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Review

Chronic Graft-versus-Host Disease: Disease Biology and Novel Therapeutic Strategies

Hisakazu Nishimori[a,b]*§, Yoshinobu Maeda[a], and Mitsune Tanimoto[a]

[a]Department of Hematology and Oncology, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, and [b]Center for Clinical Oncology, Okayama University Hospital, Okayama 700–8558, Japan

Graft-versus-host disease (GVHD) is a major complication after allogeneic hematopoietic stem cell transplantation. Chronic GVHD often presents with clinical manifestations that resemble those observed in autoimmune diseases. Standard treatment is 1–2 mg/kg/day of prednisone or an equivalent dose of methylprednisolone, with continued administration of a calcineurin inhibitor for steroid sparing. However, the prognosis of steroid-refractory chronic GVHD remains poor. Classically, chronic GVHD was said to involve predominantly Th2 responses. We are now faced with a more complex picture, involving possible roles for thymic dysfunction, transforming growth factor-β (TGF-β) and platelet-derived growth factor (PDGF), B cells and autoantibodies, and Th1/Th2/Th17 cytokines, as well as regulatory T cells (Tregs), in chronic GVHD. More detailed research on the pathophysiology of chronic GVHD may facilitate the establishment of novel strategies for its prevention and treatment.

Key words: chronic GVHD, Th17, Am80, regulatory T cell (Treg), steroid-refractory

Allogeneic hematopoietic stem cell transplantation (HSCT) is a curative modality in a substantial number of patients with hematological malignancies, bone marrow failure, immunodeficiency syndromes, and certain congenital metabolic disorders [1]. However, allogeneic HSCT is frequently complicated by graft-versus-host disease (GVHD). Based on differences in clinical manifestations and histopathology, GVHD can be divided into acute and chronic types.

The clinical manifestations of acute GVHD occur in the skin, gastrointestinal tract, and liver. Several convergent lines of experimental data have demonstrated that donor T cells and donor and/or host antigen-presenting cells (APCs) are important in the induction of acute GVHD [2–6]. Additionally, a growing body of data suggests that donor T-cell subsets, such as T-helper (Th) cells, $CD8^+$ T cells [7, 8], natural killer (NK) cells [9], NKT cells [10], and γδT cells [11], are involved in the pathogenesis of acute GVHD.

Chronic GVHD is a major cause of late death and morbidity after allogeneic HSCT [12–14]. Although half of patients respond to first-line treatment, the prognosis of steroid-refractory chronic GVHD remains poor [15]. Initially, chronic GVHD was considered to be a Th2-mediated disease, based on results from the non-irradiated parent→F_1 mouse model. Chronic GVHD in this model is mediated by autoantibody production by host B cells stimulated by donor Th2 cells. Th1 polarization in donor T cells activates donor $CD8^+$ CTLs to kill host B cells, resulting in

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*Corresponding author. Phone:+81–86–235–7227; Fax:+81–86–232–8226
E-mail:n-mori@md.okayama-u.ac.jp (H. Nishimori)
§The winner of the 2011 Hayashibara Prize of the Okayama Medical Association.

amelioration of chronic GVHD [16]. However, chronic GVHD has not fit neatly into the Th2 paradigm [17]. Recent studies have suggested that chronic GVHD may be caused by cytokines secreted by Th1 cells [18], Th17 cells [19], and/or autoantibodies [20]. However, the immune mechanisms leading to the development of chronic GVHD are still not completely understood. Moreover, evidence in steroid-refractory chronic GVHD is limited.

In this review, we outline treatments for chronic GVHD and discuss the pathophysiology of chronic GVHD, focusing on five aspects: (a) thymic dysfunction, (b) profibrotic growth factors (transforming growth factor-β (TGF-β) and platelet-derived growth factor (PDGF)), (c) regulatory T cells (Tregs), (d) B cells and autoantibodies, and (e) Th1/Th2/Th17 cytokines. Finally, we present a new strategy for the treatment of chronic GVHD using the synthetic retinoid Am80, which targets Th1 and Th17.

Clinical Significance of Chronic GVHD

Chronic GVHD often presents with clinical manifestations that resemble those observed in autoimmune diseases, such as systemic lupus erythematosus, Sjögren's syndrome, lichen planus, and scleroderma [21]. Onset usually occurs more than 100 days after HSCT [22]. The pathophysiology of chronic GVHD is complex and resembles, to some degree, the pathophysiology of autoimmune diseases, since it involves donor-derived auto-reactive T cell responses to host alloantigens. The consensus is that mild chronic GVHD can be treated with topical immunosuppressive agents or with systemic steroids alone as a first-line therapy [23]. Treatment of moderate-to-severe chronic GVHD requires systemic immunosuppression. Standard treatment is 1–2 mg/kg/day prednisone or an equivalent dose of methylprednisolone with continued administration of a calcineurin inhibitor for steroid sparing [23]. The response rate to steroids is ~50–60%, but the prognosis of steroid-refractory chronic GVHD remains poor [24].

Numerous clinical trials have evaluated approaches to secondary treatment of chronic GVHD. To date, no consensus regarding the optimal choice of agents for secondary treatment has been reached, and clinical management is generally approached through empirical trial and error. Table 1 shows the reported data for the secondary treatment of chronic GVHD [25–34]. Response rates are 26–86%, but the studies providing these data were limited almost exclusively to phase II trials or retrospective analyses. Thus, treatment of steroid-refractory chronic GVHD remains a challenge.

Biology

Thymic dysfunction. Within the thymus, T cells undergo positive and negative selection. In negative selection, self-reactive T cells are eliminated, which is called "central tolerance." Positive selection is mediated by the thymic cortical epithelium, while negative selection, via clonal deletion, is mediated primarily by thymic dendritic cells (DCs). In the acute phase, donor-derived mature T cells expanding in a thymus-independent manner in recipients are responsible for the development of GVHD, because T-cell

Table 1 Response rates in prior second-line treatment for chronic GVHD

Author	[ref.]	(published year)	Treatment	n	RR (%)
Gilman et al.	[25]	(2000)	hydroxychloroquine	40	53
Browne et al.	[26]	(2000)	thalidomide	37	38
Akpek et al.	[27]	(2001)	steroid pulse	61	76
Flowers et al.	[28]	(2008)	ECP	48	40
Olivieri et al.	[29]	(2009)	imatinib	19	79
Furlong et al.	[30]	(2009)	MMF	42	26
Kim et al.	[31]	(2010)	rituximab	37	86
Jedlickova et al.	[32]	(2010)	mTOR inhibitor	19	74
Weng et al.	[33]	(2010)	MSCs	19	74
Pidala et al.	[34]	(2010)	pentostatin	18	56

ref, reference; n, patient number; RR, response rate; ECP, extracorporeal photopheresis; MMF, Mycophenolate mofetil; mTOR, mammallian target of rapamycin; MSCs, mesenchymal stem cells.

depletion of the donor bone marrow reduces rates of acute GVHD in mice and humans [35, 36]. However, in the late phase, T cells generated *de novo* from donor-derived hematopoietic stem cells via the recipient's thymus play an important role in chronic GVHD pathophysiology. Although peripheral T cells generated in the recipient's thymus should not attack self antigen-expressing tissues, they seem to include a minor population that is potentially harmful to recipients. Indeed, Sakoda *et al.* showed that impaired thymic negative selection of the recipients allowed the emergence of autoreactive T cells and caused chronic GVHD, even in the presence of functional Tregs, in a study using a thymectomized mouse model [37]. Keratinocyte growth factor (KGF) treatment improves the restoration of thymic DCs and prevents the *de novo* generation of pathogenic $CD4^+$ T cells causing chronic GVHD [38], suggesting that protection of the thymus may contribute to improvement in chronic GVHD. Although palifermin, a recombinant human KGF that may protect the host thymus, had no significant effect in acute GVHD [39], the efficacy of palifermin treatment for chronic GVHD has not been examined. Further experiments and clinical studies will be needed to assess the role of the thymus as a target of chronic GVHD treatment.

Contribution of TGF-β and PDGF pathways. TGF-β is a pleiotropic cytokine that affects multiple cell lineages by promoting or opposing their differentiation, survival, and proliferation. Increased total plasma TGF-β1 levels correlate well with the subsequent development of liver and lung fibrosis [40, 41]. Chronic GVHD is also characterized by fibrotic changes in the skin, and it is conceivable that TGF-β1 also plays a role. In a mouse model of chronic GVHD, TGF-β has been causally related to the development of sclerodermatous skin changes [42, 43]. In humans, TGF-β1 levels are increased significantly during chronic GVHD [44]. However, in gene expression analyses, donors whose recipient did not develop chronic GVHD showed higher levels of activating components of the TGF-β signaling pathway (*EP300*, *FNBP3*, *FURIN*, *SMAD3*) and of genes induced by TGF-β (*TGFBI*, *TGIF*) but lower expression of PRF1, which is repressed by TGF-β, compared with those who developed chronic GVHD [45]. Moreover, TGF-β plays an important role in the generation and maintenance of Tregs in the periphery and enhancement of their suppressive function [46]. Thus, the *in vivo* role of TGF-β in chronic GVHD could be complex.

Members of the platelet-derived growth factor (PDGF) family play important roles during embryonic development and contribute to the maintenance of connective tissue in adults [47]. Deregulation of PDGF signaling has been linked to atherosclerosis, pulmonary hypertension, and organ fibrosis. Stimulatory antibodies to the PDGF receptor (PDGFR) recognized native PDGFR, inducing tyrosine phosphorylation, reactive oxygen species accumulation, stimulation of type I collagen gene expression, and myofibroblast phenotype conversion in normal human primary fibroblasts, resulting in sclerosis [48]. Moreover, such stimulatory antibodies were found in all patients with scleroderma [48]. These reported findings suggest that acceleration of the PDGF pathway may result in autoimmune effects. Indeed, stimulatory antibodies to the PDGFR were found selectively in all patients with extensive chronic GVHD, but in none of those without the condition [49], suggesting that the PDGF pathway is associated with chronic GVHD pathogenesis.

The tyrosine kinase inhibitor imatinib mesylate, which inhibits the constitutively active fusion gene bcr-abl, is widely used in the treatment of Philadelphia chromosome-positive leukemia. Imatinib is also a promising candidate for the treatment of fibrotic diseases and it seems reasonable to suggest that imatinib may inhibit PDGF-stimulated fibrosis, and that if TGF-β–induced fibrosis is mediated through c-abl, imatinib may represent a single therapy capable of inhibiting the activity of both TGF-β and PDGF [50]. In fact, blockade of TGF-β and/or PDGF signaling by imatinib reduced the development of fibrosis in various experimental models [50, 51]. Recently, imatinib has been investigated for the treatment for steroid-refractory chronic GVHD; results suggested its effectiveness as a salvage treatment [29, 52]. Moreover, Nakasone *et al.* showed that the incidence and severity of chronic GVHD were reduced by prophylactic administration of imatinib after SCT [53]. Thus, targeting TGF-β and/or PDGF signaling may be a useful strategy for preventing or treating chronic GVHD.

Tregs. Tregs are a T-cell subset marked by a $CD4^+$ $CD25^{hi}$ $Foxp3^+$ phenotype, and constitute ~5–10% of peripheral $CD4^+$ T cells; they play an important role in peripheral tolerance [54]. Impairment of

Tregs is associated with loss of peripheral tolerance, autoimmunity, and chronic GVHD [55, 56]. After transplant, thymic generation of naïve Tregs in adult patients was markedly impaired, and the reconstituted Tregs had a predominantly activated/memory phenotype [1]. Recently, Matsuoka *et al.* investigated the reconstitution of Tregs and conventional T cells (Tcons) after myeloablative HSCT [57]. During the lymphopenic period after HSCT, Tregs underwent higher levels of proliferation than Tcons; Tregs expanded rapidly and achieved normal levels by 9 months after HSCT. However, this Treg expansion was counterbalanced by their increased susceptibility to Fas-mediated apoptosis [57]. In patients showing prolonged $CD4^+$ lymphopenia, the Treg pool declined preferentially, resulting in a prolonged imbalance between Tregs and Tcons, which was associated with a high incidence of extensive chronic GVHD [57]. These results indicate that $CD4^+$ lymphopenia is a key factor in Treg homeostasis, and that impaired reconstitution of Tregs can result in loss of tolerance and the development of chronic GVHD.

Adoptive transfer of Tregs and regulation to increase Tregs in recipients are considered to be effective clinical strategies for GVHD. In a mouse model, donor splenic Tregs were shown to prevent chronic GVHD with autoimmune manifestations [20]. In humans, Koreth *et al.* showed that low-dose IL-2, which is required for homeostatic maintenance of natural $CD25^+$ $CD4^+$ Treg cells [58], expands the Treg population, resulting in the amelioration of human chronic GVHD [59].

Donor immunity in allogeneic HSCT harnesses beneficial graft-versus-leukemia (GVL) effects; thus, allogeneic HSCT represents a potent form of immunotherapy for hematological malignancies [60, 61]. Unfortunately, GVL effects are also closely associated with GVHD [62]. There has been a decades-long struggle to enhance GVL while suppressing GVHD. As mentioned above, "Treg therapy" may be effective for GVHD, but the infusion of Tregs may potentially increase the risk of recurrent malignancy, because Tregs are a major concern in cancer immunology, where they have documented inhibitory activity on antitumor immunity. A study by Negrin *et al.* revealed that Tregs use distinct non-overlapping mechanisms to suppress GVHD and GVL effects [63]. This suggests that Tregs can distinguish GVHD from GVL activity. More experimental and clinical studies are warranted to establish the best methods of "Treg therapy" for chronic GVHD while preserving GVL effects.

Contribution of B cells or autoantibodies. B cells or autoantibodies may be involved in the pathophysiology of chronic GVHD. A strong correlation was identified between chronic GVHD and the presence of antibodies to Y chromosome-encoded histocompatibility antigens [64]. Elevated levels of B cell-activating factor (BAFF), which promotes survival and differentiation of activated B cells, have been observed in patients with chronic GVHD; furthermore, genetic variation in BAFF was also correlated with chronic GVHD [65, 66]. She *et al.* reported that the development of human chronic GVHD was associated with an increased number of B cells expressing high levels of Toll-like receptor (TLR) 9 [67].

The idea that B cells and autoantibodies contribute to chronic GVHD is also supported by the observation that *in vivo* depletion of B cells using rituximab can suppress the progression of complex chronic GVHD [68, 69]. Rituximab is a chimeric murine/human monoclonal antibody that binds specifically to the CD20 antigen, which is expressed almost exclusively on the surfaces of B lymphocytes [70, 71]. Cutler *et al.* reported a large series of steroid-refractory chronic GVHD patients treated with rituximab [69]. The clinical response rate was 70%, including 2 patients with complete responses; the clinical responses were limited to patients with cutaneous and musculoskeletal manifestations of chronic GVHD and were durable through 1 year after therapy [69].

The Th1/Th2/Th17 paradigm. Th1 and Th2 cells are distinguished most clearly by the cytokines they produce. Interferon-γ (IFN-γ) is the defining cytokine of Th1 cells, whereas IL-4, IL-5, and IL-13 are the signature cytokines produced by Th2 cells [72]. A third subset of $CD4^+$ effector cells was identified and named Th17 cells, because the signature cytokine they produce is IL-17 [73]. In acute GVHD, several groups have reported roles of Th1/2/17 cytokines in mouse models, but with inconsistent results [74–79]. These reports indicate that donor $CD4^+$ T cells can reciprocally differentiate into Th1, Th2, and Th17 cells that mediate organ-specific GVHD (Th1: gut and liver; Th2: lung and skin; Th17: gut and skin) [74, 78, 79].

We recently showed that Th1 and Th17 cells contribute to chronic GVHD using a MHC-compatible, minor histocompatibility antigen-incompatible mouse model of chronic GVHD [21]. Th1 and Th2 responses were up-regulated early after HSCT, followed by up-regulation of Th17 cells [21]. Significantly greater numbers of Th17 cells infiltrated into the lung and liver from allogeneic recipients than from syngeneic recipients [21]. Infusion of IFN-$\gamma^{-/-}$ or IL-17$^{-/-}$ donor T cells attenuated chronic GVHD in the skin and salivary glands [21], confirming that Th1 and Th17 contribute to the development of chronic GVHD. We also identified a population of donor-derived IFN-γ/IL-17 double-positive cells following only allogeneic HSCT, not syngeneic HSCT, suggesting that this population is generated by allogeneic stimulation, but is not due to lymphopenia-induced proliferation [21].

Recently, the Th17 cell spectrum has been shown to range from "classical" to "alternative" Th17 cells. Classical Th17 cells depend on TGF-β, are more regulated, and less pathogenic. In contrast, "alternative" Th17 cells depend on IL-23, are less regulated, and more pathogenic [80]. The accumulated evidence suggests that T-bet and IFN-γ expression by Th17 cells is dependent on IL-23, but is inhibited by TGF-β and is thus a characteristic of alternative rather than classical Th17 cells [80–84]. Further investigations will be needed to clarify the difference(s) in the functions of IL-17 single-positive and IFN-γ/IL-17 double-positive cells, taking into consideration both classical and alternative Th17 cells, in chronic GVHD pathogenesis.

Retinoids for the Treatment of Chronic GVHD

Retinoic acid, the active metabolite of vitamin A, exerts multiple effects on cell differentiation and survival by binding to retinoic acid receptors (RARs) and retinoid X receptors (RXRs) [85]. All-*trans*-retinoic acid (ATRA) has been reported to suppress the differentiation of Th17 cells with reciprocal induction of Tregs [86]. Am80, a novel RARα/β-specific synthetic retinoid, has a biological activity approximately 10 times more potent than that of ATRA, and directly inhibits Th1 cytokine production [87]. Thus, we hypothesized that retinoids would down-regulate both Th1 and Th17 differentiation in donor T cells, resulting in attenuation of chronic GVHD. Recipient mice were orally administered Am80 from day 0 of HSCT. We found that Am80 significantly ameliorated the clinical and pathological chronic GVHD score, compared with controls [21]. Additionally, peripheral lymph nodes from Am80-treated recipients produced significantly less Th1 and Th17 cytokines, confirming that Am80 regulated both Th1 and Th17 responses, resulting in the attenuation of chronic GVHD [21]. We also demonstrated that Am80 was effective in the treatment setting; Am80 was orally administered to mice from day 21 of HSCT, when clinical signs of chronic GVHD had developed [21]. We are now planning a phase I/II clinical study of Am80 for the treatment of refractory chronic GVHD.

Conclusions

We reviewed many mediators that contribute to or regulate chronic GVHD. A better understanding of the biology of chronic GVHD will lead to the development of novel strategies for its prevention and treatment. Successful clinical studies of treatments for chronic GVHD would improve patient outcomes and result in the establishment of new standards of care.

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