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TLR/MyD88-mediated Innate Immunity in Intestinal Graft-versus-Host Disease

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Graft-versus-host disease (GHVD) is a severe complication after allogeneic hematopoietic stem cell transplantation. The degree of inflammation in the gastrointestinal tract, a major GVHD target organ, correlates with the disease severity. Intestinal inflammation is initiated by epithelial damage caused by pre-conditioning irradiation. In combination with damages caused by donor-derived T cells, such damage disrupts the epithelial barrier and exposes innate immune cells to pathogenic and commensal intestinal bacteria, which release ligands for Toll-like receptors (TLRs). Dysbiosis of intestinal microbiota and signaling through the TLR/myeloid differentiation primary response gene 88 (MyD88) pathways contribute to the development of intestinal GVHD. Understanding the changes in the microbial flora and the roles of TLR signaling in intestinal GVHD will facilitate the development of preventative and therapeutic strategies.

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Keywords: Graft-versus-host disease, Innate immune response, Toll-like receptor, MyD88, Myeloid derived suppressor cells (MDSCs)

INTRODUCTION

Allogeneic (allo) hematopoietic stem cell transplantation (HSCT) is an effective treatment for hematological disorders, including lymphoma and leukemia (1-4). Graft-versus-leukemia (GVL) effects, which are derived from the activation of donor T cells that recognize the allo-antigens expressed by the recipient's tumor cells, contribute to the eradication of malignant host cells (5). However, donor T cells are also reactive to allo-antigens expressed by the recipient's tissues and parenchymal cells in the gastrointestinal (GI) tract, liver, lung, and skin, and induce graft-versus-host disease (GVHD), a life-threatening complication of allo-HSCT (6,7). The suppression of severe GVHD is important for the success of allo-HSCT.

GI tract damage is a critical event in the pathogenesis of GVHD (8,9). The integrity of the GI tract and innate immunity to the intestinal microbiome both contributes to the maintenance of intestinal homeostasis; disruption of intestinal homeostasis during allo-HSCT provokes intestinal GVHD, which leads to exacerbation of the disease and systemic GVHD (9). Signaling through Toll-like receptors (TLRs) and myeloid differentiation factor 88 (MyD88), a signaling adaptor downstream of TLRs, is pivotal in innate immunity that controls response to microbial stimulation; evidence supporting the significances of their signaling in GVHD is accumulating (10,11). In this article, we will review recent research into the role of

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Abbreviations: GVHD, graft-versus host disease; allo, allogeneic; HSCT, hematopoietic stem cell transplantation; GI, gastrointestinal; MDSCs, myeloid-derived suppressor cells; BM, bone marrow

TLR/MyD88-mediated innate immunity in acute intestinal GVHD.

ACUTE AND CHRONIC GVHD

GVHD is broadly classified into acute and chronic GVHD, depending on the timing of disease incidence after allo-HSCT. Chronic GVHD was classically defined as a late complication of allo-BMT that occurs in 100 days post-transplantation. Chronic GVHD is similar to autoimmune and other immunological diseases, such as scleroderma (12,13), systemic lupus-like diseases (14), primary biliary cirrhosis (15), and immune cytopenia (16); it is characterized by tissue inflammation and fibrosis, and is mediated by cellular and CD4 T helper cell type 2-dependent humoral immunity (17,18). In 2014, revised chronic GVHD criteria were proposed, which facilitate distinction of chronic and acute GVHD, that include diagnostics in the skin (e.g., poikiloderma and sclerotic features including lichen planus-like features), mouth (e.g., lichen planus-like changes), lung (e.g., bronchiolitis obliterans), and GI tract (e.g., esophageal web, strictures or stenosis in the upper to middle third of the esophagus) (19).

Development of acute GVHD is observed within 100 days post-HSCT, with symptoms indicating damage to the skin (e.g., maculopapular rash on the palms, soles and ears, and diffuse erythematous rash over the entire body), liver (e.g., hyperbilirubinemia, jaundice, and elevated transaminases), GI tract (e.g., nausea, vomiting, abdominal cramps, anorexia, bleeding, and diarrhea), and, occasionally, lungs, eyes and oral mucosa (20). Although donor T cell-mediated adaptive immunity is an essential component of the development of acute GVHD, innate immunity also plays significant roles (6,21,22). Chemo-irradiation conditioning of recipients prior to HSCT provokes apoptosis of epithelial cells and tissue inflammation in several organs, including the intestines. The release of inflammatory cytokines activates antigen-presenting cells (APCs), which promote the activation and effector differentiation of allo-reactive donor T cells. Activated of T cells mediate cytotoxicity against allo-antigen-bearing recipient cells in damaged tissues, which increase inflammation in the target organs (Fig. 1). In particular, intestinal inflammation initiated by epithelial cell damage disrupts the epithelial barrier, which exposes innate immune cells to intestinal microbial stimuli. This innate cell stimulation by microbial antigens enhances the recruitment of activated T cells to the intestines, where they kill GI epithelial cells and cause cryptic cell degeneration, resulting in heightened intestinal inflammation and nutrient malabsorption. The degree of intestinal inflammation is associated with the severity of acute GVHD. Acute intestinal GVHD occurs in more than 50% of allo-HSCT patients (23).

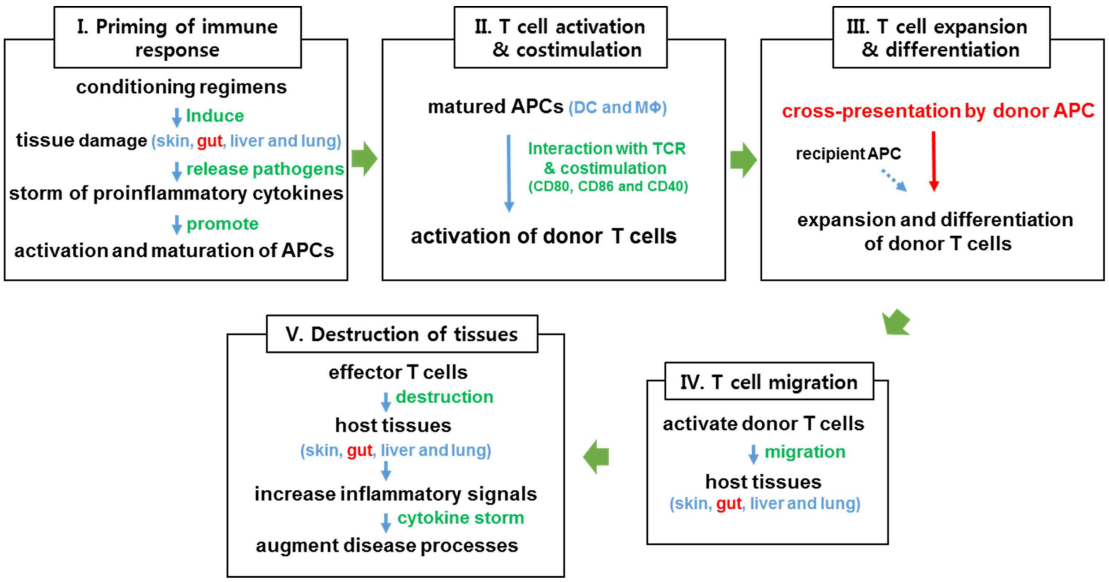

Figure 1. Schematic diagram of the development of acute GVHD. Acute GVHD can be classified into five distinct phases. Conditioning regimens (radiation or chemotherapy) induce tissue damage (I), and increase production of inflammatory cytokines, which cause the activation and maturation of APCs (II), leading to allo-reactive donor T cell priming and expansion (III). Activated donor T cells migrate to damaged host tissues (IV), where they amplify inflammatory responses and worsen GVHD (V). DC, dendritic cell; MΦ, macrophage.

GUT MICROBIOME AND INNATE IMMUNITY IN ACUTE INTESTINAL GVHD

The gut microbiome consists of diverse sets of bacteria, fungi, archaea, and viruses (24). Under physiological conditions, 10^{14} bacteria from 200 to 1500 species are approximated to exist in the colon (25,26). Alterations to or loss of intestinal microbiome diversity is related to the aggravation of acute GVHD (27,28). In a murine acute GVHD model, distinct microbes in the ileum were highly decreased (e.g., *Clostridiales* and phylum Firmicutes) or increased (e.g., *Lactobacillus johnsonii*) compared to bone marrow transplanted control mice without GVHD counterparts. *L. johnsonii* participated in the amelioration of acute GVHD by suppressing *Enterococcus* spp. (27). Inhibition of the production of the antimicrobial peptide α-defensin by Paneth cells reduced the physiological diversity of the microflora and permitted expansion of *Escherichia coli* in GVHD mice (28). Antibiotic treatment to reduce gram-negative bacteria in the GI tract ameliorat-

Table I. Studies of GVHD associated with innate immune responses through TLRs

TLRs	Treatments	Results related to acute GVHD pathogenesis	Donor/recipient	References
TLR1	SNP genotyping	SNPs in the TLR1 showed significant association with acute GVHD (e. g., SNP id: rs483307)	Donor (human)	44
	SNP genotyping	No effect on the incidence of acute GVHD by polymorphisms of the TLR1	Donor (human)	45
TLR2	Deficient	No effect on apoptosis/proliferation/neutrophilic granulocytes/survival in intestinal GVHD, donor T cells ↓	Recipient (mouse)	11
	SNP genotyping	Four SNPs in the TLR2 showed association with acute GVHD (e.g., SNP id: rs6535927)	Donor (human)	44
	SNP genotyping	No effect on the incidence of acute GVHD by polymorphisms of the TLR2	Donor (human)	45
	Deficient	No effect on acute GVHD by upregulation of TLR2 expression in G-CSF-mobilized donor grafts	Donor (mouse)	43
	Deficient	GVHD severity ↓, translocating bacteria ↓ (in TLR2/3/4/7/9^{-} in HoxB8 neutrophils)	Recipient (mouse)	31
TLR3	Deficient	GVHD severity ↓, translocating bacteria ↓ (in TLR2/3/4/7/9^{-} in HoxB8 neutrophils)	Recipient (mouse)	31
	SNP genotyping	No effect on the incidence of acute GVHD by polymorphisms of the TLR3	Donor (human)	45
TLR4	Deficient	No effects on apoptosis/proliferation/neutrophilic granulocytes/survival in intestinal GVHD, donor T cells ↓	Recipient (mouse)	11
	Agonist	GVHD severity ↑, alloreactive donor T cell proliferation ↑	Recipient (mouse)	35
	Deficient	Protection against intestinal cell apoptosis during acute GVHD by induction of tissue protective factors	Recipient (mouse)	36
	Mutation	No difference in GVHD in HLA-matched HCT with mutation in donor	Both (human)	46
	Deficient	No effect on GVHD severity	Donor (mouse)	37
TLR5	SNP genotyping	SNP in the TLR5 showed no sufficient evidence for the TLR5 importance in GVHD	Donor (human)	44
TLR6	SNP genotyping	SNP in the TLR6 showed association with acute GVHD (e.g., SNP id: rs6531656)	Both (human)	44
TLR7	Deficient	GVHD severity ↓, translocating bacteria ↓ (in TLR2/3/4/7/9^{-} in HoxB8 neutrophils)	Recipient (mouse)	31
	Agonist	Localized GVHD ↑ , infiltration of donor T cells ↑	Recipient (mouse)	39
TLR8	SNP genotyping	No effect on the incidence of acute GVHD by polymorphisms of the TLR8	Donor (human)	45
TLR9	Deficient	GVHD severity ↓, translocating bacteria ↓ (in TLR2/3/4/7/9^{-} in HoxB8 neutrophils)	Recipient (mouse)	31
	Deficient	Intestinal GVHD severity ↓ (dependent on MyD88 signaling), survival rates ↑	Recipient (mouse)	11
	Agonist	GVHD severity ↑	Recipient (mouse)	11
	Deficient	GVHD severity ↓, apoptotic cells , proliferation of cells in colon ↑	Recipient (mouse)	44
	SNP genotyping	Associated with the risk of acute GVHD by TLR9 SNPs in the donors of allogeneic HSCT	Donor (human)	45
TLR10	SNP genotyping	SNP in the TLR10 showed significant association with acute GVHD (e.g., SNP id: rs337629)	Both (human)	44

TLR, toll-like receptor; GVHD, graft-versus host disease; SNP, small nucleotide polymorphism; HSCT, hematopoietic stem cell transplantation; HoxB8, Homeobox B8.

ed acute GVHD severity (29). Shifts in the gut microbiota towards enterobacteria, enterococci, and *Bacteroides/Prevotella* spp. are associated with increased inflammatory responses in intestinal GVHD (11). Thus, the intestinal microbiota could potentially be manipulated to improve allo-HSCT outcomes.

Innate pattern recognition receptors (PRRs), such as TLRs and nucleotide oligomerization domain (NOD)-like receptors (NLRs), recognize intestinal bacterial pathogens and/or pathogenic molecules. Ligand binding by the TLRs and NLRs expressed on host and/or donor-derived APCs substantially amplifies the release of inflammatory mediators (30). The transfer of HoxB8 neutrophils that lack expression of TLR 2, 3 4, 7, and 9 reduced GVHD severity compared with the transfer of WT HoxB8 neutrophils, indicating that TLR signals promote GVHD development (31). Conditioning-induced GI damage allows the translocation of outer membrane-derived endotoxins from gram-negative bacteria (e.g., lipopolysaccharide (LPS)) into systemic circulation (11,32,33). The binding of LPS to TLR4 accelerated lethal intestinal GVHD by stimulating the production of inflammatory cytokines (e.g., TNFα, IL-1, IL-6, IL-10, IL-12, and TGFβ) from gut-associated lymphoid tissues (GALTs) and macrophages, and IFN-γ from activated donor T cells (9,34). The endogenous TLR4 agonist heparan sulfate activated dendritic cells (DCs) and aggravated acute GVHD (35). Unexpectedly, however, *Tlr4*$^{-/-}$ mic developed fulminant GVHD, and allogeneic hosts with a TLR4 mutation (C3H/HeJ mice) had increased intestinal damage compared to wild type counterparts (36,37). TLR4 signaling mediated protective effects during GVHD, characterized by reduced intestinal cell apoptosis compared to that in hosts that did not undergo TLR4 signaling (36). In addition, TLR4 ligands were not necessary for the maturation of host APCs for GVHD induction (37). Collectively, these finding suggest that TLR4 signaling is involved in both positive and negative regulation of GVHD. *Tlr9*$^{-/-}$ mice developed less severe acute GVHD post-HSCT than controls (11,38). Consistent with these findings, treatment of wild type mice with a synthetic TLR9 agonist (CpG oligonucleotides) markedly accelerated GVHD severity (39), and treatment with the TLR9-inhibitory oligonucleotide (iODN) 2088 reduced apoptosis of colonic cells in intestinal GVHD (11,39). Thus, TLR9 signaling is associated with the induction of intestinal GVHD.

Application of the TLR7/8 agonist R-848 (resiquimod) promoted substantial innate immune activation and T cell migration into target organs (40). Another TLR7/8 agonist, 3M-011, caused differential effects on GVHD depending on the timing of the treatment. Administration of 3M-011 after allogenic transplant increased GVHD mortality, but pre-treatment with 3M-011 reduced the damage to target organs by inducing IDO expression in the colon (39,41,42). Alterations to TLR2 expression on recipient lymphoid and myeloid cells from splenocytes had little effect on acute GVHD (43) (Table I). Thus, each of the TLRs is involved in acute GVHD to a different extent (43-46). Reports on the functional associations of TLRs and their adaptor molecules with GVHD are summarized in Table I and Table II.

MyD88-DEPENDENT EXPANSION OF MYELOID-DERIVED SUPPRESSOR CELLS (MDSCs) IN ACUTE INTESTINAL GVHD

MyD88 is an adaptor molecule that activates inflammatory responses downstream of TLR ligand ligation (Table II) (47-49). All TLRs, except TLR3, transduce signals through MyD88 (50). In MyD88-deficient recipient mice, the infiltration of donor T cells into the intestines and the apoptosis of colon cells were reduced, resulting in improved survival and clinical scoring for acute intesti-

Table II. Studies of GVHD associated with innate immune responses through TLR adaptor molecules

TLR adaptors	Treatments	Results related to acute GVHD pathogenesis	Donor/recipient	References
MyD88	Deficient	Acute GVHD severity ↓, apoptotic cell ↓, proliferation of cells in colon ↓	Recipient (mouse)	11
	Deficient	Intestinal GVHD ↑ , myeloid cell apoptosis ↑, donor T cells , expansion/function of MDSCs ↓	Donor (mouse)	49
	Deficient	Hepatic GVHD severity ↓, infiltration of T cells into the liver of the recipients ↓	Donor (mouse)	51
	Deficient	No effect on acute GVHD (lack of MyD88 in donor APC)	Donor (mouse)	37
TRIF	Deficient	No effect on acute GVHD, neutrophil infiltration in to colon ↑	Recipient (mouse)	11
	Deficient	No effect on acute GVHD (lack of TRIF in donor APC)	Donor (mouse)	37
MyD88/TRIF	Deficient	No effect on acute GVHD (lack of MyD88 and TRIF in donor APC)	Donor (mouse)	11

GVHD, graft-versus host disease; APC, antigen-presenting cell; MyD88, myeloid differentiation primary response 88; TRIF, TIR-domain-containing adaptor-inducing Interferon-β.

nal GVHD (11). However, MyD88-deficiency in donor bone marrow (BM) cells aggravated GVHD, resulting in increased intestinal pathology (51). The exacerbation of intestinal GVHD in recipients of MyD88-deficient BM cells was associated with insufficient expansion of MDSCs from the transplanted MyD88-deficient stem cells. These findings indicate that MyD88 signaling in donor cells promotes MDSC expansion and immune suppression in acute GVHD. The transfer of WT MDSCs into recipients of MyD88-deficient BM cells ameliorated intestinal GVHD, which supports a role for MyD88 in driving MDSC expansion in GVHD. Thus, MyD88 signaling has opposite impacts on intestinal GVHD, depending on whether MyD88 is expressed by host or donor cells.

MDSCs consist of two main subtypes: granulocytic/polymorphonuclear MDSCs and monocytic MDSCs. The phenotypes $CD11b^{+}LyG6^{+}Ly6C^{low}$ and $CD11b^{+}LyG6^{low}Ly6C^{high}$ are used to identify the respective populations in mice. MDSCs expand robustly in various pathological conditions, such as cancers (52), autoimmune diseases (53), inflammation (54), infectious diseases (55-58), and GVHD (49,51,59,60). Most MDSC biology has been studied in tumor microenvironments, and preclinical and clinical tumor therapies have been tested for their ability to block MDSC expansion and function. Inhibitors of vascular endothelial growth factor (VEGF; bevacizumab) (61), signal transducer and activator of transcription 3 (STAT3; sunitinib) (62), arginase (NOHA) (52), inducible nitric oxide synthase (iNOS; nitroaspirin) (63), and cyclooxygenase-2 (COX2; celecoxib) (64), as well as agents that induce MDSC apoptosis and necrosis (gemcitabine and IL4Rα aptamer), have been shown to decrease MDSC expansion and tumor growth (65,66). The expansion and functional enhancement of MDSCs are required for the control of acute intestinal GVHD. Arginase-1, iNOS, reactive oxygen species (ROS), and nitric oxide (NO) are mediators of the suppressive functions of MDSCs (52). Inflammatory mediators such as COX-2, G-CSF, GM-CSF, IFN-γ, IL-6, IL-10, VEFG, and prostaglandin E2 induce the differentiation and expansion of MDSCs, and inhibit the differentiation of mature myeloid cells in pathogenic environments (67,68). These mediators could be targeted to enhance the suppressive functions of MDSCs to ameliorate GVHD. The selective modulation or exploitation of MyD88-mediated signaling to induce MDSC expansion and functional enhancement could be a strategy to suppress acute intestinal GVHD.

CONCLUSION

The dysregulation of microbial homeostasis and TLR signaling-mediated inflammatory responses are involved in the pathogenesis of intestinal GVHD. Understanding the effects and cellular/molecular mechanisms of TLR/MyD88 signaling on innate immune regulation of gut bacteria and MDSCs would aid the development of specific immune modulators to treat intestinal GVHD.

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CONFLICTS OF INTEREST

There is no conflict of interest.

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