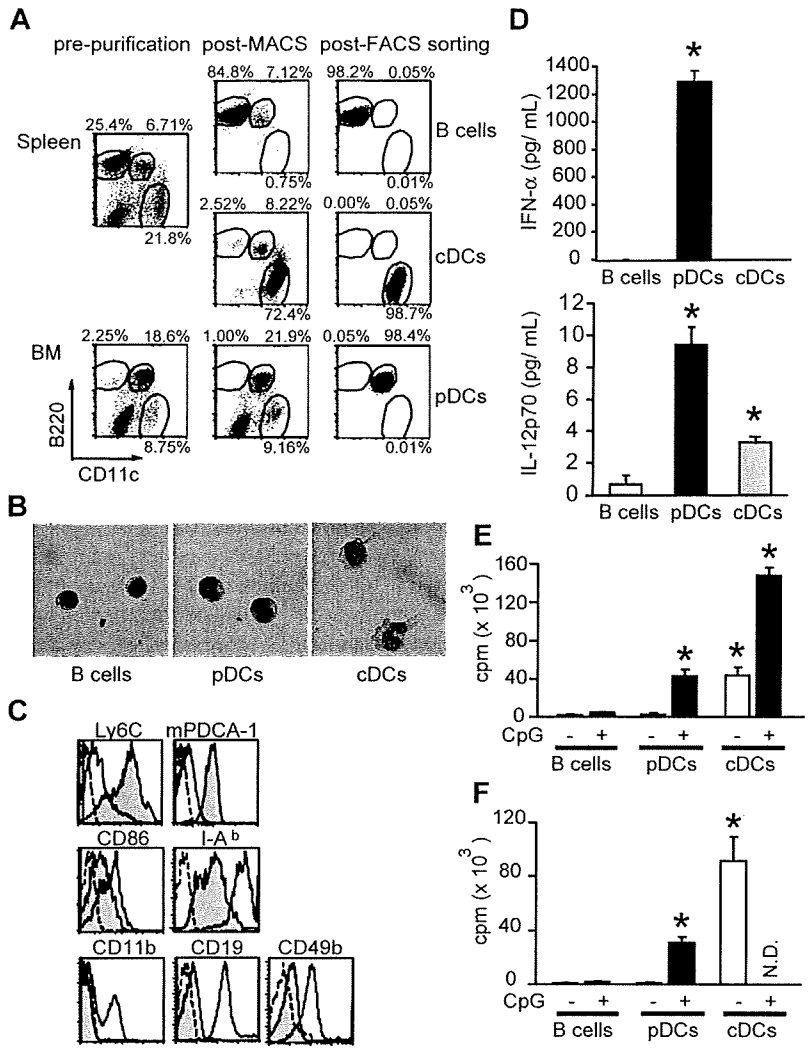

Figure 1. Isolation of pDCs and cDCs. B cells ($CD11c^-$ $B220^+$), pDCs ($CD11c^{int}$ $B220^+$), and cDCs ($CD11c^{high}$ $B220^-$) were isolated from the spleen or BM of FL-treated mice. (A) Two-dimensional counter plots of B220 and CD11c staining. Percentages of cells are shown enclosed in circles. (B) Cell morphology stained with May-Giemsa (magnification ×400). (C) Immunophenotyping. Filled histograms; pDCs, broken-lined open histograms; pDCs stained with isotype controls, solid-lined open histograms for Ly6C, mPDCA-1, CD86, $I\text{-}A^b$, and CD11b; cDCs, those for CD19 and CD49b; B cells and enriched $CD49b^+$ cells as positive controls, respectively. (D) Production of IFN-α (top) and IL-12p70 (bottom) after incubation of cells with CpG 2216 for 16 hours (mean ± SD). (E,F) Aliquots of 2×10^5 BALB/c $CD4^+$ T cells were cultured with 10^4 cells from each APC subset isolated from FL-treated (E) and FL-untreated mice (F), with or without 1 μM CpG 1668, and their proliferation 3 days later was shown as mean plus or minus SD. N.D. indicates not done. Data are representative of at least 2 similar experiments. $^*P < .05$ compared with B cells.

DCs[37,38] in the pDC fraction. After stimulation with CpG 2216, pDCs secreted high levels of IFN-α and IL-12 p70, whereas cDCs secreted moderate levels of IL-12 p70, but not IFN-α, as has been described[39](Figure 1D).

Next, we examined the allostimulatory capacity of pDCs in mixed lymphocyte reaction. Freshly isolated pDCs were poor inducers of allogeneic $CD4^+$ T-cell proliferation as previously reported[3] (Figure 1E). However, pDCs matured with CpG 1668 were capable of priming T cells nearly as effectively as cDCs (Figure 1E). Similar results were obtained when pDCs were isolated from mice without treatment with FL (Figure 1F).

MHC class II–expressing pDCs alone sufficiently stimulate donor $CD4^+$ T cells to cause GVHD in MHC class II–deficient mice

H2-Ab1$^{-/-}$ B6 mice are resistant to CD4-dependent GVHD.[26,28] We studied whether the add-back of MHC class $II^{+/+}$ pDCs could prime alloreactive T cells using this system with modification. In our previous studies, we used a bm12 → B6 model across MHC class II disparity alone. In contrast, the B6 and BALB/c strain combination used in the current study differs at both MHC class I and class II loci. To avoid the confounding effects of MHC class I and $CD8^+$ T cells, therefore, purified $CD4^+$ T cells were used to induce GVHD, while whole T cells were used in the previous studies. Contamination of $CD8^+$ T cells in the $CD4^+$ cell fraction was < 0.1%. H2-Ab1$^{-/-}$ B6 ($H\text{-}2^b$) mice were irradiated with 11 Gy TBI and injected with 2×10^6 pDCs, cDCs, or B cells isolated from wild-type (WT) B6 mice on day −1. Mice were then injected with 2×10^6 $CD4^+$ T cells from BALB/c ($H\text{-}2^d$) donors that differ at MHC and multiple minor histocompatibility antigens (miHAs) from B6 mice on day 0. On day 0, we confirmed homing of the injected cDCs and pDCs to spleen and lymph nodes (LNs), where mature DCs form long-lived contacts with T cells (data not shown).[40] Flow cytometric analysis of the mesenteric LNs (mLNs) and spleen on day +6 demonstrated the significant expansion of donor $CD4^+$ T cells ($H\text{-}2K^{d+}CD4^+$) in animals that had been repopulated with cDCs or pDCs compared with those with B cells (Figure 2A). No $CD8^+$ T-cell expansion was observed (< 0.1%), confirming the stimulation of only $CD4^+$ donor T cells in this system. Similarly, CFSE-labeled donor $CD4^+$ T cells showed robust cell division in animals preinjected with cDCs or pDCs, while those underwent some homeostatic divisions in animals preinjected with B cells (Figure 2B). Donor CD4 expansion in these recipients was associated with increased expression of IFN-γ (data not shown). Evaluation of Foxp3 expression on $CD4^+$ T cells ruled out the possibility of expansion of $Foxp3^+$ regulatory T cells in response to pDCs (data not shown). We then investigated the effects of delayed add-back of pDCs on GVHD. H2-Ab1$^{-/-}$ mice were irradiated on day −4 and injected with pDCs on day −1, followed by the injection of CFSE-labeled BALB/c $CD4^+$ T cells

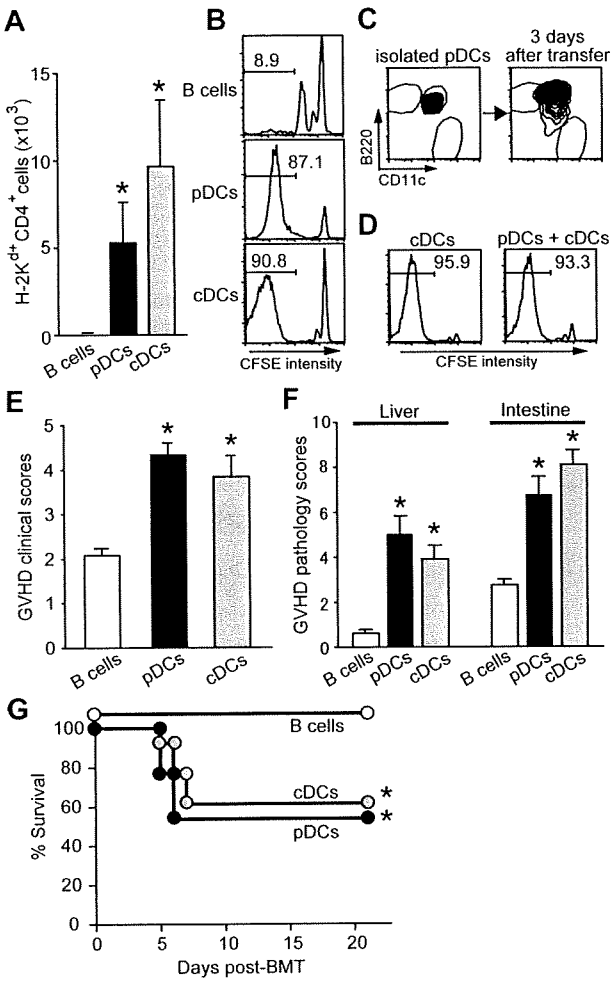

Figure 2. Host pDCs or cDCs alone are sufficient to stimulate alloreactive CD4+ T cells and induce GVHD. (A,B) Totals of 2×10^6 BALB/c (H-2K^{d+}) CD4$^+$ T cells were transferred to irradiated H2-Ab1$^{-/-}$ mice preinjected with 2×10^6 WT B cells, pDCs, or cDCs. Expansion of H-2K^{d+} donor CD4$^+$ T cells in the mLNs (mean ± SD; A) and cell division of CFSE-labeled donor CD4$^+$ T cells in the spleens (B). (C) pDCs were injected to irradiated H2-Ab1$^{-/-}$ mice. Expression levels of B220 and CD11c on I-A^{b+} cells were analyzed 3 days after transfer and compared with those before transfer. (D) Aliquots of 2×10^6 CFSE-labeled BALB/c CD4$^+$ T cells were transferred to irradiated H2-Ab1$^{-/-}$ mice preinjected with 10^6 pDCs plus 10^6 cDCs or 2×10^6 cDCs from WT B6 mice. Cell divisions of donor CD4$^+$ T cells in the spleens on day 7 are shown. (E,F) BALB/c CD4$^+$ T cells were transferred as above and GVHD clinical scores (E), and pathology scores in the liver and intestine (F) on day 6 are shown as mean plus or minus SEM. (G) Lethally irradiated H2-Ab1$^{-/-}$ mice preinjected with pDCs, cDCs, or B cells were injected with 2×10^6 CD4$^+$ T cells and 5×10^6 TCD-BM from BALB/c mice. Survival after BMT is shown. Results from 2 similar experiments were combined. *$P < .05$ compared with B cells.

on day 0. Flow cytometric analysis of the spleen showed robust division of donor CD4$^+$ T cells, thus suggesting that TBI-mediated inflammation persist at least for 3-4 days after TBI (data not shown). In these experiments, we did not have syngeneic controls, since it was apparent in a previous study of DC add-back[28] that there was no expansion of donor T cells in syngeneic controls, where H2-Ab1$^{-/-}$ B6 recipients that had been repopulated with WT B6 DCs were injected with CD4$^+$ T cells from congeneic B6-Ly5a mice.

To rule out the possibility that allostimulatory capacity of pDCs is due to FL treatment, pDCs were isolated from mice without treatment with FL, and their allostimulatory capacity was evaluated similarly. Again, CD4$^+$ T cells primed by pDCs underwent robust cell divisions (Figure S1A, available on the *Blood* website; see the Supplemental Materials link at the top of the online article) and differentiated into effectors secreting high levels of IFN-γ (Figure S1B) in H2-Ab1$^{-/-}$ mice 7 days after transfer, indicating that the ability of pDCs to prime T cells is not due to FL treatment. Therefore, we restricted our subsequent analysis to FL-expanded pDCs.

There was still a possibility that pDCs differentiate into cDCs fully endowed with APC function after transfer to H2-Ab1$^{-/-}$ mice.[41] To examine this possibility, we isolated and injected pDCs into irradiated H2-Ab1$^{-/-}$ mice. Flow cytometric analysis of the spleens, 3 days later, showed that pDCs still maintained the phenotype of pDCs (Figure 2C), thus ruling out this possibility. Next, we evaluated whether pDCs were capable of down-regulating T-cell activation induced by cDCs. To investigate this, CFSE-labeled donor CD4$^+$ T cells were transferred to H2-Ab1$^{-/-}$ mice preinjected with cDCs alone or with cDCs and pDCs. However, flow cytometric analysis of the spleen 7 days after transfer showed that the addition of pDCs did not suppress donor CD4 expansion mediated by cDCs (Figure 2D).

We then tested the hypothesis that alloantigen expression on pDCs alone is sufficient for the induction of GVHD target organ damage. H2-Ab1$^{-/-}$ mice were irradiated and injected with 2×10^6 WT pDCs, cDCs, or B cells on day −1. Mice were then injected with 2×10^6 BALB/c CD4$^+$ T cells. Mice preinjected with pDCs or cDCs developed significant GVHD, as assessed by the clinical scores[31] (Figure 2E) and pathology scores on day +6[32] (Figure 2F), compared with those with B cells. Liver histology of mice preinjected with pDCs showed standard histologic features of acute GVHD, including mononuclear cell infiltration in bile ducts and portal triads, and hepatocellular damage with acidophilic bodies (Figure 3B). Histopathology of the small and large intestine also showed significant changes in these mice, including villous atrophy with epithelial apoptosis (Figure 3E), as well as lymphocytic infiltration and granulation tissue formation (Figure 3H). To note, these pathologic features were similar to those observed in recipients preinjected with cDCs (Figure 3C, F, and I), whereas animals preinjected with B cells showed no significant pathologic signs of GVHD, as previously described[28] (Figure 3A,D,G). These results demonstrated that pDCs alone are sufficient to activate donor CD4$^+$ T cells to trigger GVHD as effectively as cDCs. Finally, we evaluated whether pDC-mediated T-cell activation induces GVHD mortality. Lethally irradiated H2-Ab1$^{-/-}$ mice preinjected with 2×10^6 WT pDCs, cDCs, or B cells on day −1 were injected with 2×10^6 CD4$^+$ T cells and 5×10^6 TCD-BM from BALB/c donors on day 0. Mice preinjected with pDCs or cDCs developed lethal GVHD (Figure 2G).

pDCs are solely sufficient to activate alloreactive CD8+ T cells in β_2m-deficient mice

We examined whether presence of allogeneic pDCs could also stimulate donor CD8$^+$ T cells in β_2m$^{-/-}$ mice with impaired cellular expression of functional MHC class I. Irradiated β_2m$^{-/-}$ C3H mice (CD90.2$^+$) were injected with WT pDCs, cDCs, or B cells on day −1, followed by the injection with 2×10^6 CD8$^+$ T cells from MHC-matched, miHA-mismatched AKR mice (CD90.1$^+$) on day 0. Flow cytometric analysis of the mLNs and spleen on day +6 showed significantly greater expansion (Figure 4A) and IFN-γ production (Figure 4B) of donor CD8$^+$ T cells (CD90.2$^-$ CD8$^+$) in mice preinjected with pDCs or cDCs than in those with B cells. Thus, alloantigen expression on pDCs can solely prime alloreactive CD8$^+$ T cells in vivo as potently as cDCs, although we were unable to examine whether these CD8$^+$ T cells could cause GVHD target organ injury because miHA expression

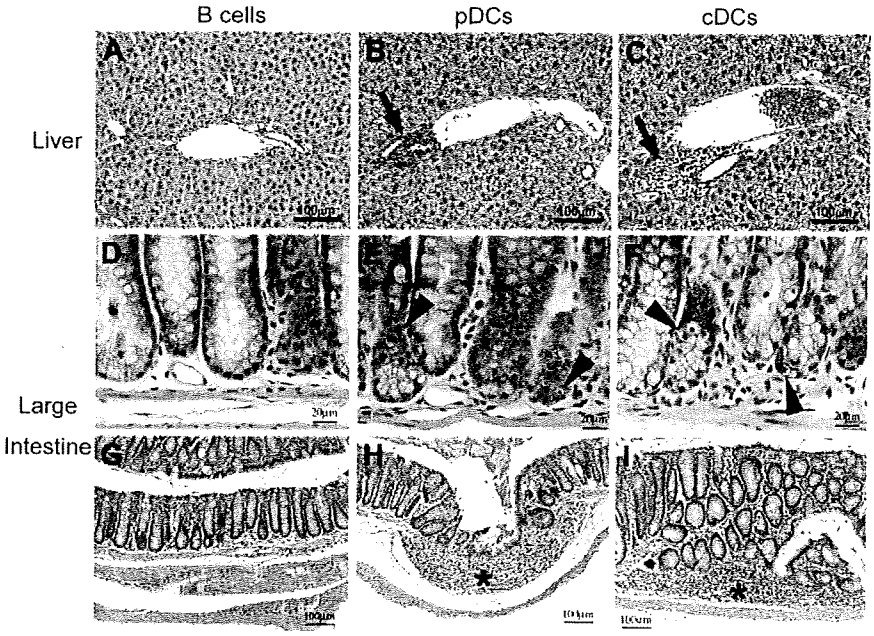

Figure 3. pDCs or cDCs alone mediate standard acute GVHD. Histologic findings of the liver (A-C) and large intestine (D-I). Periportal mononuclear infiltrates in the liver (panels B,C), and crypt cell apoptosis (panels E,F) and granulation tissue (* in panels H,I) in the large intestine are shown.

on target epithelium was required to induce GVHD in MHC-matched, miHA mismatched HSCT.[27,42]

Cognate interaction between pDCs and T cells is required for pDCs to prime alloreactive T cells

pDCs have the ability to produce large amounts of cytokines; therefore, they can be pathogenic through cytokine production as in SLE.[23] To confirm the pathogenic role of pDCs functioning as APCs in GVHD, CFSE-labeled BALB/c $CD4^+$ T cells were adoptively transferred to irradiated H2-$Ab1^{-/-}$ mice preinjected with H2-$Ab1^{-/-}$ pDCs. Flow cytometric analysis of the spleens 6 days after transfer showed that almost 90% of CFSE-labeled T cells had progressed through at least 3 cell divisions in animals preinjected with WT pDCs or cDCs, whereas only a small population did so with some homeostatic divisions in mice with H2-$Ab1^{-/-}$ pDCs or cDCs (Figure 5). These results demonstrate that cognate interaction between pDCs and T cells is required for pDCs to prime alloreactive T cells.

Irradiation is critical for pDCs to prime alloreactive T cells

Activation of pDCs is critical to prime alloreactive T cells as shown in Figure 1. Since it has been shown that irradiation induces maturation of cDCs in vivo,[27] we hypothesized that pretransplantation TBI also plays an important role in activating pDCs. To test this hypothesis, we irradiated mice with 11Gy TBI and performed a flow cytometric analysis of the spleens and mLNs 10 hours after TBI. Expression of CD86 and MHC class II was up-regulated on both pDCs and cDCs isolated from irradiated mice, compared with those from unirradiated mice (Figure 6A). Next, we investigated whether maturation of pDCs are mediated by direct effects of irradiation on pDCs or by effects of irradiation on host tissues. To examine this, 80×10^6 BM cells and splenocytes collected from B6 ($CD45.2^+$) mice were transferred to congeneic B6-Ly5a ($CD45.1^+$) mice that had been irradiated 1 hour before transfer. Flow cytometric analysis of the mLNs isolated 10 hours later showed up-regulated MHC class II expression on $CD45.2^+$ pDCs in irradiated mice compared with those isolated from unirradiated mice (Figure 6B). Collectively, irradiation, likely from the inflammation, is responsible for maturation of pDCs.

These results suggest that pDCs are incapable of priming alloreactive T cells in unirradiated mice as effectively as in irradiated mice. To test this hypothesis, we transferred CFSE-labeled BALB/c $CD4^+$ T cells to unirradiated H2-$Ab1^{-/-}$ mice

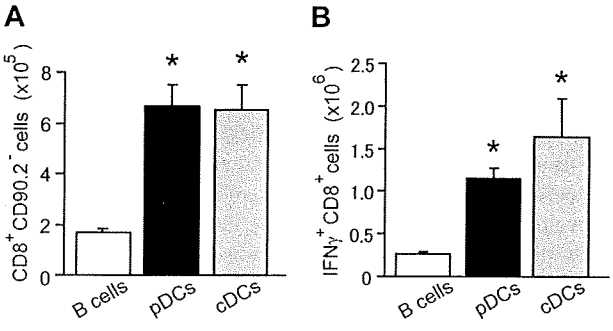

Figure 4. Alloantigen expression on pDCs alone is sufficient to stimulate alloreactive $CD8^+$ T cells. $CD8^+$ T cells from AKR ($CD90.2^-$) mice were transferred to irradiated $\beta 2m^{-/-}$ C3H ($CD90.2^+$) mice preinjected with WT B cells, pDCs, or cDCs. Expansion of $CD90.2^-$ donor $CD8^+$ T cells in mLNs (A) and IFN-γ expression on donor $CD8^+$ T cells in spleens (B) 6 days after transfer are shown as means plus or minus SEM. Data are representative of 2 similar experiments. *$P < .05$.

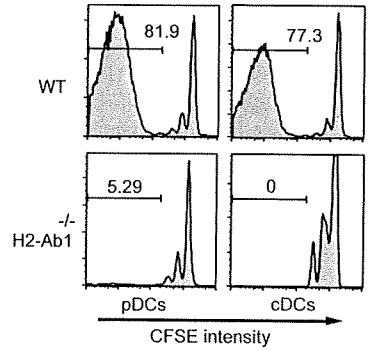

Figure 5. Cognate interaction between pDCs and T cells is required for T-cell activation. CFSE-labeled BALB/c $CD4^+$ T cells were transferred to irradiated H2-$Ab1^{-/-}$ mice preinjected with 2×10^6 pDCs and cDCs isolated from WT or H2-$Ab1^{-/-}$ B6 mice. Cell divisions of H-$2K^{d+}$ donor $CD4^+$ T cells in the spleens 6 days after transfer are shown. Data are representative of 2 similar experiments.

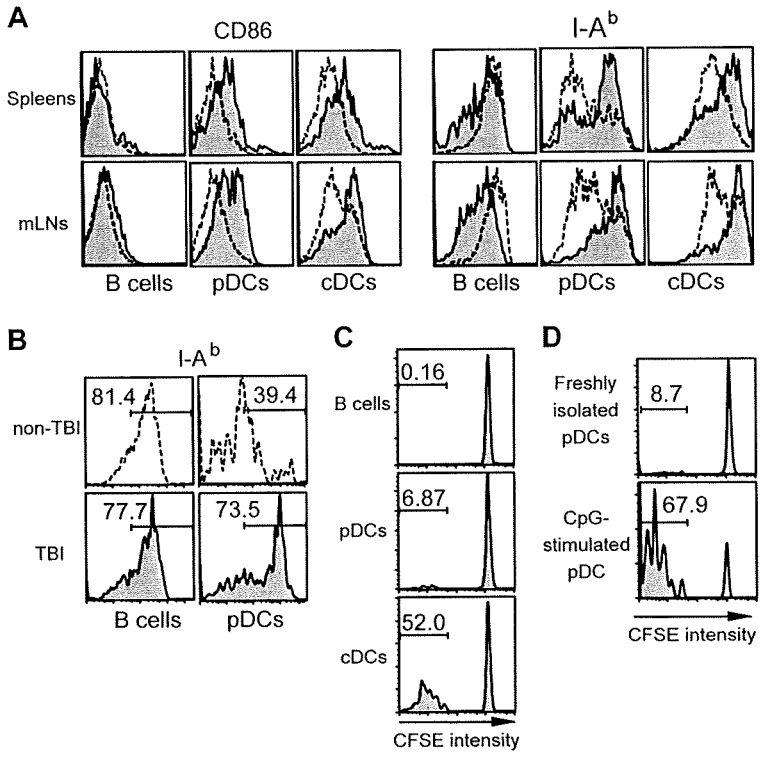

Figure 6. Host irradiation is prerequisite for pDC maturation to prime T cells. (A) Expression of CD86 and I-A^b expression on B cells, pDCs, and cDCs in the spleen and mLNs isolated from irradiated mice 10 hours after irradiation (filled histograms) and from unirradiated control mice (broken-lined open histograms). (B) Unirradiated (top) or irradiated (bottom) B6-Ly5a (CD45.1$^+$) mice were injected with 80 × 10^6 BM cells and splenocytes isolated from FL-treated B6 (CD45.2$^+$) mice. Expression of I-A^b on CD45.2$^+$ B cells and pDCs in mLNs 10 hours after injection is shown. (C) Aliquots of 20 × 10^6 CFSE-labeled BALB/c (H-2K^{d+}) CD4$^+$ T cells were transferred to unirradiated H2-Ab1$^{-/-}$ mice preinjected with 6 × 10^6 WT pDCs or cDCs. Cell division of donor CD4$^+$ T cells in the spleens on day +6 is shown. (D) Similarly, CFSE-labeled BALB/c CD4$^+$ T cells were transferred to unirradiated H2-Ab1$^{-/-}$ mice preinjected with freshly isolated pDCs or pDCs stimulated with CpG 1668 1 μM for 24 hours in vitro. Cell division of donor CD4$^+$ T cells in the spleens on day +6 is shown. Data are representative of 2 similar experiments.

preinjected with WT B cells, pDCs, or cDCs. Donor CD4$^+$ T cells were significantly proliferated in mice preinjected with cDCs 6 days after transfer (Figure 6C), although this cell division appeared to be less potent compared with that in irradiated animals. In contrast, few cell divisions were observed in mice preinjected with B cells or pDCs. We next evaluated whether maturation of pDCs was critical for T-cell activation. Isolated pDCs were cultured with CpG 1668 for 24 hours and injected into unirradiated H2-Ab1$^{-/-}$ mice, followed by the transfer of CFSE-labeled BALB/c CD4$^+$ T cells. CpG-stimulated pDCs stimulate proliferation of donor T cells even in unirradiated mice (Figure 6D).

TLR signaling is not required for pDCs to prime alloreactive T cells

Stimulation with TLR ligands is crucial for the maturation and activation of pDCs.[7,14] We, therefore, hypothesized that maturation of pDCs after TBI is mediated by TLR engagement. To test this hypothesis, we used TRIF/MyD88 DKO mice, where the TLR-dependent signaling pathway was critically abolished.[29] pDCs isolated from TRIF/MyD88 DKO mice were phenotypically identical to WT pDCs (data not shown) but did not respond to CpG or LPS stimulation (Figure 7A), as has been shown.[29] However, TRIF/MyD88 DKO cDCs and pDCs were capable of stimulating donor CD4$^+$ T-cell division in irradiated H2-Ab1$^{-/-}$ mice 6 days after transfer (Figure 7B) and induced significant pathologic GVHD (Figure 7C) as effectively as WT cDCs and pDCs. Thus, TLR signaling is not required for pDCs and cDCs to prime alloreactive T cells.

Discussion

GVHD is initiated by the interaction of alloreactive donor T cells and host DCs.[25,28] Our GVHD model system using MHC-deficient mice presents a stringent test on the allostimulatory functions of a subpopulation of APCs.[28] Using this model system with modification, we addressed whether pDCs are pathogenic in GVHD. We found that alloantigen expression on pDCs alone sufficiently stimulates donor T cells to trigger GVHD in the absence of other APC subsets. Induction of GVHD required cognate interaction of pDCs and T cells, since MHC class II expression on pDCs was absolutely required for stimulating donor CD4$^+$ T cells. It has been suggested that pDCs are involved in the pathogenesis of SLE and psoriasis through IFN-α production[23,24]; however, to our knowledge, our study is the first to directly demonstrate in vivo pathogenic role of pDCs as APCs in an antigen-specific T cell–mediated disease in the absence of other DC subsets.

Our results are in sharp contrast to previous reports suggesting that pDCs mediate tolerance in vivo. In cancer patients, pDCs are incapable of inducing antitumor immune responses but, instead, may induce regulatory T cells that inhibit immunity.[16,17] In animal model of asthma, pDCs in the lung prevent asthmatic reactions to harmless inhaled antigens.[15] In experimental cardiac transplantation, pDCs mediate tolerance and prolong survival of allografts.[11,18,19] It has been suggested that pDCs functioning as APCs generate suppressive or regulatory T cells that mediate tolerance.[12,13,16-18] In contrast, our study showed that alloantigen-presenting pDCs were incapable of suppressing activation of alloreactive T cells but, instead, induced immunity even in the absence of other APC subsets. Differences between previous studies and our study may be attributed to pretransplantation irradiation essential for performing HSCT, which was used in our study.

We have shown that TBI is critical for pDC maturation to prime T cells; TBI up-regulates expression of MHC and costimulatory molecules on pDCs. It has been shown that TBI induces phenotypic and functional maturation of cDCs.[27] Our results

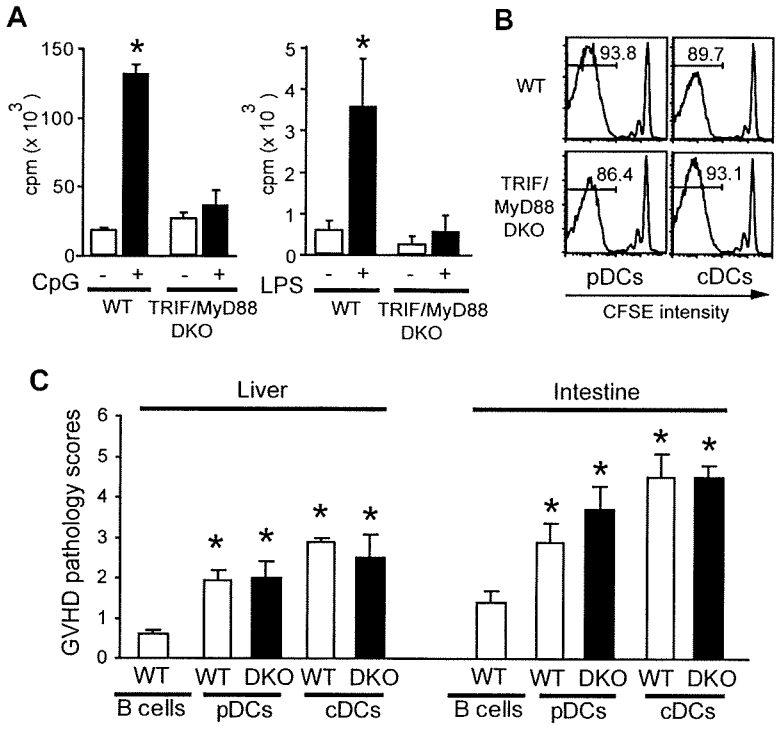

Figure 7. TLR signaling is not required for pDCs to prime alloreactive T cells. (A) A total of $2 \cdot 10^5$ BALB/c $CD4^+$ T cells were cultured with 10^4 WT or TRIF/MyD88 DKO (DKO) pDCs with or without CpG 1668 1 μM (left) or LPS 10 μg/mL (right) to determine cell proliferation. Data are shown as mean (± SD). (B,C) CFSE-labeled BALB/c $CD4^+$ T cells ($H\text{-}2K^d$) were transferred to irradiated $H2\text{-}Ab1^{-/-}$ mice preinjected with pDCs or cDCs isolated from WT or DKO B6 mice. Cell divisions of $H\text{-}2K^{d+}$ donor $CD4^+$ cells in the spleens (B) and GVHD pathology scores in the liver and intestine (C) are shown. Data from 3 similar experiments are combined and shown as means plus or minus SEM (n = 7). $^*P < .05$.

confirm and extend these findings. TBI also maturates pDCs, and this process is dependent on a TBI-mediated inflammatory environment. Although pDCs need to be in a certain state of maturation to prime naive T cells, cDCs can stimulate donor T cells even in unirradiated mice. Thus, the capacity of pDCs to prime naive T cells is far less efficient than cDCs in an uninflamed environment. Host pDCs may not be responsible for the induction of transfusion-associated GVHD in humans.[43,44]

It has been shown that TBI plays an important role in the pathogenesis of GVHD; GVHD is less severe in recipients that have had gentle rather than intensive preconditioning treatments.[45,46] TBI activates and damages host tissue to secrete endogenous factors, such as heat-shock proteins and proinflammatory cytokines like tumor necrosis factor-α and IL-1. TBI-mediated gut injury allows the translocation of exogenous microbial products, such as LPS.[27,45,47,48] DC maturation can be triggered by such endogenous and exogenous "danger signals" as well as by activated T cells, NK cells, and NKT cells.[1,3,4,49] Since many of these stimuli bind to TLRs, we hypothesized that TLR signaling is required for pDC maturation. However, pDCs from TRIF/MyD88 DKO mice with defective TLR signaling[29] were activated by TBI and activate donor T cells to cause GVHD as potently as WT pDCs. Thus, pDCs can be matured by pathways other than TLR signaling, such as nucleotide-binding oligomerization domain-like receptors, retinoic acid–inducible gene I, melanoma differentiation-associated gene 5, DNA-dependent activator of interferon-regulatory factors, C-type lectin receptors, CD40 ligands, and cytokine and chemokine receptors.[49,50]

Immature cDCs also play a role in maintaining tolerance, and thus, irrespective of the subset of DCs, DC maturation may be a control checkpoint in the initiation of immunity.[3,51] Although this simple concept has been revised by several reports showing that mature cDCs and pDCs can induce regulatory T-cell responses,[12,13] our results indicate that pDCs mature and acquire APC function to induce antigen-specific immunity in the inflamed tissue. Thus, functional outcomes of pDC–T-cell interactions depend on the immunologic context of encounter.

It is important to note that pDCs mediate clinically and histologically standard GVHD that was not indistinguishable from cDC-mediated GVHD. Thus, our results further extend the current paradigm that host DCs play a critical role in initiating GVHD.[25,28] Inactivation of host DCs can be a novel strategy to prevent GVHD.[25] Our results suggest that immunologic elimination of cDCs may not be sufficient for complete prevention of GVHD, thus providing important information for developing strategies aimed at inactivating host DCs to prevent GVHD.

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Authorship

Contribution: M.K. conducted research and wrote the paper; D.H., K.A., K.M., K.K., and H.N. conducted research; M.H., M.T., and K.A. designed the study; and T.T. designed the study and organized the data.

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ORIGINAL ARTICLE

Rituximab for the treatment of corticosteroid-refractory chronic graft-versus-host disease

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Abstract We prospectively evaluated the safety and efficacy of the anti-CD20 chimeric monoclonal antibody rituximab for the treatment of corticosteroid-refractory chronic graft-versus-host disease (GVHD) after allogeneic hematopoietic stem cell transplantation. Seven patients were treated with 375 mg/m^2 rituximab weekly for 4 consecutive weeks. Rituximab was well tolerated with no severe toxicity observed during treatment. At 1 year, 3 patients showed a partial response to rituximab therapy, 3 had stable disease, and 1 had progressive disease. Rituximab allowed a reduction in the dose of steroids in 4 patients. Responsive manifestations included mild to moderate skin and oral lesions, and immune hemolytic anemia, and thrombocytopenia. Severe manifestations involving the skin, fascia, and eye did not respond to treatment. These observations suggest that rituximab therapy may be effective for select patients with corticosteroid-refractory chronic GVHD that is not advanced.

Keywords Rituximab · Chronic GVHD · Corticosteroids · Allogeneic transplantation

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1 Introduction

Chronic graft-versus-host disease (GVHD) remains to be the major cause of late morbidity and mortality, and has a significant effect on the functional status and quality of life in long-term survivors after allogeneic hematopoietic cell transplantation (HSCT). Chronic GVHD is a pleiomorphic syndrome with highly variable clinical manifestations, involving the skin, liver, eyes, mouth, esophagus, lung, serosal surfaces, lower gastrointestinal tract, female genitalia, and fascia [1, 2]. Corticosteroids in addition to the continuous administration of a calcineurin inhibitor are the standard treatment for chronic GVHD. The prognosis of patients with corticosteroid-refractory chronic GVHD is extremely poor, and there is no standard treatment for these patients [1, 3].

Although the biological mechanisms leading to chronic GVHD are not well understood compared with those leading to acute GVHD, multiple cellular and humoral mechanisms are likely to be involved in chronic GVHD [4, 5]. Much evidence suggest that B cells and humoral immunity are likely to play a role in the pathogenesis of chronic GVHD; the B cell compartment paradoxically shows simultaneous B lymphocytopenia and B cell hyperreactivity manifested by the production of autoantibodies.

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CD86 expression is highly upregulated in B cells upon stimulation with toll-like receptor 9 in patients with chronic GVHD, as compared to that in controls [6]. Alloantibodies specific for recipient minor histocompatibility antigens have been detected in patients with chronic GVHD, usually 4–6 months after transplantation [7, 8]. Patients with antibodies to recipient minor histocompatibility antigens also have T cells specific for the same antigens [9]. A more direct role of B cells has been suggested by experiments showing that the depletion of donor B cells can protect mice from chronic GVHD [10].

Rituximab is a chimeric mouse/human anti-CD20 monoclonal antibody. It binds with high affinity to $CD20^+$ cells and specifically depletes B cells in vivo. Several phase II studies and case series studies have suggested that rituximab may be effective in the treatment of chronic GVHD [11–17]. Such beneficial effects of B cell depletion by rituximab further emphasize a potential pathogenic role of B cells in the development of chronic GVHD. However, the organ-specific responses observed between studies are substantially different, possible, in part, because previous retrospective studies involved patients who were heavily treated with different types of immunosuppressive therapy.

Ethnicity is associated with the incidence and severity of GVHD [18]. Japanese that have remained geographically isolated for significant periods of time are likely to have less genetic diversity than other ethnic populations experiencing recent and multiple immigrations. Japanese patients receiving allogeneic HSCT have a lower incidence of acute and chronic GVHD compared with patients in Western countries [19–22]. Furthermore, immunosuppressants other than calcineurin inhibitors and corticosteroids are rarely used to prevent and treat GVHD in Japan because they have not been approved for use. Thus, Japanese patients with chronic GVHD might represent a more homogeneous population in terms of genetic background and prior therapies. Here, we prospectively evaluated the safety and efficacy of rituximab in the treatment of corticosteroid-refractory chronic GVHD in Japanese patients undergoing allogeneic HSCT.

2 Patients and methods

2.1 Patients

An open-labeled and early phase II study of rituximab therapy for corticosteroid-refractory chronic GVHD was conducted. The primary objective was to determine the safety, toxicity, and efficacy of 4 courses of rituximab therapy. Eligible subjects had extensive chronic GVHD, which had shown resistance to prednisolone (PSL) at doses greater than 0.5 mg/kg for 30 days within the previous 12 months, who were receiving a stable dose of cyclosporine (CSP) or tacrolimus (TAC). The patients excluded from the study had a previous history of HSCT, an uncontrolled infection, were carriers of hepatitis B or C viruses, and younger than 18 years. This study was approved by the Institutional Review Board of each participating institute, according to the Declaration of Helsinki, and written informed consent was obtained from each participating patient.

2.2 Rituximab therapy

The patients were premedicated with acetaminophen and diphenhydramine, and then 375 mg/m^2 rituximab was intravenously administered weekly for 4 weeks. The initial rate of infusion was 25 mg/h, which was increased to 100 mg/h if there was no reaction to the infusion. During 4 courses of treatment, all patients were required to receive a stable dose of immunosuppressive agents. Following 4 courses of rituximab therapy, decisions regarding the tapering of the dose of immunosuppressive medications were prepared by the transplant physician. The recommended sequence was the withdrawal of corticosteroids and then the withdrawal of the calcineurin inhibitors based on the resolution of chronic GVHD.

2.3 Study evaluation

The diagnosis of chronic GVHD required the presence of at least one diagnostic clinical sign of chronic GVHD or diagnostic manifestation confirmed histologically or by other relevant tests in the absence of acute characteristics of GVHD [2]. The disease was classified as limited or extensive and as de novo, quiescent, or progressive GVHD [1, 23]. Chronic GVHD was staged and graded according to National Institute of Health consensus criteria [2]. The global assessment of the severity of chronic GVHD was derived by combining organ- and site-specific scores. Each organ or site was scored according to a 4-point scale (0–3), with 0 representing no involvement and 3 representing severe impairment. In addition, performance status (PS) was evaluated on this 4-point scale. For thrombocytopenia, a score of 0 was defined as platelets $\geq 140 \times 10^9/l$, 1 as platelets $\geq 100 \times 10^9/l$, 2 as platelets $\geq 50 \times 10^9/l$, and 3 as platelets $< 50 \times 10^9/l$. For autoimmune hemolytic anemia (AIHA), a score of 0 was defined as hemoglobin ≥ 12 g/dl and a negative Coombs test result. Scores of 1, 2, and 3 were defined as hemoglobin ≥ 10, ≥ 7, and < 7 g/dl, respectively. A post-treatment evaluation was performed every week until 6 weeks and then 2, 3, 4, 6, and 12 months thereafter, which included an assessment of the severity of chronic GVHD in each organ or tissue and a safety analysis. The analysis included the monitoring of

blood counts and liver and renal function test results and documenting unexpected side effects. The severity of adverse events attributable to rituximab was evaluated on the basis of the Common Terminology Criteria for Adverse Events, version 3.0. The therapeutic response was assessed 1 year after the initiation of the study, and was defined as complete response (CR), partial response (PR), stable disease (SD), or progressive disease (PD). CR was defined as the resolution of all symptoms and signs of chronic GVHD. PR was defined as a partial improvement in scores of ≥ 2 for at least one organ with no progression in any other organs and no requirement of additional systemic immunosuppressive therapy for chronic GVHD. SD was defined as no change in score and no requirement of additional systemic therapy. PD was defined as the objective worsening of the disease or the need for dose escalation of immunosuppressive agents or additional systemic treatment. Statistical analysis was performed using an unpaired 2-tailed t test.

3 Results

3.1 Patient characteristics

Seven patients (5 men and 2 women; median age 48 years, age range 24–55 years) were enrolled in this study between April 2006 and March 2007. The patients' characteristics are summarized in Table 1. All patients had extensive and corticosteroid-refractory chronic GVHD after allogeneic HSCT. The diseases for which transplantation was performed were as follows: acute myelogenous leukemia (AML, $n = 3$), chronic myelogenous leukemia (CML, $n = 2$), acute lymphoblastic leukemia (ALL, $n = 1$), and myelodysplastic syndrome (MDS, $n = 1$). Four patients underwent bone marrow transplantation (BMT) from a human leukocyte antigen (HLA)-matched or HLA-DR-mismatched unrelated donor, and 3 underwent peripheral blood stem cell transplantation (PBSCT) from an HLA-matched sibling donor. Myeloablative conditioning regimens were used in 5 patients, whereas fludarabine-based reduced-intensity conditioning regimens were used in 2. GVHD prophylaxis consisted of CSP and short-term methotrexate (MTX) ($n = 4$), TAC and short-term MTX ($n = 2$), or TAC alone ($n = 1$). All patients developed acute GVHD (grade II in 6 patients and grade I in 1 patient), which was successfully treated with 1–2 mg/kg of methylprednisolone (mPSL) or PSL and subsequently developed into quiescent and extensive chronic GVHD. On the basis of the global staging system [2], 4 patients had "severe" chronic GVHD, and 3 had "moderate" disease. The median time from transplantation to study enrollment was 42 months (range 19–112 months). The median time

Table 1 Patients' characteristics

UPN	Age/sex	Diagnosis	Donors	HLA	Stem cell source	GVHD prophylaxis	Type of onset	Prior therapy	Interval from transplantation to rituximab (months)	Interval from onset of chronic GVHD to rituximab (months)
1	24/F	CML	Sibling	Identical	PBSC	CSP+MTX	Quiescent	PSL, CSP	19	8
2	39/M	MDS	Unrelated	Identical	BM	TAC+MTX	Quiescent	PSL, pulse mPSL, CSP, TAC	42	39
3	48/M	AML	Unrelated	Identical	BM	TAC	Quiescent	PSL, TAC	46	43
4	51/M	CML	Unrelated	DR mismatch	BM	TAC+MTX	Quiescent	PSL, CSP, TAC	112	109
5	55/F	AML	Unrelated	Identical	BM	CSP+MTX	Quiescent	PSL, CSP	34	30
6	55/M	AML	Sibling	Identical	PBSC	CSP+MTX	Quiescent	PSL, CSP	47	37
7	29/M	ALL	Sibling	Identical	PBSC	CSP+MTX	Quiescent	PSL, mPSL, CSP	27	25

from the onset of chronic GVHD to study enrollment was 37 months (range 8–109 months). In all patients, prior therapy for chronic GVHD was a combination of corticosteroid and CSP or TAC. None of the patients received other immunosuppressive medications. The intervals between dose escalations of corticosteroids and rituximab administration were at a minimum of 1 month. All subjects were followed for 1 year after the initiation of rituximab therapy.

3.2 Toxicity

All patients completed a 4-week course of rituximab treatment. Only one patient developed grade 2 allergic toxicity, i.e., an infusion reaction after the first dose of rituximab. None of the patients developed grade 3 or 4 adverse events attributable to rituximab during the 4-week treatment. Later adverse events, occurring within 1 year of the initiation of therapy, included the following: grade 3 bacterial infection that required intravenous administration of cephepim in 1 patient at 2 months, grade 2 herpes simplex virus infection that required treatment with valaciclovir in 1 patient at 4 months, grade 1 hepatic injury in 1, and grade 2 renal damage in 1. These adverse events were likely related to other drugs that were used or to pronounced immune suppression related to transplantation and chronic GVHD.

3.3 Efficacy

All patients were evaluable for their response to rituximab therapy at 1 year after the study initiation (Table 2). Unique patient number (UPN) 1 developed skin sclerosis, which was initially treated with 0.5 mg/kg of PSL. Six month later, her chronic GVHD progressed to "severe" skin sclerosis and contracture. Chronic GVHD initially responded to rituximab with an improvement of symptoms, leading to successful tapering of PSL by 67% over 6 weeks. However, sclerosis progressed thereafter, and the PSL dose was increased. The PSL dose was subsequently reduced again by 67% of the initial dose at 1 year, at which time the global staging and organ-specific scores were unchanged as compared to those before rituximab therapy. The overall response at 1 year was classified with PD because of the need for an escalation in the dose of PSL. UPN 2 developed chronic GVHD in the skin and mouth, which was initially responded to 250 mg of mPSL. Skin and oral lesions were exacerbated 10 months before enrollment to this study. CSP was replaced with TAC and PSL dose was increased to 0.5 mg/kg, but chronic GVHD progressed to "moderate" cutaneous and oral disease. Rituximab therapy was started, but was not effective. However, the disease was stable during the study period without the need for an escalation in dose of CSP and PSL.

UPN 3 developed extensive chronic GVHD, including cutaneous, oral, and hepatic lesions, and autoimmune hemolytic anemia (AIHA) and immune thrombocytopenia. This patient had steroid-induced diabetes mellitus and a history of tuberculosis. The patient was initially treated with 1 mg/kg of PSL. Three months before study enrollment, PSL was increased to 0.8 mg/kg, which was maintained until study entry according to the past history of exacerbation with less doses of PSL. Rituximab therapy improved "severe" GVHD to "moderate" GVHD, and allowed an 82% reduction in the dose of PSL within 1 year of the study.

UPN 4 had a 9-year history of chronic GVHD. The most severe manifestation was slowly progressive sclerodermatous lesions in the cervical and lower facial skin and fascia, which resulted in severe flexion and rotation contracture and difficulty in mouth opening and swallowing. Rituximab therapy failed to improve these manifestations, but the disease did not progress during the study period with stable doses of CSP and PSL. However, the patient required additional immunosuppressive therapy with high-dose cyclophosphamide 17 months after rituximab therapy and died of bacterial pneumonia, which developed during cyclophosphamide-induced neutropenia.

UPN 5 had "severe" sclerodermatous skin lesions in both the upper and lower extremities. The patient also had recurrent pleural effusion and ascites and a motility disorder of the intestine. The patient was initially treated with 0.5 mg/kg of PSL. Nine months before study enrollment, the disease was deteriorated and PSL dose was increased to 0.5 mg/kg, which was discontinued before rituximab therapy because of a lack of improvement and steroid intolerance. Rituximab therapy temporally improved serositis and diarrhea, but global staging and organ-specific scores were unchanged at 1 year. The patient died of bacterial pneumonia 19 months after the initiation of rituximab therapy.

UPN 6 developed corticosteroid-refractory chronic GVHD in the skin, mouth, eyes, and muscles. Rituximab improved these symptoms, and the patient was able to discontinue PSL by 1 year. Interestingly, the patient developed conductive hearing loss due to inflammation in the bilateral middle ear at the onset of chronic GVHD. The patient recovered dramatically from deafness after the fourth dose of rituximab therapy. UPN 7 developed cutaneous chronic GVHD and treated with PSL. The disease was progressed to sclerodermatous skin disease and the patient was started on 2 mg/kg of mPSL, which was reduced due to a lack of improvement and the patient entered to this study. Sclerodermatous skin lesion improved slowly after rituximab therapy and disappeared

Table 2 Response to rituximab therapy

UPN	Pretreatment			2 months			1 year				
	Global staging	Organ/ manifestation	Score	Global staging	Score	% PSL reduction	Global staging	Score	% PSL reduction	Global response	Follow-up
1	Severe	PS	1	Severe	1	67	Severe	1	67	PD	Alive at 36 months
		Skin	2		2			2			
		Mouth	1		1			1			
		Joints and fascia	3		3			3			
2	Moderate	PS	1	Moderate	1	0	Moderate	1	0	SD	Alive at 35 months
		Skin	2		2			2			
		Mouth	2		2			2			
3	Severe	PS	1	Moderate	1	40	Moderate	1	72	PR	Alive at 34 months
		Skin	1		1			1			
		Mouth	1		1			1			
		Liver	3		2			2			
		Thrombocytopenia	2		1			1			
		AIHA	1		0			0			
4	Severe	PS	1	Severe	1	0	Severe	1	0	SD	Died of infection at 20 months
		Skin	3		3			3			
		Eye	1		1			1			
		Joints and fascia	3		3			3			
5	Severe	PS	2	Severe	2	–	Severe	2	–	SD	Died of infection at 19 months
		Skin	3		3			3			
		Eye	1		1			1			
		Intestine	1		1			1			
		Joints and fascia	1		1			1			
		Serositis	2		2			2			
		Thrombocytopenia	2		2			1			
6	Moderate	PS	2	Moderate	1	0	Moderate	1	100	PR	Alive at 30 months
		Skin	2		1			1			
		Mouth	2		1			1			
		Eye	2		1			1			
		Muscle	1		0			0			
7	Moderate	PS	1	Moderate	1	0	Moderate	1	25	PR	Alive at 23 months
		Skin	2		2			0			
		Mouth	1		1			1			
		Eye	2		2			2			
		Joints and fascia	1		1			0			

at 1 year, although dry eye and oral mucositis did not improve.

Overall, none of the patients achieved a CR, whereas a PR was noted in 3 patients. SD was noted in 3 patients and PD in 1. One year after rituximab therapy began, PSL was discontinued or reduced in 4 of 6 patients; the median reduction rate was 67% (range 0–100%). None of the 7 patients required additional immunosuppressive therapy within 1 year after the initiation of the study. At a median follow-up of 30 months, 5 patients were alive with active and continuing chronic GVHD, and 2 had died of infection after the study period.

On the basis of global staging, only 1 patient with "severe" disease improved to "moderate" disease at 1 year, whereas 3 others with "severe" disease experienced no change. Patients with severe (score 3) skin sclerosis and joint contracture related to sclerodermatous skin GVHD and fascitis did not respond to rituximab therapy. One patient with severe (score 3) hepatic GVHD responded partially to rituximab therapy. Clinical responses were

observed primarily in patients with moderate (score 2) to mild (score 1) manifestations. It is noteworthy that 6 of 11 manifestations with a score 2 responded to rituximab therapy. Improvement in the skin, mouth, eye, liver, joints and fascia, intestine, and serous membrane was observed in 2 of 7, 1 of 5, 1 of 4, 1 of 1, 1 of 4, 0 of 1, and 0 of 1 cases, respectively. Notably, all cases of immune thrombocytopenia and anemia were responded well to rituximab. However, PS improved only in 1 patient who achieved a PR.

3.4 Immunological monitoring

B cell numbers were monitored after rituximab therapy using a flowcytometric analysis. $CD19^+$ B cells were quickly eliminated within 2 weeks after the first treatment and did not repopulate at least by 12 weeks (Fig. 1). Serum levels of IgG and IgA were unchanged by 6 weeks, but gradually declined thereafter. Serum IgM levels decreased much earlier and more profound compared with those of IgG and IgA.

4 Discussion

The prognosis of corticosteroid-refractory chronic GVHD is poor, and no standard therapy for corticosteroid-refractory chronic GVHD is available [1, 3]. In the present study, we evaluated the efficacy and safety of rituximab therapy in patients with quiescent-type chronic GVHD. This condition may have been related to the ethnicity of the transplant patients. The incidence of progressive-type chronic GVHD is high, reportedly 10–70% in Western countries [24, 25]. In contrast, progressive-type GVHD is rare and quiescent-type GVHD is common in Japanese patients [22]. Rituximab therapy was well tolerated, and no severe adverse events were attributed to rituximab therapy. A 4-week course of rituximab treatment produced an overall response rate of 43% at 1 year, which is slightly lower than

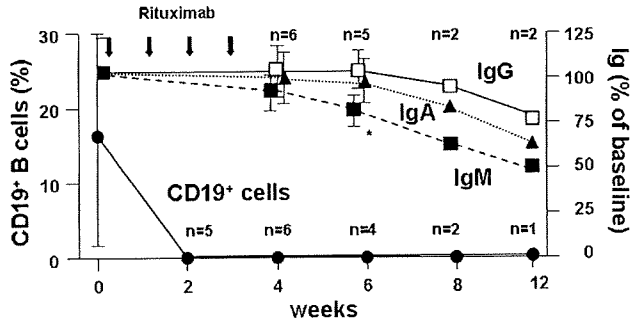

Fig. 1 Laboratory parameters over time after rituximab therapy. IgG, IgA and IgM levels are shown as percentage of baseline levels.*$P < 0.01$ compared with IgG or IgA

the overall response rate of 50–83% reported in previous studies testing the efficacy of rituximab [11–13, 15–17]. CR rates ranged from 0 to 20% in previous studies [12, 13, 16, 17]. In the present study, none of the patients achieved CR. The steroid-sparing effect is an important indicator of efficacy assessments of GVHD [26]. Rituximab therapy resulted in a median reduction in the dose of corticosteroids of 67%, which was slightly lower than the 75–86% reduction in dose observed in 3 previous studies that addressed the steroid-sparing effect of rituximab in this setting [13, 15, 16]. These results were surprising because we initially hypothesized that rituximab would be more effective in Japanese patients who tend to develop less severe chronic GVHD than Caucasians [22].

Previous studies of the efficacy of rituximab therapy for steroid-resistant chronic GVHD highlight the potential activity of rituximab against skin involvement, including scleroderma, whereas the responses to rituximab appear to be less pronounced in other organs or tissues [11–17]. These studies also suggested that the steroid-sparing effect might be more pronounced in the skin and oral lesions than others in chronic GVHD [15, 16]. In addition, hematologic abnormalities associated with chronic GVHD also respond well to rituximab therapy [11, 15, 27]. In our study, rituximab was most effective against immune thrombocytopenia and AIHA, and less effective against skin sclerosis and joint contracture related to sclerodermatous skin lesions and fascitis. This discrepancy between the current study and previous studies might have resulted because more patients with advanced sclerodermatous chronic GVHD were enrolled in our study than in the previous studies. The interval between the time of the onset of chronic GVHD and the time of study enrollment was longer in the present study (median duration 37 months) than in most of the previous studies (median duration 14–37 months) [11–13, 15, 17]. Nonetheless, our patients had undergone less immunosuppressive therapy before study enrollment than did the patients in the previous studies, most of whom had received multiple courses of immunosuppressive therapy [11, 12, 15]. Thus, the long-term duration of disease without sufficient intervention might have resulted in the development of irreversible damage in our patients.

Many advanced manifestations in chronic GVHD are potentially irreversible, including skin and joint contracture, chronic dry eye, esophageal and vaginal stricture, and bronchiolitis obliterans in the lung. The enrollment of patients with advanced chronic GVHD may not be appropriate when the endpoint of the study is the response to treatment. Alternatively, irreversible lesions could be excluded from consideration in the assessment of response [28, 29]. Such considerations were not specified in our protocol. The results of our study suggest that rituximab

may be more effective against mild to moderate manifestations than against severe manifestations of chronic GVHD. Thus, earlier treatment with rituximab or with other investigational agents for corticosteroid-refractory chronic GVHD may increase the chances of a good response. Another possible explanation for the poorer response to rituximab in our study than in previous studies, although unlikely, is that dominant immunological mechanisms associated with chronic GVHD and treatment outcomes may differ by ethnicity, because the prognostic scoring system [25], which was developed on the basis of clinical findings in Western patients, is not prognostic in Japanese patients [22].

We confirmed complete depletion of B cells after rituximab therapy. B cells were still absent 2 months after the last infusion of rituximab. In the initial multi-institutional trial evaluating a single four dose course of rituximab in patients with follicular lymphoma, the median B cell count did decline to almost undetectable levels after the first dose in the majority of patients, with recovery beginning from 6 to 9 months post-treatment, and return to normal levels between 9 and 12 months [30]. Similarly, B cells were undetectable in patients with chronic GVHD until 1 year after rituximab therapy [13]. Such a profound and prolonged B cell depletion may explain why rituximab treatment is effective in several antibody-mediated autoimmune diseases with some responses ongoing for more than 1–2 years [31]. On the other hand, rituximab therapy could results in impaired humoral immune responsiveness [32]. We also found that serum immunoglobulin levels decrease after rituximab therapy. Of note, IgM fell much more than IgG and IgA. This phenomenon was observed in patients with rheumatoid arthritis and chronic GVHD [13, 33]. This may be due to higher sensitivity of IgD^+ memory B cell subset, which produces natural mutated IgM antibodies as a first-line of defense against blood-borne antigens [33, 34], to rituximab than plasma cells.

In conclusion, the current study suggests that rituximab therapy may be effective for selective patients with corticosteroid-refractory chronic GVHD that is not advanced. A recent study indicated that that low-dose rituximab therapy is also effective [17]. However, the optimal schedule and dosing regimens for rituximab need to be determined. Furthermore, a well-designed, large-scale, prospective study is needed to conclusively address the efficacy of rituximab in the treatment of corticosteroid-refractory chronic GVHD.

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ORIGINAL ARTICLE

Cardiac and autonomic nerve function after reduced-intensity stem cell transplantation for hematologic malignancy in patients with pre-transplant cardiac dysfunction

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Abstract Recent reports have shown that cardiomyopathy caused by hemochromatosis in severe aplastic anemia is reversible after reduced-intensity allogeneic stem-cell transplantation (RIST). We comprehensively evaluated cardiac and autonomic nerve function to determine whether cardiac dysfunction due to causes other than hemochromatosis is attenuated after RIST. In five patients with cardiac dysfunction before transplant, we analyzed the changes in cardiac and autonomic nerve function after transplant, using electrocardiography (ECG), echocardiography, radionuclide angiography (RNA), serum markers, and heart rate variability (HRV), before and up to 100 days after transplant. There was no significant improvement in cardiac function in any patient and no significant alteration in ECG, echocardiogram, RNA, or serum markers. However, on time-domain analysis of HRV, the SD of normal-to-normal RR intervals (SDNN) and the coefficient of variation of the RR interval (CVRR) decreased significantly 30 and 60 days after transplant (P=0.04 and 0.01, respectively). Similarly, on frequency-domain analysis of HRV, low and high frequency power (LF and HF) significantly and temporarily decreased (P=0.003 and 0.03, respectively). Notably, in one patient who had acute heart failure after transplantation, the values of SDNN, CVRR, r-MSSD, LF, and HF at 30 and 60 days after transplantation were the lowest of all the patients. In conclusion, this study suggests that (a) RIST is well-tolerated in patients with cardiac dysfunction, but we cannot expect improvement in cardiac dysfunction due to causes other than hemochromatosis; and (b) monitoring HRV may be useful in predicting cardiac events after RIST.

Keywords Cardiac dysfunction · Heart rate variability · Reduced intensity allogeneic stem-cell transplantation · Acute heart failure

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Introduction

Allogeneic hematopoietic stem cell transplantation (allo-HSCT) is recognized as a curative treatment for hemato-

logic malignancy. However, myeloablative regimens are difficult to administer in elderly or ill patients because of increased transplantation-related toxicity. Therefore, reduced-intensity conditioning regimens for allo-HSCT have been developed for these vulnerable patients, who are ineligible for myeloablative conditioning. There are, however, no reports that describe whether reduced-intensity allogeneic stem-cell transplantation (RIST) is sufficiently well tolerated in patients with single-organ comorbidity, including cardiac, pulmonary, renal, or hepatic dysfunction. Therefore, the safety of RIST for patients with impaired organ function has not been sufficiently verified. Indeed, the existence of pretransplant organ dysfunction can worsen the prognosis after transplantation whether nonmyeloablative conditioning or myeloablative conditioning is used [1, 2]. Furthermore, in RIST utilizing melphalan and fludarabine, a high incidence of severe left ventricular failure (three of 21 patients; 14%) was reported [3]. This report casts doubt on whether RIST is safe in patients with cardiac dysfunction.

In contrast, three recent reports have shown marked improvement in cardiac function after transplant in patients with severe aplastic anemia (AA) and cardiomyopathy in secondary hemochromatosis, despite the underlying mechanism being unknown [4–6]. Conversely, the reversibility of cardiac dysfunction due to causes other than hemochromatosis has not been investigated. Hence, the primary objective of this study was to evaluate how cardiac dysfunction due to causes other than hemochromatosis changes after RIST.

Heart rate variability (HRV), which is generally recognized as an index of sympathovagal balance and autonomic cardiovascular control, has been investigated in various diseases that precipitate sudden death. Recently accumulated evidence has shown that a decrease in RR interval variability is strongly associated with sudden death and/or cardiac events after a myocardial infarction [7–10]. Furthermore, the usefulness of HRV as a clinical tool has been explored in numerous other conditions such as congestive heart failure, vasovagal syncope, hypertrophic cardiomyopathy, obstructive sleep apnea, diabetic neuropathy, and various neurological conditions [11–16]. In this study, we comprehensively analyzed the changes in cardiac function after RIST, assessed by various methods including electrocardiography (ECG), echocardiography, radionuclide angiography (RNA), natriuretic peptides and troponin T, and autonomic nerve function on 24-h Holter ECG for HRV.

Materials and methods

Patients

We prospectively enrolled five consecutive patients with pretransplant cardiac dysfunction from a group of 116 patients who had undergone allo-HSCT between September 2003 and August 2007 at our institute. In this study, the patients who were eligible for this study were those who had a left ventricular ejection fraction (LVEF) of under 50% on pretransplant radionuclide angiography, had ever been noted to have an LVEF of under 50% on echocardiography, or had a history of heart failure prior to transplant. The median age at transplantation was 42 years old (range 37–55). Pretransplant LVEFs were less than 50% in two patients, two patients had previously had an LVEF of under 50%, and the remaining patient had a history of heart failure prior to transplantation and an LVEF of under 50% at pretransplant assessment. No patient received mediastinal radiation therapy before transplantation nor did any patient have a history of hypertension, diabetes mellitus, and/or ischemic heart disease. The median level of serum ferritin prior to transplant was 189.6 ng/ml (range 58.3–1837.9), and there were no patients who were diagnosed as having cardiac hemochromatosis on echocardiography. We therefore strongly suspected anthracycline treatment as the cause of cardiac dysfunction in all five patients. As in a previous report [17], we calculated the cumulative anthracycline dose using the following ratios: daunorubicin 0.5, pirarubicin 0.8, mitoxantrone 3.4, idarubicin 1.6, and epirubicin 0.6, with cardiotoxicity of doxorubicin considered to be 1.0. These patients included three patients with non-Hodgkin's lymphoma (intravascular lymphoma, mantle cell lymphoma, and follicular lymphoma) who where refractory to chemotherapy, one patient with acute myeloid leukemia in her second complete remission (CR), and one patient with acute lymphoblastic leukemia in his first CR (Table 1). This study was approved by the Institutional Review Board. The concept, procedure, and potential risks of the study were explained, and written informed consent was obtained from all enrolled patients.

Allogeneic HSCT

Three patients received allo-HSCT from HLA-identical unrelated donors, and two patients from HLA-mismatched cord blood (Table 1). HLA matching (HLA-A, -B, and -DR) was determined by DNA genotyping in three unrelated BMTs, and only serologic typing was performed for the two cord blood transplants (CBT). Three patients received transplants from donors with matched blood types, one patient received a transplant with a major blood type mismatch, and one patient received a transplant with a minor blood type mismatch.

Reduced-intensity conditioning was employed for all five patients. Of the three patients from HLA-identical unrelated donors, two patients received reduced-intensity conditioning including fludarabine/oral busulfan (fludarabine 30 mg/m^2/day, on days −7 to −2 and oral busulfan 4 mg/kg/day, on days −4 and −3), and the remaining patient

Table 1 Patients characteristics

No.	Sex	Age	Disease	Disease status	Source	HLA Mismatch (A, B, DR)	aGVHD prophylaxis	Eligibility	Cumulative dose of anthracycline (mg/m^2)
1	F	39	AML	CR2	uBM	Allele match	CsA + sMTX	LVEF 37%	250
2	F	37	IVL	Ref	uBM	Allele match	CsA + sMTX	LVEF 46%, a history of AHF	380
3	F	55	FL	Ref	CB	2 Ag	CsA alone	a history of decreased LVEF in 45%	391.8
4	M	42	ALL	CR1	uBM	Allele match	CsA + sMTX	LVEF 48%	240
5	F	46	MCL	Ref	CB	2 Ag	CsA + sMTX	a history of decreased LVEF in 39%	150

We calculated the cumulative dose of anthracycline as daunorubicin 0.5, pirarubicin 0.8, mitoxantrone 3.4, idarubicin 1.6, epirubicin 0.6, when the intensity of the cardiotoxicity of doxorubicin was considered to be 1.0

IVL intravascular B cell lymphoma, *FL* follicular lymphoma, *MCL* mantle cell lymphoma, *Ref* chemo-refractory, *uBM* unrelated bone marrow, *Ag* antigen, *CB* cord blood, *CsA* cyclosporine, *sMTX* short-term methotrexate, *LVEF* left ventricular ejection fraction

received fludarabine/intravenous busulfan (fludarabine 30 mg/m^2/day, on days −9 to −4 and intravenous busulfan 1.6 mg/kg/day, on days −9 and −5). In contrast, the two patients who underwent CBT had fludarabine/intravenous busulfan/total body irradiation (fludarabine 30 mg/m^2/day, on days −9 to −4, intravenous busulfan 1.6 mg/kg/day, on days −9 and −5, and total body irradiation 4 Gy divided into two fractions/day, on days −3, −2, or −1).

As prophylaxis for acute graft-versus-host disease (aGVHD), four patients received both cyclosporine A (CsA) and short-term methotrexate (MTX), and one patient who received cord blood was given CsA alone. Intravenous MTX was administered on day 1 (10 mg/m^2), and on days 3 and 5 (7 mg/m^2). The doses of CsA were adjusted to a target trough level between 150 and 250 ng/ml until day 100, except where disease progression or drug toxicity occurred, and then tapered unless GVHD occurred. aGVHD was diagnosed clinically, graded according to standard criteria and confirmed by appropriate biopsies. Chronic GVHD (cGVHD) was also defined according to the standard criteria. In CBT, pre-engraftment immune reactions (PIR) were defined according to the report of Kishi et al. [18].

The evaluation of cardiac and autonomic nerve function

We evaluated cardiac function with 12-lead electrocardiograms (ECG), echocardiography, radionuclide angiography (RNA), serum markers, and autonomic nerve function with heart rate variability assessed on 24-h ECG monitoring (within the 100 days before transplant) and posttransplantation [on days 30 (±4), 60 (±6), and 100 (±12)].

The 12-lead ECG

Twelve-lead ECG was performed at rest, and the QTc interval was measured automatically using a novel recording system (FDX-6521, Fukuda Denshi, Tokyo, Japan). In brief, QT intervals were measured for 15 s at 25 mm/s. QTc intervals were then calculated by correcting the QT interval using the Bazett formula [19].

Echocardiography

Two-dimensional echocardiographic examinations were performed using a Power Vision 6000 (Toshiba, Tokyo, Japan). We assessed the following variables: (1) posterior wall (PW) and interventricular septal wall (IVS) for evaluation of left ventricular hypertrophy, (2) left ventricular end-diastolic dimension (LVDd) and end-systolic dimension (LVDs) for evaluation of left ventricular dilatation, (3) early peak flow velocity/atrial peak flow velocity (E/A) and deceleration time (DcT) for evaluation of left ventricular diastolic function. During the examination, the gain setting was optimized to a level just below background noise, and the transducer frequency was set to 2.5 or 3.5 MHz.

RNA

Radionuclide angiography was performed with the gated-equilibrium technique. Blood cells were targeted with 99 mTc-pertechnetate, facilitated by pyrophosphate and injected intravenously. ECG-gated gamma camera scanning was performed using a VertexPlus (ADAC /Philips, CA, USA) from two directions; the left anterior oblique and anterior views were obtained in order to image the left ventricle separately from the right ventricle, and the left atrium and 64×64 matrix was used. Acquisition time was 180 s, and ECG-gated images were acquired with 20 frames per cardiac cycle. LVEF and peak-filling rate (PFR) were calculated as parameters of left ventricular systolic and diastolic function.

Serum markers (natriuretic peptide measurement)

We employed human atrial natriuretic peptide (hANP) and brain natriuretic peptide (BNP) as parameters of cardiac function. Blood samples for natriuretic peptide measurement were drawn from the patients into chilled tubes containing aprotinin and 1 mg of ethylenediamine tetraacetic acid (EDTA)/ml. The tubes were immediately placed on ice and centrifuged at 4°C, and the separated plasma was stored at −80°C before assaying. According to the manufacturer's recommended protocol (SRL, Tokyo, Japan), hANP and BNP were measured with a commercially available immunoradiometric assay. The normal values of hANP and BNP are less than or equal to 40 and 20 pg/ml, respectively.

Heart rate variability

Data obtained from the 24-h ambulatory ECG recordings were analyzed with RR data analysis software (MemCalc/CHIRAM version 1, Suwa Trust, Tokyo, Japan). We employed markers including the coefficient of variance of the RR interval (CVRR), the SD of the NN interval (SDNN), and the squares of the differences between adjacent normal-to-normal RR intervals (r-MSSD) in time domain analysis and the low-frequency (LF) area, high-frequency (HF) area, and LF/HF ratio in frequency domain analysis.

Other parameters

In addition, we evaluated parameters that possibly influence HRV, such as systolic and diastolic blood pressure, heart rate, body temperature, CsA trough, and hemoglobin to assess which parameters affected HRV in this study. For analysis, we used the averaged values of each parameter on the three preceding days that were closest to the day when the HRV evaluations were conducted.

The diagnosis of acute heart failure

Clinical criteria for acute heart failure were established by referring to those used in the Framingham study [20]. We diagnosed acute heart failure when a minimum of two major criteria or when one major and two minor criteria were present concurrently. Major criteria included orthopnea, pulmonary congestion, pulmonary rales, a gallop rhythm, and jugular venous distention. Minor criteria included tachycardia (rate>120/min), shortness of breath, ankle edema, and hepatomegaly.

Statistical analysis

The Friedman test was used for detecting changes pre- and posttransplantation (30, 60, and 100 days after transplant) in all serum markers, parameters on echocardiography, RNA, ECG and HRV, systolic and diastolic blood pressure, heart rate, body temperature, CsA trough level, hemoglobin, and C-reactive protein. All P values were two-sided, and a significance level of 0.05 was used.

Results

Table 1 shows the five patients' characteristics. The median follow-up time was 324 days (range 127–1517). Engraftment was achieved in all five patients (neutrophil engraftment occurred in the three patients with unrelated BMT 13, 17, and 21 days after transplantation; in the two patients with CBT, it occurred 27 and 31 days after transplant, respectively). No patient died within 100 days of the transplant, although in one patient (no. 2), the disease relapsed within 100 days, as she had disease recurrence in the central nervous system at day 42. In this case, therefore, CsA was tapered and stopped by day 60. Although salvage chemotherapy and donor lymphocyte infusion were performed on days 65 and 76, respectively, she died from disease progression on day 127 (Table 2). An angiotensin-receptor antagonist was administered from before transplantation in one patient (no. 5), but angiotensin-converting enzyme (ACE) inhibitors, beta-blockers, and diuretics were not administered before transplantation in any patient.

Of the five patients, none developed acute heart failure early after the conditioning chemotherapy. Pre-engraftment immune reactions (PIR) occurred in the two patients who had CBT, on days 8 and 13, but neither developed acute heart failure during the period of evaluation (Table 2). Three patients had aGVHD (grades I, III, and IV, respectively). The two patients with grade III and IV aGVHD were given high-dose steroids. Diuretics were appropriately given to patients with body weight gain during PIR or aGVHD. Three patients had sepsis, two cases of which were severe. One patient (no. 3) experienced prolonged MRSA sepsis and another patient (no. 4) experienced septic shock due to catheter infection. Two patients died from disease progression. One patient (no. 3) developed acute heart failure that might partly have been caused by pneumonia and drug-induced acute renal failure 147 days after transplantation. In this patient, acute progressive dyspnea, bilateral pleural effusions, cardiomegaly, and leg edema appeared soon after pneumonia and drug-induced acute renal failure. We diagnosed her as having acute heart failure (New York Heart Association functional class IV) and performed endotracheal intubation, administered catecholamines and diuretics, and discontinued or reduced the dose of drugs thought to be nephrotoxic. Thereafter, she recovered rapidly, and she was extubated 156 days posttransplant.

Table 2 Outcomes

No.	Neutrophil engraftment	aGVHD	cGVHD	Severe infection	AHF	Outcome (Cause of death)
1	Day 21		Limited			Alive 1,517+
2	Day 17		NA			Dead 127 (relapse)
3	Day 31	IV (day 13, skin and gut)	Extensive	Prolonged MRSA sepsis >2 months from day 56	+Day 147	Alive 461+
4	Day 13	III (day 29, gut)	Limited	Septic shock at day 61		Alive 324+
5	Day 27	I (day 49, gut)	Limited			Dead 170 (relapse)

aGVHD acute graft-versus host disease, *cGVHD* chronic graft-versus-host disease, *AHF* acute heart failure after transplantation, *MRSA* methicillin-resistant *Staphylococcus aureus*

Table 3 shows the posttransplantation changes in cardiac parameters in all five patients. All parameters for RNA, echocardiography, natriuretic peptides, and ECG showed no significant changes at any of the evaluation points. On the other hand, some parameters in the analysis of HRV changed significantly (Fig. 1). SDNN and CVRR significantly decreased at 30 to 60 days after transplantation and then recovered (P=0.04 and 0.01, respectively). Moreover, LF and HF also temporarily decreased (P=0.003 and 0.03, respectively). In particular, the patients with acute heart failure had the lowest values of SDNN, CVRR, r-MSSD, LF, and HF at 30 to 60 days after transplant (Fig. 1, dashed line). LF/HF did not change significantly.

Table 4 shows changes in the parameters that might possibly have influenced HRV, including systolic and diastolic blood pressure, heart rate, body temperature, CsA trough levels, and hemoglobin. On analysis, diastolic blood pressure and body temperature showed a statistically significant change (P=0.03 and 0.04, respectively).

Discussion

In this study, we observed no significant improvement of impaired cardiac function, but in contrast, we did detect a temporary, marked decrease in HRV soon after SCT.

As mentioned earlier, there have been three Japanese reports of the use of RIST for severe AA in patients with impaired cardiac function [4–6]. Before HSCT, LVEFs in these three cases were 20–40%. In all three cases, cardiomyopathy was caused by secondary hemochromatosis attributable to heavy transfusions. Although the mechanism was not clarified, LVEFs in all three cases dramatically recovered to a normal level, a few months to years after transplant. In one case, prompt improvement in cardiac function was seen with persistent iron deposition and without normalization of hemoglobin level [4]. These reports demonstrate that cardiomyopathy is reversible in patients with secondary hemochromatosis and severe AA. It is therefore interesting to determine whether cardiac

Table 3 Posttransplant changes of cardiac parameters

	Baseline	Day 30	Day 60	Day 100	P
Radionuclide ventriculography					
LVEF (%)	48 (37–64)	51 (41–59)	45 (38–55)	47 (39–58)	0.39
PFR (EDV/s)	2.0 (1.3–3.1)	1.8 (1.6–3.2)	1.5 (1.2–3.2)	1.9 (1.3–2.7)	0.56
Echocardiography					
IVS (mm)	7.7 (6.8–9.3)	10.0 (8.0–11.0)	8.9 (7.7–12.8)	8.6 (6.8–10.7)	0.08
PW (mm)	9.6 (8.3–11.2)	10.7 (8.0–11.8)	10.7 (9.5–12.2)	10.8 (8.0–13.2)	0.42
LVDd (mm)	45 (37–58)	44 (40–56)	47 (32–53)	46 (37–54)	0.42
LVDs (mm)	34 (28–44)	34 (27–42)	35 (24–43)	33 (31–42)	0.90
E/A	0.91 (0.72–1.17)	1.24 (0.84–2.24)	1.10 (0.85–2.08)	0.87 (0.55–1.15)	0.83
DcT (ms)	185.5 (160–220)	196 (124–212)	234 (96–296)	186 (172–296)	0.62
Natriuretic peptides					
hANP (ng/ml)	15 (12–40)	25 (12–119)	35 (10–98)	29 (10–66)	0.80
BNP (ng/ml)	36 (5–100)	68 (5–183)	21 (9–180)	35 (2–107)	0.61
Electrocardiography					
QTc (ms)	435 (422–490)	427 (408–478)	422 (408–457)	415 (405–421)	0.14

LVEF left ventricular ejection fraction, *PFR* peak filling rate, *IVS* intraventricular septal wall, *PW* posterior wall, *LVDd* left ventricular end-diastolic dimension, *LVDs* left ventricular end-systolic dimension, *E/A* early peak flow velocity/atrial peak flow velocity, *DcT* deceleration time, *hANP* human atrial natriuretic peptide, *BNP* brain natriuretic peptide

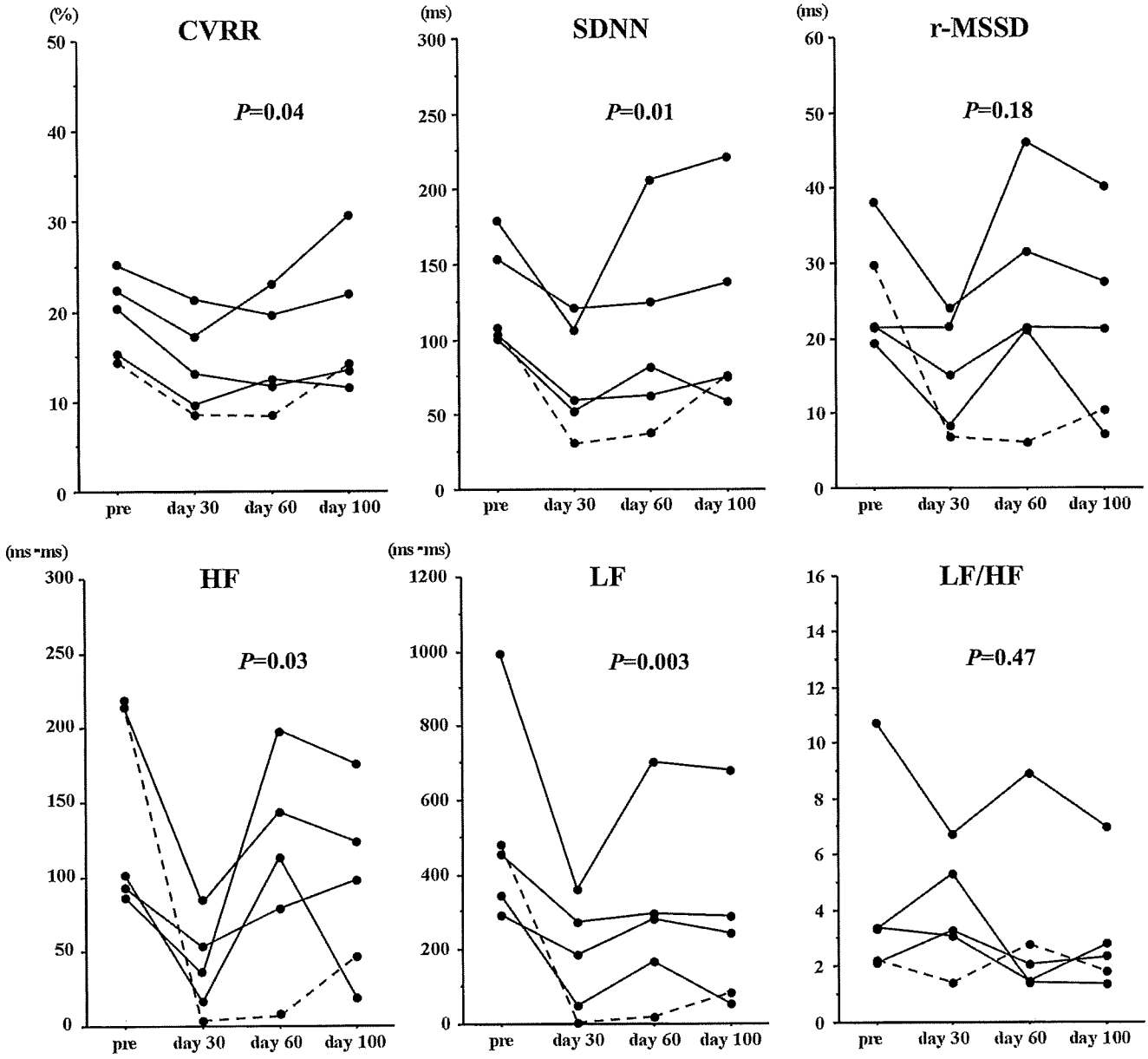

Fig. 1 Changes in HRV indicators during the first 100 days after allogeneic stem cell transplantation. The value of SDNN in the time-domain analysis and the values of HF and LF in the frequency-domain analysis significantly and temporarily decreased 30 days after transplantation. *SDNN* the standard deviation of all normal beats, *r-MSSD* the square root of the mean of the sum of squared differences between adjacent normal-to-normal intervals, *HF* high frequency power, *LF* low frequency power

dysfunction caused by other etiologies such as chemotherapy can similarly be restored. However, no significant improvement was observed in any patient in this study, suggesting that it is likely that the reversibility of cardiac function depends on the etiology of the cardiac dysfunction.

In all five patients, there were no significant changes in cardiac parameters such as left ventricular systolic and diastolic function on echocardiography and RNA, serum troponin T (data not shown), BNP and hANP, and QTc on ECG, throughout the monitoring period. One patient had acute heart failure after the completion of all assessments, but she recovered with appropriate monitoring and therapy. These results suggest that RIST is relatively well-tolerated in patients with cardiac dysfunction, provided management is adequate.

To prevent the development of acute heart failure in patients with impaired left ventricular function present before transplantation, we need to pay attention to the following points: (1) careful control of fluid intake and output by monitoring body weight and urine output, or checking for cardiomegaly and dilatation of the vena cava on chest X-ray and echocardiography; (2) careful monitoring of