Article

Clinical significance and origin of leukocytes that lack HLA-A allele expression in patients with acquired aplastic anemia

Short title: HLA-A allele-lacking leukocytes in aplastic anemia

Hiroyuki Maruyama[1]†, Takamasa Katagiri[2]†, Koichi Kashiwase[3], Takashi Shiina[4], Aiko Sato-Otsubo[5], Yoshitaka Zaimoku[1], Kana Maruyama[1], Kohei Hosokawa[1], Ken Ishiyama[1], Hirohito Yamazaki[1], Hidetoshi Inoko[4], Seishi Ogawa[5] and Shinji Nakao[1]

[1]Cellular Transplantation Biology, Kanazawa University Graduate School of Medical Science, 13-1 Takaramachi, Kanazawa, Ishikawa, 920-8640, Japan.

[2]Clinical Laboratory Science, Division of Health Sciences, Kanazawa University Graduate School of Medical Science, 5-11-80 Kodatsuno, Kanazawa, Ishikawa, 920-0942, Japan.

[3] Department of HLA Laboratory, Japanese Red Cross Kanto-Koshinetsu Block Blood Center, 2-1-67 Tatsumi, Koto-ku, Tokyo, 135-8639, Japan.

[4]Basic Medical Science and Molecular Medicine, Tokai University School of Medicine, 143

Shimokasuya, Isehara, Kanagawa, 259-1143, Japan.

[5]Department of Pathology and Tumor Biology, Graduate School of Medicine, Kyoto University,

Yoshida-Konoecho, Sakyo-ku, Kyoto, Kyoto, 606-8501, Japan.

† H.M. and T.K. were equally contributed to this work.

Corresponding to:

Shinji Nakao (snakao8205@staff.kanazawa-u.ac.jp)

Cellular Transplantation Biology, Kanazawa University Graduate School of Medical Science, 13-1

Takaramachi, Kanazawa, Ishikawa, 920-8640, Japan.

TEL: +81-762-65-2274

FAX: +81-762-34-4252

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Abstract

To gain insights into the origin and clinical significance of leukocytes that lack HLA-A allele expression due to a copy number neutral loss of heterozygosity in the short arm of chromosome 6 in patients with acquired aplastic anemia (AA), we used a high-sensitivity flow cytometry assay to investigate the presence of HLA-A allele-lacking leukocytes (HLA-LLs) in 144 AA patients. HLA-LLs accounting for 0.2-99.8% of each leukocyte population were detected in 18 of 71 (25.4%) newly diagnosed and 25 of 73 (34.2%) previously treated patients. The lineage combination patterns of the HLA-LLs in the 43 HLA-LL(+) patients were granulocytes (Gs), monocytes (Ms), B cells (Bs), and T cells (Ts; GMBT) in 13 cases, GMB in 16 cases, GM in 11 cases, and B alone in three cases. The response rate to anti-thymocyte globulin plus cyclosporine therapy (100%) and the 2-year failure-free survival rate (100%) in eight newly diagnosed HLA-LL(+) patients were significantly higher than in 23 HLA-LL(-) patients (52.2% for both rates). These data suggest that HLA-LLs are a useful marker of the presence of immune pathophysiology in AA and that T-cell attacks against hematopoietic progenitor cells, rather than hematopoietic stem cells, can trigger bone marrow failure in AA patients.

Keywords: aplastic anemia; bone marrow failure; loss of heterozygosity of the HLA haplotype; immunosuppressive therapy

Introduction

According to the widely-accepted model of acquired aplastic anemia (AA), cytotoxic T lymphocytes (CTLs) recognize auto-antigens that are presented on hematopoietic stem cells (HSCs) through their class I human leukocyte antigen (HLA) molecules, playing an important role in initiation of the autoimmune reactions that lead to bone marrow (BM) failure[1-3]. Several indirect pieces of evidence have been reported in previous studies, such as the accumulation of T cells with particular clonotypes as demonstrated by T-cell receptor analyses[2, 4, 5], as well as the presence of T-cell clones capable of killing myeloid leukemia cell lines within the BM[6, 7]. However, it is entirely unknown what kind of cells the antigen-specific T cells attack and how these T cells diminish HSCs *in vivo*.

Single nucleotide polymorphism (SNP) array analysis reveals the copy number-neutral loss of heterozygosity of the HLA haplotype due to uniparental disomy in the short arm of chromosome 6 (6pLOH) in approximately 13% of AA patients[8-10]. Flow cytometry (FCM) can be substituted for SNP array for detection of HLA-A allele-lacking leukocytes (HLA-LLs) as a result of 6pLOH, because 6pLOH was revealed in all HLA-LL(+) patients in our previous study[9]. The presence of 6pLOH is thought to offer compelling evidence of CTL involvement in the development of AA. The lack of particular class I HLA alleles that present auto-antigens represents "escape" hematopoiesis

by 6pLOH(+) HSCs that have survived CTL attack[9]. However, the origin of the HLA-LLs that serve as CTL targets and the clinical significance of HLA-LLs in patients with AA have not been thoroughly determined, due to the small number of patients analyzed via FCM before immunosuppressive therapy in our previous study.

HLA-LLs can easily be tracked using FCM with monoclonal antibodies (mAbs) specific to HLA-A alleles[9]. Studying the lineage diversity of HLA-LLs and the correlation between the presence of HLA-LLs and the response to IST may provide insight into the CTL targets responsible for the development of AA as well as the clinical significance of HLA-LLs in AA patients. To test these hypotheses, we examined a large number of AA patients for the presence of HLA-LLs, and determined the lineage combinations and the chronological changes in the HLA-LLs that occurred in association with the IST and response to IST in HLA-LL(+) and HLA-LL(-) patients with newly-diagnosed AA.

Material and methods

Patients

A total of 222 AA patients who were referred to our clinic from October 2006 to November 2013 were consecutively assessed in this study. Of these, 212 were idiopathic and 10 were hepatitis-associated; 113 were assessed pre-treatment and 109 post-treatment; 92 were diagnosed

with severe AA and 130 with non-severe AA (**Table 1**). Eighty-four of the 222 patients were included in our previous study of 6pLOH using SNP array analysis[9]. Previous treatments included rabbit anti-thymocyte globulin (ATG, Thymoglobulin, Sanofi, Paris, France) in 47 patients, cyclosporine (CsA) alone in 59 patients, and anabolic steroids alone in three patients. This study protocol (No. 287) was approved by the Ethics Committee for Human Genome/Gene Analysis Research at Kanazawa University Graduate School of Medical Science, and all patients provided their informed consent prior to participation. Severe AA was defined by the presence of a hypocellular bone marrow and at least two of three following peripheral blood criteria: absolute neutrophil count (ANC) $<0.5\times10^9$/L, platelet count $<20\times10^9$/L, and absolute reticulocyte count (ARC) $<20\times10^9$/L[11]. Patients with AA who did not meet criteria for severe AA but with at least two of three following peripheral blood criteria: ANC $<1.5\times10^9$/L, platelet count $<50\times10^9$/L, and ARC $<60\times10^9$/L, were considered as non-severe AA. ATG at a dose of 2.5 to 3.75 mg/kg per day was intravenously administered on day1 to 5. CsA was given 5 mg/kg per day orally, continued for at least six months. CsA was started on day-7 or day1 of ATG therapy. Responses to IST were evaluated according to established criteria[12] after six months of treatment, and patients who achieved complete or partial responses were considered to be responders.

Flow cytometry

Ethylenediaminetetraacetic acid-added peripheral blood (PB) was collected from participating

patients at diagnosis and/or after treatment. HLA-A allele expression on granulocytes (Gs), monocytes (Ms), B cells (Bs), T cells (Ts), and NK cells was analyzed by FCM using a FACSCanto II (Becton Dickinson, Franklin Lakes, NJ, USA) with the FlowJo program (Tree Star, Inc., Ashland, OR, USA). This study used mAbs specific for HLA-A24, A2, A26, A31, as well as the lineage marker antibodies specific for CD11b in Gs, CD33 in Ms, CD19 in Bs, CD3 in Ts, and NKp46 in NK cells. For patients in whom HLA-A alleles had not been determined, PB mononuclear cells (PBMCs) were cryopreserved, and thawed Ms were later subjected to FCM after HLA-A allele data became available. When HLA-LLs were detected in patients who were heterozygous with either HLA-A*33:03, A*01:01, or A*03:01 and either HLA-A24, A2, A26, or A31, positive results were confirmed by examining a second sample from the same patient. HLA-LLs were not assessable in patients who were homozygous for the HLA-A allele by this FCM assay because 6pLOH does not change the HLA-A allele expression pattern in these patients.

Measurement of GPI-AP$^-$ Gs was performed as previously described[13], with minor modifications. The presence of ≥0.003% fluorescein-labeled proaerolysin (FLAER)$^-$CD11b$^+$ or CD55$^-$CD59$^-$CD11b$^+$ Gs was defined as an abnormal increase, based on results obtained from 187 healthy individuals[13]. The mAbs used for this study are detailed in **Supplementary Table E1**.

Analysis of genomic copy numbers and detection of 6pLOH

DNA extracted from whole blood and sorted G populations was subjected to genomic and allele-specific copy number analyses using GeneChip® 250K arrays (Affymetrix, Inc., Santa Clara, CA, USA), as previously described[14, 15]. GPI-AP$^+$ and GPI-AP$^-$ cells were sorted from G samples obtained from patients possessing both HLA-LLs and GPI-APs$^-$ cells using FACSAria II (Becton Dickinson, Franklin Lakes, NJ, USA).

HLA-A and HLA-B gene analysis using a next generation sequencer

Genomic abnormalities responsible for the failure of class I allelic expression by isolated Bs were analyzed using a next generation Roche GS Junior System benchtop sequencer (Roche, Basel, Switzerland), as previously described[16].

Statistical Analysis

Data obtained from both treated and untreated patients were used to characterize HLA-LLs while only the data from untreated patients were used for analyzing the influence of HLA-LLs on responses to IST and prognosis of AA. All the data are reported as the mean ± S.E. of triplicate experiments. The results were analyzed using Fisher's exact test and the log-rank test. *P*-values of <0.05 were considered to indicate statistical significance. Failure-free survival was defined as the time from the first day of ATG therapy until a relapse of pancytopenia, stem cell transplantation, progression to myelodysplastic syndromes (MDS)/acute myeloid leukemia or disease-related death. Overall survival was defined as the time from the first day of ATG therapy until death. All statistical

analyses were performed using EZR, a graphical user interface for R 2.13.2 (R Foundation for Statistical Computing, Vienna, Austria)[17].

Results

Prevalence of HLA-LLs

A total of 144 (64.9%) patients were heterozygous for the HLA-A allele, and were therefore able to be assessed for the presence of HLA-LLs by FCM. The sensitivity of FCM in detecting HLA-LLs differed among the four anti-HLA-A allele-specific mAbs. Mixing experiments that used blood samples containing >90% A24 lacking (A*24:02, A*24:04, and A*24:20) or >90% A2 lacking (A*02:01, A*02:06, and A*02:07) Gs as well as normal blood samples showed FCM using fluorescence-labeled specific mAbs for these alleles to detect ≥0.5% of Gs that lacked either of the A alleles. The sensitivities of FCM using anti-A26 and anti-A31 antibodies (Abs), in combination with fluorescence-labeled anti-mouse IgM Abs, were 5% and 15%, respectively (data not shown). Eighteen (25.4%; 10 with severe AA and eight with non-severe AA) of the 71 pre-treatment patients, and 25 (34.2%; 12 with severe AA and 13 with non-severe AA) of the 73 post-treatment patients were found to be positive for HLA-LLs. The median percentages in each lineage were 34.7% (0.8-99.4%, n=40) for Gs; 34.3% (0.6-99.8%, n=40) for Ms; 10% (0.5-94.8%, n=32) for Bs; and 2% (0.2-21.8%, n=13) Ts (**Fig. 1A, B**).

Lineage diversity of HLA-LLs

The lineage combinations of HLA-LLs in the 43 HLA-LL(+) patients were GMBT in 13, GMB in 16, and GM in 11 (**Fig. 1A, Table 2, and Supplementary Table E2**). Unexpectedly, HLA-LLs were found in Bs alone in three patients (**Fig. 1A, C**). The presence of 6pLOH was confirmed via deep sequencing of isolated Bs from one of these three patients (Case 43, **Supplementary Table E3**). Two of the three patients (Cases 41, 42) showed complete responses at the time of sampling three years after ATG therapy and 20 years after CsA therapy, while Case 43 required low-dose CsA to maintain good hematopoietic function.

Blood samples from 23 of the 43 HLA-LL(+) patients were obtained for follow-up at 1-40 months after initial analysis. HLA-LLs remained detectable in all but one patient (Case 35) with the GMB pattern, in whom HLA-lacking Gs decreased from 11.7% before treatment to 0% after 12 months of ATG therapy (**Fig. 2A**). In the other 22 patients, including two newly-diagnosed patients whose samples were available once they achieved remission through IST, clone sizes and lineage combination patterns of HLA-LLs were not observed to change during the follow-up period (**Fig. 2B)**.

Table 2 summarizes characteristics of the 43 patients who possessed HLA-LLs. The clone sizes of HLA-LLs in each leukocyte lineage were consistently G≒M≥B>T. The percentages of Ms lacking HLA-A allele expression (HLA-LMs) in cryopreserved PBMCs obtained at diagnosis were

compared with those of freshly obtained PBMCs from two patients after HLA-A alleles were determined. There were no differences in the clone sizes of HLA-LMs between the two sets of samples (1.1% v. 1.0%, and 44.7% v. 48.3%).

HLA-LLs in patients possessing GPI-AP$^-$ cells

An increase in the percentage of GPI-AP$^-$ Gs, ranging from 0.003% to 99.4%, was detected in 103 of the 144 participating patients whose samples were available for the analyses of GPI-AP$^-$ cells. Twenty-five (24.3%) of the103 patients with increased GPI-AP$^-$ Gs (paroxysmal nocturnal hemoglobinuria (PNH$^+$) patients), and 18 (43.9%) of the 41 patients without these aberrant cells (PNH$^-$ patients) possessed HLA-LLs (*P*=0.026). Among the 25 PNH$^+$ patients, Gs lacking HLA-A allele expression (HLA-LGs) were detected in both GPI-AP$^+$ and GPI-AP$^-$ cells in three patients, while HLA-LGs were only detected in GPI-AP$^+$ cells in the other 22 patients (**Fig. 3**).

Response to IST and survival in patients with and without HLA-LLs

A total of 50 patients, including eight HLA-LL(+) patients, 23 HLA-LL(-) patients, and 19 patients who were homozygous for the HLA-A allele, underwent IST including ATG plus CsA after blood samples were obtained for FCM. All eight HLA-LL(+) patients, as well as 12 (52.2%) HLA-LL(-) patients (*P*=0.033), and 12 (63.2%) homozygous patients responded to treatment (**Table 3A and Fig. 4A**). Of the 47 patients who had already been treated with IST at the time of sampling, 45 including nine HLA-LL(+), 17 HLA-LL(-), and 19 homozygous patients were evaluable for responses to IST

and two patients were lost to follow up. The rate of response to IST among the nine HLA-LL(+) patients was only slightly higher than that among the 17 HLA-LL(-) patients (66.7% vs. 58.8%; *P*=1.0, **Table 3B)**. The prevalence of increased of GPI-AP$^-$ cell numbers, which is known to be associated with a good response to IST, was significantly lower in the HLA-LL(+) patients than in the HLA-LL(-) patients (33.3% vs. 76.5%; *P*=0.046). The response rate among homozygous patients (68.4%) was comparable to the response rates of the other two groups.

The median follow-up period was 817.5 days (range, 11 to 1584 days). The two-year failure-free survival rate (100%) in the previously untreated HLA-LL(+) patients were significantly higher than that of the HLA-LL(-) patients (52.2%, *P*=0.018) and that of the patients who were homozygous for the HLA-A allele (63.2%, *P*=0.022); however, there were no significant differences in the overall survival rates of the three groups (**Fig. 4B, C**).

Discussion

Our previous study used a SNP array analysis to reveal that approximately 13% of AA patients had 6pLOH(+) cells[9, 10]. However, the involvement of the lineage of HLA-LLs in 6pLOH(+) patients remained unclear due to the limited number of patients whose leukocytes were analyzed with FCM. The current study, in which a large number of AA patients were analyzed using anti-HLA-A allele specific mAbs, revealed several new aspects of HLA-LLs. First, the prevalence of HLA-LLs in

evaluable patients with newly diagnosed AA (25.4%) was higher than the prevalence of 6pLOH (15%) in newly diagnosed patents in our previous study[9]. The higher prevalence of HLA-LLs can be explained by FCM's greater sensitivity in the detection of aberrant cells (in comparison to the SNP array in the detection of 6pLOH(+) leukocytes). Another explanation is that in some patients, HLA-LLs can be caused by mechanisms other than 6pLOH. It has been shown that mutations in the coding region of class I HLA alleles have the potential to produce HLA-LLs in patients with AA[18, 19]. Some of the HLA-LLs observed in patients from the current study may have been produced by mutations of the coding region of HLA-A alleles. Given the fact that the presence of HLA-LLs cannot be evaluated in one third of AA patients (due to HLA-A homogeneity or the lack of mAbs specific to HLA-A1 and A33, as well as other HLA class I alleles, including HLA-B and HLA-C), the true incidence of HLA-class I allele-lacking leukocytes in newly-diagnosed AA patients may be higher than 25.4%.

Our previous study showed that all 11 patients with newly diagnosed 6pLOH(+) AA responded to either ATG plus CsA or CsA alone while the response rate to IST in 6pLOH(-) AA patients was 73%. The current study which involved a new cohort of HLA-LL(+) patients, including eight newly diagnosed AA patients also showed a 100% response rate to ATG, which was significantly higher than the response rate (52.2%) in HLA-LL(-) AA patients. The remarkably high response rate to IST and the significantly higher two-year failure-free survival rates of HLA-LL(+) patients in

comparison to HLA-LL(-) patients support the hypothesis that the presence of HLA-LLs is strongly associated with the CTL-mediated immune pathophysiology of AA. In agreement with this hypothesis, 6pLOH has been rarely detected in patients with inherited BM failure[20] or in patients with myelodysplastic syndromes[21] where the CTL-mediated immune mechanisms are not involved in the BM failure. These scenarios are further supported by the recent reports showing that the 6pLOH provides a common mechanism of leukemic relapse after HLA haploidentical stem cell transplantations, in which leukemic cells that lost the mismatched HLA haplotype through 6pLOH are thought to escape the immunologic surveillance of the engrafted donor T cells[22, 23]. Indeed, we successfully isolated a T-cell clone that was capable of killing hematopoietic cells in an HLA-B*40:02-restricted manner from one of the 6pLOH(+) patients (case 8)[7]. On the other hand, a recent analysis of 256 severe AA patients, including 33 6pLOH(+) patients, who were treated with horse ATG in the United States failed to show a better response rate in comparison to 6pLOH(-) patients[10]. Another recent study by Betensky et al. detected 6pLOH in eight (11.3%) of 71 patients with bone marrow failure using a SNP array analysis and found no significant difference in the response rate to IST between 6pLOH(+) patients and 6pLOH(-) patients (50% v. 77.8%)[24]. The lower response rate to IST in the two studies from the USA than ours may be attributed to the lower sensitivity of the SNP array analysis in comparison to FCM in the detection 6pLOH(+) leukocytes, which could underestimate the prevalence of 6pLOH(+) patients. The differences in the patients' age

may also have affected the response to IST. Our study included only 6% pediatric patients while 76.1% of the patients studied by Betensky et al. were children younger than 21 years old. The prognostic value of 6pLOH(+) leukocytes must be examined by prospective studies which include a greater number of AA patients.

One of the important finding of the current study is the diversity in the lineage of HLA-LLs within individual patients with AA. In contrast to our previous study, which revealed 6pLOH(+) cells in all lineages of leukocytes, many patients with HLA-LGs and HLA-LMs did not show HLA-LLs in lymphocytes. The same restricted patterns of lineage combination were observed to remain unchanged for one to 16 months in patients with newly diagnosed AA, a finding compatible with a single cell origin of the HLA-LLs. These findings suggest that the hematopoietic precursor clones that escape CTL attack are hematopoietic progenitor cells (HPCs) that lack the capacity to differentiate into lymphoid cells but are capable of self-renewing to support myelopoiesis for long periods of time. Sun et al. recently demonstrated that murine hematopoiesis is supported by a limited number of HPCs rather than HSCs[25]. We previously demonstrated that in AA patients who possess GPI-AP$^-$ cells, self-renewing *PIGA*-mutant HPCs with limited differentiation capacity produce GPI-AP$^-$ cells for many years, in a similar manner to 6pLOH(+) HPCs[26]. Unlike *PIGA*-mutant HPCs lacking all GPI-APs, many of which potentially play important roles in the regulation of hematopoiesis, 6pLOH(+) HPCs are very similar to normal HPCs. These *in vivo* observations

suggest that hematopoiesis in humans may be supported, at least in part, by HPCs.

The presence of “escape” hematopoiesis by HPCs with limited differentiation capacity provides insight into the pathogenesis of AA. In Cases 14-29, which possessed HLA-LLs in GMB, CTL targets are presumed to be HPCs capable of differentiating into GMB (**Supplementary Table E2**). The depletion of HPCs by specific CTLs, however, does not itself lead to BM failure because other HSCs that do not share antigens with the target HPCs compensate for the HPC loss by replenishing new HPCs. It is even more difficult to relate the presence of HLA-LLs in the three cases in which they were observed in Bs alone to the pathogenesis of BM failure, because CTLs are thought to target Bs or B precursors. All three of these patients had normal serum immunoglobulin levels and showed good responses to IST before sampling. A plausible mechanism for BM failure is indirect injury to BM precursors by cytokines produced by CTLs that respond to antigen-presenting B precursors or HPCs. Our previous study indicated that non-specific suppression of hematopoiesis by cytokines, rather than a decrease in the number of HSCs induced by CTLs, was the major mechanism of immune-mediated BM failure[9]. The current chronological studies of newly-diagnosed AA patients who are responsive to IST also show the persistence of HLA-LLs after successful IST at similar percentages to those observed before IST. The bystander effect has been suggested to influence BM failure caused by the immune response to abnormal HSCs with trisomy 8 by a previous study on AA patients with trisomy 8[27]. These findings suggest that CTLs specific for

HPCs can trigger BM failure, but may not play a major role in the development of AA.

In conclusion, this study revealed HLA-LLs in approximately one fourth of newly diagnosed AA patients. Considering the markedly high FFS rate and the response rate to IST in HLA-LL(+) patients, all AA patients should be screened for the presence of HLA-LLs in order to select an optimal therapy.

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Authorship Contributions

H.M. and T.K. contributed equally to this work. S.N. developed the study concept and H.M., T.K., and S.N. designed the experiments. H.M., T.K., Y.Z., K.M., K.H., K.I., H.Y., T.S., A.S.-O., H.I., and S.O. performed the experiments and analyzed the data. K.K. performed high-resolution HLA typing. H.M., T.K., and S.N. wrote the paper. All authors approved the final version of this paper.

Disclosure of Conflicts of Interest

All authors have no financial or personal relationships with other people or organizations that could inappropriately influence this study. The authors declare no competing financial interest. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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Tables

Table 1. Patient characteristics

	All (n=222)	Untreated (n=113)	Treated (n=109)
Median age, years (range)	60 (2-93)	62 (16-91)	59 (2-93)
Sex, male /female	108/114	49/64	59/50
Diagnosis			
Idiopatic AA	212	109	103
Hepatitis-asociated AA	10	4	6
Severity			
Severe	92	36	56
Non-severe	130	77	53
Patients with increased GPI-AP$^-$ cells	158 (71.2%)	84 (74.3%)	74 (67.9%)
Patients heterozygous for HLA-A allele	144 (64.9%)	71 (62.8%)	73 (67.0%)

Abbreviations: AA, aplastic anemia; GPI-AP$^-$ cells, glycosylphosphatidylinositol-anchored protein deficient cells.

Table 2. Characteristics of patients with HLA-LLs

	Patients with HLA-LLs
Cases (n)	43
Median age, years (range)	55 (18-93)
Sex, male/female	24/19
Diagnosis	
Idiopatic AA	40
Hepatitis-asociated AA	3
Severity	
Severe	22
Non-severe	21
Patients with increased GPI-AP$^-$ cells	25 (58.1%)
Untreated/Treated	18/25
Lineage combination of HLA-LLs	
GMBT	13 (30.2%)
GMB	16 (37.2%)
GM	11 (25.6%)
B	3 (7.0%)
Missing HLA-A allele	
HLA-A2	28 (65.1%)
HLA-A24	5 (11.6%)
HLA-A26	3 (7.0%)
HLA-A31	7 (16.3%)

Abbreviations: AA, aplastic anemia; GPI-AP$^-$ cells, glycosylphosphatidylinositol-anchored protein deficient cells; HLA-LLs, HLA-A allele-lacking leukocytes; G, granulocyte; M, monocyte; B, B cell; T, T cell.

Table 3. Response to immunosuppressive therapy in untreated (A) and treated (B) patients

A)

	Untreated			
	All	HLA-LL(+)	HLA-LL(-)	Homozygous for HLA-A allele
Number of patients evaluable for response to ATG plus CsA (n)	50	8	23	19
Median age, years (range)	55 (16-77)	48 (30-68)	55 (16-77)	57 (20-75)
Sex, male/female	25/25	2/6	12/11	11/8
Severity				
Severe	25	6	11	8
Non-severe	25	2	12	11
Number of patients who achieved response* (n)	28 (56.0%)	8 (100%)	12 (52.2%)	12 (63.2%)

B)

	Treated			
	All	HLA-LL(+)	HLA-LL(-)	Homozygous for HLA-A allele
Number of patients evaluable for response to ATG plus CsA (n)	45	9	17	19
Median age, years (range)	55 (2-81)	54 (22-69)	50 (2-77)	59 (25-81)
Sex, male/female	25/20	8/1	5/12	12/7
Severity				
Severe	32	6	12	14
Non-severe	13	3	5	5
Number of patients who achieved response (n)	29 (64.4%)	6 (66.7%)	10 (58.8%)	13 (68.4%)

Abbreviations: ATG, anti-thymocyte globulin; CsA, cyclosporine; HLA-LLs, HLA-A allele-lacking leukocytes.

* Responses to immunosuppressive therapy were evaluated according to the Camitta criteria after 6 months of treatment. Patients who achieved complete or partial response were considered responders.

Figure Legends

Figure 1. HLA-A allele-lacking leukocytes (HLA-LLs) in patients with aplastic anemia (AA)

A. The HLA-LLs of four cases that possessed HLA-LLs in granulocytes (Gs), monocytes (Ms), B cells (Bs), and T cells (Ts) were GMBT (Case 8), GMB (Case 19), GM (Case 37), and B alone (Case 41) are shown. Histograms shown in the red rectangles represent positive results on HLA-LLs. **B.** Scattergrams and histograms are shown for a representative case (Case 14) that possessed minor (0.8%) HLA-A24-lacking populations in GMB. No HLA-A2-lacking cells were detected in this case. **C.** Histograms of three cases who possessed HLA-LLs in Bs alone are shown.

Figure 2. Chronological changes in HLA-A allele-lacking leukocytes (HLA-LLs) of associated with immunosuppressive therapy

A. Changes in HLA-A allele-lacking granulocytes (HLA-LGs) in three cases are shown. All three cases responded to anti-thymocyte globulin (ATG) plus cyclosporine, and achieved partial response after 12 months of therapy. The percentages of HLA-LGs remained unchanged in all cases except Case 35. Enlarged figures of HLA-LGs are shown in red rectangles. **B**. The percentages of HLA-LLs in the leukocyte lineages of individual HLA-LL(+) patients, as determined at two different time points (1 and 2), are exhibited according to lineage combination patterns. The second samples were obtained 1-40 months after examination of initial samples.

Figure 3. HLA-A allele-lacking leukocytes (HLA-LLs) in glycosylphosphatidylinositol-anchored protein containing (GPI-AP$^+$) and glycosylphosphatidylinositol-anchored protein deficient (GPI-AP$^-$) granulocyte populations

A. The GPI-AP$^+$ and GPI-AP$^-$ granulocytes of two patients possessing HLA-LLs in the GPI-AP$^+$ population alone (Case 8) and in both the GPI-AP$^+$ and GPI-AP$^-$ populations (Case 19) were sorted and subjected to single nucleotide polymorphism (SNP) array analysis. The double lines compatible with 6pLOH were seen only in DNA derived from the GPI-AP$^+$ population of Case 8, while the

double lines were evident in both DNA samples derived from GPI-AP^{+} and GPI-AP^{-} populations of Case 19. **B**. HLA-LLs in different lineages of GPI-AP^{+} and GPI-AP^{-} cells of the three cases who possessed GPI-AP^{-}HLA-LLs are shown. Histograms shown in the red rectangles represent positive results for HLA-LLs.

Figure 4. Response to immunosuppressive therapy (IST) and the prognosis after IST in untreated patients with/without HLA-A allele-lacking leukocytes (HLA-LLs), and in untreated patients homozygous for the HLA-A allele

A. The IST response rate of three untreated groups. The response rate of HLA-LL(+) patients was significantly higher than that of HLA-LL(-) patients (100% vs. 52.2%; *P*=0.033). **B and C. The** overall survival and failure-free survival (FFS) in HLA-LL(+) patients, HLA-LL(-) patients and patients homozygous for the HLA-A allele. The FFS rate in HLA-LL(+) patients was significantly different from that in HLA-LL(-) patients (*P*=0.018) and patients homozygous for the HLA-A allele (*P*=0.022).

Figure 1

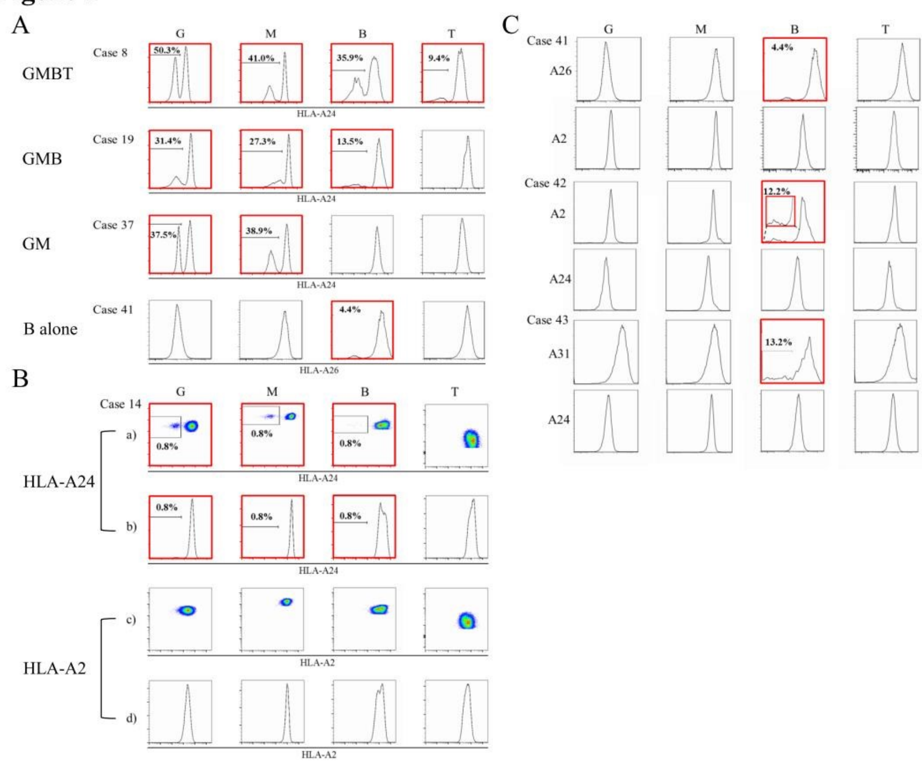
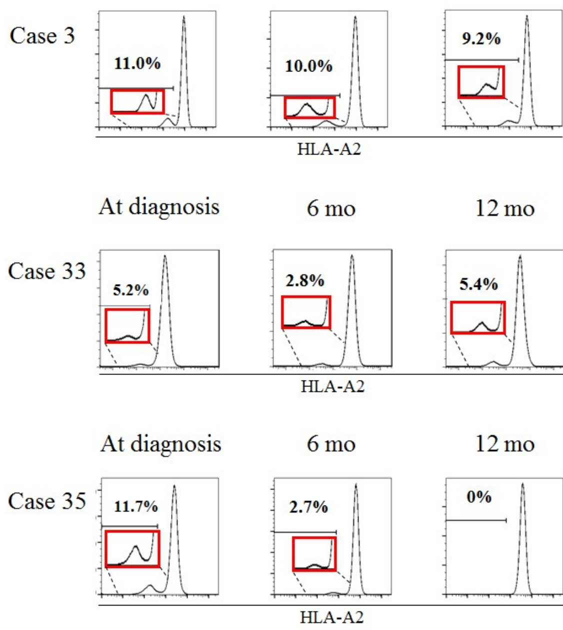


Figure 2



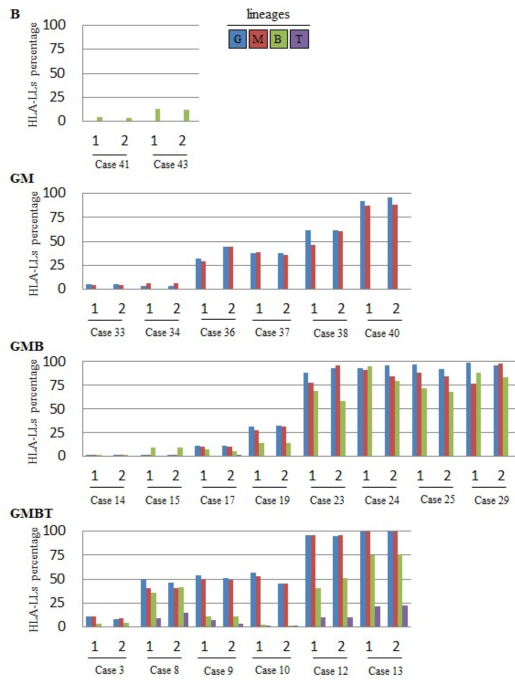


Figure 3

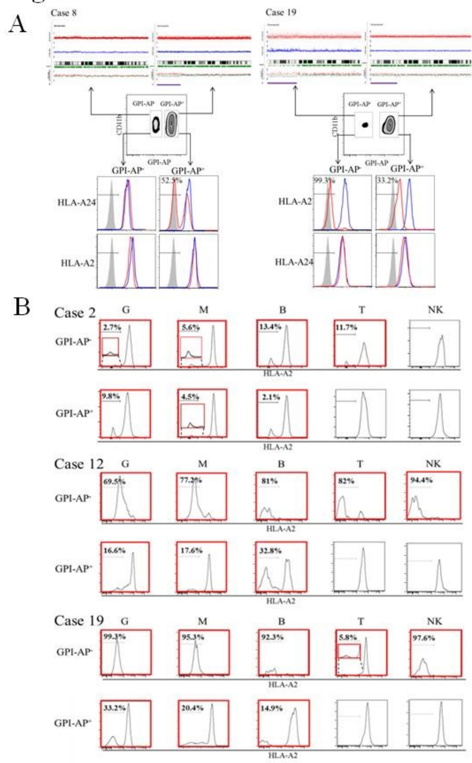
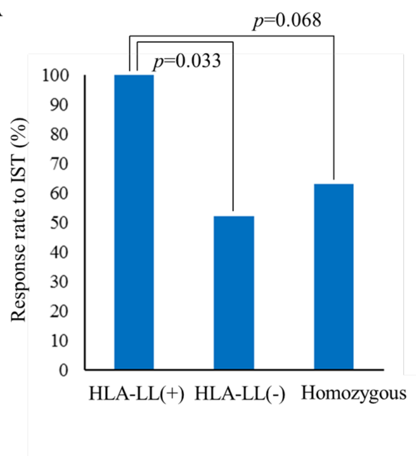


Figure 4

A



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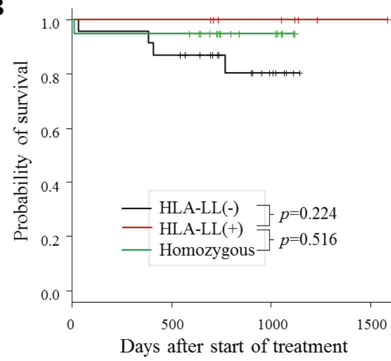

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