Biology of Blood and Marrow Transplantation

journal homepage: www.bbmt.org

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The Biology of Chronic Graft-versus-Host Disease: A Task Force Report from the National Institutes of Health Consensus Development Project on Criteria for Clinical Trials in Chronic Graft-versus-Host Disease

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A B S T R A C T

Chronic graft-versus-host disease (GVHD) is the leading cause of late, nonrelapse mortality and disability in allogeneic hematopoietic cell transplantation recipients and a major obstacle to improving outcomes. The biology of chronic GVHD remains enigmatic, but understanding the underpinnings of the immunologic mechanisms responsible for the initiation and progression of disease is fundamental to developing effective prevention and treatment strategies. The goals of this task force review are as follows:

- Summarize the current state of the science regarding pathogenic mechanisms of chronic GVHD and critical knowledge gaps.
- Develop working hypotheses/overriding concepts for chronic GVHD development.
- Define the usefulness of current preclinical models to test working hypotheses and ultimately discover and develop new therapeutic strategies.
- Identify shortcomings of preclinical models, and define criteria for the creation of additional models to address these limitations.

INTRODUCTION

Relapse of underlying malignancy and the development of chronic graft-versus-host-disease (GVHD) remain the major obstacles to improving outcomes following allogeneic hematopoietic cell transplantation (HCT). Chronic GVHD remains the prevailing cause of nonrelapse mortality in patients surviving longer than 2 years after allogeneic HCT, negatively influencing both quality of life and long-term outcomes. Unfortunately, the incidence and severity of chronic GVHD have increased over the last decade despite advances in clinical practice [1,2]. Thus, although many GVHD prevention regimens have reduced acute GVHD, chronic GVHD amelioration has been less affected [3-5], with exceptions seen with the use of antilymphocyte antibodies and high-dose cyclophosphamide in the early post-transplantation period [6-9]. Unlike acute GVHD, which is driven almost exclusively by the activation of donor T cells and the release of proinflammatory cytokines [10], the immunopathophysiology of chronic GVHD is more complex. Chronic GVHD involves multiple, distinct interactions among alloreactive and dysregulated T and B cells and innate immune populations, including macrophages, dendritic cells (DCs), and neutrophils, that culminate in the initiation and propagation of profibrotic pathways.

Over the past decade, the National Institutes of Health's consensus criteria for the diagnosis and scoring of chronic GVHD have brought consistency to the terminology and methods for reporting assessment findings in HCT recipients [11,12]. This effort has been successful in standardizing the language and documentation used by clinicians to describe clinical manifestations of disease [13-15], yet the precise mechanisms responsible for the onset and progression of chronic GVHD remain elusive. In this paper, we review the current understanding of the immunology of chronic GVHD and provide guidance for pursuing several focused areas of research over the next decade.

CLINICAL MANIFESTATIONS OF CHRONIC GVHD

Chronic GVHD presents with the following key clinical manifestations: mucocutaneous, myofascial, pulmonary, and "other," affecting essentially any organ system in the body. Characteristic features may include chronic inflammatory changes that can be relatively acellular involving ocular [16], oral, esophageal, skin, joint and fascial, and genital [12] tissues. Progression to clinically significant fibrosis involving multiple organs in the integumentary, musculoskeletal, aerodigestive, gastrointestinal, cardiorespiratory, reproductive, and peripheral nervous systems occurs in severely affected individuals. Rare but severe clinical presentations of chronic GVHD also can include polyserositis (with pericardial and pleural effusions) or polymyositis with severe muscle weakness and elevated muscle enzyme levels [17].

Because scoring is based on the degree of tissue involvement and functional impairment and not on the underlying biology, clinical disease classifications are unlikely to help translational scientists complete association analysis of large datasets. This is particularly complicated by the strong correlations between chronic GVHD and other late complications,

including metabolic syndrome, renal impairment, infections, and the development of second cancers [18-20].

Standardizing Clinical Disease Nomenclature to Facilitate Interpretation of Biological Studies of Chronic GVHD

The transplantation biology field seeks approaches to establish clinical tolerance, defined as a specific lack of immune activity to donor and host tissues with preservation of responses to foreign antigens, such as invading pathogens [21]. Tolerance could be achieved through mitigation of T cell reactivity, a process that typically occurs through 2 mechanisms, central (thymic) tolerance and peripheral (extrathymic) tolerance [22]. Known requirements for the induction or description of tolerance after HCT in the clinic are lacking. Chronic GVHD is the net result of an imbalance between relatively higher immune effector mechanisms that cause inflammation and disease and lower inhibitory (regulatory) mechanisms that may maintain tolerance (Figure 1).

The interpretation of biological studies of chronic GVHD is complicated by variability in the classification of different manifestations of disease. A rational approach for grouping patient samples is required for studies of human immune cell function. Deciphering the biology of clinical chronic GVHD and interpreting correlative biology studies conducted in affected patients is both important and challenging because of the grouping of diverse patient subsets (eg, established chronic GVHD with newly diagnosed de novo with overlap, controls with/without previous acute GVHD or with/without subsequent chronic GVHD) that customarily occurs in the context of clinical investigation. A single nomenclature and comparisons among similar clinical groups should be used (Table 1). Moreover, the biology of chronic GVHD is likely different in newly diagnosed patients (at the onset of active disease) compared with that observed later in the disease course. Thus, grouping all chronic GVHD patients together in biological analyses should be avoided whenever possible. Instead, we propose grouping chronic GVHD patients according to the presumed underlying biology that consists of inflammatory, immune dysregulatory (functionally nontolerant), or fibrotic/sclerotic manifestations (Table 2), and noting the duration of the disease.

Similarly, definitions of nomenclature regarding the terms "alloreactivity" and "autoreactivity" require consistent use. In this paper, we refer to all donor T cell responses as alloreactive in nature when donor cells respond to recipient cells and autoreactive when donor immune response occur against donor cells, such as platelets or red blood cells. Both responses are part of the spectrum of chronic GVHD, and the term "autoantibodies" has been used to describe tissue reactive alloantibodies. These definitions have caveats given the possible contribution of donor-derived antigen-presenting cells (APCs) to the T cell activation that contributes to chronic GVHD [23,24].

Factors Influencing the Development of Chronic GVHD and the Interpretation of Biological Studies

A number of clinical variables are associated with the development of chronic GVHD and may influence the underlying pathophysiology of the disease. These include, but are not
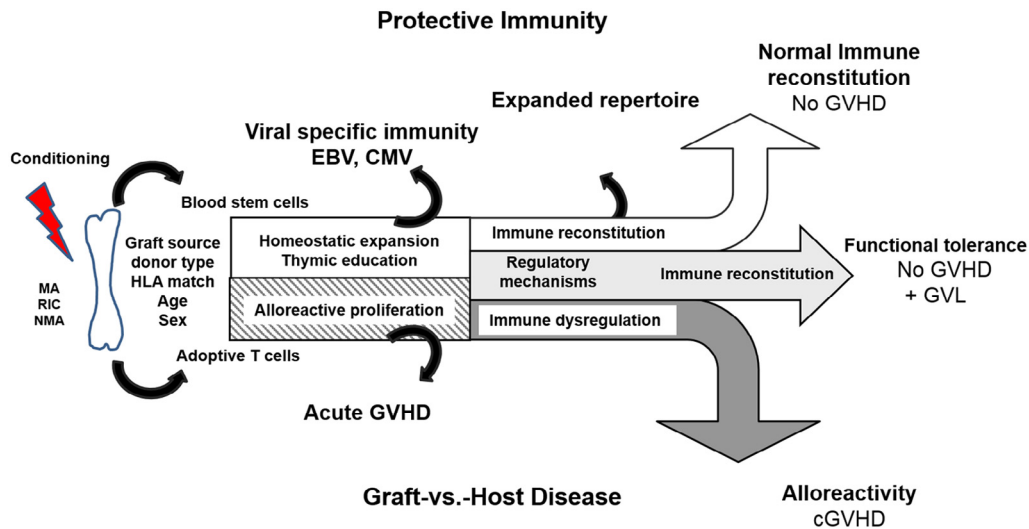
Figure 1. Pathways to functional tolerance or chronic GVHD. Several factors significantly influence the immunologic landscape that evolves after allogeneic HCT and is ultimately responsible for (1) normal immune reconstitution, including the restoration of protective, anti-infective host immunity and the reestablishment of expanded T cell and B cell repertoires; (2) functional tolerance with preservation of graft-versus-tumor effects; or (3) immune dysregulation and alloreactivity that drives the development of chronic GVHD. These factors include, but are not limited to, the following: conditioning regimen intensity; donor and host parameters, including graft source, donor type, HLA match, age, and sex; the contribution of both mature lymphocytes infused at the time of HCT and those generated from the donor stem cell graft and educated in thymic remnants of the host; and the efficiency (or lack thereof) of early and late regulatory mechanisms.
MA, myeloablative conditioning; RIC, reduced-intensity conditioning; NMA, nonmyeloablative conditioning; +GVL, presence of graft-versus-leukemia activity.

Table 1
GVHD Status Definitions and Grouping for Biological Studies Performed in Patients After Allogeneic HCT

Definition	Alloreactive and Autoreactive Effector Mechanisms	Regulatory Mechanisms	Previous Acute GVHD	Previous Chronic GVHD	Off Immune Suppression >3 mo	Physical Manifestations of Acute and/or Chronic GVHD	Normal IgG Level (Patient Not Anergic)
Resolved GVHD (immune tolerance)	+	+	±	±	+	−	+
No GVHD ("normal" immune reconstitution)	−	−	−	−	+	−	+
Active late acute GVHD	+	−	±	−	−	+	±
Active chronic GVHD, new onset (within 1 mo) and established	+	−	±	+	−	+	±
Inactive chronic GVHD	+	+	±	+	+	+	±

GVHD indicates graft-versus-host disease; HCT, hematopoietic cell transplantation; +, present; −, absent; ±, may or may not be present.

limited to, donor type, stem cell source, conditioning regimen intensity, underlying diagnosis (myelodysplastic syndrome or chronic myelogenous leukemia), in vivo T cell depletion by alemtuzumab and antithymocyte antibodies, use of post-transplantation cyclophosphamide, sex mismatch, HLA mismatch, and evidence of prior cytomegalovirus and Epstein-Barr virus infection [1,25-36]. It is also possible that, paradoxically, treatment with and subsequent withdrawal of commonly used calcineurin inhibitors may contribute to the development of chronic GVHD by blocking thymic T cell development and thymic and peripheral T cell tolerance [37-39]. Additional factors include the ages of the donor and recipient. Although early reports supported the hypothesis that increasing donor age was associated with higher rates of chronic GVHD, perhaps owing to higher numbers of memory T cells [27], recent data would suggest that it has a lesser effect [40-42]. Possibly more important is the fact that younger recipients, especially children, have a functional thymus that may have a significant influence on the development of chronic GVHD and could explain the lower rate of chronic

GVHD in younger patients [43,44]. We discuss the role of the thymus in chronic GVHD, although its role in adult recipients likely is much less prominent.

A 3-PHASE MODEL FOR CHRONIC GVHD BIOLOGY

Experimental studies have underscored the consequences of inflammation early after HCT from conditioning and activation of donor T cells. Vascular endothelial cell (EC) activation and injury promotes the migration of donor immune cells into target organs. Thymic injury and dysfunction have deleterious effects on pathways of central tolerance. Depletion of regulatory T cells (Tregs) or reduction of their suppressor function by calcineurin inhibition further impairs tolerance induction by peripheral mechanisms. Propagation of tissue injury by dysregulated donor lymphocyte populations and aberrant repair mechanisms set the stage for fibroblast activation, collagen deposition, fibrosis, and irreversible end-organ dysfunction. Figure 2 proposes a 3-phase model for the initiation and development of chronic GVHD that involves early inflammation and tissue injury (phase 1),

Table 2
Biological Subgrouping of Key Clinical Manifestations of Chronic GVHD

| Manifestations	Inflammatory (Phase 1)	Immune Dysregulatory (Phase 2)	Fibrotic/Sclerotic (Phase 3)
Mucocutaneous	Oral lichen planus-like erythema/ulcers; erythematous skin rashes; conjunctival erythema; genital/vaginal erythema, lichen planus-like or ulcerations	Chronically infected ulcers	Salivary dysfunction; limitation of mouth opening; lacrimal dysfunction; cutaneous sclerosis; labial agglutination; vaginal stenosis; phimosis
Lung	Pulmonary inflammation (clinical or subclinical)	Chronic lymphocytic bronchiolitis; chronic interstitial pneumonitis; recurrent sinopulmonary infections	Bronchiolitis obliterans syndrome; interstitial fibrosis
Myofascial	Extremity edema, fasciitis	Myositis; myasthenia gravis; chronic inflammatory demyelinating polyneuropathy	Subcutaneous deep fibrosis; joint contractures
Liver	Cholestatic or hepatitic GVHD	Autoimmune hepatitis	Advanced liver GVHD with periportal fibrosis, ductopenia
Gastrointestinal	Colitis, epithelial cell injury	Chronic colitis, malabsorption	Esophageal web, stricture formation
Hematopoietic system	Neutrophilia; elevated platelet counts; anemia of chronic disease	Lymphopenia; immune neutropenia or thrombocytopenia; eosinophilia; autoimmune hemolytic anemia	Marrow failure/fibrosis
Immune system	Acute-phase reactants	Infections, especially with encapsulated bacteria; hypogammaglobulinemia or hypergammaglobulinemia; autoimmune phenomena (renal, thyroid, polyserositis, other)	Functional asplenia; opportunistic infections

GVHD indicates graft-versus-host disease.
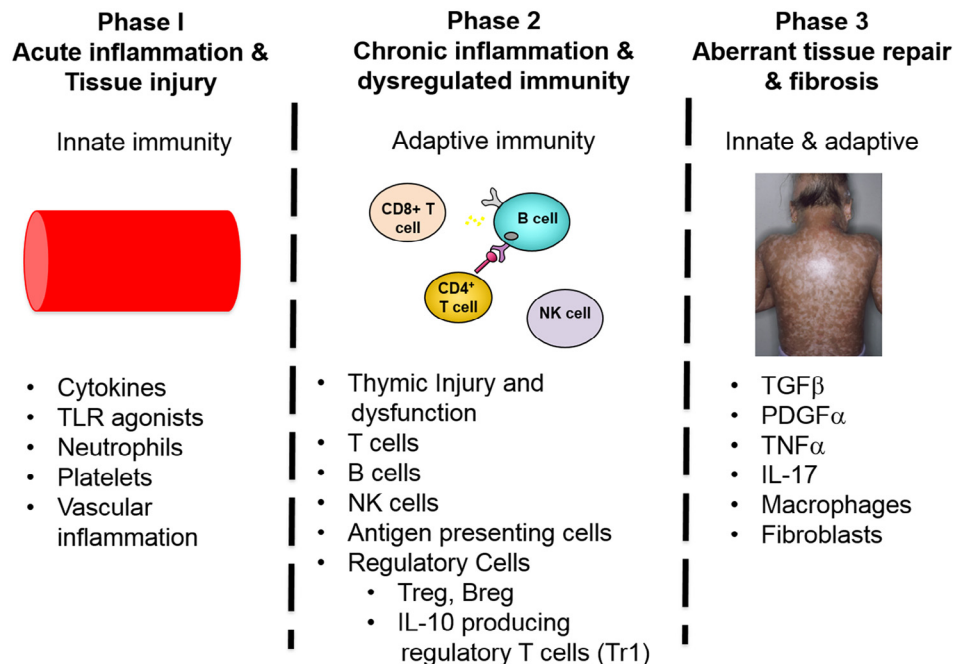
Figure 2. Biological phases of chronic GVHD. A 3-step model for the initiation and development of chronic GVHD is proposed that involves early inflammation and tissue injury (phase 1), dysregulated immunity (phase 2), and aberrant tissue repair often with fibrosis (phase 3)*. In phase 1, numerous soluble, inflammatory proteins, including cytokines and TLR agonists, are released in response to cytotoxic agents, infections, and acute GVHD. Together with cellular components of the innate immune system, these inflammatory stimuli result in diffuse, nonspecific damage to numerous organs and the vascular endothelium. Endothelial cell activation and injury set the stage for the migration of donor immune cells into secondary lymphoid organs, including the spleen and lymph nodes, and subsequently into GVHD target tissues. Phase 2 is characterized by the activation of effector populations in the adaptive immune system, including T cells, B cells, antigen-presenting cells, and NK cells with compensatory inhibition by regulatory populations including Tregs, Bregs, NKregs, and possibly Tr1 cells. The response appears to be both antigen-specific (MHCs and miHAs) and non–antigen-specific. Thymic injury and dysfunction engendered during phase I and phase II has deleterious effects on pathways of central tolerance. In Phase 3, propagation of tissue injury occurs by dysregulated donor lymphocyte populations in the context of aberrant repair mechanisms. This in turn promotes the release of profibrotic mediators, leading to macrophage and fibroblast activation, collagen deposition, fibrosis, and irreversible end-organ dysfunction. *It should be noted that although these are usually sequential events, patients in phase 1 can often go both to phase 2 and phase 3 simultaneously or sometimes only to phase 2 without phase 3.
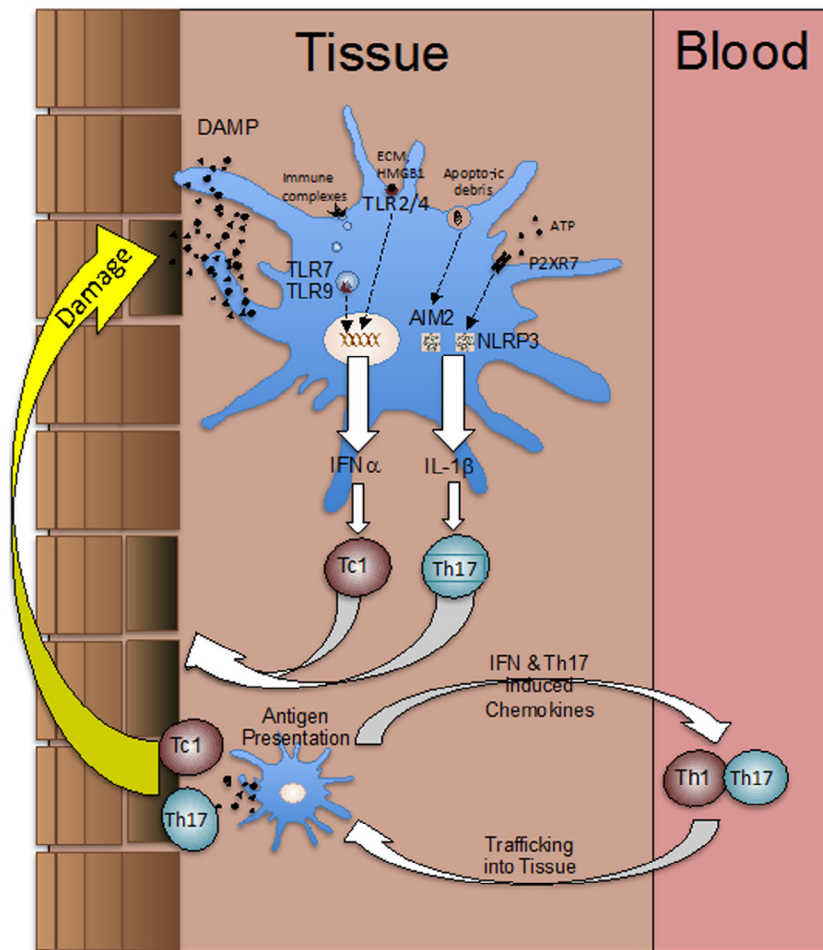
Figure 3. Phase 1: Early inflammation and tissue injury. Diagram of damage-induced activation of the innate immune system resulting in recruitment of Th1/ Tc1 and Th17 cells to a tissue site. Ongoing damage to epithelial and connective tissue releases damage-associated molecular patterns (DAMPs), including RNA, DNA, chromosomal HMGB1, extracellular matrix materials, ATP, and uric acid. RNA and DNA can be taken up into endosomes as part of immune complexes with anti–nuclear material autoantibodies (triggering TLR3, TLR7, and TLR9). ECM and HMGB1 bind to plasma membrane TLR2, TLR4, and RAGE complexes. All of these TLR pathways trigger IRF transcription factors, inducing IFNα, and TNFα and IL-6 through NFkB. NLRP3 inflammasome formation can be triggered by ATP (via P2XR7), resulting in IL-1β production. IFNα, and IL-1β plus IL-6, can induce T cell differentiation into Th1/Tc1 and Th17. IFN and IL-17 also induce chemokines (CXCL9/10 and CCL20), which recruit Th1 and Th17 cells into tissues from the blood. Cytolytic attack by these effector T cells continues a cycle of tissue damage and release of DAMP molecules.

chronic inflammation and dysregulated immunity (phase 2), and aberrant tissue repair often with fibrosis (phase 3). Strategies focusing on (1) specific depletion or functional inhibition of mature, alloreactive, T cells in the stem cell graft; (2) preserving or restoring thymic function and restoration of Treg numbers and functional capacities; and (3) mechanisms of dysregulated inflammation and repair, which lead to fibrosis, may successfully reduce the incidence and severity or halt the progression of chronic GVHD. Such approaches will promote the establishment of immune tolerance with preservation of antiinfective and antitumor immunity after HCT.

Phase I: Effect of Early Post-Transplant Inflammation and Tissue Injury on Chronic GVHD

Role of the adaptive and innate immune system responses in chronic GVHD

Acute GVHD is initiated and sustained by innate immune system pathways that mediate the response to microbial products and molecules released by cellular damage [45-48], in cooperation with the adaptive (T and B cell) system. Triggering inflammatory pathways in scavenger macrophages,

plasmacytoid and myeloid DCs, B cells, and neutrophils results in the production of key mediators that enhance antigen presentation and direct the commitment of naïve T cells into differentiated Th1/Tc1 and Th17/Tc17 T cell effector lineages (Figure 3). For example, Toll-like receptor (TLR) pathways are triggered through receptors on the plasma membrane (TLR2, TLR4) and in endosomes (TLR3, TLR7/8, TLR9). Deleting TLR or NOD-like receptor (NLR) pathways or blocking their activity with small molecule inhibitors significantly reduces acute GVHD [49-52]. Similar mechanisms appear to be in place for chronic GVHD. Hyperresponsiveness to TLR9 agonists has been described in B cells at the onset of chronic GVHD [53], but responses to a TLR9 agonist are muted in certain B cell subsets [54]. In addition, agents that inhibit TLR7, TLR8, and TLR9 signaling within the lysosome have shown variable in vivo activity in murine and human chronic GVHD [55-57].

TLR pathway activation induces IFNα production via transcriptional interferon response factors (IRFs) 3 and 7 along with IL-6 and TNFα through NFκB. IFNα can drive Th1/Tc1 commitment [58], resulting in IFNγ production. IFNα and IFNγ in turn can induce chemokines (CXCL9, CXCL10, CXCL11) that

Table 3
Potential Agents for Blockading Effects of Innate Immune Response

Target	Agent	Effects	Species	Reference(s)
TLR4	NI-0101	TLR4-specific antibody	Mouse	[323]
TLR7, TLR9	AT791; E6446	Small-molecule inhibitor	Mouse	[324]
MyD88	ST2825	Small-molecule inhibitor	Mouse	[325]
	Imatinib	Blocks TLR agonist effect	Mouse	[326]
P2X7R	A-438079	Selective P2X7R antagonist	Mouse, human	[327]
	CE-224,535			
NLRP3	Pirfenidone	Inhibits NLRP3 expression	Mouse	[328]
	Glyburide	Blocks NLRP3 activity	Mouse	[329]
IL-1β	Rilonacept	IL-1R1 fusion protein	Human	
	Canakinumab	Anti–IL-1β	Human	[330]
	VX-765, parthenolide	Caspase-1 inhibitor	Human, mouse	[331,332]
IFNα	Anti-IFNα	Reduces BAFF, CXCL9/10	Human	[333,334]
JAK	Baracitinib, ruxolitinib, INCB018424, CYT387 (JAK1,2)	Blocks JAK/STAT signaling of multiple cytokines, including IFN (IFNα/β, IFNγ), gp130 family (IL-6), IL12/IL23, and γc family (IL-2, IL-4, IL-7, IL-15)	Human, mouse	[60,97]
	Tofacitinib (JAK**1**,2,3)	Blocks JAK/STAT signaling of multiple cytokines, including γc family (IL-2, IL-4, IL-7, IL-15)	Human, mouse	[335]

recruit Th1/Tc1 cells into tissue sites of inflammation and other factors that enhance the processing and presentation of host antigens [59-63].

The assembly of inflammasome complexes containing the adapter protein ASC (apoptosis-associated speck-like protein containing C-terminal caspase recruitment domain [CARD]) and caspase I regulates antigen presentation and migratory capacity of DCs and lymphocytes, respectively [64], causing loss of myeloid-derived suppressor cell function during acute GVHD induction [65,66]. Inflammasomes also catalyze the production of active IL-1β and IL-18 from their proforms. IL-1β, in combination with IL-6, induces differentiation of pathogenic Th17 cells in humans [67,68].

Three lines of evidence suggest that similar immune pathways play a role in the initiation and persistence of chronic GVHD. First, donor T cells activated early post-transplantation appear to contribute to and may sustain chronic GVHD [69,70]. Second, tissue damage incurred during acute GVHD may persist, as evidenced by progressive onset of chronic GVHD or overlap syndrome. Even restricted areas of mild cell damage (eg, waistband pressure, varicella zoster virus reactivation) can trigger localized sclerotic cutaneous chronic GVHD [71]. Th1/Tc1 and Th17 cells are the dominant T cell effectors in lichenoid infiltrates of the skin and mucosa [62,72-77] (Figure 3). These cells can result in extensive tissue destruction, leading to release of damage molecules (eg, ATP, RNA and DNA, HMGB1) that trigger the TLR, NLR, and inflammasome pathways that propagate inflammation [78-82]. The IL-33 receptor ST2/IL1RL1, now recognized as a biomarker for both acute and chronic GVHD, is also released in response to cell damage [83-87]. Third, gene expression in circulating monocytes from clinical samples reveals that multiple IFN-inducible genes and receptors for pathogen-associated molecular patterns and damage-associated molecular patterns (known as pattern recognition receptors [PRRs]) become up-regulated at the time of onset of chronic GVHD, remaining elevated in patients with active disease [88]. These IFN-inducible and PRR genes were comparably up-regulated in patients with cutaneous lichenoid infiltrates and in those with extensive sclerotic involvement, providing a common operant mechanism across the spectrum of chronic GVHD manifestations [88]. The use of a neutralizing type I IFN receptor (IFNAR) antibody prevented skin and vascular changes in a sclerotic chronic GVHD murine model (B10.D2→BALB/c), and a similar strategy reduced Th1 activation and collagen production in a

phase I clinical trial for patients with for systemic sclerosis [89,90].

In addition, the IFNγ-inducible chemokines CXCL9, CXCL10, and CXCL11, responsible for CXCR3-expressing Th1 lymphocyte and natural killer (NK) cell recruitment into tissue, have been identified as plasma biomarkers for chronic GVHD. These chemokines are up-regulated at diagnosis and remain elevated in severely affected patients [73,85,91-94]. In particular, CXCL9 was recently found to be part of a biomarker panel that significantly correlated with the diagnosis and severity of chronic GVHD once established and had predictive power at day 100 for patients who would develop chronic GVHD within the subsequent 3 months [85]. IFNγ enhances the production of another recognized chronic GVHD biomarker, the homeostatic B cell activating factor (BAFF), and may contribute to B cell activation in chronic GVHD [95,96]. Blockade of IFNγ signaling through the use of Janus activated kinase (JAK)-1/2 inhibitors is as effective in chronic GVHD as in the acute form of the disease [97,98], although alteration of non-IFN signaling pathways regulated by JAK1/2 also may contribute to the response.

Contradictory data regarding the role of IFNγ exists in humans, where microsatellite polymorphisms within the first intron appear to be associated with decreased IFNγ production and higher chronic GVHD rates [99]. Recent studies also have demonstrated a correlation of low plasma IFNγ concentrations with the onset of chronic GVHD in humans [100]. Collectively, these observations suggest that inhibiting TLR and NLR pathways with approaches that target the adaptive immune system may represent a novel and effective approach for preventing or treating chronic GVHD (Table 3).

Endothelial damage and activation

Vascular ECs are the first host-derived cells to be exposed to the donor immune system and are the primary barrier separating donor and recipient tissue. The passage of donor cells across the vascular endothelium into inflamed tissues is tightly controlled; leukocyte transmigration through an EC monolayer is a complex, orchestrated process that involves intimate contact between the 2 cell types [101,102]. The precise mechanisms operating during GVHD development have yet to be fully defined, however. If vascular ECs express and present cognate antigen to alloreactive donor T cells, they can be susceptible to direct immune attack as well. The resulting EC activation and injury can facilitate the passage of
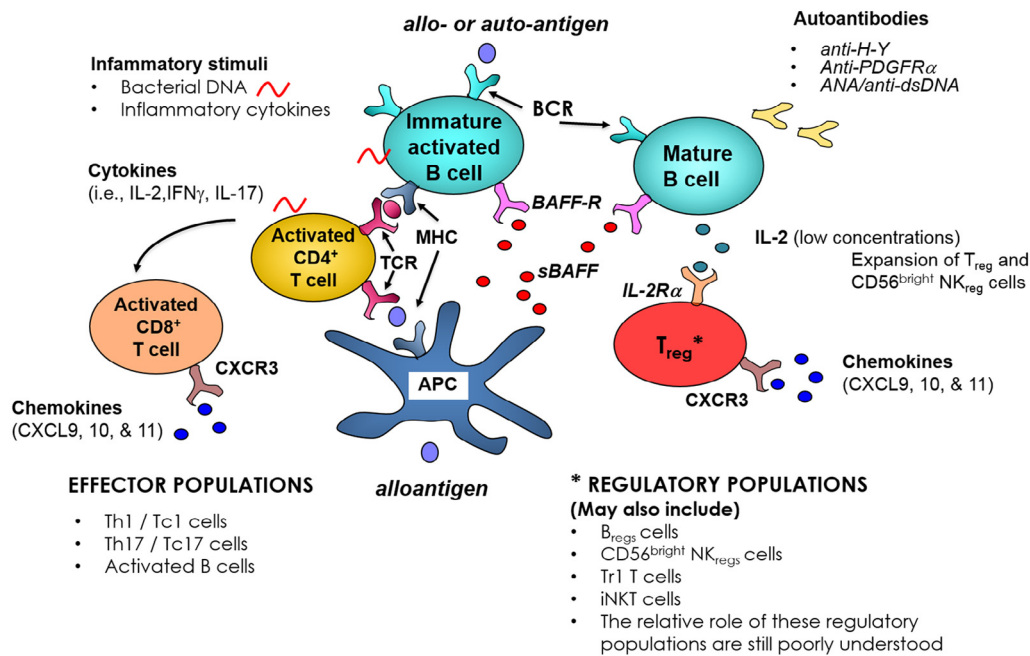
Figure 4. Phase 2: Chronic inflammation and dysregulated immunity. The production and release of inflammatory stimuli enhance interactions between antigen-presenting cells and donor-derived lymphocyte populations, including CD4+ T cells, CD8+ T cells, and B cells. Some of the responses are antigen-specific, and some derive from nonspecific inflammatory pathways. Activation of donor lymphocytes results in the generation of effector populations. The production of signature cytokines, BAFFs, and autoantibodies, along with chemokine receptor–ligand interactions, are part of the aberrant immune response contributing to inflammation and the recruitment of effector and regulatory cells into peripheral target tissues. The difference between the presence and absence of chronic GVHD appears to be related to a predominance of either dysregulated effector mechanisms when active chronic GVHD is present versus regulatory populations when chronic GVHD is absent or has resolved. The relative contributions of each remain to be determined.

donor-derived cellular and soluble effectors from the blood and into recipient tissues.

Diffuse EC damage is implicated as a direct contributor to multiple complications occurring after allogeneic HCT [103-108]. Several in vitro and in vivo systems have also demonstrated that ECs respond to stimuli such as irradiation, lipopolysaccharide, TNFα, and cytotoxic lymphocytes (CTLs), all of which are involved with inflammation early post-HCT, by either becoming activated or undergoing programmed cell death [107,109,110]. Involvement of EC activation and injury in the development of chronic GVHD is inferred by clinical and experimental data. Intimal arteritis similar to allograft vasculopathy and extensive loss of microvessels have been observed in the skin of chronic GVHD patients [111]. The latter was reported in direct association with perivascular infiltration of donor-derived CTLs, loss of ECs in the vessel lumen, and increased plasma von Willebrand factor levels [112]. Although microvessel loss was not compensated for by vascular remodeling, it appeared to be reversible if systemic GVHD was successfully treated. In animal models, EC activation and apoptosis in the setting of intense lymphocytic infiltration during acute GVHD are observed in advance of epithelial injury of the oral mucosa and lungs of mice, 2 key chronic GVHD target organs [113,114]; thus, microvascular loss and resultant tissue ischemia may contribute to the target organ fibrosis characteristic of chronic GVHD, as well as the occurrence of late cardiovascular disease reported after allogeneic HCT [18-20]. Elucidation of mechanisms that influence EC vulnerability to immune-mediated injury during chronic GVHD may uncover novel approaches to prevent the initiation and progression of disease and subsequent end-organ damage and dysfunction.

Effector T cells and chronic GVHD biology

In vivo T cell depletion using lymphocyte antibody therapy or a short, early post-transplantation course of high-dose cyclophosphamide each has been shown to reduce the incidence and severity of chronic GVHD [6-8,32,115-117]. These data suggest that chronic GVHD is dependent, at least in part, on mature donor T cells infused with the hematopoietic stem cell graft [118,119]. A subset of mature donor T cells is specific for histocompatibility antigens uniquely expressed by recipient cells.

The first suggestion that the antigen specificity of the T cells involved in acute GVHD differed from those of chronic GVHD came from the analysis of clonal T cells in rodent HCT models. Whereas all of the T cell clones from mice with acute GVHD were specific for restricted histocompatibility antigens of the host, the majority of T cell clones from mice with chronic GVHD were specific or restricted by histocompatibility antigens shared by the donor and recipient strains [120]. Although clonal T cells have been found in HCT recipients with acute GVHD and in some cases their antigen specificity has been determined, clonal T cells have not been consistently identified in patients with chronic GVHD [121].

Activated, clonally expanded donor T cells differentiate into distinct functional subsets, including Th/Tc1, Th/Tc2, and Th/Tc17 cells (Figure 3 and 4). Their effector mechanisms include the production of both cytokines and cytolytic enzymes [122] that contribute to the generation of GVHD. Of the CD4 T cell subset, Th1 cells are the most important for inducing acute GVHD through inflammatory cytokines, including IFNγ and TNFα, whereas Th2 cells and cytokines are drivers of chronic GVHD. This Th1/Th2 paradigm has been challenged and

refined, however [99,100,123,124]. In addition, the role of Th17 cells has been assessed in the context of GVHD (reviewed in [125]). The Th17 pathway of differentiation is distinct from that of Th1 or Th2 CD4+ T cell development. Th17 differentiation requires TGFβ1 and IL-6, is enhanced by IL-1β and TNFα [126,127], and is dependent on the transcription factors retinoid-related orphan receptor (ROR)γt and RORα [128]. This lineage of CD4+ helper cell development is characterized by the production of IL-17A, IL-17F, IL-22, and IL-21. A possible role for IL-17–producing CD4+ (Th17) cells and CD8+ (Tc17) cells alone or in combination with other effector subtypes was noted in several preclinical mouse models of both acute and chronic GVHD [129-132]. IL-17 is mechanistically linked to skin and lung GVHD in mice [133-135]. In the setting of chronic GVHD, the contribution of Th17/Th1 cells to tissue injury is regulated in part by signal transducer and activator of transcription (STAT)-3 [136] and IL-12/IL-23 [137] signaling along with programmed death 1 (PD-1)/PD1 ligand 1 (PDL-1) interactions [138]. Elevated Th17 cell numbers have been found in patients with acute and chronic GVHD and are associated with disease status [139]. These Th17 cells included both IFNγ− and IFNγ+ subpopulations, and the IFNγ+ Th17 cells migrated into GVHD lesions in the skin and liver. In support of these findings, the number of Th17-secreting CD8+ T cells was increased in the skin (but not the peripheral blood) in patients with lichenoid chronic GVHD, but not in patients with scleroderma-like chronic GVHD [72].

The realization that Th17 CD4+ T cells play a central role in GVHD development suggests that they might be attractive targets for prophylactic or therapeutic intervention. However, the applicability of targeting IL-17 is influenced by a report suggesting that infusion of IL-17−/− donor T cells into lethally irradiated major histocompatibility complex (MHC)-mismatched recipients led to enhanced acute GVHD as a result of robust Th1 expansion and IFNγ production by donor cells [140]. However, other studies have demonstrated that blocking Th17 cell differentiation using RORγt knockout mice as donors mitigated acute GVHD and altered T cell trafficking to GVHD target organs while maintaining robust engraftment and graft-versus-leukemia (GVL) effects [141,142]. Similar approaches can be considered to determine the effectiveness of preventing or treating chronic GVHD. Other cytokines produced by Th17 cells (ie, IL-21 and IL-22) also may contribute to Th17-mediated pathology, suggesting additional targets for prevention or therapy. The finding that IL-21/R signaling and IL-2γc cytokines are required for disease initiation and maintenance in a multiorgan system chronic GVHD model that includes bronchiolitis obliterans syndrome (BOS), suggests that IL-21 may be an optimal target for intervention, possibly obviating the need for targeting IL-17 escape mechanisms [143,144].

Phase 2. Chronic Inflammation, Dysregulated Immunity, and the Development of Chronic GVHD

Following allogeneic HCT, the production and release of inflammatory stimuli enhance interactions between APCs and donor-derived lymphocyte populations, the generation of effector and regulatory populations and their ultimate recruitment to peripheral target tissues (Figure 4). Immune dysregulation leading to chronic GVHD results from the predominance of donor-derived effector immune mechanisms that cannot be controlled by donor- or host-derived regulatory immune responses. This occurs in part because standard calcineurin-based GVHD prophylaxis, although active in preventing acute GVHD, is ineffective in preventing chronic GVHD

in most patients. Several lines of investigation have assessed whether dysregulated immunity following allogeneic HCT relates to a failure of central or peripheral tolerance mechanisms. The contribution of multiple mechanisms is supported by recent reports examining transcriptional profiles of peripheral blood mononuclear cells of HCT recipients to elucidate molecular changes associated with "operational tolerance" [145,146]. These studies defined operational tolerance as the successful discontinuation of all immunosuppressive agents and sustained absence of any clinical manifestations of acute or chronic GVHD. Nontolerant HCT recipients had ongoing chronic GVHD requiring continued systemic immunosuppressive therapy. Differential expression of several candidate genes, including those involved in NK cell cytotoxicity, antigen presentation, lymphocyte proliferation, and apoptosis, were identified in tolerant and nontolerant patients [145,146].

Other regulatory mechanisms also play important roles in establishing a balance with T cell effector cells. As we discuss below, decreased regulatory functions of B cells may contribute to chronic GVHD in some patients [147]. The CXCR3+ subpopulation of CD56+bright cytokine-producing NK cells (NKregs) is also associated with the development of chronic GVHD [93]. Murine models have identified CD4+ invariant NKT cells (iNKT) as key regulators of Treg expansion and function in vivo [148-150]. In HCT recipients, the number of Tregs in the stem cell product was inversely correlated with the subsequent development of acute GVHD [151,152].

Thymic dysfunction, lack of immune tolerance, and evolution of chronic GVHD

The thymus is the primary organ for the development of T cells and tolerance induction. Most of what is known regarding how bone marrow (BM)-derived, lymphoid-skewed precursor cells travel to the thymus and differentiate into naïve T cells in a tightly regulated stepwise process involving proliferation, differentiation, and positive and negative selection has been elucidated using murine systems (reviewed in [153]). Positive selection is regulated predominantly by cortical thymic ECs (cTECs) and results in MHC restriction of naïve T cells. Negative selection, the process whereby naïve T cells are deleted when encountering self-antigen presented within the thymus, was once believed to occur only in the medulla under the control of medullary TECs (mTECs) and DCs. However, recent studies indicate that thymic B cells and cTECs can contribute to negative selection as well [154]. An important feature of thymic negative selection is the expression of tissue-restricted peripheral self-antigens by mTECs. The expression of (most) tissue-restricted self-antigens is under the control of the autoimmune regulator (Aire) transcription factor, which is expressed by mTECs [155].

Twenty-five years ago, it was demonstrated that acute GVHD can attack the thymus, resulting in the generation of donor T cells with antihost reactivity owing to defective negative selection (failed central tolerance) [156]. This seminal observation is supported by more contemporary mouse studies [157-164]. In preclinical murine models [155-160,165-167] and in patients [168], the thymus is identified as a target organ of chronic (and acute) GVHD, resulting in a loss of thymic output and T cell selection processes. Both mTECs and thymic DCs are targeted by donor-derived T cells [159,161,165,167,169], which may have profound effects on negative selection. In mouse models, acute GVHD of the thymus results in loss of Aire-positive mTECs and a decrease in the diversity of Aire-dependent, tissue-restricted peripheral self-antigens that result in negative selection,

especially those antigens involved in chronic GVHD, such as skin, liver, salivary glands, lungs, eyes, and gastrointestinal tract [155]. This effect reduces the likelihood of central tolerance induction and allows for the development of donor-derived T cells with specificity (or cross-reactivity) for chronic GVHD target antigens [155]. Additional studies in preclinical models support the concept that alloimmunity during acute GVHD may impair effective peripheral T cell tolerance, leading to the emergence of self-reactive donor T cells capable of recognizing nonpolymorphic tissue antigens or commensally derived antigens presented by either donor or host APCs [69,70,170,171].

Whether peripheral or central pathways preferentially contribute to the development of donor CD4+ and CD8+ T cells responsible for the induction of multiorgan chronic GVHD has been explored previously [136,159,161]. In one model, CD8+ T cells generated chronic GVHD only through thymic-dependent (central) mechanisms. In contrast, CD4+ T cells produced chronic GVHD by alteration of either central or peripheral tolerance pathways. In this model, short-term, in vivo anti-CD4+ T cell depletion therapy reduced thymic damage, subsequent autoreactivity, and resultant multiorgan chronic GVHD. Regeneration of thymic architecture and function after damage (such as caused by conditioning regimen and acute GVHD) decreases with age, potentially explaining why the incidence of chronic GVHD is generally lower in pediatric HCT recipients compared with adult HCT recipients [172].

The role of Tregs in chronic GVHD development

Tregs are CD4+ T cells that express high levels of CD25 (IL-2Rα needed to form a high-affinity IL-2 receptor) and FoxP3 (the master transcription factor regulator). Tregs are vital for immune homeostasis [173], and diminution of Treg numbers or function results in autoimmune disease [174,175]. CD4+ Tregs significantly contribute to the effectiveness of GVHD prophylaxis, and their efficacy can be influenced by medications used in the post-transplantation setting [176-178]. They are important in the establishment and maintenance of both central and peripheral immune tolerance after allogeneic HCT [179,180], and post-HCT Treg recovery is a critical element of immune reconstitution [181]. Early after HCT, Tregs are derived primarily from mature memory Tregs in the stem cell product. Several clinical studies have suggested that adoptive transfer of in vitro expanded Tregs early after stem cell infusion can reduce the incidence and severity of acute GVHD [182-184].

The role of CD4+ Tregs in chronic GVHD development appears to be more complex. Increased, normal, and decreased numbers of Tregs have been reported at the onset of chronic GVHD [185-189]. Following HCT, Treg reconstitution is altered and is dependent on various factors, including thymic repopulation and recovery, "homeostatic" Treg proliferation, subsequent survival of regenerated activated Tregs, and the choice and use of immunosuppressive drugs. Antigen-specific proliferation and conversion of conventional T cells into Tregs may contribute to Treg recovery [190,191]. Recent studies have examined possible mechanisms that lead to Treg deficiency during chronic GVHD [192-194]. An imbalance between Tregs and T cell effectors, with too few Tregs, results in a skewed thymic production of naïve T cells toward conventional T cells [194]. Naïve Treg proliferation is also impaired for prolonged periods after HCT [194]. Similar to the situation in healthy adults, post-HCT Treg reconstitution is dependent primarily on homeostatic expansion of memory Tregs. Although memory Tregs proliferate at high levels after HCT, these cells develop short telomeres and have increased susceptibility to apoptosis [195]. When high levels of proliferation cannot be maintained, Treg deficiency occurs, which can impair tolerance induction and is associated with significant clinical manifestations of chronic GVHD [194].

Although Tregs are hypoproliferative in vitro, murine studies have documented their rapid expansion after adoptive transfer in HCT recipients [196]. Treg depletion from the stem cell graft exacerbates acute GVHD in mice, whereas supplemental Treg infusion can prevent disease development [197-202]. Adoptive therapy with ex vivo expanded Tregs can be used to augment the Treg pool [203-206], correct a relative Treg deficiency, and promote peripheral immune tolerance in vivo [207-211]. Tregs are not detectable in the peripheral blood beyond 2 weeks postinfusion in HCT recipients [183], however, possibly due to the suppression of IL-2 production by Tregs, the use of calcineurin inhibitors, or migration out of the blood into tissues. Murine models have identified IL-2 as the primary homeostatic cytokine that regulates CD4+ Treg development and maintains the Treg pool in vivo [212-215]. Genetic deletion of IL-2 or CD25 leads to Treg deficiency and autoimmunity [216]. Low-dose IL-2 can be administered safely for prolonged periods later after allogeneic HCT, when inflammation has partially subsided, with clinical benefit in mice [217] and patients with chronic GVHD. This approach results in the selective expansion of CD4+ Tregs in vivo without promoting the expansion of effector T cells or exacerbation of GVHD [218-220]. Chronic augmentation of systemic IL-2 at physiological concentrations increases thymic production of naïve Tregs along with the proliferation of naïve and memory Tregs [221]. In this context, *bcl2* expression is increased, thereby reducing Treg susceptibility to apoptosis. Despite these changes in Treg homeostasis, IL-2 alone might not be sufficient to increase the functional Treg pool, and a combination of low-dose IL-2 or novel IL-2 formulations with adoptive Treg therapy may be required to provide long-lasting recovery of immune tolerance after HCT [222].

B cell dysregulation and chronic GVHD

In the non-HCT setting, high avidity interactions of B cell receptors (BCRs) with autoantigens in the BM results in deletion of autoreactive B cells [223]. This does not occur in patients with chronic GVHD who develop antibody responses to minor histocompatibility antigens (miHAs) after allogeneic HCT [224-226] (Figure 4). Although in several murine models, alloimmunity is required for the development of chronic GVHD [227] and transferable autoimmunity [170,228], B cells and the production of autoantibodies appear to play key roles in some types of chronic GVHD [229]. Moreover, the frequent production of autoantibodies by patients with chronic GVHD suggests that a loss of B cell tolerance is operative [230]. Although a number of autoantibodies, including anti-nuclear antibodies (ANAs), anti–double-stranded DNA (anti-dsDNA), and platelet-derived growth factor receptor (PDGFR)-α, have been found in association with chronic GVHD [231-234], these findings have been variable. An exception is antibodies directed against Y-chromosome–encoded epitopes (H-Y antibodies) in male recipients of stem cell grafts from female donors who develop chronic GVHD [224,225,235].

Both BCR signaling and BAFF play key roles in determining B cell fate and survival. Aberrant activation of B cells relies on the presence of pivotal drivers of BAFF and BCR signaling after HCT [236,237]. Recently identified signaling pathways that are dysregulated during chronic GVHD have led to

clinical trials using targeted agents to inhibit these pathways [238-241]. As in the non-HCT autoimmune disease setting, both the availability of BAFF and the affinity of available antigen for the BCR determine the autoreactive potential of the peripheral B cell pool [242]. High BAFF levels early after HCT in combination with failure of normal checkpoints vital to B cell tolerance allow persistence and propagation of donor B cells reactive to a variety of host antigens that can secrete disease-causing alloantibodies and autoantibodies. Multiple groups have shown that chronic GVHD is closely associated with aberrant BAFF levels [96,234], an activated B cell phenotype, and aberrant BAFF/B cell ratios [239,243-245]. B cells with polyreactive BCRs that should be deleted in the periphery if B cell tolerance is achieved are potentially rescued by sufficient amounts of BAFF in patients with chronic GVHD [243]. Excessive BAFF levels in chronic GVHD have been associated with increased proportions of antigen-experienced and transitional-like B cell subsets [246,247]. These CD27+ B cells, including an IgD+CD38Hi extrafollicular B cell subset, are likely promoted by excessive amounts of BAFF, but how these cells are generated and whether they are pathological remains unknown [243]. Whether these B cell subsets are present in lesional tissue and have the ability to target sites of disease remains an area of active interest [248]. Regulatory B cells (Bregs) [249] and T follicular helper (Tfh) cells [144,250] may contribute to aberrant B cell function and represent important nodes of B cell and T cell cooperativity in chronic GVHD pathogenesis.

Potential mechanistic links between BCR activation and BAFF in disease microenvironments require further study in murine models. For example, studies in patients are almost always based on peripheral blood B cells, whereas murine studies preferentially use splenic B cells. Furthermore, the expression of B cell surface antigens differs between mice and humans [251]. In mice, germinal center (GC) reactions can be critical for chronic GVHD development [144,252] and may produce clues to pathogenic mechanisms operative in the development of clinical disease as described below. Patients with chronic GVHD are often characterized by functional hyposplenism, although patients without chronic GVHD can also have splenic dysfunction [253-255]. Secondary lymphoid organs are difficult to study in patients, and reconstitution of follicular B cells in lymph nodes is known to be delayed and atypical in patients with chronic GVHD [256]. A paucity of total CD27+ memory and IgD− post-GC B cell numbers in the peripheral blood [257], in association with increased infections, corroborates a failure of typical antimicrobial GC reactions in patients with possible support by aberrant B cells. Although robust GC formation appears to be critical for disease initiation, a recent study revealed that GC disruption is important for disease maintenance [258]. In addition to potential GC and non-GC extrafollicular reactions in secondary lymphoid organs, the BM may represent a critical site in the genesis of chronic GVHD [259-261]. Additional experiments addressing these possibilities are warranted.

Why chronic GVHD patients produce alloreactive B cells and antibodies but not clinically relevant antimicrobial responses highlights an important gap in our knowledge about B cell pathobiology. Loss of antibody titers to microbes and muted B cell responses to microbial pattern recognition receptors like lipopolysaccharide potentially contribute to this GVHD-associated immune deficiency [262]. Whereas ex vivo assays have shown that B cells are constitutively activated in chronic GVHD, B cell lymphopenia and humoral immune deficiency are distinctive characteristics of chronic GVHD

[144,238-241]. In this regard, patients with chronic GVHD appear to be similar to patients with common variable immune deficiency given their shared propensity toward B cell autoreactivity/alloreactivity in the face of profound humoral immune deficiency [230,263]. The apparent incongruence between increased B cell survival, activation, and IgG production and poor functional antimicrobial responses remains to be addressed.

Finally, B cells can have regulatory properties that suppress rather than initiate chronic GVHD. Bregs have been recently identified as a novel B cell population associated with chronic GVHD development [54,147,249]. Emergence of a population of CD19+CD21low B cells by day 100 correlates with the subsequent development of chronic GVHD [264], specifically in patients who have BOS [265] and hypogammaglobulinemia [266]. Proportions of this B cell subset may have predictive value with respect to responsiveness to extracorporeal photopheresis [267].

Step 3: Aberrant Repair, Propagation of Fibrosis, and Progression of Chronic GVHD
Wound healing, repair, and fibrosis

The immune system plays a central role in the regulation of inflammation, tissue repair, and recovery. These processes are essential for host defense, wound healing in response to epithelial damage, and the maintenance of tissue homeostasis. Dysregulated immunity and aberrant tissue repair can lead to scarring or fibrosis [268], defined as the excessive accumulation of components of the extracellular matrix (ECM) in and around inflamed or damaged tissue. Pathological fibrosis results from chronic infection, persistent immune activation, and/or impaired regenerative responses [269]. Progressive loss of normal tissue architecture and organ function is the hallmark of fibrotic disorders. Immunologic mechanisms resulting in excessive collagen deposition and the development of fibrosis are complex. Activation of ECM-producing myofibroblasts is a common feature of all fibrotic disorders regardless of the initiating event [268]. Acute inflammatory responses often initiate the fibrotic cascade. Early endothelial damage activates coagulation pathways and results in the release of chemotactic factors that recruit immune cells to sites of tissue injury.

Synergistic interactions between components of innate and adaptive immunity contribute to evolving tissue inflammation and ultimately regulate the differentiation of fibroblasts into activated, ECM-producing myofibroblasts (reviewed in [268]). Myeloid cells secrete soluble factors (TNFα, IL-1β, and IL-6), identified as important drivers of fibrosis. Tissue macrophages are also key regulators of fibrosis and a major source of TGFβ (perhaps the most significant molecule involved in fibrinogenesis), and PDGF. They are also major producers of both matrix metalloproteinases (MMPs) as well as their endogenous suppressors, tissue inhibitors of MMPs (TIMPs) [270,271]. In addition to TGFβ, classically activated (M1) macrophages secrete proinflammatory cytokines, including TNFα and IL-1β, which can activate fibroblasts and contribute to ECM generation. Alternatively activated (M2) macrophages are a distinct subset of cells that have been shown to suppress inflammation and contribute to both profibrotic and antifibrotic processes [267,272].

The contribution of the adaptive immune response to fibrosis is well recognized. The recruitment of activated Th2 and Th17 CD4+ T cells also promotes fibrosis primarily through the secretion of IL-13 and IL-17, respectively. In contrast, the role of Th1 cytokine IFNγ is more controversial, exhibiting both
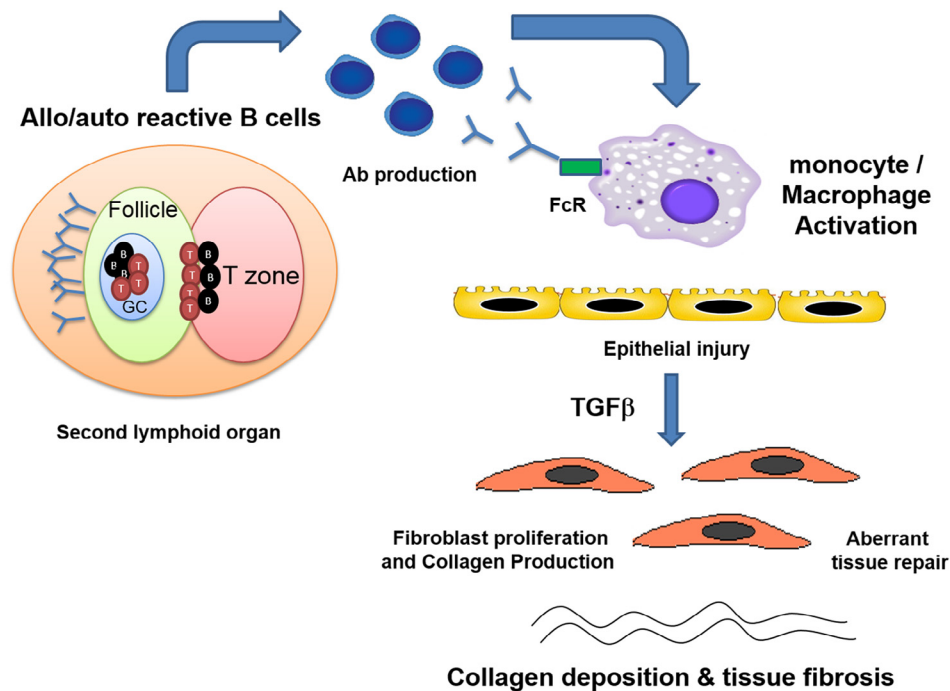
Figure 5. Phase 3: Aberrant tissue repair and pathways of antibody-mediated fibrosis. The contribution of B cells to the development of chronic GVHD has been recently highlighted in several experimental systems. One pathway emphasizes interactions between donor-derived T cells and B cells in secondary lymphoid tissues, including the spleen peripheral lymph nodes. The generation of alloreactive or autoreactive B cells and the dysregulated production of alloantibodies and autoantibodies initiates a cascade of events that involves activation of monocytes and macrophages along with endothelial and epithelial injury. The release of soluble factors, including TGFβ, and fibroblast stimulation characteristic of aberrant tissue repair result in collagen and matrix production and deposition, culminating in target organ fibrosis and dysfunction.

profibrotic and antifibrotic effects. Several experimental systems have revealed a role for B cells in the development of fibrosis that characterizes some forms of chronic GVHD [144,240,252,273]. Interactions between donor-derived T cells and B cells in secondary lymphoid tissues initiates a cascade of events resulting in the generation of alloreactive/ autoreactive B cells and the dysregulated production of alloantibodies/autoantibodies, activation of monocytes and macrophages, injury to ECs and epithelial cells, and release of soluble factors, including TGFβ. Fibroblast stimulation leads to matrix production and collagen deposition, culminating in multiorgan fibrosis and dysfunction (Figure 5). Finally, Tregs also contribute to wound healing through the production of IL-10, TGFβ, and amphiregulin. Tregs suppress inflammation, but their effects on fibrosis are variable and may depend in large part on the role of other T effector populations. Tregs may exacerbate TGFβ-induced fibrosis but suppress Th2- and Th17-driven disease [268,271].

Normal tissue repair mechanisms activated in response to an injurious event work to dampen the associated inflammatory response, limit cellular damage, reestablish tissue integrity and homeostasis, and ultimately expedite functional wound healing as opposed to progressive scarring [274,275]. Like the inflammation engendered during chronic GVHD, wound healing exemplifies a highly dynamic interface between innate and adaptive immunity [276]. The healing of damaged tissue must be tightly controlled as an inflammatory response ends. As noted, nonresolving inflammation or exuberant or excessive repair can lead to fibrosis, scarring, and organ dysfunction. Complex interactions among neutrophils, macrophages, stromal cells, coagulation pathways, lipid mediators, and ECM molecules must occur in a

synchronized fashion in distinct locations [277,278]. Mechanisms contributing to the resolution of inflammation are now understood to be biochemically distinct from traditional anti-inflammatory pathways [276,278]. Although some aspects of these pathways may be conserved, others are likely to be organ-specific.

Deposition of an ECM is paramount to the initiation and evolution of the reparative process. For example, the establishment of a provisional matrix to replace lost or damaged tissue is followed by transition to a more "mature" ECM that is ultimately remodeled to replicate functional tissue [279,280]. Importantly, the ECM plays an active role in modulating cell–ECM interactions during these changes. Functional repair, irrespective of organ involvement, is predicated in large part on reestablishment of the epithelial barrier and vascular remodeling [275,279]. The latter is paramount to maintaining blood flow and establishing a continual supply of oxygen and nutrients to damaged tissues [275,280]. In this context, epithelial and endothelial regeneration are fundamental to the restoration of vascular integrity and tissue architecture and organ function [279].

It is conceivable that these reparative pathways are operative when the inciting alloimmune stimulus and accompanying inflammation that characterizes chronic GVHD is controlled and immune tolerance is restored. The precise mechanisms involved in tissue regeneration and repair after successful treatment of chronic GVHD have yet to be rigorously studied or understood, however, and this remains a significant knowledge gap in both basic biology and clinical medicine. Similarly, determining whether the tissue destructive effects of chronic GVHD have fully resolved following the development of immune tolerance is also challenging.
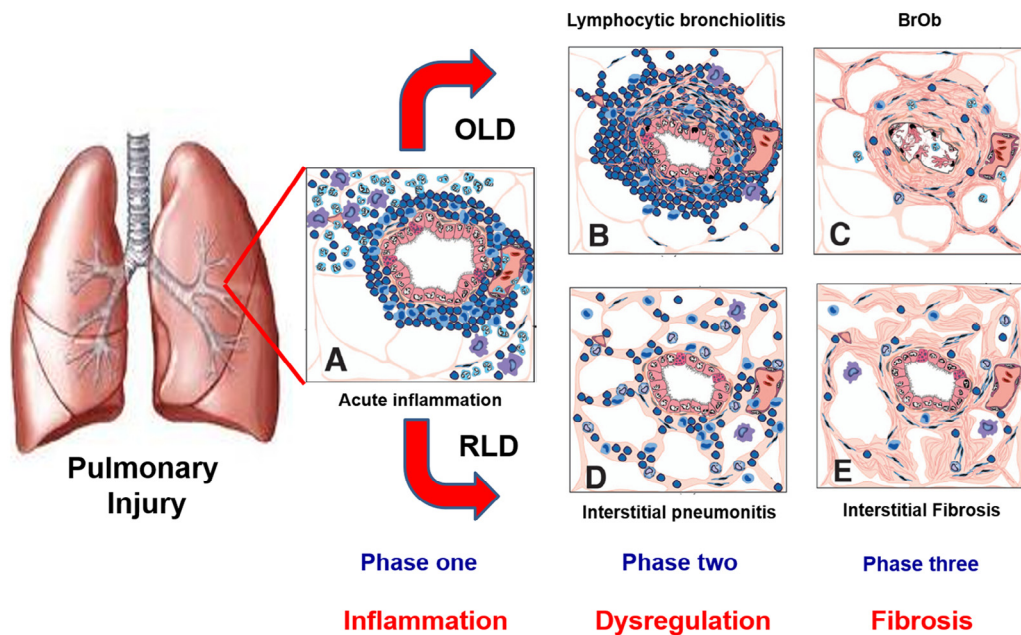
Figure 6. Pulmonary dysfunction and the triphasic model of chronic GVHD. Conceptually, the triphasic model of chronic GVHD can be applied to the development of lung dysfunction after allogeneic HCT. (A) In phase one, acute lung injury occurs as a consequence of an allogeneic immune response and results in the influx of donor immune cells into an inflamed pulmonary parenchyma. (B and D) Persistence of an inflammatory signal in the setting of dysregulated immunity promotes the transition from acute to chronic injury in phase two. If the inciting injurious event involves predominantly bronchiolar epithelial cells, then phase II is associated with the development of chronic bronchiolitis (B). If, in contrast, the principal target of chronic inflammation is the alveolar epithelium, then leukocyte recruitment and matrix deposition during phase two contribute to interstitial pneumonitis (D). (C) In the context of aberrant repair, chronic inflammation proceeds to phase three. Lung fibroblasts increase dramatically in number and contribute to the enhanced deposition of collagen and granulation tissue in and around bronchial structures, ultimately resulting in complete obliteration of small airways and fixed OLD characteristic of bronchiolitis obliterans (BrOb). (E) Fibroblast proliferation and intraseptal collagen deposition during phase three ultimately results in interstitial thickening, septal fibrosis, significant volume reduction, and the development of severe restrictive lung disease (RLD) and interstitial fibrosis.

Normalization of laboratory values, inflammation, and organ function in the liver and kidney; restoration of epidermal and mucosal integrity in the skin and oral tissues; resolution of end organ symptomatology in the gut, eyes, and lungs; and stabilization or improvement of lung function all may be considered in the decision of whether to discontinue treatment with immunosuppressive medications.

Mechanisms of pulmonary fibrosis

Declining lung function is a significant complication in the months and years after successful allogeneic HCT [15,281,282]. Two forms of chronic lung disease are commonly observed in this context: obstructive lung disease (OLD), otherwise known as BOS, and restrictive lung disease (RLD) [283-287]. The most recognized form of chronic GVHD of the lung is BOS [281,282,288]. In each scenario, collagen deposition and the development of fibrosis either in the peribronchiolar (OLD) or interstitial (RLD) space contribute to the resultant patterns of lung dysfunction [283]. The complex pathophysiology that characterizes lung fibrosis after HCT is poorly understood and represents the most significant gap in the current knowledge of this spectrum of chronic GVHD [20,289,290]. This limitation stems from the lack of correlative data obtained from afflicted HCT recipients, along with the paucity of suitable HCT animal models for either the restrictive form or the obstructive form of chronic lung injury.

Until recently, most of the knowledge of the pathogenesis of OLD/BOS was based on observations made in lung allograft recipients and from data generated in murine heterotopic tracheal transplantation models. In this context, fibrosis developing during chronic GVHD of the lung is be-

lieved to involve, at least in part, a persistent or recurrent antigenic stimulus, which elicits chronic inflammation and aberrant repair [283]. This exaggerated reparative response involves the recruitment of donor-derived immune cells, differentiation of fibroblasts into myofibroblasts, inappropriate ECM deposition, and disruption of the alveolar capillary membrane and/or obliteration of the terminal bronchioles [280]. Destruction of epithelial cells, ECs, and basement membrane integrity culminates in the uniform loss of tissue architecture, progressive fibrosis, and ultimately, irreversible loss of function. Removal of the chronic inflammatory stimulus may lead to resorption/remodeling of the ECM. Subsequent reepithelialization and reendothelialization can result in reestablishment of the alveolar capillary membrane and the terminal bronchiolar architecture and restoration of normal function [280]. Although alloreactive effector cells are required to initiate chronic GVHD in mice and patients, their role in the initial damage to the alveolar or bronchiolar epithelium and the subsequent progression to chronic pulmonary injury have not been thoroughly elucidated. For example, an early robust inflammatory phase might not be a prerequisite for subsequent fibrosis; persistent epithelial damage and subsequent "cross-talk" among epithelial cells, inflammatory cells, and fibroblasts may be sufficient for the development of fibrotic lung disease [271,291].

Conceptually, the triphasic model of chronic GVHD outlined above can be applied to the development of pulmonary dysfunction after HCT [284] (Figure 6). In phase 1, acute inflammation, which may be subclinical in nature, results in the sequential influx of lymphocytes, macrophages, and neutrophils into the pulmonary parenchyma. This injury is

initiated early after allogeneic HCT by a systemic proinflammatory environment that leads to chemokine up-regulation, leukocyte recruitment, and secretion of inflammatory cytokines in the lung [104]. In phase 2, persistence of an inflammatory signal in the setting of dysregulated immune mechanisms results in epithelial apoptosis and the transition from acute to chronic injury, either involving the peribronchiolar areas, resulting in the development of chronic bronchiolitis, or confined primarily to the interstitial space. As chronic inflammation proceeds to phase 3, lung fibroblasts contribute to the enhanced deposition of collagen and granulation tissue. When this occurs in and around bronchial structures, complete obliteration of small airways, and significant, "fixed" OLD ensues. In contrast, fibroblast proliferation and intraseptal collagen deposition may ultimately result in interstitial thickening, septal fibrosis, significant volume loss, and impaired gas exchange, which are characteristic of severe RLD.

TGFβ is a central mediator that may be necessary to initiate fibrosis but insufficient to sustain fibrosis. More recently, murine systems have shown that dysregulation of other factors, including TNFα and IL-1β [268,284,292], aberrant B cell immunity, and autoantibody/alloantibody production [144], along with disruption of the balance of M1/M2 macrophage function [132,268], may be operative as well.

MURINE CHRONIC GVHD MODELS: LABORATORY INSIGHTS AS A BRIDGE TO CLINICAL TRANSLATION

It is generally accepted that animal models incompletely replicate the complex phenotypes and clinical manifestations of chronic GVHD in humans. Moreover, current preclinical models do not encompass the full spectrum of pathological features of the clinical disease state. In keeping with observations in humans, data generated in murine models suggest that the immunologic underpinnings of chronic and acute GVHD are distinct at both cellular and molecular levels. Two publications have provided comprehensive overviews of currently available chronic GVHD systems and have described the diverse efforts to study chronic GVHD using murine models [293,294]. Model variables have been compared and contrasted in terms of methodology (strain combination, conditioning regimen, cell dose), disease presentation (target organs, pathological basis), and proposed or elucidated pathogenic mechanisms.

In this section, we focus on more recent models that have contributed to advances in our understanding of the pathophysiology of chronic GVHD, particularly related to the phases of chronic GVHD (Table 4). We discuss several new chronic GVHD models that have been instrumental in facilitating the testing of targeted strategies to prevent or treat chronic GVHD.

INFLAMMATORY MODELS AND THE TRANSITION FROM ACUTE TO CHRONIC GVHD

It is accepted that proinflammatory acute GVHD responses likely play a role in the subsequent development of chronic GVHD. This is particularly relevant to the study of "overlap syndrome" with clinical acute GVHD characteristics and progressive chronic GVHD. Chronic GVHD that is preceded by acute GVHD may differ biologically from de novo chronic GVHD. One such model using the fully allogeneic, MHC- and miHA-mismatched strain combination (C57BL/6→Balb/c) is an established system for acute GVHD (Table 4). Activated host APCs induce CD4+ and CD8+ donor-derived T cells and cause mTEC damage, resulting in the production of autoreactive CD4+ T cells. Donor graft–derived CD8+ T cells

are more potent than CD4+ T cells in inducing chronic GVHD, but recipient thymus and de novo donor-derived CD4+ T cells are required for disease penetration. Autoreactive T cells then interact with donor DCs and B cells, resulting in their expansion and consequent perpetuation of chronic GVHD and autoantibody production [70,159,295].

Similarly, a clinically relevant, G-CSF–treated, parent→F1 (C57BL/6→B6D2F1) irradiation model (Table 4), as well as the aforementioned multiorgan system chronic GVHD models (reviewed in [10,104]), implicate roles for IL-17 and tissue infiltration of F4/80+ macrophages as central mediators of skin scleroderma [296]. Targeting macrophage colony-stimulating factor (CSF) signaling may represent a novel therapeutic strategy for prevention and treatment of chronic GVHD [132]. Findings in this parent→F1 model also have been instrumental in the study of chronic fibrotic pulmonary dysfunction after allogeneic HCT, including the demonstration that TNFα plays a critical role during the transition from acute to chronic inflammation in the lungs [284,297,298]. Experimental data led to the development and completion of a clinical study using etanercept for HCT recipients with restrictive or obstructive noninfectious chronic lung disease [299].

Preclinical Models of Immune Dysregulatory Chronic GVHD

Lupus-like models

Historically, the most frequently used chronic GVHD strain combination is a semi-allogeneic, non-irradiated, parent into F1 model that result in lupus-like manifestations [300] (Table 4). Following the infusion of unfractionated parental splenocytes, GVHD is initiated by donor CD4+ T cell activation in response to host allogeneic MHC class II antigens, resulting in cognate donor CD4+ T cell help to host B cells. Acknowledged weaknesses of this model include the lack of clinical correlates of human chronic GVHD, incomplete donor engraftment, and the absence of radiation or conditioning regimen–associated tissue injury. Nonetheless, novel potential targets for chronic GVHD intervention have been illustrated through this model, including CD137 agonist therapy and anti-TNF p55 subunit blockade [300,301].

Thymic damage and treg deficiency

As noted above, murine chronic GVHD models centered on thymic damage and Treg deficiency have provided important information on the breakdown of thymic function and central tolerance, uncovering an etiologic link between acute and chronic GVHD [129,302]. In these models (Table 4), GVHD-induced alloimmunity is antigen-driven, resulting in repertoire skewing and dominant, high-frequency clonotypes that emerge owing to inadequate T cell immune regulation [69], and manifests predominantly as inflammatory (colitis) rather than fibrotic disease [69].

B cells, immune dysregulation, and multiorgan GVHD

The importance of B cells in development of chronic GVHD was first described in a sclerotic chronic GVHD miHA-disparate, MHC-identical mouse model (C57BL6→LP/J) using an anti-μ antibody [229] and subsequently in a full MHC-disparate model (C57BL/6→B10.BR) in which the development of multiorgan, chronic GVHD (including lung, liver, and colon fibrosis) is associated with CD4+ T cell and B cell infiltration [252,273]. In addition, donor-derived alloantibody (IgG) and an increased frequency of Tfh cells that support GC formation are required for BOS development. Interruption of GC formation by various approaches can effectively reverse

Table 4
Summary of Chronic GVHD Mouse Models

Model Type	Model Characteristics	Model Clinical Phenotype	Findings	Therapies Generated	References
Lupus-like	Parental haploidentical, DBA2 (H-2^d haplotype) splenocytes into unconditioned (B6 × DBA2) F1 hosts (H-2^{bd} haplotype)	• DNA and chromatin-directed autoantibody; immune complex–mediated glomerulonephritis; lack of clinical correlation to human chronic GVHD	• Partial donor chimerism • Relies on activated host B cells	• CD137 agonist therapy • Anti-TNF p55 subunit blockade	[300,301]
Thymic damage	• Acute GVHD in lethally irradiated, MHC-mismatched, recipient • Post-BM transplantation, splenic T cells transferred into unconditioned donor strain immunodeficient (Rag$^{-/-}$) recipients	• Inflammatory disease (colitis) • Not a fibrotic process	• GVHD is antigen-driven • Repertoire skewing seen • Dominant high-frequency clonotypes		[69,129,302]
Multiorgan involvement	MHC disparate model; B10.BR mice are conditioned with cyclophosphamide and TBI, followed by the infusion of BM and purified splenic T cells from B6 donors	Multiorgan involvement: • Lung • Liver • Colon • Tongue • Spleen • Thymus	• CD4$^+$ T cell and B cell infiltration in target organs • Increased frequency of T follicular helper cells by supporting GC formation and maintenance, leading to IgG deposition in target tissues, is essential for disease pathogenesis • Deficiency of T follicular regulatory cells that suppress GC formation • Macrophage-dependent disease • Multiorgan system fibrosis (lung, liver, colon) is a prominent feature	Abrogation of chronic GVHD by targeting: • Lymphotoxin-β receptor Ig targeting GC formation • B cell signaling through Bruton's tyrosine kinase and IL-2–inducible T cell kinase via ibrutinib; spleen tyrosine kinase targeting with fostatinib • Targeting Tfh cells by blocking ICOS, CD40L, IL-21, and IL-2Rgc • Phosphorylation of STAT3, Rho-associated kinase 2, RORC, Jak1/2 (ruxolitinib) • Macrophage depletion by targeting CSF-1 signaling • T follicular regulatory infusion	[98,132,144,217, 240,241,252,273, 310]
Sclerodermatous/ lupus-like	• DBA/2 (H-2^d) and BALB/c (H-2^d) miHA-only mismatched • Sublethal irradiation and DBA/2 splenocytes	Donor B and CD4$^+$ T cells required for pathogenesis	• B cells drive clonal donor autoreactive CD4$^+$ T cells • CD11b$^+$/Gr-1$^+$ PMNs and macrophages	• Anti-CD20 antibody prevents chronic GVHD	[295,336].
Profibrotic	• Classical model B10.D2→BALB/c • Donor T cells B10.D2 and BALB/c mice are MHC-matched (H-2^d haplotype) mismatched at MiHA • 700 cGy of TBI to recipients and infusion of BM and whole spleen cells	• Fibrotic changes in skin, gastrointestinal tract, liver • Bronchiolitis obliterans syndrome model • Donor B and CD4$^+$ T cells required for pathogenesis	• Mononuclear cell infiltration, increased collagen deposition • Expansion of Th1 and Th17 cells, dermafibrosis with donor CD11b$^+$ monocytes and activated macrophages • Effector T cell and pSTAT3 dependent • IL-10–producing B cells prevent disease	• Prevention and/or attenuation of established chronic GVHD • Am80, a potent synthetic retinoid • Sphingosine-1-phosphate receptor antagonist FTY720 modulates inflammatory immune cells • Bortezomib modulates pathogenic B cells	[62, 136-138, 144, 304-307, 336]
Sclerodermatous	• LP/J→C57BL/6 model of sclerodermatous chronic GVHD • HLA-matched strains • Myeloablative	Manifestations of both acute and chronic GVHD: • Skin • Lungs • Kidneys	Removal of B cells prevents T cell priming to MiHA and development of chronic GVHD	Prevention of chronic GVHD: • Treatment with anti-µ polysera • Chloroquine • Ibrutinib	[241,308]
Inflammatory with progression of acute GVHD to chronic GVHD	• MHC mismatched, strain combination (B6→Balb/c) • Established model of acute GVHD • Reduction in donor T cells develop pathognomonic findings of chronic GVHD (>60 d after HCT)	Chronic GVHD manifestations • Cutaneous fibrosis • Salivary glands • Fibrosis of the thymus • Serum autoantibodies	• Donor CD4$^+$ and CD8$^+$ alloreactive T cells damage mTECs • Donor CD8$^+$ T cells induce chronic GVHD • Recipient thymus and de novo donor CD4 T cells required • IL-17 mediates scleroderma • Macrophages critical	Targeting CSF-1 signaling	[70,159,295].
Progressive acute GVHD to chronic GVHD	• C57Bl/6 parent→irradiated B6D2F1 model with BM plus G-CSF–mobilized splenocytes or unmobilized T cells	Sclerodermatous manifestation of chronic GVHD: • Skin • Lung	• TNFα important for progression of acute GVHD to chronic GVHD of the lung	• Anti–CSF-1 • Anti-TNFα (etanercept)	[132,292,296]

GVHD indicates graft-versus-host disease; HCT, hematopoietic cell transplantation; GC, germinal center; BM, bone marrow; PMN, polymorphonuclear lymphocyte; TBI, total body irradiation; MHC, major histocompatibility vomplex; MiHA; minor histocompatibility antigen; G-CSF, granulocyte colony-stimulating factor; mTEC, medullary thymic epithelial cell.

established, antibody-dependent chronic GVHD early in the course of disease [240,241,252]. Recent work with same model has shown that targeting Tfh cells hinders GC formation and prevents chronic GVHD, or reverses its early manifestations [144].

Several important insights were also gained using an MHC-matched DBA2→BALB/c model, in which disease is manifested as lupus-like and sclerodermatous phenotypes (Table 4). Donor B cells have a marked effect on the progression of chronic GVHD by sustaining the clonal expansion of donor alloreactive CD4+ T cells [295]. Pathogenic alloreactive T cells responsible for target tissue injury and consequent recruitment of CD11b+/Gr-1+ macrophages and neutrophils appear to originate from mature (post-thymic) T cells administered with the donor graft, emerging without obvious thymus damage [70]. Anti-CD20 monoclonal antibodies (mAb) given early post-HCT prevent chronic GVHD induction and preserve GVL effects [303]. In contrast to human chronic GVHD, administration of anti-CD20 mAb after GVHD onset is not effective in depleting donor B cells or ameliorating murine chronic GVHD in an miHA-disparate model (B10.D2→Balb/c) and in 2 distinct MHC-disparate models (C57BL/6→B10.BR and C57BL/6→Balb/c) [144]. This is possibly the result of failure to deplete CD20neg plasmablasts and plasma cells.

Profibrotic Chronic GVHD Models

Important advances have occurred in various models in which tissue fibrosis is the primary histological finding. In the classical miHA-disparate, MHC-identical B10.D2→BALB/c sclerodermatous model of chronic GVHD, the phenotypic hallmarks are fibrotic changes in the skin, lung, gastrointestinal tract, and liver (Table 4) [144,304]. Donor B cells and CD4+ T cells are again important to disease development. Multiorgan injury is caused by expansion of both Th1 and Th17 CD4+ T cells, and dermal fibrosis is preceded by infiltration of donor-derived CD11b+ cells consisting of monocytes and activated macrophages [304]. When B10.D2 animals deficient in STAT3 are used as donors, Th17 cells do not develop in the recipient's spleen and liver, the overall expansion of donor CD4+ T cells is reduced [136], and FoxP3+ Treg frequency steadily increases. Selective neutralization of Th1 (IL-12)- or Th17-promoting cytokines (IL-23) subverted chronic GVHD generation [137]. This initial Th1/Th17-driven step toward the development of tissue fibrosis is amenable to down-regulation by either modulation of PDL-1 expression on tissues and/or stimulation of the PD-1 on donor T cells [138]. Several pharmacologic interventions, such as Am80, a potent synthetic retinoid [305], the proteosomal inhibitor bortezomib [306], and the sphingosine-1-phosphate receptor antagonist FTY720 [307], play significant roles in the prevention and/or attenuation of established chronic GVHD in this model. IL-10–producing Bregs also play a role in the prevention of sclerodermatous GVHD in this system [249].

The LP/J→C57BL/6 model of sclerodermatous chronic GVHD [308] is another solely miHA-disparate donor–recipient strain combination. This system is characterized by manifestations of both acute and chronic forms of skin and lung GVHD and was one of the first models to reveal a possible role for B cells in antigen presentation during chronic GVHD pathogenesis [229]. In addition to skin disease, alloimmune-mediated injury can be detected in lung and kidney as well. Ibrutinib, a Bruton's tyrosine kinase and IL-2–inducible T cell kinase, improves sclerodermatous chronic GVHD

progression-free survival and diminishes clinical and histopathological chronic GVHD in this model as well as in the C57BL/6→B10.BR multiorgan system model [241].

There are multiple roads to the development of tissue fibrosis, all converging on the production of profibrogenic molecules and fibroblast proliferation. Genetic factors that define the presentation and recognition of miHA antigens, along with the utilization of G-CSF donor cell mobilization [296], may be critical determinants of chronic GVHD susceptibility [309]. Both Th17 and Th2 cells appear to collaborate with B cells that produce autoreactive or alloreactive antibodies via IL-21 or IL-4, respectively. Indeed, donor splenocytes deficient in the retinoid-related orphan receptor γ RORC (required for lineage commitment to IL-17–producing cells) fail to cause chronic GVHD in a multiorgan system model [132], indicating that BOS develops in an IL-17–dependent fashion, similar to cutaneous chronic GVHD [296]. Antibody deposition can cross-link Fc receptors expressed on monocytes and macrophages culminating in TGF-β release and activate B cells, further fueling the antibody response. Th1 cells also may contribute to chronic GVHD generation by causing tissue injury to the thymus and mucosa of epithelial-rich organs, such as small and large intestines and liver, and through the release of inflammatory cytokines like TNFα. From a broader perspective, skin fibrosis appears to be an IL-17–dependent process, whereas aberrant B cell function and alloantibody deposition along with inflammatory cytokine release are important to lung and liver injury.

For the foreseeable future, there remains an unmet need for relevant chronic GVHD murine models, especially those that simulate the multiorgan manifestations and complex immune pathology of chronic GVHD observed in humans. Establishing a model of chronic GVHD developing in an adult or aged immune system may have significant merit, given the increasing frequency of chronic GVHD with age. Improvements in current chronic GVHD models that are limited by the general absence of immunosuppressive drugs for acute or chronic GVHD prophylaxis and treatment are also needed; such systems may increase the likelihood of identifying the most clinically relevant pathways of chronic GVHD generation and maintenance.

Finally, developing preclinical models to optimally address how interventions to abrogate chronic GVHD may influence GVL effects requires future investigation. This is a highly relevant topic, given that some clinical studies have suggested that effective GVL activity is derived primarily from chronic GVHD rather than from acute GVHD, particularly when low-intensity conditioning regimens are used. Numerous rodent experiments have demonstrated preservation of GVL effects when interventions are introduced in mice to attenuate acute GVHD, but the same question has only recently been explored in the context of chronic GVHD [303,306,310]. In this context, optimal chronic GVHD models should include several factors: the biology of tumor proliferation and kinetics of tumor elimination, whether interventions used to abrogate chronic GVHD also target the tumor used, whether mechanisms exploited by the experimental system to induce chronic GVHD (targeting of tissue-specific antigens or end-organ–specific immune cell homing, contribution of B cell responses) lend themselves to antileukemia/antitumor effects, and the magnitude of the immune response required to best study graft-versus-host and graft-verus-tumor activity. The latter will be influenced both by the number and composition (purified T cells versus whole splenocytes) of immune cells infused along with the extent of lymphocyte (T cell and B cell)

amplification associated with the development of chronic GVHD.

BIOMARKERS AND HUMAN CHRONIC GVHD BIOLOGY

Although the primary purpose of biomarkers is not to study the biology of disease, biomarker discovery and validation can still guide the understanding of disease pathogenesis. Biomarker studies have identified multiple molecules that have brought to light patterns in cellular subsets, cytokines, autoantibodies, and other factors associated with the diagnosis of chronic GVHD (reviewed in [311]. As such,

biomarker candidates can be important in guiding the design of animal models focused on understanding the pathogenesis of human disease (reviewed in [311]). Inflammatory markers, including IL-2Rα, aminopeptidase N (CD13), IL-4, IL-6, and TNFα, have consistently been identified in patients with chronic GVHD [92,100,139,234,312,313]. Some of the most promising diagnostic markers include the chemokine ligands CXCL9, CXCL10, and CXCL11, which have been reported to correlate with chronic GVHD [73,77,85,92-94].

Whether Tregs are reliable biomarkers for chronic GVHD remains to be determined. Treg numbers are low in some

Table 5
Gaps in Knowledge and Proposed Priority Research Studies for Chronic GVHD Biology

Gaps and Questions	Proposed Research Studies
Links between clinical manifestations and biology of chronic GVHD	
What is the effect of the different type of outcomes identified in Table 1 on immune responses after HCT?	⟶ Evaluate late vaccine and immune responses in humans with resolved, active, no, and inactive chronic GVHD (Table 1).
Do different clinical manifestations of chronic GVHD have distinct biology?	⟶ Study biological characteristics of the clinically annotated models proposed herein.
Are there distinct disease biology clusters that occur independently across various clinical key manifestations?	⟶ Study clustering of biological characteristics in patients independent of clinical manifestations.
How does previous chronic GVHD increase the risk of late effects, including stroke, myocardial infarction, infections, and subsequent malignancies?	⟶ Conduct human and murine studies evaluating the role of immune dysregulation and chronic inflammation in the development of late effects
Gaps in understanding chronic GVHD biology	
What are the specific contributions of donor and recipient immunity to inflammatory pathways involved in chronic GVHD induction for each pair?	⟶ Conduct in-depth clinical studies of HLA-encoded and non–HLA-encoded polymorphisms for donor–recipient pairs.
What are the target antigens of chronic GVHD in humans?	⟶ Perform large analyses to expand on current miHA target antigens recognized in humans and develop murine models based on these miHAs.
T cell and B cell repertoire analyses in peripheral blood, lymphoid tissues, and target organs are needed to better understand clonal responses of T cells and antigen identification, and are underexplored in preclinical models and in clinical studies.	⟶ Conduct detailed analyses of human T cell and B cell repertoires that develop within the different types GVHD defined in Table 1. Develop murine GVHD models that reflect human repertoire development.
What is the role of tissue densritic cells in inducing and propagating chronic GVHD?	⟶ Evaluate the role of dendritic cells in murine chronic GVHD models.
Is there a mechanistic link between early injurious events that occur early after transplant and the subsequent development of chronic GVHD?	⟶ Study whether targeted protection of early vascular and epithelial injury may reduce subsequent target organ dysfunction associated with chronic GVHD.
Do the fibrotic manifestations involve distinct pathways in various organs or do they have a singular inciting pathway that unifies different clinical phenotypes?	⟶ Conduct longitudinal clinical studies of target tissue immunobiology.
Are fibrotic manifestations end-stage changes or are they reversible? What are the mechanisms involved in functional wound healing after tolerance has been established?	⟶ Conduct longitudinal observational and therapy studies of fibrotic manifestations in mice and humans
What are the mechanisms responsible for collagen deposition and target organ fibrosis during end stages of chronic GVHD?	⟶ Develop novel approaches to target fibrinogenic pathways to treat patients with evolving organ fibrosis and dysfunction.
Gaps in understanding functional immunity and tolerance	
What are the molecular pathways underpinning development of functional immunity (and tolerance)?	⟶ Study immune function in murine models of transplant tolerance (in mice on GVHD prophylaxis and after medication wean) and conduct studies of patients with chronic GVHD who then have full resolution of clinical manifestations and are weaned off immune suppression.
How do current GVHD prophylaxis regiments influence the development of immune tolerance? Can we decipher mechanisms of chronic GVHD prevention with post-transplantation cyclophosphamide?	⟶ Incorporate GVHD prophylaxis in mouse models to decipher their role in preventing tolerance induction and/or promotion of chronic GVHD.
How does an aged immune system versus a juvenile immune system (where all animal studies are focused) respond differently to the development of acute and chronic GVHD?	⟶ Improve our understanding of the effect of both donor and recipient age on immune tolerance.
Development of new animal models	
Can steroid-refractory animal models be developed?	⟶ Study new agents for steroid-refractory disease.
A robust, large animal model of chronic GVHD is a gap in the field and would be useful for testing human reagents before clinical trials.	⟶ Expand the current primate and dog models to better evaluate chronic GVHD.
The specific antipathogen and antitumor effects of therapeutic agents on chronic GVHD therapy in preclinical models have been largely unexplored and merit further consideration for testing.	⟶ Perform murine chronic GVHD model-based studies focused on the ability to respond to pathogens and malignancy in the presence of chronic GVHD.
Biomarkers and disease pathogenesis	
What specific biomarkers and preclinical animal models would be most useful for exploring disease pathogenesis and therapy?	⟶ Perform large HCT population-based studies evaluating how a number of identified covariates influence biomarker concordance with chronic GVHD and evaluate these factors in murine GVHD models.

GVHD indicates graft-versus-host disease; HCT, hematopoietic cell transplantation; miHA, minor histocompatibility antigen.

studies but increased in others [187]. Recently, decreased Treg:T cell effector ratios have been correlated with chronic GVHD development in large series [192,221], thus this parameter has emerged as a promising biomarker for disease. As noted earlier, the consequences of thymic damage on appropriate negative selection following HCT is an area of significant interest [160,164,169]. Although some studies have not found an association between chronic GVHD and thymopoiesis [314,315], others have noted a strong correlation [168,316].

Despite efforts at categorical grouping, validation of even the most promising biomarker candidates among large, independent cohorts has yet to be consistently achieved. Association analyses may be optimized/enhanced by phenotypic classification. A possible approach would be to cluster organ manifestations by their anatomic patterns (mucocutaneous, myofascial, pulmonary, and other organ). However, although various organs involved in chronic GVHD can be affected simultaneously, disease manifestations often demonstrate a spreading behavior that progressively affects a broader array of organs and sites over a period of weeks to months. Another major challenge is that a number of clinical factors appear to influence the predictive and prognostic value of biomarkers. These include total body irradiation in the conditioning regimen, peripheral or cord blood as the stem cell source, and reduced-intensity preparative regimens [93], strongly suggesting that the biology of chronic GVHD may vary depending on the clinical scenario. Thus, given the clinical and temporal variations in the organs affected, and the protean manifestations within a given organ system, it is unlikely that a unifying biomarker will be associated with all chronic GVHD manifestations.

Establishing a clinical-biological classification system (Table 2) may promote more effective correlations among soluble or cellular biomarkers and established phenotypes. Thus, it would be feasible to study these various biological processes (inflammatory, immune dysregulatory, fibrotic/sclerotic) stratified by organ involvement, in an effort to understand the pathogenic mechanisms that explain the clinical heterogeneity of the disease. For example, a recent study separates oral chronic GVHD into oral lesions, salivary dysfunction, and impaired mouth opening, and demonstrates associations with cutaneous erythema, lacrimal dysfunction, and cutaneous sclerosis, respectively [317,318]. Along similar lines, recent work has described the incidence and features of cutaneous, soft tissue (fasciitis), or joint (contracture) sclerosis in a large cohort of patients with chronic GVHD [319]. A follow-up study by the same group revealed that 3 candidate single-nucleotide polymorphisms (SNPs) (in *BANK1*, *CD247*, and *HLA-DPA*), all of which have well-documented links to systemic sclerosis, are also associated with sclerotic GVHD. These findings support the concept that sclerotic GVHD is a distinct biological phenotype of chronic GVHD and can be distinguished from "conventional" chronic GVHD by genetic differences [320].

CONCLUDING REMARKS

Chronic GVHD remains the scourge of allogeneic HCT, negatively influencing recipients' quality of life, limiting successful outcomes, and correlating with a number of late effects. It has been more than 60 years since the seminal demonstration of acquired transplantation tolerance by Billingham, Brent, and Medawar [321,322], yet most current therapies for chronic GVHD control are empirical, globally immunosuppressive, do not specifically promote tolerance induction, and continue to be associated with significant morbidity and mortality. Cel-

lular, medicinal, and protein/antibody-based strategies aimed at restoring immune regulation, modulating T cell signaling responsible for proinflammatory and profibrotic cytokine production, blocking of T:B cell cooperativity, depleting antibody-secreting cells or monocytes/macrophages that bind pathogenic antibody, and targeting the profibrogenic process offer the greatest opportunity for preventing and reversing established chronic GVHD. Based on preclinical modeling, some of these approaches have already been or are scheduled to be clinically tested. The heterogeneity of organ involvement and clinical phenotypes, the paucity of human tissue available for analysis, inconsistent signals from biomarker studies, and profound knowledge gaps in biological mechanisms of disease have hindered the discovery and implementation of effective therapeutic strategies. Increased understanding of the initiating events, regulation of inflammation and repair, and propagation of tissue injury and fibrosis is required to achieve the long-term goal of chronic GVHD immune modulation. A multidisciplinary approach, along with prospective, longitudinal data collection and testing of relevant preclinical animal models, are needed to define the immunologic basis of disease and predict disease development and progression (Table 5). This approach will allow investigators to better define and exploit a window of opportunity that may exist for patients at high risk for chronic GVHD or in the early stages of disease development.

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