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Updated Views on Neutrophil Responses in Ischemia-Reperfusion Injury

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Abstract

Ischemia-reperfusion injury is an inevitable event during organ transplantation and represents a primary risk factor for the development of early graft dysfunction in lung, heart, liver, and kidney transplant recipients. Recent studies have implicated recipient neutrophils as key mediators of this process and have also found that early innate immune responses after transplantation can ultimately augment adaptive alloimmunity and impact late graft outcomes. Here, we discuss signaling pathways involved in neutrophil recruitment and activation after ischemia-mediated graft injury in solid organ transplantation with an emphasis on lung allografts, which have been the focus of recent studies. These findings suggest novel therapeutic interventions that target ischemia-reperfusion injury-mediated graft dysfunction in transplant recipients.

INTRODUCTION

The interruption of blood flow is inherent to organ transplantation and often followed by prolonged cold ischemic storage during transport to the recipient. Once blood flow is restored, allograft tissues become enriched in oxygen and substrates that exaggerate the ischemic injury, a phenomenon known as ischemia-reperfusion injury (IRI). Early graft dysfunction after transplantation is the clinical presentation of molecular and cellular events largely induced by IRI and encompasses a wide variety of terms, including primary graft dysfunction (PGD), early graft failure, primary dysfunction, primary nonfunction, initial

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poor function, reimplantation response and delayed graft function. Throughout this review, we will refer to the syndrome of early graft dysfunction after lung, heart, liver, or kidney transplantation as PGD.

When IRI is assessed by histologic markers (e.g. neutrophilic infiltration) in human biopsies of transplanted organs, approximately 40–50% of lung and kidney allografts and 87% of liver allografts show evidence of IRI within the first 3 hours after reperfusion[1–3]. This early inflammatory response significantly impacts graft viability and is evidenced clinically by early graft dysfunction in 20–30% of all transplanted lungs, hearts, livers, and kidneys[2–12]. PGD can adversely affect short-term post-transplant outcomes and remains the leading cause for early mortality following lung and heart transplantation[6,13,14]. Mortality following the development of PGD after kidney or liver transplantation is often circumvented by re-initiation of dialysis or immediate hepatic re-transplantation, with an estimated 2–6% liver transplant recipients requiring early re-transplantation[15–19]. Furthermore, PGD is associated with reduced long-term allograft function and recipient survival, and is a risk factor for rejection after lung, heart, liver, and kidney transplantation[6,9,20–28].

While much remains to be discovered regarding the cellular and molecular pathways underlying IRI, recipient neutrophils have been shown to play a central role in this process[29,30]. Multiple studies in both human and animal models have demonstrated that IRI-mediated injury is associated with neutrophilic infiltration into allografts and inhibition of this process may protect against post-transplant IRI[30–34]. In this review, we synthesize an expanding body of experimental findings on neutrophil recruitment, extravasation and activation following solid organ transplantation with a focus on pulmonary transplantation. These studies pave the way for future investigations and novel strategies to improve allograft function following solid organ transplantation.

Neutrophil Recruitment

In the setting of IRI, recipient neutrophils are recruited to newly reperfused tissues through numerous cellular and molecular pathways that differ between organ systems. Novel techniques of in vivo multiphoton microscopy have allowed visualization of neutrophil infiltration in various animal models of IRI, including mouse lung, heart, and kidney transplantation, as well as mouse models of warm ischemia induced by transient interruption of arterial flow to the lung, kidney, and liver[31,34–44]. Following syngeneic or allogeneic lung transplantation in mice, recipient neutrophils are rapidly recruited to the vessels within the graft, many of which extravasate during the first 2 to 3 hours after reperfusion[38,39]. Similarly, rapid recruitment of neutrophils has also been observed by intravital two-photon microscopy after syngeneic heart transplantation in mice, where neutrophils slow down, adhere to the walls of large coronary veins, and subsequently enter the myocardial tissue where they form clusters[35,36]. In mouse models of lung IRI induced by 60 minutes of hilar clamping, robust neutrophil influx occurs within the first few hours[40,41]. However, in mice that undergo transient occlusion of the renal hilum for 45 minutes, neutrophils immediately infiltrate glomeruli from the afferent arterioles early after reperfusion and can be observed infiltrating inside and outside of the peritubular capillaries and filling the tubules[44]. In a mouse model of liver IRI induced by 45 minutes of hepatic arterial clamping, Honda

reported that the number of neutrophils that accumulate in the vasculature of the affected lobules steadily increases until 4 hours after reperfusion[42]. Similarly, Jaeschke reported an 80-fold increase in neutrophilic accumulation at 24 hours in a rat model of warm hepatic IRI[34].

Unlike most other organs, resting human lungs contain a neutrophil reservoir that equates to approximately three times as many neutrophils as the peripheral circulation[45,46]. Multiple in vivo microscopic studies in animal models have illustrated this marginated pool of neutrophils within the lungs of ventilated mice and dogs at steady state as well[31,47]. While the role of this marginated pool of neutrophils remains unknown, recent work has suggested that this pulmonary reservoir of neutrophils may contribute to the defense against intravascular pathogens[48]. Other studies have found that this neutrophil pool regulates diurnal oscillations of transcription patterns in the lung[49]. Additionally, these marginated neutrophils may serve as an alternate reservoir for circulating neutrophils. To this end, Devi found that administration of plerixafor, a CXCR4 antagonist, triggered neutrophil mobilization from the pulmonary vasculature[50]. Marginated pools of neutrophils may also be encountered in other organs (i.e. liver), albeit to a lesser extent. Although most solid organs are flushed with a cold solution at the time of organ recovery, this is unlikely to eliminate many sequestered neutrophils adherent to vascular endothelium. Thus, the role of marginated neutrophil pools within donor organs, especially lungs, warrants further explorations.

The stimulus for neutrophil recruitment following IRI-mediated injury is multifactorial and largely driven by a sterile inflammatory process that is triggered after reperfusion. Inflammatory cascades after reperfusion can be initiated by several pathways of cell death, both apoptotic and non-apoptotic. The predominant pathway of cell death following transplantation appears to vary among organs and may be impacted by the severity of injury. We have previously reported that administration of ferrostatin-1, a specific ferroptosis inhibitor, inhibits neutrophil recruitment following myocardial IRI, including heart transplantation in mice[35]. Neutrophil trafficking was not impacted when cardiac grafts were deficient in receptor-interacting protein kinase 3 (RIPK3), a kinase essential in mediating necroptotic cell death. It is worth noting that the predominant pathway of cell death may change as time progresses following transplantation. To this end, Pavlosky has shown that RIPK3-deficient cardiac grafts develop lower levels of tissue necrosis, high-mobility group box 1 (HMGB1) production, and cell infiltration 12 days after transplantation (vs. earlier post-operative time points in our study[35])[51].

We have recently shown that necroptosis triggers the recruitment of neutrophils to lung allografts during severe injury induced by cold and warm ischemia and leads to impaired graft function[52]. Here, neutrophil recruitment is mediated by CXCL1 produced by donor-derived non-classical monocytes (NCMs). Donor lungs deficient in RIPK3 had significantly improved function and decreased neutrophilic extravasation[52]. Moreover, in a rat model of lung transplantation with prolonged cold ischemia, inhibition of necroptosis through administration of necrostatin-1 to both the donor and recipient resulted in significantly reduced tissue injury and improved graft function[53].

Necroptosis has also been shown to play an important role after kidney IRI. Following renal hilar clamping in mice, RIPK3 expression is markedly upregulated in proximal and distal tubules as early as 4 hours after IRI and remains elevated at 48 hours post-injury[54]. Similar to Pavlosky's findings in hearts, RIPK3-deficient kidney allografts demonstrated markedly decreased injury following transplant-mediated IRI[54,55]. However, other cell death pathways may contribute to renal IRI including cyclophilin-D-mediated necrosis and ferroptosis[54,55,56,57]. Thus, modulation of multiple cell death pathways may provide maximal protection from renal transplant-mediated IRI.

Following IRI, dying cells release damage-associated molecular patterns (DAMPs) which stimulate the release of pro-inflammatory cytokines and chemokines. For example, elevated levels of extracellular ATP are present in human samples of bronchoalveolar lavage (BAL) fluid following lung transplantation, and higher levels of ATP are found in BAL fluid from patients who develop early PGD[58]. The binding of DAMPs to toll-like receptors (TLRs) results in activation of pro-inflammatory signaling pathways that promote neutrophil recruitment. TLR4 is one such receptor that has been recognized as an important mediator of neutrophil trafficking following IRI in lung, heart, liver, and kidney transplantation[35,59–62]. Prakish showed that TLR4 expression is significantly upregulated following transient pulmonary artery ligation in mice and correlates with neutrophil infiltration in the affected lung[59]. In our previously mentioned study using a mouse orthotopic lung transplant model, we showed that neutrophil-mediated vascular leakage is dependent on TLR4 expression on vascular endothelium[52] (Figure 1). Additionally, we showed that the downstream expression of NADPH oxidase 4 (NOX4) leads to production of reactive oxygen species (ROS). TLR4-dependent signaling promotes the release of IL-1β, CXCL1, and CXCL2, as well as enhancing neutrophil recruitment to the lung graft[52,59].

The importance of TLR4 in neutrophil recruitment after IRI has been demonstrated in other organs as well. We have previously shown that neutrophil adhesion to the walls of coronary veins after murine heart transplantation is mediated by TLR4/Trif-dependent signaling in graft endothelial cells[35]. TLR4 is also highly upregulated on renal tubular epithelial cells after ischemic injury, triggering multiple downstream effects that exaggerate allograft injury[61]. Activation of TLR4 in renal IRI promotes the recruitment of neutrophils, illustrated by markedly reduced neutrophil infiltration in TLR4-deficient mice subjected to renal IRI[63]. Following renal IRI, TLR4-dependent signaling stimulates the synthesis of neutrophil chemoattractants in response to inflammatory cytokines such as IL-1β, IL-6 and tumor necrosis factor alpha (TNF-α). TLR4 signaling is also an important mediator of neutrophil recruitment in hepatic IRI. Upregulation of TLR4 following hepatic IRI is associated with increased levels of IL-6 and TNF-α[62]. Tsung employed TLR4 chimeric mice to demonstrate that TLR4 engagement on phagocytic nonparenchymal cells is required for warm IRI-mediated injury in the liver. They found that administration of HMGB1, a TLR4 ligand, resulted in increased IRI-induced hepatic injury in a TLR4-dependent fashion. Similarly, TLR4-deficient mice or treatment with an HMGB1-neutralizing antibody attenuated injury after hepatic warm IRI[64]. In a mouse model of renal hilar clamping, HMGB1 release after reperfusion was localized to areas with increased susceptibility to ischemic injury including renal tubules and peritubular capillaries[65]. Importantly, administration of a HMGB1-blocking antibody 30 minutes prior to renal ischemia markedly

reduced inflammation and preserved renal function. These findings show that TLR4 signaling is an important mediator in early responses to IRI after transplantation and suggest that targeting HMGB1 or other endogenous TLR4 ligands may minimize IRI-mediated graft dysfunction. In addition to TLR4, other TLRs have also been shown to play a role in neutrophil recruitment after transplantation. For example, we have shown that TLR9 expression by cardiac macrophages is crucial for promoting neutrophil trafficking into the graft following ischemic injury in a mouse cardiac transplantation model[36]. Using this model, we found that neutrophil influx was regulated by TLR9/MyD88-mediated production of CXCL2 and CXCL5. Thus, targeting TLR signaling pathways may provide a valuable therapeutic opportunity to minimize IRI-mediated graft injury.

Several CXC chemoattractants, namely CXCL1 and CXCL2, have been identified as key mediators of neutrophil recruitment to allografts following reperfusion. In a rat model of orthotopic lung transplantation, Belperio found that allografts and isografts exposed to 6 hours of cold ischemia had increased levels of CXCL1 and CXCL2, which peaked 8 to 16 hours after transplantation and correlated with graft neutrophilia[30]. Notably, treatment with an anti-CXCR2 antibody resulted in decreased neutrophil infiltration and attenuated lung graft injury. Expression of CXCL1 and CXCL2 also increases in mouse livers after IRI[66]. Of note, CXCL2 expression is induced within 3 hours after reperfusion, before any detectable increase in neutrophil accumulation; this is in contrast to CXCL1 expression, which increases 9 hours after reperfusion, suggesting that CXCL2 mediates the initial recruitment of neutrophils following reperfusion. In a study of canine heart transplantation, Birdsall found that potent neutrophil chemotactic factors such as C5a appear in cardiac lymphatic fluid shortly following reperfusion and correlate with rapid and robust neutrophil infiltration into the myocardium[67]. Other chemoattractants, such as IL-8, appear to aid in neutrophil recruitment to allografts as well. Oz analyzed human blood samples from the following reperfusion after elective cardiac surgery and cardiac transplantation and found significantly higher levels of IL-8 among transplant patients. Furthermore, human endothelial cells subjected to ischemia in vivo triggered a time-dependent release of IL-8, demonstrating a direct role of tissue ischemia to the local release of a neutrophil chemoattractant in humans[68].

Prior work from our laboratory has identified molecular targets that regulate acute granulopoietic responses induced by pulmonary transplant-associated IRI. Following transplantation of syngeneic or allogeneic murine lung grafts, we have discovered that a surge of granulocyte colony-stimulating factor (G-CSF) stimulates expansion of bone-marrow-resident neutrophil progenitors and leads to accumulation of neutrophils in the peripheral blood and graft tissues[32,69]. Additionally, transplantation of mouse lung allografts following prolonged cold ischemic storage is associated with significantly higher serum concentrations of G-CSF, increased neutrophils in the graft and periphery, and more severe acute graft injury[69]. Importantly, G-CSF blockade or neutrophil-depleting antibodies in the setting of extended cold ischemic storage resulted in diminished neutrophilic graft infiltration and improved allograft function. In renal transplantation, similar findings of transient granulopoiesis have been documented. For example, in human kidney transplant recipients, ischemic injury induces a transient rise in G-CSF expression in renal tubular epithelium which correlates with increased neutrophil expression of the surface glycoprotein

CD177[70]. Following kidney IRI in mice, renal G-CSF expression significantly increases, triggering systemic neutrophilia. Thus, regulation of the G-CSF-mediated granulopoietic response following IRI holds potential to minimize allograft injury after transplantation.

Many graft-resident cell populations play an important role in mediating neutrophil recruitment to newly reperfused tissues, the majority of which involve monocyte or macrophage equivalents. In lung allografts, donor-derived $Ly6G^{-}$, $NK1.1^{+}$, $CD11b^{+}$, $Ly6C^{low}$, $CD64^{-}$ NCMs are retained within the vasculature of human and murine lungs procured for transplantation and have been identified as key facilitators of neutrophil recruitment after IRI[39]. In a mouse model of lung transplantation, lung-resident NCMs are necessary for early neutrophil recruitment through the production of CXCL2 in a MyD88/TRIF-dependent fashion[39]. Here, neutralization of CXCL2 attenuates neutrophil infiltration of lung grafts, and depletion of NCMs in donor lungs, either pharmacologically or genetically, results in significantly impaired neutrophil influx and attenuated lung graft injury. Some studies suggest that alveolar macrophages (AMs) play a role in neutrophil recruitment to lungs as well. Using a mouse model of warm IRI, Prakash showed that depletion of $CD11c^{+}$ AMs via treatment with liposomal clodronate or diphtheria toxin in CD11c-DTR donors resulted in markedly diminished pulmonary neutrophil recruitment[59]. Kupffer cells, the analogous resident macrophages of the liver, appear to facilitate neutrophilic infiltration and subsequent injury following liver transplantation. In a study by Mosher using a mouse model of warm hepatic IRI, Kupffer cells were found to be major contributors to the production pro-inflammatory cytokines, which correlated with increased neutrophil infiltration into the injured liver[71]. When Kupffer cell activity was inhibited by gadolinium chloride administration 24 to 48 hours prior to the onset of ischemia, there was a marked reduction in CXC chemokine production and IRI-mediated liver injury. In a mouse model of renal IRI induced by 20 minutes of hilar clamping, Ferenbach similarly demonstrated that macrophages promote neutrophil recruitment through production of various cytokines[72]. Interestingly, they found contrasting effects between two different strategies of macrophage depletion, with significant protection induced by clodronate administration but no protective effect seen with DT-mediated depletion in CD11b-DTR mice, presumably due to preservation of a protective $CD11c^{+}$ cell population with clodronate treatment ($CD206^{+}$ $CD11c^{+}$ M2 macrophages and/or $CD11c^{+}$ resident monocytes). While this $CD206^{+}$ $CD11c^{+}$ cell population has not yet been identified as playing a protective role in the IRI of other organs, other $CD11c^{+}$ cell types have been shown to be protective after transplant-mediated ischemic injury. In a mouse model of renal transplantation, Aiello found that intragraft donor-derived $CD11c^{+}$ $F4/80^{+}$ renal macrophages transition into a reparative phenotype that orchestrates tissue repair in an IL-1 receptor 8-dependent manner[73]. Studies have suggested a dichotomous role for tissue-resident and recruited $CD11c^{+}$ dendritic cells (DCs) following liver IRI. Utilizing hepatic portal clamping, Zhang found that DC-deficient mice experienced significantly less tissue injury and reduced neutrophil infiltration, and adoptive transfer of wild-type DCs increased hepatic injury[74]. Interestingly, when $CD11c^{+}$ DC-deficient livers were transplanted into wild-type mice, hepatic necrosis and neutrophil infiltration were significantly increased compared to controls. Thus, tissue-resident and circulating DCs appear to play important and distinct roles in neutrophil recruitment and tissue injury following IRI.

Following cardiac IRI, IL-6 is generated from hypoxic myocytes and associated with increased neutrophil influx into ischemic myocardium[75]. Furthermore, IL-6 production is regulated by TNF-α secretion by mast cells in a canine model of cardiac IRI following transient coronary occlusion[76]. While the precise mechanistic pathway underlying this enhancement of neutrophil migration remains unclear, these findings highlight the multifaceted stimuli for neutrophil recruitment from both hematopoietic and non-hematopoietic tissues following IRI.

Neutrophil Extravasation

Neutrophils are rapidly recruited to the vasculature of reperfused tissues, where they subsequently extravasate out of the vessel lumen into the surrounding interstitial tissues. Neutrophils interact with the vascular endothelium through tethering, rolling, adherence, and crawling before extravasating through vessel walls in either a paracellular or transcellular fashion. IRI can induce altered expression of endothelial proteins that enhance neutrophil adhesion and subsequent extravasation. In ischemic heart grafts, endothelial cells exhibit increased expression of intercellular adhesion molecule-1 (ICAM-1), largely due to the release of proinflammatory cytokines such as TNF-α[77,78]. Using intravital two-photon microscopy, we have demonstrated that blockade of ICAM-1 receptors LFA-1 (CD11a/CD18) and Mac-1 (CD11b/CD18) prevented neutrophil adherence to and crawling on the endothelium, respectively, markedly inhibiting neutrophilic extravasation[37]. Furthermore, consistent with the notion that CXCL2 enhances integrin affinity, administration of anti-CXCL2 antibodies prevents adhesion of neutrophils to coronary veins. In a mouse model of myocardial IRI induced by transient coronary artery occlusion for 30 minutes, Bowden demonstrated that vascular cell adhesion molecule-1 (VCAM-1) is significantly upregulated on cardiac endothelium and associated with transendothelial migration of neutrophils[79]. In rat renal grafts, the expression of ICAM-1, VCAM-1, and P-selectin increases rapidly following transplantation in a TLR4-dependent manner and correlates with neutrophil influx and impaired renal function[80,81]. During hepatic IRI, ICAM-1 is transcriptionally upregulated on the surfaces of hepatocytes and sinusoidal endothelial cells[82–84]. However, ICAM-1 expression was only partially required for neutrophil migration due to the extensive vascular injury present. To this end, murine models of hepatic IRI only demonstrate partially attenuated injury after treatment with anti-ICAM-1 antibodies[85–87]. Dipeptidase-1 has also been identified as a major adhesion receptor on lung and liver endothelium for neutrophil trafficking in response to endotoxemia[88]. However, we have shown that immune responses can differ in sterile and pathogen-driven inflammation[40,89]. Thus, this receptor should be explored in transplant models to investigate its role as a therapeutic target. These findings illustrate the situational variability and tissue-specificity which exists to regulate neutrophil trafficking to injured tissues.

In both human lung allografts and murine models, we have found that IRI is associated with the mobilization of recipient classical monocytes (CMs) into injured lungs[40,90]. In mice, CMs are hallmarked by the expression of CCR2 and high levels of Ly6C. We have reported that recipient CCR2 expression mediates lung allograft dysfunction[91]. Using intravital two-photon microscopy, we have observed neutrophils tracking behind CMs ($CD115^+$, $CD11b^+$, $Ly6C^{hi}$ cells) during transendothelial migration in reperfused

lung grafts[31] (Figure 1). Furthermore, spleen-derived recipient CMs serve a crucial role for neutrophil extravasation through MyD88-dependent production of IL-1β, resulting in increased endothelial permeability through downregulation of ZO-2, a cytoplasmic protein that interacts with transmembrane tight junction proteins in pulmonary vascular endothelial cells[40]. We found that depletion of recipient CMs abrogated neutrophil extravasation and ameliorated IRI. Interestingly, in lung grafts devoid of donor NCMs, which was achieved by administration of airway liposomal clodronate prior to lung procurement or by using donors deficient in NR4A1, a transcription factor required for NCM formation, CM recruitment was significantly reduced. Accordingly, reconstitution of NR4A1-deficient grafts with NCMs resulted in CM recruitment and neutrophil extravasation into the transplanted lung. Recently, we have shown that donor NCMs following lung transplantation release IL-1β, which activates AMs and results in the release of CCL2, in turn recruiting recipient $CCR2^+$ CMs to the lung graft[92]. Thus, in addition to the aforementioned role of NCMs in mediating neutrophil recruitment, donor NCMs serve an essential role in recruiting recipient CMs from the spleen, which then mediate neutrophil extravasation (Figure 1).

Donor-derived tissue resident macrophages also play an important role in neutrophil extravasation in lung allografts following IRI. We have shown that tissue-resident macrophages in both mouse and human lungs subjected to cold ischemic storage express DNAX-activation protein 12 (DAP12), a cell-membrane associated protein that regulates the production of various neutrophil chemokines including CXCL1, CXCL2, IL-6, and TNF-α[39]. While transplantation of DAP12-deficient murine lungs resulted in similar recruitment of neutrophils in the pulmonary vessels, neutrophil extravasation was markedly impaired. Furthermore, we found that administration of wild-type macrophages into the donor bronchus of DAP12-deficient lung grafts immediately prior to reperfusion reconstituted neutrophil extravasation to levels observed in wild-type grafts. These findings highlight the necessity of DAP12 expression by pulmonary resident cells for neutrophil extravasation following IRI and that different steps of neutrophil extravasation are orchestrated by cells of both donor and recipient origin.

Tissue-resident macrophages also promote neutrophil extravasation into ischemic myocardium following cardiac transplantation. Using intravital two-photon imaging in a mouse model of IRI after cardiac transplantation, we have demonstrated that donor tissue-resident $CCR2^+$ monocyte-derived macrophages orchestrate neutrophil extravasation through TLR9/MyD88-mediated production of CXCL5. Here, MyD88 deficiency in heart-resident macrophages alone was sufficient to impair the extravasation of neutrophils[36]. Interestingly, sub-types of tissue-resident macrophages can serve a protective role against neutrophil infiltration during ischemic kidney injury[93]. In this study, the authors found that depletion of kidney-resident $CD169^+$ macrophages exacerbated neutrophil migration into injured tissue due to elevated expression of endothelial ICAM-1, as well as increased CXCL1 and CXCL2, resulting in more severe IRI-mediated tissue injury and functional impairment. Additional in vitro co-culture experiments confirmed that direct interactions between tissue-resident macrophages and endothelial cells suppress the adhesive and transmigratory activity of neutrophils through ICAM-1 suppression. As such, various tissue-resident macrophages appear to play different roles among solid organ systems, but additional investigations are warranted before these studies can be translated clinically.

Neutrophil Activation

Following neutrophil extravasation in the setting of IRI, molecular cues guide neutrophil migration through extravascular tissues and stimulate their activation. Neutrophil activation can trigger various effector pathways, including the oxidative response that consists of ROS production, such as superoxide anion and hydrogen peroxide following NADPH-oxidase complex activation, and production of halogenated oxidants, such as hypochlorous acid and chloramines via myeloperoxidase (MPO). Neutrophil granules provide a reservoir of inflammatory mediators, including azurophilic granules (MPO, neutrophil elastase) and specific granules (metalloproteinase-8 (MMP-8), MMP-9). In vitro experiments have demonstrated that following neutrophil activation some of these stored enzymes, such as MMPs, must receive additional activation signals to trigger their release, though these mechanisms remain incompletely understood[94–96]. In support of these findings, a mouse model of warm lung IRI demonstrated that recruited neutrophils remain dormant unless activated by additional signals within the lung tissue[97]. These findings were corroborated by results in a mouse model of ventilated warm lung IRI, which showed that despite the early surge of inflammatory cytokines and rapid recruitment of neutrophils to injured lungs, there was quick resolution of this process within 24 hours without any evidence of tissue damage[59]. Furthermore, in studies of IRI in rat lungs subjected to 90 minutes of hilar clamping or transplanted after cold ischemic storage, levels of MPO activity in lung tissues increased during the first 16 hours following reperfusion and fell thereafter[30,98]. These findings suggest that IRI-mediated injury is associated with rapid recruitment of neutrophils that undergo extravasation and activation within ischemic tissues, followed by a phase of inflammation resolution mediated by specialized pro-resolving lipid mediators, such as resolvins, that can be released from a variety of cells including macrophages and neutrophils[99,100].

In canine heart and rat liver IRI models, NADPH oxidase-mediated ROS production by neutrophils has been identified as an important effector arm of tissue damage[101,102]. Neutrophil elastase release by activated neutrophils can also mediate tissue damage in animal models of lung and hepatic IRI. In a mouse model of warm liver IRI, neutrophil transmigration from the vascular lumen into liver parenchyma triggers neutrophil elastase release, which degrades the extracellular matrix of the endothelium. The resultant tissue injury leads to the release of pro-inflammatory cytokines and chemokines and upregulation of TLR4, resulting in further propagation of neutrophil-mediated graft injury via the mechanisms discussed above[103]. The damaging effects of neutrophil elastase have also been demonstrated in lung IRI. In a study by Ishikawa using a rabbit model of lung IRI induced by 2 hours of hilar clamping, intravenous administration of a neutrophil elastase inhibitor resulted in reduced tissue injury and neutrophil infiltration[104]. Similarly, in a canine model of single lung transplantation, continuous infusion of a neutrophil elastase inhibitor during reperfusion resulted in significantly reduced MPO activity and successfully ameliorated lung injury, as evidenced by pulmonary function and histology[105]. Moreover, in a rat model of lung transplantation, addition of a neutrophil elastase inhibitor, sivelestat, to the organ flushing solution prior to cold ischemic storage resulted in significantly improved graft function and reduced lung tissue MPO activity[106]. These results were corroborated in a

porcine lung transplant model, where a neutrophil elastase inhibitor given during ex vivo lung perfusion resulted in improved lung function both during and after transplantation[107].

Increased levels of MMP-9 is present in human hepatic allograft tissues following reperfusion[108]. Animal studies have illustrated that increased levels of MMP-9 in acutely injured liver tissues stimulates tissue destruction through the degradation of extracellular matrix proteins[109]. In a mouse model of warm liver IRI, both neutrophils and Mac-1-positive leukocytes were found to be the major sources of MMP-9 in damaged hepatic tissues, and the expression of MMP-9 was specifically localized to leukocytes that had already initiated the process of extravasation[110]. In a rat liver model of syngeneic liver transplantation following 24 hours of ex vivo cold ischemia, vascular expression of fibronectin was significantly increased in sinusoidal endothelial cells shortly following reperfusion, and fibronectin binding to the integrin receptor α4β1 regulated the expression of MMP-9 by infiltrating neutrophils[110,111]. Moreover, blockade of fibronectin interactions disrupted the extravasation of neutrophils after IRI and down-regulated MMP-9 expression. To further evaluate the role of MMP-9 of infiltrating leukocytes, MMP-9-deficient mice and wild-type mice treated with an anti-MMP-9 neutralizing antibody were subjected to warm liver IRI. MMP-9 inhibition prevented neutrophil migration across fibronectin and reduced MPO activation in vivo[112]. Additionally, mice treated with a broad inhibitor for both MMP-9 and MMP-2 showed inferior protection against IRI-mediated hepatic damage compared to MMP-9 specific inhibition. These findings suggest that MMP-2 and MMP-9 likely serve distinct roles in liver IRI, with MMP-2 potentially promoting anti-inflammatory pathways to reduce tissue destruction.

The effects of MMPs may be organ-specific. In a rat model of orthotopic lung transplantation, MMP-2 and MMP-9 were found to be elevated following reperfusion and were associated with increased alveolar capillary permeability and neutrophil infiltration[113]. Notably, when a nonselective MMP inhibitor was administered to both donors and recipients, there was significantly reduced capillary leakage and neutrophil infiltration. In another study by Yano utilizing a rat lung transplantation model, elevated enzymatic activity of both MMP-2 and MMP-9 were seen following prolonged cold storage which correlated with markers of pulmonary graft injury[114]. In another rat model of lung transplantation utilizing warm IRI, increased levels of both MMP-2 and MMP-9 were observed, but MMP-9 expression was transient while MMP-2 expression was sustained[115]. Thus, MMP inhibition may serve as a potential target to reduce lung injury following IRI and future investigations should further define the role and appropriate timing for specific MMP inhibitors in this context.

The role of MMPs in cardiac transplantation also appears unique compared to other solid organ transplants. In a study of isolated, perfused rat hearts subjected to 20 minutes of warm ischemia, MMP-2 was found to be elevated in the coronary effluent during reperfusion and correlated with the duration of ischemia and the degree of myocardial dysfunction[116]. Furthermore, inhibition of MMP-2 improved myocardial function, whereas MMP-2 administration worsened myocardial recovery during reperfusion. In vitro studies have revealed that human troponin I and troponin T are susceptible to proteolytic degradation by MMP-2[117]. In rat hearts subjected to 20 minutes of ischemia followed

by reperfusion via the Langendorff method, MMP-2 co-localizes with troponin I in cardiomyocytes, and MMP-2 inhibition prevented the IRI-induced degradation of troponin I and improved recovery of myocardial function[117]. In canine hearts that underwent coronary occlusion for 60 minutes, levels of MMP-9 were increased in cardiac lymph and myocardium, and dual-labeling immunofluorescence determined that neutrophils were the main source of MMP-9[118]. Using a mouse model of warm cardiac IRI, Carbone found elevated myocardial and serum levels of receptor activator of nuclear factor-κB ligand (RANKL), and administration of a neutralizing anti-RANKL antibody resulted in decreased neutrophil infiltration and degranulation and significantly reduced MMP-9[119]. The authors also demonstrated in vitro that RANKL abrogated neutrophil chemotaxis and release of MMP-9.

Clinical studies of lung transplant recipients have demonstrated that patients with severe PGD following lung transplantation have higher levels of circulating mitochondrial DNA (mtDNA) than patients without PGD[120]. Notably, in a mouse model of PGD following orthotopic lung transplantation, graft injury was inhibited by pharmacologic inhibition or genetic deletion of formyl peptide receptor 1 (FPR1), a chemotactic receptor that drives neutrophil migration towards free peptides released by bacteria and mitochondria. Further analysis revealed that FPR1-mediated neutrophil trafficking is associated with the engulfment of damaged mitochondria and the activation of ROS-induced tissue injury. A novel method of in vivo single-photon emission computed tomography (SPECT) imaging was recently developed which allows visualization of activated neutrophils through administration of a synthetic FPR1 ligand (polyethylene glycolated cinnamoyl-F-(D)L-F-(D)L-F-K)[41]. Using this imaging technique in a mouse model of pulmonary hilar clamping, there is increased signal intensity from activated neutrophils, which peaks 2 hours after reperfusion and resolves by 24 hours.

Neutrophils can also promote graft injury by undergoing a unique form of cell death that involves extrusion of neutrophil extracellular traps (NETs) comprised of extracellular chromatin decorated with histones, antibacterial peptides, and serine proteases. NETs appear to serve as a marker of severity of inflammation, and increased levels of NETs have been reported in the BAL fluid of human lung transplant recipients with PGD as well as renal transplant recipients with acute antibody-mediated rejection[121,122]. Multiple experimental models of lung and liver transplantation have similarly demonstrated that NETs play a role in transplant-mediated IRI[121–124]. These studies showed that inhibition of NET formation through administration of DNAse, Cl-Amidine, or recombinant human thrombomodulin improved organ function and attenuated inflammation. In a mouse model of orthotopic lung transplantation, Mallavia found that prolonged cold ischemia triggered mtDNA release into BAL fluid[125]. Accordingly, intrabronchial DNase treatment resulted in degradation of mtDNA in BAL fluid, and in vitro studies of mtDNA-stimulated neutrophils incubated with DNase failed to produce NETs. Additionally, adoptive transfer of mtDNA in a minimal ischemia model during transplantation induced NET formation and lung injury, which was prevented in mice with TLR9 deficiency in either the lung donor or recipient.

MicroRNAs (miRNAs), particularly miR-223, have been identified as important regulators of neutrophil activation and function. However, the role of miR-223 appears to vary

among different experimental settings of acute injury. In both humans and mice with acute lung injury, pulmonary expression of miR-223 is significantly increased[126,127]. In mouse models of acute lung injury induced by infection or barotrauma, Neudecker found that miR-223 deficiency is associated with severe inflammation, while pulmonary miR-223 overexpression results in lung protection[128]. Furthermore, activated neutrophils were found to be a major source of miR-223, and in vitro co-culture of human neutrophils with epithelial cells resulted in transfer of miR-223 from neutrophils to the epithelial cells. These results collectively suggest that miR-223 may serve as a negative feedback molecule to limit acute inflammation. However, this protective effect is less clear in the setting of IRI. In a mouse model of lung IRI induced by transient pulmonary hilar occlusion, miR-223 was similarly found to be expressed at high levels in injured tissue, but here it was shown to inhibit expression of hypoxia-inducible factor-2α and β-catenin, which promoted autophagy and aggravated IRI-induced injury[127]. In a mouse model of liver IRI induced by portal clamping, Yu found that miR-223 expression levels were upregulated following reperfusion and significantly correlated with serum transaminases[129]. As such, the mechanisms whereby miRNA expression alters IRI-induced injury warrants further investigation to determine the potential of this pathway as a therapeutic target.

Purine signaling has also been implicated in guiding neutrophil responses during acute inflammation. Eltzschig showed that activated human neutrophils can promote vascular integrity by releasing adenine nucleotides which subsequently undergo metabolism and activate endothelial adenosine receptors, particularly the adenosine A_{2B} receptor[130]. A study by Li reported increased levels of circulating netrin-1, a protective neuronal guidance protein, in patients after myocardial infarction and mice following myocardial IRI[131]. Neutrophils were found to be the main source of serum netrin-1 levels following IRI, and netrin-1-mediated cardioprotection was dependent on purinergic signaling through the myeloid adenosine A_{2B} receptor. Similarly, a protective role for adenosine A_{2B} receptor has been described after renal and hepatic IRI. Utilizing a mouse model of renal IRI with ischemic preconditioning, Grenz demonstrated that mice deficient in the adenosine A_{2B} receptor had increased kidney injury, while treatment of wild-type mice with an adenosine A_{2B} receptor agonist attenuated kidney injury[132]. However, contrasting results reported by other studies suggest that adenosine A_{2B} receptor may play a pro-inflammatory role in other instances after IRI. For example, Anvari found that adenosine A_{2B} receptor-deficient mice had significantly reduced lung injury following IRI induced by hilar clamping[133]. Further experiments using bone marrow chimeras revealed that these effects were due to A_{2B} receptor activation on pulmonary resident cells. Thus, the pro- and anti-inflammatory effects associated with adenosine A_{2B} receptor signaling is likely dependent on the specific organ and inflammatory model studied.

Other innate immune cells, such as NK cells, contribute to tissue injury following IRI as well. In a mouse model of hepatic IRI induced by portal vein clamping, NK cell-deficient mice develop reduced liver injury[134]. Similarly, tissue-resident NK cells influence immune responses following renal IRI. For example, Victorino demonstrated that depletion of conventional and resident NK cells protected against renal IRI, while selective depletion of only conventional NK cells failed to confer protection[135]. These studies highlight that cellular mediators other than neutrophils also contribute to IRI-induced injury, and while

outside the scope of this article, these alternate pathways should also be considered in future investigations.

Facilitation of Alloimmunity

Cellular depletion experiments have illustrated that neutrophils provide an important link between innate immune activation and the development of alloimmunity. However, the pathways that facilitate alloimmunity as a result of IRI are less understood than the innate immune processes discussed above and likely differ between organs. For example, we have previously demonstrated that alloimmune responses following lung transplantation are initiated within the allograft itself, independent of secondary lymphoid tissues and setting it apart from other solid organs. Following lung transplantation in mice, T cell clusters are observed around donor-derived graft-resident $CD11c^+$ DCs as early as 30 hours after transplantation[136]. Using intravital two-photon microscopy, we have shown that graft-infiltrating neutrophils stimulate donor $CD11c^+$ DCs in lung allografts in a contact-dependent fashion shortly after transplantation[69]. We have demonstrated that neutrophil depletion or G-CSF inhibition in the setting of severe IRI results in decreased IL-12 production by DCs, reduced interferon-gamma (IFN-γ^+) T cells, and attenuated rejection. As described above, ischemic injury stimulates the release of inflammatory mediators which trigger neutrophil activation. To this end, higher levels of mtDNA have been shown to be associated with PGD in lung transplant recipients, thereby increasing their risk for chronic rejection[23,120]. Several other pathways have been suggested that may further clarify the mechanisms linking early neutrophil responses and lung transplant rejection, but much remains to be discovered before such pathways can be targeted clinically[137].

Little mechanistic evidence currently exists regarding how neutrophils may regulate humoral responses in transplanted organs. As mentioned previously, prior studies have elucidated that increased levels of NETs are associated with the development of acute antibody-mediated rejection in renal transplant recipients[121]. In studies of acute antibody-mediated rejection in CCR5-deficient mice, there is an initial wave of neutrophil infiltration into kidney allografts immediately after transplantation followed by a later surge of neutrophil infiltration that occurs in parallel to rising titers of donor-specific antibody titers[138,139]. Depletion of neutrophils prior to this second wave of infiltration reduces natural killer cell and monocyte proliferation within the graft and abrogates natural killer cell activation, a key mechanism underlying acute antibody-mediated rejection of kidney grafts. These findings suggest that depletion of neutrophils may inhibit myeloid cell activation and indirectly suppress alloimmune responses. In a study of cardiac allograft rejection in mice, recipients with genetic or pharmacologic inhibition of the neutrophil chemokine receptor CXCR2 exhibited significantly attenuated neutrophilic graft infiltration and inhibited of T cell infiltration into the transplanted hearts[140]. Additionally, when costimulatory blockade was combined with either peri-transplant neutrophil depletion or anti-CXCL1/2 antibodies, cardiac allograft survival was significantly prolonged. Neutrophils may also augment alloimmunity by transporting donor antigens from injured tissues to peripheral lymph nodes in a process termed ‘reverse neutrophil migration’[141,142]. Furthermore, neutrophils may acquire antigen-presenting capabilities or enhance the secretion of immunomodulatory molecules that influence lymphocyte activity[143,144].

Conclusion

Neutrophils play a pivotal role in IRI following heart, lung, kidney and liver transplantation. The recruitment, extravasation and activation of recipient-derived neutrophils is orchestrated through a variety of graft-resident and recipient-derived cell populations. Recent technological advances, including novel intravital imaging techniques, have enabled a more accurate characterization of neutrophilic responses during IRI. These newly recognized immune pathways warrant further investigation and translation to clinical trials in hopes of ameliorating IRI-mediated graft dysfunction.

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Abbreviations

AM	Alveolar macrophage
BAL	Bronchoalveolar lavage
CM	Classical monocyte
DAMP	Damage-associated molecular pattern
DAP12	DNAX-activation protein 12
DC	Dendritic cell
FPR1	Formyl peptide receptor 1
G-CSF	Granulocyte colony-stimulating factor
HMGB1	High-mobility group box 1
ICAM-1	Intercellular adhesion molecule-1
IFN-γ^+	Interferon-gamma
IRI	Ischemia-reperfusion injury
miRNA	microRNA
MMP	Metalloproteinase
MPO	Myeloperoxidase
MPT	Mitochondrial permeability transition
mtDNA	Mitochondrial DNA
NCM	Non-classical monocyte
NET	Neutrophil extracellular trap

NK	natural killer
NOX4	NADPH oxidase 4
PGD	Primary graft dysfunction
RANKL	Receptor activator of nuclear factor-κB ligand
RIPK1	Receptor-interacting protein kinase 1
RIPK3	Receptor-interacting protein kinase 3
ROS	Reactive oxygen species
SPECT	Single-photon emission computed tomography
TLR	Toll-like receptor
TNF-α	Tumor necrosis factor alpha
VCAM-1	Vascular cell adhesion molecule-1

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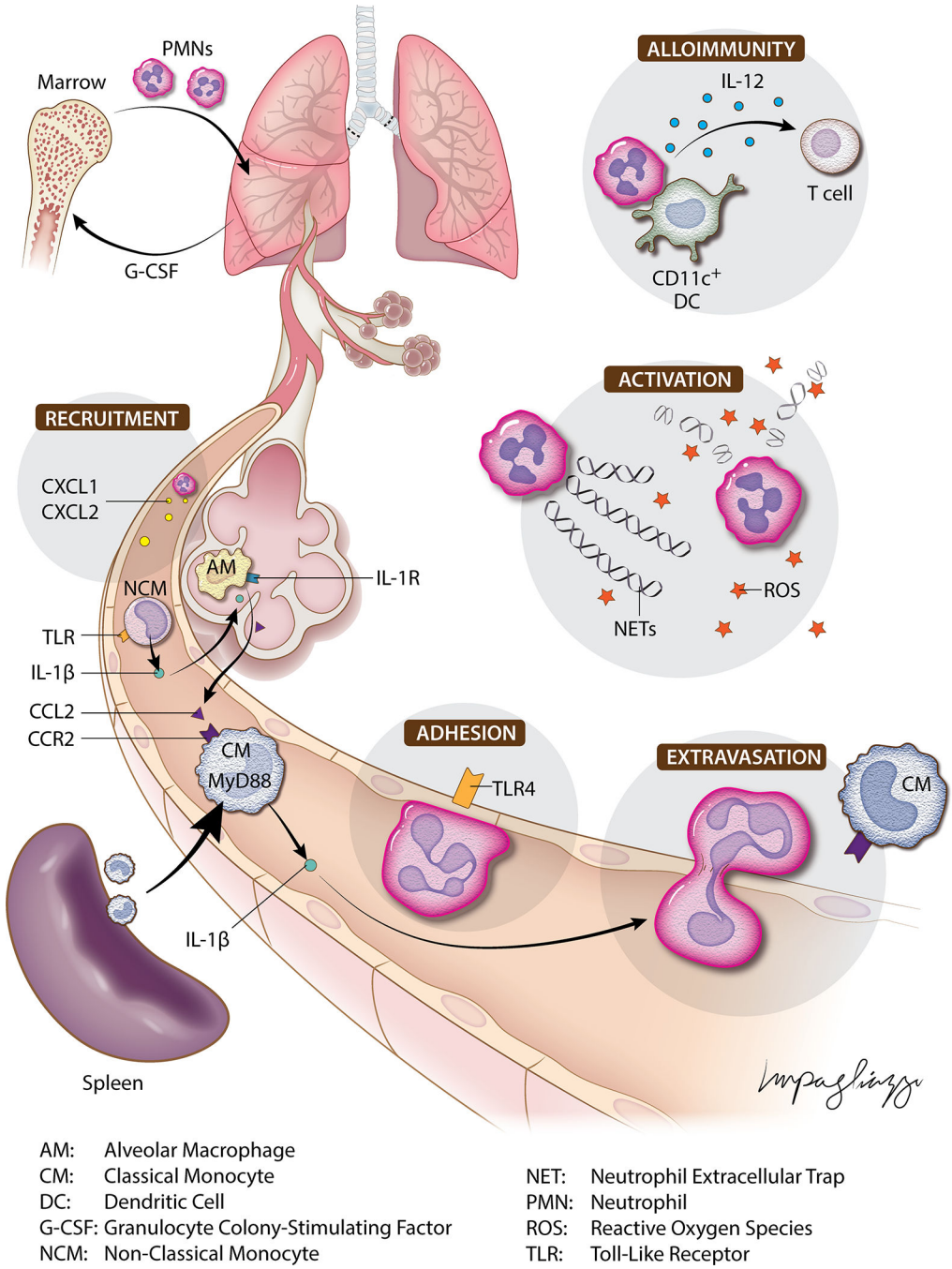

Figure 1:

Schematic illustrating neutrophil-mediated immune responses within the lung following ischemia-reperfusion injury.