Biol Blood Marrow Transplant xxx (2013) 1–9

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

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Article history:
Received 4 June 2013
Accepted 1 November 2013

Key Words:
Chronic graft-versus-host disease (GVHD)
Cyclosporine
Mammalian target of rapamycin (mTOR) inhibitor
Regulatory T cell

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

Financial disclosure: See Acknowledgments on page 8.

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http://dx.doi.org/10.1016/j.bbmt.2013.11.018

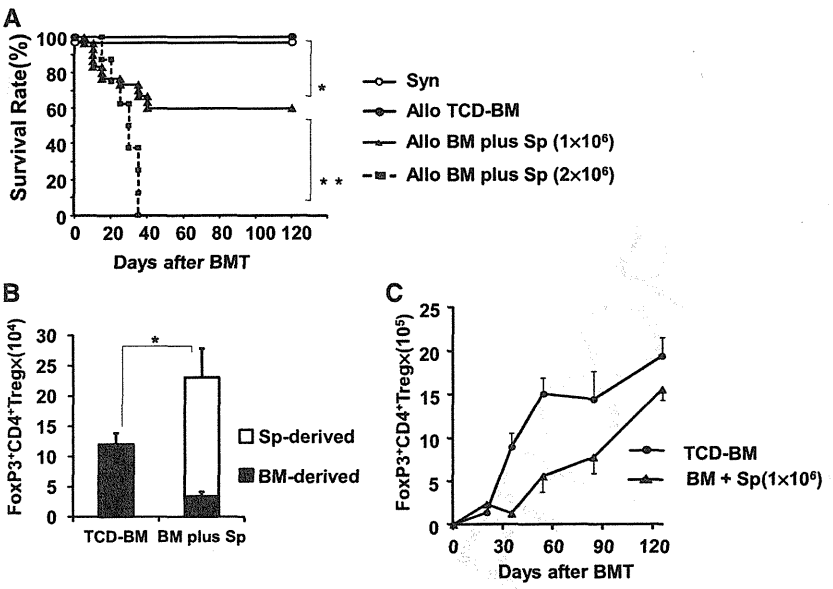

Figure 1. *Regulatory T cell reconstitution after allogeneic BMT. Lethally irradiated C3H (H-2^k) recipient mice received 10 × 10^6 T cell–depleted bone marrow (TCD-BM) cells from B6.Ly-5a (H-2^b,CD45.1) mice with/without 1 to 2 × 10^6 spleen cells from B6 (H-2^b,CD45.2) mice. The syngeneic group received 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-Ab1$^{-/-}$ 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

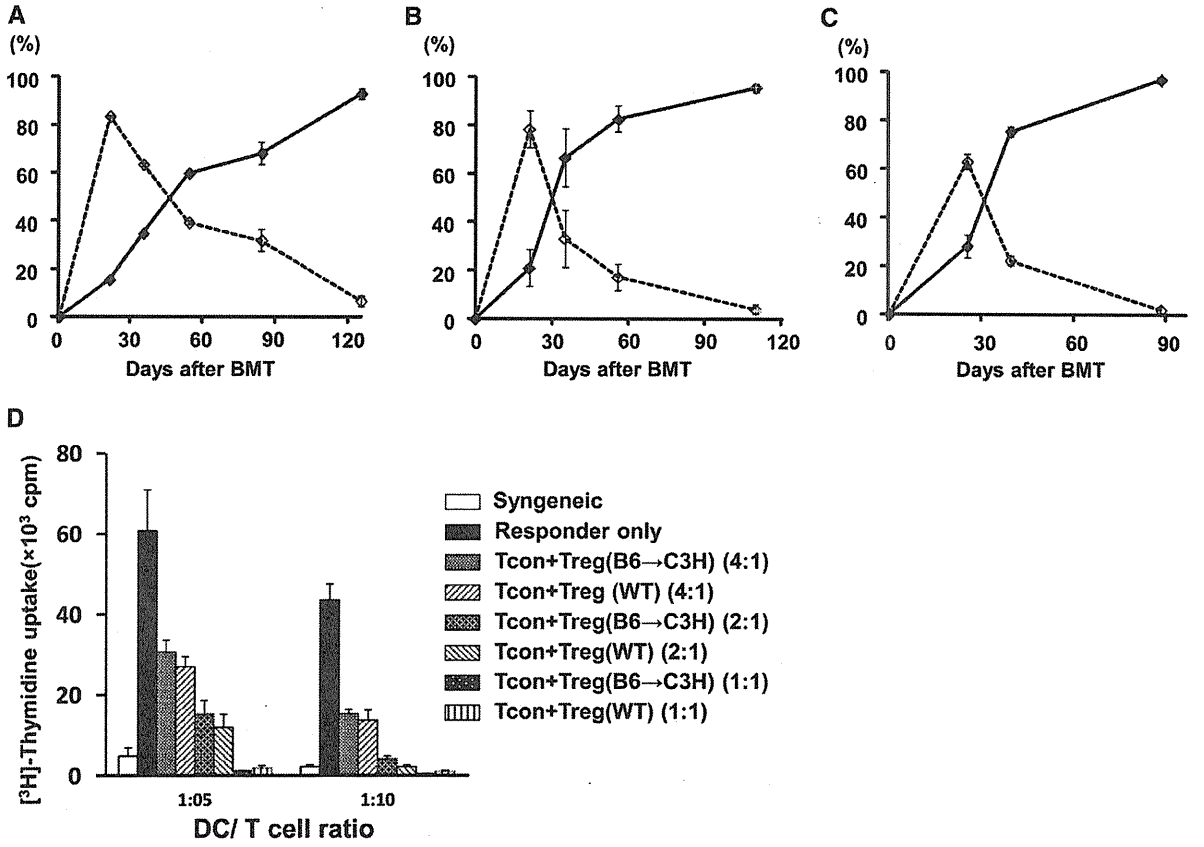

Figure 2. Donor BM–derived progenitors comprise the long-term peripheral Treg pool. Lethally irradiated C3H recipients underwent transplantation as in Figure 1: (B6 → C3H). The rates of CD45.2$^+$ spleen cell–derived (broken lines) and CD45.2$^-$ BM–derived (solid lines) Treg in CD4$^+$Foxp3$^+$ Treg 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-2^b) recipients underwent transplantation from B6 (H-2^b) donors. The rates of CD45.2$^+$ splenic T cell–derived (broken lines) and CD45.2$^-$ BM–derived (solid lines) 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 ^{3}H-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-2^b, H-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 ^{3}H-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-2^k) recipient mice received 10×10^6 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. 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

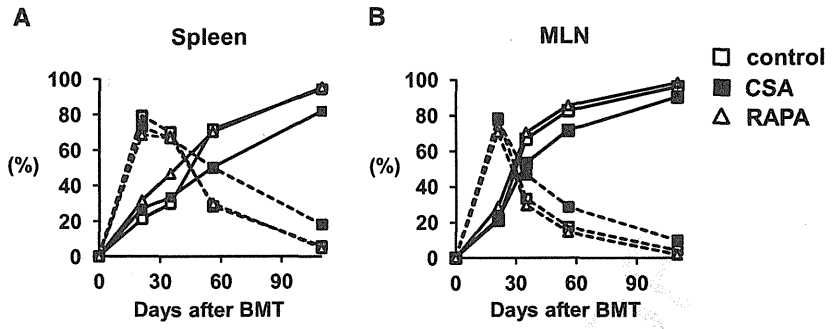

Figure 3. Effects of CSA and mTOR inhibitors on the Treg compartment. Lethally irradiated C3H recipients underwent transplantation from B6 donor mice as shown in Figure 1 and received i.p. injections of CSA (closed squares), mTOR inhibitor (rapamycin, RAPA; open triangles), or vehicle control (open squares) 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-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-2^b) into C3.SW (H-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

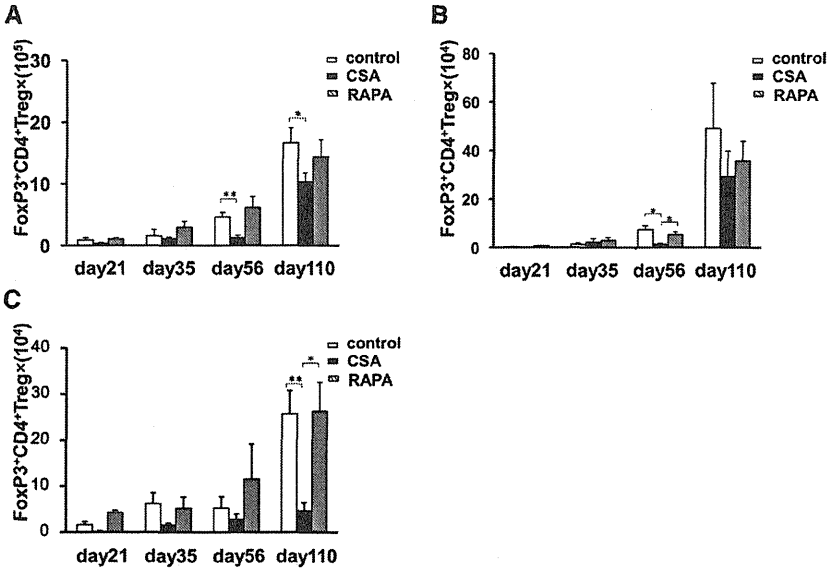

Figure 4. CSA, but not mTOR, inhibitors hampered reconstitution of BM-derived Treg. (B6 → C3H) mice received i.p. injections of CSA (black bars), mTOR inhibitor (rapamycin, RAPA; gray bars), or vehicle control (white bars) daily from day 0 to 110. The absolute numbers of Treg in the spleen (A), MLN (B), 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: $1.3 \pm .4$ versus $4.6 \pm .8 \times 10^5$, $P < .01$; day 110: 10.4 ± 1.4 versus $16.7 \pm 2.4 \times 10^5$, $P < .05$) (Figure 4A) and in the MLN (day 56: $1.3 \pm .5$ versus $7.4 \pm 1.6 \times 10^4$, $P < .03$; day 110: 2.9 ± 1.0 versus $4.9 \pm 1.9 \times 10^5$, $P = .46$) (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 $25.7 \pm 5.0 \times 10^4$, $P < .01$) (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 $16.6 \pm 2.4 \times 10^5$, $P = .73$, Supplemental Figure 1A; thymus: 17.4 ± 3.2 versus $25.7 \pm 5.0 \times 10^4$, $P = .26$, 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 ($5.5 \pm .8$ versus $1.6 \pm .3$, $P < .01$) (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 ($4.0 \pm .5$ versus $1.8 \pm .1$, $P < .01$) (Figure 5C). CSA-treated mice showed bile duct injury and

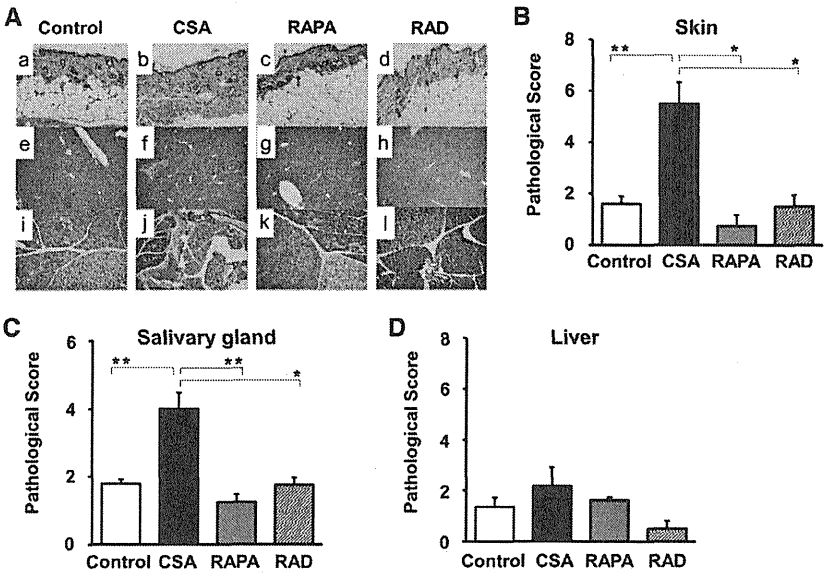

Figure 5. CSA, but not mTOR, inhibitors increased the likelihood of chronic GVHD. (A) Histological findings of the skin (a to d), liver (e to h), and salivary glands (i to l) (on day 120) from (B6 → C3H) mice given CSA, mTOR inhibitor (RAPA, RAD), or vehicle control. Sclerodermatous skin changes, such 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. $^*P < .05$; $^{**}P < .01$.

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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.

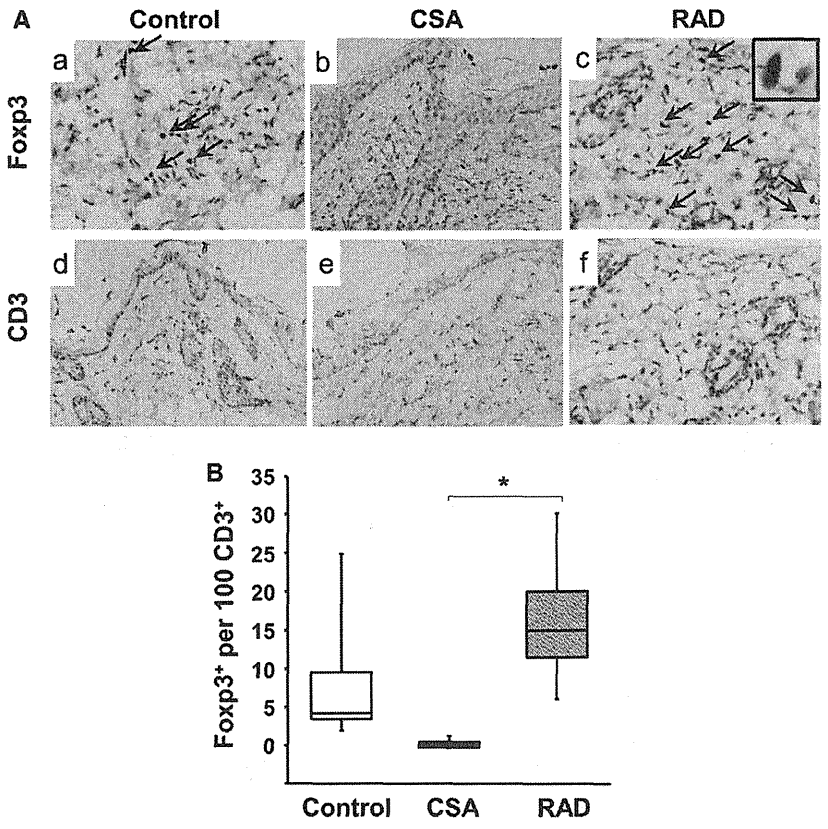

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Figure 6. CSA, but not mTOR, reduces Treg infiltration in skin tissue. (A) Lethally irradiated C3H recipients underwent transplantation from B6 donor mice as shown in Figure 1 and received vehicle control (a, d), CSA (b, e), or mTOR inhibitor (RAD; 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). $^*P < .05$.

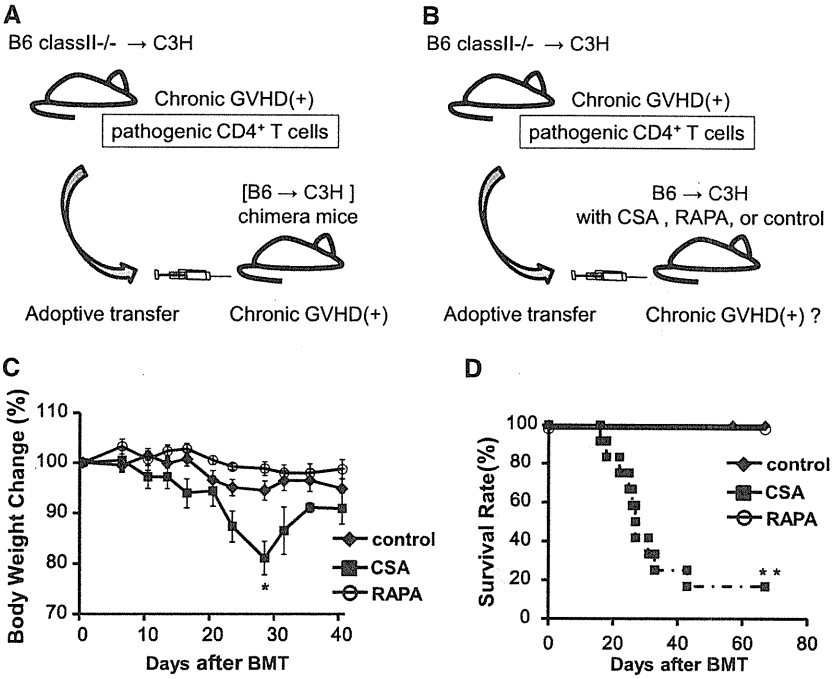

Figure 7. Adoptive transfer of pathogenic $CD4^+$ T cells caused severe chronic GVHD. (A) Lethally irradiated C3H recipients were reconstituted with TCD BM from MHC class II-deficient ($H2\text{-}Ab1^{-/-}$) B6 mice. These mice developed chronic GVHD and $CD4^+$ T cells isolated from chronic GVHD mice ([$H2\text{-}Ab1^{-/-}$→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 [$H2\text{-}Ab1^{-/-}$→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. Q4

ACKNOWLEDGMENTS

Q5 *Financial disclosure:* ■■■.

Conflict of interest statement: There are no conflicts of interest to report. Q6

SUPPLEMENTARY DATA

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

REFERENCES

1. Teshima T, Wynn TA, Soiffer RJ, et al. Chronic graft-versus-host disease: how can we release Prometheus? *Biol Blood Marrow Transplant.* 2008; 14:142-150.
2. Socie G, Stone JV, Wingard JR, et al. Long-term survival and late deaths after allogeneic bone marrow transplantation. Late Effects Working Committee of the International Bone Marrow Transplant Registry. *The New England journal of medicine.* 1999;341:14-21.
3. Baker KS, Gurney JG, Ness KK, et al. Late effects in survivors of chronic myeloid leukemia treated with hematopoietic cell transplantation: results from the Bone Marrow Transplant Survivor Study. *Blood.* 2004; 104:1898-1906.
4. Deeg HJ, Lin D, Leisenring W, et al. Cyclosporine of cyclosporine plus methylprednisolone for prophylaxis of graft-versus-host disease: a prospective, randomized trial. *Blood.* 1997;89:3880-3887.
5. Storb R, Deeg HJ, Pepe M, et al. Graft-versus-host disease prevention by methotrexate combined with cyclosporin compared to methotrexate alone in patients given marrow grafts for severe aplastic anaemia: long-term follow-up of a controlled trial. *Br J Haematol.* 1989;72:567-572.
6. Kansu E, Gooley T, Flowers ME, et al. Administration of cyclosporine for 24 months compared with 6 months for prevention of chronic graft-versus-host disease: a prospective randomized clinical trial. *Blood.* 2001;98:3868-3870.
7. Couriel DR, Saliba R, Escalon MP, et al. Sirolimus in combination with tacrolimus and corticosteroids for the treatment of resistant chronic graft-versus-host disease. *Br J Haematol.* 2005;130:409-417.
8. Johnston LJ, Brown J, Shizuru JA, et al. Rapamycin (sirolimus) for treatment of chronic graft-versus-host disease. *Biol Blood Marrow Transplant.* 2005;11:47-55.
9. Jurado M, Vallejo C, Perez-Simon JA, et al. Sirolimus as part of immunosuppressive therapy for refractory chronic graft-versus-host disease. *Biol Blood Marrow Transplant.* 2007;13:701-706.
10. Jedlickova Z, Burlakova I, Bug G, et al. Therapy of sclerodermatous chronic graft-versus-host disease with mammalian target of rapamycin inhibitors. *Biol Blood Marrow Transplant.* 2011;17:657-663.
11. Cutler C, Logan BR, Nakamura R, et al. Tacrolimus/sirolimus vs. tacrolimus/methotrexate for graft-vs.-host disease prophylaxis after hla-matched, related donor hematopoietic stem cell transplantation: results of Blood and Marrow Transplant Clinical Trials Network Trial 0402. [ASH Annual Meeting Abstracts] Blood 2012;120:739.
12. Edinger M, Hoffmann P, Ermann J, et al. CD4+CD25+ regulatory T cells preserve graft-versus-tumor activity while inhibiting graft-versus-host disease after bone marrow transplantation. *Nature Med.* 2003;9: 1144-1150.
13. Hoffmann P, Ermann J, Edinger M, et al. Donor-type CD4(+)CD25(+) regulatory T cells suppress lethal acute graft-versus-host disease after allogeneic bone marrow transplantation. *J Exp Med.* 2002;196:389-399.
14. Taylor PA, Lees CJ, Blazar BR. The infusion of ex vivo activated and expanded CD4(+)CD25(+) immune regulatory cells inhibits graft-versus-host disease lethality. *Blood.* 2002;99:3493-3499.
15. Zeiser R, Leveson-Gower DB, Zambricki EA, et al. Differential impact of mammalian target of rapamycin inhibition on CD4+CD25+Foxp3+ regulatory T cells compared with conventional CD4+ T cells. *Blood.* 2008;111:453-462.
16. Shin HJ, Baker J, Leveson-Gower DB, et al. Rapamycin and IL-2 reduce lethal acute graft-versus-host disease associated with increased expansion of donor type CD4+CD25+Foxp3+ regulatory T cells. *Blood.* 2011;118:2342-2350.
17. Fontenot JD, Rasmussen JP, Gavin MA, Rudensky AY. A function for interleukin 2 in Foxp3-expressing regulatory T cells. *Nature Immunol.* 2005;6:1142-1151.
18. D'Cruz LM, Klein L. Development and function of agonist-induced CD25+Foxp3+ regulatory T cells in the absence of interleukin 2 signaling. *Nature Immunol.* 2005;6:1152-1159.
19. Setoguchi R, Hori S, Takahashi T, Sakaguchi S. Homeostatic maintenance of natural Foxp3(+) CD25(+) CD4(+) regulatory T cells by interleukin (IL)-2 and induction of autoimmune disease by IL-2 neutralization. *J Exp Med.* 2005;201:723-735.
20. Grusby MJ, Johnson RS, Papaioannou VE, Glimcher LH. Depletion of CD4+ T cells in major histocompatibility complex class II-deficient mice. *Science.* 1991;253:1417-1420.
21. Anderson BE, McNiff JM, Matte C, et al. Recipient CD4+ T cells that survive irradiation regulate chronic graft-versus-host disease. *Blood.* 2004;104:1565-1573.
22. Reddy P, Maeda Y, Liu C, et al. A crucial role for antigen-presenting cells and alloantigen expression in graft-versus-leukemia responses. *Nature Med.* 2005;11:1244-1249.
23. Sakoda Y, Hashimoto D, Asakura S, et al. Donor-derived thymic-dependent T cells cause chronic graft-versus-host disease. *Blood.* 2007;109:1756-1764.
24. Matsumoto Y, Hof A, Baumlin Y, et al. Differential effects of everolimus and cyclosporine A on intimal alpha-actin-positive cell dynamics of carotid allografts in mice. *Transplantation.* 2004;78:345-351.
25. Kaplan DH, Anderson BE, McNiff JM, et al. Target antigens determine graft-versus-host disease phenotype. *J Immunol.* 2004;173:5467-5475.
26. Duffner UA, Maeda Y, Cooke KR, et al. Host dendritic cells alone are sufficient to initiate acute graft-versus-host disease. *J Immunol.* 2004; 172:7393-7398.
27. Maeda Y, Tawara I, Teshima T, et al. Lymphopenia-induced proliferation of donor T cells reduces their capacity for causing acute graft-versus-host disease. *Exp Hematol.* 2007;35:274-286.
28. Coenen JJ, Koenen HJ, van Rijssen E, et al. Rapamycin, not cyclosporine, permits thymic generation and peripheral preservation of CD4+ CD25+ FoxP3+ T cells. *Bone Marrow Transplant.* 2007;39:537-545.
29. Miura Y, Thoburn CJ, Bright EC, et al. Association of Foxp3 regulatory gene expression with graft-versus-host disease. *Blood.* 2004;104: 2187-2193.
30. Rieger K, Loddenkemper C, Maul J, et al. Mucosal FOXP3+ regulatory T cells are numerically deficient in acute and chronic GvHD. *Blood.* 2006; 107:1717-1723.

31. Zorn E, Kim HT, Lee SJ, et al. Reduced frequency of FOXP3+ CD4+CD25+ regulatory T cells in patients with chronic graft-versus-host disease. *Blood*. 2005;106:2903-2911.
32. Matsuoka K, Kim HT, McDonough S, et al. Altered regulatory T cell homeostasis in patients with CD4+ lymphopenia following allogeneic hematopoietic stem cell transplantation. *J Clin Invest*. 2010;120:1479-1493.
33. Bayer AL, Jones M, Chirinos J, et al. Host CD4+CD25+ T cells can expand and comprise a major component of the Treg compartment after experimental HCT. *Blood*. 2009;113:733-743.
34. Zeiser R, Nguyen VH, Beilhack A, et al. Inhibition of CD4+CD25+ regulatory T-cell function by calcineurin-dependent interleukin-2 production. *Blood*. 2006;108:390-399.
35. Dunn CJ, Wagstaff AJ, Perry CM, et al. Cyclosporin: an updated review of the pharmacokinetic properties, clinical efficacy and tolerability of a microemulsion-based formulation (neoral)1 in organ transplantation. *Drugs*. 2001;61:1957-2016.
36. Mantel PY, Ouaked N, Ruckert B, et al. Molecular mechanisms underlying FOXP3 induction in human T cells. *J Immunol*. 2006;176:3593-3602.
37. Abraham RT, Wiederrecht GJ. Immunopharmacology of rapamycin. *Annual review of immunology*. 1996;14:483-510.
38. Blazar BR, Taylor PA, Panoskaltsis-Mortari A, Vallera DA. Rapamycin inhibits the generation of graft-versus-host disease- and graft-versus-leukemia-causing T cells by interfering with the production of Th1 or Th1 cytotoxic cytokines. *J Immunol*. 1998;160:5355-5365.
39. Hess AD, Fischer AC, Horwitz L, et al. Characterization of peripheral autoregulatory mechanisms that prevent development of cyclosporin-induced syngeneic graft-versus-host disease. *J Immunol*. 1994;153:400-411.
40. Bucy RP, Xu XY, Li J, Huang G. Cyclosporin A-induced autoimmune disease in mice. *J Immunol*. 1993;151:1039-1050.
41. Wu DY, Goldschneider I. Cyclosporin A-induced autologous graft-versus-host disease: a prototypical model of autoimmunity and active (dominant) tolerance coordinately induced by recent thymic emigrants. *J Immunol*. 1999;162:6926-6933.
42. Cutler C, Li S, Ho VT, et al. Extended follow-up of methotrexate-free immunosuppression using sirolimus and tacrolimus in related and unrelated donor peripheral blood stem cell transplantation. *Blood*. 2007;109:3108-3114.
43. Alyea EP, Li S, Kim HT, et al. Sirolimus, tacrolimus, and low-dose methotrexate as graft-versus-host disease prophylaxis in related and unrelated donor reduced-intensity conditioning allogeneic peripheral blood stem cell transplantation. *Biol Blood Marrow Transplant*. 2008;14:920-926.
44. Rodriguez R, Nakamura R, Palmer JM, et al. A phase II pilot study of tacrolimus/sirolimus GVHD prophylaxis for sibling donor hematopoietic stem cell transplantation using 3 conditioning regimens. *Blood*. 2010;115:1098-1105.
45. Rosenbeck LL, Kiel PJ, Kalsekar I, et al. Prophylaxis with sirolimus and tacrolimus +/- antithymocyte globulin reduces the risk of acute graft-versus-host disease without an overall survival benefit following allogeneic stem cell transplantation. *Biol Blood Marrow Transplant*. 2011;17:916-922.
46. Schleuning M, Judith D, Jedlickova Z, et al. Calcineurin inhibitor-free GVHD prophylaxis with sirolimus, mycophenolate mofetil and ATG in Allo-SCT for leukemia patients with high relapse risk: an observational cohort study. *Bone Marrow Transplant*. 2009;43:717-723.

Int J Hematol (2013) 98:293–299
DOI 10.1007/s12185-013-1421-x

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

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Received: 18 July 2013 / Accepted: 19 July 2013 / Published online: 28 August 2013

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

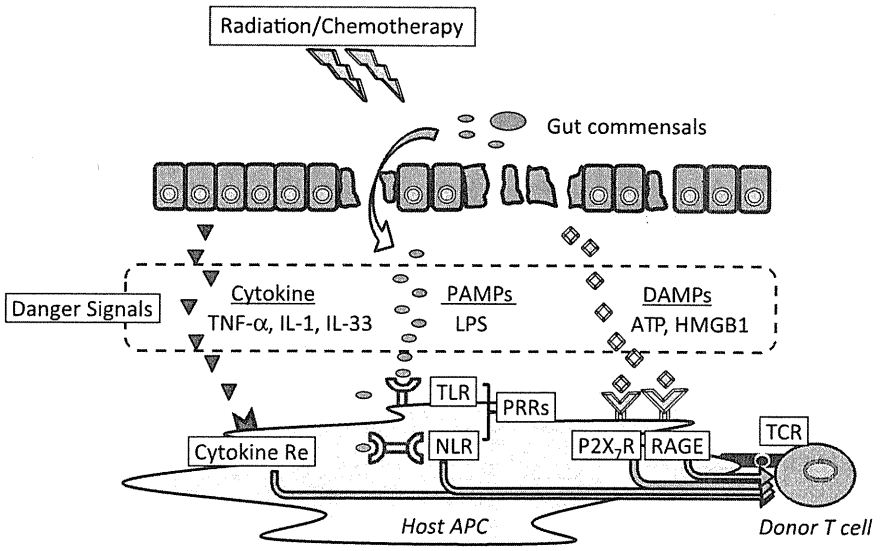

Fig. 1 Importance of innate immune response activated by danger signals in GVHD. The conditioning regimen which includes total body irradiation and/or chemotherapy leads to the damage of host tissues. Damaged cells release "danger signals" including cytokines, exogenous pathogen-associated molecular 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, *$P2X_7R$* 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.

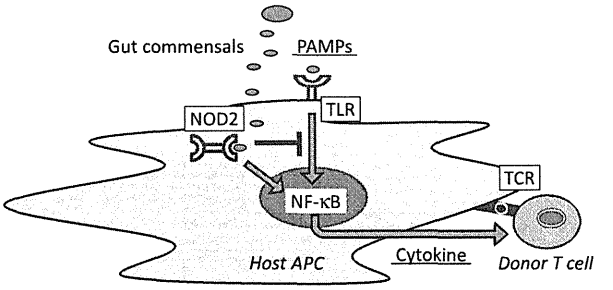

Fig. 2 PAMPs are recognized by NLRs and also by TLRs. A member of the nucleotide-binding oligomerization domain (*NOD*)-like receptors (*TLRs*), NOD2 recognizes pathogen-associated molecular patterns (*PAMPs*) as a primary sensor and induces NF-kB activation leading to enhance T-cell responses. In addition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

References

1. Barnes DW, Loutit JF, Micklem HS. "Secondary disease" of radiation chimeras: a syndrome due to lymphoid aplasia. Ann NY Acad Sci. 1962;99:374–85.
2. Billingham RE. The biology of graft-versus-host reactions. Harvey Lect. 1966;62:21–78.

3. Shlomchik WD, Couzens MS, Tang CB, et al. Prevention of graft-versus-host disease by inactivation of host antigen-presenting cells. Science. 1999;285:412–5.
4. Duffner UA, Maeda Y, Cooke KR, et al. Host dendritic cells alone are sufficient to initiate acute graft-versus-host disease. J Immunol. 2004;172:7393–8.
5. Levine JE. Implications of TNF-alpha in the pathogenesis and management of GVHD. Int J Hematol. 2011;93:571–7.
6. Shin OS, Harris JB. Innate immunity and transplantation tolerance: the potential role of TLRs/NLRs in GVHD. Korean J Hematol. 2011;46:69–79.
7. Cooke KR, Hill GR, Crawford JM, et al. Tumor necrosis factor-alpha production to lipopolysaccharide stimulation by donor cells predicts the severity of experimental acute graft-versus-host disease. J Clin Invest. 1998;102:1882–91.
8. Cooke KR, Gerbitz A, Crawford JM, et al. LPS antagonism reduces graft-versus-host disease and preserves graft-versus-leukemia activity after experimental bone marrow transplantation. J Clin Invest. 2001;107:1581–9.
9. Garantziotis S, Palmer SM, Snyder LD, et al. Alloimmune lung injury induced by local innate immune activation through inhaled lipopolysaccharide. Transplantation. 2007;84:1012–9.
10. Lorenz E, Schwartz DA, Martin PJ, et al. Association of TLR4 mutations and the risk for acute GVHD after HLA-matched-sibling hematopoietic stem cell transplantation. Biol Blood Marrow Transplant. 2001;7:384–7.
11. Elmaagacli AH, Koldehoff M, Hindahl H, et al. Mutations in innate immune system NOD2/CARD 15 and TLR-4 (Thr399Ile) genes influence the risk for severe acute graft-versus-host disease in patients who underwent an allogeneic transplantation. Transplantation. 2006;81:247–54.
12. Chakraverty R, Cote D, Buchli J, et al. An inflammatory checkpoint regulates recruitment of graft-versus-host reactive T-cells to peripheral tissues. J Exp Med. 2006;203:2021–31.
13. Taylor PA, Ehrhardt MJ, Lees CJ, et al. TLR agonists regulate alloresponses and uncover a critical role for donor APCs in allogeneic bone marrow rejection. Blood. 2008;112:3508–16.
14. Jasperson LK, Bucher C, Panoskaltsis-Mortari A, et al. Indoleamine 2,3-dioxygenase is a critical regulator of acute graft-versus-host disease lethality. Blood. 2008;111:3257–65.
15. Jasperson LK, Bucher C, Panoskaltsis-Mortari A, et al. Inducing the tryptophan catabolic pathway, indoleamine 2,3-dioxygenase (IDO), for suppression of graft-versus-host disease (GVHD) lethality. Blood. 2009;114:5062–70.
16. Crellin NK, Garcia RV, Hadisfar O, et al. Human CD4+ T-cells express TLR5 and its ligand flagellin enhances the suppressive capacity and expression of FOXP3 in CD4+ CD25+ T regulatory cells. J Immunol. 2005;175:8051–9.
17. Galkin VE, Yu X, Bielnicki J, et al. Divergence of quaternary structures among bacterial flagellar filaments. Science. 2008;320:382–5.
18. Hossain MS, Jaye DL, Pollack BP, et al. Flagellin, a TLR5 agonist, reduces graft-versus-host disease in allogeneic hematopoietic stem cell transplantation recipients while enhancing antiviral immunity. J Immunol. 2011;187:5130–40.
19. Calcaterra C, Sfondrini L, Rossini A, et al. Critical role of TLR9 in acute graft-versus-host disease. J Immunol. 2008;181:6132–9.
20. Heimesaat MM, Nogai A, Bereswill S, et al. MyD88/TLR9 mediated immunopathology and gut microbiota dynamics in a novel murine model of intestinal graft-versus-host disease. Gut. 2010;59:1079–87.
21. Elmaagacli AH, Koldehoff M, Beelen DW. Improved outcome of hematopoietic SCT in patients with homozygous gene variant of Toll-like receptor 9. Bone Marrow Transplant. 2009;44:295–302.
22. Penack O, Smith OM, Cunningham-Bussel A, et al. NOD2 regulates hematopoietic cell function during graft-versus-host disease. J Exp Med. 2009;206:2101–10.
23. Watanabe T, Asano N, Murray PJ, et al. Muramyl dipeptide activation of nucleotide-binding oligomerization domain 2 protects mice from experimental colitis. J Clin Invest. 2008;118:545–59.
24. Watanabe T, Kitani A, Murray PJ, Strober W. NOD2 is a negative regulator of Toll-like receptor 2-mediated T helper type 1 responses. Nat Immunol. 2004;5:800–8.
25. Holler E, Rogler G, Herfarth H, et al. Both donor and recipient NOD2/CARD15 mutations associate with transplant-related mortality and GvHD following allogeneic stem cell transplantation. Blood. 2004;104:889–94.
26. Holler E, Rogler G, Brenmoehl J, et al. Prognostic significance of NOD2/CARD15 variants in HLA-identical sibling hematopoietic stem cell transplantation: effect on long-term outcome is confirmed in 2 independent cohorts and may be modulated by the type of gastrointestinal decontamination. Blood. 2006;107:4189–93.
27. Granell M, Urbano-Ispizua A, Arostegui JI, et al. Effect of NOD2/CARD15 variants in T-cell depleted allogeneic stem cell transplantation. Haematologica. 2006;91:1372–6.
28. Mayor NP, Shaw BE, Hughes DA, et al. Single nucleotide polymorphisms in the NOD2/CARD15 gene are associated with an increased risk of relapse and death for patients with acute leukemia after hematopoietic stem-cell transplantation with unrelated donors. J Clin Oncol. 2007;25:4262–9.
29. Sairafi D, Uzunel M, Remberger M, et al. No impact of NOD2/CARD15 on outcome after SCT. Bone Marrow Transplant. 2008;41:961–4.
30. Gruhn B, Intek J, Pfaffendorf N, et al. Polymorphism of interleukin-23 receptor gene but not of NOD2/CARD15 is associated with graft-versus-host disease after hematopoietic stem cell transplantation in children. Biol Blood Marrow Transplant. 2009;15:1571–7.
31. Tanabe T, Yamaguchi N, Matsuda K, et al. Association analysis of the NOD2 gene with susceptibility to graft-versus-host disease in a Japanese population. Int J Hematol. 2011;93:771–8.
32. Penack O, Holler E, van den Brink MR. Graft-versus-host disease: regulation by microbe-associated molecules and innate immune receptors. Blood. 2010;115:1865–72.
33. Wilhelm K, Ganesan J, Muller T, et al. Graft-versus-host disease is enhanced by extracellular ATP activating P2X7R. Nat Med. 2010;16:1434–8.
34. Tsukamoto H, Chernogorova P, Ayata K, et al. Deficiency of CD73/ecto-5′-nucleotidase in mice enhances acute graft-versus-host disease. Blood. 2012;119:4554–64.
35. Lappas CM, Liu PC, Linden J, et al. Adenosine A2A receptor activation limits graft-versus-host disease after allogenic hematopoietic stem cell transplantation. J Leukoc Biol. 2010;87:345–54.
36. Granell M, Urbano-Ispizua A, Pons A, et al. Common variants in NLRP2 and NLRP3 genes are strong prognostic factors for the outcome of HLA-identical sibling allogeneic stem cell transplantation. Blood. 2008;112:4337–42.
37. Lee KH, Park SS, Kim I, et al. P2X7 receptor polymorphism and clinical outcomes in HLA-matched sibling allogeneic hematopoietic stem cell transplantation. Haematologica. 2007;92:651–7.
38. Brennan TV, Lin L, Huang X, et al. Heparan sulfate, an endogenous TLR4 agonist, promotes acute GVHD after allogeneic stem cell transplantation. Blood. 2012;120:2899–908.
39. Tawara I, Sun Y, Lewis EC, et al. Alpha-1-antitrypsin monotherapy reduces graft-versus-host disease after experimental allogeneic bone marrow transplantation. Proc Natl Acad Sci USA. 2012;109:564–9.
40. Marcondes AM, Li X, Tabellini L, et al. Inhibition of IL-32 activation by alpha-1 antitrypsin suppresses alloreactivity and increases survival in an allogeneic murine marrow transplantation model. Blood. 2011;118:5031–9.

41. Jarvis M, Marzolini M, Wang XN, et al. Heat shock protein 70: correlation of expression with degree of graft-versus-host response and clinical graft-versus-host disease. Transplantation. 2003;76:849–53.
42. Bogunia-Kubik K, Lange A. HSP70-hom gene polymorphism in allogeneic hematopoietic stem-cell transplant recipients correlates with the development of acute graft-versus-host disease. Transplantation. 2005;79:815–20.
43. Shi Y, Evans JE, Rock KL. Molecular identification of a danger signal that alerts the immune system to dying cells. Nature. 2003;425:516–21.
44. Brunner AM, Thomas R. Spitzer TR, Chen Yi-Bin A et al. Urate Oxidase (Rasburicase) to Inhibit Graft Versus Host Disease (GVHD) After Myeloablative HLA-Matched Allogeneic Hematopoietic Cell Transplantation (HCT). ASH (Annual Meeting Abstracts) 2012;120: Abstract 3063.
45. Milinkovic M, Antin JH, Hergrueter CA, et al. CD44– hyaluronic acid interactions mediate shear-resistant binding of lymphocytes to dermal endothelium in acute cutaneous GVHD. Blood. 2004;103:740–2.
46. Shirali AC, Goldstein DR. Activation of the innate immune system by the endogenous ligand hyaluronan. Curr Opin Organ Transplant. 2008;13:20–5.
47. Ehrchen JM, Sunderkotter C, Foell D, et al. The endogenous Toll-like receptor 4 agonist S100A8/S100A9 (calprotectin) as innate amplifier of infection, autoimmunity, and cancer. J Leukoc Biol. 2009;86:557–66.
48. Chiusolo P, Giammarco S, Fanali C, et al. Salivary proteomic analysis and acute graft-versus-host disease after allogeneic hematopoietic stem cell transplantation. Biol Blood Marrow Transplant. 2013;19:888–92.
49. Castiglioni A, Canti V, Rovere-Querini P, Manfredi AA. High-mobility group box 1 (HMGB1) as a master regulator of innate immunity. Cell Tissue Res. 2011;343:189–99.
50. Kornblit B, Masmas T, Petersen SL, et al. Association of HMGB1 polymorphisms with outcome after allogeneic hematopoietic cell transplantation. Biol Blood Marrow Transplant. 2010;16:239–52.
51. Inoue Y, Kosugi S, Miura I, et al. Successful treatment of refractory acute GVHD complicated by severe intestinal transplant-associated thrombotic microangiopathy using recombinant thrombomodulin. Thromb Res. 2011;127:603–4.
52. Luft T, Dietrich S, Falk C, et al. Steroid-refractory GVHD: T-cell attack within a vulnerable endothelial system. Blood. 2011;118: 1685–92.
53. Fuchs TA, Kremer Hovinga JA, Schatzberg D et al. Circulating DNA and myeloperoxidase indicate disease activity in patients with thrombotic microangiopathies. Blood 2012;120:1157–1164.

Synthetic retinoid Am80 ameliorates chronic graft-versus-host disease by down-regulating Th1 and Th17

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Chronic GVHD (cGVHD) is a main cause of late death and morbidity after allogeneic hematopoietic cell transplantation, but its pathogenesis remains unclear. We investigated the roles of Th subsets in cGVHD with the use of a well-defined mouse model of cGVHD. In this model, development of cGVHD was associated with up-regulated Th1, Th2, and Th17 responses. Th1 and Th2 responses were up-regulated early after BM transplantation, followed by a subsequent up-regulation of Th17 cells. Significantly greater numbers of Th17 cells were infiltrated in the lung and liver from allogeneic recipients than those from syngeneic recipients. We then evaluated the roles of Th1 and Th17 in cGVHD with the use of IFN-γ–deficient and IL-17–deficient mice as donors. Infusion of IFN-$\gamma^{-/-}$ or IL-$17^{-/-}$ T cells attenuated cGVHD in the skin and salivary glands. Am80, a potent synthetic retinoid, regulated both Th1 and Th17 responses as well as TGF-β expression in the skin, resulting in an attenuation of cutaneous cGVHD. These results suggest that Th1 and Th17 contribute to the development of cGVHD and that targeting Th1 and Th17 may therefore represent a promising therapeutic strategy for preventing and treating cGVHD. (*Blood.* 2012; 119(1):285-295)

Introduction

GVHD is a result of immune attack of host tissues, such as the skin, gut, liver, and lung, by donor T cells in transplants.[1,2] On the basis of the differences in clinical manifestations and histopathology, GVHD can be divided into acute and chronic types. Chronic GVHD (cGVHD) is the main cause of late death and morbidity after allogeneic hematopoietic stem cell transplantation.[3-5] cGVHD often presents with clinical manifestations that resemble those observed in autoimmune diseases, such as systemic lupus erythematosus, Sjögren syndrome, lichen planus, and scleroderma. It has traditionally been assumed that the predominant cytokines produced during acute GVHD are Th1 cytokines, whereas those produced during cGVHD are Th2 cytokines. Although recent studies have suggested that cGVHD could be caused by cytokines secreted by Th1 cells,[6] Th17 cells,[7] or autoantibodies,[8] or both, the immune mechanisms leading to the development of cGVHD are not completely understood.

Th17 cells are a third subset of polarized effector T cells characterized by their expression of proinflammatory cytokine IL-17 and other cytokines.[9] IL-17 belongs to a family of 6 members: IL-17A, IL-17B, IL-17C, IL-17D, IL-17E (also known as IL-25), and IL-17F. Of these, IL-17A and IL-17F are the best characterized cytokines and form heterodimers. IL-17 plays an important role in the control and clearance of various pathogens.[9] In addition, Th17 cells have been implicated in allograft rejection of solid organs and several autoimmune diseases.[10,11] Although a number of studies have addressed how Th17 cells contribute to GVHD[12] and have reported that Th17 cells are sufficient but not necessary to induce acute GVHD,[13,14] the functional role of Th17 in cGVHD is unclear.

Retinoic acid, the active metabolite of vitamin A, has multiple effects on cell differentiation and survival by ligating the receptors from 2 families, retinoic acid receptors (RARs) and retinoid X receptors, each of which exists in multiple isoforms.[15] All-*trans*-retinoic acid (ATRA) has been reported to inhibit IFN-γ synthesis by Th1 cells and to suppress the differentiation of Th17 cells by down-regulating the orphan nuclear receptor RORγt, a key regulator of Th17 differentiation.[16-19] Am80 is a novel RARα/β-specific synthetic retinoid that shows ~ 10-fold more potent biologic activity than ATRA by binding to RARα and RARβ but not to RARγ.[20] Am80 also inhibits IL-6 signaling[20,21] and reduces the severity and progression of inflammatory disease models.[20-23]

In the present study, we used the B10.D2 (H-2^d) into BALB/c (H-2^d) MHC-compatible, multiple minor histocompatibility Ag (miHA)–incompatible model of cGVHD to address the contribution of Th1/Th17 cells and the effects of retinoids on cGVHD with the use of IFN-$\gamma^{-/-}$ mice and IL-$17^{-/-}$ mice as donors. We also tested the hypothesis that the administration of Am80 ameliorates cGVHD by reducing the levels of Th1 and Th17 inflammatory cytokines and the fibrosis factor TGF-β.

Submitted January 26; 2011; accepted October 29, 2011. Prepublished online as *Blood* First Edition paper, November 10, 2011; DOI 10.1182/blood-2011-01-332478.

The online version of this article contains a data supplement.

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Methods

Mice

Female B10.D2 (H-2^d) mice were purchased from Japan SLC. BALB/c (H-2^d) recipient mice were purchased from Charles River Japan. IL-17A–deficient (IL-17$^{-/-}$) mice with the BALB/c background were generated previously.[24] IFN-γ–deficient (IFN-γ$^{-/-}$) mice were purchased from The Jackson Laboratory. IL-17$^{-/-}$ and IFN-γ$^{-/-}$ mice with the B10.D2 background were backcrossed for 8-10 generations from the original knockout mice. All experiments involving animals were performed according to the regulations of the Institutional Animal Care and Research Advisory Committee, Okayama University Advanced Science Research Center.

BM transplantation

Mice received transplants according to the standard protocols described previously.[25] Briefly, BALB/c mice received a single dose of 6.75 Gy x-ray total body irradiation. Recipient mice were injected with 2×10^6 spleen T cells and 8×10^6 T cell–depleted BM (TCD-BM) cells from B10.D2 donors. T-cell depletion and purification were performed with anti-CD90.2 Microbeads, pan T-cell isolation kit, and CD25 isolation kit and an AutoMACS system (Miltenyi Biotec) according to the manufacturer's instructions. Donor cells were injected intravenously into the recipients on day 0.

Evaluation of cGVHD

After BM transplantation (BMT), animals were weighed every 3 days and scored for skin manifestations of GVHD. The following scoring system was used[25]: healthy appearance, 0; skin lesions with alopecia < 1 cm^2 in area, 1; skin lesions with alopecia 1-2 cm^2 in area, 2; skin lesions with alopecia > 2 cm^2 in area, 3. In addition, animals were assigned 0.3 points each for skin disease (lesions or scaling) on the ears, tails, and paws. The minimum score was 0, and the maximum score was 3.9.

Tissue histopathology

Shaved skin from the interscapular region (~ 2 cm^2), the left lung, liver, and colon specimens of recipients were fixed in 10% formalin, embedded in paraffin, sectioned, mounted on slides, and stained with H&E. Slides were scored by a pathologist blind to experimental group (K.T.) on the basis of dermal fibrosis, fat loss, inflammation, epidermal interface changes, and follicular drop-out (0-2 for each category; the maximum score was 10).[25] Lung, liver, and colon slides were scored by a pathologist blind to the experimental group (T.T.). Lung slides were scored according to periluminal infiltrates, pneumonitis, and the extent of injury (0-3 for each category), and the maximum score was 9.[26] Liver slides were scored according to bile duct injury and inflammation (0-4 for each category), and the maximum score was 8.[27] Colon slides were scored according to crypt apoptosis and inflammation (0-4 for each category), and the maximum score was 8.[27]

Intracellular cytokine staining and cytokine analysis

Organs from mice were removed, processed into single-cell suspensions, and stimulated in vitro with 50 ng/mL phorbol 12-myristate 13-acetate (PMA; Sigma-Aldrich) and 100 ng/mL ionomycin (Sigma-Aldrich) at 37°C for 3 hours. Cells were then incubated with GolgiStop (BD PharMingen) for an additional 2 hours. mAbs conjugated to fluorescein isothiocyanate, phycoerythrin, peridinin-chlorophyll protein complexes, allophycocyanin, or Alexa Fluor 488 were used to assess the cell populations and were purchased from BD PharMingen or eBioscience. Cells were analyzed on a FACSCalibur flow cytometer with CellQuest software (both from Becton Dickinson) or MACS Quant flow cytometer (Miltenyi Biotec) with FlowJo software (TreeStar); both were housed in the Central Research Laboratory, Okayama University Medical School. Total peripheral lymph node (PLN) cells were adjusted to 1×10^6/mL in cultures. Supernatants were removed, and cytokine levels were measured with a BD Cytometric Bead Array (CBA) or by ELISA (R&D Systems) according to the respective manufacturer's protocol.

IFN-γ neutralization

Anti–mouse IFN-γ mAbs for in vivo experiments were prepared from mouse ascites from clones R4-6A2. Mice were treated intraperitoneally with anti–IFN-γ mAbs or rat IgG (160 μg/mouse; Sigma-Aldrich) on days 0, 5, 10, and 15 after BMT.

Administration of ATRA and Am80

Recipients were orally administered ATRA (200 μg/mouse; Wako), Am80 (1.0 mg/kg body weight; Nippon Shinyaku), or vehicle solutions daily from day 0.

Real-time RT-PCR

Total RNA was isolated from homogenized ear tissue with the use of an RNeasy mini kit (QIAGEN). cDNA synthesis was initiated by application of oligo dT primers and TaqMan Reverse Transcription Reagents (Applied Biosystems). Target cDNA levels were quantified by real-time PCR. The TaqMan Universal PCR Master Mix and the following Assay-on-Demand mouse gene-specific fluorescently labeled TaqMan MGB probes were used in an ABI Prism 5300 sequence detection system (Applied Biosystems): Mm01178820_m1 (TGF-β1). The mRNA expression of individual genes was normalized relative to GAPDH with the use of the equation $dCt = Ct_{target} - Ct_{GAPDH}$. The samples were obtained at room temperature using light microscopy (BX51; Olympus) with an objective lens (10×/0.40 NA, or 20×/0.70 NA; Olympus) and a camera (DP-70; Olympus). The images were acquired with image processing software (DP2-BSW Version 1.2; Olympus).

Statistical analyses

Group comparisons of skin cGVHD scores and pathology scores were performed using the Mann-Whitney *U* test or Kruskal-Wallis test. Cell populations, cytokine levels, mean weights, and gene expression data were analyzed with the unpaired 2-tailed Student *t* test. In all analyses, $P < .05$ was taken to indicate statistical significance.

Results

Th17 cells are increased in lymphoid organs during cGVHD development

We first assessed the kinetics of Th1/Th2/Th17 cytokine production of donor T cells generated during cGVHD. We used the most common cGVHD model: the MHC-compatible, multiple miHA-incompatible allogeneic BMT model (B10.D2 into BALB/c). Sublethally irradiated (6.75 Gy) BALB/c mice were transplanted with 2×10^6 B10.D2 spleen T cells and 8×10^6 B10.D2 TCD-BM cells. Ly9.1 was used as a marker to distinguish donors from recipients; B10.D2 and BALB/c are negative and positive for Ly9.1, respectively. Flow cytometric analysis of the spleens and PLNs on days 14 and 28 indicated that donor chimerism as determined by the negativity for Ly9.1 was > 95%. The allogeneic recipients showed pathologic damage to the skin, salivary glands, lung, and liver, as reported previously (Figure 1A).[25,27] Cells isolated from PLNs were harvested on days 14 and 28 after BMT and analyzed for cytokine expression. In the early phase (day 14), IL-17$^+$ T cells were detected more frequently in the PLNs of recipients of syngeneic BMT, whereas in the late phase (day 28), IL-17$^+$ T cells in allogeneic recipients increased and were detected significantly more frequently than in syngeneic recipients (Figure 1B). We detected consistently higher percentages of donor T cells expressing IFN-γ and IL-13 in PLNs from allogeneic recipients than from syngeneic recipients (Figure 1B). Intracellular staining showed that most of the IL-17–producing cells were CD4$^+$ T cells (Figure 1C) and that IFN-γ/IL-17 double-positive cells (Th1/Th17

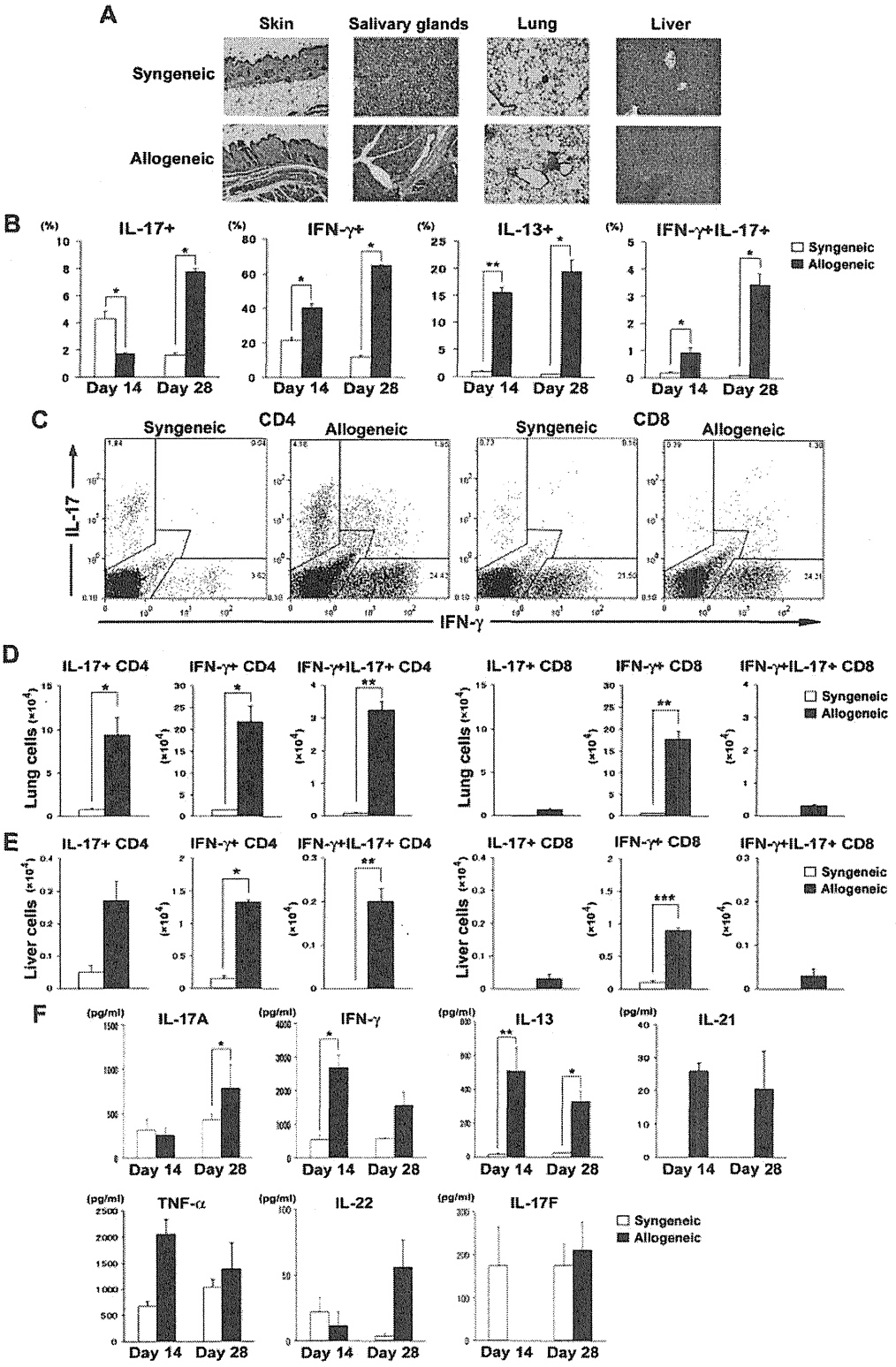

Figure 1. Th17 cells are increased in lymphoid organs during the late phase of cGVHD. Sublethally irradiated (6.75 Gy) BALB/c mice were transplanted with 2×10^6 spleen T cells plus 8×10^6 TCD-BM from WT B10.D2 mice (allogeneic group; black bars). The syngeneic group (white bars) received a transplant of the same dose of splenocytes and TCD-BM from BALB/c mice. (A) Histopathology of skin, salivary glands, lung, and liver of syngeneic and allogeneic recipients 35 days after BMT. (B) The percentages of donor-derived $CD3^+$ T cells expressing IL-17, IFN-γ, IL-13, and IFN-γ/IL-17 on days 14 and 28 are shown. (C) Representative staining for intracellular IFN-γ and IL-17 on $CD4^+$ and $CD8^+$ T cells on day 28 for syngeneic and allogeneic mice. (D-E) Absolute numbers of IL-17–, IFN-γ–, and IFN-γ/IL-17–producing $CD4^+$ and $CD8^+$ T cells in recipient lung (D) and liver (E). (F) PLN cells from syngeneic and allogeneic recipients on days 14 and 28 were stimulated with PMA and ionomycin in vitro. Five hours later, the supernatants were collected to determine cytokine levels by ELISA or CBA. Graphs indicate the levels of cytokines secreted per 1×10^6 total stimulated PLN cells. Three to 6 mice per group were used. The means (± SE) of each group are shown. Data are from 1 representative of ≥ 2 independent experiments. $^*P < .05$, $^{**}P < .01$, and $^{***}P < .005$.

cells) were exclusively detected in allogeneic recipients (Figure 1B-C). As allogeneic recipients developed GVHD-induced lymphopenia on day 28; absolute numbers of IFN-γ^+ T and IL-17^+ T cells in PLNs from allogeneic recipients were not greater than those from syngeneic recipients (IFN-γ^+ T, $51.8 \pm 17.5 \times 10^4$ vs $49.4 \pm 4.2 \times 10^4$, $P = .92$; IL-17^+ T, $5.9 \pm 2.2 \times 10^4$ vs $6.9 \pm 0.59 \times 10^4$, $P = .16$). Numbers of Th1 and Th17 cells from allogeneic recipients were significantly greater than those from syngeneic recipients in the lung (Figure 1D) and liver (Figure 1E). Cells isolated from PLNs of allogeneic recipients secreted significantly greater amounts of IL-17, IFN-γ, and IL-13 after stimulation with PMA and ionomycin (Figure 1F) or without stimulation (supplemental Figure 1, available on the *Blood* Web site; see the Supplemental Materials link at the top of the online article). These cytokine levels were also elevated in serum from allogeneic recipients 28 days after BMT (supplemental Figure 2). To confirm that our observations were not strain dependent or model dependent, we performed similar experiments in the DBA/2 into BALB/c model of cGVHD. We confirmed the up-regulated Th1 and Th17 responses in this model (supplemental Figure 3).

IL-$17^{-/-}$ donor T cells ameliorate cGVHD

We next used IL-$17^{-/-}$ mice with the B10.D2 background as donors to evaluate whether Th17 contributes to cGVHD. On transfer of IL-$17^{-/-}$ B10.D2 donor T cells into allogeneic BMT models, weight loss was mild and fur loss was clearly ameliorated in comparison to that seen in recipients of wild-type (WT) T cells (Figure 2A-B). Clinical cGVHD severity was assessed with a standard scoring system (see "Methods"). Allogeneic IL-$17^{-/-}$ BMT recipients showed significantly less skin cGVHD than WT controls ($P < .05$; Figure 2C). Histopathologic examination of the skin showed significantly reduced cGVHD pathology in recipients of IL-$17^{-/-}$ donors (3.17 ± 1.09 vs 8.50 ± 0.84; $P < .01$; Figure 2D). A dry mouth is one of the distinctive features of cGVHD, and lymphocytic inflammation, fibrosis, and atrophy of acinar tissue were observed in the salivary glands of WT BMT recipients. Histopathologic examination of the salivary glands showed reduced cGVHD pathology in the recipients of IL-$17^{-/-}$ donors (Figure 2E). Atrophy of the salivary glands as determined by their size was significantly reduced in recipients of IL-$17^{-/-}$ donors (4.21 ± 0.13 vs 3.54 ± 0.11; $P < .01$; Figure 2E). No significant differences were observed in pathology scores of the lung, liver, or colon between recipients of IL-$17^{-/-}$ and WT donors (lung, 2.6 ± 1.04 vs 0.8 ± 0.44, $P = .19$; liver, 1.5 ± 0.87 vs 1.83 ± 0.37, $P = .75$; colon, 1.6 ± 0.36 vs 2.8 ± 0.33, $P = .06$). Thus, IL-$17^{-/-}$ BMT recipients showed less cGVHD in the skin and salivary glands than did the WT controls. Flow cytometric analysis of the PLNs in the early phase (day 14) showed no differences in frequency of IFN-g^+ cells between IL-$17^{-/-}$ and WT recipients, whereas recipients of IL-$17^{-/-}$ showed fewer IFN-γ^+ cells in the late phase (day 35, $4.3\% \pm 0.8\%$ vs $18.9\% \pm 3.5\%$; $P = .01$; Figure 2F). As allogeneic WT recipients developed more severe GVHD-induced lymphopenia on day 35 than IL-$17^{-/-}$ recipients, absolute numbers of IFN-g^+ cells in PLNs from allogeneic WT recipients were not greater than those from IL-$17^{-/-}$ recipients (IFN-γ^+ T cells, $6.08 \pm 0.87 \times 10^4$ vs $4.83 \pm 1.23 \times 10^4$; $P = .48$). As expected, IFN-γ/IL-17 double-positive cells were not detected in recipients of IL-$17^{-/-}$ donors on days 14 and 35 (Figure 2G-H). No differences were observed in the IL-13^+ cells or Foxp3^+ cells between the groups (data not shown). These data suggest that donor IL-17 contributes to the pathogenesis of cGVHD.

Donor Th1 differentiation is responsible for the development of cGVHD

To test whether donor Th1 differentiation is responsible for cGVHD, we used IFN-$\gamma^{-/-}$ mice with the B10.D2 background as donors. BMT from IFN-$\gamma^{-/-}$ donors compared with WT donors significantly improved the clinical cGVHD score ($P < .05$; Figure 3A). Histopathologic examination of the skin showed significantly reduced cGVHD pathology in recipients of IFN-$\gamma^{-/-}$ donors (4.75 ± 0.54 vs 7.80 ± 0.52; $P = .02$; Figure 3B). Salivary gland atrophy was also reduced in recipients of IFN-$\gamma^{-/-}$ donors (3.81 ± 0.05 vs 2.87 ± 0.19; $P < .05$; Figure 3C). No significant differences were observed in pathology scores of the lung, liver, or colon between recipients of IFN-$\gamma^{-/-}$ and WT donors (lung, 2.4 ± 0.61 vs 3.2 ± 0.52, $P = .4$; Figure 3B; liver, 1.0 ± 0.4 vs 1.6 ± 0.32, $P = .21$; colon, 0.75 ± 0.21 vs 1.6 ± 0.67, $P = .36$). Intracellular staining of PLNs showed no differences in IL-13– or IL-17–producing cells between IFN-$\gamma^{-/-}$ and WT recipients (data not shown), although significantly greater numbers of Foxp3^+ cells were detected in the IFN-$\gamma^{-/-}$ recipients (day 35; $P < .05$; Figure 3D). To examine whether an increase in numbers of Treg cells was responsible for the reduced cGVHD in the absence of donor IFN-$\gamma^{-/-}$, mice were injected with whole T cells or CD25-depleted T cells from donors. As shown in Figure 3E, depletion of CD25^+ cells from the donor inoculum exacerbated skin scores ($P < .05$). However, CD25-depleted T cells from IFN-$\gamma^{-/-}$ mice caused less severe skin GVHD than those from WT mice ($P < .05$). These findings suggest that IFN-γ contributes to the pathogenesis of cGVHD by both Treg-independent and -dependent pathways. Next, we evaluated the role of IFN-γ in the development of skin cGVHD by administering anti–IFN-γ mAbs to recipients of WT or IL-$17^{-/-}$ donors. Anti–IFN-γ mAb treatment significantly reduced skin scores and pathology scores in recipients of WT donors (Figure 3F-G). Recipients of IL-$17^{-/-}$ donors again showed reduced skin scores, and treatment with anti–IFN-γ mAbs further reduced skin scores (Figure 3H). These findings suggest that IFN-γ contributes to cGVHD pathogenesis.

Am80 inhibits donor Th1 and Th17 cells both in vitro and in vivo

ATRA has been reported to suppress the differentiation of Th17 cells with a reciprocal induction of Treg cells.[28] Am80, a novel RARα/β-specific synthetic retinoid, has a biologic activity ~ 10 times more potent than that of ATRA[20] and directly inhibits Th1 cytokine production.[20,22,29] Therefore, we hypothesized that ATRA or Am80 down-regulates both Th1 and Th17 differentiation in donor T cells, resulting in attenuation of cGVHD. To clarify whether retinoids directly inhibit the production of cytokines, PLNs were isolated from mice 14 days after allogeneic BMT and cultured with Am80 for 24 hours to determine cytokine production. Am80 inhibited IFN-γ (Figure 4A) and IL-17 (Figure 4B) production in a dose-dependent manner. Next, BMT recipients were orally administered Am80 at a dose of 1.0 mg/kg of body weight or vehicle daily from day 0 of BMT, and cytokine expression was assessed in PLNs harvested on day 35. We detected significantly fewer IFN-γ^+ T cells in Am80-administered recipients (Figure 4C). In addition, PLNs from Am80-treated recipients produced significantly less IFN-γ after stimulation with PMA and ionomycin ($P < .01$; Figure 4D). No difference was observed in the percentage of IL-17–producing donor cells, although PLN cells from Am80-treated recipients produced significantly less IL-17 ($P < .05$) and IL-21 ($P < .01$) after stimulation with PMA and ionomycin (Figure 4D). Taken together, these data suggest that Am80 down-regulates both Th1 and Th17 cells in vitro and in vivo.