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Mammalian Target of Rapamycin Inhibitors Permit Regulatory T Cell Reconstitution and Inhibit Experimental Chronic Graft-versus-Host Disease

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A B S T R A C T

Chronic graft-versus-host disease (GVHD) remains a major late complication of allogeneic bone marrow transplantation (BMT). In a previous study, impaired thymic negative selection of the recipients permitted the emergence of pathogenic T cells that cause chronic GVHD using MHC class II-deficient (H2-Ab1 KO) B6 into C3H model and $CD4^+$ T cells isolated from chronic GVHD mice caused chronic GVHD when administered into the secondary recipients. In this study, we evaluated the kinetics of regulatory T cell (Treg) reconstitution in wild type B6 into C3H model. After myeloablative conditioning, host Tregs disappeared rapidly, followed by expansion of Tregs derived from the donor splenic T cell inoculum. However, the donor splenic T cell–derived Treg pool contracted gradually and was almost completely replaced by newly generated donor bone marrow (BM)-derived Tregs in the late post-transplantation period. Next, we compared the effects of cyclosporine (CSA) and mammalian target of rapamycin (mTOR) inhibitors on Treg reconstitution. Administration of CSA significantly impaired Treg reconstitution in the spleen and thymus. In contrast, BM-derived Treg reconstitution was not impaired in mTOR inhibitor-treated mice. Histopathological examination indicated that mice treated with CSA, but not mTOR inhibitors, showed pathogenic features of chronic GVHD on day 120. Mice treated with CSA until day 60, but not mTOR inhibitors, developed severe chronic GVHD followed by adoptive transfer of the pathogenic $CD4^+$ T cells isolated from H2-Ab1 KO into C3H model. These findings indicated that long-term use of CSA impairs reconstitution of BM-derived Tregs and increases the liability to chronic GVHD. The choice of immunosuppression, such as calcineurin inhibitor-free GVHD prophylaxis with mTOR inhibitor, may have important implications for the control of chronic GVHD after BMT.

INTRODUCTION

Chronic graft-versus-host disease (GVHD) is the most serious late complication after allogeneic hematopoietic stem cell transplantation, but the pathophysiology and treatment strategy of chronic GVHD remain poorly defined [1-3]. GVHD prophylaxis using calcineurin inhibitors, such as cyclosporine (CSA) and tacrolimus, reduces the expansion of effector T cells by blocking interleukin (IL)-2 and prevents acute GVHD, but fails to reduce chronic GVHD [4,5]. Administration of CSA for up to 24 months, longer than the standard 6 months of CSA, also did not decrease the risk of chronic GVHD [6]. Several studies have indicated that the efficacy and safety of mammalian target of rapamycin (mTOR) inhibitor, rapamycin (RAPA), in refractory chronic GVHD patients [7-10]. However, a recent randomized trial showed that the combination of RAPA and tacrolimus as GVHD prophylaxis failed to reduce chronic GVHD compared with tacrolimus and methotrexate [11].

$CD4^+CD25^+Foxp3^+$ regulatory T cells (Tregs) have been shown to play an important role in the establishment of tolerance between recipient tissues and donor-derived immunity. A series of animal studies indicated that Tregs in the inoculum can prevent acute GVHD when injected together with donor T cells [12-14]. Based on the role of Tregs in the prevention of GVHD and on their dependence on IL-2, there is considerable concern regarding the impact of blocking IL-2 signaling or IL-2 production by the immunosuppressive agents used for prophylaxis of GVHD. Zeiser et al. reported that Tregs showed relative resistance to RAPA as a result of reduced usage of the mTOR pathway and functional PTEN, a negative regulator of the phosphatidyl-inositol 3-kinase/Akt/mTOR pathway in Tregs compared with conventional T cells

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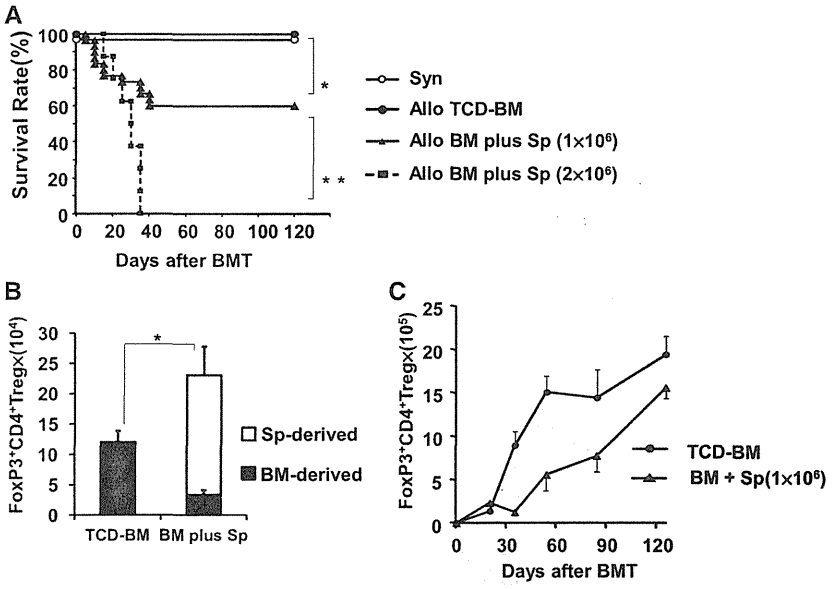

Figure 1. Regulatory T cell reconstitution after allogeneic BMT. Lethally irradiated C3H (H-2^k) recipient mice received 10×10^6 T cell–depleted bone marrow (TCD-BM) cells from B6.Ly-5a (H-2^b,CD45.1) mice with/without 1 to 2×10^6 spleen cells from B6 (H-2^b,CD45.2) mice. The syngeneic group received transplantation from C3H mice. (A) Survival: the recipients of allogeneic BM plus 1×10^6 spleen cells (BM plus Sp cells) showed a survival rate of 60% by day 120. Open circle, syngeneic; closed circle, TCD-BM cells only; triangle, -with 1×10^6 spleen cells; square, -with 2×10^6 spleen cells. (B) Origin of $CD4^+Foxp3^+$ Treg in the spleen on day 21 post transplantation: $CD45.2^+$ splenic T cell–derived (white bars) and $CD45.2^-$ BM–derived (black bars) are shown. (C) The absolute numbers of Treg in the recipients of BM plus Sp cells (triangles) and TCD-BM (closed circles) are shown. Each group consisted of 7 to 25 mice. The means (±SE) of each group are shown. Data are from a representative of at least 3 independent experiments. $^*P < .05$; $^{**}P < .01$.

[15]. In contrast to CSA, RAPA allowed expansion of adoptively transferred Treg cells and led to reduction of alloreactive T cell expansion when animals received Treg treatment in combination with RAPA. They also showed that a combination of RAPA plus IL-2 increased both expansion of donor natural Tregs and conversion of induced Tregs from donor conventional T cells, and suppressed acute GVHD [16]. These animal data suggest that RAPA and CSA have differential effects on peripheral Tregs after bone marrow transplantation (BMT).

IL-2 signaling is pivotal for Treg homeostasis in the periphery and is also essential for naturally occurring Treg development in the thymus [17-19]. T cell repopulation after BMT is composed of 2 subsets: T cells derived from the donor splenic T cell inoculum and newly arising T cells from bone marrow (BM) inoculum. It has been shown that Tregs from the former pathway play an important role in acute GVHD, whereas, no previous study evaluated whether use of CSA for an extended period affects donor BM-derived Treg generation. We hypothesized that BM-derived Tregs comprise the long-term peripheral Treg pool and that CSA, but not mTOR inhibitors, causes impaired BM-derived Treg reconstitution, which has a negative effect on chronic GVHD. In the present study, we therefore evaluated effects of different immunosuppressants on 2 distinct Treg expansion reconstitution pathways and on the development of chronic GVHD.

MATERIALS AND METHODS

Mice

Female C57BL/6 (B6: H-2^b, CD45.2^+) and C3H/HeN (C3H: H-2^k) mice were purchased from Charles River Japan (Yokohama, Japan) or from the Okayama University mouse colony (Okayama, Japan). B6-Ly5a (H-2^b, CD45.1^+) and C3.SW (H-2^b, CD45.2^+) mice were purchased from Jackson Laboratory (Bar Harbor, ME). B6-background MHC class II-deficient H2-Ab$1^{-/-}$ mice (B6.129-*H2-Ab1*^{tm1Gru} N12) were from Taconic Farms (Germantown, NY) [20]. Mice between 8 and 18 weeks of age were maintained under specific pathogen-free conditions and received normal chow and hyperchlorinated drinking water after transplantation. All experiments involving animals were approved by the Institutional Animal Care and Research Advisory Committee, Okayama University Advanced Science Research Center.

BMT

Mice underwent transplantation according to the standard protocol described previously [21,22]. Briefly, recipient mice received 2 split doses of either 500 cGy (allogeneic C3H and C3.SW recipients) or 650 cGy (syngeneic B6 recipients) total-body irradiation (TBI) 3 to 4 hours apart. Recipients were injected with 10×10^6 T cell–depleted bone marrow (TCD-BM) cells plus 1 or 2×10^6 whole spleen cells from B6 donors. [*H2-Ab1*$^{-/-}$→C3H] chimeras were produced by reconstituting lethally irradiated C3H mice with 5×10^6 TCD-BM cells from *H2-Ab1*$^{-/-}$ mice, as described previously [23]. T cell depletion was performed using anti-CD90–microbeads and an AutoMACS system (Miltenyi Biotec, Auburn, CA) according to the manufacturer's instructions. Donor cells were injected intravenously into the recipients on day 0.

Immunosuppressive Treatment

RAPA was purchased from Toronto Research Chemicals Inc. (North York, ON, Canada). Everolimus (RAD) and CSA were synthesized and provided by Novartis Pharma AG (Basel, Switzerland). Everolimus emulsion was dissolved in distilled water at a concentration of 625 μg/mL and administered to recipients by oral gavage at a dose of 5 mg/kg. RAPA and CSA were given as suspensions in carboxymethylcellulose sodium salt: CMC (C5013; Sigma-Aldrich, St. Louis, MO) at a final concentration of .2% CMC. RAPA and CSA were administered to recipients by peritoneal injection at doses of .5 and 20 mg/kg, respectively [15,24]. Immunosuppressive treatments were performed once daily, starting on day 0 and continuing until death or end of the observation period (day 110 to 125).

Adoptive Transfer

Splenocytes were isolated from [*H2-Ab1*$^{-/-}$→C3H] chimeras 6 to 11 weeks after TCD-BMT. $CD4^+$ T cells were negatively selected from splenocytes by depletion of $CD8^+$, $DX5^+$, $CD11b^+$, Ter-119^+, and $B220^+$ cells using the AutoMACS system, as described previously [23]. A total of 2×10^7 $CD4^+$ T cells per mouse were injected intravenously into recipients after immunosuppressive therapy for 70 days after BMT.

Assessment of GVHD

After BMT, survival was monitored daily, and weight changes were assessed twice per week. The degree of clinically acute GVHD was assessed twice per week using a scoring system that sums changes in 5 clinical parameters: weight loss, posture, activity, fur texture, and skin integrity

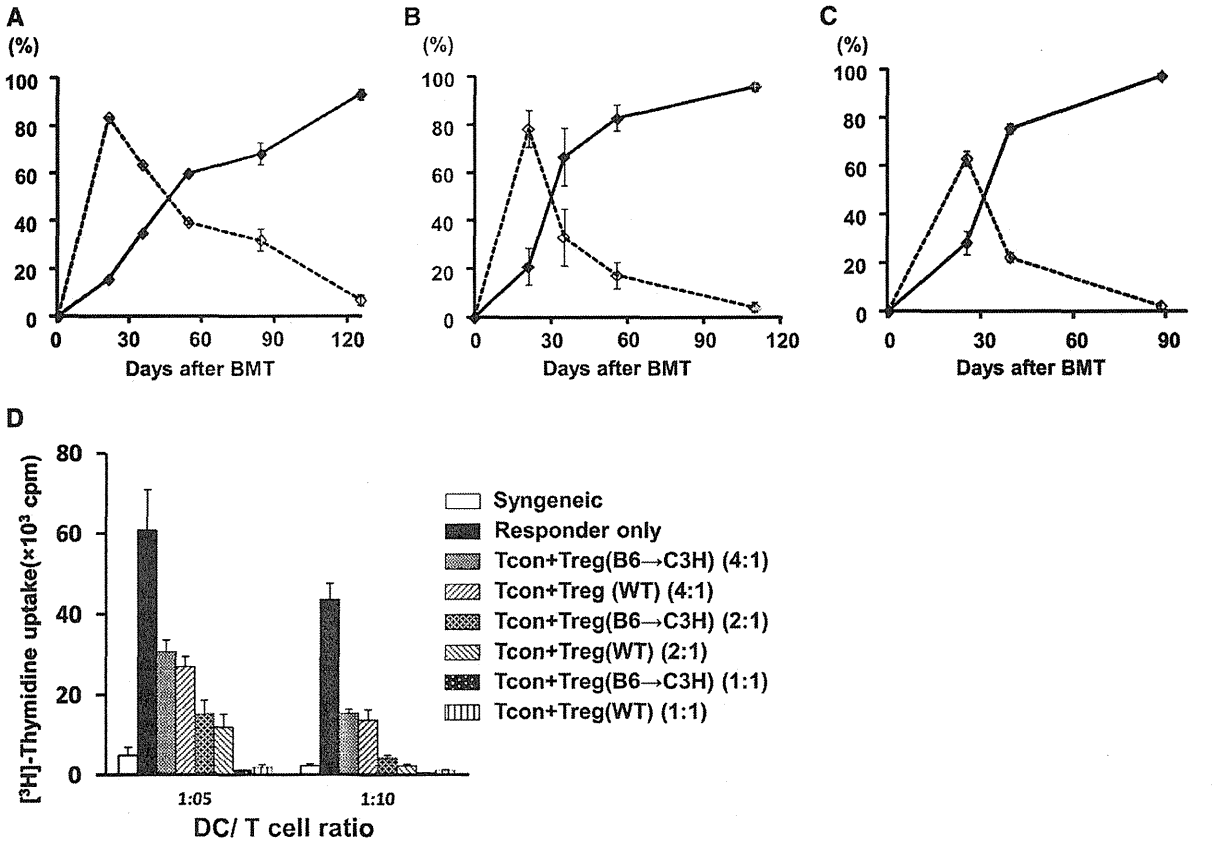

Figure 2. Donor BM–derived progenitors comprise the long-term peripheral Treg pool. Lethally irradiated C3H recipients underwent transplantation as in Figure 1: (B6 → C3H). The rates of $CD45.2^+$ spleen cell–derived (broken lines) and $CD45.2^-$ BM–derived (solid lines) Treg in $CD4^+Foxp3^+$ Treg are shown. Spleen (A) and mesenteric lymph nodes (MLN) (B) were isolated from (B6 → C3H) mice at various time points after BMT and cells were analyzed by fluorescent activated cell sorter. (C) Lethally irradiated C3.SW ($H\text{-}2^b$) recipients underwent transplantation from B6 ($H\text{-}2^b$) donors. The rates of $CD45.2^+$ splenic T cell–derived (broken lines) and $CD45.2^-$ BM–derived (solid lines) Treg in $CD4^+Foxp3^+$ Treg in the spleen are shown. Each group consisted of 20 to 23 mice. The means (±SE) of each group are shown. Data are from a representative of at least 2 independent experiments. (D) $CD25^+CD4^+$ Treg were purified from the spleens of (B6 → C3H) mice (on day 120) or naïve B6 (WT). B6 $CD4^+CD25^-$ T cells (Tcon) together with various numbers of Treg were cultured with irradiated C3H $CD11c^+$ DC as stimulators for 72 hours. Proliferative activities were determined by monitoring ^3H-thymidine uptake.

(maximum index, 10), as described previously [22]. Shaved skin from the interscapular region (approximately 2 cm^2), liver, and salivary gland specimens of recipients were fixed in 10% formalin, embedded in paraffin, sectioned, mounted on slides, and stained with hematoxylin and eosin. Skin slides were scored on the basis of dermal fibrosis, fat loss, inflammation, epidermal interface changes, and follicular drop out (0 to 2 for each category; the maximum score was 10) [21]. Liver slides were scored based on bile duct injury and inflammation (0 to 4 for each category), and the maximum score was 8 [25]. Salivary gland slides were scored based on atrophy and inflammation (0 to 3 for each category), and the maximum score was 6. All slides were scored by pathologists (T.K. and T.T.) blind to experimental group.

Immunohistochemistry

Immunohistochemical staining for Foxp3 and CD3 was performed using the high polymer (HISTOFINE simple stain, NICHIREI, Tokyo, Japan) method. Anti-Foxp3 (eBioscience) and anti-CD3 (Abcam, Cambridge, MA) were used to identify Tregs and effector T cells, respectively.

Flow Cytometry

The mAbs used were unconjugated anti-CD16/32 (2.4G2); FITC-, PE-, PerCP-, or APC-conjugated anti-mouse CD4, CD25, CD45.1, CD45.2, $H\text{-}2^b$, $H\text{-}2^d$ (BD Pharmingen, San Diego, CA); and Foxp3 (eBioscience, San Diego, CA), as described previously [26]. A Foxp3 staining kit (eBioscience) was used for intracellular staining. Cells were analyzed on a FACSAria flow cytometer with FACSDiva software (BD Immunocytometry Systems, San Diego, CA).

Mixed Leukocyte Reaction

$CD4^+CD25^-$ T cells, $CD4^+CD25^+$ T cells, and $CD11c^+$ DC were magnetically separated by AutoMACS using microbeads from a $CD4^+CD25^+$ regulatory T cell isolation kit and CD11c microbeads. $CD4^+CD25^-$ T cells (5×10^4 per well) together with various numbers of $CD25^+CD4^+$ T cells (0 to 5×10^4 per well) were cultured with irradiated (30 Gy) $CD11c^+$ DC as stimulators for 72 hours in 96-well round-bottomed plates. Cells were pulsed with ^3H-thymidine (1 μCi [.037 MBq] per well) for a further 16 hours [27]. Proliferation was determined using Topcount NXT (Packard Instruments, Meriden, CT).

Statistics

Data are given as means ± SEM. The survival curves were plotted using Kaplan-Meier estimates. Group comparisons of pathology scores were performed using the Mann-Whitney *U* test. Comparative analysis of cell ratios was performed by the unpaired 2-tailed Student *t*-test or Welch's *t*-test. In all analyses, $P < .05$ was taken to indicate statistical significance.

RESULTS

Kinetics of Treg Reconstitution after Allogeneic BMT

We first examined whether Tregs intermixed in the graft persist in the host for long periods post BMT using the MHC-mismatched model of BMT. Lethally irradiated C3H ($H\text{-}2^k$) recipient mice received 10×10^6 TCD-BM cells from B6.Ly-5a ($H\text{-}2^b$,CD45.1) mice with/without 1 to 2×10^6 spleen cells from B6 ($H\text{-}2^b$,CD45.2) mice. All of the recipients of allogeneic C3H TCD-BM cells from B6 mice and syngeneic mice survived and were resistant to induction of GVHD. Although 100% of the animals that received allogeneic BM plus 2×10^6 spleen cells died by day 35 with clinical and histopathological signs

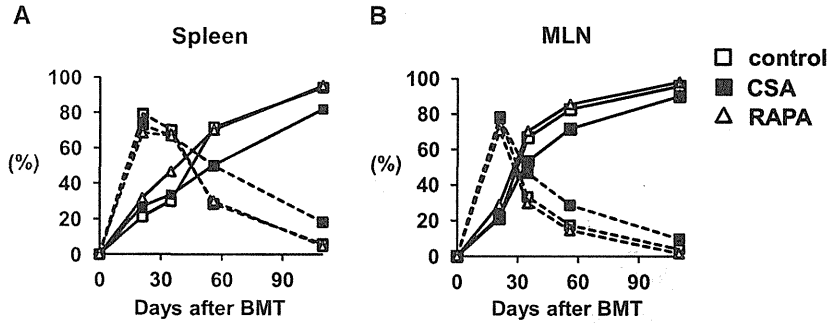

Figure 3. Effects of CSA and mTOR inhibitors on the Treg compartment. Lethally irradiated C3H recipients underwent transplantation from B6 donor mice as shown in Figure 1 and received i.p. injections of CSA (closed squares), mTOR inhibitor (rapamycin, RAPA; open triangles), or vehicle control (open squares) daily from day 0 to 110. The rates of $CD45.2^+$ splenic T cell–derived (broken lines) and $CD45.2^-$ BM–derived (solid lines) Treg in $CD4^+Foxp3^+$ Treg are shown. Spleen (A) and mesenteric lymph nodes (MLN) (B) were isolated from (B6 → C3H) mice at various time points after BMT and cells were analyzed by fluorescent activated cell sorter. Each group consisted of 16 to 23 mice. The means (±SE) of each group are shown. Data are from a representative of at least 2 independent experiments.

of severe GVHD, the recipients of allogeneic BM plus 1×10^6 spleen cells (BM plus Sp cells) showed mild clinical signs of GVHD and 60% survived by day 120 (Figure 1A); the following experiment was performed in this setting. Flow cytometric analysis of donor cell chimerism in the spleen 3 weeks after allogeneic BMT showed that 98.8% ± 0.7% of spleen cells were derived from the donor in mice, thus confirming complete donor cell engraftment. Host Tregs, as determined by $CD4^+Foxp3^+H\text{-}2^{K+}$, were not detected in the spleen on day 21 post transplantation (data not shown). On day 21 post transplantation, the majority of $CD4^+Foxp3^+$ Tregs were derived from $CD45.2^+$ splenic T cells (83.4% ± 2.2%), suggesting that splenic T cell–derived Tregs underwent homeostatic and/or alloantigen-driven expansion (Figure 1B) and the absolute number of Tregs in the spleens of the recipients of BM plus Sp cells was significantly higher than in TCD-BM recipients. From day 21 onward, due to GVHD-induced lymphopenia, the absolute number of Tregs in the spleens of recipients of BM plus Sp cells was lower than in TCD-BM recipients (Figure 1C). The rate of $CD45.2^+$ splenic T cell–derived Tregs in $CD4^+Foxp3^+$ Treg decreased gradually and most $CD4^+Foxp3^+$ Treg were $CD45.1^+$ BM-derived (93.2%) on day 125 post transplantation (Figure 2A). The rate of $CD45.1^+$ BM-derived Tregs in the mesenteric lymph nodes (MLN) was also increased and became dominant in the late post-transplantation period (Figure 2B). To exclude strain-dependent artifacts, we next evaluated the kinetics of Treg reconstitution in the B6 ($H\text{-}2^b$) into C3.SW ($H\text{-}2^b$) MHC-compatible, multiple minor histocompatibility antigen (miHA)-incompatible model of SCT. The kinetics of Treg reconstitution in the spleen was similar and most $CD4^+Foxp3^+$ Tregs were derived from $CD45.1^+$ BM (97%) on day 90 post transplantation (Figure 2C). These findings indicated that the peripheral Treg pool was restored first by expanded splenic T cell–derived mature Treg and then by new Tregs generated from donor BM-derived progenitors. Next, to examine the function of newly arising Tregs, purified $CD4^+CD25^+$ T cells on day 120 post transplantation were assessed for their ability to inhibit proliferation by responding syngeneic $CD4^+CD25^-$ B6 T cells. Their suppressive

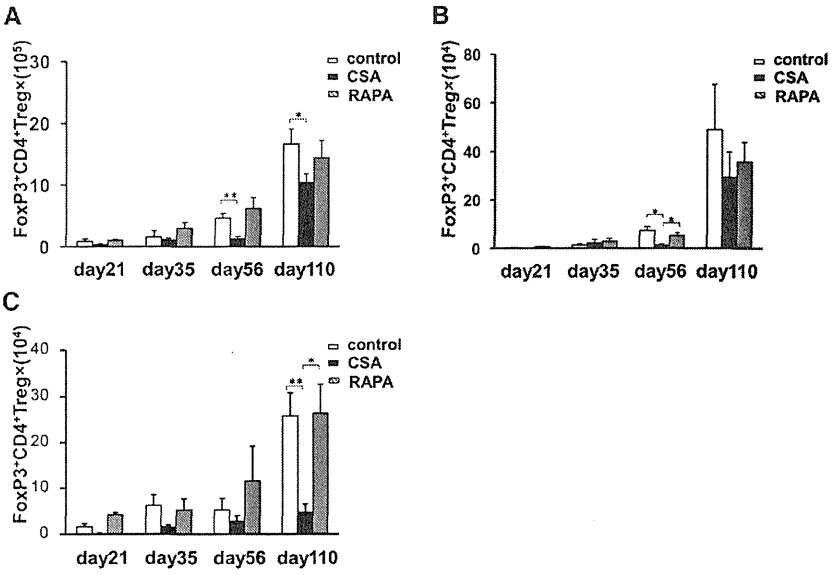

Figure 4. CSA, but not mTOR, inhibitors hampered reconstitution of BM-derived Treg. (B6 → C3H) mice received i.p. injections of CSA (black bars), mTOR inhibitor (rapamycin, RAPA; gray bars), or vehicle control (white bars) daily from day 0 to 110. The absolute numbers of Treg in the spleen (A), MLN (B), and thymus (C) are shown. Each group consisted of 19 to 26 mice. The means (±SE) of each group are shown. Data are from a representative of at least 2 independent experiments. $^*P < .05$; $^{**}P < .01$.

activity was virtually indistinguishable from that of Tregs obtained from normal B6 mice (Figure 2D). Taken together, Tregs generated from donor BM-derived progenitors comprise the long-term peripheral Treg pool and exhibit immunosuppressive activity.

CSA, but Not mTORinhibitors, Hampered Reconstitution of BM-derived Treg

Coenen et al. reported that 28 days of CSA administration hampered Treg homeostasis in normal mice [28]. We examined whether the use of CSA for an extended period affected the long-term peripheral Treg pool after BMT. C3H recipient mice underwent transplantation from B6 donor mice (as shown in Figure 1) and received i.p. injection of CSA, mTOR inhibitor (rapamycin; RAPA), or vehicle control daily from day 0. We analyzed the effects of CSA and RAPA on the Treg compartment at 21, 35, 56, and 110 days post hematopoietic cell transplantation. Mice treated with CSA or RAPA showed the same Treg reconstitution pattern as those treated with vehicle solution. On day 21 post transplantation, the majority of $CD4^+Foxp3^+$ Tregs in the spleen were $CD45.2^+$ splenic T cell–derived cells but the Treg compartments were dominated by BM-derived cells on days 56 and 110 post transplantation in all 3 groups (Figure 3A). In the MLN, these 3 groups also showed similar Treg reconstitution kinetics (Figure 3B). There were no differences in the absolute numbers of Treg among the 3 groups on day 21. From day 21 onward, however, the absolute numbers of Tregs in the CSA-treated mice were lower than those in control mice both in the spleen (day 56: 1.3 ± .4 versus 4.6 ± .8 × 10^5, $P < .01$; day 110: 10.4 ± 1.4 versus 16.7 ± 2.4 × 10^5, $P < .05$) (Figure 4A) and in the MLN (day 56: 1.3 ± .5 versus 7.4 ± 1.6 × 10^4, $P < .03$; day 110: 2.9 ± 1.0 versus 4.9 ± 1.9 × 10^5, $P = .46$) (Figure 4B). Especially in the thymus, mice treated with CSA showed a marked reduction in the absolute numbers of Tregs compared with those treated with vehicle control (day 110: 4.6 ± 1.8 versus 25.7 ± 5.0 × 10^4, $P < .01$) (Figure 4C). In contrast to mice treated with CSA, mice treated with RAPA showed no reduction in the absolute numbers of Tregs and no differences compared with control mice in the spleen or MLN at any time point post transplantation (Figure 4A,B). The absolute numbers of newly arising Tregs in the thymus were also not reduced in mice treated with RAPA (Figure 4C). We next examined the effects of another mTOR inhibitor, everolimus (RAD), which exhibits greater polarity than RAPA and has been approved in Europe for use as an immunosuppressant for prevention of cardiac and renal allograft rejection. Reconstitution of newly arising Tregs in the thymus was not impaired in mice treated with RAD, and there were no differences in the absolute numbers of spleen Tregs compared with control mice on day 110 (spleen: 15.4 ± 2.5 versus 16.6 ± 2.4 × 10^5, $P = .73$, Supplemental Figure 1A; thymus: 17.4 ± 3.2 versus 25.7 ± 5.0 × 10^4, $P = .26$, Supplemental Figure 1B). These findings suggested that CSA, but not mTOR inhibitors, hampered the long-term reconstitution of BM-derived Tregs.

CSA, but Not mTOR Inhibitors, Increased Liability to Chronic GVHD

Recent studies revealed the association of reduced Treg frequency in patients with chronic GVHD. In the present study, we examined histopathological change in CSA-treated mice where reconstitution of BM-derived Tregs was impaired. The skin of CSA-treated mice showed pathogenic features of chronic GVHD (Figure 5A), and pathogenic scores revealed significantly exacerbated chronic GVHD pathology compared with those treated with vehicle control (5.5 ± .8 versus 1.6 ± .3, $P < .01$) (Figure 5B). A dry mouth is one of the distinctive features of chronic GVHD. Lymphocytic inflammation, fibrosis, and atrophy of acinar tissue were observed in the salivary glands of CSA-treated mice (Figure 5A) and pathological scores were significantly higher in CSA-treated mice than in the controls (4.0 ± .5 versus 1.8 ± .1, $P < .01$) (Figure 5C). CSA-treated mice showed bile duct injury and

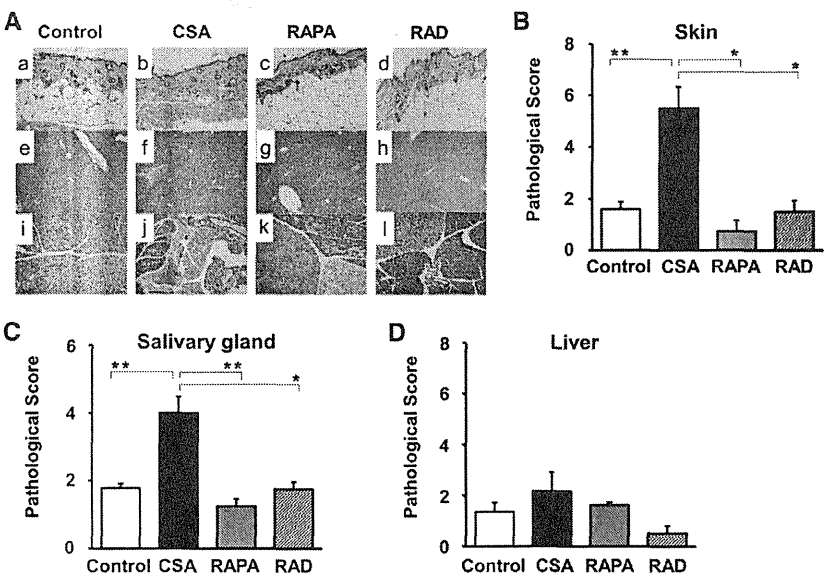

Figure 5. CSA, but not mTOR, inhibitors increased the likelihood of chronic GVHD. (A) Histological findings of the skin (a to d), liver (e to h), and salivary glands (i to l) (on day 120) from (B6 → C3H) mice given CSA, mTOR inhibitor (RAPA, RAD), or vehicle control. Sclerodermatous skin changes, such as epidermal atrophy, fat loss, follicular dropout, and dermal thickness (b); fibrosis in the portal area and peripheral mononuclear cells infiltrates in the liver (f); and fibrosis and atrophy of acinar tissue in the salivary glands (j) were observed (original magnification: × 100) Pathological scores of skin (B), salivary gland (C) and liver (D). The data are expressed as means ± SE. Data are from a representative of at least 2 independent experiments. $^*P < .05$; $^{**}P < .01$.

fibrosis in the portal area and peripheral mononuclear cell infiltration in the liver and pathological scores of the liver also tended to be worse in CSA-treated mice, as compared with those treated with vehicle control, although it was not statistically significant (Figure 5D). In contrast to mice treated with CSA, mice treated with RAPA showed no pathogenic features of chronic GVHD and there were no differences in pathogenic skin and salivary gland scores, as compared with control mice (skin: .75 ± .4 versus 1.6 ± .3, $P = .18$, Figure 5B; salivary gland: 1.25 ± .2 versus1.78 ± .1, $P = .08$, Figure 5C). Immunohistochemical staining for Foxp3 and CD3 revealed that $CD3^+$ T cells infiltrated in the skin tissue of all 3 groups, and RAD-treated mice showed abundant infiltration by $CD3^+$ T cells and $Foxp3^+$ cells (Figure 6A). In contrast to RAD, $Foxp3^+$ cells were scarcely found in skin tissue of CSA-treated mice. The ratio of Foxp3 Tregs per 100 $CD3^+$ lymphocytes in the skin tissue of CSA-treated mice was significantly lower than those in RAD-treated mice (3.23 ± .4 versus 19.5 ± 4.4, $P < .05$). CSA-treated mice tended to show poorer survival, as compared with those treated with mTOR inhibitors or vehicle control (CSA 27.6% versus control 54.2%, RAD 57.1%, RAPA 61.5%, $P = .28$, Supplemental data Figure 2). These findings suggested that CSA, but not mTOR inhibitors, hampered the reconstitution of BM-derived Treg and increased liability to chronic GVHD.

We next tested liability to chronic GVHD in CSA-treated mice using adoptive transfer experiments. Previously, Sakoda et al. demonstrated that impaired thymic negative selection of the recipients permitted the emergence of pathogenic T cells that cause chronic GVHD (Figure 7A) [23]. Lethally irradiated C3H recipients were reconstituted with TCD BM from MHC class II-deficient ($H2\text{-}Ab1^{-/-}$) B6 mice ([$H2\text{-}Ab1^{-/-} \rightarrow$C3H]). These mice developed disease conditions that showed all of the clinical and histopathological features of human chronic GVHD. $CD4^+$ T cells isolated from chronic GVHD mice ([$H2\text{-}Ab1^{-/-} \rightarrow$C3H] $CD4^+$ T cells) cause chronic GVHD when B6 antigens are provided by hematopoietic cells in the absence of B6 antigen expression on target epithelium ([B6→C3H] chimeras) [23]. In the current study, C3H mice underwent transplantation from B6 donors as shown in Figure 1 and were orally administered CSA, RAPA, or vehicle solution until 60 days post BMT, when none of the recipients showed significant signs of chronic GVHD. To test liability to chronic GVHD, these C3H-recipient mice with B6-derived antigen presenting cells received adoptive transfer of [$H2\text{-}Ab1^{-/-} \rightarrow$C3H] $CD4^+$ T cells (Figure 7B). As shown in Figure 7C and D, adoptive transfer of pathogenic $CD4^+$ T cells caused severe weight loss (CSA 81.1 ± 4.1% versus control 94.5 ± 2.1%, $P < .05$; and CSA 81.1 ± 4.1% versus RAPA 98.9 ± 1.5%, $P < .01$) and chronic GVHD in CSA-treated mice, with a mortality rate of 83%. RAPA-treated mice and controls showed resistance to induction of chronic GVHD by transfer of pathogenic $CD4^+$ T cells; the survival rate on day 62 after adoptive transfer was 100%. Taken together, these data demonstrated that CSA, but not mTOR inhibitors, increased liability to chronic GVHD.

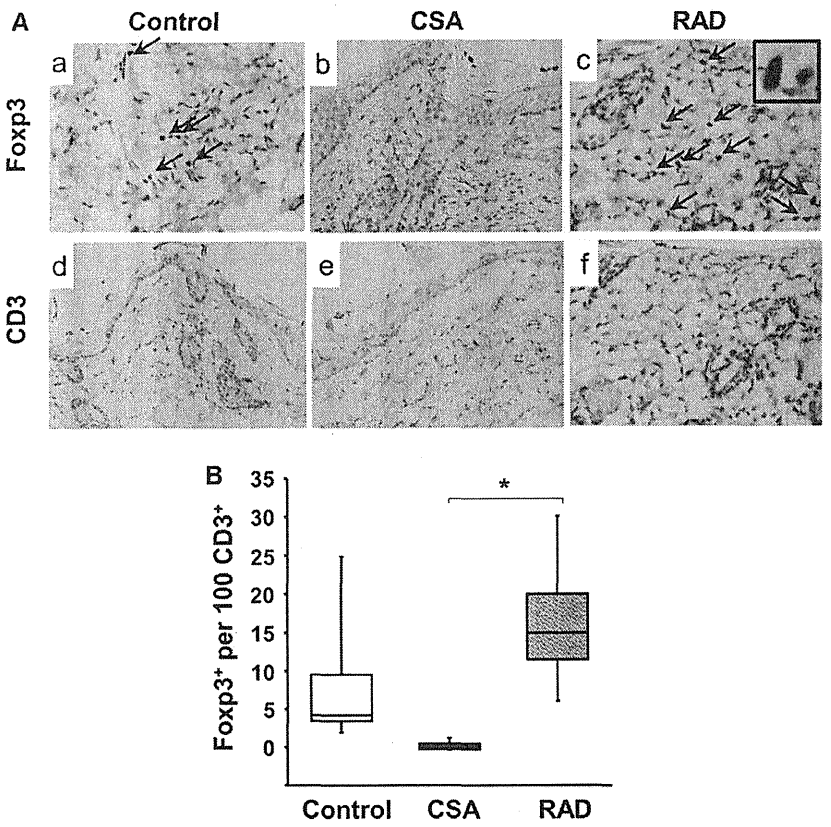

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Figure 6. CSA, but not mTOR, reduces Treg infiltration in skin tissue. (A) Lethally irradiated C3H recipients underwent transplantation from B6 donor mice as shown in Figure 1 and received vehicle control (a, d), CSA (b, e), or mTOR inhibitor (RAD; c, f), daily from day 0 to 120. Immunohistochemical staining was performed using anti-Foxp3 (a to c) and anti-CD3 (d to f) antibodies on day 120. Arrows indicate Foxp3 positive cells. (B) The ratio of Foxp3 Tregs per 100 $CD3^+$ lymphocytes. The number of CD3 and Foxp3 cells was counted in all the high-power fields. Results are expressed as mean ± SD. Pictures and data are from a representative of 2 independent experiments. (n = 3 to 4 per group). *$P < .05$.

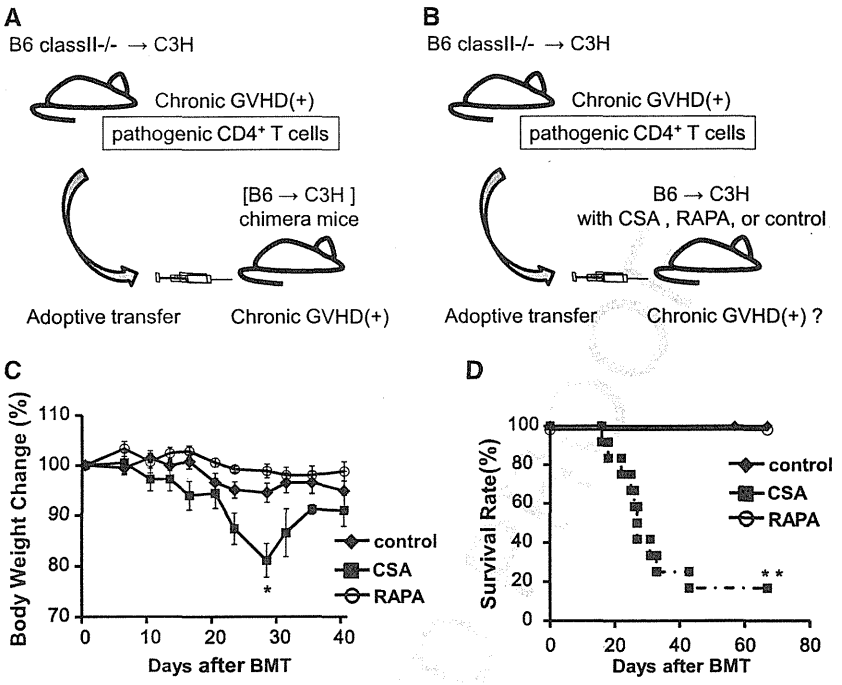

Figure 7. Adoptive transfer of pathogenic $CD4^+$ T cells caused severe chronic GVHD. (A) Lethally irradiated C3H recipients were reconstituted with TCD BM from MHC class II-deficient (H2-$Ab1^{-/-}$) B6 mice. These mice developed chronic GVHD and $CD4^+$ T cells isolated from chronic GVHD mice ([H2-$Ab1^{-/-}$→C3H] $CD4^+$ T cells) were primarily donor reactive. These pathogenic $CD4^+$ T cells cause chronic GVHD when B6 antigens are provided by hematopoietic cells in the absence of B6 antigen expression on target epithelium ([B6→C3H] chimeras). (B) C3H recipient mice underwent transplantation from B6 donors as shown in Figure 1 and received CSA, RAPA, or vehicle solution until 60 days post BMT. These C3H recipient mice received adoptive transfer of [H2-$Ab1^{-/-}$→C3H] $CD4^+$ T cells. Body weight change is shown in (C) and overall survival is shown in (D). Data from 3 similar experiments are combined (n = 8 to 12 per group). The data are expressed as means ± SE. $^*P < .05$; $^{**}P < .01$.

DISCUSSION

Patients with chronic GVHD have a lower frequency of Tregs when compared with patients without chronic GVHD [29-32]. Experimental BMT demonstrated that Tregs in the inoculum can prevent acute GVHD when injected together with donor T cells [12-14]; however, it is not known whether Tregs in the grafts persist into the late post-transplantation period and play a role in preventing chronic GVHD. Mastuoka et al. prospectively monitored $CD4^+$ T cell subsets and showed that thymic generation of naïve Treg was markedly impaired and Treg levels subsequently declined in patients with prolonged $CD4^+$ lymphopenia [32]. This resulted in a relative Treg deficiency, which was associated with a high incidence of extensive chronic GVHD. In the present study, we monitored Treg reconstitution kinetics in the spleen, MLN, and thymus according to 2 subsets, T cells derived from peripheral-expanded mature T cells and newly arising T cells from bone marrow stem cells, using 2 mouse BMT models because this is difficult to examine in a human setting. The results indicated that host Tregs disappeared rapidly in mice receiving allogeneic T cells early in the early post-transplantation period, consistent with a previous report [33]. In addition, this study showed that splenic T cell–derived Treg initially occupy a niche in lymphopenic transplantation recipients, suggesting that mature Treg underwent homeostatic and/or alloantigen-driven expansion. However, the donor splenic T cell–derived Treg pool contracted gradually and Tregs generated from donor BM-derived progenitors comprised the long-term peripheral Treg pool. The BM-derived Treg compartment was functionally competent, as determined by in vitro lymphoid suppression, indicating that these cells play a role in post-BMT immune tolerance.

Coenen et al. reported that 28 days of treatment with CSA resulted in a reduction in thymic generation of $CD4^+Foxp3^+$ T cells and peripheral $CD25^+Foxp3^+$ T cells in normal mice [28]. We assessed whether CSA affects the peripheral Treg pool after allogeneic BMT; on day 21, there were no differences in the absolute numbers of Tregs among 3 groups, and CSA had no impact on early Treg reconstitution. Consistent with our observations, Setoguchi et al. reported that in contrast to the requirement of IL-2 for physiological expansion of $CD4^+CD25^+$ Treg cells in normal nonlymphopenic mice, homeostatic proliferation in a lymphopenic environment appears to be IL-2-independent [19]. Zeiser et al. also reported that CSA administration has only a minor impact on the expansion of adoptively transferred $CD4^+CD25^+$ T cells on day 7 post transplantation [34]. However, whether prolonged use of CSA affects the long-term peripheral Treg pool has not been reported. Our data showed that CSA, but not mTOR inhibitors, hampered the long-term reconstitution of BM-derived Tregs. The numbers of Tregs in the spleen, thymus and tissue were significantly reduced in mice receiving CSA in comparison with those receiving mTOR inhibitors or PBS on day 110. CSA blocks nuclear factor of activated T cells translocation into the nucleus by inhibiting calcineurin phosphatase activity [35]. CSA inhibits the thymic generation of Tregs by impairment of TCR signaling and by reducing nuclear factor of activated T cells–dependent Foxp3 promoter activity [36]. In contrast, rapamycin-sensitive downstream targets of phosphatidyl-inositol 3-kinase are IL-2-independent, and rapamycin affects neither the initial signal transduction upon TCR triggering nor the thymic generation of Treg [37]. Immunosuppressive drugs have different mechanisms of promoting immune suppression and our data revealed

different effects on the long-term peripheral Treg pool after allogeneic BMT.

Although mouse models of chronic GVHD have provided important insights into pathophysiology of this disease, one factor that confounds the translation of findings in mouse models to the human disease is that time course of development of chronic GVHD is more rapid in most mouse models than in human. Another factor is that most patients are given immunosuppressive therapy to prevent acute GVHD [38], and these medications might influence the development of chronic GVHD. In this study, histopathological examination revealed that CSA-treated mice showed pathogenic features of chronic GVHD, whereas those treated with mTOR inhibitors showed no significant differences compared with control mice. This is the first report that long-term use of CSA induces chronic GVHD in transplant-recipient mice. This may have been due to induction of autoreactive T cells by CSA [39,40]. Wu et al. reported that CSA contributes to chronic GVHD in experimental models, which was ascribed to the disruption of clonal-deletion mechanisms in the thymus, resulting in the export of autoreactive T cells [41]. The present study demonstrated another mechanism by which CSA impaired Treg reconstitution. Adoptive transfer of the pathogenic $CD4^+$ T cells caused severe chronic GVHD in CSA-treated mice, whereas mTOR inhibitor-treated and control mice showed resistance to induction of chronic GVHD. These findings suggest that the increased liability to chronic GVHD in CSA-treated mice might be due to limited reconstitution of BM-derived Treg cells; further mechanistic studies will be required to determine if this is truly causative rather than merely an association.

Here, we assessed the differential effects of CSA and mTOR inhibitors on the long-term peripheral Treg pool after allogeneic BMT. Our findings indicated that, in contrast to mTOR inhibitors, CSA compromises homeostasis in peripheral immune compartments and thymic generation of $CD4^+CD25^+Foxp3^+$ T cells. GVHD prophylaxis with mTOR inhibitor and calcineurin inhibitor failed to reduce chronic GVHD [11,42-45]. The choice of calcineurin inhibitor–free GVHD prophylaxis with mTOR inhibitors, such as mTOR inhibitors plus IL-2 [16] or mTOR inhibitors plus antithymocyte globulin [46] may have important implications for the control of chronic GVHD after BMT.

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SUPPLEMENTARY DATA

Supplementary data related to this article can be found online at http://dx.doi.org/10.1016/j.bbmt.2013.11.018.

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Pathogenesis of graft-versus-host disease: innate immunity amplifying acute alloimmune responses

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Abstract In addition to reduced-intensity conditioning, which has expanded the eligibility for hematopoietic cell transplantation (HCT) to older patients, increased availability of alternative donors, including HLA-mismatched unrelated donors, has increased access to allogeneic HCT for more patients. However, acute graft-versus-host disease (GVHD) remains a lethal complication, even in HLA-matched donor–recipient pairs. The pathophysiology of GVHD depends on aspects of adaptive immunity and interactions between donor T-cells and host dendritic cells (DCs). Recent work has revealed that the role of other immune cells and endothelial cells and components of the innate immune response are also important. Tissue damage caused by the conditioning regimen leads to the release of exogenous and endogenous "danger signals". Exogenous danger signals called pathogen-associated molecular patterns and endogenous noninfectious molecules known as damage-associated molecular patterns (DAMPs) are responsible for initiating or amplifying acute GVHD by enhancing DC maturation and alloreactive T-cell responses. A significant association of innate immune receptor polymorphisms with outcomes, including GVHD severity, was observed in patients receiving allogeneic HCT. Understanding of the role of innate immunity in acute GVHD might offer new therapeutic approaches.

Keywords Innate immunity · Danger signals · PAMPs · DAMPs · GVHD

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Introduction

Allogeneic hematopoietic cell transplantation (HCT) is used to treat many hematologic malignancies. However, acute graft-versus-host disease (GVHD) remains the most important complication of allogeneic HCT. GVHD was initially reported by Barnes [1] and Billingham [2] who identified three prerequisites for the development of GVHD: (1) the graft must contain immunologically competent cells; (2) the recipient must be incapable of rejecting the donor cells; and (3) the recipient must express tissue antigens that are not present in the transplant donor. Mature donor T-cells were identified as the fundamental cellular mediators of GVHD and several convergent lines of experimental data have demonstrated that host and donor antigen-presenting cells (APCs), especially dendritic cells (DCs), are critical for the induction of GVHD [3, 4]. In addition to T-cells and DCs, several other cellular subsets, such as B cells, macrophages, γδ T-cells, NK cells, and NKT cells, are involved in the pathogenesis of GVHD. The past decade has brought impressive advances in our understanding of the role of innate immune responses in the pathogenesis of GVHD. A conditioning regimen that includes total body irradiation (TBI) or chemotherapy damages the host tissues [5]. Injured, stressed, or dying cells release exogenous and endogenous "danger signals" (Fig. 1). This article reviews the importance of the innate immune response activated by danger signals in GVHD.

Triggers that induce GVHD: pathogen-associated molecular patterns (PAMPs)

After a conditioning regimen, tissue damage in the gastrointestinal (GI) tract allows the transit of bacteria. To

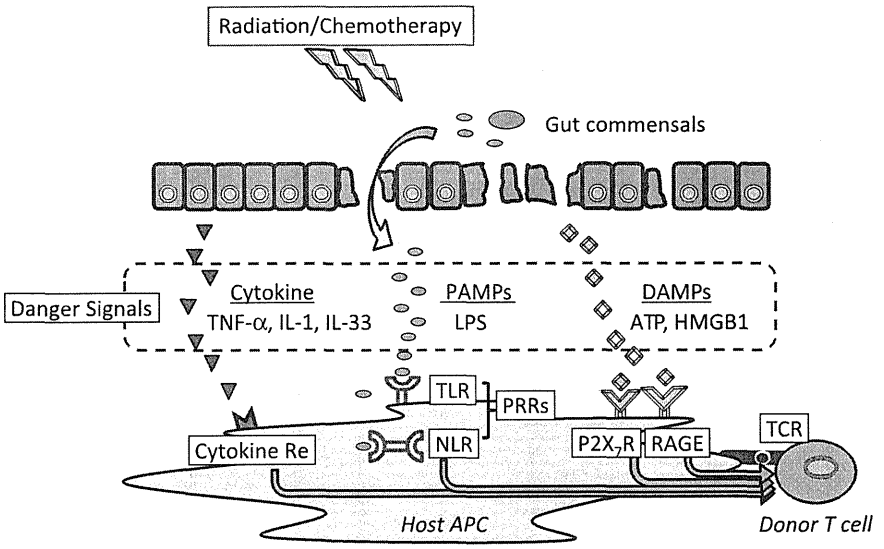

Fig. 1 Importance of innate immune response activated by danger signals in GVHD. The conditioning regimen which includes total body irradiation and/or chemotherapy leads to the damage of host tissues. Damaged cells release "danger signals" including cytokines, exogenous pathogen-associated molecular patterns (PAMPs), and endogenous damage-associated molecular patterns (DAMPs). Danger signals are responsible for initiating or amplifying acute GVHD by the enhancement of DC maturation and alloreactive T-cell responses. *LPS* lipopolysaccharide, *ATP* adenosine triphosphate, *HMGB1* high mobility group box 1 protein, *TLR* Toll-like receptor, *NLR* the nucleotide-binding oligomerization domain-like receptor, *PRRs* pattern recognition receptors, *P2X$_7$R* P2X purinoceptor 7 receptor, *RAGE* receptor for advanced glycation endproducts, *TCR* T-cell receptor, *APC* antigen-presenting cell

detect exogenous bacterial components, the host immune system identifies conserved structural moieties called pathogen-associated molecular patterns (PAMPs) that are found in microorganisms. Most innate immune cells express pattern recognition receptors (PRRs) and recognize PAMPs via PRRs, such as Toll-like receptors (TLRs) and the nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs). The binding of PAMPs by PRRs on APCs activates the innate immune response, which induces the upregulation of cytokines and MHC class II costimulatory molecules and promotes DC migration to the T-cell area of lymph nodes. TLRs are transmembrane proteins located at the cell surface or in endosomes, while NLRs are located in the cytoplasm. To date, 11 TLRs have been identified in humans and 13 in the mouse [6].

Toll-like receptors (TLRs)

Ferrara et al. [7] clarified an essential role of TLR4 ligand and the lipopolysaccharide (LPS)/TLR4 pathway in the development of GVHD. Using mouse GVHD models, they showed that HCT recipients from an LPS-resistant donor led to significantly less GVHD compared with HCT recipients from an LPS-sensitive donor, and that an LPS antagonist reduces GVHD [8]. LPS was also shown to play a role in alloimmune lung injury. Garantziotis et al. [9] reported that LPS-induced lymphocytic lung inflammation was dependent on intact TLR4 signaling in donor-derived hematopoietic cells. In the clinical setting, a trend toward a reduced incidence of severe acute GVHD was found when a TLR4 mutation associated with LPS hyporesponsiveness was present [10]. However, these associations were not statistically significant in recipients of HLA-matched sibling marrow transplants. Another study also failed to detect significant associations with polymorphisms of the genes encoding TLR4 and GVHD [11], although experimental murine GVHD models show the importance of the LPS/TLR4 pathway in systemic and pulmonary GVHD. The different effects of TLR4 signaling in humans and mouse models might be caused in part by the bacterial gut decontamination performed routinely in clinical allogeneic HCT.

TLR7/8 recognizes single-stranded RNA and induces anti-viral response. Sykes et al. [12] showed that systemic exposure to the TLR7 agonist, R-848, is sufficient to permit access of activated T-cells to peripheral tissues and induce GVHD. Blazar et al. [13] administered the TLR7/8 agonist, 3M-011 after allogeneic HCT and observed increased GVHD mortality. Interestingly, the same group showed that mice injected with 3M-011 before transplantation had reduced GVHD lethality. Ligation of TLR7/8 expressed primarily on APCs induced the expression of indoleamine 2,3-dioxygenase (IDO) which can suppress T-cell responses and promote tolerance and reduced injury in the colon [14, 15]. These results suggest that certain TLRs can contribute to immune regulatory function. For instance,

bacterial flagellin, a TLR5 agonist, regulates CD4 T-cell response by increasing the generation of regulatory T-cells (Tregs) [16] and protects epithelial cells from radiation-induced toxicity [17]. Hossain et al. [18] showed that pretransplant administration of fragellin reduced GVHD while preserving posttransplant donor immunity.

TLR9 recognizes cytosine-phosphorothioate-guanine oligodeoxynucleotides (CpG ODNs) that mimic bacterial and viral DNA and was also shown to be involved in GVHD [19, 20]. TLR9$^{-/-}$ APCs have reduced allo-stimulatory activity and TLR9$^{-/-}$ mice showed reduced gut GVHD morbidity and overall GVHD mortality [19, 20]. Ligation of TLR9 on host APCs with CpG ODNs enhanced donor T-cell responses, accelerating GVHD [13]. In the clinical setting, although the occurrence of acute GVHD was not different, TLR9 gene variants that are associated with reduced TLR9 expression were significantly associated with improved treatment-related mortality (TRM), overall survival (OS), and a lower relapse rate [21].

Nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs)

PAMPs are recognized not only by TLRs, but also by nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs), which include proteins such as NACHT-, LRR-, and PYD-containing proteins (NALPs), NOD1, and NOD2. NLRs are involved in the secretion of inflammatory cytokines, such as interleukin-1β (IL-1β) and IL-18. NOD2 recognizes muramyl dipeptide (MDP), a component of bacterial peptidoglycan, and induces NF-κB activation, leading to enhanced Th1 responses. Van den Brink et al. examined the role of NOD2 during GVHD. Unlike TLRs, they found that the use of NOD2$^{-/-}$ donor cells in wild-type recipients had no effect on GVHD [22]. Interestingly, they observed increased GVHD in NOD2$^{-/-}$ HCT recipients and demonstrated that NOD2 deficiency in host hematopoietic cells exacerbates GVHD using chimeric mice. NOD2$^{-/-}$ DCs had a higher activation status and increased ability to induce T-cell proliferation during GVHD. These findings are in line with the observation that NOD2$^{-/-}$ DCs had enhanced ability to trigger inflammatory T-cell responses, and NOD2$^{-/-}$ mice showed increased susceptibility to experimental colitis [23]. Watanabe et al. [23, 24] found that MDP activation of NOD2 regulates innate responses to intestinal microflora by downregulating multiple TLR responses and that the absence of such regulation leads to heightened Th1 responses.

In the clinical setting, several studies have shown that NOD2 single nucleotide polymorphisms (SNPs) are associated with GVHD [11, 25, 26]. Holler et al. [26] first reported an association between a greater incidence of GVHD and NOD2 SNPs of the donor or recipient. However, several other studies failed to confirm this association [27–31]. The conflicting results might be explained by multiple factors, including the NOD2 SNP frequency, overall incidence of GVHD, donor source, and intestinal microbial decontamination [32]. Moreover, NOD2 functions as a primary sensor of microbial products inducing inflammatory T-cell responses and also negatively regulates TLR-mediated responses (Fig. 2). This immunological balance might cause conflict in the association of NOD2 SNPs with GVHD.

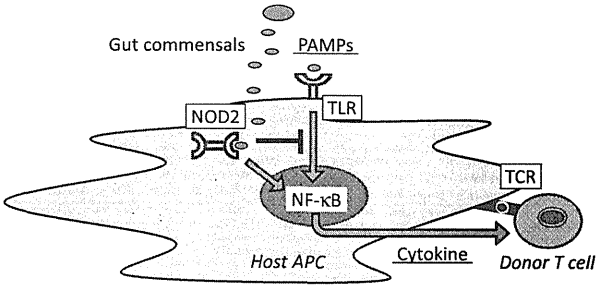

Fig. 2 PAMPs are recognized by NLRs and also by TLRs. A member of the nucleotide-binding oligomerization domain (*NOD*)-like receptors (*TLRs*), NOD2 recognizes pathogen-associated molecular patterns (*PAMPs*) as a primary sensor and induces NF-kB activation leading to enhance T-cell responses. In addition to inducing inflammatory T-cell responses, NOD2 functions negatively regulates TLR-mediated responses

Endogenous danger signals: damage-associated molecular patterns (DAMPs)

Endogenous noninfectious molecules, known as damage-associated molecular patterns (DAMPs), are released following conditioning regimen-induced tissue damage and play a critical role in GVHD (Table 1). Although the proinflammatory cytokines are not considered DAMPs, they serve as DAMPs, and the relationship between inflammatory cytokines and GVHD severity is well supported by animal models. The damaged, activated host tissues secrete cytokines, such as TNF-α, IL-1, and IL-33. The consequences of the action of these cytokines are the increased expression of MHC antigens and adhesion molecules, which recruits effector cells and enhances the recognition of host alloantigens by donor T-cells. In addition to proinflammatory cytokines, DAMPs include intracellular molecules and extracellularly located ones. These are extracellular matrix fragments released by extracellular matrix degradation during tissue damage.

Adenosine triphosphate (ATP)

Zeiser et al. [33] demonstrated that extracellular adenosine triphosphate (ATP) released by dying cells serves as a

Table 1 The role of DAMPs in the development of GVHD

DAMPs	Receptors	Observations		References
ATP	NLRP3 ($P2X_7R$)	Mouse	Blockade of ATP-$P2X_7R$ signaling pathways decreased acute GVHD	[33]
		Human	Polymorphisms of $P2X_7R$, NALP2 and NALP3 are associated with OS	[36, 37]
Heparan sulfate	TLR4	Mouse	α1-antitrypsin decreased serum heparan sulfate levels and GVHD	[39]
		Human	Serum heparan sulfate levels were associated with GVHD	[40]
HSPs	TLR2, TLR4, CD91, CD24, CD14 and CD40	Human	HSP70 expression was correlated with high graft-versus-host responses	[41]
		Human	Recipient HSP polymorphisms are associated with GVHD	[42]
Uric acid	NLRP3	Human	Rasburicase reduced the serum uric acid levels and grade II–IV GVHD	[44]
Hyaluronan	TLR2, TLR4 and CD44	Human	CD44– hyaluronan contribute to lymphocytotropism to skin GVHD	[45]
S100 proteins	RAGE	Human	S100 proteins were significantly more detected in saliva of GVHD patients	[48]
HMGB1	TLR2, TLR4, TLR9, RAGE and CD24	Human	Polymorphisms of HMBG1 are associated with GVHD, TRM and OS	[50]

DAMP damage-associated molecular pattern, *ATP* adenosine triphosphate, *P2X7R* P2X purinoceptor 7 receptor, *NALP*, *NACHT*, *LRR*, and *PYD* domains-containing protein, *TRM* treatment-related mortality, *OS* overall survival, *HSP* heat shock protein, *HMGB1* high-mobility group box 1, *RAGE* receptor for advanced glycation end products

danger signal to enhance GVHD. ATP binds to P2X purinoceptor 7 receptor ($P2X_7R$) on host APCs and induces higher expression of the costimulatory molecules CD80 and CD86 on APCs. This receptor plays a central role in IL-1β secretion via NALP3 or the cryopyrin inflammasome, thereby also allowing more potent allo-stimulatory T-cell priming. The pharmacological blockade of $P2X_7R$ decreased the incidence of acute GVHD and increased the number of Tregs. They also showed that STAT5, which has several binding sites in the Foxp3 promoter region, was involved in Treg induction in $P2X_7R$-deficient animals. CD39 dephosphorylates ATP to ADP and AMP and then CD73 dephosphorylates AMP to adenosine, which reduces inflammation. In the experimental GVHD models, the pharmacological blockade of CD73 enhanced GVHD activity [34], while an adenosine receptor agonist decreased acute GVHD [35]. In human recipients of allogeneic HCT, polymorphisms of $P2X_7R$, NALP2, and NALP3 are associated with survival differences in allogeneic HCT patients [36, 37]. Therefore, $P2X_7R$ signaling blockade might be a useful strategy for preventing acute GVHD caused by tissue damage during conditioning.

Heparan sulfate (HS)

Heparan sulfate (HS), an extracellular matrix component, can activate TLR4 on DC, which enhances DC maturation and alloreactive T-cell responses [38]. Treatment with the serine protease inhibitor α1-antitrypsin (A1AT) decreased serum levels of HS, leading to a reduction in GVHD severity. In the setting of allogeneic HCT, serum HS levels were increased and associated with the severity of GVHD. Tawara et al. [39] showed that A1AT treatment early after HCT reduced the expansion of alloreactive T-effector cells, but enhanced the recovery of Tregs and decreased mortality in experimental GVHD models. The administration of A1AT reduced serum proinflammatory cytokine levels and suppressed the LPS-induced secretion of proinflammatory cytokines in vitro, which enhanced the production of IL-10 in the host DCs. Another study showed that A1AT treatment reduces serum IL-32 levels and experimental GVHD severity [40]. These findings suggest that blocking HS release or administering A1AT might be an effective strategy for preventing GVHD.

Heat shock proteins (HSPs)

Heat shock proteins (HSPs) are ubiquitous chaperones that bind to and are involved in the folding and unfolding of other proteins. Extracellular HSPs released by dying cells activate innate immune responses via PRRs. HSPs also both induce the maturation of APCs and provide chaperoned polypeptides for triggering specific acquired immune responses. The 70 kilo Dalton HSP (HSP70) expression was correlated with high graft-versus-host responses in an in vitro-generated graft-versus-host reaction in human skin [41]. In human recipients of allogeneic HCT, recipient HSP polymorphisms are associated with a risk of acute GVHD [42].

Uric acid (UA)

Uric acid (UA) is released from dying cells and has adjuvant activity in vivo. UA enhanced DC maturation and amplified T-cell responses, and the elimination of UA in mouse models reduced the immune response [43]. Rasburicase is a recombinant urate-oxidase enzyme that catalyzes the oxidation of UA into an inactive soluble metabolite and is currently used to prevent tumor lysis syndrome. In a pilot trial, Brunner et al. [44] administered rasburicase to 23 patients beginning on the first day of conditioning therapy. They reported that rasburicase reduced the serum UA levels and there was significantly less grade II or higher acute GVHD in the rasburicase group compared with 44 comparable patients.

Hyaluronan (HA)

On tissue injury, high-molecular-weight (HMW) hyaluronan (HA), which is distributed ubiquitously in the extracellular matrix, is broken down into lower-molecular-weight (LMW) species. Milinkovic et al. [45] showed that hyaluronidase digestion of acute GVHD skin sections completely blocked CD44+ lymphocyte adherence to endothelium, suggesting that CD44– HA interactions contribute to lympho cytotropism to skin in acute GVHD. In addition to facilitating the recruitment of CD44+ leukocytes, LMW HA acts as an endogenous danger signal, leading to the activation of both innate and acquired immunity [46], although its relationship with GVHD is still underdetermined.

S100 proteins

S100 proteins are calcium binding and there are at least 21 different types of S100 protein. S100A8 and S100A9 are secreted by activated phagocytes and induce proinflammatory cytokines and adhesion molecules in endothelial cells [47]. Since a change in salivary constituents could reflect innate and adaptive immune responses during the development of GVHD, Chiusolo et al. [48] performed a proteome analysis of saliva from allogeneic HCT recipients with or without acute GVHD and healthy volunteers. They found significant differences among the three groups in terms of the frequency and levels of the proteins S100A8, S100A9, and S100A7, although further studies are needed to clarify the role of these proteins in the pathophysiology of acute GVHD.

High mobility group box 1 protein (HMGB1)

High mobility group box 1 protein (HMGB1) is expressed ubiquitously and located mostly in cell nuclei. HMGB1 is released on tissue damage as an endogenous DAMP and is actively produced by immune cells. Extracellular HMGB1 acts as a key molecule of innate immunity, downstream from persistent tissue injury, orchestrating inflammation, stem cell recruitment/activation, and eventual tissue remodeling [49]. Kornblit et al. [50] investigated HMGB1 polymorphisms and found associations between the HMGB1 genotype and outcome after allogeneic HCT following myeloablative (MA) conditioning, but not following nonmyeloablative (NMA) conditioning. The difference in the results between MA and NMA might be explained by a differential effect of HMGB1 depending on the intensity of the conditioning regimen. Inoue et al. [51] reported that a case of refractory acute GVHD complicated by thrombotic microangiopathy was treated successfully with recombinant thrombomodulin (rTM), which possesses the ability to neutralize HMGB1. TM is a membrane glycoprotein expressed mainly by vascular endothelial cells and is involved in coagulation and inflammation. Although the role of HMGB1 in GVHD is not completely clear, targeting innate immune cells and endothelial cells might lead to improved therapeutics in refractory acute GVHD complicated by thrombotic microangiopathy.

Conclusions and further directions

Despite improvements in clinical care, acute GVHD remains a major cause of morbidity and mortality for allogeneic HCT recipients and there is no standard treatment for patients with steroid-refractory GVHD. Most of the current prevention and treatment of acute GVHD targets donor T-cells. The blockade of DAMPs signaling involving ATP, HS, UA, and HMGB1 might be a useful strategy for preventing acute GVHD by reducing DC maturation and alloreactive T-cell responses. In addition to innate immune cells, endothelial cell dysfunction might lead to refractory GVHD treatment [52]. Luft et al. [52] revealed that rising levels of soluble TM, a marker of endothelial damage, were associated with steroid-refractory GVHD, suggesting that its pathogenesis involves progressive microangiopathy. Recently, DAMPs such as extracellular DNA, histones, and S100A8/A9 cause thrombotic microangiopathy [53]. An understanding of the role of PAMPs/DAMPs during the development of GVHD and also microangiopathies might offer new therapeutic approaches for steroid-refractory GVHD.

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Review

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

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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 1-2mg/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 $\gamma\delta$T 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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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 1–2mg/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].