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Biomarkers in chronic graft-versus-host disease

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Abstract

Chronic graft-versus-host disease (cGVHD) is a leading cause of allogeneic hematopoietic stem-cell transplantation-related mortality and morbidity. It is an immune-mediated disorder that can target almost any organ in the body, often with devastating consequences. The immune-suppressive medications currently used to treat it are equally toxic and are often not very effective. At this time, our understanding of its pathophysiology is limited. The discovery of potential biomarkers offers new possibilities in the clinical management of cGVHD. They could potentially be used for diagnosing cGVHD, for predicting or evaluating response to therapy and for unique insights into the pathophysiology underlying the clinical manifestations of cGVHD. Understanding the biological origins of these biomarkers can help us construct a more comprehensive and clinically relevant model for the pathogenesis of this disease. In this article, we review existing evidence for candidate biomarkers that have been identified in the framework of how they may contribute to the pathophysiology of cGVHD. Issues regarding the discovery and application of biomarkers are discussed.

Keywords

allogeneic hematopoietic stem-cell transplantation; biomarkers; chronic graft-versus-host disease; graft-versus-leukemia

Hematopoietic stem-cell transplantation (HSCT) has become a curative therapy for increasing numbers of diseases, both malignant and nonmalignant. To date, it is the only successful cellular immunotherapy for high-risk malignancies such as leukemia, taking advantage of the graft-versus-leukemia (GVL) effect. In pediatrics, it offers curative therapy for nonmalignant blood disorders such as thalassemia [1], immune dysregulation [2], congenital bone marrow failure syndromes [3], inborn errors of metabolism [4] and autoimmune conditions [5]. To meet the need for donors, the use of unrelated donors has lead to an increase in the occurrence of both acute and chronic graft-versus-host disease (cGVHD), where immune cells from the donor respond against multiple organs in the patient.

There are two main categories of graft-versus-host disease (GVHD), acute and chronic, each with two subcategories [6]. The acute GVHD (aGVHD) category includes classic aGVHD occurring within 100 days after transplantation and persistent, recurrent or late aGVHD

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(features of aGVHD occurring beyond 100 days, often during withdrawal of immune suppression). The broad category of cGVHD includes classic cGVHD (without features or characteristics of aGVHD) and an overlap syndrome in which diagnostic or distinctive features of cGVHD and aGVHD appear together.

Acute GVHD is characterized by skin, gastrointestinal and hepatic involvement. It induces an erythematosus rash that can progress to bullae in its most severe form. Gastrointestinal involvement usually involves a watery, secretory diarrhea and hepatic involvement typically targets the biliary epithelium. Based on the close concordance of mouse GVHD models with human aGVHD, we have a very good understanding of aGVHD pathophysiology. aGVHD is thought to occur in three sequential phases: activation of antigen-presenting cells (APCs) via a cytokine storm caused by recipient conditioning tissue damage; donor T-cell activation; and target cell apoptosis [7].

Tailored conditioning regimens, effective antimicrobial therapy and better supportive care have resulted in a significant reduction in the early morbidity and mortality associated with HSCT [8]. As a consequence, cGVHD has become the leading cause of transplantation-related morbidity and mortality [9,10]. In adults with cGVHD there is approximately 60% mortality after 8 years [11], and in children mortality is 20% after 15 years [12].

However, cGVHD is irrevocably linked to the GVL effect, in which the immune system cells from a normal donor attack cancer cells. The beneficial effect of cGVHD on the incidence of leukemia relapse is well established [13–15]. The challenge of allogeneic stem-cell transplantation for treatment of hematological malignancies is to prevent GVHD without losing the GVL effect [16]. Furthermore, in patients receiving transplants for nonmalignant diseases, avoiding GVHD is essential in justifying the benefits of transplant in light of its morbidity and mortality.

Chronic GVHD involves multiple organs. Diagnostic signs and symptoms of cGVHD include: skin (poikiloderma and lichen planus-like, sclerotic, morphea-like and lichen sclerosus-like features), mouth (lichen-type features, hyperkeratotic plaques), genitalia (lichen planus-like features, vaginal scarring or stenosis), GI tract (esophageal web, strictures or stenosis in the upper-to-mid third of the esophagus), lung (bronchiolitis obliterans), and muscles, fascia and joints (fasciitis and joint stiffness or contractures secondary to sclerosis). There are many other distinctive and common features of cGVHD that involve the skin, nails, mouth, eyes, liver, muscle, fascia and hematopoietic and immune system. In particular, the effect on the immune system can be devastating and the majority of deaths in patients with cGVHD are attributed to infections [10]. Persistently decreased cellular immunity [17] and functional asplenia [18], features of cGVHD, render patients highly susceptible to opportunistic bacterial, viral and fungal infections. Many of the clinical signs resemble autoimmune and immunological disorders such as scleroderma, Sjögren's syndrome, systemic lupus erythematosus (SLE), inflammatory bowel disease and rheumatoid arthritis [19–21]. The diagnosis of cGVHD requires at least one diagnostic manifestation of cGVHD or at least one distinctive manifestation, with the diagnosis confirmed by pertinent biopsy, laboratory tests or radiology in the same or another organ. The limitations of these criteria are that they are not response criteria and do not distinguish between disease activity and irreversible damage [6].

At this time, treatment of cGVHD remains unsatisfying. Corticosteroids are first-line therapy, but they are not always effective and their long-term use is associated with serious complications [22,23]. Therefore, new steroid-sparing approaches are being pursued. A number of drugs, including calcineurin inhibitors [24], sirolimus [25], rituximab [26–29], mycophenolate mofetil [30], thalidomide [24], hydroxychloroquine [31], pentostatin [32]

and extracorporeal photophoresis [33] have been used with varying results, often in combination with corticosteroids. Unfortunately, all of these medications have their own serious side effects that potentially contribute to morbidity. None of these agents have been shown to be superior to corticosteroids alone [34]. Direct comparison between different treatments is further hampered by differences in study design and end points. One of the most fundamental challenges clinicians face is deciding which intervention is appropriate and for how long to continue therapy after clinical resolution of symptoms. We are limited by our lack of insight into the basic biology of cGVHD and a shortage of comprehensive instruments to properly diagnose cGVHD.

The official NIH definition of a biomarker is a characteristic that is objectively measured and evaluated as an indicator of normal biologic processes, pathogenic processes or pharmacologic responses to a therapeutic intervention [35]. The following applications of biomarkers could be useful in cGVHD clinical trials or management:

- Predicting response to therapy;
- Measuring disease activity and distinguishing irreversible damage from continued disease activity;
- Predicting the risk of developing cGVHD;
- Diagnosing cGVHD;
- Predicting the prognosis of cGVHD;
- Evaluating the balance between GVHD and GVL effects (GVL or graft-versus-tumor);
- Serving as a surrogate end point for therapeutic response [36].

Biomarkers could also be used to elucidate the biologic mechanisms of a disease. Understanding the biological origins of these biomarkers helps us construct a more comprehensive and clinically relevant model for the pathogenesis of this disease. In turn, understanding mechanisms will provide potential targets for therapies. The ideal aim is to use biomarkers to have both prognostic and diagnostic uses. For example, they could initially be used to establish disease status and stage of disease. Then they could be used to stratify diseased populations. Biomarkers would be used to predict the likely course of disease and the response to medications.

This article will provide a detailed description of potential biomarkers categorized according to mechanisms that are thought to contribute to the pathogenesis of cGVHD. We believe that understanding the underlying mechanism/origin of a biomarker is the ideal way to then evaluate its prognostic and diagnostic use.

Allogeneic disparity as a cGVHD biomarker: HLA disparity

Chronic GVHD is significantly increased in patients receiving unmanipulated bone marrow and peripheral blood stem cells from unrelated class I mismatched donors, the mismatch either detected by low- or high-resolution typing [37]. An HLA-A/-B mismatch also induces a significantly higher incidence of cGVHD than in HLA-matched patients receiving marrow transplants whereas a HLA-DR or -DQ mismatch does not [38]. These findings vary from study to study where others have shown that HLA-A mismatching is associated with a higher incidence of cGVHD in bone marrow transplants, and the possibility that HLA-DR and -DP are also mismatches [39]. Similar observations have been made in reduced-intensity conditioning stem-cell transplantation for hematological malignancies where increased HLA disparity correlates to the development of cGVHD [40]. These correlations may be

associated with the donor source since HLA mismatch was not associated with cGVHD in unrelated-donor peripheral blood stem-cell transplantation in one study [41]. To further support the impact of donor source as a major factor, the association of HLA disparity with cGVHD after cord-blood transplantation is less clear. Several studies have demonstrated that the incidence of cGVHD did not correlate with the extent of HLA disparity [42,43]. More often, given the low incidence of cGVHD associated with cord-blood transplantation, risk factor analysis cannot be performed [44,45]. Chronic GVHD following umbilical cord blood transplant may be more responsive to therapy, leading to a lower nonrelapse mortality [46].

Allogenenic disparity as a cGVHD biomarker: disparities in minor histocompatibility antigens

By focusing on HLA-identical bone marrow transplants, several groups have identified minor histocompatibility antigens (mHAgs) associated with occurrence of cGVHD. Using tetrameric HLA-class and I-mHAg HA-1 and HY peptide complexes, one group demonstrated a significant increase in HA-1 and HY-specific cytotoxic T lymphocytes during cGVHD, which decreased after successful GVHD treatment [47]. It has been well established that the use of parous female donors results in an increased risk of cGVHD [48], supporting the view that donor immune cells specific for male mHAg encoded by Y-chromosome genes contributes to cGVHD [49]. A large retrospective review concluded that mHAg incompatibility at HA-1, HA-2, HA-3, HA-8 and CD31 has no detectable effect on the outcome of HLA-matched unrelated donor HSCT [50].

Allogeneic disparity as a cGVHD biomarker: non-HLA polymorphisms

There is increasing evidence that non-HLA gene polymorphisms can influence the risk of cGVHD. The overwhelming majority of genetic variation in humans consists of single-nucleotide polymorphisms (SNPs). SNPs found in coding regions of the genome produce functional differences in gene products. This would lead to altered functions at any step in possible biological pathways in cGVHD. By identifying specific genes with SNPs that alter the severity or occurrence of cGVHD, we can then study the properties of the gene products and how they contribute to the pathophysiology of cGVHD. This approach is becoming even more appealing as analysis of polymorphisms performed pre-HSCT can predict the development of cGVHD, allowing for risk classification of HSCT recipients. Candidate polymorphisms are summarized in Table 1.

A number of recipient polymorphisms have been identified. Two SNPs in the human heparanase gene, associated with high recipient heparanase levels, were significantly correlated with extensive cGVHD [51]. Heparanase is an enzyme that degrades polymeric heparan sulfate molecules into shorter chain-length oligosaccharides. The authors hypothesized that heparan sulfate degradation fragments activate T cells, monocytes and dendritic cells, resulting in the synthesis of cytokines and matrix metalloproteinases known to be involved in GVHD. Upregulation of heparanase has also been found in the colonic epithelium of inflammatory bowel disease [52] and in the synovial fluid of patients with rheumatoid arthritis [53].

A second recipient biomarker associated with tissue damage has been described. Polymorphisms in the *PARP1* gene have been associated with a higher risk of cGVHD [54]. PARP1 has a role in repair of ssDNA breaks. Variations in DNA repair can influence the amount of tissue damage caused by the conditioning regimen prior to stem-cell transplant. Tissue damage is thought to be one of the initiating events in the pathogenesis of GVHD.

Additional recipient polymorphisms are associated with the inflammatory response. A specific *MADCAM1* gene SNP was associated with a significantly higher risk of cGVHD [55]. The protein encoded by this gene is an endothelial cell adhesion molecule that interacts preferentially with receptors on myeloid cells to direct leukocytes into mucosal and inflamed tissues.

Serum haptoglobin levels in patients with cGVHD are higher than in patients without cGVHD [56]. In addition, patients with cGVHD had a higher incidence of haptoglobin 2–2 phenotype in comparison to patients without cGVHD. This is an important finding as haptoglobin has been shown to modulate the immune system, as demonstrated by an *in vitro* inhibitory effect on Th2 cytokine release promoting Th1 activation over Th2 [57], inhibition of cathepsin B and L [58], and inhibition of monocyte and macrophage function [59].

A relationship between the Fc receptor-like (FCRL) 3 gene SNP and the occurrence of cGVHD has been identified [60]. The same SNP is associated with susceptibility to rheumatoid arthritis, autoimmune thyroid disease and SLE [61]. FCRL molecules are preferentially expressed in B cells and can exert immunoregulatory functions either through tyrosine-based inhibitory and/or activation-like motifs in their cytoplasmic tails. The authors propose that host B cells that highly express *FCRL3* have a protective effect against cGVHD [60], providing another piece of evidence implicating B cells in the pathogenesis of cGVHD.

Two donor polymorphisms associated with development of cGVHD involve the high mobility group box 1 (*HMGB1*) and the chemokine *CCR9* genes. There is a successive increase in the incidence of limited or extensive cGVHD with the donor carrying 0, 1 or 2 minor alleles of the *HMGB1* 3814C > G, 1177G > C and 2351insT genotype [62]. *HMGB1* is an endogenous damageassociated molecular pattern. It diffuses freely from necrotic cells and is tightly sequestered in the nucleus of apoptotic cells, providing an endogenous danger signal for the organism to distinguish between programmed and nonprogrammed cell death [63]. Extracellular HMGB1 exhibits inflammatory cytokine-like activity and acts as a potent mediator of APC activation and proliferation of T cells [64]. The 2351insT minor allele is associated with increased function of HMGB1. Increased extracellular expression of HMGB1 is a feature of autoimmune diseases that share clinical features with cGVHD such as Sjögren's syndrome [65] and SLE [66]. The 926AG SNP in the *CCR9* gene, which encodes for a chemokine differentially expressed by T lymphocytes of the small intestine and colon, was significantly associated with the incidence of chronic skin GVHD [67]. The authors were able to show more active homing of CCR9–926AG T cells to Peyer's patches.

Cytokines have been an intense field of study and numerous polymorphisms have been identified in both donor and recipient. There have been numerous studies evaluating the role of IL-10 and IL-10β receptor SNPs on the incidence of cGVHD after HSCT. IL-10Rβ rs1800872 A/A homozygous patients were protected from cGVHD when the patient and donor had similar IL-10 production levels [68]. Another group identified an IL-10 promoter gene polymorphism – known to be associated with a lower production of IL-10 cytokine – correlated with development of cGVHD [69]. They identified a recipient haplotype that was associated with a significantly shorter duration of systemic immunosuppressive therapy, making this one of the few potential biomarkers that could predict response to therapy. Others have shown that the donors of the patients with cGVHD more frequently possessed a greater number of alleles in the *IL-10* gene [70].

Other cytokine polymorphisms associated with cGVHD are IL-1, TNF-α, IL-6 and IFN-γ. Polymorphisms of the promoter gene *TNFA-238GA* have also been associated with the development of extensive cGVHD [71,72]. An IL-6 polymorphism at position −174 of the recipient and donor was associated with the increased incidence of cGVHD [73] as well as

IL-1α gene polymorphisms [74,75]. Microsatellite polymorphisms within the first intron of the *IFN-γ* gene appear to be associated with decreased IFN-γ production and have higher rates of cGVHD [76].

Immune effector populations as a cGVHD biomarker: Th1 & Th2 shifts in response

There are data suggesting that cGVHD may be a Th2-mediated process, as indicated by overproduction of cytokines such as IL-4 and IL-5 [77]. Eosinophilia, which is associated with cGVHD, can occur with Th2-mediated processes. Consistent with the finding that IL-10 polymorphisms have a strong impact on development of cGVHD, higher levels of IL-10 at the fourth month post-transplant is associated with development of cGVHD, potentially owing to a Th2 predominance [78]. Similarly, high levels of Th1 cytokines, by $CD8^+$ T cells, such as TNF-α, IFN-γ and IL-1, have been found in skin lesions and in the peripheral blood of cGVHD patients [79–82]. Further support for the importance of Th1/Th2 shifts is that a lower level of IFN-γ at the third month post-transplant is present in patients that develop cGVHD [80]. Unfortunately, this observation is not consistent in that others have shown an increased IFN-γ mRNA expression associated with extensive cGVHD [83,84].

As a whole, these results suggest that there are no distinctive Th1 or Th2 cytokine profiles for cGVHD. A possible explanation for this discrepancy is that underlying pathological mechanisms are temporally regulated in cGVHD. Our group has recently found that there are different cytokine profiles associated with early (3–8 months) and late (≥9 months) cGVHD [85]. We found that early-onset cGVHD was characterized by decreased expression of IFN-γ and IL-2 mRNA after nonspecific phorbol 12-myristate 13-acetate-ionomycin stimulation. By contrast, late-onset cGVHD was characterized by decreased expression of IL-4 and IL-2 mRNA after anti-CD3 stimulation of T cells. Receiver operator characteristic curve analysis revealed that IFN-γ production could determine the absence of early cGVHD, and IL-4 and IL-2 the absence of late cGVHD.

Immune-modulatory populations as cGVHD biomarkers: T cells

It is clear that there are several different immune cell populations, whose function and numbers are altered in cGVHD. The potential to manipulate specific immune cell populations *ex vivo* and *in vivo* to modulate pathogenic immune responses has proven fertile ground for the development of new therapies. These are summarized in Table 2.

The role of regulatory T cells (Tregs) as a biomarker in cGVHD remains unclear, as results are contradictory, demonstrating normal, decreased and increased levels in cGVHD. Some groups have shown no significant difference in the number of Foxp3-expressing $CD4^+CD25^{high}$ T cells in patients with or without GVHD [86]. Others have shown that patients with cGVHD had markedly elevated numbers of $CD4^+CD25^{high}$ T cells as compared with patients without GVHD [87]. *In vitro*, the $CD4^+CD25^{high}$ T cells were hyporesponsive to polyclonal stimulation and suppressed the proliferation and cytokine synthesis of $CD4^+CD25^-$ cells. The author's conclusion was that these results indicate the cGVHD does not occur as a result of Treg deficiency.

Contrasting data demonstrate that Tregs are correlated with less cGVHD. Several groups have demonstrated significantly decreased Foxp3 mRNA expression [88] and decreased numbers of $CD4^+CD25^+$ $Foxp3^+$ T cells [78,89,90] in patients with cGVHD. One group has shown that Treg levels declined in patients with prolonged $CD4^+$ lymphopenia after transplant and that this pattern was associated with a high incidence of extensive cGVHD

[91]. The authors suggest that the decrease is caused by limited generation of naive Tregs in the thymus and increased susceptibility of the $CD45RA^{-}$ activated/memory phenotype to apoptosis. Another group suggests that alloantigen-driven expansion may be the key to the effectiveness of $CD4^{+}CD25^{+}$ Tregs in cGVHD [92]. Tregs could attenuate cGVHD through a number of possible mechanisms, including direct lysis of cytotoxic cells, inhibition of inflammatory cytokine production and secretion of immunomodulatory cytokines. The ability to manipulate the number and function of Tregs could prove to be a potent therapeutic tool. It would also be interesting to determine whether increasing Treg activity may in turn negatively reduce the GVL effect.

The ability to comparatively analyze the different results regarding Tregs is hindered by different study end points, patient selections, control populations and heterogeneous immunosuppressive therapy. Some of the conflicting observations may also be explained by the fact that Tregs lack clear defining markers and functional assays are difficult to perform. For example, expression of Foxp3 is a normal consequence of $CD4^{+}$ T-cell activation [93] and IL-2 is responsible for T-cell antigen receptor-activated Foxp3 expression by both $CD4^{+}$ and $CD8^{+}$ human T cells [94]. These findings provide evidence that Foxp3 cannot be used as an exclusive marker of Tregs. Therefore, it is possible that elevated Foxp3 expression may represent Tregs and/or activated $CD8^{+}$ or $CD4^{+}$ effector T cells, potentially providing an explanation for the contradictory results regarding Tregs in cGVHD. For example, our laboratory found elevated Foxp3 mRNA expression in patients with early-onset cGVHD (3–8 months post-HSCT), but in late-onset cGVHD (≥9 months) elevated Foxp3 mRNA expression is only found in control patients without cGVHD, providing evidence for differing temporal T-cell mechanisms on cGVHD [85].

T cells play a role in cGVHD and identification of specific markers may be used as biomarkers. A higher percentage of donor $CD4^{+}$ effector memory cells ($CCR7^{+}/CD62L^{low}$) may be associated with cGVHD [95]. A second population associated with cGVHD is markedly higher levels of blood effector $CD8^{+}/CCR7^{-}/CD45RA^{+}$ cells compared with patients without cGVHD [96]. These $CD8^{+}$ cells have low CD8 coreceptor expression, reduced proliferative potential, and a high content of perforin and granzyme A. They also have a lower cell turnover and propensity to apoptosis. Histopathologically, there is infiltration of both $CD4^{+}$ and $CD8^{+}$ T cells in oral lichenoid lesions of cGVHD [97].

Recently, the possibility that the Th17 population may be a biomarker for cGVHD has been evaluated. One group has shown an increased Th17 population in patients with active cGVHD. The percentage of these cells drastically decreased in patients with inactive cGVHD. IFN-γ^{+} Th17 cells were also able to infiltrate liver and skin GVHD lesions. Most interestingly, the authors observed an inverse relationship between the proportion of Th17 and Tregs [98].

Immune-modulatory populations as cGVHD biomarkers: dendritic cells

Dendritic cells (DCs) may be altered in cGVHD and potentially act as a biomarker. They serve as a link between the innate and adaptive immune system by processing antigen material and presenting it to other immune cells. Two functional subsets have been described in humans: myeloid (DC1: $CD11c^{+}HLADR^{+}lin^{-}$) and plasmacytoid DCs (DC2: $CD123^{+}HLADR^{+}lin^{-}$). The proposed distinction at the functional level is that the former type is for immunity and the latter for regulation or tolerance [99].

Higher numbers of plasmacytoid DCs or DC2 in donor bone marrow grafts were significantly associated with a lower risk of developing cGVHD after transplant [100]. However, they also observed a strong, direct correlation between high donor graft numbers of DC2 and the risk of relapse. Another study found that a low plasmacytoid DC count in

the recipient's peripheral blood on day 28 (≤4.5/μl) was significantly associated with the development of cGVHD in patients who underwent HLA-matched related G-CSF mobilized allogeneic peripheral blood stem-cell transplant [101]. By contrast, a preliminary study involving peripheral blood stem-cell transplant from HLA-related donors found that cGVHD correlated with higher DC2 numbers in the graft in ten patients with cGVHD, compared with 12 patients without cGVHD [102]. There are no validated DC populations that can currently be used as a biomarker.

Immune-modulatory populations as cGVHD biomarkers: monocytes

Monocytes potentially also play a role in cGVHD and may be used as a biomarker. One group demonstrated that patients with cGVHD showed increased numbers of marrow monocytes when compared with patients without cGVHD [103]. Furthermore, the marrow-derived monocytes of cGVHD patients had greater CD86 expression in both the marrow and peripheral blood, and treatment with prednisone resulted in decreased CD86 expression. At this time, such a population cannot be considered a validated biomarker for use in cGVHD.

Immune-modulatory populations as cGVHD biomarkers: eosinophils

There are numerous reports of eosinophilia in adult patients associated with cGVHD [104–106] and one showing that an absolute eosinophil count of higher than 500×10^6/l often predates or coincides with cGVHD in children [107]. Their data suggested that by 2 years after stem-cell transplantation, patients without eosinophilia do not appear to develop cGVHD. Eosinophilia does appear to meet criteria as a biomarker for use clinically and will require further validation.

Immune-modulatory populations as cGVHD biomarkers: natural killer & natural killer T cells

One group has developed a novel, reduced-intensity, preparatory regimen using fractionated total lymphoid irradiation and anti-thymocyte serum, which has been shown in a murine model to alter the host immune profile to favor regulatory natural killer (NK) T cells. This population is thought to suppress GVHD by polarizing donor conventional T cells toward secretion of non-inflammatory cytokines such as IL-4 and by promoting expansion of donor $CD4^+CD25^+Foxp3^+$ Tregs [108–110]. Using a total lymphoid irradiation and ATG-containing human protocol to enhance the presence of NK T cells, they went on to demonstrate relatively lower rates of acute and cGVHD and preserved graft-versus-tumor reactions cells [111].

Natural killer cells are also an important immune-regulatory cell population that may serve as biomarkers for cGVHD. Higher bone marrow NK cell doses in patients receiving an HLA identical bone marrow transplant has been associated with a decreased incidence of cGVHD [112]. This correlation has been confirmed by another group who observed that NK and $CD3^+CD152^+$ cell counts were inversely correlated to the onset of cGVHD in the third to sixth month post-HSCT [113]. Expression of inhibitory NK cell receptors such as CD158b and CD94/NKG2A on peripheral $CD3^-$ and $CD3^+$ cells were increased in parallel with GVHD [114]. In the T-cell depleted donor product of haploidentical HSCT, NK cells are an even more important biomarker. Mismatched transplants may trigger NK-cell alloreactivity, and pretransplant infusion of alloreactive NK cells can kill leukemic cells, recipient T cells and DCs, protecting the recipient from GVHD [115].

Immune-modulatory populations as cGVHD biomarkers: B cells

B cells appear to play a major role in cGVHD and appear to have the highest potential to be consistent biomarkers for cGVHD. The role of B cells was first described by our group in a murine model [116], with validation in humans after the successful treatment of steroid-refractory cGVHD with rituximab, an anti-CD20 monoclonal antibody [25–29]. Recently, a prospective, multicenter Phase II trial of weekly rituximab followed by monthly rituximab maintenance therapy for the treatment of steroid-refractory cGVHD was concluded [117]. It was the largest prospective study to date with 37 patients, both pediatric and adult. They reported an overall response rate of 86%, with a complete response rate of 25%. Most importantly, it allowed patients to reduce or discontinue steroid use earlier.

It appears that there are several potential mechanisms through which B cells can contribute to pathogenesis, including alloantibody production, cytokine production and antigen presentation. An association between circulating autoantibodies and cGVHD was first reported in 1980 [106]. Subsequently, antibody responses to the Y chromosome-encoded mHAgs following sex-mismatched HSCT were shown to correlate with the development of cGVHD [118,119]. Antinuclear autoantibody (ANA) has also been shown to be more frequent in patients with extensive cGVHD, with the nucleolar pattern of immunofluorescence of ANA correlating with the degree and extension of cGVHD [120]. Patients who developed these autoantibodies had higher $CD20^{+}$ cell blood counts than negative patients post-transplant. Other studies have confirmed the prevalence of ANAs in cGVHD [121]. Other autoantibodies have also been described including smooth muscle, cardiolipin and dsDNA [122–124]. A recent predictive study has shown anti-dsDNA as the marker with the highest sensitivity and specificity [125].

More recently, stimulatory antibodies to the PDGF receptor were found selectively in all patients with extensive cGVHD [126]. Higher levels were detected in patients with generalized skin involvement and/or lung fibrosis. The antibodies recognized PDGF receptor, induced tyrosine phosphorylation, accumulation of reactive oxygen species (ROS) and stimulated type 1 collagen gene expression through the Ha–Ras–ERK1/2–ROS signaling pathway. All of these markers require further validation before they can be considered clinically useful.

B-cell cytokines are a second potential biomarker. B-cell activating factor (BAFF) is a ligand of the tumor necrosis family. Along with cGVHD, elevated serum levels of BAFF have been found in patients with SLE [127], rheumatoid arthritis [128] and Sjögren's syndrome [129] – autoimmune diseases that share clinical features with cGVHD. BAFF overexpression has been found to rescue self-reactive B cells normally deleted with relatively low stringency and facilitate their migration into otherwise forbidden microenvironments [130]. Using BAFF transgenic mice, the authors were able to show that BAFF overexpression resulted in self-reactive B cells normally deleted in the bone marrow around the late T2 stage of peripheral development were rescued from deletion, matured and colonized the splenic follicle. This may explain the autoimmune features associated with cGVHD.

A number of groups have evaluated soluble BAFF as a possible biomarker for cGVHD. BAFF levels were significantly higher in adult patients with active cGVHD compared with those without disease [131]. Treatment with high-dose prednisone (≥30 mg/day) was associated with reduced BAFF levels in patients with active cGVHD. A predictive study showed that 6-month BAFF levels ≥10 ng/ml were strongly associated with subsequent development of cGVHD. Soluble BAFF was elevated in patients with both early- and late-onset cGVHD compared with controls as part of a Children's Oncology Group Phase III

cGVHD therapeutic trial [125]. Recently it was demonstrated that clinical response to rituximab in cGVHD was associated with naive B-cell reconstitution and decreased BAFF/B-cell ratios [132].

The cell type responsible for producing BAFF in patients with cGVHD remains to be determined. However, there are clues in other autoimmune diseases. In the salivary glands of patients with primary Sjögren's syndrome, B cells produce BAFF. Furthermore, receptors for BAFF were observed on transitional B lymphocytes, creating the potential for an autocrine pattern of self-stimulation [133]. $CD4^+$ and $CD8^+$ T cells from patients with active SLE expressed intracellular BAFF, whereas those from normal subjects did not. The authors propose that BAFF may play a pathogenic role in SLE by stimulating T-cell-dependent B-cell autoantibody production [134]. The use of belimumab, a BAFF-specific inhibitor, has shown promising efficacy in the treatment of active SLE disease [135]. It is only a matter of time before anti-BAFF antibodies will likely be evaluated in cGVHD. Taking into account the time of post-transplant and immunosuppressive treatments, soluble BAFF appears to be one of the most promising biomarkers for cGVHD.

When the role of B cells was first described in a murine cGVHD model, both donor and recipient B cells appeared to be important. Moreover, it was their role as APCs that appeared to be most important [116]. Whether this is true in human cGVHD remains to be determined. In murine studies evaluating the role of B cells in cGVHD, it was shown that chloroquine could inhibit B-cell TLR9 signaling as one possible mechanism for inhibition of GVHD. The role of TLR9-expressing B cells was confirmed in human GVHD with a recent study of CpG oligodeoxynucleotide responsive B cells in cGVHD [136]. A significantly greater percentage of phosphorothioate-modified CpG-stimulated B cells from cGVHD patients demonstrated an increased expression of CD86 compared with controls. This response had a significant correlation between B-cell TLR9 expression and CD86 upregulation using the entirely TLR9-dependent native phosphodiester CpG. The response to CpG oligodeoxynucleotide of this B-cell population at 2 months post-cGVHD therapy appeared to also serve as a surrogate marker for therapeutic response at 9 months post-HSCT.

Chronic GVHD is associated with a lower blood marginal zone B cell/IgM memory B-cell population [137] and elevated numbers of $CD21^{negative/low}$ B cells in patients with active cGVHD [138]. Elevation of this population has been reported in a number of other autoimmune conditions such as rheumatoid arthritis and common variable immunodeficiency [139]. In cGVHD patients with hypogammaglobulinemia, they observed a significant $CD19^+$ B-cell deficiency with significantly higher $CD19^+CD21^{low}$ immature B-cell proportions, significantly higher $CD19^+CD21^{int\text{-}high}CD38^{high}IgM^{high}$ transitional B-cell proportions, significantly lower $CD19^+CD10^-CD27^-CD21^{high}$ naive B-cells and significantly lower $CD19^+CD27^+IgD^+$ nonclass switched and $CD19^+CD27^+IgD^-$ class switched memory B cells compared with cGVHD patients with high or normal gammaglobulinemia [140]. These populations require further validation.

An interesting caveat to the discussion regarding B-cell involvement is that there is a universal assumption that pathogenic B cells are donor-derived. An association has been observed between mixed chimerism state, high levels of pathogenic IgG autoantibodies and subsequent development of cGVHD-like lesions in a murine reduced-intensity conditioning transplantation model [141]. They found that the persistence of host B cells was associated with the appearance of cGVHD-like lesions. They were also able to confirm host origin of autoantibodies.

Inflammatory cGVHD biomarkers

Inflammation is an important component in cGVHD and inflammatory biomarkers show the potential to have a strong correlation with diagnosis, disease activity and therapeutic response. Soluble CD13/aminopeptidase N was found using proteomic analysis, and we were able to validate it in children and adults as a biomarker for early-onset cGVHD [125]. We also found that soluble CD13 did not correlate with induction of anti-CD13 antibodies present in a proportion of patients after allo-HSCT [142]. CD13 is a type II integral membrane protein with both receptor function and enzyme activity. It can alter T-cell function through degradation of peptides bound to MHC class II molecules [143]. Soluble CD13 can be secreted by myeloid and B cells in order to attract T cells. CD13 induces *in vitro* chemotactic migration of human lymphocytes [144]. The chemotactic activity was greater for $CD4^+$ T lymphocytes than for $CD8^+$ lymphocytes. The enzymatic activity of CD13 was responsible for the chemotactic activity because bestatin, an inhibitor of CD13, abolished the chemotactic activity. CD13 also appears to participate in the mechanism of lymphocyte involvement in inflamed joints of rheumatoid arthritis patients as a lymphocyte chemoattractant [145]. Several groups have also shown that early-onset cGVHD is characterized by elevated soluble IL-2Rα [125,146,147], suggesting high levels of T-cell activation.

Donor chimerisms as a cGVHD biomarker

Chimerisms of most post-HSCT cGVHD populations are generally thought to be donor in origin. This is supported by the fact that patients with early complete donor hematopoietic chimerism (by day 100 post-transplant) developed significantly more severe cGVHD, measured by the need for three drug treatments to control the disease [148]. They concluded that achievement of early complete donor hematopoietic chimerism in peripheral blood is strongly predictive of severe extensive cGVHD.

Nonimmune blood-based cGVHD biomarkers

Low platelet count is predictive for poor survival in cGVHD patients after allogeneic HSCT [149]. This may be due to their role in the inflammatory response as part of antigen presentation. Alkaline phosphatase may be a useful predictive factor for the development of progressive- or quiescent-type cGVHD in patients who experience aGVHD after allogeneic HSCT [150,151].

Optimal source to derive cGVHD biomarkers

The majority of biomarkers studied thus far are blood-based with a few originating from biopsy studies. Saliva is easily collected and has been an area of focus. Elevated labial saliva sodium concentration was significantly associated with the occurrence of cGVHD in nonirradiated transplant recipients [152]. The same group also found that patients with active extensive GVHD had significantly depressed levels of salivary IgA [153] and increased concentrations of salivary albumin and IgG [154]. Such changes were also reversible with decreased inflammation. Other groups are evaluating urine-based biomarkers [155]. A concern regarding the collection of blood samples is that if they are transported, which is often unavoidable in multicenter trials with central laboratories, it is possible that results may be affected by release of cytokines and cellular proteins. There is a strong need for robust biomarkers.

Current issues & future directions for cGVHD biomarkers

At this time, there are many promising biomarkers but none of them have been validated in large, prospective, multi-institutional clinical trials to allow for clinical application. These trials are critically needed in order to provide clinicians with biomarkers that can be used for diagnosis, prognostication and potential therapies for specific subtypes, the ultimate goal being personalized treatment of cGVHD.

There are still many outstanding challenges to evaluating potential biomarkers, including:

- Identification of candidate biomarkers: biomarkers can be identified through hypothesis-driven and discovery-based methods. At this time, the majority of biomarkers have been found through the hypothesis-driven approach. The emergence of new microarray technology and advances in proteomics provides investigators with powerful new tools for large-scale testing of biological samples. Samples need to include easily collected biofluids such as blood, urine or saliva along with current gold-standard tissue biopsies with accurate and comprehensive clinical data, taken at well-defined time points;
- Design of appropriate clinical trials to evaluate cGVHD biomarkers: there are many confounding factors that limit the interpretation of previous biomarker studies and which need to be resolved in future studies. These include the type of graft (peripheral blood, bone marrow or umbilical cord blood), mobilization with granulocyte colony-stimulating factor, conditioning regimen, *in vivo* T-cell depletion, presence and immunosuppressive treatment of aGVHD, and time of onset of cGVHD after transplantation. One of the most important factors to consider, especially in immune cell subset analysis, is the gradual immune reconstitution that occurs in all patients post-stem-cell transplant. This requires that samples be taken from time-matched controls without cGVHD;
- Animal models that mimic clinical manifestations of human cGVHD: we need to be able to prove that the origin of biomarkers is a direct consequence of cGVHD-triggering events. Good animal models are needed to show that hypothesized mechanisms resulting in biomarkers will also cause signs and symptoms of cGVHD. Manipulating potential pathogenic mechanisms may eventually provide us with the ideal model systems that will allow the testing of novel therapies;
- Methodological considerations: there is a need for standardized biomarker assays. At this time, techniques for testing are often institution- or investigator-specific. The development of large, multi-institutional clinical trials would require the standardization of assays;
- Functional data versus phenotype of immune cell subsets: the true value of understanding the patterns of specific immune cells is not simply in their numbers, but how their function or dysfunction contributes to the pathophysiology of cGVHD. Knowing this information will only make a specific biomarker more powerful.

Summary

A variety of biomarkers will soon be validated and used in the treatment of cGVHD. We hope that these biomarkers will allow early and accurate diagnosis of cGVHD, biological classification of chonic GVHD and predict response to therapy.

Expert commentary

There is significant evidence for numerous potential biomarkers that highlight the diverse immune dysregulation that underlie cGVHD. Differences between the donor and host preferentially trigger pathological pathways and inflammatory responses that overcome regulatory immune cell populations, leading to widespread tissue damage. The key to better understanding cGVHD is discovering the triggers for pathological cell populations, in the specific biological milieu that occurs post-transplantation.

Five-year view

The majority of biomarkers presented in this article still require validation. Clinical validation will require large, cooperative trials that employ standardized diagnostic and response to therapy criteria along with consistent treatments. We believe that the most exciting work that will occur in the next 5 years will be efforts to use these biomarkers to help understand the pathogenesis of cGVHD. This in turn will lead to the discovery of new therapeutic targets. We also believe that new technologies in DNA sequencing and proteomics will increase our ability to find new biomarkers in cGVHD.

Key issues

- The pathophysiology of chronic graft-versus-host disease (cGVHD) remains poorly understood.
- The use of biomarkers offers new possibilities in the clinical management of cGVHD through improved prediction of risk and prognosis, more consistent diagnosis, better measurement of disease activity and serving as effective surrogates for therapeutic response.
- There is increasing evidence for the role of genetic polymorphisms affecting the function of gene products implicated in cGVHD.
- There are several outstanding controversies as to the beneficial or pathogenic role of certain immune cell populations in cGVHD, such as regulatory T cells and Th1/Th2 balances that need to be addressed in further studies.
- New and emerging evidence implicates a major role for B cells in the pathogenesis of cGVHD.
- Almost all potential biomarkers remain to be validated.
- Biomarkers will provide new insights into underlying pathogenesis, allowing identification of novel therapeutic targets.

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• of interest

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Table 1

Summary of non-human leukocyte antigen genetic associations with chronic graft-versus-host disease.

Gene	Polymorphisms	Associated with donor or recipient	Effect on cGVHD	Ref.
Heparanase	SNP rs4693608, SNP rs4364254	Recipient and donor	Increased risk of extensive cGVHD associated with pairs where the recipient possessed the genotype associated with higher heparanase expression, while donors were carriers of genotype associated with lower expression	[51]
Poly (ADP-ribose) polymerase 1	SNP rs1805410	Recipient	Homozygous minor genotype associated with a higher risk of cGVHD	[54]
Mucosal addressin cell adhesion molecule-1	SNP rs2302217 A/A	Recipient	Developed cGVHD more frequently than patients with other phenotypes	[55]
Haptoglobin	Haptoglobin 2–2 phenotype	Recipient	Increased expression of haptoglobin after cGVHD onset. Patients with cGVHD had a higher incidence of HP 2–2 phenotype	[56]
Fc receptor-like 3 gene	169C/C genotype	Recipient	This genotype was significantly less frequent in cGVHD patients	[60]
High mobility group box 1 protein	3814C>G, 1177G>C and 2351insT	Donor	Cumulative incidence of developing limited or extensive cGVHD showed a successive increase with the donor carrying 0, 1 or 2 minor alleles	[62]
C–C chemokine receptor type 9	SNP rs12721497 (G926A)	Donor	Significantly associated with the incidence of chronic skin GVHD	[67]
IL-10Rβ	IL-10Rβ A/A	Recipient and donor	When the IL-10Rβ A/A homozygous patients received a graft with the same IL-10 production level, they were protected from cGVHD	[68]
IL-10 promoter	1082*A/819*T/592*A (ATA), 1082*A/819*C/592*C (ACC)	Recipient	ATA/ATA homozygote had a sevenfold increasing risk of developing cGVHD compared with ACC/ACC. Duration of systemic IST significantly shorter in recipients without ATA haplotype	[69]
IL-10	Allele 13 or more, which contain 26 or more CA repeats	Donor	Donors of the patients with cGVHD more frequently possessed a greater number of alleles (allele 13 or more which contain 26 or more CA repeats) in the *IL-10* gene	[70]
TNF-α	238GA	Recipient and donor	Increased occurrence and severity of cGVHD in patients with 238GA and patients with donor 238GA	[71,72]
IL-6	Polymorphism at position 174	Recipient and donor	Associated with the increased incidence of cGVHD	[73]
IL-1α	Allele 2 at IL-1a-889 and IL-1a variable number tandem repeat	Recipient	Associated with the occurrence of cGVHD	[74,75]
IFN-γ	First intron; 3/3 genotype	Recipient	Increased risk of cGVHD	[76]

cGVHD: Chronic graft-versus-host disease; IST: Immunosuppressive therapy; SNP: Single-nucleotide polymorphism.

Table 2

Summary of immune cell subset numbers in chronic graft-versus-host disease.

Cell type	Phenotype	cGVHD	Ref.
T cells	Regulatory ($CD4^+CD25^+Foxp3^+$)	Unclear	[80,86–92]
	$CD8^+/CD4^+$	Increased number of activated $CD8^+/CD4^+$ T cells capable of infiltrating target tissues	[95–97]
	Th17	Increased. Infiltrate target tissues	[98]
	NK	Decreased	[107]
Plasmacytoid dendritic cells	lin-, $HLA\text{-}DR^+$, $CD123^+$	Higher numbers in bone marrow grafts associated with lower risk. By contrast, higher numbers in peripheral blood grafts associated with higher risk	[100–102]
Monocytes	$CD45^+$, $CD14^+$	Increased in cGVHD	[103]
Eosinophils		Increased in cGVHD	[104–107]
NK cells	$CD16^+$,$CD56^+$	Appear to be protective. Limit proliferation of donor T cells	[112–115]
B cells	$TLR9^+$	Increased	[136]
	IgM memory	Decreased	[137]
	$CD21^{lo/-}$	Increased	[138]

cGVHD: Chronic graft-versus-host disease; NK: Natural killer; TLR: Toll-like receptor.