REVIEW

Biology of chronic graft-*vs*-host disease: Immune mechanisms and progress in biomarker discovery

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Abstract

Chronic graft-*vs*-host disease (cGVHD) is the leading cause of long-term morbidity and mortality following allogeneic hematopoietic stem cell transplantation. It presents as a chronic inflammatory and sclerotic auto-immune-like condition that most frequently affects the skin, oral mucosa, liver, eyes and gastrointestinal tract. Both clinical and animal studies have shown that multiple T cell subsets including Th1, Th2, Th17, T follicular helper cells and regulatory T-cells play some role in cGVHD development and progression; B cells also play an important role in the disease including the production of antibodies to HY and nuclear antigens that can cause serious tissue damage. An array of cytokines and chemokines produced by different types of immune cells also mediate tissue inflammation and damage of cGVHD target tissues such as the skin and oral cavity. Many of these same immune regulators have been studied as candidate cGVHD biomarkers. Recent studies suggest that some of these biomarkers may be useful for determining disease prognosis and planning long-term clinical follow-up of cGVHD patients.

Key words: Chronic graft-*vs*-host disease; Biomarker; Allogeneic hematopoietic stem cell transplantation; Cytokine

Core tip: Chronic graft-*vs*-host disease (cGVHD) is a frequent long-term medical complication of allogeneic hematopoietic stem cell transplantation which can have a devastating impact on overall health and quality of life. This immune-mediated disorder manifests as an inflammatory and autoimmune-like disorder that can affect multiple tissues in an individual patient. Both clinical and animal studies demonstrate that multiple T cell subsets, as well as B cells, and their secreted cytokines play important roles in cGVHD initiation and progression. In the last decade many molecular biomarkers have been identified that correlate with cGVHD onset and/or progression, and some might have applications clinically in the near future.

INTRODUCTION

Allogeneic hematopoietic stem cell transplantation (allo-HSCT) is utilized primarily as a curative treatment for both hematological and non-hematological malignancies[1], although it has been used successfully in small-scale clinical trials as a stem cell therapy for some inherited diseases such as Recessive Dystrophic Epidermolysis Bullosa[2]. In the case of hematologic malignancies, the graft-*vs*-leukemia or graft-*vs*-tumor (GVL or GVT) effect mediated by donor-derived T cells helps to eliminate malignant cells in the transplant recipient[3]. However, a major long-term complication of allo-HSCT is chronic graft-*vs*-host disease (cGVHD), which occurs in 30%-70% of patients, with adults more frequently affected than pediatric patients[4]. Chronic GVHD manifests as an autoimmune-like inflammatory disease that can affect a single organ, but more typically it presents as a multi-organ disease affecting the skin (75% of patients), oral mucosa (51%-63% of patients), liver, eyes and gastrointestinal tract (22%-51% of patients)[4]. Oral mucosal disease can include salivary gland pathology or sclerosis of the lamina propria or submucosa. Other tissues including the lung, esophagus, joints, muscles and genitalia can also be involved (Table 1). cGVHD is often preceded by acute GVHD, which typically occurs within 100 d after transplantation, although the acute form can persist longer.

In allo-HSCT patients, cGVHD is the most common cause of non-relapse mortality (NRM, which refers to mortality not related to the primary malignancy or disease) among patients surviving more than two years[5]. Other important contributing factors to patient mortality are viral or bacterial infection and secondary malignancies (Figure 1)[4,6]. A recent analysis by the Center for International Blood and Marrow Transplant Research (CIBMTR) of more than 26000 allo-HSCT patients demonstrated that the incidence of cGVHD is increasing worldwide, making it imperative that we fully understand the etiology of this disease[7].

This review will focus on the pathobiology of cGVHD, which has features of both alloimmune and autoimmune disease and involves altered activities and function of various T cell populations [T helper (Th) 1, Th2, Th17, T follicular helper cells and regulatory T-cells] as well as of B cells. Equally important are the various cytokines and chemokines produced by immune cells and their target tissues, which cause inflammation and tissue damage. A second productive area of cGVHD research is biomarker discovery; high-throughput approaches including mass spectrometry have led to the identification of a number of molecular markers from blood and saliva that correlate with active disease. Not surprisingly, many of these markers are associated with altered host immunity and/or tissue inflammation. This review will not discuss current primary and secondary therapeutic strategies for cGVHD; for an in-depth discussion of this topic, the reader is referred elsewhere[8-10].

CLINICAL FEATURES

According to the NIH consensus criteria published in 2005, cGVHD can be subclassified into: (1) Classic cGVHD presenting with manifestations that can be ascribed only to cGVHD; and (2) Overlap syndrome that has diagnostic or distinctive cGVHD manifestations together with features typical of acute GVHD[11]. Acute GVHD occurs in 40%-60% of patients receiving allo-HSCT and is one of the major risk factors for subsequent cGVHD. To improve cGVHD classification, the NIH severity score was developed which documents the number of organs involved and numerically scores the degree of functional impairment. Generally, patients are assessed as having mild, moderate or severe disease on a scale of 1 to 4 for each tissue[11-13]. However, clinical symptoms of cGVHD often overlap with other autoimmune diseases such as lichen planus and scleroderma and the degree of organ involvement is highly variable, which can make diagnosis challenging[8,14]. Table 1 lists signs and symptoms that are considered to be diagnostic of cGVHD as well as some of the commonly observed clinical features that are considered to be insufficient for disease diagnosis. As many as three or more tissues can be affected in a single patient, as reflected in the NIH global severity classification of cGVHD[13]. Skin manifestations that are considered diagnostic include poikiloderma (altered pigmentation with erythema), lichen planus-like lesions, sclerosis and morphea-like features (Table 1). Distinctive features (often observed in skin cGVHD but not sufficient for diagnosis) include depigmentation, papulosquamous lesions, ichthyosis and pruritis. Skin appendages are often targeted as well but these signs are not considered diagnostic: Symptoms can include scalp hair thinning or alopecia, sweat impairment and nail dystrophy or onycholysis (nail loss)[11,13].

Cutaneous cGVHD can occur in two forms termed lichenoid and sclerodermatous[15]. Lichenoid lesions usually occur early in the course of the disease, presenting as erythematous papules or plaques, with a squamous surface. Typical affected sites include the face, ears, palms and soles. Sclerodermatous cGVHD, which generally develops as a later complication, appear as sclerotic, shiny, white or yellow plaques with patchy hyperpigmentation or a poikilodermal appearance[15]. Sclerodermatous cGVHD can be localized or generalized and affect underlying tissues including the fascia, ligaments and peripheral nerves, causing pain and morbidity for the affected patient.

Oral symptoms vary but commonly involve lichenoid changes, xerostomia as a result of salivary gland damage,

Table 1 Signs, symptoms and prevalence of chronic graft-*vs*-host disease in selected organs and tissues

Organ or tissue	Prevalence, %[1]	Diagnostic features[2]	Distinctive features[3]
Skin	75%	Poikiloderma	Depigmentation
		Lichen planus-like features	Papulosquamous lesions
		Sclerosis	
		Morphea-like features	
Mouth	51%-63%	Lichen planus-like features	Xerostomia
			Mucoceles
			Mucosal atrophy
			Ulcers
			Pseudomembranes
Liver	29%-51%	None[4]	None[4]
Eye	22%-33%		Dry, gritty or painful eyes
			Cicatricial conjunctivitis
			Keratoconjunctivitis-Sicca syndrome
GI tract and esophagus	7%-45%	Esophageal web	
		Strictures or stenosis in upper esophagus	
Lung	4%-19%	Bronchiolitis obliterans	Air trapping and bronchiectasis on chest CT scan
Muscles, fascia and joints	6%	Fascitis	Myositis or polymyositis
		Sclerosis	
		Joint stiffness or contractures	
Genitalia	1%	Lichen planus-like features	Erosions, Fissures, Ulcers
		Lichen sclerosus-like features	

[1]Frequency of tissue involvement at initial cGVHD diagnosis (from Lee *et al*[4]); [2]Clinical symptoms that are sufficient for cGVHD diagnosis. Information adapted from references 8 and 13; [3]Clinical symptoms that are frequently seen in cGVHD, but insufficient for cGVHD diagnosis. Information adapted from references 8 and 13; [4]While no diagnostic or distinctive features have been identified for liver cGVHD, hepatitis is often seen (and also sometimes in acute GVHD) with elevated serum levels of bilirubin, alkaline phosphatase and alanine aminotransferase (ALT)[13]. cGVHD: Chronic graft-*vs*-host disease; CT: Computed tomography.
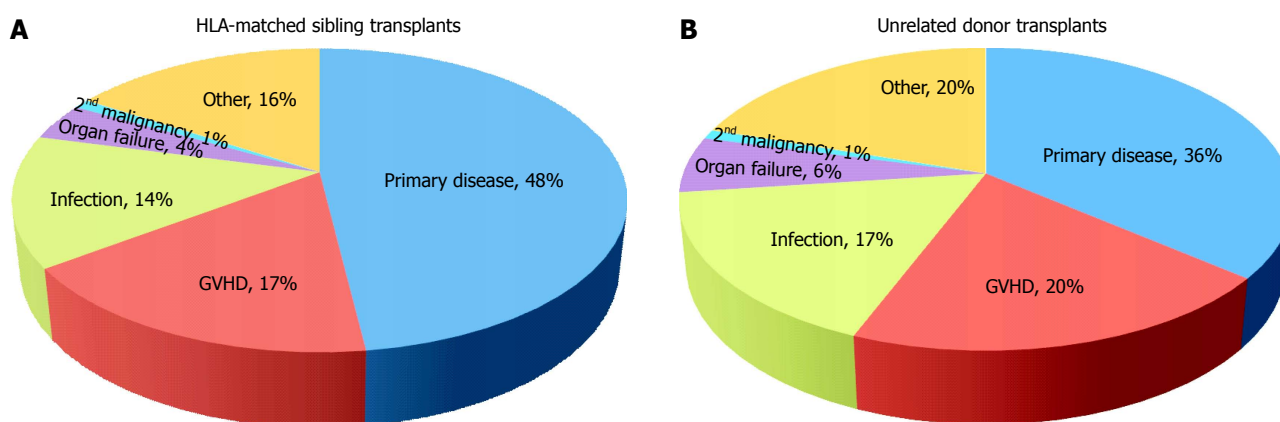

Figure 1 Causes of death among allogeneic hematopoietic stem cell transplantation patients. Pie charts show causes of death among patients who received a cell graft from (A) an HLA-matched sibling or (B) an unrelated donor. Data is from the Center for International Blood and Marrow Transplant Research, for allogeneic hematopoietic stem cell transplants performed in 2012-13[88] (Available from: URL: http//www.cibmtr.org/Data/Resources/pages/index.aspx). GVHD: Graft-*vs*-host disease.

mucositis, erythema, mucoceles and restricted mouth opening (trismus) due primarily to sclerosis[16-18]. However, under current guidelines only lichen planus-like features are considered to be diagnostic (Table 1)[13]. Oral sensitivity and pain are often observed, which in more severe cases manifests as dysphagia (difficulty with swallowing) and weight loss. In one recent study of 210 cGVHD patients, 29% of cases were classified as malnourished by measurement using the Patient-Generated Subjective Global Assessment tool. Malnutrition was correlated with a lower body-mass index and poorer overall survival[19]. Gingivitis and tooth decay also occur because of xerostomia and altered oral immunity

related to immunosuppression and reduced salivary IgG production[20].

Clinical symptoms seen in other involved tissues such as the liver, eyes, gastrointestinal tract, lungs, muscles/fascia and genitalia are summarized in Table 1, and have been reviewed extensively elsewhere[9,13]. Neurological manifestations of cGVHD are rare, but when present can include Myositis and Myasthenia gravis that affect the peripheral nervous system, and less commonly, various complications that affect the central nervous system[21]. Clinical features of cGVHD do not seem to vary with patient age, graft source (typically either bone marrow or PBSCs) and type of pre-transplant conditioning[4,9].

Most cases of cGVHD occur 4-6 mo after allo-HSCT, but 5%-10% of patients are diagnosed more than one year following allogeneic transplantation.

RISK FACTORS

The best documented risk factors for cGVHD are a history of acute GVHD (seen in 40%-60% of cGVHD patients), the use of PBSCs for grafting, a female donor-male recipient combination, older patient age and the use of HLA-mismatched or unrelated donors[13,22,23]. The increasing use of PBSCs (which contain more T cells compared to aspirated bone marrow) is one factor that influences the incidence and severity of cGVHD, since alloreactive T cells are a major player in cGVHD pathobiology[7]. These risk factors appear to largely explain the increasing incidence of cGVHD worldwide in allo-HSCT; however, additionally, a significant decline in early NRM appears to be contributing to the increased incidence of cGVHD in long-term survivors[7]. Notably, the frequency of GVHD-associated mortality is similar in HLA-matched sibling transplants compared to transplants performed using an unrelated donor (Figure 1).

A number of studies have also implicated certain genetic polymorphisms in addition to HLA antigen disparity between donor and recipient in the risk of GVHD risk (reviewed in Pidala *et al*[24]). For example, polymorphisms in a considerable number of genes that encode cytokines, chemokines or their receptors are associated with increased risk of cGVHD. These include genetic variants in the donor and/or recipient *IL-10* genes[25-27], donor *IL-1α* gene[28], recipient *IL-6* gene[29], recipient MHC class I-related chain A (*MICA*) gene (Val allele)[30], and donor and recipient *IL-1* receptor antagonist (*IL-1ra*) genes[25,28]. For MICA, which acts as an activating ligand for the NKG2D receptor on certain types of T-cells, cGVHD incidence was positively correlated with serum MICA levels in patients post-HSCT; on the other hand, the presence of MICA antibodies prior to transplantation conferred protection against cGVHD[30]. A smaller number of genetic polymorphisms have been associated with decreased risk of cGVHD[24].

Baron *et al*[31] utilized gene expression profiling of donor CD4+ and CD8+ T cells to develop a "GVHD-predictive signature", demonstrating the central importance of the TGF-β signaling pathway in regulating donor T cell function[31]. Remarkably, the so-called "dangerous donor" trait derived from T cell gene expression profiling not only predicted early (acute) GVHD, but also cGVHD occurrence in the recipient at one year post-transplantation. These observations reinforces other studies in humans and mice showing that the growth factor TGF-β has pleiotropic effects on T cells, including inhibition of Th1 cell differentiation and promoting expansion of regulatory T cells that are protective against cGVHD[9,32,33]. It also suggests that the grafted stem cells can have a long-term, dominant influence on the transplant recipient's T cell profile and consequently the overall health of the patient.

PATHOBIOLOGY OF CGVHD: ROLE OF T CELLS, B CELLS AND THEIR CYTOKINES

T cells

While the mechanisms that cause the inflammation and tissue damage of acute GVHD are now quite well understood, the pathobiology of cGVHD is more complex and less well understood. Many investigators believe that the destructive immunological and autoimmune mechanisms that cause cGVHD are distinct from acute GVHD, irrespective of whether or not the cGVHD evolves from acute GVHD[6,34]. Activated donor T cells are the most important cell population in cGVHD, since T cell depletion from the graft prevents cGVHD in both human and animal studies[35]. The use of rabbit anti-thymocyte globulin (ATG) in conditioning regimens prior to transplant reduces the risk of subsequent acute and cGVHD, either by depleting donor T cells or by interfering with their activation by recipient alloantigens[22,36]. The major T cell subsets proposed to be involved in cGVHD include CD4+ T cells, CD4+ regulatory T cells (Tregs) and CD8+ T cells (Table 2).

Th1, Th2 and Th17 cells: Alloreactive CD4+ T cells that react to foreign (donor-derived) antigens include several Th cell subsets, primarily Th1, Th2 and Th17 cells. A central role for Th1 cells in acute GVHD is well established[6]; however, the importance of Th1 (and Th2) cells in cGVHD is still a matter of debate, even though Th1 cytokines such as interferon-γ (IFN-γ) can be found in skin and other tissues of affected patients[37]. Infusion of murine IFN-γ-null donor T cells reduced cGVHD symptoms in skin and salivary glands, indicating a role for Th1 cells in certain tissues[38]. A role for Th2 cells has been suggested because of the role of Th2 cytokines such as IL-4 and IL-13 (Table 2) in the production of antibodies to both self and non-self-antigens in patients; murine studies support the involvement of the Th2 cytokines IL-4 and IL-10 in stimulating B cell expansion in cGVHD[39].

Th17 cells produce several cytokines including IL-17, IL-21 and IL-22, which have potent pro-inflammatory functions in cGVHD[40]. IL-17A, produced mainly by CD8+ T cells, stimulates scleroderma which is an important feature of cutaneous cGVHD; however, current data suggests that co-expressed Th1 cytokines such as tumor necrosis factor-α (TNF-α) contribute to the observed pathology[41]. Improvement in cGVHD symptoms correlates with a reduction in Th-17 cell numbers in peripheral blood[42]. In liver cGVHD, there are increased numbers of Th17 cells and an increased Th17/Treg ratio observed in liver biopsies, suggesting that Th17 cells are an important driver of clinical liver disease[43].

T follicular helper cells: T follicular helper (TFH) cells promote differentiation of naïve B cells into memory B

Table 2 Immune cell types and their function in chronic graft-*vs*-host disease

Cell type	Subtypes	Key cytokines or markers	Brief summary of disease involvement
CD4^{+} T cells	Th1	IFN-γ, TNF-α	Pro-inflammatory. Important in acute GVHD, but role in cGVHD unclear
	Th2	IL-4, IL-13	Stimulate antibody production. Role in clinical cGVHD poorly defined
	Th17	IL-17; also IL-21, IL-22, TNF-α	Pro-inflammatory. IL-17 levels correlate with disease severity; IL-17 induces scleroderma of skin and lung
	Tregs	TGF-β, required for Treg proliferation and (differentiation)	Produced mostly in thymus. Suppress autoreactive T cells. Lower levels of Tregs present in cGVHD patients, associated with thymic damage and loss of self-tolerance in cGVHD
	T follicular helper cells[1]	Express CCR5, PD-1 and ICOS	Promote abnormal B cell maturation into long-lived active plasma cells, and IgG secretion
CD8^{+} T cells		CXCL9, CXCL10	Mediate graft-*vs*-tumor effect of transplant. Serum CXCL9 levels elevated in cGVHD patients
B cells (total)		Increased BAFF/B-cell ratio, elevated serum BAFF levels	Decreased in active cGVHD. Remaining B cells are resistant to apoptosis
Naïve and transitional B cells		CD19	Decreased in active cGVHD
Memory B cells (total)		CD19, CD27	Decreased in active cGVHD. Cells essential for a normal immune response to bacterial pathogens or opportunistic infections
Regulatory B cells		IL-10	Decreased in active cGVHD. Function to maintain tolerance and help prevent autoimmune disease
Plasma cells		CD27, CD38	Increased in active cGVHD. Cells secrete immunoglobulins including IgGs and are resistant to apoptosis

[1]Mainly classified into Th2 and Th17 subtypes[44]. cGVHD: Chronic graft-*vs*-host disease; BAFF: B-cell activating factor; CD3: Cluster of Differentiation molecule 13, 19, 27 and 38; CCR5: Chemokine (C-C Motif) receptor 5; CXCL: Chemokine (C-X-C motif) ligand; ICOS: Inducible T-Cell Co-Stimulator; IFN-γ: interferon gamma; IL: Interleukin; PD-1: Programmed cell death 1; Th: T helper cell; TGF-β: Transforming growth factor beta; TNF-α: Tumor necrosis factor alpha.

cells and class switching of *IgG* genes in the germinal center, within secondary lymphoid organs. A recent study showed that TFH cells were unusually active with prolonged survival in cGVHD patients, which correlates with the aberrant survival of B cells and hypersecretion of immunoglobulins[44]. The increased survival of Th2- and Th17-type TFH cells was correlated with increased cellular expression of the pro-survival marker Bcl-2. Overall, the study by Forcade *et al*[44] suggests that aberrant B cell activity including production of antibodies is driven, at least in part, by abnormal TFH cell activity. Studies using a murine model have confirmed the importance of TFH cells in cGVHD pathogenesis, particularly for the development of bronchiolitis obliterans syndrome which is a signature feature of lung cGVHD[45] (Table 1).

Tregs: Tregs, which are CD4^{+} CD25^{+} and also express the transcription factor FOXP3, suppress autoreactive T cells and are important for immune system homeostasis. Specifically, Tregs are essential for the establishment and maintenance of tolerance after allo-HSCT[46]. Tregs are depleted in both acute and cGVHD, demonstrating their importance as suppressors of inflammation and disease development[23]. Impaired Treg production and function has been linked to thymic damage as a result of CD4^{+} lymphopenia following allo-HSCT, at least in myeloablative patients[47]. The presence of Tregs in the skin and oral mucosa of cGVHD patients in a functional (*e.g.*, CXCR3^{+})

state suggests they may play a role in limiting tissue damage by alloreactive T cells[48]. Pharmacological approaches used to treat steroid-refractory cGVHD that increased Treg cell numbers have shown promise clinically in treating cutaneous cGVHD[49]. Further, in a study of allo-HSCT patients with acute leukemia, direct infusion of Tregs together with conventional T cells protected against GVHD in almost 90% of engrafted patients, while still maintaining the GVT anti-tumor effect conferred by conventional T cells[50]. These studies suggest that manipulation of Tregs might be a feasible approach to reducing or preventing GVHD without compromising the anti-tumor surveillance capacity of the patient's immune system.

CD8^{+} T cells: CD8^{+} T cells are another immune cell population present in tissues affected by cGVHD, including the skin and oral mucosa[17]. Donor CD8^{+} cells mediate the GVT effect of allo-HSCT that typically results in the eradication of malignant cells from the patient. Among the cytokines produced by CD8^{+} cells are CXCL9 and CXCL10; CXCL9 is elevated in the serum of early-stage cGVHD patients, with CXCL9 levels being correlated with disease severity[51] (Tables 2 and 3).

B cells
In addition to T cells, there is increasing evidence that B cells play a number of important roles in cGVHD pathogenesis[52]. Patients with active cGVHD consistently have

Table 3 Candidate biomarkers of chronic graft-*vs*-host disease[1]

Gene/protein	Function	Biofluid[3]	Ref.
BAFF, soluble; BAFF/B cell ratio	Growth factor, promotes B cell expansion and activation	Blood	[59,60,77]
CXCL9[2]	Chemokine produced by activated T cells	Blood	[51,79]
CD-13, soluble	Antigen presentation	Blood	[59]
C-reactive protein[3]	Acute phase protein	Blood	[12]
Cystatin B	Inhibitor of cathepsin proteases	Saliva	[80]
IL-1ra	Inhibitor of IL-1 receptor signaling	Saliva	[80]
IL-2R, soluble	IL-2 receptor, marker of activated T cells	Blood	[59,76]
IL-6	Pro-inflammatory Th2 cytokine	Blood	[42,75]
IL-10	Th2 cytokine	Blood	[73]
IL-15	Enhances anti-tumor function of CD8$^+$ T cells	Blood	[78]
Lactoperoxidase	Anti-microbial enzyme	Saliva	[81]
Lactoferrin	Iron-binding glycoprotein	Saliva	[81]
MICA, soluble	Stimulates T cell activity *via* NKG2D receptor	Blood	[30]
TGF-β	Anti-inflammatory cytokine; stimulates activity of Tregs	Blood	[33]
TNF-α	Pro-inflammatory Th1 cytokine	Blood	[73-75]

[1]This table only includes proteins identified in human biofluids. Antibodies are discussed in the text; [2]Blood markers were measured in either plasma or serum isolated from peripheral blood, depending on the study. For saliva, whole unstimulated saliva collected from oral cGVHD patients was used; [3]Increased CRP levels were especially associated with joint/fascia and skin involvement, compared to the non-cGVHD control group. cGVHD: Chronic graft-*vs*-host disease; BAFF: B-cell activating factor; CXCL: Chemokine (C-X-C motif) ligand; CD-13: Cluster of Differentiation molecule 13 (or aminopeptidase N); IL-1ra: Interleukin 1 receptor antagonist; IL-2R: Interleukin 2 receptor; MICA: MHC class I-related chain A; TGF-β: Transforming growth factor beta; TNF-α: Tumor necrosis factor alpha; CRP: C-reactive protein.

lower numbers of naïve and transitional B cells as well as total B cells[53,54] (Table 3). Regulatory B cells that secrete the anti-inflammatory cytokine IL-10 (and form a subpopulation within the transitional and memory B cell compartments) were also less frequent in cGVHD patients and displayed a deficiency in IL-10 production[55]. Together with Tregs, regulatory B cells play a central role in graft tolerance and the prevention of autoimmune disease and hence represent a topic worthy of further investigation in relation to cGVHD[56]. CGVHD patients are susceptible to pneumococcal infection which can cause severe or fatal infections in long term transplant survivors[57]. This susceptibility to infections is associated with the abnormal B cell profile, including decreased numbers of memory B cells that are critical for a normal immune response including IgG production[53,58]. Like many other autoimmune conditions, cGVHD patients frequently produce allo- and auto-antibodies to DNA and/or other antigens such as male HY antigen, which can correlate with disease onset and severity (see below). Activated B cells secrete an array of Th1 and Th2 cytokines that can regulate the function of T cell populations including Tregs. Levels of B cell activation factor (BAFF), a cytokine that promotes the survival and differentiation of activated B cells, are consistently increased relative to B cell numbers in patients with cGVHD[59,60]. As discussed above, the increased activity of TFH cells appears to play a significant role in producing the abnormal B cell profile characteristic of cGVHD[44].

Perhaps the best evidence that B cells are functionally important in human cGVHD are the numerous clinical observations with Rituximab, a humanized monoclonal antibody that targets the membrane protein CD20 of B cells, causing their cell death. Rituximab (and other anti-CD20 drugs) are effective in the treatment of steroid-refractory cGVHD, resulting in rapid and selective depletion of B cells and diminished activation of cytotoxic T cells; concurrently, the number and activity of Tregs are elevated[52,61,62]. In one study, the prophylactic use of Rituximab after allo-HSCT significantly reduced the incidence of both acute and cGVHD as well as NRM[63]. Hence, inhibiting B cell function has profound effects on both B and T cell homeostasis, with significant benefits to cGVHD patients especially in cases where other primary and/or secondary treatments have been unsatisfactory.

ANIMAL MODELS OF CGVHD

Several types of murine models have been utilized for studies of GVHD pathobiology including: (1) a bone marrow transplantation (BMT) model involving lethal radiation (total body irradiation, TBI) and transplantation of syngeneic marrow into treated mice[64]; (2) a parent-into-F1 model where donor spleen cells are infused into non-irradiated mice[39]; and (3) a transgenic model utilizing a self-antigen, membrane-associated chick ovalbumin, expressed under the control of the K14 promoter (K14-mOVA), where autoreactive skin disease is promoted by adoptive transfer of CD8 T cells from a second mouse strain, OT-1, that has an engineered T cell receptor specific for an ovalbumin peptide[65,66]. These animal models each demonstrate one or more manifestations of clinical cGVHD including the presence of anti-DNA antibodies, sclerosis, weight loss and chronic inflammation of skin and mucosal tissues associated with elevated Th1, Th2 and/or Th17 cytokines. The K14-mOVA adoptive transfer model has been used to test the efficacy of novel anti-inflammatory biologics that target the Janus kinase (JAK)[67] and Histone Deacetylase 6[68] enzymes, which were shown to be effective at suppressing and/or

reversing cutaneous disease. Tofacitinib, the JAK inhibitor, blocked the expansion and activation of CD8^{+} cells thereby reducing IFN-γ secretion by CD8^{+} cells and keratinocytes as well as preventing the downstream consequences of interferon signaling such as chemokine production and keratinocyte apoptosis[67]. Another JAK inhibitor, Ruxolitinib (INCB018424), reduced murine GVHD (acute GVHD) symptoms and the levels of pro-inflammatory cytokines by both impairing differentiation of CD4^{+} T cells into IFN-γ and IL-17-producing cells, and by promoting the production of protective Tregs[69]. Notably this JAK 1/2 inhibitor reduced GVHD symptoms and improved overall animal survival while still maintaining the anti-tumor (GVT) effect[70].

While these pre-clinical models have been valuable in defining the immune mediators of cGVHD, the animal models do not typically parallel the evolution of the human disease, especially the common clinical presentation of classic cGVHD[8]. Additionally, in mice receiving intensive conditioning regimens (especially radiation), there is a well characterized scenario of inflammatory cytokine release, T cell activation and homing to target organs where tissue destruction occurs through the action of PBMCs and their associated cytokines. However, in humans the preparative regimen is only one factor involved in GVHD initiation, and its influence may be diminished in patients who now receive reduced-intensity conditioning prior to allo-HSCT[8]. Some recently described animal models exhibit systemic disease with multi-organ involvement including the lung, which appears to more closely resemble human cGVHD[71]. Despite some weaknesses, animal models will undoubtedly continue to provide insight into specific aspects of cGVHD pathobiology and will be essential for preclinical testing of new therapies for acute and cGVHD.

CGVHD BIOMARKERS

An emerging area of cGVHD research involves the discovery and validation of biomarkers that might eventually be used in clinical diagnosis or treatment planning. To date, most studies have focused on protein and immune cell biomarkers, even though RNAs (including mRNAs and micro RNAs) might also have utility as disease biomarkers[54,60]. As defined at the first meeting of the NIH Biomarker Working Group in 2006, cGVHD biomarkers could be used in disease management or clinical trials to: (1) predict response to therapy; (2) measure disease activity; (3) predict the risk of developing cGVHD; (4) diagnose cGVHD or predict prognosis; and (5) serve as a surrogate end point for therapeutic response[37].

To date, researchers have utilized mass spectrometry-based discovery approaches as well as Luminex and antibody arrays to screen clinical samples for potential serum and saliva protein biomarkers. Biomarkers identified to date can be broadly divided into proteins that function as cytokines and chemokines, immune (*e.g.,* cytokine) receptors and other types of immune or non-immune proteins (Paczesny *et al*[72] for a recent review).

Identified serum biomarkers that might indicate overall disease (and/or altered immune cell) activity include B cell activation factor (BAFF), MICA and anti-MICA antibodies, TNF-α, IL-15 and Chemokine (C-X-C motif) ligand 9 (CXCL9). Salivary biomarkers, associated mainly with oral cGVHD, include IL-1ra, cystatin B, lactotransferrin and lactoperoxidase (Table 3). Cellular markers primarily comprise immune cell populations that are altered in cGVHD (Table 2).

Serum biomarkers

Chronic cGVHD onset and/or persistence is associated with increased levels of TNF-α, BAFF, IL-6, sIL-2R (soluble IL-2 receptor alpha), and IL-10, and decreased levels of TGF-β and IL-15 (Table 3). Several studies have reported elevated levels of the pro-inflammatory cytokine TNF-α in acute and cGVHD, with measured levels correlating with cGVHD severity[73-75]. IL-6 shows a similar trend and correlation with cGVHD severity[42,75]. Soluble IL-2 receptor alpha (sIL-2Rα) is another example of a serum marker that is increased in pediatric and adult patients with cGVHD[59,76]. BAFF, a growth factor that promotes B cell differentiation and immunoglobulin production, is increased in both pediatric and adult patients with cGVHD; levels of this growth factor are often reported relative to the number of B cells in blood samples[51,59,60,77]. High levels of BAFF protein were present in allo-HSCT patients who subsequently developed cGVHD, confirming its role in alloimmunity[77]. CXCL9 levels are also elevated in newly diagnosed cGVHD patients and were correlated with disease severity in three different cohorts studied at two transplant centers[51].

Other markers besides BAFF and CXCL9 have been shown to have potential predictive value in allo-HSCT patients for determining future disease. For example, elevated levels of soluble MICA protein post-allo-HSCT were associated with an increased risk of cGVHD (by contrast, as stated above, the presence of MICA antibodies before transplantation conferred some protection from cGVHD)[30]. Similarly, Pratt *et al*[78] have shown that patients with low serum levels of IL-15 at day 7 post-transplant had 3-fold higher risk of developing cGVHD subsequently. IL-15 levels were observed to be inversely correlated with CD8 T cell levels, which are important for the GVT effect but also influence the development of cGVHD (see above).

In addition to intrinsic, host-dependent (*e.g.,* immune) factors, the levels of biomarkers such as BAFF and CXCL9 can be modified by extrinsic factors including immunosuppressive drugs such as corticosteroids[51,54]. Hence, as recognized by many investigators, independent validation of promising biomarker candidates is essential. CXCL9 was recently validated as a cGVHD biomarker in a multicenter United States study of allo-HSCT patients[79].

Salivary biomarkers

Two recent studies utilizing mass spectrometry approaches identified a total of 82 and 102 salivary proteins, re-

spectively, that showed altered expression in oral cGVHD[80,81]. IL-1 receptor antagonist (IL-1ra) exhibited reduced expression in patients with oral cGVHD[80]. The changes in IL-1ra expression coupled with higher levels of IL-1 family cytokines[16] in saliva likely enhance oral inflammation and subsequent tissue damage. In particular, IL-6 levels have been shown to correlate with oral cGVHD severity[16]. Changes in expression of salivary lactoperoxidase and lactotransferrin have also been reported, indicative of impaired innate immunity in oral cGVHD[81] (Table 3). The alterations in the salivary proteome among proteins involved in innate and acquired immunity are consistent with the clinical features of oral cGVHD, in particular patient susceptibility to bacterial and viral infections[4,18]. Changes in inorganic salivary components, especially Na^+ and Cl^- ions and inorganic phosphate, also occur in concert with cGVHD onset, correlating with hyposalivation and damage to the salivary glands[82,83].

Other biofluids
Certain Th2 and Th17 cytokines, in particular IL-6, IL-10, IL-17A and TNF-α are elevated in the tear fluid of cGVHD patients and correlated with systemic cGVHD regardless of ocular symptoms; levels of three of these cytokines (IL-6, IL-10 and TNF-α) also were significantly correlated with ocular cGVHD parameters[84].

Cellular biomarkers
In addition to protein biomarkers, a large number of immune cell populations have been studied as potential cGVHD biomarkers. Some of the best studied are listed in Table 2. As discussed in the Pathobiology section above, $CD4^+$ $IL-17^+$ Th17 cells are elevated in active cGVHD while Tregs that express the markers CD4, CD25 and FoxP3 are typically decreased[42,47]. There are also complex changes in the B cell population of cGVHD patients. Overall, total B cell counts are decreased in cGVHD patients as are the levels of naïve, transitional and regulatory B cells. In contrast, differentiated $CD38^+$ $CD27^+$ IgG-secreting plasma cells are increased in patients with active cGVHD (Table 2)[53,54].

Antibodies
Antibodies including autoantibodies are another group of well-studied potential biomarkers that are produced in cGVHD patients by an aberrant B cell population. Up to 80% of allogeneic transplants involving a female donor-male recipient combination produce antibodies against Y-chromosome-encoded HY proteins, and these antibodies appear to predict the development of cGVHD[8,37]. Antibodies to Platelet-derived Growth Factor (PDGF) Receptor, double stranded DNA and anti-nuclear antibody (ANA) are also common in cGVHD patients[59]. Anti-PDGF receptor antibodies cause accumulation of reactive oxygen species and stimulate type I collagen expression, suggesting a role for these antibodies in skin and lung fibrosis[85,86]. Patients with classic cGVHD were found to have higher levels of ANA and anti-DNA antibodies compared to patients who had a prior history of acute GVHD where B cells have limited involvement[59].

CONCLUSION
CGVHD is a chronic inflammatory and autoimmune-like condition that involves a complex interplay between the immune systems of the transplant donor and recipient. Despite significant progress in understanding the risk factors, and the development of effective second-line treatments for steroid-refractory cGVHD, the incidence of cGVHD is increasing worldwide[7]. While donor-derived T cells are still considered to be the preeminent mediators of cGVHD, aberrant B cells clearly play a significant role in promoting autoimmunity and inflammation, and con-ferring susceptibility to serious, often life-threatening infections. The enhanced activity of T follicular helper cells in cGVHD also appears to play a key role in the aberrant B cell activity and the resulting autoimmune-like features of cGVHD, including the presence of antibodies that target HY and nuclear proteins[44].

In addition to the significant progress in our under-standing of cGVHD immunobiology and pathobiology, guidelines for biomarker development and validation were recently updated. The updated guidelines include recommendations for biomarker identification, verification, qualification, and application with terminology based on Food and Drug Administration and European Medicines Agency guidelines[72]. Suggested areas of focus for vali-dation include biomarkers that are prognostic, stratify cGVHD risk or are predictive of future disease. Biobank repositories that can serially collect peripheral blood and cell samples from allo-HSCT patients in a standardized format will also be an important tool for pre-clinical biomarker validation[72]. The French National Cryostem Project is one example of such a national effort[87,88] which, together with multicenter collaborations[51,79], should enable protein biomarkers to be added to the clinician's toolkit for cGVHD patient care in the not-too-distant future.

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