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The Role of Neutrophils in Transplanted Organs

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Abstract

Neutrophils are often viewed as non-specialized effector cells whose presence is a simple indicator of tissue inflammation. There is new evidence that neutrophils exist in subsets and have specialized effector functions that include extracellular trap generation and the stimulation of angiogenesis. The application of intravital imaging to transplanted organs has revealed novel requirements for neutrophil trafficking into graft tissue and illuminated direct interactions between neutrophils and other leukocytes that promote alloimmunity. Paradoxically, retaining some neutrophilia may be important to induce or maintain tolerance. Neutrophils can stimulate anti-inflammatory signals in other phagocytes and release molecules that inhibit T cell activation. Here we will review the available evidence of how neutrophils regulate acute and chronic inflammation in transplanted organs and discuss the possibility of targeting these cells to promote tolerance.

Introduction

Neutrophils are usually the first leukocytes to infiltrate transplanted organs and are a well-established marker of transplant injury (1). Most work on neutrophils in transplanted organs has focused on their destructive role during Ischemia Reperfusion Injury (IRI), a form of sterile tissue damage that is exacerbated by the massive release of oxidative and proteolytic effector activity by these cells. Advances in our understanding of the underlying mechanisms of sterile inflammation have revealed that both neutrophil infiltration and activation are augmented by the release of damage associated molecular patterns (DAMPs) from necrotic cells and the extracellular matrix (ECM) (2). Accordingly, DAMPs induce the expression of inflammatory cytokines via stimulating pattern recognition receptors (PRRs) on macrophages. These include ELR+ CXC chemokines (e.g., CXCL8 in humans & CXCL1, CXCL2 in rodents) and IL-1β that play a key role in neutrophil recruitment by activating vascular endothelium (3). Neutrophils also express PRRs, which when engaged by

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DAMPs, induce the generation of reactive oxygen species (ROS) and hydrolytic enzymes that exacerbate graft damage. From this perspective graft-infiltrating neutrophils respond they would during an infection since DAMPs contain the analogous structural features of pathogen associated molecular patterns.

However, this is a rather limited view of neutrophil function as there is accumulating evidence that they also play a critical role in adaptive immunity. Similar to professional antigen presenting cells (APCs), neutrophils have the capacity to leave peripheral sites and deliver antigen to lymph nodes (4). Additionally, neutrophils can induce the differentiation of T lymphocytes via expression of MHC and co-stimulatory molecules (5). Finally, and perhaps the least described role of neutrophils is their contribution to the resolution of inflammation. Through their own apoptotic death, neutrophils can induce the expression of anti-inflammatory molecules in other myeloid cells (6) while subsets of neutrophils can inhibit T cell activation (7) as well as promote angiogenesis (8). Here we will review neutrophil biology in the context of acute and chronic transplant inflammation as well as examine approaches to developing neutrophil targeted therapies to promote graft survival.

Acute Injury

Neutrophil recruitment begins as a passive process triggered initially by vascular endothelium. Neutrophils make contact with the vascular endothelium through a series of distinct dynamic behaviors known as tethering, rolling, adherence, and crawling before finally extravasating through the vessel (3). While these behaviors are likely common to all leukocytes, early studies suggested that targeting adhesion molecules had profound effects on neutrophil graft infiltration. For example, in a rat intestinal transplantation model treatment with a recombinant P-selectin glycoprotein ligand-1 reduced graft neutrophilia and ameliorated tissue injury (9).

Although these observations have illuminated some important signals neutrophils use to traffic into grafts only recently have there been direct observations of their dynamic behavior. These advancements have been forwarded by the use of intravital 2-photon (2P) microscopy for transplanted organs. In a beating mouse heart transplant model Li et al. visualized the effects of antibody-mediated blockade of the integrins Mac-1 and LFA-1 on neutrophil intravascular behavior and extravasation. Here LFA-1 blockade completely prevented adherence and crawling while Mac-1 blockade allowed adherence but slowed intravascular crawling and transendothelial migration speed (10). In a mouse lung transplant model intravital 2P microscopy revealed that neutrophils track behind inflammatory Ly6C^{hi} monocytes while in the process of transendothelial migration (11). This relationship is likely critical for extravasation as clodronate-mediated depletion of monocytes induced neutrophils to accumulate along the luminal surfaces of vascular endothelium. Future studies will be required to determine how inflammatory monocytes promote neutrophil transendothelial migration and whether monocyte-dependent neutrophil extravasation occurs in other organs.

Neutrophils commit the bulk of their effector responses following exit from the vasculature. In the transplant setting one of the most destructive activities is the generation of reactive oxygen species (ROS) (12). Activated neutrophils primarily employ the oxidant-generating

complex system NADPH oxidase to generate superoxide, which promotes macromolecule peroxidation and irreversible cellular damage. NADPH oxidase activity requires two membrane-spanning proteins ($gp91^{phox}$, $p22^{phox}$) to assemble with three cytosolic components of the complex ($p40^{phox}$, $p47^{phox}$, $p67^{phox}$). In this regard, chronic granulomatous disease patients who encode hypofunctional mutations in $gp91^{phox}$ or $p47^{phox}$ exhibit significant protection from transiently induced upper limb IRI (13). Moreover, in canine heart and rat liver IRI models neutrophil NADPH oxidase-mediated ROS generation was the predominant contributor to tissue damage (14, 15). Neutrophil-mediated graft tissue damage is also driven by the release of tissue-digesting enzymes such as metalloproteinase-9 (MMP9) and neutrophil elastase (NE). MMP9 and NE, which have been observed to accumulate in acutely injured liver and lung transplants (16, 17), break down homeostatic barriers that promote graft function by hydrolyzing ECM proteins collagen, elastin and fibronectin. Accordingly, mice deficient in MMP9 are protected from steatotic liver IRI (18). The lack of MMP9 also promoted vascular integrity and inhibited the proteolysis of platelet endothelial cell adhesion protein, which is reported required for maintaining endothelial cell-cell junctions.

Neutrophils can also promote graft inflammation through undergoing a unique form of programmed cell death termed “NETosis” (19). These senescent neutrophils display extracellular chromatin (NETs) decorated with histones, antibacterial peptides and serine proteases. NETs were first described to have antimicrobial functions but later studies revealed that they cause extensive endothelial cell damage in response to sterile inflammation. NETs have been reported in the bronchoalveolar lavage fluid of human lung transplant recipients with primary graft dysfunction and in experimental lung transplant-mediated IRI and liver IRI mouse models (20, 21). In both studies the authors used DNAse to dissolve NETs, which led to improved organ function and attenuated acute inflammation measured up to one day. However, whether NETs exclusively promote inflammation remains controversial. Shauer et al reported that NETs in a chronic gout model can trap and degrade ELR+ chemokines and inflammatory cytokines (e.g., IL-6, IL-1β) due to their high concentrations of proteases (22). Neutrophil depletion, inhibition, or DNAse digestion of NETs actually worsened chronic tissue injury. Thus, future studies will be needed to determine the long-term consequences of NETs on graft survival.

Acute Cellular Rejection

Neutrophil depletion experiments have revealed the importance of neutrophils in promoting alloimmune responses. For example, in a mouse skin transplant model neutrophil depletion slowed acute rejection by attenuating the recruitment of alloreactive memory CD8+ T cells (23). Neutrophils may stimulate the recruitment of activated CD8+ T cells through their expression Fas ligand, which can induce expression of the T cell chemoattractants CCL1, CCL2 and CCL5 (24). Additionally, intravital imaging of allografts has uncovered the importance of neutrophil cross-talk with other graft-resident leukocytes in stimulating alloimmune responses. In a model orthotopic lung transplant model, neutrophil depletion was shown to promote immunosuppression-mediated allograft acceptance leading to less intragraft antigen-presenting cell (APC) IL-12 production and reduced T_h1 cell alloimmunity (25). Intravital visualization of infiltrating neutrophils in acutely rejecting

grafts showed that they made prolonged interactions with donor-derived graft-resident CD11c+ APCs. Additionally, when these infiltrating neutrophils were co-cultured with dendritic cells (DC) they induced IL-12 and MHC Class II expression in a manner that required contact and TNF-α on their plasma membrane. By contrast, neutrophils from resting mice had little plasma membrane TNF-α and did not activate DCs suggesting that IRI licenses neutrophils to promote alloimmune responses (25).

It remains conceivable that neutrophils additionally stimulate alloimmunity as also they infiltrate infected allografts. This possibility was recently investigated in a mouse lung transplant model where *Pseudomonas aeruginosa* infection prevented the maintenance of tolerance (26). Infiltrating neutrophils were visualized to interact with lung allograft resident T cells that were also in contact with CD11c+ APCs. These interactions were shown to stimulate alloimmune responses through *Pseudomonas*-dependent upregulation of neutrophil CD80 and CD86, which, in turn, provided a sufficient trans-costimulation signal to expand the intragraft T_h1 and T_h17 compartment (26). The interplay between infection and alloimmunity is of considerable interest and has been reviewed elsewhere (27).

Antibody-mediated Rejection

Unlike for T cell mediated-alloimmunity, there is comparatively little mechanistic data on how neutrophils regulate humoral responses to transplanted organs. Presently, there are no reports analyzing the effects of depleting neutrophils on antibody-mediated rejection. Clinical pathological findings show that graft neutrophilia is linked to antibody-mediated transplant rejection (28, 29). In mouse heart and lung transplant models, antibody-mediated rejection induced infiltrating neutrophil activity that played a significant role in tissue destruction (29, 30). In this regard neutrophils are conceivably being recruited to grafts by complement fixed antibodies or by platelets bound to vascular endothelium injured by alloantibodies (31). Accordingly, neutrophils express the complement receptor 1 which promotes adhesion to complement decorated immune complexes. Activated platelets can stimulate endothelial cell P-selectin upregulation that can additionally promote neutrophil recruitment. How alloantibodies contribute to neutrophil effector responses in grafts is even less clear. Early work in an anti-MHC Class I antibody-mediated acute lung injury model suggested that Fcγ receptor-mediated recognition of immunoglobulin complexes could lead to neutrophil effector responses within grafts (32). However, in a later report kidney transplant recipients with alloantibodies and a hypofunctional mutation in the inhibitory Fcγ IIB receptor, which negatively regulates Fcγ receptors that promote inflammation, there was no impact on graft survival (33). It is also possible that neutrophils control B cell differentiation following transplantation. Recent work has shown the existence of a neutrophil subset infiltrating the lymph node peri-marginal zones that secretes high levels of BAFF, APRIL, CD40L and IL-21, which are B cell stimulating factors involved in antibody class switching, plasma cell differentiation and survival (34). Future studies will need to be conducted to determine if B cell inducing neutrophils exist in transplant recipients and, if so, whether they play a role antibody-mediated rejection.

Chronic rejection

Infiltrating neutrophils are commonly observed in chronically rejecting allografts. The predominant view is that neutrophilia is an effector arm of IL-17 expression in chronic rejection, mainly through the accumulation of T_h17 cells (35). IL-17 is a potent driver of both neutrophil infiltration and production by stimulating the production of ELR+ CXC chemokines and granulopoietic cytokines (e.g., G-CSF, GM-CSF), respectively. The impact of IL-17-mediated neutrophilia on chronic rejection was recently reported in lung transplant recipients where a mutation in the IL-17 receptor that promotes airway neutrophilia was shown to increase the risk for chronic rejection (36). How neutrophils promote chronic rejection remains poorly described. Studies linking TLR signaling pathways to chronic rejection suggest that, similar to acute settings, neutrophil-mediated injury is triggered by DAMPs. For example, the ECM DAMP hyaluronic acid (HA) accumulates in airway lesions of human lung recipients with bronchiolitis obliterans syndrome (BOS). Analysis of the low molecular weight form of HA in a mouse lung transplant model showed that it stimulated neutrophil infiltration in a TLR2/4 dependent manner in addition to ROS burst (37). Moreover, proteolytic enzymes commonly related to ECM degradation and neutrophils migration, such as metalloproteinases (e.g., MMP9, MMP8), have been found to be increased in patients affected by BOS (17). Neutrophils may also promote chronic inflammation by influencing the maintenance of parenchymal tissues. A recent report showed the accumulation of neutrophil-derived alpha and beta defensin peptides in BOS patients. These peptides induced airway epithelial cells to produce inflammatory cytokines (e.g., IL-8, IL-1β) and pro-fibrotic growth factors (e.g., VEGF, EGF) suggesting a link between neutrophil recruitment and the induction of gene expression patterns within parenchymal cells known to promote tissue remodeling (38).

Resolution of Inflammation

The resolution of graft inflammation is dependent on the efficient removal of neutrophils from inflamed tissue. This is largely controlled by induction of neutrophil apoptotic death (39). Importantly, neutrophil apoptosis not only terminates effector activity but also is a passive anti-inflammatory signal that is propagated to other immune cells. The externalization of phosphatidyl serine (PS) is a signal for the uptake or ‘efferocytosis’ of neutrophil carcasses by phagocytes (e.g., macrophages) and leads to several complementary mechanisms that can resolve inflammation (6, 39). First, the engulfment of apoptotic neutrophils prevents secondary necrosis, which can result in the uncontrolled release of damaging proteolytic and oxidative mediators. Second, efferocytosing phagocytes produce large amounts of the anti-inflammatory cytokines IL-10 and TGF-β, which not only act to inhibit inflammatory gene expression, but also help to enforce tolerance through generation and maintenance of peripheral regulatory CD4+ T cells (40). Lastly, efferocytosing phagocytes convert arachidonic acid, a lipid involved in the exacerbation of IRI, into pro-resolving lipid mediators that inhibit the activation and recruitment of neutrophils (41). In the latter case treatment with the pro-resolving lipid lipoxin A4 ameliorated hepatic tissue injury, reduced evidence of histopathological allograft rejection and improved liver function in a rat model of liver allotransplantation (42). Lipoxin A4 also increased the ratio of T_h2 to

T_h1 cytokine expression, which is a CD4+ T cell helper cell profile often associated with allograft tolerance.

Apoptotic neutrophils can also modulate inflammation though the release of small extracellular vesicles called Neutrophils Derived Microvesicles (NDMV). NDMVs are small vesicles ranging in size from 0.1–1.0 μm that promote intercellular communication by allowing transfer of membrane and cytosolic proteins, lipids, and RNA. Notably, NDMV function as intercellular reservoirs of pro-resolving lipid mediator precursors which can be metabolized to an active form at the inflammation site (43). In addition, recognition of the PS expressed on NDMVs membranes stimulated macrophages to express the pro-resolving cytokine TGF-β and to be less responsive to LPS (44).

Perhaps the most unrecognized activity of neutrophils is the potential to inhibit alloimmunity by preventing T cell activation. For example, activated neutrophils can suppress T cell activation by the release of NE and cathepsin G (45). These serine proteases cleave and inactivate IL-2 and IL-6 and promote the shedding of their cognate receptors from T-cells. Neutrophils also may act preemptively to prevent T cell alloimmunity. Pillay et al demonstrated that $CD16b^{bright}CD62^{lo}$ neutrophil subsets in acutely injured patients could bind to T cells via Mac-1 and release hydrogen peroxide into the immunological synapses, resulting in the suppression of proliferation (7). Future studies will be required to determine if neutrophil subsets with regenerative or anti-inflammatory activity exist in transplant recipients.

Finally, neutrophils may also inhibit graft inflammation by promoting wound healing and tissue repair. Neutrophils use their integrin receptors to form dense clusters around necrotic tissue foci and seal them off from healthy tissue (46). Distinct neutrophil subsets then may help re-establish graft perfusion. Christoffersson et al identified a circulating pro-angiogenic $CD11b+Gr\text{-}1+CXCR4^{hi}$ neutrophil subset that was recruited in a vascular endothelial growth factor-A dependent manner to avascular mouse pancreatic islet transplants (8). Neovascularization was dependent on high levels of MMP9, a protease that is normally required for revascularization. Notably, a CD49+ $VEGFR1^{hi}$ $CXCR4^{hi}$ neutrophil subset with an analogous function was also found in humans (47).

Neutrophil-based therapy

At present, there are no clinically available agents that specifically inhibit neutrophil function. Given the immunosuppression status of transplant recipients, if such therapies are developed, rigorous infection monitoring will be needed because neutrophil impairment or neutropenia profoundly increases susceptibility to fungal and bacterial infections. However, it may be possible to target molecules on neutrophils that augment alloimmunity without detrimentally attenuating pathogen surveillance. In a mouse lung transplant model, CTLA4Ig was used to block the effects of *Pseudomonas*-mediated CD80/86 upregulation on neutrophils (26). Notably, CTLA4Ig treatment prevented acute cellular rejection and peribronchiolar fibrosis without inhibiting *Pseudomonas* clearance. It remains to be determined if CTLA4Ig modulates neutrophil function in humans. However, a recent 84-month study evaluating a humanized version of CTLA4Ig in kidney recipients showed

improved graft function and survival without increasing the occurrence of serious infectious complications (48).

Blunting neutrophil chemotaxis may be a safe option for chronic rejection. In lung transplant recipients with no ongoing signs of infection, treatment with the macrolide antibiotic azithromycin inhibited expression of the ELR+ CXC chemokine IL-8, blunted airway neutrophilia and improved graft function (49). Of note azithromycin did not appear to alter rates of infection suggesting direct effects on neutrophil infiltration. However, application of this therapy remains controversial as a later data show its effectiveness is restricted to certain subtypes of neutrophilic chronic airway rejection (50).

Targeting neutrophil effector function may represent another therapeutic avenue to promote allograft survival. Sivelestat, a specific inhibitor of NE, has been shown to attenuate organ dysfunction in an experimental model of liver IRI (51). Several agents show promise to block NADPH-dependent ROS production. PR39, an anti-microbial peptide that prevents NADPH oxidase assembly is reported effective in protecting heart function and inhibiting neutrophil infiltration in a rat cardiac IRI model (52). Additionally, Apocynin has been shown to reduce neutrophilia, prevent signs of oxidative damage, and improve pulmonary function in a mouse lung IRI model (53). How Apocynin precisely functions is unclear, although it preferentially targets phagocytic NADPH oxidase over the vascular isoforms of this enzyme.

Finally, the induction of neutrophil apoptosis could promote tolerance. Extracorporeal Photophoresis (ECP) is an anti-inflammatory treatment in which circulating peripheral leukocytes are incubated with the DNA cross-linking agent psoralen, exposed to UV light, and then transferred back into the patient (54). Although the therapeutic mechanisms of ECP remain obscure a recent report has shown that it predominantly accelerates neutrophil apoptosis and results in the release of arginase-1 (55). Argininase-1 mediated arginine depletion inhibits T cell activation and has been reported to promote the tolerance of corneal allografts in mice (56). There also reports demonstrating therapeutic benefits of ECP for recipients with chronic rejection of lung, heart, liver and kidney transplants (54). As with ECP and other therapies that potentially involve neutrophils more studies will be necessary to determine what mechanisms should be targeted to promote transplant survival.

Conclusions

Neutrophils are increasingly recognized as important components of the inflammatory circuitry that regulate the link between innate and adaptive immunity. From the moment of engraftment, they exert a wide range of effector functions that result in the exacerbation of IRI and the promotion of rejection through bidirectional cross-talk with other immune cells. There is also substantial contrasting evidence that neutrophils have inducible anti-inflammatory properties or exist in regulatory subsets suggesting that they could be utilized to protect the allograft from injury and promote tolerance. However, it remains to be determined if targeting neutrophils is a workable approach to promote tolerance. When compared to other leukocyte populations few reports directly address the role of neutrophils

in transplants. Future mechanistic studies will be required to better understand the role of neutrophils in transplanted organs.

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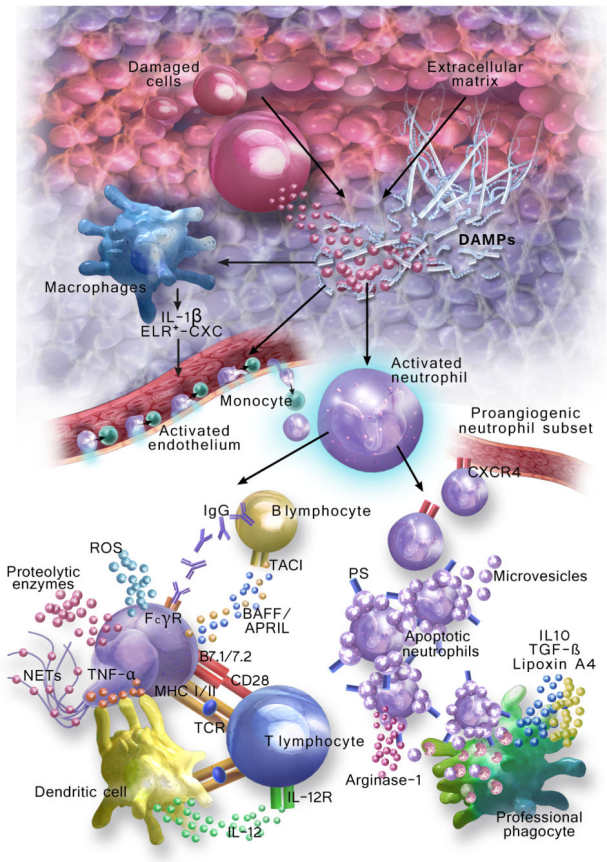

Figure. Pathways of neutrophil recruitment and activation that impact innate and adaptive immune responses to transplanted organs

Early inflammatory events after transplantation lead to the release of DAMPs that can originate from the damaged cells or from the extra-cellular matrix. DAMPs, in turn, stimulate resident macrophages to secrete inflammatory mediators (e.g., ELR+-CXC, IL-1ß) that activate vascular endothelium to trigger the adherence of circulating neutrophils. Monocytes may also be required for neutrophil transendothelial migration. Once in extravascular spaces DAMPs contribute to neutrophil activation, which leads to graft injury through ROS burst, proteolytic enzyme release and NET generation. Neutrophils also engage in cross-talk with other leukocytes to stimulate alloimmunity. Neutrophil membrane TNF-α induces dendritic cells to augment MHC II and IL-12 expression. Neutrophils can also directly present antigens and co-stimulatory signals to T lymphocytes. Finally, they may amplify antibody-mediated rejection through FcΓ-receptor dependent mechanisms and release soluble factors (e.g., BAFF, APRIL) involved in shaping B lymphocyte maturation and differentiation through the TACI. Alternatively, neutrophils could help to resolve graft inflammation. Apoptotic neutrophils release Arginase-1, which metabolically suppresses T cell activation, and shed microvesicles containing anti-inflammatory mediators. Additionally, the externalization of phosphatidyl serine (PS) stimulates the clearance of apoptotic neutrophils by professional phagocytes, which induces the production of anti-inflammatory cytokines (e.g., IL10, TGF-ß) and pro-resolving lipids such as Lipoxin A4.

Finally, a proangiogenic subset of CXCR4^{hi} neutrophils has been shown to promote re-vascularization.

Table

Neutrophil function in transplanted organs

Organ	Species	Mechanisms of neutrophil trafficking into grafts	References
Intestine	Rat	P-selectin mediated intra-graft trafficking	(9)
Heart	Mouse	Mac-1 and LFA-1 mediated intragraft trafficking	(10)
Lung	Mouse	Monocyte dependent neutrophil extravasation	(11)
Organ	**Species**	**Mechanisms of neutrophil-dependent graft injury**	**References**
Heart, Liver	Dog, Rat	NADPH oxidase dependent tissue injury	(14,15)
Liver	Mouse	NE elastase dependent tissue injury	(16)
Lung, Liver	Human, Mouse	MMP 9 dependent digestion of ECM and disruption of vascular integrity	(17,18)
Lung, Liver	Human, Mouse	NET dependent tissue injury	(20,21)
Lung, Kidney	Mouse, Human	Fcy receptor-dependent tissue injury	(32,33)
Organ	**Species**	**Mechanisms of molecular and cellular cross-talk**	**References**
Skin	Mouse	Fas-ligand and perforin-dependent recruitment of activated CD8+ T cells	(24)
Lung	Mouse	Contact dependent dendritic cell maturation	(25)
Lung	Mouse	T_h1 and T_h17 compartment expansion through neutrophil costimulatory signals	(26)
Lung	Human	IL-17 dependent neutrophil infiltration	(36)
Lung	Human	Neutrophil defensin-mediated epithelial cell up-regulation offactors	(33)
Organ	**Species**	**Mechanisms of neutrophil mediated wound healing**	**References**
Pancreatic Islets	Human, Mouse	CXCR4 ^{hi} subset promoting revascularization	(8,47)