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Chronic Allograft Rejection – a Fresh Look

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Abstract

Purpose of review—New developments suggest that the graft itself, and molecules expressed within the graft microenvironment dictate the phenotype and evolution of chronic rejection.

Recent findings—Once ischemia-reperfusion injury, cellular and humoral immune responses target the microvasculature, the associated local tissue hypoxia results in HIF-1 α -dependent expression of pro-inflammatory and pro-angiogenic growth factors including vascular endothelial growth factor (VEGF) as a physiological response to injury. However, these genes, and notably VEGF, can promote the recruitment of alloimmune T effectors and T regulatory cells into the graft. Semaphorin family molecules may also bind to neuropilin-1 on T cell regulatory cell subsets to stabilize functional responses. mTOR/Akt signaling within endothelial cells regulates cytokine and alloantibody-induced activation and proliferation and their pro-inflammatory phenotype. Inhibition of mTOR and/or Akt results in an anti-inflammatory phenotype and enables the expression of coinhibitory molecules that limit local T cell reactivation and promote immunoregulation. Ligation of neuropilin-1 on T regulatory cells also inhibits Akt-induced responses suggesting common theme for enhancing local immunoregulation and long-term graft survival.

Summary—Events within the graft initiated by changes within the microvasculature and mTOR/Akt-induced signaling promotes the development of chronic rejection. Semaphorin-neuropilin biology represents a novel avenue for targeting this biology and warrants further investigation.

Keywords

chronic allograft rejection; endothelial cells; T cells; Vascular Endothelial Growth Factor; Neuropilins

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Introduction

Current state-of-the-art immunosuppressive strategies have been most successful at inhibiting acute allograft rejection and have significantly improved short-term graft survival rates [1]. However, these same strategies have failed to interrupt the development of chronic allograft rejection, the leading cause of graft failure following solid organ transplantation [2]. It has therefore become most apparent that long term success following transplantation will only become a reality if we develop a better understanding of factors and molecules leading to the development of chronic rejection, and consequently design therapeutic strategies to prevent or interrupt this process. By definition, chronic rejection is an insidious process involving delayed type hypersensitivity mechanisms, characterized by mononuclear cell infiltrates, an obliterative vasculopathy and progressive fibrosis [3, 4]. While this process is well-established to be mediated by the recipient's immunological response to donor antigen that is initiated and coordinated by CD4⁺ T cells, it also involves other complex issues including adaptive and immunoregulatory responses within the graft [5-7]. New insights into roles for the innate and the adaptive immune response and T cell and B cell activation pertinent to rejection are reviewed in detail in other sections of this issue. Here, we focus our discussion on how the graft itself, and events within the graft are critical determinants of the initiation and evolution of chronic rejection.

Key events within the intragraft microenvironment that sustain chronic allograft rejection

We recently described a paradigm in which changes in the integrity of the microvascular endothelium (EC) within a graft creates a microenvironment leading to the local expression of cytokines and growth factors, including Vascular Endothelial Growth Factor (VEGF), that are mechanistic to promote and/or sustain chronic allograft rejection [6, 8]. In this model, we propose that direct injury to the graft EC resulting from ischemia-reperfusion injury, cellular and humoral alloimmune targeting elicits a cascade of events initiated by local tissue hypoxia and the local overexpression of VEGF. Direct targeting of the EC, for instance by cytotoxic T cells or alloantibodies, will clearly result in local hypoxia and tissue injury [9**, 10-11]. Nevertheless, it is somewhat underappreciated that mononuclear cell infiltrates stimulate EC proliferation and a leukocyte-induced angiogenesis reaction [12, 13] that also promotes local tissue hypoxia [6, 14-16]. Also, the binding of alloantibody to EC and the local effects of cytokines and growth factors can stimulate proliferative signaling within activated EC [10, 17-20]. Furthermore, during chronic rejection the number of pericytes that colocalize with EC is decreased thereby compromising the structural integrity of the microvascular bed [6, 21, 22]. We [6, 8], and others [22, 23] suggest that disruption of EC homeostasis creates abnormal microvascular blood flow patterns within a graft that result in local hypoxia. All of these events (injury, proliferation, pericyte loss and hypoxia) have potential to precede the process of endothelial-to-mesenchymal transition (EndMT) [24, 25] resulting from the dedifferentiation of EC into fibroblasts that ultimately results in collagen production and fibrosis development. Consistent with this model, we propose that early stabilization of the microvasculature has potential to inhibit the progression of chronic rejection. This possibility will be discussed in more detail below.

Nicolls group recently characterized time-dependent changes in the microvasculature that occur during chronic allograft rejection [22]. Consistent with older studies from our laboratory [14, 16, 18, 26], they demonstrated that remodeled microvasculature is markedly disorganized within grafts and, similar to tumors [27], is associated with interstitial infiltrates and local tissue hypoxia. In chronic rejection, many CD31⁺ vessels were found to lack pericytes indicating that their structural integrity may be disrupted [22]. Several proangiogenic growth factors, including VEGF, are induced by local tissue hypoxia through the transcriptional activity of Hypoxia-inducible factor 1 α (HIF-1 α) [23, 28]. Nicolls *et al.* demonstrated that these changes are associated with tissue hypoxia following orthotopic tracheal transplantation, and examined roles for HIF-1 α in the remodeling repair response. Using a conditional knockout, it was observed that deletion of HIF-1 α results in accelerated graft failure [22], while transient overexpression of HIF-1 α within the graft (using a viral vector) was found to limit intragraft hypoxia, preserve and promote microvascular integrity and prolong graft function. Thus, HIF-1 α -dependent events within a graft may result in physiological protective responses that sustain microvascular integrity. Consistent with this possibility, overexpression of HIF-1 α within grafts was also associated with the induced expression of several proangiogenic factors including CXCL12 (Stromal cell-derived factor 1, SDF-1), placental growth factor and VEGF. Since these angiogenesis growth factors also promote the recruitment of infiltrates and have potent proinflammatory properties [26, 29-31]. It is thus not surprising that hypoxic and/or HIF-1 α -expressing grafts had extensive inflammatory infiltrates [15, 22]. Overall, it appears that early injury and local hypoxia can drive a cycle of events that result in ongoing inflammation through the induction of pro-angiogenic factors.

Central role for VEGF in the chronic rejection intragraft microenvironment

There is significant data to suggest that the overexpression of VEGF may be central to the pathological intragraft microenvironment. As discussed above, overexpression of VEGF within the graft may be a consequence of microvascular injury, local tissue hypoxia and HIF-1 α -dependent responses. Intragraft VEGF may also result from delivery by infiltrating leukocytes within the local microenvironment. Indeed, several recent studies indicate that VEGF levels can be monitored as an index of risk for the development of rejection and/or graft failure [32*, 33-36]. Most notably, in animal models, delivery and/or forced overexpression of VEGF within cardiac allografts mobilizes bone marrow derived monocyte/macrophages and accelerates the development of allograft vasculopathy [37]. There is a general consensus that VEGF is a dominant factor mediating vascular remodeling, and it is possible that its expression is a key physiological response to enable repair following acute and chronic injury [38]. However, its newly described effects on the chemotaxis and activation of effector T cells [39-41] and its potential effects on immunoregulatory T cells [42-45] have broad biological and clinical implications for the understanding of chronic rejection.

The VEGF receptors Flt-1 (VEGF receptor 1) and KDR (VEGF receptor 2) may be expressed by T cell subsets [40-45], and VEGF-VEGFR interactions promote lymphocyte chemotaxis *in vitro* and *in vivo* [26, 40, 41]. Furthermore, VEGFR-expressing T cells have been found to accumulate within rejecting human allografts *in vivo* [41], suggesting that

locally expressed VEGF may interact with VEGFRs expressed on circulating subsets of T cells [39, 41]. VEGF-VEGFR interactions facilitate the transmigration of select populations of previously activated and/or memory CD4⁺ and CD8⁺ T cells across activated EC *in vitro* [41], the intragraft accumulation of T cell infiltrates [26, 39, 41] and the development of allograft vasculopathy in the humanized SCID mouse [39]. In models of acute rejection, antibodies to VEGF or to the classical VEGFRs (Flt-1 and KDR) prolong graft survival [26, 39, 41, 46-48] further suggesting that VEGF has a potent proinflammatory function in association with alloimmunity. Since the induced expression of VEGF, and VEGFR-mediated signals require activation of the Akt/mTOR signaling pathway [49, 50], these observations also suggest that mTOR inhibitors (mTORi) may have potential to attenuate VEGF-dependent biology within the graft. As will be discussed below, it is also possible that cell intrinsic modulation of the Akt/mTOR signaling pathway may interrupt the progression of chronic rejection [51].

Semaphorin-Neuropilin interactions within the graft microenvironment and the development of chronic rejection

The neuropilins (NRP) were originally identified as receptors for the semaphorins, but they are now well established as non-classical VEGF receptors expressed by multiple cell types including subsets of T cells and antigen presenting cells (APC) [40, 42, 45, 52*, 53]. They are single spanning transmembrane glycoproteins that function as chemorepulsive mediators in neurons [54]. NRP-1 was subsequently found to be a receptor for the class 3 semaphorin, Semaphorin 3A (Sema3A), while another semaphorin, Sema3F also known to inhibit axonal guidance, was observed to be selective in its binding for NRP-2 [55, 56]. Klagsbrun *et al.* coincidentally identified NRP-1 and NRP-2 as VEGF receptors on endothelial cells [57, 58], and they are now known to play a major role in vasculogenesis, angiogenesis and tumor growth [59, 60]. Nevertheless, little is reported on the interactions between VEGF and its accessory neuropilin receptor on T cells, and the biological implications of the recruitment of NRP-expressing T cells into allografts is not known.

Recent studies indicate that the NRPs may have significant biological effects in T cells, and that they function to elicit regulatory signaling responses. NRP-1 is now well-established to be most selectively expressed by the immunoregulatory CD4⁺ T cell subset (Treg) [40, 45, 61**]. Some reports suggest that its expression is dominant on natural thymic-derived Treg (nTreg) [45, 62], while other studies suggest that its expression is not necessarily selective to nTreg [40, 61**, 63, 64] and may be prominent on expanded pooled populations of Foxp3⁺ Treg, including *in vitro* induced iTregs. Interestingly, VEGF-NRP-1 interactions may stimulate chemoattraction and have been reported to guide NRP-1⁺ Treg into tumors [63]. Knockdown of NRP-1 on CD4⁺Foxp3⁺ Treg was found to restrict the infiltration of Treg into tumors, which limits local immunoregulation and results in an efficient anti-tumor immune response. Whether this effect is entirely related to NRP-1 expression, rather than other VEGFRs on T cells [44] is not known, but it is likely that this biology is of great relevance in the development of chronic rejection. For instance, if intragraft hypoxia stimulates the overexpression of VEGF, it is entirely possible that this response may enable the recruitment of NRP-expressing Treg which have potential to promote intragraft

immunoregulation. In this manner, intragraft VEGF has potential to promote the local recruitment of both effectors and regulatory T cells and thus, and the relative expansion of NRP-1⁺Foxp3⁺ Treg in individual patients will determine outcome.

Another possibility is that local intragraft expression of semaphorins may interact with NRP receptors on T cells and elicit a regulatory response. The class 3 family of semaphorins, were identified as negative mediators of axon migration and as angiogenesis inhibitors via their interactions with the NRPs [65]. They are produced by multiple cell types, including T cells [66] and epithelial cells [67, 68]. They bind to a dimeric NRP complex and recruit and activate cell surface Plexins (notably Plexin A1-4) to mediate downstream regulatory signaling [69, 70]. It has also been reported that Sema3A, 3F and 3C [66, 71] as well as Sema4A [61**] bind to NRP-1, and the signaling cascade augments the ability of Treg to suppress T effector cell activation/proliferation *in vitro*. Delgoffe *et al.* reported that selective knockout of NRP-1 on T cells abrogated Treg function *in vitro* and *in vivo* [61**]. Solomon *et al.* found that the lack of NRP-1 on T cells was associated with increased severity of disease in a model of experimental allergic encephalitis (EAE) and that Treg deficient for NRP-1 fail to suppress T effectors *in vitro* and *in vivo* [72]. Yuan *et al.* demonstrated that adoptive transfer of CD4⁺CD25⁺NRP-1⁺ T cells into a fully mismatched mouse model of cardiac allotransplantation resulted in long-term graft survival [73]. In addition, they observed that the frequencies of Foxp3⁺ T cells correlated with graft survival and hyporesponsiveness to subsequent *in vitro* restimulation. Collectively, these observations indicate that local overexpression of VEGF within a graft has potential to recruit NRP-1⁺ Treg into the local site. Its ability to, and relative binding to locally produced semaphorins may inhibit migration and promote immunoregulation. However, the unanswered question is whether the production of semaphorins within a graft is required for optimal intragraft immunoregulation, and whether expression of soluble semaphorin 3 family molecules (for instance by infiltrating activated effector T cells) or membrane bound semaphorin 4 family [61**] by APC's and other cell types inhibits NRP-1⁺ Treg recruitment and activation.

New insights into mTOR signaling as a modulator of the chronic rejection intragraft microenvironment

An implication of an endothelial-based injury/repair model of chronic rejection is that microvascular stability must be sustained in order to maintain a physiological intragraft microenvironment. We thus suggest that the design of therapeutics that target EC activation and/or augment pro-resolution intracellular signaling pathways in EC will inhibit the development of chronic rejection. To this end, activity of the mTOR/Akt signaling pathway is critical for endothelial activation, survival and proliferation [6, 50, 74]. Both intracellular mTOR complexes (mTORC1 and mTORC2) are also signaling intermediaries between inflammation [75*, 76**, 77*] and the production of VEGF [78] which, as discussed above, may be a central mediator of chronic rejection. Targeting mTOR has been shown to reduce VEGF expression [74], VEGF-induced angiogenesis [50, 74] and leukocyte-induced angiogenesis in association with inflammation [8, 78]. Several observations also suggest that mTOR-dependent activation of Akt is implicated in the production of VEGF [78], in

angiogenesis [50], and in other EC-responses pertinent to pro-inflammation [75*, 76**]. These include TNF- α -induced expression of the chemokines MCP-1 [79] and CXCL10 [80], and the adhesion molecule VCAM-1 [75*]. Furthermore, Pober's group recently demonstrated that the inhibition of Akt in TNF- α -activated EC by mTORi reduces their interactions with T cells *in vitro* and *in vivo*, which translates into a lower recruitment of leukocytes *in vivo* [75*]. In another report, Pober's group demonstrated that mTORi-treated EC fail to costimulate the reactivation of memory CD4⁺ T cells, suggesting that once T cells interact with EC in the course of recruitment, the effect of the interaction is anti-inflammatory [76**]. This anti-inflammatory effect was observed to be partially dependent on the induced expression of the coinhibitory molecules programmed death-ligand 1 (PD-L1; CD274) and PD-L2 (CD273) on mTORi-treated EC, both of which are well established to support immunoregulation [81].

The importance of alloantibody-targeting of EC in the pathogenesis of chronic rejection has recently been revisited as molecular studies indicate that persistent donor specific antibody (DSA)-mediated graft injury is dominated by an endothelial transcript [10, 11]. Indeed, it appears that the pathogenicity individual donor specific alloantibodies is associated with their effect(s) on the targeting of EC and their ability to elicit an EC-dependent intracellular signaling response [11, 20]. Consistent with this observation, Reed's group has demonstrated that alloantibody-mediated activation of EC involves mTOR/Akt-dependent signaling [17, 82], and that this response facilitates monocyte recruitment into the graft in part via endothelial expression of P-selectin [83*]. They further observed that treatment with mTORi's inhibit alloantibody-induced EC activation and proliferation [84**]. Collectively, these observations suggest that mTORi's represent EC-targeted therapeutic agents with high potential to inhibit activation and proliferation as well as their pro-inflammatory phenotype, and thus to stabilize the microvascular bed and inhibit the progression of chronic rejection.

Another possibility is that harnessing cell intrinsic mechanisms of EC stability have potential to limit activation responses associated with inflammation. We recently speculated that signaling pathways that elicit the process of inflammation resolution following activation may lead to targeted anti-inflammatory therapeutics. Our initial approach involved the identification of endogenous inhibitors of the mTOR signaling pathway in EC. We recently reported that an adaptor called DEP domain-containing mTOR-interacting protein (DEPTOR) that is known to modulate mTOR signaling [85] has marked effects on EC activation responses [77*]. DEPTOR is expressed at high levels in different microvascular EC beds *in vitro* and *in vivo*, and its expression is markedly reduced in EC following activation [77*]. Knockdown of DEPTOR in EC using siRNA resulted in a dramatic activation response that included the overexpression of chemoattractant chemokines and adhesion molecules and a phenotype that supported the adhesion of leukocytes *in vitro*. In addition, we found that DEPTOR was functional to regulate an angiogenesis response, which is consistent with its effects to modulate mTORC1 activity. In pilot experiments, we also find that forced overexpression of DEPTOR in EC inhibits the expression of TNF- α -induced adhesion molecules, which translates into a prolonged survival of cardiac allografts [unpublished data]. These findings exemplify the importance

of endogenous inhibitors of the mTOR pathway and the potential that they can be harnessed to stabilize the intragraft microvascular bed and thus improve graft survival.

Conclusion

This review and opinion defines our current understanding of, and how intragraft events dictate the phenotype of rejection. We have focused our opinion on mTOR signaling in EC and have not taken into consideration other pathways such as the cytokine-induced NF- κ B activation response that can also be targeted by novel compounds such as N-octanoyl dopamine [86, 87]. Also, we have not emphasized the critical impact of the relative balance between host alloimmunity versus tolerance on the evolution chronic rejection, or how host immunocompetence may impact events within the intragraft microenvironment. For instance, mTOR signaling, while key to microvascular stability, is well established to function in T effector, and *not* in T regulatory cell activation [88]; thus, mTORi's may regulate both the graft EC as well as host immunity. Powells group has published extensively on this biology using knockout [89] and knockdown [90] approaches, and more recently demonstrated a key function for mTOR in Th1 and Th17 differentiation, but not in Treg expansion and activity [90]. In contrast, Chi's group demonstrated that total deletion of mTORC1 in Tregs, using a FoxP3^{cre}Raptor^{fl/fl} mouse model resulted in a striking deficiency in Treg function, indicating that mTORC1 may also function in Treg [91**]. However, since pharmacological mTORi's including rapamycin only partially inhibit mTORC1, it is likely that low mTORC1 activity [64, 92] or oscillatory activity [93] is required to promote Treg expansion, stability and function. This is important as low mTORC1 activity may also be required to maintain EC survival, and the phenotype of mTORi-treated EC has potential to sustain Treg activity within the graft [76**]. This possibility also suggests that mTORi's allow for immunoregulation as a consequence of interactions between the graft microenvironment and the host.

Another area of great importance involves the function of neuropilin family molecules on T cells, notably NRP-1⁺Foxp3⁺ Treg. A significant body of data suggests that ligation of NRP-1 by semaphorin family molecules results in the inhibition of intracellular Akt/mTOR signaling [61**, 94]. While it is likely that semaphorins are expressed by multiple cells within allografts, it is theoretically possible that expression levels vary in different organs and/or that their function within the graft microenvironment will augment Treg stability and function. Understanding how the expression of semaphorins within a graft may interact with Treg has significant therapeutic implications for the future.

Finally, as discussed above, the intragraft expression of cytokines and chemokines are well established to mediate chemoattraction of different subsets of T cells, including T effector and Treg cells into the graft. Contrary to expectations, using a single-cell visualization microfluidic model, we recently observed a process whereby these same chemokines may elicit a process of chemoinhibition [95*], and that select receptor-mediated signals and/or molecules dictate directionality of an individual leukocyte migration response [95*]. It is entirely possible that there are population-based leukocyte responses, but this study indicates that individual leukocytes may migrate in opposite directions in response to an identical chemokinetic gradient. These observations further suggest that receptor-mediated signals

within subsets of leukocytes dictate the directionality of migration, and that monitoring intragraft factors that elicit a chemoinhibitory response will have great impact for the development of future therapeutics.

In conclusion, the failure of potent immunosuppressive agents to impact the initiation or the progression of chronic rejection has promoted a ‘fresh look’ into this disease process. In the absence of true tolerance, we suggest that the phenotype of the intragraft microenvironment can dictate the phenotype of rejection and the chronic rejection process. Our focus on events within the graft indicate that new molecules, regulated by HIF-1 α -dependent events, and semaphorins and neuropilins have high potential to impact this process. We also strongly believe that mTOR/Akt signaling can dictate the proinflammatory versus a tolerogenic phenotype of the graft EC a scritical determinants of the initiation and evolution of chronic rejection.

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with CAV vs. age matched controls. However, levels of VEGF-A, VEGF-C and PF-4 had the best performance as biomarkers of all grades including grade 1 (mild) CAV. This study endorses the concept that endothelial injury and HIF-1 α -dependent expression of pro-angiogenic factors (as outlined in [22]) is a major component of chronic rejection. [PubMed: 23260712]

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migratory responses in leukocyte subsets in response to cytokines and/or to cells treated with immunosuppressive therapeutic agents. [PubMed: 25183261]

Abbreviations

APC	antigen presenting cells
DEPTOR	DEP domain-containing mTOR-interacting protein
DSA	donor specific antibody
EC	endothelial cell
HIF-1α	Hypoxia-inducible factor 1 α
iTreg	induced peripheral Treg
mTORC	mTOR complex
mTORi	mTOR inhibitors
nTreg	natural thymic-derived Treg
NRP	neuropilins
PD-L	programmed death-ligand
Sema	Semaphorin
Treg	regulatory T cell
VEGF	Vascular Endothelial Growth Factor
VEGFR	VEGF receptor

Key points

- Key events within the intragraft microenvironment sustain chronic allograft rejection.
- VEGF plays a central role within the intragraft microenvironment.
- Semaphorin-Neuropilin interactions within the graft microenvironment may influence the development of chronic rejection.