Escape hematopoiesis by HLA-B5401-lacking hematopoietic stem progenitor cells in men with acquired aplastic anemia

Leukocytes that lack HLA class I alleles derived from hematopoietic stem and progenitor cells (HSPC) that undergo copy number-neutral loss of heterozygosity of the short arm of chromosome 6 (6pLOH) or HLA allelic mutations are often detected in patients with aplastic anemia (AA). The presence of HLA class I allele-lacking leukocytes provides compelling evidence that cytotoxic T-lymphocytes (CTL) are involved in the development of AA,[1-5] but the precise mechanisms underlying HLA lack and clonal hematopoiesis by such HLA(–) HSPC is unknown.

We recently showed that *B*54:01* was one of three HLA alleles that were most likely to be possessed by 6pLOH$^+$ patients [29% (5/17)] when only patients not carrying *HLA-B*40:02* were analyzed.[5] To gain insight into the mechanism underlying clonal hematopoiesis by HLA-B5401-lacking HSPC, we studied the role of *HLA-B*54:01* in the pathogenesis of AA in a larger number of patients as well as HSPC derived from induced pluripotent stem cells (iPSC) that were generated from an AA patient whose monocytes lacked B5401.

A total of 733 AA patients were enrolled in an observational study to determine the prevalence of HLA class I allele-lacking leukocytes by GeneChip 500 K arrays (Affymetrix, Japan) and droplet digital polymerase chain reaction using a QX200 AutoDG Droplet Digital PCR System (Bio-Rad, Hercules, CA, USA) or a next-generation sequencer (MiSeq; Illumina, San Diego, CA, USA) as previously described.[1,5] Informed consent was obtained from the study participants for the genetic analyses and iPSC generation. The diagnosis and severity of AA were determined according to standard criteria.[6] The characteristics of the AA patient (KANA6) who was selected to generate iPSC from monocytes are described in the *Online Supplementary Material* and in *Online Supplementary Figures S1* and *S2* and *Online Supplementary Tables S1* and *S2*. This study was approved by the ethics committee of the Kanazawa University Institute of Medical, Pharmaceutical, and Health Sciences.

The generation of iPSC (*Online Supplementary Figure S3*), induction of HSPC from iPSC, and transplantation of the induced iPSC-HSPC human CD34$^+$ cells into sub-lethally (150 cGy) irradiated 57BL/6.Rag2^{null}Il2rγ^{null} NOD-Sirpa (BRGS) young mice were carried out according to the methods we described previously. Details are provided in the *Online Supplementary Methods* and the monoclonal antibodies and primer sets used for this study are listed in *Online Supplementary Tables S3-S6*. Statistical analyses of the patients' clinical parameters, 6pLOH determination and calculation were performed as described previously.[1,5] For all iPSC experiments, statistical analyses were performed using the GraphPad Prism software package, version 5.02 (San Diego, CA, USA). The results were analyzed using a Student *t*-test.

The presence of 6pLOH was evaluable in 618 (84.3%) of 733 patients with AA, and 6pLOH was detected in 107 (17.3%) of the patients. The 6pLOH$^+$ patients were assessed for the allelic frequency in their lost haplotypes. Consistent with our previous report,[5] *HLA-B*40:02* was most strongly involved in 6pLOH (46.7%), and *HLA-B*54:01* (10.3%) was the second most frequent HLA-B allele in the lost haplotype (*Online Supplementary Table S7*). When only 6pLOH$^+$ patients not carrying *HLA-B*40:02* were analyzed, the frequency of *HLA-B*54:01* (19.3%) was the highest among all HLA-B alleles included in the lost haplotype (*Online Supplementary Table S8*).

Of the 733 patients with AA in this study cohort, 115 (15.7%) had *HLA-B*54:01*, which is a significantly higher frequency than that previously reported in a general

Table 1. Clinical characteristics of aplastic anemia patients with loss of heterozygosity of 6p carrying *HLA-B*54:01*.

Case	Age (years)	Sex	Severity	Treatments	Response to IST	Missing haplotype				Retained haplotype				PNH cells
						A	B	C	DRB1	A	B	C	DRB1	
1	65	M	VSAA	ATG/CsA	NR	24:02	54:01	01:02	04:05	31:01	15:01	03:03	08:02	(+)
2	71	M	NSAA	AS	NA	11:01	54:01	01:02	04:05	02:06	35:01	03:03	15:01	(+)
3	69	M	NSAA	ATG/CsA/AS	CR	11:01	54:01	01:02	04:05	02:01	40:01	04:01	15:01	(+)
4	77	M	SAA	ATG/CsA	PR	24:02	54:01	01:02	04:05	24:02	15:01	03:03	15:01	(+)
5	71	M	SAA	NA	NA	24:02	54:01	08:01	09:01	02:01	40:06	01:02	15:01	(-)
6 (KANA6)	53	M	NSAA	CsA/AS	CR	24:02	54:01	01:02	04:01	01:01	37:01	06:02	01:01	(+)
7	81	M	SAA	NA	NR	24:02	54:01	01:02	01:01	24:02	52:01	12:02	15:02	NT
8	80	M	NSAA	CsA/ELT	PR	24:02	54:01	03:03	15:02	02:06	35:01	01:02	04:05	(+)
9	18	M	SAA	ATG/CsA	NA	11:01	54:01	03:04	04:05	26:05	40:06	15:02	09:01	(+)
10	55	M	SAA	ATG/CsA/ELT	PR	02:07	54:01	01:02	04:05	24:02	15:18	07:04	12:01	(+)
11	77	F	NSAA	CsA	NA	24:02	54:01	01:02	15:02	24:02	15:07	03:03	04:03	(+)
12	32	M	VSAA	ATG/CsA	PR	11:01	40:02	01:02	08:03	24:02	54:01	03:04	12:01	(-)
13	11	M	SAA	BMT	NR	02:01	40:02	03:04	09:01	24:02	54:01	01:02	04:05	NT
14	30	M	SAA	BMT	NR	31:01	40:02	03:04	15:01	24:02	54:01	01:02	04:05	NT
15	20	F	SAA	ATG/CsA	PR	02:06	39:01	08:03	15:01	24:02	54:01	07:02	04:05	(+)
16	51	F	NSAA	Romiplostim	PR	02:06	46:01	01:02	09:01	24:02	54:01	01:03	15:01	(-)

PNH cells: paroxysmal nocturnal hemoglobinuria cells (glycosylphosphatidylinositol-anchored protein deficient, GPI-AP$^-$ cells); IST: immunosuppressive therapy; M: male; F: female; VSAA: very severe aplastic anemia; NSAA: non-severe aplastic anemia; SAA: severe aplastic anemia; ATG: antithymocyte globulin; CsA: cyclosporine A; AS: anabolic steroids; ELT: eltrombopag; BMT: bone marrow transplantation; NR: no response; NA: not available; CR: complete response; PR: partial response; NT: not tested; B5401(-) leukocytes, leukocytes that lost B5402 expression as a result of 6pLOH or *B*54:01* mutations..

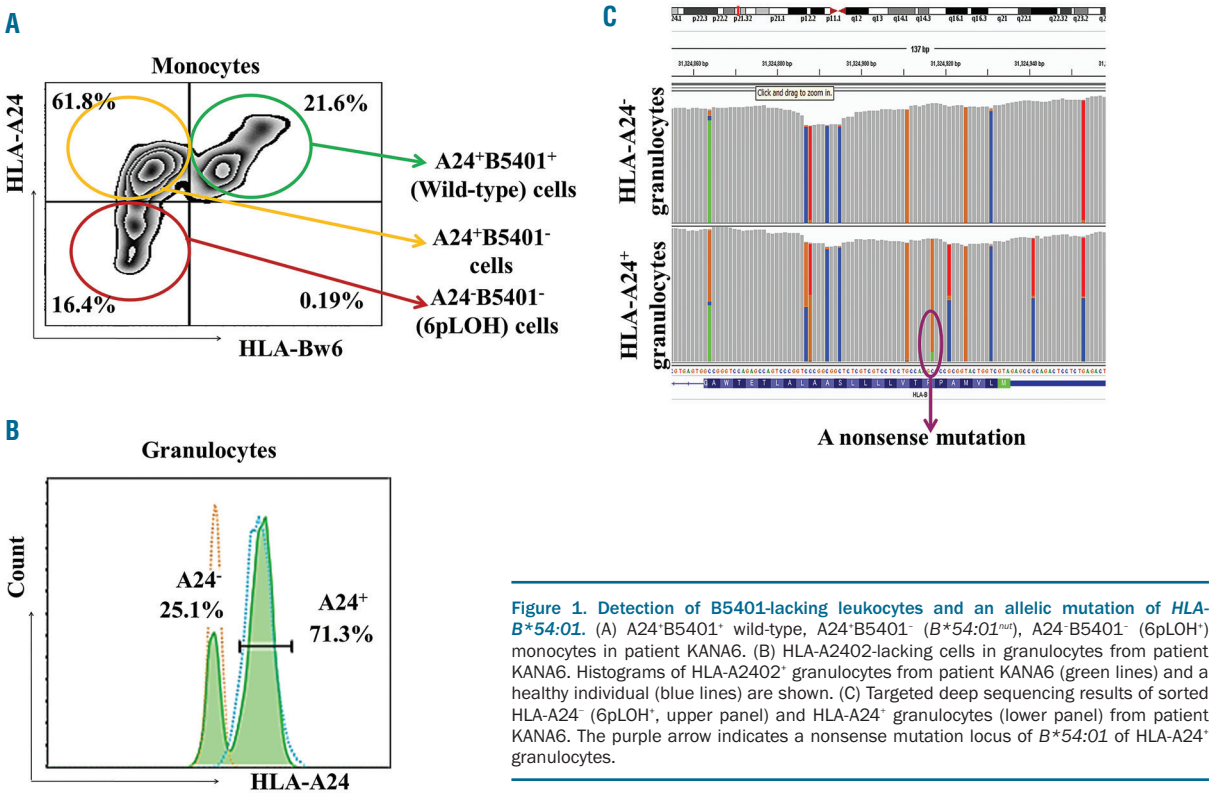
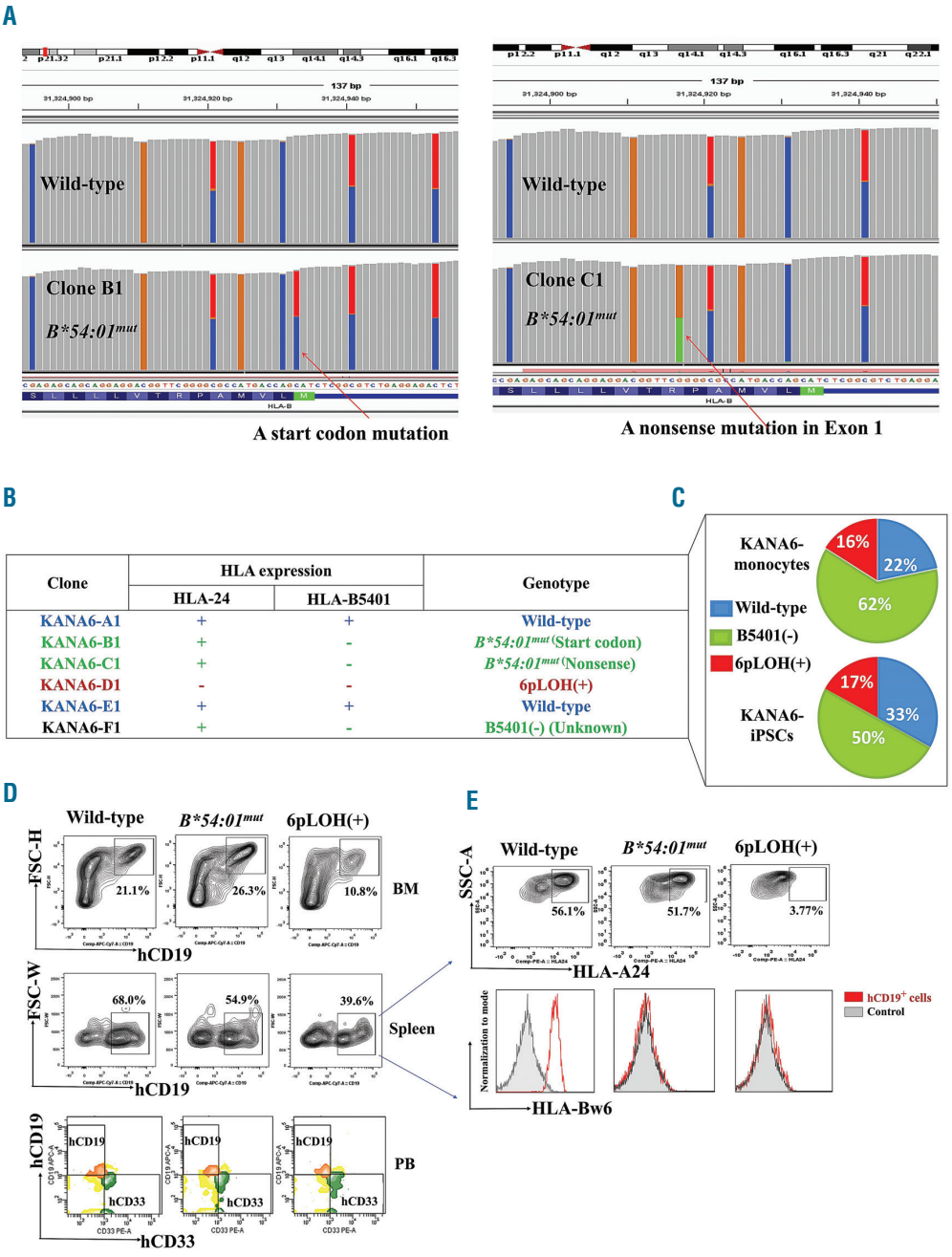


Figure 1. Detection of B5401-lacking leukocytes and an allelic mutation of *HLA-B*54:01*. (A) A24$^+$B5401$^+$ wild-type, A24$^+$B5401$^-$ (*B*54:01^{mut}*), A24$^-$B5401$^-$ (6pLOH$^+$) monocytes in patient KANA6. (B) HLA-A2402-lacking cells in granulocytes from patient KANA6. Histograms of HLA-A2402$^+$ granulocytes from patient KANA6 (green lines) and a healthy individual (blue lines) are shown. (C) Targeted deep sequencing results of sorted HLA-A24$^-$ (6pLOH$^+$, upper panel) and HLA-A24$^+$ granulocytes (lower panel) from patient KANA6. The purple arrow indicates a nonsense mutation locus of *B*54:01* of HLA-A24$^+$ granulocytes.

Japanese population (7.5%).[7] Among the 97 patients carrying *HLA-B*54:01* whose leukocytes were evaluable for 6pLOH, 16 (16.5%) were 6pLOH$^+$. This HLA-B allele was included in the lost haplotype in all 11 (100%) 6pLOH$^+$ patients who did not possess *HLA-B*40:02*- or *HLA-A*02:06*-containing haplotypes. Notably, ten of the 11 6pLOH$^+$ patients with *HLA-B*54:01* were male (Table 1). Five of the 11 patients with 6pLOH involving *HLA-B*54:01*-containing haplotypes were heterozygous for the Bw phenotype (Bw4/Bw6) and were therefore evaluable for the presence of B5401 (Bw6)-lacking leukocytes. Flow cytometry using anti-A24 and anti-Bw6 antibodies revealed that about 60% of the monocytes were A24$^+$Bw6$^-$, while 16% were A24$^-$Bw6$^-$ (6pLOH) in the peripheral blood of a patient, KANA6 (Figure 1A). Such A-allele$^+$Bw6$^-$ leukocytes were undetectable in any of the other four patients. Targeted deep sequencing of the HLA class I alleles in sorted HLA-A24$^+$ (non-6pLOH) granulocytes from patient KANA6 and whole granulocytes from the other four 6pLOH$^+$ patients revealed a nonsense mutation of *HLA-B*54:01* in patient KANA6's granulocytes (Figure 1B, C) but no mutation in any of the other four patients (*data not shown*). None of the missing *B*54:01*-containing haplotype of the four patients had high risk HLA-A alleles such as *A*02:06* and *A*31:01:1* Taken together, these findings suggest that *B*54:01* is an important target allele of CTL among different alleles in the lost haplotype.

To characterize HSPC clones that produce B5401$^+$ and B5401$^-$ leukocytes in patient KANA6, we generated six iPSC clones from patient KANA6's monocytes and induced CD34$^+$ cells from each iPSC. Genotyping and phenotyping of iCD34$^+$ cells revealed two clones (A1 and E1) to be wildtype, one clone (D1) to be 6pLOH$^+$, and two clones (B1 and C1) to have a start codon and a nonsense mutations of *B*54:01*, respectively (Figure 2A and *Online Supplementary Figures S4-S6*). The start loss mutation of *B*54:01* in clone B1 was detected in the sorted A24$^+$ granulocytes from patient KANA6 at a variant allele frequency of 2% when we reanalyzed the sequencing results. The remaining clone (F1) did not show any somatic mutation in *B*54:01* despite the absence of Bw6 expression. Figure 2B summarizes the features of the six iPSC clones. The proportions of the three different monocyte subpopulations in the peripheral blood were similar to those of iPSC clones that were established from the patient's monocytes (Figure 2C), suggesting that B5401$^-$ leukocytes are an oligoclonal population consisting of 6pLOH$^+$ cells and several B5401$^-$ only cells with different *B*54:01* mutations.

*B*54:01^{mut}* HSPC may have distinct proliferation or differentiation capacities from wildtype HSPC, given that they support hematopoiesis with a few cells for a long time. To test this hypothesis, we compared the clonogenic potentials of iCD34$^+$ cells derived from wildtype (E1), *B*54:01^{mut}* (C1), and 6pLOH$^+$ (D1) clones. All three clones showed similar differentiation patterns in the presence of StemPro culture medium (*Online Supplementary Figure S7*) OP9- and WEHI-conditioned medium (*Online Supplementary Figures S8* and *S9*) and gave rise to various types of colonies, including CFU-GM, BFU-E, CFU-M, and CFU-GEMM, with similar compositions at comparable plating efficiencies (*Online Supplementary Figure S10A,B*). Among mice injected with wildtype, *B*54:01^{mut}*, and 6pLOH$^+$ iCD34$^+$ cells at 12 weeks of transplantation, all showed multilineage reconstitution by myeloid cells (CD33$^+$), B cells (CD19$^+$), and T cells (CD3$^+$) in the bone marrow (*Online Supplementary Figure S11A*), spleen (*Online Supplementary Figure S11B*), and peripheral blood (*Online Supplementary Figure S11C*), with comparable percentages of CD33$^+$, CD19$^+$, or CD3$^+$ cells in the mouse organs (Figure 2D and *Online Supplementary Figure S12A, B*). hCD45$^+$ cells collected



Clone	HLA expression		Genotype
	HLA-24	HLA-B5401	
KANA6-A1	+	+	Wild-type
KANA6-B1	+	-	*B*54:01*^{mut} (Start codon)
KANA6-C1	+	-	*B*54:01*^{mut} (Nonsense)
KANA6-D1	-	-	6pLOH(+)
KANA6-E1	+	+	Wild-type
KANA6-F1	+	-	B5401(-) (Unknown)

Figure 2. Characterization of hematopoietic stem and progenigor cells derived from KANA6's induced pluripotent stem cells with different HLA genotypes. (A) Targeted deep sequencing results for a B5401^{-} induced pluripotent stem cell (iPSC) clone B1(left panel) and iPSC clone C1 (right panel) showing a start codon and nonsense mutations, respectively, in *B*54:01*. The results of the HLA-B allelic sequencing of a wild-type iPSC clone (clone E1) are shown as a control. The arrow indicates a mutation locus of the *B*54:01*^{mut} clone. (B) A summary of the six iPSC clones generated from patient KANA6's monocytes. (C) Pie charts showing that the proportions of three different monocyte subpopulations in the peripheral blood are similar to those of patient KANA6's iPSC clones. (D) Engraftment of myeloid and lymphoid lineage cells in different organs. Human B (hCD19^{+}) and myeloid (hCD33^{+}) cells gated on hCD45^{+} cells in the bone marrow (BM), spleen, and peripheral blood (PB) of mice transplanted with wild-type, *B*54:01*^{mut} or 6pLOH^{+} iCD34^{+} cells. (E) HLA expression profiles of human B cells isolated from the spleen of the transplanted mice with the indicated iCD34^{+}, HLA-A2402 (upper panels), and HLA-Bw6 (lower panels) expression by CD19^{+} lymphoid cells in the spleen.

from the spleen in the mice transplanted with wildtype, *B*54:01*^{mut}, or 6pLOH^{+} iCD34^{+} cells showed corresponding phenotypes, indicating that each mouse carried human cells with similar HLA profiles to the parental iCD34^{+} cells (Figure 2E).

The current study suggests that *HLA-B*54:01* may be critically involved in the presentation of auto-antigens that are unique to male AA patients. The reason for a male predominance in the incidence of *HLA-B*54:01*-lacking leukocytes remains unclear. It has been well documented that androgens play important roles in hematopoiesis by enhancing the G1-S transit rate in the cell cycle, telomerase activity, and survival of human HSPC.[8] High androgen levels in men may activate their HSPC and thereby make them more susceptible than women to CTL attack restricted by B5401. Androgen

receptors on prostate cancer cells have been shown to be upregulated by deprivation of androgens and serve as a target of CTL.[9,10] Androgen receptors on HSPC may also be upregulated by a change in the serum androgen level and may induce CTL specific to HSPC in men carrying *HLA-B*54:01*.

Similarly to our previous study in an AA patient with HLA-B4002-lacking leukocytes,[11] iCD34$^+$ cells derived from iPSC that were generated from patient KANA6's monocytes showed similar clonogenic capacities both *in vivo* and *in vitro*, regardless of their phenotype. B5401-lacking HSPC showed a hematopoietic reconstitution ability similar to that of wildtype HSPC under *in vivo* conditions of a lack of pathogenic T cells. These findings suggest that HSPC that have undergone 6pLOH or allelic mutations in *B*54:01* do not have a proliferative advantage over wildtype HSPC; instead they acquire a survival advantage by escaping the CTL attack specific to antigens presented by B5401.

This study using cell reprogramming technology confirmed our previous findings that mechanisms underlying the lack of class I HLA molecules in HSPC targeted by CTL include not only 6pLOH but also allelic mutations of the target class I alleles that are involved in auto-antigen presentation.[5] The establishment of iPSC clones with different allelic mutations in HLA which were not revealed by targeted deep sequencing of mature granulocytes from patient KANA6 suggests that studying monocyte-derived iPSC may be useful for dissecting the clonal architecture of patients with bone marrow failure and clarifying the mechanisms underlying the progression to advanced clonal diseases *in vivo* and at the molecular level.[12,13] Our xenograft model may also be useful for future studies examining the sensitivity of HSPC with different B5401 phenotypes to CTLs specific to auto-antigens.

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