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Gut microbiota injury in allogeneic haematopoietic stem cell transplantation

Yusuke Shono[1,2], **Marcel R. M. van den Brink**[1,3,4,*]

[1]Department of Immunology, Sloan Kettering Institute, Memorial Sloan Kettering Cancer Center, New York, New York, USA.

[2]Department of Medicine, State University of New York Downstate Medical Center, Brooklyn, New York, USA.

[3]Weill Medical College of Cornell University, New York, New York, USA.

[4]Adult BMT Service, Department of Medicine, Memorial Sloan Kettering Cancer Center, New York, New York, USA.

Abstract

Allogeneic haematopoietic stem cell transplantation (allo-HSCT) is considered to be the strongest curative immunotherapy for various malignancies (primarily, but not limited to, haematologic malignancies). However, application of allo-HSCT is limited owing to its life-threatening major complications, such as graft-versus-host disease (GVHD), relapse and infections. Recent advances in large-scale DNA sequencing technology have facilitated rapid identification of the microorganisms that make up the microbiota and evaluation of their interactions with host immunity in various diseases, including cancer. This has resulted in renewed interest regarding the role of the intestinal flora in patients with haematopoietic malignancies who have received an allo-HSCT and in whether the microbiota affects clinical outcomes, including GVHD, relapse, infections and transplant-related mortality. In this Review, we discuss the potential role of intestinal microbiota in these major complications after allo-HSCT, summarize clinical trials evaluating the microbiota in patients who have received allo-HSCT and discuss how further studies of the microbiota could inform the development of strategies that improve outcomes of allo-HSCT.

Allogeneic haematopoietic stem cell transplantation (allo-HSCT) is a combination of stem cell therapy, conventional therapy (with chemotherapy, radiation or antibodies) and immunotherapy[1]. Allo-HSCT consists of a conditioning regimen, including chemotherapeutic agents with or without total body irradiation and/or antibodies, that

* VandenbM@mskcc.org.

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destroys cancer cells and allows the recipient immune system to then accept an immune system-reconstituting infusion of donor HSCs. In addition, allogeneic donor T cells can attack residual tumour cells, resulting in graft-versus-tumour (GVT) activity. However, these alloreactive donor T cells can also attack target organs of the host, including the skin, liver, gut, thymus, central nervous system, ovary or testis as well as the haematopoietic system[1-6] (called graft-versus-host disease (GVHD)) (FIG. 1).

The impact of the gut microbiota on anticancer immune responses, including chemotherapy as well as allo-HSCT, is receiving increased attention. Recent studies in mice and humans suggest important relationships between the microbiota and outcomes in allo-HSCT recipients, and several clinical trials targeting the microbiota in allo-HSCT recipients are ongoing. Associations between certain bacteria and allo-HSCT outcomes have been found repeatedly despite the use of different levels of classification[7,8]. Studies in the 1970s and 1980s showed that the commensal microorganisms residing in the intestinal lumen, collectively termed the gut microbiota, play an important role in the pathophysiology of GVHD[9-11]. Recent advances in sequencing and identification of the taxonomic composition of the host microbiota have enabled detailed evaluation of microbiota–host interactions. This Review provides an update on our current knowledge of the roles of the gut microbiota in the most common complications after allo-HSCT for cancer therapy — infections, GVHD and relapse[7,12-16]. We also discuss potential roles of the microbiota in immune reconstitution as well as effects of dietary changes on the microbiota and how these factors affect GVHD after allo-HSCT. Finally, we summarize various strategies to target the microbiota to achieve better outcomes in allo-HSCT. The interplay between the gut microbiota and the host immune system (including Paneth cells, intestinal epithelial cells (IECs), short-chain fatty acids (SCFAs), antimicrobial peptides (AMPs), regulatory T (T_{reg}) cells and other immune cells) during GVHD has been discussed extensively elsewhere, so we do not cover this topic in detail. Similarly, in this Review, we do not discuss the roles of the microbiota in chemotherapy or immunotherapy in the context of antitumour activities (reviewed in REF.17).

Infectious complications of allo-HSCT

Microbial diversity predicts overall survival.

Healthy individuals have a diverse intestinal bacterial flora, which predominantly comprises members of the Firmicutes and Bacteroidetes phyla as well as populations of Proteobacteria, Actinobacteria, Verrucomicrobia and Fusobacteria[18]. During the course of an allo-HSCT, the diversity of the intestinal flora significantly decreases[19,20]. The changes can be rapid and are thought to be due to the effects of antibiotics, intestinal inflammation and changes in diet. Most patients are exposed to prophylactic and therapeutic antibiotics during neutropenia, which occurs in the first weeks after allo-HSCT[21]. They generally manifest loss of appetite owing to preconditioning treatment regimens, drug toxicity and GVHD[22,23] (FIG. 2). In a retrospective analysis of faecal specimens collected from 80 allo-HSCT recipients at the time of neutrophil engraftment — a critical time point indicating the establishment of donor haematopoiesis — a low diversity of the faecal flora was associated with significantly increased mortality (52%) compared with a high diversity of the faecal flora (8%)[24].

Specifically, a greater abundance of Gammaproteobacteria (including Enterobacteriaceae) was associated with higher mortality, whereas a greater abundance of Lachnospiraceae and Actinomycetaceae was associated with a more favourable outcome.

Infections following antibiotic treatment.

In allo-HSCT, simultaneous use of prophylactic antibiotics and antibiotic treatment of febrile neutropenia (FN) affect microbial diversity, which in turn increases susceptibility to infections (FIG. 2). Whereas a healthy individual usually carries on the order of 1,000 different species[25,26], near dominance of the intestinal flora, particularly by a single species of Proteobacteria, *Enterococcus* or *Streptococcus*, is frequently observed after allo-HSCT[19]. In particular, exposure to the antibiotic vancomycin or metronidazole is associated with *Enterococcus* domination[19,27]. Importantly, domination of *Enterococcus* or Proteobacteria was correlated with the increased risk of developing bacteraemia with vancomycin-resistant *Enterococcus* (VRE) or Gram-negative bacteria, respectively[19]. By contrast, exposure to fluoroquinolone antibiotics (ciprofloxacin or levofloxacin) decreased the risk of Proteobacteria domination[19]. Enterobacteriaceae and other Gram-negative bacteria from the phylum Proteobacteria are associated with increased mortality[24], and intestinal domination by Gammaproteobacteria was identified as a significant predictor of pulmonary complications (including infections) after allo-HSCT in a single-centre observational study irrespective of whether a patient received an antibiotic or had a pretransplant comorbidity[28]. These results are consistent with a hypothesis that antibiotic-induced elimination of commensal microbiota can result in expansion of pathogenic facultative bacteria in the gut consortium. Major microbiota injuries observed in studies of patients after allo-HSCT are summarized in FIG. 3.

Antibiotic treatment can also increase susceptibility to infections with the intestinal pathogen *Clostridium difficile*, especially in the setting of allo-HSCT, which involves major risk factors for *C. difficile* infection, such as immune suppression, chemotherapy and irradiation[29,30]. *C. difficile* infection early after allo-HSCT was positively correlated with high-intensity chemotherapy but not with antibiotic administration[31]. By contrast *Clostridium scindens* can contribute to resistance to *C. difficile* infection through secondary bile acid synthesis[32], which serves as an example of colony resistance (microbiota-mediated inhibition of specific bacteria) in allo-HSCT recipients.

Immune reconstitution after allo-HSCT is critical for the prevention of infectious complications. This could also be affected by the microbiota (BOX 1).

Clinical interventions.

Given the impact of microflora diversity loss in the gut in the course of allo-HSCT, interventions to maintain intestinal diversity might contribute to improved outcomes. One strategy could be to limit exposure to antibiotics that eliminate obligate anaerobic commensal bacteria; however, this is difficult to accomplish when patients require broad-spectrum antibiotics for FN, bacteraemia or sepsis. Another approach could be to restore microbiota diversity by faecal microbiota transplantation (FMT) with pretransplant faecal matter from the host or from a third party. Three case reports of FMT treatment for patients

with refractory *C. difficile*-associated disease after allo-HSCT have been published showing the effectiveness and safety of this method[33]. A clinical trial to restore diversity and prevent *C. difficile* infection during allo-HSCT is currently underway[34] (TABLE 1). In this study, recipients who show sufficient microbial diversity before allo-HSCT and a post-transplant loss of the protective phylum Bacteroidetes will receive their own pretransplant flora at the time of engraftment. However, there is concern for possible FMT-related sepsis in an immunocompromised host through bacterial translocation or norovirus infections, as reported in inflammatory bowel disease (IBD) or patients infected with *C. difficile*[35,36]. In addition, a recent case report in a patient with *C. difficile* infection demonstrated overgrowth of methane-producing bacteria contracted from the FMT donor, resulting in IBD-like symptoms without evidence of infections and gastrointestinal pathologic abnormalities[37]. Given these caveats, larger studies and long-term follow-up to evaluate the outcomes of FMT are required.

GVHD and the microbiota

Disruption of the intestinal barrier and homeostasis.

The gut is a primary GVHD target organ and plays a major role in the development of GVHD[38]. Conditioning regimens for allo-HSCT disrupt the equilibrium between host and microbiota, resulting in mucositis, organ dysfunction and increased susceptibility to infections (FIG. 2). For example, bacterial lipopolysaccharide (one of the bacterial ligands) can pass through the damaged intestinal barrier during conditioning and activate the innate immune system[39]. The innate immune system plays an important role in the activation of alloreactive donor T cells, which are an absolute requirement for the development of GVHD. Pathways involved in the interplay between the gut microbiota and the host's innate immune system during GVHD have been extensively described elsewhere[21,40-53], and these relationships are summarized in FIG. 4. IECs maintain physical and biochemical barriers to separate intestinal tissues and luminal contents[54].

Not only bacteria themselves but also their metabolites can be excluded from the bases of the crypts where intestinal stem cells reside[55]. Paneth cells constitute a group of IECs, which reside in the intestinal crypts and secrete AMPs, leading to an AMP gradient along the intestinal crypt[56]. Studies in mice and humans show a marked reduction of Paneth cells during GVHD[12,14,57], causing bacteraemia with impaired barrier function. Paneth cells also constitute the niche for leucine-rich repeat-containing G protein-coupled receptor 5 $(LGR5)^{+}$ intestinal stem cells and exert trophic effects through the epidermal growth factor (EGF), WNT3 and NOTCH signalling pathways[58] as well as through interleukin-22 (IL-22), which increases epithelial regeneration and improves GVHD-related mortality in mouse models[59]. Indole-3-aldehyde is a bacterial metabolite of tryptophan that activates the aryl hydrocarbon receptor on innate lymphoid cells (ILCs). It mediates colonization resistance and induces IL-22 secretion by a specific group of ILCs[60,61]. The secretion of the AMP regenerating islet-derived protein 3γ (REG3γ) by Paneth cells (a biomarker of intestinal GVHD[62]) is regulated by IL-22, and REG3γ plays a critical role in the host defence against bacterial pathogens, possibly by maintaining flora diversity[63,64]. A phase II clinical trial utilizing recombinant human IL-22 in combination with standard systemic corticosteroid treatment

for intestinal GVHD is ongoing[65] (TABLE 1). A report in a mouse model of GVHD showed that recombinant R-spondin 1, an essential growth factor for intestinal stem cells, restored gut microflora dysbiosis by normalizing faecal levels of the AMP α-defensin and promoted the restoration of Paneth cells[66]. These findings indicate the importance of protecting gut microbiota to maintain the intestinal barrier and homeostasis.

Microbiota injury during GVHD.

Studies in mouse models of allo-HSCT conducted in the 1970s showed that recipient germ-free mice kept in continuous germ-free conditions throughout the course of allo-HSCT, or decontaminated with high-dose antibiotics starting 1 week before transplant manifested significantly reduced mortality from GVHD[10]. In this study, the protective effect of a germ-free environment was most evident when bone marrow alone was used as a stem cell source, and the effect was less when splenocytes (which include alloreactive T cells) were added to induce GVHD. This finding might indicate that GVHD is a result of the balance of innate immune system activation and the number of alloreactive T cells. Thereafter, in a clinical report of 130 patients with aplastic anaemia who were undergoing allo-HSCT from human leukocyte antigen (HLA)-identical siblings, gut decontamination and laminar-airflow isolation were shown to lower the cumulative incidence of grade II–IV GVHD[11]. In these early reports, no specific bacterial species were reported to be responsible for the severity of GVHD or transplant-related mortality (TRM). With recent advances in DNA sequencing and improved techniques of culturing gut-resident microorganisms, changes in the gut microbiota composition during GVHD after allo-HSCT have been studied in more detail. As mentioned, changes in intestinal microbiota after allo-HSCT can occur very rapidly[19]; this warrants careful longitudinal studies to better assess and define these changes in the ecology of the intestinal microbiota.

Loss of bacterial diversity affects GVHD-related outcomes after allo-HSCT. A subgroup analysis of the previously mentioned study[24] revealed that loss of bacterial diversity in stool specimens collected within 7 days after neutrophil engraftment was associated with increased mortality from GVHD. Similarly, an evaluation of 64 patients after allo-HSCT demonstrated decreased bacterial diversity in stool specimens collected on day 12, which was correlated with increased GVHD-related mortality[13]. In another study, ileal bacterial densities in control and GVHD mice were not different; however, there was an increased abundance of Lactobacillales and a reduced abundance of Clostridiales during GVHD, which was confirmed in stool specimens of patients with GVHD[14]. Treatment with ampicillin, which targets Lactobacillales, worsened GVHD, and re-introduction of *Lactobacillus johnsonii* after ampicillin treatment improved GVHD. Other reports described an expansion of *Enterococcus* spp., which belong to the same order of Lactobacillales as *Lactobacillus* spp., during GVHD in mice[67] and humans[7], which may potentially contribute to increased inflammation and reduced gut integrity, given the evidence that *Enterococcus* spp. can compromise epithelial barrier integrity[68] and stimulate tumour necrosis factor (TNF) production from macrophages[69].

By contrast, Clostridiales play important anti-inflammatory homeostatic roles, including upregulation of T_{reg} cells through the production of the SCFA butyrate[70-74]. Butyrate

increased the recovery of IEC damage after allo-HSCT, and the re-introduction of a mixture of 17 strains of human Clostridiales isolates, which produce high levels of butyrate, prolonged the survival of mice with GVHD[75]. Clinical data indicate that increased abundance of the *Blautia* genus, which belongs to the class Clostridia, is significantly associated with less GVHD-related mortality and improved overall survival[13].

Other preclinical studies have reported an association between expansion of *Escherichia coli*[12] and *Bacteroides* spp. or *Prevotella* spp.[67] and worse GVHD. Also, the Muribaculaceae family could play a role in IL-17-mediated GVHD, possibly through antagonistic and protective roles in GVHD pathology[76]. Recent studies showed that patients with GVHD have more Firmicutes and Proteobacteria and fewer Bacteroidetes than those without GVHD[77,78] (REF. 78 is an abstract from the 57th American Society of Haematology Annual Meeting). Most of these studies report associations between these specific microorganisms and the severity of GVHD but lack mechanistic insights.

Little is known regarding the role of donor microbiota in the development of GVHD. One study in mouse models showed no difference in the severity of GVHD between recipients of an allograft from germ-free mice versus those receiving allografts from specific pathogen-free (SPF) mice[79]. Dietary changes after allo-HSCT may also contribute to microbiota injury and GVHD. See BOX 2 for a description of oral nutrition versus total parenteral nutrition and the impact of such diets on microbiota.

We hypothesize that the outcome of allo-HSCT depends on the complex interactions among various bacterial species (that is, ecology), and it is important to note that perturbation of microbiota ecology by GVHD or antibiotic treatment may also result in impaired colonization resistance, resulting in the expansion of certain microbial pathogens. Data are limited regarding the composition of favourable microbiota ecologies and allo-HSCT, and it would be of great interest to explore this unique ecology in allo-HSCT.

Manipulation of the microbiota — the effect of antibiotics on GVHD.

The finding that higher bacterial diversity is associated with improved overall survival after allo-HSCT raises questions regarding the practice of bacterial decontamination in allo-HSCT recipients[49]. As mentioned above, initial studies in mice and humans indicated that gut decontamination with broad-spectrum antibiotics followed by treatment in sterile laminar-airflow isolation could decrease the incidence of acute GVHD in allo-HSCT recipients[9-11]. In addition, a more recent randomized study[80] showed that the addition of metronidazole (which targets anaerobic bacteria) to ciprofloxacin as antibacterial prophylaxis resulted in reduced GVHD. However, other clinical studies did not confirm the benefit of decontamination for reducing GVHD[81-83]. Subsequently, the practice of laminar-airflow isolation was abandoned by most centres in the early 1990s.

A more recent retrospective study of 15 paediatric patients reported that the use of antibiotics effective against anaerobic bacteria resulted in a significant increase in GVHD with a reduction of gut anti-inflammatory Clostridia[84]. In this report, addition of clindamycin to levofloxacin in a mouse model of GVHD reduced Clostridia and exacerbated GVHD, recapitulating the findings from patients. In addition, a recent retrospective study of

500 patients from Canadian centres[85] showed that gut decontamination before allo-HSCT resulted in higher incidence of grade II–IV acute GVHD. It is difficult to determine the causes for these different results; one possible explanation maybe the emergence of antibiotic-resistant enterococci, which makes successful gut decontamination very difficult.

Indeed, a recent retrospective study of 112 children undergoing allo-HSCT showed that failure of successful prophylactic decontamination monitored by microbiological surveillance, including stool culture, was associated with a significant increase in acute GVHD[86]. Colonization with antibiotic-resistant bacteria has been reported in the course of allo-HSCT[19,27] and is associated with increased incidence of acute GVHD[87]. Meanwhile, a study of the prophylactic use of the broad-spectrum antibiotic meropenem during episodes of neutropenia (not for the purpose of prophylactic gastrointestinal decontamination) reported a favourable effect on morbidity by reducing febrile episodes without increasing the risk of infection from multidrug-resistant bacteria after allo-HSCT[88]. This study did not evaluate the risk of the broad-spectrum antibiotic use for the development of GVHD, and overall, no consensus has emerged regarding the ideal choice of antibiotic coverage for fever and neutropenia in allo-HSCT recipients. A recent retrospective single-centre study[15] tried to address this question by analysing the effects of different-spectrum antibiotics for treating FN on the intestinal flora and transplant-related outcomes and found that the use of antibiotics with relatively more broad-spectrum activity, such as piperacillin–tazobactam, compared with the use of antibiotics with lesser broad-spectrum activity, such as cefepime, increased GVHD-related mortality. Administration of antibiotics with more broad-spectrum activity resulted in increased loss of commensal flora, including Bacteroidetes, *Lactobacillus* spp. and Clostridia. Further investigation in a mouse model of GVHD demonstrated that administration of antibiotics with more broad-spectrum activity could increase GVHD mortality. This was associated with similar changes in the intestinal microbiota, as seen in patients, as well as an increased abundance of *Akkermansia muciniphila*, which contributed to the degradation of the mucous layer in the colon. Corroborating these findings, metagenomic shotgun sequencing revealed increased levels of mucus-degrading bacterial enzymes in the stool specimens of GVHD mice treated with antibiotics with more broad-spectrum activity. These mice also showed worse GVHD of the colon, increased numbers of colon-infiltrating allogeneic $CD4^+$ T cells and increased levels of IL-23 in the colon. A phase II randomized clinical trial is currently underway to evaluate the contribution of broad-spectrum antibiotics and microbiota injury to the development of GVHD by comparing piperacillin–tazobactam with cefepime as a treatment for FN[89] (TABLE 1).

Another report confirmed the deleterious effect of antibiotics in a xenogeneic GVHD mouse model: a cocktail treatment of ampicillin, clindamycin, vancomycin and cefoperazone in the drinking water after allo-HSCT resulted in the development of more-aggressive GVHD[90]. Regarding the timing of systemic antibiotic administration and GVHD-related mortality, a retrospective study of 621 patients at two transplant centres showed that early exposure to antibiotics (between day −7 and day 0 of allo-HSCT) resulted in significant reduction of Clostridiales and increased GVHD-related mortality[91]. Taken together, these results suggest that broad-spectrum antibiotics can exacerbate colonic GVHD, and this supports the hypothesis that selecting limited-spectrum antibiotics can prevent microbiota injury and reduce colonic GVHD and GVHD-related mortality.

Although the use of fluoroquinolone prophylaxis to reduce neutropenia-related complications in patients with cancer has been disputed with the emergence of resistant organisms[92-95], clinical practice guidelines[96] recommend use of a fluoroquinolone for cancer patients with neutropenia to prevent febrile events and blood infections, a practice that was found in a meta-analysis to be associated with a reduction in all-cause mortality[97]. As an alternative to a fluoroquinolone (ciprofloxacin), a recent retrospective analysis in allo-HSCT recipients compared rifaximin (a poorly absorbed member of the rifamycin family widely utilized in IBD[98,99] with less activity against Enterobacteriaceae) with ciprofloxacin plus metronidazole and found significantly reduced gut GVHD, reduced TRM and improved overall survival[100].

Taken together, the above findings suggest the need for well-designed prospective studies to optimize the use of antibiotics in neutropenic allo-HSCT recipients to protect from bacteraemia or sepsis while limiting injury to the intestinal microbiota that could increase GVHD mortality. A randomized phase II clinical trial evaluating the impact of gut decontamination with vancomycin plus polymyxin B on microbial diversity after allo-HSCT is now underway[101] (TABLE 1). In this paediatric population (4–23-year-old recipients of HLA-matched allo-HSCT), the effects of decontamination on immune reconstitution and GVHD are being analysed. Future studies should be performed together with detailed assessment of bacterial susceptibility to antibiotics, as the 16S ribosomal RNA sequence cannot provide this information. An example is the simultaneous use of metagenomic and metatranscriptomic sequencing in a study, which demonstrates a higher overall number and expression levels of antibiotic resistance genes in the microbiome after antibiotic treatment[102].

The metabolome and GVHD.

Microbial metabolites can affect host immune responses and protect against the development of inflammatory diseases[103,104]. Bacterial fermentation in the colon results in the production of carbohydrate metabolites, including SCFAs, such as acetate, butyrate and propionate[105]. Butyrate and to a lesser extent propionate have been reported to induce T_{reg} cells by regulating molecules that improve the homing of T_{reg} cells to gut mucosa and increase forkhead box protein 3 (FOXP3) expression in the colon[73,106]. An initial report showed that acetate can ameliorate inflammation in the gut, lung and joints and that this effect was lost in mice deficient for its receptor G protein-coupled receptor 43 (GPR43; also known as FFAR2)[107]. Moreover, SCFAs are important energy sources for the microbiota itself as well as for IECs and are known to inhibit histone deacetylase (HDAC) activity[108]. A recent study analysed a possible role of SCFAs during GVHD[75]. The concentration of butyrate in the intestinal tissue of recipient mice was significantly decreased at day 7 after allo-HSCT, and this reduction coincided with impaired integrity of IEC junctions. When butyrate was given by intragastric gavage, integrity of IEC junctions was preserved, mitigating GVHD severity. The above-mentioned findings indicate that local changes of microbial metabolites can reduce intestinal epithelial tissue damage and mitigate GVHD severity. On the other hand, it should also be noted that butyrate may have cell type-specific effects, as it appears to inhibit the proliferation of intestinal stem cells[55].

Relapse and the microbiota

The gut microbiota are potent modulators of systemic immune responses, and there is mounting evidence that microbiota can affect cancer immunosurveillance[50,109-115]. Several studies showed that intestinal microbiota can influence the immune response to systemic cancer chemotherapy, radiotherapy and immunotherapy[17,116] and that disruption of the gut microbiota is associated with resistance to cancer therapy[109,114]. GVHD (especially chronic GVHD) is inversely associated with relapse[1,2,117], as the allogeneic T cells causing GVHD also contribute to GVT activity. Because the development of GVHD is associated with changes in the intestinal flora as described above, studies to analyse changes in the intestinal microbiota and relapse after allo-HSCT seem warranted. A retrospective observational analysis of 541 patients undergoing allo-HSCT at a single centre identified a cluster of bacteria dominated by *Eubacterium limosum* that could serve as a biomarker of relapse risk: higher abundance of this cluster was associated with less relapse[16]. In this report, multiple stool specimens collected between day 0 and day 21 were analysed by 16S ribosomal RNA sequence. A time-weighted average abundance over the 3-week period was utilized to accommodate variegated collection time points and numbers of samples for each point. This methodological approach reduces biases that could occur when sampling only a single time point in the setting of the rapid microbiota shifts after allo-HSCT. *E. limosum* is a common member of the human microbiota[118] and is known for its beneficial effect in an experimental model of colitis, which occurs through an increased butyrate production in the caecum[119]; however, no mechanism has been explored with regard to how this bacterium serves in the setting of allo-HSCT and GVHD. This finding may serve as a potential diagnostic biomarker or perhaps as a therapeutic target (for example, using a probiotic strategy discussed in the following section) to prevent relapse and improve overall survival after allo-HSCT, although these results need to be validated in multicentre observational and prospective clinical trials.

Targeting the microbiota

Various factors during allo-HSCT, including conditioning regimen, diet, infections, antibiotic use and immune reactions, result in some of the most drastic and rapid perturbations of the intestinal flora seen in any patient group[19,20] (FIG. 5). Strategies targeting the microbiota to improve allo-HSCT can be summarized into four broad types: antibiotic, probiotic, prebiotic and postbiotic. In addition, the microbiota has potential for diagnostics[120].

Antibiotic strategies.

Antibiotic strategies include selecting more narrow-spectrum agents (for example, cefepime or rifaximin) that potentially spare beneficial anaerobic bacteria contributing to an anti-inflammatory milieu after allo-HSCT (for example, *Blautia* spp.) and minimizing the duration of antibiotic use that may result in an outgrowth of certain bacteria (for example, mucus-degrading bacteria such as *A. muciniphila*). Several strategies are being developed to prevent antibiotic-induced dysbiosis for purposes of preventing *C. difficile*-associated diarrhoea. If successful, such approaches, which include orally administered β-lactamases or activated charcoal, could be explored in the setting of GVHD[121,122].

Prebiotic strategies.

Prebiotics are indigestible compounds, commonly oligosaccharides, that commensal bacteria have an advantage in metabolizing; this metabolism increases the production of metabolic products through bacterial fermentation[33]. Prebiotics can be administered to patients. Encouraging oral intake in general, or the administration of gastric nutritional supplementation and/or flora-targeted nutritional supplements such as inulin-type fructans and arabinoxylans (for example, arabinose and xylose), can also be considered. A phase I study administering fructooligosaccharide in patients undergoing allo-HSCT is ongoing[123] (TABLE 1). Another clinical study evaluating the effect of a dietary supplement containing potato-based starch in allo-HSCT recipients is recruiting patients[124] (TABLE 1) and is based on the observation that this starch can increase the production of butyrate by the microbiota in healthy subjects[125].

Probiotic strategies.

A probiotic strategy consists of directly introducing one strain or selected strains of microorganisms that confer a benefit. This can be achieved by FMT (including nasoduodenal, trans-colonoscopic or enema-based applications or even oral intake of microbiota capsules).

Most patients show a decrease in bacterial diversity in the weeks after allo-HSCT, and up to one-half of bacteraemia episodes after allo-HSCT are preceded by intestinal bacterial domination of a pathobiont[7,19], suggesting the potential effectiveness of administering probiotics that prevent pathobiont colonization. As mentioned above, there have been some case reports describing the effectiveness of FMT for refractory *C. difficile*-associated disease after allo-HSCT[33], and a phase II clinical trial testing this is currently underway[34] (TABLE 1). A recent clinical study indicated the safety and efficacy of FMT in patients with steroid-refractory or steroid-dependent gut GVHD[126]. In this small pilot study, three out of four patients receiving FMT from their spouses or relatives achieved complete responses after the treatment, which was correlated with an increase in T_{reg} cells in the peripheral blood. Another study showed successful FMT in three patients with grade IV acute GVHD involving the lower gastrointestinal tract after the failure of established immunosuppressive therapies, including corticosteroids[127]. In this case report, healthy adult subjects were the faecal donor sources, and after one to six doses of FMT, all patients showed improved diversity of the gut microbiota. Treatment with oral, capsulized, frozen FMT (‘crapsules’)[128] is a potential method for maintaining bacterial diversity after allo-HSCT, and a phase I study to determine the feasibility of the treatment in allo-HSCT recipients is ongoing[129] (TABLE 1). Data are limited for determining the optimum faecal donor, and it is still undetermined whether it is better to use FMT from the stem cell donor or from a healthy random volunteer or sibling. Further studies are needed to prove the effectiveness of FMT in reducing GVHD and infection or in enhancing overall survival in allo-HSCT recipients.

The surrounding environment of the patients may also affect the diversity of the gut microbiota after allo-HSCT[130], and an ongoing clinical study aims to compare the incidence of acute grade II—IV GVHD between patients receiving patient-centred medical home care and those receiving standard hospital care[131] (TABLE 1). Administration of *Lactobacillus*

spp. was also shown to ameliorate GVHD in murine GVHD models[14,132], and a clinical trial is ongoing to analyse the benefit of oral *Lactobacillus rhamnosus* str. GG administration in the improvement of GVHD[133] (TABLE 1). Of note, a case report describes a potential risk of probiotic use in a patient who was immunocompromised and experienced sepsis after excessive consumption of probiotic *Lactobacillus acidophilus*-enriched yogurt after autologous transplant[134]. Other probiotic bacteria, such as *Bifidobacteria* spp., have been associated with improved responses to cancer immunotherapy[112] and resistance to *E. coli* infection[135] in animal models. As a potential strategy for passive bacterial modification in the gut in order to improve GVHD outcome, oral immunoglobulin administration can be used to reduce pathogenic bacteria such as *E. coli* and increase the amount of beneficial probiotic bacteria such as *Lactobacillus reuteri*. In a mouse study[136], immunoglobulin yolk antibodies from hens immunized with pathogenic *E. coli, Clostridium perfringens* and *Salmonella enterica* subsp. *enterica* serovar Typhimurium were administered starting 2 days before allo-HSCT, and this method led to reduced clinical GVHD scores and improved survival. In the near future, probiotic bacteria could likely be genetically engineered to produce certain metabolites or AMPs that mediate a benefit to the host.

Postbiotic strategies.

Postbiotic interventions involve providing bacterial metabolic products directly, bypassing the bacteria completely. This approach might be safer than probiotic strategies in allo-HSCT recipients who are severely immunocompromised. Methods include administering SCFAs (for example, butyrate) or their analogues or introducing secondary bile acids that have been shown to suppress *C. difficile*[32,75]. A pilot study of five patients with recurrent *C. difficile* infection indicated a potential benefit of sterile faecal filtrates — a safer method than FMT — that contain bacterial components as well as metabolites but do not contain live microorganisms to restore microbial diversity[137]. As a potential organic compound to modulate microbiota other than SCFAs, indole and its derivatives inhibit the growth of Gram-negative bacteria and *Candida* spp.[61,138]. In addition, various microbiota-derived aryl hydrocarbon receptor ligands have been utilized to stimulate mucosal immune cells to attenuate intestinal inflammation in a mouse colitis model[139], and potentially, this could improve gut integrity and prevent gut GVHD.

Diagnostics.

Detailed studies of the alterations of the microbiota in GVHD or malignant relapse following allo-HSCT are ongoing and suggest changes in the abundance of one or more bacteria (for example, *Blautia* spp.[13] and *E. limosum*[16]) at certain time points during allo-HSCT. These could lead to the development of bacterial diagnostic or prognostic biomarkers. Also, a clinical report suggests that genotyping of fucosyltransferase 2 (FUT2) can serve as a biomarker for GVHD and bacteraemia after allo-HSCT[140]. The *FUT2* gene is known to regulate ABH blood group antigens in the mucous layer and is reported to play a role in shaping host microbiota[141-143]. The *FUT2* genotype influences the risk of Crohn's disease as well as the risk of GVHD and bacteraemia after allo-HSCT, possibly through alterations of glycosylation of intestinal surface proteins and microbiota composition of the host[140,141]. Furthermore, 3-indoxyl sulfate, a urinary metabolite produced by Clostridia, is another potential biomarker of gut microbiota health, and it predicts reduced gut GVHD and

survival in patients[7]. Loss of bacterial diversity correlates with mean urinary 3-indoxyl sulfate levels, and the Firmicute families Lachnospiraceae and Ruminococcaceae (both in the Clostridia class) were identified as important contributors to the production of 3-indoxyl sulfate. These bacterial families are linked to reduced intestinal inflammation[138]. Given the rapid turnaround time of the assay testing for 3-indoxyl sulfate levels, it could potentially be applied to evaluate the health of microbiota in patients in clinical trials where the aim is to target restoration of injured microbiota during GVHD.

Conclusions and future directions

There has been great progress since the 1970s in discerning the role of the microbiota in allo-HSCT. Preliminary studies that may lead to novel strategies to improve outcomes after allo-HSCT include those investigating FMT, prebiotic (oligosaccharide and potato-based starch) administration, the use of different antibiotics, gut decontamination and the role of *E. limosum* in relapse. Given that microbiota composition can vary owing to geographic differences[144,145], it is critical to carry out multicentre studies. Further studies are needed to better characterize the changes in the microbiota during allo-HSCT and GVHD in a prospective multicentre fashion, the variables that affect these changes of intestinal microbiota, and the effects of bacterial ligands[146] and their metabolites on both the immune and non-immune phenotypes of the host. Novel approaches that protect the microbiota or restore it after injury need to be evaluated using multi-institutional studies; such new findings should change the current standard care routines for allo-HSCT recipients.

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Alloreactive donor T cells

T cells that have been activated after encountering antigens within the context of major histocompatibility complex (MHC) molecules other than those present during thymic selection. After allogeneic haematopoietic stem cell transplantation, T cells can be allogeneically activated by encounter with an unknown (foreign) MHC molecule (major histocompatibility mismatch) or with a known (matched) MHC molecule presenting a foreign antigen (minor histocompatibility mismatch).

Neutropenia

A condition of abnormally low numbers of neutrophils within the blood. This is an immune system condition that may lead to infections.

Febrile neutropenia

(FN). The development of fever, often with other signs of infections, occurring during the period of low neutrophil counts (neutropenia) after allogeneic haematopoietic stem cell transplantation or chemotherapy for cancer. Neutropenia typically occurs 8–12 days after chemotherapy and typically lasts for 10–25 days.

Bacteraemia

The presence of bacteria in the blood (usually resulting from impaired gut barrier function owing to a conditioning regimen and/or graft-versus-host disease in allogeneic haematopoietic stem cell transplantation) that leads to sepsis.

Facultative bacteria

A category of bacteria that can thrive under aerobic or anaerobic conditions.

Gut consortium

A group of microbiota species or strains. Interactions among the members of a consortium may form an ecological community in which members compete with one another or in which products of one member are utilized by or are toxic to another member.

Sepsis

The immune reaction of the host to bacteria.

Bacterial ligands

Components of the bacterial cell surface that interact with the host cell receptor. They interact with phagocytes and stimulate subsequent evasion of innate immune responses.

Innate lymphoid cells

(ILCs). A population of immune lymphoid cells that are morphologically similar to B cells and T cells but lack pattern-recognition receptors and rearranged antigen receptors (and thus are unable to directly mediate antigen-specific responses). They secrete a high concentration of cytokines and play key roles in effector and regulatory functions in innate immunity, metabolic homeostasis and tissue remodelling.

Germ-free mice

Also known as gnotobiotic mice, these mice are maintained under strictly sterile conditions in which absolutely no bacteria are present in or on the animals. Individual bacterial strains or groups of strains (consortia) can be introduced in a controlled fashion (axenic conditions).

Aplastic anaemia

A form of bone marrow failure in which the bone marrow cannot make enough new blood cells. It is characterized by peripheral pancytopenia (decrease of red blood cells, white blood cells and platelets) and bone marrow hypoplasia.

Laminar-airflow isolation

A method that provides a low-pathogen environment and prevents exogenous infection during hospitalization of patients with neutropenia and other immunodeficient diseases. It involves filtered air moving along separate parallel flow planes to patient rooms with no or minimal crossover of air streams (or lamina).

Specific pathogen-free (SPF) mice

Mice housed under standard laboratory conditions. These mice are free of specific pathogens but otherwise have a normal microbiota. The lists of specific pathogens tested vary by supplier and institution and typically include opportunistic organisms that do not cause illness in immunocompetent hosts but can be harmful to immunocompromised mice, for example, after allogeneic haematopoietic stem cell transplantation.

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Oral nutrition

The intake of food through the mouth and oesophagus to provide nutrients for health.

Total parenteral nutrition

A method to feed a person intravenously, completely bypassing the usual process of eating and digestion. The nutritional formulae typically include glucose, amino acids, lipids and salts, with the supplementation of vitamins and minerals as needed.

16S ribosomal RNA sequence

A method used for identification, classification and quantification of microorganisms (the 16S ribosomal RNA subunit is an essential, highly conserved component in the 30S ribosomal complex in prokaryotes).

Enteral nutrition

A method of delivering a person's calorific nutritional requirements by use of a tube (tube feeding) into the stomach, achieved by syringe, gravity or pump.

Pathobiont

A potentially harmful (disease-causing or pathological) organism that under normal conditions lives as a component of a healthy microbiota population (symbiont).

Box 1 |

Microbiota and immune reconstitution

Given the recent findings of the role of microbiota in immune homeostasis[21,49], it is possible that intestinal microbiota could affect immune reconstitution after allogeneic haematopoietic stem cell transplantation (allo-HSCT) and could be targeted as a strategy to increase post-transplant immunity. Defects in haematopoiesis have been reported in germ-free mice; specifically, the intestinal microbiota promotes steady-state myeloid cell development and increases the differentiation potential to granulocyte and/or monocyte progenitors (GMPs)[147]. Similarly, mice deficient in myeloid differentiation primary response protein MYD88 or nucleotide-binding oligomerization domain-containing protein 1 (NOD1) signalling (pathways involved in bacterial sensing and resulting immunomodulation) manifested reduced numbers of myeloid cells and GMPs[148-150], and antibiotic-treated mice in steady-state showed multilineage repression of haematopoiesis across all progenitor subtypes in the bone marrow[151]. How the intestinal microbiota controls immune responses through distant sites such as the bone marrow remains to be fully revealed. A syngeneic mouse transplant model indicates the occurrence of delayed immune reconstitution in antibiotic-treated recipients (M.R.M.v.d.B. and Y.S., unpublished observations). Studies are warranted to explore the effects of microbiota changes on haematopoiesis and immune reconstitution in allo-HSCT recipients.

A study comparing the immune systems of specific pathogen-free (SPF) mice commonly used in laboratory experiments with those of feral mice (or mice from city pet shops) found that SPF mice possess fewer effector T cells in the intestinal mucosa than feral mice and that SPF mice are similar to human neonates[152]. Co-housing of laboratory ‘clean’ SPF mice with ‘dirty’ feral mice resulted in more effector and memory T cell populations, as typically seen in adult humans, indicating that the microbiota plays an important role in shaping the immune system. In another example, vaccine-induced murine immune responses are altered in dirty mice co-infected with chronic viruses and helminths and recapitulate the responses seen in adult humans (evaluated by gene expression changes)[153], which could provide an avenue to better stimulate the human immune response to vaccination.

Box 2 |

Effect of dietary changes on GVHD

Owing to the conditioning regimen, most allogeneic haematopoietic stem cell transplantation (allo-HSCT) recipients will incur some degree of nausea, mucositis and anorexia, resulting in a deterioration of their nutritional status in the first weeks after allo-HSCT[22,23]. Impaired nutritional status after allo-HSCT negatively affects patient prognosis[154]. Fat-restricted or carbohydrate-restricted diets result in the increase of Bacteroidetes in humans[155]. Other reports show that an energy restriction by 35% for 6 weeks results in increased bacterial diversity[156]. It is important to maintain proper oral intake of diet in allo-HSCT recipients, as changes in the microbiota induced by reduced dietary intake after allo-HSCT can be related to increased graft-versus-host disease (GVHD) and poor outcomes of allo-HSCT.

Total parenteral nutrition (TPN) is widely used to support allo-HSCT recipients, and various recommendations to improve the nutritional status of patients with GVHD after allo-HSCT have been made, including the use of TPN and a diet progression protocol for patients with gastrointestinal GVHD[157-161]. The detailed effects of TPN on microbiota changes have not been documented, and it is still under debate whether oral nutrition should be preferred over TPN to prevent microbiota injury and GVHD[157,162,163]. Oral nutrition might be more beneficial than TPN with respect to the protection of gastrointestinal digestive as well as barrier functions, and two small studies showed that TPN treatment was associated with higher acute GVHD incidence and worse survival[164,165]. Moreover, in one retrospective study in allo-HSCT recipients, loss of *Blautia* spp. was associated with receiving TPN for more than 10 days, which possibly contributed to increased GVHD mortality[13]. One prospective randomized multicentre study comparing enteral nutrition with TPN with regard to transplant-related mortality and GVHD is ongoing[166] (TABLE 1). Nutritional assessment in patients after allo-HSCT is very challenging, as body weight is not a reliable indicator of nutrition when patients experience excessive fluid retention or loss caused by dynamic inflammatory status (including GVHD) after allo-HSCT. Establishing an accurate nutritional assessment of patients through multidisciplinary efforts involving nurses, haematologists, dieticians, nutritionists, medical assistants and nurse practitioners is necessary for future research to elucidate the impact of dietary changes on GVHD through microbial modulation.

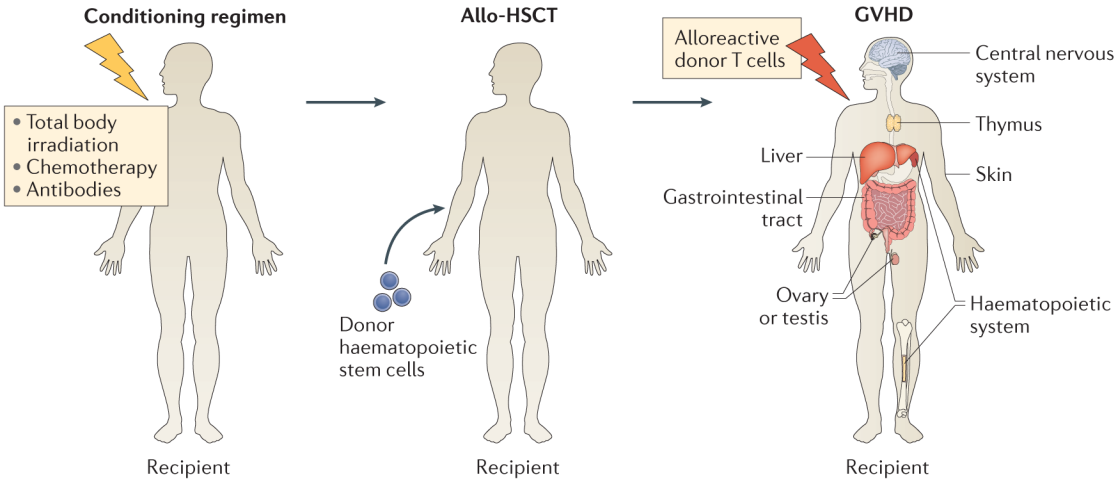

Figure 1 |. Allo-HSCT and GVHD.

Patients undergoing allogeneic haematopoietic stem cell transplantation (allo-HSCT) receive a pretransplant regimen including chemotherapeutic agents with or without total body irradiation and/or antibodies. Patients receive donor haematopoietic stem cells after this pretransplant regimen. Graft-versus-host disease (GVHD) occurs when alloreactive donor T cells attack host tissues, including the skin, liver, gastrointestinal tract, central nervous system, thymus, ovary or testis as well as the haematopoietic system.

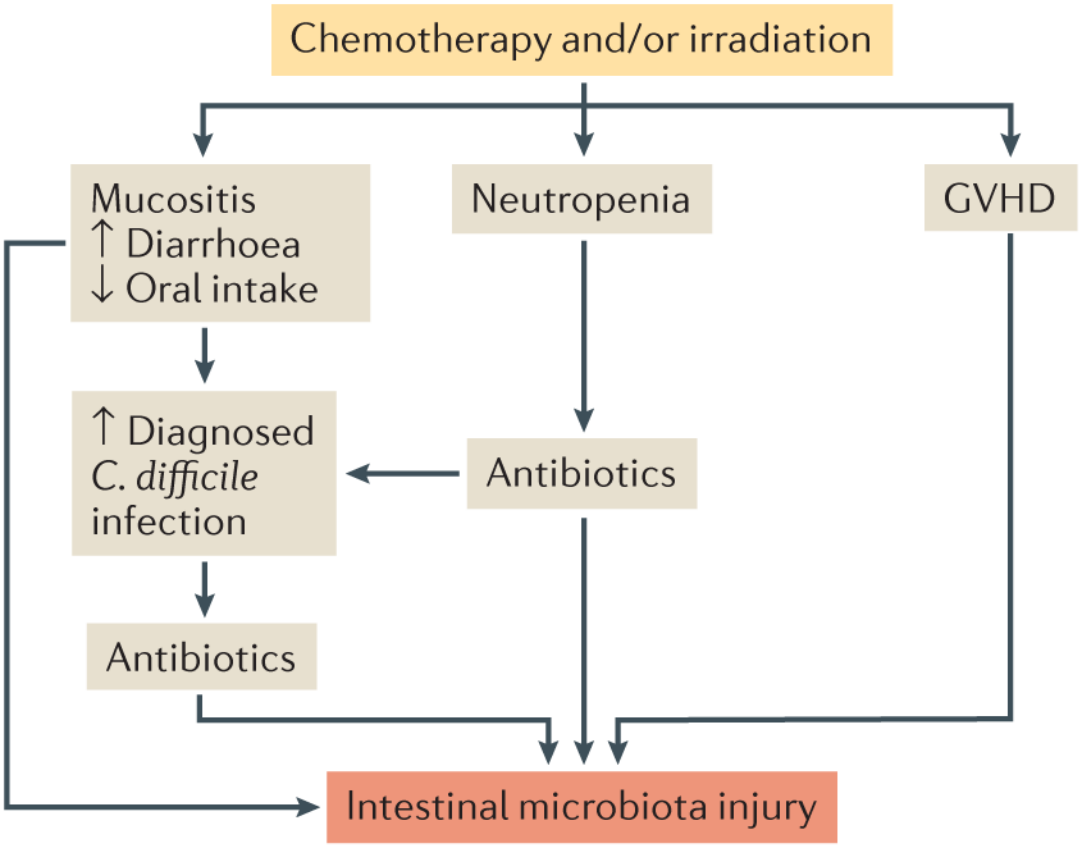

Figure 2 |. Intestinal microbiota injury after allo-HSCT.
Major factors altering microbiota homeostasis after allogeneic haematopoietic stem cell transplantation (allo-HSCT) include conditional chemotherapy and/or irradiation, antibiotic therapy, graft-versus-host disease (GVHD), mucositis and infection (for example, with *Clostridium difficile*). Patients experience neutropenia after a conditioning regimen, and this often requires treatment with antibiotics. These factors contribute to microbiota dysbiosis, which is represented by decreased bacterial diversity, lower numbers of anaerobic commensal bacteria and expansions of pathobionts.

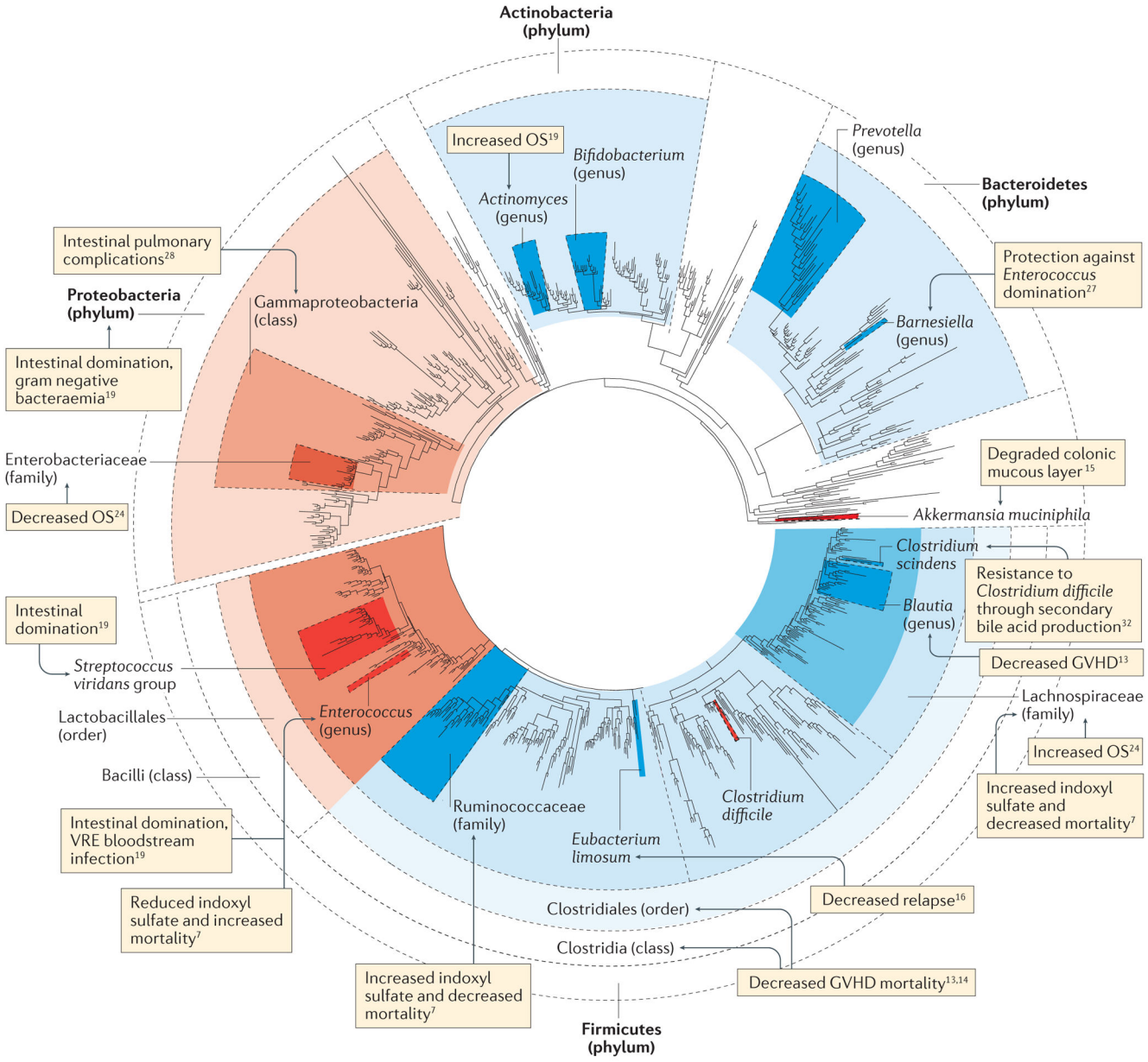

Figure 3 | Studies on microbiota injuries in allo-HSCT recipients.

A phylogenetic tree of typical gut bacterial species is shown. Shaded bacterial clades represent various levels of taxonomic classification. The blue shading denotes beneficial associations, whereas the red shading denotes association with negative effects. Allo-HSCT, allogeneic haematopoietic stem cell transplantation; GVHD, graft-versus-host disease; OS, overall survival; VRE, vancomycin-resistant *Enterococcus*. Figure adapted with permission from REF 167, Taylor & Francis Ltd (www.tandfonline.com), and updated by A. L. Gomes and Y.S.

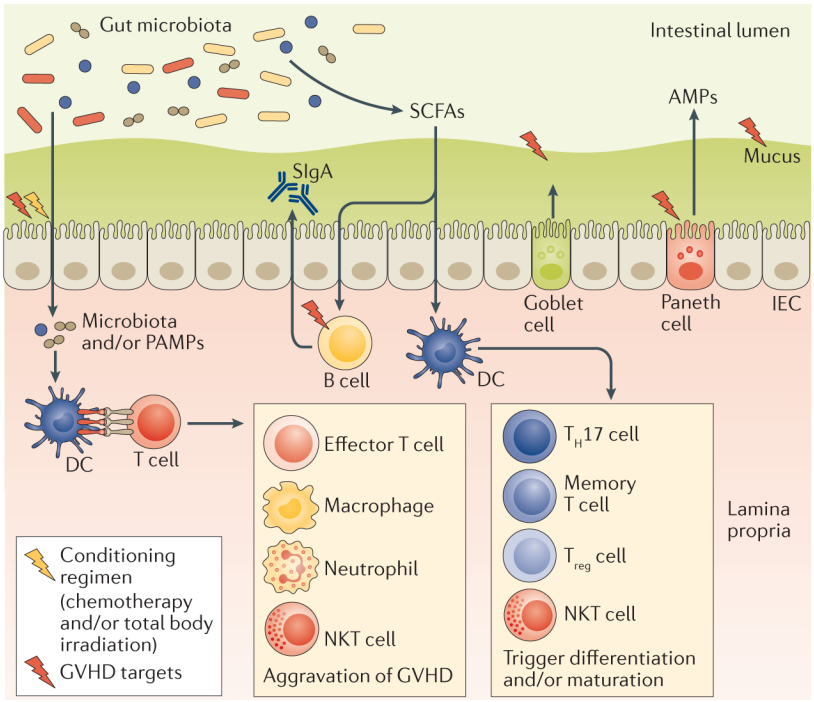

Figure 4 |. The interplay between gut microbiota and host physiology and immunity.

The host immune system maintains gut microbiota diversity and prevents outgrowth of pathobionts. Under steady-state conditions, the gut epithelial surface maintains an intact barrier that prevents bacterial invasion into the host tissue; this is accomplished by antimicrobial peptides (AMPs) produced by Paneth cells that create a sterility gradient, mucus produced by goblet cells that separates the microbiota from host epithelial tissue and secretory immunoglobulin A (SIgA) that neutralizes biologically active microbial antigens. Short-chain fatty acids (SCFAs) are bacterial fermentation products. They can be used as an energy source and also regulate the differentiation, recruitment and activation of immune cells. In the setting of allogeneic haematopoietic stem cell transplantation (allo-HSCT), total body irradiation and chemotherapy reduce the integrity of the intestinal surface. Intestinal bacteria and their components (pathogen-associated molecular patterns (PAMPs)) translocate into the lamina propria and are recognized by antigen-presenting cells such as dendritic cells (DCs), leading to activation of effector cells and aggravation of graft-versus-host disease (GVHD). B cells, Paneth cells and the mucous layer are known to be targets of GVHD, and damage to these creates a vicious circle exacerbating gut inflammation and bacterial translocation and resulting in worse survival after allo-HSCT. IEC, intestinal epithelial cell; NKT, natural killer T; T_H17, T helper 17; T_{reg}, regulatory T.

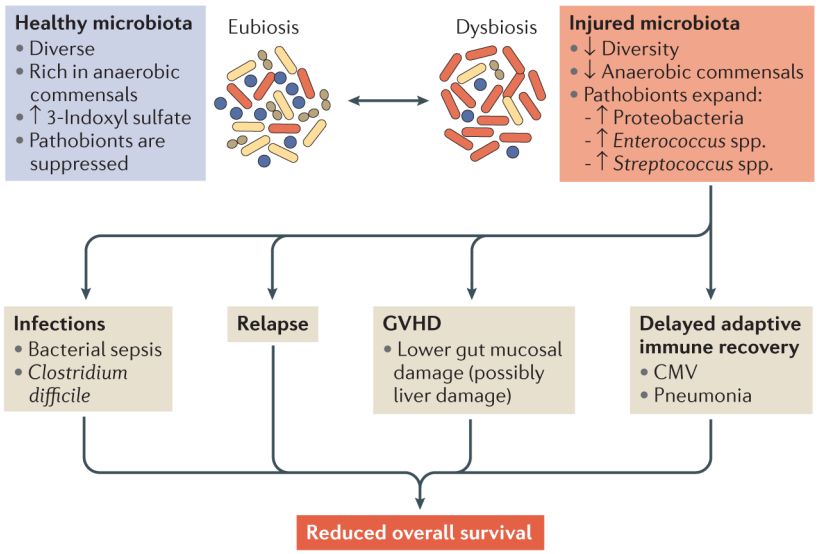

Figure 5 |. Microbiota injury and complications after allo-HSCT.

The top panels summarize the profiles of healthy microbiota before and after allogeneic haematopoietic stem cell transplantation (allo-HSCT) compared with those of injured microbiota after allo-HSCT. Microbiota injury results in infections, malignant relapse, graft-versus-host disease (GVHD) and possibly delayed immune reconstitution that contributes to additional infections (cytomegalovirus (CMV) infection and/or certain types of pneumonia that require adaptive immunity to eradicate). All of these can then contribute to reduced overall survival of patients after allo-HSCT.

Table 1 |

Summary of ongoing clinical trials investigating microbial interventions in allo-HSCT

Trial title	Aims and interventions	Phase	Trial number	Refs
Antibiotic treatment and its impact on microbiota				
Gut decontamination in paediatric allo-HSCT	To study the effect of vancomycin–polymyxin B treatment on patient flora and on GVHD and survival	II	NCT02641236	101
Choosing the best antibiotic to protect friendly gut bacteria during the course of stem cell transplant	To compare the effect between piperacillin–tazobactam and cefepime on patient flora (7 ± 2 days after initiation of antibiotics)	II	NCT03078010	89
Strategies for GVHD treatment				
Medical home care for HSC transplantation	To compare the incidence of acute grade II–IV GVHD between patients receiving patient-centred medical home care and those receiving standard hospital care	II	NCT02218151	131
Study of IL-22 IgG2-Fc (F-652) for subjects with grade II–IV lower GI acute GVHD	To examine combination treatment with corticosteroid for new-onset grade II–IV GVHD in the lower GI tract	I/II	NCT02406651	65
Lactobacillus rhamnosus str. GG in reducing incidence of GVHD in patients who have undergone donor stem cell transplant	To study the impact of *Lactobacillus rhamnosus* str.GG on reducing incidence of GVHD	Pilot	NCT02144701	133
Fructooligosaccharides in treating patients with blood cancer undergoing donor stem cell transplant	To study side effects and best dose of fructooligosaccharides for potential use for GVHD treatment	I	NCT02805075	123
Modification of the intestinal microbiome by diet intervention to mitigate acute GVHD	To study the effect of potato-based starch on butyrate production and rates of acute GVHD	II	NCT02763033	124
Gluten-free diet in preventing GVHD in patients undergoing donor stem cell transplant	To evaluate the impact of gluten-free diet on GVHD prophylaxis	Pilot	NCT03102060	168
FMT trials				
Autologous FMT for prophylaxis of *Clostridium difficile* infection in recipients of allo-HSCT	To restore diversity and prevent *Clostridium difficile* infection during allo-HSCT	II	NCT02269150	34
FMT after HSC transplantation	To assess the feasibility of empiric FMT soon after allo-HSCT; secondary outcome measures include GVHD and survival	Early I	NCT02733744	129
Stool transplantation to reduce antibiotic-resistance transmission (START)	To investigate the outcomes of FMT aiming at eradicating gut colonization with multidrug-resistant bacteria in patients with blood disorders	NA	NCT02461199	169
Nutrition and transplant outcome				
Randomized prospective multicentre study to compare enteral nutrition with parenteral nutrition as feeding support in patients presenting with malignant haemopathy who underwent an allogeneic HSC transplantation (NEPHA)	To compare between enteral nutrition and parenteral nutrition groups after myeloablative allo-HSCT; occurrence of transplant-related mortality, GVHD and infections will be assessed	III	NCT01955772	166

Allo-HSCT, allogeneic haematopoietic stem cell transplantation; FMT, faecal microbiota transplantation; GI, gastrointestinal; GVHD, graft-versus-host disease; HSC, haematopoietic stem cell; IL-22, interleukin-22; NA, not available.