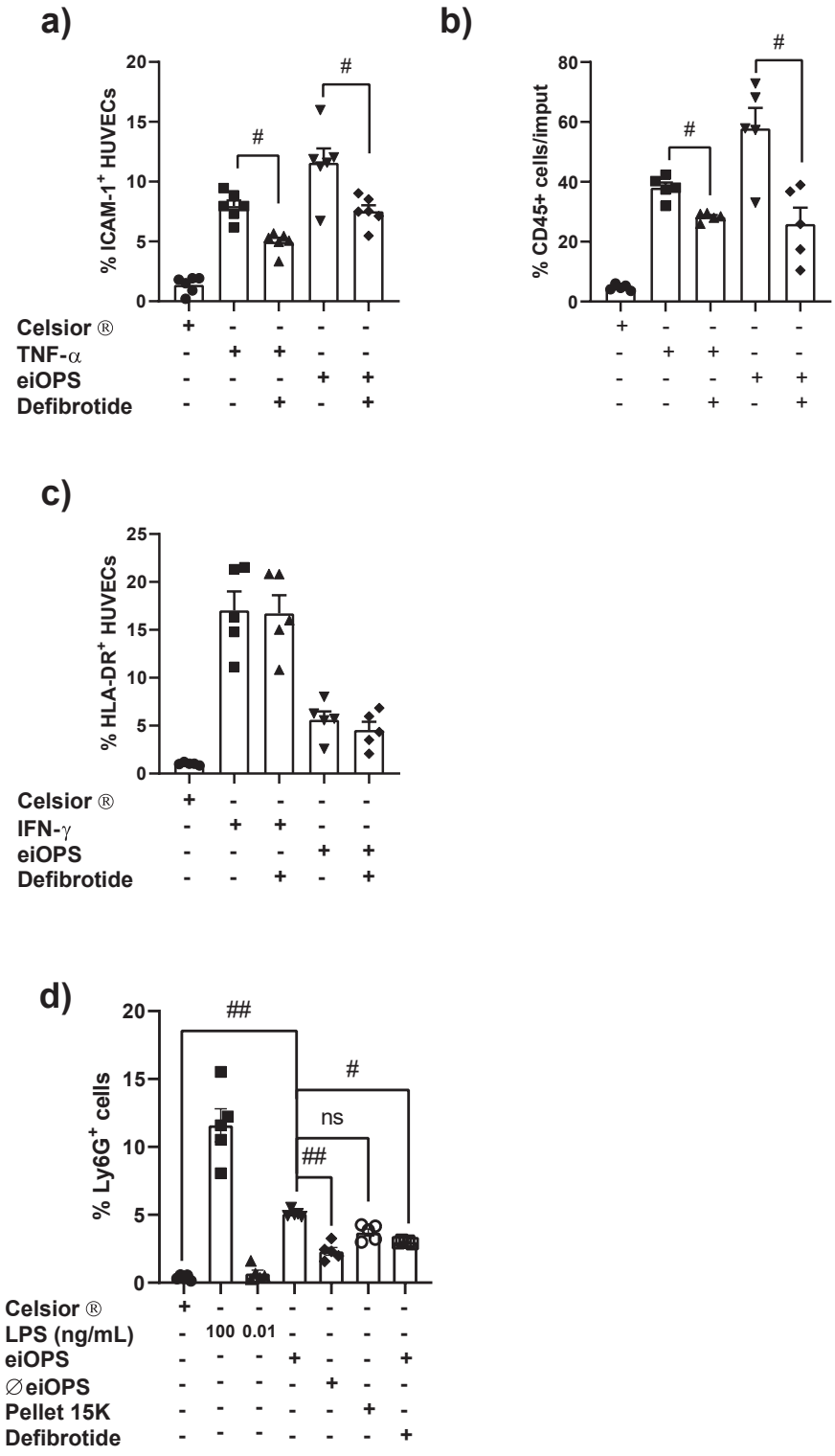

Fig. 4. Defibrotide impairs eiOPS-induced endothelial-cell activation. (a) Expression of membrane ICAM-1 in HUVECs as detected by flow cytometry. (b) In total, $7.5x10^4$ HUVECs were cultured on transwell polycarbonate membranes and incubated for 16 h in the presence of Celsior® as the negative control, with 10 ng/mL TNF-α (diluted in Celsior®) or with non-diluted eiOPS coming from a different pool of donors in the presence or absence of 100 μg/mL defibrotide; n = 6. After that, 100 ng/mL recombinant human CCL2, CXCL10, and CX3CL1 were added to the lower chamber, whereas $3x10^5$ human PBMCs were added to the upper chamber. After 16 h of incubation at 37 °C, migrated cells in the lower chamber were recovered and stained for flow cytometry, as reported in Fig. 2a; n = 5. Percentages of $CD45^+$ human leukocytes migrated to the bottom chamber of Corning® Transwell polycarbonate membrane cell culture inserts (5-μm pore size) with respect to total cells (input) added to the upper chamber were calculated.(c) Expression of membrane HLA-DR in HUVECs as detected by flow cytometry. In total, 1×10^5 HUVECs were incubated for 16 h in the presence of Celsior® as the negative control, with 10 ng/mL IFN-γ (diluted in Celsior®) or with non-diluted eiOPS coming from a different pool of donors in the presence or absence of 100 μg/mL defibrotide; n = 5. d) Neutrophil infiltration in the peritoneum of mice as detected by flow cytometry. Mice were intraperitoneally infiltrated with 300 μL of Celsior® as the negative control, two different concentrations of LPS (100 or 0.01 ng/mL), or non-diluted eiOPS from a different pool of donors, before and after (ø) total mitochondria removal by means of centrifugation at 15,000 *xg* for 15 min. Pellet obtained after centrifugation, was resuspended in Celsior® and injected intraperitoneally (Pellet 15k). One group of mice was treated with 25 mg/kg/day of defibrotide from day − 3 until the end of the experiment. Neutrophils were detected as $CD45^+F4/80^-Ly6G^+$ cells. Each dot represent a single mouse. n = 5. Results are presented as means ± SEMs. $^{\#\#}p \leq 0.01$; $^{\#}p \leq 0.05$, unpaired Student's t-test.

Furthermore, we showed a measurable myriad of inflammatory chemokines released during cold ischemic storage present in eiOPS. Chemokines are critical factors in regulating leukocyte recruitment from the blood into the liver. Kupffer cells, the resident liver macrophages, are important sensors of tissue injury. They can be activated by DAMPs released from damaged hepatocytes, cholangiocytes, or stellate cells [45]. After activation, Kupffer cells produce large amounts of pro-inflammatory molecules, including cytokines and chemokines [46]. Although up to 10 chemokines were detected in eiOPS, only CCL3, CCL4, and CXCL10 correlated moderately with the recruitment of leukocytes. CCL3 and CCL4, ligands for CCR5, mediate the recruitment of lymphocytes across the portal endothelium, whereas CXCL10, ligand for CXCR3, together with CXCL9, CXCL11, and CXCL16, mediates the recruitment across the liver sinusoidal endothelium [47]. Most effector T cells infiltrating the chronically inflamed human liver express high levels of CXCR3 and CCR5, which is consistent with a Th1 predominance and a tissue-infiltrating phenotype [48]. Moreover, CCR5 ligands are highly expressed on vascular and portal endothelia. Nevertheless,

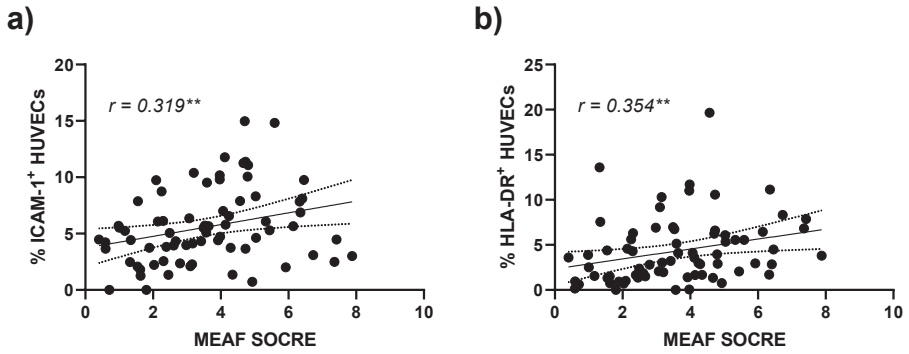

Fig. 5. Early allograft dysfunction correlates with eiOPS-mediated endothelial-cell activation. Spearman's correlation between MEAF score and membrane expression of ICAM-1 (a) and HLA-DR (b) in HUVECs activated with eiOPS.

although eiOPS from DCD livers exhibited a higher ability of chemoattraction, principally when employing the SRR method, this did not completely match with the concentration of chemokines found in eiOPS, what was mostly low for this organ extraction method. It may indicate that the chemokines may have been not completely active in the eiOPS samples or even that other molecules with the ability to attract leukocytes may have been present. In this regard, extracellular NFPs and free mitochondria, which were found in higher concentrations in DCD organs, have been shown to act as chemoattractants of neutrophils via FP receptors [44] or considered inflammatory in terms of macrophage inflammasome activation and neutrophil recruitment [6], respectively.

Our data suggest that released mitochondria in eiOPS activate the endothelium, and this could compromise the outcomes of transplanted livers. eiOPS-mediated EC activation, as reflected by the upregulation of the membrane expression of ICAM-1 and HLA-DR, displayed a positive correlation with a score model (MEAF score) for the continuous grading of early allograft dysfunction. The MEAF is a continuous score from 0 to 10 that reflects graft function [24] and has been independently validated [49], improving other recognized binary scores [50]. Current approaches to the prolonging of allograft survival focus on immune suppression in the transplant recipient; however, a direct targeting of the graft vasculature may create new opportunities for preventing allograft injury and loss. Defibrotide, an anti-thrombotic, pro-fibrinolytic, and anti-inflammatory drug [14,51] with a demonstrated protective role of endothelial-cell activation [22], appears as a promising therapeutic alternative to mitigate the damage produced by ischemia in donated organs. Defibrotide is currently used in hepatic VOD/SOS treatment, a clinical syndrome due to sinusoidal congestion, which can be caused by alkaloid ingestion. The most frequent cause of VOD/SOS is the endothelial damage produced after chemotherapy prior to HSCT [52], but it has also been reported after solid organ transplantation [53]. The relationship between defibrotide protection against graft-versus-host disease after allogeneic HSCT and its ability to inhibit EC activation has been established, showing a significant reduction in cell surface expression of P-selectin, E-selectin, VCAM-1 and ICAM-1, key endothelial adhesion molecules involved in alloreactive immune cells trafficking to tissues [20,21,54,55]. Moreover, the successful treatment of a patient with VOD/SOS with defibrotide after living-donor liver transplantation has been reported [56]. In this work, we also demonstrated the efficacy of defibrotide in inhibiting the expression of adhesion molecules in ECs, which directly influenced the transmigration of leukocytes through the endothelial barrier. However, it is worth noting that transmigration inhibition was only partial, both in in vitro experiments and in an in vivo mouse model of peritoneal neutrophil infiltration. The limited effect of defibrotide on HLA class II antigen upregulation would be consistent with the involvement of distinct pathways required to activate adhesion molecules or HLA in ECs. Consequently, defibrotide may not fully mitigate antigen presentation mediated by activated ECs.

We also found both a higher eiOPS-mediated HLA-DR expression in HUVECs and leukocyte transmigration in SRR, compared with NRP or even DBD explanted livers. NRP is a commonly used alternative to the SRR procedure [57] that involves the cannulation of the femoral vasculature after death and is frequently employed in the clinic to reperfuse and reoxygenate abdominal organs, which has been found to reduce the appearance of complications, improving DCD liver graft survival [58,59]. Improved transplant outcomes may be achieved by focusing on inflammatory factors in deceased donors prior to organ procurement. Thus, NRP may be helpful as the first delivery method for treatment with defibrotide already in the donor [60]. Additionally, ex vivo liver preservation may be a perfect platform to test therapeutic drugs thanks to extracorporeal normothermic machine perfusion (NMP) [61], which is thought to keep organs at body temperature while supplying oxygen and nutrients until implantation [62].

Within the liver, there are endothelial cells which form a complete monolayer, as well as sinusoidal endothelium, which is fenestrated and lacks a basement membrane [63]. Although HUVECs have been widely used as a laboratory model system for studying the function and pathology of endothelial cells, including liver organoid development [64], and the protective effect of defibrotide does not differ depending on the origin of the ECs [16,20,21,55], investigating how eiOPS impacts liver sinusoidal endothelial cells could provide valuable information in the future.

Thus, our study shows that during static cold ischemic storage, the release of mitochondria induces the activation of the vascular endothelium, and this activation could influence transplant progression. Controlling mitochondria release or blocking EC activation by means of defibrotide may be an effective approach to reduce the damage evoked by organ storage and could improve both the quality of marginal livers and the outcome of liver grafts. Nevertheless, the efficacy of defibrotide treatment could potentially be enhanced by incorporating a compound that can also inhibit HLA-DR expression in ECs.

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Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: P. P. is scientific consultant of Glenmak Ltd. P.P. is co-inventor on patent application to use NLRP3 inflammasome as biomarker of disease, which have been licensed to Viva in vitro diagnostics S.L, a company co-funded

by P.P., A.B.-M. and L.M-A. A.B-M. is co-inventor on provisional patent application of an in vitro method for predicting organ transplant rejection, but declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest. The rest of authors of this manuscript have no conflicts to disclose.

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Author contribution

All authors read and approved the final version of the manuscript. A.B-M. participated in the design of the work. A.B-M., D.G-B., P.P., V.L-L., F.A. and P.R. participated in the discussion and review of the manuscript and verified the underlying data. A.B-M., F.V-L., D.G-B., SV.M., and D.V-C. carried out all the experiments and statistical analysis. A.R-Z., P.R., V.L-L. and F.A. organized the intra-operating room collection of samples. M.J-A. and L.M-A. collected all the clinical data from patients. JA.P. carried out the follow-up of the patients.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.biopha.2023.115529.

References

[1] C. Enderby, C.A. Keller, An overview of immunosuppression in solid organ transplantation, Am. J. Manag Care 21 (1 Suppl) (2015) s12-23.
[2] W.G. Land, Emerging role of innate immunity in organ transplantation part II: potential of damage-associated molecular patterns to generate immunostimulatory dendritic cells, Transpl. Rev. 26 (2) (2012) 73–87.
[3] F. Lucas-Ruiz, S.V. Mateo, M. Jover-Aguilar, F. Alconchel, L. Martinez-Alarcon, C. de Torre-Minguela, et al., Danger signals released during cold ischemia storage activate NLRP3 inflammasome in myeloid cells and influence early allograft function in liver transplantation, EBioMedicine 87 (2023), 104419.
[4] Y. Zhai, R.W. Busuttil, J.W. Kupiec-Weglinski, Liver ischemia and reperfusion injury: new insights into mechanisms of innate-adaptive immune-mediated tissue inflammation, Am. J. Transpl. 11 (8) (2011) 1563–1569.
[5] L. Amari, M. Germain, Mitochondrial extracellular vesicles - origins and roles, Front Mol. Neurosci. 14 (2021), 767219.
[6] M. Zhu, A.S. Barbas, L. Lin, U. Scheuermann, M. Bishawi, T.V. Brennan, Mitochondria released by apoptotic cell death initiate innate immune responses, Immunohorizons 2 (11) (2018) 384–397.
[7] A. Meyer, G. Laverny, L. Bernardi, A.L. Charles, G. Alsaleh, J. Pottecher, et al., Mitochondria: an organelle of bacterial origin controlling inflammation, Front Immunol. 9 (2018), 536.
[8] J. Pollara, R.W. Edwards, L. Lin, V.A. Bendersky, T.V. Brennan, Circulating mitochondria in deceased organ donors are associated with immune activation and early allograft dysfunction, JCI Insight 3 (15) (2018).
[9] L. Lin, H. Xu, M. Bishawi, F. Feng, K. Samy, G. Truskey, et al., Circulating mitochondria in organ donors promote allograft rejection, Am. J. Transpl. 19 (7) (2019) 1917–1929.
[10] B.J. Hunt, K.M. Jurd, Endothelial cell activation. A central pathophysiological process, BMJ 316 (7141) (1998) 1328–1329.
[11] P. Abrahimi, R. Liu, J.S. Pober, Blood vessels in allotransplantation, Am. J. Transpl. 15 (7) (2015) 1748–1754.
[12] J.S. Pober, J. Merola, R. Liu, T.D. Manes, Antigen presentation by vascular cells, Front Immunol. 8 (2017), 1907.
[13] G. Piotti, A. Palmisano, U. Maggiore, C. Buzio, Vascular endothelium as a target of immune response in renal transplant rejection, Front Immunol. 5 (2014) 505.
[14] K.J. Palmer, K.L. Goa, Defibrotide. A review of its pharmacodynamic and pharmacokinetic properties, and therapeutic use in vascular disorders, Drugs 45 (2) (1993) 259–294.
[15] S. Corbacioglu, E.J. Jabbour, M. Mohty, Risk factors for development of and progression of hepatic veno-occlusive disease/sinusoidal obstruction syndrome, Biol. Blood Marrow Transpl. 25 (7) (2019) 1271–1280.
[16] S. Fernandez, M. Palomo, P. Molina, M. Diaz-Ricart, G. Escolar, A. Tellez, et al., Progressive endothelial cell damage in correlation with sepsis severity. Defibrotide as a contender, J. Thromb. Haemost. 19 (8) (2021) 1948–1958.
[17] D. Garcia-Bernal, E. Richardson, I. Vlodavsky, C. Carlo-Stella, M. Iacobelli, R. Jara, et al., Endothelial dysfunction and its critical role in COVID-19-associated coagulopathy: Defibrotide as an endothelium-protective, targeted therapy, EJHaem 2 (3) (2021) 680–681.
[18] E. Richardson, D. Garcia-Bernal, E. Calabretta, R. Jara, M. Palomo, R.M. Baron, et al., Defibrotide: potential for treating endothelial dysfunction related to viral and post-infectious syndromes, Expert Opin. Ther. Targets 25 (6) (2021) 423–433.
[19] C. Stein, D. Castanotto, A. Krishnan, L. Nikolaenko, Defibrotide (Defitelio): a new addition to the stockpile of food and drug administration-approved oligonucleotide drugs, Mol. Ther. Nucleic Acids 5 (8) (2016), e346.
[20] D. Garcia-Bernal, M. Palomo, C.M. Martinez, J.E. Millan-Rivero, A.I. Garcia-Guillen, M. Blanquer, et al., Defibrotide inhibits donor leucocyte-endothelial interactions and protects against acute graft-versus-host disease, J. Cell Mol. Med 24 (14) (2020) 8031–8044.
[21] J. Martinez-Sanchez, H. Hamelmann, M. Palomo, E. Mir, A.B. Moreno-Castano, S. Torramade, et al., Acute graft-vs.-host disease-associated endothelial activation in vitro is prevented by defibrotide, Front Immunol. 10 (2019), 2339.
[22] N. Orlando, G. Babini, P. Chiusolo, C.G. Valentini, V. De Stefano, L. Teofili, Pre-exposure to defibrotide prevents endothelial cell activation by lipopolysaccharide: an ingenuity pathway analysis, Front Immunol. 11 (2020), 585519.
[23] C. de Torre-Minguela, A.I. Gomez, I. Couillin, P. Pelegrin, Gasdermins mediate cellular release of mitochondrial DNA during pyroptosis and apoptosis, FASEB J. 35 (8) (2021), e21757.
[24] E. Pareja, M. Cortes, D. Hervas, J. Mir, A. Valdivieso, J.V. Castell, et al., A score model for the continuous grading of early allograft dysfunction severity, Liver Transpl. 21 (1) (2015) 38–46.
[25] F. Lucas-Ruiz, S.V. Mateo, M. Jover-Aguilar, F. Alconchel, L. Martínez-Alarcón, C. De Torre-Minguela, et al., Danger signals released during cold ischemia storage activate NLRP3 inflammasome in myeloid cells and influence early allograft function in liver transplantation, eBiomedicine (2022).
[26] M. Araake, T. Uchiyama, K. Imanishi, X.J. Yan, Activation of human vascular endothelial cells by IFN-gamma: acquisition of HLA class II expression, TSST-1-binding activity and accessory activity in T cell activation by the toxin, Int Arch. Allergy Appl. Immunol. 96 (1) (1991) 55–61.
[27] H.F. Langer, T. Chavakis, Leukocyte-endothelial interactions in inflammation, J. Cell Mol. Med 13 (7) (2009) 1211–1220.
[28] H.P. Jersmann, C.S. Hii, J.V. Ferrante, A. Ferrante, Bacterial lipopolysaccharide and tumor necrosis factor alpha synergistically increase expression of human endothelial adhesion molecules through activation of NF-kappaB and p38 mitogen-activated protein kinase signaling pathways, Infect. Immun. 69 (3) (2001) 1273–1279.
[29] M.H. Ravindranath, F. El Hilali, E.J. Filippone, The impact of inflammation on the immune responses to transplantation: tolerance or rejection? Front Immunol. 12 (2021), 667834.
[30] Haraldsen, G. Sollid, L.M. Bakke, O. Farstad, I.N. Kvale, D. Molberg, et al., Major histocompatibility complex class II-dependent antigen presentation by human intestinal endothelial cells, Gastroenterology 114 (4) (1998) 649–656.
[31] K.A. Muczynski, D.M. Ekle, D.M. Coder, S.K. Anderson, Normal human kidney HLA-DR-expressing renal microvascular endothelial cells: characterization, isolation, and regulation of MHC class II expression, J. Am. Soc. Nephrol. 14 (5) (2003) 1336–1348.
[32] A. D'Souza, A. Burch, K.M. Dave, A. Sreeram, M.J. Reynolds, D.X. Dobbins, et al., Microvesicles transfer mitochondria and increase mitochondrial function in brain endothelial cells, J. Control Release 338 (2021) 505–526.
[33] F. Puhm, T. Afonyushkin, U. Resch, G. Obermayer, M. Rohde, T. Penz, et al., Mitochondria are a subset of extracellular vesicles released by activated monocytes and induce type I IFN and TNF responses in endothelial cells, Circ. Res 125 (1) (2019) 43–52.
[34] K.P. Croome, C.B. Taner, The changing landscapes in DCD liver transplantation, Curr. Transpl. Rep. (2020) 1–11.
[35] S. Grazioli, J. Pugin, Mitochondrial damage-associated molecular patterns: from inflammatory signaling to human diseases, Front Immunol. 9 (2018), 832.
[36] M. Pizzuto, C. Lonez, A. Baroja-Mazo, H. Martinez-Banaclocha, P. Tourlomousis, M. Gangloff, et al., Saturation of acyl chains converts cardiolipin from an antagonist to an activator of Toll-like receptor-4, Cell Mol. Life Sci. 76 (18) (2019) 3667–3678.
[37] C.T. Chu, J. Ji, R.K. Dagda, J.F. Jiang, Y.Y. Tyurina, A.A. Kapralov, et al., Cardiolipin externalization to the outer mitochondrial membrane acts as an elimination signal for mitophagy in neuronal cells, Nat. Cell Biol. 15 (10) (2013) 1197–1205.
[38] E.M. Fock, R.G. Parnova, Protective effect of mitochondria-targeted antioxidants against inflammatory response to lipopolysaccharide challenge: a review, Pharmaceutics 13 (2) (2021).
[39] B. Banoth, S.L. Cassel, Mitochondria in innate immune signaling, Transl. Res 202 (2018) 52–68.
[40] K. Nakahira, S. Hisata, A.M. Choi, The roles of mitochondrial damage-associated molecular patterns in diseases, Antioxid. Redox Signal 23 (17) (2015) 1329–1350.
[41] P. Perez-Trevino, M. Velasquez, N. Garcia, Mechanisms of mitochondrial DNA escape and its relationship with different metabolic diseases, Biochim Biophys. Acta Mol. Basis Dis. 1866 (6) (2020), 165761.

[42] A. Kaczmarek, P. Vandenabeele, D.V. Krysko, Necroptosis: the release of damage-associated molecular patterns and its physiological relevance, Immunity 38 (2) (2013) 209–223.
[43] J.S. Riley, G. Quarato, C. Cloix, J. Lopez, J. O'Prey, M. Pearson, et al., Mitochondrial inner membrane permeabilisation enables mtDNA release during apoptosis, EMBO J. 37 (17) (2018).
[44] M.A. Panaro, A. Acquafredda, M. Sisto, S. Lisi, A.B. Maffione, V. Mitolo, Biological role of the N-formyl peptide receptors, Immunopharmacol. Immunotoxicol. 28 (1) (2006) 103–127.
[45] F. Marra, F. Tacke, Roles for chemokines in liver disease, Gastroenterology 147 (3) (2014), 577-94 e1.
[46] S. Cao, M. Liu, T.S. Sehrawat, V.H. Shah, Regulation and functional roles of chemokines in liver diseases, Nat. Rev. Gastroenterol. Hepatol. 18 (9) (2021) 630–647.
[47] P.L. Shields, C.M. Morland, M. Salmon, S. Qin, S.G. Hubscher, D.H. Adams, Chemokine and chemokine receptor interactions provide a mechanism for selective T cell recruitment to specific liver compartments within hepatitis C-infected liver, J. Immunol. 163 (11) (1999) 6236–6243.
[48] E.J. Kunkel, J. Boisvert, K. Murphy, M.A. Vierra, M.C. Genovese, A.J. Wardlaw, et al., Expression of the chemokine receptors CCR4, CCR5, and CXCR3 by human tissue-infiltrating lymphocytes, Am. J. Pathol. 160 (1) (2002) 347–355.
[49] M. Rayar, G.B. Levi Sandri, C. Cusumano, N. Lombard, M. Lakehal, V. Desfourneaux, et al., A score model for the continuous grading of early allograft dysfunction severity, Liver Transpl. 22 (6) (2016) 859–860.
[50] K.M. Olthoff, L. Kulik, B. Samstein, M. Kaminski, M. Abecassis, J. Emond, et al., Validation of a current definition of early allograft dysfunction in liver transplant recipients and analysis of risk factors, Liver Transpl. 16 (8) (2010) 943–949.
[51] R. Pescador, L. Capuzzi, M. Mantovani, A. Fulgenzi, M.E. Ferrero, Defibrotide: properties and clinical use of an old/new drug, Vasc. Pharmacol. 59 (1–2) (2013) 1–10.
[52] M. Senzolo, G. Germani, E. Cholongitas, P. Burra, A.K. Burroughs, Veno occlusive disease: update on clinical management, World J. Gastroenterol. 13 (29) (2007) 3918–3924.
[53] M. Sebagh, M. Debette, D. Samuel, J.F. Emile, B. Falissard, V. Cailliez, et al., "Silent" presentation of veno-occlusive disease after liver transplantation as part of the process of cellular rejection with endothelial predilection, Hepatology 30 (5) (1999) 1144–1150.
[54] S. Corbacioglu, S. Cesaro, M. Faraci, D. Valteau-Couanet, B. Gruhn, A. Rovelli, et al., Defibrotide for prophylaxis of hepatic veno-occlusive disease in paediatric haemopoietic stem-cell transplantation: an open-label, phase 3, randomised controlled trial, Lancet 379 (9823) (2012) 1301–1309.
[55] M. Palomo, E. Mir, M. Rovira, G. Escolar, E. Carreras, M. Diaz-Ricart, What is going on between defibrotide and endothelial cells? Snapshots reveal the hot spots of their romance, Blood 127 (13) (2016) 1719–1727.
[56] T.B. Lee, K. Yang, H.J. Ko, J.R. Shim, B.H. Choi, J.H. Lee, et al., Successful defibrotide treatment of a patient with veno-occlusive disease after living-donor liver transplantation: a case report, Med. (Baltim.) 100 (25) (2021), e26463.
[57] I. Jochmans, A.J. Hessheimer, A.P. Neyrinck, D. Paredes, M.I. Bellini, J.H. Dark, et al., Consensus statement on normothermic regional perfusion in donation after circulatory death: report from the European Society for Organ Transplantation's Transplant Learning Journey, Transpl. Int 34 (11) (2021) 2019–2030.
[58] A.J. Hessheimer, E. Coll, F. Torres, P. Ruiz, M. Gastaca, J.I. Rivas, et al., Normothermic regional perfusion vs. super-rapid recovery in controlled donation after circulatory death liver transplantation, J. Hepatol. 70 (4) (2019) 658–665.
[59] C.J.E. Watson, F. Hunt, S. Messer, I. Currie, S. Large, A. Sutherland, et al., In situ normothermic perfusion of livers in controlled circulatory death donation may prevent ischemic cholangiopathy and improve graft survival, Am. J. Transpl. 19 (6) (2019) 1745–1758.
[60] F. Lucas-Ruiz, A. Penin-Franch, J.A. Pons, P. Ramirez, P. Pelegrin, S. Cuevas, et al., Emerging role of NLRP3 inflammasome and pyroptosis in liver transplantation, Int J. Mol. Sci. 23 (22) (2022).
[61] F. Dengu, S.H. Abbas, G. Ebeling, D. Nasralla, Normothermic machine perfusion (NMP) of the liver as a platform for therapeutic interventions during ex-vivo liver preservation: a review, J. Clin. Med 9 (4) (2020).
[62] C.J. van Beekum, T.O. Vilz, T.R. Glowka, M.W. von Websky, J.C. Kalff, S. Manekeller, Normothermic machine perfusion (NMP) of the liver - current status and future perspectives, Ann. Transpl. 26 (2021), e931664.
[63] K. Elvevold, B. Smedsrod, I. Martinez, The liver sinusoidal endothelial cell: a cell type of controversial and confusing identity, Am. J. Physiol. Gastrointest. Liver Physiol. 294 (2) (2008) G391–G400.
[64] A. Chhabra, H.G. Song, K.A. Grzelak, W.J. Polacheck, H.E. Fleming, C.S. Chen, et al., A vascularized model of the human liver mimics regenerative responses, Proc. Natl. Acad. Sci. USA 119 (28) (2022), e2115867119.