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Phenotype-directed allele-specific flow cytometry identifies HLA-loss relapse after haploidentical hematopoietic cell transplantation for adult T-cell leukemia/lymphoma

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Authors' contributions: TK and YZ contributed equally as co-first authors. YZ designed the study. TK collected the patient's clinical data and was primarily responsible for the patient's clinical care. YZ, HK, SY, TY, TI, HM, HT, and TM provided clinical care for the patient. YZ and KH performed the investigations. TK and YZ wrote the manuscript. All authors reviewed and approved the final version of the manuscript.

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Running heads: HLA-loss relapse in ATL after haploidentical HCT

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Main Text

HLA-loss relapse after haploidentical hematopoietic cell transplantation (HCT) is a well-established immune escape mechanism in acute leukemias, in which relapsed cells lose the mismatched HLA alleles targeted by donor-derived alloreactive T cells.[1,2] This mechanism is primarily caused by copy-neutral loss of heterozygosity (LOH) involving chromosome 6p, where the HLA loci are inherited as a haplotype.

Adult T-cell leukemia/lymphoma (ATL) is a difficult-to-treat mature T-cell neoplasm caused by infection with human T-cell leukemia virus type 1.[3,4] Allogeneic HCT is a potentially curative option for selected patients; however, HLA-loss relapse in ATL remains poorly characterized. We describe a patient with acute-type ATL in whom phenotype-directed HLA allele-specific flow cytometry rapidly identified a selective loss of mismatched HLA expression in low-burden relapsed ATL cells after haploidentical HCT, thereby informing the decision to withhold donor lymphocyte infusion (DLI) from the original donor. The study was approved by the institutional review board of Kanazawa University Hospital and was conducted in accordance with the Declaration of Helsinki.

A 59-year-old woman was admitted with fever and malaise. Her peripheral blood contained 14% abnormal lymphocytes, and the serum soluble interleukin-2 receptor (sIL-2R) level was 6363 U/mL. The patient was diagnosed with acute-type ATL. Positron emission tomography/computed tomography (PET/CT) showed diffuse hypermetabolic bone marrow involvement. She received three cycles of CHOP chemotherapy followed by one cycle of VCAP-AMP-VECP,[5] but PET/CT revealed progressive disease with an increased splenic uptake. Five months after diagnosis, she underwent peripheral blood HCT from an HLA-haploidentical related donor in a non-remission state. The conditioning regimen consisted of fludarabine (125

mg/m²), melphalan (80 mg/m²), and total body irradiation (4 Gy). Graft-versus-host disease (GVHD) prophylaxis consisted of post-transplant cyclophosphamide (80 mg/kg), tacrolimus, and mycophenolate mofetil. Neutrophil engraftment was achieved on day 17, and a short tandem repeat analysis performed on day 30 showed complete donor chimerism. Post-transplant complications included grade 1 acute skin GVHD, BK virus-associated hemorrhagic cystitis, and organizing pneumonia, which required corticosteroid therapy. PET/CT on day 115 showed a complete metabolic response, and the sIL-2R level decreased to 258 U/mL (Figure 1A).

Relapse was suspected on day 181 because the sIL-2R level increased to 1853 U/mL and low-level circulating abnormal lymphocytes were noted. A short tandem repeat analysis of peripheral blood showed a small recipient-derived peak in the magnetic bead-sorted $CD3^+$ T-cell fraction, whereas granulocytes showed complete donor chimerism (Figure 1B). Flow cytometry using the CADM1/CD7 profile identified $CD3^+CD4^+CD7^-CADM1^+$ ATL-phenotype cells,[6,7] accounting for 1.1% of circulating white blood cells and 2.7% of $CD3^+$ T cells. Cytospin preparations of cells isolated from this population by fluorescence-activated cell sorting (FACS) revealed the enrichment of large atypical lymphoid cells with irregular or multilobated nuclei, consistent with relapsed ATL (Figure 1C).

The recipient and donor HLA genotypes are shown in Figure 2A. To evaluate whether ATL cells had lost the recipient-specific mismatched haplotype, we examined HLA allele-specific surface expression by flow cytometry using monoclonal antibodies against HLA-A2, encoded on the mismatched haplotype, and HLA-A24, encoded on a shared haplotype. At diagnosis, $CD3^+CD4^+CD7^-CADM1^+$ ATL-phenotype cells in cryopreserved peripheral blood mononuclear cells expressed both HLA-A2 and HLA-A24. At relapse, however, this ATL fraction lacked HLA-A2 expression while retaining strong HLA-A24 expression, whereas non-ATL cell

fractions retained HLA-A2 (Figure 2B). Targeted sequencing of FACS-sorted ATL cells confirmed LOH involving the recipient-specific mismatched HLA haplotype (Figure 2C).

Based on these findings, DLI from the original donor, which had been planned to treat post-transplant relapse, was withheld. Prior acute GVHD, corticosteroid-treated organizing pneumonia, and concurrent respiratory syncytial virus infection further supported this decision by increasing the expected risks associated with DLI. The disease subsequently progressed, with hypercalcemia, acute kidney injury, marked sIL-2R elevation, and increasing circulating abnormal lymphocytes. She received three cycles of dose-reduced CHP-like chemotherapy; however, abnormal lymphocytes persisted. At 10 months after HCT, she died of respiratory failure due to progressive ATL.

To our knowledge, HLA-loss relapse after haploidentical HCT has not been previously documented in ATL with both phenotypic and genetic evidence. Although HLA alterations have been reported in ATL at diagnosis,[8] paired analysis of diagnostic and relapsed ATL cells in the present patient demonstrated post-transplant selection of an HLA-loss clone under donor-derived immune pressure.

HLA-loss detection has immediate therapeutic implications. DLI aims to augment donor-derived alloreactivity against leukemic cells, to which the recognition of mismatched HLA alleles contributes after haploidentical HCT. When relapsed cells lose all mismatched HLA alleles through 6p LOH, DLI from the original donor is biologically unlikely to exert meaningful graft-versus-ATL effects, while the risk of GVHD remains. This is supported by a multicenter study showing that HLA-loss relapse abrogates the efficacy of DLI from the original donor, whereas second allogeneic HCT from a different donor is associated with a survival advantage.[2] Accordingly, recommendations from the European Society for Blood and Marrow

Transplantation advise considering HLA-loss testing in impending or overt relapse after HCT with an HLA mismatch in the graft-versus-host direction, particularly before DLI or a second allograft.[9] Nevertheless, a recent international survey found that HLA-loss testing was performed in only 17.7% of cases before DLI and 21% before a second allogeneic HCT.[10]

HLA allele-specific flow cytometry provided a practical approach for evaluating HLA loss in this low-burden relapse. DNA-based methods, including recently developed next-generation sequencing-based assays, detect HLA-loss relapse by comparing recipient chimerism at markers within and outside the HLA region.[10-13] However, their sensitivity and interpretability may be limited when low-burden tumor cells are diluted by donor-derived cells or non-malignant recipient cells. In contrast, flow cytometry enables the direct assessment of HLA allele expression within phenotypically defined tumor populations and provides rapid results, often within one day. HLA allele-specific flow cytometry has been established as a sensitive method for detecting minor and heterogeneous HLA-loss hematopoietic populations in acquired aplastic anemia.[14,15] The present case shows that this flow-based HLA-loss analysis can be adapted to detect post-transplant HLA-loss relapse in hematological malignancies, thereby supporting timely decisions regarding DLI from the original donor.

HLA allele-specific flow cytometry has several limitations. Informative antibodies are mainly available for HLA-A and HLA-B and cover only a subset of their alleles. Even when a targetable HLA allele is present, its loss cannot be detected when all applicable antibodies also recognize the retained haplotype, as can occur with HLA homozygosity or serologically cross-reactive HLA alleles. Using the eight commercially available antibodies, the expected coverage for HLA-loss detection ranged from 45% to 74% across populations, reflecting population-specific differences in HLA-A/B haplotype frequencies (Figure 3). However, coverage could be further

expanded through the development of additional HLA allele-specific antibodies and the validation and incorporation of other commercially available antibodies.

In addition to these antibody-related constraints, the flow cytometry-based assay requires viable cells and a phenotypically definable malignant population. Flow cytometry alone cannot define the genomic extent or mechanism of HLA loss. Nevertheless, by providing a sorted malignant-cell population for confirmatory genomic analysis, it can overcome the dilution-related sensitivity limitations of bulk DNA-based assays. Accordingly, flow cytometry should complement, rather than replace, DNA-based HLA-loss assays.

In summary, HLA-loss relapse through immune escape can occur in ATL after haploidentical HCT. Even when the circulating tumor burden is low, phenotype-directed HLA allele-specific flow cytometry can rapidly identify HLA-loss relapse and guide timely decisions regarding DLI and donor selection for subsequent cellular therapy.

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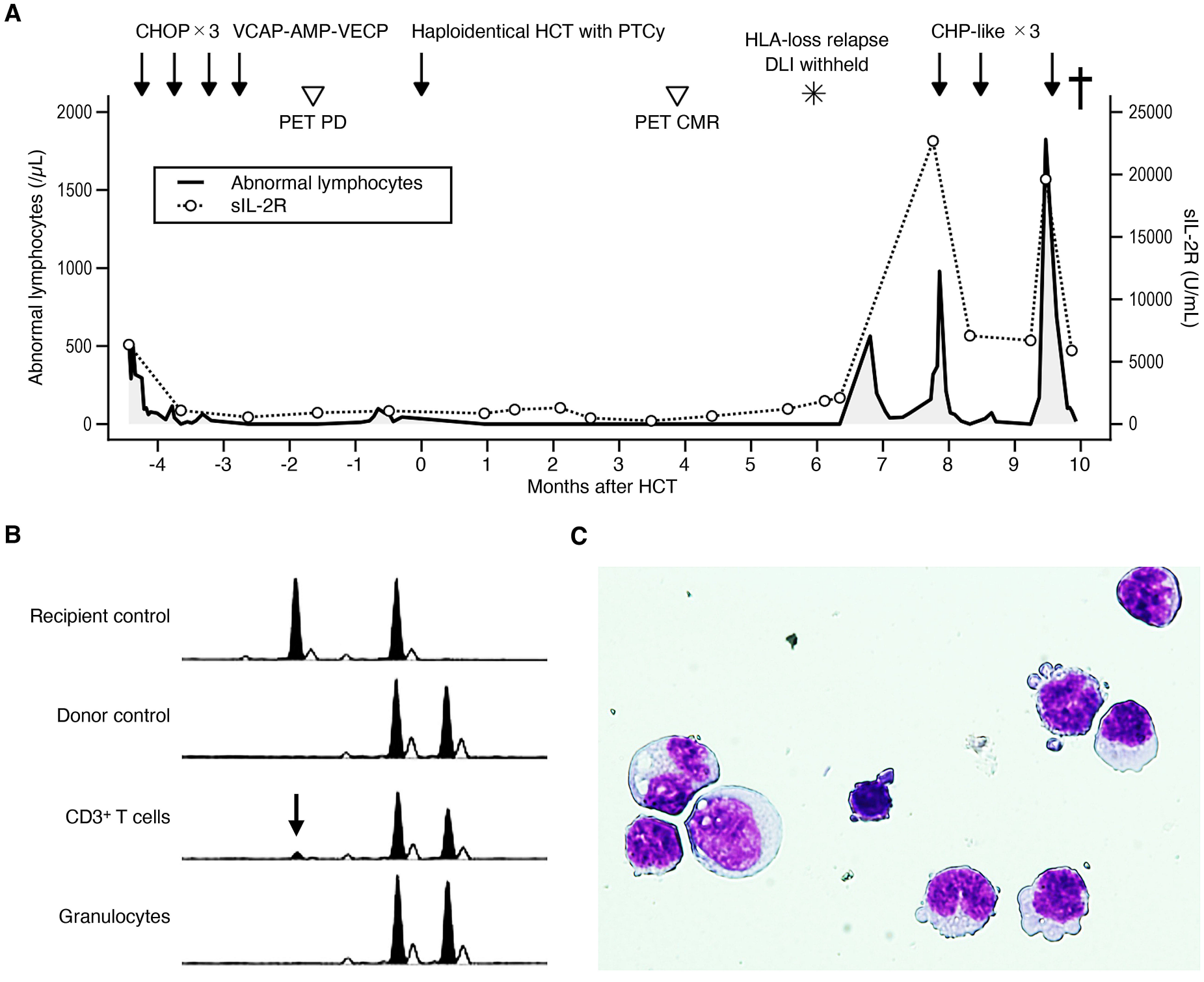
Figure Legends

Figure 1. Clinical course and identification of relapsed ATL cells. **A** Clinical course after diagnosis and haploidentical hematopoietic cell transplantation (HCT). **B** Short tandem repeat analysis at relapse showing a small recipient-derived signal in the magnetic bead-sorted $CD3^+$ T-cell fraction and complete donor chimerism in granulocytes. **C** May-Giemsa-stained cytospin preparation of sorted $CD3^+CD4^+CD7^-CADM1^+$ adult T-cell leukemia/lymphoma (ATL)-phenotype cells from the relapse sample analyzed in Figure 2B, showing enriched large atypical lymphoid cells with irregular or multilobated nuclei. Abbreviations: CHOP, cyclophosphamide, doxorubicin, vincristine, and prednisolone; CHP-like, cyclophosphamide, doxorubicin, and prednisolone-based chemotherapy; CMR, complete metabolic response; DLI, donor lymphocyte infusion; PD, progressive disease; PET, positron emission tomography; PTCy, post-transplant cyclophosphamide; sIL-2R, soluble interleukin-2 receptor; VCAP-AMP-VECP, multidrug chemotherapy consisting of vincristine, cyclophosphamide, doxorubicin, prednisolone, ranimustine, vindesine, etoposide, and carboplatin.

Figure 2. HLA allele-specific flow cytometry and sequencing analyses at diagnosis and relapse. **A** HLA genotypes of the recipient and haploidentical donor. Mismatched haplotypes are shown in bold. **B** Flow cytometric analysis of the HLA-A2 and HLA-A24 expression in ATL-phenotype and non-ATL control cell populations at diagnosis and relapse. $CD7^-CADM1^+$ ATL-phenotype cells retained both HLA-A2 and HLA-A24 at diagnosis but selectively lost HLA-A2 at relapse, whereas non-ATL fractions retained HLA-A2. **C** Targeted sequencing of sorted ATL cells showed retained HLA heterozygosity at diagnosis and loss of heterozygosity involving the recipient-specific mismatched HLA haplotype at relapse. Colored vertical bars indicate the fractions of reads carrying nucleotides that differ from the hg19 reference genome, with colors

corresponding to A, T, G, and C. Amino acid residues predicted from the hg19 reference sequence are shown above each HLA gene. Abbreviations: PBMC, peripheral blood mononuclear cells.

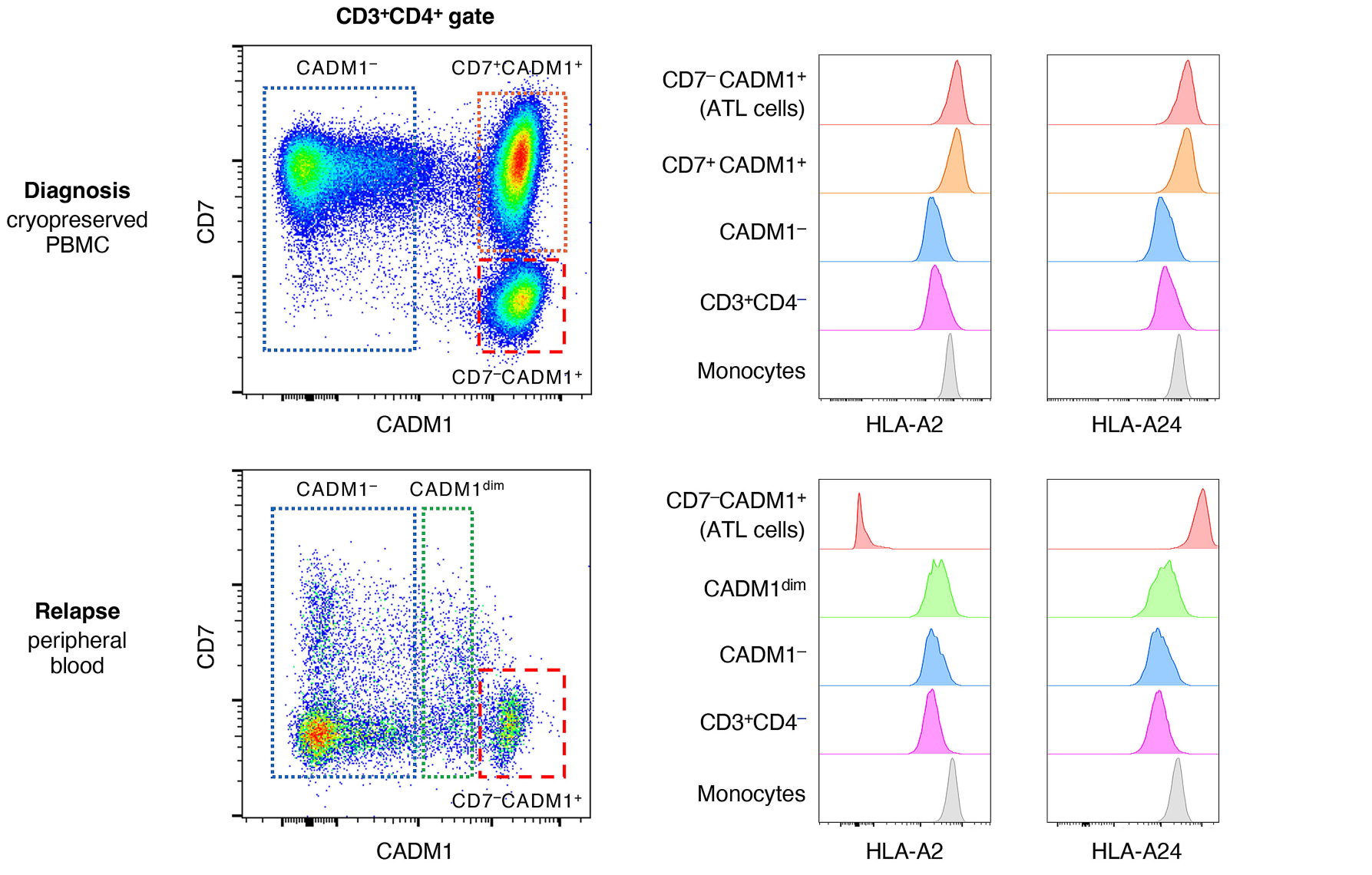
Figure 3. Expected population-specific coverage of HLA-loss detection using eight HLA class I allele-specific monoclonal antibodies. Stacked bars show the expected proportions of mismatched HLA-A/B haplotypes detectable using selected commercially available HLA allele-specific antibodies, including A2 (REA517), A2/A28 (REA142), A3 (REA950), A9 (REA127), B8 (REA145), B7/B27 (REA176), and B12 (REA138) from Miltenyi Biotec, and A30/A31 (BIH0087) from One Lambda. The panel was restricted to antibodies with validated and relatively specific reactivity and does not represent all available HLA class I antibodies. All possible combinations of potentially lost and retained haplotypes were evaluated and weighted by the product of their population-specific haplotype frequencies. An antibody was considered usable when it recognized an HLA antigen encoded by the lost haplotype but not by the retained haplotype. “Undetectable (no targetable HLA)” indicates absence of an HLA antigen recognized by any antibody in the panel, whereas “Undetectable (shared specificity)” indicates that all applicable antibodies also recognized the retained haplotype. “A2 & A2/A28” indicates combinations detectable by both A2-reactive antibodies, whereas “Multiple” indicates combinations detectable by two or more antibodies in any other combination. Populations are ordered by the combined proportion of the two undetectable categories. Haplotype-frequency data were obtained from HLA-net for European populations (https://hla-net.eu/), the HLA Laboratory for the Japanese population (https://hla.or.jp/), and the NMDP dataset for ancestry groups in the United States (https://zenodo.org/records/17966993).


A
CHOP × 3
VCAP-AMP-VECP
Haploidentical HCT with PTCy
HLA-loss relapse
DLI withheld
CHP-like × 3
PET PD
PET CMR
Abnormal lymphocytes
sIL-2R
Abnormal lymphocytes (/μL)
sIL-2R (U/mL)
Months after HCT
B
Recipient control
Donor control
CD3+ T cells
Granulocytes
C

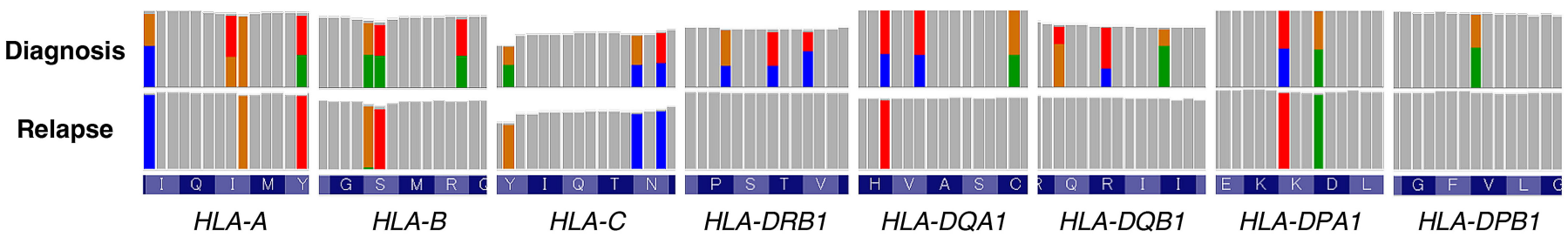
A

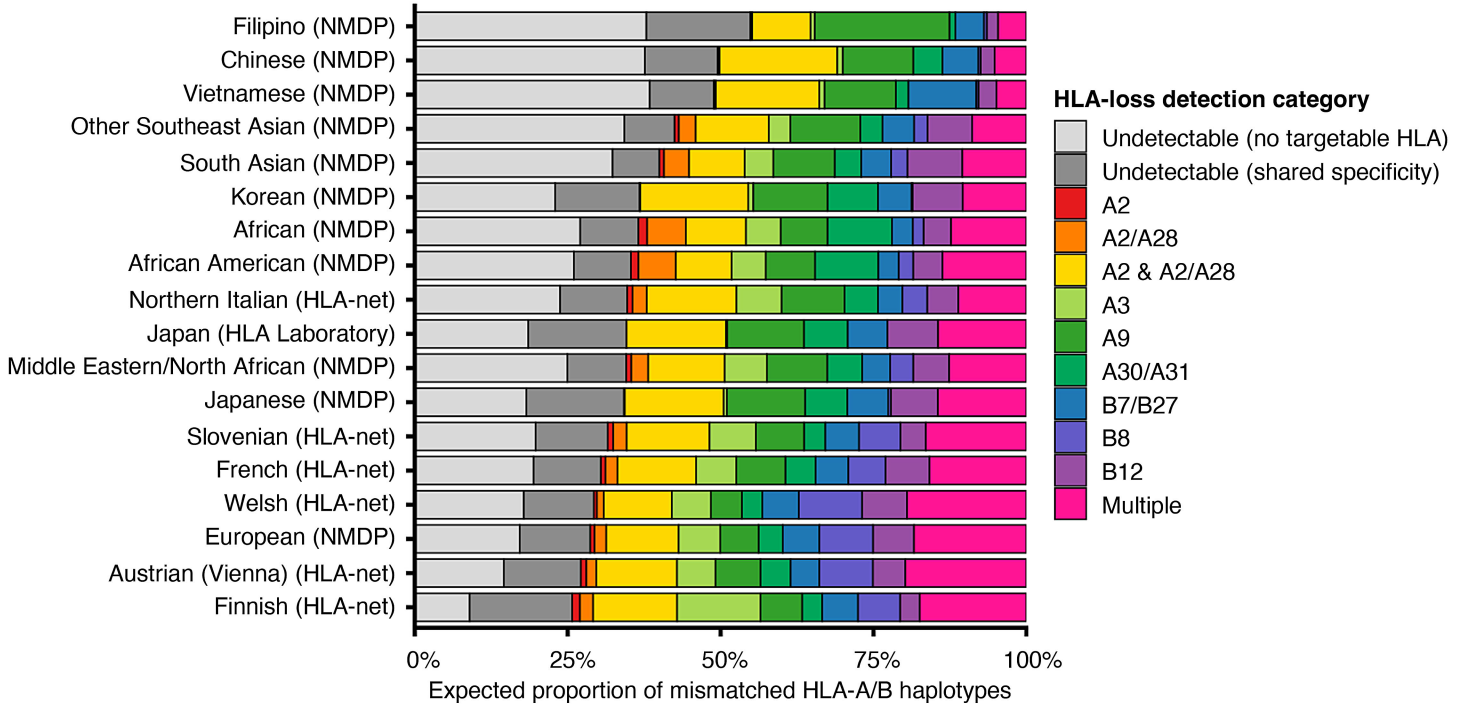
	HLA-A	HLA-B	HLA-C	HLA-DRB1	HLA-DQA1	HLA-DQB1	HLA-DPA1	HLA-DPB1
Recipient	***02:01***	***51:01***	***14:02***	***14:03***	***05:01***	***03:01***	***02:02***	***05:01***
	24:02	*52:01*	*12:02*	*15:02*	*01:03*	*06:01*	*02:01*	*09:01*
Donor	***02:06***	***44:03***	***14:03***	***13:02***	***01:02***	***06:04***	***01:03***	***04:01***
	24:02	*52:01*	*12:02*	*15:02*	*01:03*	*06:01*	*02:01*	*09:01*

B



C




Filipino (NMDP)
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African American (NMDP)
Northern Italian (HLA-net)
Japan (HLA Laboratory)
Middle Eastern/North African (NMDP)
Japanese (NMDP)
Slovenian (HLA-net)
French (HLA-net)
Welsh (HLA-net)
European (NMDP)
Austrian (Vienna) (HLA-net)
Finnish (HLA-net)
0%
25%
50%
75%
100%
Expected proportion of mismatched HLA-A/B haplotypes
HLA-loss detection category
Undetectable (no targetable HLA)
Undetectable (shared specificity)
A2
A2/A28
A2 & A2/A28
A3
A9
A30/A31
B7/B27
B8
B12
Multiple