

# The Microbiome, Systemic Immune Function, and Allograft Transplantation

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## SUMMARY

Diverse effects of the microbiome on solid organ transplantation are beginning to be recognized. In allograft recipients, microbial networks are disrupted by immunosuppression, nosocomial and community-based infectious exposures, antimicrobial therapies, surgery, and immune processes. Shifting microbial patterns, including acute infectious exposures, have dynamic and reciprocal interactions with local and systemic immune systems. Both individual microbial species and microbial networks have central roles in the induction and control of innate and adaptive immune responses, in graft rejection, and in ischemia-reperfusion injury. Understanding the diverse interactions between the microbiome and the immune system of allograft recipients may facilitate clinical management in the future.

## INTRODUCTION

Human microbial communities have diverse impacts on human physiology, including the development and maintenance of systemic immune function (1–3). Broad changes occur in the microbial flora of individuals undergoing solid organ transplantation (SOT) and hematopoietic stem cell transplantation (HSCT) via exposures to microbes, antimicrobial agents, vaccinations, infections, immunosuppression, surgery, and immune and inflammatory processes. These changes impact the outcomes of transplantation depending on the context in which they occur, including the nature, site, and intensity of infection and of immunosuppression. Several studies have examined alterations in allograft function after infection with individual viral and bacterial pathogens. This review examines the contributions of specific microbes in allograft function and the experimental and clinical data demonstrating the dynamic relationship that exists between the microbiome and systemic immune function relevant to transplantation.

Microorganisms in tissues and on barrier surfaces, including skin, airways, and gastrointestinal (GI) tract, are collectively termed the “microbiome” (4, 5). Microbial communities are distinct in different anatomic sites and under different clinical conditions (6–8). Exposures may be chronic, as for colo-

nizing organisms, or acute, in association with infection of normally sterile sites or disruption of otherwise stable microbial flora. The gut microbiome comprises an estimated  $10^{14}$  bacteria, fungi (including *Candida* species), and viruses (1, 9, 10). Viruses may alter host gene expression (e.g., endogenous retroviruses) or infect prokaryotic symbionts (e.g., bacteriophages) or eukaryotic cells as latent or productive infectious agents (e.g., herpesviruses) (11). In contrast to the case for bacteria, genetic sequencing and bioinformatics analyses of viral sequence diversity are relatively incomplete.

Many organisms comprising the human microbiome are unknown and often difficult to cultivate *in vitro*. Culture-independent nucleic acid sequencing tools and bioinformatics systems have revolutionized studies of microbial communities. Two major sequencing methods have been employed. Bacterial 16S ribosomal gene sequence data and fingerprinting methods (18S for fungi) resolve microbes to the taxon level and quantify the relative abundance of each species in a sample. Alternatively, next-generation sequencing or “shotgun” metagenomic sequencing uses direct sequencing of total DNA and has been used to examine viral sequences as well as genes in structural and metabolic pathways (12). Gene expression is further characterized with metatranscriptomic and metaproteomic analyses. With these tools and computational data analyses, the microbiome can be characterized in terms of specific organisms and relative abundance in anatomic sites and for changing populations over time with clinical syndromes and therapies.

Published 9 December 2015

Citation Nellore A, Fishman JA. 2016. The microbiome, systemic immune function, and allograft transplantation. *Clin Microbiol Rev* 29:191–199.  
doi:10.1128/CMR.00063-15.

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## SPECIFIC ORGANISMS CONTRIBUTE TO ALLOGRAFT REJECTION VIA INNATE AND ADAPTIVE IMMUNE MECHANISMS

In the face of the introduction of new organisms (“infections”), innate immune cells and proinflammatory mediators contribute to tissue injury and prime adaptive immune responses and/or may stimulate cross-reactive cellular alloimmunity. The relative contributions of each type of response to allograft injury are difficult to assess. “Heterologous immunity” describes memory T cell responses to previously encountered pathogens that cross-react with alloantigens (13). Prior infectious exposures may, in sum, enhance the risk for cross-reactive alloimmune responses. Thus, in a murine skin graft tolerance model, tolerance was more difficult to achieve in mice with immunity to a number of viruses (vaccinia virus [VV], herpes simplex virus [HSV], and vesicular stomatitis virus [VSV]) than in uninfected mice or mice infected with only one virus (14). Similarly, mice remotely infected with *Leishmania major* and undergoing tolerance induction for skin transplantation rejected grafts more rapidly than did uninfected controls. This effect was attributed to cross-reactive CD4 memory responses to *Leishmania* (15). Latent infections of B6 mice with murine gamma herpesvirus 68, a murine Epstein-Barr virus (EBV) homolog, accelerate BALB/c skin allograft rejection mediated by long-lasting viral antigen-specific CD8 memory responses (16, 17). In transplant recipients receiving calcineurin inhibitor-based immunosuppression, persistent alloimmune responses generated by chronic viral infections do not become “exhausted” and may contribute to graft injury (18–21). The lack of antiviral “exhaustion” may also occur with other viruses (cytomegalovirus [CMV] or EBV) capable of establishing latency with intermittent reactivation. The clinical significance of heterologous immunity requires further definition. Heterologous immune responses are typically restricted to single HLA molecules, limiting the breadth of these responses against HLA-diverse allografts (13, 22). Initial memory T cell infiltrates in rejecting allografts are not antigen specific, suggesting that heterologous immunity against the broad array of prior antigenic exposures rather than against single microbes might participate in allograft rejection (23). Microbial by-products, short-chain fatty acids, may modulate the balance between latent and lytic states of herpesviruses in the human host (24). Thus, interactions between bacterial and viral components of the microbiome may affect viral activation and the risk for alloimmune responses.

Microbial activation of innate immune responses has been implicated in acute and chronic allograft injury. In human transplant recipients with hypomorphic Toll-like receptor 4 (TLR4), an innate immune receptor, rates of bronchiolitis obliterans syndrome (BOS) in lung transplant recipients and of renal allograft rejection are reduced (25, 26). Inhaled lipopolysaccharide (LPS), a TLR4 agonist and mediator of sepsis syndromes, induces acute alloimmune lung injury in fully major histocompatibility complex (MHC)-mismatched murine bone marrow transplants but not in syngeneic recipients. Another innate immune activator, viral mimetic poly I:C, has also been shown to promote alloimmune lung injury (27, 28). In mice undergoing cardiac transplantation using a nondepleting (costimulatory blockade) tolerance-inducing regimen, early infection with *Listeria monocytogenes* produces allograft rejection (29, 30). Rejection occurs despite prior immunization against *L. monocytogenes* or treatment with ampicillin. In

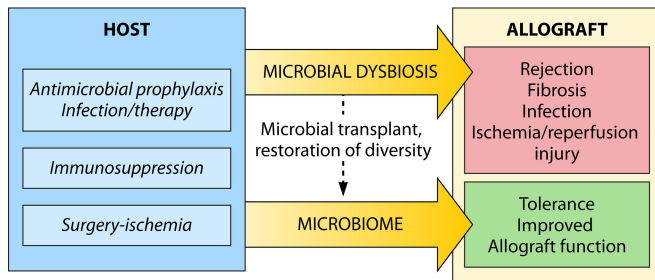
these animals, expression of type I interferons (IFNs) and bacterial expression of the virulence factor listeriolysin are required for cardiac allograft rejection (29, 30). Similarly, mice infected with *Staphylococcus aureus* reject skin allografts under a protocol that generally produces graft tolerance (31). In contrast, MyD88 knockout mice that lack a key signaling pathway of innate immune responses survive otherwise lethal challenges with heat-killed *S. aureus* and accept concurrent skin allografts, consistent with the role of innate immune responses to *S. aureus* in stimulating remote allograft rejection (31). In general, skin, lung, and intestinal transplants have been unsuccessful using tolerance induction based in costimulatory blockade. This may reflect TLR stimulation by commensal and environmental organisms (32).

Effects of viral infection on inflammation and graft function are complex and virus specific. Viral infection of grafts may increase allograft injury via innate immune mechanisms, possibly by increased expression of inflammatory mediators with recruitment of leukocytes (33). The *Herpesviridae* (e.g., CMV and EBV) are most often implicated in these processes, though hepatitis C virus (HCV) and pulmonary respiratory viral infections are also linked to allograft injuries (34–42). Posttransplantation, CMV has been implicated in endothelial inflammation, vasculopathy, and graft rejection (43). *In vitro*, CMV upregulates interleukin-8 (IL-8), transforming growth factor  $\beta$  (TGF- $\beta$ ), and other growth factors to increase fibrosis and inflammation (44, 45). Rat CMV (RCMV) upregulates genes associated with angiogenesis and wound repair with vasculopathy in rat cardiac allografts (46). Aggressive prophylaxis against CMV infection attenuates coronary artery vasculopathy in human cardiac allograft recipients and graft rejection in kidney recipients (47–49).

In addition to the effect of MHC-mismatched grafts on the intragraft efficacy of antiviral adaptive immune responses, CMV tends to suppress the host antiviral response (50). CMV reduces mobilization of monocytes and dendritic cells to inflammatory sites and decreases viral antigen presentation to T lymphocytes. CMV reduces expression of MHC class I receptors in infected host cells, facilitating immune evasion by decreasing antigen priming of T cells and cytolytic T cell responses (51). Murine cytomegalovirus (MCMV) downregulates the expression of natural killer (NK) cell receptors for MCMV, evading NK cell immune surveillance (51). The role of gamma-delta T cells at the interface of innate and adaptive immune mechanisms in the host response to CMV and in graft survival remains to be further defined (52). EBV similarly reduces expression of MHC class I and MHC class II receptors on infected cells, avoiding cellular immune responses (53). Both CMV and EBV encode a viral anti-inflammatory homolog of IL-10, further reducing host antiviral responses (54, 55). Allograft vasculopathy in murine cardiac transplant models (parental to F<sub>1</sub>) infected with lymphocytic choriomeningitis virus (LCMV) in the complete absence of T and B lymphocytes (RAG<sup>-/-</sup>) was found to be mediated by NK cells; depletion of NK cells abrogated vasculopathy (56). Thus, pathogen-specific effects on allograft survival relate to the timing, duration, and intensity of innate and adaptive responses (57).

## DYSBIOSIS, INDUCTION THERAPIES, AND IMMUNE RECONSTITUTION IN ALLOGRAFT PATHOLOGY

As opposed to the impact of specific infections common to the immunocompromised host, the effects of microbial networks on allograft function have only recently been explored. Early studies



**FIG 1** Bidirectional effects of the microbiome in allotransplantation. The process of allotransplantation disrupts the composition of the microbiome through a variety of mechanisms, including surgery, immunosuppressive agents, and antimicrobial therapies. Microbial dysbiosis has been associated with the development of chronic rejection, injury from ischemia-reperfusion, and infection. Conversely, microbiome homeostasis is associated with improved allograft outcomes. The role of microbial manipulation (e.g., by fecal transplantation) as a therapeutic measure to improve allograft outcomes is under investigation.

indicate bidirectional effects: allotransplantation induces microbial dysbiosis, and microbiome homeostasis has a key role in the control of allograft function (Fig. 1).

### Dysbiosis after Allograft Transplantation

Changes in the composition of the microbial profiles of pre- and posttransplant patients have been analyzed. The salivary microbiome in kidney and heart transplant recipients is disrupted compared with normal oral flora in favor of colonization with opportunistic pathogens, including *Pseudomonas*, *Acinetobacter*, and *Enterobacteriaceae* species (58). Such changes are termed “dysbiosis.” A comprehensive study of the blood, oral, urinary, and rectal microbiomes of kidney transplant patients before and after transplantation shows major shifts in composition by 1 month posttransplantation, with relative stability thereafter (59). In another study, the rectal microbiomes of five kidney transplant recipients in the first 90 days posttransplant showed significant increases in *Bacteroides* species and in species from the phylum *Proteobacteria* (60).

Some of these changes in the microbiome are due to the surgical process and some of these changes may be attributed to immunosuppressive regimens. For example, following small bowel transplants, analysis of ileal microbiome samples reveals inversion of the microbial composition from strict anaerobes to facultative anaerobes, likely due to the end ileostomy, which allows introduction of increased oxygen levels into the small bowel (61). Intestinal and hepatic ischemia-reperfusion injuries have been associated with a microbial dysbiosis and pathological predominance of *Enterobacteriaceae* (62).

### Microbial Shifts in Induction Therapy and Immunosuppression

Allotransplantation often utilizes antibody-based T cell depletion at the time of transplantation, which is called “induction therapy.” Changed microbiome profiles have been identified in relation to the use of T cell-depleting agents (antithymocyte globulin), non-depleting therapies (basiliximab), early steroid withdrawal programs, or prolonged steroid use, but without statistical significance in small samples (62). All such studies are confounded by effects of perioperative and posttransplantation antimicrobial therapies. T cell-depleting therapies generally deplete central memory subsets, while effector memory and regulatory T cell (Treg) subsets persist (63). Immune reconstitution following depletion is shaped by

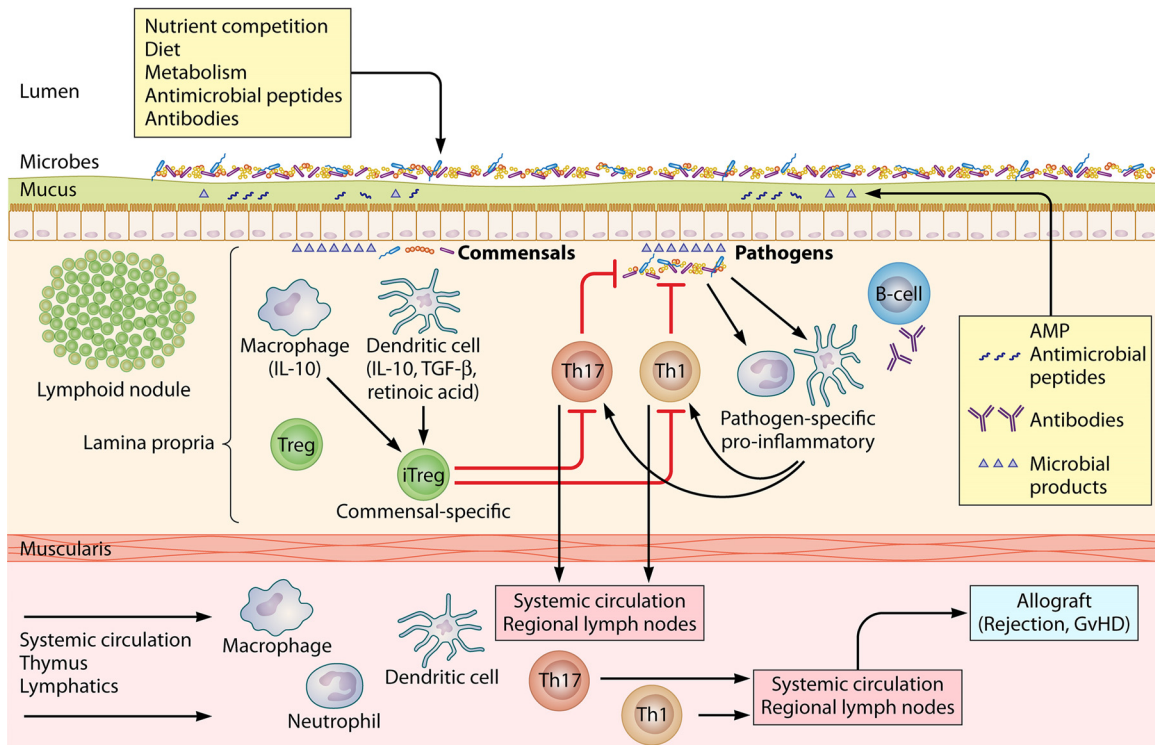
antigen exposures and subsequent immunosuppressive regimens (64). Lymphopenia induces a compensatory repopulation of immune cells, termed “homeostatic proliferation,” which favors the emergence of memory T cells and may predispose to graft rejection. Rapid proliferation of lymphocytes is antigen specific, likely driven to a great degree by commensal bacterial antigens (65). A role for commensal organisms is suggested by the absence of postdepletional T cell proliferation in germfree, immunodeficient mice compared with conventionally raised mice (66, 67).

### IMMUNOLOGIC CONSEQUENCES OF DYSBIOSIS AFTER ALLOTRANSPLANTATION: A CONCEPTUAL FRAMEWORK

How does the microbiome shape adaptive immune responses after allotransplantation? This concept is best considered in the context of recent data on the role of the localized microbiome in shaping systemic immune responses. T cell responses are classified according to surface markers and cytokine secretion patterns. Th1 responses include IFN- $\gamma$ , Th2 responses include IL-4 and IL-5, and antimicrobial and proinflammatory Th17 responses are characterized by IL-17, IL-21, and IL-23 secretion. Tregs secrete IL-10. Subsets of Tregs include natural, thymus-derived Tregs with T cell receptors (TCRs) targeting self-antigens and induced Tregs (iTregs) derived from circulating CD4<sup>+</sup> cells activated in the presence of antigen, TGF- $\beta$ , and retinoic acid.

Select bacterial components mediate the maturation of mucosal and systemic T lymphocytes. For example, polysaccharide A (PSA) from *Bacteroides fragilis* mediates Th1-Th2 balance and development of invariant natural killer T (NKT) cells in the colonic lamina propria in germfree mice (68, 69). PSA also ameliorates murine colitis (70). In contrast, in gnotobiotic mice, *Clostridium* spp., “*Candidatus* Arthromitus,” and Gram-positive segmented filamentous bacteria (SFB) induce the development of proinflammatory Th1 and Th17 effector cells in the small intestinal lamina propria (71, 72). These effector cells participate in beneficial host defenses against GI pathogens, inducing the production of antimicrobial peptides and proinflammatory chemokines and cytokines via recruitment of neutrophils, macrophages, and dendritic cells. They may also participate in allograft rejection, particularly in the absence of tolerogenic iTregs.

In the lamina propria, iTreg TCRs are specific for intestinal microbial antigens (73). Under normal conditions, iTregs limit mucosal Th2 responses to commensal organisms and Th1 and Th17 responses to pathogenic bacteria, protecting the host from excessive tissue injury (74–76). Development of iTregs is stimulated by specific organisms, including capsular antigens of *B. fragilis* and a network of spore-forming clostridia (77, 78). Importantly, iTregs and Th17 cells share a developmental requirement for TGF- $\beta$  signaling with the cofactor retinoic acid via the retinoic acid receptor-related orphan receptor alpha (ROR $\alpha$ ) and ROR $\gamma$  (79). In the presence of microbial antigens, generally those derived from noncommensals and proinflammatory cytokines, including IL-1 $\beta$  and IL-6, the action of retinoic acid is suppressed, iTreg development is blocked, and Tregs may be reprogrammed into IFN- $\gamma$ - or IL-17-secreting effector T cells (Fig. 2) (80). In addition, antibacterial agents against Gram-positive bacteria have been shown to block local TLR and MyD88 innate immune signaling pathways that are essential for iTreg development (81, 82). Thus, recipient dysbiosis and/or antimicrobial agents, both common in allotransplantation, may alter iTreg phenotype and function toward proinflammatory adaptive immune responses. These shifts



**FIG 2** The microbiome in alloimmunity. Under normal conditions, inflammatory responses to gastrointestinal flora are muted via both innate and adaptive immune mechanisms. Subsets of Tregs include both natural, thymus-derived cells with T cell receptors targeting self-antigens and induced Tregs (iTregs) derived from circulating  $CD4^+$  cells activated in the presence of microbial antigens, transforming growth factor  $\beta$  (TGF- $\beta$ ), and retinoic acid. In the presence of noncommensal microbial antigens, such as those from *Clostridium* spp. and Gram-positive segmented filamentous bacteria (SFB), and proinflammatory cytokines, iTreg development is blocked and Tregs may be reprogrammed into proinflammatory Th1 and Th17 effector cells. These effects may be blocked by antibacterial agents. Recipient dysbiosis and receipt of antimicrobial agents may shift iTreg development toward proinflammatory adaptive alloimmune responses. GvHD, graft-versus-host disease.

may predispose to the development of alloactive memory T cell responses, particularly after T cell depletion induction therapy.

The microbiome also shapes relevant innate immune responses. In the intestine, molecules derived from gut microbes, including glycoproteins, LPS, and nucleic acids, are termed pathogen-associated molecular patterns (PAMPs) or microbe-associated molecular patterns (MAMPs). MAMPs and PAMPs interact with the innate immune system via pattern recognition receptors (PRRs), including Toll-like receptors (TLRs) or Nod-like receptors (NLRs), on intestinal epithelial cells. Intestinal epithelial cells promote tolerogenic innate immune responses by conditioning intestinal dendritic cells to promote the development of regulatory T cell responses after these dendritic cells encounter antigen from commensal microorganisms (Fig. 2). Intestinal macrophages also respond to MAMP and PAMP signals via the development of inflammasomes, a complex of proteins that coordinate inflammatory processes. However, normal flora do not present antigen to intestinal macrophages as efficiently as newly introduced microbes in terms of the induction of inflammatory responses. Thus, innate immune responses to commensal organisms mediated by dendritic cells or by macrophages via dendritic cells are blunted (1, 2, 83, 84).

Innate immune responses to the microbiome have been explored in the context of liver transplantation. Under normal conditions, the liver contributes to containment of proinflammatory responses to commensal organisms (85). Conversely, in animal models of liver failure and in human studies of nonalcoholic ste-

atohepatitis (NASH), liver disease has been associated with increased systemic immune responses to commensal microorganisms, possibly due to diminished filtration of bacteria and microbial products (85). Resident innate immune cells of the liver include natural killer (NK) cells, NKT cells, and macrophages (Kupffer cells); these interact with portal blood and participate in hepatic responses to ischemia-reperfusion (86). Germfree mice lack MAMPs in the portal circulation, and this absence of MAMPs appears to correlate with lower levels of hepatic leukocyte adhesion molecule expression and reduced numbers of Kupffer cells. Consistent with these studies, treatment of rats with polymyxin B prior to liver transplantation reduced intestinal levels of *Enterobacteriaceae*; this reduction was correlated with decreased portal circulation endotoxin levels, decreased hepatic Kupffer cell tissue factor activity, and decreased posttransplant hepatonecrosis (87). Thus, after ischemia-reperfusion, MAMPs may participate in recruitment of Kupffer cells to the liver allograft, contributing to graft injury after liver transplantation (88). This may reflect exaggerated inflammatory responses elicited by microbial flora in the portal circulation that upregulate expression of gastrointestinal TLRs and responses to danger-associated molecular patterns (DAMPs) from damaged cells after surgery.

#### MICROBIOME HOMEOSTASIS AMELIORATES ALLOGRAFT INJURY

Emerging evidence suggests that preservation of the pretransplant host microbiome in the posttransplantation period improves al-

lograft outcomes. The persistence or resilience of the original pattern of the microbiome, notably in microbial diversity, through the process of allotransplantation is termed “microbiome homeostasis.” Microbiome homeostasis may promote tolerant host immune responses and avoid allograft injury. This has been best demonstrated in lung transplantation. In lung transplant patients undergoing protocol biopsies and bronchoalveolar lavage (BAL), *de novo* recipient colonization with *Pseudomonas aeruginosa* after transplantation was associated with development of early bronchiolitis obliterans syndrome (BOS), a form of chronic lung allograft injury (89). In another series, lung recipients with *Firmicutes* and *Bacilli* had more BOS than did those with *Proteobacteria* and *Gammaproteobacteria* (90). Willner et al. examined the relationship between lung transplant outcomes and the BAL fluid microbiome by 16S rRNA sequencing in a subset of cystic fibrosis patients. In those recipients who maintained the pretransplant colonizing flora in the posttransplant period, including those with *Pseudomonas*, there was a statistically significant decrease in BOS after lung transplantation (91). Conversely, colonization with new, pathogenic bacteria was associated with BOS (91).

Microbiome homeostasis may also ameliorate the effects of ischemia-reperfusion injury after transplantation. Short-chain fatty acids, the metabolic by-products of the intestinal microbiome, have been shown to attenuate ischemia-reperfusion injury (92). Short periods of hepatic ischemia followed by liver transplantation have been shown to ameliorate subsequent ischemic-reperfusion injury (93). This “ischemic preconditioning process” has been associated with restoration of pretransplant intestinal microbiota, including *Clostridium* and *Bifidobacterium* species. Amelioration of ischemia-reperfusion injury by ischemic preconditioning may be due to maintenance of normal intestinal flora, consistent with the potential importance of microbiome stability in prevention allograft injury. However, it is uncertain that the same effects would apply to organs not dependent upon the portal circulation.

#### POTENTIAL CLINICAL APPLICATIONS OF MICROBIOME MANAGEMENT IN ALLOTRANSPLANTATION

Manipulation of the microbiome may allow control of immune responses. In Pstip2<sup>cmo</sup> mice, spontaneous development of autoimmune osteomyelitis was prevented by diets rich in fat and cholesterol, with reductions in intestinal *Prevotella* levels and proinflammatory cytokines (94). Similarly, Lathrop et al. blocked the development of murine autoimmune arthritis using vancomycin treatment of SFB (73). These studies suggest that microbial modulation may durably impact proinflammatory immune responses. However, clinical applications of microbial manipulation are in their infancy. With respect to transplantation, the microbiome may serve as a marker of allograft health or a target for modulation of alloimmunity. Monitoring of the microbiome in intestinal transplant recipients may serve as a biomarker for allograft rejection; decrement of the *Firmicutes* population has been shown to be significantly correlated with acute rejection (95). However, similar associations between acute rejection and a specific microbiome composition(s) have not yet been identified in recipients of other organs. Recent studies in kidney transplant recipients did not demonstrate a clear correlation of the diversity in various microbiome sites with acute rejection events (59). Others have examined the microbiome profiles of small numbers of kidney transplant recipients with acute rejection and found significant

dysbiosis following graft rejection compared with that in nonrejecting hosts (60). This analysis was confounded by the use of antimicrobial agents around the time of the diagnosis of rejection, possibly altering the microbial composition.

Loss of microbial diversity has implications for the risk for infections in the immunocompromised host. In stem cell transplantation, decreased microbial diversity and predominance of enterococcus in enteric flora preceded enterococcal bacteremia (6). Similarly, Fricke et al. demonstrated that decreased normal *Firmicutes* in rectal swabs in renal recipients preceded systemic infection of these hosts (59). Lee et al. reported an association between the absence of common intestinal microbiota, *Bacteroides* and *Ruminococcus*, and development of posttransplant diarrhea (60). In eight liver transplant recipients who did not preserve pretransplant intestinal microbiota, five developed posttransplant infections (96). Microbial shifts may prove to be useful diagnostic markers of acute rejection or impending infection in transplant patients; further studies are required to elucidate how to best use such data.

Modulation of the microbiome by fecal transplantation has demonstrated some success in the management of refractory *Clostridium difficile* infection both in immunocompetent and immunocompromised hosts; heightened surveillance for infection after fecal transplantation in immunocompromised hosts is recommended (97, 98). Specific microorganisms may contribute to resistance to *C. difficile* infection (99). Fecal transplantation may also assist in eradication of multidrug-resistant enteric flora (100) (101). Such changes in the composition of the microbiome will alter metabolism of antirejection immunosuppressants, including tacrolimus or cyclophosphamide (102, 103). As was noted, microbial by-products may affect the control of latency in herpesviral infections; these may achieve therapeutic applications if confirmed in clinical trials (30).

#### THE MICROBIOME IN CLINICAL ALLOTRANSPLANTATION

Complex interactions exist between the allograft recipient’s microbiome, the immune system, and the allograft. There is emerging evidence that alteration of the pretransplant microbiome by the allotransplantation process results in poor clinical outcomes in both solid organ and stem cell recipients (6, 91). Viral and other new infections may contribute to detrimental immune responses to allografts. There are few studies that assess the impact of the networks of organisms and microbial products on immunity and graft function. Manipulation or reconstitution of the microbiome may be used to alter colonization patterns and as a diagnostic marker of impending posttransplant infections or of graft rejection. Therapeutic targets in the human microbiome may improve transplant outcomes in the future.

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