frontiers | Research Topics

Immunology of machine perfused organs and tissues

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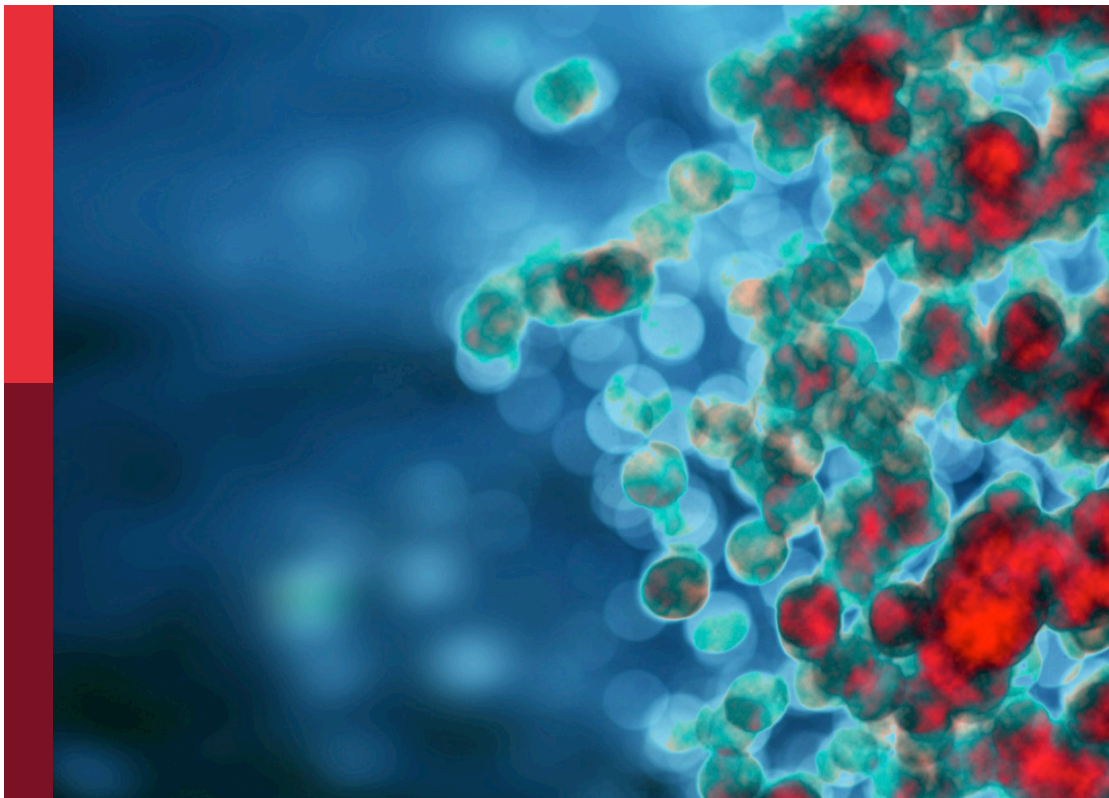
Published in
Frontiers in Immunology



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ISSN 1664-8714
ISBN 978-2-83251-100-8
DOI 10.3389/978-2-83251-100-8

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Immunology of machine perfused organs and tissues

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Citation

Hautz, T., Schneeberger, S., Brandacher, G., eds. (2023). *Immunology of machine perfused organs and tissues*. Lausanne: Frontiers Media SA.

doi: 10.3389/978-2-83251-100-8

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frontiers | Frontiers in Immunology

TYPE Editorial
PUBLISHED 06 December 2022
DOI 10.3389/fimmu.2022.1104268

Check for updates

OPEN ACCESS

EDITED AND REVIEWED BY
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SPECIALTY SECTION
This article was submitted to Alloimmunity and Transplantation, a section of the journal Frontiers in Immunology

RECEIVED 21 November 2022
ACCEPTED 25 November 2022
PUBLISHED 06 December 2022

CITATION
Hautz T, Brandacher G and Schneeberger S (2022) Editorial: Immunology of machine perfused organs and tissues.
Front. Immunol. 13:1104268.
doi: 10.3389/fimmu.2022.1104268

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Editorial: Immunology of machine perfused organs and tissues

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KEYWORDS

organ transplantation, machine perfusion, extended criteria donor, immunology, inflammation, ischemia reperfusion injury, immunomodulation, regeneration

Editorial on the Research Topic
Immunology of machine perfused organs and tissues

The clinical realization of extracorporeal, machine-based organ and tissue preservation opens a novel chapter in medicine. This technology allows for *ex-situ* organ/tissue perfusion, while still mimicking an *in-vivo* physiologic environment, in order to bridge the time between organ recovery and transplantation. It further minimizes the duration of ischemia and allows for real time assessment of organ viability, quality, and function during this state (1, 2). Most importantly, this innovative technology provides the unique possibility of organ/tissue reconditioning, immunomodulation, regeneration, and treatment outside the human body (3).

However, the impact of *ex-situ* machine perfusion on the immunological status of an organ is still largely unknown. Advances in multi-color flow cytometry, next-generation sequencing technologies, and platforms at single-cell resolution have recently opened new opportunities to better characterize intragraft cell compositions and provide novel insights into immune responses in machine perfused organs.

This Research Topic provides a collection of articles highlighting immune responses during *ex-situ* machine perfusion of organs. Original research articles include an array of studies carried out in human donor organs, as well as in pre-clinical large (porcine) and small (rat) animal models providing exciting data on the impact of machine perfusion on organ immune status and antigenicity under various conditions. In addition, this Research Topic includes relevant and interesting review articles summarizing the existing literature on immune activation during organ machine perfusion.

The concept of extracorporeal circulation has originally been established as a tool for cardiothoracic surgery, which has subsequently been extended to isolated machine perfusion of (thoracic) organs. The review article by Hatami et al. gives an overview about the activation of inflammatory and oxidative stress responses during

extracorporeal live support systems (ECLP) and *ex-situ* organ perfusion (ESOP) and presents the underlying mechanisms. It further discusses possible therapeutic interventions during ECLP and ESOP to ameliorate inflammation and oxidative stress. The technology of organ machine perfusion was introduced to not only limit ischemic time of organs prior to transplantation, but also to reduce ischemia/reperfusion injury (IRI)-associated inflammation and cell death contributing to delayed graft function (DGF) or early allograft dysfunction (EAD). Panconesi et al. offer a review focusing on the impact of different machine perfusion approaches on IRI-associated responses in the liver. In this context, the authors also describe the subcellular processes and proinflammatory downstream mechanistic effects of IRI. The authors furthermore highlight protective mechanisms observed for different perfusion approaches and discuss possible treatment strategies and the delivery of specific agents to modulate posttransplant inflammation. Knijff et al. review the impact of hypothermic machine perfusion (HMP), thereby focusing on how HMP ameliorates IRI in kidney and liver transplantation. In a study of normothermic machine perfusion (NMP) including six discarded human donor livers, Lee et al. provide first insights into changes of the immune profile for up to six hours of NMP. Time-dependent, dynamic changes were observed for individual leukocyte subsets detected in liver tissue and perfusate during NMP, while cytokine levels continuously increased. These observations suggest that NMP significantly alters the immunogenicity of *ex-situ* perfused organs. Jennings et al. showed that oxygen carriers used in a rat model of normothermic *ex-situ* liver perfusion affect the phenotype of liver-resident immune cells. Interestingly, the synthetic hemoglobin-based oxygen carrier, Oxyglobin, revealed the lowest level of immune cell activation and allogenic proliferation compared to human or rat packed red blood cells (RBC), and was hence stated as the "optimal oxygen carrier" for liver NMP. The role of the hepatic immune cell repertoire in inflammatory conditions and IRI is further discussed in a review by Fodor et al. It highlights the detrimental but also regenerative potential of various immune cell subtypes, and how specific immune cell functions need to be considered when actively modifying the immune status of donor livers during machine perfusion. The mini-review by Langford et al. expands the technology of *ex-situ* organ perfusion beyond immunomodulation and suggests it as a useful tool to deliver therapeutic agents in order to rehabilitate/repair diseased and extended criteria organs of e.g. obese donors, which in the long-run could help to increase the number of transplantable organs.

Mellati et al. advocate NMP of kidneys as a suitable preservation technique and a model of reperfusion to study inflammation and immune activation when testing for novel therapies. In their study, the authors assessed the impact of alpha-1 antitrypsin (AAT) on IRI and inflammation in porcine kidneys subjected to cold static storage, +/- NMP, followed by normothermic reperfusion. Utilizing a model of NMP in porcine and human kidneys, Jager et al. investigated activation of the complement cascade. The authors observed complement activation products in the perfusate during kidney NMP, which was associated with increased levels of pro-inflammatory cytokines and a reduced creatinine clearance, and hence suggest inhibition of complement as a promising strategy to diminish kidney graft injury during NMP. Hosgood et al. analyzed the effect of free heme during *ex-situ* NMP of discarded human donor kidneys. The authors conclude that a great amount of heme was detected in the perfusate, especially when older RBCs were used. In summary, genes associated with apoptosis, inflammation and oxidative stress were upregulated during NMP, but no correlation with free heme was found.

Finally, we hope that this special Research Topic will contribute to further advance the knowledge in the field of *ex-situ* machine perfusion and its impact on graft immunogenicity. In the future, this innovative platform may provide a unique opportunity in order to create an immunologically "masked" organ/tissue, hence reducing the risk of rejection and eventually systemic immunosuppression levels. Replacement of donor cells with patient-specific, non-immunogenic cells could allow for the generation of intra organ/tissue chimerism, which might further reduce alloimmunity. All these exciting new opportunities for *ex-situ* organ manipulations may pave the way for the realization of "personalized organs" for transplant in the future.

Author contributions

TH, GB and SS contributed to this article by providing substantial, direct and intellectual input. All authors approved the submitted version.

Acknowledgments

We would like to thank all the authors for contributing their time and expertise to this Research Topic. We are also grateful to the editors and reviewers of the articles for their precious assistance.

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frontiers in Immunology

REVIEW
published: 04 March 2022
doi: 10.3389/fimmu.2022.831930

Inflammation and Oxidative Stress in the Context of Extracorporeal Cardiac and Pulmonary Support

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Extracorporeal circulation (ECC) systems, including cardiopulmonary bypass, and extracorporeal membrane oxygenation have been an irreplaceable part of the cardiothoracic surgeries, and treatment of critically ill patients with respiratory and/or cardiac failure for more than half a century. During the recent decades, the concept of extracorporeal circulation has been extended to isolated machine perfusion of the donor organ including thoracic organs (*ex-situ* organ perfusion, ESOP) as a method for dynamic, semi-physiologic preservation, and potential improvement of the donor organs. The extracorporeal life support systems (ECLS) have been lifesaving and facilitating complex cardiothoracic surgeries, and the ESOP technology has the potential to increase the number of the transplantable donor organs, and to improve the outcomes of transplantation. However, these artificial circulation systems in general have been associated with activation of the inflammatory and oxidative stress responses in patients and/or in the exposed tissues and organs. The activation of these responses can negatively affect patient outcomes in ECLS, and may as well jeopardize the reliability of the organ viability assessment, and the outcomes of thoracic organ preservation and transplantation in ESOP. Both ECLS and ESOP consist of artificial circuit materials and components, which play a key role in the induction of these responses. However, while ECLS can lead to systemic inflammatory and oxidative stress responses negatively affecting various organs/systems of the body, in ESOP, the absence of the organs that play an important role in oxidant scavenging/antioxidative replenishment of the body, such as liver, may make the perfused organ more susceptible to inflammation and oxidative stress during extracorporeal circulation. In the present manuscript, we will review the activation of the inflammatory and oxidative stress responses during ECLP and ESOP, mechanisms involved, clinical implications, and the interventions for attenuating these responses in ECC.

Keywords: inflammation, oxidative stress, extracorporeal life support, *ex-situ* organ perfusion, cardiac and pulmonary function

OPEN ACCESS

Edited by:
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Specialty section:
This article was submitted to Alloimmunity and Transplantation, a section of the journal Frontiers in Immunology

Received: *09 December 2021*
Accepted: *08 February 2022*
Published: *04 March 2022*

Citation:
Hatami S, Hefler J and Freed DH (2022) Inflammation and Oxidative Stress in the Context of Extracorporeal Cardiac and Pulmonary Support. Front. Immunol. 13:831930. doi: 10.3389/fimmu.2022.831930

INTRODUCTION

Extracorporeal circulation systems (ECC), have been an essential part of life-support in critically ill patients and cardiothoracic surgeries (1). During the recent couple of decades, this technology has been extended to *ex-situ* organ perfusion (ESOP) to improve the protection, availability, and assessment of the donor hearts and lungs. These technologies have, without a doubt, been revolutionary, leading to significant improvements in surgical treatments, patient management, and donated thoracic organ utilization (2, 3). However, its application has met some limitations as well, with systemic inflammation and oxidative stress being among the most important challenges. These reactions negatively affect patient outcomes in extracorporeal life support (ECLS) (4–6), and may negatively affect organ preservation in ESOP (7, 8). In this review, we discuss the development of inflammatory and oxidative stress responses in ECLS and ESOP, involved mechanisms, and interventions to attenuate these responses.

HISTORY OF THORACIC ORGAN-ORIENTED EXTRACORPOREAL CIRCULATION

Extracorporeal Life Support

The first experiments that would lead to the development of ECLS began with César Julien Jean Le Gallois in the early 19th century, who showed a decapitated rabbit could be kept alive through pulmonary inflation using a syringe. Later on, Eduard Brown-Séquard successfully stimulated isolated extremities by syringe reperfusion with blood, oxygenated through agitation while in contact with air. These experiments, alongside the advances in technology, including a device capable of infusing blood under pressure (by Ludwig and Schmidt in 1868) and development of film and bubble oxygenators (by von Schroder in 1882, and Frey and Gruber in 1885), facilitated the development of extracorporeal perfusion (9–11). These advances not only improved organ perfusion devices as "tools for studying the organs", but also helped in subsequent development of the heart lung machine (cardiopulmonary bypass, CPB) by Gibbon in 1953, which allowed open-heart surgeries that had not been feasible before (12). With the subsequent success of CPB in in 1950s and 60s, and further advances in bioengineering of oxygenators, such as introduction of silicone rubber membrane oxygenators that could support oxygenation for days rather than hours, the ECLS was also extended to extracorporeal membrane oxygenation (ECMO), designed to provide longer periods of support. It provides augmented oxygenation (venovenous, VV-ECMO) and/or cardiac output (venoarterial, VA-ECMO) in patients of all ages who suffer the conditions associated with cardiopulmonary failure. The application of ECMO has also expanded to extracorporeal cardiopulmonary resuscitation, and bridging to lung transplantation, although it has been most successful in treatment of newborns with severe respiratory failure (5, 13, 14).

Ex-Situ Thoracic Organ Perfusion

After the pioneering work of isolated (*ex-situ*)-perfused heart method by Cyon in 1866, the method was further adopted for perfusion of mammal heart by Langendorff in 1895 (3). With the introduction of isolated working heart preparation less than a century later in 1967 by Neely and Morgan, *ex-situ* heart perfusion (ESHP) has been since widely utilized for studying the heart (15).

During the recent few decades, ESOP has emerged again for its potential to preserve donated hearts and lungs in a more physiologic setting, and offering a venue for assessment and potentially improvement of donor organs. This technology has enhanced organ preservation and viability assessment, and has facilitated transplantation of sub-optimal and extended-criteria hearts and lungs (2, 16). Still, the optimal *ex-situ* preservation of thoracic organs function and viability has been a matter of active research. Formation of edema and tissue injury, as well as diminished functional status of the organ during extended *ex-situ* perfusion periods, limit the optimal *ex-situ* preservation time, and create an obstacle for potential advantages of this preservation/evaluation method. Meanwhile, the implications of the artificial materials/surfaces used in *ex-situ* organ perfusion in development of cellular injury and stress has been mostly neglected (3, 17, 18).

SIMILARITIES AND DIFFERENCES BETWEEN ECLS AND ESOP SETTINGS AND APPARATUSES

The apparatuses/settings used for ESOP share many aspects with ECLS, particularly with CPB system. All these systems rely on artificial, synthetic materials/components including pumps and oxygenators for supporting circulation and oxygenation. However, there are obvious differences between those, including exclusive, isolated perfusion of the procured organ in ESOP as opposed to systemic perfusion in ECLS, or lesser blood-air interface in ECMO (closed circuit) compared to CPB. The similarities and differences of ESHP with ECLS systems are summarized in **Table 1** (4, 5, 19, 20).

INFLAMMATION AND OXIDATIVE STRESS IN ECLS

It is well-established that ECLS leads to systemic inflammation and oxidative stress yet, the extent of these phenomena, clinical significance, and successful therapeutic intervention is still a matter of debate, mostly due to the significant heterogeneity in study design (e.g., patient populations), and the conflicting results observed (5, 21). The highlighted studies on induction of inflammatory and oxidative stress responses related to ECLS are summarized in **Supplementary Table 1** (22–50). A strong body of evidence suggests that inflammation and oxidative stress occur early after initiation of ECLS and progress over time.

TABLE 1 | Differences between extracorporeal life support techniques and *ex-situ* thoracic organ perfusion.

	ECMO	CPB	ESOP
Application of artificial materials/components	Yes	Yes	Yes
Connection to body	Connected	Connected	Non-connected
Duration	Days to weeks	Minutes to hours	Minutes to hours
Hemodilution	No	Yes	Yes
Anticoagulation	Low-dose heparin	High-dose Heparin	High-dose Heparin
Reversal of anticoagulation	No	Yes (protamine)	No
Hypothermia	No	Yes	Variable
Air-blood interface	No (closed-circuit)	Yes (there are some closed-circuit variants)	Yes
Pulsatility	Variable with mode	No	Variable (dependent on device)

ECMO, extracorporeal membrane oxygenation; CPB, cardiopulmonary bypass; ESOP, ex-situ thoracic organ perfusion.

However, there are studies showing minimal or no change in the markers of immune system activation during application of ECLS or a delayed response detectible either later during ECC, or after its termination. On the other hand, among several different markers (e.g. cytokines, markers of oxidative stress) that may be induced in ECLS, only a few of them such as tumor necrosis factor alpha (TNF-α), interleukin (IL)-8, and IL-6 have been shown to have a correlation with organ function and/or patient outcomes (51–54). Regardless, clinical and experimental studies mostly suggest that the systemic responses precede the tissue-specific induction of inflammation or oxidative stress at the institution of ECLS, and pre-existing comorbidities play a key role in exacerbation of these responses.

INFLAMMATION AND OXIDATIVE STRESS IN *EX-SITU* THORACIC ORGAN PERFUSION

Despite the considerable paucity of data, the few existing studies by our team and others **Supplementary Table 1** suggest that similar to ECLS, ESOP is also associated with inflammation and oxidative stress (19, 55, 56). The extent and implications of these reactions on donor organ viability in ESOP may be critical. Donor organs routinely endure various ischemic times, promoting inflammation and oxidative stress, which may be even more severe in extended criteria donations, such as donation after circulatory death (DCD). Moreover, the absence of *in vivo* mechanisms to replenish and refine blood components in the setting ESOP may make perfused organs more vulnerable to oxidative stress. The importance of this has been strongly demonstrated by studies reporting successful, extended 24-hour and 72-hour ESHP in animal models involving cross-circulation with a live animal (57, 58), and the recent report on improved pulmonary function of *ex-situ*-perfused human lungs with xenogeneic (porcine) cross-circulation (59).

The limited available studies of ESOP have mainly focused on mitigation of the inflammatory responses in DCD or static cold storage (SCS) hearts and lungs that have been subjected to significant warm or cold ischemic times respectively (60–62). Thus, they may already be facing severe degrees of ischemia/reperfusion injury (IRI) during subsequent *ex-situ* perfusion, masking the inflammation and oxidative stress related to ECC itself. In the recent experimental studies of ESHP and ESLP by our team in a porcine model, we observed that perfusion of healthy hearts and lungs, which had not experienced the insults related to brain death or circulatory death was also accompanied by significant induction of various inflammatory mediators such as various interleukins (ILs) including IL-1β, IL-6, IL-8, IL-18, and TNF-α and/or markers of oxidative stress such as oxidized low density lipoprotein (ox-LDL), and malondialdehyde (MDA) (19, 56, 63). Clarifying the effects of activation of these responses on the quality of donor organs and eventually the outcomes of transplantation, warrants more experimental and clinical studies.

THE PATHOPHYSIOLOGY OF INFLAMMATORY AND OXIDATIVE STRESS RESPONSES DURING EXTRACORPOREAL CIRCULATION

The ECLS and ESOP systems, though different in many ways, all expose the body/organ to various types of non-physiological conditions, which may affect organ function and viability and patient outcomes (5, 21, 64). Various alterations are induced to elements of the blood/perfusate, as well as the exposed tissues/organs during ECC, which are discussed in the following sections and are also briefly reflected in **Figure 1**.

COMPLEMENT & CONTACT ACTIVATION SYSTEM

According to the Vroman effect, within minutes after the contact between the blood and the artificial material (biomaterial) of the circuit, the sequential absorption of plasma proteins starts, with fibrinogen forming a surface for other plasma proteins to bind to the biomaterial. These proteins include but are not limited to the contact activation pathway molecules, albumin, and complement component protein 3 (C3). Formation of the protein layer on the biomaterial surface and activation of the complement system, boosts the interaction between the platelets and polymorphonuclear leukocytes (PMN) leading to release of different pro-inflammatory cytokines (65). Activation of the contact system leads to sequential cleavage of intrinsic coagulation pathway elements and activation of

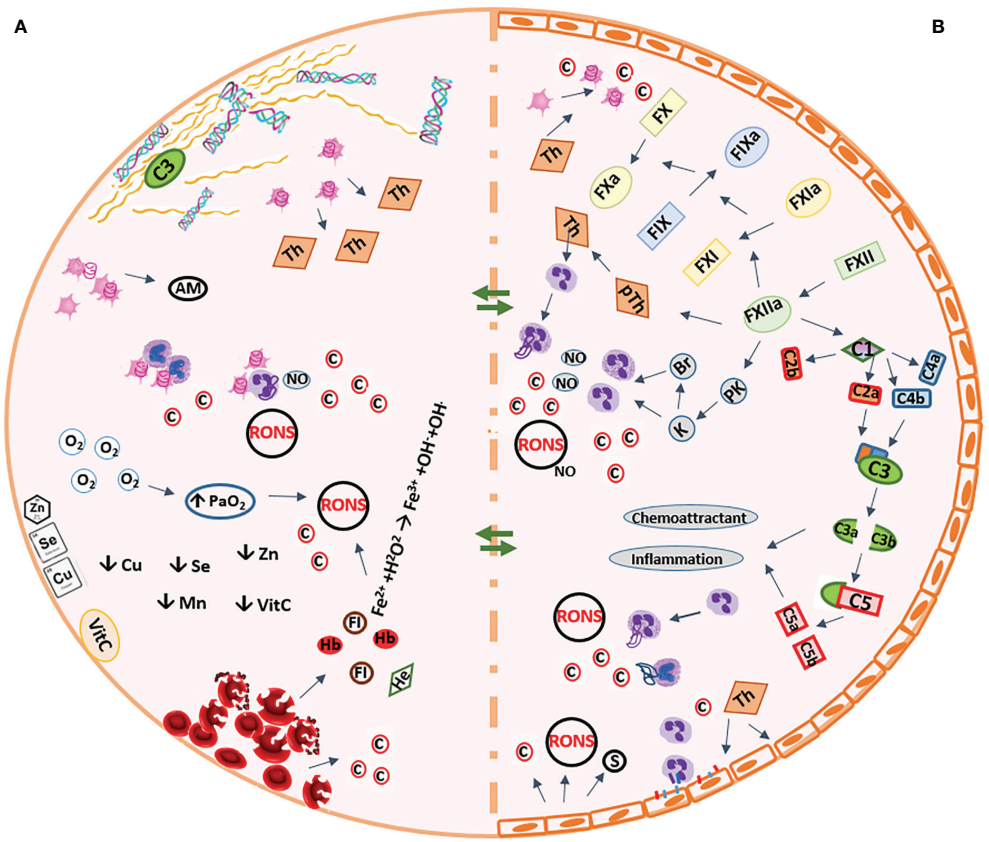

FIGURE 1 | Summary of the pathophysiologic conditions occurring during extracorporeal circulation. **(A)** Artificial biomaterial of the circuit **(B)** Vascular system of the organs AM, adhesion molecules; Br, bradykinin; C, complement compartment, c, cytokines; Cu, copper; Fe^{2+}, ferrous ion; Fe^{3+}, ferric ion, Fl, free iron; H_2O_2, hydrogen peroxide; He, heme; FIX, factor-9; FX, factor-10; FXI, factor-11; FXII, factor-12; K, kallikrein; Mn, manganese; NO, nitric oxide; O_2, oxygen; OH^-, hydroxyl; OH., hydroxyl radicalPaO$_2$, partial pressure of arterial oxygen; PK, pre-kallikrein; pTh, prothrombin; RONS, reactive oxygen and nitrogen species; s, selectins; Se, selenium; Th, thrombin; VitC, vitamin C; Zn, zinc; , monocyte; , neutrophil; , red blood cell.

factors XII-X, leading to activation of intrinsic coagulation pathway and production of kallikrein and bradykinin (**Figure 1**). Increased concentrations of kallikrein and bradykinin can stimulate the release of cytokines and nitric oxide (NO) from the neutrophils and promote inflammation (5, 66). The activated factor X (Xa, common factor between intrinsic and extrinsic coagulation pathways) converts prothrombin to thrombin. Despite administration of heparin, thrombin is formed during extracorporeal circulation, and links coagulation with inflammation as it activates endothelial cells and induces production of ROS, selectins (on platelets and endothelial cells) and platelet activating factor, activates neutrophils and platelets and induces production and release of pro-inflammatory cytokines including IL-1α, IL-6, and IL-8 (5, 67, 68).

During ECC, activation of the complement system triggers inflammatory responses and increases capillary permeability and changes in vascular tone. The circulating products of complement system activation and cleavage, including cleaved compartments 3, 4, and 5 (C3a, C4a, and C5a, anaphylatoxins), can activate platelets and endothelial cells. Of the three initiating pathways of complement activation, the classical pathway (CP) and lectin pathway (LP) are induced by antigen-antibody complex formation, while the alternative pathway (AP) can be directly induced by the contact with the biomaterial of the circuit. The AP seems to be the main pathway activated during CPB, as a result of the contact of the blood with biomaterial of the circuit and cleavage of C3 (activation of the common complement pathway). However, the CP can also be activated during ECC due to the activation of the complement protein C1 by factor XIIa of the activated contact system, protamine-heparin complex formation (after administration of protamine at the end of CPB to prevent bleeding), and endotoxin released from the intestine during ischemia and reperfusion (6, 65).

Compared to CPB, there is a significant gap in the literature about the complement system activity during ECMO and its effects on the outcomes of the patients, despite the ECC times being usually longer in ECMO. Moreover, most of the existing literature are from the 1990s with much older technology (older versions of pumps and oxygenators). In the few existing, more recent studies on ECMO, a fairly rapid elevation in the concentrations of the complement system components has been reported (69, 70).

There is a distinct paucity of literature on the activation of the complement system related to *ex-situ* perfusion, despite these platforms being used to study the contribution of complement activation and inhibition in the pathophysiology and outcomes of IRI.

BLOOD CELLS AND ENDOTHELIUM

Platelets

In 1995, Gemmell et al. showed that the contact of the blood with biosynthetic surfaces massively activated platelets, even in the presence of an anticoagulant (71). Platelet activation, triggered by tissue factor-induced thrombin, and carried on through the protease-activated receptors (PAR) on the platelet surface, leads to massive production of thrombin and thus further exacerbates the inflammatory response (65). In addition to thrombin as the main platelet activating factor, complement activation (through CP), and physical characteristics of the circuit also contribute to activation of platelets (5, 72).

Platelets, neutrophils, and endothelial cells interact mainly through CD40 on endothelial cell and CD40 ligand (CD40L) expressed on activated platelets. These interactions facilitate tissue migration of neutrophils and induce production, and release of chemokines and adhesion molecules in endothelial cells. Also, during ECC, the number of circulating, activated platelets bound to PMNs and monocytes increase, possibly triggering the pro-inflammatory effect of these immune cells (73, 74). Additionally, the activation of platelets may cause shape alterations leading platelets to release the contents of their granules into the circulation, including various chemokines such as chemokine (C-C motif) ligand 3, 5, 7, 17 and chemokine (C-X-C motif) ligand 4, 5, 7, and 8), pro-inflammatory cytokines such as IL-1β and CD-40 ligand, and adhesion molecules such as von Willebrand factor, and P-selectin, which can further promote the inflammatory response to ECC by other platelets, leukocytes and endothelial cells (5, 75).

Red Blood Cells and Hemolysis

Particularly in smaller patients, ECLS involves transfusion of significant amounts of packed red blood cells (pRBCs) that may play a key role in induction of oxidative stress, particularly with aged pRBC, which typically contain lower levels of elements supporting the antioxidative defence of the cell, such as selenium (Se) (67). Hemolysis occurs inevitably, in a time-related fashion during ECC due to the blood passing through different artificial compartments of the circuit, including the oxygenator, pump, and reservoir, at varying speeds (76, 77).

Lysed RBCs release cell-free hemoglobin, haem and iron. Being potent damage association molecular patters (DAMPs), cell-free haemoglobin and haem will induce or exacerbate inflammatory responses and oxidative stress through triggering innate immunity, complement system, and endothelial cells contributing to end organ damage. Additionally, the Fenton reaction, converting haem-iron from a ferrous to ferric state, generates highly active hydroxyl radicals that further promote oxidative stress and related modifications to lipids and proteins, altering the cellular membrane polarity and permeability, making the cells more susceptible to lysis (67, 78, 79). Furthermore, RBCs release various pro-inflammatory cytokines and chemokines to the circulation as a result of hemolysis, or stress responses in intact cells. These inflammatory molecules include but are not limited to IL-1 family, TNF family, IL-6, interferon (INF)-α2 and INF-γ, and C-C motif chemokines (such as monocyte chemoattractant protein-1 (MCP-1), and MCP-3) and C-X-C motif chemokines (such as IL-8) (80).

Scavenging systems, including organs such as the liver and spleen that uptake and metabolise the haemopexin complex, monocytes and tissue macrophages that uptake haemoglobin-haptoglobin complex, and various antioxidative enzymes such as haem oxygenase-1 (HO-1) at least partially temper hemolysis-related pathophysiologic conditions. However, when the hemolytic insult is severe, the defensive systems may become exhausted or diminished and incapable of preventing pathologic conditions related to hemolysis (78, 81).

In ESOP, there has been an ongoing effort to replace the blood-based perfusates with acellular ones, aiming to eliminate dependence on donor blood and the problems associated with banked blood as well as bypass hemolysis and related problems (82–84). Kappler et al. have shown that during four-hour ESHP in a porcine model, the free hemoglobin in the perfusate increased throughout the perfusion by approximately 0.02 mmol/L per hour (85).

Theoretically, the *ex-situ*-perfused thoracic organs may be more vulnerable to the hemolysis-related redox and inflammatory alterations due to absence of the organs involved in scavenging hemolysis products, and possible diminished antioxidant defence particularly in longer *ex-situ* perfusion times.

Leukocytes

In general, similar to the other pathologic inflammatory conditions, leukocytes are considered one of the main players in the inflammatory and oxidative stress responses induced during ECLS.

During application of ECLS, neutrophils and monocytes are activated mainly as a result of activation of complement system. Several other factors, including the contact system, thrombin, histamine, heparin, cytokines, neutrophil-activating peptide 2 released from activated platelets, and interactions with platelets, also contribute to the activation of neutrophils. In addition, thrombin triggers monocyte activation both directly (through thrombin receptors on monocytes) and indirectly, (by triggering formation of platelet-monocyte conjugation, similar to the complement system) (86, 87). Activated neutrophils release the contents of their granules, including lysozymes, myeloperoxidase, elastase, hydrogen peroxide, and reactive oxygen and nitrogen species (RONS) (6, 53, 64, 73). Similarly, during ECLS, activated monocytes (also increased in number) produce and release various pro-inflammatory cytokines including TNF-α, and IL-1β, and prostaglandins mainly with inflammatory effects such as prostaglandin (PG)-F2α and PG-E2 (88–90). While non-classical monocytes induce oxidative stress in the vascular system and cause endothelial dysfunction,

intermediate monocytes may be involved in induction of the systemic inflammatory responses related to ECC (91). Paradoxically, some monocytes express haemoglobin scavenger receptor (CD163) and may exert anti-inflammatory effects by removal of the potent pro-oxidant haemoglobin-haptoglobin complexes formed due to hemolysis during ECC (92).

In clinical ECLS studies, observations regarding lymphocytic sub-populations have been conflicting. While some studies have reported no change in either the number of lymphocytes, or their activation during application of ECLS (6, 93, 94), others report evidence of an initial increase in the absolute number T-cells, natural killer cells, and suppressor T-cells during ECLS, followed by a reduction to lower than normal values in the days after ECLS is discontinued (95). Evidence on the alterations in other subpopulations of leukocytes during ESOP is currently lacking.

Endothelial Cells

The endothelium is an active player in different physiologic functions, including controlling vascular tone and permeability, hemostasis, and immune system responses. Conditions that are related to inflammation and oxidative stress (such as ECC), lead to stimulation and activation of the endothelial cells similar to SIRS (5, 96).

Stimulated by various factors including anaphylatoxins, thrombin, and pro-inflammatory cytokines (most potently TNF-α and IL-1β) during ECLS, the expression of P-selectin and E-selectin on the surface of endothelial cells, and L-selectin of neutrophils increase, mediating the low-affinity, reversible rolling of the neutrophils along the vascular endothelium. Further interactions between the endothelial cells and leukocytes mediates transmigration of leukocytes into the extravascular compartment and exacerbation of the inflammatory responses (6, 93). On the other hand, the neutrophil elastase introduced into the endothelial cells leads to generation of superoxide anion by xanthine oxidase. Production of superoxide anion in turn reduces the intracellular ferritin-bound ferric iron (Fe^{3+}) to unstable ferrous iron (Fe^{2+}) that will cause/exacerbate oxidative stress through the Fenton reaction. The production of NO by nitric oxide synthase (NOS), which decreases the adhesion between neutrophil and endothelial cells, and scavenges superoxide to create peroxynitrite anion, partially compensates for this. However, in high concentrations or longer exposure times, peroxynitrite may directly incorporate a nitro group ($-NO_2$) to various tyrosine (Tyr) residues of different proteins/enzymes. Some of these residues include the tyrosines located near charged amino acids or on a loop structure, Tyr34 in manganese superoxide dismutase (MnSOD) and Tyr430 in prostacyclin synthase. This Incorporation can inhibit/alter their enzymatic function and structure and negatively affect normal cellular processes (97–99). The circulating pro-inflammatory cytokines can also directly stimulate endothelial cells leading to a pathologic increase in permeability causing tissue edema and impaired oxygen exchange causing multiple organ dysfunction (4, 5, 97).

Endothelial damage is accepted as an important phenomenon occurring during ESLP, that can decrease the quality of the potential lung graft. The limited data have reported induction of various markers of endothelial activation and damage, including intracellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), syndecan-1, hyaluronan, and heparan sulphate in the perfusate during

ESLP of human lungs, which had a negative correlation with the outcomes of preservation and transplantation (100–102). In a recent experimental ESHP study of healthy porcine hearts by our team, we demonstrated that the perfusate soluble ICAM-1 and VCAM-1 significantly increased over time alongside different pro-inflammatory cytokines, while the cardiac function and vascular tone (measured by increased coronary flow) decreased, and myocardial tissue developed significant edema (19). These observations strongly suggest an interaction between ECC, inflammation, activation and damage of the coronary endothelial cells, and the functional decline of the *ex-situ*-perfused organs.

CYTOKINES

Activation of the innate immune system by pathogens or cellular stressors/stimuli or tissue damage results in the production of pro-inflammatory cytokines that may be accompanied by anti-inflammatory cytokines including IL-10 (66). Cytokines are small-sized proteins with an integral role in immune system responses. Besides, they have various functions in the cell including signaling (5, 103). Generated in clusters, cytokines not only stimulate other cells (immune and non-immune) and alter their function and/or structural integrity, but also induce the production of more cytokines leading to aggravated concentrations of circulating cytokines known as "cytokine storm" (5, 66, 104). The severely induced immune system and high cytokine concentrations cause cellular damage, compromises organ function, and may lead to multiple organ dysfunction syndrome and death (66, 105). As discussed earlier in the manuscript, and demonstrated in the **Supplementary Table 1**, various cytokines have been reported to increase in the setting of ECLS and ESOP. Evidence suggests that the high level of cytokines induced in ECLS is associated with poor short-term and long-term patient outcomes (105–107). Despite the known effects of cytokines in further induction of the immune system and inflammatory and oxidative processes, the effects of cytokine removal during ECLS have been unclear. While application of the technique of cytokine removal with hemoadsorption (CytoSorb device) has been reported to be safe, its beneficial effects on cytokine removal and patient outcomes have been debatable in both CPB and ECMO studies. In a pilot study, Poli et al. reported that using the CytoSorb device during CPB did not decrease pro-inflammatory or anti-inflammatory cytokines, and did not improve clinical outcomes (108). Similarly, Bernardi et al. reported that application of the CytoSorb during CPB in patients undergoing cardiac surgery was not associated with a decrease in the level of pro-inflammatory cytokine IL-6, catecholamine requirements, or 30-day mortality (107). Stupica

et al. showed that corticosteroid treatment during cardiac surgery with CPB led to significantly lower circulating levels of IL-6, IL-8 and TNF-αcompared to CytoSorb-applied group, and naïve CPB control group. Yet, the reduction of cytokine values did not improve clinical outcomes, including cardiac function, in any of the groups (109). However, Nemeth et al. showed that while cytokine adsorption during cardiac transplantation surgery and CPB did not diminish the inflammatory responses but was associated with a tendency to improve patient outcomes such as shorter mechanical ventilation and hospital and ICU stay, as well as lower vasopressor requirements (110).

In a case report by Bruengar et al. ECMO support in a patient with cardiogenic sepsis shock, hemoadsorption with CytoSorb successfully removed IL-6 along some other inflammatory molecules such as C-reactive protein, and lead to lower than the usual need for vasopressor agents (111). However, Lother et al. showed that incorporation of cytokine adsorber into the ECMO circuit did not change the vasopressor or inotrope requirements in critically ill patients (112).

It must be remembered that in the ECLS setting the baseline cytokine levels, duration of ECLS, duration of application of hemoadsorption, as well as genetics and original condition and diseases of the patients affect the levels of cytokines (103, 113). Additionally ECMO is mostly applied in critically ill patients with an ongoing inflammatory state related to the underlying diagnosis, thus, the effects of cytokine removal during ECMO may not be attributable to attenuation of the circuit-related cytokine production, but rather the original inflammatory responses related to the illness.

In contrast to ECLS, much less is known about the effects of circulating cytokines produced during ESOP on the outcomes of organ perfusion and eventually transplantation as only a few preclinical studies have made conflicting observations. Kakishita et al. showed that removal of cytokines from the perfusate with an absorbent membrane during ESLP of porcine lungs did not improve pulmonary functional status or edema formation (8). On the other hand, in a pig model Iskender et al. showed that cytokine removal by an absorbent device (CytoSorb) during 12 hours ESLP reduced lung tissue injury and edema, tissue neutrophil infiltration and improved compliance (18). To our knowledge there are currently no studies investigating the effects of active cytokine removal with techniques such as CytoSorb during ESHP. However, Sanda et al. showed that reduced concentrations of circulating cytokines with adding methylprednisolon to the perfusate in a DCD pig model of ESHP did not improve the functional status of the heart during ESHP (61).

Theoretically, circulating cytokines during ESOP not only may directly damage the donated organs and negatively affect the outcomes of transplantation, but also can make it difficult to assess the viability of the perfused organ in case of a cytokine-related reversible functional decline. Moreover, in any of the discussed settings, it is almost impossible to support a single type of cytokine as the one responsible for inflammation-related effects. Moreover, it must be taken into account that despite being an important force in inflammatory and oxidative stress responses, cytokine production is merely one aspect of the highly sophisticated, orchestrated immune and stress responses (5, 103). Thus, robust preclinical, clinical, and transplantation studies are necessary to evaluate the direct effects of circulating cytokines during ECLS and ESOP and the potential benefits of focused cytokine removal.

HYPEROXIA

Hyperoxia is considered as one of the main reasons behind generation of ROS and oxidative stress during ECLS, and occurs routinely during CPB as a result of the aimed supra-physiological partial pressure of oxygen (PaO_2) in an effort to prevent hypoxia (67, 114). The oxidative stress caused by hyperoxia serves to further exacerbate inflammatory responses seen during CPB (114). Clinical studies on pediatric patients have suggested that hyperoxic CPB (PaO_2: 150 to180-200 mmHg) -related induction of pro-inflammatory cytokines, markers of oxidative stress (such as 8-isoprostane) and markers of tissue injury (i.e., DAMPS, such as protein S100) may be more severe in cyanotic patients (e.g., single ventricle pediatric patients). However, while the plasma pro-inflammatory cytokines may not be different between hyperoxic and normoxic CPB in non-cyanotic patients, hyperoxic CPB may still cause higher oxidative stress and tissue damage (115, 116).

In general, a considerable number of the patients undergoing ECMO are hypoxic/hypoxemic before the application of ECMO (e.g.' as a result of acute respiratory distress syndrome) thus, the hyperoxia during ECMO may be lead to an exaggerated production of RONS (117). Also, hyperoxia is a more prominent condition in venovenous ECMO (VV-ECMO) where the hyperoxic blood returns to the venous system, directly perfusing the damaged pulmonary vessels (21). Experimentally, a positive correlation has been observed between the tissue expression of markers of oxidative stress, with PaO_2 during ECMO (118). Also, higher expression of pro-inflammatory cytokines and pulmonary tissue edema, and lower expression of the anti-inflammatory IL-10 in PaO_2 ≥300 mmHg compared to PaO_2 100-299 mmHg during 120 minutes of ECMO has been observed (119). Clinically, it has been suggested that PaO_2 ≥193 mmHg during the first 48 hour of ECMO may be associated with higher risk of mortality, and some adverse outcomes, such as need for renal dialysis (120, 121).

The optimal PaO_2 is even more uncertain in ESHP. During the 20th century, ESHP was conduced with blood-free perfusates that would maintain a pH of 7.40 using 95% oxygen flow and PaO_2≥450 mmHg that was much higher than physiologic values (PaO_2:75-100 mmHg). Later, with addition of RBC to the perfusate to reach a hematocrit of 35%, the oxygen delivery of the perfusate to the heart improved with more physiologic values of PaO_2 (122). More recently, in the experimental studies using a RBC-containing perfusate (either whole blood or separated RBCs), the PaO_2 is maintained mostly in the range of 100≤PaO2<200 mmHg, which is able to provide a physiological oxygen saturation (20, 123, 124). Interestingly, in

ESLP, while most common approach in ESLP has been using a deoxygenated perfusate containing 6% oxygen, In a rat model of ESLP and lung transplantation, Noda et al. demonstrated that the perfusate containing 40% or 60% oxygen, when compared to 6%, or 100%, showed significantly lower inflammation expression of the inflammatory cytokine genes in the lung tissue during ESLP, while the group with 40% oxygen in perfusate showed the best post-transplantation pulmonary function (125). These results suggest that potentially, while hyperoxia may simply refer to administration of oxygen in percentages higher than room air (FIO_2>21%) it is yet to be well defined as it has been described it in a wide range by different studies (100>PaO_2≥485 mmHg). More over, the efficient and safe oxygenation values may highly depend on the setting, pre-existing conditions, and in the case of ESOP, depending on the organ's specific needs, thus more studies are warranted to define the safe and efficient oxygenation values in different types of ECC.

ISCHEMIA-REPERFUSION INJURY

The pathophysiology of the IRI has been well described before (126, 127). With cross-clamping the aorta and application of cardioplegic solution during cardiac surgery involving CPB, the heart undergoes global ischemia. Also, not only do the lungs become partially ischemic as a result of loss of flow through the bronchial arteries, the peripheral organs may be subjected to global hypoperfusion. The abrupt reperfusion with removal of the cross-clamp leads to IRI, affecting the patients outcomes negatively (6, 128).

The application of ECMO is not necessarily associated with IRI. Though, given the indications of ECMO (respiratory and/or cardiac failure), the organs may have been subjected to different degrees of ischemia/hypoxia and hypoperfusion, thus the hyperoxic perfusion *via* ECMO leads to massive production of RONS that contribute significantly to exacerbation of oxidative stress and inflammatory responses (67). Electron spin resonance spectroscopy evaluation has suggested that production of the reactive oxygen occurs throughout the application of ECLS, going beyond the RONS production in the context of IRI (129).

In ESOP, when compared to standard donor organ preservation method (i.e., SCS), the duration of cold ischemia is considerably shorter. However, ischemia still occurs with the hypoperfusion period following the withdrawal of the life support in the donor, during procurement of the organ, and during mounting of the organ on the *ex-situ* perfusion apparatus (20). More over, in case of DCD, longer warm ischemic times are expected due to the mandatory "no touch" period that is required to confirm the irreversible loss of circulatory function before organ procurement proceeds. Thus, the *ex-situ*-perfused organs are naturally affected by IRI during early perfusion and may also further trigger inflammatory responses and tissue damage that will follow during *ex-situ* perfusion (130, 131). IRI in the setting of ESOP is a contributor to ROS during early perfusion.

ABSORPTION OF ANTIOXIDANTS/ DECREASED ANTIOXIDATIVE POTENTIAL

Growing evidence suggest that the bioavailability of micronutrient and trace elements such as copper, zinc, selenium, manganese, necessary for the function of the antioxidative enzymes, as well as the non-enzymatic antioxidants such as, uric acid, vitamins C and E, decrease during ECC (132–134). The decreased values of micronutrients/trace elements during ECC may be related to acute-phase redistribution as a result of the increased inflammatory response, absorption to the ECC circuit, and excretion in bodily fluids/perfusate (21, 67, 133). Such a decline can lead to exacerbation of inflammatory responses and oxidative stress and related damage to the exposed tissues/organs. The status of micronutrients and trace elements during ESOP and its association with inflammation and oxidative stress and outcomes of ex-situ organ preservation and transplantation has been almost unexplored so far. In a porcine model of extended ESLP for 24 hours, Buchko et al. showed that continuous supplementation of the perfusate with the total parenteral solutions that also contain micronutrients, lead to lower inflammation, and improved graft function compared to the controls, however, the specific effects of the micronutrients/trace elements had not been explored (135). Also, to our best knowledge, there is no data available on the potential alterations in the levels of micronutrients and trace elements during ESHP, and implications of their decline, or supplementation on the preservation of the donated heart during ESHP, or the outcomes of transplantation.

CLINICAL SIGNIFICANCE OF INFLAMMATION AND OXIDATIVE STRESS

Despite the induction of inflammatory responses and oxidative stress during ECLS being reported frequently, the clinical significance of these findings has been a matter of debate, since the existing data is very limited, and is mostly related to preclinical studies. Among a vast range of the markers of inflammation and oxidative stress reported to increase during or after ECLS, IL-6, IL-8 induced during CPB have been associated with higher incidence of post operative cardiac dysfunction, infection, and longer ICU and hospital length of stay, with Il-6 specifically being associated with pulmonary dysfunction after CPB. Similarly, increased levels of IL-6, IL-8, and TNF-α are reported to be associated with higher in hospital mortality in patients undergoing ECMO, particularly VV-ECMO (4).

In the ESOP setting, it has been demonstrated that lower induction of the markers of inflammation and/or oxidative stress in tissue and/or the perfusate during *ex-situ* perfusion is associated with better preservation of functional status of the perfused heart or lung in preclinical studies (56, 136). Andreasson et al. have reported that lower levels of inflammatory cytokines in the perfusate and BAL in ESLP of a group of human donor lungs initially deemed non-transplantable, was associated with better recovery during ESLP and subsequent successful transplantation.

Their findings suggest a predictive value for combined perfusate IL-8 and IL-1β during ESLP for prediction of the early outcomes of lung transplantation (137). In a single center study by Sage et al. a scoring system based on the perfusate levels of IL-6 and IL-8 can be used to predict the early outcomes of lung transplantation including the incidence of primary graft dysfunction (138). Although these studies provide very useful information about the implications of inflammation and oxidative stress-induced during ESLP on recovery of the lungs during ESLP and the outcomes of transplantation, the results may also be affected by the variables related to the donor's organ characteristics and recipient factors. In the setting of ESHP, preclinical studies report induction of the markers of oxidative stress and inflammation during ESHP in both perfusate and myocardial tissue even in the negligible damaged animal hearts (as opposed to DCD models) (19, 63, 139), even with a leukocyte-depleted perfusate (140). In a study of ESHP of DCD rat hearts by Lu et al., melatonin attenuated the induction of IL-11β, IL-18, IL-6 and TNF-α as well as the markers of oxidative injury such as malondialdehyde and 4-hydroxynonenal in the myocardial tissue, resulting in superior functional status (141). However, in a DCD porcine model of ESHP, Sandha et al. showed that attenuating of the induction of the inflammatory responses during ESHP, although lead to lower edema formation in the heart, was not associated with improvement of myocardial function during ESHP. This observations may be at least partially related to the warm ischemic insult occurring around the procurement time in DCD, and not exclusively reflecting the effects of ECC. Yet, there is a scarcity of data on the implications of these responses on the outcomes of ESHP and heart transplantation in preclinical studies, and to our best knowledge, there is no clinical data on this issue. More studies, with adjustment to the donor and recipient conditions are warranted to evaluate the clinical significance of these phenomena that occur during ESHP on the outcomes of subsequent heart transplantation.

TARGETING INFLAMMATION AND OXIDATIVE STRESS IN EXTRACORPOREAL CIRCULATION

Circuit Optimization

With the identification of inflammation and oxidative stress in ECLS, attention was turned to 1) determining the components of the circuit that may have a prominent role in this phenomenon, and 2) how to improve the general aspects of the circuits to prevent or minimize these effects.

The surface modification of the circuit, intended to replicate the anti-thrombotic and anti-inflammatory properties of the endothelium, include application of biomimetic surfaces (e.g.' using heparin or direct thrombin inhibitors), biopassive surfaces (e.g.' using phosphorylcholine, or albumin), as well as more experimental attempts at endothelization of circuit components (142–144). Both ECLS and ESOP systems typically make use of polyvinyl chloride (PVC) tubing (with the latter taking the lead from the former).

The most commonly used biomimetic approach, which seeks to replicate the antithrombotic and anti-inflammatory properties to endothelium, is heparin-bonding. Though there has been little direct study of heparin-bonding in ESOP circuitry, it has been shown to reduce cellular activation and release of inflammatory mediators in clinical studies of ECLS, as well as *in vivo* models, and is even associated with improved clinical outcomes, such as decreased ICU stay and lower rates of post-operative atrial fibrillation (145–148). Priming the circuit with a heparin-albumin solution can have a similar effect, given the capacity of PVC to absorb plasma proteins (149). Nitric oxide (NO)-bounded materials have also been used in ECLS as a means to prevent platelet activation and aggregation (150). Though, their direct effect on inflammation has not been well studied, they are known the have inherent bactericidal properties (151).

Biopassive approaches, by contrast, seek to make the circuitry more inert. A common technique is coating with phosphorylcholine, the main phospholipid component of cell membranes. It is thought that their formation of a biomembrane surface leads to reduced thrombin formation, though their effect on inflammation is not well known. Plasticisation of circuit components with the amphiphilic polymer, poly-2-methoxyethylacrylate, has also been shown to reduce platelet adhesion and aggregation in ECLS, as well as decrease complement activation and inflammatory markers (152–154).

Considering the aforementioned, currently the best evidence exists for heparin-coated and third-generation heparin-polymer coated ECLS circuits (commercially-available), which have been shown to improve biocompatibility and attenuate inflammation, in both experimental and clinical studies, by suppressing various pathways involved. They have even been directly linked to improved clinical outcomes, though they are more expensive (142–144).

Application of miniaturized extracorporeal circuits (MECC), with lower surface area compared to conventional circuits (shorter tubing length, eliminated venous reservoir and suction device), being fully closed to prevent air-blood interface (even as a CPB circuit), and lower requirements for priming volume and blood transfusion, has been associated with significantly lower inflammation and oxidative stress. The patient outcomes using MECC circuit have been either similar with the conventional circuit, or have shown some beneficial effects including lower incidence of post-operative atrial fibrillation (AF), myocardial infarction, renal failure, and shorter duration of ICU stay and intubation time. However, they may not be suitable for highly complicated procedures/surgeries (21, 64, 155, 156).

Regardless of the size of the circuit, there is limited data on the relative contribution of each circuit component to initiating inflammation and oxidative stress. It has been suggested that using smaller oxygenators and connectors, smaller pumps needing higher pump speeds, and additional in-line filters are associated with higher shear stress and hemolysis during perfusion, exacerbating oxidative stress and inflammation (67, 157).

There is very limited scientific data on the effects of circuit optimization in ESOP. This may be partially related to the fact

that the concept of clinical ESOP with modern technology as a method for preservation and potential recovery of donor heart and lung is still in its infancy. Moreover, there is incredible variability in the custom-built circuits used in experimental studies on ESOP, also affected by the size of the experimental animal model used (3, 158) making it very difficult to evaluate/compare these circuits and their individual components for induction of inflammation/oxidative stress during perfusion. While there are currently four different devices commercially available for clinical normothermic ESLP, there is only one for clinical normothermic ESHP (3, 159). Studies using clinically relevant platforms and circuits are necessary for evaluation of biocompatibility in ESOP setting in terms of reducing inflammation and oxidative stress.

Leukocyte Filtration

Considering the importance of the role attributed to leukocytes in induction of inflammatory and oxidative stress responses during ECC, eliminating this phenomenon is believed to bear a critical potential for protection of the organs in this setting. Continuous leukocyte filtration with arterial line filters incorporated into the circuit has shown mixed results in terms of attenuation of inflammation, oxidative stress, and organ dysfunction during and after CPB. It has also been reported that placing the leukocyte filter in venous line, while still efficiently filtering the leukocytes, may cause lower leukocyte damage and thus, better attenuate inflammation (160–164).

In critically ill neonates placed on ECMO, transfusion of leukoreduced blood *via* leukocyte filtration is associated with improvement of patient outcomes, including survival (165, 166). On the other hand, prolonged leukocyte filtration, despite continuous reduction of circulating leukocyte count, may increase hemolysis and leukocyte damage, which can induce or exacerbate inflammation and oxidative stress. However, it is difficult to isolate the effects of leukoreduction in ECMO, as they may simply be related to the underlying condition (167).

Leukocyte filters are routinely incorporated into ESLP circuits to attenuate ECC-induced inflammation and oxidative stress (168). However, existing experimental studies have shown contradictory results in terms of attenuating inflammation and improving the outcomes of organ preservation and transplantation (55, 56, 168). Interestingly, our group has also reported similar number of trapped leukocytes in the leukocyte filter between cellular and acellular perfusates, and comparable histological findings between ESLP with or without leukocyte filter (168). These findings may suggest that either most of the cells trapped in the leukocyte filter during ESLP originate from the lung and/or the efficiency of leukocyte filter in ESLP is sub-optimal.

In clinical ESHP, leukoreduced donor blood is routinely used for the perfusate to prime the circuit (169). Leukocyte reduced or depleted perfusate (either using leukocyte filters or centrifuged blood) has been also frequently used in experimental ESHP studies, aiming to reduce inflammation and oxidative stress (57, 123, 170). However, our group has shown that ESHP with either a leukocyte filter or a perfusate containing leukocyte depleted blood is still accompanied with considerable systemic and tissue inflammation and oxidative stress during perfusion without benefiting functional preservation (19, 140).

Pharmacological Interventions

The optimal pharmacological prevention/attenuation of the inflammation and oxidative stress in the setting of ECC is still a matter of debate. The studies performed to address this phenomena, have applied various agents belonging to the general class of anti-inflammatory medications, as well as the experimental agents. Interestingly while most of these studies are performed in the clinical CPB setting, there a paucity of studies targeting inflammatory and oxidative responses in ECMO, or ESOP. The most widely studied agents in the setting of ECC have been discusses in the following sections.

Corticosteroids

The prophylactic administration of steroid, with the aim of improving the post-operative patient outcomes in surgeries involving CPB, has shown mixed results in clinical trials **Supplementary Table 2**. Multiple studies have found that corticosteroid-related attenuation of the inflammation and tissue injury and oxidative stress, is associated with improvement of post-operative contractile function of the heart, lower incidence of post-operative atrial fibrillation (AF), decreased requirement for ventilator and circulatory support, and shorter length of hospital and ICU stay without increasing the risk of infections (171–180). Still, some other studies have not detected any beneficial effects for corticosteroid administration in improving post-operative primary and secondary outcomes (173–176, 180). Despite the mixed findings, meta-analytical studies suggests that prophylactic corticosteroids in patients undergoing cardiac surgery involving CPB significantly decreases the length of stay in ICU and hospital and incidence of post-operation AF in both adult and pediatric patient populations, and decreased inflammation has been suggested to be one of the key element in these effects (181–183). The prophylactic steroid administration in this setting is currently recommended by the American Heart Association and the American College of Cardiology (184).

Corticosteroids (mainly methylprednisolone) are also commonly administered in critically ill patients undergoing ECMO, where they are believed to provide benefit through reducing the systemic inflammatory response and compensating for any cortico-adrenal insufficiency. The related clinical trials in general have supported its effect in improving secondary outcomes such as decreasing the need for mechanical ventilation and the length of ICU stay alongside decreasing the inflammatory responses. Moreover, a meta-analysis by Meduri et al. showed that decreased values of inflammatory cytokines in critically ill patients with ECMO who were treated with methylprednisolone for a prolonged period, and early during the course (for more than two weeks, starting in the first two weeks) was also associated with reduced incidence of in-hospital mortality (185, 186)

The addition of corticosteroid (methylprednisolone) to the perfusate during EVOP has been a traditional approach in both experimental and clinical protocols for ESOP (56, 187, 188). The main reason for this intervention has been the attempt to

attenuate/prevent the deleterious effect of IRI and related inflammation and oxidative stress that donated organs are subjected to due to ischemia and other stresses occurring during the process of donation and organ procurement. In a rat model of brain death and ESLP, Van Zanden et al. showed that the addition of methylprednisolone to the perfusate led to lower expression of IL-6 and IL-1β genes in the tissue, and lower perfusate IL-6 and improved positive inspiratory pressures compared to controls (189). In a study by Martens et al. administration of methylprednisolone both before the arrest, and during the ESLP in a DCD porcine model led to a significant reduction in IL-1β, IL-8, and TNF-α, together with superior pulmonary compliance in treated lungs compared to the controls. However, the results of this study might have been also affected by pre-arrest administration (preconditioning) of the organs by methylprednisolone, rather than only targeting the inflammatory responses during ESLP (190). Conversely, In the setting of ESHP, the few existing experimental studies have reported little or no beneficial effects for corticosteroids added during ESHP in preventing cardiac functional decline, tissue damage or edema during perfusion (61, 191). However the addition of corticosteroids to the perfusate during ESOP, has been considered as a safe and inexpensive attempt to attenuate the inflammation in the perfused hearts and lungs, regardless of the donation condition and basic status of the donated organ.

Statins

During the past few decades the anti-inflammatory and antioxidative effects of statins has been recognized (192, 193). Clinical trials and meta-analyses have suggested that preoperative prescription of a statin in patients undergoing cardiac surgery with CPB can attenuate the inflammatory responses and tissue damage induced by extracorporeal circulation, and may improve patient outcomes (4, 193, 194). However, a more recent meta-analysis by An et al. revealed that preoperative administration of statins has the beneficial effect of decreasing the incidence of post-operative AF, but not myocardial infraction or stroke in patients undergoing cardiac surgery requiring CPB (195). Interestingly, the meta-analysis by Putzu et al. suggested an increased risk of acute kidney injury and in hospital mortality with preoperative statins, while the earlier meta-analysis by this group also failed to show any improvement in patient outcomes such as mortality and incidence of post-operative AF (4, 196, 197).

Statins have been also used adjacent to VV ECMO for treatment of respiratory pathologies such as ARDS (186), however the effect of statins on inflammatory and oxidative stress occurring during extracorporeal circulation has not been investigated. Similarly, in ESHP, statins have been investigated to attenuate myocardial IRI for their anti-inflammatory and antioxidant effects, and have shown beneficial effects for this purpose (198, 199), but not specifically for attenuating ECC-related inflammation and oxidative stress in the setting of ESOP.

Phosphodiesterase Inhibitors

The anti-inflammatory effects of phosphodiesterase inhibitors (PDEIs) in ECLS have been somewhat controversial. Experimental and clinical studies suggest that PDEIs can improve myocardial energetics, and ameliorate inflammation, and may also have protective effects on endothelial and pulmonary tissue as well (200–204).

The PDEIs have been also investigated in EVLP to address conditions such as pulmonary hypertension and IRI, and have shown beneficial effects including vasorelaxation (205–207). However, experimental administration of PDEIs in EVLP of discarded human donor lungs had no effect on inflammation, and did not improve the physiological status of the lungs, or prevent tissue edema (208). Although cardioprotective and therapeutic properties of different families of PDEIs have been evaluated in the setting of ESHP (209, 210), their effect on mitigating the inflammatory responses and/or improving the functional preservation of the *ex-situ*-perfused heart is still unknown.

Complement Inhibition

Experimental and clinical studies evaluating different complement inhibitors including APT070 and TP10 (C3 and C5 activation inhibitor) and Pexelizumab (a recombinant antibody binding to C5) have reported a significant reduction in active complement proteins, and better protection of the lungs and myocardium during CPB, and improvement in patient outcomes (211–213). However, some of these studies failed to show an association between diminished complement activation and inflammation. It has been shown that heparin-coated ECMO circuits, lead to attenuation in complement activation due to complement-inhibitory effects of heparin (214).

As a technique to study the physiology and pathology of the heart, ESHP has been used to evaluate the effects of complement activation and inhibition on the myocardium (215, 216), but there is a paucity of data on the effect of complement inhibition during ESHP with blood derived perfusates, or during ESLP.

Serine Protease Inhibitors

Clinical and experimental studies on ECLS suggest that serine protease inhibitors (SPIs) may attenuate the ECC-related inflammation by decreasing the activation of the contact system (through preservation of kalikrein inhibitor activity), coagulation pathway (inhibition of plasmin), complement system (decreased production/activation of complement factors), and by modulating leukocyte activation, which may lead to organ-protective effects during ECC **Supplementary Table 2** (184, 217–220). Though, there are still some conflicting reports in the literature, such as the observational study by Mangano et al., which reported significantly higher incidence of adverse cardiac, cerebral, and renal events in patients receiving aprotinin (184, 221).

There is evidence from *ex-situ* perfusion of animal and discarded human lungs that SPIs may decrease systemic and tissue inflammation, improve *ex-situ* protection of the lungs, and attenuate tissue edema (222, 223). The anti-inflammatory and tissue protecting effects of SPIs has been assessed in the context of ESHP to protect the myocardium from IRI, and have shown cardioprotective effects against it (224, 225), but not in attenuation of the inflammatory and oxidative stress responses related to ECC specifically.

Antioxidants

Administration of different antioxidants either before, or during ECLS may lower production of RONS, attenuate oxidative stress, inflammation, and related tissue damage, and improve heart and lung protection and function, and patient outcomes (226–230). Although some studies could not detect any improvement in the pro-inflammatory profile, organ function, or patient outcomes with administration of some well known antioxidants, such as vitamins and coenzyme Q10 in the context of CPB application (231–233), a meta-analysis of the clinical trials revealed that application of NAC, polyunsaturated fatty acids (PUFA), and vitamin C in surgeries applying CPB is associated with lower risk of post-operative AF, and improved patient outcomes (mortality in NAC, and length of hospital stay in PUFA) (234). Similarly, a recent meta-analysis reported that perioperative administration of vitamin C is associated with lower incidence of post-operative arrhythmia, shorter mechanical ventilation period and shorter length of hospital and ICU stay, though not improved mortality (235).

There is a significant paucity of data on the effects of antioxidants in ECMO treatment, despite the fact that the endogenous antioxidant defence system is impaired in the critically ill patients requiring ECMO (67). Experimental and clinical studies are needed to address the effects of antioxidants administered during ECMO treatment on systemic inflammation and oxidative stress, organ damage, and patient outcomes.

Experimental administration of antioxidative agents either in the perfusate, or *via* inhalation during ESLP can decreased generation of RONS, oxidative stress-related protein modifications, inflammation, improve lung tissue protection and attenuate tissue edema, and improve post-transplantation respiratory function of the lung (236–239). Most of these ESLP studies have been performed in discarded lungs that had endured significant ischemia thus, severe IRI would be expected upon reperfusion with ESLP, which may mask the effects of antioxidants on extracorporeal related inflammation and oxidative stress. However, in a rat model of minimally damaged donor lungs (which has not endured clinically relevant ischemia), administration of the antioxidant 2% hydrogen through inhalation was reported to decrease expression of IL-6, IL-1β, and TNF-α mRNA compared to controls, and to improve metabolic profile of the lungs and post-transplantation pulmonary function (240). The results of this study not only support the detrimental effects of ECC-related oxidative stress and inflammation on preservation of the lung during ESLP, and on outcomes of transplantation, but also support the beneficial effects of antioxidative support for the lungs during ESLP, regardless of the perioperative insults.

With increasing interest in the use of ESHP to facilitate the transplantation of DCD hearts, IRI has been a focus of research in this field. Animal models of *ex-situ* perfusion of the hearts subjected to long ischemic times have reported that administration of antioxidants in ESHP reduces oxidative stress and related modifications, inflammation, and myocardial edema, and may be associated with better preservation of coronary endothelium, myocardial tissue integrity, and organelles such as mitochondria in cardiac tissue (241–244). While these studies are focused on attenuating the myocardial IRI, to our knowledge there are no available studies on the cardioprotective effects of antioxidants against the ECC-related oxidative stress during ESHP.

CONCLUSION

Inflammation and oxidative stress are induced during ECLS, as well as ESOP. While these phenomena have been explored in more details in ECLS, many aspects remain obscure in ESOP studies. Similarly, the implications of these reactions are not yet fully understood, and there is a considerable scarcity of information on the effect of ongoing inflammation and oxidative stress in ESOP on functional viability of the *ex-situ* perfused hearts and lungs, and on the outcomes of transplantation in these organs. There has been a significant variability in both experimental and clinical study designs and the anti-inflammatory and antioxidative interventions applied in the ECLS setting, as well as the existing limited studies in ESOP. The variability of the custom-built circuits in experimental extracorporeal perfusion studies may also contribute to some of the controversial findings in these studies. Increased knowledge of the ECC-related inflammation and oxidative stress and their outcomes, particularly in ESOP, and targeting those with clinically-translatable methods will help in 1) providing a safer life support for the critically ill patients or the patients undergoing cardiac surgery;, and 2) improving ESHP protocols to offer optimal organ preservation and potentially improvement of the graft condition.

AUTHOR CONTRIBUTIONS

All the authors contributed to the content of this manuscript. SH and JH have performed the literature review and summarizing, as well as categorizing the studies and sections and preparation of the study tables. SH has written the manuscript. DF is the corresponding author and has been involved with the selection of the topic, and has reviewed and guided the preparation of the manuscript. All authors contributed to the article and approved the submitted version.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.831930/full#supplementary-material

Supplementary Table 1 | Highlighted studies on inflammatory and oxidative stress responses induced during extracorporeal life support, and *ex-situ* thoracic organ perfusion. BAL, bronchoalveolar lavage; Bcl-2, B-cell lymphoma-2; CABG, coronary artery bypass grafting; CD40, cluster of differentiation 40; Clin, clinical

study; CPB, cardiopulmonary bypass; C3a, cleaved complement component C3 fragment a; C4a, cleaved complement component C4 fragment a; C5b9, cleaved complement component C5 fragment b9; ECMO, extracorporeal membrane oxygenation; Exp, experimental study; DBD, donation after brainstem death; DCD, donation after circulatory death; ESHP, *ex-situ* heart perfusion; ESLP, *ex-situ* lung perfusion; HO-1, hemoxygenase-1; IL-, interleukin; MDA, malondialdehyde; MCP-1, monocyte chemoattractant protein-1; MIF, macrophage migration inhibitory factor; MMp-2, matrix metalloproteinase-2; NADPH, Reduced nicotinamide adenine dinucleotide phosphate; PGF2, Prostaglandin F2; PMN, polymorphonuclear; SIRS, systemic inflammatory response syndrome; TNF-α, tumor necrosis factor α; TXA, tranexamic acid; VCAM-1, vascular cell adhesion molecule-1; VA-, venoarterial; VV-, venovenous

Supplementary Table 2 | Pharmacologic interventions to reduce oxidative stress & inflammation in extracorporeal circulation. C3a, cleaved complement component C3 fragment a; CD11b, cluster of differentiation 11b; CK, creatine kinase; CK-MB, MB isoenzyme of creatine kinase; CPB, cardiopulmonary bypass; ECMO, extracorporeal membrane oxygenation; ESHP, *ex-situ* heart perfusion; ESLP, *ex-situ* lung perfusion; ICU, intensive care unit; IL-, interleukin;LDH, lactate dehydrogenase; MIP-1, macrophage inflammatory protein-1, MMP, matrix metalloproteinase-2; MPO, myeloperoxidase; NADPH, Reduced nicotinamide adenine dinucleotide phosphate; PAR, poly(adenosine diphosphate-ribose); PGF2, Prostaglandin F2; PMN, polymorphonuclear; POD, post-operative day; RONS, reactive oxygen and nitrogen species; SIRS, systemic inflammatory response syndrome; TNF-α, tumor necrosis factor α; VCAM-1, vascular cell adhesion molecule-1; VA-, venoarterial

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frontiers in Immunology

MINI REVIEW
published: 25 March 2022
doi: 10.3389/fimmu.2022.830992

Revisiting the Principles of Preservation in an Era of Pandemic Obesity

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The current obesity epidemic has caused a significant decline in the health of our donor population. Organs from obese deceased donors are more prone to ischemia reperfusion injury resulting from organ preservation. As a consequence, these donors are more likely to be discarded under the assumption that nothing can be done to make them viable for transplant. Our current methods of organ preservation—which remain relatively unchanged over the last ~40 years—were originally adopted in the context of a much healthier donor population. But methods that are suitable for healthier deceased donors are likely not optimal for organs from obese donors. Naturally occurring models of acute obesity and fasting in hibernating mammals demonstrate that obesity and resilience to cold preservation-like conditions are not mutually exclusive. Moreover, recent advances in our understanding of the metabolic dysfunction that underlies obesity suggest that it may be possible to improve the resilience of organs from obese deceased donors. In this mini-review, we explore how we might adapt our current practice of organ preservation to better suit the current reality of our deceased donor population.

Keywords: obesity, cold storage, fasting, hibernation, metabolism

OPEN ACCESS

Edited by:
Stefan Schneeberger, Innsbruck Medical University, Austria

Reviewed by:
Jeanette Villanueva, Victor Chang Cardiac Research Institute, Australia
Rupert Oberhuber, Innsbruck Medical University, Austria

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Specialty section:
This article was submitted to Alloimmunity and Transplantation, a section of the journal Frontiers in Immunology

Received: *07 December 2021*
Accepted: *02 March 2022*
Published: *25 March 2022*

Citation:
Langford JT, DiRito JR, Doilicho N, Chickering GR, Stern DA, Ouyang X, Mehal W and Tietjen GT (2022) Revisiting the Principles of Preservation in an Era of Pandemic Obesity. Front. Immunol. 13:830992. doi: 10.3389/fimmu.2022.830992

INTRODUCTION

Since Joseph Murray and David Hume performed the first successful transplant of a deceased donor organ in 1962, the rate of obesity in the U.S. has more than tripled from 12.8% to 42.4% (1, 2). In this same time span, abdominal solid organ transplantation has gone from a highly experimental technique to a reliable cure for end-stage organ failure; 1-year survival rates now exceed 90% for liver and 95% for kidney (3, 4). However, in current practice, these high success rates are dependent on selecting relatively healthy donor organs. Unfortunately, the continuing rise in obesity rates within our donor pool (**Figure 1**) often forces patients to choose between two undesirable options: accept the added risk that comes with an organ from a less healthy donor or risk dying on the waitlist before a better offer comes.

For recipients who choose to accept an organ from an obese donor, the added risk of post-transplant complications can be substantial (5, 6). In one study, livers from deceased donors with BMI >35 had a 156% increased likelihood of early allograft dysfunction (6). A recent retrospective

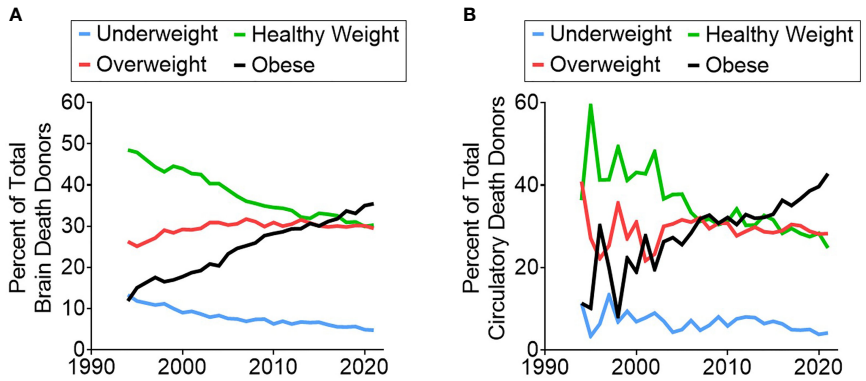

FIGURE 1 | Trend showing the increasing rate of obese donors over time in both **(A)** brain death donors and **(B)** circulatory death donors.

analysis of 14 years of registry data—from 2000-2014—revealed significantly higher risk of death-censored graft failure in renal transplants when receiving an organ from a mildly obese donor, BMI 30-35, (HR = 1.10) or a very obese donor, BMI >35 (HR = 1.22) (5). This elevated risk was particularly pronounced in deceased donor organs suggesting that donor obesity sensitizes their organs to injury during cold-storage preservation. Given these adverse outcomes, it is not surprising that organs from obese donors are more likely to be discarded (7). However, the practice of throwing away all less-than-ideal organs is not a viable long-term strategy in our current era of pandemic obesity.

The current rationale for disproportionately discarding organs from obese donors is that obesity renders these organs more susceptible to ischemia reperfusion injury (IRI), thereby increasing the risk of complications to an unacceptable degree. This line of reasoning assumes that our current approaches to organ preservation—which remain relatively unchanged since they were initially developed ~40 years ago—are as optimal for organs from obese donors as they are for young healthy donors. In this Mini-Review, we propose an alternative perspective effective preservation of organs from obese donors will require new methods specifically tailored to the mechanisms of injury present in these organs. In support of our perspective we present protective adaptations in hibernating animals that allow these obese animals to survive cold storage like conditions. We further discuss how these adaptations relate to historic and contemporary studies on the benefits of fasting in animals and humans. We conclude by suggesting how these mechanistic insights might be translated to restorative interventions within the context of organ preservation.

THE MODERN WESTERN DIET AND ITS IMPACT ON ABDOMINAL ORGAN TRANSPLANT

Nearly 50% of all adults in the U.S. have a chronic disease related to poor-quality diet (8). What constitutes a "healthy" diet should simply be based on scientific evidence. However, public recommendations can be complicated by multiple factors including politics, corporate interests, and complex scientific evidence that is difficult to interpret (9). While there is no common consensus on the best diet, there is agreement that both the total calories and macronutrient breakdown make a critical difference. Since 1970, Americans have increased their daily food intake by ~300 calories (10). Macronutrient breakdown has also changed substantially; our diets have become increasingly carbohydrate-based with a reduced percentage of calories from protein and fat. These changes to our diet have been linked to the concomitant rise of obesity and its associated pathologies (8).

The excess macronutrients in obese individuals leads to increased oxidative stress and can cause a predisposition to systemic inflammation and mitochondrial dysfunction (11–13). Abundant adipose tissue increases oxidative stress and the production of inflammatory cytokines (e.g. IL-6, TNF-alpha, MCP-1, and resistin) while decreasing anti-inflammatory mediators (e.g. adiponectin, Omentin, IL-10) (11, 14). Additionally, oxidative stress can also cause an increase in circulating free fatty acids and abnormal fat deposition in non-adipose tissues. This can result in further mitochondrial dysfunction and various forms of cell death such as lipotoxicity (15). Both effects can prime organs for damage during cold storage and reperfusion. For example, livers in cold storage from obese rats are shown to have increased rates of sinusoidal endothelial cell death compared to their lean counterparts (16). Inflammation can also prime transplanted organs for rejection by activating both the recipient immune system and the endothelium of the graft leading to an increase in the attachment and extravasation of T cells (17).

The metabolic and inflammatory dysfunction that occurs in organs from obese individuals are potentially reversible through lifestyle and diet changes. Many diets, such as the ketogenic diet or intermittent fasting, are suspected to not only promote weight loss but also repair the broken metabolic pathways caused by the average American diet (18). As one example, obese children put on a diet and lifestyle modification plan for three months had an

increase in adiponectin and a decrease in inflammatory factors (C-reactive protein, IL-6) despite no significant weight loss (19). These findings suggest that it is possible to reduce or even reverse the underlying metabolic dysfunction and inflammation associated with the oxidative stress of obesity.

It is not ethically permissible to alter the diet of an organ donor with a prolonged intervention prior to organ retrieval. We therefore are restricted to evaluating if it is possible to target these pathologies during the preservation period that follows organ recovery. Or to state the question more succinctly: Can we better preserve obese organs to improve their resilience? To address this question, it is instructive to investigate existing examples from nature. Hibernating animals provide a unique model of a species that have evolved mechanisms to allow their organs to tolerate cold storage like conditions even when they are in an obese state.

HIBERNATORS AS A ROAD MAP FORWARD

Hibernating mammals demonstrate that obesity and resilience to cold hypoxia are not mutually exclusive. Some hibernating species (e.g., ground squirrels and marmots) are known to double their body mass prior to hibernation; adipose tissue can make up as much as 80% of body mass in these animals when they enter hibernation (20). Although this weight gain occurs in a shorter timeframe than most obese humans, their weight gain is still associated with insulin resistance, hyperinsulinemia, elevated triglyceride levels and buildup of fat stores (21). However, in contrast to obese organ donors, many hibernating animals have evolved physiologic adaptations that limit damage to their organs during hibernation. These adaptations—which include reductions in both immune activity and cell death—allow these animals to withstand levels of hypoxia and hypothermia that can be just as severe as cold-storage for deceased donor organs.

When animals, such as ground squirrels and bats, enter hibernation their organs are subjected to hypoxia and hypothermia. Nevertheless, they can withstand the IRI that occurs as they wake. The primary cell type involved in tissue destruction in IRI are neutrophils (22). It is therefore notable that hibernation triggers a stark ~90% reduction in the number of circulating leukocytes, with a significant decrease in mature neutrophils (23, 24). While this leaves the animals more susceptible to infection, It is believed that this trade-off ensures their organs can better withstand the IRI that results from the repeating cycles of torpor and arousal that occur during hibernation (23). While the mechanisms that drive IRI are complex and multi-factorial, it appears that preventing neutrophil induced tissue destruction may play a pivotal role in allowing hibernators to avoid organ damage despite their obese state.

Down regulation of cell death pathways—in particular ferroptosis (an iron-dependent form of cell death)—has also been shown to be a key adaptation of hibernating animals for resilience to cold hypoxia. Renal cells of hibernating animals are less susceptible to ferroptosis when compared to the same cells derived from human or rat tissues. However, when non-hibernating cells were given ferrostatin, an inhibitor of ferroptosis, cell survival was similar to the hibernating cell lines (25). In the 1970s, when UW solution was first created, our understanding of cell death was limited, but there are now many described forms of regulated cell death (26). Some regulated cell death modes are known to be immune-stimulatory such as pyroptosis and immunogenic cell death (26). Developing therapeutic methods to regulated cell death pathways may be crucial to improving the resilience of organs from obese donors.

The preceding examples demonstrate that hibernators adapt to increase resilience prior to entering hibernation. The key question then is what serves as the trigger for these adaptations? Emerging evidence suggest that the switch from the overfed state to a fasting state that occurs in parallel with the beginning of hibernation facilitates this process (27). Hindle and colleagues found that most of the differences in the liver proteome that distinguished active vs hibernating animals was similar to the signatures observed in fed versus fasted animals. The switch to the fasted state that occurs with beginning of the hibernation period is believed to prime these animals with the capacity to withstand the stress of IRI and support the metabolic reactivation during period of arousal (27). Fasting has also been shown to make organs of non-hibernating animals more resilient to cold ischemic injury (28).

FASTING AS A PATHWAY FORWARD

In animal models of stroke and myocardial infarction, fasting has been shown to protect against damage associated with IRI (29, 30). The improvement in outcomes from these preliminary studies led to the evaluation of whether other organ systems could also gain new resilience against ischemia from fasting. Mitchell et al. evaluated warm IRI in both kidney and liver in fasted and fed mice (31). In kidney models, mice that were fasted showed improved survival outcomes and decreased levels of acute tubular necrosis, serum urea and serum creatine. Similar results were observed in liver models where fasted mice had lower levels of ALAT and hemorrhagic necrosis (31). The benefits of fasting have also been demonstrated in animal transplant models (28, 32, 33).

The benefits of fasting in transplant were incidentally discovered by Southard and Belzer in 1993 (28). They initially thought fasting would mimic conditions of donors with extended ICU stays prior to donation and lead to adverse outcomes. However, rats fasted for 4 days had markedly improved survival compared to fed rats after both warm and cold ischemia insults (28). After 60 minutes of warm ischemia none (0/8) of the rats that received livers from fed donor survived, while 89% (8/9) of the rats that received livers from fasted rats survived. After 44 hours of cold ischemia, only 29% (2/7) of rats that received livers from fed rats survived, while 83% (5/6) of rats that received livers from fasted rats survived. These impressive survival benefits have been

confirmed in additional follow up studies (32, 33). While the benefits of fasting have been shown in animal models, fasting is not a feasible intervention in human organ donors. Therefore, if we wish to modulate these pathways to improve preservation of organs from obese donors, we need to understand what pathways to modulate pharmacologically that will enable us to target these pathways prior to or during cold storage.

Studies to understand the potential mechanisms for the survival benefits of fasting have found similar mechanisms to hibernators: reductions in both inflammation as well as cell death. For example, fasted rats given intraperitoneal injections of zymosan—a glucan that produces sterile inflammation—had lower levels of TNF-α compared to fed controls (32). It is postulated that the decreased production of inflammatory factors from donors leads to a lesser immune response in recipients and as a result, less injury. Sun et al. demonstrated that fasting also decreases apoptosis in rat donor livers. Apoptosis was the same at 24 hours of cold storage in both fasted and fed rats, but after 6 hours of reperfusion there were significantly more dead sinusoidal endothelial cells in the fed group than in the fasted group (33). The difference in post reperfusion cell death demonstrates that there may be underlying metabolic changes that make fasted tissues more resilient to IRI. This suggests that intervening on these pathways could provide an avenue to improving obese donor organs' resilience to the stresses of cold storage. This is one of many strategies that may work to improve the current practice of cold preservation.

IMPROVING CURRENT COLD STORAGE TECHNIQUES

It appears fasting in both hibernating and non-hibernating animals reduces inflammation and limits cell death during reperfusion injury. These are the exact vulnerabilities that obesity appears to exacerbate. The stresses of cold storage can potentially be mitigated by adding therapeutic these specific to target these specific pathways in cold storage solutions or altering the storage conditions themselves.

Ketone bodies, which are a naturally produced from breakdown of fatty acids in a fasted state. They not only function as a fuel source for cells, but also act as signaling molecules (18). In particular, β-hydroxybutyrate, which is one of three endogenously produced ketones, has been shown to be protective against ischemia-reperfusion injury in a mouse model *in vivo* and human cells *in vitro* (34). TUNEL positive cells were decreased by inducing endogenous production of β-hydroxybutyrate *via* fasting or by administration of exogenous β-hydroxybutyrate. This effect is believed to be mediated through anti-pyroptotic effects by inducing FOXO3, an up-stream transcription factor for pyroptosis.

One strategy that may be of interest is intervention in the peroxisome proliferator-activated receptor (PPAR) pathway. Obesity is associated with the development of metabolic syndrome which is a cluster of conditions including increased fat around the waist, elevated cholesterol, elevated triglyceride and increased risk of heart disease and type 2 diabetes. PPAR agonists are medications which are currently used to treat elevated triglycerides or blood glucose but have been shown to be effective in managing metabolic syndrome (35). Interestingly, PPARs are upregulated in fasting and play an important role in metabolic regulation (36). Not only are PPARs directly involved in metabolic syndrome and fasting but activation of PPARα has been shown to play a critical role in decreasing apoptosis and inflammation during renal IRI in a mouse model (37). Pre-treatment of mice with a PPARα activator, docosahexaenoic acid, significantly decreased the apoptotic and inflammatory responses compared to untreated wild type mice while PPARα knockout mice had increased apoptosis and inflammation (37).

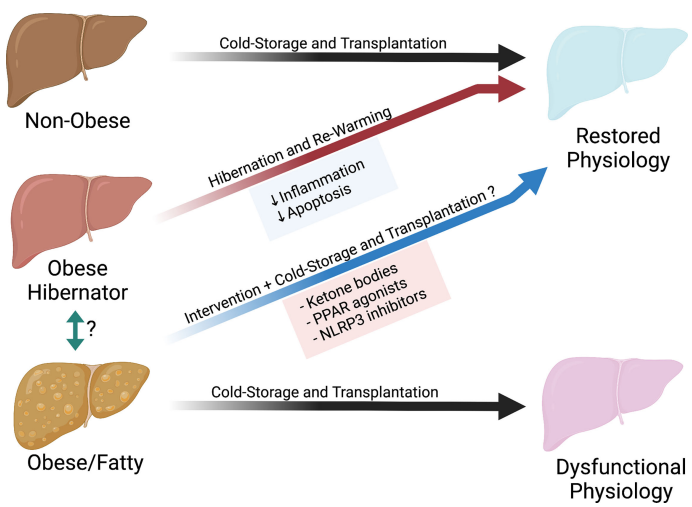

FIGURE 2 | Potential pathway to restore organs from obese donors.

Another important pathway to consider is the NLRP3 Inflammasome. The NLRP3 inflammasome is a multi-protein complex consisting of NLRP3 (sensor), ASC (adaptor), and Caspase-1 (effector). It is activated by a wide range of stimulus and is associated with sterile inflammation in obesity (38). It then causes the release of IL-1β, IL-18 and leads to pyroptotic cell death (39). In particular, NLRP3 has been shown to be important in ischemia reperfusion of the brain and heart in animal models (40). Using a model of *ex-vivo* ischemia reperfusion demonstrated that using INF4E, a NLRP3 inflammasome inhibitor, reduced infarct size, lactate dehydrogenase release and improved left ventricular pressure. Interestingly, fasting decreases NLRP3 inflammasome in humans and is believed to be due to SIRT3- mediated activation of superoxide dismutase 2 (41). By targeting the obesity-induced inflammation and its consequences we could potentially target several pathways to improve outcomes from obese donor organs.

CONCLUSIONS

Our understanding and applied practices of cold storage has been based on ideal organs from a healthy population. However, as obesity rates increase in the population, it is of paramount importance to understand the effects of cold storage on obese organs (**Figure 2**). We can build on the previous work of Belzer, Southard and others by incorporating different fields into transplant science, such as hibernating animal models or the nutritional science of fasting. This will allow us to expand our understanding on the modes of failure during cold storage. We can then implement new therapeutics and new technologies such as machine perfusion to treat and rehabilitate these organs. This has the potential to both improve outcomes and increase the number of transplantable organs for patients on the waitlist.

AUTHOR CONTRIBUTIONS

Concept and Design: JL and GT. Writing: JL. Critical Revisions: JD, ND, GC, DS, XO, WM, and GT. All authors contributed to the article and approved the submitted version.

FUNDING

Supported by the National Institutes of Health Grant T32GM086287 and R01DK124420.

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Conflict of Interest: GT is the co-founder of Revalia Bio.

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REVIEW
published: 29 April 2022
doi: 10.3389/fimmu.2022.848352

The Effect of Hypothermic Machine Perfusion to Ameliorate Ischemia-Reperfusion Injury in Donor Organs

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Specialty section:
This article was submitted to Alloimmunity and Transplantation, a section of the journal Frontiers in Immunology

Received: *04 January 2022*
Accepted: *04 April 2022*
Published: *29 April 2022*

Citation:
Knijff LWD, van Kooten C and Ploeg RJ (2022) The Effect of Hypothermic Machine Perfusion to Ameliorate Ischemia-Reperfusion Injury in Donor Organs. Front. Immunol. 13:848352. doi: 10.3389/fimmu.2022.848352

Hypothermic machine perfusion (HMP) has become the new gold standard in clinical donor kidney preservation and a promising novel strategy in higher risk donor livers in several countries. As shown by meta-analysis for the kidney, HMP decreases the risk of delayed graft function (DGF) and improves graft survival. For the liver, HMP immediately prior to transplantation may reduce the chance of early allograft dysfunction (EAD) and reduce ischemic sequelae in the biliary tract. Ischemia-reperfusion injury (IRI), unavoidable during transplantation, can lead to massive cell death and is one of the main causes for DGF, EAD or longer term impact. Molecular mechanisms that are affected in IRI include levels of hypoxia inducible factor (HIF), induction of cell death, endothelial dysfunction and immune responses. In this review we have summarized and discussed mechanisms on how HMP can ameliorate IRI. Better insight into how HMP influences IRI in kidney and liver transplantation may lead to new therapies and improved transplant outcomes.

Keywords: hypothermic machine perfusion, ischemia-reperfusion injury, HIF, cell death, endothelial dysfunction, immune response

INTRODUCTION

Several countries use hypothermic machine perfusion (HMP) as standard preservation method for kidney transplantation instead of storing the organ on ice. It has been shown by several clinical studies that HMP is superior to static cold storage (SCS), but the underlying mechanisms are still unknown. Ischemia-reperfusion injury (IRI) is unavoidable during transplantation and is one of the main causes for delayed graft function (DGF) (1). Since HMP is known to affect DGF, it is possible that this positive effect is due to its influence on IRI. To gain a better insight into the effect of HMP on ameliorating IRI. In this review we evaluated the effect of HMP on four main aspects that are involved in the pathophysiology of IRI to gain a better insight into the effect of HMP on ameliorating IRI. This was done by analysing studies comparing static cold storage with HMP.

HISTORY OF MACHINE PERFUSION

Since the early beginning of solid organ transplantation, cold preservation has been the gold standard in kidney preservation. Lowering the temperature of the donor organ below 10°C decreases metabolism by approximately 90%, allowing maintenance of donor organ viability and safe preservation. Initially kidneys were cold perfused on a machine, but when better organ preservation solutions were developed, simple SCS in a box with melting ice became the standard in organ preservation due to its simple and effective way to transport the graft. In the past decades, due to the persistent donor kidney shortage, most centres have increasingly been accepting older and higher risk donor kidneys. These kidneys are from donation after circulatory death (DCD) donors or from donors with increased co-morbidity, i.e. hypertension, diabetes or atherosclerotic disease (2). This change of practice often resulted in compromised function and lower graft survival. Also, it became clear that the conventional method of SCS did not suffice for this type of donor organs and improved strategies in preservation appeared to be necessary. This insight resulted in a revisit of continuous machine perfusion, now using novel technologies in medical engineering including oxygenation, both hypo- and normothermic temperatures and modifications of perfusion solutions. Following initial clinical trials demonstrating better outcomes for higher risk donor kidneys (3), several countries have now implemented HMP as the preferred preservation method. In 2009, the first study was published reporting on the results of an international randomized controlled trial with a paired kidney design. One kidney was preserved with HMP whilst for the contralateral kidney SCS was used. In this trial, the method of HMP overall reduced DGF from 26.5% to 20.8% and increased 1 year graft survival from 90% to 94% (4). When analyzing the different subgroups of donor types (ECD, donation after brain death (DBD), DCD), HMP remained superior in decreasing DGF. Subsequent clinical studies and registry reports have been performed and confirmed the beneficial effect of HMP over SCS. Meta-analysis showed that HMP reduced DGF [relative risk (RR) 0.81 with 0.71-0.92 95% confidence interval (CI)] in all deceased donor types in kidney transplantation (DBD fixed-effects analysis with RR 0.84 with 0.69-1.03 95% CI and DCD random-effects analysis with RR of 0.80 with 0.62-1.04 95% CI) (5). A more recent meta-analysis showed a reduced risk of delayed graft function when kidneys were preserved with HMP versus SCS (RR 0.77, 95% CI 0.67-0.90) as well as a trend towards improved graft survival (6). HMP was also shown to be cost effective, because lower DGF and graft failure rates decreased the need for a return to (chronic) dialysis. Beneficial effects of HMP were also shown in a meta-analysis for the liver, by a lower incidence of early allograft dysfunction (EAD), less biliary complications and ischemic cholangiopathy, as well as lower aspartate aminotransferase levels (7). Although short term outcomes in kidney transplantation have improved over the last decades, long term outcomes are still moderate with 70% of DBD and DCD grafts failing between 5 and 10 years posttransplant (8). Two important factors that influence long term outcome in kidney transplantation are the quality of the graft and the immunosuppression administered (9). HMP may potentially alter immunogenicity of the donor organ, thereby improving the quality of the graft. Also, HMP offers a great platform for therapeutic options to potentially reduce the amount of immune suppressive drugs.

ISCHEMIA-REPERFUSION INJURY

Ischemia followed by reperfusion is inevitable in the context of organ transplantation. The temporary cut-off of the donor organ from the blood supply at the time of procurement until the recipient operation will cause hypoxia, leading to inhibition of the electron transport chain and subsequent lower ATP production. Lower ATP levels will cause a shift toward anaerobic metabolism with dysfunction of sodium-potassium, calcium and sodium-hydrogen pumps. This will result in an imbalance in cellular osmolarity, with as a consequence cell swelling. Anaerobic metabolism also leads to metabolic acidosis from the increased lactic acid levels; decrease of antioxidative agents; and detachment of ribosomes resulting in less protein synthesis. During reperfusion of the graft, there will be a second wave of injury, further damaging the graft, including the release of reactive oxygen species (ROS). The lower levels of antioxidants are unable to neutralize the ROS, leading to cell death. Subsequently, the innate immune system is activated by the profound release of damage-associated molecular patterns (DAMPs) from dying cells. A more detailed review about IRI can be found elsewhere (10). In this review IRI is divided into three main phases: hypoxia, reoxygenation and reperfusion. Although all three phases can be characterized by distinct molecular mechanisms, they are also closely interrelated (**Figure 1**).

INFLUENCE ON HYPOXIA INDUCIBLE FACTOR LEVELS

The first phase of ischemia-reperfusion injury is hypoxia. When oxygen levels are normal, hypoxia inducible factor (HIF) α proteins (HIF-1α, HIF-2α) are rapidly produced, but also degraded. Prolyl hydroxylases (PHD) enzymes use oxygen as a cofactor to mark HIF - α proteins for degradation by the proteasome (**Figure 2**). During hypoxia, PHD enzymes are no longer able to trigger the break down, resulting in increasing HIF-α levels. HIF-α translocates to the nucleus where it binds to the HIF-1β subunit. Together the HIF protein binds to HIF response elements on the DNA, thereby influencing many genes that regulate angiogenesis, metabolism, cell growth and survival (11, 12).

Most studies reported lower levels of HIF proteins (HIF-1α, HIF-2α, HIF-3α and HIF-1β) in HMP-stored donor organs compared with SCS (13–18). Besides lower levels of HIF proteins, improvements on transplant outcome (less damage, better kidney function) were also observed when HMP was applied. However, there is a lot of variability between these studies. Studies were performed with canine, porcine or human organs with HMP duration varying between 3 and 24h. Organs

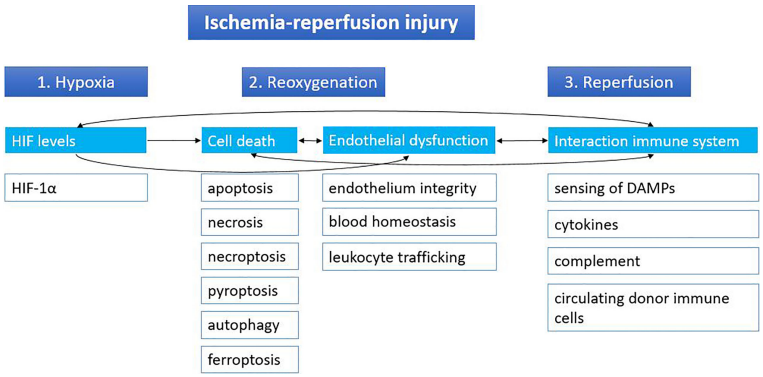

FIGURE 1 | Stages of ischemia-reperfusion injury. Ischemia-reperfusion injury can be divided into three stages based on distinct molecular mechanisms: hypoxia, reoxygenation and reperfusion. Cut-off of the donor organ leads to hypoxia, which leads to changes in HIF levels. During reoxygenation, reactive oxygen species lead to massive cell death and endothelial dysfunction. When the graft is reperfused in the recipient, the immune system of the recipient can react to the graft and its immune cells and vice versa. Arrows indicate the relations between the different mechanisms. DAMPs, damage-associated molecular patterns; HIF, hypoxia inducible factor.

that were subjected to HMP or SCS were liver, limb or kidney. Also, two studies were found where higher levels of HIF-1α and HIF-2α (and downstream factor vascular endothelial growth factor A) were found in HMP perfused human livers. Burlage et al. (19) showed in a human liver model better endothelial cell function with increased HIF-1α levels as measured by Krüppel-like factor 2 (Klf2), endothelial nitric oxide synthase (eNOS), nitric oxide (NO) and thrombomodulin (19). Ito et al. showed in a human liver model that HIF-1α expression increased after HMP (20). They also analysed HIF-1α levels in clinical liver patients and found that higher levels were associated with significantly better graft survival. Unfortunately, to date only an abstract is available of this study, so details about this study are lacking. HIF upregulation that occurs during hypoxia can be explained as a protective mechanism to better handle the ischemic injury (20–23). Studies reporting lower levels of HIF proteins focus more on the hypoxia part, suggesting that lower levels of HIF mean less hypoxia. The two studies reporting higher HIF levels can be compared at best with the studies of Guarrera et al. (17) and Henry et al. (18) that also use a human liver model. Unfortunately, the first study only provides an abstract and does not include the duration of HMP, making it difficult to compare with other studies. The study of Henry et al. provided both clinical outcomes (beneficial for HMP with a shorter hospital stay and a trend towards less EAD and analysis of biopsies and serum (less oxidative stress, inflammatory markers, adhesion molecules and cellular infiltration in the HMP group). Taking along the previously mentioned studies that reported lower levels

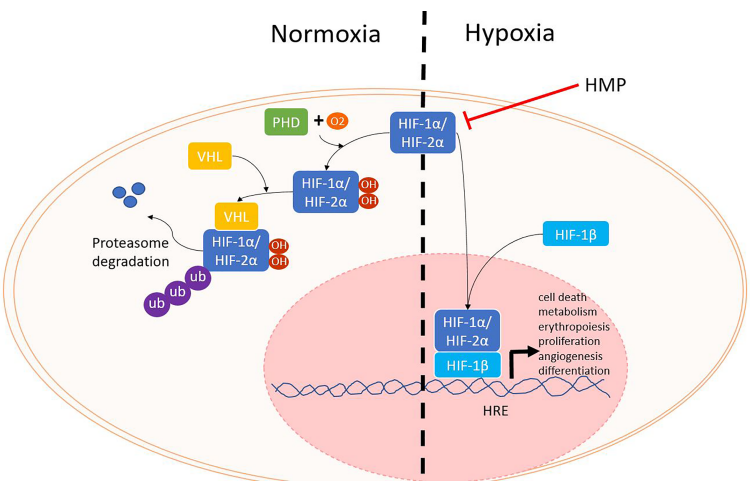

FIGURE 2 | HIF proteins during normoxia and hypoxia. When oxygen levels are sufficient, PHD enzymes use oxygen as a cofactor to hydroxylate HIF-α. Next, VHL will mark HIF-α for degradation by the proteasome. During hypoxia, HIF-α is not degraded and can translocate with HIF-1β to the nucleus where it can bind to HRE and lead to the activation of several different genes. HIF, hypoxia inducible factor; HRE, HIF response element; PHD, prolyl hydroxylase; VHL, von Hippel Lindau.

of HIF, it is likely that HMP reduces the level of HIF proteins despite the great heterogeneity between studies. Nevertheless, these results illustrate the complexity of interpreting data on HIF levels, next to the different molecular consequences of HIF.

As mentioned before, HIF can regulate expression levels of many different genes. Those gene products can have contradictory effects, making it difficult to predict the effect in changing HIF levels. For example, HIF activation can lead to upregulation of both pro- and anti-apoptotic genes. A study by Ravall et al. showed that in a renal cell carcinoma cell line there were opposing effects of HIF-1α and HIF-2α (24). When HIF-1α was expressed, HIF-2α was suppressed and tumour growth was inhibited. When HIF-2α was expressed, HIF-1α was suppressed and tumour growth was increased.. HIF-1α regulates many glycolytic enzymes and is expressed in many different tissues whereas HIF-2α regulates more broadly hypoxia inducible genes like cyclin D, transforming growth factor (TGF)-α and matrix metalloprotease 2 and its expression is more regulated (25, 26). The HIF-3α unit is less studied, but it is thought to inhibit HIF-1α and HIF-2α (26). The expression of the different HIF proteins can vary between different tissues, which could result in different hypoxia responses. In the kidney HIF-1α is expressed in the tubular epithelium, while HIF-2α is expressed in glomerular cells and peritubular interstitial cells (27, 28). HIF-2 has been associated with regulating erythropoietin synthesis (29). Kapitsinou et al. showed in a mouse model that HIF-2α endothelial inactivation resulted in increased expression of cellular infiltration and renal injury markers (27). This was associated with elevated *Vcam1* expression. HIF-2 appears to protect against IRI and could therefore be a potential therapeutic target.

As stated earlier, the different phases of IRI are overlapping. HIF activation has been linked to increased thrombotic factors (30), fibrosis (31) and activating the innate immune cells (32). A rat study showed the effects of changing HIF levels on cell death and inflammation in a hepatic IRI model (33). Overexpression of HIF-1α lead to a protective effect by reducing necrosis, apoptosis, neutrophil infiltration and inflammatory cytokines IL-6 and TNF-α. Inhibition of HIF-1α had the opposite effect and aggravated the IRI injury. These results show the diversity of effects of changing HIF levels, where the protective effect was seen with increasing levels, whereas HMP mainly showed that a decrease lead to better outcomes. Future studies with a paired kidney design, where one kidney is put on HMP while the other is preserved SCS, both with similar cold ischemia times, could provide better insight into changing HIF levels.

INFLUENCE ON CELL DEATH

During the second phase of IRI the reoxygenation leads to the release of ROS, leading to massive cell death. There are different forms of cell death (i.e. necroptosis, pyroptosis, autophagy), but all result in the release of DAMPs that can activate an immune response. However, different forms of cell death could still lead to different DAMPs being released, leading to a different immune response. Apoptosis is generally considered not to activate the immune system. Macrophages that engulf apoptotic cells are stimulated to secrete anti-inflammatory TGF-β and IL-10 (34). Nevertheless, DAMPs can still be released during this form of regulated cell death, as macrophages can also be stimulated to secrete high mobility group box 1 (HMGB1), a well-known DAMP (35, 36). Also, the release during apoptosis of oxidized mitochondrial DNA has been shown to initiate an immune response by activating the nucleotide binding domain and leucine rich repeat (NLR) pyrin domain containing 3 (NLRP3) (37). Necrosis - on the other hand - is also a form of cell death where many DAMPs are released. Necrosis is characterized by organ swelling and membrane rupture, leading to release of the cellular content (10). When comparing the effects of HMP with SCS, the amount of cell death by apoptosis and necrosis was reduced in HMP (**Figure 3**) (38–45). Less cell death most likely translates to less release of DAMPs, leading to less activation of the immune system. Therefore this could contribute to better outcomes with hypothermic machine perfused organs versus static cold stored ones. The ratio of anti-apoptotic Bcl2/pro-apoptotic Bax increased when organs were perfused with HMP versus SCS (41, 42). A higher Bcl2/Bax ratio will prevent the occurrence of apoptosis (46). Apoptosis is initiated from an extrinsic pathway that activates caspase 8 and 10 or *via* an intrinsic pathway activating caspase 9. Once activated, the cascade of caspases leads to a regulated dismantling of the cell. Caspases also play a role in inflammation by recognizing bacterial products such as lipopolysaccharide, leading to pyroptosis and activating pro-inflammatory cytokines by cleavage. It has been suggested that caspase 4 and 5 can directly recognize lipopolysaccharide in the cytosol (47). Caspase 3 has been found to be downregulated after reperfusion while caspase 12 was found upregulated when organs were perfused with HMP versus SCS (18, 38–40, 42, 48–51). Caspase 3 is an essential effector caspase that plays a role in both the intrinsic and extrinsic pathway. Caspase 12, on the contrary, is known as a negative regulator of inflammation by inhibition of caspase 1, which is responsible for cleavage of pro-IL-1β and IL-18 to their active form (47, 52).

Most research about the effect of HMP on cell death is focused on apoptosis and necrosis. Other forms of cell death such as necroptosis, pyroptosis or autophagy have not yet been studied extensively, although it is known that different forms of cell death may activate different immune pathways. Necroptosis has been associated with IRI in liver transplantation (53), ischemic brain injury (54) and renal IRI (55, 56). A study performed in rabbit kidneys showed that the expression of receptor-interacting protein kinase 3 (RIPK3) was significantly lower in the HMP group versus the SCS group both at the protein and mRNA level (51). The RIPK1-RIPK3 complex is an important regulator of necroptosis by forming the necroptosis-inducing complex called the necrosome (57).

The influence of HMP on pyroptosis has been investigated in liver perfusion in a rat study (58). The investigators studied the effect of hypothermic oxygenated machine perfusion (HOPE) following a period of SCS on the inflammasome pathway that plays a role in pyroptosis. The HOPE group showed less pyroptosis, likely by blocking the interaction between

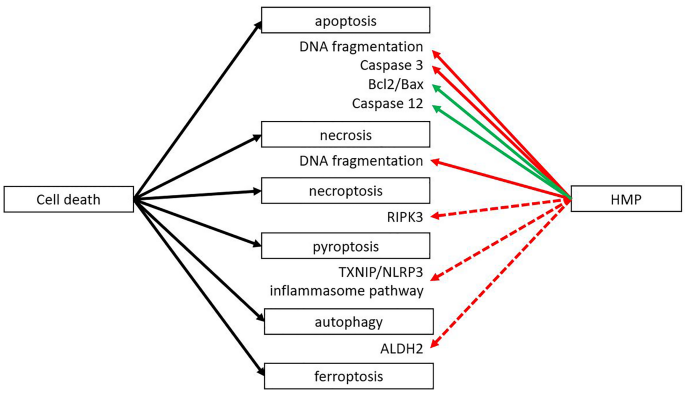

FIGURE 3 | HMP reduces several forms of cell death. Different forms of cell death can occur after ischemia-reperfusion injury. HMP has been found to have an inhibiting effect on apoptosis and necrosis at the end of machine perfusion and after reperfusion in a NMP setting or autotransplantation (red line). The inhibiting effect on apoptosis is *via* inhibition of pro-apoptotic processes and increase of anti-apoptotic factors (green line). There are studies suggesting HMP could also have an inhibiting effect on necroptosis, pyroptosis and autophagy, but more research is needed (red dotted line). The effect of HMP on ferroptosis is yet unclear. ALDH2, aldehyde dehydrogenase; RIPK, receptor-interacting protein kinase; TXNIP/NLRP3, thioredoxin-interacting protein/NOD-like receptor protein.

thioredoxin-interacting protein and NLRP3. The role of decreasing pyroptosis on transplant outcome was studied by Noda et al. in lung perfusion. They showed that lung perfusion of rat heart-lung blocks showed significantly better lung function and lower IL-6 levels when perfused with a leukocyte filter (59). Leukocytes trapped in the filter were analysed for cell death and they found that 26% of the cells were pyroptotic (caspase 1 positive, 7AAD negative), 16% apoptotic (Annexin V positive, 7AAD negative) and 40% necrotic (Annexin V positive, 7AAD positive). Administering a caspase-1 inhibitor during perfusion to inhibit pyroptosis showed better lung function and lower mRNA levels of proinflammatory cytokines IL-6, TNF-α and IL-1β, comparable with perfusion with a leukocyte filter. A limitation of this study is that only mRNA levels of cytokines were measured instead of protein levels. Caspase 1 plays an important role in activation and secretion of IL-1β and has also been linked to TNF-α and IL-6 secretion from macrophages (60).Therefore, it may be of interest to study whether the effects of blocking pyroptosis are similar on the protein level as found to be on the mRNA level.

The role of autophagy during IRI has also been studied in various organs (61–63). According to van Erp et al. the degree of autophagy in the donor can also be influenced by age and gender (64). During the first ischemic phase, it is proposed that autophagy acts as a protection mechanism whereas during the reperfusion stage excessive autophagy results in cell death (65). A study in rabbits showed higher levels of phosphorylated aldehyde dehydrogenase 2 (ALDH2) in HMP-perfused kidneys versus SCS (63). ALDH2 influences autophagy *via* expression of 4-HNE that regulates the Akt/mTOR autophagy pathway, suggesting that under HMP conditions, autophagy increases when compared to SCS. Administering an ALDH2 agonist to enhance autophagy resulted in (i) better kidney function as shown by lower serum creatinine, (ii) lower oxidative stress levels measured by malonaldehyde and superoxide dismutase 2, and (iii) better inflammatory profile as demonstrated by lower levels of TNF-α, IL-6 and higher levels of IL-10. Administering an antagonist, to decrease autophagy, lead to the opposite results. A study by Zeng et al. looked into the role of HOPE in upregulating autophagy to alleviate liver IRI in a rat model (44). HOPE increased expression of autophagy-related proteins and was associated with better liver function as measured by alanine transaminase, aspartate transaminase and lactate dehydrogenase compared with SCS. Administration of the autophagy inhibitor 3-MA attenuated the protective effect. Perfusion with 100% nitrogen showed similar results as under SCS conditions, indicating that the effect of HOPE treatment was not due to a washout during perfusion. It also demonstrated that a minimal oxygen level is required for the protective effect of HMP. According to Boteon et al. autophagy is important for removal of harmful substances and providing energy during cell stress (66). Normothermic machine perfusion (NMP) has been suggested to increase autophagy by maintaining normal calcium levels and by providing shear stress. Although more research is needed to evaluate the effect of HMP on autophagy, it is likely similar to NMP. During HMP the perfusate contains calcium and there are also low levels of shear stress detectable.

Another form of cell death is ferroptosis, which is iron-dependent. Excessive amounts of iron can lead to the generation of ROS through the Fenton reaction (67). Several studies showed that ferroptosis could play a role in IRI in the liver and kidney (68–70). Ferroptosis was also shown in several cell lines exposed to continuous cold stress, therefore it could be interesting to look into the role of ferroptosis during machine perfusion at subnormothermic (25-35°C) or normothermic (37°C) temperatures as well (71). Currently, no studies have looked into the role of HMP on ferroptosis.

Most studies use a TUNEL (Terminal deoxynucleotidyl transferase dUTP nick end labelling) assay to measure apoptosis *via* detection of DNA fragmentation. However, this assay can also detect necrosis, pyroptosis and possibly other forms of cell death

(72–74). Knowing which forms of cell death are affected and which are not, is also important to identify specific targets to reduce IRI. A combination of multiple forms of cell death could also be possible. Recently, a new protein complex was identified as the PANoptosome (75). It drives the three main forms of programmed cell death, namely pyroptosis, apoptosis and necroptosis. The PANoptosome contains RIPK1, caspase 8, NLRP3 and apoptosis-associated speck-like protein (ASC) containing a caspase recruitment domain. Therefore, it contains molecules that are critical for programmed cell death. The different pathways can be activated together or separately and crosstalk between pathways occurs (75, 76).

In conclusion, it appears that HMP reduces the amount of cell death by decreasing apoptosis and necrosis. It is possible that HMP also reduces other forms of cell death like necroptosis, autophagy, pyroptosis and ferroptosis, but so far not many studies have looked into this. The effect of HMP on cell death will likely affect other mechanisms as well. Cell death plays an important role in activating the innate immune system *via* the release of DAMPs and caspases are critical in cleaving several cytokines into their active form. If cell death occurs in endothelial cells, this will disrupt the glycocalyx.

INFLUENCE ON ENDOTHELIAL DYSFUNCTION

The endothelium plays a major role in inflammation with leukocyte adhesion and vascular health. Multiple studies have shown that IRI leads to the upregulation of adhesion molecules like E-cadherin and intercellular adhesion molecule 1 (ICAM-1) that enable leukocyte adhesion and neutrophil infiltration (77–79). Following HMP and reperfusion, many studies showed better endothelial function as measured by less transmigration of immune cells (**Figure 4**). In addition, less fibrosis after kidney preservation was measured by Sirius Red staining as well as by lower levels of pro-fibrotic transforming growth factor β (39, 80–82). A porcine model was used to study the effects of HMP on fibrosis, with the exception of Liu et al. (82) who used a rabbit model. Fibrosis is significantly associated with chronic graft dysfunction (11). Endothelial dysfunction has been mentioned as an important initiator and maintainer of fibrosis (83, 84). Therefore, we suggest that the level of fibrosis may indicate endothelial dysfunction. P-selectin is important for the rolling of leukocytes to ultimately invade the tissue and ICAM-1 is important for adhesion of leukocytes to endothelial cells. Lower expression of P-selectin and ICAM-1 were observed when HMP was applied versus SCS (13, 17, 18, 43, 49, 85). Reduced leukocyte rolling and adhesion with HMP was shown in liver models in rat (49), mouse (43), dog (13) and human (17, 18, 85). Most studies showed lower expression of P-selectin and ICAM-1 after perfusion (13, 17, 18, 85) or reperfusion (13, 43, 49), while Henry et al. (18) also showed a decrease in P-selectin levels after transplantation. Less cellular infiltration was also shown by a reduction in invading neutrophils and monocytes as measured by myeloperoxidase, CD68, Ly6G and reduced levels of monocyte chemoattractant protein-1 (18, 43, 80, 86, 87). A reduction in cellular infiltration was seen across various organisms (mouse, rabbit, human, pig) after perfusion, in reperfusion models and after transplantation. This was supported by a decrease in chemokines CXCL14 and IL-8 as shown after perfusion in a canine or human liver (13, 18, 85). Henry et al. (18) showed both after perfusion and transplantation an downregulation of CXCL14 and IL-8. Endothelial-to-mesenchymal transition (EMT) was observed less frequently as measured by lower levels of EMT marker vimentin after perfusion of porcine kidneys (80, 81). EMT is an important factor that contributes to fibrosis and chronic graft failure (88). A downregulation of the thrombotic von Willebrand-factor was also observed after reperfusion in liver transplantation in mice or pigs (44, 45). The vascular tone of the endothelium is important for sufficient flow. Vasodilation can be regulated by eNOS phosphorylation, leading to an increase in NO levels, which were found to be upregulated after reperfusion of HMP-treated organs (19, 89–91). This was true for both kidneys and livers and in different organisms (pig, rat, human). For the porcine kidney transplantation, higher levels of eNOS were also found after transplantation (90). In conclusion, a beneficial effect of HMP on

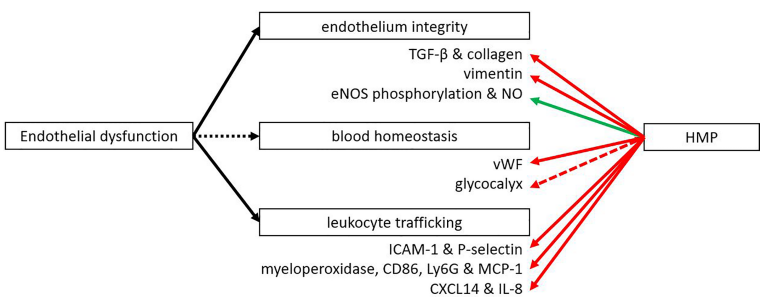

FIGURE 4 | HMP influences several functions of the endothelium. After HMP or reperfusion (NMP or autotransplantation) there is less leukocyte trafficking compared to SCS treated organs (red line). Also less thrombotic factors, fibrosis and EMT were observed. However, the vascular tone was improved when organs were treated with HMP instead of SCS (green line). More studies are needed to confirm the effect of HMP on the glycocalyx (red dotted line). eNOS, endothelial nitric oxide synthase; ICAM, intercellular adhesion molecule; Ly6G, lymphocyte antigen 6 complex locus G6D; MCP-1, monocyte chemoattractant protein-1; NO, nitric oxide; TGF-β, transforming growth factor β; vWF, von Willebrand factor.

endothelial integrity and function has been demonstrated at different levels.

Many of these beneficial effects on the endothelium are most likely caused by the activation of Klf2, which gets activated upon flow-mediated shear stress. Besides regulation by flow, Klf2 can also be negatively regulated by proinflammatory cytokines such as TNF-α and H2O2 resulting from oxidative stress (92). Klf2 can regulate many different processes in the endothelium like angiogenesis, vascular tone, thrombosis, inflammation, immune regulation and oxidative stress. It is known to downregulate HIF-1α, vascular endothelial growth factor, endothelin-1, vascular cell adhesion molecule and E-selectin, while thrombomodulin and eNOS are some of the upregulated markers (92, 93). It has already been shown by multiple studies that Klf2 gets upregulated during HMP by the shear stress created from the pump (82, 94, 95). The upregulation of eNOS phosphorylation is important for NO levels. NO is an important vasodilator, allowing for sufficient blood flow through the organ. Besides regulating vascular tone, NO also plays a role in endothelial cell migration, proliferation, angiogenesis and it has anti-inflammatory properties by inhibiting leukocyte adherence (96).

When discussing the endothelium, another essential factor is the glycocalyx, a thin layer consisting of proteoglycans that covers the endothelium. This cover is important for leukocyte and platelet adhesion, coagulation and transferring shear stress to endothelial cells (97). The glycocalyx is also an important place where several enzymatic reactions take place due to the docking function of the glycosaminoglycans. Damage to the glycocalyx is a direct consequence of IRI, as demonstrated in several studies (98–100). A large part of the damage to the glycocalyx happens during the reperfusion phase (101). One study looked in more detail into the glycocalyx degradation during human liver transplantation and found that syndecan-1, a biomarker of glycocalyx degradation, was released during reperfusion (102). However, heparan-sulphate levels, another biomarker for glycocalyx degradation, were lower in effluent veins compared with portal venous blood, suggesting binding or uptake of heparan-sulphates. This uptake might suggest repair of the damaged graft. This is supported by a study in kidney transplantation, where the thickness of the glycocalyx increased in time after reperfusion (103). Therefore, it could be beneficial to perfuse donor grafts for a longer period of time to give the glycocalyx time to repair itself, although more research is needed to confirm this. One study in a porcine model of brain death showed that HMP could potentially be used as a platform to restore the glycocalyx by infusion of corline heparin conjugate, heparin molecules that strongly bind to tissue with heparin affinity. By labelling the heparin molecules, binding to the damaged endothelium could be demonstrated (104). Unfortunately, no results on function or outcome were reported.

All in all, there appear to be many functions of the endothelium that are influenced by HMP. Most studies focussed on the infiltration of immune cells and fibrosis and noticed a reduction when using HMP versus SCS. Endothelial dysfunction also affects other mechanisms of IRI. ICAM-1 has also been studied in cancer cells, where they found that an upregulation of ICAM-1 increased cell survival (105). For P-selectin it was found that inhibition of P-selectin reduced apoptosis in endotoxin-induced liver injury in a mouse model (106). Besides leukocyte recruitment, activation of the coagulation system can also lead to an immune response. Components of the clotting system like fibrin can enhance adherence of immune cells and facilitate migration (107). Cross-talk between the complement system and the coagulation system can further activate the immune system (108).

INFLUENCE ON INNATE IMMUNE RESPONSE

Both the innate and adaptive immune system play an important role in transplantation. An eligible donor-recipient match has to be found, immunosuppressive drugs have to be taken daily and there is the risk of allograft rejection of the donor organ. The innate immune system plays an important role in IRI. Toll like receptor 4 (TLR4), one of the best characterised TLRs, is able to sense DAMPs that are released during IRI. HMBG1 is one of the most described DAMPs and it has been found to play a role in IRI by activating TLR4 (109). HMGB1 normally resides within the nucleus, where it plays a role in transcription and chromatin modelling (110). It can be actively released by immune cells like dendritic cells or macrophages or passively released upon cell death. Extracellular HMGB1 can bind to TLR4, TLR2 or the receptor for advanced glycation end-products (RAGE) to promote inflammation (110, 111). Several TLRs have been found upregulated in ischemia, mostly TLR2 and TLR4. Activation of TLR4 leads to increased expression of proinflammatory cytokines and adhesion molecules, attraction of neutrophils and macrophages, and activation of circulating immune cells (11, 112–114). Several studies have shown that TLR4 expression strongly correlates with renal graft dysfunction in rats and that TLR4 knockout mice are protected against IRI (14). Both TLR4 and HMGB1 were found to be downregulated when comparing HMP versus SCS (**Figure 5**) (41, 44, 45, 51). Besides TLR4 and HMGB1, pro-inflammatory cytokines TNF-α, IL-1β, IL-6 and IL-2 were reported to be downregulated after HMP treatment (17, 18, 42, 43, 45, 51, 85, 86, 91). The possibility of further reducing pro-inflammatory cytokine levels during HMP was shown in a study that compared porcine kidneys in a reperfusion model with or without a cytokine filter (115). Kidneys that were perfused with a cytokine filter showed lower levels of IL-6 and IL-8 and higher blood flow. No effect on kidney function based on creatinine clearance was found, but it might be that more processes play a role in improving kidney function. For instance, a cytokine filter is non-specific which means that anti-inflammatory cytokines are removed as well. A shortcoming of most studies reporting on changes in the immune system after HMP is that many only look at pro-inflammatory cytokines while anti-inflammatory cytokines like IL-10 are not as often investigated.

Next to the release of DAMPs or inflammatory mediators, molecular alterations in the donor organ following IRI can also result in the activation of innate immunity *via* the complement system. The complement system can be activated *via* the

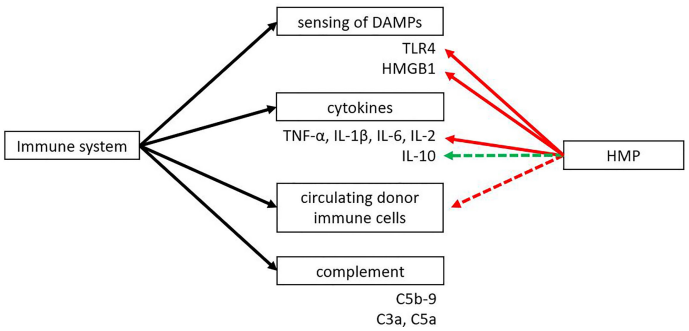

FIGURE 5 | The effect of HMP on dampening the immune response. HMP has shown to reduce sensing of DAMPs and pro-inflammatory cytokines compared to SCS (red line). Some studies suggest there might be an upregulation of anti-inflammatory cytokines and a reduction in circulating donor immune cells (green and red dotted line). The effect of HMP on the complement system is still unknown. DAMPs, damage-associated molecular patterns; HMGB1, high-mobility group box 1; TLR, Toll-like receptor.

classical, lectin or alternative pathway. Activation of the complement system by DAMPs released from IRI can lead to the cleavage of C3 and C5 and the formation of the membrane attack complex. Cleavage products C3a and C5a can act as anaphylatoxins to activate immune cells (116, 117). A complexity in complement research is that activation of the complement system can vary between organs. IRI in the liver leads to activation of the classical pathway, while IRI in the heart activates the classical and lectin pathway (117). Therefore, more studies should look into the effect of hypothermic machine perfusion on complement activation.

Probably due to the short time of perfusion and the cold temperature not many studies have looked into the effect of HMP on the adaptive immune system. Several studies have looked into the circulating cell types during machine perfusion and found large amounts of immune cells with flow cytometry (118, 119). Although the consequence of circulating immune cells during machine perfusion is not clear yet, it could have a beneficial effect by immunodepletion of the graft.

Just like the other mechanisms, immune activation also influences the previous discussed mechanisms involved in IRI. Jantsch et al. (120) showed that TLR activation on mouse dendritic cells can also lead to stabilization of HIF-1α under normoxic conditions. This increase in HIF-1α resulted in the transcription of inflammatory target genes *Ptgs2* and *Nos2*, whereas increase of HIF-1α by hypoxia lead to increased transcription of HIF-1α target genes *Glut1* and *Pgk1*. Besides regulation by PHD, pro-inflammatory cytokines have also been reported to regulate HIF-1α (121). TNF-α and IL-1β have been proposed to increase HIF-1α levels *via* various mechanisms at pre- and posttranscriptional levels. However, most information is obtained from cell studies, so further investigations in transplantation models are needed to confirm this. Immune activation can also lead to cell death and endothelial dysfunction. Activation of the complement system leading to the membrane attack complex can induce cell death *via* lysis (117). In an IRI mouse model it was also shown that activation of the terminal complement pathway lead to shedding of the glycocalyx, indicated by accumulation of glycocalyx components syndecan-1, hyaluronan and heparan sulphate (122).

INTERVENTIONS DURING HYPOTHERMIC MACHINE PERFUSION

Machine perfusion provides an ideal platform to use for interventions. Current interventions that are being exploited are RNA silencing, stem cell therapy and complement blockade (123). One study looked into the use of lentiviral vectors encoding short hairpin RNAs that target the β2-microglobulin of the major histocompatibility complex 1 during sub-normothermic *ex vivo* rat kidney perfusion (124). They found decreased transcription levels of β2-microglobulin and pro-inflammatory cytokine levels, while increased levels of anti-inflammatory cytokines were found. Genetic modification showed no additional cell death, showing feasibility of this technique.

Mesenchymal stromal cells (MSCs) are also an emerging topic in this field. Due to the immune modulating properties of MSCs both the innate and adaptive immune response could be controlled. MSCs could also be beneficial for tissue repair (125). The TRITON study was a single centre randomized prospective study where MSCs were infused 6 and 7 weeks after renal transplantation in combination with reduced immunosuppressive drugs. It showed that MSC therapy was safe and feasible and also showed higher numbers of regulatory T cells in peripheral blood (126). Due to the size of the MSCs they will get stuck in the lung capillaries when given to the recipient systemically *via* the blood stream. Therefore, *ex vivo* machine perfusion could be an attractive way to give MSC therapy. In a renal porcine autotransplantation model it was shown by the MePEP consortium that giving MSC therapy during NMP was safe and feasible and MSCs ended up in the renal cortex (127). However, in the short follow-up time of 14 days no beneficial effects on function could be shown despite prolonged warm and cold ischemia times. Thompson et al. (128) added multipotent adult progenitor cells added during NMP of discarded human

kidneys. They showed that this therapy may increase urine production and microvascular perfusion, upregulate anti-inflammatory cytokines and downregulate injury markers and pro-inflammatory cytokines (128).

Activation of the complement system already occurs early-on in deceased donors as shown by Damman et al., as elevated C5b-9 levels were found in the plasma of deceased donors (129). The elevated levels were also associated with biopsy-proven acute rejection. A phase 1 study where complement is inhibited using C1-INH is being conducted to try to decrease systemic inflammation and DGF incidence in expanded criteria donors as a donor pre-treatment strategy (NCT02435732). Complement inhibitor C1-INH is a serine protease that can regulate the classical, lectin and alternative pathway (130). Machine perfusion could provide an ideal environment to target the complement system specifically in the graft instead of the whole body. The EMPIRIKAL trial was the first study to look into the administration of complement inhibitor Mirococept at time of transplantation to prevent DGF (ISRCTN49958194) (131, 132). The inhibitor is given during a 15min flush of the kidney while the organ is on ice slush. Mirococept is designed to inhibit the complement system at the C3 level. Because the first dose of 10mg did not show a significant difference from the control, the study was stopped to conduct a dose study first. A dose finding study was initiated in normothermic machine perfused porcine kidneys showing that 80mg of Mirococept was the optimal dose (132). It showed to be safe as minimal washout into the circulation occurred and no detrimental effects on flow parameters or histology were observed, opening the path to further clinical development. In this study the inhibitor was not given during HMP, but during a short flush. It would be interesting to study if Mirococept, when given during HMP where would be circulating through the organ for several hours, could be administered in a lower dose.

NEW AREAS OF INTEREST

With HMP being implemented and clinically used in kidney and liver preservation in several countries, research into machine perfusion has emerged. Due to novel technology, nowadays also *ex-vivo* NMP has become feasible, although its clinical implementation is still in its infancy. The potential advantages of NMP over HMP are its ability to restore cellular function, upregulate protective repair mechanisms and allow better assessment of function. At the same time it provides a platform for cell therapy i.e. administration of mesenchymal stem cells (133). The first clinical trial performed by Hosgood and Nicholson et al. showed that 1-hour normothermic perfusion of human donor kidneys with subsequent transplantation is safe and feasible (134). Due to better assessment of human DCD kidneys, that were declined for transplantation, this group was able to reverse that decision and successfully transplant several kidneys, thereby increasing the donor pool.

A topic of interest in current HMP research is the presumed positive effect of addition of oxygen during cold machine perfusion. Due to the low temperature (approximately 10°C), the metabolism of the graft is reduced by 90%. Initially, it was thought that addition of oxygen was not necessary at that level of reduced metabolism and might actually increase detrimental ROS formation. New insight obtained by pre-clinical work in kidney and liver (135–137) showed improved recovery of transplanted organs when oxygenated perfusion had been used. Recently, the COMPARE trial by the COPE consortium showed in a multicentre clinical context that oxygenated HMP of older DCD donor kidneys was better than non-oxygenated HMP in terms of kidney function, graft survival and rejection rate (ISRCTN32967929) (138). The POMP trial of the COPE consortium compared in higher-risk ECD kidneys conventional SCS to a preservation of first SCS, then followed by a brief period of oxygenated HMP. This large clinical study did not detect any difference in function or survival. This suggests that a brief period of 4.5h oxygenated HMP at the end of preservation is not sufficient for oxygenated HMP to have beneficial effects. It might be best to start oxygenated HMP as soon as possible after organ procurement (139). To date, several clinical trials investigating hypothermic oxygenated machine perfusion in liver and kidney transplantation are ongoing.

As in many other fields, studies looking into machine perfusion are still mainly performed in animal models. Choosing the right animal model however is of importance to be able to translate the newfound knowledge to the human situation. Lerink et al. showed that there is still a big translational gap in many preclinical IRI models (140). This was also shown in the above mentioned EMPERIKAL trial that was first tested in a rat model. Based on those results, the dose range for humans was decided. However, when tested in the pig it was shown that the human dose should have been 12 times higher than estimated from the rat study (132). The porcine transplantation model appears to be the best simulation of human conditions due to similar physiology, size and immune system. Several groups have started to use slaughterhouse pig organs to test various aspects of organ perfusion which reduces the need for animal house pigs n the early exploratory stages (141). In a next step, including experimental transplantation, animal house models will be required before phase 1 studies in humans can be ethically justified.

SUMMARY

In conclusion, machine perfusion and in particular HMP appears to influence many different pathways involved in IRI. The goal of this review was to compare HMP with the old gold standard SCS. Studies are now available including clinical evidence that HMP has beneficial effects on outcomes such as immediate function and survival but also on important mechanisms that are involved in IRI: HIF levels, cell death, endothelial dysfunction and the innate immune response. To obtain better insight in the mechanisms of injury and repair, future studies should focus on analysis of the effects of HMP on all four mechanisms. This will allow the discovery of underlying relationships and clinically

relevant pathways. It will also lead to the development of targeted interventions to increase viability whilst possibly modulating the graft and rendering it less immunogenic. This may help to reach the goal to enhance function and prolong survival avoiding chronic graft dysfunction after 5-10 years due to progressive scarring of the transplanted organ.

AUTHOR CONTRIBUTIONS

LK, CK, and RP came up with the conception and design. LK wrote the manuscript and CK and RP revised the manuscript. All authors have read and agreed to the published version of the manuscript.

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ORIGINAL RESEARCH
published: 02 May 2022
doi: 10.3389/fimmu.2022.849742

The Effects of Free Heme on Functional and Molecular Changes During *Ex Vivo* Normothermic Machine Perfusion of Human Kidneys

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OPEN ACCESS

Edited by:
Stefan Schneeberger, Innsbruck Medical University, Austria

Reviewed by:
Fausto Chiazza, University of Eastern Piedmont, Italy
Maria Irene Bellini, Sapienza University of Rome, Italy

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Specialty section:
This article was submitted to Alloimmunity and Transplantation, a section of the journal Frontiers in Immunology

Received: *06 January 2022*
Accepted: *04 April 2022*
Published: *02 May 2022*

Citation:
Hosgood SA, Elliott TR, Jordan NP and Nicholson ML (2022) The Effects of Free Heme on Functional and Molecular Changes During Ex Vivo Normothermic Machine Perfusion of Human Kidneys. Front. Immunol. 13:849742. doi: 10.3389/fimmu.2022.849742

Normothermic machine perfusion (NMP) is a technique of kidney preservation designed to restore cellular metabolism after cold ischemia. Kidneys are perfused with an oxygenated banked red blood cell (RBC) based solution for 1h at 36°C. During NMP, RBCs can become damaged, releasing free heme into the perfusate. This can act as a damage-associated molecular pattern (DAMP) activating inflammatory signalling pathways. The aim of this study was to measure the levels of free heme during NMP, assess the effect on kidney function and determine any association with inflammatory and stress related gene expression. Levels of free heme were measured in perfusate samples from a series of donation after circulatory death (DCD) kidneys undergoing NMP as part of a randomised controlled trial (RCT). The age of RBCs and levels of free heme were correlated with perfusion parameters. Changes in gene expression were analysed in a series of kidneys declined for transplantation using the NanoString nCounter Organ Transplant Panel and qRT-PCR. Older units of RBCs were associated with higher levels of free heme and levels increased significantly during NMP (Pre 8.56 ± 7.19μM vs 26.29 ± 15.18μM, $P<0.0001$). There was no association with levels of free heme and perfusion parameters during NMP ($P > 0.05$). Transcriptional and qPCR analysis demonstrated the upregulation of differentially expressed genes associated with apoptosis (FOS and JUN), inflammatory cytokines (IL-6, SOCS3, ATF3), chemokines (CXCL8, CXCL2, CC3/L1) and oxidative stress (KLF4) after NMP. However, these did not correlate with levels of free heme ($P >0.05$). A significant amount of free heme can be detected in the perfusate before and after NMP particularly when older units of red cells are used. Although transcriptional analysis demonstrated significant upregulation of genes involved with apoptotic, inflammatory and oxidative pathways these were not associated with high levels of free heme.

Keywords: normothermic perfusion, kidney, free heme, hemolysis, immune response

INTRODUCTION

Normothermic machine perfusion (NMP) technologies are being trialled in clinical practice to improve early graft function and assess the quality of kidneys for transplantation (1–3). An oxygenated red cell-based solution is circulated through the kidney under near physiological conditions. Cellular function is restored to replenish ATP and minimize the effects of cold ischemia.

NMP can be carried out using a number of different strategies. Experimentally, prolonged durations of NMP have shown benefit in improving early graft function (4). However, in clinical practice shorter durations performed after hypothermic preservation at the recipient center are logistically more practical. Preliminary studies have demonstrated the safety and feasibility of this technique in clinical practice (5) and the results of a RCT in DCD kidneys (1) are expected later this year.

Compatible packed red blood cells (pRBCs) from a local blood bank are used in most NMP circuits as an oxygen carrier. RBCs are also important regulators of vascular tone and nitric oxide bioavailability (6). Although pRBCs can be stored for up to 42 days, over time their condition deteriorates (7). During storage the destruction of the RBC membrane causes the release of haemoglobin and free heme (8, 9). This is further exacerbated during NMP when RBCs come into contact with artificial surfaces and by the perfusion pump (10, 11). Excess free heme is highly toxic and has the ability to induce oxidative stress, inflammation, cellular injury and apoptosis (10, 12). Free heme also binds nitric oxide derived from the endothelium reducing nitric oxide availability to cause vasoconstriction, thrombin formation, fibrin deposition, platelet activation and aggregation that leads to organ dysfunction (10, 12).

The aim of this study was to measure the levels of free heme during NMP and assess the effect on kidney function and to determine any association with inflammatory and stress related gene expression.

MATERIALS AND METHODS

Kidneys undergoing 1h NMP from a RCT (ISRCTN15821205) were used to determine the effect of free heme on perfusion parameters (n = 42). The study protocol and trial documents were approved by the NHS Health Research Authority East of England, Cambridge Central Research Committee (15/EE/0356).

Human kidneys rejected for transplantation and offered for research were used to investigate the transcriptional effects of free heme on gene expression and cellular damage (n = 15). Consent for research was obtained by specialist nurses in organ donation (SNODs). The study was approved by the National Research Ethics committee and Research and Development office at the University of Cambridge (NRES: 15/NE/0408).

Normothermic Machine Perfusion

NMP was carried out for 1 hour after a period of static cold storage as previously described (1). In brief, the NMP was performed using adapted cardiopulmonary bypass technology (Medtronic) and consisted of a centrifugal pump, membrane oxygenator, heat exchanger, venous reservoir and PVC tubing.

The circuit was primed with one unit of compatible packed red blood cells (RBCs) mixed with a priming solution (**Supplementary Table 1**). Supplements were added to support kidney function. In the research kidneys creatinine (1500μmol/L) was added to the perfusate to measure creatinine clearance. Kidneys were flushed with 500ml cooled Ringer's solution (4°C) prior to being connected to the NMP circuit

The renal artery was cannulated and kidneys were perfused at a pump speed of 1450 RPM. The perfusate temperature was maintained between 35 and 37°C and mean arterial pressure of 75–85mmHg. Renal blood flow (RBF), arterial pressure, temperature and urine output were monitored throughout.

Quality Assessment Score

During NMP the quality of each transplanted kidney was graded. The NMP quality assessment score was derived from three factors: macroscopic appearance, mean RBF and total urine output. Kidneys with a quality assessment score of 4 or less were considered suitable for transplantation provided there were no other preclusions to transplant (13).

Perfusate and Urine Analysis

Perfusate samples were collected before and after NMP. Samples were centrifuged at 1600 RPM for 10 minutes at 4°C. Supernatant was removed, flash frozen in liquid nitrogen and stored at −80°C. Urine samples were collected after 1h NMP. Samples were centrifuged at 1600 RPM for 10 minutes at 4°C. Supernatant was removed, flash frozen in liquid nitrogen and stored at −80°C.

Free Heme

Free heme was measured in the perfusate samples before and after NMP using the Heme Assay Kit (Sigma-Aldrich, St Louis, USA) following manufacturer's instructions.

Tissue Samples

In the research kidneys, wedge biopsies of cortex were taken before and after NMP. Biopsies were divided into three for fixation in 10% formalin (CellStor, CellPath, Powys, UK) for paraffin wax embedding, flash frozen in liquid nitrogen and fixed

Abbreviations: ATF3, activating transcription factor 3; ATP, adenosine triphosphate; CC3/L1, C-C motif chemokine ligand 3 like 1; CXCL8, C-X-C motif chemokine ligand 8; CXCL2, C-X-C motif chemokine ligand 2; DAMP, damage-associated molecular pattern; DBD, donation after brain death; DCD, donation after circulatory death; ERK, extracellular signal-regulated kinase; FOS, Fos proto-oncogene; HMGB1, high mobility group box 1; HO-1, hemeoxygenase 1; IL-6, interleukin 6; IL-10, interleukin 10; JNK, c-Jun N-terminal kinases; JUN, Jun proto-oncogene; KLF4, Kruppel Like Factor 4; MAPKs, mitogen-activated protein kinases; NF-κB, nuclear factor kappa B; NGAL, neutrophil gelatinase-associated lipocalin; NLR, nod like receptor; NLRP3, NLR family pyrin domain containing 3; NMP, normothermic machine perfusion; pRBCs, packed red blood cells; RBCs, red blood cells; RBF, renal blood flow; RCT, randomised controlled trial; RT, reverse transcription; SOCS3, suppressor of cytokine signalling 3; TLR4, toll-like receptor 4; TNF, tumor necrosis factor; VEGF, vascular endothelial growth factor.

in RNALater Stabilisation Solution (Invitrogen, ThermoFisher, California, USA).

Apoptosis was measured using TUNEL staining on 4μm sections of paraffin fixed tissue. TUNEL staining was carried out following manufacturer's instructions (abcam, Cambridge, UK). Sections were viewed at ×200 magnification and ten fields of view were imaged. TUNEL positive cells were counted in each field of view and an average number of positive cells was calculated for each sample. Apoptotic cells were identified by dark brown staining.

Injury Markers

Protein lysates were prepared from tissue samples collected before and after NMP. Tissue (5mg) was homogenised in RIPA lysis buffer (Sigma-Aldrich, St Louis, USA) with protease inhibitor cocktail (Sigma-Aldrich, St Louis, USA) using mechanical homogeniser. Samples were left on ice for 30 mins and then centrifuged for 20 minutes at 4°C 12000 RPM.

The protein lysates were used to measure oxidative stress in the research kidneys using the Protein Carbonyl content assay kit (RayBiotech, Georgia, USA) following manufacturer's instructions.

Human neutrophil gelatinase-associated lipocalin (NGAL) was measured in the urine at the end of NMP following manufacturer's instructions (Cohesion Bioscience, London, UK).

NanoString Analysis

Gene expression profile was performed using the Human Organ Transplant panel from NanoString technologies. Total RNA was isolated from kidney biopsies and 770 genes related to organ transplant pathways were screened according to manufacturer's instructions. Normalization, differential of expression and pathways analysis were performed within the nSolver Software Advanced Analysis. Normalisation was based on the selection of a panel of housekeeping genes through geNorm pairwise variation statistic. Differential of expression was displayed in a volcano plot with p-value adjusted by the Benjamini–Hochberg false discovery rate correction method. Further analysis was performed within the R software: the heatmap and principal component analysis were plotted with the top 25 and 100 differentially expressed genes respectively.

Gene Expression

Gene expression of IL-6, TLR4, HO-1, HMGB1, FOS and JUN (ThermoFisher, California, USA) (**Supplementary Table 2**) was quantified using RT qPCR on RNAlater fixed tissue in 15 of the research kidneys. RNAlater fixed tissue was ground in lysis buffer (Qiagen, Maryland, USA) using a micropestle. The samples were homogenised. Chloroform was added and the samples were shaken vigorously and centrifuged for 15 minutes at 12000g and 4°C. The upper phase was collected and RNA extraction was carried out using RNAEasy Mini Kit following manufacturer's instructions (Quiagen, Maryland, USA). The RNA elution was immediately stored on ice to prevent RNA degradation. RNA concentration and quality was measured using NanoDrop Spectrophotometer ND-1000.

Reverse transcription (RT) was carried out on RNA samples using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Fisher Scientific, California, USA) following manufacturer's instructions. Briefly, each sample was mixed with the master mix provided and RT was carried out in the thermal cycler (BioRad T100) at 25°C for 10 minutes, 37°C for 120 minutes, 85°C for 5 minutes and 4°C until used in qPCR.

qPCR was carried out on the BioRad CFX RealTIme System. Samples were analysed in triplicate and each experimental well contained SsoAdvanced Universal Probes Supermix (BioRad, Hertfordshire, UK), primer (**Supplementary Table 2**), cDNA and RNAase free water. PCR consisted of 95°C for 10 seconds followed by 60°C for 30 seconds for a total of 40 cycles.

Fold increase of expression was quantified using the delta delta ct method with 18s used as the gene of reference to which samples were normalized. Gene expression was presented as a fold change after NMP compared to baseline biopsies taken prior to NMP.

Statistical Analysis

Continuous data were tested for normality and the appropriate statistical test carried out. Data were compared using Student's t-test or Wilcoxon test. Correlations to determine associations between levels of free heme measured in the perfusate before and after NMP and age of pRBC, levels of potassium, lactate, functional perfusion parameters and gene expression were calculated using Pearson's correlation matrix.

For the transplanted and research kidneys donor demographics and ischemic times were recorded.

To determine any differences between subpopulations of research kidneys dependent on donor type demographics, perfusion parameters, protein expression and gene expression in donation after brain death (DBD) and DCD kidneys were compared.

Values are presented as the mean ± SD for parametric data and median and inter-quartile range (IQR) for non-parametric distributed data. P values of >0.05 were considered significant. Statistical analysis was carried out using Prism statistical software version 9 (GraphPad, California, USA).

RESULTS

Clinical Series

A total of 42 kidneys underwent NMP with pRBCs. Donor demographics and ischemic times are listed in **Table 1**. Levels of free heme and lactate increased significantly during NMP (P<0.0001, 0.0004; **Table 1**) but levels of potassium remained stable (P =0.2795; **Table 1**).

The majority of kidneys were of good quality and had a quality assessment score of 1 and 2 (**Table 1**). The mean RBF was 73.1 ± 20.5ml/min/100g, total urine output 130 ± 104ml and urine NGAL 16.1 ± 13.2ng/ml (**Table 1**).

Correlation With Perfusion Parameters in the Clinical Kidneys

Older units of pRBCs were associated with higher levels of free heme, higher levels of potassium and lactate before and after 1h

TABLE 1 | Donor demographics and perfusion outcomes (n=42) of DCD kidneys that underwent 1h NMP prior to transplantation.

Demographics		N=42	Statistical significance
Donor age (years)		54 ± 14	
Donor gender		36	
% Female			
Donor cause of death *(n)*	*ICH*	12	
	Hypoxic brain damage	17	
	Intracranial thrombosis	3	
	Respiratory failure	2	
	Pneumonia	1	
	Cardiac arrest	6	
	Pulmonary embolism	1	
Terminal creatinine (μmol/L)		78.8 ± 43.5	
Warm ischemic time (minutes)		11.4 ± 2.5	
Cold ischemic time (minutes)		824.2 ± 268.1	
Free heme (uM)	*Pre NMP*	8.56 ± 7.19	<0.0001
	1h NMP	26.29 ± 15.18	
Lactate (mmol/L)	*Pre NMP*	8.26 ± 2.17	0.0004
	1h NMP	10.13 ± 3.63	
Potassium (mmol/L)	*Pre NMP*	10.01 ± 2.81	0.2795
	1h NMP	10.53 ± 2.83	
QAS (n)	*1*	21	
	2	16	
	3	4	
	4	1	
RBF (ml/min/100g)		73.10 ± 20.49	
Urine output (ml)		130.2 ± 104.4	
NGAL (ng/ml)		16.06 ± 13.22	
Oxygen consumption (ml/mg/g)		45.67 ± 17.86	

Data are presented as mean and standard deviation from the mean. Parameters measured pre and post NMP were compared and statistical significance determined (Student's paired t test). ICH, intracerebral hemorrhage; NGAL, neutrophil gelatin lipocalin; NMP, normothermic machine perfusion; QAS, quality assessment score; RBF, renal blood flow.

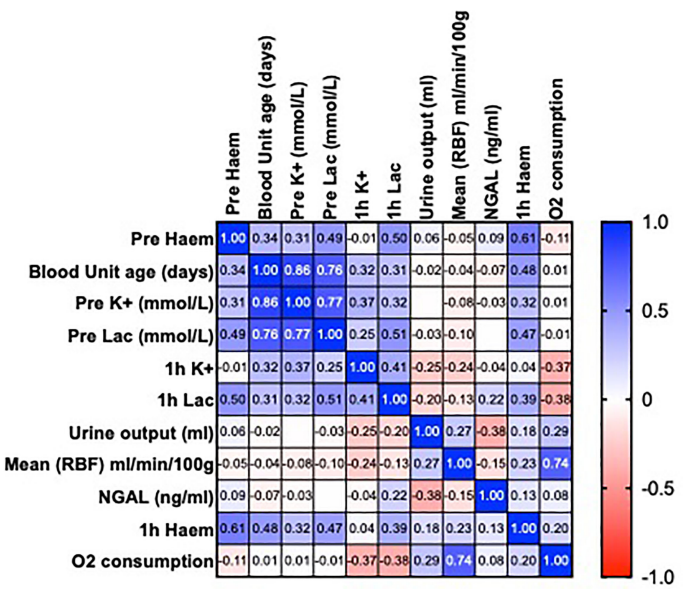

FIGURE 1 | Heatmap showing Pearson's correlation matrix to examine the associations of the age of packed red blood cells and levels of free heme on perfusion parameters before and after 1h of normothermic machine perfusion (NMP). Values are R.

TABLE 2 | Donor demographics and ischemic times for research kidneys (n=15) that underwent 1h NMP.

Donation type	Donor age (Years)	Left/Right Kidney	WIT (mins)	CIT (mins)	Reason for decline	Cause of death	Urine output (ml)	Urine NGAL (ng/ml)	Mean RBF (ml/min/100g)	Age of pRBCs (days)
DBD	64	Right	na	2051	Enlarged lymph nodes	Cardiac arrest	35	–	73.3	21
DBD	60	Left	na	653	Suspected malignancy	ICH	120	14.01	102.7	23
DBD	72	Right	na	1380	Past medical history	ICH	92	16.32	69.5	33
DCD	53	Left	17	1674	Cut ureter	ICH	92	39.46	78.2	19
DBD	75	Left	na	2034	Remuzzi score	ICH	164	10.16	104.8	14
DBD	45	Right	na	1545	Suspected malignancy	ICH	116	15.22	82.0	37
DCD	68	Right	10	1773	Remuzzi score	ICH	120	33.70	92.2	35
DBD	58	Left	na	1248	Dissection renal artery	Hypoxic brain damage	14	10.27	53.2	17
DBD	60	Left	na	1483	Damage to ureter	Trauma - unknown cause	30	46.61	40.9	35
DCD	74	Right	10	1327	Remuzzi score	ICH	145	21.11	128.5	29
DBD	56	Left	na	1154	Suspected malignancy	Septicaemia	10	17.31	32.3	21
DBD	76	Right	na	536	Diseased aortic patch	ICH	28	24.19	31.6	37
DBD	75	Left	na	711	Atheroma renal artery	ICH	25	8.41	28.2	18
DCD	59	Right	18	1140	Damage to renal artery	ICH	164	9.58	81.3	22
DCD	67	Right	9	1647	Damage to renal vein	ICH	90	16.24	34.0	19

CIT, cold ischemic time; DBD, Donation after brain death; DCD, donation after circulatory death; ICH, intracerebral hemorrhage; NMP, normothermic machine perfusion; WIT, warm ischemic time. na, not applicable.

NMP (P <0.05). Baseline levels of free heme were associated with significantly higher levels of baseline potassium (P = 0.044), baseline lactate (P = 0.001), 1h lactate (P = 0.001) and 1h free heme (P <0.0001).

There was no association with the age of pRBCs or levels of free heme and functional parameters (urine output, RBF, oxygen consumption and NGAL; P >0.05) (**Figure 1**).

Research Kidneys

The donor characteristics and ischemic times for the research kidneys (10 DBD and 5 DCD) are detailed in **Table 2**. The average cold ischemic time before NMP was 1395 ± 498 minutes (**Table 2**). Mean urine output was 83 ± 55ml, NGAL 3.1 ± 4.2ng/ml and RBF 68.9 ± 31.2 ml/min/100g. The average age of pRBCs was 25 ± 8 days (**Table 2**). Levels of free heme in the perfusate increased significantly during NMP (P = 0.001; **Figure 2**).

Transcriptional Gene Expression

NanoString nCounter Organ Transplant Panel was used to examine the transcriptional changes in gene expression in four human DBD research kidneys that underwent 1h NMP.

Seventeen differentially expressed genes were significantly upregulated after NMP. These included genes associated with apoptosis (FOS and JUN), inflammatory cytokines (IL-6, SOCS3, ATF3), chemokines (CXCL8, CXCL2, CC3/L1) and oxidative stress (KLF4). Several genes associated with anti-inflammatory properties (IL-10) and endothelial cell recovery (VEGF) were also upregulated though did not reach statistical significance (**Figures 3A, B**). Pathway analysis showed that the top five significantly upregulated pathways after 1h NMP were involved in nod like receptor (NLR) signalling, oxidative stress, apoptosis and cell cycle regulation, Th17 mediated biology and TNF family signalling (**Figure 3C**). Principal component analysis demonstrated a distinct separation of the pre- and post-1h NMP samples (**Figure 3D**)

Quantification (qPCR)

PCR analysis of the 15 research kidneys showed a significant increase in expression of FOS (P < 0.0001), median fold increase 59 (3-364), IL-6 (P = 0.0001), median fold increase 11 (3-81), JUN (P = 0.0001), median fold increase 11 (1-146) and TLR4 (P = 0.0353), median fold increase 1.4 (0.3-16.2). There was no significant fold change in HO-1 (P = 0.2293) and HMGB1 (P = 0.446; **Table 3**).

Correlations With Gene Expression and Perfusion Parameters in the Research Kidneys

pRBC age and levels of free heme pre and post NMP did not correlate with changes in gene expression (IL-6, FOS, JUN, HO-1, HMGBI, TLR4 P >0.05; **Figure 4**) in the discard series. There were associations between the expression of IL-6 and FOS and JUN (P = 0.048, 0.015, respectively; **Figure 4**), HO-1, FOS, JUN and HMGB1 (P <0.05; **Figure 4**) and HMGB1, FOS, JUN and TLR4 (P<0.05; **Figure 4**)

pRBC age and levels of free heme pre and post NMP also did not correlate with each other or with perfusate levels of IL-6 or levels of functional parameters (P>0.05; **Figure 4**). There was a significant positive correlation between levels of IL-6 and urinary NGAL (r=0.824, ***P<0.0003; **Figure 4**).

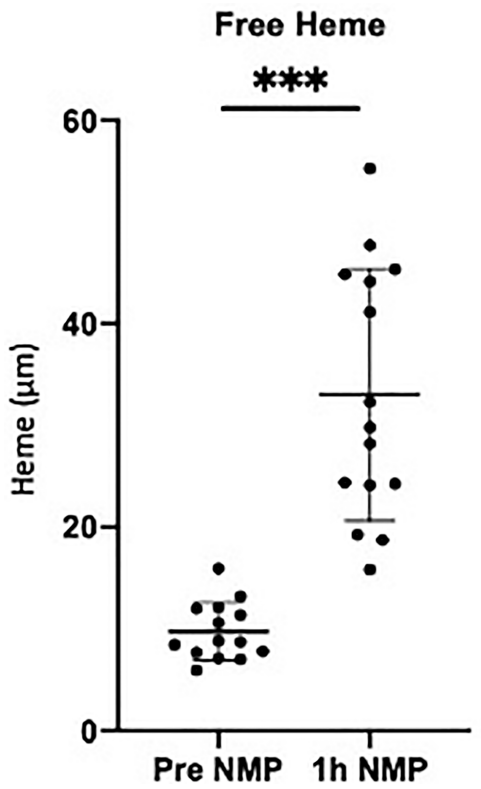

FIGURE 2 | Levels of free heme measured in the perfusate pretransplant and post 1h NMP in a series of human kidneys that were rejected for transplanted and offered for research. Data presented as mean (SD). ***P < 0.0003

Oxidative Stress and Apoptosis

There was a numerical increase in levels of protein carbonyl after NMP; however, this did not reach statistical significance (P = 0.115) (**Figure 5**).

The number of apoptotic cells significantly increased during NMP compared to baseline samples (P = 0.027). The majority of positive staining was found in the tubular epithelial cells (**Figures 6A, B**).

Comparison of DCD and DBD Research Kidneys

A subanalysis was carried out to determine any differences between the DCD and DBD kidneys. There was no significant difference in the mean donor age (DBD 66 ± 10y, DCD 62 ± 9y; P = 0.241) and the cold ischemic time was matched between groups (DBD 1512 ± 267 min, DCD 1360 ± 573 min; P = 0.588).

Baseline and 1h NMP levels of free heme were similar between DCD and DBD kidneys (baseline P = 0.699, 1h NMP P = 0.254).

DBD kidneys had a significantly greater fold change in IL-6 gene expression compared to the DCD kidneys after 1h NMP (DCD 6.08 (4.09-14.83), DBD 29.51 (8.41-52.25); P = 0.029). There were no significant differences between groups in the five other target genes (FOS, JUN, HO-1, HMGB1, TLR4; P > 0.05).

DISCUSSION

The NMP system uses adapted clinical grade pediatric cardiopulmonary bypass technology designed to minimize hemolysis. However, factors such as the heat generated by the centrifugal pump, shear stress, negative pressures, turbulent flow and gravity assisted venous drainage contribute to hemolysis and release of free heme. In cardiac bypass procedures excess levels of heme are associated with organ damage and increased mortality (11, 14). The use of banked RBCs for NMP is problematic in that during storage the deformability and surface charge of RBCs decreases and fragility and aggregability increases (15, 16). This makes them more susceptible to damage during NMP. During storage there is also an accumulation of lipids and RBC derived microparticles or macrovesicles that act as damage-associated molecular patterns (DAMPs), which can stimulate the immune system and production of reactive oxygen species (17, 18). In the clinical series, we found that older units of pRBCs had significantly higher levels of free heme at baseline and levels increased further during NMP. Furthermore, older units of pRBCs also had higher levels of potassium and lactate before and after NMP. This is presumably due to leakage from the fragile RBCs during storage. Perfusate levels of lactate increased significantly during perfusion but it is difficult to determine whether this was due to further damage of the red cells or increased lactate production due to inadequate tissue oxygenation (19). Interestingly, in the discard kidney series the age of pRBCs was not associated with increased levels of free heme but levels increased significantly during NMP. This may be due to the small sample size and variation within this group. Nonetheless, in both the transplanted and discarded kidneys no association was found between the levels of free heme and renal perfusion parameters including oxygen consumption which suggests no detrimental effect and adequate tissue oxygenation. There was also no evidence of significant oxidative damage at the level of protein expression or in the number of apoptotic cells within this short NMP timeframe.

To determine the molecular effects of heme, changes in transcriptional gene expression in a number of the discarded kidneys were assessed. There was significant upregulation of cellular stress related genes that are consistent with ischemic injury. Genes upregulated after NMP included heat shock proteins, oxygen free radicals (KLF4), hypoxia and inflammatory genes (IL-6, SOCS3, ATF3) and chemokines (CXCL8, CXCL2, CC3/L1). FOS and JUN gene transcription were amongst the top five significantly upregulated differentially expressed genes. They regulate activator protein 1, a prominent transcription factor involved with regulating cellular fate involving the mitogen-activated protein kinases (MAPKs) pathways (ERK, p38 and JNK). Together with the NLR pathway, the MAPK pathway is involved in the downstream signalling of the innate immune response and pro-inflammatory cytokine production with activation of NF-κB/NLRP3 inflammasome (20).

To quantify the findings from the transcriptional data, a number of genes were investigated using qRT-PCR. In agreement with the NanoString transcriptomic data, there was

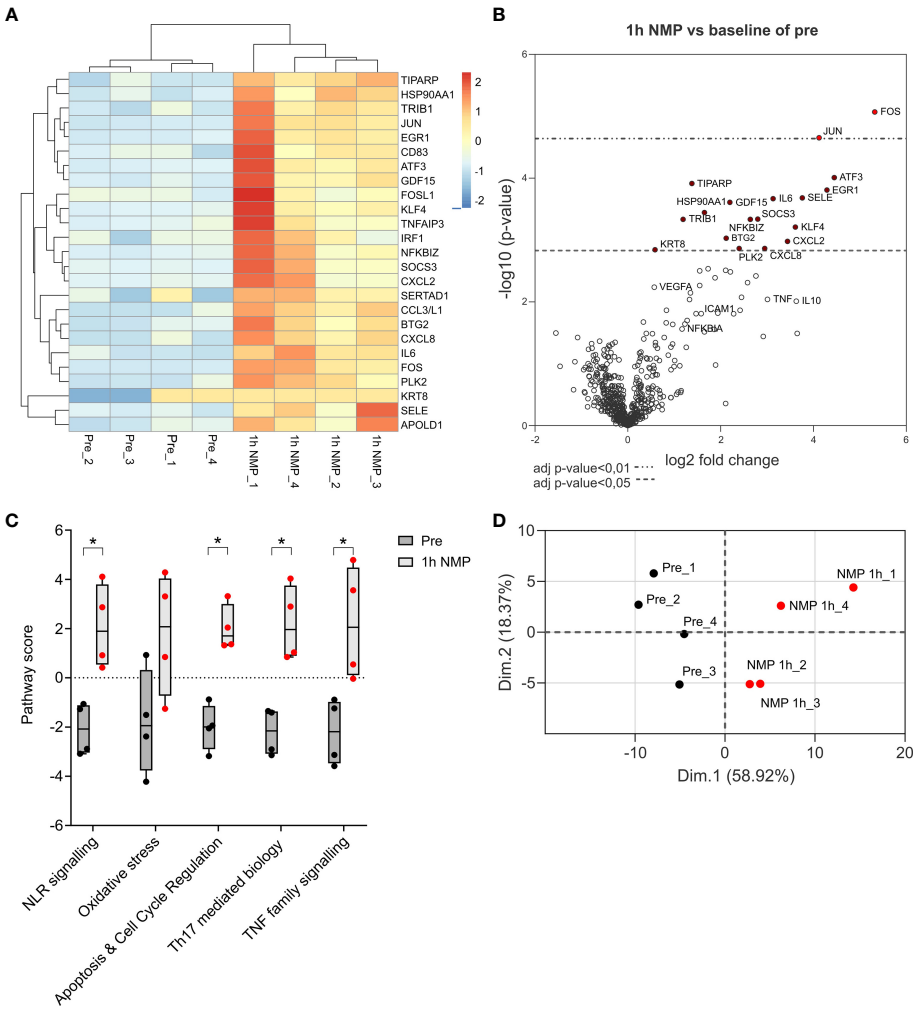

FIGURE 3 | Nanostring nCounter Organ Transplant Panel analysis of 4 cortex samples of kidneys comparing transcriptional analysis of differential expressed genes after 1h NMP compared to baseline pre samples. Total RNA was isolated and processed. **(A)** Heatmap of the top 25 significantly differentially expressed genes. **(B)** Volcano plot represents differential gene expression after 1h NMP. Difference in gene expression level with p-value adjusted <0.01 or <0.05 are marked by red dots. **(C)** Top 5 pathways up-regulated after NMP. Statistical significance was calculated by Mann Whitney test (*, p<0.05). **(D)** Principal component analysis on the top 100 differentially expressed genes.

a significant upregulation of IL-6, FOS and JUN. We also measured HMGB1, a nuclear protein that in situations of stress and ischemia, translocates from the nucleus to the cytoplasm and is excreted into the extracellular space to act as a DAMP (21, 22). HMGB1 has proinflammatory interactions with downstream receptors such as toll like receptor-4 (TLR4). The HMGB1/TLR4 pathway is well recognized in the pathophysiological process of renal ischemic injury and acute kidney injury activating NF-κB mainly through the MyD88 pathway (23). In this study we found a significant upregulation

TABLE 3 | Expression of genes in tissue measured using qPCR.

Gene name	Fold change (median (IQR))	Significance (p value)
FOS	59.03 (21.91–98.13)	<0.0001
IL-6	11.02 (6.32–41.46)	0.0001
JUN	11.24 (5.71–17.07)	0.0001
TLR4	1.44 (1.00–3.04)	0.0353
HO-1	1.15 (0.65–3.04)	0.2293
HMGB1	1.03 (0.49–3.07)	0.4457

Measured as median fold change (IQR) post NMP compared to samples taken prior to NMP. Wilcoxon test for significance (p < 0.05)

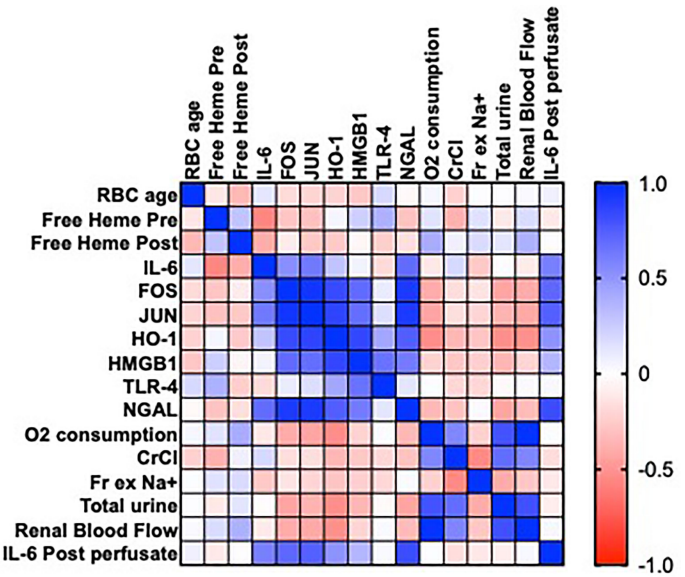

FIGURE 4 | Heatmap showing Pearson's correlation matrix in a series of 15 human kidneys rejected for transplantation to examine the associations of the age of packed red blood cells (pRBC) and levels of free heme on gene expression (IL-6, FOS, JUN, HO-1, HMGB1 and TLR-4) after NMP, measures of renal function (oxygen consumption, creatinine clearance [CrCl], fractional excretion of sodium [Fr Ex Na+], total urine output, renal blood flow), levels of urinary neutrophil gelatinase associated lipocalin (NGAL) and perfusate levels of IL-6 post NMP. Values are R.

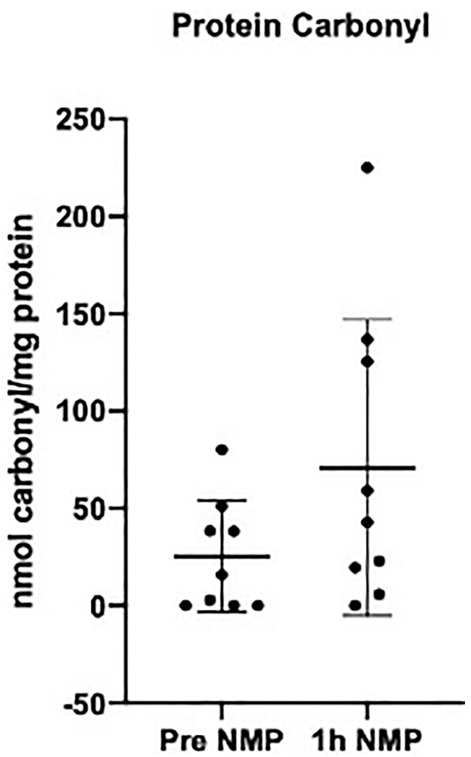

FIGURE 5 | Levels of protein carbonyl quantifying oxidative stress measured in the perfusate pretransplant and post 1h NMP in a series of human kidneys that were rejected for transplant and offered for research. Data presented as mean (SD).

of TLR4 after NMP but not HMGB1. Dexamethasone is a glucocorticoid receptor that suppress NF-κB and MAPK-ERK activation and the subsequent translocation of HMGB1 (24). It is added to the perfusate during NMP as an anti-inflammatory agent and may therefore account for our findings.

There was no association in levels of free heme and gene expression in the series of discarded kidneys. However, based on the known effects of free heme we cannot dismiss the possibility of its contribution to the stimulation of these inflammatory and oxidative stress pathways. With such a short perfusion time, we were unable to quantify the protein expression of these pathways. As a DAMP, free heme influences the expression of many genes and, in the NMP environment in the absence of plasma proteins, free heme can interact with TLR4 to upregulate inflammatory mediators (23, 25, 26). Pathway analysis showed upregulation of NLR, oxidative stress, apoptosis, Th17 and TNF family signalling, all of which can be stimulated by free heme (27). Surprisingly, hemeoxygenase-1 (HO-1), an important enzyme rapidly stimulated to counteract the detrimental effects of free heme by catalyzing heme to carbon monoxide, ferrous iron and biliverdin (28), was not upregulated during NMP. Again, this may be due to the addition of dexamethasone during NMP which can suppress its expression (29).

The upregulation of inflammatory mediators during NMP has been associated with prolonged early graft dysfunction (30) and therefore efforts should be made to improve the NMP environment, including reducing levels of free heme, this may be particularly important for longer periods of NMP. Younger units of pRBCs would be advantageous but free heme, potassium, lactate and microparticles can be removed by washing the RBCs

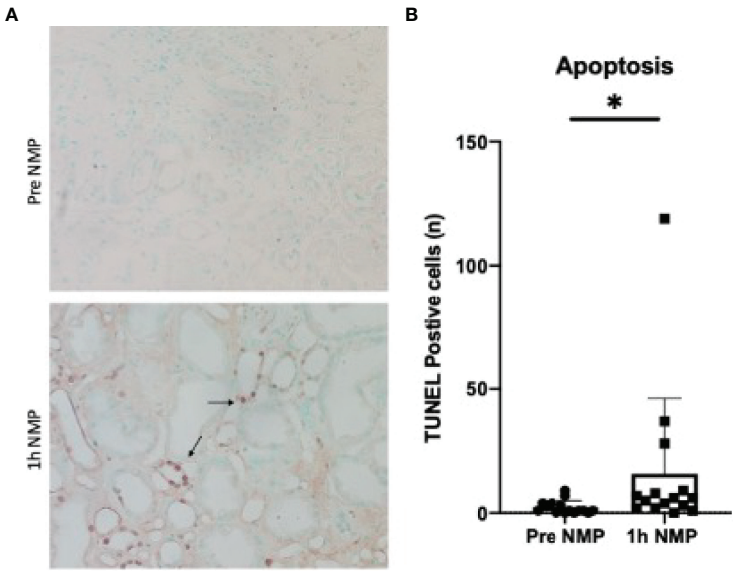

FIGURE 6 | **(A)** TUNEL staining of apoptotic cells in pre and post cortical biopsies. Positive cells stained brown (highlighted by arrows). **(B)** Quantification of TUNEL positive staining. Average number of cells per 10 fields of view. Data is presented as mean (SD).*P = 0.027

before use. However, this may affect the quality of the RBCs increasing their fragility and susceptibility to mechanical stress during NMP (8). Dilution of the RBCs with plasma or albumin can reduce inflammation and oxidative damage by binding to free heme (29).

The addition of an oxygen carrier during NMP is considered essential for adequate oxygen delivery to the tissues. Venema L et al, found that the addition of RBCs during more prolonged periods of NMP of porcine kidneys resulted in higher levels of oxygen consumption and function compared to conditions without RBCs (19). Artificial oxygen carriers such as hemopure are an alternative to RBCs and are being trialled in kidney NMP (31, 32). Subnormothermic temperatures or the combination of subnormothermic temperature and artificial oxygen carriers have recently been shown to reduce inflammation and pulmonary vascular resistance during ex vivo lung perfusion (EVLP) (33) and should be explored in kidney NMP.

Another strategy is to use an oxygenated acellular solution to rewarm kidneys in a controlled manner. Zlatev H et al. recently published the results of six extended criteria donor kidneys demonstrating the safety and feasible of the technique (34). Nonetheless, this has not be trialled for longer periods of perfusion.

This is the first study to examine the effects of free heme during kidney NMP. It involved two populations of kidneys: to determine the effects of free heme on kidney function during NMP a series of DCD kidneys included in a RCT were used; to determine the effect of free heme at a molecular level a series of DBD and DCD kidneys rejected for transplantation and offered for research were used. Although the research kidneys had longer cold ischemic times and were from DBD and DCD donors, levels of free heme and functional parameters during NMP were similar to the RCT kidneys. The population of DCD research kidneys were further disadvantaged with more of the DCD kidneys being perfused with older units of pRBCs. The small sample size limits any conclusion on the effect of free heme on DCD compared to DBD kidneys in this study but previous analysis of transcriptional gene expression after NMP has found results to be similar between DBD and DCD kidneys (30).

In conclusion, high levels of free heme are found in units of stored RBCs and increase significantly during 1h of NMP. There was no direct association between levels of free heme, kidney function or the upregulation of inflammatory and stress related genes during perfusion. However, this may have consequences for longer periods of perfusion and levels of hemolysis should be minimized during NMP.

DATA AVAILABILITY STATEMENT

The data presented in the study are deposited in the GEO repository, accession number (GSE197304) https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE197304)

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by East of England, Cambridge Central Research Committee (15/EE/0356), National Research Ethics committee

and Research and Development office at the University of Cambridge (NRES: 15/NE/0408). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

SH conceived the research idea, planned and conducted the experiments, analysed the results and co-wrote the manuscript; TE conducted the experiments, analysed the results and co-wrote the manuscript; NJ conducted the experiments, analysed the results and revised the manuscript; MN conceived the research idea and revised the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

The research was funded Kidney Research UK and by the National Institute for Health Research Blood and Transplant Research Unit (NIHR BTRU) in Organ Donation and Transplantation at the University of Cambridge in collaboration with Newcastle University and in partnership with NHS Blood and Transplant (NHSBT) Grant RG75628.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.849742/full#supplementary-material

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frontiers | Frontiers in Immunology

ORIGINAL RESEARCH
published: 01 June 2022
doi: 10.3389/fimmu.2022.850271

Kidney Normothermic Machine Perfusion Can Be Used as a Preservation Technique and a Model of Reperfusion to Deliver Novel Therapies and Assess Inflammation and Immune Activation

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OPEN ACCESS

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Innsbruck Medical University, Austria

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Specialty section:
This article was submitted to Alloimmunity and Transplantation, a section of the journal Frontiers in Immunology

Received: 07 January 2022
Accepted: 27 April 2022
Published: 01 June 2022

Citation:
Mellati A, Lo Faro L, Dumbill R, Meertens P, Rozenberg K, Shaheed S, Snashall C, McGivern H, Ploeg R and Hunter J (2022) Kidney Normothermic Machine Perfusion Can Be Used as a Preservation Technique and a Model of Reperfusion to Deliver Novel Therapies and Assess Inflammation and Immune Activation. Front. Immunol. 13:850271. doi: 10.3389/fimmu.2022.850271

Ischaemia–reperfusion injury (IRI) is an inevitable process in transplantation and results in inflammation and immune system activation. Alpha-1 antitrypsin (AAT) has anti-inflammatory properties. Normothermic machine perfusion (NMP) can be used to deliver therapies and may help in assessing the effects of IRI and immunity. This study investigated the effects of AAT on IRI and inflammation in pig kidneys when administered during preservation, followed by normothermic reperfusion (NR) with autologous whole blood, as a surrogate for transplant. Two different models were used to deliver AAT or placebo to paired slaughterhouse pig kidneys: Model 1: 7-h static cold storage (SCS) + 3-h NR (n = 5 pairs), where either AAT (10 mg/ml) or placebo was delivered in the flush following retrieval; Model 2: 4-h SCS + 3-h NMP + 3-h NR (n = 5 pairs), where either AAT or placebo was delivered during NMP. Injury markers and cytokines levels were analysed in the perfusate, and heat shock protein 70 KDa (HSP-70) was analysed in biopsies. AAT delivered to kidneys showed no adverse effects on perfusion parameters. HSP-70 fold changes were significantly lower in the AAT group during NMP ($P < 0.01$, paired t-test) but not during NR. Interleukin-1 receptor antagonist (IL-1ra) fold changes were significantly higher in the AAT group during NR model 1 ($p < 0.05$, two-way ANOVA). In contrast to the AAT group, significant upregulation of interleukin-1 beta (IL-1β) and interleukin-6 (IL-6) between t = 90 min and t = 180 min and interleukin-8 (IL-8) between baseline and t = 90 min was observed in the control group in NR model 2 ($p < 0.05$, Tukey's multiple comparison test). However, overall inflammatory cytokines and injury markers showed similar levels between groups. Delivery of AAT to pig kidneys was safe without any detrimental effects. NMP and NR provided excellent methods for comparison of inflammation and immune activation in the delivery of a novel therapy.

Keywords: normothermic machine perfusion, alpha-1 antitrypsin, inflammatory response, immune activation, cytokines, ischaemia–reperfusion injury, kidney transplantation

INTRODUCTION

Chronic kidney disease (CKD) resulting in end-stage renal failure leads to the need for renal replacement therapy (RRT) that includes dialysis or kidney transplantation (1, 2). Kidney transplantation results in improved patient survival and improved quality of life and is more cost-effective (3). However, there is a gap between the availability of organs and the number of patients waiting, resulting in an organ shortage (4). To meet these demands, there is a need to use non-standard organs including those from older donors with comorbidities, so-called expanded criteria donors (ECDs). Donation after circulatory death (DCD) donor kidneys are more prone to delayed graft function (DGF; the need for dialysis after transplant) due to a period of warm ischaemia sustained during retrieval (5). The severity of ischaemia–reperfusion injury (IRI) sustained following transplant in non-standard organs increases the chance of poor long-term outcomes (6–8).

IRI is unavoidable in kidney transplantation, and numerous studies have been involved in understanding mechanisms of IRI using *in vitro* and *in vivo* models (9). IRI consists of pathophysiological conditions including cell death, microvascular dysfunction, transcriptional reprogramming, innate and adaptive immune system activation, and endothelial-to-mesenchymal transition (9, 10). The efficacy of various pharmacological treatments has been tested on renal IRI in several studies. These studies investigate potential protective and improving effects of pharmacological agents on renal IRI through inhibition of different mechanisms including cell death, endothelial cell injury, inflammatory responses, complement activation, and free radical production, as well as supporting mitochondria protection and antioxidant capacities *in vitro*, animal models (*in vivo* and *ex vivo*), and clinical trials (11–17).

While *in vitro* IRI models are restricted by cell type numbers and simulating pathophysiological conditions involved in IRI, small animal species like rodents and rabbits are also limited by size, anatomical differences, and lack of natural heterogeneity due to inbreeding when it comes to translatability to human. Large animal models such as pigs have been used in IRI and transplantation studies due to similarities in genetics, anatomy, and pathophysiology with human as well as their suitability for needed surgical procedures (18). Use of slaughterhouse pigs could be preferable when considering cost and ethics due to the use of non-consumption organs compared to animal house pigs that also have been shown to be a valid DCD model (19).

Normothermic machine perfusion (NMP) is an established preservation method in the research setting, although the mechanisms of action and the impact on inflammation and immunity are unclear. NMP provides near-physiological conditions using oxygenated perfusate at 37°C that creates the opportunity for assessment of organ viability and interventional therapeutics prior to kidney transplantation (20). Alpha-1 antitrypsin (AAT) is a protease inhibitor that is used as an augmentation therapy for patients with AAT deficiency. There is evidence that AAT has anti-inflammatory and tissue protective effects, which may be beneficial in areas such as organ transplant and organ preservation (21–25).

The aim of this study was to investigate the effects of AAT delivered during static cold storage (SCS) or NMP followed by assessment using normothermic reperfusion (NR) on inflammation and immune activation in ischemia–reperfusion in pig kidneys.

MATERIALS AND METHODS

Study Design

Preclinical studies were performed using paired slaughterhouse pig kidneys to simulate the warm ischaemic injury sustained in DCD kidneys. Due to the use of slaughterhouse kidneys in this study, no ethics committee approval was needed.

Two models were designed to deliver AAT or placebo (equivalent human albumin concentration to treatment group) during different preservation methods: Model 1: 7-h SCS + 3-h NR (n = 5); Model 2: 4-h SCS + 3-h NMP + 3-h NR (n = 5). In model 1, following retrieval either the drug or placebo was delivered at the beginning of SCS, and in model 2, the drug or placebo was delivered at the beginning of NMP as the main

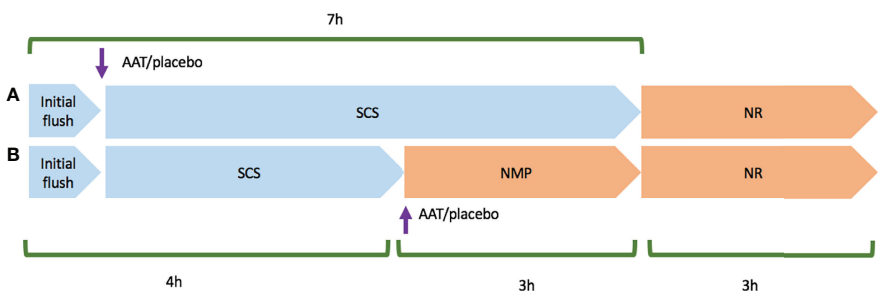

FIGURE 1 | Schematic planning of model 1 and model 2 experiments. Cold ischaemia time started upon the initial flush using Soltran. **(A)** Represents model 1 experiment that Alpha-1 antitrypsin (AAT) or placebo was added in the second flush, and then kidney pairs were stored in static cold storage (SCS) in a mixture of Soltran + Alpha-1 antitrypsin (AAT) or placebo. **(B)** Represents model 2 experiment that following retrieval, kidney pairs were transferred to the lab in cold storage, and then AAT or placebo was added to NMP circuits once both normothermic machine perfusion preservation (NMP) circuits were primed. Both models were followed and completed by 3-h normothermic reperfusion with whole blood (NR).

preservation phase. Both models were completed with 3-h NR as an experimental surrogate for transplantation (**Figure 1**). It was decided that the drug would be given at the beginning of preservation to expose the organ as early as possible. As NMP could not be initiated in the slaughterhouse, the kidneys in model 2 needed a short period of SCS for transport back to the lab and preparation of NMP.

Retrieval and Surgical Procedures

Model 1 (7-h SCS + 3-h NR): Pairs of porcine kidneys and blood were obtained from a local slaughterhouse. Pigs were slaughtered following Home Office guidance using electrocution and exsanguination *via* jugular vessels. Autologous blood was collected in a container with 20,000 units heparin, and pigs were de-haired in a 60°C hot wash for 5 min. Afterward, both kidneys were flushed with ∼1,000 ml heparinised Soltran (5,000 IU in 1,000-ml Soltran). Here, 4-mm punch biopsies were taken from the upper pole of both kidneys for further analysis. Left and right kidneys were randomly allocated to control or treatment using the randomization function in Microsoft Excel software. A 500-ml solution containing 6.74% AAT (Kamada) and Soltran (final concentration: 10 mg/ml) for treatment and 20% human albumin (Grifols) with 0.9% saline and Soltran (final concentration: 10 mg/ml) for control was produced. Kidneys were flushed with 100 ml of either treatment or control and suspended in the remaining 400 ml of solution. They were then cold stored at <4°C on melting ice for 7 h to simulate the approximate average cold-stored preservation time in clinical practice.

Model 2 (3-h NMP + 3-h NR): Warm allogeneic blood was directly collected into a heparinised funnel (10,000 IU) connected to leucocyte filters to obtain leucocyte depleted blood for the normothermic preservation phase. Kidneys were retrieved as for model 1 and transferred to the lab using SCS. Baseline tissue biopsies were taken immediately prior to the start of NMP.

Perfusate Preparation for Normothermic Machine Perfusion and Normothermic Reperfusion

Normothermic Reperfusion

Whole blood was filtered through medical gauze to remove debris. Filtered blood was diluted to a haematocrit of 30% with 0.9% saline with the following additional supplements: Amoxicillin-clavulanate (1,200 mg) to avoid bacterial contamination, mannitol (10 mg) as a free radical scavenger and osmotic diuretic, insulin (5 IU) to facilitate physiological glucose absorption, and verapamil (0.75 mg) to reduce vasoconstriction. Also, 5% glucose and 10% calcium gluconate were added during NR to maintain physiological values. In this study, 500 ml of perfusate was used for each circuit.

Normothermic Machine Perfusion Preservation

A red blood cell (RBC)-based perfusate was prepared using allogeneic leucocyte-depleted blood. Filtered blood was centrifuged at 3,000 × g for 20 min at room temperature (RT), and the plasma supernatant was removed and discarded. To make 1,000 ml perfusate, 750 ml 5% human albumin (Grifols), 9 ml 10% calcium gluconate, 60 ml demineralised water, 18 ml 5% glucose, and 15 ml 8.4% sodium bicarbonate were mixed together, and then isolated RBCs were gradually added to the mixture to reach 25% haematocrit. Afterward, in addition to 500 ml of prepared RBC-based perfusate, amoxicillin-clavulanate (1,200 mg), insulin (5 IU), mannitol (10 mg), creatinine (1,000 μM), and verapamil (0.75 mg) were added to each circuit.

Perfusion Circuits

The Kidney Assist device (Organ Assist) was used for NMP and NR. This consisted of a membrane oxygenator (Hilte Lt 2500, Medos Medizintechnik or Terumo FX05, Terumo Corp.), a centrifugal pump head (DP3, Medos Medizintechnik AG), and tubing. In this study, 95% O_2/5% CO_2 carbogen cylinder (BOC) (0.5 ml/min rate) was used for gas delivery. An ultrasonic flow sensor probe (Em-tec-Gmbh) measured flow rate on the arterial line, and a pressure transducer (Edwards Lifesciences) was connected to the arterial line at the same height as the renal artery. Temperature was set at 37°C and perfusion pressure at 70 mmHg.

Perfusate samples were taken and centrifuged at 1,800 × g for 12 min at 4°C and stored at -80°C freezer. Urine was recirculated during NMP and NR in model 2 but not during NR in model 1. Tissue biopsies were taken at baseline (abattoir for model 1 and before the start of NMP for model 2) and end of NMP and NR. All tissue biopsies were bisected; half was stored in formalin, and half was frozen at -80°C.

Transferring From Normothermic Machine Perfusion to Normothermic Reperfusion

Once NMP was completed, arterial cannulas were clamped and disconnected from the circuit and kidneys were promptly flushed with Soltran to remove the perfusate. They were then stored cold until the NR phase (temperature <4°C). Simultaneously, NMP circuits were rinsed using 0.9% saline to remove the residual perfusate and primed using NR whole-blood perfusate. This procedure from the end of the NMP phase to the start of the NR phase took ∼60 min.

Arterial Blood Gas Values and Biochemistry Analyses

Arterial blood gas values were measured using ABL90 FLEX blood gas analyser. Perfusate samples were sent to the clinical biochemistry lab (John Radcliffe Hospital, Oxford, UK) to determine aspartate aminotransferase (AST) and lactate dehydrogenase (LDH) activity.

Neutrophil Gelatinase-Associated Lipocalin

NR perfusate samples were used to determine the concentration of neutrophil gelatinase-associated lipocalin (NGAL) using a pig Lipocalin-2 ELISA kit (Abcam, Cat. No.: ab207924). In brief, perfusate samples were added in duplicate to the precoated kit wells. Standards and blanks were also added to the plate, and the

plate was incubated for 1 h at RT. Afterward, all wells were properly washed using diluted wash buffer and then a biotinylated pig-NGAL primary antibody was added, followed by incubation for 1 h at RT. After washing, horseradish peroxidase (HRP)-streptavidin was added to all wells and the plate was incubated for 1 h at RT on the shaker. 3,3′,5,5′-Tetramethylbenzidine (TMB) and hydrogen peroxide substrates were added to all wells once washing steps were completed, and after 10-min incubation in the dark, the stop solution was added to the plate and the plate was read using a Bio-Rad iMark microplate reader.

Kidney Injury Molecule-1

Kidney injury molecule-1 (KIM-1) concentration was measured in NMP and NR samples using Porcine KIM-1 ELISA kit (FineTest, Cat. No.: EP0102). Briefly, the precoated ELISA plate was washed twice using wash buffer, and samples and standards were plated and incubated at 37°C for 90 min. Then, the plate was washed twice, and 100-µl primary antibody (1:100 dilution) was added to each well followed by incubation for 60 min at 37°C. After 3 washes, 100-µl HRP-streptavidin conjugate (1:100) was added to each well. Following this, the plate was incubated again at 37°C for 30 min. Next, the plate was washed and 90-µl TMB solution was added to each well, and the plate was again incubated in the dark at 37°C for 25 min. Then, 50 µl of stop solution was added to each well, and the plate was read in Bio-Rad iMark microplate reader at 450 nm.

Cytokines

Cytokine concentration was measured in NR samples using a Millipore Luminex kit (Merck, Cat. No.: PCYTMAG-23K). Briefly, samples (baseline, t = 90 min, and t = 180 min) were thawed on ice and centrifuged at 10,000 × g for 5 min and then were diluted to 1:2 (model 1) and neat (model 2). Then, 200-µl assay buffer was added to each well, and the plate was left on a shaker for 10 min. After decanting assay buffer, 25 µl of serially diluted standards, quality controls, assay buffer (background), and samples were added to the wells based on the plate layout. Then, 25-µl assay buffer was added to the sample wells, and 25 µl Serum Matrix was added to background, standards, and control wells. Afterward, 25-µl beads were added to each well, and then the plate was incubated on a shaker overnight in the dark at 2°C–8°C. The next day, the plate was washed 3 times and 50-µl detection antibody was added to each well. After 2-h incubation at RT, 50 µl streptavidin-phycoerythrin was added per well, followed by 30-min incubation. The plate was washed 3 times, and 100-µl Drive Fluid was added to each well. The plate was run in Magpix Luminex machine controlled with xPONENT software. All results were analysed using Milliplex Analyst software.

Heat Shock Protein 70KDa

The expression of heat shock protein 70 KDa (HSP-70) was measured using Western blot technique. In brief, tissue biopsies were homogenised using Radioimmunoprecipitation assay buffer (RIPA buffer) (Merck) in a sonicator, and then the protein concentration was determined by Pierce™ Bicinchoninic acid (BCA) Protein Assay Kit (Thermo Fisher Scientific). Equal amounts of protein from kidney cortical biopsies were loaded on 26 wells of a 4%–12% sodium dodecyl sulfate–polyacrylamide gel (SDS_PAGE gel), and gel electrophoresis was performed at 80 V for 15 min and then 150 V for up to 2 h at RT. Then, separated proteins were transferred to a Polyvinylidene fluoride membrane (PVDF membrane) membrane at 60 V for 3 h at 4°C. After transfer, the membrane was washed in Tris-buffered saline, 0.1% Tween 20 (TBS-T) and blocked in 5% semi-skimmed milk for 60 min on shaker at RT. The membrane was washed using TBS-T and incubated by primary antibodies HSP-70 (Cat. No.: C92F3A-5, Bioquote-stressmarq) and β Actin (Cat. No.: ab8226, Abcam) diluted in 5% milk-TBST overnight at 4°C. Next day, blots were washed with TBST and then were incubated with IRDye 680RD (Cat. No. 926-68070, Li-Cor) under agitation for 1 h at RT. Blots were washed in TBST and rinsed with 1× TBS and then scanned by Odyssey DLx Imager (LI-COR Biosciences). The scanned blots were analysed by ImageJ software and GelAnalyzer software for quantitation.

Myeloperoxidase Staining

Immunohistochemistry staining of myeloperoxidase-positive (MPO+) cells was performed by staining MPO on paraffin-embedded kidney slices with 4-µm thickness. Antigen retrieval was performed using Epitope Retrieval Solution for 20 min (Bond, AR9961). Then, sections were stained using rabbit anti-human MPO antibody (1:400, Agilent Technologies, Cat. No.: A039829-2). All staining steps were performed using automatic Leica Microsystem machine.

Statistical Analyses

The area under the curve (AUC) was calculated for continuous variables. Fold changes were calculated by dividing values by baseline values except where baseline values were below detection range to compare control vs. treatment group. Data were assessed for normality and were analysed using paired t-test or 2-way ANOVA to compare control vs. treatment group. Changes over time were also analysed using 2-way ANOVA. Results are reported as mean ± standard deviation (SD) of the mean. In this study, p values <0.05 were considered as statistically significant. Analyses were performed using GraphPad Prism 9.0.

RESULTS

Machine Perfusion Characteristics and Haemodynamics

All kidneys in model 1 and model 2 experiments were perfused successfully and without any adverse events. The mean flow rate decreased during the first 30 min followed by an increase to a steady state in NR phase of model 1. As for model 2 (both NMP and NR), mean flow rate increased from the beginning of perfusion to steady state. Flow rate and intrarenal levels were similar between AAT and control groups in NR (models 1 and 2) and NMP phases (**Figure 2**).

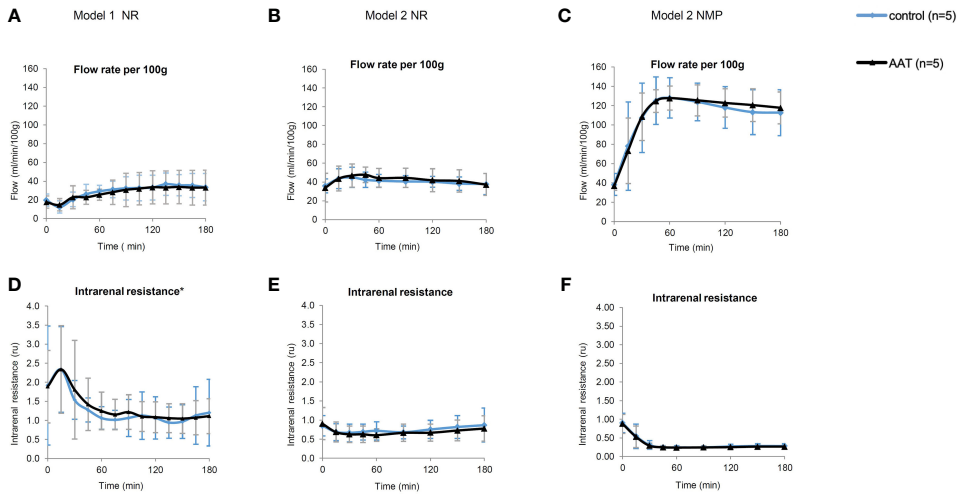

FIGURE 2 | Perfusion parameters including flow rate (ml/min/100 g) and intrarenal resistance (ru). **(A–C)** represent flow rate, and **(D–F)** represent intrarenal resistance during the normothermic reperfusion phase (NR) in models 1 and 2 as well as during the nomothermic machine perfusion preservation (NMP) phase in model 2, respectively. All results are shown as mean ± SD. *One data point (t = 15 min) in the graph **(D)** is excluded due to a technical error in the device.

Kidney Injury Biomarkers

KIM-1 and NGAL that are released following tubular injury in plasma were measured as biomarkers of acute kidney injury (26–28). KIM-1 concentration in plasma increased over time during NR in models 1 and 2 ($p < 0.0001$ for both models 1 and 2 and $p < 0.001$ for NMP, 2-way ANOVA) (**Figure S1**) with similar levels in both AAT and control groups. In addition, NGAL levels in plasma increased over time in both models 1 and 2 ($p < 0.001$ and $p < 0.05$, respectively, 2-way ANOVA) with similar levels in both AAT and control groups (**Figure S2**). These results suggest that the addition of AAT does not reduce tubular injury following ischaemia–reperfusion. Furthermore, an increase in the levels of both KIM-1 and NGAL during warm perfusion suggests increasing tubular injury during preservation.

AST and LDH are markers of cellular injury, and lactate is a marker of ischaemic injury (28). All 3 increased throughout the 3-h NR (models 1 and 2) and 3-h NMP. The AUC of LDH and lactate levels in model 1 and model 2 (NMP and NR) showed similar levels in the AAT and control groups (**Figure S3**). The AUC of the AST levels during NMP in the treatment and control groups was 144.8 ± 40.9 vs. 179.8 ± 64.5, respectively ($p = 0.08$, paired t-test).

Heat Shock Protein 70 KDa

HSP-70 that increases following ischemia–reperfusion has a direct relationship with elevated levels of tissue injury due to oxidative stress (29–31). Similar levels of HSP-70 were observed in the AAT and control groups in the NR phase of both model 1 and model 2. A significant increase in levels of HSP-70 protein expression was observed in the control group compared to that in the treatment group ($p < 0.01$, paired t-test), showing less oxidative stress in the AAT group at the end of NMP (**Figure 3**).

Cytokines

A Luminex assay was used to analyse the inflammatory profile during NR in both model 1 and model 2 experiments.

As for the model 1 experiment, interleukin-1 alpha (IL-1α), interleukin-12 (IL-12), and tumour necrosis factor alpha (TNF-α) significantly decreased over time ($p < 0.01$ for IL-1α and IL-12, and $p < 0.05$ for TNF-α, 2-way ANOVA). The levels of interleukin-6 (IL-6), interleukin-8 (IL-8), interleukin-18 (IL-18), interleukin-1 receptor antagonist (IL-1ra), and interleukin-10 (IL-10) significantly increased over time ($p < 0.0001$ for IL-6, IL-8, and IL-18, and $p < 0.01$ and $p < 0.05$ for IL-1ra and IL-10, respectively, 2-way ANOVA). IL-1ra fold changes showed a significant increase in the AAT group compared to those in the control group in model 1 ($p < 0.05$, 2-way ANOVA). In addition, IL-1ra levels showed a significant increase when levels at t = 90 vs. t = 180 and t = 0 vs. t = 180 were compared in the treatment group in model 1 ($p < 0.05$, Tukey's multiple comparison test). This suggests that the addition of AAT during cold storage leads to upregulation of IL-1ra after 90 min during NR. Interleukin-1 beta (IL-1β) and interferon gamma (INF γ) levels stayed steady during NR.

In model 2, there was a significant decrease in levels of IL-12 ($p < 0.0001$, 2-way ANOVA). In contrast, a significant increase in levels of IL-1α, IL-1β, IL-6, IL-8, IL-1ra, and IL-10 was observed over time ($p < 0.05$ for IL-1α and IL-1β, $p < 0.01$ for IL-8 and IL-10, $p < 0.001$ for IL-6 and IL-1ra, 2-way ANOVA). In this model, IL-1β and IL-6 significantly increased between t = 90 min and t = 180 min in the control group ($p < 0.01$ and $p < 0.05$, respectively, Tukey's multiple comparison test). In contrast, in the AAT group, similar significant increases between t = 90 min and t = 180 min were not observed. This suggests that addition of AAT during NMP caused inhibition of IL-1β and IL-6 upregulation in NR after 90 min. In addition, IL-8 levels significantly increased

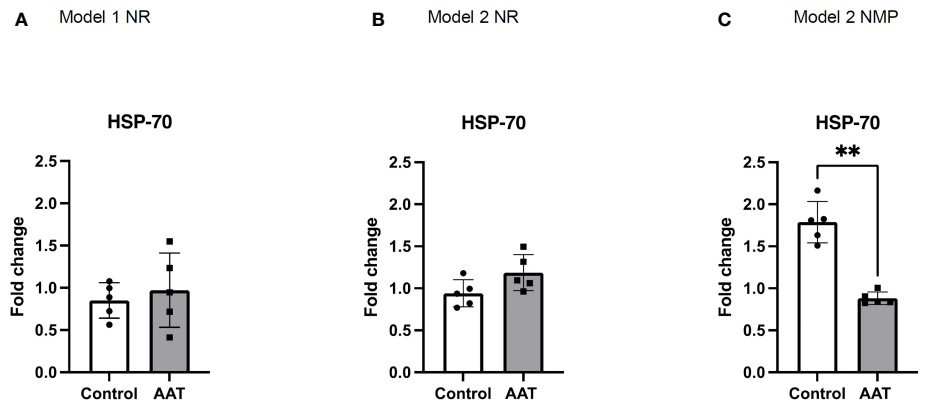

FIGURE 3 | Fold changes of heat shock protein-70 KDa (HSP-70) protein expression in tissue biopsies in normothermic reperfusion (NR) phase following 7-h static cold storage (SCS) in model 1 **(A)** and following 4-h SCS + 3-h normothermic machine perfusion preservation (NMP) in model 2 **(B)** as well as in NMP phase in model 2 **(C)**. Values for HSP-70 were normalised to β actin, and results were normalised to baseline values to calculate fold changes of HSP-70 at the end of NR and NMP. Results are shown as mean ± SD and **p < 0.01.

from baseline to t = 90 min in the control group ($p < 0.05$, Tukey's multiple comparisons test), while this significant difference between baseline and t=90 min was not observed in the AAT group. This suggests that addition of AAT in NMP inhibited IL-8 upregulation in NR between baseline and t = 90 min. IL-18, TNF-α, and INF γ levels stayed steady without any significant changes. No significant differences were observed between the control and treatment group except for IL-1ra in model 1 (**Figure 4**).

Myeloperoxidase-Positive Cells Before Preservation and After Normothermic Reperfusion

Light microscopy of tissue slices showed the number and distribution of stained MPO+ cells present in the tissue in biopsies taken after completion of NR. **Figure 5** shows biopsies taken prior to the start of preservation and biopsies taken after NR. There was an increase in the number of MPO+ neutrophils in the tissue following NR in both models and in both control and treatment groups.

DISCUSSION

In this study, the efficacy of AAT on IRI has been assessed by delivery of the drug during different preservation methods (NMP and SCS) followed by reperfusion with autologous whole blood. Although NMP has great potential for delivery of the pretransplant treatments, it is much simpler to deliver potential treatments during SCS, and as such, both methods were tested in this series of experiments. Much like other groups, we have used paired kidneys to test the addition of a novel therapy and then used NR, with whole blood as an experimental surrogate for transplant (32–34).

NMP is performed with RBCs suspended in albumin, without leucocytes, complement, platelets, and the immune compartment of the plasma, such as cytokines, chemokines, and other mediators. This allows the technique to be used as a preservation method designed to minimise the influence and injury caused by the immune system during perfusion, although there are still some immune cells present within the graft (35, 36). NR, using whole blood, offers an experimental surrogate to transplant, as the perfusate contains all blood components, and it is possible to be conducted after a long period of cold storage or short normothermic perfusion.

The addition of AAT during two different kidney preservation techniques was safe and feasible. There were indications that AAT might have some early anti-inflammatory and tissue-protective effects with increased levels of IL-1ra and inhibition of IL-1β, IL-6, and IL-8 upregulation during NR. There was also a decrease in AST levels and HSP-70 levels during NMP, which leans toward NMP being a more effective method of delivery for AAT than SCS. However, the majority of cytokines and tissue injury markers assessed were similar in both groups and in both models. The absence of a clear difference in outcomes when AAT was delivered during SCS compared with NMP was surprising. One might expect the normothermic environment to be more favourable to the activity of the enzymatic properties of AAT. However, from a logistical and resource perspective, the delivery of a therapy during SCS is more desirable than the complexities and cost of delivery during NMP. Other groups have demonstrated the merits of drug delivery during SCS (17), and preclinical testing of optimal preservation methods of delivery using NR as a transplant surrogate may be a helpful way of informing future trials.

Roles of pro- and anti-inflammatory cytokines following IRI have been studied widely using *in vivo* models (37–42). IL-1α and IL-1β stimulate an increase of macrophage chemoattractants (37, 41). IL-8 and TNF-α both participate in stimulation of neutrophil tissue infiltration in response to ischaemia–reperfusion (38, 39). This was also observed in our study when following IL-8 upregulation, infiltration of MPO+ cells into tissue

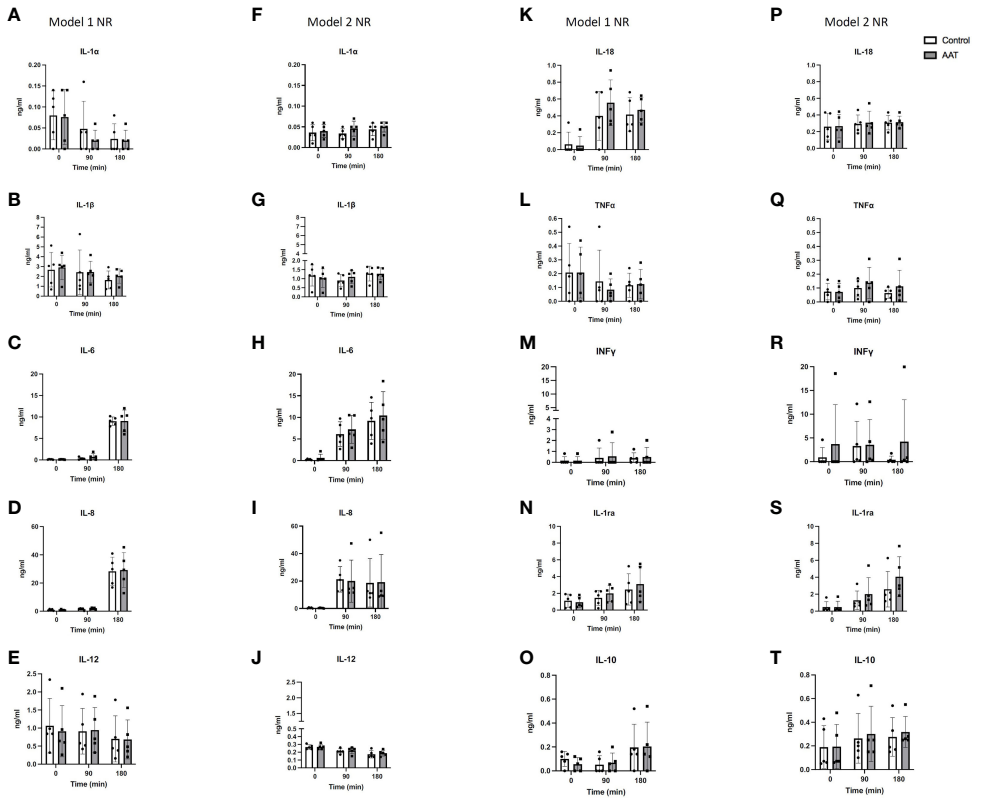

FIGURE 4 | Cytokine concentrations in the perfusate over 3-h normothermic reperfusion (NR) with whole blood following 7-h static cold storage (SCS) in model 1 as well as following 4-h SCS + 3-h normothermic machine perfusion preservation (NMP) in model 2 experiments. Panels **(A–E, K–O)** represent results for model 1 experiments; and panels **(F–J, P–T)** represent results for model 2 experiments. Results are shown as mean ± SD.

was observed. IL-10 and IL-1ra have shown anti-inflammatory and tissue-protective effects by reducing inflammation and apoptosis following IRI (40, 41). In this study, following NR, increasing levels of IL-6, IL-8, IL-10, and IL-1ra and decreasing levels of IL-12 were observed in both models. IL-12 and IL-8 have stimulatory effects on the production of INF γ from T cells and NK cells, and INF γ contributes to Major Histocompatibility Complex (MHC) class I and II upregulation (42). INF γ and IL-12 have been reported to be upregulated in the late phase of IRI, which might explain the decreasing levels of IL-12 and no changes in INF γ levels in the NR phase of both models (42). Interestingly, in a study of leucocyte mobilisation of donor kidney during NMP preservation following 2-h SCS, inflammatory cytokines were upregulated even though a leucocyte-depleted perfusate was used (43). In another study, in the effluent of a high-volume flush following 2-h SCS, INF γ, IL-1β, IL-8, IL-18, and TNF-α were detected (44). These studies in combination with our findings from the NR phase of both models suggest that donor-derived inflammatory events during preservation might aggravate reperfusion injury during transplantation. As such, it is logical to deliver interventional therapeutics with anti-inflammatory and tissue-protective properties in the preservation phase to reduce donor-derived inflammatory events and test their efficacy during NR.

MPO is a lysosomal enzyme and mainly expressed in neutrophils that allows the infiltration of leucocytes to be assessed and is an excellent measure of early innate immune activation during NMP (45). In this study, there was an absence of MPO+ cells following retrieval and prior to preservation in control and treatment groups in both models. Following NR, there was significant tissue infiltration of neutrophils in both control and treatment groups. This is consistent with findings from other studies using NMP and of renal IRI (46–48). Although there was no suggestion that AAT reduced tissue infiltration of neutrophils in this model, it is clear that both NMP and NR are associated with tissue inflammation.

Our models of kidney preservation followed by NR have some limitations. Firstly, the reperfusion phase was only 3 h, while some of the consequences of IRI and changes in inflammatory markers and immunity would occur much later. For example, upregulation of IL-6 in late IRI has anti-inflammatory properties in contrast to its pro-inflammatory role in early IRI (49), or upregulation of INF γ and IL-12 may be observed in the late phase of IRI (42). In IRI studies using *in vivo* models, a significant decrease of injury markers in the AAT group was only observed in late IRI events, and the 3-h reperfusion in this study may have missed these changes (22). In addition, a lack of

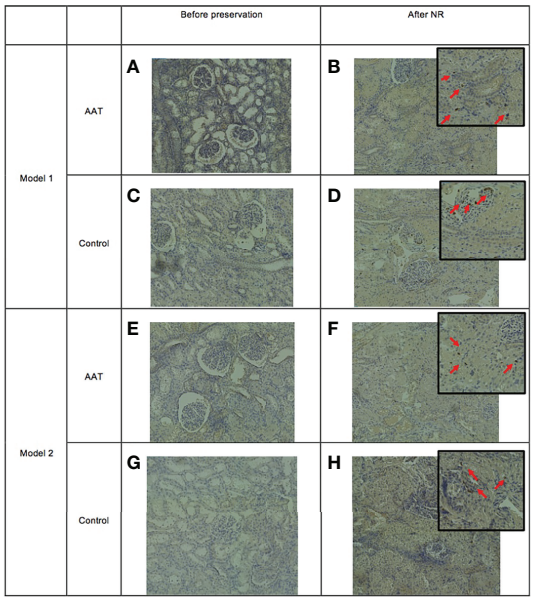

FIGURE 5 | Infiltration of myeloperoxidase-positive (MPO+) cells into tissue following normothermic reperfusion (NR). Panels **(A, C)** represent alpha-1 antitrypsin (AAT) and control groups before static cold storage (SCS), and panels **(B, D)** represent tissue following NR in model 1 (7-h SCS + 3-h NR). Panels **(E, G)** are AAT and control groups before normothermic machine perfusion preservation (NMP), and panels **(F, H)** represent tissue following NR in model 2 (4-h SCS + 3-h NMP + 3-h NR).

complete immune system and other organs in this setup limits our insights into IRI events and changes following interventional therapies. Slaughterhouse pig kidneys are from young healthy animals unlike the older comorbid patients who comprise the donor pool, which is a recognised limitation of the model. However, they are an ethical and sustainable resource that offers an excellent experimental model. We acknowledge that the use of a slaughterhouse model presents more variability in comparison to an animal house model, where the environment is more controlled. However, we have refined this method of retrieval over a number of years and previously described the strengths and limitations (50). We have shown acceptable reproducibility in preclinical dose–response studies where kidneys underwent normothermic perfusion as a basis for a clinical trial (17) and have also demonstrated that this method of multiple organ procurement is adequate for comparing different preservation methods in pig pancreases (51). We have not included histological evaluation in this study due to findings from a previous work on slaughterhouse pig kidneys. Experiments of similarly short duration demonstrated global acute tubular injury and were not sufficient to see histological differences between groups.

This study showed that AAT can be effectively delivered during cold storage and NMP to injured pig kidneys. Although some inflammatory cytokine levels were shown to be lower in the AAT-treated kidneys, the majority of cytokines and tissue injury markers were not different and further data are required to determine whether AAT has an important anti-inflammatory effect.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding authors.

ETHICS STATEMENT

Ethical review and approval were not required for the animal study because organs were retrieved from slaughterhouse animals after death (which occurred following standard Home Office procedures), and therefore no additional ethics were required.

AUTHOR CONTRIBUTIONS

Research design: AM, LLF, RP, JH. Performance of research: AM, RD, PM, KR, SS, CS, HM. Drafting of article: AM. Critical revision: All authors. All authors contributed to the article and approved the submitted version.

FUNDING

This research was funded by Kamada Pharmaceuticals that also supplied alpha-1 antitrypsin for use in this project.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.850271/full#supplementary-material

Supplementary Figure 1 | Kidney injury molecule-1 (KIM-1) measured in plasma and perfusate in NR model 1 **(A, B)**, NR model 2 **(C, D)** and NMP model 2 **(E)**. A, C and E show KIM-1 concentration, and B and E show KIM-1 fold changes at time points=90 min and 180 min when values were normalised to baseline values.

Supplementary Figure 2 | Neutrophil gelatinase-associated lipocalin (NGAL) measured in plasma and perfusate samples in NR model 1 **(A, B)** and NR model 2 **(C, D)**. **(A, C)** show NGAL concentration, and **(B, D)** show NGAL fold changes at time points= 90 min and 180 min when all final values were normalised to baseline values. Data were analysed using paired t-test and a P value<0.05 was considered as statistically significant. Results are shown as mean ± SD and each data point shows result from each kidney.

Supplementary Figure 3 | Area under the curve of aspartate aminotransferase (AST) and lactate dehydrogenase (LDH) activity as well as Lactate concentration over 3h NR in model 1 **(A, D, G)** and model 2 **(B, E, H)** as well as 3h NMP in model 2 **(C, F, I)**. Data were analysed using paired t-test and a P value<0.05 was considered as statistically significant. Results are shown as mean ± SD and each data point shows result from each kidney.

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frontiers | Frontiers in Immunology

ORIGINAL RESEARCH
published: 02 June 2022
doi: 10.3389/fimmu.2022.788935

The Effect of Normothermic Machine Perfusion on the Immune Profile of Donor Liver

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Background: Normothermic machine perfusion (NMP) allows viability assessment and potential resuscitation of donor livers prior to transplantation. The immunological effect of NMP on liver allografts is undetermined, with potential implications on allograft function, rejection outcomes and overall survival. In this study we define the changes in immune profile of human livers during NMP.

Methods: Six human livers were placed on a NMP device. Tissue and perfusate samples were obtained during cold storage prior to perfusion and at 1, 3, and 6 hours of perfusion. Flow cytometry, immunohistochemistry, and bead-based immunoassays were used to measure leukocyte composition and cytokines in the perfusate and within the liver tissue. Mean values between baseline and time points were compared by Student's t-test.

Results: Within circulating perfusate, significantly increased frequencies of CD4 T cells, B cells and eosinophils were detectable by 1 hour of NMP and continued to increase at 6 hours of perfusion. On the other hand, NK cell frequency significantly decreased by 1 hour of NMP and remained decreased for the duration of perfusion. Within the liver tissue there was significantly increased B cell frequency but decreased neutrophils detectable at 6 hours of NMP. A transient decrease in intermediate monocyte frequency was detectable in liver tissue during the middle of the perfusion run. Overall, no significant differences were detectable in tissue resident T regulatory cells during NMP. Significantly increased levels of pro-inflammatory and anti-inflammatory cytokines were seen following initiation of NMP that continued to rise throughout duration of perfusion.

Conclusions: Time-dependent dynamic changes are seen in individual leukocyte cell-types within both perfusate and tissue compartments of donor livers during NMP. This suggests a potential role of NMP in altering the immunogenicity of donor livers prior to

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Edited by:
Theresa Hautz,
Innsbruck Medical University, Austria

Reviewed by:
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Specialty section:
This article was submitted to Alloimmunity and Transplantation, a section of the journal Frontiers in Immunology

Received: *03 October 2021*
Accepted: *02 May 2022*
Published: *02 June 2022*

Citation:
Lee ACH, Edobor A, Lysandrou M, Mirle V, Sadek A, Johnston L, Piech R, Rose R, Hart J, Amundsen B, Jendrisak M, Millis JM, Donington J, Madariaga ML, Barth RN, di Sabato D, Shanmugarajah K and Fung J (2022) The Effect of Normothermic Machine Perfusion on the Immune Profile of Donor Liver. Front. Immunol. 13:788935. doi: 10.3389/fimmu.2022.788935

transplant. These data also provide insights for future work to recondition the intrinsic immune profile of donor livers during NMP prior to transplantation.

Keywords: normothermic machine perfusion, liver transplantation, immune profile, flow cytometry, immunohistochemistry

INTRODUCTION

Liver normothermic machine perfusion (NMP) systems have gained clinical approval following recent clinical trials and are now in wider use (1). These devices may decrease the critical organ shortage by allowing the assessment and use of livers from extended criteria donors (2–7).

Liver NMP may also serve as a platform to condition organs prior to transplantation to minimize rejection or optimize organ function (8, 9). The liver contains high numbers of lymphohematopoietic cells that are transferred with donor livers to recipients during transplantation (10, 11). These donor leukocytes persist in the allograft following transplantation and have been shown to be important determinant of outcome (12). Using cell therapy or pharmacological treatment of allografts during NMP, the intrinsic immune function of donor livers could be manipulated to optimize organ survival, minimize primary graft dysfunction and offset rejection (8). Previous studies have demonstrated the favorable intrinsic immune properties of livers (13). In contrast with other solid-organ allografts, 8-33% of clinical liver transplant recipients who withdraw from immunosuppression exhibit operational tolerance, with an even higher incidence reported among pediatric patients (13, 14). The window of opportunity provided by NMP to recondition the immune profile of transplant livers therefore represents an important and clinically translatable research area.

It has been suggested that liver NMP may provide a less inflammatory environment than conventional cold storage, as NMP reduces the number of INF-gamma and IL-17-producing T cells and enlarges the regulatory T cell in the liver perfusates collected after NMP when compared to those collected after cold storage (15). In addition, studies using normothermic *ex vivo* lung perfusion demonstrated removal of passenger non-classical monocytes from perfusates, suggesting altered immunogenicity of transplant lungs with *ex vivo* perfusion (16, 17). These studies have analyzed the cellular and cytokine profile of circulating perfusate during NMP. Importantly the effect of NMP on the tissue-resident immune compartment that is transferred within the allograft has not been defined. In this study we determined serial changes in donor-liver resident leukocyte populations during NMP. In addition, we serially determined the leukocyte characteristics and cytokine profile within NMP perfusate. The results of this study reveal the effect of NMP on the immune profile of donor-livers and provide important insights into future strategies to manipulate the immune properties of livers during *ex vivo* perfusion.

METHODS

Human Donor Liver Procurement

The study was reviewed and approved by the University of Chicago IRB under Request for Research on Decedents. The use of expired human donors' organs for this research study was approved by Gift of Hope Tissue and Organ Network, Itasca, Illinois. Six non-transplantable human donor livers available for research were included in our study. Families of all human liver donors had consented donation of livers for research. Four donors were donation after cardiac death (DCD) and two were donation after brain death donors (DBD). Donors with history of hepatitis B or C infection and DCD donors who took longer than 90 minutes to expire after withdrawal of care were excluded from our study (**Table 1**). All liver grafts were procured by certified abdominal transplant surgeons registered as procuring surgeons

TABLE 1 | Liver donor medical history and evaluation.

ID	Donor Age	Donor Gender	BMI	Donation Type	DCD time to expire (min)	Cold Ischemia Time (min)	Donor PMH	Reason for no transplant
6	73	Female	49.4	DBD	NA	291	Hypertension, 40 pack year smoker, HLD, OSA, MI, Schizophrenia, rhabdomyolysis	Cirrhosis on gross examination
7	57	Male	30.2	DCD	61	720	Hypertension, hyperlipidemia, obesity	Expedited DCD, not enough time to place liver
8	54	Male	19.9	DCD	67	664	Heart failure, cardiomyopathy, bradycardia, hyperlipidemia, COPD/ emphysema, drug use, GERD, depression, CVA, >20 pack year smoker	DCD, prolonged time to expire
9	45	Male	23.9	DCD	12	407	ETOH abuse (1/5 vodka/day), COPD, HTN, Anxiety, >20 pack year smoker, recent cellulitis of left hand	70% macrosteatosis
10	49	Male	42.5	DBD	NA	422	Unknown	40% macrosteatosis
11	39	Male	42.1	DCD	9	393	25 pack year smoker, asthma, polysubstance abuse, obesity, depression	40% macrosteatosis

by Gift of Hope Tissue and Organ Network, Itasca, Illinois, in standard clinical fashion. The liver grafts were then transported to our laboratory in standard cold storage fashion.

Normothermic Machine Perfusion

Normothermic machine perfusion (NMP) of liver was performed using a circuit design compatible with existing clinical NMP devices (**Figure 1**). The circuit consisted of Sarns disposable centrifugal pump (Terumo, Ann Arbor, MI), CAPIOX FX Advance oxygenator with integrated hardshell venous reservoir (Terumo, Ann Arbor, MI), a soft venous reservoir bag (Terumo, Ann Arbor, MI), a collection basin coated with PTFE, and a combination of ¼ inch and 3/8-inch ECMO cannulas (Medtronic, Minneapolis, MN) (**Figure 1**). To simulate physiological pressures and flow rates, the portal vein was perfused by gravity *via* a soft venous reservoir bag that was hung 20 centimeters above the portal vein. The hepatic artery was cannulated and received inflow directly from the centrifugal pump. The inferior vena cava was cannulated from the superior cut end, and directed into the hardshell venous reservoir. The hardshell reservoir directed perfusate to the centrifugal pump, oxygenator and heater. Perfusate was subsequently divided between the hepatic artery and the soft venous reservoir bag prior to the portal vein. The common bile duct was cannulated separately with a pigtail cannula. Circuit perfusate consisted of 3-4 units of packed red blood cells obtained from the University of Chicago blood bank. Perfusate fluid was supplemented with 3 liters of DME-H21 high glucose (4500 mg/L) medium (Thermo Fisher Scientific, Waltham, MA) containing 5% bovine serum albumin, with an eventual hematocrit goal of 10-15%.

Perfusion was initiated at room temperature. Perfusate temperature was incrementally increased to a goal temperature of 36.8-37°C over 20 minutes. Sweep gas composed of 5L-7L/min O2 and 0.5-1L/min of CO_2 was initiated to oxygenate inflow perfusate to the liver at the same time providing a physiologic level of dissolved CO_2. Further titration of sweep gas was performed based on serial blood gas analysis. The centrifugal pump flow rate, or the total perfusate flow rate, was adjusted to achieve a desired ratio of portal vein flow rate to hepatic artery flow rate of 1:2 to 1:3. Six hours of perfusion was selected based on a clinically applicable time for machine perfusion. In addition, previous clinical and pre-clinical studies assessed donor organ viability during NMP when organs were perfused for an average of 4 hours (3–7).

Physiological Measurements During NMP

Physiological parameters of the perfused liver were measured during NMP. The portal vein and hepatic artery pressures and flow rates were measured hourly. Three mL perfusate samples were collected hourly at both upstream and downstream of the organ for blood gas analysis to assess oxygenation, lactate and acid base status using an iSTAT point-of-care blood gas analyzer (Abbott Labs, Chicago, IL). Another 10 mL perfusate sample was collected hourly downstream of the organ for complete blood count and comprehensive metabolic panel including liver enzyme levels.

Sample Processing

Before initiation of perfusion, perfusate samples from retrograde flush with cold phosphate-buffered saline (about 300 mL) and tissue samples from wedge biopsies (about 3 g) were obtained. The samples collected prior to initiation of perfusion were labeled as 0^{th} hour. During perfusion, perfusate samples (about 300 mL) and tissue samples (about 3 g) were obtained at each time point (1^{st} hour, 3^{rd} hour, and 6^{th} hour of perfusion). Perfusate samples were spun down at 400G and treated with

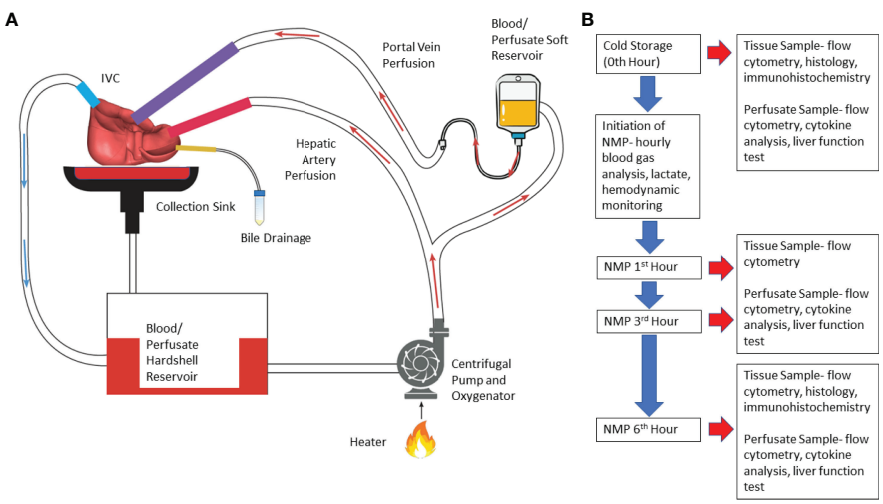

FIGURE 1 | Illustration of normothermic machine perfusion set up and experimental design. **(A)** Schematic illustration- Perfusion of the circuit is propelled by a centrifugal pump; the perfusate passes through the heater and oxygenator where the perfusate is oxygenated by sweep gas that contains high concentration of O_2 and low concentration of CO_2; effluent perfusate from hepatic vein drains directly into the cannula which routes the perfusate to venous reservoir. **(B)** Experimental Design- Timeline of perfusion and sample collection. (NMP, normothermic machine perfusion).

ammonium-chloride-potassium buffer (Gibco|Thermo Fisher Scientific, Waltham, MA) to lyse remaining red blood cells. Liver tissue was mechanically disrupted and enzymatically digested at 37°C for 90 minutes using 100U/mL of Type II collagenase (Gibco|Thermo Fisher Scientific, Waltham MA) in Hank's Balanced Salt Solution (Gibco|Thermo Fisher Scientific, Waltham MA) prior to filtration through 100-micron filters (Fisher Scientific, Waltham, MA). Samples were then washed and resuspended in phosphate-buffered saline.

Flow Cytometry

Flow cytometry was used to determine the donor leukocyte profile in both the graft and perfusate compartments. Isolated cells were washed and stained with Zombie UV viability dye (Biolegend, San Diego, CA) in phosphate-buffered saline and then stained with the following markers: anti-CD3 Alexa Fluor 700 (Biolegend), anti-CD4 Alexa Fluor 488 (Biolegend), anti-CD8 Brilliant Violet 785 (Biolegend), anti-CD14 Brilliant Violet 650 (Biolegend), anti-CD16 Brilliant Violet 570 (Biolegend), anti-CD19 Brilliant Violet 510 (Biolegend), anti-CD45 Alexa Fluor 532 (Invitrogen, Carlsbad, CA), anti-CD56 PE-Vio770 (Miltenyl Biotec, Bergisch Gladbach, Germany), and anti-CD66b PE-dazzle (Biolegend) (**Supplemental Table 1**). Cells were identified and analyzed using an Aurora full spectrum flow cytometer (Cytek Biosciences, Fremont, CA), which offered the computational capability to subtract tissue autofluorescence from the actual stained samples. Analysis of flow cytometry results was performed using FlowJo Software (Version 10; FlowJo LLC, Ashland, OR) (**Supplemental Figure 1**).

Histology and Immunohistochemistry

About 300 mg of tissue samples were taken as wedge biopsy prior to perfusion (0^{th} hour) and at the end of perfusion (6^{th} hour). Tissue samples were fixed in 10% neutral buffered formalin (Scigen, Gardena, CA) for 24-36 hours prior to embedding in paraffin. Sections were stained with hematoxylin and eosin for qualitative evaluation for sinusoidal and portal triad structural integrity.

Sections were also stained with anti-CD3 (BioCare medical, Pacheco, CA), CD4 (BioCare Medical), DAPI (Akoya Biosciences, Marlborough, MA) and FOXP3 (BioCare medical) (**Supplemental Table 2**), and slides were scanned by Vectra Polaris System (Akoya Biosciences) and analyzed with Inform Automated Image Analysis Software (Akoya Biosciences). A minimum of five regions of interest (ROI) (931 um x 698 um) were selected within each slide, in which number of tissue regulatory T cells (Treg cells) and CD3+ T cells were counted. The percentage of Treg cells out of all CD3+ T cells was calculated for each ROI.

Cytokine Analysis

3mL of perfusate downstream from the organ were collected in ethylenediaminetetraacetic acid coated tube prior to perfusion and at 1^{st} hour, 3^{rd} hour, and 6^{th} hour of perfusion. Samples were spun down at 1200 RPM to obtain supernatant, which were immediately frozen on dry ice. The samples were then processed using Fisher Scientific Multiplex Assay, and the various cytokine levels were measured using Luminex 200 instrument system (Fisher Scientific).

Statistical Analysis

Using flow cytometry, the frequency each immune cell type based on the total number of CD45+ cells was calculated in both perfusate and liver tissue. To illustrate the time dependent, dynamic effect of NMP on each individual cell type in either compartment, percentage change of each cell type frequency at various time points (1^{st} hour, 3^{rd} hour, or 6^{th} hour) relative to 0^{th} hour (cold storage) was calculated.

$$\text{Percentage Change at Time Point(x)} = 100\,\% \times \frac{\text{cell frequency at time point (x)} - \text{cell frequency at 0th hour}}{\text{cell frequency at 0th hour}}$$

Paired student's t-test was used to determine whether the percentage changes for each immune cell type at various time points referenced to 0^{th} hour were statistically significant. All values were presented as mean ± standard deviation. A P value < 0.05 was used for statistical significance.

Mean and standard deviation of concentration (pg/mL) of each cytokine at each time were calculated for all organs. Student's t-test was used to determine if concentration changes between time points were significant.

Using immunohistochemistry, the percentage of regulatory T cells out of total number of CD3+ T cells were calculated for all ROI's from each tissue slide taken from 0^{th} hour and 6^{th} hour. A mean regulatory T cell frequency was assigned to each organ for each time point by averaging all ROI values. Paired Student's t-test was used to compare regulatory T cell frequencies of all organs between time points 0^{th} hour and 6^{th} hour.

RESULTS

Normothermic Machine Perfusion Maintains Donor-Livers Under Physiological Conditions

All 6 donor-livers included in the study were successfully maintained on normothermic machine perfusion. Cold ischemia times varied from 291 to 720 minutes. Perfusion pressures and flow rates were constant and within physiologic range (**Figures 2A–D**). Lactate concentration fell to <3 by 6 hours in all livers except one (0.5^{th} h: 7.41 ± 2.58 versus 6^{th} h: 2.53 ± 1.01, P = 0.009) (**Figure 3A**). Following initiation of normothermic machine perfusion, acidosis within the circulating perfusate improved (0.5^{th} h: 7.35 ± 0.18 versus 6^{th} h: 7.44 ± 0.15, P = 0.047) (**Figure 3B**). Perfusate baseline lactate concentration varied. Aspartate aminotransferase level or alanine aminotransferase level within the perfusate stayed relatively constant throughout the duration of NMP (**Figures 3C, D**). Histological examination of liver tissue after 6 hours of perfusion showed structural integrity of underlying liver tissues with intact portal triad and sinusoids in all 6 livers. In the 2 DCD livers included in this study with longer than 60 minutes of warm ischemia time, focal superficial subcapsular hepatic necrosis was seen on histology. This was absent in the other four livers (**Figure 4**).

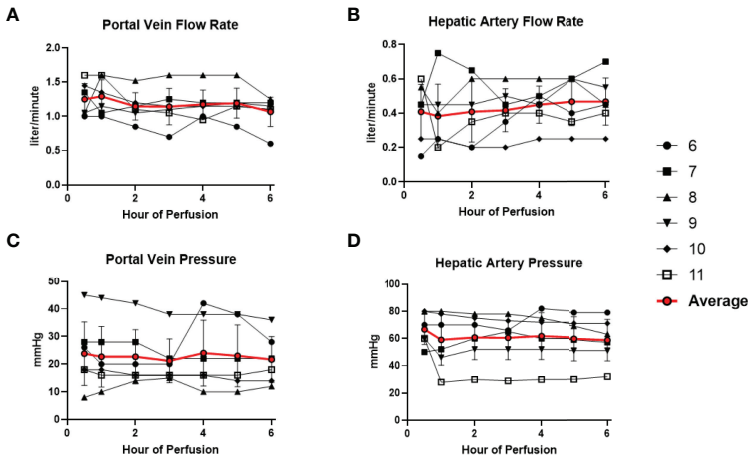

FIGURE 2 | Normal physiologic parameters in all 6 livers were maintained during normothermic machine perfusion. **(A)** Portal vein flow rate (liter/minute) remained stable. **(B)** Hepatic artery flow rate (liter/minute) remained stable. **(C)** Portal vein pressure (mmHg) remained stable. **(D)** Hepatic artery pressure (mmHg) remained stable.

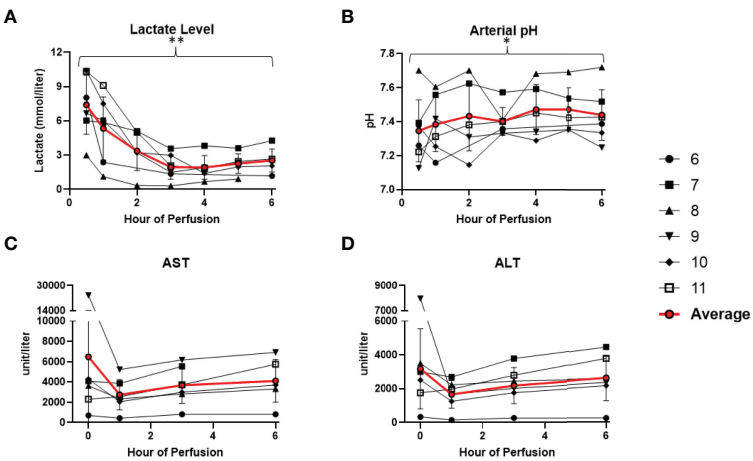

FIGURE 3 | Metabolic parameters improved or remained stable in all 6 livers during normothermic machine perfusion. **(A)** Lactate level (mmol/liter) decreased during perfusion. **(B)** Hepatic artery pH normalized during perfusion. **(C)** Aspartate aminotransferase level (unit/Liter) remained stable. **(D)** Alanine aminotransferase level (unit/Liter) remained stable. (* P<0.05; ** P<0.01).

Cellular Composition of Perfusate and Liver Tissue Is Altered During NMP

Following organ procurement and cold flush of donor-livers, the most abundant resident leukocytes in digested liver tissue were neutrophils (26.6 ± 19.7%), classical monocytes (10.0 ± 4.2%), and macrophages (9.9 ± 7.3%). By contrast, the composition of leukocytes within the perfusate consisted of predominantly neutrophils (55.6 ± 16.3), NK cells (13.1 ± 6.5%), and eosinophils (7.7 ± 10.8%) (**Figure 5**).

Changes in leukocyte composition were seen following NMP. After 6 hours on NMP, resident neutrophils remained the most abundant leukocyte in the tissue, but there was a decrease in the frequency of neutrophils within the overall leukocyte compartment (19.1 ± 17.8%). The second and third most abundant cells types in the liver tissues were macrophages (13.1 ± 10.0%) and NK cells (10.3 ± 7.1%). Similar to analysis of the tissue resident leukocytes, neutrophils were the most abundant leukocytes in the perfusate following 6 hours of NMP, with a lower percentage of neutrophils detected at 6 hours compared to prior to initiation of NMP (36.4 ± 24.5%). The second most abundant cell type were eosinophils (32.6 ± 22.6%), while CD4 T cells constituted a greater relative population following NMP (10.7 ± 4.7%) (**Figure 5**).

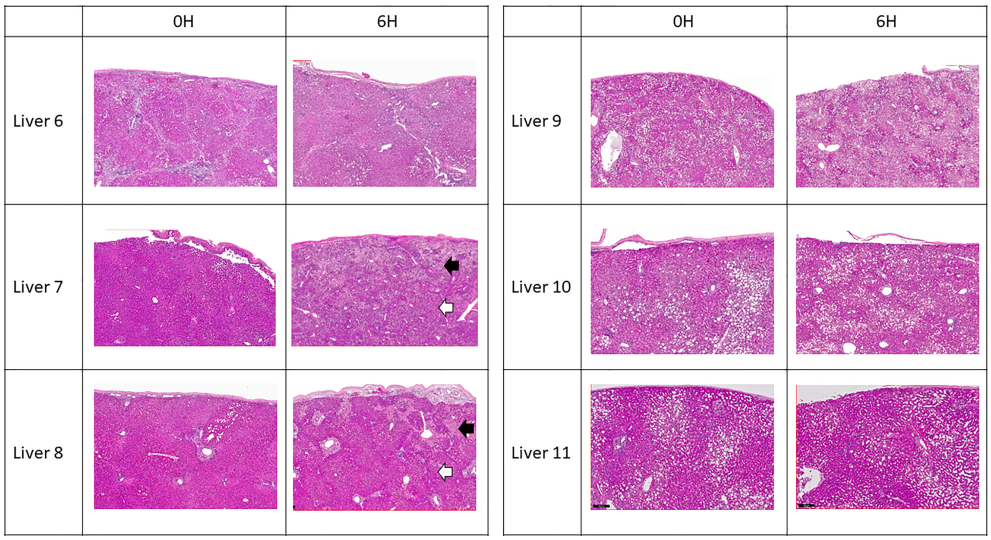

FIGURE 4 | Representative H & E histology of liver tissue. Liver 6 exhibited cirrhotic change. Livers 9, 10 and 11 exhibited variable degree of microsteatotic change. At the end of perfusion, histology from livers 6, 9, 10, and 11 showed completely viable tissue with no structural change. Livers 7 and 8 exhibited patchy subcapsular hepatocyte necrosis (black arrows) with viable liver parenchyma underneath (white arrows).

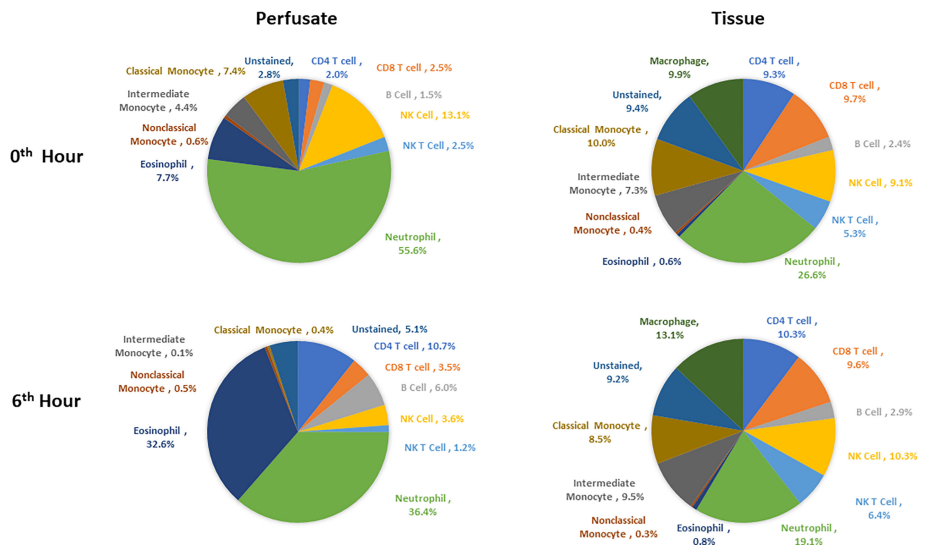

FIGURE 5 | Composition of leukocytes of perfusates and liver tissues prior to perfusion and at the end of perfusion (N=6). Neutrophils were the most abundant cell types in both perfusates and tissues both prior to and at 6th hour of perfusion. Percentages were obtained by dividing the count of each immune cell type by CD45+ cell count for each tissue type at each time point.

Temporal Changes in Tissue Resident Leukocyte Composition During Normothermic Machine Perfusion

Dynamic changes in immune cell composition are seen during the course of NMP. To serially determine the changes in tissue leukocyte composition during NMP, the percent change of each cell type was evaluated at various time points relative to prior to initiation of NMP.

Overall, there was no significant changes within the T cell and NK cell compartments within the tissue among tissue CD8 T cells, CD4 T cells, NK cells, and NK T cells. Tissue B cells constituted a significant higher proportion of leukocytes at the end of normothermic perfusion, with no significant differences observed at earlier time points (+26.3 ± 16.0%) (**Figures 6**, **7**).

Among cells of myeloid lineage, the frequency of tissue neutrophils was significant lower at the 6th hour of NMP

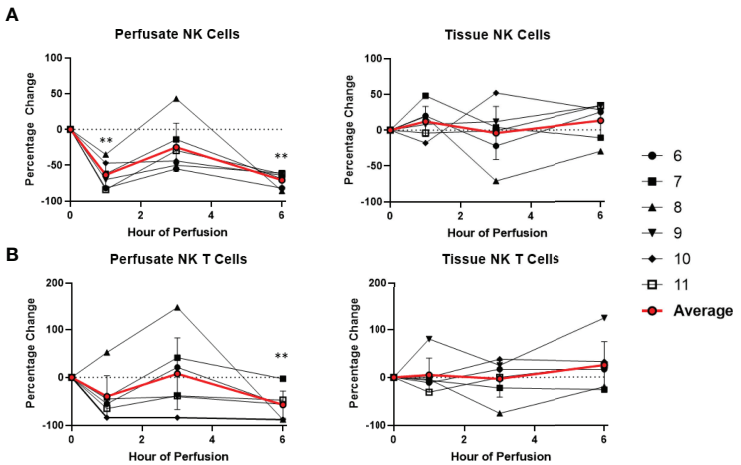

FIGURE 6 | Percentage change of frequency of cells from NK cell lineage during perfusion. A percentage change of 0% suggests there is no change in the innate immune cell frequency from time point 0th hour. Average values of percentage changes (red line) as well as standard deviations were calculated (vertical bar). A two-sided, paired t-test was performed to compare percentages of each time point to those at time 0H. (** P<0.01). **(A)** Perfusate NK cell frequency decreased at the 1st hour of perfusion (-63.4 ± 17.8%) and remained decreased at the 6th hour of perfusion (-70.7 ± 9.8%), without changes to tissue NK cell frequency. **(B)** Perfusate NK T cell frequency decreased at the 6th hour of perfusion (-56.8 ± 28.5%) without changes to tissue NK T cell frequency.

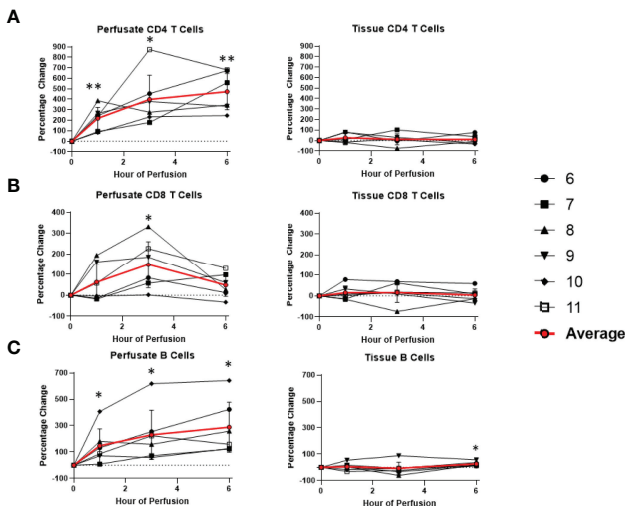

FIGURE 7 | Percentage change of frequency of cells from lymphocyte lineage during perfusion. A percentage change of 0% suggests there is no change in the innate immune cell frequency from time point 0th hour. Average values of percentage changes (red line) as well as standard deviations were calculated (vertical bar). A two-sided, paired t-test was performed to compare percentages of each time point to those at time 0H. (* P<0.05; ** P<0.01). **(A)** Perfusate CD4 T cell frequency increased at the 1st hour of perfusion (+218.9 ± 104.6%) and continued to increase at the 6th hour (+472.6 ± 172.5%) without changes to tissue CD4 T cell frequency. **(B)** Perfusate CD8 T cell frequency increased at the 3rd hour of NMP (+147.4 ± 111.3%) and returned to baseline at the 6th hour without changes to tissue CD8 T cell frequency. **(C)** Perfusate B cell frequency increased at the 1st hour of perfusion (+147.4 ± 127.5%) and continued to increase at the 6th hour of perfusion (+288.5 ± 189.2%), while tissue B cell frequency increased at 6th hour of perfusion (+26.3 ± 16.0%).

(-24.5 ± 21.3%) (**Figure 8**). The frequency of tissue intermediate monocytes dropped in the tissue at the 1st hour (-30.7 ± 19.7%). By the third hour of perfusion, the frequency of tissue intermediate monocyte was similar to baseline (**Figure 9**). No significant change existed in frequencies of tissue eosinophil, classical and non-classical monocytes during NMP compared to cold storage baseline (**Figures 8**, **9**).

Temporal Changes in Perfusate Leukocyte Composition During Normothermic Machine Perfusion

Dynamic changes in the composition of perfusate leukocytes were seen during NMP. Among cells of lymphoid lineage, perfusate CD4 T cell frequency significantly increased at the 1st hour of NMP (+218.9 ± 104.6%) and continued to increase at the 6th hour (+472.6 ± 172.5%). Perfusate B cell frequency likewise increased throughout duration of NMP, starting at the 1st hour (+147.4 ± 127.5%) and continuing to the 6th hour (+288.5 ± 189.2%) (**Figure 7**). On the other hand, a significantly lower proportion of perfusate NK cell was observed (-63.4 ± 17.8%) at the 1st hour of perfusion and remained decreased at the 6th hour of perfusion (-70.7 ± 9.8%) (**Figure 6**). Similarly, perfusate NK T cell frequency decreased at the 6th hour of NMP (-56.8 ± 28.5%) (**Figure 6**). Perfusate CD8 T cell frequency at the 6th hour was comparable to that seen prior to initiation of NMP (**Figure 7**).

Among cells of myeloid lineage, perfusate eosinophil frequency increased throughout the duration of perfusion, from (+249.3 ± 175.3%) at the 1st hour to (+763.1 ± 533.4%) at the 6th hour (**Figure 8**). In contrast, perfusate classical monocytes and intermediate monocyte frequencies both immediately decreased following initiation of NMP at the 1st hour (-91.6 ± 5.8% and -98.3 ± 1.5%, respectively), and remained

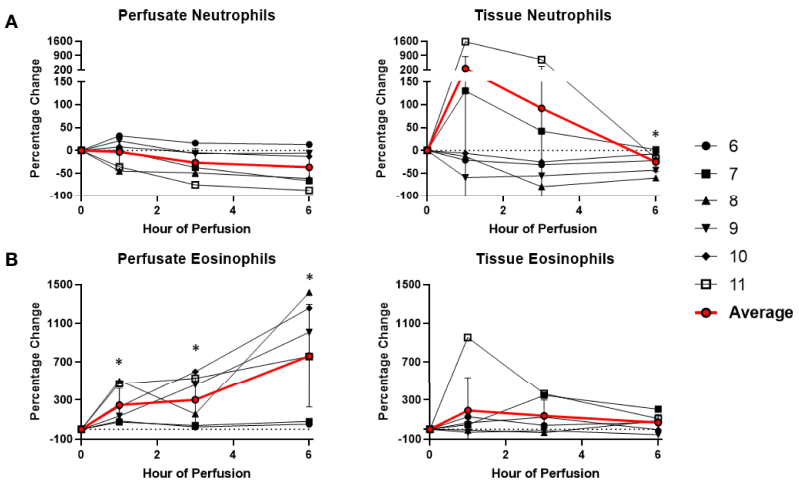

FIGURE 8 | Percentage change of frequency of cells from granulocyte lineage during perfusion. A percentage change of 0% suggests there is no change in the innate immune cell frequency from time point 0th hour. Average values of percentage changes (red line) as well as standard deviations were calculated (vertical bar). A two-sided, paired t-test was performed to compare percentages of each time point to those at time 0H. (* P<0.05). **(A)** No changes were observed in perfusate neutrophil frequency but tissue neutrophil frequency decreased at the 6th hour of perfusion (-24.5 ± 21.3%). **(B)** Perfusate eosinophil frequency increased at the 1st hour of perfusion (+249.3 ± 175.3%) and continued to increase at the 6th hour of perfusion (+763.1 ± 533.4%), without changes to tissue eosinophil frequency.

decreased throughout NMP (**Figure 9**). No significant change was observed in perfusate neutrophil and non-classical monocyte frequencies throughout NMP (**Figures 8**, **9**).

Effect of NMP on Tissue Resident T Regulatory Cells

Given that FoxP3, a critical marker for regulatory T cells (Treg cells), is a transcription factor, immunohistochemistry was used to identify tissue Treg cells. At baseline, 4.1 ± 2.5% of tissue CD3+ T cells were Treg cells. At the end of perfusion, 2.5 ± 2.2% of tissue CD3+ T cells were Treg cells. Compared to baseline, there was no significant T reg cell frequency change at end of perfusion (**Figure 10**). Within individual livers changes to Treg composition were observed, with increase in liver 8 and decrease in liver 9, 10 and 11.

The Effect of NMP on Cytokine Secretion

Among pro-inflammatory cytokines, IL-4, IL-17A, IFN-gamma, and TNF-alpha were below the limit of detection in cold storage. At one hour after initiation of NMP, there was significant increase in IL-2, IL-4, and GM-CSF compared to cold storage. Compared to their levels at 1 hour, IL-1b, IL-2, IL-4, IL-6, G-CSF, IFN-gamma, GM-CSF, TNF-alpha, CXCL2, and CXCL1 continued to increase at 3rd hour of NMP. By the 6th hour of perfusion, compared to cold storage, there were significant increases in IL-4, IL-6, IL-8, G-CSF, GM-CSF, CCL2, CXCL2, CXCL1 and granzyme A. No significant differences were detected in levels of IL-1b, IL-2, IL-17A, IFN-gamma, TNF-alpha and granzyme B between cold storage and at 6th hour of perfusion, despite significant temporary increases in levels of IL-1b, IL-2 and TNF-alpha at the 3rd hour of perfusion (**Table 2**).

With regards to anti-inflammatory cytokines, IL-10 was below the limit of detection in cold storage. However, IL-10 consistently increased throughout duration of NMP starting at the 1st hour of perfusion. At the 6th hour of perfusion, IL-10 had more than 20,000-fold increase compared to baseline cold storage (**Table 2**).

DISCUSSION

NMP devices allow *ex vivo* viability assessment and have been shown to allow successful transplantation of livers that would otherwise be discarded based on specific high-risk criteria (2, 4, 5). In this study we demonstrate serial changes in composition of passenger leukocytes of livers maintained on NMP. Within the donor-liver tissue, a significant increase in cell frequency of B cells and decrease in cell frequency of neutrophils were detected at the end of NMP with stable cell frequencies of other cell lineages. When serial perfusate was analyzed, duration of NMP was associated with increases in frequencies of CD4 T cell, B cells and eosinophils and significant decreases in frequencies of NK cells, classical monocytes and intermediate monocytes. Cytokine analysis demonstrated increased levels of cytokines associated with lymphocytes (IL-2, IL-4, IL-6), granulocytes (CXCL2, CXCL1, G-CSF, IL-8), NK cells (granzyme A, interferon-gamma), monocytes/macrophages (CCL-2, GM-CSF, interferon gamma), as well as increased levels of anti-

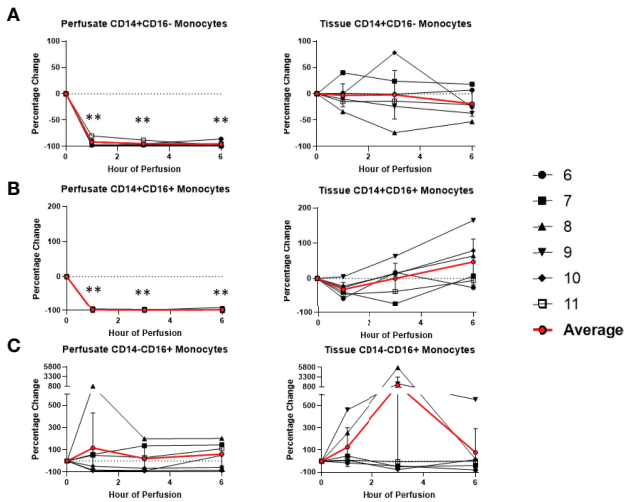

FIGURE 9 | Percentage change of frequency of cells from monocyte lineage during perfusion. A percentage change of 0% suggests there is no change in the innate immune cell frequency from time point 0th hour. Average values of percentage changes (red line) as well as standard deviations were calculated (vertical bar). A two-sided, paired t-test was performed to compare percentages of each time point to those at time 0H. (** P<0.01). **(A)** Perfusate classical monocyte frequency decreased at the 1st hour of perfusion (-91.6 ± 5.8%) and remained decreased at the 6th hour of perfusion (-95.6 ± 4.3%), with no changes observed in tissue classical monocyte frequency. **(B)** Perfusate intermediate monocyte frequency decreased at the 1st hour of perfusion (-98.3 ± 1.5%) and remained decreased at the 6th hour of perfusion (-98.5 ± 2.7%), while tissue intermediate monocyte frequency decreased at the 1st hour of perfusion (-53.2 ± 17.6%) and returned to baseline at the 3rd hour of perfusion. **(C)** No changes were observed in perfusate non-classical monocyte frequency and tissue non-classical monocyte frequency.

inflammatory cytokine associated with lymphocytes and macrophages (IL-10) during NMP.

Previous ex vivo perfusion studies have demonstrated an effect of normothermic machine perfusion on the immune profile of organs. NMP reduced the number of INF-gamma and IL-17-producing T cells and enlarged the regulatory T cell in the liver perfusates collected after NMP compared to cold storage (15). In addition, lungs maintained on *ex vivo* lung perfusion (EVLP) demonstrate a rapid increase in perfusate monocytes at onset of perfusion with abundant non-classical monocytes attached to the leukocyte filter at end of EVLP, suggesting EVLP may remove passenger non-classical monocytes (16). In contrast to previous work assessing leukocyte populations in perfusate during or after machine perfusion of organs, we have characterized the immune profile of liver tissue during machine perfusion in liver. Given that the liver tissue rather than the perfusate is transferred and the immune cells within the graft has been shown to have important immunological effects, our results may have important implications on future work.

We have demonstrated that NMP significantly increased the proportion of T cells that are detectable in the perfusate throughout the course of perfusion. This may suggest that donor tissue T cells are mobilized into the perfusate during NMP. This finding may not be surprising, considering the migration of donor passenger T cells from the donor liver allograft into recipient circulation has been demonstrated in the literature prior to clinical use of NMP (10, 11). However, tissue T cell frequency stayed relatively unchanged throughout the course of NMP. There are several possible explanations for this including that the liver contains a large reservoir of T cells relative to the perfusate. As a result, the increase in detectable T cells within the perfusate during perfusion may represent a small amount of the liver resident T cells. An alternative explanation is that perfusate T cells continuously migrate back into the donor liver tissue, creating a dynamic T cell trafficking loop between the perfusate and tissue compartments. The increased levels of T helper type 1 cell cytokines (IL-2, IFN-gamma), T helper type 2 cell cytokines (IL-4, IL-6) and T cell-associated anti-inflammatory cytokine (IL-10) demonstrate that multiple counteracting molecular pathways are present and may orchestrate a balanced cell migration equilibrium.

We have demonstrated that tissue neutrophil frequency significantly decreased at end of normothermic perfusion, though no significant change was observed in the perfusate neutrophils. The drop in tissue neutrophil frequency suggests that tissue neutrophils are activated and mobilized during NMP. This observation be may explained by ischemia-reperfusion injury of the circuit. In our study, cold ischemia time of donor-livers ranged from 4 hours to 12 hours. The ischemic

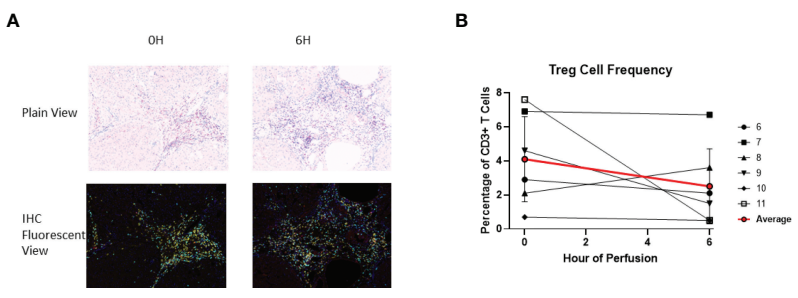

FIGURE 10 | Identification of Treg using IHC (DAPI+CD3+CD4+FoxP3+). **(A)** Representative Slides (Liver 6) from before (0th hour) and following perfusion (6th hour). DAPI (Blue), CD3 (Cyan), CD4 (Yellow), FoxP3 (Red). **(B)** Treg cell frequency out of total CD3+ T cells both before and following perfusion.

TABLE 2 | Cytokine concentrations (pg/mL) in human livers during 6 hour of normothermic machine perfusion.

	Hour 0 of NMPConcentration (pg/mL)	Hour 1 of NMPConcentration (pg/mL)	1H vs 0H P-value	Hour 3 of NMPConcentration (pg/mL)	3H vs 0H P-value	3H vs 1H P-value	Hour 6 of EVLPConcentration (pg/mL)	6H vs 0H P-value	6H vs 1H P-value
IL-1b	8.39 ± 11.08	1.92 ± 0.77	0.226	37.14 ± 26.06	0.086	0.029	252.16 ± 261.41	0.095	0.085
IL-2	1.47 ± 0.43	3.04 ± 1.28	0.023	13.54 ± 7.44	0.016	0.020	37.45 ± 45.91	0.140	0.150
IL-4	0.60 ± 0.80	5.21 ± 3.44	0.037	25.44 ± 3.13	0.000	0.000	24.27 ± 2.91	0.000	0.000
IL-6	14.10 ± 8.12	567.37 ± 628.36	0.106	26368.84 ± 17769.95	0.021	0.020	24707.60 ± 17412.95	0.025	0.025
IL-8 (CXCL8)	43.40 ± 73.04	2815.07 ± 3103.37	0.105	2835.61 ± 1563.92	0.012	0.989	785.97 ± 267.48	0.002	0.197
IL-10	0.19 ± 0.12	186.28 ± 128.71	0.023	3223.56 ± 2154.59	0.020	0.025	4686.07 ± 2901.50	0.015	0.016
IL-17A (CTLA-8)	0.09 ± 0.13	0.22 ± 0.03	0.194	4.00 ± 5.32	0.163	0.180	19.22 ± 21.92	0.108	0.110
G-CSF (CSF-3)	1.58 ± 2.38	0.94 ± 1.80	0.614	1088.88 ± 318.49	0.001	0.001	2482.11 ± 688.53	0.000	0.000
IFN-gamma	0.20 ± 0.12	0.21 ± 0.16	0.941	6.90 ± 5.30	0.038	0.035	220.75 ± 245.05	0.100	0.100
GM-CSF	2.04 ± 0.95	5.91 ± 2.99	0.025	27.11 ± 5.76	0.000	0.000	53.31 ± 35.74	0.023	0.030
TNF-alpha	0.03 ± 0.07	205.68 ± 233.06	0.104	2633.87 ± 2027.38	0.034	0.031	1488.60 ± 1331.19	0.054	0.056
MCP-1 (CCL2)	220.74 ± 117.06	916.22 ± 736.96	0.109	3200.63 ± 3030.23	0.075	0.169	1622.62 ± 706.26	0.006	0.136
MIP-2a (CXCL2)	4.56 ± 4.83	526.88 ± 589.60	0.105	2040.39 ± 1209.96	0.013	0.022	1930.59 ± 996.82	0.008	0.017
GROa (CXCL1)	1.28 ± 2.70	76.63 ± 65.93	0.054	377.16 ± 106.55	0.001	0.001	297.82 ± 69.17	0.000	0.000
Granzyme A	29.80 ± 30.54	61.97 ± 55.69	0.291	87.56 ± 54.01	0.012	0.310	114.26 ± 66.80	0.022	0.019
Granzyme B	5.04 ± 1.19	9.97 ± 4.53	0.078	9.54 ± 4.24	0.071	0.880	125.79 ± 139.88	0.112	0.127

The following cytokines were presented in abbreviations in the table: interleukin-1b (IL-b), interleukin-2 (IL-2), interleukin-4 (IL-4), interleukin-6 (IL-6), interleukin-8 (IL-8) or C-X-C motif chemokine ligand 8 (CXCL8), interleukin-10 (IL-10), interleukin 17-A (IL-17A) or cytotoxic T-lymphocyte-associated antigen 8 (CTLA-8), granulocyte colony-stimulating factor (G-CSF) or colony stimulating factor 3 (CSF-3), interferon-gamma (IFN-gamma), granulocyte-macrophage colony-stimulating factor (GM-CSF), tumor necrosis factor-alpha (TNF-alpha), monocyte chemotactic protein-1 (MCP-1) or C-X-C motif chemokine ligand 2 (CXCL-2), and growth-regulated oncogene alpha (GROa) or C-X-C motif chemokine ligand 1 (CXCL1). Data are presented as mean ± standard deviation.

period was followed by reperfusion on NMP, where there was a shift from metabolic distress caused by ischemia to an excessive innate immune response triggered by reperfusion. The reperfusion phase may induce cell damage within the donor-livers and result in the release of endogenous molecules that served as chemoattractant to neutrophils, as observed by increasing levels of CXCL1 and CXCL2 in the perfusates during NMP (18). Of note, reperfusion of donor-livers in recipients is historically known to cause excessive neutrophil influx to the liver from the vasculature, causing injury following reperfusion (18). However, in NMP, such endogenous source of circulating recipient neutrophils is lacking, and may explain the paradoxical decrease in donor-liver tissue neutrophils that we observed. Interestingly, we did not observe increased perfusate neutrophil cell frequency during NMP, contrary to what we would expect. One potential explanation may be that the exposure of circulating neutrophils in perfusates to non-endothelialized surfaces of the perfusion circuit may cause proinflammatory state, resulting in adherence of neutrophils to circuits. Our findings are consistent with previous studies demonstrating reduced numbers of neutrophil clusters in liver tissue following NMP compared to cold storage with the decrease attributed to neutrophil circulation and possibly to an anti-inflammatory hepatic environment during NMP (15). Neutrophils have been recognized as a key player in liver injury following ischemia reperfusion, and elimination of excessive neutrophils or inhibition of their function may lead to reduction of liver injury and inflammation following transplantation (17). The effect of selective depletion of tissue neutrophils following NMP will be determined in future studies.

However, unlike previous studies, the current study initiated NMP after transport with associated cold ischemia time (4, 5, 15). Despite subjecting each donor liver to different length of cold ischemia time, we observed similar findings in all livers with increased frequencies of perfusate CD4 T cells, B cells and eosinophils and decreased frequency of tissue neutrophils during NMP. This can likely be explained by the controlled oxygenated rewarming employed by our study after substantial cold ischemia time. In prior animal studies, NMP with controlled oxygenated rewarming of liver over an extended period of time after cold storage was shown to result in significantly improved recovery and histopathology upon reperfusion as compared to cold-stored only livers (19, 20). This suggested beneficial role of NMP even after significant cold ischemia time, and the protective role of controlled oxygenated rewarming against liver injury following due to prolonged cold ischemia time.

There are limitations in our study. First of all, the livers included in our study did not meet transplant criteria prior to perfusion. Using these discarded livers, we were able to take serial wedge biopsies to characterize the organ-resident immune profile. While this study would not be possible in livers used to clinical transplant, the livers used are similar in profile to high-

risk livers that have been investigated in clinical trials (VITTAL) (5). All livers included in our study showed ability to clear elevated levels of lactate and transaminases that accumulated during cold ischemia. The observed increased cytokine concentrations throughout perfusion suggested that decreased levels of lactate and transaminases during machine perfusion were not due to potential dilution alone, but may instead suggested preserved liver metabolism (21). The results of the study may therefore be applicable to the cohort of organs that will be placed in machine perfusion systems in the future for feasibility assessment. Future studies will determine whether changes to immune profile of organs influences liver survival during NMP. Secondly, we utilized stored pRBC's that were discarded from clinical use or expired from local blood bank and permissively utilized perfusates with low hematocrits to preserve blood product usage. The maximal time that pRBC's were used for clinical care was 42 days from original date of donation. With our perfusion system, all livers' perfusion parameters were stable with viable liver parenchymal cells on histology. We acknowledge that expired pRBC's may alter immunology. However, recent preclinical trials on NMP similarly utilized stored pRBC's (22). Third of all, we chose to demonstrate our data with cell frequency rather than absolute amounts. This allows comparison between time points and is compatible with flow for detailed cell phenotype data. Cell frequency also allows better reproducibility of results across experiments (23). Finally, it is possible that perfusate passenger leukocytes were consumed by the perfusion circuit during NMP due to cell lysis or cell adhesion to the perfusion tubes, thus contributing to the trends we have observed. However, this phenomenon may be present in clinical NMP as well.

In summary, our study demonstrates changes to the intrinsic immune profile of liver donor-livers during NMP. Furthermore, the changes in immune profiles of the perfusate and the organ don't seem to mirror each other. Given that the organ is transplanted while the perfusate is discarded, our findings may be important in future NMP studies to address this. The results of our study have important implications on future work on novel targeted therapies to recondition donor livers using NMP to expand the organ donor poor, prevent delayed graft failure and induce tolerance.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

AL - study design, data acquisition, data analysis, and writing of manuscript. AE - data acquisition and writing of manuscript. ML- data acquisition and writing of manuscript. VM - data acquisition and writing of manuscript. AS - data acquisition and writing of manuscript. LJ - data acquisition, data analysis, and writing of manuscript. RP - data acquisition. RR - data acquisition. JH - data acquisition. BA - data acquisition. MJ - data acquisition. JM - study design, supervision, and manuscript revision. JD - acquisition of funding and supervision. MM - acquisition of funding, study design, and supervision. RB - acquisition of funding and supervision. DS - acquisition of funding and supervision. KS - acquisition of funding, study design, supervision, and manuscript revision. JF - acquisition of funding, supervision, and manuscript revision. All authors contributed to the article and approved the submitted version.

ACKNOWLEDGMENTS

We thank Peter Friend and Fungai Dengu for their scientific input and critical review of the manuscript. We thank the Gift of Hope Organ and Tissue Donor Network as well as Human Tissue Resource Center, Human Immunologic Monitoring Core Facility, and Cytometry and Antibody Technology Core Facility at the University of Chicago for their assistance in completion of this study.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.788935/full#supplementary-material

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frontiers | Frontiers in Immunology

ORIGINAL RESEARCH
published: 22 June 2022
doi: 10.3389/fimmu.2022.833243

The Immunological Effect of Oxygen Carriers on Normothermic *Ex Vivo* Liver Perfusion

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Introduction: Normothermic *ex vivo* liver perfusion (NEVLP) is an organ preservation method that allows liver graft functional assessment prior to transplantation. One key component of normothermic perfusion solution is an oxygen carrier to provide oxygen to the liver to sustain metabolic activities. Oxygen carriers such as red blood cells (RBCs) or hemoglobin-based oxygen carriers have an unknown effect on the liver-resident immune cells during NEVLP. In this study, we assessed the effects of different oxygen carriers on the phenotype and function of liver-resident immune cells.

Methods: Adult Lewis rat livers underwent NEVLP using three different oxygen carriers: human packed RBCs (pRBCs), rat pRBCs, or Oxyglobin (a synthetic hemoglobin-based oxygen carrier). Hourly perfusate samples were collected for downstream analysis, and livers were digested to isolate immune cells. The concentration of common cytokines was measured in the perfusate, and the immune cells underwent phenotypic characterization with flow cytometry and quantitative reverse transcription polymerase chain reaction (qRT-PCR). The stimulatory function of the liver-resident immune cells was assessed using mixed lymphocyte reactions.

Results: There were no differences in liver function, liver damage, or histology between the three oxygen carriers. qRT-PCR revealed that the gene expression of nuclear factor κ light chain enhancer of activated B cells (NF-kB), Interleukin (IL-1β), C-C motif chemokine ligand 2 (CCL2), C-C motif chemokine ligand 7 (CCL7), and CD14 was significantly upregulated in the human pRBC group compared with that in the naive, whereas the rat pRBC and Oxyglobin groups were not different from that of naive. Flow cytometry demonstrated that the cell surface expression of the immune co-stimulatory protein, CD86, was significantly higher on liver-resident macrophages and plasmacytoid dendritic cells perfused with human pRBC compared to Oxyglobin. Mixed lymphocyte reactions revealed increased allogeneic T-cell proliferation in the human and rat pRBC groups compared to that in the Oxyglobin group.

OPEN ACCESS

Edited by:
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Innsbruck Medical University, Austria

Reviewed by:
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Specialty section:
This article was submitted to Alloimmunity and Transplantation, a section of the journal Frontiers in Immunology

Received: *10 December 2021*
Accepted: *20 May 2022*
Published: *22 June 2022*

Citation:
Jennings H, Carlson KN, Little C, Verhagen JC, Nagendran J, Liu Y, Verhoven B, Zeng W, McMorrow S, Chlebeck P and Al-Adra DP (2022) The Immunological Effect of Oxygen Carriers on Normothermic Ex Vivo Liver Perfusion. Front. Immunol. 13:833243. doi: 10.3389/fimmu.2022.833243

Conclusions: Liver-resident immune cells are important mediators of rejection after transplantation. In this study, we show that the oxygen carrier used in NEVLP solutions can affect the phenotype of these liver-resident immune cells. The synthetic hemoglobin-based oxygen carrier, Oxyglobin, showed the least amount of liver-resident immune cell activation and the least amount of allogeneic proliferation when compared to human or rat pRBCs. To mitigate liver-resident immune cell activation during NEVLP (and subsequent transplantation), Oxyglobin may be an optimal oxygen carrier.

Keywords: liver, transplantation, normothermic perfusion, oxygen carrier, immune system

INTRODUCTION

The rising incidence of chronic liver disease coincides with the rising demand for liver transplantation from a limited pool of organ donors (1, 2). To address this critical organ shortage, clinicians have utilized marginal organs from elderly donors, donation after cardiac death (DCD), and donors with multiple comorbidities, all of which are associated with poorer clinical outcomes in part due to their poor tolerance of the standard preservation method, static cold storage (SCS) (3, 4). In contrast to SCS, normothermic *ex vivo* liver perfusion (NEVLP) is an organ preservation method that maintains the liver at physiologic temperature, thereby preserving metabolic functions. Therefore, NEVLP allows liver graft functional assessment and the opportunity for organ modification prior to transplantation. Clinical studies indicate improved transplant outcomes for organs preserved by machine perfusion (5, 6), and the efficacy of NEVLP has been demonstrated in two randomized clinical trials (7, 8).

To preserve metabolic function during NEVLP, an oxygen carrier, nutrients, and medications are perfused through the liver (9–11). A common NEVLP oxygen carrier is packed red blood cells (pRBCs) (12). However, the use of pRBCs entails certain drawbacks, including hemolysis in centrifugal/rotary pump perfusion systems (13), immune-mediated phenomena, blood-borne pathogen transmission (14), cross-matching difficulties, and pRBC being a limited resource (15). Hemoglobin-based oxygen carriers, such as Hemopure and Oxyglobin (HbO2 Therapeutics), have been presented as a promising alternative to pRBC in *ex vivo* normothermic perfusion procedures (11, 16, 17). Oxyglobin is a polymerized hemoglobin synthesized from bovine RBCs and purified to avoid blood-borne transmissions (16). However, studies comparing pRBC and acellular perfusates lack focused investigation of the immunological effects of various oxygen carriers on the organ being perfused.

Liver-resident immune cells are key players in understanding the development of transplant tolerance or rejection (18, 19). It has been previously shown that NEVLP increases the expression of damage-associated molecular pattern (DAMP) proteins in the liver (20). Furthermore, we found that NEVLP-associated inflammation in the liver affects the phenotype of liver-resident dendritic cells (DCs), which may have undesirable downstream immunological consequences (21). Therefore, reducing the inflammatory environment within the liver during NEVLP for experimental or clinical purposes is of critical importance.

In this study, we performed NEVLP on Lewis rat livers and assessed the immunological effects of three different oxygen carriers by measuring inflammatory cytokine secretion and gene expression signatures. In addition, given the role that liver-resident DCs play in allograft immunogenicity, we specifically evaluated the effect of different oxygen carriers on the phenotype of these cells and assessed their stimulatory function in mixed lymphocyte reactions (MLRs).

MATERIALS AND METHODS

Animals

Male Lewis and Brown Norway rats (Charles River Laboratories, Wilmington, MA, USA) aged 4–15 weeks weighing 320 g ± 11 g [mean ± standard error of the mean (SEM)] were used in all experiments. Animals were housed in specific pathogen-free conditions in animal care facilities at the University of Wisconsin (UW)-Madison in accordance with institutional guidelines. The study protocol was approved by the Institutional Animal Care and Use Committee at the UW-Madison, and all animals were treated ethically.

Animal Surgery and Liver Procurement

Animals were randomly assigned to one of three treatment groups: naive (n = 4), SCS (n = 4), NEVLP with human pRBC (n = 6), NEVLP with rat pRBC (n = 7), and NEVLP with Oxyglobin (n = 8) (**Figure 1**). Surgeries were performed under inhaled 5% isoflurane (Phoenix, St. Joseph, MO, USA) anesthesia for induction and 2%–3% isoflurane maintenance. After disinfection with Betadine (Purdue Pharma LP, Stamford, CT, USA), the abdominal cavity was opened by a midline and transverse incision and the portal vein was exposed. The common bile duct was cannulated with a 24-gauge angiocatheter (BD Biosciences). The hepatic artery and gastrosplenic and duodenopancreatic branches of the portal vein were isolated and ligated. Heparin (400U, Fresenius Kabi, Lake Zurich, IL, USA) was injected through the inferior vena cava and allowed to circulate for 5 min. The portal vein was then cannulated with a 1.3-mm miniball cannula with basket tip (Harvard Apparatus, Holliston, MA, USA) and flushed with 20 ml of cold 0.9% saline (Baxter). Livers were then explanted and weighed. Naive livers were processed immediately, SCS

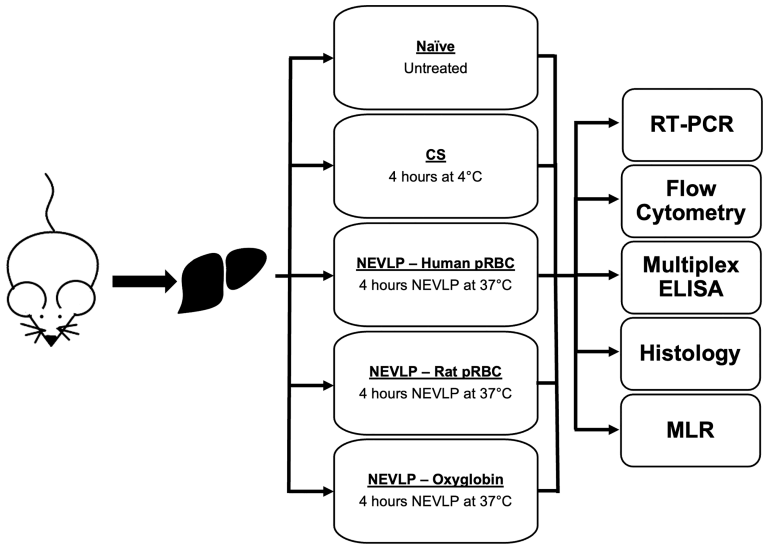

FIGURE 1 | Experimental design. pRBC, packed red blood cells; NEVLP, normothermic ex vivo liver perfusion; CS, Cold storage.

livers were stored on ice for 4 h and then processed, and NEVLP livers were connected to the perfusion machine with minimal cold ischemic time (<5 min).

Perfusate Composition

NEVLP perfusate was composed of William's E Media (Quality Biological, Gaithersburg, MD, USA; 65 ml for pRBC oxygen carriers and 50 ml for Oxyglobin) with the following additives: 3,250U each penicillin/streptomycin (Life Technologies Corporation, Grand Island, NY, USA), 500U heparin (Fresenius Kabi, Lake Zurich, IL, USA), 1 mg insulin (Sigma-Aldrich), 1.25 mg hydrocortisone (Pfizer, New York, NY, USA), and 15 mg papaverine (American Regent, Shirley, NY, USA). In addition, the total perfusate volume of 100 ml contained sodium pyruvate 0.65 mM (Sigma-Aldrich, St. Louis, MO, USA), L- glutamine 1.30 mM (Sigma-Aldrich), and human albumin 1% (Baxter).

The oxygen carriers added to the above perfusate were human pRBC, rat pRBC, or Oxyglobin. Human pRBC (30 ml; American Red Cross, Madison, WI, USA) of blood type A were leukoreduced, irradiated, and used within expiration date. Pooled rat pRBC was collected from the aortic puncture of donor Lewis rats into citrate phosphate dextrose adenine (CPDA) and centrifuged for 10 min at 2,200g. Plasma and buffy coat were discarded, and pRBC was resuspended in AS-5, then drop-filtered through an Acrodisc WBC filter (Pall Gellman), stored at 4°C, and used within 1 week (30 ml). Oxyglobin (46 ml) was kindly provided by HbO2 Therapeutics, LLC (Souderton, PA, USA).

Machine Perfusion

NEVLP was performed as previously described (17). Briefly, warmed oxygenated perfusate was circulated through the portal vein at 1.8 ml/min/g liver by peristaltic pump. Temperature, pressure, flow rate, and oxygen saturation were monitored throughout perfusion (Hugo Sachs Elektronic, Harvard Apparatus). The oxygenator was supplied by 95% O_2/5% CO_2 gas to maintain >95% saturation of the perfusate; the temperature of the liver was maintained at 37°C.

An i-STAT point-of-care analyzer (CG4+ and CHEM8+ cartridges, Abbott Point of Care Inc., Abbott Park, IL, USA) was used for hourly inflow perfusate testing. Samples for qPCR and ELISA were collected from the inflow port and centrifuged for 15 min at 20,000g, and supernatants were stored at -80°C until further processing.

Liver Damage and Inflammation Assessment

For liver damage analysis, liver-type arginase 1 (ARG1), aspartate transaminase 1 (AST), α-glutathione S-transferase (GSTα), sorbitol dehydrogenase (SDH), and 5′-nucleotidase (5′-NT) were measured in the perfusate by Milliplex™ Rat Liver Injury Panel (Millipore Sigma, Billerica, MA, USA) according to the manufacturer's recommendations. Data were acquired on the Luminex MAGPIX (Austin, TX, USA) and analyzed in the Belysa software package v1.0.19. For perfusate cytokine analysis, the LEGENDplex™ Rat Inflammation Panel (BioLegend, San Diego, CA, USA) was used to detect IL-1α, IL-1β, IL-6, IL-10, IL-12p70, IL-17A, IL-18, IL-33, CXCL1, CCL2 [Monocyte Chemoattractant Protein-1 (MCP-1)], granulocyte-macrophage colony-stimulating factor (GM-CSF), interferon (IFN-γ), Tumor necrosis factor (TNF-α), according to the manufacturer's recommendations. Data were acquired on a BD FACS Calibur and analyzed in LEGENDplex™ software package v7.1.21.

Histopathology and Immunohistochemistry

Prior to liver digestion, left median lobe liver sections were fixed in 4% paraformaldehyde and embedded in paraffin. Slides cut at 4-μm thickness were stained with hematoxylin and eosin (H&E).

An expert liver pathologist (YL) blindly scored the severity of histologic damage to the liver according to the Suzuki criteria (21). Representative micrographs were collected at appropriate magnification using an Olympus DP73 equipped microscope (Olympus, Tokyo, Japan).

Liver-Resident Immune Cell Isolation

Liver-resident immune cells were isolated as previously described (21). Briefly, 100 ml of warmed 1× Hank's balanced salt solution (HBSS, Worthington, Lakewood, NJ, USA) containing 25 mM N-2-Hydroxyethylpiperazine-N'-2-Ethanesulfonic Acid (HEPES) (Sigma-Aldrich), 4.2 mM $NaHCO_3$ (Sigma-Aldrich), and 83 μM Ethylenediaminetetraacetic acid (EDTA) (Sigma-Aldrich) was perfused through the portal vein. Subsequently, 100 ml of warm 1× HBSS with 25 mM HEPES, 4.2 mM $NaHCO_3$, 1.26 mM $CaCl_2$ (Sigma-Aldrich), 490 μM $MgCl_2$ (Sigma-Aldrich), 406 μM $MgSO_4$ (Sigma-Aldrich), and 50 mg of Collagenase Type IV (Worthington) was perfused through the liver for 15 min to disrupt the extracellular matrix. Tissue was then mechanically disrupted and passed through a 100-μM cell strainer. Hepatocytes were pelleted using centrifugation at 70g for 3 min, and non-parenchymal cells (NPCs) in the supernatant were transferred to a fresh tube. After a second hepatocyte-removal step, the NPCs were pelleted at 300g centrifugation for 5 min. RBCs were lysed by incubation in 2 ml of ACK buffer (Sigma-Aldrich) for 2 min at room temperature. NPCs underwent a 25:50 Percoll gradient to isolate liver-resident immune cells for downstream analyses.

Flow Cytometry

An aliquot of isolated liver-resident immune cells was incubated at room temperature with mouse anti-rat Fc block (BD Biosciences), followed by fluorochrome-labeled monoclonal antibodies (**Supplementary Table S1**). Data were acquired on an Aurora spectral flow cytometer (Cytek, Bethesda, MD, USA) calibrated according to the manufacturer's recommendations using appropriate controls. Data were analyzed in FCS Express 7 (DeNovo software, Pasadena, CA, USA).

Quantitative Reverse Transcription Polymerase Chain Reaction

An aliquot of isolated liver-resident immune cells was immediately lysed using the RNeasy Mini Kit (Qiagen, Valencia, CA, USA) RLT buffer; mRNA was isolated following the manufacturer's protocol. Reverse transcription was performed using an iScript cDNA synthesis kit (Bio-Rad) according to the manufacturer's instructions. The NCBI primer design tool was used to identify accurate forward and reverse primers, which were then synthesized as 20–25-base pair DNA oligonucleotides (Integrated DNA Technologies, Newark, NJ, USA; **Supplementary Table S2**). Quantitative reverse transcription polymerase chain reaction (qRT-PCR) was performed using an Applied Biosystems 7500 fast detection system (Applied Biosystems, Foster City, CA). Total cDNA was amplified in 20 μl of PCR mix containing 250 nM of each primer, 1× SYBR Green Supermix (Bio-Rad), 5 ng cDNA, and nuclease-free water (Ambion, Inc., Austin, TX, USA) for 40 cycles. Gene expression was interpreted relative to the glyceraldehyde 3-phosphate dehydrogenase housekeeping gene using the $2^{-\Delta Ct}$ method.

Mixed Lymphocyte Reactions

Freshly isolated lymph node-derived lymphocytes from naive Brown Norway rats were collected as responder cells for MLRs. Cryopreserved liver-resident immune cells, isolated from the livers of control and experimental Lewis rats, were recovered to serve as allogeneic stimulator cells. In Phosphate buffered saline (PBS), at a concentration of 1×10^6 cells/ml, stimulators were labeled with a 1:1,000 dilution of Cell Trace Violet (CTV), while responders were separately labeled with a 1:1,000 dilution of Cell Trace Far Red (CTFR) (Thermo Fisher Scientific, Waltham, MA, USA). Stimulator cells then received 20 grays of irradiation prior to culture. In a 96-well plate, 2×10^5 responders were plated with 0.5×10^5 irradiated stimulators in RPMI media supplemented with 10% fetal bovine serum (FBS). Negative controls were established using irradiated CTFR-labeled leukocytes derived from naive Brown Norway livers. Non-specific stimulation using activating plate-bound anti-CD3 (1ug/mL; BD Biosciences) and soluble anti-CD28 (1 μg/ml; BD Biosciences) served as positive controls. All experimental conditions were performed in triplicate. After 4 days of culture at 37°C, cells were collected and flow cytometry data were acquired using an LSR II FACS machine (BD Biosciences). Data analysis was performed using FlowJo software (Tree Star, San Carlos, CA, USA).

Statistical Analysis

Statistical analyses were performed in GraphPad Prism v.8.3.1. Dunnett's multiple comparisons test or two-tailed t-tests were used as appropriate unless otherwise indicated. A p value <0.05 was considered significant.

RESULTS

Liver Function During Normothermic *Ex Vivo* Liver Perfusion

Liver perfusion quality and liver function were assessed throughout each NEVLP experiment. All NEVLP livers demonstrated uniform perfusion and were free of gross ischemia (**Figure 2A**). Perfusate lactate concentrations were measured hourly, and the rate of lactate clearance was calculated between each subsequent hour of perfusion. There were no significant differences in lactate clearance rate observed between the groups (**Figure 2B**). Total bile production was

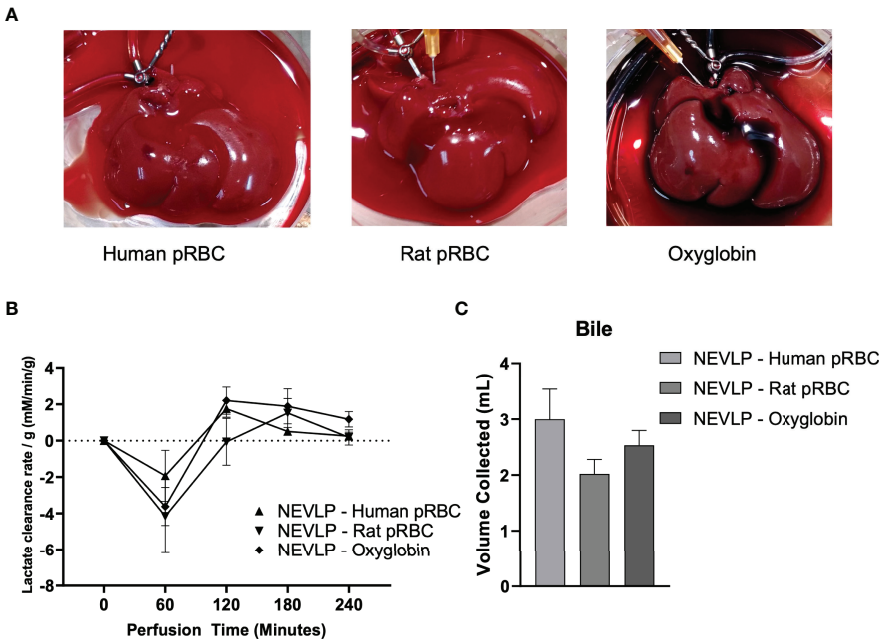

FIGURE 2 | Normothermic machine liver preservation. Livers in the normothermic ex vivo liver perfusion arms underwent 4 h of normothermic preservation with human pRBC, rat packed red blood cells, or Oxyglobin as the oxygen carrier. Oxygen saturation of the perfusate was maintained at >95% throughout the duration of each perfusion, and no significance was found in portal vein pressure between groups (data not shown). Perfusate samples were collected once hourly for assessment of lactate, liver damage, and cytokine concentrations. **(A)** Representative livers undergoing perfusion from each treatment group. **(B)** Lactate clearance of livers undergoing perfusion with different oxygen carriers. **(C)** Bile was collected from the bile duct during perfusion. Due to technical failures with bile duct cannulation (2 in the human pRBC, 1 in the rat pRBC, and 2 in the Oxyglobin groups), bile production data consist of n = 4 for human pRBC and n = 6 for rat pRBC and Oxyglobin groups. Data are shown as mean ± SEM. pRBC, packed red blood cells; NEVLP, normothermic ex vivo liver perfusion.

measured at the conclusion of most experiments (**Figure 2C**), with no significant differences observed between groups.

Liver Injury and Cytokine Production During Normothermic *Ex Vivo* Liver Perfusion

To evaluate liver injury caused by NEVLP, we analyzed the perfusate samples for markers of liver damage. Levels of ARG1, SDH, 5′-NT, and GSTα, markers of tissue injury, did not reveal any significant differences between groups, although all trended higher over the course of perfusion. In contrast, AST levels in the human pRBC group trended higher than the other groups at 0 and 60 min and were significantly higher at 240 min ($p < 0.001$; **Figure 3A**).

To evaluate the damage to the liver caused by NEVLP using different oxygen carriers, we performed a histopathological analysis of liver architectural damage following the Suzuki criteria. Representative H&E images demonstrate similar findings between all groups, showing that oxygen carriers during machine perfusion had little effect on liver architecture damage (**Figures 3B, C**).

To determine the cytokine production capacity of liver-resident immune cells during NEVLP, we assessed the perfusate for various immune-related cytokines. During NEVLP, samples were collected at 0, 60, and 240 min of perfusion, and cytokines were evaluated with a multiplex ELISA using the cell-free perfusate supernatants. There were no significant changes in pro-inflammatory or anti-inflammatory cytokines during the perfusion between different oxygen carriers (**Supplementary Figure S1**).

Quantitative Reverse Transcription Polymerase Chain Reaction Indicates Increased Pro-Inflammatory Cytokine Production in Human pRBC-Based Perfusates

Gene expression levels of IL-1β, CCL2, CD14, NF-κB, IL-1α, IL-6, IL-12a, TNF-α, CCL7, CXCL1, and IFN-α were measured in isolated liver NPCs by qRT-PCR. NF-κB and inflammatory markers IL-1β, CCL2, CCL7, and CD14 were significantly upregulated in the human pRBC NEVLP group compared to those in naive liver, whereas other groups were indistinguishable from naive (**Figure 4**). IL-1α, IL-6, IL-12a, TNF-α, CXCL1, and IFN-α expression levels were not significantly different between groups.

CD86 Expression Is Higher in Human pRBC-Based Perfusate by Flow Cytometry

Isolated liver NPCs were analyzed using flow cytometry using a gating strategy we recently described to identify liver-resident

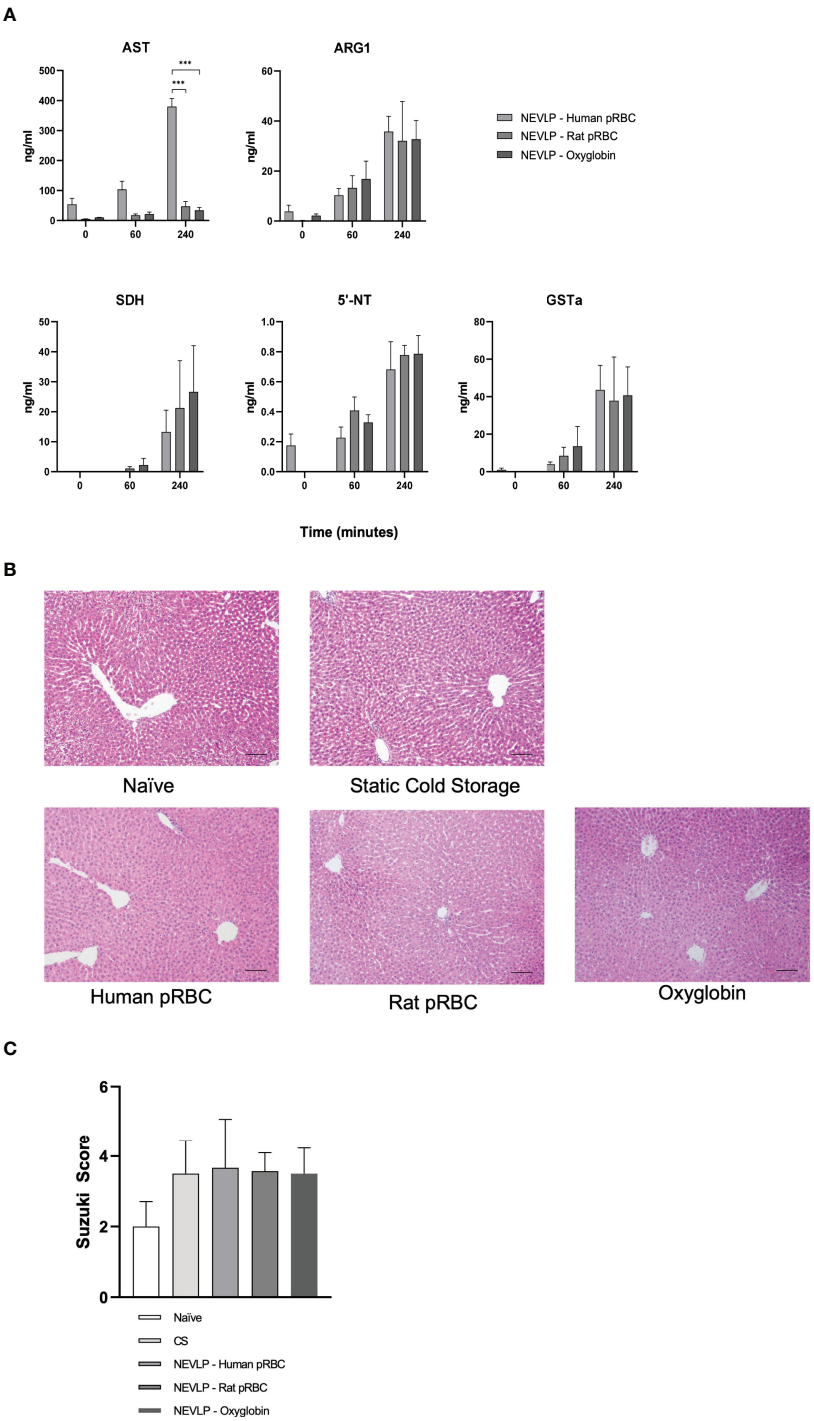

FIGURE 3 | Oxygen carriers have no effect on liver damage during NEVLP. Liver injury was assessed by the presence of liver damage-associated enzymes in the perfusate and histology. **(A)** Markers of liver damage were measured in the perfusate hourly and at the conclusion of NEVLP. These experiments showed elevations in AST in the human RBC oxygen carrier group. Perfusate samples from one human pRBC and one rat pRBC NEVLP experiment were mislabeled, and timing could not be confirmed. Therefore, these samples were omitted from perfusate analysis (data consist of n = 5 for human pRBC, n = 3 for rat pRBC, and n = 5 for the Oxyglobin group). **(B)** Representative micrographs of livers stained with H&E for each treatment group. **(C)** Quantitative analysis of liver damage using the Suzuki criteria. No significant differences were found between treatment groups, and overall levels of damage were low. ***$p < 0.001$.

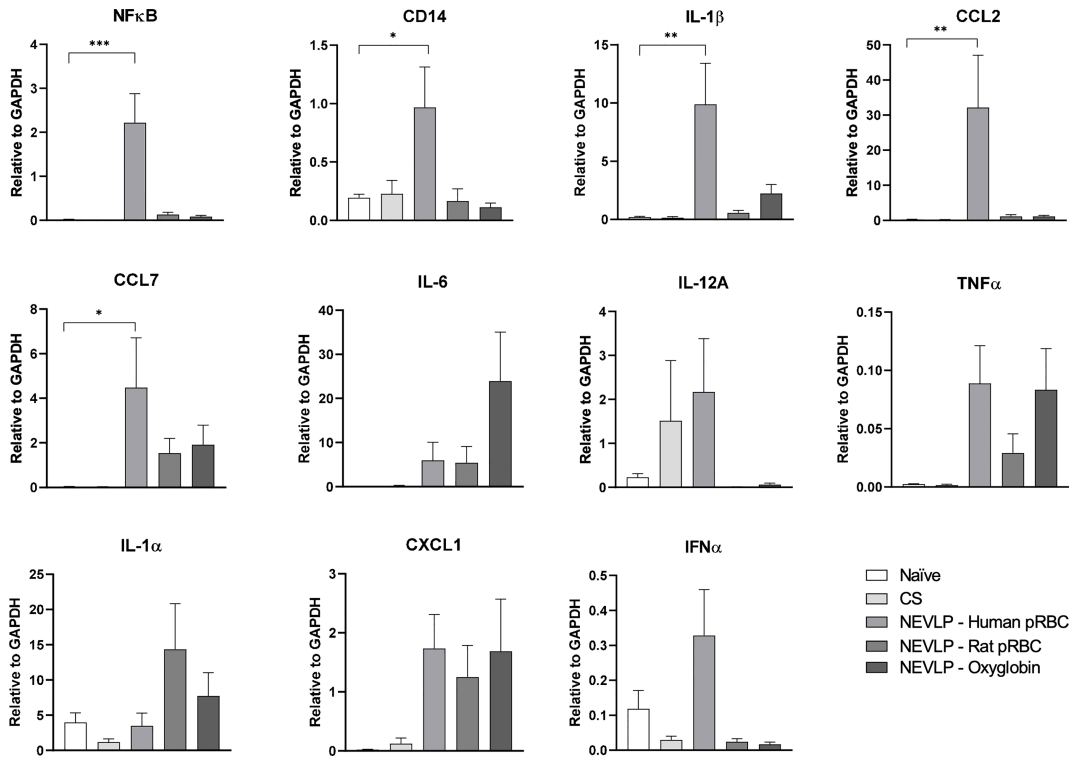

FIGURE 4 | Inflammatory markers are elevated when using human pRBC for NEVLP. qRT-PCR was used to measure the gene expression of inflammatory and anti-inflammatory markers in NPCs. There were no significant differences in the majority of the markers assessed; however, the levels of IL-1, CCL2, CCL7, CD14, and NF-κB were higher in the human pRBC compared to those in naive. One human pRBC and one rat pRBC NEVLP experiment had poor cell yield after liver digestion and NPCs were prioritized for flow cytometry; therefore, data consist of naive (n = 4), SCS (n = 4), NEVLP with human pRBC (n = 5), NEVLP with rat pRBC (n = 6), and NEVLP with Oxyglobin (n = 8). *p < 0.05, **p < 0.01, ***p < 0.001.

classical DCs (cDCs), plasmacytoid DCs (pDCs), and macrophages (22). CD86 surface expression on macrophages and pDCs was significantly higher in the rat pRBC NEVLP group compared to that in the Oxyglobin group ($p < 0.01$ and $p < 0.05$, respectively). MHCII, CD11b/c, CD40, CD80, and PD-L1 expression levels were different from those in naive samples; when comparing between each of the oxygen carrier groups, none reached statistical significance (**Figure 5**).

Decreased Allogeneic Stimulation in Oxyglobin-Based Perfusate

Isolated liver NPCs were assessed for their ability to stimulate allogeneic responder cells in MLRs. Liver-resident immune cells isolated from the Oxyglobin group were the least stimulatory in MLRs when compared to either human or rat pRBCs (**Figure 6**).

DISCUSSION

In this study, our goal was to examine the immunological effects of common oxygen carriers used during NEVLP on the liver and liver-resident immune cells. Although prior studies have investigated the effect that different oxygen carriers have on organ function during normothermic perfusion (reviewed in Bodewes et al. (16), this is the first study to assess the effects of oxygen carriers on the tissue-resident immune cells.

We first show that using human pRBCs, rat pRBCs, or the hemoglobin-based oxygen carrier, Oxyglobin, as oxygen carriers in the perfusate, that each can maintain a functional liver during NEVLP. This is demonstrated by the lactate clearance and bile production of the livers during perfusion (**Figure 2**). Although the enzyme markers of liver damage increased throughout perfusion, in accordance with prior studies (20, 21), these increases were mostly independent of the oxygen carrier as shown by multiplex ELISA (**Figure 3**). The notable exception to this trend was AST in the human pRBC group, which increased rapidly and significantly throughout NEVLP when compared to those of both rat pRBC and Oxyglobin. However, given no differences in the four other liver damage-associated analytes measured, the rise in AST may be more indicative of hemolysis of the human pRBCs as opposed to liver damage (23). Although the human pRBCs were used within the expiration date, preanalytical variability in human donors, such as age and storage time (24), may have made these cells susceptible to breakdown with subsequent release of AST. However, prior studies have shown that rat RBCs show decreased deformability and membrane rigidity when compared

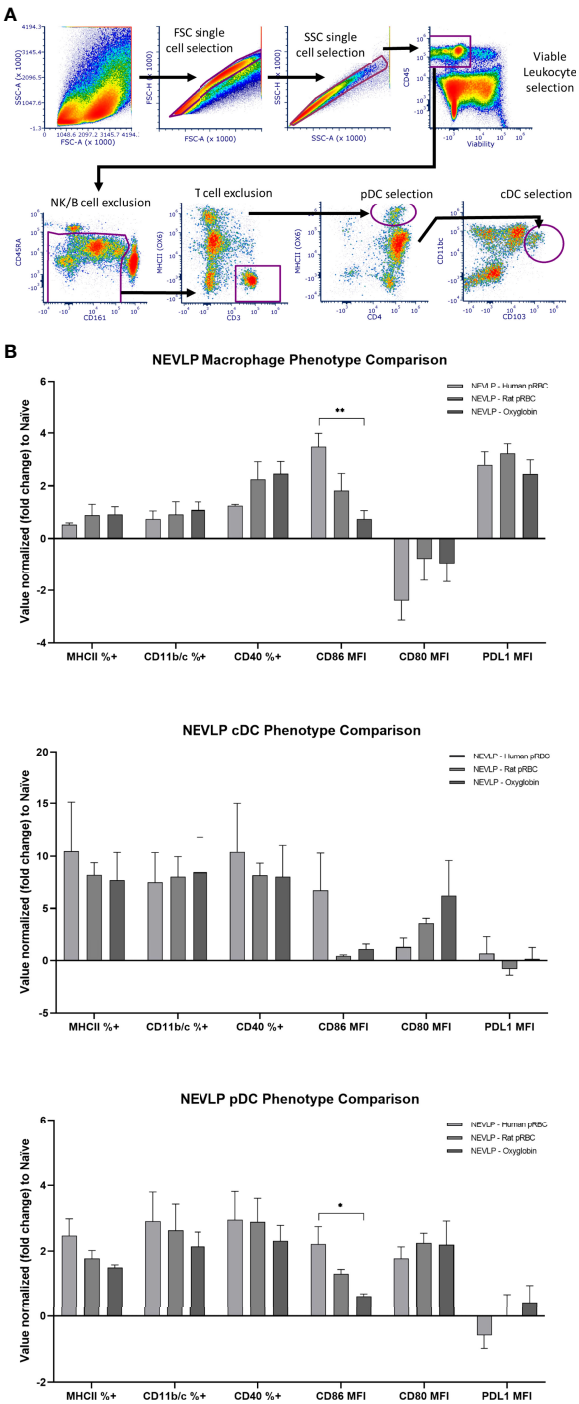

FIGURE 5 | CD86 expression is higher on macrophages and pDCs after NEVLP perfused with human pRBCs. **(A)** Liver-resident immune cells were identified in the NPC compartment using the following gating strategy: after single cells and live cells were identified, immune cell identification with CD45, T-cell and B-cell exclusion using CD3 and CD45RA, and NK cell exclusion of CD161hi/MHC II- cells were done. cDCs (CD45+, CD103+, CD11bc+), pDCs (CD45+, CD4+, MHCII+, CD103-, CD11bc-), and macrophages (CD45+, CD4+, MHCII+, CD103lo, CD11bc+) were then identified as shown. **(B)** Populations were gated into positive vs. negative phenotypes when appropriate. Median fluorescence intensity (MFI) was used for signals showing variability on a gradient. Data consist of naive (n = 4), SCS (n = 4), NEVLP with human pRBC (n = 6), NEVLP with rat pRBC (n = 5), and NEVLP with Oxyglobin (n = 7). *p< 0.05, **p < 0.01.

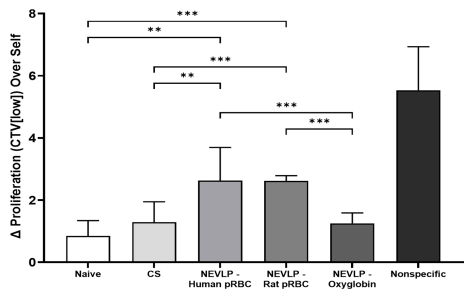

FIGURE 6 | Allogeneic MLR responses are elevated after NEVLP with human pRBC or rat pRBC. Lymph node-derived Brown Norway leukocytes were cocultured with irradiated Lewis liver-resident immune cells isolated from control (naive, CS) or experimentally perfused (NEVLP-Human pRBC, NEVLP-Rat pRBC, NEVLP-Oxyglobin) livers. The MLR response was measured after 4 days in culture by the delta (Δ) increase of T-cell (CD3+) proliferation over an autologous (Brown Norway liver-resident immune cell stimulator) internal control. Statistical comparisons between allogeneic conditions (Lewis-stimulated) are shown (**$p < 0.01$, ***$p < 0.001$). The nonspecific positive control, stimulated with T cell-activating antibodies (anti-CD3/anti-CD28), yielded a significant ($p < 0.001$) increase in T-cell proliferation compared to all allogeneic conditions. Triplicate data were derived from n = 4 for Oxyglobin, n = 3 for human pRBC, and n = 2 for naive, CS, rat pRBC, and nonspecific groups.

to human RBCs, which may make them more susceptible to hemolysis (25). Nevertheless, all oxygen carrier groups showed low levels of tissue damage histologically, further supporting that RBC-based and hemoglobin-based oxygen carriers can be used in NEVLP (16, 26).

In the current study, there were no significant variations in pro-inflammatory or anti-inflammatory cytokines produced by the liver during NEVLP between the different oxygen carriers (**Supplementary Figure S1**). However, there were significant differences in the gene expression levels of certain inflammatory genes based on the oxygen carrier present during NEVLP. Specifically, in the human pRBC group, there were significantly upregulated levels of CD14, CCL2, NF-κB, and IL-1β when compared to naive liver, indicating Toll-like receptor signaling and inflammasome priming (27) (**Figure 4**). This inflammation associated with human pRBC as a perfusate component is consistent with previous reports of DAMPs released during machine perfusion (20). In contrast, inflammatory gene expression levels in the syngeneic rat pRBCs and Oxyglobin groups were not significantly different from those in naive livers.

Given the importance of liver-resident immune cells on transplantation rejection (18, 19), we sought to assess how oxygen carriers in the NVELP perfusate affect their phenotype and stimulatory function. Using spectral flow cytometric analysis of liver-resident immune cells, we demonstrated that NEVLP with human pRBCs led to a significant increase in CD86 expression on both macrophages and pDCs when compared to that in the Oxyglobin group (**Figure 5**). A higher expression of this costimulatory protein indicates that the liver-resident immune cells were activated in this group (28). These data suggest a less activated immune phenotype on liver-resident immune cells when rat pRBCs or Oxyglobin is used as an oxygen carrier. The activated immunophenotype of liver-resident macrophages and pDCs correlated with the stimulatory function seen in the MLRs with allogeneic responder cells. In these experiments, Oxyglobin showed no significant differences in stimulation over the CS or naive groups, while the human and rat pRBC groups demonstrated significantly higher stimulation indices (**Figure 6**).

Normothermic perfusion can create an inflammatory environment within the organ with subsequent increase in DAMPs and activation of tissue-resident immune cells (20, 21, 29, 30). These activated immune cells can have negative downstream consequences, and mitigating their inflammatory potential is of major clinical interest. For example, Noda et al. (30) reported using a leukocyte filter to remove these activated lymphocytes to improve lung function after normothermic preservation, and Scheuermann et al. (20) found that sub-normothermic temperatures could decrease inflammation markers. In our prior study, we added anti-inflammatory cytokines to the NEVLP circuit to keep liver-resident immune cells in a quiescent state. However, the effects of oxygen carriers on the inflammation observed in normothermic perfusion have not been fully elucidated.

One of the most common normothermic preservation oxygen carriers is pRBCs (12). However, there are potential issues with pRBCs as oxygen carriers, including immune-related phenomena and transmission of blood-borne infections (14, 31). In addition, RBCs can undergo loss of structural integrity over the course of machine perfusion, resulting in hemolysis and the release of free hemoglobin (13). Extracellular heme is a DAMP that engages inflammasome activation as a key mediator of inflammation in macrophages (32) and sustain a cycle of oxidative stress (33). Therefore, hemolysis of human pRBCs in the current study could explain the higher levels of CD14, CCL2, NF-κB, and IL-1β. However, as mentioned above, rat pRBCs may be more prone to hemolysis (25), yet there were no significant differences in the levels of any inflammatory markers in the liver-resident immune cells in this NEVLP group. Although this study did not identify the mechanism of increased inflammation in the human pRBC group, it is worth noting the effects that the oxygen carrier can have on subsequent immunological analysis.

Acellular hemoglobin-based oxygen carriers have been found to be an acceptable alternative to pRBCs for *ex vivo* organ perfusion (16). In this study, the hemoglobin-based oxygen carrier, Oxyglobin, was associated with the least amount of immune activation on liver-resident immune cells during NEVLP. As such, this study provides further support for the use of Oxyglobin as an oxygen carrier for use in NEVLP. However, hemoglobin-based oxygen carriers have been associated with vasoconstriction and the formation of methemoglobin during longer perfusions. Therefore, the decreased immune activation with Oxyglobin must be balanced with the limitations of this oxygen carrier in NEVLP (34).

Limitations of this study include the relatively short perfusion time, which was sufficient to observe differences in gene expression signatures and immune cell activation but not long enough to observe differences in cytokine production between oxygen carrier groups. Indeed, our perfusion time of 240 min is

much shorter than the average clinical machine perfusion time of 548 min (7). Longer preservation times in a large animal and human model will be required in future studies to determine subtle differences in liver cytokine production. Another limitation is that although we demonstrated phenotypic and functional differences in liver-resident immune cells between oxygen carrier groups, we did not assess the relevance of these findings *in vivo*. Future studies using an allogeneic transplant model are required to investigate the *in vivo* effects of activated liver-resident immune cells following NEVLP.

In conclusion, we demonstrate that human pRBC, rat pRBC, and Oxyglobin are suitable oxygen carriers for use in NEVLP; however, Oxyglobin is associated with less liver-resident immune activation.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary files, further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by the Institutional Animal Care and Use Committee at UW-Madison.

AUTHOR CONTRIBUTIONS

HJ: Research design, performance of the research, data analysis, and writing the paper; KC: Research design, performance of research, and data analysis; CL: Performance of research, data analysis, and writing the paper; JV: Performance of research; JN: Data analysis and writing the paper; YL: Performance of the research; BV: Performance of the research; WZ: Performance of the research SM: Performance of the research and data analysis PC: Performance of the research, data analysis, and writing the paper; DA-A: Research design, performance of the research, data analysis, and writing the paper. All authors contributed to the article and approved the submitted version.

FUNDING

This research was supported by the National Institute of Health (NIH) K08AI155816 awarded to DA.

ACKNOWLEDGMENTS

We would like to thank the UW Carbone Cancer Center Flow Cytometry Laboratory and Experimental Animal Pathology Laboratory, which are supported by NIH/NCI P30 CA014520, and the Wisconsin Institutes for Medical Research small animal vivarium for their support. In addition, we thank the UW-Madison, Department of Surgery, Histology Core Lab, Dr. Susan Thibeault, PhD, CCC-SLP, PI of the DOS Histology Core, along with certified Histotechnician, Sierra Raglin HTL (ASCP), and Lab Supervisor, Sara Dutton Sackett, PhD, for their assistance in histopathology. Oxyglobin [hemoglobin glutamer-200 (bovine)] was generously provided by HbO2 Therapeutics.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.833243/full#supplementary-material

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REVIEW
published: 08 July 2022
doi: 10.3389/fimmu.2022.855263

Impact of Machine Perfusion on the Immune Response After Liver Transplantation – A Primary Treatment or Just a Delivery Tool

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The frequent use of marginal livers forces transplant centres to explore novel technologies to improve organ quality and outcomes after implantation. Organ perfusion techniques are therefore frequently discussed with an ever-increasing number of experimental and clinical studies. Two main approaches, hypothermic and normothermic perfusion, are the leading strategies to be introduced in clinical practice in many western countries today. Despite this success, the number of studies, which provide robust data on the underlying mechanisms of protection conveyed through this technology remains scarce, particularly in context of different stages of ischemia-reperfusion-injury (IRI). Prior to a successful clinical implementation of machine perfusion, the concept of IRI and potential key molecules, which should be addressed to reduce IRI-associated inflammation, requires a better exploration. During ischemia, Krebs cycle metabolites, including succinate play a crucial role with their direct impact on the production of reactive oxygen species (ROS) at mitochondrial complex I upon reperfusion. Such features are even more pronounced under normothermic conditions and lead to even higher levels of downstream inflammation. The direct consequence appears with an activation of the innate immune system. The number of articles, which focus on the impact of machine perfusion with and without the use of specific perfusate additives to modulate the inflammatory cascade after transplantation is very small. This review describes first, the subcellular processes found in mitochondria, which instigate the IRI cascade together with proinflammatory downstream effects and their link to the innate immune system. Next, the impact of currently established machine perfusion strategies is described with a focus on protective mechanisms known for the different perfusion approaches. Finally, the role of such dynamic preservation techniques to deliver specific agents, which appear currently of interest to modulate this posttransplant inflammation, is discussed together with future aspects in this field.

Keywords: ischemia reperfusion injury, innate immune activation, machine perfusion, mitochondrial injury, hypothermic oxygenated perfusion, marginal livers

OPEN ACCESS

Edited by:
Stefan Schneeberger, Innsbruck Medical University, Austria

Reviewed by:
Jeanette Villanueva, Victor Chang Cardiac Research Institute, Australia
Andrew S. Barbas, Duke University, United States

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Specialty section:
This article was submitted to Alloimmunity and Transplantation, a section of the journal Frontiers in Immunology

Received: *15 January 2022*
Accepted: *31 May 2022*
Published: *08 July 2022*

Citation:
Panconesi R, Flores Carvalho M, Dondossola D, Muiesan P, Dutkowski P and Schlegel A (2022) Impact of Machine Perfusion on the Immune Response After Liver Transplantation – A Primary Treatment or Just a Delivery Tool. Front. Immunol. 13:855263. doi: 10.3389/fimmu.2022.855263

INTRODUCTION

The introduction of an effective immunosuppression was a milestone in the history of organ transplantation. Various modifications of the pharmaceuticals used to modulate the immune system have been reported for most solid organs since that. The general drawback remains however with the putative risk to develop de-novo malignancies, chronic renal failure, and cardiovascular or metabolic diseases (1, 2). In an attempt to reduce such side effects, the weaning of immunosuppression (IS) and the induction of graft tolerance are important targets nowadays (1, 3, 4). Human liver allografts display several unique immunological features, including transplantation across a positive cross match, less vigorous immunosuppressive regimens, the lack of a significant benefit from HLA matching, and the overall low rates of chronic rejection. While some of these characteristics can be explained by the unique regenerative capacity of the liver, only in liver transplantation a significant proportion of patients can be eventually discontinued from maintenance IS without immediate rejection, a phenomenon known as operational tolerance (5). Tailored posttransplant protocols will include biopsies and IS regimens, which are required to individualize such concepts for a successful IS minimization (6). This approach starts however with preventive measures, applied ideally before graft reperfusion to reduce the initial "inflammatory hit" during ischemia-reperfusion. This early activation of the innate immune system is the direct consequence of ischemia-reperfusion-injury (IRI), which is the initial trigger of tissue inflammation in the transplanted organ and also in the entire recipient (7). This complex interplay between graft and recipient triggers various less well explored crosstalks between implanted organ and recipient's immune system and promotes the establishment of an inflammatory milieu with a chronic component (8, 9). The level of this inflammation strictly depends on the initial donor and organ quality and can be modulated through a different preservation strategy (10). The potential role of machine perfusion technology and the effect on this initial posttransplant inflammation, which is crucial for recipient outcomes, is therefore highly relevant (9). While the first randomized controlled trials (RCT) are now available and demonstrate a reduction of posttransplant complications with the use of machine perfusion, the impact on basic mechanisms of early IRI and subsequent immune response, remains not well explored.

This review focus therefore on the mechanisms of how the innate immune response is triggered after liver transplantation and the role of machine perfusion techniques, individual and as a tool to deliver therapeutic agents to the organ.

THE CORNERSTONES OF THE ISCHEMIA-REPERFUSION-INJURY CASCADE

Ischemia-reperfusion injury (IRI) is a paradoxical cascade of injury, which occurs during re-oxygenation of an ischemic organ (11, 12). It is now well recognized, that the predominant effector of this injury is an early burst of reactive oxygen species (ROS), occurring across numerous types of tissues, including liver, kidney, lung, heart, muscle, and brain (7, 13–17). While there are several sources of cellular ROS, for example the xanthine oxidase pathway or NADPH oxidase systems, superoxide production during IRI is reported to be the result of a dysregulation of the electron transport chain, with electrons leaking at various sites when oxygen is re -introduced following a period of ischemia (18, 19). The level of previously accumulated succinate, the most important Krebs cycle metabolite in this context, plays a crucial role and associates with the level of released ROS from mitochondrial complex I (20). With prolonged cellular ischemia, succinate accumulates to a higher extent, which also correlates with the energy content of liver cells (11). When mitochondrial succinate levels are high, they trigger an immediate metabolism of this detrimental compound by mitochondrial complex II and the TCA-cycle as soon as oxygen is reintroduced and the respiratory chain reestablishes an electron flow. This is however initially uncoordinated and retrograde and therefore triggers the release of reactive oxygen species (ROS) at complex I (11, 18). Cells, which are severely affected by ROS may die and release further molecules, including Danger-associated molecular pattern (Damps) and cytokines, which trigger the inflammatory response from other surrounding cells, initially less affected (**Figure 1**) (8). Such Damps are released from all severely injured liver cells in combination with mitochondrial DNA and represent the first level of innate immune system activation (9, 11, 21–23). With the presentation of surface markers on activated macrophages, recipient T cells are attracted and activated, which represent the next level of contribution to the innate immune response (7, 8, 24). The next subchapter provides further details on specific innate immune signaling among involved cells.

MECHANISM OF INNATE IMMUNE SIGNALING

Mitochondrial ROS are a consequence of mitochondrial disruption in all liver cells, macrophages and T cells. Once oxygen is reintroduced after a period of ischemia, mitochondrial electron transfer complexes I and III serve as the major sites of ROS production (18, 25). Released ROS are directly antibacterial, but also signal the release of inflammatory cytokines. Following their release, ROS molecules first injure subcellular structures within the same cell, which releases further proinflammatory molecules (26). Cells severely affected by the cascade of ischemia-reperfusion-injury (IRI) leak pro-inflammatory molecules, which in turn activate other cells, initially less affected (**Figure 1A**). Such pro-inflammatory molecules include the release of damage-associated molecular patterns (DAMPs), which activate local antigen presenting cells (21). DAMP receptors are toll-like-receptors (TLR) or receptors for advanced glycation end products (RAGE), which mediate downstream cytokines release with subsequent production of more ROS by non-parenchymal liver cells, mostly Kupffer cells. ROS and DAMP release promotes two main signals, first

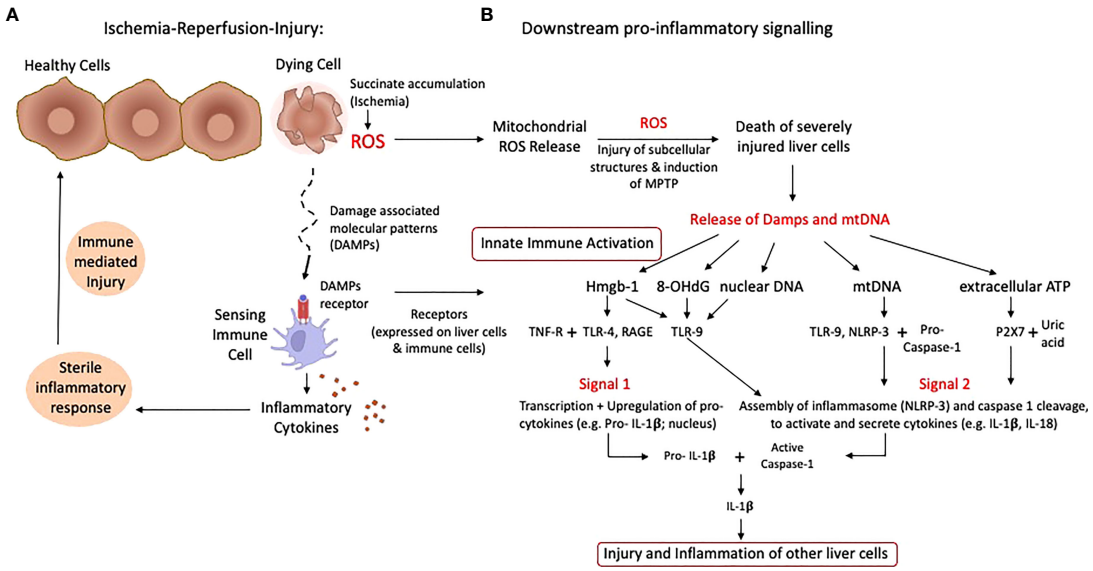

FIGURE 1 | Schematic presentation of ischemia-reperfusion-injury with pro-inflammatory signaling. The rapid succinate metabolism at complex II leads to the initial key event of ROS release when oxygen is reintroduced into ischemic tissues. Based on the level of accumulated succinate during ischemia, a number of cells are severely affected and die with subsequent release of mitochondrial DNA and Damps **(A)**. Specific receptors are expressed on liver and immune cells, which trigger transcription and upregulation of pro-cytokines, creating an inflammatory milieu. Various Damps molecules instigate proinflammatory signals and the assembly of the inflammasome (NLRP-3) plus caspase cleavage, which activates available pro-cytokines **(B)**. Such features lead to the injury of previously less activated and affected cells, which releases additional damps and cytokines. ATP, Adenosine-trisphosphate; Damps, danger associated molecular patterns; Hmgb-1, High mobility group box protein-1; IL, Interleukin; ROS, reactive oxygen species; TLR, toll like receptor.

transcription and upregulation of pro-cytokines in the nucleus (e.g., IL-1β) and second, the assembling of the inflammasome (NLRP-3) and capsase-1 cleavage to finally activate and cleavage such pro-cytokines (**Figure 1B**) (8, 27, 28). TLR-signaling also involves multiple adaptor proteins MyD88, TIRAP, TRIF, TRAM, and the activation of the transcription factors NFκB, AP-1 and IRF3, which ultimately lead to a maturation of dendritic cells with consecutively presentation of antigens together with the expression of co-stimulatory molecules, which attract immune cells (**Figure 2**) (21, 29, 30).

T cells appear as main contributors in this context and become activated (T cell receptor signaling), thereby inducing a further increase in mitochondrial ROS production (31, 32). Interestingly, T cell subtypes that lack the Complex-III component RISP are more protected from ROS release. Subsequently, such T cells have a decreased expression of the activation markers CD-25 and CD-69 (31). Pharmacological blocking of this T cell activation process antagonizes their IL-2 production. One subtype of such immune cells appear as regulatory T cells, which are described with a reduced expression of CD-25 and subsequent less IL-2 production. Such T regs are therefore protective from IRI-injury and may help to reduce inflammation (33). The antagonism of CD-25 in Treg deficient mice was shown to protect murine kidneys from IRI-injury (34). Despite such known protective effect, the role of Tregs was described controversial in different stages of brain IRI. While Tregs were found to reduce inflammation and promote tissue repair in the later stage of resolution after IRI, during the acute phase stroke pathogenesis was rather pronounced by Treg (34). Additionally, in other models as for example hindlimb ischemia and reperfusion, Treg depletion through anti-CD25 led to higher levels of inflammation and to a higher number of newly developed vessels.

TCR signaling also leads to the release of calcium, stored in the endoplasmic reticulum, which can be taken up by mitochondria to drive the Krebs cycle enzyme activity, thereby increasing the amounts of NADH and Krebs cycle intermediates, including succinate (35). Mitochondrial calcium influx contributes to mitochondrial ROS generation in T cells, and succinate drives further mitochondrial ROS generation in macrophages (31).

Mitochondrial ROS also function as a signal in B cell activation. B cell activation by antigen and helper T cells induces several processes such as somatic hypermutation, to diversify the B cell receptor for antigen, and class-switch recombination (CSR), which enables B cells to express a particular type of immunoglobulin (36). The ligation of the BCR stimulates calcium release into the cytoplasm, which promotes ROS production. Stimulation with LPS and IL-4 or with anti-CD40 and IL-4 generates a population of B cells with increased mitochondrial mass, membrane potential and ROS production compared with naive B cells (37). Undifferentiated cells within this population undergo CSR after differentiation. Mitochondrial ROS may also act to induce CSR by inhibiting the synthesis of heme, a molecule that decreases CSR by antagonizing BACH2, a key transcription factor for CSR57 (36).

In summary, these observations indicate that T and B cells require ROS production for the generation of an appropriate immune response. Innate immune cells are similarly reliant on

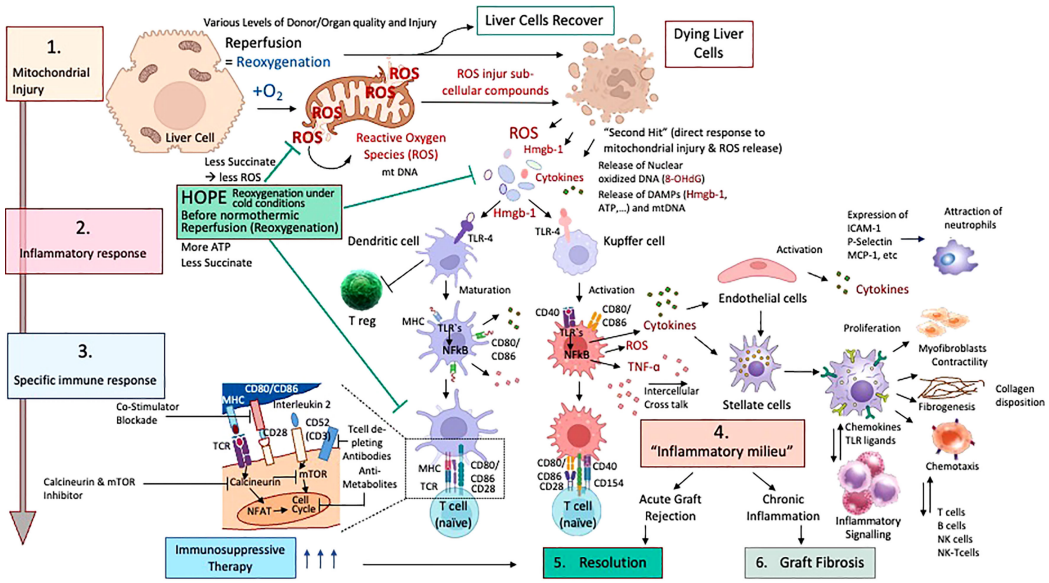

FIGURE 2 | Cascade of ischemia-reperfusion-injury with specific downstream activation of the innate immune system; Different stages of ischemia reperfusion injury (IRI) with innate immune activation and resolution with or without pharmacological treatment in liver transplantation are presented. According to the accumulated succinate, ROS are released at reperfusion (1) with subsequent release of mitochondrial DNA and Damps molecules, which link IRI to innate immune response. (2) Subsequently, dendritic cells and macrophages become activated and present their well-known surface receptors to circulating T cells, which become in turn activated (3). Recipient neutrophils and other immune cells are also attracted by activated endothelial cells in the organ. With the involvement of other liver and recipient immune cells, including stellate cells and fibroblasts, T and B cells plus killer cells, the inflammatory milieu achieves a chronic stage (4) and is resolved by pharmacological upstream treatment (5). Chronic processes may also become very advanced and severe with subsequent fibrosis of the implanted organ (6) if resolution mechanisms fail.

ROS production, with Krebs cycle intermediates such as succinate, citrate, fumarate and itaconate emerging as important regulators of the production of this noxious agent, as well as other events (38). Overall, mitochondrial ROS, originating from a variety of mitochondrial sources, are key signaling molecules for immune-cell function.

SUCCINATE AS REGULATOR OF IRI-ASSOCIATED INFLAMMATION

During ischemia, various Krebs cycle metabolites with important inflammatory signaling function accumulate in all involved cells. These metabolites are present because of the occurrence of break points in the Krebs cycle during ischemia. The interruption at the enzyme succinate dehydrogenase (SDH), which represents mitochondrial complex II, and converts succinate to fumarate, is associated with an accumulation of succinate, a metabolite with proinflammatory activity and roles in immunity (18).

Mitochondrial succinate, once accumulated to a significant extend, triggers two main metabolic features at reoxygenation. First, when high succinate levels are present, mitochondria aim for a rapid metabolism and immediate reestablishment of the electron flow, which is however reverse (RET) with subsequent ROS production from complex I, thereby instigating the inflammatory IRI-cascade (**Figure 1**, **2**) (11, 39, 40). Succinate oxidation by SDH, also leads to HIF-1α activation and HIF-1α-dependent gene expression; a notable example of a gene affected in this way is IL1B, which encodes IL-1β (41). Succinate also limits the production of anti-inflammatory cytokines, particularly of IL-10. Inhibition of SDH with dimethylmalonate (DMM) profoundly inhibits LPS-induced ROS generation and the expression of IL-1β and a range of proinflammatory genes in macrophages, and boosts the expression of IL-10 and anti-inflammatory genes (8, 10, 24, 42). DMM administration in mice treated with LPS results in decreased IL-1β expression and a boost in IL-10 expression systemically (31, 35). LPS treatment boosts the mitochondrial membrane potential in macrophages, which is dependent on increased glycolytic ATP production. Limiting of LPS-induced membrane hyperpolarization, which is required for RET, inhibits IL-1β induction in response to LPS in macrophages (31). The induction of IL-1β by LPS and succinate is also impaired in macrophages, that express the enzyme alternative oxidase (AOX), which limits RET. All of these observations not only confirm a role for mitochondria-derived succinate as a critical proinflammatory mediator, but also shed light on the mechanism by which LPS drives ROS production, and provide further rationales for why macrophages favor aerobic glycolysis and decrease Oxidative Phosphorylation (OXPHOS)

in response to LPS. These metabolic alterations repurpose mitochondria from ATP synthesis to ROS production to promote and maintain a proinflammatory state (18, 21, 23, 25, 43). An antibacterial role of SDH was also suggested by the

observation that the inhibition of SDH during bacterial infections rendered mice more susceptible to infection. Taken together, these findings identify complex II (SDH) as one key control point IRI-associated inflammation.

MITOCHONDRIAL SIGNALING ACTIVATES NLRP-3 INFLAMMASOME

Mitochondrial antiviral signaling protein (MAVS) is another key signaling protein activated by the different RNA sensors, including RIG-I and MDA5 (29). It in turn activates pathways, that regulate the transcription factor NF-κB and IRFs to promote proinflammatory gene expression (8, 29, 44, 45). Interaction with the outer mitochondrial membrane is essential for a fully functional MAVS molecule. Mitochondrial ROS can induce and drive MAVS oligomerization, leading to the production of type I interferon, independent of RNA sensing, which in turn suggests that MAVS might be a key sensor of mitochondrial ROS, that acts to promote the recipient defense and inflammation (25, 29, 31). Furthermore, MAVS associates with Inflammasome (NLRP-3) and promotes its oligomerization, which leads to caspase-1 activation (8, 23, 43, 46–48). Activation of NLRP-3 with the synthetic Toll-like-receptor (TLR) -7 ligand imiquimod has recently been shown to occur as a result of the mitochondria ROS production from complex I and the quinone oxidoreductase NQO_2 (31, 48). This effect was independent of potassium efflux, which highlights the importance of mitochondrial ROS for NLRP-3 activation (49, 50). Finally, NLRP-3 is also regulated by cardiolipin, a lipid of the inner mitochondrial membrane, which translocates to the outer membrane, where it recruits NLRP-3 after mitochondrial membrane depolarization (31). This interaction appears crucial for NLRP-3 activation, and suggests that mitochondria function as signaling hubs and activate the innate immunity. NLRP-3 activation in turn leads to mitochondrial damage and subsequent mitochondrial ROS release, which represents a feedback loop between NLRP-3 and mitochondria (23, 48). Mitochondria are therefore critical for signaling by the described three major innate immune signaling pathways.

MITOCHONDRIA: THE NEW THERAPEUTIC TARGET?

A relatively novel strategy to decrease the posttransplant inflammation and the need of IS is to approach the crosslink between organ ischemia-reperfusion injury (IRI) and immune response. Instead of focusing on the key instigator of the IRI-cascade in mitochondria, most teams target the suppression of proinflammatory molecules, released in response to ROS later after reperfusion (**Table 1**). Others focus on the suppression of defense mechanisms. Preventive or therapeutic measures with impact on IRI-associated inflammation were applied for example in the donor as direct treatment or as an additive to the donor flush solution (56). Others have added specific molecules during cold storage or as a treatment in the recipient. Most molecules were however not yet transferred into clinical practice, although the results seen in experimental studies appeared quite promising with impact on IRI-associated inflammation, the effect got either lost or was limited when explored in transplant models (40, 57, 58). The reason behind could be the target of too peripheral individual genes or molecules of the IRI-cascade. Although the underlying mechanisms of IRI are increasingly linked to tissue succinate accumulation during ischemia with subsequent ROS release after reoxygenation, this key metabolite is rarely addressed in studies (40). A significant protection from IRI-associated complications could be achieved through the prevention of succinate accumulation before or during donation (e.g., before ischemia) or also by a slow succinate oxidation before normothermic reperfusion at transplantation (40). Already established succinate levels could be eliminated through a slow activation of complex II, before reintroducing oxygen at normothermic temperatures (**Figure 3**) (40). Reduced cellular succinate stores prevent the initial burst of ROS release, which therefore eliminates the initial danger event of the IRI- cascade (40). In addition to succinate, other compounds in mitochondria could be the target to reduce IRI. Malonate could for example be used to block complex II (SDH) and limit succinate accumulation and also impact on the rapid succinate oxidation at reperfusion (11, 18, 38). Next, the inhibition of SDH with DMM may also provide a therapeutic benefit by limiting ROS production and proinflammatory responses, and boosting the anti-inflammatory response (19, 31). If the reprogramming would sustain, it could also offer the possibility of inducing remission in chronic inflammatory diseases. In addition, the blockage of the mitochondrial permeability transition pore (MPTP) with Cyclosporin, as tested in mice, could be advanced into human practice. However, despite promising results of a randomized controlled trial (RCT), the effect of this pharmaceutical was limited in a recent phase III study in hearts (59, 60).

Various other molecules and drugs are currently developed to be used to reduce downstream inflammatory processes induced by ROS, Damps and mitochondrial DNA. Most studies appear however older and involve only animal models with only very few that have explored the effect in a transplant model (56).

RNA INTERFERENCE AS TOOL TO REDUCE IRI-ASSOCIATED INFLAMMATION

Next, the natural process of RNA interference (RNAi) with silencing of specific genes was recently explored and introduced into transplantation settings. Various experimental studies were presented with the interference of the specific RNA, targeting genes of IRI-injury and immune activation. Unfortunately, in most studies, RNAi is explored in the donor 1 to 72hrs before ischemia (61). This approach appears however

TABLE 1 | Clinical studies with the impact of machine perfusion on immune response within the last 3 years.

Authors, Year study type & Country	Number and Type of livers	Type & Duration of Donor warm ischemia time (min)	Duration of cold ischemia before perfusion	Duration of Perfusion	Duration of Follow-up	Main Findings	Discussion
Clinical studies with the impact of hypothermic machine perfusion on the immune system							
Van Rijn et al, 2021, Randomized controlled trial, Europe (51)	78 DCD livers each arm (D-HOPE vs. CS)	Total DWIT cDCD D-HOPE: 29 (IQR: 22-33); CS group: 27 (IQR: 21-35) Asystolic DWIT cDCD D-HOPE: 11 (IQR: 8-13); CS group: 11 (IQR: 8-15),	6hrs 11min (IQR: 5hrs 16min – 6hrs 55min)	2hrs 12 min (IQR: 2hrs – 2hrs 33min)	6 months	D-HOPE reduces acute rejections: D-HOPE 11.5% vs cold storage control 20.5%	Follow up of 6 months
Czigany et al, 2021, Germany (+Prague) (52)	23 DBD livers each arm (HOPE vs. CS)	None	DBD HOPE: median 6.3hrs (IQR: 5.2-7.8hrs); DBD SCS: median: 8.4hrs (IQR: 7.8-9.7hrs)	DBD HOPE: median 2.4hrs (IQR: 1.7-3.4hrs)	12 months	HOPE treatment reduced the acute rejection rate from 26% (CS control) to 17% (HOPE group), primary endpoint is reduced Peak ALT levels (p=0.03), other endpoints: shorter ICU (p=0.045) and hospital stay (p=0.002), less major complications ≥ Clavien Grade IIII (p=0.036), cumulative complications (CCI: p=0.021), estimated costs (p=0.016)	Study was not powered for complications, DBD grafts
Retrospective Studies							
Ravaioli et al, 2020, Italy (53)	Extended DBD/ HOPE=10, SCS controls (n=30) None	None	14.5hrs (IQR: 10.8-22hrs)	2.2hrs (IQR: 1-3.5hrs)	12 months	Tendency toward a lower ACR rate: 10% HOPE group and 13.3% CS control; No PNF and lower rate of EAD, lower recipient transaminases after HOPE treatment and 100% graft survival compared control,	Low case number, matched cohort study, DBD
Schlegel et al., 2019, UK, Switzerland (54)	cDCD/SRR/ HOPE=50; DBD/ SCS=50 (control), cDCD/SRR (unperfused) =50	Total DWIT HOPE: median 36 (IQR:31-40); SRR: 25.5 (IQR:21-31); Functional DWIT HOPE: median 31 (IQR:27-36); SRR; median 17 (IQR:15-19); Asystolic DWIT HOPE: median 19 (IQR:17-21); SRR: median 12.5 (10-15)	cDCD/HOPE: median 4.4hrs (IQR: 3.5-5.2hrs); SRR group: 4.7hrs (IQR: 4.3-5.3hrs)	Median 2hrs (IQR: 1.6-2.4hrs)	5 years	cDCD/HOPE with less acute rejection; 4% HOPE group, 28% CS group, p=0.0019, SRR DCD: 22% (n=11/50) with 10% (n=1/69) graft loss; HOPE: 8% (n=4/50) with 0% graft loss; Less PNF, HAT and ischemic cholangiopathy result in an improved five-year survival of HOPE treated extended DCD liver grafts	Matched cohort study, retrospective
Patrono et al, 2019, Italy (55)	Extended DBD/D-HOPE, macro-steatotic=25, DBD/ SCS=50 (control)	None	311min ±53 (mean, SD)	186min ±49 (mean, SD)	6 months	Lower rate of acute rejections with 8.6% (HOPE group) and 16% CS control, lower rate of post-reperfusion syndrome, acute kidney injury grade 2-3, and EAD, lower rates of anastomotic strictures: CS: 12% (n=6/50); D-HOPE: 16% (n=4/25), SCS: 8% (n=4/50), 2 symptomatic patients; D-HOPE: 8% (n=2/25), both asymptomatic	DBD grafts

Studies are summarized according to the literature within the last 3 years concerning transplantation of controlled DCD or DBD livers procured with standard cold storage and machine perfusion, included were studies with a cold storage control group, either DCD or DBD and with information on acute liver rejection or other parameters relevant for the immune response; ACR, acute cellular rejection; DBD, donation after brain death; DCD, donation after circulatory death; DWIT, donor warm ischemia time; EAD, early allograft dysfunction; HOPE, hypothermic oxygenated perfusion; IQR, interquartile range; SCS, standard cold storage; SRR, super rapid retrieval; concerns DCD donors.

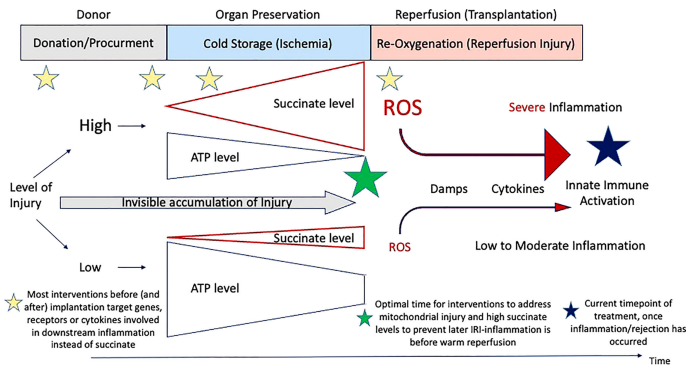

FIGURE 3 | Timing of treatment modalities to address IRI-associated inflammation. Overview on the current timings when therapeutics are administered from the donor to implantation. Most molecules target genes or downstream receptors beyond the instigating processes, instead of succinate and subsequent ROS release.

rather unpractical in clinical settings, where donor treatment is prohibited in most countries (62). Only very few preclinical studies involve other treatment routes, including administration through portal vein infusion (61, 63).

In addition to RNAi other molecules, including antioxidants and antibodies are administered to block specific molecular reactions. Li et al. added the CD47m antibody into the liver flush solution during donation. CD47 is a member of the immunoglobulin superfamily and is involved in various pathways related to ROS molecules. Recipients were found with lower rates of acute rejection and a lower severity in this allogenic mouse model (64).

In summary, various treatments are currently explored, frequently targeting rather peripheral processes instead of mitochondrial components. Multiple routes are used, including donor injections, organ flush and recipient treatment. The modulation of the current standard cold storage preservation, with the implementation of machine perfusion technologies, might serve as most attractive tools to supply pre-injured organs with beneficial compounds before implantation in the future. However, prior to successful utilization of machine perfusion as delivery tool, underlying mechanisms of protection and injury should be explored and understood.

THE ROLE OF ORGAN PERFUSION TECHNIQUES FOR IMMUNE MODULATION

Novel organ perfusion strategies appear of interest and various groups have promoted the technological development in the last decade (51, 54, 55, 65–68). Despite this advancement, available data on the solitary impact of machine perfusion (MP) on ROS-mediated allograft oxidative injury and subsequent immune response remain scarce (5, 42).

Novel organ perfusion approaches can be divided into two main strategies, first in-situ perfusions, which include regional perfusion, mainly done at normothermic temperatures and secondly, ex-situ techniques, where organs undergo perfusion on a tailored device either after cold storage (e.g., endischemic) or instead (69). Normothermic regional perfusion (NRP) is applied in the donor immediately after circulatory death (DCD). Leading countries, where this technology is routinely used in DCD liver donors include Spain, France, Italy and a few centers in the United Kingdom (70–73). The early graft evaluation during NRP in the donor helps to select livers with too high injury. This selection is mainly based on the macroscopic liver appearance, the perfusion quality, pH, liver transaminases and lactate measured from the NRP-circuit. DCD liver preserved with NRP were shown to achieve immediate function and recipients experienced reduced levels of biliary complications after transplantation (68, 74). With the overall aim to push regulatory and donor risk boundaries, the limitations of all perfusion techniques are increasingly described. With NRP, DCD livers were well preserved provided that the national regulations of donor and recipient risk were respected. French centres for example described a higher number of graft loss with the use of NRP-treated grafts when the donor warm ischemia time or recipient lab MELD exceeded national criteria (72). With additional cold storage time following NRP and liver procurement, the exact metabolic situation of such organs appears not well known at the time of implantation (69). In this context, prolonged cold ischemia of more than 7hrs was recently described as risk factor for graft loss in Spain, when combined with NRP (75). This risk was even more pronounced when such DCD grafts were implanted into recipients waiting for a retransplantation (75). Comparable to any other technique of normothermic reperfusion, where oxygen is reintroduced into ischemic tissue, during initial NRP, the cells and more specifically mitochondria have the main goal to metabolize high succinate concentrations rapidly with subsequent ROS release from mitochondrial complex-I and downstream inflammation. This has been recently demonstrated in the setting of clinical NRP in Italy with the quantification of cytokine levels in the recirculating NRP-

perfusate (76). Based on the known link between IRI and innate immune activation, more data are required to describe the impact of NRP on the innate immune system, and ideally in context of an increased utilization of risky donors.

In contrast, two main ex-situ machine perfusion techniques are currently explored (51, 67, 68). First, the normothermic machine perfusion (NMP) technique using a blood-based perfusate at 37°C, which is ideally applied after liver procurement and instead of cold storage, replacing cold ischemia with a subsequent reduction of liver injury and IRI (62). And secondly, a logistically less challenging perfusion strategy, applied after cold ischemia in the recipient centre using a hypothermic oxygenated perfusion (HOPE) prior to implantation (11, 77). Of note, although various groups have explored the use of such perfusion techniques as delivery tool for molecules or stem cells in ex-vivo models of liver perfusion, the underlying mechanisms of such perfusion techniques are not well enough understood and deserve more recognition (78).

Perfusion techniques done at warm temperatures were found to trigger the same ROS-induced inflammation as seen with the IRI-cascade after liver transplantation. The level of IRI might however be dependent on the perfusate components. The literature describing such features of liver NMP in a transparent way is very scarce (28, 79, 80). Normothermic perfusion strategies induce an inflammatory environment with the release of ROS and DAMPs and consecutive activation of toll-like-receptors (28, 81). Downstream activation of the innate immune system after NMP of livers can be likewise expected, promoting acute organ rejection, particularly when NMP is applied after a relevant period of standard cold storage (82, 83). Recent data from normothermic kidney perfusion support such mechanistical insights (84).

Unfortunately, markers of immune response are frequently not reported in clinical and experimental studies with normothermic perfusion. We were therefore also not able to add clinical studies to **Table 1**, which assess the impact of NMP on the immune system.

Jassem et al. have recently compared the expression of immune-related pro-inflammatory genes between cold stored livers (n=12) and grafts that underwent NMP instead (n=27) from brain death donors (DBD) (85, 86). Cold stored livers showed a higher expression of genes involved in innate immune activation, neutrophil chemotaxis and platelet activity with higher levels of recipient plasma cytokines (85). Livers, exposed to NMP demonstrated less amounts of specific T cells, known for their production of Interleukins 2, 4 and 17, and Interferon y. The proportion of regulatory T cells was found slightly higher in the NMP group, particularly the tissue-resident subtype $CD4^{pos}CD25^{high}CD127^{neg}FOXP3^{pos}$ was described with higher concentrations throughout prolonged normothermic perfusion (85). Of note, the replacement of cold storage by NMP led to a lower liver injury in the NMP group, while grafts in the control group were exposed to the entire duration of cold ischemia before implantation and exploring the immune response presented here (67, 85, 87). The work by Scheuermann et al. presents one of the very few studies actually quantifying the inflammatory injury during NMP, compared to other perfusion techniques. Authors have demonstrated that liver perfusion at lower temperatures, e.g., subnormothermic, induces less inflammation compared to NMP (88).

In contrast, hypothermic oxygenated perfusion (HOPE), is performed at 6-10°C using an artificial perfusate, which is highly oxygenated (60-80kPa) (89, 90). HOPE therefore was shown to reprogram mitochondrial during the introduction of oxygen at such low temperatures. During HOPE the electron flow is reestablished with subsequent steady and slow metabolism of accumulated succinate and ATP reloading at the same time. While the respiratory chain is functioning the high load of NADH at complex I is also reduced by metabolism to NAD, which is a surrogate marker of a functioning complex I and energy recharge (91). Mitochondria, which underwent reoxygenation at hypothermic temperatures are switched and can handle the reperfusion with blood at normothermic temperatures without thew known detrimental rapid succinate metabolism and with much lower ROS production (11, 81, 92, 93). HOPE-treatment achieves this by increasing the activity of mitochondrial complex proteins (11). Based on these significant changes during HOPE, mitochondria are prepared and experience less oxidative stress and subsequently trigger less Damps release with less TLR activation and a reduced innate immune response (28, 79). Ex-vivo graft treatment by HOPE was consecutively also found to prevent downstream T-cell activation in allogenic liver and kidney transplant models, without any additional immune suppressive treatment (17, 42). Combined experimental protocols allowed to reduce the immunosuppression to one third of the normal dosage when IS was combined with HOPE. An excerpt of the results found after transplantation with various IS and HOPE combinations in this allogeneic liver model is shown in **Figure 4** (42). Normal livers from Lewis rats were procured and with minimal cold ischemia transplanted into Brown Norway recipient rats, which is well-described in the literature as a known model of allogeneic liver transplantation. Of note, recipients without any immunosuppression achieved poor survival, expectedly shorter than two weeks in most. In contrast, HOPE treatment in these allogeneic rodent livers led to similar survival rates as seen in recipient, which received the normal immunosuppressive treatment with Tacrolimus (42). In combination with a reduced Tacrolimus dosage, allogeneic liver recipients achieved prolonged survival when combined with HOPE treatment. Of note, similar findings are described in the literature for allogeneic models of kidney transplantation using endischemic HOPE (17). These findings from preclinical studies are now also paralleled by an increasing body of clinical studies with and without a randomized design. **Table 1** summarizes clinical studies reporting an impact of HOPE on the immune activation after human liver transplantation. The recently presented RCT from the Groningen group found as a secondary endpoint a 9% reduction of acute rejections after DCD liver transplantation with dual HOPE treatment compared to cold storage controls (51). A similar tendency was described by another RCT from Germany, where the team described a reduction of acute rejections from 26% in the cold storage study arm to 17% after HOPE (52). Earlier retrospective studies have already demonstrated similar results. The Zurich group compared the effect of HOPE in extended DCD liver grafts with unperfused, cold stored controls from the United Kingdom. Of note, livers with HOPE presented an acute

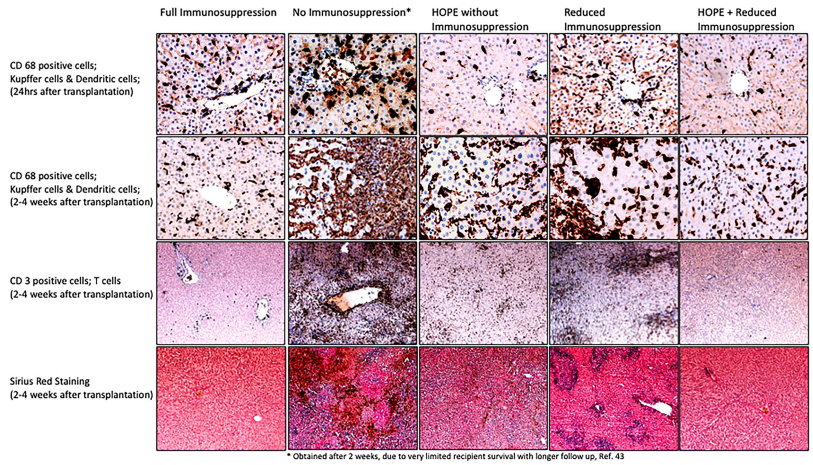

FIGURE 4 | Impact of HOPE on innate immune response after allogeneic liver transplantation; To address the accumulated succinate with a slow oxidation at complex II is a key mechanism to avoid the massive ROS release and subsequent IRI cascade with complications after transplantation. Machine perfusion is therefore a well explored new method to improve and assess metabolic processes. Hypothermic oxygenated perfusion (HOPE) before implantation was shown to reduce the accumulated succinate and to improve complex I and II function. Using a model of allogeneic liver transplantation, the protective effect of HOPE on the innate immune system was demonstrated with a lower number of activated of Kupffer cells (through less Damps release) and subsequently a lower number of infiltrating T cells in transplanted livers, compared to untreated controls (without HOPE and without immunosuppression). Of note, HOPE treatment achieved the best protection from Kupffer cell and dendritic cell activation early after implantation, e.g., at 24hrs, also compared to the group with full dosage of immunosuppression, which requires time until the most effective blood levels are seen. The protective effect of HOPE was still present 4 weeks after implantation, although the delayed immune response became visible. HOPE treatment was therefore combined with a low dose of immunosuppression, which led to acute rejection when applied alone. HOPE with reduced immunosuppression protected recipients from innate immune activation and acute rejection, similarly to recipients which received the full dose of tacrolimus (immunosuppression). These images were obtained from samples from the study presented in reference (42) (samples and histological images were not published before in this reference). CD, cluster of differentiation.

rejection in 4%, which is a significant reduction from 28% seen in unperfused controls (p=0.0019)(**Table 1**) (90). Although the impact of machine perfusion on costs is not well enough explored yet, a more than 10-20% reduction of acute rejections will most likely reduce transplant related costs with less requirements of liver graft biopsies and readmissions. In addition to the impact on biliary and overall complications, further results are awaited with regard to costs. Another important factor is the perfusion duration of HOPE. A recent multi-centre study has collected outcomes of 93 human liver transplants (50 DCD, 43 DBD) with a prolonged HOPE treatment of >4hrs and a median overall preservation of 10hrs. Outcomes were excellent and comparable to other series reported with shorter endischemic HOPE (94). Of note, prolonged HOPE of >4hrs was also shown after more than 10hrs of cold storage in extended criteria donor livers in Germany and in combination with high MELD recipients in Brasil (95). A prospective study on prolonged HOPE is currently ongoing in the Netherlands to confirm the safe prolongation to compensate logistical issues (96).

MACHINE PERFUSION AS A TOOL TO DELIVER SPECIFIC MOLECULES

Recirculating perfusates may provide two main benefits during machine perfusion. First, injured and dying cells release specific molecules, which might signal functional deficits and could be used to assess organ viability (97). And secondly, perfusate could be used to carry specific molecules into the cells and subcellular compounds to prevent production and release of IRI-associated compounds. Various therapies, such as pharmacological agents, genes, stem cells and nanoparticles were administered into organs through this route in preclinical studies (56, 57). The technique of normothermic perfusion is more frequently applied because of the assumed better transport and uptake of such molecules at 37°C, when compared to hypothermic perfusion settings. This last subchapter describes studies within the last 3 years, where machine perfusion was used to deliver compounds with an impact on the innate immune response (**Table 2**). Only very few studies explore the impact in a transplant model. Cao et al. exposed DCD livers with 30 minutes of donor warm ischemia time to 4hrs of NMP with the addition of bone marrow-derived mesenchymal stem cells, which were found to inhibit the release of Hmgb-1 with subsequent reduction of TLR-4 activation. Authors demonstrated a clear effect on the early innate immune response through NMP with such stem cells compared to unperfused cold stored controls (98). This work was paralleled by Yu et al, who blocked NLRP-3 in a pig DCD liver transplant model. All experimental groups underwent hypothermic perfusion and the NLRP-3 blocker was administered either during perfusion or after implantation in the recipient. Groups with additional NLRP-3 blockage were found with lower innate immune response independent from the administration route compared to hypothermic perfusion alone. Of note the

TABLE 2 | Experimental studies exploring the impact of machine perfusion with or without specific perfusate additives on the immune system in liver transplantation.

Authors, Year study type & Country	Number and Type of livers, species	Donor warm ischemia time (min)	Duration of cold ischemia before perfusion	Type and Duration of Perfusion	Additives to Perfusate	Model of Liver Transplantation (yes/no)	Duration of Follow-up	Main Findings	Discussion
Experimental studies with liver perfusion and transplantation									
Schlegel et al, 2014 (42)	Rat livers, allogeneic model with full Tacrolimus, compared to HOPE without any Tacrolimus, and 1/3 of Tacrolimus with/without HOPE	n.a.	60min	1hr HOPE	none	Yes	4 weeks	HOPE protects from acute T cells mediated rejection, reduces T cell infiltration and CD40/CD86 expression, HOPE plus reduced IS was equally protective compared to full IS, lack of perfusate oxygen leads to the same injury as unperfused, untreated controls	B cell response was not addressed
Experimental studies with liver perfusion with the use of specific perfusate additives and subsequent transplantation									
Cao et al, 2020 (98)	30 rat livers, 5 groups of 6	30min	4 hours (SCS only)	4 hours NMP	BMMSCs and Heme-oxygenase 1 (HO-1)-modified BMMSCs	Yes	1, 7, 14 days	HO-1/BMMSCs combined with NMP exerted protective effects on DCD donor liver and significantly improved recipient prognosis. The effect of HO-1/BMMSCs was greater than that of BMMSCs and was mediated *via* Hmgb-1 expression and TLR-4 pathway inhibition.	Demonstrated the role of monocytes, requires further investigation needed on protective mechanism of BMMSCs, perfusion model can't be translated into clinical practice
Yu et al, 2019 (99)	Pig livers, n=36, all DCD	30min	HMP + additive: 275 min HMP + post op additive: 268 min HMP + no additive: 274 min	HMP 2 hours all groups	MC950 (NLRP-3 Inhibitor)	Yes	3 days	The HMP-Postop group suffer the lightest ischemia reperfusion injury (IRI), and functioned best after transplantation. Model for the Early Allograft Function Score degree of injury in the hepatocytes and rate of apoptosis was lowest in the HMP-Postop group. The HMP-Postop group had the lowest downstream inflammation, and the level of IL-1β was lowest. Postop group functioned better than control group, but not comparable with HMP-Postop group.	Short follow up, unknown reference for dosage of additive
Experimental studies with liver perfusion and the use of specific perfusate additives without transplantation									
Carlson et al, 2021 (100)	22 Rat livers, 4hrs NMP vs. 4hrs SCS naiüve (n=4), CS (n=4), NEVLP (n=7), and NEVLP with anti-inflammatory cytokines (NEVLP-Cyt, n=7)	n.a.	SCS group only: 240 min	4hrs NMP at 37° (NMP and NMP +additive groups only)	IL-10 & TGF-β (20ng/mL)	No	n.a.	Pro-inflammatory gene expression during NMP, dominant in macrophages and dendritic cells, increased MHC II, CD40, CD86 expression, IL-10&TGF-β in NMP perfusates reduced immune activation	No transplant model, confirms induction of inflammation and immune system during NMP
Laing RW et al, 2020 (78)	6 Human livers, 2 DBD and 4 DCD	Not available	500 min	6 hrs NMP at 37°	MSC	No	n.a.	demonstrated that cells can be delivered directly to the target organ, prior to host immune cell population exposure and without compromising	No transplant model, small sample,

(Continued)

TABLE 2 | Continued

Authors, Year study type & Country	Number and Type of livers, species	Donor warm ischemia time (min)	Duration of cold ischemia before perfusion	Type and Duration of Perfusion	Additives to Perfusate	Model of Liver Transplantation (yes/no)	Duration of Follow-up	Main Findings	Discussion
								the perfusion. Transendothelial migration occurs following arterial infusion. MAPC cells appear to secrete a host of soluble factors that would have anti-inflammatory and immunomodulatory benefits in a human model of liver transplantation.	
Boteon et al, 2019 (101)	Hepatocytes and discarded human livers, n=10 2x (3 DBD + 2 DCD)	Treated group: 12min Control: 13 min	Median 737 min	12 hours NMP	Defatting cocktail	No	n.a.	Treatment reduced tissue triglycerides by 38% and macrovesicular steatosis by 40% over 6 hours Treatment down-regulated the expression of markers for oxidative injury as well as activation of immune cells (CD14; CD11b) and reduced the release of inflammatory cytokines in the per- fusate (tumor necrosis factor α; interleukin 1β)	No transplant model, Heterogeneous sample, higher risk in control group

N.A. means not applicable.

group where the recipient received the NRLP-3 blocker was superior to all other groups. This work demonstrated first, that the addition of molecules with a blocking effect on specific receptors is also effective during HMP or HOPE and secondly, that the already protective HOPE-effect on mitochondria can be even more enhanced with additional reduction of downstream inflammatory pathways (99). Other groups have for example demonstrated, that a selective cytokine blockage, e.g., IL-10 and TGF-β also reduces

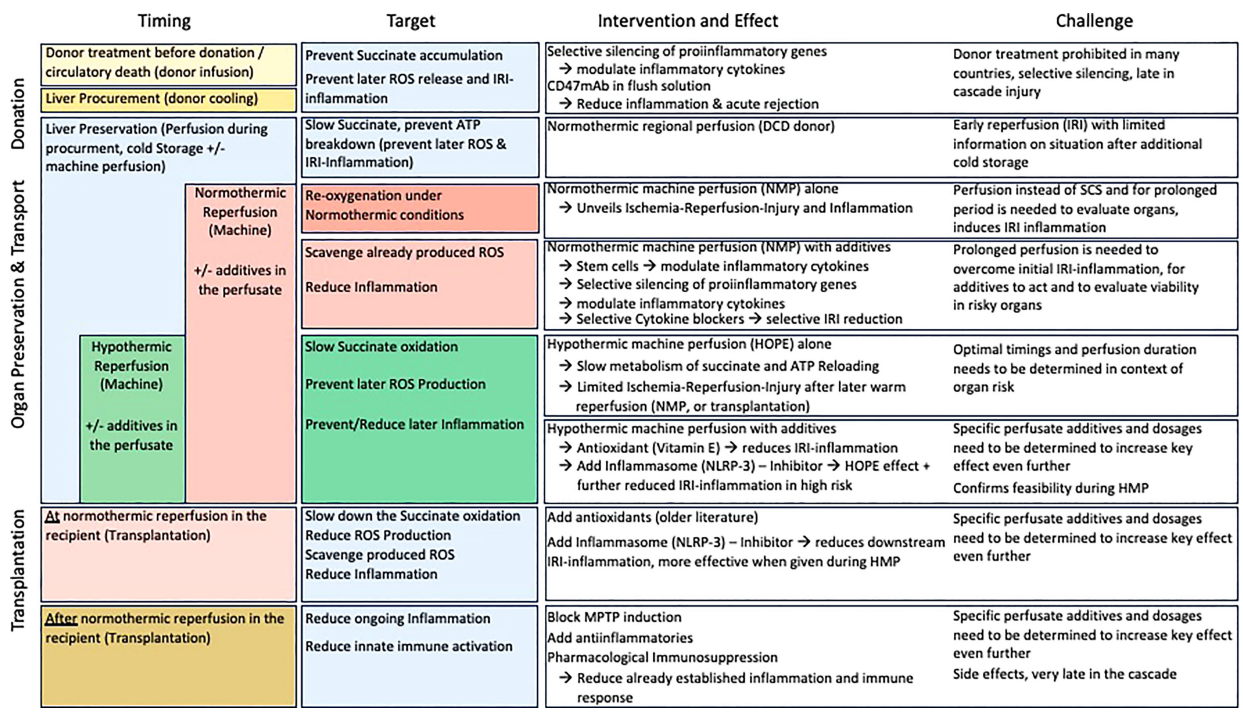

FIGURE 5 | Therapeutic strategies to target ischemia-reperfusion injury and improve outcomes after liver transplantation; This figure provides an overview of currently applied pharmacological and non-pharmacological modalities with impact on IRI associated features. From the donor, procurement to preservation and reperfusion (transplantation) modalities, their targets and results as well as challenges are presented. The majority of strategies affects individual genes or receptors, which might lead to an even higher proinflammatory response by other genes not affected.

immune responses, however mainly in models with liver machine perfusion but without subsequent transplantation (100). Another approach is the addition of stem cells to perfusates. Laing et al. have added mesenchymal stem cells to NMP perfusates and show feasibility and comparable cell settling in perfused livers, when administered through different inflow vessels (78). Other agents, including defatting cocktails, were found to reduce the overall inflammation induced by NMP. Boteon et al. demonstrated an effect on the early innate immune response during NMP. Both, CD14, as found in macrophages and neutrophils, and pro-inflammatory cytokines were reduced by defatting cocktails in NMP perfusates (101). Another attractive approach is increasingly discussed with RNA interference. The group of Paulo Martins has recently demonstrated the first successful administration of siRNA during both, hypothermic and normothermic perfusion. The hepatocyte transfection was achieved through siRNA coating with lipid nanoparticles (102, 103). Of note, this is another evidence that such treatments are feasible in both perfusion approaches, warm and cold (53, 55, 103). The impact of such selective gene down regulation might however trigger responsive upregulation of other genes and subsequent cytokine and mediator release, where more studies also to identify the best pathways in humans are needed (**Figure 5**).

SUMMARY AND FUTURE PERSPECTIVES

Machine perfusion is an attractive tool to directly treat human livers prior to implantation and also to deliver specific molecules with impact on outcomes. The underlying mechanisms will however require more studies in the future to understand the individual impact of different perfusion strategies on the immune system. This review has mainly focused on the innate immune response, where an effect of HOPE perfusion is described in the current literature. The potential effect on the B cell response remains however entirely unknown. Most administered compounds during MP remain currently experimental and studies to identify the best route and dosage for administration are still lacking, particularly in humans. This is also valid for the spectrum of nanoparticles with various available types and formulations to be explored in the future.

AUTHOR CONTRIBUTIONS

RP, MC and AS designed the review and the figures. RP, MC, DD and AS: wrote the first draft. All coauthors discussed the content, revised the manuscript and approved the final version.

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GLOSSARY

ALT	Alanine Aminotransferase
AP-1	Activator Proteine-1
AS	Anastomotic strictures
AST	Aspartate-Aminotransferase
ATP	Adenosine-trisphosphate
BACH2	transcription regulator protein
CD	Cluster of differentiation
CIT	Cold ischemia time
CS	cold storage
CSR	Class switch recombination
Damp`s	Danger associated molecular pattern`s
DBD	donation after brain death
DCD	Donation after circulatory death
DMM	dimethylmalonate
DWIT	donor warm ischemia time
EAD	Early allograft dysfunction
ECD	Extended Criteria Donor
FMN	Flavin-mononucleotide
HA	Hepatic artery
GSH	Glutathione
HAR	hexa-ammineruthenium
HAT	Hepatic artery thrombosis
HMGB-1	High mobility group box-1 protein
HMP	Hypothermic machine perfusion
HOPE	Hypothermic oxygenated perfusion
H&E	Hematoxylin and Eosin
IC	Ischemic cholangiopathy
ICAM-1	Intercellular adhesion molecule-1
IL	Interleukin
IRF3	Interferon regulatory factor 3
IRI	Ischemia-reperfusion injury
IS	Immunosuppression
ITBL	Ischemic type biliary lesions
KC`s	Kupffer cells
LPS	Lipopolysaccharide
LT	Liver Transplantation
MAVS	Mitochondrial antiviral signaling (protein)
MELD	Model of end stage liver disease
MPS	Machine perfusion solution
MPT pore	Mitochondria permeability transition pore
mtROS	mitochondrial reactive oxygen species
MyD88	Myeloid differentiation primary response 88
NAD/NADH	nicotine adenine dinucleotide (oxidized/ reduced)
NFkB	nuclear factor kappa-light-chain-enhancer
NOX4, NMP	Normothermic machine perfusion
NRP	normothermic regional perfusion
PNF	Primary non function
PV	portal vein
RCT	Randomized controlled trial
RET	reverse electron flow
ROS	reactive oxygen species
SCS	standard cold storage
SDH	Succinate dehydrogenase
SEC	sinusoidal endothelial cells
TIRAP	Toll-interleukin 1 receptor (TIR) domain containing adaptor protein
TLR-4 (9)	Toll-like-receptor-4
8-OHdG	8-hydroxy-2-deoxy Guanosine

frontiers | Frontiers in Immunology

ORIGINAL RESEARCH
published: 13 July 2022
doi: 10.3389/fimmu.2022.831371

Complement Is Activated During Normothermic Machine Perfusion of Porcine and Human Discarded Kidneys

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Specialty section:
This article was submitted to Alloimmunity and Transplantation, a section of the journal Frontiers in Immunology

Received: *08 December 2021*
Accepted: *14 June 2022*
Published: *13 July 2022*

Citation:
Jager NM, Venema LH, Arykbaeva AS, Meter-Arkema AH, Ottens PJ, van Kooten C, Mollnes TE, Alwayn IPJ, Leuvenink HGD, Pischke SE and PROPER study consortium (2022) Complement is Activated During Normothermic Machine Perfusion of Porcine and Human Discarded Kidneys. Front. Immunol. 13:831371. doi: 10.3389/fimmu.2022.831371

Background: The gap between demand and supply of kidneys for transplantation necessitates the use of kidneys from extended criteria donors. Transplantation of these donor kidneys is associated with inferior results, reflected by an increased risk of delayed graft function. Inferior results might be explained by the higher immunogenicity of extended criteria donor kidneys. Normothermic machine perfusion (NMP) could be used as a platform to assess the quality and function of donor kidneys. In addition, it could be useful to evaluate and possibly alter the immunological response of donor kidneys. In this study, we first evaluated whether complement was activated during NMP of porcine and human discarded kidneys. Second, we examined the relationship between complement activation and pro-inflammatory cytokines during NMP. Third, we assessed the effect of complement activation on renal function and injury during NMP of porcine kidneys. Lastly, we examined local complement C3d deposition in human renal biopsies after NMP.

Methods: NMP with a blood-based perfusion was performed with both porcine and discarded human kidneys for 4 and 6 h, respectively. Perfusate samples were taken every hour to assess complement activation, pro-inflammatory cytokines and renal function. Biopsies were taken to assess histological injury and complement deposition.

Results: Complement activation products C3a, C3d, and soluble C5b-9 (sC5b-9) were found in perfusate samples taken during NMP of both porcine and human kidneys. In addition, complement perfusate levels positively correlated with the cytokine perfusate levels of IL-6, IL-8, and TNF during NMP of porcine kidneys. Porcine kidneys with high sC5b-9 perfusate levels had significantly lower creatinine clearance after 4 h of NMP.

In line with these findings, high complement perfusate levels were seen during NMP of human discarded kidneys. In addition, kidneys retrieved from brain-dead donors had significantly higher complement perfusate levels during NMP than kidneys retrieved from donors after circulatory death.

Conclusion: Normothermic kidney machine perfusion induces complement activation in porcine and human kidneys, which is associated with the release of pro-inflammatory cytokines and in porcine kidneys with lower creatinine clearance. Complement inhibition during NMP might be a promising strategy to reduce renal graft injury and improve graft function prior to transplantation.

Keywords: kidney transplantation, normothermic machine perfusion, complement system, ischemia-reperfusion injury, immunology

INTRODUCTION

Normothermic machine perfusion (NMP) is a preservation technique that recently has been introduced to assess organ quality prior to transplantation. The increasing gap between demand and supply resulted in the necessity to transplant kidneys with a lower graft quality and has resulted in an increased risk of delayed graft function and inferior renal function compared to standard criteria donor kidneys (1, 2). The exact mechanism is unknown, but it is hypothesized that the higher immunogenicity of older donors and increased comorbidities lead to inferior results after transplantation. The increased use of extended criteria donor kidneys led to the idea of tailoring the preservation method to the renal graft, which resulted in the use of NMP. During NMP, the renal graft is perfused with an oxygenated perfusion solution at 37°C (**Figure 1**). Maintaining a kidney at a normothermic temperature has many advantages. It not only provides the possibility of evaluating kidney quality but also reconditions prior to transplantation. Furthermore, it would be possible to characterize immunological responses of donor kidneys. Limited studies have shown the potential of machine perfusion to reduce the immune response in the donor kidney. Pig studies showed reduced graft immunogenicity by initiating an inflammatory cytokine storm leading to leukocyte mobilization and removal prior to renal transplantation. NMP of porcine kidneys resulted in the modulation of pro-inflammatory gene expression levels and a decrease in the number of leukocytes in the kidney prior to transplantation (3, 4). However, less is known about the pathophysiology of these immunological responses and how to reduce the immunogenicity of kidneys during NMP.

Kidneys are equipped with a sophisticated localized immune system. Previous studies have shown that severe injury, such as brain death or ischemia–reperfusion injury, induces a robust local immune response. As part of the local immune response, the complement system is activated (3, 5–7). To avoid inappropriate complement activation under normal conditions, complement is strictly regulated by regulatory proteins. Inappropriate activation of the complement system can have deleterious effects on kidneys and needs to be prevented when possible (8, 9). It is known that the complement system can be activated through contact with foreign surfaces, which is the case

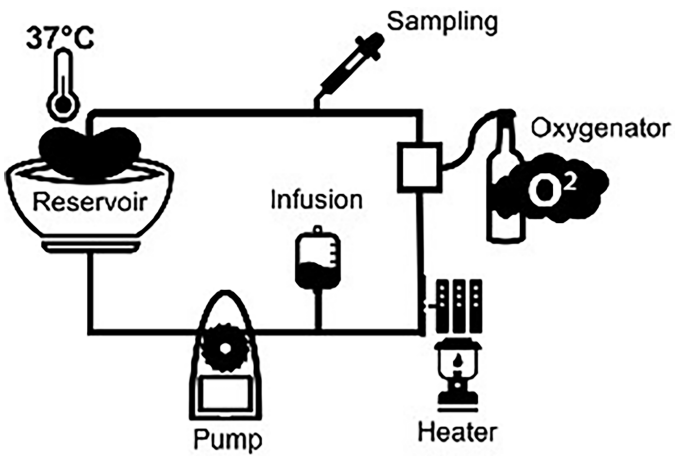

FIGURE 1 | Normothermic machine perfusion setup.

during extracorporeal membrane oxygenation (ECMO), cardiopulmonary bypass (CPB), and hemodialysis (HD) (10, 11). An NMP system consists of the same components as an ECMO, CPB, and HD system. Therefore, it is likely that NMP also results in complement activation. In addition, complement activation also plays a prominent role in renal graft injury, which is started in the donor and continued in the recipient (12, 13). Given the importance of complement in renal graft injury, modulating the complement system is a potential promising strategy to improve renal transplant outcome (13, 14). This study aimed to examine whether complement gets activated during NMP of porcine and human discarded kidneys. The effect of complement activation on the pro-inflammatory response and renal function during NMP of porcine kidneys was investigated.

MATERIALS AND METHODS

Normothermic Machine Perfusion of Porcine Kidneys

In the preclinical phase, porcine kidneys (from female, Dutch landrace pigs) and autologous heparinized whole blood were used, as described previously (15). In short, kidneys ($n = 20$) were exposed to a warm ischemia time of 30 min to induce ischemic injury. Subsequently, kidneys were cold flushed with 180 ml of cold (4°C) saline (Baxter BV, Utrecht, The Netherlands) and stored for 24 h by either static cold storage (SCS) or with hypothermic machine perfusion (HMP) with a mean arterial pressure of 25 mmHg with no active oxygenation or with 21% or 100% oxygen (15). Kidneys of the HMP groups were cannulated for connection to the HMP device (Kidney Assist Transport, Organ Assist, Groningen, The Netherlands) and perfused with 500 ml of University of Wisconsin machine perfusion solution (UW-MP solution, Bridge to Life Ltd., London, United Kingdom). SCS preserved kidneys were submerged in 500 ml of University of Wisconsin cold storage solution (UW-CS, Bridge to Life Ltd.) and stored on ice. Next, kidneys were flushed with 50 ml of cold saline solution to remove the remaining UW solution and connected to the NMP device (**Figure 1**). Thereafter, all kidneys were pressure-controlled perfused with a mean arterial pressure of 75 mmHg at a normothermic temperature (37°C) for 4 h (Kidney Assist Transport, Organ Assist). The perfusion solution was leukocyte-depleted blood diluted with Ringer's lactate solution and several additives (**Table 1**). Renal artery and ureter were cannulated with a 12F and 8F cannula respectively. The perfusion solution was oxygenated *via* an oxygenator (Medos Medizin AG, Stolberg, Germany) with a mixture of 95% O_2/5% CO_2 at a flow rate of 0.5 L/min. During NMP, there was a continuous supply of nutrients, which was administered at a rate of 20 ml/h (**Table 1**). In case the glucose levels dropped below 5 mmol/L, glucose 5% was administered. To differentiate between the activation of complement by the kidney versus by the machine setup, i.e., tubing and oxygenator, we included a small ($n = 2$) kidney-free NMP control group where NMP was performed with heparinized pig blood for 4 h without a kidney.

Normothermic Machine Perfusion of Human Discarded Kidneys

Human discarded kidneys were included within the Prolonged *ex situ* NMP for kidney regeneration (PROPER) trial registered in the Dutch Trial Register as NL8446. The PROPER trial is a Dutch initiative from three University transplant centers—The University Medical Center Groningen (UMCG), Leiden University Medical Center (LUMC), and Rotterdam Erasmus Medical Center (Erasmus MC)—to introduce and clinically evaluate prolonged NMP. As part of the PROPER trial, the current study investigated the role of complement activation during NMP in human discarded kidneys. The ten analyzed kidneys were included between January 1, 2019 and August 1, 2019. The included kidneys were retrieved from donation after brain death (DBD) and donation after circulatory death (DCD) donors, but discarded postretrieval. Reasons for discarding are described in **Table 3**. Kidneys were cold flushed with University of Wisconsin solution (UW-CS, Bridge to Life Ltd.) and preserved *via* SCS or with HMP. As a perfusion solution, matched banked red blood cells (RBCs) were used, which were washed prior to NMP with 2 L of 0.9% NaCl by using a CellSaver (Fresenius C.A.T.S. plus, Fresenius Kabi GmbH, Bad Homburg, Germany) and afterwards diluted with 0.9% NaCl (**Table 1**).

TABLE 1 | Composition of the perfusion solution used for normothermic machine perfusion.

Perfusion solution—Porcine kidneys	Perfusion solution—Human discarded kidneys
500 ml of Leukocyte-depleted blood	2 packed washed red blood cells
1,000 mg/200 mg of Amoxicillin/Clavulanate	1000 mg of Cefazoline
6 mg Mannitol	20 ml of 15% Mannitol
10 ml of 8.4% Sodium bicarbonate	Approximately 15–20 ml 8.4% Sodium bicarbonate, to correct pH before perfusion
100 µl of 20 mg/ml Sodium Nitroprusside	20 ml of 10% Calcium Gluconate
300 ml of Ringer's lactate	500 ml of NaCl
90 mg of Creatinine	100 ml of 20% Albumin
6 mg of Dexamethasone	
Infusion solution—Porcine kidneys	Infusion solution—Human discarded kidneys
80 ml of 10% Aminoplasmal	50 ml of 10% Aminoplasmal [23.3 ml/h]
17 IU Novorapid	Cernevit multivitamins (0.5 ml added to aminoplasmal)
	0.5 mg of Flolan [5.8 ml/h]
	Glucose 5% [8 ml/h]

Upon arrival, the renal artery and ureter were cannulated with a 12F and 8F cannula, respectively. After preparation, the kidney was weighed and flushed with 200 ml of Ringer's lactate solution and connected to the NMP device (Kidney Assist Transport, Organ Assist). Kidneys were pressure-controlled perfused with a mean arterial pressure of 75 mmHg for 6 h. The perfusion solution was oxygenated *via* an oxygenator (Medos Medizin AG) with a mixture of 95% O_2/5% CO_2 at a flow rate of 0.5 L/min. During NMP, there was a continuous supply of nutrients. Specifics on infusion and the perfusion solution can be found in **Table 1**.

Perfusate Samples

Perfusate samples during NMP were taken at baseline, 30 min, 1 h, 2 h, 3 h, and 4 h. Samples at time points 5 h and 6 h were only taken during NMP of human discarded kidneys. Samples were collected in EDTA tubes (Biosciences, Plymouth, UK) and stored on ice and centrifuged at 4°C, 2,500 × *g* for 20 min. Plasma was stored at −80°C.

Renal Function

Creatinine concentration was measured in perfusate and urine, using routine procedures at the clinical chemistry lab of the University Medical Center Groningen. Creatinine clearance (L/min/100 g) was calculated with the following formula ((urine creatinine concentration (mmol/L) * urine flow (ml/min)/perfusate creatine concentration (mmol/L))/kidney weight (g))) *100.

Renal Morphology After NMP of Porcine Kidneys

Renal biopsies were taken after 4 h of NMP. Paraffin sections (4 μm) were stained with hematoxylin-eosin (H&E). Histological injury was scored on the basis of two criteria: proximal tubular cell necrosis and proximal tubular cell edema. Histological injury was scored blinded by two independent examiners under the supervision of a pathologist. Proximal tubular cell necrosis and proximal tubular cell edema were scored as described previously (15): (1) no necrosis/edema; (2) minor signs of necrosis/edema; (3) moderate signs of necrosis/edema; (4) severe necrosis/edema; and (5) complete necrosis/edema. The different biopsies were randomly assigned to the examiners, and in case of discrepancy, the supervising pathologist was consulted.

Renal C3d Deposition After NMP of Human Discarded Kidneys

Renal biopsies were taken after 6 h of NMP. Frozen sections (4 μm) were fixed with acetone and endogenous peroxidase was blocked by incubating all sections in 30% H_2O_2. Next, the sections were incubated with primary antibody against C3d (Clone A0063, Dako, CA, USA). Subsequently, sections were incubated with a goat-anti-rabbit-FITC antibody. Finally, sections were incubated with 4' 6-diamidino-2-phenylindole (DAPI) to stain the nuclei and mounted with Citifluor. Tissue images were acquired using Leica confocal microscope (Leica, Wetzlar, Germany). Sections were quantified by two independent examiners. An immunofluorescence density (ID) score was calculated based on (1) the intensity of the staining in the renal cortex (scored from 1 to 5) and (2) the percentage area of the renal cortex with C3d deposition (scored from 0 to 100).

Complement Assays for Perfusate Samples After NMP of Porcine Kidneys

To measure complement activation products in the perfusate samples, complement activation products at the level of C3 were measured. In the perfusate samples retrieved from NMP with porcine kidneys, C3a was measured using a highly specific porcine ELISA described earlier, using antibodies reacting with the C3a fragment (16). The terminal pathway activation was measured by soluble C5b-9 (sC5b-9) using the monoclonal antibody aE11 as capture antibody (17). It reacts efficiently with a neoepitope exposed in both human- and pig-activated C9, and the assay was performed as described previously. The level was related to the International Complement Standard #2, defined to contain 1,000 complement arbitrary units (AU) per ml.

Complement Assays for Perfusate Samples After NMP of Human Discarded Kidneys

C3 and C3d were measured in perfusate samples after NMP of human discarded kidneys. C3d was measured as described previously (18). Briefly, samples were polyethylene glycol (PEG) precipitated. PEG precipitation is necessary since free C3d shares epitopes with intact C3. Based on the measured values, a C3d/C3 ratio was calculated, which is a measure for complement activation at the level of complement C3. All samples were 1:1 diluted with 22% PEG in 0.1 M borate/EDTA buffer (pH 8.32) and incubated on ice for 3 h. Afterwards, they were centrifuged and supernatants were used for C3d quantification. Rabbit anti-human C3d was used as coating antibody (Dako). A rabbit anti-human C3d-DIG was used as capture antibody and a rabbit anti-DIG-HRP (Roche) was used as detection antibody. All samples were measured in duplicate. Values are expressed as ng/ml. A standard curve was made in a 6-fold dilution and reference samples was made from stock solution.

Quantification of Porcine and Human Cytokines

To detect and quantify pro-inflammatory cytokines, tumor necrosis factor (TNF), interleukin-6, and interleukin-8 in the perfusate samples of porcine kidneys, we used commercial porcine immunoassay kits according to the manufacturer's instructions (R&D Systems, Minneapolis, MN, USA). For the detection and quantification of interleukin-6 and interleukin-8 in perfusate samples of human kidneys, we used commercial human immunoassay kits according to the manufacturer's instructions (R&D Systems, Minneapolis, MN, USA).

Statistical Analysis

Statistical analysis was performed using GraphPad Prism Software v8.3.1. Differences between time points were tested

using Kruskal–Wallis test followed by a Mann–Whitney *U post-hoc* test. Differences between two groups were tested by a Mann–Whitney *U* test. Spearman correlation coefficients were calculated to determine which cytokines were significantly associated with complement C3a and sC5b-9 levels. $P < 0.05$ was considered significant. Data are presented as median ± interquartile range (IQR).

RESULTS

Complement Is Activated During Normothermic Machine Perfusion of Porcine Kidneys

Complement activation was assessed by the quantification of perfusate levels of C3a and sC5b-9 during 4 h of NMP. C3a levels increased after 2 h of NMP with a steep increase to 4 h NMP and were significantly increased compared to C3a perfusate levels at 30 min of NMP (**Figure 2A**). Complement sC5b-9 perfusate levels significantly increased after 2 and 4 h of NMP (**Figure 2B**). The preservation method before NMP, hypothermic machine perfusion with or without oxygen, did not influence C3a or sC5b-9 perfusate levels during 4 h of NMP (**Supplementary Figures 1A, B**). In addition, significantly less complement was activated in kidney-free NMPs (**Supplementary Figures 2A, B**).

High Levels of Pro-Inflammatory Cytokines Are Released Normothermic Machine Perfusion of Porcine Kidneys

Perfusate levels of IL-6 and IL-8 were significantly higher after 2 and 4 h than baseline levels. Both increased exponentially between 2 and 4 h of NMP (**Figures 3A, B**, respectively). There was a significant release of TNF after 2 h of NMP, which further increased after 4 h of NMP (**Figure 3C**). TNF increased linearly throughout the NMP period. Different preservation techniques prior to NMP did not affect cytokine perfusate levels during, NMP (**Supplementary Figures 3A–C**).

Complement and Cytokine Perfusate Levels Strongly Correlate During Normothermic Machine Perfusion of Porcine Kidneys

Increase in complement C3a perfusate levels strongly correlated with the increase of perfusate levels of IL-6 and IL-8 (**Figures 4A, B**). In addition, there was a significant but moderate correlation between complement sC5b-9 perfusate levels and cytokine perfusate levels after 4 h of NMP (**Table 2**).

Kidneys With High sC5b-9 Perfusate Levels Have Significantly Lower Creatinine Clearance

Next, subgroup analysis was performed for sC5b-9 perfusate levels after 4 h of NMP of porcine kidneys (**Figure 3B**). Two subgroups (n = 10/group) were formed based on the median value of sC5b-9 after 4 h of NMP (median 105 AU/ml) and the creatinine clearance for these two subgroups was calculated. Kidneys with sC5b-9 perfusate levels above 105 AU/ml after 4 h of NMP had a significant lower creatinine clearance than kidneys with sC5b-9 levels below this value (**Figure 5**).

Porcine Kidneys With High sC5b-9 Perfusate Levels Have Significantly More Histological Injury

Histological injury was examined in porcine kidneys after 4 h of NMP. All porcine kidneys had proximal tubular cell necrosis and edema after NMP. After initial examination, the kidneys were divided in subgroups, based on low or high sC5b-9 perfusate levels (n = 10/group). Kidneys with high sC5b-9 perfusate levels had significantly more proximal tubular cell necrosis and edema than kidneys with low 2C5b-9 perfusate levels (**Figure 6**).

Complement Is Activated During Normothermic Machine Perfusion of Human Discarded Kidneys

To translate the experimental results seen during NMP of porcine kidneys, complement activation during NMP of

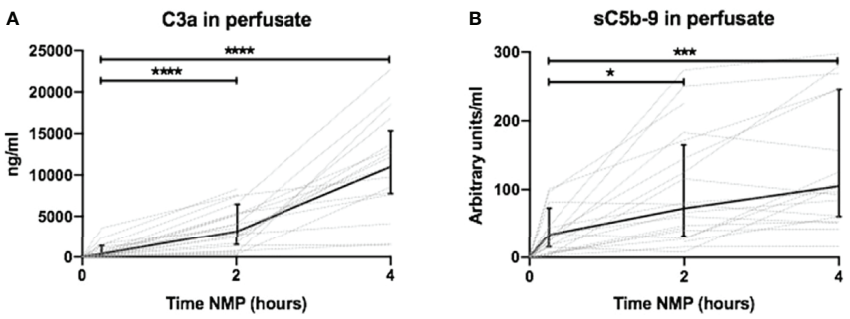

FIGURE 2 | Dynamics of complement perfusate levels during 4 h of normothermic machine perfusion of porcine kidneys. Dynamics of **(A)** C3a and **(B)** sC5b-9 perfusate levels during 4 h of normothermic machine perfusion of porcine kidneys. Perfusate levels of both C3a and sC5b-9 increase during normothermic machine perfusion of porcine kidneys. Dotted lines: increase of complement perfusate levels per individual perfused kidney. Solid lines and bars: median ± interquartile range of complement perfusate levels for all kidneys (n = 20). *$p < 0.05$, ***$p < 0.001$, ****$p < 0.0001$. NMP, normothermic machine perfusion; sC5b-9, soluble C5b-9.

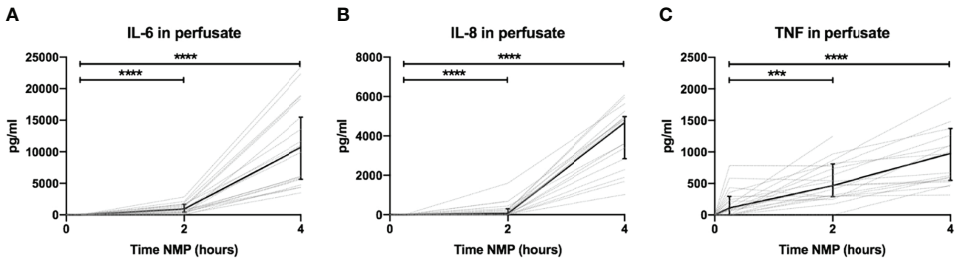

FIGURE 3 | Cytokine perfusate levels during normothermic machine perfusion of porcine kidneys. Dynamics of pro-inflammatory cytokines **(A)** IL-6, **(B)** IL-8, and **(C)** TNF in perfusate during normothermic machine perfusion of porcine kidneys. Cytokine perfusate levels significantly increased during 4 h of normothermic machine perfusion. Dotted lines: increase of cytokine levels in perfusate per individual perfused kidney. Solid lines and bars: median ± interquartile range of cytokine perfusate levels for all kidneys (n = 20). ***p < 0.001, ****p < 0.0001. IL-6, interleukin-6; IL-8, interleukin-8; NMP, normothermic machine perfusion; TNF, tumor necrosis factor.

human discarded kidneys was investigated. These human discarded kidneys were retrieved from five DBD and five DCD donors. Baseline characteristics of these kidneys are shown in **Table 3**. To investigate whether complement was activated during NMP of human discarded kidneys, complement C3 and C3d perfusate levels were measured. Complement C3 perfusate levels did not change during 6 h of NMP (**Figure 7A**). In contrast, C3d perfusate levels significantly increased during 6 h of NMP, with the biggest increase between 4 and 6 h of NMP (**Figure 7B**). In addition, the C3d/C3 ratio significantly increased after 6 h of NMP compared to baseline levels (30 min after NMP) (**Figure 7C**).

Kidneys Retrieved From Brain-Dead Donors Have Significantly Higher Complement Perfusate Levels Than Kidneys Donated After Circulatory Death

To investigate the role of different types of donors, C3d/C3 ratio during NMP of kidneys retrieved from DBD *versus* DCD donors were compared. Kidneys retrieved from DBD donors had a significantly higher C3d/C3 ratio at 6 h of NMP than kidneys retrieved from DCD donors (**Figure 8**). During NMP of kidneys retrieved from DBD donors, there was a significant increase of the C3d/C3 ratio over time. In contrast, the C3d/C3 ratio during NMP of kidneys from DCD donors did not significantly change over time. No differences in the C3d/C3 ratio were seen based on different preservation methods (SCS *versus* HMP) or based on the cold ischemia time (shorter *versus* longer than 15 h) (data are not shown).

Kidneys Retrieved From Brain-Dead Donors Have Significantly More Renal C3d Deposition

In addition, C3d deposition was examined in human renal biopsies taken after 6 h of NMP (**Figures 9A, B**). C3d deposition was seen in all renal biopsies, independent of the type of donor. However, subgroup analyses demonstrated that renal biopsies derived from DBD donors have significantly more C3d deposition than renal biopsies derived from DCD donors after 6 h of NMP (**Figure 9C**).

High Levels of Cytokines Are Released During Normothermic Machine Perfusion of Human Discarded Kidneys

Lastly, we assessed the release of pro-inflammatory cytokines during NMP of human discarded kidneys. No IL-6 was seen during the first 2 h of NMP. In contrast, there were significantly high levels of IL-6 after 6 h of NMP (**Figure 10A**). Likewise, high

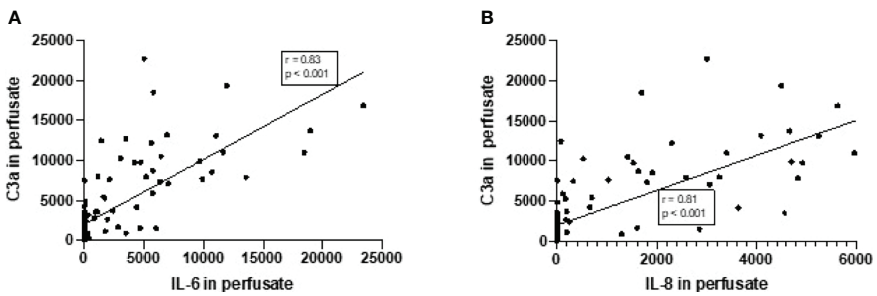

FIGURE 4 | Correlation between complement C3a perfusate levels and cytokine perfusate levels in porcine kidneys after 4 h of normothermic machine perfusion. Correlation between **(A)** complement C3a and IL-6 levels in perfusate and **(B)** correlation between complement C3a and IL-8 levels in perfusate after 4 h of NMP of porcine kidneys. Spearman's correlation coefficient (r) and p-values are indicated. IL-6, interleukin-6; IL-8, interleukin-8; NMP, normothermic machine perfusion.

TABLE 2 | Correlation between complement perfusate levels and cytokine perfusate levels after 4 h of normothermic machine perfusion of porcine kidneys.

Perfusate levels	Correlation with C3a		Correlation with sC5b-9	
	r	*p*-value	*r*	*p*-value
IL-6	0.83	<0.0001	0.52	<0.0001
IL-8	0.81	<0.0001	0.48	<0.0001
TNF-alpha	0.66	<0.0001	0.54	<0.0001

r, correlation coefficient.

levels of IL-8 were observed in perfusate after 6 h of NMP of human discarded kidneys (**Figure 10B**).

DISCUSSION

In this study, complement activation during NMP was assessed by using porcine and human discarded kidneys. Complement was activated during NMP of porcine kidneys, reflected by increase of complement activation fragments in the perfusate. Furthermore, levels of complement activation during NMP of porcine kidneys positively correlated with pro-inflammatory cytokine levels. Porcine kidneys with high sC5b-9 perfusate levels after 4 h of NMP had a significantly lower creatinine clearance and significantly more histological injury than kidneys with low sC5b-9 perfusate levels. In line with our findings in porcine kidneys, discarded human kidneys showed the same trend of complement activation during NMP. Looking at the different types of donors, kidneys retrieved from brain-dead donors showed significantly higher complement activation during NMP than kidneys retrieved after circulatory death. In addition, kidneys retrieved from brain-dead donors had significantly more renal C3d deposition after NMP. Lastly, an enormous release of pro-inflammatory cytokines IL-6 and IL-8 was seen during NMP of human discarded kidneys.

TABLE 3 | Baseline characteristics of human discarded kidneys perfused during 6 h of normothermic machine perfusion.

Characteristic	Summary
Female donor (*n*; %)	1; 10
Reason for decline (*n*; %)	2; 20
- Artheriosclerosis	1; 10
- High donor age	1; 10
- Dissection renal artery	1; 10
- High transaminase levels	1; 10
- Suspicious for malignancy	4; 40
- Medical reasons (not further specified)	
Donor type (*n*; %)	5; 50
- DBD donor	5; 50
- DCD donor	
Organ preservation method (*n*; %)	4; 40
- Static cold storage	6; 60
- Hypothermic machine perfusion	
Cold ischemia time (*n*; %)	4; 40
- < 15 h	6; 60
- > 15 h	

DBD, donation after brain death; DCD, donation after circulatory death.

Complement was immediately activated at the start of NMP. Based on other *ex situ* setups, our results are in line with studies describing complement activation in ECMO, CPB, and HD (19, 20). All these studies reveal an increase in complement C3 activation and sC5b-9 formation within the first 15 min (21, 22). The rapid increase in complement activation products could be due to the initial blood-to-material contact, already described decades ago. This blood-to-material contact could result in immediate adsorption of serum proteins, i.e., complement C3 and immunoglobulin G (19, 23). After the initial activation of complement, levels of complement continued to rise to the end of NMP, consistent with an imbalance between activation and inhibition of the system. The absence of a negative feedback loop to inhibit further complement activation could be the reason for the ongoing complement activation. *In vivo* complement activation is regulated *via* plasma and membrane-bound regulators, which avoid inappropriate complement activation (24). However, complement activation is not regulated by membrane-bound regulators during *ex situ* machine perfusion. In addition, the bio-incompatibility of the NMP system may add to the total activation of complement. Kidney-free NMP confirmed that an initial rise in complement activation is due to blood-to-material contact. However, complement activation levels were significantly higher during NMP compared to the kidney-free system, indicating that the kidney plays an important role in complement activation. Of note, human kidney NMP was performed with a plasma- and leucocyte-free perfusate, but also here complement activation and cytokine production were observed, adding to the notion that the kidney might be regarded as an immunologically active organ on its own (25). Future studies should investigate if complement proteins are produced in the kidney and add fuel to an ongoing complement activation during NMP.

So far, the consequences of complement activation during NMP are unknown. This study shows that complement activation is strongly correlated with the release of the pro-inflammatory cytokines IL-6, IL-8, and TNF. Our results are in line with previous results obtained with porcine kidney NMP, demonstrating the release of pro-inflammatory cytokines IL-6, IL-1β, and IL-18 (26). Interestingly, the dynamics of IL-6 and IL-8 differed from TNF. TNF perfusate levels increase from the start of NMP, while IL-6 and IL-8 perfusate levels started to increase after 2 h of NMP. This might imply that IL-6 and IL-8 might be produced *via* a TNF-dependent pathway (27). Together, these cytokines might propagate further release of adhesion molecules and polymorphonuclear cells contributing to a pro-inflammatory state causing tissue damage (28)..

So far, the consequences of the inflammatory response seen during NMP on renal graft function remains unknown (29). Activation of complement during NMP could theoretically be beneficial, because it could exhaust the complement activation capacity of the renal graft prior to ischemia reperfusion in the recipient. In addition, local complement activation in the renal graft can contribute to the restoration of homeostasis by clearing of cell debris and necrotic sells. However, complement activation

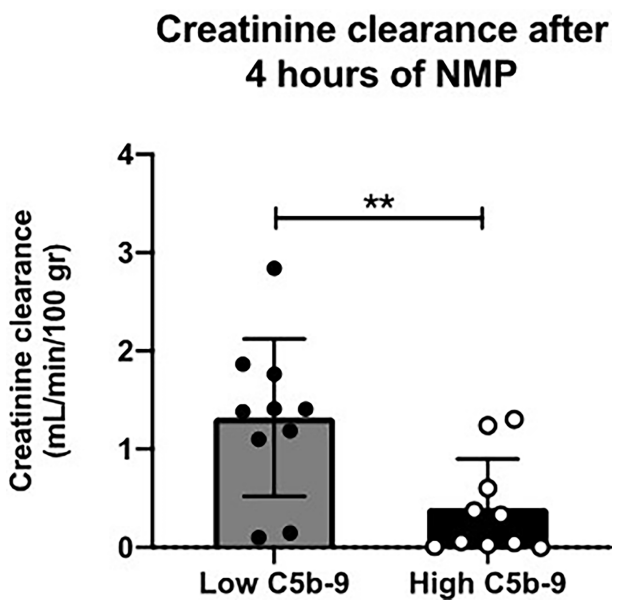

FIGURE 5 | Creatinine clearance after 4 h of normothermic machine perfusion of porcine kidneys with low *versus* high sC5b-9 perfusate levels. Kidneys (n = 10) with low sC5b-9 perfusate levels after 4 h of normothermic machine perfusion (NMP) had significantly higher creatinine clearance than kidneys (n = 10) with high sC5b-9 perfusate levels after 4 h of NMP. Subgroups were based on the median sC5b-9 perfusate level after 4 h of NMP, which was 105 AU/ml. $^{**}p < 0.01$. NMP, normothermic machine perfusion; sC5b-9, soluble C5b-9.

during NMP could also have undesirable effects resulting in increased cytokine concentrations and decreased renal function. In our study, we demonstrated that the significant increase in sC5b-9 perfusate levels result in a significant lower creatinine clearance and more histological injury. These results could implicate that complement activation during NMP has adverse effects. However, the human discarded kidneys used in our study are not transplanted into a recipient. Until kidneys after NMP get

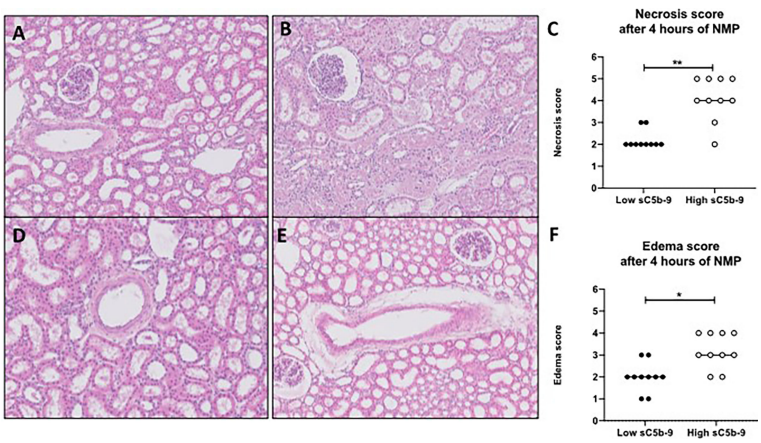

FIGURE 6 | H&E-stained biopsies of porcine kidneys after 4 h of normothermic machine perfusion. **(A–C)** Proximal tubular necrosis score (scored from 1 to 5) was assessed histologically after 4 h of normothermic machine perfusion (NMP). **(A)** Representative picture of a low necrosis score (score 2) after 4 h of NMP. **(B)** Representative picture of a high necrosis score (score 5) after 4 h of NMP. **(C)** Graph of the overall necrosis score after 4 h of normothermic machine perfusion. Kidneys with low sC5b-9 perfusate levels (n = 10) had significantly lower necrosis scores than kidneys with high sC5b-9 perfusate levels (n = 10). **(D, E)** Edema score (score from 1 to 5) was assessed histologically after 4 h of NMP. **(D)** Representative picture of a low edema score (score 1). **(E)** Representative picture of a high edema score (score 4). **(F)** Graph of the overall edema score after 4 h of normothermic machine perfusion. Kidneys with low sC5b-9 perfusate levels (n = 10) had significantly lower edema scores than kidneys with high sC5b-9 perfusate levels (n = 10). Subgroups were based on the median sC5b-9 perfusate levels after 4 h of normothermic machine perfusion. $^{*}p < 0.05$, $^{**}p < 0.01$. Images were obtained by using Leica Confocal Software. All magnifications ×100. sC5b-9, soluble C5b-9; NMP, normothermic machine perfusion.

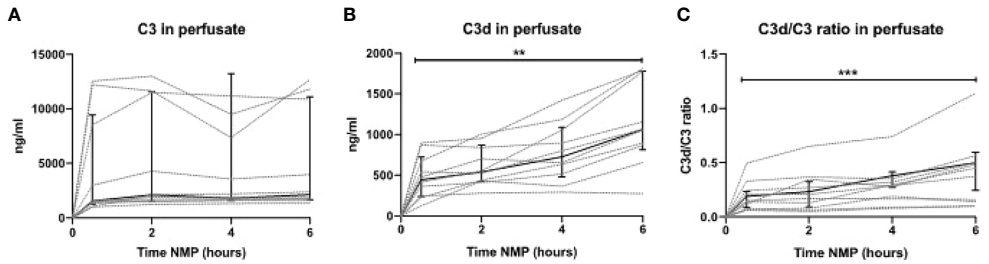

FIGURE 7 | Complement perfusate levels during normothermic machine perfusion of human discarded kidneys. **(A)** Complement C3 (in ng/ml) and **(B)** C3d levels (in ng/ml) and **(C)** the calculated C3d/C3 ratio in perfusate during 6 h of normothermic machine perfusion of human discarded kidneys. Dotted lines: increase of complement per individual kidney. Solid lines and bars: median ± interquartile range for all kidneys (n = 10). $^{**}p < 0.01$, $^{***}p < 0.001$. NMP, normothermic machine perfusion.

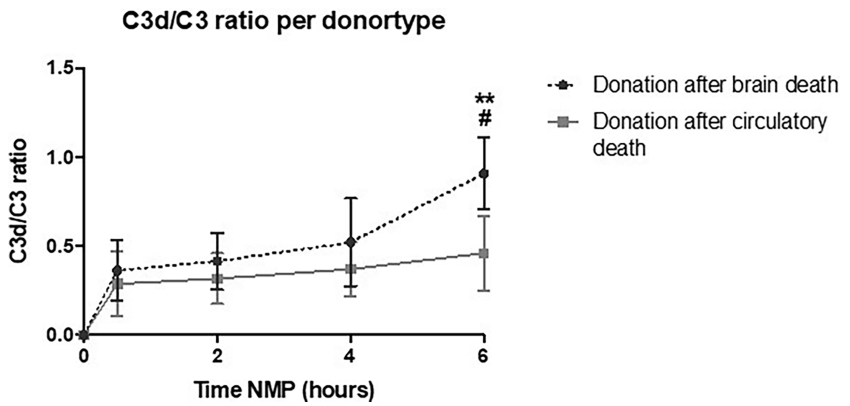

FIGURE 8 | Perfusate C3d/C3 ratio of human kidneys retrieved from brain-dead donors is significantly higher after 6 h of normothermic machine perfusion. Shown are the differences in C3d/C3 ratio during normothermic machine perfusion based on donor type. Kidneys retrieved from brain-dead donors showed significantly higher C3d/C3 ratio after 6 h of normothermic machine perfusion than kidneys retrieved after circulatory death. Data shown as median ± interquartile range. The asterisk denotes a significant difference between baseline C3d/C3 ratio and C3d/C3 ratio after 6 h of NMP of kidneys from brain-dead donors. The hashtag denotes a significant difference in C3d/C3 ratio between the two groups, brain-dead donors and donation after circulatory death. $\#p < 0.05$, $^{**}p < 0.01$. NMP, normothermic machine perfusion.

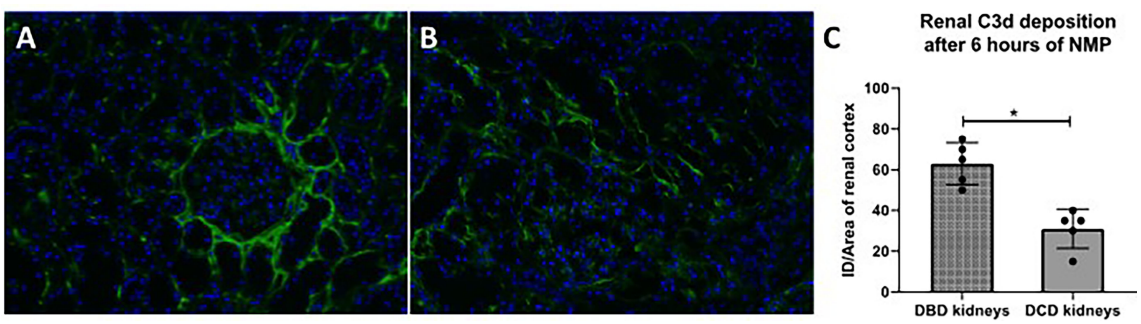

FIGURE 9 | C3d deposition in human discarded kidneys after 6 h of normothermic machine perfusion. Confocal microscopy of human discarded kidneys procured from **(A)** brain-dead donor and **(B)** a circulatory death donor after 6 h of normothermic machine perfusion. **(C)** Difference in C3d deposition after 6 h of normothermic machine perfusion in kidneys derived from brain-dead versus circulatory death donors. Nuclei were counterstained with DAPI. Original magnification ×100. DBD, donation after brain death; DCD, donation after circulatory death. ID, immunofluorescence density; NMP, normothermic machine perfusion.

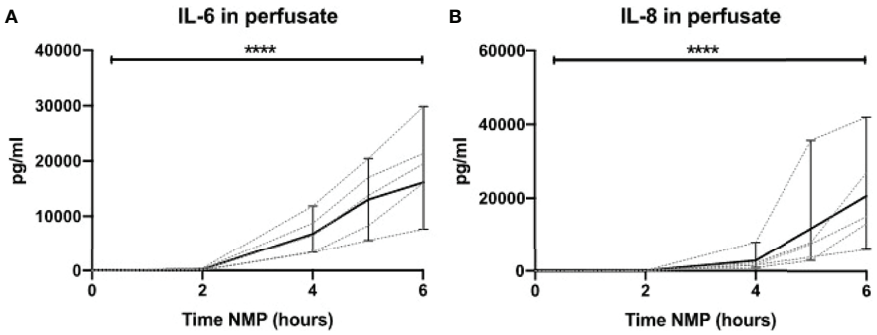

FIGURE 10 | Cytokine perfusate levels during normothermic machine perfusion of human discarded kidneys. Dynamics of pro-inflammatory cytokines **(A)** IL-6 and **(B)** IL-8 in perfusate during normothermic machine perfusion of human discarded kidneys. Cytokine perfusate levels significantly increased during 6 h of normothermic machine perfusion. Dotted lines: increase of cytokine levels in perfusate per individual perfused kidney. Solid lines and bars: median ± interquartile range of cytokine perfusate levels for all kidneys (n = 10). ****$p < 0.0001$. IL-6, interleukin-6; IL-8, interleukin-8; NMP, normothermic machine perfusion.

evaluated in a recipient, no conclusions can be drawn whether activation of complement during NMP is desirable or not. Another potential strategy to investigate the role of complement activation during NMP would be the use of a complement inhibitor, which should be investigated in a future study.

Aiming at translation, we measured complement activation levels during NMP of discarded human kidneys. Like NMP of porcine kidneys, complement activation levels were significantly increased during NMP of human kidneys. In addition, we demonstrated that kidneys retrieved from DBD donors showed a significantly higher C3d/C3 ratio than kidneys retrieved after circulatory death. This might be due to the increased inflammatory response in DBD donors compared to DCD donors. Brain death leads to activation of the immune system, which results in local and systemic inflammation (30). This might lead to an increased inflammatory response in kidneys retrieved from DBD donors during NMP (13, 31). Kidneys retrieved from brain-dead donors might thus specifically benefit from treatment with a complement inhibitor during NMP (18, 32, 33). No differences in complement activation during NMP were seen between preservation with SCS or HMP. This is remarkable, because the outcome of kidneys after HMP is superior to SCS in deceased donor renal transplantation, reflected by the lower incidence of delayed graft function after HMP (34). We speculate that the lack of differences between complement activation in HMP *versus* SCS is due to the attenuated complement activation at low temperatures, which resumes function after rewarming (35). In accordance, multiple studies describe low or no complement activation under other hypothermic conditions (35, 36).

This work has some limitations. First, the number of kidneys included in the human cohort is small and therefore might impact the statistical analyses. In addition, we did not correlate complement activation during NMP of human kidneys to renal injury or renal dysfunction. Creatinine clearance was not possible to assess in human kidneys as the perfusate does not contain creatinine and cannot be added sterile. Therefore, we were not able to include this analysis in this study. Further research should focus on the functional consequences of complement activation during NMP of human kidneys, which is possible only in the recipient after transplantation of an NMP-treated kidney, and test the efficacy of a complement inhibitor during NMP.

In conclusion, this study showed that complement was significantly activated during NMP of both porcine and human kidneys and associated with lower renal function, tissue damage, and inflammation. Complement inhibition during NMP might thus be a promising strategy to reduce renal injury and improve renal graft function prior to transplantation.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

Ethical review and approval was not required for the study on human participants in accordance with the local legislation and institutional requirements. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements. Ethical review and approval was not required for the animal study because Slaughterhouse waste material was used.

AUTHOR CONTRIBUTIONS

NJ and LV conducted preclinical porcine experiments, analyzed data, and wrote the manuscript. AA performed human kidney NMP. AM-H and PO assisted with analyses of the samples. TM

and SP helped with setting up the complement analyses and supervised the data analysis. NJ, LV, AA, SP, IA, and HL designed the research. SP and HL also had a supervising role. All authors read and approved the manuscript in the submitted version.

FUNDING

This study received the following funding: South-Eastern Norway Regional Health Authority (SEP; 2020117).

ACKNOWLEDGMENTS

We want to acknowledge Kristin Pettersen for the development of the porcine C3a assay that was used in this manuscript.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.831371/full#supplementary-material

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frontiers | Frontiers in Immunology

TYPE Review
PUBLISHED 13 October 2022
DOI 10.3389/fimmu.2022.982018

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OPEN ACCESS

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SPECIALTY SECTION
This article was submitted to Alloimmunity and Transplantation, a section of the journal Frontiers in Immunology

RECEIVED 30 June 2022
ACCEPTED 30 September 2022
PUBLISHED 13 October 2022

CITATION
Fodor M, Salcher S, Gottschling H, Mair A, Blumer M, Sopper S, Ebner S, Pircher A, Oberhuber R, Wolf D, Schneeberger S and Hautz T (2022) The liver-resident immune cell repertoire - A boon or a bane during machine perfusion?
Front. Immunol. 13:982018.
doi: 10.3389/fimmu.2022.982018

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The liver-resident immune cell repertoire - A boon or a bane during machine perfusion?

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The liver has been proposed as an important "immune organ" of the body, as it is critically involved in a variety of specific and unique immune tasks. It contains a huge resident immune cell repertoire, which determines the balance between tolerance and inflammation in the hepatic microenvironment. Liver-resident immune cells, populating the sinusoids and the space of Disse, include professional antigen-presenting cells, myeloid cells, as well as innate and adaptive lymphoid cell populations. Machine perfusion (MP) has emerged as an innovative technology to preserve organs *ex vivo* while testing for organ quality and function prior to transplantation. As for the liver, hypothermic and normothermic MP techniques have successfully been implemented in clinically routine, especially for the use of marginal donor livers. Although there is evidence that ischemia reperfusion injury-associated inflammation is reduced in machine-perfused livers, little is known whether MP impacts the quantity, activation state and function of the hepatic immune-cell repertoire, and how this affects the inflammatory milieu during MP. At this point, it remains even speculative if liver-resident immune cells primarily exert a pro-inflammatory and hence destructive effect on machine-perfused organs, or in part may be essential to induce liver regeneration and counteract liver damage. This review discusses the role of hepatic immune cell subtypes during inflammatory conditions and ischemia reperfusion injury in the context of liver transplantation. We further highlight the possible impact of MP on the modification of the immune cell repertoire and its potential for future applications and immune modulation of the liver.

KEYWORDS

liver transplantation, machine perfusion, liver-resident immune cells, immune activation, innate immunity, adaptive immunity, ischemia reperfusion injury

10.3389/fimmu.2022.982018

Introduction

Liver transplantation (LT) still remains the only treatment option for a variety of liver diseases eventually resulting in end-stage organ failure. Extended criteria donors (ECD) are increasingly used for transplantation to meet the high demand of organs. However, this poses a risk of early allograft dysfunction (EAD), primary non-function (PNF) and biliary complications [1-6]. Moreover, ECD livers are more susceptible to ischemia reperfusion injury (IRI), compared to standard criteria donor grafts.

While hypothermic conditions reduce cellular activity and metabolism during organ ischemia, accumulated toxins and reactive oxygen species (ROS) are released upon reperfusion, which initiates pro-inflammatory cascades, activates immune cells, releases damage associated molecular patterns and ultimately results in apoptosis and tissue necrosis (1–3). To limit organ damage during organ preservation, machine perfusion (MP) has emerged as an alternative to static cold storage (SCS). Normothermic machine perfusion (NMP) keeps a liver *ex vivo* in a complete functional state, close-to physiologic conditions and allows for comprehensive graft viability assessment before transplantation (4–9). An improved metabolic function, reduced expression of key markers of IRI and decreased activation of the immune response of NMP livers, compared to SCS livers, was previously demonstrated (2, 3).

The liver is essentially involved in balancing the innate and the adaptive immune system. Its anatomic position and distinctive vascular system allow for its unique ability to continuously exchange immunological information (Figure 1) (10, 11). Upon inflammation, the innate immunity including the complement system, pre-formed antibodies, as well as hepatic natural killer (NK) cells, macrophages and neutrophils, induces the inflammatory cascade and further initiates the adaptive immune response. Central to the hepatic adaptive immune system are T and B lymphocytes, which are able to recognize and reply to pathogens in an antigen-specific way, while natural killer T (NKT) cells function as a bridge between innate and adaptive immunity (12–15). To date, there is little evidence whether MP alters the quantity, activation state and function of hepatic immune-cells (16). The migration of donor passenger T cells from the donor liver allograft into recipient circulation has been demonstrated prior to the clinical use of NMP (17). A study by Jassem et al. reports an anti-inflammatory effect of NMP of donor livers and the promotion of liver regeneration (2). Recently, changes of the intrinsic immune profile of donor livers during NMP were confirmed. Specifically, it was suggested that liver-resident T cells and neutrophils are mobilized and released into the perfusate during NMP (18). Moreover, hypothermic oxygenated perfusion (HOPE) impressively reduced the number of liver-resident T cells and decreased cytokine levels, resulting in downregulation of the immune system and thereby preventing rejection and cholangiopathy after LT (19, 20). At this point it also remains speculative if liver-resident immune cells primarily exert a pro-inflammatory effect on the machine perfused organ, or in part may be essential to induce liver regeneration and counteract liver damage.

This review summarizes the function of various immune cell populations during hepatic immune responses with particular attention to inflammatory conditions in the context of LT. It discusses the potential role of the hepatic immune cell

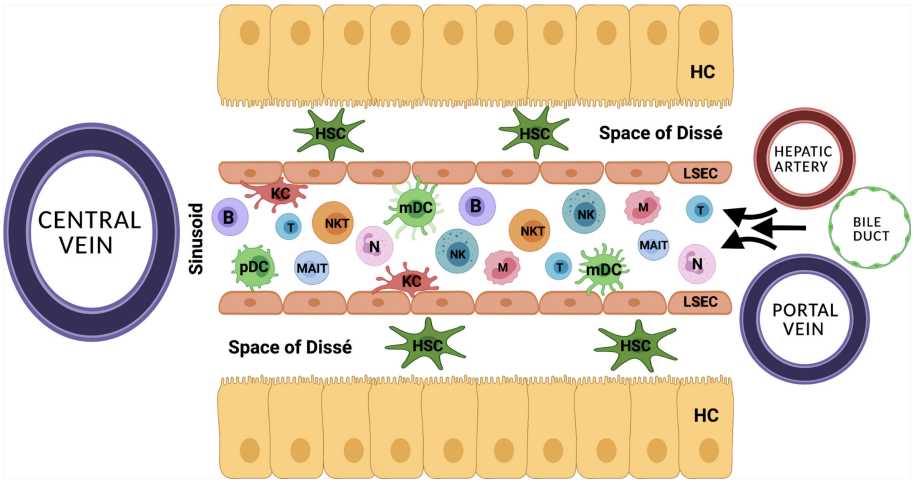

FIGURE 1
The distinctive anatomical vascular system allows for continuous exchange of immunological information. HC, hepatocyte; HSC, hepatic stellate cell; KC, Kupffer cell; B, B cell; T, T cell, NK, natural killer cell; NKT, natural killer T cell; N, neutrophil; MAIT, mucosa associated invariant T cell; mDC, myeloid dendritic cell; pDC, plasmacytoid dendritic cell; LSEC, liver sinusoidal endothelial cell.

microenvironment during MP and if active modification of the immune cell repertoire may be advantageous during MP.

Neutrophils are key players of immune cell activation as well as regeneration

Though initially considered a rather uniform, pro-inflammatory immune type, advances in analytical techniques suggest a variety of neutrophil subsets. The two most prominent neutrophil subtypes are N1 and N2. Their function is quite similar to their macrophage counterparts M1 and M2, resembling a pro-inflammatory and an anti-inflammatory, regenerative phenotype, respectively. *In vitro*, polarization of neutrophils toward an N1-like phenotype can be conducted with lipopolysaccharide (LPS), interferon gamma (IFNγ), and interferon beta (IFNβ). N2 cells differentiate upon treatment with L-lactate, adenosine, transforming growth factor beta (TGF-β), interleukin 10 (IL-10), prostaglandin E2 (PGE2), and granulocyte colony stimulating factor (G-CSF) (21). aged neutrophils tend to overactivation (22), whereas chronical exposure to pro-inflammatory conditions causes reduced inflammatory effector functions. These exhausted neutrophils, e.g., from patients with decompensated liver cirrhosis, reactivate their effector functions upon stimulation with toll like receptors (TLR) 7/8 and partially with TLR4 agonists (22, 23). Upon reperfusion of an organ, danger- or death-associated molecular patterns (DAMPs) released by ischemic tissue are detected by Kupffer cells (KC) and endothelial cells, creating a CXCL1/CXCL2 gradient to guide neutrophils chemotactically towards the site of injury. Moreover, KC secret IL1β, which induces the expression of intercellular adhesion molecule (ICAM)-1 in endothelial cells. In the liver, ICAM-1 enables binding of neutrophils to endothelia *via* MAC1 and subsequent transepithelial migration. When reaching the site of injury, neutrophils migrate towards DAMP signals, while disregarding the CXCL1/CXCL2 gradient (24, 25). Additionally, activation of the complement system has shown to promote neutrophil recruitment and subsequent tissue injury (26). The effects of complement on neutrophil migration in the context of liver MP are still unexplored, but might be relevant, as complement is produced in the liver itself and the organ is not perfused with whole blood ex situ. A recent study on human livers demonstrated that tissue neutrophil frequency significantly decreased at end of NMP, while no significant change was observed in the perfusate neutrophils (18). Thus, it was supposed that tissue neutrophils were activated and mobilized during NMP, based on a an augmented innate immune response triggered by reperfusion, which is known to cause excessive neutrophil influx to the liver from the vasculature (26). However, in liver NMP, the observed paradoxical decrease in donor-liver tissue neutrophils and the contemporanely constant perfusate neutrophil cell frequency may be explained by the continuous exposure of circulating neutrophils to non-endothelialized surfaces of the perfusion circuit, causing a proinflammatory state, resulting in adherence of neutrophils to circuits (2, 18). At the site of injury, neutrophils are involved in enhancing tissue damage and inflammation as well as tissue regeneration and immune suppression. As primary functions, neutrophils produce ROS and cytokines, perform formation of neutrophil extracellular traps (NET)osis, phagocytosis, proteolysis, and induce angiogenesis (Figure 2) (23, 24), which damage hepatocytes and enhance local inflammation. Delaying neutrophil exit in a Cathepsin-C (*Ctsc*) deficient mouse model also delayed revascularization of thermal liver injury (27). As NETs are extracellular structures, they act as DAMPs and consequentially enhance immune reactions (24, 28). In lung and kidney transplants, NETosis has been shown to be involved in graft rejection (24). Moreover, they promote coagulation and thereby disturb perfusion (24). Since, however, anti-coagulated blood is used for MP the significance of this process during *ex vivo* organ perfusion is questionable. On the other side, neutrophils participate in the clearance of debris by phagocytosis (24) (Figure 3). Thereby, they reduce the amount of free DAMPs, which prevents subsequent inflammatory reactions. In alcoholic liver disease, neutrophils are mediators of liver damage, but they can diminish inflammation by clearing necrotic debris and induce hepatocyte regeneration *via* HGF (23). Similarly, antibody mediated depletion of neutrophils resulted in reduced clearance of debris as well as delayed vascularization and healing, in a model of thermal liver injury (29). These findings also highlight the role of neutrophils for tissue regeneration. Neutrophils participating in tissue revascularization, might display a targetable subset during MP (30). In a mouse model of pancreas transplantation, MMP9 turned out to be a key mediator for this process (27). This indicates that protein degradation is a key player not only in damaging hepatocytes, but also to induce revascularization and subsequent wound healing, additional to growth factor secretion. Further, neutrophils induce M2 polarization in macrophages. Targeting neutrophils is not common in LT or to prevent IRI. However, there have been efforts to reduce tissue damage by preventing neutrophil invasion. Inhibition of CXCL1 or CXCR2 diminished neutrophil migration and tissue damage. Besides, deactivation of matrix metalloprotease 9 (MMP9) demonstrated tissue protective effects in a mouse model of hepatic IRI, which has been attributed to impaired invasion (24, 26). On the other hand, MMP9 has been identified as a key player in revascularization (27). Hence, if MMP9 inhibition is beneficial for minimizing IRI in LT, this effect might be restricted to preventing neutrophil activity in the damaged tissue. As there is no neutrophil recruitment from the blood compartment during liver MP, it is questionable whether inhibition of neutrophil recruitment can show significant effects in an *ex vivo* liver

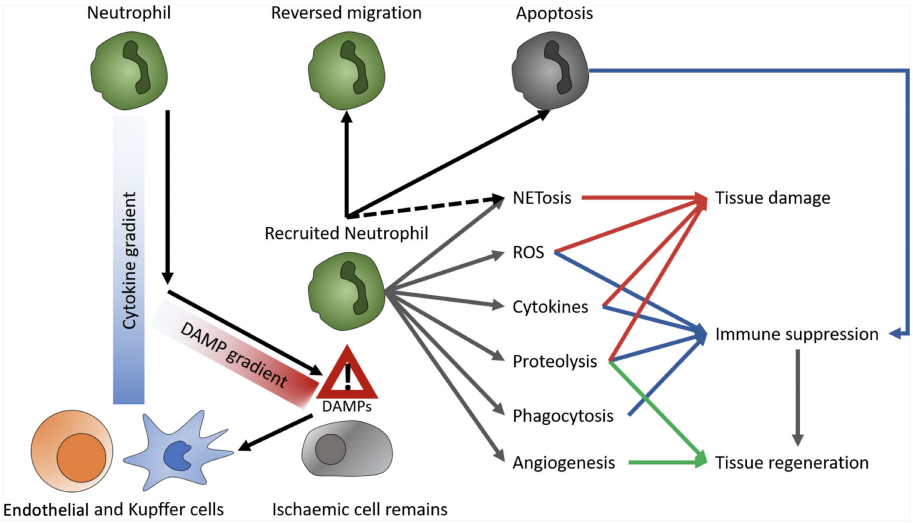

FIGURE 2
Role of neutrophils. DAMPs, danger- or death-associated molecular patterns; ROS, reactive oxygen species; NETs, neutrophil extracellular traps.

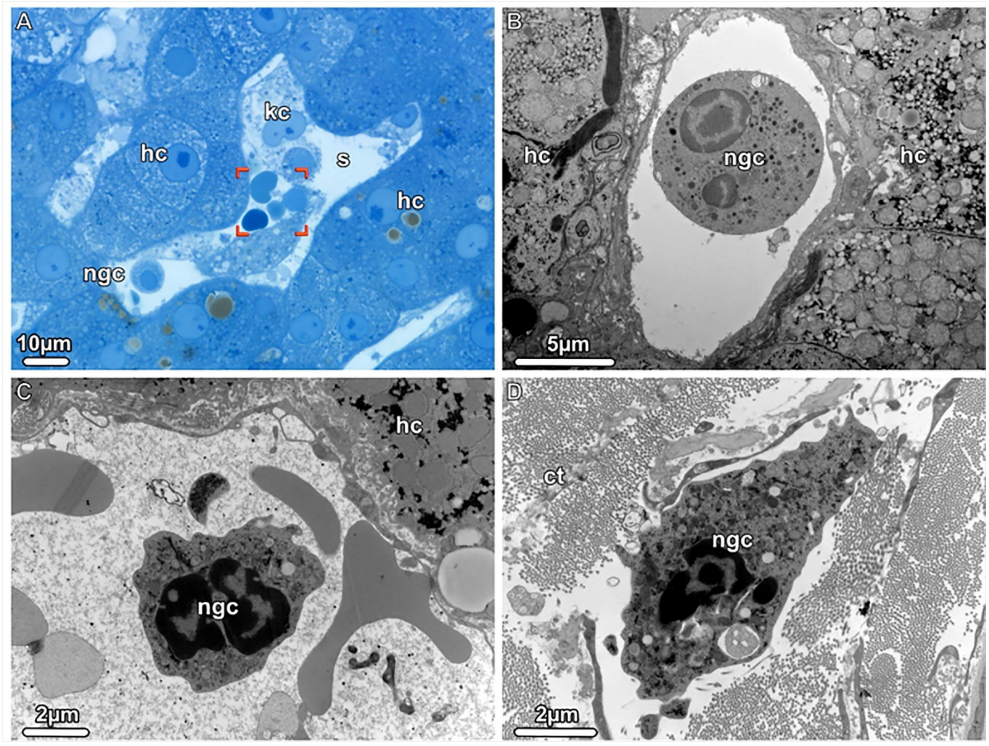

FIGURE 3
Neutrophil granulocytes detected during donor liver NMP, Light (LM) and Transmission electron microscopy (TEM). **(A)** LM. An overview of a liver sinusoid (s) containing Kupffer cells (kc), neutrophil granulocytes (gnc) and erythrocytes (brackets) is shown. **(B, C)** TEM. The sinusoids harbor mature as well as young neutrophilic granulocytes (ngc), both with a segmented nucleus. **(D)** TEM. A young neutrophil granulocyte (ngc) is seen in the dense connective tissue (ct) surrounding the liver, scattered between bundles of collagen fibrils. hc, hepatocytes.

perfusion setting. In analogy to preventing invasion, promoting neutrophil evasion into the perfusate might diminish immune mediated IRI. Both, intravascular and tissue resident neutrophils may be recruited into the blood flow (31). Additionally, neutrophils follow a chemotactic hierarchy, by which some migratory signals overwrite others (24). Hence, baiting neutrophils out of an organ prior to any in-tissue activities might diminish IRI. Alternatively, inducing reverse migration, for instance by treatment with LTB4 (30, 32), could reduce the number of locally active neutrophils.

In general, it appears that a total absence of neutrophils is not desirable to prevent IRI and tissue damage, also in MP. Instead, some activity is necessary for clearing debris, healing and regeneration. To provide a regenerative immune environment in general, *ex vivo* conditioning of donor livers and organs might be beneficial. Besides, elimination of excessive neutrophils may lead to reduction of liver injury and inflammation following LT (33).

Kupffer cells are specialized hepatic antigen-presenting cells

In 1876, von Kupffer identified liver-resident macrophages for the first time (34). These macrophages comprise about 90% of the total population of fixed macrophages in human body and form a third of the non-parenchymal liver cells (35). They are co-localized with sinusoidal endothelial cells, hepatic stellate cells and NK cells in the hepatic sinusoids (14) (Figure 4). Depending on their distinct location, the function, morphology and number of KC changes (36). They have been described as the immunological sentinels of the liver and, depending on their surface marker phenotype or cellular functions, they are distinguished as having inflammatory as well as immunoregulatory properties.

The main role of KC is to clear the portal circulation from foreign materials and pathogens. In addition to their utility as antigen presenting cells (APCs), they are able to scavenge gut-derived pathogens, damaged erythrocytes and regulate iron and lipid metabolism (37). When doing so, KC release a battery pro-inflammatory cytokines such as IL-1, IL-6, IL-12, IL-18, tumor necrosis factor-alpha (TNF-α) and IFN-γ (14). With regard to LT, KC play a relevant mediating role in IRI, converting the liver into a highly inflammatory micromilieu and leading to PNF and EAD (3). During hepatic hypoxia, the resulting cellular stress triggers the release of endogenous DAMP molecules, which subsequently induces KC activation, release of cytokines and inflammatory mediators, in order to attract neutrophils and produce ROS (38). During IRI, the activation of TLR4 on KC enhances TNF secretion, which is further associated with hepatocyte apoptosis (39). Moreover, the activation of the complement system during IRI is responsible for KC-induced oxidative stress, triggering the formation of ROS and neutrophil recruitment to the ischemic liver (40). In the early state of hepatic IRI, KC produce inducible nitric oxide synthase (iNOS), which leads to reduced capillary perfusion and increased liver injury (41). On the other side, KC contribute to immune regulation, tissue repair and liver regeneration (42). After LT, KC act as APCs by increasing the expression of MHC class II and identifying recipient T cells migrating to the liver, which leads to T cell apoptosis and therefore play an important role during graft tolerance and survival (43, 44). In case of bacterial infection, KC produce anti-inflammatory cytokines such as IL-10, preventing activation of CD4+ T cells and limiting the adaptive immune response (45). Moreover, presentation of specific antigens by KC, induce IL-10 producing regulatory T cells, promoting antigen-specific tolerance. Contrarily, during acute liver injury, KC produce pro-inflammatory cytokines like IL-1, IL-6 and TNF-α as well as chemokines such as MIP-1α and RANTES (46). Human liver scRNAseq studies from three

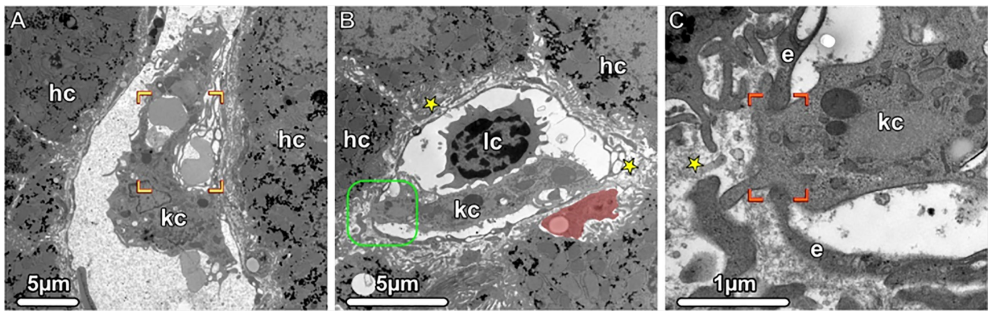

FIGURE 4
Kupffer Cells, Transmission electron microscopy (TEM). **(A, B)** Liver sinusoids with Kupffer cells (kc), lymphocytes (lc) and erythrocytes are shown. The damaged erythrocytes (brackets) are phagocytosed by a Kupffer cell, and in the Disse space (asterisks), an ito cell (highlighted in light red) is visible storing lipid droplets. The boxed area is shown in higher magnification in **(C)**. A portion of the Kupffer cell (kc) penetrates the fenestrated endothelium (e and brackets), thus gaining accesses to the Disse space (asterisks). hc, hepatocytes.

different groups have defined human KC facilitating the spatial mapping of these cells in human liver (47–49). Through identification of several markers, two distinct populations were distinguished, seeming to segregate into pro-inflammatory and immunoregulatory phenotypes. Specifically, MARCO (MAcrophage Receptor with COllagenous structure) is only expressed in non-inflammatory KC, while an inflammatory character was suggested by enriched expression of LYZ, CSTA and CD7454 (47).

Liver HOPE, which was shown to protect from IRI, downregulates activation of KC in a rat model (19). In contrast, increased levels of cytokines associated with KC activation (CCL-2, GM-CSF, IL-10, IFN-γ) were detected during human NMP together with the induction of an overall proinflammatory state (18). Based on the evidence that KC are key regulators of homeostasis, immune activation, tolerance induction (50), and that NMP triggers KC activation in human livers (18), targeting myeloid inflammation may help to improve organ function upon LT.

Natural killer cells as early source of immunoregulatory cytokines

NK cells were initially described in 1975 based on their ability to kill tumor cells without prior sensitization (51). Following activation, NK cells offer a bridge between innate and adaptive immune system by augmenting early adaptive immune responses through the production of TNF-α and IFN-γ (52). The liver contains two NK cell subsets: conventional NK cells which circulate freely and liver resident NK cells (53). According to their surface markers, NK cells are divided into CD56bright and CD56dim subsets, where nearly to 90% are CD56dim, characterized by a high cytotoxicity. CD56bright NK cells, expressing a distinctive panel of chemokine receptors, are particularly enriched in the liver where they constitute over 50% of the total hepatic NK population, compared with 10–15% in peripheral blood [6]. They are located primarily in the sinusoids, produce a great amount of cytokines, but display low natural cytotoxicity (54, 55).

In the landscape of LT, NK cells have classically been described as proinflammatory, due to the increased expression of the activation marker CD69 and the natural cytotoxicity receptor NKp44, contributing to the release of inflammatory cytokines and cytolysis of donor cells. Hepatic NK cells express tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), which is a potent inducer of hepatocyte cell death. The effect of TRAIL expression on NK cells during hepatic IRI was investigated and confirmed. Mice lacking TRAIL displayed significantly higher levels of liver injury and neutrophil infiltration (56). Additionally, it was assumed liver resident NK cells are responsible for the innate immune response in the early phase of IRI through self/non-self-recognition (57). Previous studies have demonstrated that viral infections induce NK cell accumulation and activation in the liver (58–60). Activated NK cells also work against biliary epithelial cells and contribute to hinder fibrosis through killing of hepatic stellate cells (61). A relative loss of a subpopulation of CD56+CD16– NK cells was observed in fibrotic human liver tissue using scRNAseq [24]. The interaction between liver NK cells and KC might trigger the production of IFN-γ and TNF-α, contributing to the development of fulminant hepatitis [87]. Furthermore, TRAIL+ NK cells could eliminate immature DC (62) thereby impact the advancing of certain liver diseases.

There is also evidence that NK cell populations have important immunoregulatory functions (58). A high proportion of hepatic NK cells express the inhibitory receptor NKG2A. In contrast to their peripheral blood counterparts, they are capable of twice the cytotoxicity level, resulting in depleted activation of T cells and tolerance induction after LT (63). During hepatitis C virus (HCV) infection, they can inhibit DC activation by producing the suppressive factors transforming growth factor-beta (TGF-ß) and IL-10. Subsequently, the resulting tolerogenic DC trigger the expansion of regulatory T cells, contributing to the induction of an immunotolerant state (64). Diverse studies have investigated the role of NK cells during graft rejection (14, 65). The absence of recipient-derived NK cells or the decrease in IFN-γ production after LT has been shown to be advantageous during both allograft rejection and tolerance induction in a rat model (66). In addition, 13 genes that are highly expressed in NK cells were found to be present in LT recipients with graft tolerance, which further indicates/provides further evidence that NK cells are involved in tolerance induction (67). The conflicting role of NK cells is still not fully understood; however, it is likely that NK cells play a role in the development of tolerance, thus providing a novel rationale for minimizing immunosuppression in recipients of livers with greater proportions of NK cells (52). A fist analysis on human liver NMP, showed that the composition of leukocytes within the perfusate after organ procurement and cold flush consisted mainly of neutrophils (about 55%) and NK cells (about 13%). While no significant changes regarding the NK cell compartments within the tissue were observed, duration of NMP was associated with significant decreases in frequencies of NK cells when serial perfusate was analyzed (18). In the light of the growing use of MP in routine LT, future studies focusing on immune interactions at the time of LT and during rejection episodes, combined with cell dynamics during MP, should clarify the role of NK cells in rejection and tolerance.

Dendritic cells are key players in induction and regulation of immune responses

In 1973 Cohn and Steinman discovered a specific type of immune cell, the dendritic cell (DC) (68), which plays an important role as sentinel of the immune system, as they are deployed throughout the body and monitor their surroundings for antigens and danger signals derived from pathogens or tissue damage. DC can be categorized into two separate lineages: conventional/myeloid DC (mDC) are specialized APCs capable of beginning and driving specific T cell immune responses, whereas plasmacytoid DC (pDC) are able to rapidly produce type 1 interferons and regulate inflammatory responses (12). All populations of DC have now been identified in human liver tissue using flow cytometry (69) and scRNAseq, with markers such as LILRA4, XCR1 and CD1c in order to distinguish them (12). Human mDC express high levels of CD11c and are classified according to their expression of CD1a (also known as blood dendritic cell antigen [BDCA] 1) versus CD141 (also known as BDCA3) (70). The BDCA1+ DC differentiate under the influence of interferon regulatory factor 4 (IRF4), express high levels of CD1b, CD14, and SIRP-α and promote T helper (Th) 2 responses. BDCA3+ DC develop under IRF8, express XCR1, CLEC9F, BTLA4 and secrete IL12-promoting Th1 responses in CD4+ T cells (71). In the healthy liver 70% are BDCA1+, while 30% are BDCA3+ (69). On the other side, pDC display an accentuate response to viral pathogen associated molecular patterns (PAMPs) and the synthetize IFNα (72). They express CD123, CD14 and CD303 (also known as BDCA2), besides initiating antiviral immune response and secreting IFNα/β (72, 73).

In the healthy liver, DC are mainly immature cells, capable to capture and process antigens (12). In the context of LT, mDC and pDC have been explored as key players of graft rejection and immune tolerance. This was also observed in a transgenic murine model, in which depletion of DC resulted in loss of liver tolerance and allograft rejection (74). Simultaneously, DC can also promote liver graft rejection, according to studies where donors were treated with Fms-like tyrosine kinase receptor 3 (Flt3)-ligand, causing not only increased DC numbers, but also augmented CD80 and CD86 expression (75, 76). Although mDC can induce graft rejection, their baseline state is likely to promote liver tolerance, as a consequence of several mechanisms driving a close interaction with hepatic stellate cells (HSCs) (72). There is less evidence concerning the role of liver DC in IRI, but it was shown that this innate immune pathology follows very different rules from the T cell tolerance experienced in LT. In this context, pDC appear to be key players of the immunopathology. Their expression of TLR4 and TLR9 renders them highly responsive to DAMPs released by ischemia-injured cells, and their response is to secrete IFNα, IL6, and TNF-α, which further augment tissue injury. Contrarily, mDC suppress IRI, through the secretion of anti-inflammatory cytokines (72). Future research should examine how these populations differ functionally in regulating hepatic immunity, how they contribute to liver disease development (37) and which role they may assume in the context of liver MP. Dendritic cell–derived extracellular vesicles (DC-EVs) have emerged as a novel immunomodulatory agent in LT. DC therapies are able to induce a tolerogenic immune environment through secretion of anti-inflammatory cytokines and induction of T cell anergy, nearly to attenuating hypoxic injury and promoting allograft survival (77–79). The administration of EVs directly to the liver during NMP may guarantee their targeted delivery, providing time for modulation of the immune environment prior to LT and maximizing the therapeutic potential (77, 80).

The liver adaptive immune system: the specific role of lymphocyte subtypes in hepatic immune activation

The highly specialized liver adaptive immunity, consisting of humoral and cellular immunity, is able to provide long-term protection with immunological memory, while promoting self-tolerance (12). The liver possess an immunosuppressive microenvironment, which means that hepatic adaptive immune cells become readily tolerogenic, endorsing the death of effector cells and the "education" of regulatory cells (81).

Based on different functions and phenotypes, the most relevant T lymphocytes implicated in adaptive immunity include CD4 T cells and CD8 T cells, additionally characterized into several subgroups. CD4 T cells counted various functional categories. While helper T (Th)1, Th2, Th17 and follicular helper T (Tfh) cells, mostly support innate and adaptive immune responses, the regulatory T (Treg) cells (CD4+, CD25high, CD127low, FoxP3+), usually overrule the augmented inflammatory reaction resulting from innate and adaptive immunity and restore immune homeostasis. Multiple immunosuppressive mechanisms have been attributed to Tregs such as the secretion of anti-inflammatory cytokines, depletion of crucial growth factors, and direct cytotoxic killing of effector cells (13). The liver immune response is mostly associated with a strong CD4 and CD8 T cell reaction. CD8 T cells play a key role in this context, because they recognize peptides from intracellular pathogens in the context of MHC I. Subsequently they initiate diverse effector mechanisms, including the production of cytokines, such as IFN-γ and TNF-α, and further cytolytic mechanisms, by releasing granule contents

like perforin and granzyme and by triggering Fas-mediated apoptosis (12, 82). Jassem et al. observed reduced numbers of proinflammatory cytokines IFN-γ and IL-17 producing CD4 and CD8 T cells on human livers subjected to NMP (2).

A main characteristic of the adaptive immune system is to form a pool of memory T cells, enabling an effective immune response after pathogen re-exposition. The liver displays intrahepatic tissue resident memory (TRM) cells, which require a different cytokine milieu and have diverse phenotypes compared to their counterparts in the blood (83, 84). While in mice the non-circulating liver TRM account for 40–60% of the liver-resident T cells, this amount is significantly higher in humans, where it ranges between 60 and 80%. In order to infiltrate the liver, memory T cells express liver-specific homing markers like CD103, LFA-1, CXCR6 or CXCR3. However, TRM have the ability to return back into the bloodstream by upregulating CCR7 and S1PR1 (83). After activation, CD8+ TRM cells produce TNF-α and IFN-γ, acquiring the ability to directly lyse target cells. The proinflammatory cytokine expression on TRM cells is elevated in comparison to the circulating memory T cells, denoting an efficient effector function at the tissue-site of infection. Additionally, CD8 TRM cells recruit other immune cells by chemokine production after antigen recognition (85). In comparison to CD8 TRM cells, the CD4 TRM cell amount in the human liver is low, potentially due to a reversed CD8/CD4 ratio compared to the blood (86). T cells are highly involved in the pathogenesis of IRI, which includes not only CD4 T cells, but also CD8 and γδ T cells. Recently, it was demonstrated that NMP significantly increased the proportion of T cells in the perfusate throughout the course of perfusion. This may suggest that donor tissue T cells are mobilized into the perfusate during NMP (18). However, tissue T cell frequency remains mostly unchanged throughout the course of NMP. It was suggested that perfusate T cells permanently migrate back into the liver tissue, generating a dynamic T cell trafficking loop between perfusate and tissue compartments (18).

B cells have been considered a main component of the adaptive immune response, also contributing to mediate graft injury. They comprise about 5% of the liver lymphocytes. While immature, chronically activated B cells are effective APCs, thought to augment T cell-mediated rejection, mature, late lineage B cells produce donor-specific antibodies and contribute to both acute and chronic allograft injury (87). B cells additionally produce cytokines and chemokines modulating the extent of the alloimmune response. As described for T cells, also B cells should also be capable of both augmenting and suppressing immune responses (88). B cell dysfunction has been implicated in the pathogenesis of numerous immune mediated liver diseases, such as autoimmune hepatitis (AIH), IgG4-related hepatobiliary disease (IgG4-HBD), primary biliary cholangitis (PBC) and primary sclerosing cholangitis (PSC) (87, 89, 90). The role of regulatory B cells (Breg) has been established in the context of autoimmunity (88), however, the lack of molecular markers is still a limiting factor for their further characterization. They are often identified by the production of IL-10. Moreover, there is evidence that Breg and Treg may collaborate in order to promote tolerance, through the mediating effect of IL-10 (88). In the context of liver NMP, continuously increasing frequencies of B cells were detectable within the perfusate over the entire NMP course (18). The authors concluded that this could probably be explained by the controlled oxygenated rewarming after substantial cold ischemia time. Likewise, NMP with controlled oxygenated rewarming of liver after cold storage resulted in significantly improved recovery upon reperfusion associated with cold-stored only grafts (91).

Perspective and conclusion

To date there is conflicting data whether MP exerts a pro- or an anti-inflammatory effect on donor livers prior to LT. While there is evidence that a great amount of leukocytes is mobilized into the perfusate during liver MP and an increase in proinflammatory cytokines are found with prolonged perfusion in some studies, also upregulation of regenerative pathways and primarily anti-inflammatory mediators in the course of human liver NMP are observed. Previously, refining the perfusate composition with anti-inflammatory agents, as prostaglandin E1, antiplatelet and fibrinolytic factors during *ex vivo* warm liver MP improved the outcome after LT in a pig model (92). Further, liver-resident immune cells gained an activated phenotype during NMP on gene and protein levels in a rat model, which could be reduced through therapeutic intervention with anti-inflammatory IL-10 and TGF-β (93). Moreover, a time-dependent increase in DAMPs levels and inflammatory cytokines during MP, particularly pronounced at higher preservation temperatures, was shown in another rat model (94). In the past, differential centrifugation, sedimentation, cell washing, freezing and thawing, and filtration have been used to leukodeplete the perfusate used for MP (95). The efficacy of leukocyte depletion filters (LDFs) was previously evaluated in the context of normothermic *ex vivo* lung perfusion (96) and in LT for oncological disease, where some types of LDFs could reduce the risk for reintroducing tumor cells (97). While in human organ perfusion, leukocyte-depleted packed red blood cells are immediately available from blood banks, controlling white blood cells-mediated damage, the use of whole blood-based perfusate still remains a limiting factor in experimental animal models of MP (98). Active mobilization and elimination of hepatic immune cells using leukocyte depletion filters (LDFs) during liver MP is an option and may seem reasonable as this reduces the antigenic load of the organ, hence diminishing acute rejection after LT. On the other hand,

specific subtypes of immune cells have been shown to be critically involved in regenerative, healing and tolerogenic processes and hence indiscriminate withdrawal may be contra productive. However, how much elimination is needed to balance destructive versus regenerative processes in the liver while on the perfusion device? In this context it might be advisable to specifically and actively promote migration and trafficking of highly proinflammatory immune cells by strongly activating the inflammatory cascade. The high levels of proinflammatory cytokines could then be filtered from the perfusate together with the correspondingly acting cells. Future investigations should consider the possible application of leucocyte filtering during MP as therapeutic strategy.

With the development of prolonged organ perfusion, and the possibility of the MP systems to add substances and therapeutics directly into the perfusate which then circulate directly through the liver, it may also be an option to administer factors affecting the maturation state of immune cells or inducing a regulatory and/or regenerative phenotype. Moreover, *ex vivo* expanded subtypes of immune cells exerting an advantageous effect during liver MP may be administered into the perfusate for therapeutic purposes in the future.

To take the next steps it is of uttermost importance to elucidate and understand (i) the role of hepatic immune cells during MP (ii) how MP influences the immune cell repertoire, and (iii) how this affects the immune microenvironment and milieu. This should lay the groundwork for active immune modulation and induction of regeneration during liver MP as a future goal.

Author contributions

Designed and outlined the review: FM, SaS, HT; designed the figures: FM, SaS, MA, GH, BM; drafted the review: FM, SaS, GH, MA, HT; critically revised review and approved final version: all.

Acknowledgments

We thank all members of the NMP team at the Department of Visceral, Transplant and Thoracic Surgery as well as the organLife team.

Conflict of interest

The authors 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.

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