Immune-mediated hematopoietic failure after allogeneic hematopoietic stem cell transplantation: A common cause of late graft failure in patients with complete donor chimerism

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Short title: Immune-mediated hematopoietic failure after stem cell transplantation

Abstract

Late graft failure (LGF) without evidence of residual recipient cells is a serious complication after allogeneic hematopoietic stem cell transplantation (allo-SCT) and often requires stem cell infusion from the same donor when the patient fails to respond to conventional therapies. We screened the peripheral blood (PB) of 14 patients who developed donor-type LGF at 2–132 months after allo-SCT for the presence of the markers for immune-mediated bone marrow (BM) failure. Increased glycosylphosphatidyl inositol-anchored protein-deficient (GPI-AP$^-$) leukocytes, which accounted for 0.009–0.147% of the total granulocytes, were detected in five (severe aplastic anemia, n=2; follicular lymphoma, n=1; acute lymphoblastic leukemia, n=1; and myelodysplastic syndromes [MDS], n=1) and 4.7–81.2% HLA-allele lacking leukocytes (HLA-LLs) were detected in two (acute myeloid leukemia, n=1; and MDS, n=1) patients. Three of the five patients with increased GPI-AP$^-$ leukocytes were treated with antithymocyte globulin (ATG) and two patients achieved transfusion independence. These results suggest that immune-mechanisms that are similar to acquired aplastic anemia underlie condition of approximately half of the patients with donor-type LGF and that in patients with increased GPI-AP$^-$ cells, donor-derived hematopoiesis may be restored by ATG therapy alone without donor stem cell infusion.

Keywords: late graft failure, GPI-AP$^-$ cells, HLA allele-lacking leukocytes, antithymocyte globlin

Introduction

Late graft failure (LGF) without evidence of residual recipient cells is a serious complication after allogeneic hematopoietic stem cell transplantation (allo-SCT). Various factors, such as infections and chronic graft-versus-host disease (GVHD), are involved in the development of such donor-type LGF[1,2]. It is therefore difficult to identify the exact cause and to restore the graft function without performing a second transplant. One exceptionally treatable condition is immune-mediated bone marrow (BM) failure that is similar to acquired aplastic anemia (AA). This condition occurs during donor-derived hematopoiesis, and can be diagnosed by identifying immune markers in the peripheral blood (PB). The markers include glycosylphosphatidylinositol-anchored protein (GPI-AP)-deficient blood cells (GPI-AP$^-$ cells,[3,4]) and HLA allele-lacking leukocytes (HLA-LLs) due to copy number-neutral loss of heterozygosity in the short arm of chromosome 6 (6pLOH[5,6]) or missense mutations in the HLA alleles[7], which can be found in 50% and 25% of patients with newly diagnosed AA, respectively. To determine how often such immune mechanisms are involved in the development of donor-type LGF, we retrospectively analyzed the laboratory data and clinical courses of patients who were referred to our clinic to undergo a closer examination for donor-type LGF.

Methods

Patients

During the eight-year period from 2007 to 2015, 17 patients with LGF after allo-SCT were referred to our clinic to undergo a closer examination for LGF. All patients showed initial engraftment but subsequently developed loss of a previously functioning graft defined by at least two cytopenic lines [8]. The platelet count increased to 60×10^9-205×10^9/L before the development of LGF. Three of the 17 patients were diagnosed with LGF due to mixed chimerism, while the other 14 patients were diagnosed with donor-type LGF based on the absence of recipient-derived T cells, which was demonstrated by the amplification of short tandem repeat markers or a fluorescence *in situ* hybridization (FISH) analysis of the sex chromosomes. None of the 14 patients had apparent signs of GVHD, infections, or drug reactions at the time of LGF development. Eleven of the 14 patients were dependent on transfusions (eight on red blood cell and eight on platelet transfusions).The medical records (including the prevalence of increased GPI-AP^- cells and HLA-LLs) and treatment outcomes of the 14 patients with donor-type LGF were reviewed. The study protocol (No. 287) was approved by the Ethics Committee for Human Genome/Gene Analysis Research at Kanazawa University Graduate School of Medical Science. All of the patients provided their informed consent prior to participation in the study.

The detection of GPI-AP^- cells and HLA-LLs

PB was collected from the patients at the time of the diagnosis and from the donor in Cases 1, 3 and 6. To detect GPI-AP$^-$ PNH-type cells, we performed high-sensitivity flow cytometry of the granulocytes and erythrocytes, as described previously[3]. The presence of ≥0.003% CD55$^-$CD59$^-$CD11b$^+$ granulocytes or FLAER$^-$CD11b$^+$ granulocytes and ≥0.005% CD55$^-$CD59$^-$glycophorin-A$^+$ RBCs was defined as an abnormal increase based on the reference ranges in healthy individuals[9]. In our previous study, none of 51 SCT recipients without graft failure showed an increase in the percentage of GPI-AP$^-$ cells [4]. For patients who did not show increased GPI-AP$^-$ cells, we attempted to detect HLA-LLs. The expression of HLA-A on granulocytes, monocytes, B and T cells was analyzed by flow cytometry using a FACS Canto II (Becton Dickinson, Franklin Lakes, NJ, USA) with the FlowJo program (Tree Star, Ashland, OR, USA). This study used monoclonal antibodies specific for HLA-A24, A2, A26, and A31 as well as the lineage marker antibodies specific for CD33 in granulocytes and monocytes, CD19 in B cells and CD3 in T cells, as described previously[5]. HLA-A allele lacking leukocytes were not detected in any of 8 SCT recipients who did not develop LGF.

The SNP array analysis and the deep sequencing of the HLA-A gene

PB was collected from the patient and the donor in Case 6. The DNA extracted from isolated granulocyte was subjected to genomic and allele-specific copy number analyses using GeneChip® 250K arrays (Affymetrix, Inc., Santa Clara, CA, USA), as previously described[10,11]. The genomic abnormalities responsible for the failure of HLA-A allelic

expression were also analyzed using Illumina MiSeq and HiSeq sequencing systems (Illumina, San Diego, CA, USA).

The statistical analysis

Overall survival (OS) was calculated as the number of months from the diagnosis of LGF until death or the last follow-up examination. The survival time was estimated by the Kaplan-Meier method and compared by a log-rank test. Two-sided *P* values were calculated, and *P* values of <0.05 were considered to indicate statistical significance. All of the statistical analyses were performed using the EZR software package (Saitama Medical Center, Jichi Medical University), a graphical user interface for the R software program (The R Foundation for Statistical Computing, version 2.13.0)[12].

Results

The prevalence of the immune markers in patients with LGF

GPI-AP$^-$ granulocytes ranging from 0.009–0.147% were detected in 5 of the 14 patients (35.7%, Figure 1). Five patients who did not show an increase in GPI-AP$^-$ granulocytes and who were heterozygous for the HLA-A allele were subjected to FCM to detect HLA-LLs. 4.7–81.2% HLA-LLs were detected in 2 of the 5 (40%) patients (Figure 2). All of the 14 patients showed prominent thrombocytopenia at the onset of LGF, which gradually progressed to pancytopenia; their platelet counts were 7–40×10^9/L (median 18.5×10^9/L) and their neutrophil counts were 0.3–3.8×10^9/L (median 0.9×10^9/L).

The clinical courses of the seven patients

Table 1 summarizes the clinical characteristics of the seven patients who possessed immune markers. High-sensitivity flow cytometry detected small populations of CD55$^-$ CD59$^-$ cells or FLAER$^-$ cells in the granulocytes at the development of LGF. The numbers denote the proportion of GPI-AP$^-$ cells in the CD11b$^+$ granulocytes.

Case 1's clinical course was previously reported[4]. Briefly, 0.147% GPI-AP$^-$ granulocytes (Figure 1) and 0.019% GPI-AP$^-$ erythrocytes were detected in the PB of the patient after 2nd stem cell transplantation (SCT) for severe aplastic anemia (SAA) when the patient developed donor-type LGF. An increase in GPI-AP$^-$ cells was not detected in the PB of the donor. We treated the patient with antithymocyte globulin (ATG) and cyclosporine. The patient achieved

a complete hematological recovery and has been in complete remission for 10 years after the ATG therapy.

Case 2 had SAA. The patient was negative for increased GPI-AP$^-$ cells and did not respond to ATG plus cyclosporine therapy. He underwent BM transplantation (BMT) from an unrelated donor whose HLA allele showed a 2-loci mismatch. Although his platelet count once increased to 96×10^9/L on day 44 after BMT, it gradually decreased to 15×10^9/L in parallel with the progression of anemia and leukocytopenia. A PB sample obtained at 7 months after BMT showed 0.009% GPI-AP$^-$ granulocytes (Figure 1). At 9 months after BMT, he was treated with rabbit ATG therapy (Thymoglobulin, 3.5 mg/kg × 5 days) and his pancytopenia improved after 2 months of the therapy (neutrophil count, 0.7×10^9/L to 1.1×10^9/L; hemoglobin, 5.5 g/dL to 7.8 g/dL; platelet count, 15×10^9/L to 23×10^9/L; and reticulocyte count, 9.1×10^9/L to 59.9×10^9/L). Although the patient was transfusion-independent for 6 weeks, his pancytopenia progressed again at 4 months after ATG therapy. The patient required red blood cell transfusions twice a month and platelet transfusions once a week as of 24 February 2017. A blood analysis at his most recent examination revealed the following findings: neutrophil count, 0.7×10^9/L; hemoglobin, 6.7 g/dL; platelet count, 18×10^9/L and reticulocyte count, 25.5×10^9/L.

Case 3 received PB stem cell transplantation (PBSCT) from an HLA-haploidentical son for the treatment of follicular lymphoma that was refractory to chemotherapy. When the

patient developed donor-type LGF at 7 years after PBSCT, 0.125% GPI-AP$^-$ granulocytes (Figure 1) were detected in the PB of the patient. The patient's donor was negative for increased GPI-AP$^-$ cells. She received rabbit ATG therapy (3.75 mg/kg × 5 days) and cyclosporine (6mg/kg/day) at 2 weeks after the development of pancytopenia, but the severe neutropenia persisted and she did not respond to G-CSF. At 6 weeks after ATG therapy, she received an infusion of PBSCs (3.6×10^6/kg CD34$^+$ cells) from the original donor to promote hematological recovery. Her neutrophil count increased to 2.7×10^9/L 68 days after the PBSC infusion without conditioning, but decreased to 0.1×10^9/L again on day 134 after the infusion. She eventually received a conditioning regimen consisting of fludarabin (162 mg/m^2), busulfan (6.4 mg/kg) and ATG (5 mg/kg) followed by a third PBSCT from the original donor at 6 months after ATG therapy. Her post-transplant course was uncomplicated and her complete blood cell counts were normal at 57 months after the third PBSCT. GPI-AP$^-$ cells were undetectable when her blood was examined at one month after PBSCT.

Case 4 was a 57-year-old woman who received HLA 2-loci-mismatched umbilical cord blood transplantation (CBT) for the treatment of acute lymphoblastic leukemia. When she developed donor-type LGF at 7 years after CBT, 0.011% GPI-AP$^-$ granulocytes were detected in her PB (Figure 1). Because she declined ATG therapy, cyclosporine (6 mg/kg/day) was administered for 11 months without an appreciable response. In the four years since developing LGF, she has required red blood cell transfusions monthly due to persistent

pancytopenia.

Case 5, a 25-year-old man, received a BMT from an HLA-matched unrelated donor for the treatment of MDS-refractory cytopenia of multilineage dysplasia (RCMD). His platelet count increased to 114×10^9/L on day 44, but gradually decreased thereafter. When he was diagnosed with donor-type LGF on day 175 after the BMT, 0.016% GPI-AP$^-$ granulocytes were detected in his PB (Figure 1). However, the patient chose to undergo a second BMT from an HLA-matched unrelated donor rather than IST, following conditioning with fludarabin (125 mg/m^2) and melphalan (140 mg/m^2). The second BMT was successful and a sustained hematologic recovery was achieved. A laboratory analysis on October 26, 2016 revealed the following findings: neutrophil count, 2.7×10^9/L; hemoglobin, 9.1 g/dL; and platelet count, 96×10^9/L.

Case 6 was a 23-year-old woman who underwent PBSCT from her HLA-haploidentical father for the treatment of acute myeloid leukemia with *FLT3*-internal tandem duplication. After neutrophil engraftment on day 19, she developed a generalized skin rash and high fever. After severe acute GVHD (grade II) was diagnosed, methylprednisolone (2 mg/kg/day) was started on day 24 and her skin rash quickly resolved. Platelet engraftment occurred on day 27 and her platelet count reached 119×10^9/L on day 38. However, her platelet count gradually decreased thereafter, and when her platelet count fell to 33.0×10^9/L on day 89, her PB was negative for GPI-AP$^-$ cells. Since the patient's donor was heterozygous for the HLA-A allele

(A2 and A24), we examined her PB granulocytes for the presence of HLA-LLs—15% of the total granulocytes were negative for HLA-A2. HLA-A2-lacking leukocytes were not detectable in the PB of her donor (Figure 2A).

Since her thrombocytopenia was mild, she was observed and the administration of tacrolimus and prednisolone—which had been prescribed for acute GVHD treatment—was continued. Her platelet counts showed a slight increase and stabilized at around 100×10^9/L thereafter. The HLA-A2-lacking leukocytes gradually increased and accounted for 81.2% of the total granulocytes on day 167, but the percentage subsequently decreased and became undetectable on day 287 (Figure 2B). Her platelet count remained stable at 31 months after PBSCT. To identify the mechanisms responsible for the lack of the HLA-A allele, the patient's granulocytes obtained on day 118 were subjected to a single nucleotide polymorphism (SNP) array analysis and granulocytes obtained on day 126 were used for the deep sequencing of the HLA-A gene. The donor's granulocytes were used as a control. We did not detect a copy number-neutral 6pLOH or structural gene mutations of HLA-*A*02:01* (Supplementary Figure 1).

Case 7 was a 63-year-old man who received a HLA-2-loci-mismatched CBT for the treatment of RCMD. Neutrophil and platelet engraftment were achieved on days 18 and 43 after CBT, respectively, and his platelet count increased to 139×10^9/L on day 134. When the patient developed donor-type LGF at 21 months after CBT, 4% of the total monocytes were

HLA-A26-lacking (Figure 2A). He was treated with cyclosporine (6 mg/kg) for one month without effect and died of systemic adenovirus infection at two months after the initiation of treatment.

The clinical courses of the seven patients (Cases 8-14) without immune markers

The PB of seven patients did not show an increase in either of GPI-AP$^-$ cells or HLA-LLs. They developed LGF at 3 to 84 months after SCT. Four of them suffered from severe pancytopenia and required frequent transfusions. Cases 8-10 were treated with cyclosporine and responded; however, Case 10 developed pancytopenia 5 months after the discontinuation of cyclosporine. Since a low percentage of recipient-derived lymphocytes appeared at the time of relapse, the patient was treated with donor lymphocyte infusion (DLI) and a good hematopoietic function was restored. Case 11, who was heavily dependent on red blood cell transfusions, responded to a dose escalation of tacrolimus, which was given as prophylaxis against GVHD. Case 12 received DLI from the original donor because graft failure caused by residual recipient lymphocytes, which were not be detected by a FISH analysis of the patient's sex chromosomes, could not be excluded, and the pancytopenia resolved. Case 13 did not respond to anabolic steroids and died of diffuse pulmonary hemorrhage. Case 14's LGF, which was associated with acute GVHD, was improved by corticosteroids and iron chelation therapy (Table 2).

The HLA alleles of the patients and the donor

Table 3 shows the HLA alleles of the patients and their donors. Four donors of the seven patients possessed one of the four HLA class I alleles (A*02:01 and A*02:06) that we previously identified as being associated with susceptibility to AA[5]. An HLA-A allele (A*02:01) that was lost in the donor-derived granulocytes in Case 6 was not shared by the recipient; however, there was no evidence of residual recipient T cells. Figure 3 shows the OS of the 14 patients with donor-type LGF.

DISCUSSION

The present study identified seven patients with donor-type LGF who possessed GPI-AP$^-$ cells or HLA-LLs that are known to reflect the immune pathophysiology of BM failure. In keeping with the proposed significance of the immune markers, two of the three patients who were treated with ATG showed a sustained or transient improvement of their hematopoietic function. The prevalence of the immune markers among the patients with donor-type LGF was 50%. Even the patients who were negative for the immune markers may have had immune-mediated BM failure because a good hematopoietic function was restored in four of the seven patients who were treated with cyclosporine or tacrolimus. It is therefore suggested that immune-mediated BM failure, which is similar to AA, may underlie donor-type LGF.

The mechanism responsible for the development of immune-mediated BM failure is not clear. Cases 1 and 2 originally had SAA, while Cases 5 and 7 had MDS-RCMD, which could have been diagnosed as non-severe AA. The BM microenvironment of these patients may predispose SCT recipients to developing immune mediated BM failure. However, this hypothesis does not explain the development of BM failure in the other three patients who had hematologic malignancies before SCT. Alternatively, the donors might have had genes that were associated with susceptibility to immune-mediated BM failure. We previously revealed that four class I HLA alleles (A*02:01, A*02:06, A*31:01 and B*40:02) are associated with susceptibility to AA. HLA-DRB1*15:01 and 15:02 have also been shown to

be associated with immune-mediated BM failure[11]. With the exception of Case 7, all of the subjects in the present study possessed one of these high-risk HLA-alleles. Of note, Case 6 did not possess any of the high-risk class I alleles but the patient's donor possessed HLA-*A*02:01*, which was missing in 15% of the total granulocytes when the patient developed thrombocytopenia. In these patients with donor-type LGF, certain triggers (such as infections or drugs) might have induced the breakdown of immune tolerance to hematopoietic stem cells, leading to the development of BM failure.

Both an SNP array analysis and the deep sequencing of the HLA-A gene failed to detect mutations in the HLA-*A*02:01* that would have accounted for the lack of HLA-A2 in the Case 6's granulocytes. Our recent analyses of the HLA-*B*40:02* gene in AA patients possessing HLA-*B*40:02*-lacking granulocytes revealed various mutations in the aberrant granulocytes of all patients[13]. However, we previously experienced some patients whose granulocytes, which lacked HLA-*A*31:01*, did not show any mutations involving this A allele and did not express its mRNA (unpublished observation). Thus, epigenetic mechanisms may have been involved in the transient appearance of HLA-A2-lacking granulocytes in Case 6.

LGF has been defined as hematopoietic failure secondary to neutrophil engraftment. Some patients who meet this criterion do not achieve platelet engraftment and require regular platelet transfusions. We excluded patients with persistent thrombocytopenia from the present study because their hematopoietic failure is primarily caused by the poor engraftment of

donor HSCs due to the infusion of an insufficient number of HSCs. In reality, none of eight patients with persistent thrombocytopenia showed an increase in GPI-AP$^-$ cells or HLA-LLs (unpublished observation). Similar to acquired AA, thrombocytopenia was the first sign of LGF in the seven patients with increased numbers of GPI-AP$^-$ cells or HLA-LLs. It is therefore important to bear in mind that progressive thrombocytopenia precedes immune-mediated hematopoietic failure in donor hematopoiesis.

In conclusion, immune-mediated BM failure similar to AA is a common in patients with donor-type LGF after allo-SCT; GPI-AP$^-$ cells or HLA-LLs were detected in half of the patients. This immune-mediated BM failure may be potentially curable by ATG, without the need for a second SCT. When donor-type LGF occurs without preceding infections, GVHD or exposure to drugs, the patient's blood should be screened for the presence of GPI-AP$^-$ cells and HLA-LLs using high-sensitivity flow cytometry.

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Table 1. The characteristics of the seven patients who had GPI-AP^{-} cells or HLA-LLs.

Table 1

A

	Age/ Sex	Diagnosis	Graft			Time from SCT to donor-type LGF
			source	donor	HLA	
Case 1	63/M	Severe aplastic anemia	PBSCs	Sibling	Identical	2 months after a second PBSC Infusion
Case 2	53/M	Severe aplastic anemia	BM	URD	2-loci mismatched (1-loci; Host→Graft)	7 months
Case 3	56/F	Follicular lymphoma	BM	Son	Haploidentical	7 years
Case 4	57/F	Acute lymphoblastic leukemia	CB	URD	2-loci mismatched	7 years
Case 5	25/M	MDS-RCMD	BM	URD	Identical	2 months
Case 6	23/F	Acute myeloid leukemia	PBSCs	Father	Haploidentical	2 months
Case 7	63/M	MDS-RCMD	CB	URD	2-loci mismatched	2 years

Abbreviations; MDS indicates myelodysplastic syndrome; RCMD, refractory cytopenia with multilineage dysplasia; PBSC, peripheral blood stem cell; BM, bone marrow; CB, cord blood; URD, unrelated donor; HLA, human leukocyte antigen; SCT, stem cell transplantation; and LGF, late graft failure.

B

	Markers for aplastic anemia	CBC at diagnosis of LGF				The result of bone marrow aspiration or biopsy	Therapy	Outcome of hematologic al function
		WBC x10^9/L	ANC x10^9/L	Hb g/dL	Platelet x10^9/L			
Case 1	GPI-AP$^-$ cells	5.3	0.9	7.5	22	Hypocellular marrow	ATG + CsA	Improvement
Case 2	GPI-AP$^-$ cells	2.3	0.7	5.5	15	Hypocellular marrow	ATG	Transient improvement
Case 3	GPI-AP$^-$ cells	1.4	0.3	9.1	14	Hypocellular marrow	ATG + CsA followed by donor's PBSC infusion and SCT	Improvement
Case 4	GPI-AP$^-$ cells	3.5	1.6	5.8	21	Hypocellular marrow	CsA and anabolic steroid	No Response
Case 5	GPI-AP$^-$ cells	1.2	0.6	6.0	10	Hypocellular marrow	2nd SCT on day 411 after 1st SCT	Improvement
Case 6	HLA-A2 lacking cells	4.3	2.8	11.1	32	Hypocellular marrow	Tacrolimus	Stable
Case 7	HLA-A26 lacking cells	8.5	3.8	7.5	23	Hypocellular marrow	CsA	Dead

Abbreviations; GPI-AP indicates glycosylphosphatidyl-inositol anchored proteins; CBC, complete blood count; WBC, white blood cell; ANC, absolute neutrophil count; Hb, hemoglobin; ATG, antithymocyte globulin; and CsA, cyclosporine.

Table 2. The characteristics of the seven patients who did not have GPI-AP^{-} cells or HLA-LLs.

Table 2

A

	Age/ Sex	Diagnosis	Graft			Time from SCT to donor-type LGF
			source	donor	HLA	
Case 8	35/F	Severe aplastic anemia	BM	Sibling	Identical	42 months
Case 9	56/F	Aplastic anemia	PBSCs	Sibling	Identical	7 years
Case 10	13/M	Severe aplastic anemia	BM	Sibling	Identical	22 months
Case 11	44/M	Acute myeloid leukemia	BM	URD	1-loci mismatched ; Host→Graft	7 months
Case 12	35/F	Severe aplastic anemia	BM	Sibling	Identical	6 months
Case 13	40/F	Mixed phenotype acute leukemia	PBSCs	Mother	1-loci mismatched	5 months
Case 14	71/M	Acute myeloid leukemia	CB	URD	2-loci mismatched	3 months

Abbreviations as in Table 1A.

B

	Markers for aplastic anemia	CBC at diagnosis of LGF				The result of bone marrow aspiration or biopsy	Therapy	Outcome of hematological function
		WBC x10^9/L	ANC x10^9/L	Hb g/dL	Platelet x10^9/L			
Case 8	None	2.0	0.8	5.8	18	Hypocellular marrow	CsA	Improvement
Case 9	None	4.5	1.4	7.0	19	Hypocellular marrow	CsA	Improvement
Case 10	None	2.8	0.9	11.1	40	Hypocellular marrow	CsA	Transient improvement
Case 11	None	2.6	1.0	6.5	13	Hypocellular marrow	Dose escalation of Taclolimus	Improvement
Case 12	None	1.6	0.4	6.6	7	Hypocellular marrow	DLI	Improvement
Case 13	None	3.2	2.6	7.9	7	Hypocellular marrow	Anabolic steroid	Dead
Case 14	None	1.3	0.7	7.7	24	Hypocellular marrow	corticosteroids and an iron chelation therapy	Improvement

Abbreviations as in Table 1B. DLI indicates donor lymphocyte infusion.

Table 3. The HLA alleles of the donors and recipients of the seven patients who had GPI-AP^{-} cells or HLA-LLs.

Table 3

	HLA (donor)				HLA (recipient)			
	A	**B**	**C**	**DRB1**	**A**	**B**	**C**	**DRB1**
Case 1	11	52	4	4	11	52	4	4
	24	62	-	15	24	62	-	15
Case 2	02:06	48:01	01:02	04:07	02:01	48:01	01:02	04:07
	-	55:02	08:01	11:01	02:06	55:02	08:22	11:01
Case 3	02:01	15:01		04:05	02:01	15:01		12:02
	24:02	55:02		12:02	33:03	44:03		13:02
Case 4	02:06	15:01		11:01	02:06	15:01		14:01
	24:20	35:01		14:06	24:02	35:01		90:12
Case 5	11:01	15:01	04:01	04:06	11:01	15:01	04:01	04:06
	24:02	52:01	12:02	15:02	24:02	52:01	12:02	15:02
Case 6	02:01	07:02	01:02	01:01	24:02	07:02	07:02	01:01
	24:02	46:01	07:02	12:02	24:07	38:02	-	15:02
Case 7	24:02	07:02		01:01	02:18	07:02		01:01
	26:01	51:01		08:03	24:02	46:01		08:03

Figure Legends

Figure 1. The Analysis of the GPI-AP$^-$ cells at the development of LGF.

Figure 2. The analysis of HLA-LLs at the development of LGF and the clinical course of Case 6.

(A) HLA-allele lacking leukocytes (HLA-LLs) were detected by flow cytometry. The numbers denote the proportion of HLA-LLs in the specific leukocyte compartment. In Case 6, HLA-A2-lacking leukocytes were detected in the granulocytes at the development of LGF, but none of them were detected in the donor. In Case 7, HLA-A26-lacking leukocytes were detected in the monocytes at the development of LGF.

(B) In Case 6, acute GVHD developed on day 24 and thrombocytopenia developed on day 45 after SCT. The proportion of HLA-A2-lacking leukocytes in granulocytes was followed. The percentage became undetectable on day 287.

Figure 3. The OS of the 14 patients who were diagnosed with donor-type LGF according to the status of their GPI-AP$^-$ cells or HLA-LLs.

Figure 1

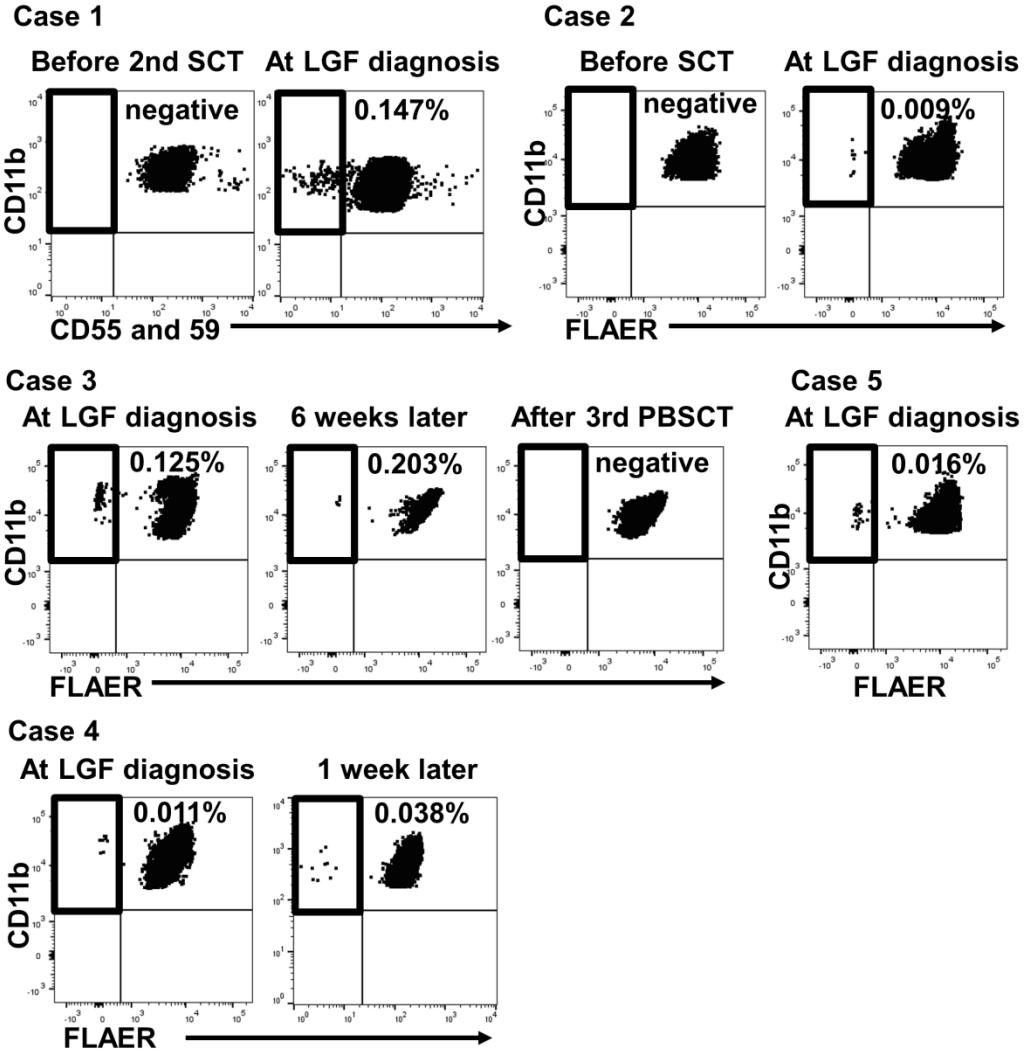

Case 1
Before 2nd SCT
At LGF diagnosis
negative
0.147%
CD11b
CD55 and 59
Case 2
Before SCT
At LGF diagnosis
negative
0.009%
CD11b
FLAER
Case 3
At LGF diagnosis
6 weeks later
After 3rd PBSCT
0.125%
0.203%
negative
CD11b
FLAER
Case 5
At LGF diagnosis
0.016%
CD11b
FLAER
Case 4
At LGF diagnosis
1 week later
0.011%
0.038%
CD11b
FLAER

Figure 2

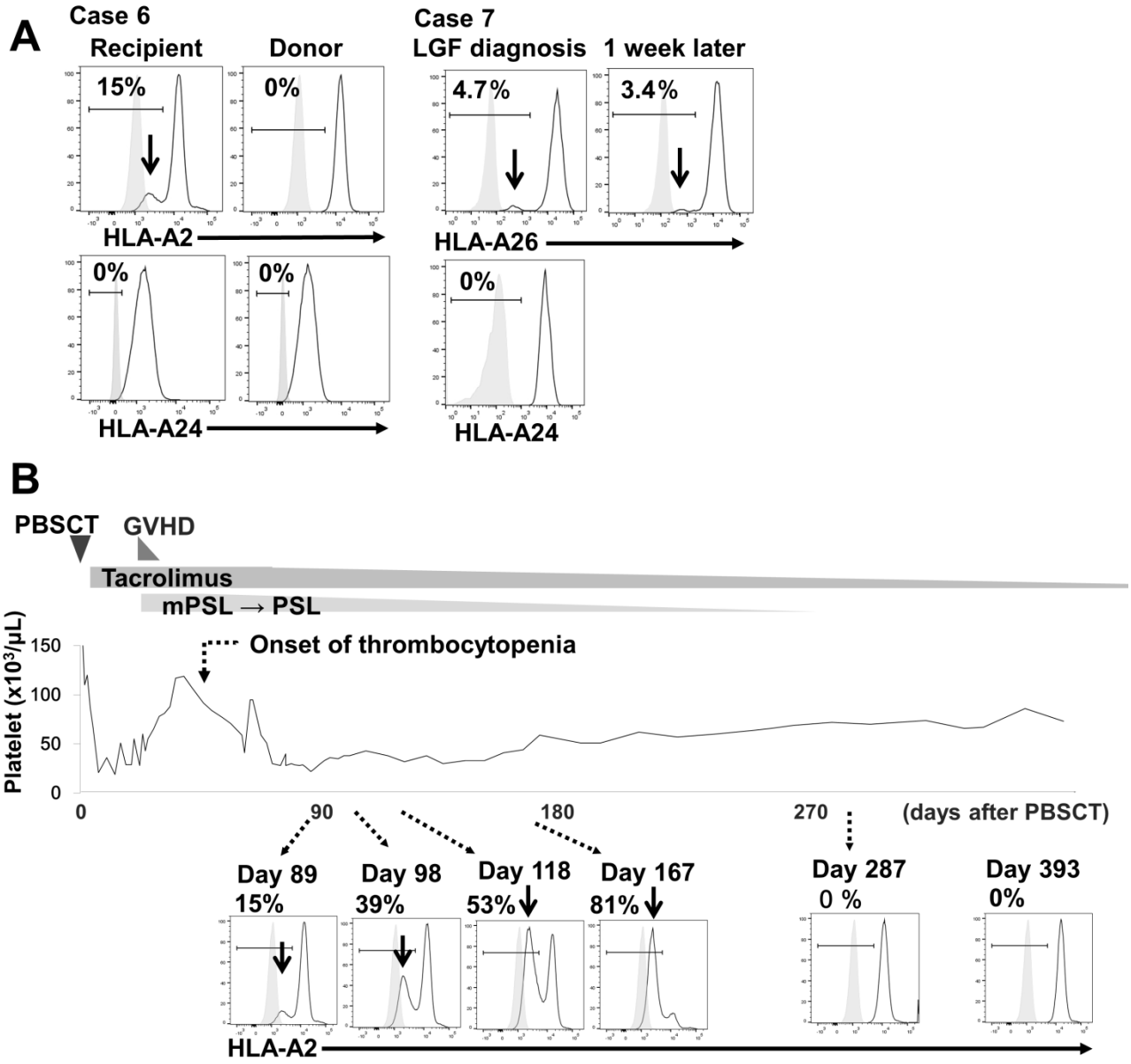
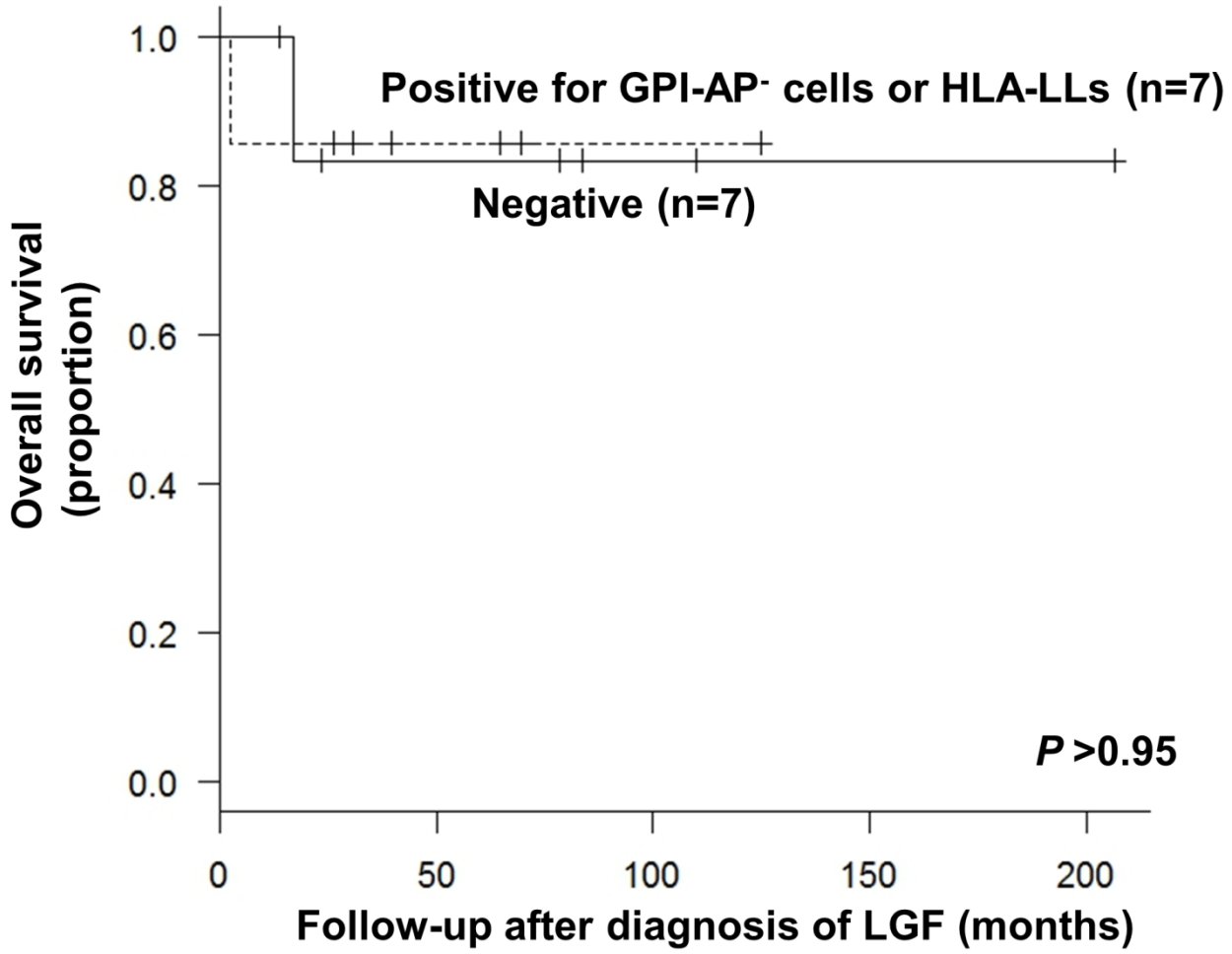
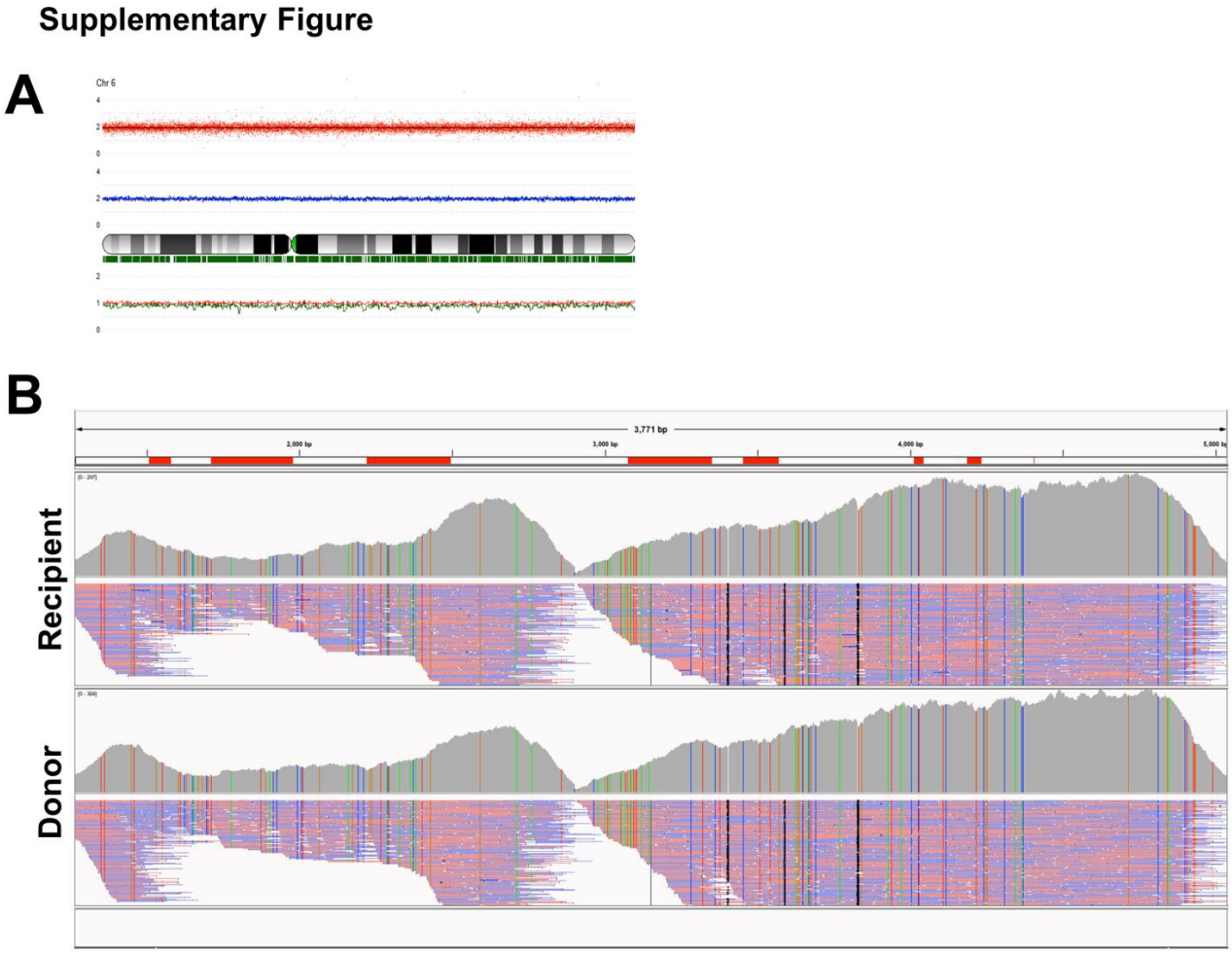

A
Case 6
Recipient
Donor
15%
0%
HLA-A2
0%
0%
HLA-A24
Case 7
LGF diagnosis
1 week later
4.7%
3.4%
HLA-A26
0%
HLA-A24
B
PBSCT
GVHD
Tacrolimus
mPSL → PSL
Onset of thrombocytopenia
Platelet (x10³/μL)
150
100
50
0
0
90
180
270
(days after PBSCT)
Day 89
15%
Day 98
39%
Day 118
53%
Day 167
81%
Day 287
0 %
Day 393
0%
HLA-A2

Figure 3


Positive for GPI-AP- cells or HLA-LLs (n=7)
Negative (n=7)
Overall survival (proportion)
1.0
0.8
0.6
0.4
0.2
0.0
P >0.95
0
50
100
150
200
Follow-up after diagnosis of LGF (months)

Supplementary Figure 1. SNP array analysis of chromosome 6 and deep sequencing of the HLA-A*02:01 gene



(A) A copy number-neutral 6pLOH was not detected in the recipient's granulocytes.
(B) Alignment view of HLA-A*02:01 allelic sequences from the recipient's granulocytes (Recipient) and the donor's granulocytes (Donor). The allelic sequences were phased by phase-defined HLA gene sequencing pipeline[1]. In HLA-A gene region, allelic sequences of phased HLA-A*02:01 sequence of recipient and donor were completely identical and rare somatic mutations were not observed either.

Supplemental Reference

1. Hosomichi K, Jinam TA, Mitsunaga S, Nakaoka H, Inoue I. Phase-defined complete sequencing of the HLA genes by next-generation sequencing. BMC genomics 2013;14:355.