Table 2. Details of the rescue transplantation

No	Interval between 2 transplantations (days)	Donor relationship	Donor Sex/ Age	HLA disparity	Conditioning treatment	Cell dose CD34 cells × 10^6/kg	Cell dose CD3 cells × 10^8/kg	Engraftment of rescue graft	days	Hematological recovery Neu>0.5 ×10^9/l (days)	Hematological recovery PLT>20 ×10^9/l (days)	GvGVHD effect	survival (days)	Cause of death
1	94	Sibling	F/25	2/2	chemo	3.90	1.61	+	15	9	101	complete	+3304	-
2	40	Sibling	F/22	0/0	chemo	6.60	5.81	NE		8	-	NA	10	TMA
3	145	Mother	F/58	1/1	chemo	3.76	5.38	NE		-	-	NA	13	Renal failure
4	40	Offspring	M/12	3/3	chemo	16.50	4.19	+	20	10	-	partial	135	GVHD
5	481	Mother	F/55	2/2	chemo+TBI	3.90	2.50	+	34	not decreased	not decreased	complete	+2714	-
6	213	Sibling	F/23	1/0	chemo+TBI	5.20	6.71	+	29	10	36	complete	831	Cardiac failure
7	47	Mother	F/45	2/2	chemo	3.60	3.57	−		26	-	partial	76	Pneumonia
8	98	Mother	F/47	3/3	chemo+TBI	6.20	3.11	+	11	10	-	partial	23	Pneumonia
9	227	Mother	F/48	2/3	chemo+TBI	4.51	2.06	+	17	not decreased	not decreased	complete	+2170	-
10-1	59	Mother	F/51	2/2	chemo+TBI	2.80	2.12	−		not decreased	-	transient	+42	-
10-2	101	Mother	F/51	2/2	chemo+TBI*	2.30	2.27	+	14	9	32	complete	+2086	-
11	63	Sibling	M/37	0/0	chemo	7.10	1.71	−		not decreased	not decreased	partial	33	VOD
12	32	Mother	F/ 51	3/0	chemo+TBI	23.00	3.49	−		not decreased	-	partial	46	GVHD
13	36	Offspring	M/22	2/3	chemo+TBI	7.16	3.22	+	52	8	9	complete	+1637	-
14	59	Sibling	F/12	3/1	chemo+TBI	18.60	8.10	+	14	10	-	transient	72	TTP
15	49	Offspring	M/16	3/3	chemo	17.10	4.30	+	8	8	-	complete	163	Hepatic failure
16	39	Sibling	F/27	3/2	chemo	14.00	2.66	+	107	not decreased	16	complete	+490	-

chemo, chemotherapy consisting of fludarabine 30 mg/m2 and anti-T-lymphocyte globulin; TBI, total body irradiation 3Gy; NE, not evaluable; not decreased, neutrophils or platelet counts did not decrease below 0.5 x 109/l or 20 x 109/l, respectively; GvGVHD effect, graft-versus-GVHD effect; complete, complete response; partial, partial response; TMA, thrombotic microagiopathy; VOD, hepatic veno-occlusive disease; TTP, thrombotic thrombocytopenic purpura.

*Thiotepa 10 mg/kg and TBI 4 Gy were given in addition to fludarabine and ATG.

interleukin-2 receptor level [17,18], as an indicator, and was thereafter continued carefully.

Acute GVHD was graded according to standard criteria [19] and GVHD beyond 100 days after transplantation was diagnosed based on the proposed National Institutes of Health criteria [20]. Patient status before rescue transplantation was assessed by the Karnofsky performance rating. We defined the response to treatment as follows: complete response: loss of all symptoms of acute GVHD; partial response: improvement of at least one GVHD grade; stable disease: no change in GVHD grade; progressive disease: worsening of GVHD. Regarding the assessment of GVHD after the rescue transplantation, if the symptoms of patients were considered to have been caused mainly by a complication other than GVHD, their GVHD stages were downgraded by one stage, according to the recommendation in the 1994 consensus conference on acute GVHD grading [21]. A diagnosis based on autopsy directly reflected the assessment of response.

Each patient was isolated in a laminar air-flow room and standard decontamination procedures were followed. Oral antibiotics (ciprofloxacin, vancomycin, amphotericin B) were administered to sterilize the bowel. Patients with negative cytomegalovirus (CMV) IgG titers received blood products from CMV seronegative donors. Intravenous immunoglobulin was administered at a minimum dose of 100 mg/kg every 2 weeks until day 100. Cotrimoxazole was given for at least 1 year for prophylaxis of *Pneumocystis jirovecii* infections. Acyclovir was administered at a dose of 1000 mg/day for 5 weeks after transplantation to prevent herpes simplex infections.

Ganciclovir 7.5 mg/kg divided in three doses per day was administered from day −10 to day −3 as prophylaxis for CMV infection. Thrombotic microangiopathy was diagnosed according to Zeigler's criteria [22], and based on the recommendations reported by Nishida et al. [23].

Chimerism analysis

Chimerism between the donor and recipient was analyzed as described previously [13]. Chimerism analysis was continued twice a week after transplantation until donor engraftment or rejection. Blood samples were analyzed to determine the degree of donor/recipient chimerism in the T-cell or neutrophil-enriched cell fraction, using polymerase chain reaction amplification of informative microsatellite regions, which identified differences between the donor and recipient (based on polymorphisms found in pretransplantation donor/recipient samples) [24]. To remove monocytes, KAC-2 silica beads (Japan Immunoresearch Laboratories Co., Ltd., Gunma, Japan) were mixed with heparinized peripheral blood and incubated at 37°C for 1 hour. To enrich T cells, a negative selection system (RosetteSep; StemCell Technologies) was used [25]. To obtain a T-cell–enriched cell fraction, a cocktail containing anti-CD16, anti-CD19, anti-CD36, and anti-CD56 antibodies was added to the blood samples after they were treated with Silica beads. After Ficoll-Paque (GE Healthcare, Little Chalfont, Buckinghamshire, UK) density gradient centrifugation, $CD3^+$ cells were recovered from the Ficoll: plasma interface with a purity >95%. Neutrophils were recovered from the Ficoll:RBC interface with a purity >99%.

Statistical analysis

The protocol was designed as a phase II study with sufficient power to detect a response rate of ≥20% with a standard error of 10%. Comparison of patients who did or did not achieve rescue donor engraftment for the response for GVHD was evaluated using the χ^2 test. Survival data from patients achieving rescue donor engraftment or not were compared based on the results of log-rank tests. Results were considered significant at $p < 0.05$.

Data were "locked" for analysis on May 31, 2010.

Results

Engraftment of rescue donor grafts

To treat GVHD, patients received peripheral blood stem cells from a second allogeneic donor with a median of 6.40×10^6 (range, $2.30–23.00 \times 10^6$) $CD34^+$ cells/kg, including a median of 3.22×10^8 (range, $1.61–8.10 \times 10^8$) $CD3^+$ cells/kg, without T-cell depletion. As shown in Table 1, 16 patients received 17 rescue transplantations to treat GVHD. Because of a poor performance status at transplantation, two patients (nos. 2 and 3) died early (days 10 and 13, respectively) and could not be evaluated for the effects of rescue transplantation; therefore, data from 15 transplantations were analyzed.

Among the 15 transplantations that could be evaluated, rescue donor grafts engrafted in 11 cases, but not in 4 cases. T-cell engraftment preceded neutrophil engraftment (data not shown). In chimerism analysis, all patients showed 100% first donor chimerism in both T-cell and myeloid cell components before the rescue transplantation. It was difficult to obtain continuous chimerism data between first and second (rescue) donors within 1 week after transplantation because of lymphocytopenia. Changes of T-cell chimerism of patients, in whom the chimeric status could be consecutively measured, are shown in Figure 1. In the four patients rejecting a rescue graft, although transiently increasing up to 35% on day 4, rescue donor-derived T cells, thereafter decreased and became undetectable up to 2 weeks after transplantation. Regarding patients who achieved engraftment, donor T-cell chimerism rapidly or gradually increased after transplantation, and full T-cell chimerism of the rescue donor was achieved in a median of 15 days (range, 7–106 days).

Regarding neutrophil recovery, in 6 of the 15 patients, absolute neutrophil counts did not decrease to $<0.5 \times 10^9$/L, and in the remaining 9 patients, absolute neutrophil counts increased to $>0.5 \times 10^9$/L at a median of 10 days (range, 8–26 days). The platelet counts did not decrease to $<20 \times 10^9$/L in three patients (nos. 5, 10–2, and 12). Among the remaining 12 patients, platelet recovery occurred in 5 patients at a median of 32 days (range, 9–101 days), but not in the remaining 7 patients because of early death or subsequent transplantation.

Graft-versus-GVHD effects

Clinical effects of rescue transplantation are shown in Table 3. For successful graft-versus-GVHD treatment, engraftment of the rescue donor graft was mandatory in our murine model [11], in which immunosuppressive agents were not used. In the present clinical study, in which immunosuppressive agents

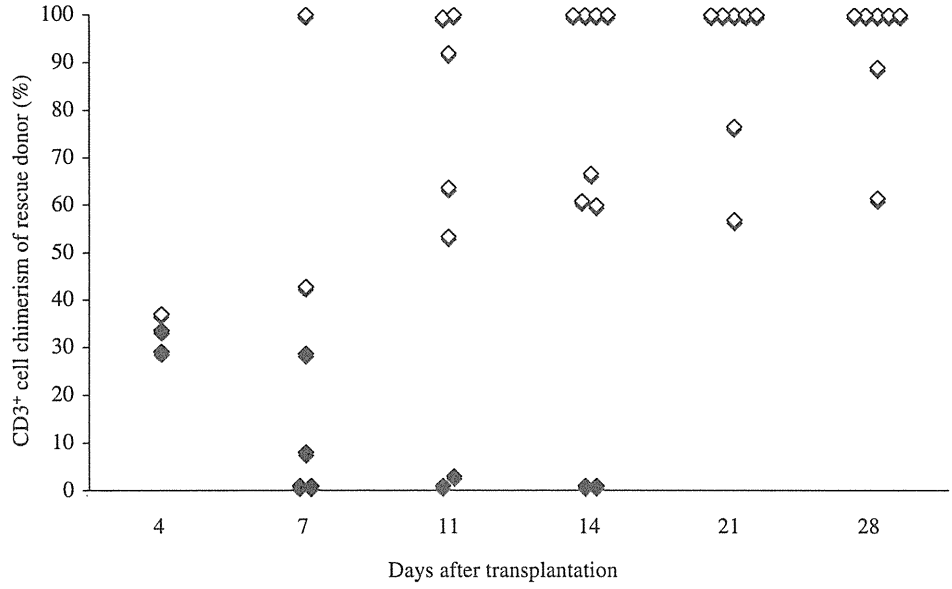

Figure 1. T-cell chimerism between first and second (rescue) donors in patients who did or did not achieve rescue donor engraftment. Open or closed diamonds denote patients who did or did not achieve rescue donor engraftment, respectively.

were naturally used in the transplantation, the response rate for patients achieving rescue donor engraftment or not was 90.9% (eight complete response, two partial response, and one stable disease) and 50% (one complete response, one partial response and one stable disease), respectively. Patients achieving rescue donor engraftment tended to show a higher response than patients not achieving engraftment ($p = 0.080$, χ^2 test). For the response of each organ, patients achieving rescue donor engraftment showed a significantly higher response with cutaneous GVHD than patients not achieving engraftment ($p = 0.016$), but there was no significant difference in response for intestinal and hepatic GVHDs between patients who did and did not achieve rescue donor engraftment. Regardless of achieving engraftment of the rescue donor graft, most GVHD symptoms began to improve during the conditioning treatment, and continued to improve by 1 week after transplantation. Thereafter, in patients who achieved rescue donor engraftment, the majority of GVHD symptoms continued to improve and disappeared within 40 days after transplantation, whereas in patients not achieving engraftment, some GVHD

Table 3. Change of the severity of GVHD

		stage			
No.	engraftment	skin	gut	liver†	grade
1	yes	3 → 0 (19)*	1 → 0 (0)	0 → 0	II → 0 (19)
4	yes	3 → 0 (6)	4 → 2 (12)	0 → 0	III → III
5	yes	3 → 0 (19)	0 → 0	0 → 0	II → 0 (19)
6	yes	3 → 0 (21)	0 → 0	0 → 0	II → 0 (21)
8	yes	0 → 0	3 → 1 (9)	3 → 0 (11)	III → II (11)
9	yes	3 → 0 (4)	0 → 0	0 → 0	II → 0 (4)
10-2	yes	3 → 0 (7)	2 → 0 (15)	0 → 0	III → 0 (15)
13	yes	4 → 0 (38)	2 → 0 (20)	0 → 0	IV → 0 (38)
14	yes	2 → 0 (−5)	3 → 0 (30)	1 → 1	III → II (30)
15	yes	3 → 0 (10)	3 → 0 (30)	0 → 0	III → 0 (30)
16	yes	2 → 0 (−6)	2 → 0 (5)	0 → 0	III → 0 (5)
7	no	4 → 1 (2)	0 → 0	0 → 0	IV → I (2)
10-1	no	3 → 1 (5)	4 → 1 (5) → 2 (19)	0 → 0	III → II (5) → III(19)
11	no	3 → 0 (−5)	2 → 0 (−5)	3 → 0†	III → 0†
12	no	2 → 0 (10)	2 → 0 (15)	2 → 3 (4) → 2(13)	III → III

*Numbers in parentheses denote the day after rescue transplantation when the stage or grade of GVHD was changed.
†Staging of hepatic GVHD was decided based on the serum bilirubin levels. Patient No.11 had an increased bilirubin level and died on day 33, but the main cause of death of the patient was diagnosed from autopsied samples with hepatic veno-occusive disease without no evidence of GVHD.

symptoms disappeared and others became stable or rebounded. Once a complete response was achieved, no rebound of GVHD occurred. In 8 patients who achieved rescue donor engraftment and who had a complete response, the median time for achieving a complete response was 19 days (range, 4–38 days) after transplantation. Among three patients not achieving a complete response despite rescue donor engraftment, one patient (no. 4) showed a complete response for cutaneous GVHD, but had continued diarrhea. The diarrhea was diagnosed to be mainly caused by thrombotic microangiopathy because of partial improvement of the symptom by tapering the immunosuppressants [23]. In another patient (no. 8), the serum bilirubin level was normalized after rescue transplantation and diarrhea had also improved (stage 3 → stage 1) by day 23 when the patient died of aspergillus pneumonia. The remaining patient (no. 14) showed a complete response of cutaneous and gut GVHDs, but serum bilirubin levels continued to increase. The aggravation of jaundice was diagnosed to be caused by thrombotic thrombocytopenic purpura based on the presence of severe hemolysis and renal failure. In four patients who rejected rescue donor grafts, one patient (no. 11) showed a complete response of cutaneous and intestinal GVHDs, but showed a progressive increase in serum bilirubin levels and died on day 33. The patient was diagnosed from autopsied liver samples with hepatic veno-occlusive disease with no evidence of GVHD. Patient no. 7 achieved a partial response (stage 4 → stage 1) of cutaneous GVHD but died of pneumonia on day 76. Patient no. 12 showed a complete response for cutaneous and intestinal GVHDs, but showed no response of hepatic GVHD, and died of aggravated GVHD on day 46. The remaining patient (no. 10–1) showed a partial response of cutaneous GVHD and also showed partial improvement of intestinal GVHD by day 5, when diarrhea rebounded and was progressively aggravated; therefore, he underwent a second rescue transplantation, after which he achieved rescue donor engraftment and ultimately had a complete response.

Regarding chronic GVHD, only 1 of the 10 patients who survived for >100 days developed limited-type chronic GVHD (skin lesion).

Adverse effects (Table 4)

CMV antigenemia occurred in 11 of 15 transplants (73.3%). The median peak number of CMV antigen-positive leukocytes was 15.4 per 50,000 white blood cells (15.4/50,000), with a range of 2.8/50,000 to 285.7/50,000. No CMV disease was observed.

Three patients developed bacterial infections: one (no. 7) had fatal pneumonia from *Enterococcus cloacae*, and one (no.16) had *Escherichia coli* sepsis, and one (no. 15) had sinusitis, all were successfully treated with administration of antibiotics. Two patients developed aspergillus pneumonia: one patient (no. 13) was successfully treated by antibiotics and another patient (no. 8) with a pulmonary aspergillus lesion before rescue transplantation died of aggravated pneumonia and brain fungal embolism. One patient (no. 14) developed fatal thrombotic thrombocytopenic purpura and one (no. 11) fatal hepatic veno-occlusive disease. One patient (no. 10–1) developed pancreatitis, which was improved by conventional treatment. Ten patients (62.5%) developed liver dysfunction with an increase to more than three times the normal upper limit of the transaminase level. The majority of cases of liver dysfunction were due to steroid- or drug-induced toxicities, and the transaminase level in these patients was normalized after tapering or discontinuation of the causative drugs. Other adverse events are shown in Table 4.

Relapse, cause of death, and overall survival

No patients had recurrence of the original disease. Two patients died early because of a poor performance status at rescue transplantation. Among them, 1 patient (no. 2) had severe GVHD accompanied by sepsis hyperbilirubinemia (10.2 mg/dL), and died of multiorgan failure on day 10. Another (no. 3) developed renal failure after the start of conditioning treatment. Despite receiving hemodialysis, he died of renal failure on day 13.

Overall survival at 6 months and 3 years was 44.6% (95% confidence interval [CI], 19.8–86.8%), and 37.2% (95% CI, 12.4–62.0%), respectively. Patients who achieved rescue donor engraftment showed a significantly improved survival rate compared with those who rejected grafts (log-rank test, $p = 0.013$) (Fig. 2). Six of the eight patients who achieved a complete response survived without any GVHD symptoms or relapse of the original diseases, with a median follow-up of 2128 days (range, 490–3304 days). Two of these patients needed no immunosuppressive agents and the others a small dose of steroids. Two of the patients who achieved a complete response died of cardiac failure on day 831 (no. 6) and of hepatic failure on day 163 (no. 15). Three patients who achieved rescue donor engraftment and who did not achieve a complete response died of multiorgan failure, including thrombotic microangiopathy on day 135 (no. 4), fungal pneumonia on day 23 (no. 8), and thrombotic thrombocytopenic purpura on day 72 (no. 14), as described previously. On the other hand, no long-term survivors were observed in patients who rejected rescue donor grafts. The causes of death for patients who rejected grafts were as described here. Performance status at rescue transplantation was important because no long-term survivors were observed among patients with $\leq 20\%$ Karnofsky performance score.

Discussion

In the present study, we clearly showed that severe, steroid-refractory GVHD was successfully treated by allogeneic SCT using grafts from a second allogeneic donor. The response rate was 80.0% (90.9% for patients achieving engraftment and 50.0% for patients rejecting graft).

Table 4. Adverse events (%)

Infection	bacteria	bacteremia	1 (5.9)
		others	2 (11.8)
	fungus		2 (11.8)*
	virus	cytomegalovirus	0 (0)
		herpes zoster	2 (11.8)
	pneumocystis jiroveci		0 (0)
Hypoxemia			1 (5.9)
Hemorrhagic cystitis			2 (11.8)
Thrombotic thrombocytopenic purpura			1 (5.9)
Thrombotic microangiopathy			2 (11.8)
Venoocclusive disease			1 (5.9)
Pancreatitis			1 (5.9)
Liver dysfunction†			10 (58.8)
Hypertension			4 (23.5)
Aseptic necrosis			2 (11.8)
Cataract			2 (11.8)
Hyperglycemia‡			8 (47.1)
Nephrotoxicity§			1 (5.9)
Insufficiency of adrenal gland			1 (5.9)

*One patient had aspergillus pneumonia before transplantation.
†An increase to > 3 times the normal upper limit of transaminase.
‡Insulin dose of >30U/day was needed to control blood sugar.
§Nephrotoxicity that needed hemodialysis.

Although patients who were enrolled in the present study had a severe GVHD after HLA-mismatched SCT, which is known to be very difficult to control [26], the overall survival at 6 months and 3 years was 44.6% and 37.2%, respectively. Furthermore, the GVHDs were not only steroid-resistant, but also heavily treated: these patients were refractory to a median of four lines of GVHD-specific treatments (12 patients received tumor necrosis factor blockade, 12 ATG, 11 mycophenolate mofetil, and 9 a pulse therapy of mPSL). The rationale for graft-versus-GVHD treatment is that allogeneically harmful lymphocytes responsible for GVHD are all eliminated by retransplantation using a second allogeneic graft [11]. In the realization of the graft-versus-GVHD concept, there are two major barriers to be overcome: organ toxicity by conditioning treatment and new development of GVHD by a second allogeneic graft.

Regarding the organ toxicities of conditioning treatment, patients with severe GVHD are in a poor state of health due to GVHD-related organ damage, and therefore cannot

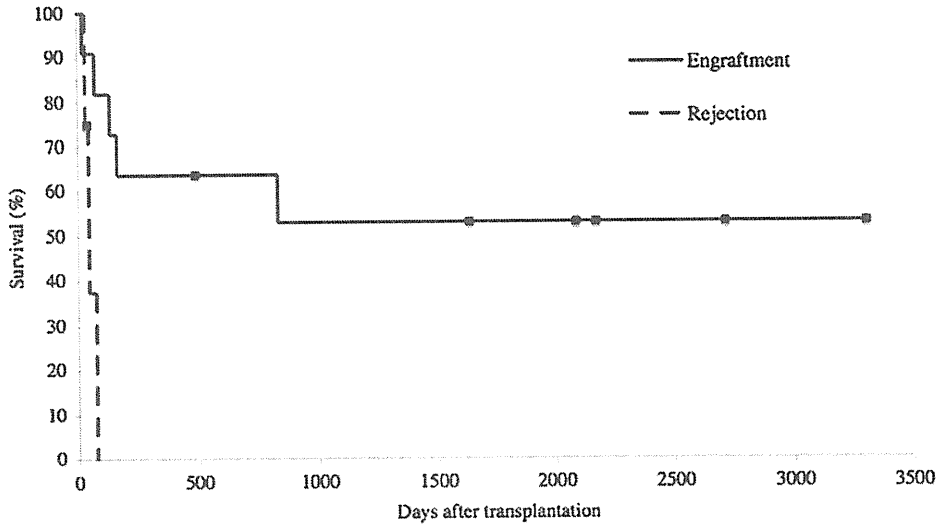

Figure 2. Overall survival of patients with refractory GVHD who did or did not achieve rescue donor engraftment. Patients achieving rescue donor engraftment showed a significantly improved survival rate compared with those rejecting grafts (p = 0.013). The survival rate of patients (n = 11) who achieved rescue donor engraftment was 63.6% (95% CI, 34.6–92.6%) at 6 months and 53.0% (95% CI, 22.0–84.0%) at 3 years, respectively.

Table 5. Relationship between first (GVHD-induced) and second (rescue) donors in graft-versus-GVHD treatment

No.	First donor	Second donor	Relationship of 2 donors (second to first donors)	HLA disparity in the direction of 1st to 2nd donors	GVH-target HLA antigens in 2nd donor*	Engraftment of rescue grafts
14	first son	second son	HLA-matched sibling	0	–	+
15	younger sister	youngest sister	HLA-matched sibling	0	–	+
17	brother	sister	HLA-matched sibling	0	–	+
1	mother	sister	Daughter and mother	2	–	+
9	sister	mother	Mother and daughter	3	–	+
10	brother	mother	Mother and son	3	–	+
16	UCB	son	Unrelated	2	–	+
5	first son	second son	HLA-haploidentical sibling	3	A24B48DR14	+
6	mother	brother	Son and mother	2	A26B59	+
7	mother	sister	Daughter and mother	2	B7DR1	+
11	brother	mother	Mother and son	2	B52DR15	+/–†
12	son	brother	Uncle and nephew	2	B54DR4	–
8	father	mother	Spouse	5	A11B35DR4	–
13	father	mother	Spouse	6	A33B44DR13	–

*HLA determinants of 2nd donors that could be major targets for GVH reaction in first (GVHD-induced) transplantation.
†The patient rejected the first rescue transplantation, but achieved engraftment of the second rescue transplantation.

usually tolerate an intensified conditioning treatment. However, as shown in our murine BMT model, recipients with severe GVHD were in a profoundly immunosuppressive state as a result of GVHD-related activation-induced cell death [27,28] and, therefore, with the help of unmanipulated (T-cell replete) grafts, could easily accept second allografts, even under minimal conditioning treatment, which was advantageous for recipients with serious organ damage.

Regarding GVHD induced by second allogeneic grafts, we demonstrated that second GVHD could be suppressed by conventional GVHD prophylaxis consisting of FK506 and a small dose of mPSL. This was fully expected because, in the unmanipulated HLA-haploidentical reduced-intensity SCT that we recently developed, using a conditioning treatment consisting of fludarabine + busulfan + ATG, and GVHD prophylaxis consisting of FK506 + mPSL 1 mg/kg, the actual incidence of GVHD was only 10% [13]. We consider that, in addition to in vivo T-cell purging by ATG, reduced-intensity conditioning, and a small dose of mPSL effectively suppressed inflammatory cytokine production in the transplantation period, which was shown to be closely involved in the pathophysiology of GVHD [29]. The molecular and cellular mechanisms of the high resistance to GVHD development have not been fully determined: however, in our murine studies, significantly reduced interferon-γ levels and a significantly increased percentage of $CD3^+CD4^+foxp3^+$ cells [30,31] were observed in day 7 spleens of second rescue BMT recipients compared with recipients of first BMT with severe GVHD. In addition, antigen-presenting cells (APCs) in the recipient spleen were found to have already been replaced by those of first-donor origin at the time of the second BMT. When APCs are replaced with first donor-derived cells from host cells, the first donor APCs need to cross-present host antigens to second donor T cells to induce GVHD; however, it was reported using major histocompatibility complex–matched, minor antigen-mismatched, murine BMT systems that this cross-presentation was insufficient to induce GVHD [32]. Although the present study includes mostly HLA-mismatched donor/recipient combinations, the limited ability of first donor-derived APCs to cross-present host antigens is considered to reduce the magnitude of the GVH reaction, at least compared with the first transplantation, in which host-type APCs directly present host antigens.

For successful graft-versus-GVHD treatment, engraftment of the rescue donor graft was mandatory in our murine model in which immunosuppressive agents were not used. In the present clinical study, even in patients who rejected the rescue graft, some GVHD symptoms improved within a week after the second transplantation because of conditioning treatment, including immunosuppressive agents and possibly because of the alloreactive response of second donor grafts to dampen first donor lymphocytes. Although these effects may have potential to completely control GVHD coupled with GVHD prophylaxis after second transplantation, as observed in patient no. 11, basically as long as the alloresponse from first donor-derived lymphocytes is maintained, GVHD symptoms continue or are aggravated, as shown in most patients who rejected second grafts. In fact, 8 of the 11 patients achieving rescue donor engraftment had a complete response, and 6 of the 8 patients survived without GVHD symptoms, with a median follow-up of 2128 days. These results strongly suggest, also in humans, that the engraftment of second donor grafts contributes to enduring control of GVHD and longer survival of patients with severe, refractory GVHD. Regarding HLA disparity between the first and second (rescue) donors, when rescue donors did not have HLA determinants that could be major targets for the GVH reaction in transplantation inducing GVHD, no rejection occurred (Table 5). As the extent

of HLA disparity in the direction of the first to second donor became greater, rejection tended to occur more frequently. When the 2 donors were HLA-matched siblings, 100% rescue donor chimerism was gradually achieved over 2 to 3 months.

Furthermore, as suggested in our murine model, the timing of rescue transplantation was another key factor for obtaining a positive graft-versus-GVHD effect. In particular, graft-versus-GVHD treatment in the late stage of GVHD is not effective. When organ damage due to GVHD proceeds fully, although the cell components involved in GVHD are all eliminated, recovery from severe organ damage is difficult, as shown by the lack of long-term survivors among recipients with a low PS score ≤20%. Thus, graft-versus-GVHD treatment may be started as one of the treatments for steroid-refractory GVHD before patients are heavily treated.

As patients did not show relapse of the original disease after successful graft-versus-GVHD treatment, and the majority of GVHD patients treated by autologous SCT had a relapse of the original disease [8–10], this strongly suggests GVL effects of second rescue allografts. In autologous transplant settings for GVHD, autografts can reintroduce malignant cells into the recipients in addition to the absence of GVL effects. Furthermore, in rescue transplantation of GVHD by allogeneic grafts, there is a possibility that malignant cells may have been eliminated by allogeneic NK cells as ATG was integrated into the conditioning treatment [33]. Thus, graft-versus-GVHD treatment has a unique feature in that it exerts GVL effects together with treating GVHD, which indicates the achievement of separating GVL from GVHD, a goal in allogeneic SCT.

We have proposed here the novel concept of graft-versus-GVHD and clinically showed that, using reduced-intensity conditioning and T-cell–replete grafts mostly from an HLA-mismatched donor, the second allogeneic SCT succeeded in eliminating harmful lymphocytes responsible for GVHD without the new development of GVHD. Thus, this graft-versus-GVHD strategy may be a promising treatment for refractory GVHD, although our results will have to be confirmed in a large-scale study.

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Conflict of interest disclosure

No financial interest/relationships with financial interest relating to the topic of this article have been declared.

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CASE REPORT

Strategy for bone marrow transplantation in eculizumab-treated paroxysmal nocturnal hemoglobinuria

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Abstract Although the recent introduction of eculizumab has had a significant impact on the management of paroxysmal nocturnal hemoglobinuria (PNH), bone marrow transplantation (BMT) remains the only therapeutic option for patients who develop severe aplasia in the clinical course of PNH. However, information regarding BMT for eculizumab-treated PNH patients is scarce, and two major points—the optimal duration of eculizumab therapy, and the optimal BMT conditioning regimen—remain unclear. Here, we describe the clinical course of a PNH patient who was successfully treated with unrelated reduced-intensity BMT. Eculizumab was discontinued 2 weeks prior to the initiation of the conditioning regimen, which consisted of fludarabine 180 mg/m^2, cyclophosphamide 100 mg/kg, rabbit anti-thymocyte globulin 2 mg/kg, and TBI 3 Gy. Complete donor chimerism was rapidly achieved in association with a rapid decrease in the proportion of PNH erythrocytes. The patient became transfusion-free immediately after BMT, and had no recurrence of hemolysis. The present case suggests that discontinuation of eculizumab before BMT and the use of a highly lymphoablative conditioning regimen may act as a successful treatment strategy in BMT for PNH. Further studies are warranted to evaluate the efficacy and safety of this treatment strategy.

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1 Introduction

Paroxysmal nocturnal hemoglobinuria (PNH) is a rare hematologic disorder resulting from clonal expansion of multipotent hematopoietic stem cells with a phosphatidylinositol glycan complementation class A (*PIG-A*) gene mutation [1, 2]. This mutation results in deficiency of all glycosyl phosphatidylinositol-anchored proteins (GPI-APs) expressed by hematopoietic cells. Deficiency of CD55 and CD59, which are GPI-anchored complement regulatory proteins, activates the complement cascade on the cell surface and thus causes hemolysis by the terminal complement complex (the membrane-attack complex). Apart from hemolytic anemia, the significant clinical manifestations of PNH include thrombosis and bone marrow failure. Recent studies have suggested the role of endothelial cell activation and dysfunction in the increased risk of thrombosis in PNH patients [3].

The recent introduction of eculizumab, a humanized monoclonal antibody directed against the terminal complement protein C5, has had a significant impact on the management of PNH [4–7]. Eculizumab stabilizes hemoglobin levels by inhibiting complement-mediated intravascular hemolysis. Moreover, eculizumab reduces the risk of thrombosis, partly by suppressing the activation of both the plasma hemostatic system and the vascular endothelium [3]. As a result, eculizumab treatment significantly improves the quality of life of PNH patients. Moreover, the significance of the introduction of eculizumab was confirmed by a recent study that found that the length of

survival of patients treated with eculizumab did not significantly differ from that of age- and sex-matched normal controls [8]. However, eculizumab treatment has an inherent drawback: the lack of any effects on the underlying stem-cell abnormality. As a result, eculizumab treatment needs to be continued indefinitely; more importantly, the problem of bone marrow failure that may manifest as a terminal feature of PNH [9] remains unresolved.

Therefore, bone marrow transplantation (BMT) remains the only treatment option for patients who develop severe aplasia in the clinical course of PNH, even in the eculizumab era. However, information regarding BMT in eculizumab-treated PNH patients remains scarce. Whereas several positive effects with eculizumab treatment, including the amelioration of inflammation and hemostatic activation are expected, several concerns also exist. Eculizumab treatment is known to be associated with a substantially increased risk of infection, particularly with encapsulated organisms [10]. Moreover, the effects of terminal complement inhibition with eculizumab on donor hematopoietic engraftment or those on graft-versus-host disease remain unknown. On the other hand, discontinuation of eculizumab in the presence of PNH erythrocytes may result in recurrence of hemolysis, which would complicate the clinical course after BMT.

Here, we report an eculizumab-treated PNH patient who successfully received unrelated reduced-intensity BMT.

2 Case report

A 51-year-old woman was diagnosed with classical PNH in 1991 based on the presence of hemolytic anemia and PNH blood cells. Treatments with androgen and prednisolone were not effective, and the patient became red blood cell transfusion dependent by April 2007. Neutrophil and platelet counts also gradually decreased, which required the intermittent use of granulocyte-colony stimulating factor (G-CSF). In May 2008, cyclosporine therapy was started, with an aim toward improving bone marrow failure. However, pancytopenia continued to progress, requiring more frequent red blood cell transfusions. Moreover, the patient developed deep vein thrombosis, which was associated with the elevation of D-dimer level (6.5 μg/mL). In June 2010, eculizumab treatment was initiated. Lower-extremity edema exacerbated transiently after the initiation of eculizumab treatment; however, this subsided with repeated administrations of eculizumab. Although the patient experienced improvements in hemolysis soon after treatment initiation, which decreased the need for red blood cell transfusions, she remained dependent on blood transfusions and G-CSF administration due to bone marrow failure. Based on these considerations, the necessity of BMT was strongly indicated. Because the patient lacked human leukocyte antigen (HLA)-matched related donors, an HLA genotypically 8/8 matched unrelated donor was located. ABO blood type was also matched. Informed consent was obtained after thorough explanation including the fact that BMT for eculizumab-treated PNH has not been established.

The patient's laboratory data on admission for BMT indicated pancytopenia (WBC 690/μL, Neut. 15%, Lym. 75.5%, Mon. 7.5%, Eos. 2%, RBC 162 × 10^4/μL, Hb 5.4 g/dl, Ht 15.9%, Ret 2.4%, and PLT 13 × 10^3/μL). Hemolysis was not evident (T-Bil 1.2 mg/dL, AST 15 U/L, and LDH 246 U/L), D-dimer levels were within the normal range, and renal function was not impaired (BUN 21 mg/dL and CRE 0.87 mg/dL). The proportion of PNH cells, as defined by CD59 negativity, was more than 90% in polymorphonuclear neutrophil (PMN), and approximately 20% in erythrocytes, likely reflecting dilution from red blood cell transfusions. While C3 and C4 levels were within the normal range, a CH50 assay (50% hemolytic unit of complement) showed an absence of detectable levels of complement activity, reflecting the effect of eculizumab. The results of bone marrow aspiration revealed hypocellular marrow with no chromosomal abnormalities.

Eculizumab was discontinued 2 weeks prior to the initiation of the BMT conditioning regimen, which consisted of fludarabine 30 mg/m^2 for 6 days (day −7 to −2), cyclophosphamide 50 mg/kg for 2 days (day −6 and −5), anti-thymocyte globulin (ATG, Thymoglobulin) 1 mg/kg for 2 days (day −2 and −1), and 3 Gy of total body irradiation (TBI) on day 0. The graft-versus-host disease prophylaxis consisted of continuous intravenous infusion of tacrolimus with a target level of 10–15 ng/mL and mycophenolate mofetil 30 mg/kg with oral administration. The patient was hospitalized in a single room ventilated with a high-efficiency particulate air filtration system. Broad-spectrum intravenous antibiotics were used prophylactically during neutropenia. G-CSF 300 μg/m^2 was administered from day 5 until neutrophil recovery.

The clinical course of the patient is shown in Fig. 1. BMT was performed in April 2011, using infused bone marrow containing 3.0 × 10^8/kg of nucleated cells. Neutrophil engraftment was achieved on day 10, and platelet count reached 20 × 10^3/μL without transfusions on day 24. No red blood cell transfusion was required after BMT. Although the LDH level mildly elevated from the initiation of the conditioning regimen until the day of BMT, it thereafter gradually decreased to normal levels.

The kinetics of donor chimerism in PMN fractions and T cell fractions, those of the proportions of PNH PMNs and PNH erythrocytes, and those of the CH50 levels are shown in Fig. 2. Complete donor chimerism was achieved in both

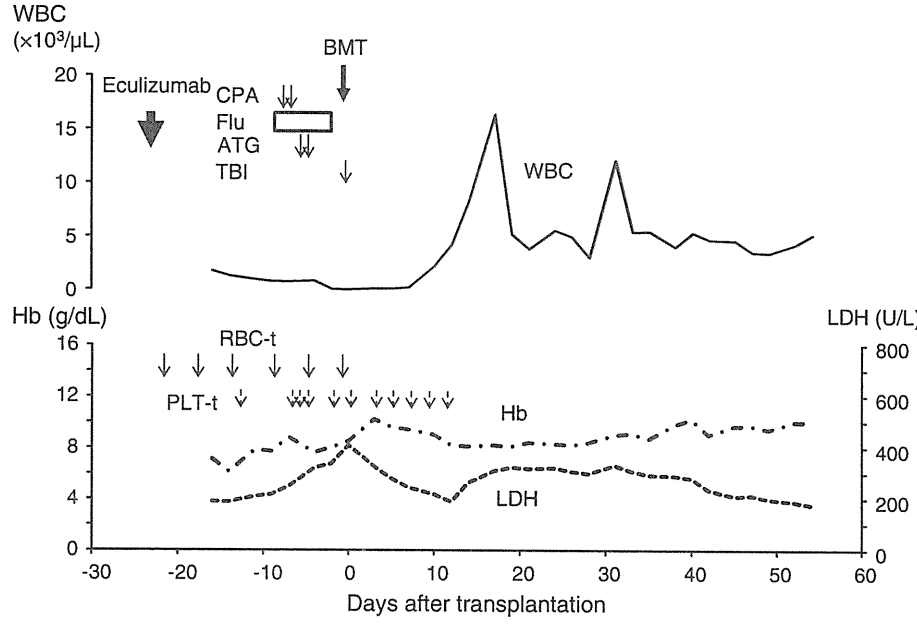

Fig. 1 Clinical course of the patient. *CPA* cyclophosphamide, *Flu* fludarabine, *ATG* anti-thymocyte globulin, *TBI* total body irradiation, *BMT* bone marrow transplantation, *WBC* white blood cells, *RBC-t* red blood cell transfusion, *PLT-t* platelet transfusion, *LDH* lactate dehydrogenase, *Hb* hemoglobin

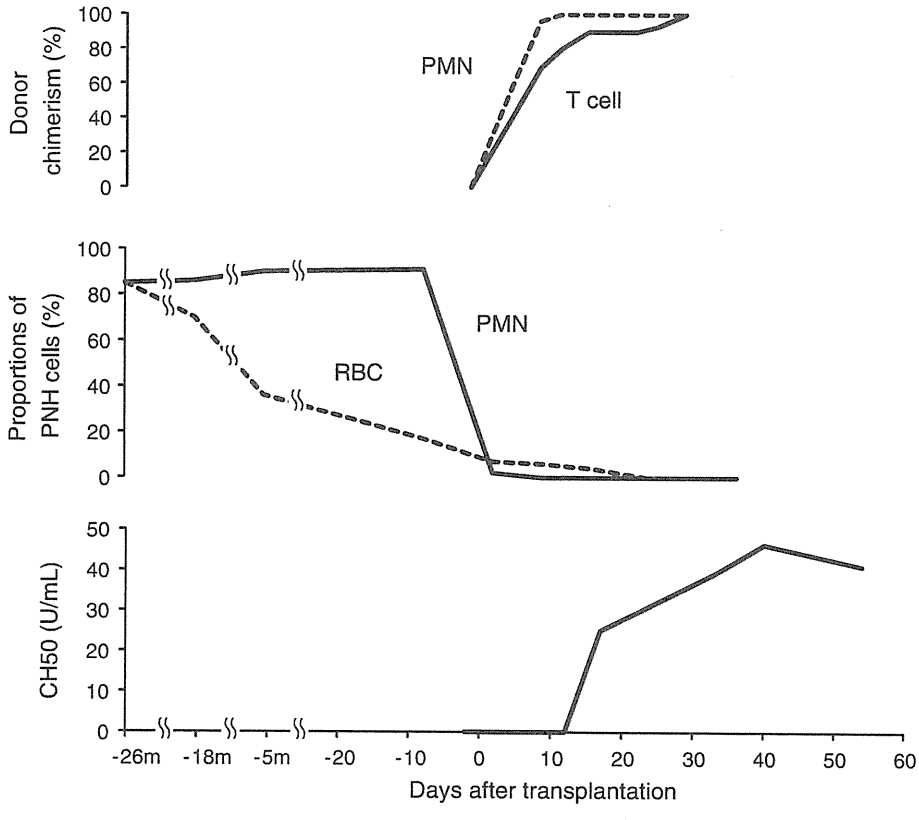

Fig. 2 Kinetics of donor chimerism, proportions of PNH blood cells, and complement activity. The proportions of PNH cells, as defined by CD59 negativity, were examined by flow cytometry in each fraction of blood cells. *PMN* polymorphonuclear neutrophils, *PNH* paroxysmal nocturnal hemoglobinuria, *RBC* red blood cells, *CH50* 50% hemolytic unit of complement, *m* month

fractions by 3 weeks after BMT. Simultaneously, the proportions of PNH PMNs and PNH erythrocytes both rapidly decreased after BMT, with PNH erythrocytes decreasing to an undetectable level by day 25. CH50 level remained undetectable until day 12 (5 weeks after the discontinuation of eculizumab), but recovered thereafter.

The clinical course of the patient after BMT was uneventful, with the exception of transient mild elevation

of liver transaminases (NCI-CTC grade 2) and cytomegalovirus antigenemia, which was preemptively treated with ganciclovir. Mycophenolate mofetil was tapered starting on day 17 and discontinued on day 30. Tacrolimus was switched to oral administration on day 35 and thereafter tapered by approximately 20% per month. The patient was discharged on day 57. At present (over 4 months after BMT), the patient is well without any signs of PNH recurrence.

3 Discussion

We have described here a patient who received unrelated reduced-intensity BMT for eculizumab-treated PNH. To our knowledge, this is the first report of successful BMT for eculizumab-treated PNH. There are 2 major points regarding BMT for eculizumab-treated PNH that remain unclear: the duration of eculizumab therapy and the optimal BMT conditioning regimen.

The decision regarding the time point at which eculizumab therapy may be discontinued poses a dilemma: whereas continuation of eculizumab after BMT may have unknown impacts on the clinical course (e.g., impaired engraftment) or may result in the development of severe infection, early discontinuation of eculizumab may result in recurrence of hemolysis. Patients receiving eculizumab have been found to have increased susceptibility to infection from encapsulated organisms, particularly meningococcus [10]. Due to this increased susceptibility, administration of the meningococcal vaccine and prophylactic use of antibiotics are recommended for all patients receiving eculizumab treatment at major institutes [8]. Moreover, patients who receive allogeneic stem-cell transplantation have also been found to have increased susceptibility to infection from encapsulated organisms, which are often fatal in this situation [11]. Thus, continuation of eculizumab after BMT may result in doubled susceptibility to infection from encapsulated organisms.

On the other hand, early discontinuation may result in the recurrence of hemolysis. Although no cases of severe hemolytic crisis after discontinuation of eculizumab have been reported, previous studies have shown that decreased levels of eculizumab may result in the recurrence of hemolysis [12]. In a one-compartmental model, the half-life of eculizumab after intravenous infusion was 272 h [13]. The approved dose of eculizumab is 900 mg every 2 weeks, which has been demonstrated to be capable of maintaining the required serum concentration of eculizumab (>35 μg/mL) in a majority of the patients.

To prevent the recurrence of hemolysis, we based the treatment of our patient on our hypothesis that the recurrence of hemolysis is likely to occur in the situation where complement activity recovers after the discontinuation of eculizumab in the presence of recipient-derived PNH erythrocytes due to graft failure or mixed chimerism. Our strategy thus was based on the following points: (1) discontinuation of eculizumab therapy 2 weeks before initiation of the conditioning regimen, and (2) use of a highly lymphoablative conditioning regimen for achieving rapid and complete donor hematopoiesis.

Although our patient experienced a mild elevation in the LDH level approximately 2 weeks after discontinuation of eculizumab, this elevation may be attributable to the influence of the conditioning regimen. Moreover, the fact that complement activity as measured by the in vitro CH50 assay remained undetectable until day 12 (5 weeks after eculizumab discontinuation) suggests the continued effect of previously administered eculizumab on complement activity.

The optimal conditioning regimen for BMT in PNH patients remains unknown [14]. The largest retrospective study of BMT for PNH has included 26 patients, where a majority of donors were HLA-matched siblings [15]. Among the 11 patients who received reduced-intensity conditioning regimen, only 1 patient had graft failure. However, interpretation of the data is difficult due to the heterogeneity of the conditioning regimen used in the study as well as the high treatment-related mortality (63% at 1 year).

Reduced-intensity conditioning regimen has a great advantage over myeloablative conditioning regimen with lower regimen-related toxicity particularly for elderly patients. Moreover, previous studies have demonstrated that PNH cells can be immunologically eradicated following hematopoietic stem-cell transplantation with nonmyeloablative or reduced-intensity conditioning [15, 16]. Thus, we determined to use reduced-intensity conditioning in the patient. However, previous studies have shown the high incidence of graft failure after nonmyeloablative or reduced-intensity BMT in patients with non-malignant disease. A recent study conducted by the European Group for Blood and Marrow Transplantation showed that stem-cell transplantation (unrelated BMT in majority) using a conditioning regimen consisting of fludarabine 120 mg/m^2, cyclophosphamide 1200 mg/m^2, ATG (thymoglobulin) 7.5 mg/kg, and TBI 2 Gy resulted in graft failure in 17% of patients [17].

Based on these previous findings, we used a stronger lymphoablative regimen, consisting of fludarabine 180 mg/m^2, cyclophosphamide 100 mg/kg, ATG (thymoglobulin) 2 mg/kg, and TBI 3 Gy. Using this regimen, our patient experienced only minor regimen-related toxicity. Complete donor chimerism was rapidly achieved in association with a rapid decrease in the proportion of PNH erythrocytes. Consequently, the patient became transfusion-free immediately after BMT. Collectively, the conditioning regimen used

here provided a smooth recipient-to-donor transition of erythropoiesis, and thus prevented the recurrence of hemolysis after the recovery of complement activity.

In conclusion, the present case suggests the usefulness of a treatment strategy for BMT in eculizumab-treated PNH that involves the discontinuation of eculizumab prior to the initiation of conditioning regimen and the use of a highly lymphoablative conditioning regimen. Further studies are warranted for evaluating the efficacy and safety of this treatment strategy. Recent studies showing the effect of eculizumab in ameliorating inflammation and hemostatic activation suggest the positive impact of eculizumab treatment after BMT in preventing complications, including transplant-associated thrombotic microangiopathy. Studies concerning the post-transplantation use of eculizumab may also be warranted with careful monitoring for infectious diseases.

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

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Case of the Month

Dasatinib-induced rapid regression and complete molecular remission of multiple subcutaneous tumours presenting as relapsed chronic myeloid leukaemia after cord blood transplantation

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A 47-year-old male in chronic myeloid leukaemia (CML) blast crisis was transferred to our hospital for cord blood transplantation (CBT) in July 2008. His disease began in the chronic phase in July 2002, but his treatment was very unusual because he discontinued medicating himself many times. As an apparent result, an additional chromosomal abnormality of +8, +idem, and +der (12) appeared from March 2007, and the disease entered the accelerated phase. Although dasatinib was administered at 100 mg/day from July 2008, there was no response and multiple subcutaneous nodules appeared as extramedullary CML invasions. There were no detectable ABL kinase mutations during the course of his disease. We began idarubicin (12 mg/m^2/day for 3 days) and cytarabine (100 mg/m^2/day for 1 week) chemotherapy, which led to a partial reduction in the subcutaneous nodules. This treatment was followed by a double CBT in October 2008. Pre-transplant conditioning included cytarabine (3 g/m^2/day for 2 days), cyclophosphamide (60 mg/kg/day for 2 days), granulocyte-colony stimulating factor (5 μg/kg for 2 days), total body irradiation (3 Gy/day for 4 days) and prophylaxis for acute graft-versus-host disease with continuous infusion of cyclosporine and short-term methotrexate, as previously reported [1]. The graft sources were human leukocyte antigen (HLA)-matched and one-HLA-mismatched cord blood containing, respectively, 2.9×10^4 and 4.6×10^4 cells/kg CD34-positive cell doses. Neutrophils engraftments >500 and platelets >20,000/μL were noted on day 30 and 51, respectively. Chimerism analysis on day 29 showed 100% donor-type HLA-matched cord blood. The post-transplant course was uneventful without signs of acute and chronic graft-versus-host disease. After CBT, all extramedullary lesions disappeared and bone marrow examination on day 43 showed complete haematological and cytogenetic responses. However, *BCR-ABL* real-time quantitative-polymerase chain reaction (RQ-PCR) analysis (frequently employed to monitor molecular response in Japan [2]) of bone marrow detected 130 copies/μg mRNA. On day 50, the subcutaneous nodules recurred and exacerbation of extramedullary lesions were diagnosed based on pathological examination of the biopsy specimens. Cyclosporine was discontinued and we started a combination of interferon-alpha and local radiotherapy. Radiotherapy partially reduced the local nodules; however, new nodules appeared continuously (Fig. 1a). Because the disease progressed with an increasing number of new extramedullary lesions, dasatinib was restarted at 140 mg/day in February 2009. All extramedullary lesions diminished rapidly by 10 days (Fig. 1b), and completely disappeared after one month. Moreover, *BCR-ABL* transcripts in bone marrow could not be detected concurrently by RQ-PCR analysis, indicating complete molecular remission for the first time that persisted for more than 3 months until the patient died from an accident.

CML relapse following stem cell transplantation (SCT) is frequently treated by dose reduction or discontinuing immunosuppressants combined with donor lymphocyte infusion; however, the results are generally poor, especially in extramedullary relapse cases [3]. Although a second SCT is the most proactive approach for inducing a graft-versus-leukaemia effect on extramedullary lesions, the response rate is too low for high transplant-related mortality [4]. Some reports have suggested that imatinib was effective after SCT; however, imatinib is presently regarded as first-line CML therapy, and CML cells have often previously acquired resistance to imatinib, which is consistent with our case [5,6]. Thus, new strategies with second-generation tyrosine kinase inhibitors such as dasatinib are expected to be effective against post-transplant relapses.

Dasatinib therapy has been shown to effectively treat extramedullary lesions [7]. In the case described here, dasatinib

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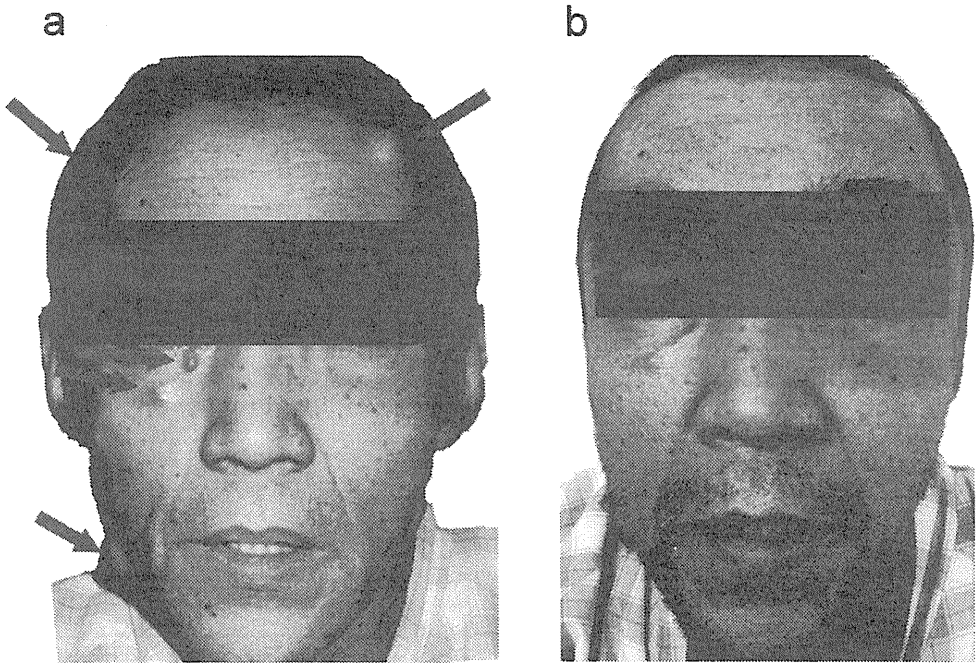

Fig. 1. Photographs were taken before (a) and 10 days after (b) dasatinib therapy, respectively.

was much more effective than anticipated in treating this post-transplant extramedullary relapse. Unfortunately, the patient's untimely death due an unrelated cause obviously prevented longer term observation. It is important to note, however, that dasatinib enabled the patient to achieve complete molecular remission for the first time. Furthermore, there were no side effects for at least three months. A recent report indicated that dasatinib might suppress regulatory T cell function, while another pointed out that dasatinib might enhance graft-versus-leukaemia effect [8]. It is impossible, based on our case alone, to conclude that dasatinib increased the graft-versus-leukaemia effect. It is certain, however, that our patient's successful response to dasatinib indicates that it could effectively treat extramedullary CML relapse after SCT.

Conflict of interest

The authors declare no conflict of interest.

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c-Maf plays a crucial role for the definitive erythropoiesis that accompanies erythroblastic island formation in the fetal liver

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c-Maf is one of the large Maf (musculoaponeurotic fibrosarcoma) transcription factors that belong to the activated protein-1 super family of basic leucine zipper proteins. Despite its overexpression in hematologic malignancies, the physiologic roles c-Maf plays in normal hematopoiesis have been largely unexplored. On a C57BL/6J background, *c-Maf*$^{-/-}$ embryos succumbed from severe erythropenia between embryonic day (E) 15 and E18. Flow cytometric analysis of fetal liver cells showed that the mature erythroid compartments were significantly reduced in *c-Maf*$^{-/-}$ embryos compared with *c-Maf*$^{+/+}$ littermates. Interestingly, the CFU assay indicated there was no significant difference between *c-Maf*$^{+/+}$ and *c-Maf*$^{-/-}$ fetal liver cells in erythroid colony counts. This result indicated that impaired definitive erythropoiesis in *c-Maf*$^{-/-}$ embryos is because of a non–cell-autonomous effect, suggesting a defective erythropoietic microenvironment in the fetal liver. As expected, the number of erythroblasts surrounding the macrophages in erythroblastic islands was significantly reduced in *c-Maf*$^{-/-}$ embryos. Moreover, decreased expression of VCAM-1 was observed in *c-Maf*$^{-/-}$ fetal liver macrophages. In conclusion, these results strongly suggest that c-Maf is crucial for definitive erythropoiesis in fetal liver, playing an important role in macrophages that constitute erythroblastic islands. (*Blood*. 2011;118(5): 1374-1385)

Introduction

In mouse embryogenesis, red blood cells are produced in the yolk sac in a process called primitive erythropoiesis. Erythropoiesis then takes place in the fetal liver around embryonic day (E) 10 onward and in BM and spleen after birth. This process, which is characterized by enucleated red blood cells, is called definitive erythropoiesis.[1] During terminal erythroid differentiation, erythroblasts are associated with a central macrophage, which forms a specialized microenvironment, the so-called erythroblastic islands. In the erythroblastic islands, a central macrophage provides favorable proliferative and survival signals to the surrounding erythroblasts, and it eventually engulfs the extruded nuclei of maturing erythrocytes.[2-5] Inhibition of the interaction between macrophage and erythroblasts usually leads to embryonic anemia accompanied by accelerated apoptosis of erythroid cells. Targeted disruption of the gene *palld*, which encodes actin cytoskeleton associated protein (palladin) prevented effective erythroblast-macrophage interactions because of differentiation defects in the macrophage. Therefore, mouse embryos homozygous for the mutant gene experience severe anemia and succumb in the embryonic period.[6] Meanwhile, it has been reported that a series of adhesion molecules are also involved in the process of forming erythroblastic islands. Erythroblast macrophage protein (EMP) is a transmembrane protein expressed in both erythroblasts and macrophages, and it mediates the erythroblast-macrophage interaction. Indeed, the targeted deletion of EMP causes lethal anemia in mouse embryos because of the suppressed formation of erythroblastic islands.[7] Furthermore, a previous report showed that the interaction between very late Ag-4 ($\alpha_4\beta_1$ integrin) on erythroblasts and VCAM-1 on macrophages plays a key role in maintaining the islands.[8] For example, administration of Abs raised against either $\alpha_4\beta_1$ integrin or VCAM-1 caused disruption of the island structure.[8] However, the molecular mechanism, in terms of transcriptional regulation of island-affiliated genes in macrophages, remains largely unknown.

The large Maf transcription factor c-Maf is a cellular homolog of v-maf, which was isolated from a chicken musculoaponeurotic fibrosarcoma induced by avian retrovirus AS42 infection.[9] The large Maf transcription factors contain an acidic domain that promotes transcriptional regulation and a basic region/leucine zipper domain that mediates dimerization, as well as DNA binding to either Maf recognition elements (MAREs) or the 5′ AT-rich half-MARE.[10-12] Each large Maf protein has been shown to play a distinct role in cellular proliferation and differentiation in both pathologic and physiologic situations.[9,13-19] In B-lymphoid and T-lymphoid lineages, aberrant expression of c-Maf works as an oncogene, as shown in patients with multiple myeloma and angioimmunoblastic T-cell lymphoma and in a transgenic mouse model.[20-23] Physiologic c-Maf expression is indispensable for the proper regulation of IL-4 and IL-21 gene expression in T-helper cells.[24,25] In macrophages, c-Maf has been reported to regulate IL-10 expression, which is essential for differentiation of regulatory T cells.[26] In addition, combined deficiency of MafB and c-Maf

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enables long-term expansion of differentiated, mature macrophages.[27] We recently reported that c-Maf is abundantly expressed in fetal liver macrophages and that it regulates expression of F4/80, which mediates immune tolerance.[28] However, the physiologic consequences of c-Maf deletion on terminal erythroid differentiation in erythroblastic islands have been largely unexplored.

In the present study, we demonstrate that *c-Maf*–deficient mice exhibit embryonic anemia, which is associated with a failure to retain erythroblastic islands. Moreover, we found significantly reduced expression of VCAM-1 in *c-Maf*–deficient macrophages, which presumably accounts for the deficiency in island maintenance and subsequent embryonic anemia. Thus, these results suggest that c-Maf is indispensable for definitive erythropoiesis in fetal liver, because it activates VCAM-1 expression in macrophages, and this causes the maintenance of erythroblastic islands.

Methods

Mice

c-Maf–deficient mice were originally generated on a 129/Sv background[14] and have been backcrossed onto a C57BL/6J background for > 7 generations. In staging the embryos, gestational day 0.5 (E0.5) was defined as noon of the day a vaginal plug was found after overnight mating. Mice were maintained in specific pathogen-free conditions in a Laboratory Animal Resource Center. All experiments were performed according to the Guide for the Care and Use of Laboratory Animals at the University of Tsukuba.

Hematologic analysis of fetal liver cells, embryonic blood, and adult blood

Single-cell suspensions prepared from fetal livers were washed and resuspended in 1 mL of 2% FBS/PBS. The cell number was counted with the use of a hemocytometer. Collection of embryonic peripheral blood was performed as described previously.[29] Peripheral blood samples from adult mice were obtained from retro-orbital venous plexus with the use of heparin-coated microtubes. Blood counts were determined with an automated hemocytometer (Nihon Kohden). Blood smears were prepared with the wedge technique and were stained with May-Grünwald-Giemsa and then photographed with a Keyence Biorevo BZ-9000 microscope. Images were processed with Photoshop software (Adobe).

Flow cytometry

Freshly isolated fetal liver cells were immunostained at 4°C in PBS/2% FBS in the presence of 5% mouse serum to block Fc receptors. Cells were incubated with FITC-conjugated anti–TER-119 and allophycocyanin (APC)–conjugated anti-CD44 Abs, followed by a 5-minute incubation with phycoerythrin PE-conjugated Annexin V (BioVision) at room temperature. To quantify the presence of VCAM-1 and integrin αV on macrophages, FITC-conjugated anti–Mac-1, Alexa Fluor 647–conjugated anti–VCAM-1 and PE-conjugated anti-integrin αV Abs were used for analyses. All Abs except PE-conjugated annexin V were from eBioscience. Flow cytometry was performed on a Becton Dickinson FACS LSR with CellQuest software. The isolation of erythroblasts at different stages of maturation was performed as described previously.[30] Data were analyzed with FlowJo (Tree Star Inc) analysis software.

Cell cycle analysis

Cell cycle analysis was performed with propidium iodide (PI). Further details are provided in supplemental Methods (available on the *Blood* website; see the Supplemental Materials link at the top of the online article).

Histology and TUNEL assay

Whole E13.5 embryos were fixed in 10% formalin neutral buffer solution (Wako). Paraffin-embedded tissue was sectioned, mounted, and stained with H&E. TUNEL assays were performed with an in situ Apoptosis Detection Kit according to the manufacturer's protocol (TaKaRa). Images were captured by a digital camera system with the use of a Leica DM RXA2 microscope (Leica Microsystems).

CFU assay

CFU assays were performed in MethoCult GF M3434 (StemCell Technologies) for erythroid burst-forming unit (BFU-E) and granulocyte, erythroid, megakaryocyte, and macrophage CFU (CFU-GEMM), and M3334 for erythroid CFU (CFU-E), following the manufacturer's instructions. CFU-E was scored 2 days after plating. BFU-E and CFU-GEMM were scored 8 days after plating.

Preparation of "native" erythroblastic islands and "reconstituted" erythroblastic islands

Native erythroblastic islands were isolated from fetal livers with the use of a previously described protocol.[31] Full details are provided in supplemental Methods.

Microarray analysis of fetal liver macrophages

Microarray analysis was performed as described previously.[32] Further details are provided in supplemental Methods. The data have been deposited in the Gene Expression Omnibus database under the accession number GSE23305.

Real-time RT-PCR analysis of fetal liver macrophages

Macrophages were prepared from fetal livers of *c-Maf*$^{+/+}$ or *c-Maf*$^{-/-}$ mice. Separation of fetal liver macrophages with the use of Mac-1^{+} magnetic beads and the MACS system was performed as described previously.[28] RNA extraction and quantitative RT-PCR were performed as described previously.[28] Primers used for PCR and the PCR conditions are available in supplemental Table 1.

Luciferase reporter assay

The *VCAM-1* 0.7 kilobase (kb) promoter (VCAM-1 Luc) or VCAM-1 mut Luc ligated to a luciferase reporter[33] was transiently cotransfected with c-Maf expression plasmids in macrophage cell line J774 with the use of FuGENE 6 (Roche). Twenty-four hours after the transfection, cells were collected, and reporter gene assays were performed with the Dual Luciferase Kit (Promega). Transfection efficiency was normalized to the expression of *Renilla* luciferase.

Reconstitution of hematopoietic system with fetal liver cells

The donor cells for hematopoietic reconstitution were prepared from E14.5 fetal livers of *c-Maf*$^{+/+}$ or *c-Maf*$^{-/-}$ (C57BL/6J-Ly5.1) mice. Fetal liver cells (2×10^6 cells) were injected into the tail vein of 8- to 10-week-old C57BL/6J-Ly5.2 mice that were previously exposed to x-rays at a 10-Gy dose.

Phenylhydrazine stress test

Mice were injected subcutaneously on days 0, 1, and 3 with 50 mg/kg phenylhydrazine (PHZ) hydrochloride solution in PBS as previously described.[34] Blood was obtained from the retro-orbital plexus on days 0, 3, 6, 8, and 10.

Statistical analysis

Data were represented as mean ± SEM. Statistical significance between any 2 groups was determined by the 2-tailed Student *t* test, in which *P* values < .05 were considered significant.

Table 1. Genotypic analysis of neonates and embryos from *c-Maf*$^{+/-}$ intercross on a C57BL/6J background

Embryonic stage	No. of each *c-Maf* genotype (no. of dead embryos)			Total no. of embryos
	c-Maf$^{+/+}$	*c-Maf*$^{+/-}$	*c-Maf*$^{-/-}$	
E12.5	13	30	15	58
E13.5	57	88	46 (5)	191
E14.5	75	135	55 (12)	265
E15.5	35	58	16 (3)	109
E16.5	11	15	4 (3)	30
E18.5	14	18	4 (3)	36
Neonate	13	29	0	42

Embryos were isolated at the indicated time points of gestation and within 7 days of birth (postnatal), and analyzed for viability. Genotypes of embryos were determined by PCR.

Results

Lethal erythropoietic deficiency in *c-Maf*$^{-/-}$ embryos

The *c-Maf*$^{-/-}$ mice, as originally generated, exhibited perinatal mortality within a few hours after birth on a 129/Sv background.[14] However, unexpectedly, on a C57BL/6J genetic background, c-Maf deficiency resulted in embryonic lethality from E15.5 onward, and almost all *c-Maf*$^{-/-}$ embryos died before E18.5 (Table 1). The fetal livers from *c-Maf*$^{-/-}$ embryos at E13.5 appeared pale compared with those from healthy *c-Maf*$^{+/+}$ control embryos (Figure 1A). As expected, the hematocrits of peripheral blood in the *c-Maf*$^{-/-}$ embryos (E13.5~E15.5) were markedly reduced (E13.5, 13.4% ± 1.8%; E14.5, 14.5% ± 1.0%; E15.5, 16.9% ± 1.0%), compared with the *c-Maf*$^{+/+}$ controls (E13.5, 18.7% ± 0.6%; E14.5, 23.1% ± 1.7%; E15.5, 29.6% ± 1.8%; Figure 1B). May-Grünwald-Giemsa staining of peripheral blood smears showed a significant reduction of enucleated red blood cells in *c-Maf*$^{-/-}$ embryos (E13.5, 6.68% ± 1.0%; E14.5, 17.2% ± 1.9%; E15.5, 29.8% ± 5.6%; Figure 1C-D), whereas *c-Maf*$^{+/+}$ control embryos retained a normal population of enucleated red blood cells (E13.5, 25.5% ± 4.7%; E14.5, 54.1% ± 1.1%; E15.5, 86.9% ± 2.8%; Figure 1C-D). Considering that the enucleated red blood cells are mainly derived from definitive erythropoiesis, these data suggest that c-Maf–deficient embryos suffer from impaired definitive erythropoiesis on the C57BL/6J genetic background. A previous study showed that placental insufficiency causes embryonic lethality.[35] To assess the effect of c-Maf on the placenta, immunofluorescence staining of placenta was performed with an anti–c-Maf Ab (supplemental Figure 1B). The expression of c-Maf was not detected in placenta. Compared with the head tissue as a positive control, c-Maf mRNA expression was 5-fold less in the placenta (supplemental Figure 1C). In addition, no obvious abnormalities in the *c-Maf*$^{-/-}$ placenta were observed after H&E staining (supplemental Figure 1D).

Increased apoptotic cell death in *c-Maf*$^{-/-}$ fetal liver

We next examined the cellular viability, as well as the cell cycle status, of *c-Maf*$^{-/-}$ fetal liver cells, in the hope of elucidating the molecular and cellular basis of their erythropoietic deficiency. The fetal liver size in *c-Maf*$^{-/-}$ embryos was smaller; thus, consistently fewer fetal liver cells were harvested from *c-Maf*$^{-/-}$ embryos between E13.5 and E15.5 (E13.5, 3.37 ± 0.5 × 10^6 cells; E14.5, 7.61 ± 1.0 × 10^6 cells; E15.5, 6.54 ± 2.7 × 10^6 cells) than from age-matched *c-Maf*$^{+/+}$ control embryos (E13.5, 6.89 ± 0.4 × 10^6 cells; E14.5, 16.6 ± 2.3 × 10^6 cells; E15.5, 32.5 ± 2.4 × 10^6 cells; Figure 2A-B). Of note, H&E staining of E13.5 *c-Maf*$^{-/-}$ fetal liver sections showed an increased number of pyknotic nuclei, which are indicative of apoptosis, compared with the *c-Maf*$^{+/-}$ control embryo (Figure 2C). In addition, TUNEL-positive apoptotic cells were remarkably increased in E13.5 *c-Maf*$^{-/-}$ fetal liver sections (Figure 2C). Moreover, flow cytometric analysis of PI-stained fetal liver cells showed an increased abundance of a sub-G_0/G_1 population early apoptotic cell fraction in *c-Maf*$^{-/-}$ fetal liver (8.77% ± 2.5%) compared with *c-Maf*$^{+/+}$

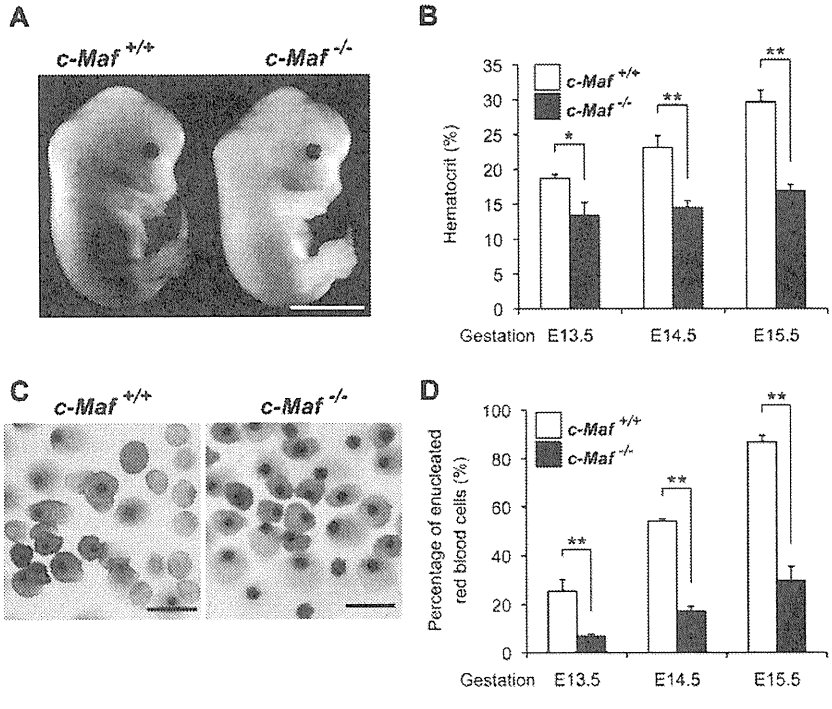

Figure 1. *c-Maf*$^{-/-}$ embryos are anemic. (A) Gross appearance of E13.5 embryos. The *c-Maf*$^{-/-}$ embryo is paler and smaller than its *c-Maf*$^{+/+}$ littermate. Scale bar represents 5 mm. The picture was taken with a NIKON coolpix 5200 digital camera in macro mode and processed with the Adobe Photoshop CS4 software. (B) Hematocrit values for *c-Maf*$^{+/+}$ (n = 6) and *c-Maf*$^{-/-}$ (n = 6) embryos at E13.5, *c-Maf*$^{+/+}$ (n = 8) and *c-Maf*$^{-/-}$ (n = 7) embryos at E14.5, and *c-Maf*$^{+/+}$ (n = 12) and *c-Maf*$^{-/-}$ (n = 9) embryos at E15.5. Data are presented as mean ± SEM. The mean hematocrit values for *c-Maf*$^{-/-}$ embryos were significantly lower than values for *c-Maf*$^{+/+}$ at E13.5, E14.5, and E15.5. (C) Blood smears from E13.5 embryos stained with May-Grünwald-Giemsa stain. The blood smear from a *c-Maf*$^{-/-}$ embryo contains far fewer enucleated red blood cells. Images were acquired by a Biorevo BZ microscope (Plan Apo 20×0.75 DIC N2) at room temperature and processed with the Adobe Photoshop CS4 software. Scale bars represent 20 μm. (D) The percentage of enucleated red blood cells in peripheral blood for *c-Maf*$^{+/+}$ (n = 5) and *c-Maf*$^{-/-}$ (n = 7) embryos at E13.5, *c-Maf*$^{+/+}$ (n = 4) and *c-Maf*$^{-/-}$ (n = 4) embryos at E14.5, and *c-Maf*$^{+/+}$ (n = 8) and *c-Maf*$^{-/-}$ (n = 8) embryos at E15.5. A minimum of 200 cells was counted for each sample. Data are presented as mean ± SEM. The percentage of enucleated red blood cells is significantly reduced in *c-Maf*$^{-/-}$ embryos than in *c-Maf*$^{+/+}$ embryos at E13.5, E14.5, and E15.5. *$P < .05$ and **$P < .01$.

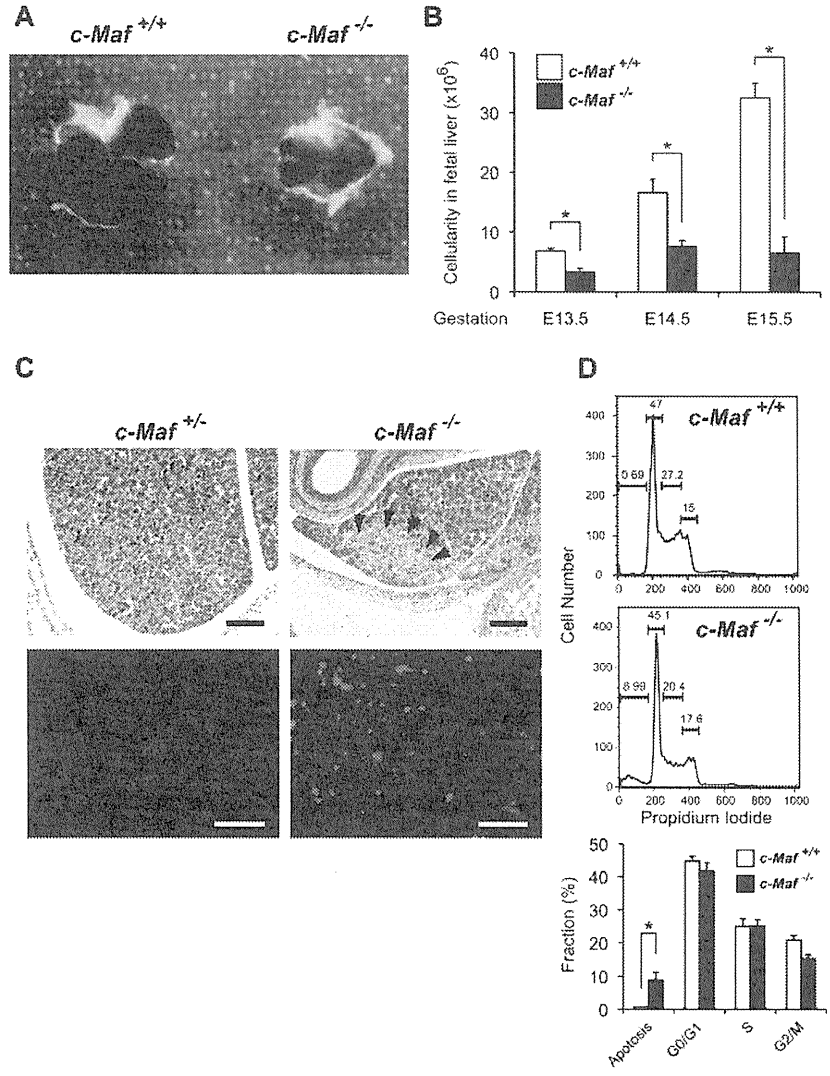

Figure 2. Increased number of apoptotic cells is observed in *c-Maf*$^{-/-}$ fetal liver. (A) Gross appearance of E13.5 fetal liver. The *c-Maf*$^{-/-}$ fetal liver is smaller than a *c-Maf*$^{+/+}$ fetal liver. Scale bar represents 100 μm. The picture was taken with a NIKON coolpix 5200 digital camera in macro mode and processed with the Adobe Photoshop CS4 software.(B) The mean total number of fetal liver cells in *c-Maf*$^{+/+}$ (n = 14) and *c-Maf*$^{-/-}$ (n = 10) embryos at E13.5, *c-Maf*$^{+/+}$ (n = 6) and *c-Maf*$^{-/-}$ (n = 4) embryos at E14.5, and *c-Maf*$^{+/+}$ (n = 8) and *c-Maf*$^{-/-}$ (n = 8) embryos at E15.5. The mean number of fetal liver cells is significantly reduced in *c-Maf*$^{-/-}$ embryos. Data are presented as mean ± SEM. (C) H&D staining of *c-Maf*$^{+/-}$ and *c-Maf*$^{-/-}$ fetal liver sections (top). Arrowheads indicate pyknotic nuclei, which indicate apoptotic cells. TUNEL assays showed increased apoptosis in the *c-Maf*$^{-/-}$ fetal liver (bottom panel). Images were acquired by a Leica DM RXA2 microscope (Lecia HC PL Fluotar 20×/0.50 PH2) at room temperature and processed with the Adobe Photoshop CS4 software. Scale bars in the top panel represent 200 μm. Scale bars in the bottom panel represent 50 μm. (D) The fraction of cells in different phases of the cell cycle was measured by PI staining followed by flow cytometric analyses. The percentage of cells in sub-G_0/G_1, G_1, S phase, and G_2/M are indicated. The sub-G_0/G_1 phase represents the apoptotic population. The apoptotic population was increased in *c-Maf*$^{-/-}$ fetal liver.

control cells (0.67% ± 0.01%; Figure 2D). However, the cellular population of each phase of the cell cycle, that is, G_0/G_1, S, and G_2/M, was not significantly affected (Figure 2D). Overall, these observations suggest that the fetal liver hematopoietic cells from *c-Maf*$^{-/-}$ embryos are prone to undergo apoptotic cell death.

Impaired fetal liver erythropoiesis because of a non–cell-autonomous effect of c-Maf deficiency

Given the significant disturbance of erythropoiesis, we next attempted to delineate the maturation status of erythroid lineage cells in *c-Maf*$^{-/-}$ fetal liver. To this end, we examined fetal liver erythropoiesis by flow cytometry with the use of the erythroid markers CD44 and TER-119, which distinguish various stages of erythroid-cell differentiation (Figure 3A). By modifying a method reported by Chen et al[30] to isolate erythroblasts at different maturation stages from adult BM, we isolated erythroblasts from fetal liver cells and analyzed their structure (Figure 3B). Decreased numbers of mature erythroid compartments (regions II, III, IV, and V in Figure 3C) were observed in *c-Maf*$^{-/-}$ fetal liver than in *c-Maf*$^{+/+}$ fetal liver. These results showed a reduction of basophilic erythroblasts, polychromatic erythroblasts, orthochromatic erythroblasts, reticulocytes, and mature red cells in *c-Maf*$^{-/-}$ fetal liver. Previously, Zhang et al[36] reported a method to study erythropoiesis with the use of an anti-CD71 Ab and an anti–TER-119 Ab. Similar results were obtained with their method. Decreased numbers of mature erythroid compartments (region 3 to region 5 in supplemental Figure 2A-B) were observed in *c-Maf*$^{-/-}$ fetal liver in combination with anti-CD71 Ab and anti–TER-119 Ab. These observations prompted us to quantify the apoptotic cell population at each stage of erythroid cells by annexin V staining.

In good agreement with the preferential decrease of the mature erythroid compartments (regions II-V), we observed a highly increased number of annexin V-positive cells in the most mature erythroid compartment (region V) of *c-Maf*$^{-/-}$ fetal liver compared with the *c-Maf*$^{+/+}$ fetal liver. In contrast, the premature erythroid compartments (regions I-IV) of *c-Maf*$^{-/-}$ fetal liver cells exhibited a comparable number of annexin V-positive cells with the *c-Maf*$^{+/+}$ control (Figure 3D; supplemental Figure 2C). To examine the state of globin regulation, the Mac-1$^-$ cells from E13.5 fetal liver were sorted and analyzed for mRNA of Hbb (hemoglobin beta chain) genes by real-time RT-PCR analysis. Expression profiles showing switching of Hbb genes indicated that the definitive Hbb gene Hbb-b1 (hemoglobin, beta adult major chain) was significantly down-regulated, whereas the primitive globin genes Hbb-bH1

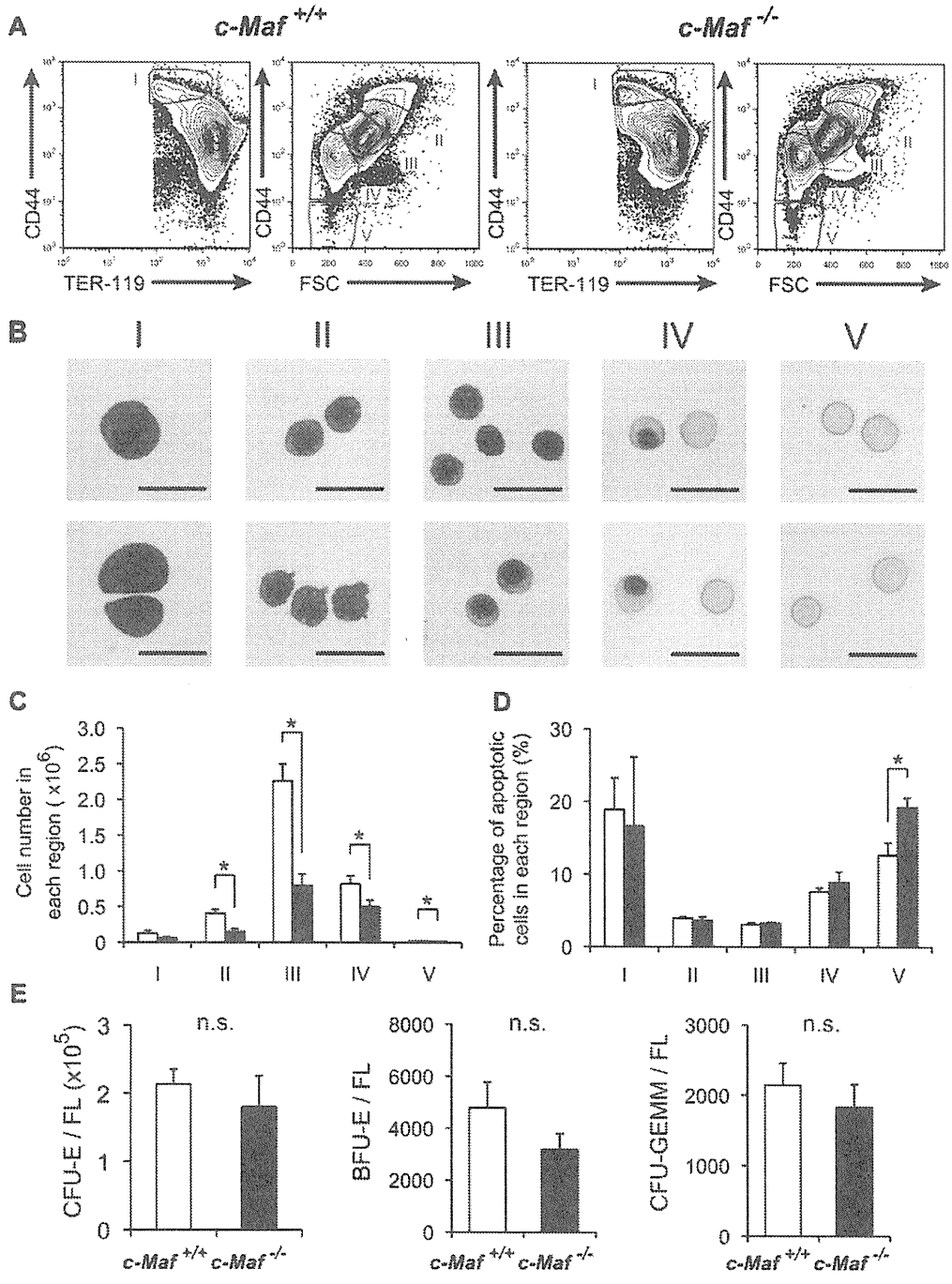

Figure 3. Definitive erythropoiesis in fetal liver is impaired in *c-Maf*$^{-/-}$ embryos but *c-Maf*$^{-/-}$ fetal liver cells can form erythroid colonies. (A) Flow cytometric analysis of fetal liver cells isolated from E13.5 embryos labeled with a FITC-conjugated anti–TER-119 mAb and an APC-conjugated anti-CD44 mAb. Regions I to V are defined by a characteristic staining pattern and forward scatter (FSC) intensity of cells as indicated. (B) Representative images of erythroblast structure on stained cytospins from the 5 distinct regions shown in Figure 3A of wild-type fetal liver. Images were acquired by a Biorevo BZ microscope (Plan Apo 20×0.75 DIC N2) at room temperature and processed with the Adobe Photoshop CS4 software. Scale bar represents 20 μm. (C) Comparison of *c-Maf*$^{+/+}$ and *c-Maf*$^{-/-}$ fetal livers in region I to region V. □ represents *c-Maf*$^{+/+}$; ■, *c-Maf*$^{-/-}$; n = 8∼10 per group; $^*P < .05$. (D) The ratio of annexin V$^+$ cells from region I to region V was compared in *c-Maf*$^{+/+}$ and *c-Maf*$^{-/-}$ fetal livers. □ represents *c-Maf*$^{+/+}$; ■, *c-Maf*$^{-/-}$; n = 3 per group; $^*P < .05$. (E) In vitro colony assay with the use of fetal liver cells from *c-Maf*$^{+/+}$ (□) and *c-Maf*$^{-/-}$ (■) embryos at E13.5. A total of 20 000 fetal liver cells were plated and cultured with methylcellulose media. The numbers of CFU-E–, BFU-E–, and CFU-GEMM–derived colonies per fetal liver are shown. No significant difference (n.s.) was found in the number of CFU-E–, BFU-E–, or CFU-GEMM–derived colonies per fetal liver in *c-Maf*$^{+/+}$ embryos and *c-Maf*$^{-/-}$ embryos; n = 6∼7 per group; data are presented as mean ± SEM. FL indicates fetal liver.

(hemoglobin z, beta-like embryonic chain) and Hbb-y (hemoglobin y, beta-like embroyonic chain) were not significantly suppressed in the *c-Maf*$^{-/-}$ fetal liver erythroid fraction compared with the *c-Maf*$^{+/+}$ control (supplemental Figure 3).

To examine the colony formation potential of hematopoietic progenitors in *c-Maf*$^{-/-}$ fetal liver, conventional set of CFU assays were performed. As a result of CFU assays, there were no significant differences between *c-Maf*$^{+/+}$ and *c-Maf*$^{-/-}$ in the