THE INTRAGRAFT MICROENVIRONMENT AS A CENTRAL DETERMINANT OF CHRONIC REJECTION OR LOCAL IMMUNOREGULATION/ TOLERANCE

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Abstract

Purpose of review—Chronic rejection is associated with persistent mononuclear cell recruitment, endothelial activation and proliferation, local tissue hypoxia and related biology that enhance effector immune responses. Despite similarities, the tumor microenvironment elicits signals/factors that inhibit effector T cell responses and promote local immunoregulation. The identification of immunoregulatory check points and/or secreted factors that are deficient within allografts is of great importance in the understanding and prevention of chronic rejection.

Recent findings—The relative expression of immunomodulatory molecules (cell surface and secreted) on microvascular endothelial cells are deficient within rejecting allografts, and this deficiency is of functional importance in shaping the phenotype of rejection. Furthermore, the expression of co-inhibitory molecules/factors that enhance local immunoregulation are modulated, and the identification of intracellular regulatory signals or secreted factors are the subject of ongoing research. For example, semaphorins may interact with endothelial cells and regulatory T cells to promote local tolerance. Additionally, metabolites and electrolytes within the allograft microenvironment may regulate local effector and regulatory cell responses.

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Summary—Multiple factors within allografts shape the microenvironment either towards local immunoregulation or proinflammation. Promoting the expression of intragraft cell surface or secreted molecules that support immunoregulation will be critical for long-term graft survival and/or alloimmune tolerance.

Introduction

In this review, we discuss new findings indicating that events within the allograft itself shape a microenvironment that promotes either proinflammation or local immunoregulation/ tolerance. It is increasingly appreciated that select families of immunoregulatory molecules (*i.e.*, immune checkpoints) within tissues may function to elicit a local pro-tolerogenic state within the tumor microenvironment. Thus, the presence or absence of 'checkpoint' molecules within the allograft itself, and/or local intragraft events have potential to support systemic alloimmune tolerance. Although a detailed description of potential local immune checkpoints is beyond the scope of this review, we will focus our opinion on how the intragraft microenvironment may be shaped by the integrity and phenotype of the microvascular endothelium to support either pro-inflammatory or pro-tolerogenic events.

Role of the microvasculature in shaping the intragraft microenvironment: A paradigm for understanding local immunoregulation and tolerance

The graft vascular endothelial cell (EC) is uniquely located to serve as an interface between the allograft and the recipient's immune system. To this end, it is not surprising that microvascular injury occurs as a consequence of all forms of rejection [1–4]. Indeed, in an endothelial-based model, initial injury results from ischemia-reperfusion, leukocytic infiltration and subclinical rejection, and the EC response is characteristically associated with activation as well as proliferation [5–9]. Furthermore, an increasing body of evidence indicates that the response of the microvasculature to injury (*e.g.* activation, protection and/or repair *vs.* dysfunctional angiogenesis) is critical in both the initiation as well as the progression of chronic rejection [4,5,8,10]. EC injury also results in cell death and microvascular loss, which in turn results in impaired delivery of oxygen and nutrients within the graft parenchyma/tissue. The resultant (and inevitable) local tissue hypoxia is likely a driving force that regulates local immunity in part through direct effects on infiltrates [4,11,12]. For example, hypoxia may elicit its effect on infiltrating T cells via hypoxia-inducible factor (HIF)-1α regulated expression of proinflammatory cytokines and intracellular signals associated with the dedifferentiation of T regulatory cells (Treg) and the enhanced differentiation of effector T cells [11]. These collective events within the microenvironment are most predictive of the initiation of chronic rejection [4,6,7,9]. Also, cell-mediated and humoral alloimmune responses and hypoxia/HIF-1α lead to a characteristic EC activation response that includes the expression of MHC class I and II molecules, pro-inflammatory cytokines, chemokines and growth factors that collectively

serve to modify the phenotype of both acute and chronic rejection [3,13]. Thus, the degree of initial microvascular injury, local tissue hypoxia and the phenotypic response of the EC to inflammation (including pathological leukocyte-induced angiogenesis) shape the intragraft microenvironment for subsequent events (illustrated in the cartoon in Figure 1).

Leukocyte-induced angiogenesis is a pathological reaction that was initially identified following the local injection of allogeneic splenocytes into the skin of nude mice [14,15]. Following injection, the angiogenesis reaction was dose and cell number-dependent and occurred within a period of 3 to 6 days. Interestingly, it failed to occur following the injection of syngeneic cells and was found to be dependent on the alloactivation of effector CD4+ T lymphocytes [16]. Monocyte/macrophages were subsequently identified to produce and deliver angiogenesis factors into tissues, and pathological angiogenesis is generally considered a somewhat characteristic response in association with cell-mediated immunity and chronic inflammatory disease [17,18]. The local expression of Vascular Endothelial Growth Factor (VEGF) is central to all these effects, and may be produced locally and/or by mononuclear cell infiltrates including CD4+ T cells in the course of chronic inflammation [18–22]. Consistent with these observations, we have found that the early expression of VEGF is a feature of rejection [21,23], and it has high potential as a biomarker of evolving disease [21,24,25].

Unexpectedly, it was reported that the leukocyte-induced angiogenesis reaction that occurs at early times in the rejection process is associated with local hypoxia [5,7,10], likely due to sluggish and chaotic blood flow patterns, as observed in tumors [4,6,12,26,27]. This is most suggestive that dynamic changes within the EC of an allograft (destruction and/or proliferation) have potential to create abnormal microvascular networks and local areas of tissue hypoxia. These events serve as a stimulus for HIF-1α-mediated signaling in multiple cell types (including leukocytic infiltrates), the inducible expression of growth factors (such as VEGF), leukocyte chemoattraction and modified local CD4+ T cell activation responses [9,11,28,29]. Remarkably, all these events (angiogenesis, local hypoxia, mononuclear cell infiltrates) are described in tumors and are integral to the formation of the tumor microenvironment [12,26,27], yet this microenvironment supports immunoregulation and *not* effector T cell activation. We thus hypothesize that select immunoregulatory molecules that are produced within the microenvironment of tumors are deficient within allografts, and further, that their induced expression may target effector alloimmunity and promote local immunoregulation. For example, as will be discussed below, co-inhibitory programmed death ligand (PDL)1/2 expression on microvascular EC and/or the expression of the class 3 semaphorin family of neuronal guidance molecules have great potential to enhance tolerance. Thus, we propose a new paradigm in which chronic rejection evolves from a deficiency and/or lack of production of local immunoregulatory checkpoints within the graft microenvironment. Additionally, we speculate that forced expression of families of tumor associated 'immune checkpoints' within an allograft will promote local immunoregulation and augment systemic tolerance.

Immunogenic function of microvascular endothelial cells as a determinant of the phenotype of rejection

In a seminal study, Chalasani *et al.* challenged existing dogma that the nature and magnitude of an effector immune response alone determines the phenotype of rejection [30]. They used an adoptive model in which fully MHC mismatched cardiac allografts were exposed to a constant number of effector T cells. Adoptive transfer into newly transplanted recipients resulted in acute rejection, whereas transfer of the identical effector T cell population into recipients of healed-in allografts (day 50–70 post-transplantation) resulted in a chronic rejection response. Importantly, they also observed that ischemia-reperfusion injury/ retransplantation of healed-in allografts recapitulated the acute rejection response. These observations are most suggestive that the state of the local tissue microenvironment within the allograft, and not only the effector T cell population are determinants of the phenotype of rejection (acute *vs.* chronic). While these authors did not address the mechanistic basis for this response, it is most intriguing to consider the identity of select factors/cell surface molecules present or deficient within each type of microenvironment that shape these different outcomes.

An explanation supported by a long-standing literature [31–33] is that the state of activation of intragraft microvascular EC regulates the immunogenicity of the local microenvironment and is a central determinant of the phenotype of rejection (acute *vs.* chronic). Indeed, several studies have demonstrated that the expression of multiple cell surface and secreted molecules on EC regulate local interactions with infiltrating T effector cell and/or T regulatory cells [34–38]. For example, there is a substantial body of evidence demonstrating that microvascular EC express MHC class I and II and costimulatory molecules that function in the reactivation of both CD4^{+} and CD8^{+} T cells through both direct and indirect pathways [31,38–42]. Abrahimi *et al.* found that blocking MHC class II on the graft EC was of critical importance in both CD4^{+} and CD8^{+} T cell-mediated rejection *in vivo* [43]. Costimulatory molecules on EC, including LFA-3 and ICOSL [39,44,45], also support T cell reactivation responses, and the expression of adhesion molecules, *e.g.* E-selectin, ICAM-1 and VCAM-1 [33,46] facilitate the recruitment and infiltration of T cells into the allograft. Pober's groups proposed a model (that has stood the test of time) whereby EC recruit and reactivate antigen-specific effector memory T cells during the early stages of inflammation [32,47]. These alloactivated T cells release effector cytokines, *e.g.* IFN-γ, within the graft that is central to all subsequent EC-dependent mechanisms of rejection. In multiple studies they used a human artery interposition model in humanized mice to further study the role of allograft EC in chronic rejection [48–50]. Systemic administration of human IFN-γ, which only interacted with the donor human vessel and not with mouse cells, sustained endothelial MHC-antigen expression in healed-in allografts up to 30 days post-transplant and resulted in the development of allograft vasculopathy. Pro-inflammatory leukocytes, including innate immune cells follow IFN-γ-inducible gradients into the vessel wall and stimulate the recruitment and proliferation of vascular smooth muscle cells within the intima [8,48,50]. Moreover, using this model, they showed that human allograft EC produce the proinflammatory cytokines IL-1α and IL-6 respectively [51,52], which promote the recruitment and differentiation of Th17 cells within the graft. Neutralizing of IL-1α or IL-6

respectively resulted in an expansion of Tregs and reduced effector T cell-mediated injury. Lion *et al.* also demonstrated that the local production of IL-6 by EC serves as a key factor to influence CD4$^+$ differentiation [53] suggesting that allograft EC-derived cytokines can shift the microenvironment towards a proinflammatory phenotype.

An increasing body of literature has emphasized the importance of the expression and function of co-inhibitory molecules by EC as a component of local tissue immunoregulation, most notably within tumors [35,54–61] Some additional studies are also most suggestive that the expression of co-inhibitory molecules on intragraft EC may be of great importance in the expansion of Tregs and the prevention of chronic allograft rejection [58,62]. For example, blockade or knockout of PDL1 within donor cardiac allografts results in accelerated chronic rejection and graft loss [58,62]. Using bone marrow chimeras, it was concluded that this accelerated rejection effect is likely related to its expression and function on graft EC [58,62]. CD95/FasL is another EC cell surface molecule that regulates CD4+ and CD8+ effector immunity, but has limited effects on the killing of Treg cells [63,64]. In this manner, its expression on EC has been found to be of functional importance in local immunoregulatory responses within tumors [63] as well as within allografts [64]. Tumor EC expression of CD95/FasL is induced by several secreted factors known to be expressed within allografts [63], and importantly, the inability of FasL to elicit killing of Tregs is due to their high endogenous level of expression of anti-apoptotic c-FLIP [63]. Collectively, these novel observations are suggestive that the state of activation and phenotype of the intragraft EC may play a dominant role to shape effector/regulatory interactions within a graft.

The identity of intragraft cell surface molecules and/or soluble factors and signals that function as 'alloimmune check points' (similar to those within tumors [61]) will have high relevance for the development of therapeutics to manipulate the immunogenicity of the graft. To this end, Pober's group demonstrated that intracellular mTOR-induced signaling functions to inhibit the immunoregulatory phenotype of EC. Specifically, the treatment of EC with rapamycin (an mTOR inhibitor that is used clinically) augmented the inducible level of expression of co-inhibitory PDL1/2 [37], which functions to augment the expansion of FoxP3$^+$ Tregs following co-culture *in vitro* and following transplantation *in vivo*. In another report, Wang *et al.* determined that the inhibition of mTOR reduces the ability of EC to interact with effector T cells and is associated with reduced T cell recruitment [56]. Thus, intracellular signals, molecular events and/or soluble factors regulate the immunogenicity of EC and have potential to shape the intragraft microenvironment to promote local immunoregulation and sustain alloimmune tolerance [65].

Class 3 semaphorins as novel intragraft factors with potential to shape the immunogenicity of endothelial cells and the intragraft microenvironment towards immunoregulation

Axonal/neuronal guidance molecules belong to at least four families, namely the Netrins, Semaphorins, Ephrins and Slits, and all families are implicated in lymphocyte activation and in immunomodulation [66–69]. Furthermore, members of these families are increasingly

being appreciated as modulators of cell mediated immune responses, vascular disease and alloimmunity due to high expression levels of their receptors on leukocyte subsets [70–73] and EC [74–78]. Below, we wish to briefly discuss the class 3 semaphorin family (SEMA3) that have potential to regulate the alloimmune response [72,73,79].

Semaphorins were originally described as chemorepellent factors due to their primary function to elicit cytoskeletal collapse in neurons [69,76,77,80]. More recent studies have demonstrated that this family, including SEMA3 family members, regulate a wider array of cellular activation responses, most notably PI-3K/Akt/mTOR intracellular signaling, migration and proliferation [81–83]. Over the past 10 years, several studies have identified SEMA3 receptors, including neuropilin (NRP)-1, NRP-2 and Plexin A family molecules on tumor cells suggesting that they function within the tumor microenvironment to limit cellular migration and growth [80,81,84,85]. Three members of the Class 3 family (SEMA3A, -3C and -3F) also bind NRP's that are expressed on immune cell types including EC [75–78,81], antigen presenting cells [86,87] and CD4^{+} T cells [70]. In general, ligation of the NRP receptor alone is not sufficient to elicit a signal, but serves as co-receptor to activate Plexin A family receptors that in turn elicit potent inhibitory signaling responses [88,89]. Indeed, deletion of Plexin A on CD4+ cells is associated with profound effector T cell activation and autoimmunity [88] Both NRPs also bind additional molecules including VEGF, Placental Growth Factor and Sema4A and thus may serve to augment binding/crosslinking of their classical receptors. In this manner, these molecules may compete with SEMA3's for their NRP binding domain and regulate SEMA-NRP-elicited inhibitory signaling responses [81,90]. As will be discussed below, two members, namely SEMA3A and SEMA3F have high potential to interact with NRP-1 and NRP-2, respectively, on T cells and EC; thus, it is not surprising that they have potential to inhibit T cell activation and migration and to serve as regulators of the immune responses.

NRP-1 is expressed on CD4^{+} Tregs and APCs [70,91,92] and is reported to function in T cell/APC interactions [93] and in the regulation of cellular migration [91] and suppression [82]. Battaglia *et al.* compared NRP-1^{+} and NRP-1^{−} Tregs and found that NRP-1^{+} Tregs had higher levels of expression of FoxP3 and Treg activation markers [70], and were more suppressive *in vitro*. In cervical cancer patients, a lower frequency of NRP-1^{+} within the Treg population was associated with reduced tumor mass following chemoradiotherapy. Yadav *et al.* identified NRP-1 as a marker of thymic-derived Tregs [92] and demonstrated that NRP-1^{low} Tregs were insufficient to control autoimmunity. Furthermore they identified an association between NRP-1 expression and levels of Foxp3 and Treg function in an experimental autoimmune encephalomyelitis model. Consistent with these reports, Delgoffe *et al.* observed that signaling via NRP-1 in Tregs inhibits Akt activity, promotes Treg survival and enhances their immunoregulatory function *in vitro* and *in vivo* [82]. Hansen *et al.* demonstrated that NRP-1 is critical for the migration of Tregs into tumors via its binding to tumor-derived VEGF, and they observed that their local accumulation results in suppression of anti-tumor immune responses [91]. Thus, there is substantial data to suggest that NRP-1 and perhaps its SEMA ligands are of great importance in the local pro-tolerogenic microenvironment but additional studies will be required to identify their respectively roles within allografts in the augmentation of alloimmune tolerance.

We recently evaluated the biological effect of SEMA3F on T cells, EC and several tumor cell lines [81]. Similar to Delgoffe [82], we found that it is potent to inhibit PI-3K and Akt activity in part through disruption in the formation of the mTORC2 complex [81]. We also found that this regulatory effect required both NRP-2 and Plexin A1 expression on each cell type. Consistent with our findings, another group reported that semaphorin-plexin interactions regulate TOR signaling (in *C. elegans*) [94] and are potent to disrupt actin cytoskeleton and morphological changes in various cell types. These findings are most suggestive that SEMA3F-NRP interactions also serve to enhance immunoregulation locally within tissues.

Furthermore, in our studies [81], we also noted that SEMA3F-NRP-2 signals suppressed hypoxia-induced VEGF expression, which, as discussed above is characteristic of the chronic rejection process [4]. Within EC, SEMA3-NRP-2 interactions are anti-angiogenic, and stabilize the microvasculature and inhibit vascular leak in association with cell mediated immunity [77,78,84]. Mucka *et al.* observed massive tissue swelling and edema in NRP-2 knockout mice compared with wild-type littermates in association with delayed-type hypersensitivity [78]. They also demonstrated that the injection of SEMA3F into skin was potent to inhibit vascular leak induced by VEGF. Collectively, these studies are most suggestive that SEMA3F functions to stabilize the phenotype and integrity of microvascular EC during inflammation, which, as discussed above, is of relevance to chronic rejection (also illustrated in the cartoon in Figure 1). Since the SEMA3 family molecules are well established to be expressed within tumors, we postulate that they are deficient within the allograft microenvironment, and thus their local regulatory effects are lacking. It will be most intriguing to assess if forced expression of regulatory semaphorins within a graft results in enhanced immunomodulation and vascular stability and thus support the hypothesis that they are functional in the pro-tolerogenic allograft microenvironment.

The microenvironment can regulate the immune response by targeting immune metabolism

A final consideration is that the metabolic state of the local microenvironment also regulates T effector and Treg cells. Eil *et al.* reported that elevated potassium levels inhibit T cell effector function, and that augmenting potassium efflux enhances activation responses *in vitro* and *in vivo* [95]. Kleinewietfeld *et al.* demonstrated that high sodium levels facilitate the differentiation of Th17 effector cells and accelerates the onset and severity of experimental autoimmune encephalomyelitis [96]. Furthermore, Hernandez *et al.* demonstrated that high sodium diet/levels impair immunoregulation via the induction of IFN-γ in Tregs *in vitro* and *in vivo* [97]. Knock-down/neutralization of IFN-γ restored their immunosuppressive function even in the presence of high sodium levels. Safa *et al.* translated these observations within transplantation models and demonstrated that a high sodium diet accelerates allograft rejection in part via impaired alloimmune Treg function [98].

It is also becoming increasingly appreciated that Tregs favor select substrates to meet their metabolic needs and that their metabolic requirements subsequently influence the immune

response [99]. Two major integrated pathways are utilized by T effector and Tregs: one that involves glucose metabolism/glycolysis, and another that involves oxidative phosphorylation. This latter metabolic pathway requires either pyruvate, fatty acids or glutamine to generate ATP within mitochondria. Chang *et al.* demonstrated that oxidative phosphorylation is required for the initial activation of naive $CD4^+$ T cells [100], whereas T effector cells generally use glycolysis to meet their energy requirements. Interestingly, the consumption of glucose by tumor cells within a tumor microenvironment reduces available substrate and thus restricts glycolysis, which in turn translates into impaired local T effector function [101]. Also, as discussed above, local hypoxia may additionally modulate T cell activation through effects on metabolism, and notably glycolysis [12]. Furthermore, Beier *et al.* demonstrated that the inhibition of oxidative phosphorylation impairs both T effector and Treg function [102]. However, Treg function is disproportionally altered and restricts long-term allograft survival in a transplant model. Lee *et al.* systemically inhibited glycolysis and glutamate metabolism (alone or in combination) and observed prolonged survival in fully MHC mismatched cardiac and skin allograft transplantation models [103]. Together, these findings indicate that the balance of electrolytes and availability of metabolic substrates within the intragraft microenvironment may also shape the local pro-tolerogenic immune response.

Conclusion

In this review, we discuss a working model whereby the relative lack of expression of cell surface and/or secreted immunoregulatory molecules within allografts are a determinant of the phenotype of rejection. We speculate that the induced expression of EC cell surface co-inhibitory molecules and secreted immunoregulatory cytokines/factors such as the semaphorins will support local immunoregulation as evidenced within the microenvironment of tumors. The class 3 semaphorin family members and their interactions with neuropilin receptors are intriguing as potential local factors that modulate both the local microenvironment (via interactions with EC) and also local immune responses (via interactions with T regulatory and other immune cells). There is also high potential for local metabolic changes in the graft to shape the local immune response towards tolerance. Collectively, these findings are most suggestive that there is a great need to focus future research on the intragraft microenvironment as a determinant of chronic rejection or local immunoregulation/ tolerance.

Abbreviations

> **EC**   endothelial cell

HIF	hypoxia-inducible factor
NRP	neuropilin
PDL	programmed death ligand
Sema	semaphorin
Treg	regulatory T cell
VEGF	vascular endothelial growth factor

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Key points

- Distinct cell surface and secreted molecules within the intragraft microenvironment shape either a pro-inflammatory or a pro-tolerogenic immune response.

- Dynamic changes in the endothelium influence the phenotype of the microenvironment.

- Underappreciated factors within the microenvironment such as semaphorins, electrolytes and metabolites, can influence the behavior of effector and immunoregulatory T cells and thus the phenotype of rejection and/or graft survival.
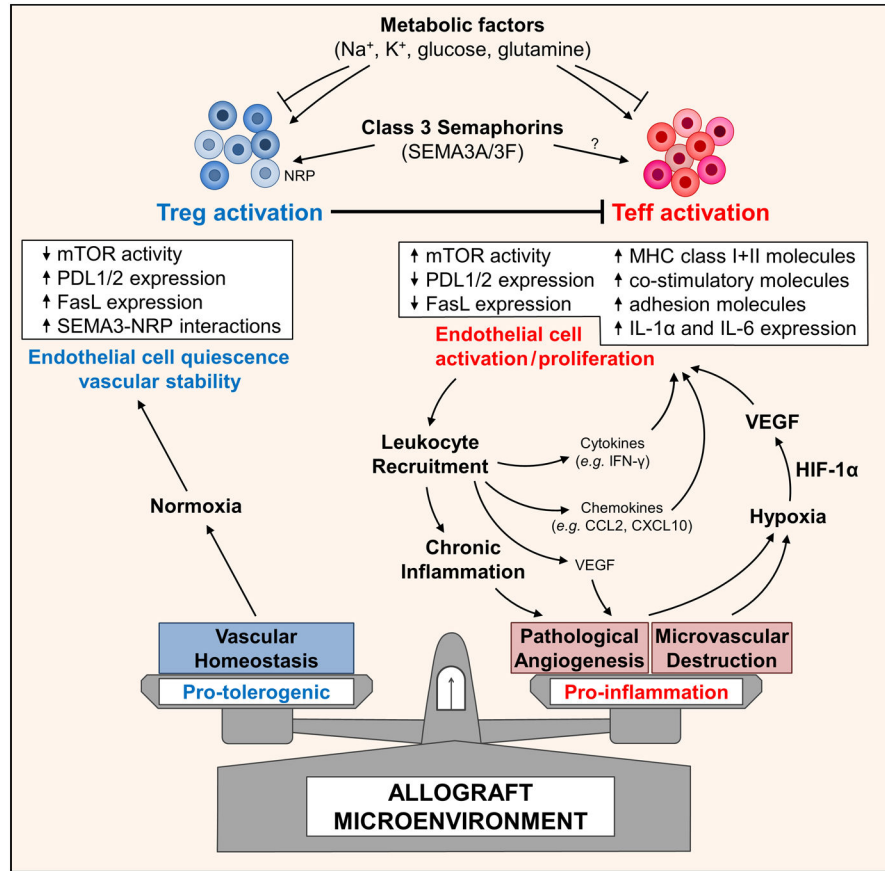
Figure 1. Cartoon illustration of a working model of how the allograft microenvironment may be shaped to support alloimmune tolerance

Initial inflammatory infiltration into a graft target the microvasculature and may either result in injury or pathological leukocyte-induced angiogenesis. Early injury and microvascular loss results in local tissue ischemia. Uncontrolled pathological angiogenesis also leads to an abnormal microvascular bed, sluggish/chaotic blood flow patterns and may result in tissue hypoxia. Local tissue hypoxia drives HIF-1α-dependent activation of growth factors, cytokines and chemokines (including VEGF-A) but also facilitates the dedifferentiation of T regulatory cells (Teffs) into effectors (Teffs). Within the tumor microenvironment, immune checkpoints expressed on EC, and secreted factors within the microenvironment promote Treg activation, expansion and function, and inhibit T effector cell activity. Also electrolytes and metabolic substrates within the microenvironment regulate local immunity including T regulatory cell responses. Thus, similar to tumors, novel cell surface molecules (*e.g.* neuropilins (NRP) and secreted molecules, *e.g.* class 3 semaphorins that are likely to be deficient within allografts have potential to shape the environment and local immunoregulation.