antigenemia was significantly lower for those received foscarnet <40 mg/kg ($P = 0.01$).

3.5 Survival

The overall survival of all patients who received foscarnet was 34% at a median follow-up of 3 years (Fig. 5a). Patients with CMV disease showed significantly lower survival than those who received preemptive or prophylactic therapy (Fig. 5b, $P = 0.0004$). No significant difference in prognosis was found between the patients with and without preceding other anti-viral agents ($P = 0.21$). A total of 170 patients died, and the main causes of death were disease recurrence in 47, bacterial sepsis in 27, acute/chronic graft-versus-host disease in 25, and fungal infection in 10. The cumulative incidence of transplant-related mortality at 1 year was 30% (95% confidence interval 25–35%). Three patients eventually died of CMV disease, and the cumulative incidence of CMV-associated death at 1 year was 1.0% (95% confidence interval 0.3–2.6%).

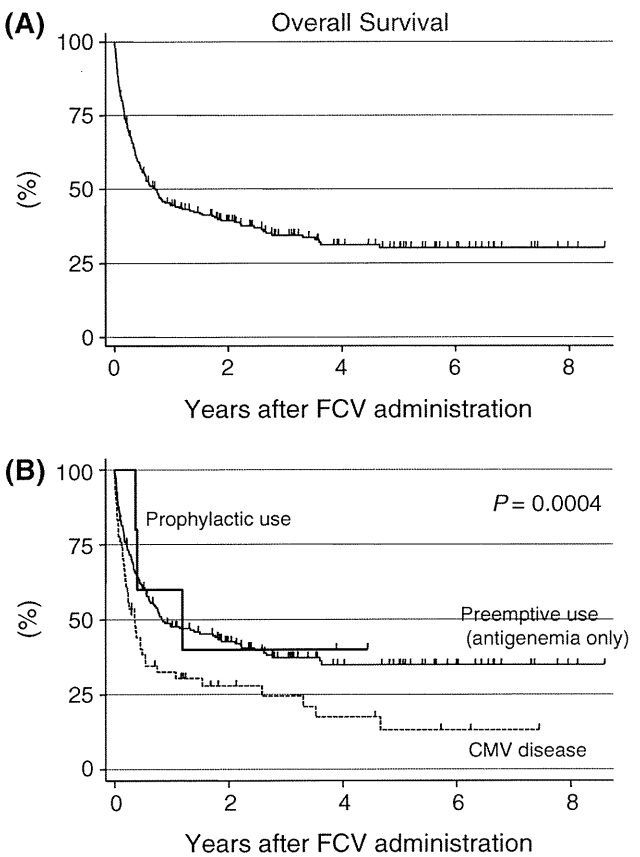

Fig. 5 Overall survival (OS) of patients who received foscarnet therapy. **a** The 3-year OS was 34%. **b** The prognosis of patients with CMV disease was significantly poorer than those of patients who had received preemptive or prophylactic use ($P = 0.0004$)

3.6 Adverse events

Adverse events (irrespective of causal association) of NCI-CTCAE grade 3 or higher are listed in Table 3. The most common adverse event was electrolyte abnormalities, which occurred in 35 patients (11%). The other major toxic events included neutropenia in 27 patients, thrombocytopenia in 26 patients, and bone marrow dysfunction in 11 patients. Renal and hepatic damage developed in 11 and 10 patients, respectively. Adverse events associated with foscarnet included neutropenia in 5 patients; electrolyte abnormalities in 4 patients; thrombocytopenia, renal dysfunction and sensory disturbance in 2 patients each; and bone marrow dysfunction in 1 patient. No patient died of an adverse reaction associated with foscarnet. The total number of patients who developed a grade 3 adverse reaction or higher was 56 (28%) in the patients who received prior ganciclovir and 21 (17%) in those who did not ($P = 0.03$). The rate of adverse events did not differ among the 5 dose categories (Table 4). The duration of foscarnet medication was not different between patients who developed adverse event of grade 3 or more (median 16 days, range 2–121) and those did not (median 20 days,

Table 3 Adverse events during foscarnet treatment

	Prior GCV $N = 198$		No prior GCV $N = 122$		Total $N = 320$	
	N	%	*N*	%	*N*	%
Graft failure	2	1.0	2	1.6	4	1.3
Neutropenia	19	9.6	8	6.6	27	8.4
Grade 3	7	3.5	2	1.6	9	2.8
Grade 4	12	6.1	6	4.9	18	5.6
Thrombocytopenia	19	9.6	7	5.7	26	8.1
Grade 3	6	3.0	0	0.0	6	1.9
Grade 4	13	6.6	7	5.7	20	6.3
BM dysfunction	7	3.5	3	2.5	10	3.1
Grade 3	4	2.0	1	0.8	5	1.6
Grade 4	3	1.5	2	1.6	5	1.6
Renal damage	6	3.0	5	4.1	11	3.4
Grade 3	4	2.0	5	4.1	9	2.8
Grade 4	2	1.0	0	0.0	2	0.6
Electrolyte abnormality	27	13.6	8	6.6	35	10.9
Grade 3	20	10.1	7	5.7	27	8.4
Grade 4	7	3.5	1	0.8	8	2.5
Neurological	3	1.5	1	0.8	4	1.3
Grade 3	3	1.5	1	0.8	4	1.3
Grade 4	0	0.0	0	0.0	0	0.0
Liver damage	9	4.5	1	0.8	10	3.1
Grade 3	7	3.5	0	0.0	7	2.2
Grade 4	2	1.0	1	0.8	3	0.9

BM bone marrow

Table 4 Adverse effects according to foscarnet dose

Dose level (mg/kg)	0–39 $N = 18$ (%)	40–79 $N = 106$ (%)	80–99 $N = 88$ (%)	100–159 $N = 60$ (%)	160– $N = 48$ (%)	Total $N = 320$ (%)
Any grade 3 or higher	33	23	17	25	35	24
Grade 3 or higher, possibly by foscarnet	28	12	13	17	17	15
Grade 3 or higher, definitely by foscarnet	0	2.8	3.4	8.3	6.3	4.4

range 1–322, $P = 0.50$). The difference was not evident for patients with possible and definite association with foscarnet ($P = 0.84$ and $P = 0.22$, respectively). When the adverse events were compared between HLA-matched and -mismatched transplant, the rates were significantly higher in the HLA-matched transplant. Any grade 3 or more toxicity was developed in 36 of 108 HLA-matched and 33 of 160 HLA-mismatched transplant ($P = 0.02$). Of these, 31 and 24, respectively, were possibly due to foscarnet use (29 vs. 15%, $P = 0.009$).

4 Discussion

The present study demonstrated that foscarnet is effective for patients with CMV infection who are not suitable for ganciclovir therapy. Sixty percent of the patients had a history of prior ganciclovir, but had demonstrated problems of ineffectiveness and/or adverse reactions. The remaining 40% had poor bone marrow function, and therefore foscarnet had been selected as the up-front use. In both situations, most of the patients were preemptively treated, and prophylactic use was seen in <2% of cases in our series.

The initial dose of foscarnet had two convergent doses, which were 90 and 180 mg/kg. The former corresponds to the maintenance dose, and the latter is the initial dose which was used in most prospective studies [18, 19]. The dose of foscarnet was significantly higher in patients with secondary therapy. This might have resulted from a higher number of more severe patients with CMV infection being present in the secondary therapy group. On the other hand, no dosage differences were found between the various purpose groups (preemptive/prophylactic/treatment). The lack of a correlation between foscarnet dose and creatinine clearance suggested that foscarnet was used irrespective of the renal function of the patient.

The most important adverse reaction of foscarnet was previously described as renal damage including electrolyte abnormalities. In that study, one-third of patients developed renal insufficiency and/or electrolyte disturbance [15]. However, a later study showed that these adverse events occurred less frequently [19]. In our series of patients, electrolyte abnormalities were recognized in 11% of patients, and renal insufficiency was found in no >3% of patients, which was consistent with the findings in the literature [24]. Thus, foscarnet seems to be a safer drug than was initially predicted.

In the preemptive use of foscarnet, >80% of patients showed CMV antigenemia disappearance in both the initial and secondary therapy groups. Foscarnet was highly effective in this setting, but its efficacy was decreased in CMV disease. The efficacy of foscarnet did not correlate with its dose, which was contradictory to a previous dose-finding study [25]. Our findings suggest a need to explore appropriate therapeutic strategies for this agent. Recently, "low-dose" administration of foscarnet at 60 mg/kg/day has been reported to be effective for CMV preemptive treatment [26, 27], which could be an option for future clinical trials. A prospective trial comparing ganciclovir alone and a combination of ganciclovir and foscarnet (half doses of both) was performed for HSCT and organ transplant patients [28]. The efficacy was equivalent for both arms, but adverse events were more frequent in the foscarnet combined arm.

In conclusion, our study shows that foscarnet is a safe and effective agent for treating CMV antigenemia after allogeneic HSCT. It remains to be determined how CMV infections should be treated, as well as how to improve the survival of affected patients.

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

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

Introduction

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

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

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

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

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Methods

Mice

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

BM transplantation

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

Evaluation of cGVHD

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

Tissue histopathology

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

Intracellular cytokine staining and cytokine analysis

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

IFN-γ neutralization

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

Administration of ATRA and Am80

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

Real-time RT-PCR

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

Statistical analyses

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

Results

Th17 cells are increased in lymphoid organs during cGVHD development

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

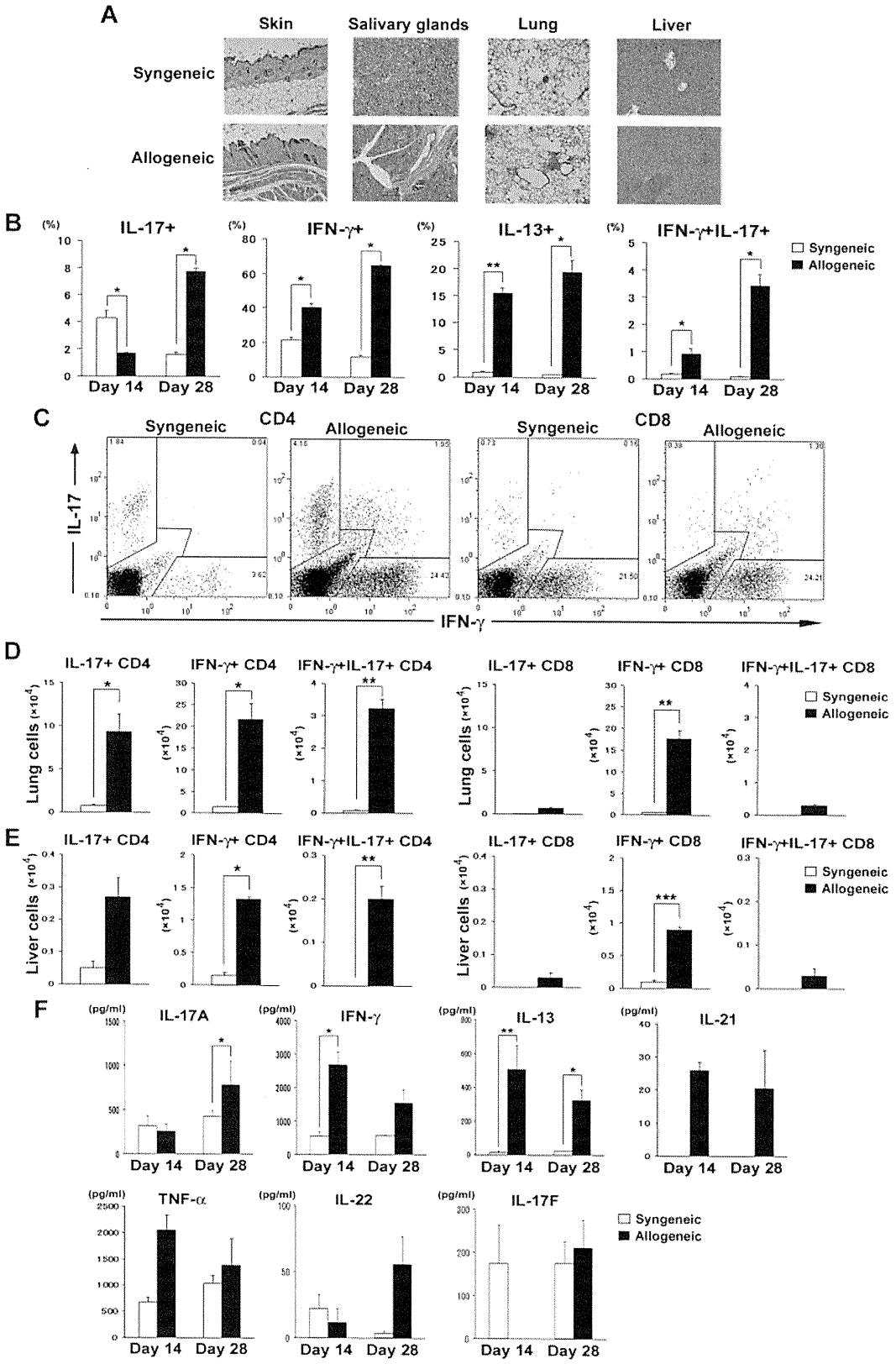

Figure 1. Th17 cells are increased in lymphoid organs during the late phase of cGVHD. Sublethally irradiated (6.75 Gy) BALB/c mice were transplanted with 2×10^6 spleen T cells plus 8×10^6 TCD-BM from WT B10.D2 mice (allogeneic group; black bars). The syngeneic group (white bars) received a transplant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

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

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

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

Donor Th1 differentiation is responsible for the development of cGVHD

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

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

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

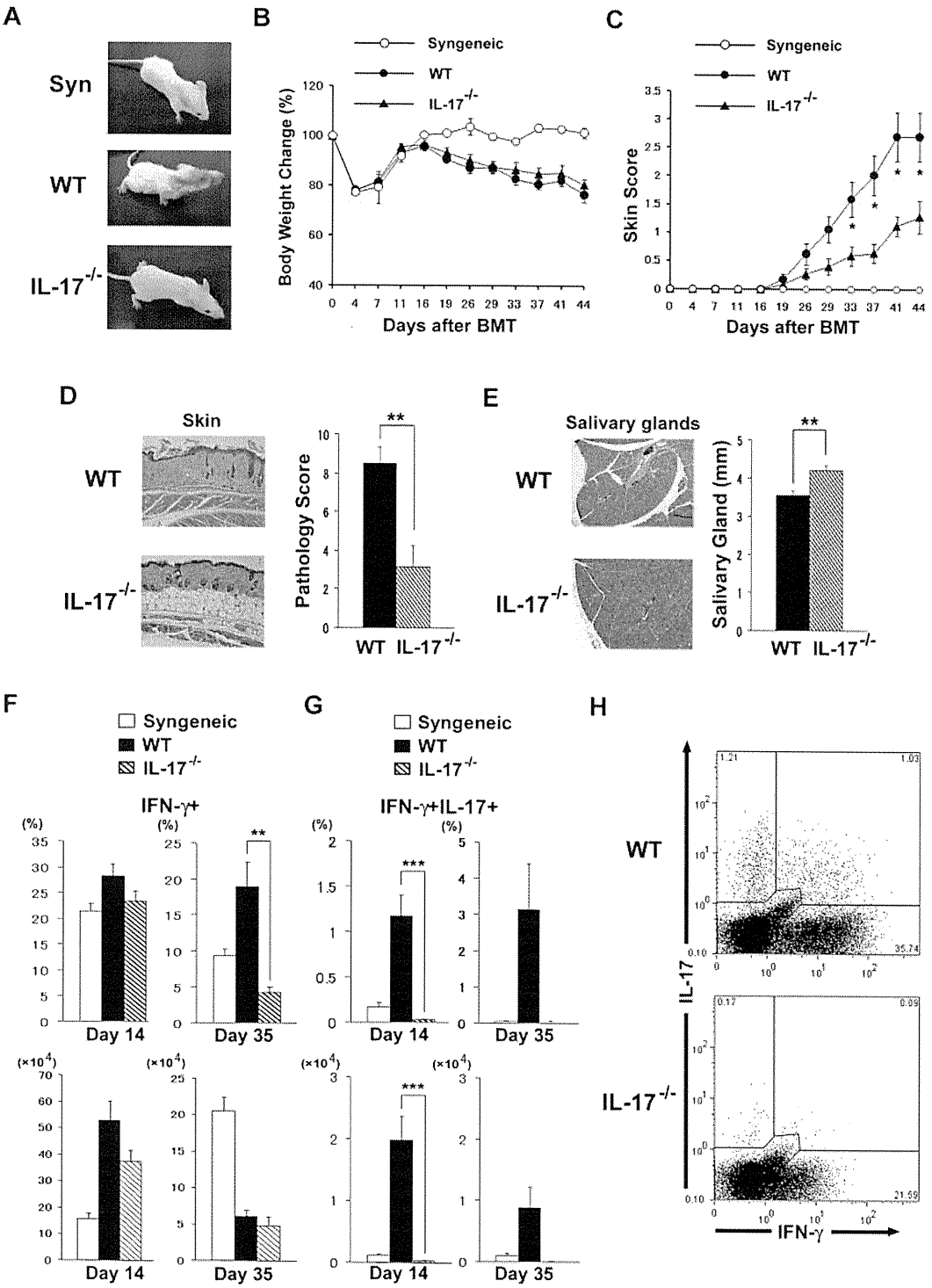

Figure 2. IL-17$^{-/-}$ donor T cells ameliorate cGVHD. Sublethally irradiated BALB/c recipients were transplanted from WT, IL-17$^{-/-}$ B10.D2, or syngeneic BALB/c donors. (A) Gross observation of the skin lesions from recipients of syngeneic, WT, and IL-17$^{-/-}$ donors 28 days after BMT. The recipients were analyzed for body weight (B) and cGVHD skin scores (C); data from 2 independent experiments were combined (n = 14 per group). Pathology score of skin (D) and the longest diameter of the salivary gland (E) on day 35 of BMT are shown. Four to 6 recipients were examined in each group. (F-G) PLN cells of the recipients of syngeneic (white bar), WT (black bar), or IL-17$^{-/-}$ (striped bar) donors were stained for intracellular IFN-γ and IL-17 on days 14 and 35 after BMT. The percentages and absolute numbers of IFN-γ$^+$ cells (F) and IFN-γ$^+$/IL-17$^+$ cells (G) are shown. Data from 2 replicated experiments were combined (n = 6-11 per group). (H) Representative staining for intracellular IFN-γ and IL-17 on CD4$^+$ T cells of WT or IL-17$^{-/-}$ mice on day 35 is shown. Data represent the means ± SEs. *$P \leq .05$, **$P \leq .01$, and ***$P \leq .001$.

Administration of Am80 ameliorates cGVHD

Next, we examined whether ATRA or Am80 can down-regulate cGVHD. BALB/c recipients were orally administered ATRA (200 μg/mouse) or Am80 from day 0 of BMT. We found that ATRA tended to decrease the clinical cGVHD score (Figure 5A), whereas Am80 significantly ameliorated the clinical score compared with controls ($P = .01$; Figure 5B). Histopathologic examination of the skin on day 16 showed significantly reduced cGVHD damage in Am80-treated animals (day 16, 4.8 ± 0.4 vs 7.4 ± 0.4; $P < .01$; Figure 5C), No differences were observed in pathology scores of the lung, liver, or colon between the 2 groups (Figure 5C). Because it has been reported that Am80 can induce Treg cells,[29] we

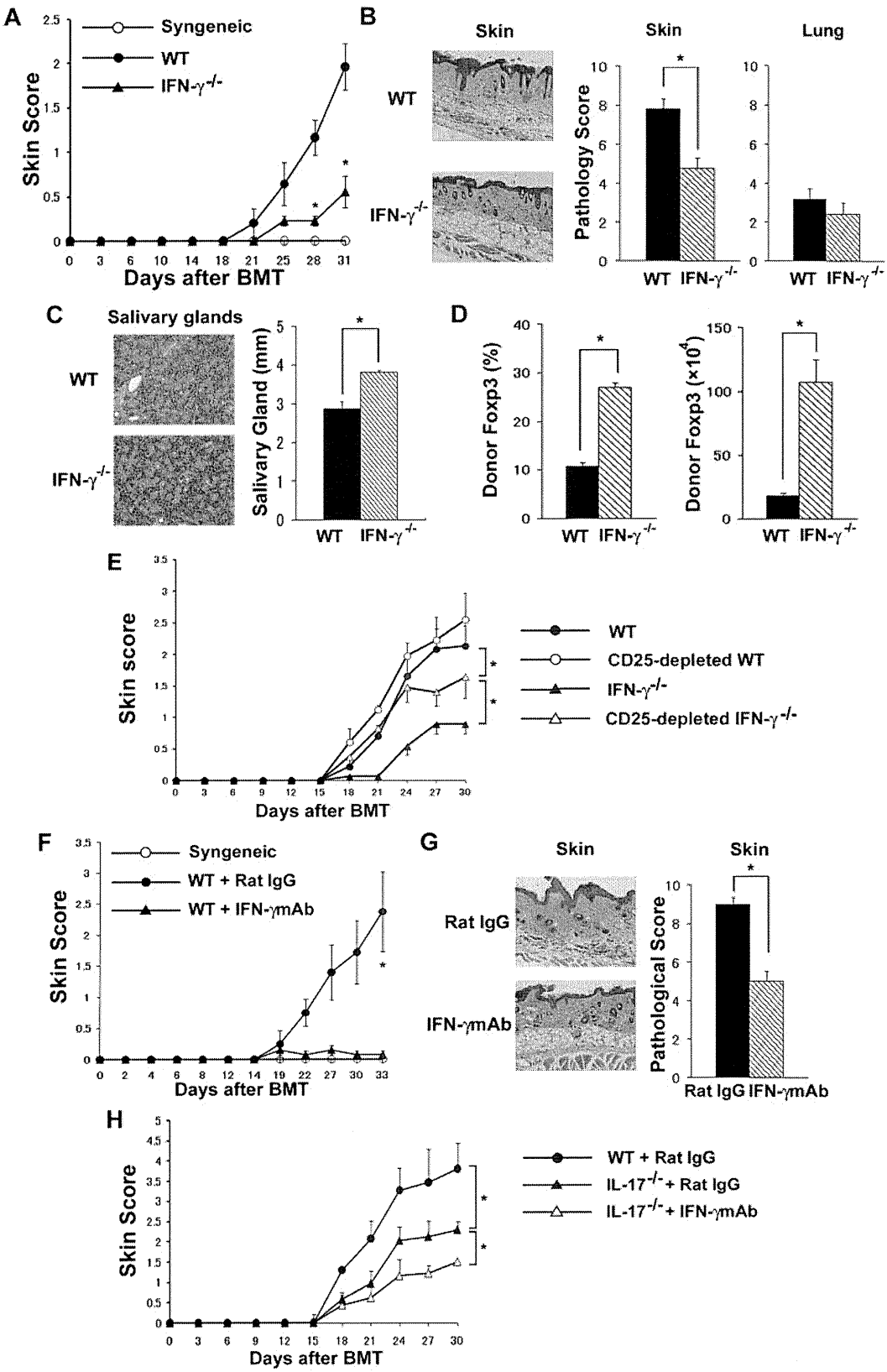

Figure 3. Donor Th1 differentiation and IFN-γ production are responsible for exacerbated cGVHD. (A-D) Sublethally irradiated BALB/c recipients were transplanted from WT or IFN-$\gamma^{-/-}$ B10.D2 donors. Clinical skin cGVHD scores (A), pathology score of skin and lung (B), and the longest diameter of the salivary gland (C) on day 35 after BMT are shown. Four to 6 recipients were examined in each group. Data are from 1 representative of 3 independent experiments. (D) PLN cells of the recipients on day 35 were stained for intracellular Foxp3. The percentages and the absolute number of $CD4^+Foxp3^+$ Treg cells are shown. Four to 6 recipients were examined in each group. Data are from 1 representative of 2 independent experiments. (E) Sublethally irradiated BALB/c recipients were transplanted 8×10^6 TCD-BM cells plus 2×10^6 total spleen T cells or CD25-depleted T cells from WT or IFN-$\gamma^{-/-}$ B10.D2 donors. The skin cGVHD scores are shown ($n = 6$ per group). Data are from 1 representative of ≥ 2 independent experiments. (F-H) Sublethally irradiated BALB/c recipients were transplanted from WT or IL-$17^{-/-}$ B10.D2 donors. The recipients were injected with anti–IFN-γ mAbs or rat IgG (160 μg/mouse) on days 0, 5, 10, and 15 after BMT and were assessed for the clinical signs of cGVHD every 3 days. The clinical skin cGVHD scores (F), histopathology, and pathology score of the skin (G) on day 35 of BMT from WT donors. Four mice per group were used. Data are from 1 representative of ≥ 2 repeated experiments. (H) The clinical skin cGVHD scores after BMT from WT or IL-$17^{-/-}$ donors are shown. Six mice per group were used. Data are from 1 representative of 2 independent experiments. The means (± SEs) of each group are shown; $^*P < .05$.

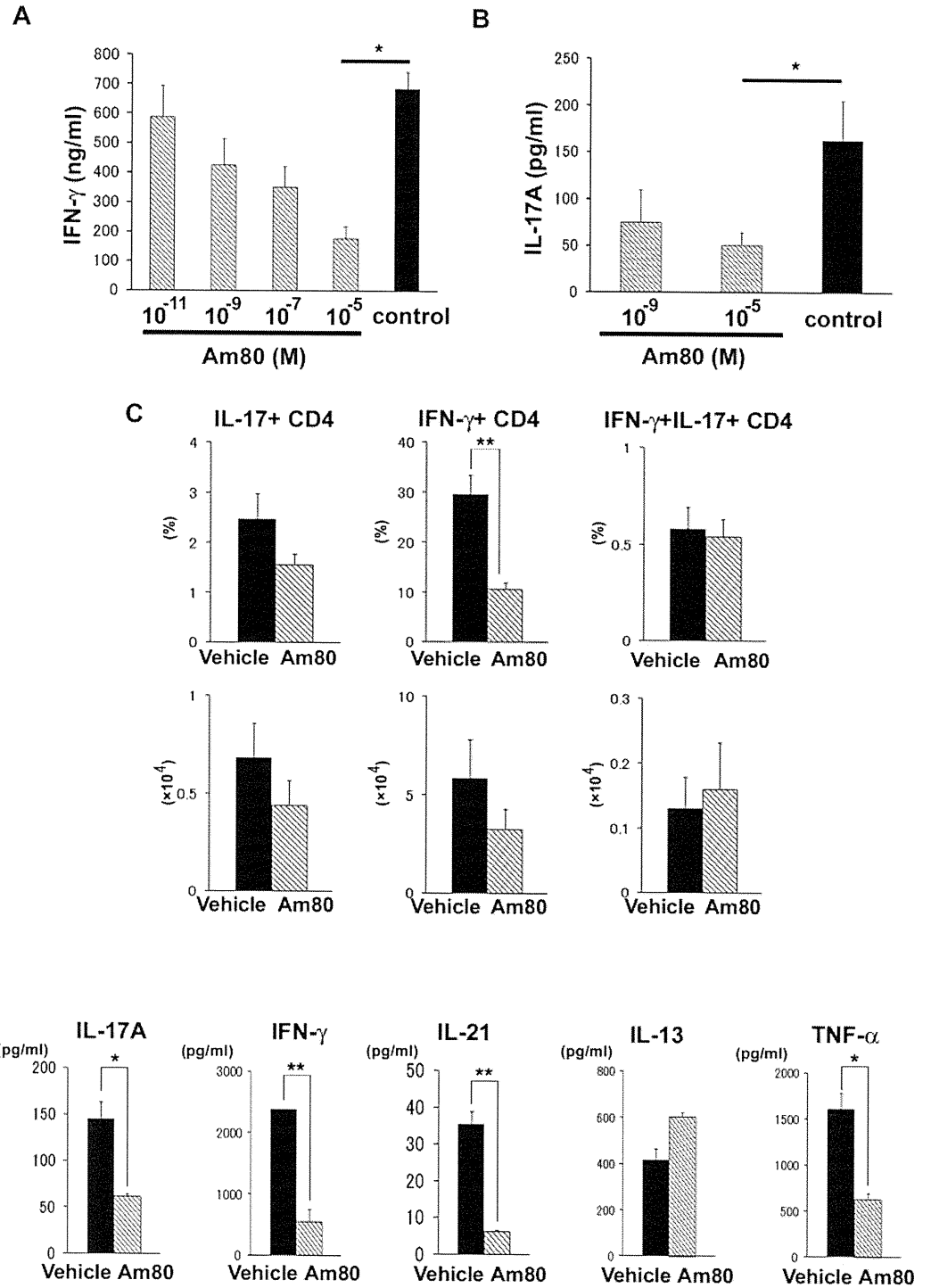

Figure 4. Am80 inhibits donor Th1 and Th17 cells in vitro and in vivo. Sublethally irradiated BALB/c recipients were transplanted from WT B10.D2 donors. (A-B) PLN cells from recipients (n = 3-6 per group) on day 14 were treated with Am80 or vehicle solution for 24 hours, the supernatants were collected, and ELISA was performed to determine the cytokine levels. Graphs represent the levels of cytokines secreted per 1×10^6 whole stimulated PLN cells. The data are from 1 representative of ≥ 3 independent experiments. (C-D) After BMT, recipients (n = 4-6 per group) were administered oral Am80 (1.0 mg/kg of body weight) or vehicle solution daily from day 0. PLNs of the recipients were stained for intracellular IFN-γ and IL-17. (C) The percentage and absolute number of IFN-γ^+ and IL-17^+–producing $CD4^+$ T cells. Data are from 1 representative of ≥ 2 repeated experiments. (D) PLN cells from recipients (n = 3-6 per group) treated with Am80 or vehicle on day 16 were stimulated with PMA and ionomycin. Five hours later, the supernatants were collected to determine cytokine levels by CBA. Graphs represent the levels of cytokines secreted per 1×10^6 whole stimulated PLN cells. The data are from 1 representative of ≥ 3 independent experiments. The means (± SEs) of each group are shown; $^*P < .05$ and $^{**}P < .01$.

quantified the frequency of Foxp3-expressing $CD4^+$ T cells in the PLNs after BMT. Recipients administered Am80 showed a decreased frequency of $Foxp3^+$ cells (day 17, 12.3% ± 2.5% vs 23.5% ± 2.6%; $P = .02$; Figure 5D). Foxp3 mRNA expression of the target organ (the ear) was also decreased in the Am80 recipients (data not shown). To confirm that the effects of Am80 are independent of Treg cells, mice were injected with whole T cells or CD25-depleted T cells from donors. As shown in Figure 5E, depletion of $CD25^+$ cells from the donor inoculum did not exacerbate skin cGVHD in Am80-treated mice, thus suggesting that the effects of Am80 treatment are not associated with Treg cells.

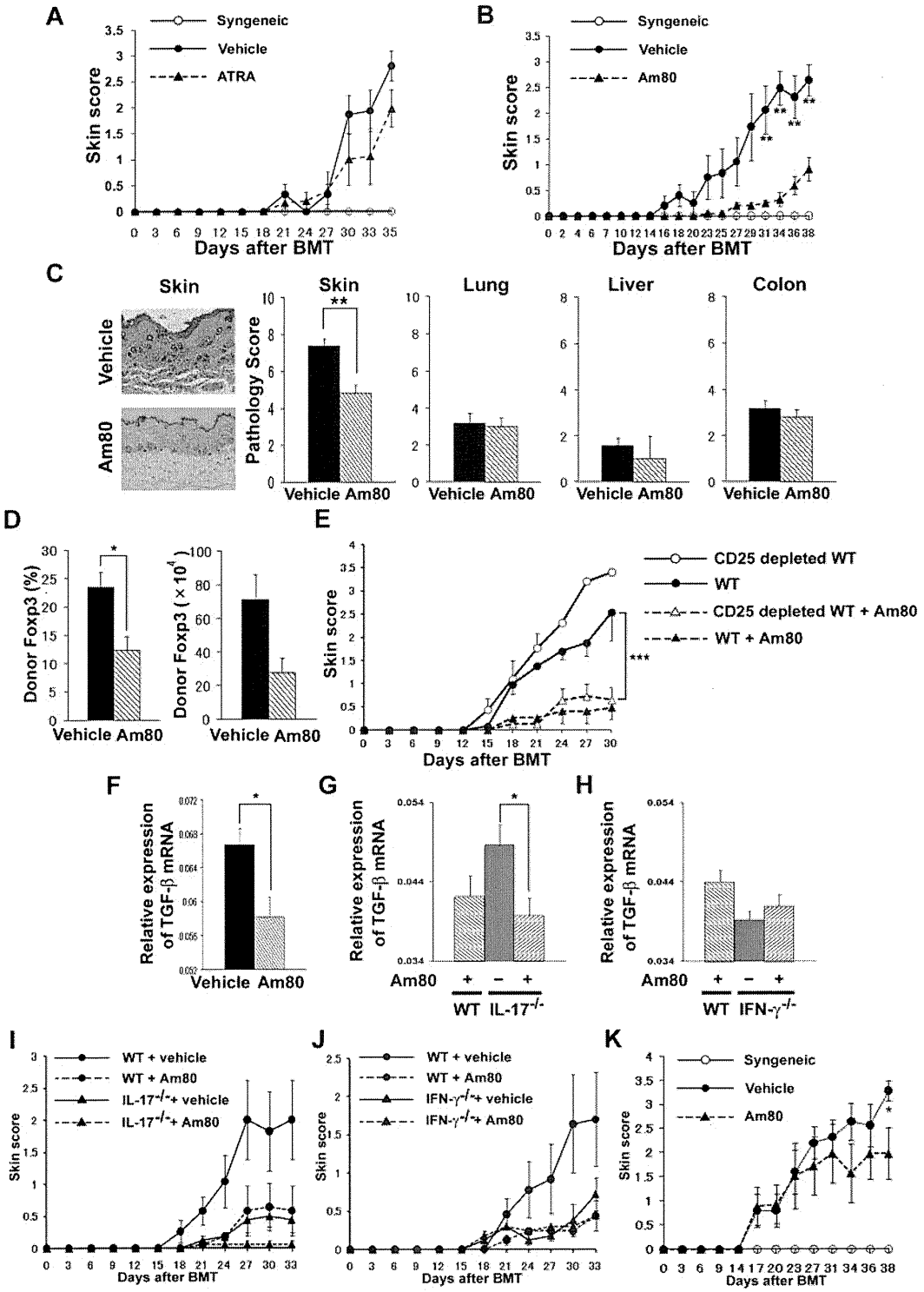

Figure 5. Administration of Am80 ameliorates cGVHD. (A-D) Sublethally irradiated BALB/c recipients were transplanted from WT B10.D2 donors. The recipients received daily administration of ATRA (200 μg/mouse; A), Am80 (1.0 mg/kg of body weight; B), or vehicle solution orally after BMT and were assessed for clinical signs of cGVHD every 3 days. The skin cGVHD scores are shown. (C) Representative histopathology of skin and pathology score of skin, lung, liver, and colon in each group (n = 5-6 per group) on day 16 after BMT are shown. (D) PLN cells of the recipients on day 16 were stained for intracellular Foxp3. The percentages and absolute numbers of $CD4^+Foxp3^+$ Treg cells are shown. Data are from 1 representative of ≥ 2 independent experiments. (E) Sublethally irradiated BALB/c recipients were transplanted with 8×10^6 TCD-BM cells plus 2×10^6 total spleen T cells or CD25-depleted T cells from WT or IFN-$\gamma^{-/-}$ B10.D2 donors. After BMT, recipients were given Am80 or vehicle solution. The skin cGVHD scores are shown. There were 6 recipients in each group; the data are from 1 representative of ≥ 2 independent experiments. (F-K) Sublethally irradiated BALB/c recipients were transplanted from WT (F), IL-$17^{-/-}$ (G), and IFN-$\gamma^{-/-}$ (H) donors. After BMT, recipients were given Am80 or vehicle solution. TGF-β mRNA expression in the ears on day 35 after BMT (F-H) and skin cGVHD scores (I-J) are shown. Data are from 1 representative of ≥ 2 independent experiments (n = 5 per group). (K) The skin cGVHD scores of BMT recipients treated with Am80 or vehicle solution orally daily after day 21 of BMT; data from 3 independent experiments were combined (n = 12-14 per group). $^*P < .05$, $^{**}P < .01$, and $^{***}P < .005$.

TGF-β is a critical mediator of fibrosis in cGVHD skin lesions.[30] TGF-β mRNA expression was decreased in the ear of the Am80 recipients (day 17, $P = .02$; Figure 5F). We then assessed TGF-β mRNA expression in recipients of IL-$17^{-/-}$ or IFN-$\gamma^{-/-}$

donors treated with Am80. Am80 further reduced skin scores and TGF-β expression in recipients of IL-17$^{-/-}$ donors (Figure 5G-I) but not in recipients of IFN-γ$^{-/-}$ donors (Figure 5H,J). These results suggest that the effects of Am80 are more dependent on IFN-γ than on IL-17.

Finally, we examined whether Am80 could be used for the treatment of cGVHD. Am80 was orally administered to mice from day 21 of BMT, when mice had developed clinical signs of cGVHD. Am80 significantly improved clinical scores ($P = .016$; Figure 5K).

Discussion

The results of the present study showed that Th1 and Th17 cells contribute to cGVHD with the use of a MHC-compatible, miHA-incompatible model of cGVHD. In addition, we demonstrated that Am80 down-regulates both Th1 and Th17 cells in vitro and in vivo, resulting in attenuation of cGVHD.

For many years, the best defined subsets of effector T cells of the CD4$^+$ Th lineage were the Th1 and Th2 cells. A third subset of CD4$^+$ effector cells was identified and named Th17 cells, because the signature cytokine that they produce is IL-17.[31] Although the role of Th17 in acute GVHD has been evaluated by several groups with inconsistent results,[32-35] few studies have addressed the role of Th17 in cGVHD. Initially, cGVHD was hypothesized to be a Th2-mediated disease on the basis of the results in a nonirradiated P→F1 model of cGVHD. cGVHD in this model is mediated by host B-cell autoantibody production stimulated by donor Th2 cells. Th1 polarization of donor T cells activates donor CD8$^+$ CTLs to kill host B cells, resulting in amelioration of cGVHD.[36] However, the relevance of this model is unclear in clinical BMT in which host B cells are eliminated by conditioning. Such different effector mechanisms between the models may be associated with distinct requirement of Th subsets for cGVHD between the studies. In the present study, we assessed the kinetics of Th1, Th2, and Th17 cells during the development of cGVHD in the B10.D2→BALB/c model. Th1 and Th2 responses were up-regulated early after BMT, followed by a subsequent up-regulation of Th17 cells. Significantly greater numbers of Th17 cells were detected in the lung and liver from allogeneic recipients than in those from syngeneic recipients. We then evaluated the role of Th17 in cGVHD with the use of IL-17$^{-/-}$ mice as several groups had used,[32-34,37,38] although interpretation of the results deserves caution because the Th17 lineage is uniquely regulated by RORγt,[13,14] and other cytokines such as IL-21 and IL-22 produced by Th17 cells may also contribute to Th17-mediated GVHD. On transfer of IL-17$^{-/-}$ B10.D2 donor T cells, cGVHD was significantly ameliorated compared with that in recipients of WT T cells, suggesting that Th17 contributes to cGVHD in this model. In particular, Th17 plays a significant role in skin cGVHD. This agrees with the recent observation by Hill et al[37] that donor pretreatment with G-CSF induces Th17 differentiation of donor T cells and induces skin GVHD after peripheral blood stem cell transplantation. In an adoptive transfer model of autoimmune cGVHD, Th17 cells infiltrated target tissues.[39] However, a subsequent study showed the absence of donor Th17 cells did not abrogate GVHD pathology,[38] in contrast to our results. In the absence of donor IL-17, Th1 responses were preserved in that study but were reduced in our study. Such difference in Th1 responses may produce different outcomes between the studies. In mouse models of acute GVHD, Yi et al showed enhanced Th1 differentiation of donor T cells by increased production of IL-12 from dendritic cells in the absence of IL-17.[33] By contrast, Kappel et al showed reduced numbers of IFN-γ–positive CD4$^+$ T cells and IFN-γ secretion in culture in the absence of IL-17.[34] These results together with our results suggest that IL-17 may induce IFN-γ, although such a hierarchy of Th1/Th17 pathways may be context or model dependent or both and will need to be studied in the future. Nonetheless, it should be noted that cGVHD still developed in the absence of donor IL-17 cells in our study. Taken together, it is probable that Th17 is not an absolute requirement for cGVHD, and either Th1 or Th17 is sufficient to cause cGVHD.

We demonstrated that IFN-γ$^{-/-}$ donor mice and injecting anti–IFN-γ mAb ameliorated cGVHD. Thus, Th1 and Th17 responses play a pathogenic role in cGVHD in this model. These results were consistent with a recent study reporting that cGVHD is mediated by Th1 and Th17 responses because of the progressive loss of CD4$^+$CD25$^+$Foxp3$^+$ T cells during acute GVHD in mice.[39] These results were also consistent with clinical studies showing that Th1 cells and Th17 cells increased in patients with active cGVHD.[40-43] Increased transcription of IFN-γ has also been detected in the affected skin and oral mucosa of patients with cGVHD.[41,44] In this study, we found no differences in Th17 cells between IFN-γ$^{-/-}$ and WT recipients, although significantly greater numbers of Treg cells were detected in IFN-γ$^{-/-}$ recipients. CD25-depleted T cells from IFN-γ$^{-/-}$ mice induced more severe skin cGVHD compared with CD25-replete IFN-γ$^{-/-}$ T cells, but still less severe cGVHD compared with CD25-depleted T cells from WT mice (Figure 3E), suggesting that IFN-γ contributes to the pathogenesis of cGVHD by both Treg-independent and -dependent pathways. Neutralization of IFN-γ ameliorated cGVHD in the absence of donor IL-17 (Figure 3H), suggesting again that both Th1 and Th17 responses contribute to the pathogenesis of cGVHD.

We found that donor-derived Th17 cells were generated in recipients of syngeneic transplantation in addition to allogeneic transplantation. However, the kinetics of Th17 development differed between the syngeneic and allogeneic settings; Th17 cells developed in the early phase after syngeneic transplantation. Kappel et al speculated that Th17 development may be the result of increased immune reconstitution of syngeneic hosts compared with allogeneic hosts with GVHD.[34] We additionally identified a population of donor-derived IFN-γ$^+$IL-17$^+$ cells after allogeneic BMT. It has been shown that a subset of IL-17–producing cells can also produce IFN-γ in vivo.[34,45] Such CD4$^+$IFN-γ$^+$IL-17$^+$ T cells have been postulated to play a causative role in the pathogenesis of experimental autoimmune encephalomyelitis (EAE).[46] IFN-γ$^+$IL-17$^+$ cells were only detected after allogeneic BMT, but not after syngeneic BMT, suggesting that this population is generated by allogeneic stimulation, but not because of lymphopenia-induced proliferation. Further investigations are required to clarify the difference in function between IL-17 single-positive and IFN-γ/IL-17 double-positive cells.

ATRA suppresses Th17 differentiation and effector function by RARα signaling,[18] but ATRA can also bind to RARβ and RARγ, which can form a variety of homodimers and heterodimers with 3 retinoid X receptors.[15] Nonselective receptor binding is thought to be a main cause of the side effects associated with the administration of ATRA and other pan-RAR agonists. Am80 is a synthetic RAR agonist that shows high affinity to RARα/β. In addition to a greater specificity for RARα, Am80 offers several other advantages over ATRA as a therapeutic agent, including less toxicity, greater stability, fewer potential side effects, and superior bioavailability. Am80 is effective in autoimmune disease models of collagen-induced arthritis,[20,47] EAE,[21,29] 2,4-dinitrofluorobenzene–

induced contact dermatitis,[22] and atherosclerosis.[23] Because retinoids can down-regulate Th1 and Th17 cells and can ameliorate autoimmune diseases, we hypothesized that these retinoids would attenuate cGVHD. We demonstrated that Am80 down-regulated Th1 and Th17 differentiation of donor T cells in BALB/c recipients of B10.D2 donors, resulting in reduced cGVHD. Our results suggest that combined blockade of Th1 and Th17 responses may represent a promising strategy to prevent or treat cGVHD, as has been suggested for acute and chronic GVHD.[32,39,48] Most recently, Yu et al used mice deficient for both T-bet and RORγt as T-cell donors and clearly showed that blockade of both Th1 and Th17 differentiation is required to prevent acute GVHD.[14] In addition, TGF-β mRNA expression in the skin decreased in the Am80 recipients of WT and IL-17$^{-/-}$ but not IFN-γ$^{-/-}$ donors. These results suggest that Am80 down-regulates TGF-β and that this effect is more dependent on IFN-γ than on IL-17. Unexpectedly, those recipients administered Am80 had a significantly lower frequency of Foxp3^{+} cells. These results differ from those of in vitro studies performed by Mucida et al,[28] in which retinoic acids were shown to be capable of inhibiting the IL-6–driven induction of Th17 cells and to promote Treg cell differentiation. Thus, retinoic acids enhance Treg differentiation and inhibit both Th17 and Th1 in vitro; however, the effects of retinoids may be more complex in vivo, because retinoids can affect not only T cells but also other immunoregulatory cells. For example, previous in vivo studies reported that Am80 suppressed Treg cells in experimental models of EAE[29] and collagen-induced arthritis,[47] similar to our study. In our study, Am80 suppressed TGF-β expression, a key cytokine in Treg development, which may have resulted in the suppression of Treg.

In conclusion, both Th1 and Th17 contribute to the development of cGVHD. Am80 down-regulates TGF-β and also regulates both Th1 and Th17 cells in vitro and in vivo, resulting in attenuation of cGVHD. Thus, administration of Am80, which is currently available as medication for acute promyelocytic leukemia in Japan,[49] may represent effective strategy for prevention and treatment of cGVHD.

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Authorship

Contribution: H.N. conducted the experiments, analyzed the data, and wrote the manuscript; Y.M. designed the experiments, supervised the research, and wrote the manuscript; H.S., K.K., Y.Y., S.K., and H.U. performed the research; K.T., T. Tanaka, and T.Y. performed histopathologic analyses of the organs; Y.I. provided vital new reagents for the study; and T. Teshima and M.T. supervised the research.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

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ORIGINAL ARTICLE

Reduced-intensity conditioning by fludarabine/busulfan without additional irradiation or T-cell depletion leads to low non-relapse mortality in unrelated bone marrow transplantation

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Abstract In reduced intensity, allogeneic stem cell transplantation from unrelated donors (u-RIST), graft-versus-host disease (GVHD), graft failure, and non-relapse mortality (NRM) are persistent problems. Although anti-thymocyte globulin, alemtuzumab, and total body irradiation (TBI) have been explored as conditioning modalities for u-RIST, the necessity for T-cell depletion or TBI to prevent GVHD or facilitate engraftment in u-RIST has not been determined. We here report the use of u-RIST with bone marrow grafting, following a simple conditioning regimen of 180 mg/m^2 fludarabine and 8 mg/kg of oral or intravenous busulfan without TBI or T-cell depletion. The study population was exclusively Japanese patients with a history of prior chemotherapy. We retrospectively analyzed 31 consecutive patients (median age 53 years). Twenty-five patients (81%) were transplanted from HLA-A, -B, and -DRB1 allele-matched donors. In all patients, neutrophil engraftment was achieved. The cumulative incidence of grade II–IV acute GVHD was 42%. However, 77% of patients with acute GVHD improved with, and could be managed by, initial, systemic, high-dose steroid treatment alone. Two-year overall and event-free survival was 62 and 53%, respectively. The NRM of 10% at 2 years was relatively low. Our results suggest that u-RIST without TBI or T-cell depletion may improve the prognosis after u-RIST in certain patient populations.

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Keywords Reduced-intensity conditioning · Bone marrow transplantation · Unrelated donor · Non-relapse mortality

1 Introduction

Although in recent times various approaches to reduced-intensity conditioning have increasingly been used in allogeneic hematopoietic stem cell transplantation, optimal conditioning for unrelated reduced intensity, allogeneic hematopoietic stem cell transplantation (u-RIST) has not yet been adequately established. In u-RIST, high rates of graft-versus-host disease (GVHD), graft failure, and non-relapse mortality (NRM) remain major problems, contributed by the elderly population and/or co-morbidity. Most of the reported conditioning regimens for u-RIST include T-cell depletion with anti-thymocyte globulin (ATG) [1–7] or alemtuzumab [5, 8–10], or low-dose total body irradiation (TBI) [11–18]. Recently, it was reported that the addition of ATG to GVHD prophylaxis resulted in a decreased incidence of acute and chronic GVHD without an increase in relapse or NRM, in a large, prospective, randomized phase 3 trial of myeloablative allogeneic hematopoietic stem cell transplantation with matched, unrelated grafts derived from peripheral blood [19]. However, it has not yet been determined whether additional TBI or T-cell depletion is necessarily required to facilitate engraftment or control GVHD in u-RIST, particularly using bone marrow-derived grafts.

The TBI may in fact evoke additional toxic effects, and induce or enhance acute GVHD via tissue damage, and thereby provoke release of inflammatory cytokines, as has been discussed [20]. Therefore, a regimen that involves TBI may cause a high incidence of NRM, particularly in

elderly patients and/or patients with co-morbidity. On the other hand, intensive T-cell depletion by ATG or alemtuzumab may cause excessive suppression of graft-versus-lymphoma or graft-versus-leukemia effects, or delay immune reconstitution, which results in an increased rate of relapse, progression, and NRM due to a high incidence of opportunistic infections [21–24].

In this study we therefore evaluated the impact on outcomes of simple conditioning, without irradiation or T-cell depletion, application being limited to u-RIST with bone marrow transplantation in Japanese patients with a history of prior chemotherapy.

2 Patients and methods

2.1 Patients

We retrospectively evaluated 31 consecutive patients with hematological malignancies, who had experienced some chemotherapy, and excluded patients without any prior chemotherapy from the analysis. The evaluable patients underwent allogeneic bone marrow transplantation at our institute between September 2002 and December 2009.

Table 1 shows the patients' characteristics and the transplantation details. The median age at transplantation was 53 years (range 22–69 years). These patients included eleven individuals with acute myeloid leukemia, nine with acute lymphoblastic leukemia (ALL) (including seven Ph-positive ALL), two with refractory anemia with excess blasts II [RAEB II, a variant of myelodysplastic syndrome (MDS)], six with non-Hodgkin's lymphoma, and three with the lymphomatous type of adult T-cell leukemia/lymphoma. All patients had experienced chemotherapy prior to transplantation with a median of six courses of prior chemotherapy (range 1–19). Reduced-intensity conditioning was chosen because of age above 50 years ($n = 17$), cardiac dysfunction ($n = 7$), prior allogeneic hematopoietic stem cell transplantation ($n = 4$), prior intense chemotherapy ($n = 2$), or the patient's choice ($n = 1$). We regarded the following patients as being at standard disease risk: acute leukemia in first or second complete remission, refractory anemia due to MDS, low-grade lymphoma of any status, or aggressive lymphoma in complete remission ($n = 20$). The other patients ($n = 11$), including those with aggressive lymphoma without remission ($n = 4$), MDS RAEB ($n = 2$), AML from chronic myelomonocytic leukemia ($n = 1$), fourth complete remission of AML ($n = 1$), AML without remission ($n = 1$), third complete remission of ALL ($n = 1$), and ALL without remission ($n = 1$), were regarded as "high risk".

As it was difficult to obtain informed consent for this retrospective study, we made the context of this study known to the public by putting up a notice at our hospital and on our website in accordance with the ethical guidelines for epidemiological research compiled by both the Ministry of Education, Culture, Sports, Science and Technology and the Ministry of Health, Labour and Welfare in Japan. This retrospective study was approved by the Institutional Review Board.

Table 1 Characteristics of patients

No. of patients, *n*	31
Median recipient age at transplantation, years (range)	53 (22–69)
Age >60/≤60	9/22
Recipient sex, *n* (male/female)	21/10
Disease, *n*	
AML	11
ALL	9 (Ph + ALL: 7)
MDS	2 (RAEB-II: 2)
NHL	9 (FL: 2, DLBCL: 2, ALCL: 1, IVLBCL: 1, ATL/L: 3)
Median prior chemotherapy, *n* (range)	6 (1–19)
CMV serostatus, *n*	
Recipient-/donor-	1
Other	30
Sex mismatch, *n*	
Female donor to male recipient	4
Other	27
ABO incompatibility, *n*	
Match	18
Mismatch	13
Relapse risk, *n*	
Standard	20
High	11
HLA matching (at HLA-A, B, DR), *n*	
Serologically 6/6 matched	31
Allele 6/6 matched	25
Allele 5/6 matched	6 (DRB1 mismatch: 5, A allele mismatch: 1)
GVHD prophylaxis, *n*	
CsA + sMTX	31
Conditioning regimen, *n*	
Oral Bu 8 mg/kg + Flu 180 mg/m^2	12
Intravenous Bu 8 mg/kg + Flu 180 mg/m^2	19

AML acute myeloid leukemia, *ALL* acute lymphocytic leukemia, *GVHD* graft-versus-host disease, *MDS* myelodysplastic syndrome, *RAEB* refractory anemia with excess blasts, *NHL* non-Hodgkin's lymphoma, *FL* follicular lymphoma, *DLBCL* diffuse large B-cell lymphoma, *ALCL* anaplastic large cell lymphoma, *IVLBCL* Intravascular large B-cell lymphoma, *ATL/L* Adult T-cell leukemia/lymphoma, *CMV* cytomegalovirus, *Flu* fludarabine, *Bu* busulfan, *sMTX* short-term methotrexate, *CsA* cyclosporine A

2.2 Donors and HLA typing

Unrelated bone marrow donors were selected through the agency of the Japan Marrow Donor Program Donor Center (JMDP). HLA serotyping and genotyping for HLA-A, -B, and -DR were performed in all the patients and donors. HLA, -A, -B, and -DR compatibility between patients and donors was determined by standard serological techniques and high-resolution DNA typing as previously reported [25]. HLA-C compatibility was not considered for donor selection because HLA-C allele typing was not routinely performed for unrelated donor selection before April 2004 in the JMDP. On HLA serotyping, all of the 31 recipient–donor pairs were serologically matched for HLA-A, B, and DR, and in addition, 25 pairs were matched for 6 of 6 HLA-A, B and DR alleles (Table 1). Of six HLA allele-mismatched pairs, five were mismatched at one DRB1 allele, and the other was mismatched at a single A allele. In 14 of the 31 pairs, the HLA-C allele was available for both the recipient and donor. Ten of 14 patients had 8/8 HLA-matches with the donor, three had a mismatched donor for a single allele (two at the C allele and one at the DRB1 allele), and one patient had a mismatched donor for two alleles (at the C and DRB1 alleles).

2.3 Conditioning regimen and GVHD prophylaxis

The conditioning regimen consisted of intravenous fludarabine (Flu) 30 mg/m^2/day for 6 days (total 180 mg/m^2), oral busulfan (Bu) 4 mg/kg/day divided into four doses per day for 2 days (total 8 mg/kg) ($n = 12$), or intravenous Bu 1.6 mg/kg/day divided into two doses per day for 5 days (total 8 mg/kg) ($n = 19$). Recently, we used 0.8 mg/kg IV Bu q12hr for 5 days because we aimed to reduce toxicity by increasing the Bu dosing interval and to ensure engraftment by increasing the total dose of Bu. In this study we regarded intravenous Bu 8 mg/kg as reduced-intensity conditioning although intravenous Bu 8 mg/kg is categorized as myeloablative conditioning according to the CIBMTR definition [26]. Non-T-cell-depleted bone marrow was infused on day 0.

As prophylaxis for GVHD, all patients received cyclosporine A (CsA) and short-term methotrexate (sMTX). CsA was started from day −1 at a dose of 3 mg/kg/day divided into two doses per day, and MTX was administered intravenously on day 1 (10 mg/m^2) and on days 3 and 5 (7 mg/m^2). The CsA dosage was adjusted to a target trough level between 150 and 300 ng/ml until day 100, except where disease progression or drug toxicity occurred, and then tapered over 3–6 months unless GVHD occurred.

Granulocyte colony-stimulating factor was administered intravenously from day 1 or day 5 in all patients until neutrophil engraftment. As antibiotic prophylaxis, polymyxin B sulfate, acyclovir, and fluconazole were used for prevention of bacterial infection, herpes viral infection, and fungal infection, respectively, after the conditioning regimen started. Sulfamethoxazole/trimethoprim was started for prophylaxis of *Pneumocystis jiroveci* pneumonia after neutrophil engraftment.

2.4 Chimerism analysis

We assessed donor–recipient chimerism by polymerase chain reaction (PCR)-based amplification of a polymorphic short tandem repeat (STR) region. Chimerism was serially evaluated in the T-cell fraction of peripheral blood samples on days 30, 60, 90 and thereafter at appropriate points after transplantation. According to the manufacturer's recommended protocol (BML, Tokyo, Japan), T-cell chimerism analysis with PCR-based STR analysis was undertaken with a commercially available assay. Briefly, peripheral blood samples were collected, and the T-cell enriched fraction was prepared by negative selection using a RosetteSep kit (Stem Cell Technologies, Vancouver, Canada) for other cell lineages (CD16, CD19, CD36, CD56, CD66b, and glycopholin A). DNA was then extracted from selected cells using a QIAamp DNA Blood Mini Kit (QIAGEN, Hilden, Germany). Mutiplex PCR was performed using a GeneAmp 9700 thermal cycler (Applied Biosystems, Foster City, CA, USA). Separation and detection of the amplified PCR products were performed, and the area under the curve for each STR allele was automatically processed using the CEQ 8000 Genetic Analysis System (Beckman Coulter, Fullerton, CA, USA). The range of error of chimerism was regarded as 10%.

2.5 GVHD diagnosis and treatment

Acute GVHD was diagnosed clinically, graded in accordance with the standard criteria [27] and confirmed by appropriate biopsy of the lesion. Chronic GVHD was also defined in accordance with the traditional criteria [28]. We initially treated the patients who developed acute GVHD with methylprednisolone or prednisolone at a dose of 1–2 mg/kg/day.

2.6 Statistical analysis

Neutrophil recovery was defined as the first of three consecutive days with an absolute neutrophil count (ANC) $\geq 0.5 \times 10^9$/L. Platelet recovery was defined as the first of three consecutive days with a platelet count $\geq 20 \times 10^9$/L, without platelet transfusion in the preceding 7 days. Full donor T-cell chimerism was defined as more than 90% donor T cells in the patient's peripheral blood. Primary graft failure was defined as a lack of neutrophil recovery in the absence of disease relapse or progression affecting

hematopoietic recovery. Secondary graft failure was defined as the proportion of donor T cells being less than 10% in the peripheral blood, excluding disease relapse or progression-related loss of donor T cells. Event-free survival (EFS) was defined as survival without disease relapse or progression, or death from any cause. NRM was defined as any death except for death related to relapse or progression. Cumulative incidence curves of NRM and relapse were analyzed under the assumption that they represented competing risks. Death not associated with GVHD was treated as a competing risk event in constructing the cumulative incidence curve for acute GVHD. The Kaplan–Meier method was used for overall survival (OS) and EFS. The log-rank test was used for comparing survival between high- and standard-risk groups. The Cox proportional hazard model was used to determine the significant factors that influenced OS in univariate and multivariate analyses. We defined statistical significance as a P value less than 0.05. Statistical analyses were performed using STATISTICA version 6.0 and R version 2.9.1.

3 Results

3.1 Engraftment and T-cell chimerism

All patients received bone marrow from unrelated donors. The median total number of nucleated cells in the marrow graft was 2.82 (range 1.26–4.14) $\times$ 10^8 per kg of the recipient's body weight. All 31 patients achieved neutrophil engraftment, and the median time to neutrophil engraftment was 16 days (range 12–26 days) after transplantation. Twenty-nine patients (94%) achieved platelet engraftment, and the median time to platelet engraftment was 24 days (range 18–291 days) after transplantation.

Twenty patients (65%), 26 patients (84%), and 28 patients (90%) achieved complete donor T-cell chimerism by 30, 60, and 90 days after transplant, respectively. Of three patients who did not achieve complete donor T-cell chimerism by 100 days, one patient achieved complete donor T-cell chimerism on day 236, and in the other two patients, grafts were rejected due to disease progression. Only nine of twelve patients with oral Bu conditioning achieved complete donor T-cell chimerism. On the other hand, all 19 patients who received intravenous Bu achieved complete donor T-cell chimerism by day 100 [$P = 0.049$ (Fisher's exact test)]. None had experienced graft rejection except for relapse-related graft rejection.

3.2 Regimen-related toxicities and infections

Regimen-related toxicities until 28 days after transplantation were graded in accordance with the National Cancer Institute Common Toxicity Criteria, version 3.0 and are shown in Table 2. There were 17 episodes of grade III toxicity and two episodes of grade IV toxicity. Of grade III or IV toxicities, Grade III mucositis was frequently observed (42%). One patient had a grade III hemorrhage, consisting of a subdural hematoma at 14 days after transplantation. He underwent emergency surgery to remove the hematoma and recovered. Grade IV toxicities were observed in two patients. One patient experienced grade IV hepatic toxicity due to hemophagocytic syndrome, but he recovered following a single administration of 50 mg/m^2 etoposide. The other experienced grade IV pulmonary toxicity due to engraftment syndrome and needed endotracheal intubation. Steroid administration was very effective for engraftment syndrome, and the patient could be extubated, but died from disease progression and cytomegalovirus (CMV) pneumonia. No hepatic veno-occlusive disease was documented in this study.

Table 2 Grade III and IV regimen-related toxicities up to 28 days after transplantation

	Grade, no. of episodes (% of patients)		
	I–II	III	IV
Cardiac	0 (0)	0 (0)	0 (0)
Mucositis	7 (23)	13 (42)	0 (0)
GI (nausea or diarrhea)	8 (26)	2 (6)	0 (0)
Hepatic	5 (16)	0 (0)	1 (3)
Pancreas	1 (3)	0 (0)	0 (0)
Central nervous system	0 (0)	1 (3)	0 (0)
Pulmonary	1 (3)	0 (0)	1 (3)
Renal	1 (3)	0 (0)	0 (0)
Hemorrhage	0 (0)	1 (3)	0 (0)

GI gastrointestinal

Infectious complications up until day 100 are summarized in Table 3. Culture-negative febrile neutropenia was documented in 17 patients (55%). Nine patients (29%) had bacteremia including eight Gram-positive organisms and one Gram-negative organism. Twenty-four patients (77%) became positive for CMV antigenemia: five of them developed CMV colitis and one developed CMV pneumonia. Hemorrhagic cystitis was documented in seven patients. Urinary PCR for viral DNA was performed in six of these seven patients. Adenovirus was detected in the urine of two patients, BK virus in another two, and both adenovirus and BK virus in the remaining two patients.

3.3 Graft-versus-host disease

Acute GVHD occurred in 18 patients (58%) with a median time to development of acute GVHD of 23.5 days (range 11–55 days). Five patients had grade I, eight had grade II,

Table 3 Infectious complications in the first 100 days of transplantation

	No. of episodes	No. of patients (%)
Culture-negative febrile neutropenia	18	17 (55)
Bacterial		
Bacteremia	10	9 (29)
Pneumonia	2	2 (6)
Viral		
CMV antigenemia	–	24 (77)
CMV colitis	5	5 (16)
CMV pneumonia	1	1 (3)
Hemorrhagic cystitis	7	7 (23)
VZV reactivation	2	2 (6)
HSV reactivation	1	1 (3)
Fungal		
Pneumonia	2	2 (6)
Pneumocystis pneumonia	1	1 (3)

CMV cytomegalovirus, *VZV Varicella zoster* virus, *HSV Herpes simplex* virus

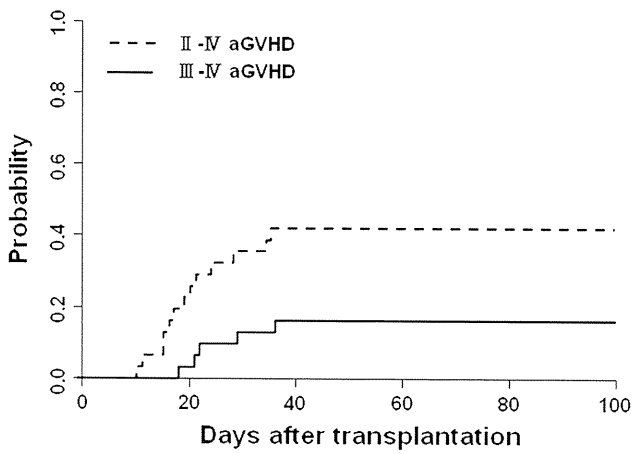

Fig. 1 Cumulative incidence of acute graft-versus-host disease (GVHD). The cumulative incidence of grades II–IV and III–IV acute GVHD are displayed

two had grade III, and three had grade IV GVHD. The cumulative incidence of grade II–IV and III–IV acute GVHD by day 100 after transplantation was 42 and 16%, respectively (Fig. 1). Systemic high-dose steroid was administered to all 13 of these patients as initial treatment of grade II–IV acute GVHD, and in 10 patients (77%) acute GVHD improved and was manageable without any additional treatment. In the other three patients (23%), acute GVHD was refractory to the steroid treatment, and they received secondary therapies including mycophenolate mofetil, pentostatin, or intra-arterial infusion of steroid for acute GVHD involving the gut. Two of them died of acute GVHD, and the third died of disease relapse although acute

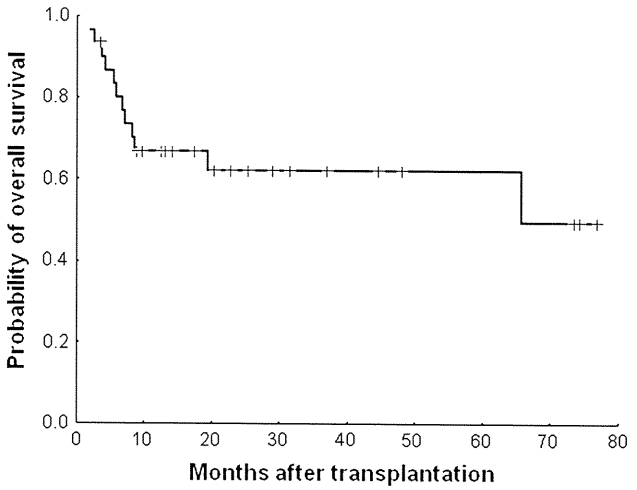

Fig. 2 Kaplan–Meier curve of overall survival. Kaplan–Meier product estimates of overall survival are presented

GVHD subsided. Chronic GVHD was observed in 15 of 25 evaluable patients (60%), and the extensive type of chronic GVHD was present in seven patients (28%).

3.4 Survival and cause of death

The median follow-up period for all 31 patients was 14.1 months (range 1.8–76.7 months). Of these patients, 19 were alive at 25.2 months of median follow-up (range 3.4–76.7 months). Of 12 deaths, nine patients died of disease relapse or progression, and three died of non-relapse-related causes including acute GVHD/thrombotic microangiopathy (day 55), acute GVHD/bacterial pneumonia (day 102), and chronic GVHD/bacterial pneumonia (day 162). Of 13 patients with disease relapse or progression, three patients underwent allogeneic hematopoietic stem cell transplantation again, and two underwent donor lymphocyte infusion, and nine died during the follow-up period.

In all patients, OS and EFS were 67 and 53% at 1 year, and 62% and 53 at 2 years, respectively (Fig. 2). In the standard disease-risk group, OS and EFS were 84 and 72% at 1 year, and 75 and 72% at 2 years. In the high disease-risk group, OS and EFS were 33 and 18% at 1 and 2 years, respectively (Log rank test: $P = 0.06$ and 0.009, respectively). The NRM at day 100 and 1 year were very low, 3 and 10%, respectively, and the rates of relapse at day 100 and 1 year were 13 and 37%, respectively.

Table 4 shows the results of univariate and multivariate analyses for factors influencing OS. On univariate analysis, elderly patients (>60) had a significantly worse outcome [HR 7.0, 95% confidence interval (CI) 20–24, $P = 0.002$], and patients with high-risk disease tended to have worse outcome (HR 2.9, 95% CI 0.9–9.4, $P = 0.07$). On multivariate analysis, older age (>60) remained significantly