

# The emerging roles of the gut microbiome in allogeneic hematopoietic stem cell transplantation

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## ABSTRACT

Allogeneic hematopoietic stem cell transplantation (allo-HSCT) is used for the treatment of hematologic cancers and disorders. However, graft-versus-host disease (GVHD) in which the donor immune cells attack the genetically-disparate recipient is a significant cause of morbidity. Acute GVHD is an inflammatory condition and the gastrointestinal system is a major organ affected but is also tied to beneficial graft-versus-tumor (GVT) effects. There is increasing interest on the role of the microbiome on immune function as well as on cancer progression and immunotherapy outcomes. However, there are still significant unanswered questions on the role the microbiome plays in GVHD progression or how to exploit the microbiome in GVHD prevention or treatment. In this review, concepts of HSCT with the focus on GVHD pathogenesis as well as issues in preclinical models used to study GVHD will be discussed with an emphasis on the impact of the microbiome. Factors affecting the microbiome and GVHD outcome such as obesity are also examined. The bridging of preclinical models and clinical outcomes in relation to the role of the microbiome will also be discussed along with possibilities for therapeutic exploitation.

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## Current concepts in hematopoietic stem cell transplantation

Hematopoietic stem cell transplantation (HSCT) is primarily used for the treatment of hematological malignancies such as leukemia and lymphoma, but also for hematopoietic disorders<sup>1</sup>. Before receiving the donor cells, recipients are treated with cytoreductive or immunosuppressive conditioning regimens including either total body irradiation, chemotherapy, or a combination of both to eradicate the malignant or abnormal cells, create a niche for the donor hematopoietic stem cells (HSCs) as well as facilitate engraftment and reconstitution.<sup>2</sup> There are two principal types of HSCT: allogeneic HSCT (allo-HSCT), in which there is genetic disparity between the donor HSCs and the recipient, and autologous HSCT (auto-HSCT) in which the HSC's are from the recipient. The primary advantage of allo-HSCT is the potent antitumor response due to a graft-versus-tumor (GVT) effect generated by donor T cells to the tumor. However, this

beneficial GVT is also tied to one of the primary disadvantages of an allo-HSCT revolving around the development of recipient tissue damage in the gastrointestinal (GI) tract, skin, and liver due to the attack by the allo-reactive donor T cells, called graft-versus-host disease (GVHD). GVHD represents a major cause of morbidity following allo-HSCT. The extent of genetic disparity, the types and extent of cytoreductive conditioning used on the recipient as well as the presence of co-morbidities all impact the occurrence and severity of GVHD.<sup>3</sup> GVHD represents a major limitation in allo-HSCT and necessitates the need for immunosuppression which also impacts the beneficial GVT effects resulting in higher relapse.

The cytoreductive conditioning regimens play a crucial role in HSCT outcome but also result in significant toxicities, particularly in older patients. The myeloablative regimen consists of intensive radiation therapy alone or in combination with chemotherapy but can result in significant GI,

hepatic and pulmonary toxicities also fueling the GVHD processes. Therefore, non-myeloablative immunosuppressive conditioning using agents such as fludarabine as well as lower doses of cytoreductive agents are now used in order to reduce toxicities and facilitate engraftment of donor HSCs, especially for children or older patients and this has greatly increased application of allo-HSCT to these patient cohorts.<sup>4,5</sup> Outside of direct tissue attack, delayed hematopoiesis and immune reconstitution post-transplant which also result during GVHD are additional life-threatening complications of allo-HSCT due to susceptibility to opportunistic infections. Immune cell reconstitution is critical but is also dependent on the age of the recipient but NK cells (30–100 d) followed by T cells (from 100 d but can take years, especially in older patients), and B cells (1–2 y) all impact susceptibility to opportunistic infections as well as relapse.<sup>6</sup> Increased susceptibility to bacterial infections such as *Pseudomonas*, *Enterobacter*, *Klebsiella*, parasites, fungi, and reactivation of latent viruses such as Cytomegalovirus, Herpes Simplex, and Epstein Barr virus all rise.<sup>7</sup> To counteract these conditions, the uses of hematopoietic growth factors (granulocyte colony-stimulating factor, G-CSF) to promote myeloid recovery, administration of prophylactic antibiotics, and anti-viral therapies (for CMV reactivation) are routinely applied.<sup>8,9</sup> Patient demographics are being increasingly appreciated as factors such as obesity, diet, age and other co-morbidities clearly affect the allo-HSCT outcome but are still poorly understood as well as the impact of these different prophylactic regimens on the various aspects of HSCT and recovery.

### Graft-versus-host disease

Although allo-HSCT is a potential treatment for hematological malignancies, GVHD still remains one of the most difficult obstacles for the success of this approach.<sup>3</sup> GVHD is the immunological phenomena mediated by the donor-derived T cells in the graft targeting the major histocompatibility complex (MHC) and minor-MHC antigen differences (which in allo-HSCT exist despite more sophisticated typing and matching to ensure greater compatibility) and immunosuppressed recipient resulting in host multi-organ organ attack

including the GI system, lung, liver, skin resulting in tissue damage, organ failure, and mortality. GVHD is the cause of 15–30% of deaths post-allogeneic HSCT.<sup>10</sup> GVHD prevention can be successful by simply removing donor T cells from the HSC graft but unfortunately, it also abrogates beneficial GVT and the dominant procedures use T cell-replete grafts. An effective means to treat ongoing GVHD outside of blanket immunosuppression is still lacking especially with severe GVHD due to the nature of a “cytokine storm” that occurs and these immunosuppressive regimens also impact GVT and therefore relapse. Therefore, understanding the mechanisms of GVHD induction as well as evaluation of multiple approaches to ameliorate this disease is in need.

There are two principal types of GVHD with distinct features in pathobiology and clinical phenotypes: acute and chronic GVHD. Clinically, acute GVHD (aGVHD) usually appears at the first 100 d after transplant, while chronic GVHD (cGVHD) was classically defined as occurring later although this is now shown not to be absolute and both processes can also occur at the same time.<sup>11,12</sup> The pathologic processes of aGVHD and cGVHD are clearly distinct. aGVHD is primarily an inflammatory disease and is due to many factors such as tissue toxicities arising after conditioning regimen which culminates with donor T cell-mediated target tissue damage due to MHC disparities and massive pro-inflammatory cytokine release originally called a “cytokine storm” and which is extremely difficult to treat.<sup>13</sup> The primary aGVHD target organs are the GI tract or gut, liver, lung, and skin with severe acute gut GVHD representing a major cause of early morbidity. It is the pro-inflammatory cytokine storm consisting of IL-1, TNF, IL-6, and other cytokines and recruitment of innate cells such as macrophages which amplify the process that makes aGVHD extremely difficult to control or treat. In contrast, cGVHD typically results later after HSCT and is associated with tissue fibrosis resembling an autoimmune-like syndrome attacking skin and mucosal tissues.<sup>3</sup> As opposed to aGVHD, cGVHD is associated with a dominant role for donor B cells in its progression. The last phase of cGVHD involves in tissue fibrosis regulated mainly by macrophages with transforming growth factor  $\beta$  and platelet-derived growth factor  $\alpha$ . These pathological changes can

severely impact quality of life and cGVHD is notoriously difficult to treat as it can become resistant to steroids or immunosuppressive regimens resulting in extremely limited options for treatment. It is important to note that these processes are not exclusionary and a patient can present with both types necessitating determination by biopsy.

Cytoreductive conditioning regimens are the predominant cause of mucositis early post-HSCT, followed by the development of acute GVHD.<sup>14</sup> Radiation and chemotherapeutics such as cyclophosphamide cause extensive DNA damage and apoptosis in the GI tract, along with reduced proliferation of intestinal stem cells. This leads to an increase in intestinal permeability, which results in bacterial translocation from the microbiome in the gut, culminating in systemic infection and reduced survival post-HSCT.<sup>13</sup> Endotoxin from bacterial translocation further activates myeloid cells via toll receptor engagement and fuels the allo-reactive processes of the donor T cells and exacerbates the gut damage causing the cascade to progress. The gut tissue damage by the conditioning regimens, also promotes recruitment of allo-reactive donor T cells to the intestinal tract.<sup>15</sup> Acute GVHD with the destruction of the intestinal mucosa leads to a failure of fluid absorption, particularly in the ileum, and voluminous diarrhea in patients.<sup>14</sup> One of the earliest symptoms of GI GVHD is the tissue damage that happens in the duodenum, leading to early bleeding, appetite loss, nausea, vomiting, and weight loss.<sup>16–18</sup> The continuous development and use of anti-fungi, anti-viral, and GVHD prophylaxis has helped in reducing the incidence of severe GI bleeding after HSCT.<sup>19</sup> The pathology of aGVHD in the gut is distinctive with neutropenic enterocolitis diagnosed by the thickening of the ileum and colon wall using abdominal computed tomographic (CT) scan.<sup>20</sup> CT scan with intravenous and oral contrast confirms thickened bowel segments in patients with acute GVHD.<sup>21</sup> Similar to CT scan, magnetic resonance enterography (MRE) also could be used as an alternative method for assessing GI acute GVHD with its capability to detect long-segment bowel wall thickening, submucosal edema, and mucosal hyperemia.<sup>22</sup> Treatment options primarily consist of blanket immunosuppression although more targeted

approaches including cytokine blockade or blocking T cell homing to the gut via blockade of specific integrins are being evaluated.

### Graft-versus-tumor effect

Allo-HSCT is used as a cancer immunotherapy because it generates the GVT effect in which donor-derived cells attack the host hematologic malignant cells.<sup>1,23,24</sup> Although GVHD and GVT effect share several biology processes such as antigen-presenting cell activation and donor T cell activation possibly to similar if not identical antigens, it has been difficult even in preclinical models to delineate the two processes which nonetheless remains the primary goal in studies. Lymphodepletion has been shown to enhance the efficacy of adoptively transferred tumor-specific CD8 + T cells by using antibody to remove the  $\gamma_C$  cytokine-responsive endogenous cells.<sup>25</sup> Interestingly, in cancer immunotherapy, depletion of the patient's lymphocytes has been increasingly studied to improve efficacy of adoptive immune cell transfer. Lymphodepleting conditioning regimen prior to adoptive cell transfer in patients with metastatic melanoma significantly improved the efficacy of therapy with in vitro expanded tumor-infiltrating lymphocytes.<sup>26</sup> These cytoreductive conditioning regimens are also routinely applied with chimeric antigen receptor (CAR)-T cell therapy and similar toxicities are being observed including impact on cytokine storm or "cytokine release syndrome."<sup>27,28</sup> Outside of merely creating a niche for the transferred T cells, with T cell immunotherapy outside of HSCT, it has been postulated that bacterial translocation due to conditioning regimen potentially contributed to improve efficacy by activating dendritic cells augmenting the function of adoptively transferred CD8 T cells.<sup>29</sup> It has been demonstrated that an intact microbiome is essential to maintain anti-tumor effects in preclinical models. Use of gnotobiotic or germ-free mice as well as mice treated with antibiotics all have reduced responses to immunotherapies indicating an important role of the microbiome in immune regulation and function.<sup>30,31</sup> Additionally, it was observed that certain types of bacteria potentially contribute to modulate the clinical outcomes with immunotherapies. *Bifidobacterium* spp. is associated with delayed

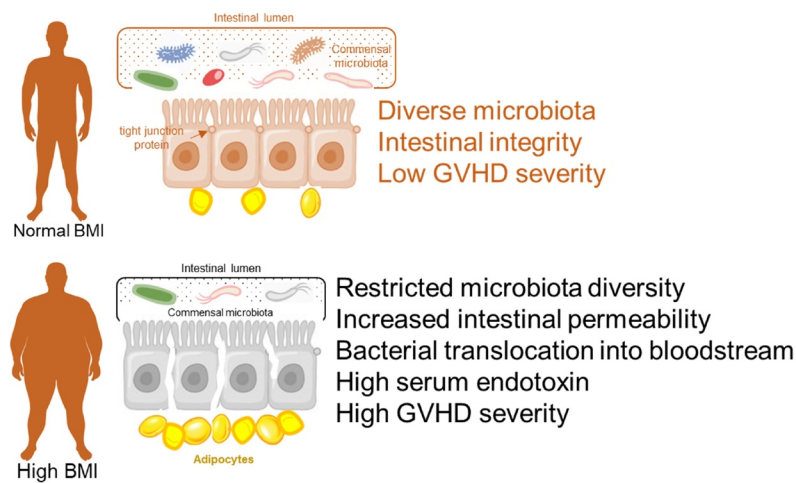
tumor progression and increased responses to programmed death-ligand 1 (PD-L1) checkpoint blockade.<sup>32</sup> Using another preclinical model, *Bacteroides* spp. is found to be required for optimal anti-tumor responses of cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) checkpoint blockade.<sup>33</sup> These results indicate that the microbiome can play pivotal roles in cancer immunotherapy efficacy regarding T cell immunotherapies as well as potential GVT effects post-allo-HSCT.

### Significant differences between HSCT in murine models and humans

The inbred laboratory mouse model is the most common preclinical model used to study allo-HSCT. The development of inbred laboratory mouse husbandry and genetic modification tools facilitated the broad use of murine models of GVHD and GVT. Mice are inbred (genetically identical), fed with controlled diets, and housed in highly regulated specific pathogen free (SPF) environments which provide researchers the ability to generate more reproducible data. However, these factors also give rise to significant discrepancies between the clinical scenario which is not as controlled given that humans are outbred and exposed to multiple pathogens throughout life and often as they age, have the presence of co-morbidities including obesity. Another one of these variables that is increasingly appreciated concerns the microbiome of laboratory mice and its difference between humans. Outside of clear species differences and dietary habits, there are multiple studies providing evidences of how mouse gut microbiota shifts in various housing conditions and affects the immune system. In addition, compared to mice housed under SPF conditions, the use of germ-free mice often used in microbiome transfer studies, lack all microorganisms and have various immunological abnormalities with significant defects in immune development. These include deficits in development of lymphoid organs, altered mucosal immunity, impaired innate cell number and function, and adaptive responses being in a naïve state as well as demonstrated decreases of total CD4+ helper T cells while shifting to T<sub>H</sub>2 phenotype.<sup>34,35</sup> Gnotobiotic mice are animals with defined

microorganisms and are also used in studies to study the impact of particular bacterial species. However, the complexity of handling protocols, quality control of bacterial colonization, and extremely high cost are the main limitations for extensive application of this model in current research and similar immune deficits.<sup>36–38</sup> It is also important to keep in mind that, even under SPF housing conditions, mice obtained from different vendors display distinct microbiome profiles which has been demonstrated to significantly affect their responses to cancer immunotherapies.<sup>32</sup> Importantly, these differences were found to be normalized when mice are co-housed or even bedding from one are transferred given that mice exhibit coprophagy (the eating of their feces) which normalizes the microbiome within a cage.<sup>32</sup> Interestingly, feral mice and mice obtained from pet stores have extensive bacterial and pathogen exposure and present with notably more diverse microbiota than SPF mice. Furthermore, the immunological profile of such mice has been demonstrated to more appropriately model the complexity of human immune system demonstrating the importance of the microbiome on immune functions but also the complexities in attempting to model reflecting the human condition.<sup>39</sup>

Outside of the not insignificant species differences, human populations exhibit considerable MHC diversity, wide ranges in age and different pathogen exposure as well as the existence of pre-existing conditions (i.e. diabetes, obesity) all of which can be influenced by the microbiome and have profound immunological consequences. Both obesity<sup>40</sup> and aging<sup>41</sup> are associated with a heightened inflammatory state which predisposes individuals to after HSCT to cytokine-induced pathologies due to increased gut permeability and bacterial translocation<sup>42,43</sup> and condition regimen complications targeting the GI tract (i.e. radiation toxicity and bacterial translocation).<sup>44</sup> Obesity (BMI > 30) has reached pandemic proportions in the U.S. with greater than one-third of U.S. adults are obese according to Center for Disease Control and Prevention. Obesity is associated with meta-inflammation exacerbated by metabolic complications such as glucose intolerances, diabetes, hypertension etc. The inflammation in the obese environment is also



**Figure 1.**

fueled by adipocyte factors and the effects on the immune system have been called “inflammaging” due to suppressed adaptive immune responses.<sup>45</sup> This is in part due to alteration of immune subsets<sup>46,47</sup> as well as increased production of inflammatory cytokines such as IL-1 $\beta$ , TNF- $\alpha$ , IL-6 from macrophages, monocytes, and T cells.<sup>48</sup> Obesity also impacts the microbiome with less diversity being reported.<sup>49,50</sup> Obesity of the recipient has been shown to correlate with poor outcomes after allogeneic HSCT in both mouse preclinical models and human clinical outcome data<sup>50</sup> in which high BMI was associated with a significantly greater risk of grade II–IV acute GVHD<sup>51</sup> (Figure 1). However, there are also clinical studies suggesting that obesity was associated with a higher survival rate after allo-HSCT<sup>52</sup> and auto-HSCT.<sup>53</sup> As there can be significant differences in HSCT procedures (such as patient selection, conditioning, regimens, stem cell sources, immunosuppression applied) as well as the presence of co-morbidities such as cancer, all of these can impact outcomes.<sup>54</sup> Apart from obesity, aging also plays a critical role in inducing microbiota changes. The decreased abundances of *Bifidobacterium* and *Lactobacillus*, together with an increased abundance of *Enterobacteriaceae* are the key microbiota shifts in elderly individuals.<sup>55</sup> Possible reasons for microbiome shifts in aging could be explained based on diet adjustment, lessened exercise, reduced mobility, residence locations, less muscle mass (sarcopenia), etc.<sup>56</sup> Although obesity and aging correlate with

restricted microbiota diversity in mouse and human<sup>50,55,57,58</sup> and the less-diverse microbiome profile has been proved to be associated with poor outcomes after HSCT,<sup>59–61</sup> recent studies suggested that there are certain types of bacteria that could serve as beneficial factor for health and longevity.<sup>56,62</sup> *Bifidobacteria* supplementation reduced the accumulation of aging biomarkers (carbonyls and lipofuscin),<sup>63</sup> while transfer of *Christensenella minuta* to germ-free mice significantly reduced adiposity gain.<sup>64</sup> Thus, there is an interplay between the gut microbiome on body weight gain and immune outcomes but also in reverse where the diet can impact the microbiome.

Humans also have been exposed to various immunological challenges and pathogens throughout life (i.e. cytomegalovirus, EBV) that significantly affect HSCT outcomes. Clinical data have suggested that obese patients are at higher risk of infection-associated complications and transplantation-related toxicity post-HSCT, especially in allogeneic HSCT.<sup>65,66</sup> These data indicate the necessity to modify our current murine HSCT models to be more reflective of human conditions (Table 1). One excellent example of this is the study with co-housed laboratory mice with pet store mice gave rise to significant alterations in the immune profile of laboratory mice with increased resistance to infection and altered T cell kinetics.<sup>39</sup> The problem arises with regard to the costs of using so-called “dirty” mice and lack of ability to therefore control all the variables allowing for reproducible data, particularly between different laboratories.

In order to mirror the clinical HSCT scenario more accurately, large-animal models, primarily in canines and non-human primates, have been studied and represent the key models for GVHD studies and the development of treatment protocols for HSCT. However, extended use of these models are hampered due to high costs, difficulties in experimental control of MHC disparities, limited validated immune reagent availability and immune monitoring capabilities, time and limited sample size, as well as difficulty to use in cancer studies which are the predominant reasons for allo-HSCT. GI tract GVHD in rhesus macaques has been characterized with diarrhea as a clinical symptom, significant lymphocyte infiltration and loss of normal tissue architecture with mucosal damage with histology assessment and represents a useful GVHD model.<sup>74–76</sup> In canine models, GI tract acute GVHD displayed a distinct punctate GI hemorrhage with blunted villous architecture, sloughing, mucosal destruction progressing from crypt abscess formation to denudation, and CD3 infiltration representing typical GI acute GVHD symptoms also representing a useful model but mechanistic studies are extremely difficult to perform.<sup>77,78</sup> Thus, both small animal and large animal models offer advantages and disadvantages in HSCT studies (Table 1) and these need to be constantly taken in consideration when evaluating data and attempting to extrapolate to the clinical situation.

### Methodological approach to study intestinal microbiota

Given the predominance of gut GVHD and the importance of the microbiome on immune functions, particularly in the gut, there has been considerable interest in defining the gut microbiome in both preclinical and clinical samples. The most commonly used method in microbiome studies uses sequencing of 16S ribosomal RNA (rRNA), the highly conserved gene in all bacteria. Sequencing of regions of hyper-variability in 16S rRNA gene helps us identify different bacterial taxa but this technique could result into inaccuracies at species level classification. The most common

**Table 1.** Comparison of HSCT in mice and humans.

	Mice	Humans
Genotype	Inbred (genetically homogeneous)	Outbred with tremendous human leukocyte antigen (HLA) diversity
Age	Predominantly young (8–12 weeks old), equivalent to early adolescence in humans	Variable age
Environment	specific-pathogen-free (SPF)	Numerous pathogen exposures (acute, chronic and latent)
Microbiome	Husbandry-related factors such as mouse transfer, water decontamination could affect microbiome change <sup>67,68</sup>	Preexisting disease (i.e., obesity <sup>50,57,58</sup> ), environmental exposure, and antibiotic use all have effects on the human microbiome <sup>69</sup>
Diet	Changes in diet can alter the intestinal microbiome <sup>70–72</sup>	Diet types (omnivorous, vegan, and vegetarian) determine microbiome profiles <sup>73</sup>
Recipient health status	Healthy lean (body weight below 30 g)	Highly variable, with an increasing obesity (BMI >30 kg/m <sup>2</sup> ) and preexisting diseases
HSCT	conditioning regimens	Lethal dose of total body radiation (single or split doses), chemotherapy rarely applied
HSCT	Myeloablative and non-myeloablative regimens application	Modeling of HSCT for human cancers, including xenogeneic transplant models
Treatment of a variety of disease states ranging from cancer to GVHD	hematopoietic disorders	
GVHD	pathogenesis	Donor bone marrow cells do not give rise to GVHD. Donor T cells (mostly naïve T cells) are required to induce GVHD.
Adult HSC sources are sufficient to cause GVHD	depending on the level of HLA compatibility and the conditioning regimen used	
GVHD type	Mostly either acute or chronic, crucially dependent on strain combination used and the type of conditioning.	GVHD can be mixed (acute and chronic)
Graft-versus-tumor (GVT) effect	Numerous studies of approaches to enhance GVT effect and limit GVHD, although the emphasis is on GVHD prevention or treatment and only short-term results	Emphasis has been to improve GVT either through adoptive cell therapies or improved immune reconstitution following HSCT, without exacerbating GVHD

hyper-variable regions in 16S rRNA is V3-V4 region. A simple DNA sequencing procedure involves bacterial DNA extraction from samples, bacterial DNA amplification from variable regions

of 16S rRNA using polymerase chain reaction (PCR), library preparation, DNA sequencing, bioinformatics, and biostatistics analyses.<sup>79,80</sup> Shotgun next-generation metagenomic sequencing (shotgun NGS sequencing) is also a DNA sequencing method but amplifies all genomic DNA in a sample (including bacterial DNA and non-bacterial DNA) by gene fragmentation, tagging, PCR amplification, sample pooling in equal proportions, DNA sequencing, and bioinformatics analysis to classify results into taxonomic levels.<sup>81</sup> Because of broader sequencing capabilities, shotgun NGS sequencing allows researchers to identify multiple bacteria, fungi, viruses and many other types of microorganisms in their samples.<sup>82</sup> Diversity is one of the most important parameters in microbiome study. Microbiome diversity has been well-established as a “biomarker” for HSCT outcome. High microbiome diversity correlates with better survival rate, lower incidence and severity of GVHD.<sup>59,60,83,84</sup> Alpha-diversity (definition of the species composition within samples) and beta-diversity (quantification of the overall compositional differences between groups of subjects) are key factors to study the gut microbial ecosystem.<sup>85</sup> In alpha-diversity, Shannon diversity index was used to describe evenness and diversity by measuring both the number of species and the inequality between species abundances. In contrast, Shannon evenness index is independent of species richness and provides information about how evenly the microbes are distributed in a sample. Beta diversity represents the differences between microbial communities from different environments with the main focus on the differences in taxonomic abundances from different samples.<sup>86</sup> Because of new molecular techniques, statistic and bio-informatics analysis methods are discovered every few years; microbiome characterization is expected to reach the next level soon with potential linkage with microbiome alteration factors such as antibiotics, strain dynamics, and homeostasis.

### Microbiota modification and HSCT outcomes

Commensal bacteria are the results of the co-evolution and symbiotic relationship between the host and microorganisms. They impact the host metabolism as well as provide protection against

pathogenic bacterial growth. A diverse microbiota is required to maintain host-microbe homeostasis in various environmental changes because species-rich bacterial communities could compensate for missing ones. In HSCT, together with microbiome diversity as an indicator for outcomes,<sup>60</sup> some pathogenic bacterial taxa have been shown to increase in the GVHD recipients such as Enterococcaceae, *Akkermansia muciniphila*, and Lactobacillales.<sup>50,61,84,87</sup> In contrast, *Blautia* has been shown as the beneficial bacterial genus for HSCT<sup>88</sup> as well as the correlation of increased GVHD with reduced abundances of some bacterial genera such as *Faecalibacterium*, *Bacteroides*, and *Parabacteroides*.<sup>89</sup> Certain bacterial taxa also have been shown to contribute to GI tract recovery after radiation. *Lachnospiraceae* and *Enterococcaceae* are essential to maintain GI tract integrity and facilitate immune reconstitution long-term after radiation exposure.<sup>90</sup> Therefore, multiple selective microbiota alteration approaches have been extensively studied to ameliorate GVHD and improve HSCT outcomes (Figure 2). It is important to note that the conditioning regimens applied in HSCT often involve cytoreductive conditioning in the form of radiation and chemotherapeutics which cause significant damage to the GI system thus exacerbating the impact of the microbiome on outcome, particularly during the initial period of immune deficiency in the recipient immediately following HSCT.

### Antibiotics

Following the rapid development of cutting-edge techniques for microbiota analysis, the important role of the microbiome in health and diseases has been extensively revealed. These achievements have opened many research directions to modify microbiota component to establish highly effective microbiome therapies<sup>91</sup> in pre-clinical models and in clinics. Early pre-clinical studies indicated a significant reduction of GVHD with antibiotics<sup>92</sup> and transplantation in germ-free conditions.<sup>93</sup> However, there are also conflicting clinical data demonstrating poorer outcome in patients receiving prophylactic antibiotics. We and others demonstrated in murine HSCT models that prior administration of broad spectrum antibiotics reduced acute GVHD

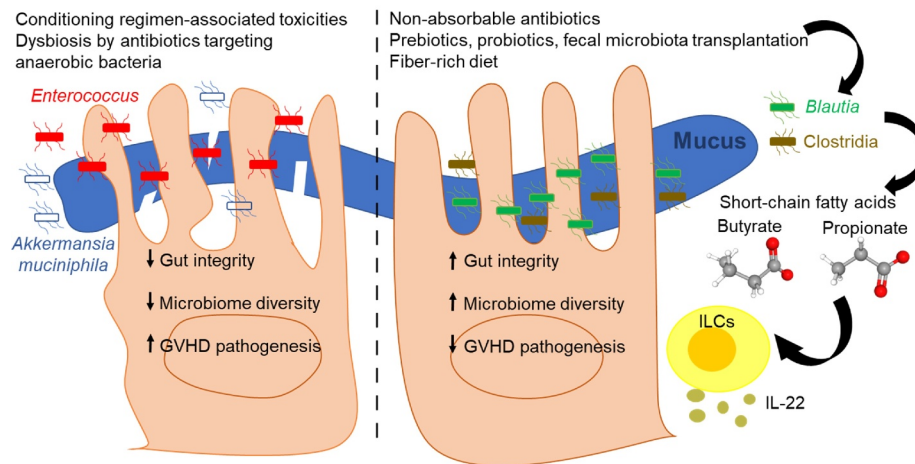


Figure 2.

post-allogeneic HSCT in part due to significantly decreased bacterial translocation into bloodstream.<sup>50,94</sup> Interestingly, increased expression of MHC class II in the intestinal epithelial cells after radiation was also reduced, likely affecting donor T cell priming for GVHD induction in HSCT.<sup>95</sup> Other possible mechanisms include reduced migration of neutrophils<sup>96</sup> or donor T cells<sup>50</sup> into the mesenteric lymph nodes where priming for gut GVHD occurs. However, there is also recent evidence that antibiotics with specific activity targeting the ribosome demonstrated a direct immunosuppressive role against  $T_H17$  cells, suggesting that antibiotics with no specific target on bacteria can directly exert immunological regulatory effects so caution must be exercised before assuming that the microbiome was the mechanism underlying the effects.<sup>97</sup>

The microbiome recovery after antibiotic treatment and HSCT is another parameter which can be variable and is impacted by factors intrinsic to the patient as well as diet and regimens applied all affect the gut and microbiome repopulation dynamics. It was reported that the recovery of the gut microbiota started around day 50, but even by day 100 post-HSCT, the composition and bacterial abundance were less diverse compared to the pre-transplant microbiota.<sup>98</sup> In allogeneic HSCT patients, sterile food, antibiotic treatment, and skin cleansing significantly reduced mortality.<sup>99</sup> Promising results representing the correlation of complete gut microbiota decontamination with better outcomes post-HSCT also came from studies

using prophylactic antibiotic administration. Vossen et al. used the combination of neomycin, polymyxin B, cephaloridin, and amphotericin B to target all bacteria, yeasts, and fungi and demonstrated reductions in both infections and GVHD in children.<sup>100</sup> A follow-up study comparing the efficacy of successful and unsuccessful microbiota decontamination in children before and after HSCT in GVHD prevention also suggested the remarkable reductions of moderate and severe acute GVHD in patients with complete microbiota decontamination.<sup>101</sup> It is important to notice that although the use of broad-spectrum antibiotics dramatically protected patients against infection and transplant-related mortality, antibiotics could lead to substantial microbiota disruption and dysbiosis.<sup>102</sup> Additionally, the type of antibiotics determines the composition of microflora and seems to correlate with GVHD incidence and outcomes. Antibiotics targeting anaerobic bacteria are associated with higher rates of GVHD-related mortality targeting the intestinal tract due to the development of mucus-degrading bacteria, but other organs such as skin and liver remained unaffected.<sup>87</sup> The protective effect of antibiotics in HSCT could be explained by the reductions of inflammation and decreased radiation sensitivity in other murine models treated with antibiotics. Treatment with poorly absorbed ampicillin and non-absorbed neomycin reduced metabolic endotoxemia and inflammation in obese leptin-deficient mice (*ob/ob*) or mice fed with high-fat diet,<sup>103</sup>



indicating the benefit of antibiotic treatment on controlling obesity-associated “meta-inflammation”. More importantly, antibiotic treatment prevented bacterial translocation from the intestine into the bloodstream after radiation, a critical step for the activation of innate immune cells (e.g. dendritic cells) and cytokine storm triggering, therefore reducing gut permeability and tissue damage due to radiation sensitivity.<sup>29,104</sup> These evidences provide different perspectives of how one might consider choosing the “right type” of antibiotics to preserve the beneficial bacteria taxa without compromising treatment efficacy.

Besides the type of antibiotics, timing of treatment also affects outcomes post-HSCT. Short-term discontinuous administration of ampicillin followed by a recovery period before HSCT resulted in worse survival outcomes in mice underwent allogeneic MHC mismatched HSCT.<sup>84</sup> Continuous prophylactic treatment using broad-spectrum antibiotic cocktail with ampicillin, vancomycin, and neomycin demonstrated a protective effect in gut permeability and GVHD outcomes in high-fat diet fed obese mice.<sup>50</sup> Short-term treatment with a similar antibiotic combination after HSCT also showed a complete protection from diarrhea, weight loss, and death in mice.<sup>94</sup> However, there are evidences about prophylactic antibiotics resulted into microbiome disruption and higher transplant-related mortality than those who had antibiotics administered on or after day 0 or untreated patients indicating that more still needs to be delineated on the impact of different antibiotics as well as timing of administration.<sup>102</sup>

### Diet, prebiotics, and probiotics

Dietary intake has been shown to influence the component of the trillions of intestinal microorganisms.<sup>70,71,105</sup> GVHD in the digestive tract has been shown to correlate with malnutrition, protein losing enteropathy, magnesium derangements, and deficiencies of zinc, vitamin B12 and vitamin D.<sup>106</sup> Prebiotics are indigestible carbohydrates or substances metabolized by beneficial intestinal bacteria that help inhibiting the growth of other pathogenic bacteria, boosting the immune responses, and reducing the risk of various diseases.<sup>107</sup> Short-chain fatty acids (SCFAs) are the

product from the fermentation process of bacteria that recently been linked with prebiotics metabolism and health benefit.<sup>108</sup> One of the most important SCFAs is butyrate, which has been shown to mitigate GVHD through the signaling by G-protein-coupled receptor 43 (GPR43) on the intestinal epithelial cells.<sup>109</sup> Propionate and tryptophan has been shown to markedly increase in mice that survived long-term radiation exposure and administration of these metabolites also protected mice from cytokine storm and GI tract permeability.<sup>90</sup> Dietary fiber changed the gut microbiota profiles by increasing diversity (Bacteroidaceae and Bifidobacteriaceae), which enhances SCFAs production.<sup>110</sup> Microbiome changes also are dependent on the components of diets. The use of the “Western Diet,” which involves high fat and high sugar as opposed to only high fat, reduces Bacteroidetes and Actinobacteria, but increases Firmicutes, Proteobacteria, and Deferribacteres,<sup>72</sup> while a high-fat only diet increases *Bacteroides*, *Enterobacteriaceae*, *Escherichia*, *Klebsiella*, and *Shigella*.<sup>111</sup> With regard to diets, the length of time on the diet impacts outcome as does the composition of the diet with high-fat only diets ranging in the percent of fat (from 25–60%) in the diet.

Prebiotics consist of starch, fructans (inulin and fructo-oligosaccharides), and galacto-oligosaccharides that contribute to the increase of genus *Bifidobacterium* and *Lactobacilli* and the decrease of the genus *Bacteroides*<sup>112</sup> which potentially beneficial for HSCT patients (<https://doi.org/10.1101/2020.04.08.20058198>) or cancer treatment with checkpoint blockade.<sup>32</sup> Enteral supplementation of glutamine, fiber, and oligosaccharide in a retrospective study of allogeneic HSCT showed less mucositis grade 3–4, reduced weight loss and diarrhea, and improved survival percent at day 100 post-HSCT.<sup>113</sup> In a recent article studying impact of pre-biotics and HSCT outcomes, resistant starch and prebiotics mixture (glutamine, polydextrose, and lactosucrose) were used pre-transplant and throughout 4 weeks post-HSCT. Patients that consumed prebiotics had reduced mucosal tissue damage and ameliorated acute GVHD.<sup>114</sup>

Probiotics are live microorganisms administered to improve health and have long been used as part of traditional diets. Supplementation of probiotics

restores commensal flora in the intestine and has been shown to promote growth of beneficial species, thereby improving intestinal microbiome diversity and HSCT outcomes. In murine models, probiotics has been studied with some positive results. Oral administration of *Lactobacillus rhamnosus* GG in drinking water before and after HSCT resulted in reduced bacterial translocation, improved survival, and reduced acute GVHD pathogenesis.<sup>115</sup> Administration of 17 butyrate-producing *Clostridia* spp. strains by oral gavage before and after allogeneic HSCT with an MHC mismatched model demonstrated a significant increase of butyrate in the intestine and better survival rate.<sup>116</sup> A cocktail of *Clostridium bolteae*, *Ruminococcus gnavus*, *Ruminococcus torques*, and *Blautia producta* delivered by oral gavage also showed significant survival in mice.<sup>117</sup> However, the efficacy of probiotics for HSCT in human is still questionable. The use of probiotics-enriched yogurt in a case report of a patient with autologous HSCT for treating mantle-cell lymphoma resulted in unexpected *Lactobacillus acidophilus* sepsis.<sup>118</sup> In line with that, another clinical study reported the bloodstream infections with *Lactobacillus* bacteremia in patients with autologous and allogeneic HSCT within the first 100 d post-HSCT which implied that the toxicities from immunosuppression by conditioning regimens and mucosal disruption could contribute to bacteremia from probiotics consumption.<sup>119</sup> In contrast, a study in children and adolescents undergoing allogeneic HSCT suggested that administration of probiotics with *Lactobacillus plantarum* was safe and feasible with no bacteremia case recorded.<sup>120</sup>

### Fecal microbiota transplantation

Aside from probiotics, another approach to introduce beneficial bacteria for HSCT patients for microbiome preservation or restoration is fecal microbiota transplantation (FMT). This is the method that is potentially applicable if patients have *Clostridium difficile* infections post-HSCT. Early clinical studies used FMT to treat patients with *Clostridium difficile* infections with high efficacy and minimal complications.<sup>121–127</sup> FMT was also performed in steroid-resistant acute GVHD patients with no adverse effect and resulted in the

restored microbiome diversity, an increase of peripheral regulatory T cells and better progression-free survival.<sup>128–130</sup> Most recently, when FMT was used in patients with steroid-refractory or steroid-dependent intestinal GVHD, researchers observed a significant increase of microbiome alpha-diversity, increased abundances of butyrate-producing Clostridiales and *Blautia*.<sup>131</sup> Taken together, FMT is a promising approach for intestinal GVHD patients and worth to be investigated further in a larger scale.

### Conclusions and future directions

Although microbiome characterization in HSCT studies has been investigated in the past decades, there are many questions remain unanswered regarding the “cause and effect” of specific bacterial taxa with the immune system, cell metabolism, and physiological consequences. Although obesity or high-fat diet consumption has been shown to affect microbiota profile and vice versa, little is known about whether microbiome changes in obesity, or high-fat diet exposure in either short-term or long-term could influence the HSCT outcomes. Most of the current concepts are descriptive and there is an urgent need on finding mechanism of how the different bacteria interact and how it affects immune parameters. The inbred laboratory mouse is still the cornerstone of preclinical HSCT modeling, but there is clearly a need to have it better reflect the human condition and involve human modifying factors such as obesity and prior infectious challenges, particularly in performing microbiome studies. This is also important with regard to finding a balance between having sufficient incorporation of the variables involved in human HSCT and also allowing for reproducibility of results. The gut pathology in the preclinical GVHD models does reflect the clinical scenario with common immune pathways and even similarities in microbiome content. Nonetheless, much more is needed to be known before targeted manipulation of the intestinal microbiome-immune system axis could be successfully applied as a therapeutic approach to limit HSCT complications such as GVHD and opportunistic infections, improve GVT effect and survival outcomes for patients. This not only applies to aGVHD affecting the gut but also in

other organs and cGVHD given the linkage of the gut microbiome throughout every organ and immune parameter.

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