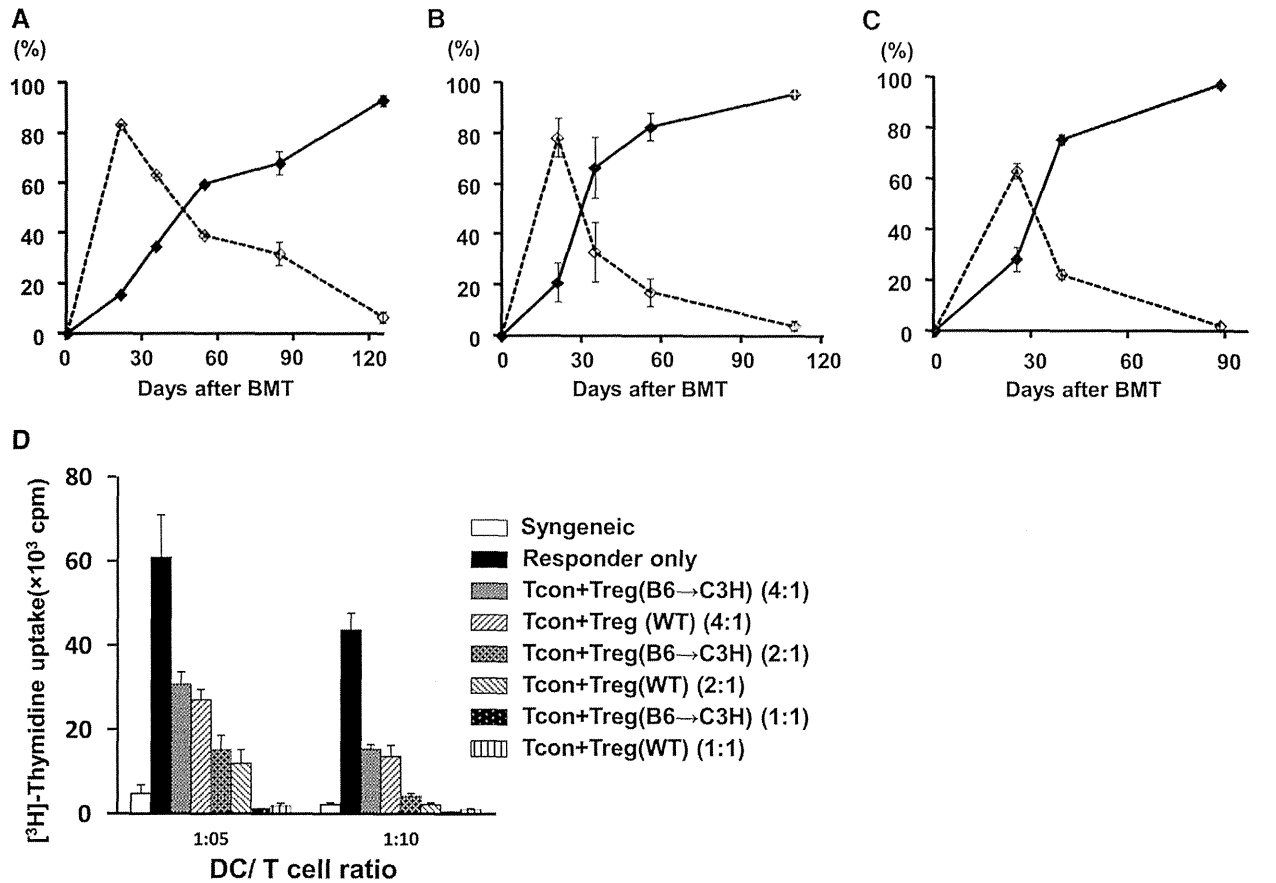

Figure 2. Donor BM–derived progenitors comprise the long-term peripheral Treg pool. Lethally irradiated C3H recipients underwent transplantation as in Figure 1: (B6 → C3H). The rates of CD45.2$^+$ spleen cell–derived (broken lines) and CD45.2$^-$ BM–derived (solid lines) Treg in CD4$^+$Foxp3$^+$ Treg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

parameters: weight loss, posture, activity, fur texture, and skin integrity (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

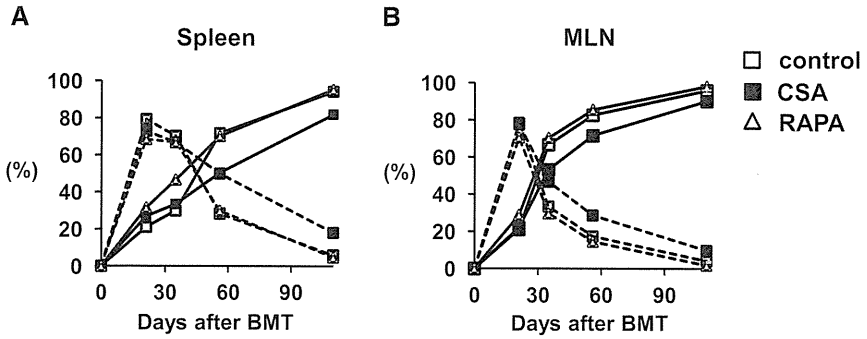

Figure 3. Effects of CSA and mTOR inhibitors on the Treg compartment. Lethally irradiated C3H recipients underwent transplantation from B6 donor mice as shown in Figure 1 and received i.p. injections of CSA (closed squares), mTOR inhibitor (rapamycin, RAPA; open triangles), or vehicle control (open squares)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

the animals that received allogeneic BM plus 2×10^6 spleen cells died by day 35 with clinical and histopathological signs of severe GVHD, the recipients of allogeneic BM plus 1×10^6 spleen cells (BM plus Sp cells) showed mild clinical signs of GVHD and 60% survived by day 120 (Figure 1A); the following experiment was performed in this setting. Flow cytometric analysis of donor cell chimerism in the spleen 3 weeks after allogeneic BMT showed that 98.8% ± 0.7% of spleen cells were derived from the donor in mice, thus confirming complete donor cell engraftment. Host Tregs, as determined by $CD4^+Foxp3^+H\text{-}2^{k+}$, were not detected in the spleen on day 21 post transplantation (data not shown). On day 21 post transplantation, the majority of $CD4^+Foxp3^+$ Tregs were derived from $CD45.2^+$ splenic T cells (83.4% ± 2.2%), suggesting that splenic T cell–derived Tregs underwent homeostatic and/or alloantigen-driven expansion (Figure 1B) and the absolute number of Tregs in the spleens of the recipients of BM plus Sp cells was significantly higher than in TCD-BM recipients. From day 21 onward, due to GVHD-induced lymphopenia, the absolute number of Tregs in the spleens of recipients of BM plus Sp cells was lower than in TCD-BM recipients (Figure 1C). The rate of $CD45.2^+$ splenic T cell–derived Tregs in $CD4^+Foxp3^+$ Treg decreased gradually and most $CD4^+Foxp3^+$ Treg were $CD45.1^+$ BM-derived (93.2%) on day 125 post transplantation (Figure 2A). The rate of $CD45.1^+$ BM-derived Tregs in the mesenteric lymph nodes (MLN) was also increased and became dominant in the late post-transplantation period (Figure 2B). To exclude strain-dependent artifacts, we next evaluated the kinetics of Treg reconstitution in the B6 ($H\text{-}2^b$) into C3.SW ($H\text{-}2^b$) MHC-compatible, multiple minor histocompatibility antigen (miHA)-incompatible model of SCT. The kinetics of Treg reconstitution in the spleen was similar and most $CD4^+Foxp3^+$ Tregs were derived from $CD45.1^+$ BM (97%) on day 90 post transplantation (Figure 2C). These findings indicated that the peripheral Treg pool was restored first by expanded splenic T cell–derived mature Treg and then by new Tregs generated from donor BM-derived progenitors. Next, to examine the function of newly arising Tregs, purified $CD4^+CD25^+$ T cells on day 120 post transplantation were

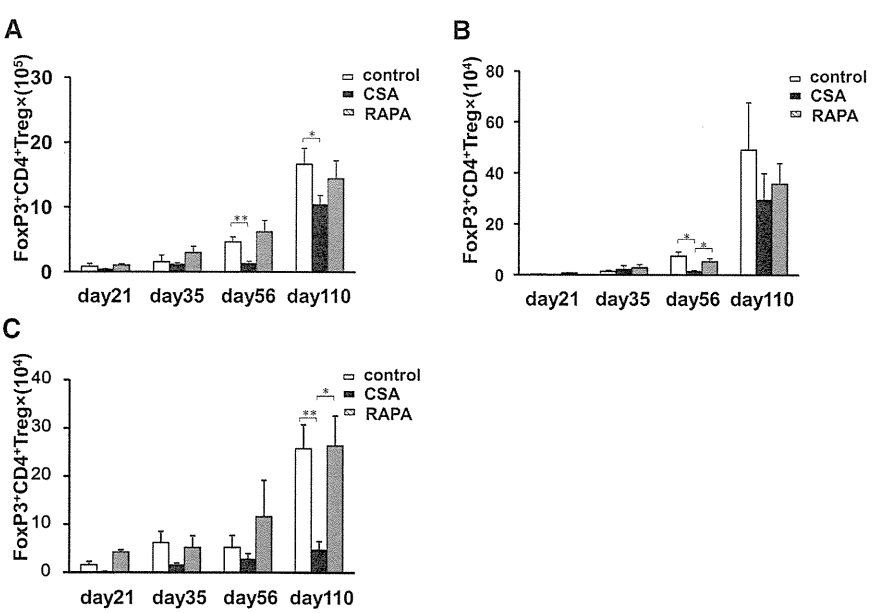

Figure 4. CSA, but not mTOR, inhibitors hampered reconstitution of BM-derived Treg. (B6 → C3H) mice received i.p. injections of CSA (black bars), mTOR inhibitor (rapamycin, RAPA; gray bars), or vehicle control (white bars) daily from day 0 to 110. The absolute numbers of Treg in the spleen (A), MLN (B), 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. $^*P < .05$; $^{**}P < .01$.

assessed for their ability to inhibit proliferation by responding syngeneic $CD4^+CD25^-$ B6 T cells. Their suppressive 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

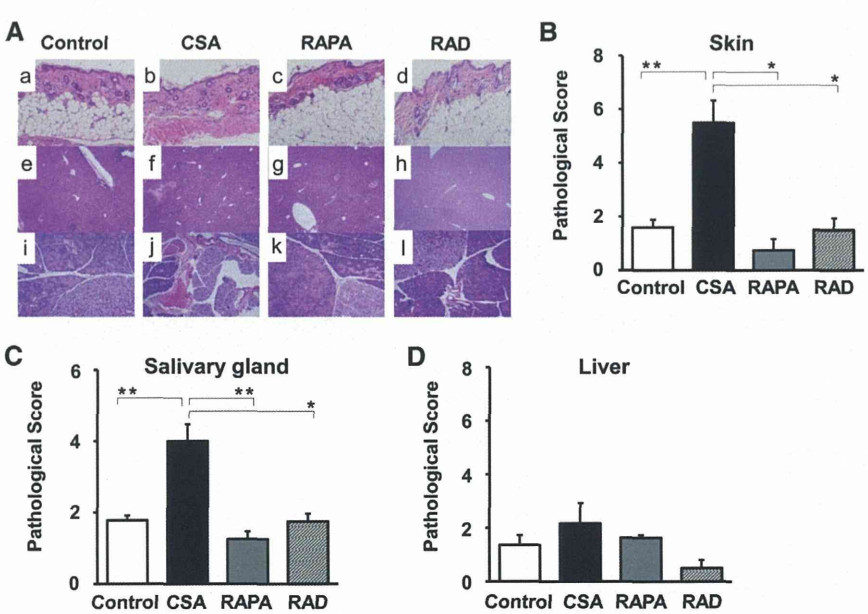

Figure 5. CSA, but not mTOR, inhibitors increased the likelihood of chronic GVHD. (A) Histological findings of the skin (a to d), liver (e to h), and salivary glands (i to l) (on day 120) from (B6 → C3H) mice given CSA, mTOR inhibitor (RAPA, RAD), or vehicle control. Sclerodermatous skin changes, such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

mice than in the controls (4.0 ± .5 versus 1.8 ± .1, $P < .01$) (Figure 5C). CSA-treated mice showed bile duct injury and 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 ([$H2\text{-}Ab1^{-/-}$→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 ([$H2\text{-}Ab1^{-/-}$→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 [$H2\text{-}Ab1^{-/-}$→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

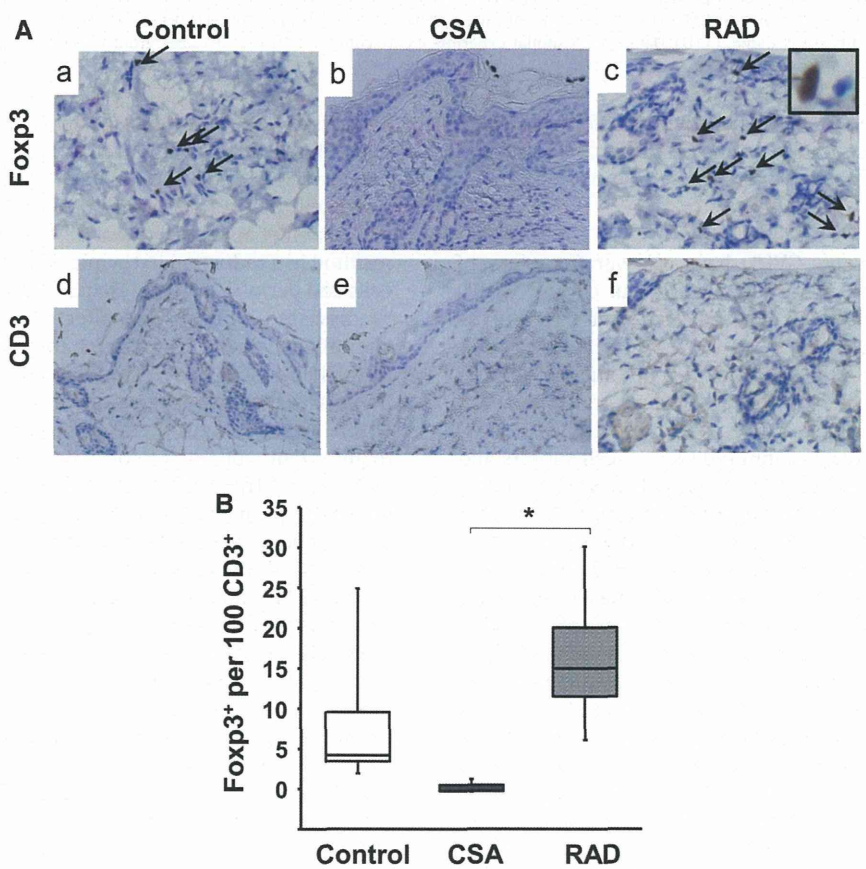

Figure 6. CSA, but not mTOR, reduces Treg infiltration in skin tissue. (A) Lethally irradiated C3H recipients underwent transplantation from B6 donor mice as shown in Figure 1 and received vehicle control (a, d), CSA (b, e), or mTOR inhibitor (RAD; c, f), daily from day 0 to 120. Immunohistochemical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

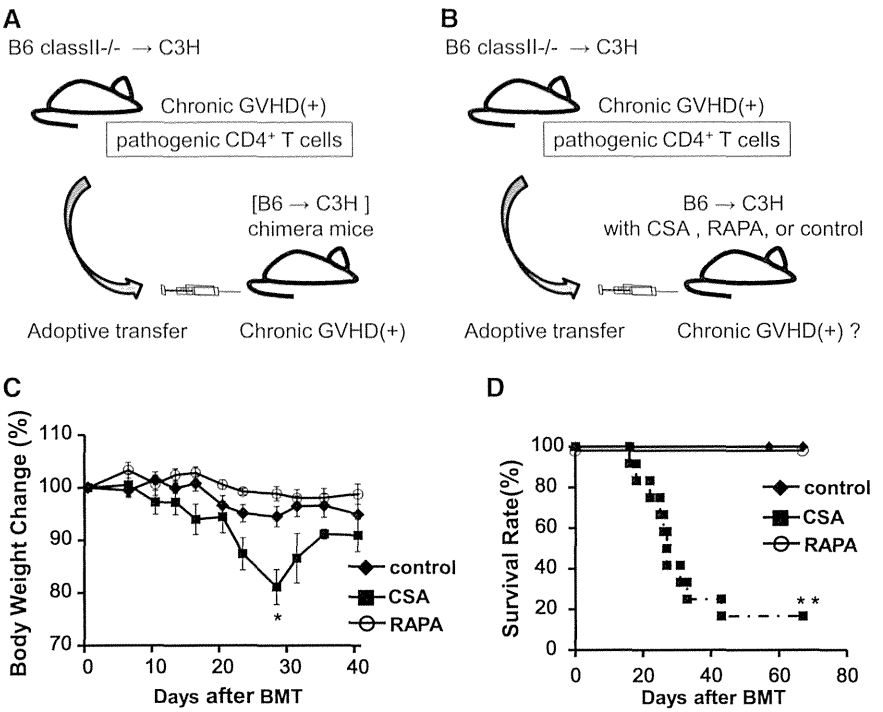

Figure 7. Adoptive transfer of pathogenic CD4+ T cells caused severe chronic GVHD. (A) Lethally irradiated C3H recipients were reconstituted with TCD BM from MHC class II-deficient (H2-Ab1$^{-/-}$) B6 mice. These mice developed chronic GVHD and CD4+ T cells isolated from chronic GVHD mice ([H2-Ab1$^{-/-}$→C3H]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

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.

DISCUSSION

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

Coenen et al. reported that 28 days of treatment with CSA resulted in a reduction in thymic generation of CD4+Foxp3+ T cells and peripheral CD25+Foxp3+ T cells in normal mice [28]. We assessed whether CSA affects the peripheral Treg pool after allogeneic BMT; on day 21, there were no differences in the absolute numbers of Tregs among 3 groups, and CSA had no impact on early Treg reconstitution. Consistent with our observations, Setoguchi et al. reported that in contrast to the requirement of IL-2 for physiological expansion of CD4+CD25+ Treg cells in normal nonlymphopenic mice, homeostatic proliferation in a lymphopenic environment appears to be IL-2-independent [19]. Zeiser et al. also reported that CSA administration has only a minor impact on the expansion of adoptively transferred CD4+CD25+ T cells on day 7 post transplantation [34]. However, whether prolonged use of CSA affects the long-term peripheral Treg pool has not been reported. Our data showed that CSA, but not mTOR inhibitors, hampered the long-term reconstitution of BM-derived Tregs. The numbers of Tregs in the spleen, thymus and tissue were significantly reduced in mice receiving CSA in comparison with those receiving mTOR inhibitors or PBS on day 110. CSA blocks nuclear factor of activated T cells translocation into the nucleus by inhibiting calcineurin phosphatase activity [35]. CSA inhibits the thymic generation of Tregs by impairment of TCR signaling and by reducing nuclear factor of activated T cells–dependent Foxp3 promoter activity [36]. In contrast, rapamycin-sensitive downstream targets of phosphatidyl-inositol 3-kinase are IL-2-independent, and

rapamycin affects neither the initial signal transduction upon TCR triggering nor the thymic generation of Treg [37]. Immunosuppressive drugs have different mechanisms of promoting immune suppression and our data revealed different effects on the long-term peripheral Treg pool after allogeneic BMT.

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

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

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SUPPLEMENTARY DATA

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

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.

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ORIGINAL ARTICLE

Single nucleotide polymorphisms of cytarabine metabolic genes influence clinical outcome in acute myeloid leukemia patients receiving high-dose cytarabine therapy

Jun Amaki · Makoto Onizuka · Ken Ohmachi · Yasuyuki Aoyama · Ryujiro Hara · Akifumi Ichiki · Hidetsugu Kawai · Ai Sato · Mitsuki Miyamoto · Masako Toyosaki · Shinichiro Machida · Minoru Kojima · Yukari Shirasugi · Hiroshi Kawada · Yoshiaki Ogawa · Kiyoshi Ando

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Abstract Cytarabine arabinoside (Ara-C) is the most important agent for treating acute myeloid leukemia (AML). Here, we genotyped 11 single nucleotide polymorphisms (SNPs) of seven Ara-C metabolism-related genes in 39 AML patients who had received high-dose Ara-C as a single-agent treatment. Univariate analysis identified three SNPs that were significantly associated with shorter time-to-relapse (TTR): CTPS rs12144160 GG compared to AA/AG, DCTD rs9990999 AG/GG compared to AA, and SLC29A1 rs693955 CC compared to AA/AC. Multivariate analysis of TTR revealed the SLC29A1 rs693955 CC genotype and first induction failure to be significantly associated with a shorter TTR. The DCTD rs9990999 AG/GG and SLC29A1 rs693955 CC genotypes were also significantly associated with shorter duration of neutropenia. The results of our study suggest that SNP analysis can be an important tool in improving drug responsiveness and enabling a better understanding of this condition and the development of tailor-made treatments for AML patients who benefit from consolidated high-dose Ara-C therapy.

Keywords Cytarabine · Single nucleotide polymorphism · Acute myeloid leukemia

J. Amaki · M. Onizuka (✉) · K. Ohmachi · Y. Aoyama · R. Hara · A. Ichiki · H. Kawai · A. Sato · M. Miyamoto · M. Toyosaki · S. Machida · M. Kojima · Y. Shirasugi · H. Kawada · Y. Ogawa · K. Ando
Division of Hematology/Oncology, Department of Internal Medicine, Tokai University School of Medicine, 143 shimokasuya, Isehara, Kanagawa 259-1143, Japan
e-mail: moni5@mac.com

Introduction

Acute myeloid leukemia (AML) is hematological malignancy characterized by the rapid growth of abnormal white blood cells that accumulate in the bone marrow and interfere with the production of normal blood cells. Treatment strategies for AML that use one of the various cytarabine arabinoside (Ara-C) agents have remained the general choice of clinicians for more than 40 years. Multiple clinical trials have demonstrated complete remission (CR) rates of 50–60 % and overall survival rates of 30–40 % among AML patients receiving such Ara-C-based therapy [1–3]. However, many studies indicate that AML describes a heterogeneous collection of diseases characterized by distinct chromosomal abnormalities and cytogenetic mutations, and as such, the most suitable general treatment for AML is still unclear. Gene variations in leukemic cells significantly associated with prognosis have now been identified, with consequent prognostic improvement [4–7], and we propose that similar improvements in AML treatment could be achieved by better understanding the genetic polymorphisms related to the pharmacokinetics of Ara-C.

Ara-C is one of the nucleotide-analog therapeutic agents, which are transported into cells by nucleoside transporters including solute carrier family 29 member 1 (SLC29A1) [8]. Intracellular Ara-C is phosphorylated into Ara-C monophosphate (Ara-CMP) by deoxycytidine kinase (DCK) and eventually to Ara-C triphosphate (Ara-CTP), which competes with deoxycytidine triphosphate (dCTP) for incorporation into DNA and subsequent blocking of DNA synthesis causing cell death. In turn, cytidine deaminase (CDA) and deoxycytidylate deaminases (DCTD) catalyze the conversion of Ara-C and Ara-CMP into an inactive form [9], and 5′-nucleotidase cytosolic II (NT5C2) activity opposes that of DCK by dephosphorylating Ara-CMP.

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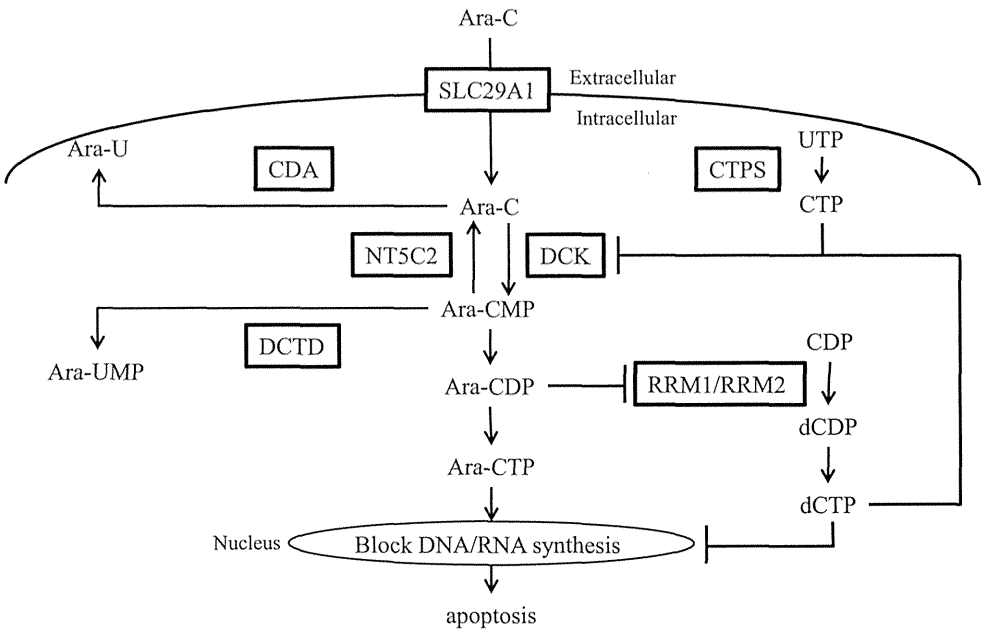

Fig. 1 Metabolic pathway of Ara-C. Ara-C is transported into cells by SLC29A1. Intracellular Ara-C is phosphorylated by DCK. Ara-CTP competes with dCTP for incorporation into DNA. CDA catalyzes the conversion of Ara-C into Ara-U, and DCTD catalyzes the conversion of Ara-CMP into Ara-UMP. CTPS catalyzes the conversion of UTP to CTP. Exhaustion of the CTP/dCTP pools due to facilitated Ara-C phosphorylation causes incorporation of Ara-CTP into DNA by reducing the feedback inhibition of DCK. RRM1/RRM2 is an enzyme involved in DNA synthesis. The enzyme regulates intracellular dCTP pools, which in turn, have been implicated in the development of Ara-C resistance

In addition, cytidine-5′-triphosphate synthetase (CTPS) and ribonucleotide reductase (RRM1/RRM2) are enzymes that regulate the intracellular CTP/dCTP pools, with exhaustion of the CTP/dCTP pools via facilitated Ara-C phosphorylation causing incorporation of Ara-CTP into DNA by reducing the feedback inhibition of DCK [10] (Fig. 1).

Understanding the pharmacogenetic response to Ara-C could lead to personalized treatment strategies and improved outcomes in AML patients. Indeed, each of the Ara-C metabolism-associated genes exhibits a significant degree of genetic variation, particularly via single nucleotide polymorphisms (SNPs), and several studies of individual SNPs in Ara-C metabolic genes have reported that genetic background plays an important role in the clinical outcomes of AML patients receiving Ara-C-based therapy [11–13]. In one such study, Gamazon et al. [11] conducted a meta-analysis of genome-wide association studies involving 523 lymphoblastoid cell lines from individuals of European, African, Asian, and African American ancestry, and identified 18 of 33 SNPs associated with either cytarabine 50 % inhibitory concentration in leukemia cells or clinical response parameters among patients randomized to receive low-dose or high-dose Ara-C plus daunorubicin and etoposide. In addition, Kim et al. [13] reported that the *SLC29A1* rs3734703 AA/AC genotype in combination with *TYMS* rs2612100 AA genotype was significantly associated with shorter relapse-free survival in Korean AML patients received an induction regimen of Ara-C and idarubicin followed by sequential consolidation therapy with Ara-C and anthracyclines or hematopoietic stem cell transplantation (HSCT). However, in these reports, the patient background included anthracycline agents or HSCT and thus might not accurately reflect the influence of genetic polymorphism on Ara-C metabolism.

Analyzing the combined effects of SNPs may provide evidence of drug response. Accordingly, we hypothesized that sensitivity to Ara-C could be influenced by SNP located in Ara-C metabolic genes and thus focused on high-dose Ara-C as single-agent therapy. In this study, we simply examined the association between SNPs in such genes and the clinical outcome of AML patients receiving high-dose Ara-C without HSCT.

Materials and methods

Study patients

We selected de novo AML patients who received high-dose Ara-C as consolidation therapy, and whose bone marrow or peripheral blood samples were stored in our laboratory. We excluded one patient diagnosed as M3 subtype and

one patient who received HSCT in the first CR. All subjects enrolled in this study provided informed consent for genetic analysis. This study was approved by the Institutional Review Board of Tokai University Hospital.

SNP selection

Seven Ara-C metabolic genes, *CDA*, *CTPS*, *DCK*, *DCTD*, *NT5C2*, *RRM1* and *SLC29A1*, were evaluated. SNPs were selected based on SNP frequency data from the International Hap-Map project (http://hapmap.ncbi.nlm.nih.gov/) and The National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/). SNP frequency data were identified according to the HapMap Genome Browser release #27 (Phase 1, 2 and 3—merged genotypes and frequencies) of Japanese in Tokyo, Japan (JPT) database to estimate linkage disequilibrium (LD) blocks by Haploview software (version 4.2) [14]. The criteria for SNP selection were thus as follows: (1) minor allele frequency (MAF) >0.25 in JPT; (2) one or two SNPs were selected from each evaluated gene; (3) SNP reported by previous study [15], or located with each different LD blocks of the highest MAF. Allele frequency data of rs9937 was lacking in the JPT, so we instead search SNP frequency data of the CHB + JPT data in NCBI. Finally, eleven SNPs (*CDA*; rs10916827, rs477155, *CTPS*; rs4132440, rs12144160, *DCK*; rs12648166 *DCTD*; rs9542, rs9990999, *NT5C2*; rs3736922, *RRM1*; rs9937, *SLC29A1*; rs693955, 9394992) in each of the gene locus were selected.

SNP genotyping

Genomic DNA was isolated from bone marrow or peripheral blood individually using a QIAamp mini DNA kit (Qiagen, Valencia, CA, USA). SNP genotyping was performed using the TaqMan platform in 96-well plates and read with the Sequence Detection Software on a 7500 Real-Time PCR System according to the manufacturer's instructions (Applied Biosystems, Foster City, CA, USA). Primers and probes were supplied by Applied Biosystems.

Definition of clinical response and hematological toxicity

All clinical information about the patients was obtained from our institution database.

Overall survival (OS) was measured from the date of diagnosis to the date of death from any cause. Time to relapse (TTR) was measured from the date of diagnosis to the date of relapse. Relapse was defined as the presence of more than 5 % blast cells in the bone marrow or reappearance of blast cells in the peripheral blood. Hematological toxicity was measured by duration of neutropenia and thrombocytopenia during each high-dose Ara-C course. Neutropenia was defined as an absolute neutrophil count less than 500/μl. Thrombocytopenia was defined as an absolute platelet count less than 50,000/μl.

Statistical analysis

For SNP analysis, we tested two genetic models: the dominant model (major allele homozygous + heterozygous vs. minor allele homozygous) and the recessive model (major allele homozygous vs. heterozygous + minor allele homozygous). For univariate analysis, OS probabilities were estimated by the Kaplan–Meier method, and differences in the distributions between the dominant and recessive model of each SNP were evaluated using the log-rank test. TTR was estimated by the cumulative incidence method, and Gray's test was used to compare distribution differences between the genotypes [16]. Death without experiencing a relapse was considered a competing event with experiencing first relapse.

For multivariate analysis, the Fine–Gray regression model [5] was used for the sub-distribution hazard of a competing risk to analyze the effect of baseline risk factors on the cumulative incidence function of relapse. Factors associated with a two-side P value of less than 0.05 in the univariate SNP analysis and known prognostic risk factors for the Japanese population [17], such as age >50 years, performance status >2, myeloperoxidase (MPO) positivity of blasts >50 %, and first induction failure, were included in the multivariate analysis. We used a stepwise regression based on P values and retained only the statistically significant variables in the final model. This analysis did not include the factor of the DCTD rs9990999 AA genotype, because no patients with DCTD rs9990999 AA genotype experienced relapse. Thus, the DCTD rs9990999 AA genotype could not be treated as a single multivariate factor for the time-to-event analysis. For hematological toxicity analysis, comparisons between the dominant and recessive models of each SNP were performed using Student's t test.

All statistical analyses were performed with EZR (Saitama Medical Center, Jichi Medical University), which is a graphical user interface for R (The R Foundation for Statistical Computing, version 2.13.2) [18]. More precisely, it is a modified version of R commander (version 1.6–3) that includes statistical functions frequently used in biostatistics. For all analyses, P values were two-tailed, and a P value of less than 0.05 was considered significant.

Results

Patient characteristics and treatments

Thirty-nine AML patients were eligible for this study. Table 1 summarizes the patients' characteristics. The

Table 1 Patient characteristic

Factor	Group	Number (%) $n = 39$ (100 %)	Median (range)
Age			54.00 y/o (23.00, 71.00)
Gender	Male	26 (66.7)	
	Female	13 (33.3)	
FAB	M1	8 (20.5)	
	M2	22 (56.4)	
	M4	4 (10.3)	
	M5	5 (12.8)	
Karyotype	Normal	14 (35.9)	
	t(8;21)	12 (30.8)	
	inv(16)	2 (5.1)	
	del(7)	2 (5.1)	
	Complex	2 (5.1)	
	Others	7 (17.9)	
FLTIII-ITD mutation	Negative	16 (41.0)	
	Positive	3 (7.7)	
	Unknown	20 (51.3)	
WBC count at diagnosis			20,800/μl (900, 474,800)
MPO positivity of blasts	≤50 %	10 (25.6)	
	>50 %	29 (74.4)	
Performance status	0	24 (61.5)	
	1	12 (30.8)	
	2	2 (5.1)	
	4	1 (2.6)	
First induction regimen	DNR + Ara-C	8 (20.5)	
	IDA + Ara-C	31 (79.5)	
No. of induction	1	33 (84.6)	
	>1	6 (15.4)	
HSCT after relapse		7 (17.9)	
Relapse rate		19 (48.7)	
Time to relapse			306 days (152, 1271)
Non-relapse morality		4 (10.3)	
Overall survival			833 days (55, 3931)

FAB French–America–British classification, *WBC* white blood cell, *MPO* myeloperoxidase, *PS* performance status, *HSCT* hematopoietic stem cell transplantation

median age of patients was 54.0 years (range 23.0–71.0 years) and the male/female proportion was 26/13. The most frequent French–American–British (FAB) subtype was M2 (56.4 %) followed by M1 (20.5 %). M0, M6, and M7 subtypes were not represented in this population. In total, 14 patients had a good cytogenetic risk based on karyotype; 12 patients had t(8; 21) (q22;q22) and 2 patients had inv(16) (p13q22). Another 4 patients had a poor cytogenetic risk; 2 patients had del(7) and 2 patient had a complex karyotype. A total of 14 patients (35.9 %) were of normal karyotype, and 7 patients had an unspecified karyotype. Three of the 19 patients in whom the FLT3 internal tandem duplication (ITD) mutation information was identified had a FLT3/ITD mutation; however, the FLT3/ITD mutation was not examined for the other 20 patients and data on other molecular abnormalities were not available. Median white blood cell (WBC) count at diagnosis was 20,800/μl (range 900–47,4800/μl). Eight patients had received their first induction regimen consisting of Ara-C with daunorubicin, and another 31 patients received an induction regimen consisting of Ara-C with idarubicin. Thirty-three patients (84.6 %) achieved CR after the first induction regimen, and six patients (15.4 %) needed two or more induction regimens to achieve CR. Once patients achieved complete remission, the patients received consolidation therapy consisting of high-dose Ara-C. Seventeen patients received three courses of Ara-C dose of 2.0 g/m^2 for 5 days. Two patients received two courses of Ara-C dose of 2.0 g/m^2 for 5 days and one patient was died in the second course of consolidation therapy due to severe infection. Three patients died in the first course. Other patients received various doses and durations of Ara-C treatment according to the physician's clinical decision, as follows: one patient received three courses of Ara-C dose of 3.0 g/m^2 for 5 days; one patient received three courses of Ara-C dose of 3.0 g/m^2 for 4 days; two patients received four courses of Ara-C dose of 3.0 g/m^2 for 3 days; one patient received two courses and two patients received three of Ara-C dose of 2.0 g/m^2 for 3 days; and, one patient received two courses and four patients received three of Ara-C dose of 1.0 g/m^2 for 5 days. Nineteen patients (48.7 %) relapsed during the follow-up period and the median TTR was 306 days (range 152–1271 days). The median follow-up period overall survival was 833 days (range 55–3931 days).

Results of SNP genotyping

All SNP genotyping was successful among the AML patients, as summarized in Table 2.

The SNP genotypes could be divided into three groups, and comparison between groups only was analyzed statistically, due to insufficient minor allele frequency.

SNP effect on treatment outcomes

The effects of the two SNP genetic models on OS and TTR are summarized in Table 3. In the univariate analysis, no SNP was a significant prognostic factor for OS. However, three SNPs, individually, had associations with TTR (Fig. 2). The *CTPS* rs12144160 GG genotype was

Table 2 SNP genotype frequency and analysis model in our study

Gene; ref.SNP	Chromosome	Location	Genotype	*n*	Analysis model	
CDA; rs10916825	Chr1	Intron	AA	17	Dominant model	AA vs. AG + GG
			AG	20	Recessive model	AA + AG vs. GG
			GG	2		
CDA; rs477155	Chr1	Intron	AA	6	Dominant model	GG vs. GA + AA
			AG	20	Recessive model	GG + GA vs.AA
			GG	13		
CTPS; rs4132440	Chr1	Intron	AA	18	Dominant model	AA vs. AG + GG
			AG	18	Recessive model	AA + AG vs. GG
			GG	3		
CTPS; rs12144160	Chr1	Intron	AA	7	Dominant model	GG vs. GA + AA
			AG	20	Recessive model	GG + GA vs. AA
			GG	12		
DCK; rs12648166	Chr4	Intron	AA	10	Dominant model	AA vs. AG + GG
			AG	21	Recessive model	AA + AG vs. GG
			GG	8		
DCTD; rs9542	Chr4	Intron	CC	7	Dominant model	TT vs. TC + CC
			CT	18	Recessive model	TT + TC vs. CC
			TT	14		
DCTD; rs9990999	Chr4	Intron	AA	6	Dominant model	GG vs. GA + AA
			AG	14	Recessive model	GG + GA vs. AA
			GG	19		
NT5C2; rs3736922	Chr10	Intron	AA	9	Dominant model	GG vs. GA + AA
			AG	19	Recessive model	GG + GA vs. AA
			GG	11		
RRM1; rs9937	Chr11	Exon	AA	15	Dominant model	AA vs. AG + GG
			AG	16	Recessive model	AA + AG vs. GG
			GG	8		
SLC29A1; rs693955	Chr6	Intron	AA	1	Dominant model	CC vs. CA + AA
			AC	15	Recessive model	CC + CA vs. AA
			CC	23		
SLC29A1; s9394992	Chr6	Intron	CC	20	Dominant model	CC vs. CT + TT
			CT	16	Recessive model	CC + CT vs. TT
			TT	3		

significantly associated with shorter TTR compared to the AA/AG genotype (2-year relapse rate 0.694 [95 % CI 0.258–0.907] vs. 0.363 [95 %CI 0.177–0.552], $P = 0.0209$). The *DCTD* rs9990999 AG/GG genotype was significantly associated with shorter TTR compared to the AA genotype (2-year relapse rate 0.529 [95 % CI 0.331–0.692] vs. NA [NA–NA], $P = 0.0255$). The *SLC29A1* rs693955 CC genotype was significantly associated with shorter TTR compared to the AA/AC genotype (2-year relapse rate 0.683 [95 % CI 0.416–0.848] vs. 0.131 [0.019-0.353], $P = 0.00261$). There were 14 patients with CBF leukemia and 21 patients with intermediate risk group. The *SLC29A1* rs693955 CC genotype with intermediate risk ($n = 14$) was significantly associated with shorter TTR compared to the AA/AC genotype ($n = 7$) (2-year relapse rate 0.701 [95 % CI 0.295–0.902] vs. 0.214 [0.002–0.689], $P = 0.0498$). There was no statistical significance of the comparison for other SNPs and TTR regarding cytogenetic risk groups.

Multivariate analysis of TTR revealed that the *SLC29A1* rs693955 CC genotype (HR 7.659 [95 % CI 1.98–29.63], $P = 0.0096$) and first induction failure (HR 3.613 [95 % CI 1.37–9.55]) were significantly associated with shorter TTR (Table 4).

SNP effect on hematological toxicity

The total number of high-dose Ara-C was 109, and febrile neutropenia (FN) observed in 83 cases including 4 mortalities due to exacerbation of infections. The dead cases were

excluded from the duration analysis because they did not recover from the neutropenia or the thrombocytopenia. The duration of hematological toxicity analysis was therefore analyzed for 105 treatment courses. The mean durations of neutropenia and thrombocytopenia were 14.1 days (95 % confidential interval [CI]; 12.8–15.4 days) and 14.3 days (95 %CI; 12.6–16.0 days), respectively. Administration of granulocyte colony-stimulating factor (GCSF) was noted in 55/109 courses. Table 5 summarized the statistical analysis of SNPs compared to duration of neutropenia or thrombocytopenia. Figure 3 shows the three candidate SNPs and the association with duration of neutropenia. The *DCTD* rs9990999 AG/GG genotype was significantly associated with shorter duration of neutropenia compared to the AA genotype (13.2 ± 5.77 vs. 19.5 ± 9.11 days, $P = 0.000497$). The *SLC29A1* rs693955 CC genotype was significantly associated with shorter duration of neutropenia compared to the AA/AC genotype (13.1 ± 5.69 vs. 15.8 ± 7.87 days, $P = 0.0386$) and also thrombocytopenia (12.7 ± 7.89 vs. 16.9 ± 9.15 days, $P = 0.0116$). The frequency of GCSF administration was 1 in 16 of the *DCTD* rs9990999 AA genotypes and 54 in 93 of AG/GG genotypes, and was similar between the *SLC29A1* AA/AC genotype (20/42) and CC genotype (32/67). The *SLC29A1* AA/AC genotype required longer duration of GCSF administration than CC genotype (16.1 ± 5.54 vs. 13.1 ± 4.58 days, $P = 0.0377$). Concerning neutropenia without GCSF, the *DCTD* rs9990999 AG/GG genotype ($n = 39$) was significantly associated with shorter duration of neutropenia compared to the AA genotype ($n = 15$) (15.1 ± 5.44 vs. 19.5 ± 9.11 days, $P = 0.0314$), and the *SLC29A1* rs693955 CC genotype ($n = 32$) was significantly associated with shorter duration of neutropenia compared to the AA/AC genotype ($n = 22$) (14.8 ± 5.33 vs. 18.5 ± 5.27 days,

Table 3 Summary of *P* value of SNPs about OS and TTR

Gene	SNP	*P* Value (OS)		*P* value (TTR)	
		Dominant	Recessive	Dominant	Recessive
CDA	rs10916825	0.931	0.954	0.994	0.399
CDA	rs477155	0.252	0.0916	0.619	0.069
CTPS	rs4132440	0.615	0.228	0.891	0.967
CTPS	rs12144160	0.448	0.179	0.119	**0.0209**
DCK	rs1268166	0.991	0.805	0.232	0.896
DCTD	rs9542	0.345	0.334	0.828	0.22
DCTD	rs9990999	0.371	0.473	**0.0255**	0.579
NT5C2	rs3736922	0.984	0.544	0.916	0.843
RRM1	rs9937	0.601	0.633	0.483	0.845
SLC29A1	rs693955	0.74	0.814	**0.00261**	0.699
SLC29A1	rs9394992	0.921	0.2	0.626	0.248

Value of $P < 0.05$ were considered statistically significant

Bold value donate statistical significance

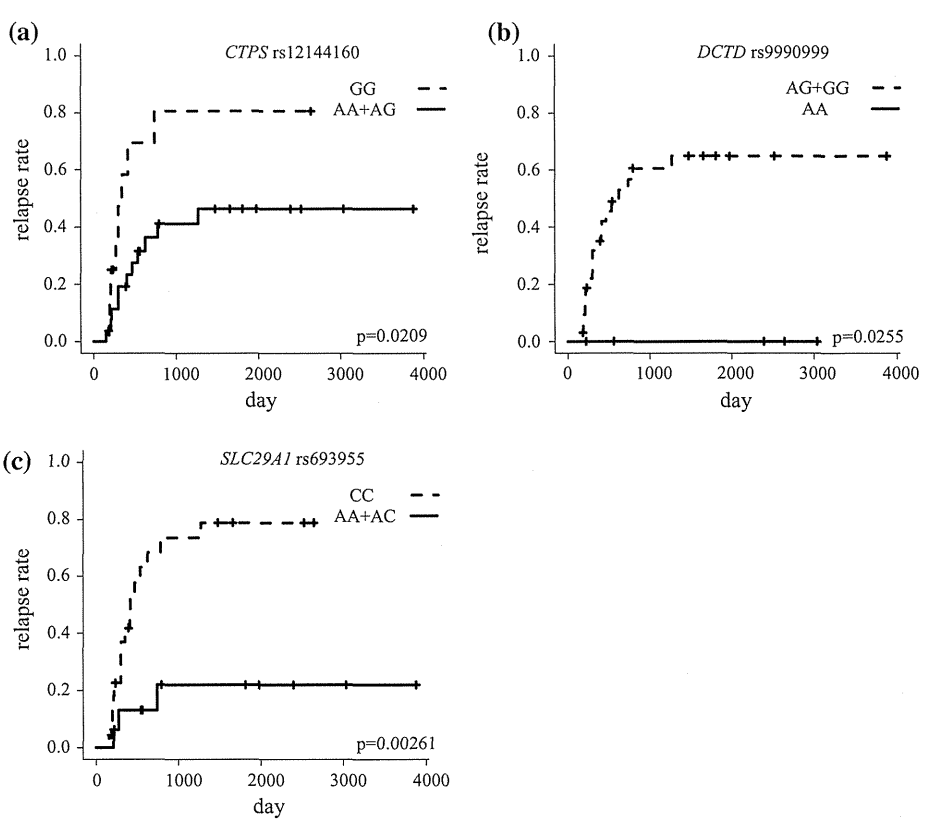

Fig. 2 Significant effect of SNP on time to relapse. Time to relapse was estimated by the cumulative incidence method, and Gray's test was used to compare differences between genotypes with respect to the SNP distributions. Death without experiencing a relapse was considered a competing event with experiencing first relapse. **a** SNP effect of *CTPS* rs12144160. **b** SNP effect of *DCTD* rs9990999. **c** SNP effect of *SLC29A1* rs693955

Table 4 Competing-risk model of variables associated with the cumulative incidence of relapse

Factor	Group	*n*	Univariant			Multivariant	
			2 year Relapse rate	Median TTR	*P* value	Hazard ratio	*P* value
Age	≤50	13	0.500 (0.231–0.614)	643	0.557		
	>50	26	0.433 (0.220–0.629)	NA			
No. of induction	1	33	0.414 (0.230–0.590)	1271	0.271		
	>1	6	0.667 (0.122–0.925)	291.5		3.613 (1.37–9.55)	**0.0032**
Karyotype	CBF-leukemia	14	0.429 (0.166–0.670)	NA	0.346		
	Others	25	0.539 (0.289–0.736)	745			
MPO positive blasts	≤50 (%)	10	0.594 (0.084–0.897)	647	0.518		
	>50 (%)	29	0.432 (0.242–0.609)	1271			
Performance status	1–2	38	0.437 (0.264–0.598)	782	0.357		
	3–4	1	NA (NA–NA)	415			
WBC count at diagnosis	≤20000/μl	19	0.369 (0.141–0.601)	782	0.274		
	>20000/μl	20	0.533 (0.280–0.733)	415			
CTPS rs12144160	AA/AG	27	0.363 (0.177–0.552)	NA	**0.0209**		
	GG	12	0.694 (0.258–0.907)	350			
DCTD rs9990999	AA	6	0.000 (0.000–0.000)	NA	**0.0255**		
	AG/GG	33	0.529 (0.331–0.692)	647			
SLC29A1 rs693955	AA/AC	16	0.131 (0.019–0.353)	NA	**0.00261**		
	CC	23	0.683 (0.416–0.848)	415		7.659 (1.98–29.63)	**0.0096**

Bold value donate statistical significance

Value of $P < 0.05$ were considered statistically significant

MPO myeloperoxidase, *WBC* white blood cell, *CBF* core binding factor

Table 5 Summary of *P* value of SNPs about duration of neutropenia and thrombocytopenia

Gene	SNP	Neutropenia (WBC <500/μl)		Thrombocytopenia (Platelet <50,000/μl)	
		Dominant	Recessive	Dominant	Recessive
CDA	rs10916825	0.175	0.734	0.832	0.636
CDA	rs477155	**0.00249**	0.619	0.0576	0.919
CTPS	rs4132440	0.648	0.327	0.680	0.460
CTPS	rs12144160	0.544	0.628	0.0681	0.909
DCK	rs1268166	0.419	0.689	0.0816	0.756
DCTD	rs9542	**0.00265**	**0.00989**	0.154	0.736
DCTD	rs9990999	0.0257	**0.000497**	0.0895	0.0558
NT5C2	rs3736922	0.861	0.962	0.859	0.721
RRM1	rs9937	0.926	0.884	0.705	0.911
SLC29A1	rs693955	**0.0386**	**0.000155**	**0.0166**	0.0995
SLC29A1	rs9394992	**0.0177**	0.994	**0.0116**	0.477

Value of $P < 0.05$ were considered statistically significant

Bold value donate statistical significance

$P = 0.0468$). The frequency of FN was 12 of 16 *DCTD* rs9990999 AA genotypes, 71 of 93 AG/GG genotypes, 30 of 42 *SLC29A1* AA/AC genotypes, and 53 of 67 CC genotypes. In addition, the four cases that died during high dose Ara-C treatment all had the same *SLC29A1* rs693955 AA/AC genotype. However, there was no severe acute neurotoxicity caused by high-dose Ara-C therapy observed in the patients, and other acute, severe non-hematologic side effects were not documented in the available clinical information.

Discussion

The present study suggested that the three SNPs, rs12144160 in the *CTPS* gene, rs9990999 in the *DCTD* gene, and rs693955 in the *SCL29A1* gene could influence outcomes in AML patients receiving high-dose Ara-C treatment.

The catalytic conversion of UTP to CTP is accomplished by the CTPS enzyme encoded by *CTPS*, and increased Ara-C sensitivity results from decreased CTP/dCTP pools caused by inhibition of CTPS with cyclopentenyl cytosine in myeloid leukemia and T-lymphoblastic leukemia cell lines [19, 20]. Although Ara-C resistance caused by clustered mutations within the coding region of CTPS have been identified in Chinese hamster ovary cells, no mutations were identified in the regions indicated from recurrent or resistant acute leukemia in 36 patients [10]. In our study,

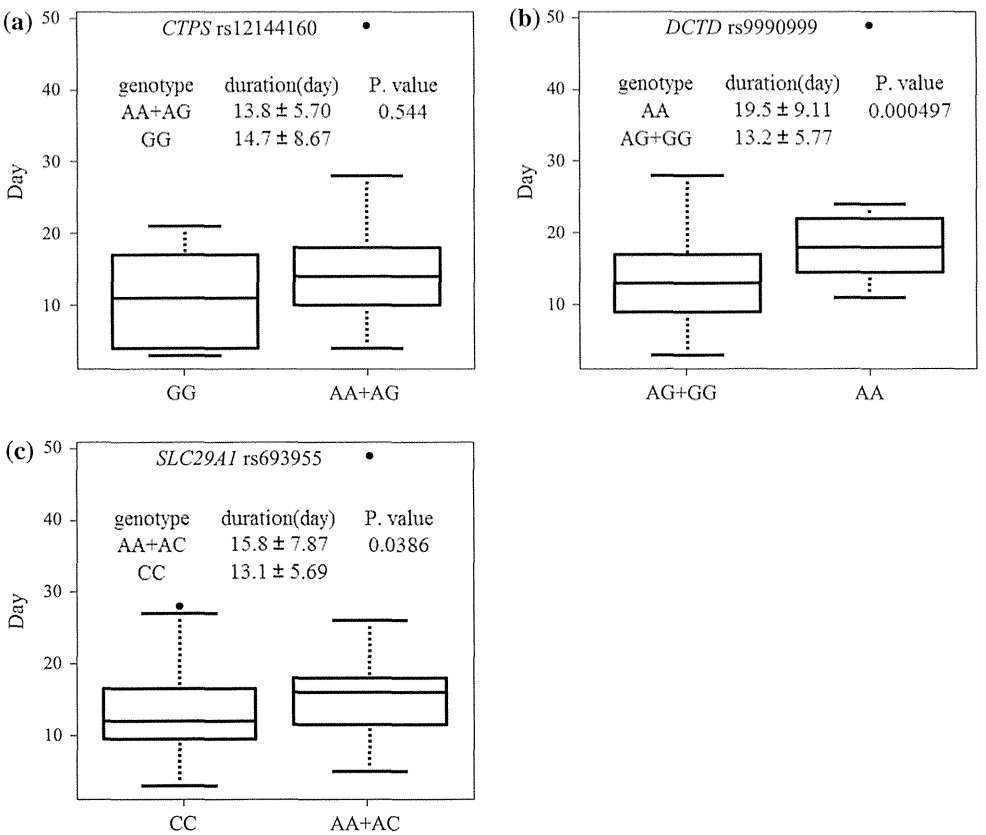

Fig. 3 Duration of neutropenia. Three SNPs associated with TTR are shown in this figure. **a** SNP effect of CTPS rs12144160. **b** SNP effect of DCTD rs9990999. **c** SNP effect of SLC29A1 rs693955. CTPS rs12144160 was not associated with hematological toxicity. The DCTD rs9990999 AA genotype was associated with a longer duration of neutropenia than the AA or CC genotype. The SLC29A1 rs693955 AA genotype was associated with a longer duration of both neutropenia and thrombocytopenia than the AA or CC genotype

the *CTPS* rs12144160 GG genotype was significantly associated with shorter TTR compared to the AA/AG genotype, suggesting that the GG genotype patients had higher levels of CTPS expression or activity than those with the AA/AG genotype. However, rs12144160 is located in an intronic region and therefore might affect RNA expression rather than enzyme activity.

SNP rs9990999 in the *DCTD* gene was a significant prognostic factor for TTR in this study, and the AG/GG genotype was significantly associated with shorter TTR compared to the AA genotypes. Interestingly, the patient with an AA genotype showed a long duration of neutropenia and did not experience relapse in our study. The protein encoded by the *DCTD* gene catalyzes the deamination of Ara-CMP to Ara-UMP and is allosterically activated by dCTP and inhibited by dTTP. Schröder et al. [9] reported that the expression level of DCTD was not associated with Ara-C sensitivity, while a non-synonymous SNP, the A172G mutation causing Asn58Asp, on the coding regions and causing loss of activity for gemcitabine monophosphate was identified in Caucasian and African ethnic groups in vitro assays [21], although the minor allele frequency was too low for meaningful association analysis with clinical response to Ara-C in this previous study [12]. However, nonsynonymous SNPs, including A172G, have not been observed in the DCTD gene in a Japanese population. There is also no evidence of functional SNPs in the *DCTD* gene, although our findings showed some kind of gene function for rs9990999 in TTR and neutrophil toxicity.

Our univariate and multivariate analysis found that the *SLC29A1* rs693955 CC genotype was significantly associated with shorter TTR and shorter duration of hematological toxicity. The *SLC29A1* gene encodes the human equivalent of nucleoside transporter 1 (hENT1), a protein that resides in the plasma membrane to mediate the cellular uptake of cytotoxic nucleosides as Ara-C from the surrounding medium. Although multiple alternatively spliced variants have been found for the *SLC29A1* gene, they all encode the same protein, and thus a deficiency in hENT1 expression might be the basis of cellular resistance to Ara-C [22]. SNPs have been previously detected in the *SLC29A1* gene from Japanese populations and some SNPs have been implicated in the efficacy of Ara-C [23] and the

mRNA expression [24, 25]. However, hENT1 is a limiting determinant of Ara-C efficacy, and the simple diffusion rate of Ara-C exceeds its pump-mediated transport in high plasma concentrations of Ara-C [26]. Although it is less likely that Ara-C is taken up into the cell by SLC29A1 at the 50-μM plasma concentration reached by high-dose Ara-C, rs693955 located in the *SLC29A1* gene might still influence clinical outcomes, based on our multivariate analysis identifying first induction failure and rs693955 CC genotype as independent prognostic factors. Suzuki et al. [25] reported that mRNA levels in the rs6932345 wild-type (A>C) and rs747199 wild-type (G>C) were higher than in the mutation carriers, and LD block analysis from the HapMap database linked the rs693955 C allele with the rs747199 C allele at a frequency of 14.8 %. Conversely, the rs693955 A allele was not linked with the rs747199 G allele. Thus, we proposed that patients with the rs693955 CC genotype have lower expression levels of hENT1 and consequently, shorter TTR and lower hematological toxicity. In addition, Pérez-Torras et al. [27] reported that overexpression of hENT1 in a relatively low transporter activity background increased the uridine uptake, resulting in a decreased amount of mRNA encoding key nucleotide metabolism enzymes, such as DCK and RR, and reduced cell cycle progression in the cell lines derived from human pancreatic adenocarcinomas. Nucleotide metabolism with the rs693955 CC genotype might be easy to change by the similar action of hENT1 overexpression. High intracellular concentrations of Ara-C or the product of Ara-C metabolism due to high-dose Ara-C therapy may have caused the decrease in nucleotide metabolism enzymes and cell cycle progression. The rs693955 CC genotype patient in this study might therefore have a phenotypic resistance to Ara-C, leading to the rapid recovery from cytopenia and the early relapse.

DCK is required for the first phosphorylation of deoxyribonucleosides and Ara-C is the most important enzyme in the activation pathway of Ara-C. However, in our study, the *DCK* rs12648166 was not associated with therapeutic outcomes. Our study does not include all SNPs on the *DCK* locus, but only one SNP was available based on our SNP selection criteria, and it is possible other SNPs on the *DCK* locus with a lower MAF might be functional polymorphisms.

Interestingly, the *DCTD* rs9990999 AG/GG and *SLC29A1* rs693955 CC were associated with shorter time to relapse and shorter duration of neutropenia. This finding suggested that the sensitivity to Ara-C of de novo leukemic cells is not very different from the sensitivity to Ara-C of normal hematopoietic stem cells. Braess et al. [28] reported CDA activities and Ara-C deamination in a variety of the most commonly used leukemic cell lines, fresh blasts, and normal bone marrow cells. However, the cell lines herein had different CDA activity profiles and degrade between 18.5 and 96.5 % of Ara-C to Ara-U. Formation of Ara-CTP is therefore significantly influenced by the differences in cell type-dependent cytidine deaminase activity. In contrast to the cell lines, fresh leukemic blasts and normal bone marrow mononuclear cells show low Ara-C degradation, and cultured cell lines are exposed to unknown selective pressure during a year or even several decades. Therefore, the biological reactions of cultured cells to Ara-C exposure seem to be relatively changed from the primary source leukemia cells. Conversely, the de novo leukemic cell without exposure to Ara-C might maintain a similar Ara-C metabolism to normal cells.

The results of our study suggested that SNP analysis could lead to better drug responsiveness and improved treatments for AML patients who benefit by receiving consolidation therapy with high-dose Ara-C. To our knowledge, this is the first report showing the relationship between SNPs and clinical outcomes in AML patient receiving high-dose Ara-C as single-agent therapy. Previous studies investigated the relationships between SNPs located on Ara-C metabolic genes and clinical response or toxicity with various doses of Ara-C based therapy including hematopoietic stem cell transplantation. *RRM1* rs1042919 and rs1561876 were related to intracellular Ara-CTP concentration, CR rate, and OS [29]. *CDA* rs2072671 and rs532545 were related to OS in a FLT3-ITD mutation-positive normal karyotype AML patient, and *NT5C2* rs10883841 was related to OS in a FLT3-ITD mutation-negative case [30]. In addition, the *SLC29A1* rs3734703 AA/AC genotype in combination with *TYMS* rs2612100 was significantly associated with relapse-free survival and *DCK* rs469436 was associated with OS in AML patients [13]. Nevertheless, few studies have determined SNP functions, and it is possible that *CDA* rs2072671 caused CDA protein variants (p.Lys27Gln) that may be related to the loss of CDA activity [31]. Our study did not include such SNPs because their MAF was lower than 0.25. For particular SNPs to be extracted as therapeutic surrogate markers, the functional meaning of these candidate SNPs must be determined and validated in future cohort studies. It might also be applicable to improve other nucleotide analog treatments which have similar pharmacokinetics system of Ara-C, such as gemcitabine, fluorouracil, and azacitidine by conducting SNP analysis associated with the Ara-C pharmacokinetics. However, our study was limited due to the small sample size and the inherent selection bias, since the patients examined here were already in the first remission state and were receiving high-dose Ara-C as a consolidation therapy. All the meaningful SNPs in our study were in introns. We could therefore not explore the SNP functions based on RNA expression, elongation, or splicing variants of the gene. It is therefore necessary to identify functional

SNPs that could be related to LD with the SNPs in our study by genome sequencing and thereby confirm the differences by RNA expression level or enzyme activity. Further studies are therefore needed to reveal the SNP functions and validation cohort studies are warranted.

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Conflict of interest The authors declare that they have no conflict of interest.

References

1. Kumar CC. Genetic abnormalities and challenges in the treatment of acute myeloid leukemia. Genes Cancer. 2011;2:95–107.
2. Döhner H, Estey EH, Amadori S, Appelbaum FR, Büchner T, Burnett AK, European LeukemiaNet, et al. Diagnosis and management of acute myeloid leukemia in adults: recommendations from an international expert panel, on behalf of the European LeukemiaNet. Blood. 2010;115:453–74.
3. Miyawaki S, Ohtake S, Fujisawa S, Kiyoi H, Shinagawa K, Usui N, et al. A randomized comparison of 4 courses of standard-dose multiagent chemotherapy versus 3 courses of high-dose cytarabine alone in postremission therapy for acute myeloid leukemia in adults: the JALSG AML201 Study. Blood. 2011;117:2366–72.
4. Bullinger L, Döhner K, Bair E, Fröhling S, Schlenk RF, Tibshirani R, et al. Use of gene-expression profiling to identify prognostic subclasses in adult acute myeloid leukemia. N Engl J Med. 2004;350:1605–16.
5. Sakamaki H, Miyawaki S, Ohtake S, Emi N, Yagasaki F, Mitani K, et al. Allogeneic stem cell transplantation versus chemotherapy as post-remission therapy for intermediate or poor risk adult acute myeloid leukemia: results of the JALSG AML97 study. Int J Hematol. 2010;91:284–92.
6. Naoe T, Kiyoi H. Gene mutations of acute myeloid leukemia in the genome era. Int J Hematol. 2013;97:165–74.
7. Krivtsov AV, Figueroa ME, Sinha AU, Stubbs MC, Feng Z, Valk PJ, et al. Cell of origin determines clinically relevant subtypes of MLL-rearranged AML. Leukemia. 2013;27:852–60.
8. Wiley JS, Jones SP, Sawyer WH, Paterson AR. Cytosine arabinoside influx and nucleoside transport sites in acute leukemia. J Clin Investig. 1982;69:479–89.
9. Schröder JK, Seidelmann M, Kirch HC, Seeber S, Schütte J. Assessment of resistance induction to cytosine arabinoside following transfer and overexpression of the deoxycytidylate deaminase gene in vitro. Leuk Res. 1998;22:619–24.
10. Whelan J, Smith T, Phear G, Rohatiner A, Lister A, Meuth M. Resistance to cytosine arabinoside in acute leukemia: the significance of mutations in CTP synthetase. Leukemia. 1994;8:264–5.
11. Gamazon ER, Lamba JK, Pounds S, Stark AL, Wheeler HE, Cao X, et al. Comprehensive genetic analysis of cytarabine sensitivity in a cell-based model identifies polymorphisms associated with outcome in AML patients. Blood. 2013;121:4366–76.
12. Mahlknecht U, Dransfeld CL, Bulut N, Kramer M, Thiede C, Ehninger G, et al. SNP analyses in cytarabine metabolizing enzymes in AML patients and their impact on treatment response and patient survival: identification of CDA SNP C-451T as an independent prognostic parameter for survival. Leukemia. 2009;23:1929–32.
13. Kim KI, Huh IS, Kim IW, Park T, Ahn KS, Yoon SS, et al. Combined interaction of multi-locus genetic polymorphisms in cytarabine arabinoside metabolic pathway on clinical outcomes in adult acute myeloid leukaemia (AML) patients. Eur J Cancer. 2013;49:403–10.
14. Barrett JC, Fry B, Maller J, Daly MJ. Haploview: analysis and visualization of LD and haplotype maps. Bioinformatics. 2005;21:263–5.
15. Rha SY, Jeung HC, Choi YH, Yang WI, Yoo JH, Kim BS, et al. An association between RRM1 haplotype and gemcitabine-induced neutropenia in breast cancer patients. Oncologist. 2007;12:622–30.
16. Gray RJ. A class of k-sample tests for comparing the cumulative incidence of a competing risk. Annal Statist. 1988;16:1141–54.
17. Fine JP, Gray RJ. A proportional hazards model for the subdistribution of a competing risk. Am Statist Assoc. 1999;94:496–509.
18. Kanda Y. Investigation of the freely available easy-to-use software 'EZR' for medical statistics. Bone Marrow Transplant. 2013;48:452–8.
19. Verschuur AC, van Gennip AH, Leen R, Voûte PA, van Kuilenburg AB. Cyclopentenyl cytosine increases the phosphorylation and incorporation into dna of arabinofu-ranosyl cytosine in a myeloid leukemic cell-line. Adv Exp Med Biol. 2000;486:311–7.
20. Verschuur AC, Brinkman J, Van Gennip AH, Leen R, Vet RJ, Evers LM, et al. Cyclopentenyl cytosine induces apoptosis and increases cytarabine-induced apoptosis in a T-lymphoblastic leukemic cell-line. Leuk Res. 2001;25:891–900.
21. Gilbert JA, Salavaggione OE, Ji Y, Pelleymounter LL, Eckloff BW, Wieben ED, et al. Gemcitabine pharmacogenomics: cytidine deaminase and deoxycytidylate deaminase gene resequencing and functional genomics. Clin Cancer Res. 2006;12:1794–803.
22. Gati WP, Paterson AR, Larratt LM, Turner AR, Belch AR. Sensitivity of acute leukemia cells to cytarabine is a correlate of cellular es nucleoside transporter site content measured by flow cytometry with SAENTA-fluorescein. Blood. 1997;90:346–53.
23. Kim SR, Saito Y, Maekawa K, Sugiyama E, Kaniwa N, Ueno H, et al. Thirty novel genetic variations in the SLC29A1 gene encoding human equilibrative nucleoside transporter 1 (hENT1). Drug Metab Pharmacokinet. 2006;21:248–56.
24. Suzuki Y, Homma M, Abei M, Hyodo I, Kohda Y. Impact of solute carrier family 29 member 1 (SLC29A1) single nucleotide polymorphisms on mRNA expression in peripheral blood mononuclear cells. Biol Pharm Bull. 2013;36:144–6.
25. Myers SN, Goyal RK, Roy JD, Fairfull LD, Wilson JW, Ferrell RE. Functional single nucleotide polymorphism haplotypes in the human equilibrative nucleoside transporter 1. Pharmacogenet Genom. 2006;16:315–20.
26. Capizzi RL, Yang JL, Cheng E, Bjornsson T, Sahasrabudhe D, Tan RS, et al. Alteration of the pharmacokinetics of high-dose ara-C by its metabolite, high ara-U in patients with acute leukemia. J Clin Oncol. 1983;1:763–71.
27. Pérez-Torras S, García-Manteiga J, Mercadé E, Casado FJ, Carbó N, Pastor-Anglada M, et al. Adenoviral-mediated overexpression of human equilibrative nucleoside transporter 1 (hENT1) enhances gemcitabine response in human pancreatic cancer. Biochem Pharmacol. 2008;76:322–9.
28. Braess J, Pförtner J, Kern W, Hiddemann W, Schleyer E. Cytidine deaminase—the methodological relevance of AraC deamination for ex vivo experiments using cultured cell lines, fresh leukemic blasts, and normal bone marrow cells. Ann Hematol. 1999;78:514–20.

29. Cao X, Mitra AK, Pounds S, Crews KR, Gandhi V, Plunkett W, et al. RRM1 and RRM2 pharmacogenetics: association with phenotypes in HapMap cell lines and acute myeloid leukemia patients. Pharmacogenomics. 2013;14:1449–66.
30. Falk IJ, Fyrberg A, Paul E, Nahi H, Hermanson M, Rosenquist R, et al. Decreased survival in normal karyotype AML with single-nucleotide polymorphisms in genes encoding the AraC metabolizing enzymes cytidine deaminase and 5′-nucleotidase. Am J Hematol. 2013;88:1001–6.
31. Carpi FM, Vincenzetti S, Ubaldi J, Pucciarelli S, Polzonetti V, et al. CDA gene polymorphisms and enzyme activity: genotype–phenotype relationship in an Italian-Caucasian population. Pharmacogenomics. 2013;14:769–81.

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LETTER TO THE EDITOR

Feasibility of umbilical cord blood transplantation with a myeloablative, reduced toxicity-conditioning regimen

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Umbilical cord blood transplantation (UCBT) with reduced-intensity conditioning (RIC) has been reported in older patients and/or those with comorbidities who have no suitable related or unrelated donors.[1–4] However, RIC for UCBT has not yet been standardized due to a high rate of graft failure and TRM. To reduce regimen-related toxicity, non-TBI containing regimens that employ agents such as fludarabine (Flu) and either melphalan (Mel) or Bu have been attempted, but are associated with a high rate of graft failure.[5,6] In contrast, with CBT using a conditioning regimen consisting of Flu, Mel and TBI, a high rate of TRM has been documented.[4] A less toxic conditioning regimen of sufficient intensity to achieve engraftment is therefore required. Flu/Bu-based conditioning has been reported to be less toxic than Flu/Mel-based conditioning.[7] Barker *et al.*[2] reported a high rate of graft failure for Flu/oral Bu with 200 cGy TBI. In addition, unlike oral Bu, i.v. Bu is not influenced by variable dose-to-dose bioavailability[8] and does not depend on intestinal absorption, which can be disrupted by gastrointestinal complications. It has been suggested that a high Bu concentration is associated with an increased risk of TRM.[9] Conversely, a low area under the curve (AUC) regarding Bu level could increase the risk of graft rejection and/or disease relapse. An adequate plasma Bu concentration can lead to sufficient immunosuppression that facilitates engraftment, with a lower rate of disease relapse and less toxicity. We therefore hypothesized that Flu TBI combined with an increased dose of i.v. Bu rather than oral Bu might be a more optimal regimen.

We retrospectively analyzed all patients at our institute who had undergone UCBT with Flu, i.v. Bu, and low-dose TBI between March 2005 and June 2011. Ten patients with a median age of 61 (range 40–68) were evaluated (Table 1). The preparative conditioning regimen consisted of Flu 30 mg/m^2/day on days −9 to −4 (total dose, 180 mg/m^2), i.v. Bu 0.8 mg/kg every 12 h on days −9 to −5 (total dose, 8 mg/kg) and 400 cGy TBI in two fractions on day −1. In all patients, a single cord unit was transplanted. In most patients, CYA (15 mg/kg twice a day from day −1) and short-term MTX (10 mg/m^2 on day 1, 7 mg/m^2 on day 3 and 7 mg/m^2 on day 5) were used as GVHD prophylaxis. This study was approved by the Institutional Review Board.

The median follow-up time of survivors was 5.2 years (range 1.7–6.3). In two patients who had active disease and infection at the time of transplantation, engraftment could not be evaluated due to early death at day 9 or day 14 after UCBT without hematopoietic recovery. Of the remaining eight patients, seven achieved neutrophil engraftment after a median duration of 22 days (range 13–31). Platelet recovery was documented in six patients after a median duration of 53 days (range 24–234). In all evaluable patients, complete donor T-cell chimerism in the peripheral blood, as assessed by CD3+ cells, was confirmed at day 30 and was sustained at days 60 and 90. Two of the three patients (Nos. 6 and 8) had active disease and infection at the start of conditioning. The one patient in our study (No. 9) in whom neutrophil recovery was not observed more than 28 days after UCBT died on day 34 from pneumonia and alveolar hemorrhage due to *Stenotrophomonas maltophilia*. The same pathogen was also detected in blood culture. TRM and relapse rate at 5 years were 10% and 50%, respectively. Overall survival was 40% at 5 years.

Although oral mucositis was the most frequent regimen-related toxicity, diarrhea of more than grade 2 was seen in just one patient (Table 2). Grade III to IV gut GVHD was also only observed in one patient (Table 1). In addition, no patient developed grade 4 toxicity in any organ. Veno-occlusive disease was also not observed in any patient.

Table 1. Patient characteristics and outcomes

	Sex	Age	Disease	Status	Nucleated cell dose (10^7/kg)	CD34+ cell dose (10^5/kg)	HLA match	GVHD prophylaxis	Neutrophil engraftment	Donor chimerism at day 30	Acute GVHD (grade/site)	Chronic GVHD	Outcome	Cause of death	Survival (days)
1	F	66	NHL	2nd relapse	3.4	1.6	6/6	CyA alone	Yes (day13)	>90%	III/gut	N.A.	Death	Relapse / PD	59
2	M	64	ATL	Refractory to chemotherapy	2.3	1.3	4/6	CyA alone	Yes (day22)	>90%	III/liver	limited	Death	Relapse / PD	211
3	F	55	FL	1st relapse	2.8	0.8	4/6	CyA alone	Yes (day31)	>90%	II/gut	extensive	Alive	—	2291+
4	F	68	AML	1st CR	2.5	0.4	4/6	CyA+sMTX	Yes (day22)	>90%	II/gut	limited	Alive	—	2210+
5	F	46	MCL	Refractory to chemotherapy	3.6	0.7	4/6	CyA+sMTX	Yes (day27)	>90%	No	limited	Death	Relapse/PD	173
6	M	40	AML from MDS	PD with no prior therapy	2.3	0.8	4/6	CyA+sMTX	No	N.A.	N.A.	N.A.	Death	Relapse/PD	14
7	F	61	AML	1st CR	4.4	1.6	4/6	CyA+sMTX	Yes (day15)	>90%	No	No	Alive	—	1558+
8	M	49	AML from MDS	PD after allo-HCT	4.0	0.6	4/6	FK+MMF	No	N.A.	N.A.	N.A.	Death	Relapse/PD	9
9	M	61	ALL	2nd CR	4.7	1.4	4/6	CyA+sMTX	No	N.A.	N.A.	N.A.	Death	Pneumonia	34
10	F	62	ATL	PR	3.9	0.7	4/6	CyA+sMTX	Yes (day25)	>90%	II/gut	No	Alive	—	635+

Abbreviations: ATL = adult T cell leukemia; allo-HCT = allogeneic hematopoietic stem cell transplantation; FK = tacrolimus; FL = follicular lymphoma; MCL = mantle cell lymphoma; MDS = myelodysplastic syndrome; MMF = mycophenolate mofetil; NA = not applicable; NHL = non-Hodgkin lymphoma; PD = progressive disease; sMTX = short-term methotrexate.

Table 2. Regimen-related toxicities up to 28 days after UCBT

	Maximal Grade, n			
	1	*2*	*3*	*4*
Cardiac	0	1	0	0
Oral mucositis	2	2	6	0
Nausea	2	3	1	0
Diarrhea	2	0	1	0
Liver	3	1	0	0
Renal	1	0	0	0
Lung	0	0	0	0

Regimen-related toxicities were assessed according to the National Cancer Institute's Common Toxicity Criteria, version 4.0, for all adverse events.

In this study, all evaluable patients with the exception of one achieved engraftment and complete T-cell donor chimerism by day 30. Three of four patients (75%) in CR or PR at the time of UCBT have remained in long-term remission (Table 1).

Barker *et al.*[2] reported on a large pilot trial of UCBT with Flu, TBI (200 cGy) and either oral Bu (8 mg/kg) or CY (50 mg/kg). In that study, the CY group had a better outcome than the Bu group, with only 76% sustaining engraftment in the Bu group. The Minnesota group reported a 92% neutrophil engraftment rate with the use of Flu, CY and TBI for conditioning for UCBT.[3] However, most of these patients received two cord units, which is still not permissible in many countries. From the limited data available from their study related to transplantation of a single cord unit, the relapse rate at 3 years was high, at 41%. Non-myeloablative conditioning with/without the use of antithymocyte globulin might be associated with an increased risk of relapse. Uchida *et al.*[4] reported a conditioning regimen consisting of Flu (125 mg/m^2), Mel (80 mg/m^2) and TBI 400 cGy, for transplantation of a single unit of cord blood. TRM was 45% and OS was 23% at 2 years. Severe gastrointestinal mucositis induced by the combination of Mel and TBI possibly caused severe gut infection and GVHD, which may have contributed to the high TRM.

In our study, Grade III to IV gut GVHD was observed in just one patient, suggesting the possibility that the lower gastrointestinal toxicity of this conditioning regimen could decrease the incidence of not only severe gut infection but also GVHD; a 10% rate of TRM at 5 years appears to be an acceptable outcome compared with the previous report.[10] Despite the small sample size of our study, a myeloablative dose of i.v. Bu (8 mg/kg) and 400 cGy TBI could be an option in older patients and/or those with comorbidities.

CONFLICT OF INTEREST

Dr Masayuki Hino has received honoraria from Kyowa Hakko Kirin Co., Ltd, Otsuka Pharmaceutical Co. and Ltd, Sanofi K.K. He has also received research grant and travel support to attend the meeting from Kyowa Hakko Kirin Co., Ltd. Dr Hirohisa Nakamae has received research grant, lecture fees and travel support from Kyowa Hakko Kirin Co., Ltd. He has also received honorarium and travel support from Otsuka Pharmaceutical Co., Ltd. Dr Mika Nakamae has received research grant and travel support from Kyowa Hakko Kirin Co., Ltd. Dr Yoshiki Terada and Hideo Koh have received travel support from Kyowa Hakko Kirin Co., Ltd. Dr Takahiko Nakane has received lecture fee and travel support from Kyowa Hakko Kirin Co., Ltd. Dr Mitsutaka Nishimoto has received travel support from Kyowa Hakko Kirin Co., Ltd. He has also received lecture fees from Otsuka Pharmaceutical Co. and Ltd. The remaining authors declare no conflict of interest.

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M Nishimoto, H Nakamae, M Nakamae, A Hirose, K Hagihara, H Koh, T Nakane, Y Terada and M Hino
Hematology, Graduate School of Medicine, Osaka City University, Osaka, Japan
E-mail: hirohisa@msic.med.osaka-cu.ac.jp

REFERENCES

1 Cutler C, Ballen K. Reduced-intensity conditioning and umbilical cord blood transplantation in adults. *Bone Marrow Transplant* 2009; **44**: 667–671.
2 Barker JN, Weisdorf DJ, DeFor TE, Blazar BR, Miller JS, Wagner JE. Rapid and complete donor chimerism in adult recipients of unrelated donor umbilical cord blood transplantation after reduced-intensity conditioning. *Blood* 2003; **102**: 1915–1919.
3 Brunstein CG, Barker JN, Weisdorf DJ, Defor TE, Miller JS, Blazar BR *et al.* Umbilical cord blood transplantation after nonmyeloablative conditioning: impact on transplantation outcomes in 110 adults with hematologic disease. *Blood* 2007; **110**: 3064–3070.
4 Uchida N, Wake A, Takagi S, Yamamoto H, Kato D, Matsuhashi Y *et al.* Umbilical cord blood transplantation after reduced-intensity conditioning for elderly patients with hematologic diseases. *Biol Blood Marrow Transplant* 2008; **14**: 583–590.
5 Narimatsu H, Watanabe M, Kohno A, Sugimoto K, Kuwatsuka Y, Uchida T *et al.* High incidence of graft failure in unrelated cord blood transplantation using a reduced-intensity preparative regimen consisting of fludarabine and melphalan. *Bone Marrow Transplant* 2008; **41**: 753–756.
6 Horwitz ME, Morris A, Gasparetto C, Sullivan K, Long G, Chute J *et al.* Myeloablative intravenous busulfan/fludarabine conditioning does not facilitate reliable engraftment of dual umbilical cord blood grafts in adult recipients. *Biol Blood Marrow Transplant* 2008; **14**: 591–594.
7 Shimoni A, Hardan I, Shem-Tov N, Rand A, Herscovici C, Yerushalmi R *et al.* Comparison between two fludarabine-based reduced-intensity conditioning regimens before allogeneic hematopoietic stem-cell transplantation: Fludarabine/melphalan is associated with higher incidence of acute graft-versus-host disease and non-relapse mortality and lower incidence of relapse than fludarabine/busulfan. *Leukemia* 2007; **21**: 2109–2116.
8 Andersson BS, Kashyap A, Gian V, Wingard JR, Fernandez H, Cagnoni PJ *et al.* Conditioning therapy with intravenous busulfan and cyclophosphamide (IV BuCy2) for hematologic malignancies prior to allogeneic stem cell transplantation: A phase II study. *Biol Blood Marrow Transplant* 2002; **8**: 145–154.
9 Ljungman P, Hassan M, Bekassy AN, Ringden O, Oberg G. High busulfan concentrations are associated with increased transplant-related mortality in allogeneic bone marrow transplant patients. *Bone Marrow Transplant* 1997; **20**: 909–913.
10 Cahu X, Rialland F, Touzeau C, Chevallier P, Guillaume T, Delaunay J *et al.* Infectious complications after unrelated umbilical cord blood transplantation in adult patients with hematologic malignancies. *Biol Blood Marrow Transplant* 2009; **15**: 1531–1537.