

Cytokine mediators of chronic graft-versus-host disease

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Substantial preclinical and clinical research into chronic graft-versus-host disease (cGVHD) has come to fruition in the last five years, generating a clear understanding of a complex cytokine-driven cellular network. cGVHD is mediated by naive T cells differentiating within IL-17-secreting T cell and follicular Th cell paradigms to generate IL-21 and IL-17A, which drive pathogenic germinal center (GC) B cell reactions and monocyte-macrophage differentiation, respectively. cGVHD pathogenesis includes thymic damage, impaired antigen presentation, and a failure in IL-2-dependent Treg homeostasis. Pathogenic GC B cell and macrophage reactions culminate in antibody formation and TGF- β secretion, respectively, leading to fibrosis. This new understanding permits the design of rational cytokine and intracellular signaling pathway-targeted therapeutics, reviewed herein.

Introduction

Allogeneic stem cell or bone marrow transplantation (hereafter referred to as BMT) remains a cornerstone curative therapy for high-risk hematological malignancy and severe immune deficiencies (1). Chronic graft-versus-host disease (cGVHD) is a multi-system inflammatory disease characterized by tissue fibrosis and mucosal lichenoid plaques that develop late after BMT and now represents the major cause of procedural morbidity and nonrelapse mortality (2, 3). While cGVHD has been historically defined by its time of onset (more than 100 days after BMT), it is now classified on the basis of clinical diagnostic features that typically involve cutaneous and/or pulmonary fibrosis (scleroderma and bronchiolitis obliterans [BO], respectively), oral lichenoid lesions, and myofascial manifestations, although it can affect virtually any organ in the recipient (4, 5). These changes to diagnosis and severity criteria have been generated in the last decade in an attempt to address difficulties with reproducible clinical staging and response criteria (6, 7) that have previously hindered the testing of therapeutics in appropriate controlled clinical trials.

Our understanding of cGVHD has improved dramatically in the last five years and is now conceptualized as a complex immunological process incorporating multiple facets of adaptive and innate immunity, including B cells, T cells, and macrophages together with their interactions with target tissues. Cytokines can be secreted by most cell lineages and orchestrate cellular responses that include migration, activation, and growth. This Review focuses on the cytokines that coordinate the cellular and molec-

ular determinants of cGVHD, outlining the pivotal soluble and surface-expressed mediators controlling disease at a cellular and extracellular level. Given the complexity of cGVHD, we will discuss cytokine effects in the context of relevant cellular mediators of disease and outline potential therapeutic approaches based on insights gained in preclinical models.

Since this Review cannot cover all aspects of the pathogenesis of GVHD, there are multiple additional reviews, both within this series in the *JCI* and elsewhere, focused on acute (8, 9) and chronic GVHD (10–12) that can provide a broad overview of the GVHD disease process. It should be noted that most of our recent understanding of cGVHD pathogenesis, particularly in relation to cytokine biology, has been developed in murine systems, and recent reviews have highlighted the pros and cons of these studies (1, 13). Where information exists, these broad pathogenic principles have been confirmed in patients undergoing BMT, and thus, this Review will focus on cytokine-dependent regulation of disease in mice and patients.

Modeling cGVHD clinical manifestations in mice

The incidence of moderate to severe cGVHD has increased over the last two decades because of the widespread use of granulocyte CSF-mobilized peripheral blood stem cells (G-PBSCs) over unmanipulated BM grafts. It is now clear that the enhanced and accelerated engraftment seen with G-PBSCs versus BM is countered by higher levels of cGVHD (14, 15). Other risk factors for cGVHD include the use of HLA-mismatched and unrelated donors, recipient age, and absence of antithymocyte globulin in conditioning (16). The increasing use of G-PBSC-mismatched donors and the routine transplantation of patients over 60 years old have led to a dramatic increase in the burden of cGVHD (14).

It is notable that cGVHD may develop in the context of preceding acute GVHD (aGVHD), whether effectively treated or developing as a continuum from acute disease (17). Indeed, prior aGVHD is a powerful and important risk factor for subsequent cGVHD (18). Furthermore, it has recently been appreciated that GVHD “break-

Authorship note: B.R. Blazar and G.R. Hill contributed equally to this work.

Conflict of interest: G.R. Hill has received funding from Roche for clinical trials of IL-6 inhibition. B.R. Blazar declares a conflict of interest in patents submitted for methods of treating and preventing alloantibody-driven chronic graft-versus-host diseases with ibrutinib (US Provisional Application No. 14/558,297) and with the ROCK2 inhibitor KD025 (US Provisional Application No. 61/977,564).

Reference information: *J Clin Invest.* 2017;127(7):2452–2463.
<https://doi.org/10.1172/JCI90593>.

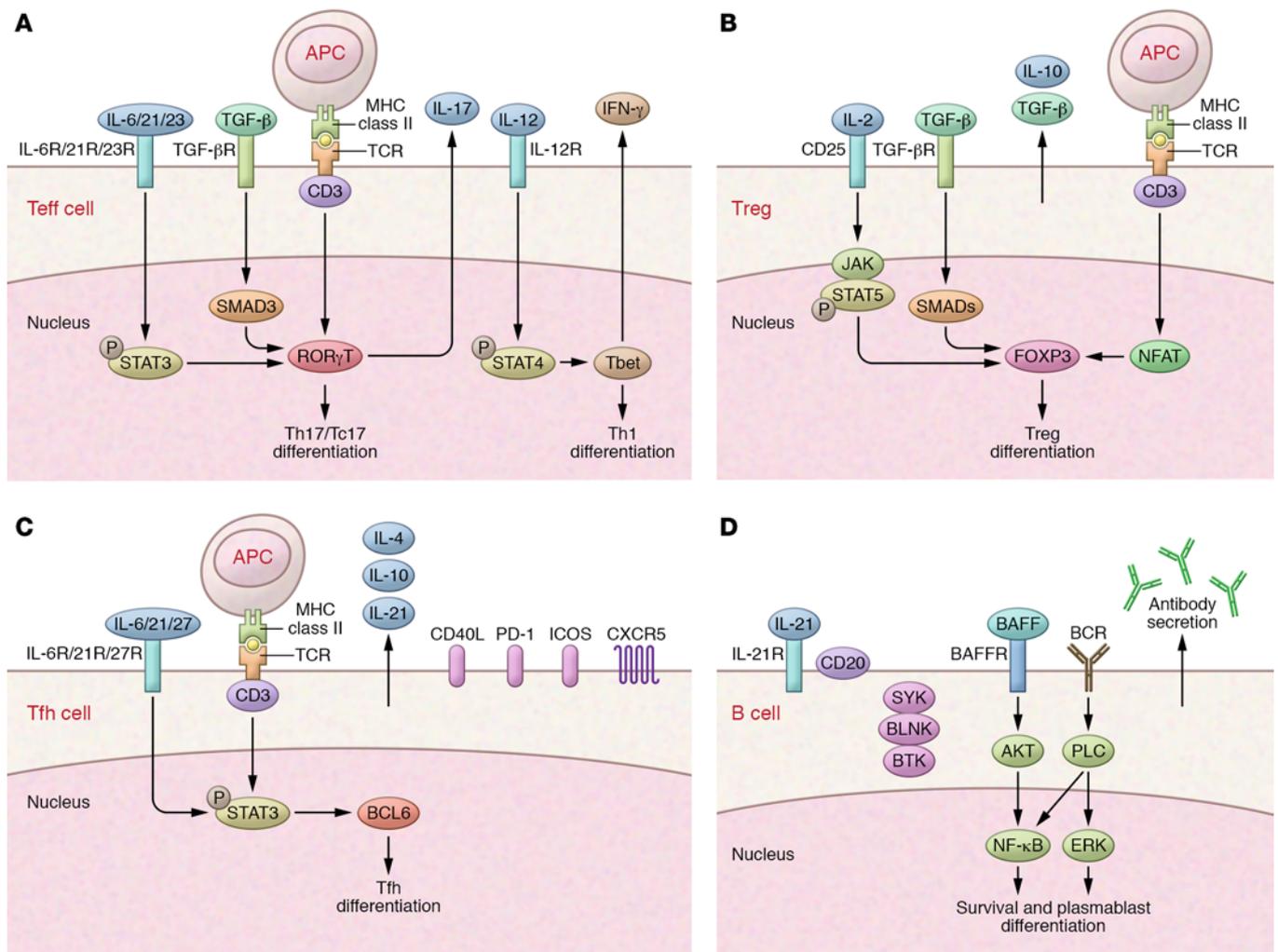


Figure 1. Cytokines and signaling pathways associated with the effector populations of cGVHD. (A) T effector cells respond to MHC/peptide on antigen presenting cells (APC) and, in the context of IL-6-, IL-23-, and IL-21-stimulated STAT3 phosphorylation, transcribe *RORC* to drive Th17 and Tc17 differentiation with secretion of IL-17 and related cytokines. IL-12 stimulation promotes T cell-specific T-box transcription factor (*TBET*) transcription and IFN- γ secretion that signals in an autocrine fashion to enhance Th1 differentiation. *Tbet* expression and IFN- γ secretion are also characteristic of Th17/Tc17 cells after BMT. (B) Tregs respond to antigen within MHC class II and concurrent TGF- β and IL-2 signaling, which promotes STAT5 phosphorylation, SMAD signaling, and subsequent *FOXP3* transcription. Immune regulation by secreted IL-10 and TGF- β ensues. (C) Tfh, identified by ICOS, CXCR5, and PD-1 expression, respond to antigen presented within MHC class II in the context of IL-6, IL-27, and IL-21 stimulation to drive STAT3 phosphorylation and *BCL6* transcription, with subsequent secretion of IL-21. (D) BAFF and IL-21 drive GC B cell expansion and, in the context of B cell receptor signaling, subsequent SYK and BTK transcription promotes survival and allo- and autoantibody secretion. BLNK, B cell linker; NFAT, nuclear factor of activated T-cells; BCR, B cell receptor; PLC, phospholipase C.

ing through” prophylaxis (usually immune suppression with calcineurin inhibitors) may have distinct immunological features from GVHD that develops in the absence of calcineurin inhibitors (19); this is an important potential consideration for therapy.

Historically, mouse cGVHD studies were often generated in the absence of conditioning therapy by infusion of parental splenocytes into semiallogeneic F₁ hosts, resulting in a lupus-like reaction (reviewed in refs. 20, 21). However, these models did not well simulate the wider spectrum of clinical cGVHD, and, since no conditioning or donor hematopoietic cells were infused, host immune elements were major contributors to disease pathogenesis. The dominant disease manifestations were glomerulonephritis with scleroderma that was associated with single-stranded DNA autoantibodies. Today, mouse models of BMT typically use

total-body irradiation-based conditioning and BM grafts together with purified splenic and/or lymph node-derived T cells to induce GVHD (22). More recently, granulocyte CSF-mobilized (G-CSF-mobilized) splenocytes have been used to model G-PBSCs, which generally results in more severe cGVHD compared with models using unstimulated splenocytes (23).

While models are characteristically defined as giving rise to aGVHD or cGVHD, in practice, it is not always possible to clearly distinguish the two pathologies. Indeed, donor T cell dose, donor/recipient strain combinations, and environmental conditions dictate the extent to which the recipient experiences aGVHD and/or cGVHD. Thus, early mortality (in the first 2 weeks) after BMT with high T cell doses is typically a result of CD4-dependent aGVHD of the gastrointestinal (GI) tract (1), whereas disease features typ-

ical of cGVHD occur 4–8 weeks after BMT with low T cell doses that avoid early mortality and cause chronic T cell stimulation and subsequent antibody production (23–28). We believe that clinically relevant cGVHD is not unique to models of GVHD in response to minor histocompatibility antigens but rather reflects the nature of the immune response in the model, e.g., CD4 versus CD8 T cells and their respective differentiation and cytokine/chemokine expression patterns. Nevertheless, as in clinical practice, features of aGVHD in the GI tract and liver and cGVHD in skin and lung often coexist, reflected by the current NIH criteria (5). Clinical cGVHD can affect almost any target tissue, making modeling in preclinical systems challenging. Nevertheless, the predominant and diagnostic organ pathologies that develop are usually scleroderma or BO, although, for potentially important reasons that are yet to be defined, disease seldom coexists in these two organs within animal systems. Additional features of cGVHD have been described in the tongue, salivary and lacrimal glands, and eye; such pathology requires histological confirmation and grading in severity (29–31). Pulmonary function tests are also particularly informative for determining the severity of BO; however, these tests are technically challenging and available in a limited number of laboratories (27, 28). To date, reliable models and functional determinants of manifestations of cGVHD such as Sjögren's syndrome are generally lacking and represent an important unmet need in the field.

aGVHD: setting the stage for cGVHD

As noted, aGVHD is often a portent of cGVHD, suggesting that the pathophysiology of the two processes is linked. Recent lessons from the translation of preclinical approaches to prevent GVHD, predominantly post-transplant cyclophosphamide (32), which depletes alloreactive T cells while sparing Tregs, and selective ex vivo naive T cell depletion (33), have resulted in dramatic reductions in cGVHD, but not aGVHD (34, 35). These data indicate that the expansion and differentiation of naive T cells in the donor graft are central to cGVHD pathogenesis. The initial stages of T cell differentiation occur in an inflammatory and lymphopenic environment, a result of the chemoradiotherapy used in conditioning (36). Thus, elevated levels of proinflammatory cytokines, particularly IL-6 and to a lesser extent IL-1 and TNF (37), act in concert with products of luminal damage-associated and pathogen-associated molecular patterns to modify and augment both alloantigen presentation and T cell differentiation (38, 39). While this inflammatory storm favors differentiation of IFN- γ -secreting CD4⁺ and CD8⁺ T cells (Th1 and Tc1, respectively) and IL-17-secreting CD4⁺ and CD8⁺ T cells (Th17 and Tc17, respectively) (Figure 1), and resultant target organ apoptosis (36, 40–43), in the clinic this process is highly modified by pharmacological immune suppression (37). Thus, the process is modulated and can be delayed relative to that seen in preclinical systems where pharmacological immune-suppressive agents are seldom given after BMT (44). Indeed, the intense early post-BMT TNF/IL-1/IFN- γ dysregulation seen in animal systems is not fully recapitulated in the clinic; therefore it is possible that murine systems may overestimate the Th1/Tc1 dominance of aGVHD.

Although aGVHD and cGVHD impair donor B cell differentiation in the BM and aGVHD causes peripheral B cell depletion (1,

45–47), it is also now clear that aberrant B cell expansion is a feature of cGVHD (28, 29). In addition to B cell depletion, the thymus is a primary target of aGVHD (48), setting the scene for aberrant T cell selection and differentiation later after BMT (30). In cGVHD it is therefore possible that donor T cells may be both auto- and alloreactive (25); indeed, T cells from animals with cGVHD can induce disease in syngeneic recipients (30).

Adaptive immunity: cytokine-dependent T and B cell differentiation

IL-6 and Th17/Tc17. T cell differentiation after BMT is characterized by a number of pathogenic and protective paradigms. Unlike other proinflammatory cytokines, IL-6 dysregulation after BMT occurs in response to conditioning and is largely independent of immune suppression (37, 44). IL-6 is a pleiotropic cytokine that can be produced by most cells; production by monocytes and macrophages dominates (49). The cytokine signals through a trimer receptor complex that involves IL-6R and gp130 (50). IL-6R expression is relatively limited in distribution to some T cells, monocyte-macrophages, and hepatocytes, while gp130 is ubiquitously expressed (49). Classical IL-6 signaling through this receptor complex results in phosphorylation of STAT3, which is critical for the generation of cGVHD (51, 52). In T cells, this pathway drives expression of the transcription factor (TF) RAR-related orphan receptor- γ (ROR γ t) and the generation of cytokine gene products characteristic of Th17/Tc17 differentiation (41, 53). This differentiation pattern appears to be augmented by stem cell mobilization with G-CSF, which provides a link between cGVHD predilection and G-PBSC grafts (23). Recently, the use of cell fate-reporter systems has made it clear that Th17/Tc17 differentiation after BMT is highly promiscuous and plastic in nature (41). In particular, IL-17 secretion is transient, and, despite continuously elevated ROR γ T expression, the dominant cytokine signature over time is IFN- γ and TNF (Th1 cytokines). Importantly, the Tc17 lineage appears to have limited capacity for cytotoxicity with low levels of granzyme B production and minimal capacity to mediate graft-versus-leukemia effects (41, 54). Tc17 also express high levels of T-bet and, over time, after both preclinical and clinical BMT (37), exhibit concurrent dysregulation of both IFN- γ and IL-17, consistent with a role for this lineage in cGVHD. Indeed both scleroderma and BO fail to develop in animals in the absence of IL-17A and/or RORC (23, 24), consistent with a central role for this pathway in disease.

In the clinic, the nature of cells infiltrating target organs remains poorly defined, likely reflecting differences over time and within individual organs. Nevertheless, Th1/Tc1, Th17/Tc17, and ROR γ T are all seen at cGVHD sites (55–57), as well as STAT3 phosphorylation, which has been suggested to predict GVHD (37, 57, 58). Recently, nonhuman primate systems demonstrated the dominance of the Th17/Tc17 differentiation pattern in animals developing GVHD that breaks through immune suppression (19), akin to the breakthrough observed in patients. A number of studies have demonstrated the importance of IFN- γ in sclerodermatous cGVHD, with or without IL-17A (59–62). In contrast, a protective role for Th1 cytokines has been described in lung disease, including BO (42, 44, 63). Other Th17/Tc17 cytokines include granulocyte-macrophage CSF (GM-CSF) and CSF-1, both of which play critical roles in monocyte-macrophage biology (64, 65) (their role

Table 1. Open clinical trials for systemic treatment of chronic GVHD (accessed October 30, 2016)

Treatment	Mechanism	Design	Sponsor	Country	ClinicalTrials.gov identifier
Signaling targets					
Carfilzomib	Proteasome inhibitor	MC	Fred Hutchinson Cancer Research Center	USA	NCT02491359
Ixazomib	Proteasome inhibitor	MC	Fred Hutchinson Cancer Research Center	USA	NCT02513498
Baricitinib	JAK1/2 inhibitor	SC	National Cancer Institute	USA	NCT02759731
Ibrutinib ^a	BTK/ITK inhibitor	MC	Pharmacyclics	USA	NCT02195869
Fostamatinib	Syk inhibitor	SC	Duke University	USA	NCT02611063
Entospletinib	Syk inhibitor	MC	Gilead Sciences	International	NCT02701634
KD025	Rho-associated coiled-coil kinase 2 (ROCK2) inhibitor	MC	Kadmon Corp.	USA	NCT02841995
Vismodegib	Hedgehog inhibitor	MC	University of Utah	USA	NCT02337517
LDE225	Hedgehog inhibitor	MC	Massachusetts General Hospital	USA	NCT02086513
Suppressor cell-based therapies					
Dose-escalated IL-2	Induction of Tregs	SC	Dana-Farber Cancer Institute	USA	NCT02318082
IL-2 + Tregs	Induction of Tregs	SC	Dana-Farber Cancer Institute	USA	NCT01937468
IL-2 + extracorporeal photopheresis	Induction of Tregs	SC	Dana-Farber Cancer Institute	USA	NCT02340676
Tregs	Infusion of Tregs	MC	Instituto de Medicina Molecular	Portugal	NCT02385019
	Infusion of Tregs	MC	University Hospital of Liege	Belgium	NCT01903473
	Infusion of Tregs	SC	Stanford University	USA	NCT01911039
Mesenchymal stem cells (MSCs)	Suppressive population	SC	Emory University	USA	NCT02359929
	Suppressive population	MC	Guangdong General Hospital	China	NCT02291770
	Suppressive population	MC	Nanfeng Hospital of Southern Medical University	China	NCT01765660
	Suppressive population	MC	Chinese Academy of Medical Sciences	China	NCT01526850
UCB MSCs	Suppressive population	SC	Samsung Medical Center	South Korea	NCT01549665
DCs	Suppressive population	SC	Washington University	USA	NCT02611180
Photodynamic ex vivo cell depletion and repetitive reinfusion	Depleting alloreactive T cells and sparing Tregs	SC	Maisonneuve-Rosemont Hospital	Canada	NCT02519816
Other chronic GVHD targets					
Ofatumumab	Anti-CD20 antibody	MC	H. Lee Moffitt Cancer Center	USA	NCT01680965
Abatacept	CTLA4-Ig fusion protein	MC	Beth Israel Deaconess Medical Center	USA	NCT01954979
Ponesimod	S1P1 receptor modulator	MC	Actelion	USA	NCT02461134
Pomalidomide	Multiple mechanisms	SC	National Cancer Institute	USA	NCT01688466
Brentuximab	CD30 antibody-drug conjugate	SC	Massachusetts General Hospital	USA	NCT01940796
AZD9668	Neutrophil elastase inhibitor	SC	National Cancer Institute	USA	NCT02669251
Hydrogen-rich water	Antiinflammatory	SC	Navy General Hospital	China	NCT02918188

^aActive but not recruiting. SC, single center; MC, multicenter; UCB, umbilical cord blood.

in cGVHD is described below), and IL-22, a cytokine known to be important in the protection of the GI tract from GVHD when secreted by recipient innate lymphoid cells (ILCs) (66, 67). Whether IL-22 within the GI tract is from ILCs or from Th17 or a divergent Th22 lineage has yet to be determined. IL-22 also has proinflammatory properties (68, 69), and a pathogenic role of donor T cell-secreted IL-22 has been described in GVHD. This function is related, at least in part, to the suppression of Tregs and host type I IFN/STAT1 signaling (70, 71). Ongoing clinical trials of exogenous IL-22 IgG2-Fc fusion protein to treat patients with lower-GI aGVHD (NCT02406651, ClinicalTrials.gov) will determine the extent to which the intestinal reparative effects might outweigh potential pathogenic proinflammatory effects of IL-22.

The inhibition of IL-6 has shown impressive protection from aGVHD in preclinical models in association with inhibition of Th17 differentiation, Treg expansion (72), and direct inhibition

of target cell apoptosis (73). Promising clinical data have also suggested an effect in patients, with low incidence of aGVHD seen in those receiving IL-6R inhibition for 3–4 weeks after BMT. In this setting, protection was associated with modification of myeloid and T cell responses (37). Importantly, short-term IL-6 inhibition did not seem to influence the development of cGVHD, suggesting that long-term IL-6 inhibition would be required to prevent cGVHD. Alternatively, these findings indicate that IL-17 differentiation late after BMT is independent of IL-6 (see below).

IL-21 and Tfh. The differentiation of Tfh is defined by the BCL6 TF and surface expression of CXCR5 and programmed death-1 (PD1) (74). Tfh cells express high levels of IL-21, which drives germinal center (GC) B cell formation and antibody secretion (28, 74). While autoantibodies are widely seen in patients with cGVHD, their role (i.e., cause versus effect) in disease remains unclear. In contrast, alloantibody generation in conjunction with

GC B cell expansion has been observed in cGVHD models (28, 29, 75). Inhibition of IL-21/IL-21R signaling prevented GC expansion and alloantibody formation, as well as disease (23, 28). Furthermore, serum transfer experiments confirmed the ability of alloantibody to induce cGVHD (76), and H-Y alloantibodies correlated with disease in clinical sex-mismatched donor/recipient pairs (77, 78). Whether alloantibody is present in all patients and involved in disease universally is currently unclear. Likewise, whether the major pathogenic sources of IL-21 are Tfh, Th17, or a recently described IL-6-dependent CD8⁺ T cell lineage also is unclear (79).

B cells. B cell generation is disrupted during GVHD, leading to elevations in immature and transitional B cells and defects in memory populations, which exhibit enhanced sensitivity to B cell activating factor (BAFF) (80, 81). BAFF is produced primarily by myeloid cells, stromal cells, and some lymphoid cells and is a transmembrane protein that can be cleaved into a soluble form. The BAFF receptor (BAFFR) is expressed by B cells and plasma cells and determines immature B cell survival and maturation (82). BAFF/B cell ratios are elevated in patients with active cGVHD (83–85), and this elevation is associated with alterations in GC B cells and the presence of plasma cell-like populations, consistent with a state of B cell hyperactivation (84). Given that inhibitors of this pathway are in clinical use, it is important to study this pathway in depth in relevant preclinical models. Moreover, since plasmablasts and plasma cells produce copious amounts of antibodies, strategies described below to target these cell populations may be useful in overcoming BAFF-mediated signaling events.

IL-2 and Tregs. The differentiation of T cell lineages with regulatory capacity has been widely described and has focused principally on FoxP3⁺ Tregs (86), but also more recently on FoxP3-IL-10⁺ Treg type 1 (Tr1) cells (87). Tregs may be generated centrally in the thymus (tTregs) or induced peripherally (iTregs) under the influence of TGF- β and antigen presentation within MHC class II. FoxP3 expression appears to be much more stable in tTregs, but both Treg subtypes are characterized by expression of the high-affinity IL-2 receptor CD25 (88). Thymic Treg production (26, 30) and peripheral Treg homeostasis are perturbed, and Treg numbers are reduced in cGVHD (89, 90). In preclinical BMT models, Treg depletion induces cGVHD, and adoptive Treg transfer can ameliorate disease (25, 91). Donor DCs are essential for maintaining donor Treg homeostasis after BMT (25, 92). Importantly, aGVHD profoundly impairs antigen presentation within MHC class II (93), providing a causal link between aGVHD and the development of Treg deficiency and subsequent cGVHD (25).

A number of elegant studies have demonstrated that exogenous IL-2 administration can enhance Treg numbers and induce responses in about half of patients with steroid-refractory cGVHD (94–97). It appears that ongoing treatment is necessary to maintain responses (96), yet it is currently unclear whether adoptive Treg transfer, with or without IL-2, can further improve response rates, which will be clarified by several ongoing worldwide clinical trials (Table 1). The ability of alternative cytokines (either long-acting IL-2 mutants or other common γ chain cytokines) to enhance regulatory responses remains a burgeoning area of interest.

IL-10. The role of the Tr1 lineage in cGVHD is presently unclear, but these cells are known to regulate immune responses via IL-10, which is an important mechanism by which Tregs and B cells mod-

ulate GVHD (98–100). Whether the regulatory effects of IL-10 are principally from myeloid cells (101), Tr1 or Treg populations (102, 103), or so-called regulatory B cells (104, 105) is unclear and deserves further study. IL-10 signals through IL-10R, which is principally expressed on immune cells, and can promote Treg differentiation and licensing, regulate TNF secretion, and inhibit effector T cell proliferation, most likely owing to inhibitory effects on antigen-presenting cells (reviewed in ref. 106). IL-10 is also induced by nonhematopoietic cells following G-CSF-mediated stem cell mobilization (107, 108) and enhances Treg expansion early after BMT (109). Thus, IL-17 induction and IL-10 induction appear to be divergent mechanistic pathways by which the transplantation of G-PBSCs results in a profound augmentation of cGVHD but has relatively little effect on the incidence of aGVHD. Because IL-10R is also expressed on Th17 (110) and CD8⁺ T cells (111), and high-dose exogenous IL-10 (112), in striking contrast to low doses (113), can drive rather than protect mice against aGVHD lethality, it is possible that during aGVHD the immunoregulatory properties of IL-10 in some patients may be offset by effects on Th17 and CD8⁺ cytotoxic T cells. Although progress has been made on Tr1 characteristic cell surface antigens (114), investigation of Tr1 in this process is currently limited by a lack of pivotal TFs required for lineage development and/or stable surface phenotypes. These are needed to analyze the role of these cells in the BMT setting in order to optimize the potential therapeutic benefits of IL-10 (115, 116).

Cytokine-driven effector pathways in cGVHD

CSF-1 and macrophages. CSF-1 is the master regulator of the cells of the mononuclear phagocytic system. CSF-1 signaling controls the differentiation (117), proliferation (118), migration and survival (119) of tissue-resident macrophages and their precursors (120), and contributes to DC homeostasis (121). The CSF-1 receptor (CSF-1R), a class III RTK belonging to the PDGF family, is expressed at high levels on monocytes and macrophages. CSF-1R is also broadly expressed at low levels on multiple lineages, including hematopoietic (122) and neural stem cells (123), DCs, microglia (124), osteoclasts (125), and Paneth cells (126). As such, CSF-1 signaling contributes to embryonic development, homeostasis, innate and acquired immunity, and tissue repair. Consistent with these roles, impaired CSF-1 signaling is implicated in multiple disease states.

In macrophages, CSF-1R ligation leads to the autophosphorylation of the intracellular tyrosine residues and subsequent phosphorylation of several kinase signaling systems, including PI3K (127), MEK, and the Tek family kinases Tec and Bruton's tyrosine kinase (BTK) (128). Other components of the CSF-1 signaling pathways include SHIP1, ERK1/2, AKT, p38, JNK, and ERK5, which together regulate diverse downstream functional decisions, including proliferation, differentiation, and survival (reviewed in ref. 129 and shown in Figure 2).

Although some studies have shown that the pretransplant conditioning regimen leads to the release of inflammatory cytokines by host macrophages (36), others have demonstrated that host macrophages that persist after BMT can reduce GVHD by engulfing donor T cells (120, 130, 131) and pre-BMT CSF-1 can expand macrophages, thereby suppressing aGVHD lethality (131). In cGVHD, accumulating evidence demonstrates a critical role for CSF-1-dependent monocytes and macrophages in fibrogenesis. In most

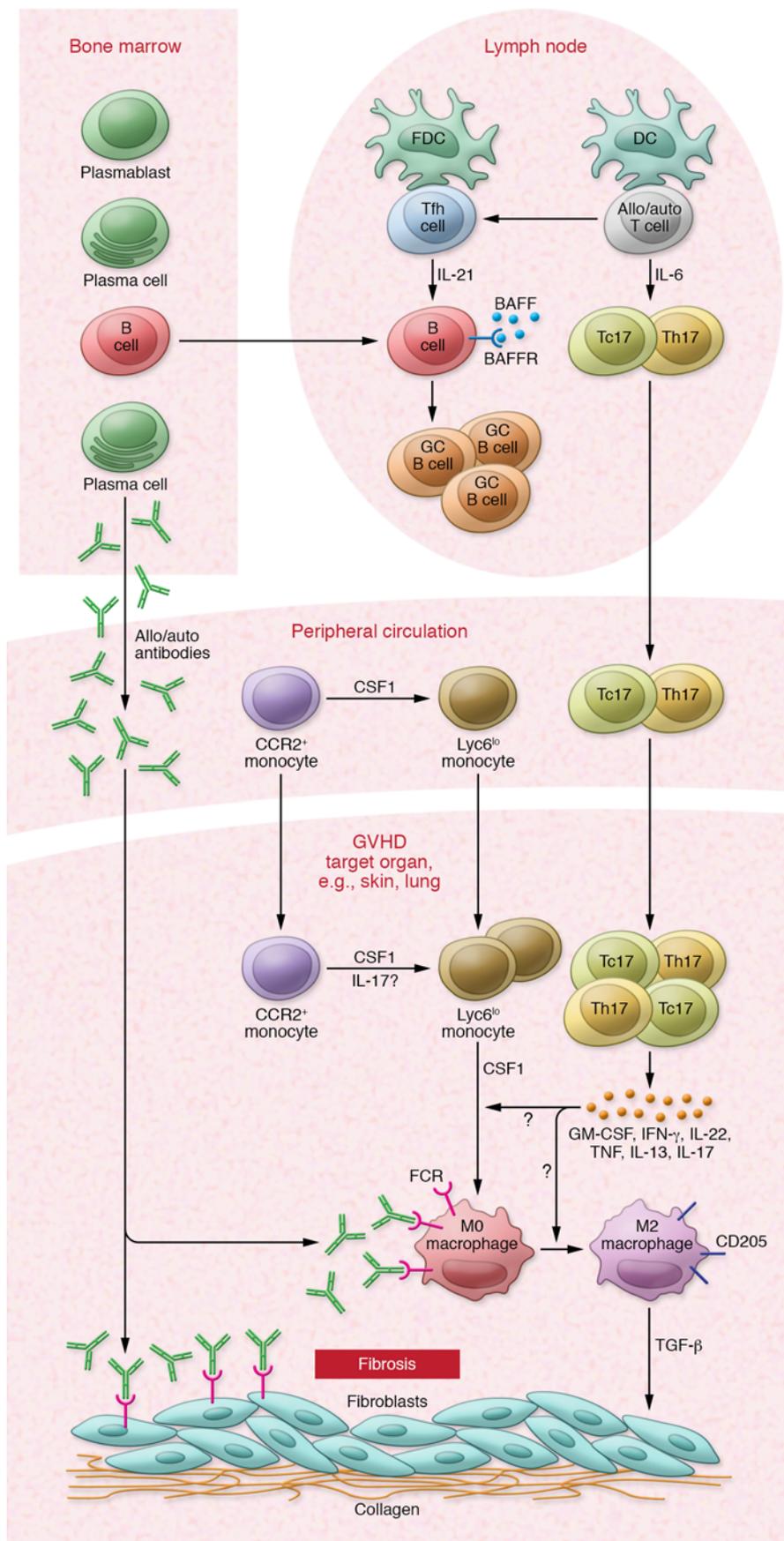


Figure 2. Overview of the cytokine-driven cellular network driving cGVHD. cGVHD is now recognized as a Th17/Tc17- and Tfh-mediated disease. DC priming of naive allogeneic T cells in the setting of elevated IL-6 production after BMT drives ROR γ t expression and Th17/Tc17 differentiation and accumulation in target tissues. Increased Tfh, GC B cells, and antibody, which accumulate in target tissues, contribute to cGVHD pathology. Tfh express high levels of IL-21, which, together with elevated levels of BAFF, drive GC B cell formation and antibody secretion. CSF-1-dependent macrophages are late-phase mediators of cGVHD pathology. Th17/Tc17 are polyfunctional and coexpress high levels of multiple cytokines, including IFN- γ , TNF, IL-22, CSF-1, and GM-CSF, which are implicated in promoting the migration and differentiation of Ly6C^{lo} monocytes into pathogenic M2 macrophages. Plasma cell-derived allo- and autoantibodies can bind to Fc receptors on macrophages, potentially contributing to their polarization. Through their secretion of TGF- β , Ly6C^{lo}-derived macrophages promote fibroblast activation and collagen production, leading to tissue fibrosis. Adapted with permission from *Blood* (11).

fibrotic tissues, including clinical cGVHD lesions, macrophages are abundant and are found in close proximity to collagen-producing myofibroblasts (55, 132). In multiple preclinical models of cGVHD, macrophage sequestration into GVHD target organs has been shown to be both IL-17- and CSF-1-dependent (23, 24). Critically, the disruption of CSF-1 signaling following BMT using an anti-CSF-1R mAb (M279) depleted tissue macrophages and attenuated cGVHD-associated cutaneous and pulmonary fibrosis. Importantly, CSF-1R blockade also specifically ablated Ly6C^{lo} monocytes, the established tissue-resident macrophage precursors (120). The infiltrating macrophages, which were of donor origin, exhibited an antiinflammatory M2-skewed phenotype, and were capable of promoting fibrosis via their secretion of TGF- β and possibly other profibrotic proteins (24).

IL-17 may contribute directly to myeloid cell sequestration and differentiation, as both monocytes and macrophages express high levels of the IL-17A receptor (133) and IL-17 signaling in these cells elicits multiple functions including chemotaxis and activation (134, 135). However, Tc17, which are implicated in mediating macrophage sequestration, also express other proinflammatory cytokines, including GM-CSF (41), which may contribute syner-

gistically to macrophage differentiation/polarization at localized sites. The factors driving aberrant macrophage differentiation and the potential role of alloantibody in this process are unclear but require investigation. Notably, Tec and BTK are essential regulators of macrophage CSF-1 signaling (128), contributing to both survival and GM-CSFR α expression, thus representing a therapeutically targetable downstream pathway.

TGF- β . The TGF- β family of growth factors controls proliferation, differentiation, and survival in many cell types. TGF- β is secreted by cells of multiple lineages, including cells of mesenchymal (136) and hematopoietic (137) origin. The relative contribution of TGF- β isoforms appears to be contextual, with TGF- β 1 as the predominant isoform in the immune system (138). TGF- β is secreted in latent form and is unable to engage its receptors (139). The proteolytic degradation of the latent peptides renders TGF- β biologically active. Once activated, signaling is elicited through an oligomeric complex composed of type I and type II serine/threonine kinase receptors. TGF- β first binds to the constitutively phosphorylated TGF-RII, which in turn binds to and phosphorylates TGF-RI, leading to the activation of the cytoplasmic signaling molecules Smad2 and Smad3 (140, 141), which translocate to the nucleus to regulate target gene expression. Additionally, other noncanonical TGF- β -activated pathways, including MAP kinase, Rho-like GTPase, and PI3K/AKT pathways, collectively contribute to signaling outcomes (142). TGF- β signaling mediates diverse biological responses and plays a role in tissue regeneration (143) and the maintenance of immune tolerance (138). Aberrant TGF- β expression and signaling are implicated in the profound immune dysregulation that occurs after BMT and in cGVHD-associated fibrotic manifestations (144–147).

Following injury, TGF- β signaling promotes both the production and degradation of various extracellular matrix proteins and is therefore instrumental in tissue repair (143). In all tissues, fibroblasts are the primary producers of extracellular matrix. During injury, TGF- β signaling promotes fibroblast migration, proliferation, and differentiation into α -smooth muscle actin-expressing (α -SMA-expressing) myofibroblasts, which produce large amounts of collagen. Notably, profibrogenic PDGF synergizes with TGF- β , resulting in augmented expression of both α -SMA and collagen by myofibroblasts (148). In preclinical models and patients, cGVHD is associated with elevated TGF- β levels in the serum and target organs, and TGF- β is negatively correlated with survival (144, 147, 149, 150). Furthermore, cGVHD patients harbor elevated levels of agonistic PDGFR antibodies, although whether these are involved in disease pathogenesis remains contentious (151, 152). Myeloid lineages that accumulate within cGVHD target organs are the primary TGF- β -producing populations (24, 146). Importantly, TGF- β neutralization effectively prevents both skin and lung fibrosis in murine models (2, 146). Although TGF- β blockade has yet to be investigated clinically, dual blockade of TGF- β and PDGFR pathways using tyrosine kinase inhibitors has shown promising results in patients with steroid-refractory cGVHD (153, 154).

TGF- β is also a key immunoregulatory cytokine that contributes to the maintenance of peripheral tolerance, as evidenced by the multiorgan inflammation and autoimmunity that occur in TGF- β cytokine- or receptor-deficient mice (138, 155). TGF- β mediates immunosuppression through inhibition of lymphocyte

proliferation, differentiation, and effector function. T cell-intrinsic TGF- β signaling dampens T cell expansion via multiple mechanisms, including the suppression of IL-2 production and promotion of apoptosis (156, 157). TGF- β also potently inhibits Th1 and Th2 differentiation via downregulation of the TFs T-bet and GATA3 (158, 159). In concert with IL-2, TGF- β promotes the conversion of naive CD4⁺ T cells into FoxP3-expressing iTregs. Consistent with the importance of this cytokine in the generation of and regulation by Tregs (160), TGF- β inhibition early after BMT promotes aGVHD (146). Thus, following BMT, TGF- β signaling elicits both protective antiinflammatory, immunosuppressive, and pathogenic profibrogenic responses in a temporal manner, indicating that carefully timed therapeutic targeting of this pathway will be required. Alternately, targeting the cellular source of TGF- β in inflamed tissues or specific downstream signaling pathways may provide a means of selective inhibition of some but not all of the cytokine responses; the use of canonical and noncanonical signaling pathways supports the feasibility of the latter approach. For example, although TGF- β signaling is critical for tTreg generation, Smad2 and Smad3 are not required (161), suggesting that targeting these signaling components could preferentially diminish fibrogenic responses while sparing Tregs.

Pharmacological therapeutics that target cytokine signaling pathways

Steroids continue to be the mainstay of first-line cGVHD therapy; however, steroids are associated with substantial morbidity and mortality, especially when given long-term as is typically required for cGVHD patients. Guided by both rodent studies and clinical biomarker analyses, new cGVHD-targeting pathways have been elucidated, leading to new pharmacological and cellular approaches to treat this disease. A particularly successful approach has been to reuse drugs known to have validated immunological and antiinflammatory properties as well as an acceptable safety profile in inflammatory disease. Similarly, cellular therapies useful in aGVHD are currently being tested in cGVHD. A tabular summary of active trials in cGVHD is provided in Table 1. Those and related completed trials involving pharmacological agents directly regulating cytokine production or responses will be discussed below.

Bortezomib (Velcade) is the first therapeutic proteasomal inhibitor to be tested in humans for treatment of multiple myeloma. Studies in murine aGVHD indicated that bortezomib and a related compound promoted alloantigen-specific T cell deletion and inhibited proinflammatory responses associated with NF- κ B upregulation (162, 163). In a sclerodermatous minor histocompatibility antigen-mismatched model of cGVHD, bortezomib reduced serum and skin IL-6 levels and proved efficacious in treating skin manifestations of cGVHD in patients in preliminary studies (164). A phase II study of bortezomib combined with prednisone resulted in organ-specific complete response rates of 73% for skin, 53% for liver, 75% for the GI tract, and 33% for diseased joint, muscle, or fascia, and permitted a 60% median prednisone dose reduction by week 15 (165). Carfilzomib, a peptidylepoxyketone, and ixazomib, a peptide analog, both inhibit the 20S proteasomal subunit β type 5 and are being tested for efficacy in cGVHD therapy (Table 1). Ruxolitinib, which is approved for the treatment of myelofibrosis, and baricitinib are selective JAK1/2 inhibitors. JAK1/2 signal-

ing regulates T cell activation and survival through the IL-2 common chain, a constituent of the receptor complexes for IL-2, IL-4, IL-7, IL-9, IL-15, and IL-21; targeting JAK1/2 signaling ameliorated murine aGVHD and cGVHD (166). In a preclinical antibody-mediated, multiorgan system model of cGVHD that includes BO, ruxolitinib therapy reversed active cGVHD manifestations (167). In 41 patients with steroid-resistant cGVHD, the overall response rate to ruxolitinib was 85% (167).

Ibrutinib targets B cell malignancies by inhibiting BTK and IL-2-inducible tyrosine kinase (ITK). Ibrutinib has been approved to treat various types of lymphoid malignancies and is known to inhibit malignant B cell responses to soluble factors in the tumor environment, including BAFF, IL-6, IL-4, and TNF- α . In a preclinical sclerodermatous cGVHD model, ibrutinib delayed progression, improved survival, and ameliorated cGVHD manifestations. In the antibody-driven cGVHD model, ibrutinib treatment restored pulmonary function and reduced GC reactions and tissue immunoglobulin deposition; using knockout donor cells, it was established that both BTK and ITK were critical for cGVHD development (27). Moreover, ibrutinib treatment reduced activation of T and B cells from patients with active cGVHD. Based on positive phase Ib/II data, ibrutinib is entering phase III trials for treatment of steroid-dependent or refractory cGVHD (Table 1).

Spleen tyrosine kinase (Syk) is an enzyme that regulates T and B cell signaling pathways and has been implicated in hematological malignancies, including those with *ITK* translocations. Fostamatinib is a prodrug inhibitor and entospletinib is an ATP-competitive inhibitor of Syk. In the BO model, Syk was necessary in donor B cells but not donor T cells for disease progression, and fostamatinib treatment reversed disease in both nonsclerodermatous and several sclerodermatous models (168). Syk upregulation was observed in B cells from cGVHD mice and patients, and Syk inhibition in vitro effectively induced apoptosis of human cGVHD B cells. Syk inhibitors are currently in both phase I and phase II double-blind randomized trials for treatment of cGVHD (Table 1).

Rho-associated coiled-coil kinase 2 (ROCK2) has been implicated in IL-21 and IL-17 regulation. Treatment with the selective ROCK2 inhibitor KDO25 ameliorated cGVHD in multiple models. In an antibody-mediated cGVHD model, spleens of KDO25-treated mice had decreased frequency of Tfh cells and increased frequency of T follicular regulatory cells along with both increased phospho-STAT3 and decreased phospho-STAT5 (52). In cGVHD patients with active disease, KDO25 inhibited IL-21, IL-17, and IFN- γ secretion and phospho-STAT3. KDO25 has entered phase IIa clinical trials for cGVHD therapy (Table 1).

Sonic hedgehog and its receptor patched are expressed on resting and activated human peripheral CD4⁺ T cells (169). In the absence of hedgehog proteins, patched-1 inhibits the coreceptor smoothed (Smo). Smo induces the TFs Gli-1 and Gli-2, which

activate the *SOCS1* promoter. Thus, Smo antagonists mimic hedgehog ligand absence and lead to reduced *SOCS1* promoter activity and increases in IFN- γ and phospho-STAT1. Hedgehog signaling is activated in human and murine cGVHD (170). Treatment with LDE223, a highly selective small-molecule Smo antagonist, almost completely prevented sclerodermatous cGVHD development and was useful in cGVHD therapy (170). Vismodegib and LDE225 are Smo antagonists being tested in steroid-refractory chronic GVHD patients in ongoing studies. The direct targeting of a number of cytokines is now possible, and inhibition of IL-21, IL-17A, CSF-1R, or TGF- β appears to be a logical therapeutic strategy to move forward into well-designed phase I/II clinical trials.

Summary

Our understanding of the pathophysiology of cGVHD has dramatically improved over the last five years, as has our ability to undertake informative clinical trials in this setting. We are now in an exciting period in which a number of new and established therapeutics that focus on the elimination of the aberrant cytokine responses that drive fibrosis can be rapidly tested within the constraints of well-designed clinical trials to both prevent and treat cGVHD.

Acknowledgments

We thank members of our laboratories, our collaborators, and the scientific community for providing the foundation for this Review. We apologize to those investigators whose work we were unable to cite because of space restrictions. Importantly, we thank the patients who have participated in clinical studies that have fostered the advancement of the new therapies for this devastating disease. This work was supported by Australian National Health and Medical Research Council (NH&MRC) grant APP1031728 (to KPAM); and by National Cancer Institute grants P01-CA142106-06A1 and P01-CA047741-20, National Institute of Allergy and Infectious Diseases grants P01-AI056299 and R01-AI11879, and Leukemia and Lymphoma Society Translational Research grants 6458-15 and 6462-15 (to BRB). GRH is an NH&MRC Senior Principal Research Fellow and Queensland Health Senior Clinical Research Fellow.

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