

T-cell receptor diversity is selectively skewed in T-cell populations of patients with Wiskott-Aldrich syndrome

Junfeng Wu, MD,^a Dawei Liu, MS,^a Wenwei Tu, MD, PhD,^b Wenxia Song, PhD,^{a,c} and Xiaodong Zhao, MD, PhD^a

Chongqing and Hong Kong, China, and College Park, Md

Background: Wiskott-Aldrich syndrome (WAS) is a severe disorder characterized by thrombocytopenia, eczema, immunodeficiency, and increased risk of autoimmune disease and lymphoid malignancies. The immunodeficiency caused by a lack of WAS protein expression has been mainly attributed to defective T-cell functions. Whether WAS mutations differentially influence the T-cell receptor (TCR) diversity of different T-cell subsets is unknown.

Objective: We aimed to identify the degree and pattern of skewing in the variable region of the TCR β -chain (V β) in different T-cell subsets from patients with WAS.

Methods: The TCR repertoire diversity in total peripheral T cells, sorted CD4⁺ and CD8⁺ T cells, and CD45RA⁺ (CD45RA⁺CD45RO⁻ cells) and CD45RO⁺ (CD45RA⁻CD45RO⁺ cells) CD4⁺ and CD8⁺ T cells from patients with WAS and age-matched healthy control subjects was analyzed and compared by using spectratyping of complementarity-determining region 3. The complementarity-determining region 3 of TCR β transcripts in CD45RA⁺CD4⁺ and CD45RA⁺CD8⁺ T cells, CD45RO⁺CD4⁺ T cells, CD8⁺ terminally differentiated effector memory T (Temra) cells, and naive CD8⁺ T cells (CD8⁺CD45RO⁻CCR7⁺ cells) from patients and control subjects were analyzed and compared by using high-throughput sequencing.

Results: The TCR repertoire diversity in CD45RO⁺CD4⁺ T cells and CD8⁺ Temra cells of patients with WAS was significantly skewed in comparison with that seen in age-matched control subjects.

Conclusion: Our results indicate that WAS gene mutations selectively influence TCR repertoire development or expansion in CD45RO⁺ (memory) CD4⁺ T cells. (J Allergy Clin Immunol 2014;■■■:■■■-■■■.)

Key words: Wiskott-Aldrich syndrome, T-cell receptor repertoire diversity, high-throughput sequencing

Abbreviations used

CDR3: Complementarity-determining region 3
D50: Diversity 50
Ds: Simpson index of diversity
H': Shannon-Wiener index
HTS: High-throughput sequencing
PE: Phycoerythrin
TCR: T-cell receptor
Temra: Terminally differentiated effector memory T
WAS: Wiskott-Aldrich syndrome
WASP: Wiskott-Aldrich syndrome protein

Wiskott-Aldrich syndrome (WAS) is a complex and severe immune disorder characterized by thrombocytopenia, eczema, immunodeficiency, and increased risk of autoimmune diseases and lymphoid malignancies.¹ WAS affects 1 to 10 in every million male newborns.² The disease is caused by mutations in the WAS gene on the X-chromosome, which encodes the Wiskott-Aldrich syndrome protein (WASP).³ WASP is a hematopoietic specific regulator of actin nucleation and is activated in response to signaling arising at the cell membrane.⁴ The average life expectancy of patients with mutations that abolish WASP expression is approximately 15 years.⁵ WAS gene mutations can also cause X-linked thrombocytopenia, which is considered an attenuated form of WAS and is characterized by low platelet counts and minimal or no immunodeficiency⁶; however, X-linked thrombocytopenia can evolve into WAS over time.⁷ Because WASP is expressed in most hematopoietic cells, WAS mutations affect multiple cell lineages. However, the most prominent immunologic symptoms are thought to be mainly attributable to defective T-cell function.

WASP plays a key role in T-cell activation on engagement of the T-cell receptor (TCR)⁸⁻¹³ and the costimulatory molecule CD28.¹⁴ T cells from patients with WAS are defective in signaling, which leads to impaired cell proliferation and IL-2 production.^{9,11,13,15} WASP is also critical for T-cell chemotaxis induced by *in vitro* stimulation¹⁶ and *in vivo* homing to secondary lymphoid organs.¹⁷ Patients with WAS have a high risk of T-cell lymphopenia, probably caused by abnormal T-cell development and increased apoptosis.¹⁸⁻²⁰

Although defective T-cell activation has been implicated as a major cause of immune deficiency in patients with WAS, whether the signaling defects lead to alterations in the T-cell repertoire has not been extensively studied. Two previous studies suggested that the TCR diversity in patients with WAS mutations is skewed.^{21,22} However, because these studies analyzed the TCR diversity in total peripheral T cells, whether WAS mutations differentially influence the TCR diversity of different T-cell subsets is unknown.

From ^athe Ministry of Education Key Laboratory of Child Development and Disorders, Children's Hospital of Chongqing Medical University; ^bthe Department of Paediatrics and Adolescent Medicine, LKS Faculty of Medicine, University of Hong Kong; and ^cthe Department of Cell Biology and Molecular Genetics, University of Maryland, College Park.

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Corresponding author: Xiaodong Zhao, MD, PhD, Division of Immunology, Children's Hospital, Chongqing Medical University, Chongqing 400014, China. E-mail: zhaodx530@aliyun.com.

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TABLE I. Patients' characteristics

Patient ID	Age at onset	Age at diagnosis	Age at sample collection	Clinical score	Protein expression	Platelet count ($\times 10^9/L$)	WAS gene mutation location	Mutation	Codon change or predicted splicing abnormality
P1	9 d	2 mo	2 mo	3	Absent	46	Exon 1	71 C>T	R13X
P2	2 d	2 mo	2 mo/8 mo	3	Absent	13	Intron 8	IVS8-1 G>A	Predicted splicing abnormality
P3	1 d	4 mo	5 mo	4	Absent	1	Intron 9	IVS9+1 G>A	Predicted splicing abnormality
P4	20 d	3 mo	6 mo	3	Absent	25	Intron 6	IVS6+5 G>A	Predicted splicing abnormality
P5	1 d	1 y, 9 mo	1 y, 7 mo	4	Absent	16	Exon 10	1326 del G	R432fsX444
P6	1 y, 3 mo	2 y, 6 mo	2 y, 6 mo	2	Reduced (single peak)	8	Exon 11	1412 C>T	P460S
P7	40 d	3 y, 4 mo	5 y, 1 mo/5 y, 8 mo	3	Absent	11	Intron 8	IVS8+1 G>A	Predicted splicing abnormality
P8	1 mo	3 mo	7 mo	2	Reduced (single peak)	20	Exon 2	264 A>G	D77G
P9	14 d	1 y, 8 mo	1 y, 8 mo	3	Absent	33	Exon 7	665 C>T	R211X
P10	1 d	24 d	5 y	3	Absent	44	Exon 7	665 C>T	R211X
P11	3 d	4 y, 4 mo	6 y, 5 mo	4	Absent	13	Exon 8	797 del C	Q55fsX60
P12	2 d	8 mo	1 y, 4 mo	4	Absent	15	Exon 4	431 G>A	E133K
P13	1 d	1 y	1 y, 5 mo/1 y, 7 mo	4	Reduced (double peak)	13	Intron 8	IVS8+1_+4 del gtga	Predicted splicing abnormality
P14	1 d	11 mo	1 y, 5 mo/1 y, 8 mo	4	Absent	14	Exon 10	1199-1216 18-bp del/49-bp ins	ND
P15	1 d	1 y, 9 mo	1 y, 11 mo	4	Absent	11	Exon 4	408 G>A	G125E
P16	1 d	2 y, 5 mo	2 y, 6 mo	5A	Absent	12	Exon 7	665 C>T	R211X
P17	40 d	2 y, 5 mo	2 y, 7 mo	3	Reduced (single peak)	18	Exon 4	454 C>T	Q152X
P18	3 d	10 mo	10 mo/1 y	2	Reduced (single peak)	7	Exon 2	290 C>T	R86C
P19	40 d	8 mo	1 y	2	Absent	35	Exon 2	201 C>T	A56V
P20	2 d	1 y	1 y/1 y, 9 mo	2	Reduced (single peak)	23	Exon 2	290 C>T	R86C
P21	10 d	7 y, 6 mo	7 y, 6 mo	2	Absent	12	Exon 3	239 T>C	W97R
P22	8 mo	12 y, 3 mo	13 y, 5 mo	5A	Absent	14	Exon 2	168 C>T	T45M
P23	9 mo	22 y, 1 mo	22 y, 7 mo	4	Absent	29	Exon 2	288 T>C	I85T
P24	1 mo	7 mo	8 mo	3	Absent	13	Intron 2	IVS2-2 A>C	Predicted splicing abnormality
P25	8 d	4 mo	1 y	3	Absent	26	Intron 8	IVS8+1 G>A	Predicted splicing abnormality
P26	2 mo	3 mo	3 y, 2 mo	3	Absent	25	Exon 1	71 C>T	R13X

5A, Clinical score of 5 with autoimmune disease; *del*, deletion; *ins*, insertion; ND, not determined.

In this study we analyzed TCR diversity in total peripheral T cells, sorted CD4⁺ and CD8⁺ T cells, and sorted CD45RA⁺ (CD45RA⁺CD45RO⁻) and CD45RO⁺ (CD45RA⁻CD45RO⁺) CD4⁺ and CD8⁺ T cells by using spectratyping complementarity-determining region 3 (CDR3). Furthermore, the CDR3 region of TCR β transcripts in CD45RA⁺CD4⁺ and CD45RA⁺CD8⁺ T cells, CD45RO⁺CD4⁺ T cells, CD8⁺ terminally differentiated effector memory T (Temra) cells (CD45RO⁻CCR7⁻), and naive CD8⁺ T cells (CD45RO⁻CCR7⁺) of patients with WAS and age-matched control subjects was analyzed by using high-throughput sequencing (HTS). Compared with age-matched healthy control subjects, TCR diversity was severely skewed in CD45RO⁺CD4⁺ T cells and CD8⁺ Temra cells of patients with WAS.

METHODS

Patients and control subjects

A total of 26 Chinese patients with WAS mutations were enrolled in this study. The diagnosis of WAS was made based on clinical signs and symptoms,

WAS mutations, and WASP expression measured by means of flow cytometry analysis, as previously described (Table I).²³ Patients were assigned a clinical score by using a previously described scoring system (Table I).^{24,25} Healthy control subjects consisted of 35 age-matched subjects (average age). Pediatric control subjects were represented by blood samples from male patients left over after the completion of diagnostic tests. Criteria for control samples included an absence of related immune diseases and current infections. This study was conducted in accordance with the tenets of the Declaration of Helsinki and was approved by the Ethics Committee of Chongqing Medical University.

Flow cytometric analysis and cell sorting

PBMCs were isolated from freshly drawn heparin-treated blood by means of Ficoll density gradient centrifugation. PBMCs were stained for T-cell surface markers with antibodies (anti-CD3–perCP-CY5.5, anti-CD4–phycoerythrin [PE]-CY7, anti-CD8–PE, anti-CD45RA–fluorescein isothiocyanate, anti-CD45RO–allophycocyanin, anti-CD8–fluorescein isothiocyanate, and anti-CCR7–PE; BD Biosciences, San Jose, Calif), and CD4⁺ T cells, CD8⁺ T cells, CD45RA⁺ T cells, CD45RO⁺ T cells, CD8⁺ Temra cells, and naive

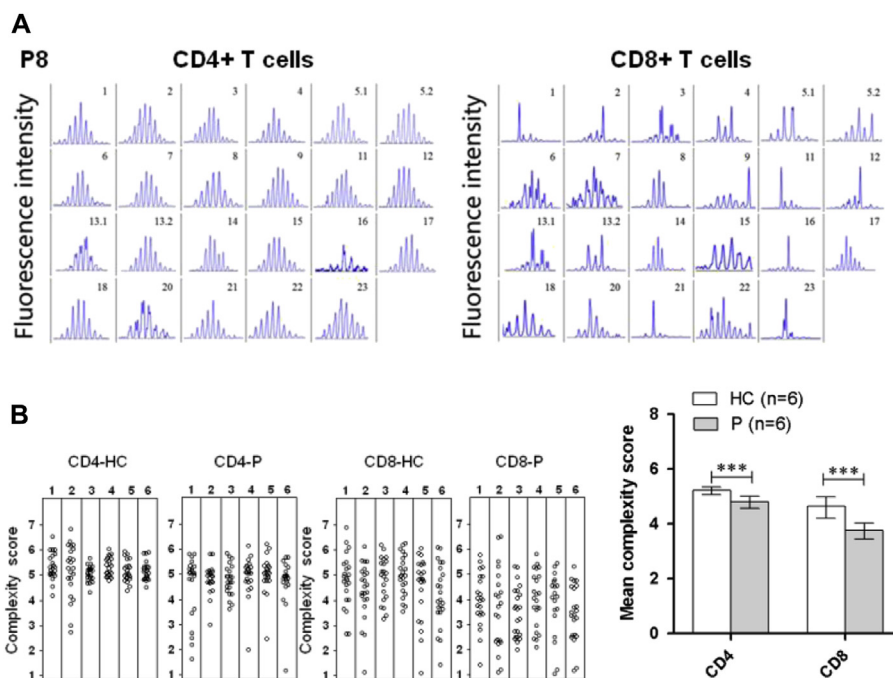


FIG 1. Analysis of T-cell repertoires in sorted CD4⁺ and CD8⁺ T cells. **A**, CDR3 size distribution of TCR Vβ subfamilies. The CDR3 size distribution in sorted CD4⁺ and CD8⁺ T cells from a patient with WAS (P8) is shown. **B**, Complexity scores of TCR Vβ. The complexity scores of CD4⁺ and CD8⁺ T cells for each healthy control subject and patient are shown (top). The mean (with range) complexity scores for CD4⁺ and CD8⁺ T cells are shown in the histogram (bottom). HC, Healthy control subjects; P, patients. ***P < .0001.

CD8⁺ T cells were sorted with a FACSria II (BD Biosciences). Table E1 (available in this article's Online Repository at www.jacionline.org) lists the proportions of the various sorted subsets in each patient and the ranges of the proportions of the corresponding cell subsets in the age-matched healthy control subjects. There were no significant differences in the proportions of different subsets of T cells between the patient and control groups, with the exception of the proportion of CD8⁺ Temra cells, which was significantly higher in patients with WAS than in control subjects ($P = .001$), and the proportion of naive CD8⁺ T cells, which was significantly less in patients with WAS than in age-matched healthy control subjects ($P = .008$). However, there were no significant differences in the numbers of T-cell subsets obtained from sorting between patients and control subjects. The purity of sorted cell subtypes exceeded 95%, as assessed by using flow cytometry (see Fig E1 in this article's Online Repository at www.jacionline.org).

RNA extraction and cDNA synthesis

RNA was prepared from 0.5 to 2×10^6 T cells from each sample. Because the blood sample from a single pediatric patient was insufficient for analyzing the diversity of TCR Vβ in all T-cell subset, blood samples from different patients were used for the analysis of total T cells (patients P1-P7, $n = 7$), sorted CD4⁺ and CD8⁺ T cells (patients P2 and P7-P11, $n = 6$), sorted CD45RA⁺ and CD45RO⁺ CD4⁺ and CD8⁺ T cells (patients P12-P23, $n = 12$), CD8⁺ Temra cells (patients P13, P14, P18, P20, and P24-P26; $n = 7$), and naive CD8⁺ T cells (patients P13, P14, P18, P20, and P26; $n = 6$). Two blood samples were obtained from P2, P7, P13, P14, P18, and P20, one of which was used to analyze the TCR Vβ diversity in total peripheral T cells (patients P2 and P7) or CD45RA⁺ and CD45RO⁺ T cells (patients P13, P14, P18, and P20) and the other of which was used to analyze sorted CD4⁺ and CD8⁺ T cells (patients P2 and P7) or CD8⁺ Temra and naive CD8⁺ T cells (patients P13, P14, P18, and P20). The clinical scores of the patients in the 4 groups were similar to one another, thereby making the results comparable.

RNA extraction and cDNA synthesis was done according to the manufacturers' instructions (see the Methods section in this article's Online Repository at www.jacionline.org).

CDR3 spectratyping

CDR3 spectratyping was performed, as described previously (see the Methods section in this article's Online Repository).²⁶

High-throughput TCR repertoire sequencing

The diversity of the TCR repertoire was profiled by using HTS of rearranged TCRβ loci from cDNA. Because the cell numbers were limited, CD45RO⁺CD8⁺ T cells were not included in the HTS analysis. The TCRβ CDR3 region was defined according to the International Immunogenetics collaboration,²⁷ starting with the second conserved cysteine encoded by the 3' portion of the Vβ gene segment and ending with the conserved phenylalanine encoded by the 5' portion of the Jβ gene segment. TCRβ CDR3 regions were amplified and sequenced by BGI Tech (Shenzhen, China) by using previously described protocols (see the Methods in this article's Online Repository).²⁸

The diversity of the TCR repertoire was calculated based on the Simpson index of diversity (Ds),²⁹ the Shannon-Wiener index (H'),^{30,31} and the diversity 50 (D50) value (see the Methods in this article's Online Repository).³²

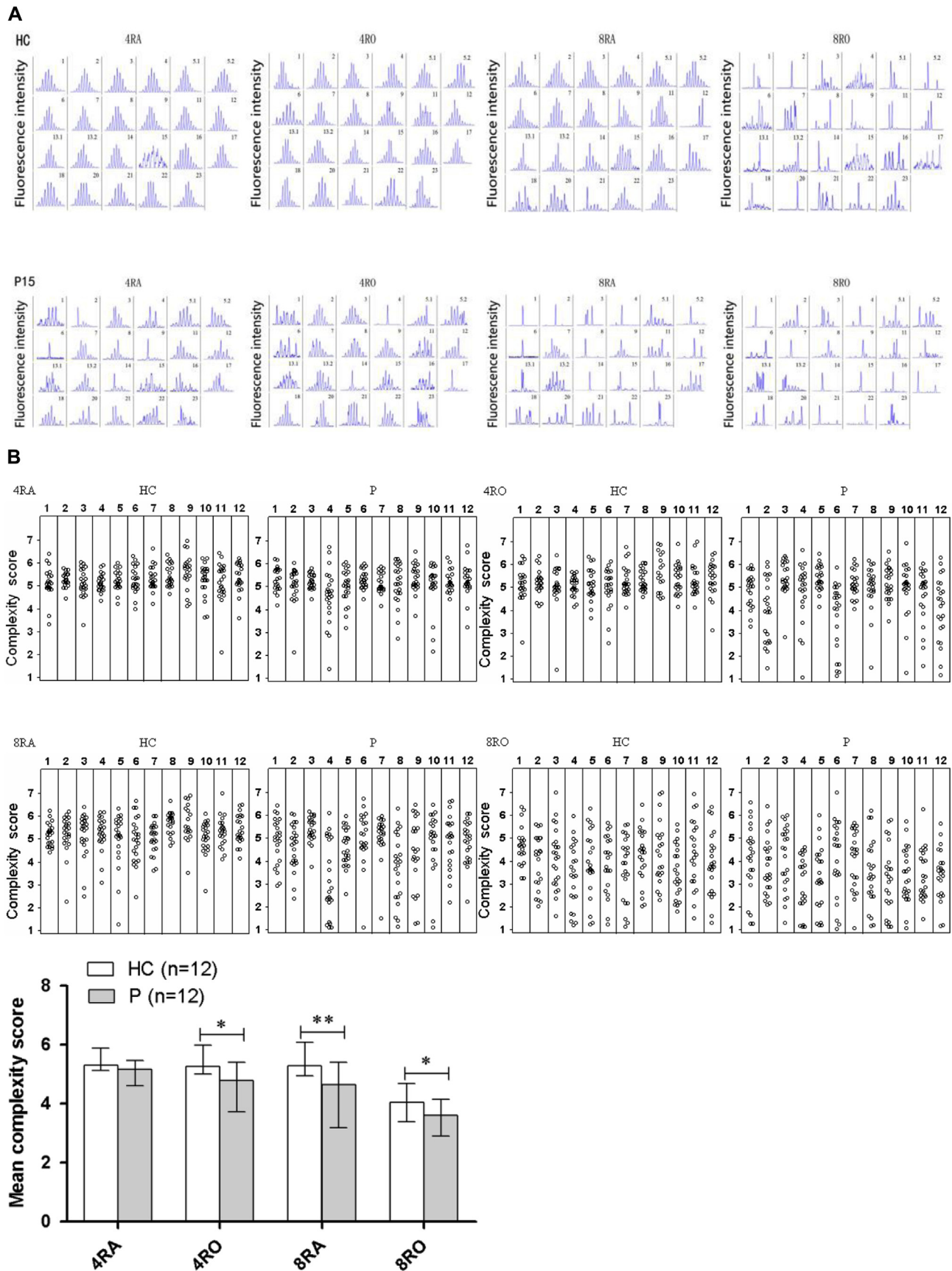
Statistical analysis

Because of the small sample size in this study, analysis of differences among data groups was performed with the Mann-Whitney test.²⁹ P values of less than .05 were considered significant. Statistical analyses were conducted with GraphPad Prism software (GraphPad Software, San Diego, Calif).

RESULTS

Clinical features and genetic mutations of patients

This study included a total of 26 patients and 35 healthy donors. The demographic and clinical features of the patients are shown in Table I. The age at onset of symptoms of WAS ranged from 1 day to 1.25 years. The median age at WAS



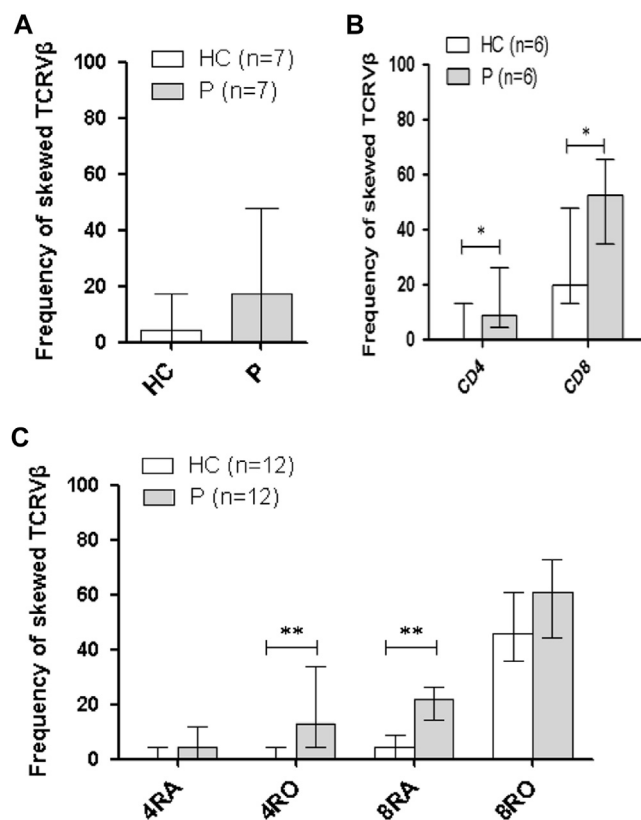


FIG 3. Frequency of skewed TCR Vβ repertoires. Shown are the median (with range) frequencies of skewed TCR Vβ subfamilies in total peripheral T cells (A), CD4⁺ and CD8⁺ T cells (B), and CD45RA⁺CD4⁺, CD45RA⁺CD8⁺, CD45RO⁺CD4⁺, and CD45RO⁺CD8⁺ T cells (C) from the control and patient groups. HC, Healthy control subjects; P, patient; 4RA, CD4⁺CD45RA⁺ T cells; 8RA, CD8⁺CD45RA⁺ T cells; 4RO, CD4⁺CD45RO⁺ T cells; 8RO, CD8⁺CD45RO⁺ T cells. **P* < .05 and ***P* < .01.

diagnosis was 11.5 months (range, 24 days to 22.1 years). The clinical scores of the 26 patients with WAS in this study ranged from 2 to 5. Among the 26 patients, only 2 had a score of 5; one (patient P16) of the 2 patients had autoimmune hemolytic anemia, whereas the other (patient P22) had IgA nephropathy. Genetic mutations and WASP expression levels were analyzed by using sequencing analysis and flow cytometry, respectively. WAS mutations in the patients with WAS were diverse, including null mutations (nonsense, deletions, and insertions), missense mutations, splice anomalies, and complex mutations. WASP expression was undetectable in 77% (20/26) of patients with WAS. Five patients (patients P6, P8, P17, P18, and P20) expressed reduced levels of WASP in PBMCs compared with those seen in age-matched control subjects. One patient (patient P13) exhibited reduced WASP expression in a proportion of PBMCs and no WASP expression in the remaining PBMCs. For this patient, somatic reversions were excluded by means of extensive genetic analysis.

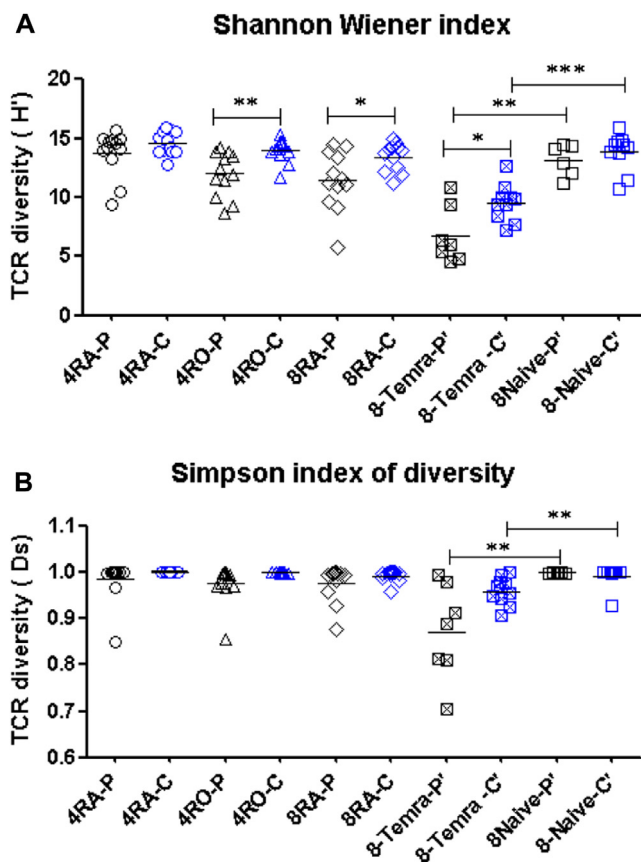


FIG 4. HTS for TCR diversity analysis. TCR diversity was measured by using H', which is a reflection of the change in richness (A), and Ds, which is a reflection of the change in evenness (B), in the indicated groups. Symbols represent individual subjects, and bars depict group means. **P* < .05 and ***P* < .01.

Patients with WAS mutations have altered TCR CDR3 size patterns

Blood samples were collected from patients between 2 months and 22 years old, 69% (18/26) of whom were younger than 3 years. The length of TCR CDR3 was first analyzed in total peripheral T cells from 7 patients and 7 age-matched healthy control subjects by means of PCR with a set of Vβ primers and a fluorescent Cβ primer. The majority of Vβ subfamilies in T cells from control subjects exhibited 6 or more peaks with a Gaussian distribution, indicating a polyclonal Vβ repertoire. By contrast, a number of Vβ subfamilies in T cells from patients with WAS displayed skewed spectratyping profiles, indicating clonal or oligoclonal proliferation of T cells (see Fig E2, A, in this article's Online Repository at www.jacionline.org). By using these data, the mean complexity score was calculated based on the amplification of all 23 Vβ segments. Consistent with the spectratyping profiles, the mean complexity score of patients (4.76; range, 4.08-5.06) was significantly lower than that of control subjects (5.23; range,

CD4⁺CD45RA⁺ (4RA), CD4⁺CD45RO⁺ (4RO), CD8⁺CD45RA⁺ (8RA), and CD8⁺CD45RO⁺ (8RO) T cells from a control donor and from a patient with WAS (P15) are shown. B, Complexity scores of TCR Vβ. The complexity scores of CD45RA⁺CD4⁺, CD45RA⁺CD8⁺, CD45RO⁺CD4⁺, and CD45RO⁺CD8⁺ T cells for each healthy control subject and patient are shown (top). The mean (with range) complexity scores are shown in the histograms below. HC, Healthy control subjects; P, patients. **P* < .05 and ***P* < .01.

4.95-5.48; $P = .002$; see Fig E2, B). This observation suggests that WAS mutations can cause reductions in TCR diversity.

TCR V β repertoires in both CD4⁺ and CD8⁺ T cells of patients with WAS are skewed

To determine which T-cell subtypes contribute to the altered TCR spectratype profiles in patients with WAS, the TCR receptor diversity was analyzed in sorted CD4⁺ and CD8⁺ T cells from 6 patients and 6 control subjects by using V β spectratype analysis and complexity scoring. As shown in Fig 1, A, most TCR V β subfamilies in patients' CD4⁺ T cells showed a Gaussian distribution, whereas the TCR repertoire was mostly skewed in CD8⁺ T cells. The mean complexity scores of both CD4⁺ (4.81; range, 4.56-5.01) and CD8⁺ (3.76; range, 3.43-4.02) T cells from patients with WAS were significantly lower than those in control CD4⁺ (5.22; range, 5.07-5.34) and CD8⁺ (4.65; range, 4.12-4.99) T cells (CD4, $P = .002$; CD8, $P = .002$; see Fig 1, B). These results indicate that the TCR V β diversity in both CD4⁺ and CD8⁺ T cells from patients with WAS was skewed.

TCR V β repertoires of CD45RA⁺CD8⁺ and CD45RO⁺CD4⁺ and CD45RO⁺CD8⁺ T-cell subpopulations are skewed in patients with WAS

The CDR3 size diversity was determined in sorted CD45RA⁺CD4⁺, CD45RA⁺CD8⁺, CD45RO⁺CD4⁺, and CD45RO⁺CD8⁺ cells in 12 patients and 12 healthy control subjects to further characterize the effect of WAS mutations on the diversity of T-cell subpopulations. Amplification of all 23 V β segments showed that the majority of V β subfamilies in the CD45RA⁺CD4⁺, CD45RA⁺CD8⁺, and CD45RO⁺CD4⁺ T-cell subpopulations from healthy control subjects exhibited a Gaussian distribution; however, CD45RO⁺CD8⁺ T cells were mostly skewed (Fig 2, A). In patients with WAS, CD45RA⁺CD8⁺ T cells and CD45RO⁺CD4⁺ and CD45RO⁺CD8⁺ T cells showed skewed distributions of V β subfamilies, but CD45RA⁺CD4⁺ T cells displayed a normal distribution similar to that of the control cells (Fig 2, A). In the patient group the mean complexity scores of CD45RA⁺CD8⁺ and CD45RO⁺CD4⁺ and CD45RO⁺CD8⁺ T cells, but not CD45RA⁺CD4⁺ T cells, were significantly less than those of the healthy control group. These data indicate that the TCR diversity of the CD45RA⁺CD8⁺, CD45RO⁺CD4⁺, CD45RO⁺CD8⁺ T-cell populations was skewed in patients with WAS.

Degree of V β skewing in patients with WAS mutations

The frequency of unevenly distributed V β subfamilies was determined in total peripheral T cells in patients with WAS mutations and in the control group. The median frequency of skewed TCR V β subfamilies was 17.39 (range, 0-47.83), which was not significantly different from the median frequency in healthy control subjects (4.35; range, 0-17.39; Fig 3, A). Because the degree to which skewing is attributable to each T-cell subset could not be determined in total PBMCs, the frequency of skewed TCR V β subfamilies was determined in sorted CD4⁺ and CD8⁺ T cells. Compared with control subjects, the frequency of TCR V β subfamilies was significantly higher both in patients' CD4⁺ T cells ($P = .03$) and CD8⁺ T cells ($P = .01$; Fig 3, B). Sorted CD45RA⁺CD4⁺, CD45RA⁺CD8⁺, CD45RO⁺CD4⁺, and CD45RO⁺CD8⁺ T cells were analyzed separately to determine

whether patients' CD45RA⁺ or CD45RO⁺ T cells were the source of the frequent V β skewing. Only CD45RA⁺CD8⁺ and CD45RO⁺CD4⁺ T cells in the patient group showed significantly higher frequencies of skewed TCR V β subfamilies when compared with those from the control group (Fig 3, C). These results further demonstrate that TCR V β repertoire diversity in CD45RA⁺CD8⁺ and CD45RO⁺CD4⁺ T cells is significantly skewed in patients with WASP deficiency.

Diversity analysis of the T-cell repertoire by using HTS

By using HTS, approximately 1.58 billion effective sequence reads were generated corresponding to the sequenced cDNA of CDR3, for 105 distinct T-cell populations (CD45RA⁺CD4⁺, CD45RO⁺CD4⁺, and CD45RA⁺CD8⁺ T-cell subsets from 12 control subjects and 12 patients and CD8⁺ Temra and naive CD8⁺ T cells from 10 control subjects and 7 patients, which were identified by using CD45RO and CCR7 as surface markers; see Fig E1, B). Because patient P24 experienced chronic cytomegalovirus infection, the CD8⁺ T-cell population was predominantly composed of Temra (CD45RO⁺CCR7⁻) and effector memory T cells, and the number of naive CD8⁺ T cells was not sufficient for further analysis (see Fig E1, C); therefore naive CD8⁺ T cells were only analyzed in 6 patients with WAS. Table E2 in this article's Online Repository at www.jacionline.org summarizes the CDR3 sequence reads for different subsets of T cells. The productive reads from CD45RO⁺CD4⁺ T cells and the number of unique peptides from CD45RA⁺CD8⁺ T cells were significantly lower in the patient group than in the control group ($P = .03$ and $P = .02$, respectively). The total clonotypes and unique peptides from CD8⁺ Temra cells were significantly lower than from naive CD8⁺ T cells in both patients ($P = .008$ and $P = .008$, respectively) and control subjects ($P = .005$ and $P = .002$, respectively), which indicates that TCR repertoire diversity in CD8⁺ Temra cells was significantly skewed from that of naive CD8⁺ T cells.

To quantitatively evaluate the overall diversity of patients' TCR repertoires, we used the Ds.²⁹ This index is a function of the relative number of clonotypes present (richness), as well as the relative abundance and distribution of each clonotype (evenness).^{29,33} H'³¹ is a diversity index because it gives each clonotype a weight proportional to its abundance.³⁴ Both the Ds and H' values of the patient group were lower than those of the control group in all T-cell subsets (Fig 4), which indicates that the TCR repertoires of patients were less diverse than those of control subjects. Both the Ds and H' values of CD8⁺ Temra cells were significantly lower than those of naive CD8⁺ T cells (Fig 4) in both patients and control subjects, suggesting that the TCR repertoire of Temra cells was significantly less diverse than that of naive CD8⁺ T cells in both patients and control subjects. The H' scores of CD45RO⁺CD4⁺ T cells, CD45RA⁺CD8⁺ T cells, and CD8⁺ Temra cells in patients were significantly lower than those seen in control subjects ($P = .003$, $P = .022$, and $P = .03$, respectively; Fig 4, A). These data confirm the results found by means of spectratyping that TCR diversity in CD45RO⁺CD4⁺ and CD45RA⁺CD8⁺ T cells was significantly skewed in patients with WAS compared with that seen in control subjects.

In addition, the magnitude of the clonotypic expansion was evaluated by using D50,³² which represents the percentage of dominant and unique T-cell clones or CDR3 amino acid

sequences that account for 50% of the total T cells in a given sample. The D50 values of CD45RO⁺CD4⁺ and CD45RA⁺CD8⁺ T cells in patients with WAS were significantly lower than those in control subjects (see Fig E3 in this article's Online Repository at www.jacionline.org), suggesting a skewed TCR repertoire in these 2 T-cell subsets in patients with WAS.

Patterns of Vβ skewing in patients with WAS

In addition to analyzing the frequency of skewed Vβ profiles, we identified individual Vβ clonotypes and subfamilies that are potentially involved in the disease process. Conventional CDR3 spectratyping does not assess the diversity of TCR CDR3 at the sequence level but measures the diversity of TCRβ or TCRβ CDR3 lengths in subsets of αβ T cells that use a specific Vβ or Vβ gene segment.²⁸ Here, using HTS analysis, we investigated whether any single VDJ segment is used predominantly by patients' T cells. No significant differences were found in the V, D, and J gene uses between healthy control subjects and patients (see Fig E4 in this article's Online Repository at www.jacionline.org). Furthermore, no CDR3 amino acid sequences were identified that were expressed predominantly either in the patient cohort or in the control subjects (see the Results section and Fig E5 in this article's Online Repository at www.jacionline.org).

DISCUSSION

This study found that the diversity of the TCR Vβ repertoire in patients with WAS is decreased and skewed. These results are in agreement with the results of previous studies.^{21,22} In one study a comparison of patients with WAS (n = 13) with adult control subjects (n = 10) showed decreased and skewed T-cell repertoires in patients with WAS,²¹ whereas the other study found a significant skewing of T-cell repertoires in young patients with WAS before gene therapy.²² Our study further analyzed the effects of WAS mutations on the TCR diversity of different T-cell subsets in patients with WAS. Using both spectratyping and HTS approaches, we show that WAS mutations specifically affect the TCR diversity of CD45RO⁺CD4⁺ cells, suggesting skewed diversity of memory CD4⁺ T cells in patients with WAS.

Previous studies show that elderly people have a markedly restricted TCR repertoire, which is associated with decreased immunocompetence and increased susceptibility to infection and cancer.³⁵⁻³⁷ This indicates that restrictions in the peripheral TCR repertoire can lead to impaired immune responses. Accumulated infections throughout the lifespan likely cause the reduction in TCR diversity.³⁷⁻³⁹ However, most of the patients with WAS in this study were young. Whether infection alone can decrease TCR diversity in young children has not yet been determined. Furthermore, WASP-deficient T cells are known to be defective in signal transduction, TCR-induced proliferation, and cytokine production,^{8,9,12,15} which can directly interfere with T-cell clonal expansion. These data together support the notion that recurrent infections in young patients with WAS is unlikely to be the only or major cause of the skewed T-cell repertoire. Because WASP has been implicated in the maturation of human T and B cells and in the function of mature T cells,²⁰ the reduction of TCR diversity in memory CD4⁺ T cells in young patients might reflect the role of WASP in extrathymic T-cell maturation. Diverse memory T cells are crucial in immune protection against repeated infection and cancer. In addition, previous studies showed that skewed TCR Vβ diversity in autoimmune diseases, such as autoimmune

lymphoproliferative syndrome⁴⁰ and Hashimoto thyroiditis,⁴¹ correlated with the severity of the autoimmunity. Skewed TCR diversity can also contribute to the onset of autoimmunity.⁴²

Taken together, these findings suggest that decreased and skewed TCR Vβ diversity might be involved in the pathogenesis of immunodeficiency and autoimmunity in patients with WAS while raising the hypothesis that the activation-dependent memory response might be the major T-cell defect in patients with WAS. If this is the case, the diversity of the TCR repertoire could be used to predict the level of impairment of the immune response to vaccines and infections in patients with WAS. However, the involvement of infection cannot be excluded. The molecular mechanisms underlying this accelerated repertoire contraction remain unknown.

Using HTS, we further found that the skewed TCR diversity of the CD45RA⁺CD8⁺ T-cell population was mainly due to the contribution of CD8⁺ Temra cells and not naive CD8⁺ T cells. Chronic viral infections in immunodeficient patients, such as those with WAS, might lead to the expansion of Temra cells and might shape the TCR pattern. Patient P24, who experienced a chronic cytomegalovirus infection, showed significantly lower Ds and H' values than control patients, providing an example of the role of chronic infection in the skewed TCR of CD8⁺ Temra cells.

Two parameters were used to quantify TCR diversity measured by using spectratyping (the mean complexity score and the frequency of skewed TCR diversity), and 2 additional parameters were used to assess TCR diversity measured by HTS (Ds and H'). The diversity indices from spectratyping and HTS were not always consistent. Although the inconsistency might be due to the limited numbers of samples, different parameters also reflect different aspects of the data. Combining these parameters increases the credibility of the results.

Because WAS is a rare disease, a limited number of patients were available for our analyses. No significant differences in TCR diversity skewing were found in certain T-cell subsets, such as CD45RA⁺CD4⁺ T cells. This finding should be further addressed with a larger sample size. To our knowledge, this study represents the first detailed analysis of the degree of skewing and the pattern of TCR Vβ repertoire diversity in sorted CD4⁺; CD8⁺; CD45RA⁺CD4⁺; CD45RA⁺CD8⁺; CD45RO⁺CD4⁺; and CD45RO⁺CD8⁺ T cells; CD8⁺ Temra cells; and naive CD8⁺ T cells from patients with WAS in comparison with age-matched healthy control subjects. The skewed diversity of CD45RO⁺CD4⁺ T cells in patients with WAS indicates a selective effect of WAS mutations on the generation and maturation of the memory CD4 T-cell subset. The shrinkage of TCR repertoire diversity in this T-cell subset is an effect of perturbed immune responses/homeostasis in patients with WAS and could potentially provide a quantitative indication of a patient's immune response to vaccines and infections. Further studies are required to explain the persistence and progression of TCR restriction in patients with WAS and its underlying molecular mechanisms.

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Clinical implications: Selectively skewed TCR repertoire diversity might affect immune responses and homeostasis in patients with WAS.

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METHODS

RNA extraction and cDNA synthesis

Total RNA was extracted from T cells by using the QIAamp RNA Blood Mini Kit (Qiagen GmbH, Hilden, Germany) and eluted in 30 to 50 μ L of diethyl pyrocarbonate-treated water. cDNA was synthesized from equal quantities of total RNA (1 μ g) in 40- μ L reactions by using the PrimeScript RT Reagent Kit (Takara, Otsu, Japan), according to the manufacturer's instructions.

CDR3 spectratyping

Briefly, each TCR V β fragment was amplified with one of the 23 V β -specific primers and a 5' FAM-labeled C β primer.^{E1} TCR V β 10 and V β 19 are pseudogenes and were excluded from the study.^{E2} The V β 24 subfamily was also excluded because amplification products could not be reliably obtained, size fractionation of the PCR products did not produce profiles with adequate signal-to-noise ratios, or both. Forty-five cycles of amplification (94°C for 1 minute, 65°C for 1 minute, and 72°C for 1 minute) were used. PCR products were diluted 1:10, and 1 μ L was mixed with 12 μ L of deionized formamide and 0.5 μ L of size standard and heated (95°C for 3 minutes). The size distribution of each fluorescent PCR product was determined by using electrophoresis in Performance-Optimized Polymer 4 with an Applied Biosystems 310 automated sequencer, and data were analyzed with GeneScan software (Applied Biosystems, Foster City, Calif).

To define each profile as normal or skewed, a previously reported complexity scoring system was used, as follows^{E3,E4}:

Complexity score = (Sum of all the peak heights/Sum of the major peak heights) $\times$ Number of major peaks.

Major peaks were defined as those with amplitudes of at least 10% of the sum of all the peak heights. The number of TCR V β subfamilies with skewed CDR size patterns, as shown by a complexity score of less than 4, was determined for each subject.

High-throughput TCR repertoire sequencing

Briefly, a multiplexed PCR method was used to amplify all possible rearranged genomic TCR β sequences from cDNA samples by using 48 forward primers, each specific to a TCR V β segment, and 13 reverse primers, each specific to a TCR J β segment. Reads 60 bp in length were obtained with the Illumina HiSeq System (Illumina, San Diego, Calif). Sequences that did not match CDR3 sequences were excluded from the analysis. A standard algorithm was used to identify which V, D, and J segments contributed to each TCR V β CDR3 sequence.^{E5}

The diversity indices D_s and H' were calculated according to the following formulas:

$$D_s = 1 - \sum_{i=1}^c \frac{ni(ni-1)}{n(n-1)}$$

$$H' = - \sum_{i=1}^s \frac{mi}{N} \ln \frac{mi}{N}$$

In both D_s and H' , ni is the clonal size of the i^{th} clonotype (ie, the number of copies of a specific clonotype). C (in D_s) and S (in H') are the number of different clonotypes, and n (in D_s) and N (in H') are the total number of TCR β sequences analyzed. D_s uses the relative frequency of each clonotype to calculate a diversity index ranging from 0 to 1, where 0 represents the minimum and 1 represents the maximum diversity. A sample of TCRs containing more than 1 clonotype will have maximum diversity when all clonotypes have equal clone size.

To measure clonotypic expansion, the D50 of each patient was analyzed, where D50 was defined as the minimum percentage of distinct clonotypes or CDR3 peptides accounting for at least half of the total clonotypes or CDR3 peptides in a population or subpopulation of immune system cells; the higher the number, the greater the level of diversity.^{E6}

RESULTS

Diversity analysis of the T-cell repertoire by using HTS

The D50 value,^{E6} which represents the percentage of dominant and unique T-cell clones or CDR3 amino acid sequences that account for 50% of the total T cells in a given sample, was determined to evaluate the magnitude of clonotypic expansion. A high D50 value indicates a high level of diversity in the T-cell clones or CDR3 amino acid sequences. The D50 values of CD45RO⁺CD4⁺ and CD45RA⁺CD8⁺ T cells in patients with WAS were significantly lower than those in control subjects (Fig E3, A and B), suggesting a skewed TCR repertoire in these 2 T-cell subsets in patients with WAS. The D50 value of CD8⁺ Temra cells was significantly lower than that of other T-cell subsets from both patients and control subjects (Fig E3), suggesting that a few clones might dominate the TCR repertoire of CD8⁺ Temra cells.

Patterns of V β skewing in patients with WAS

Based on the results of HTS analysis, we further determined whether there were any CDR3 amino acid sequences that are dominantly expressed in patients or control subjects. Most of the CDR3 amino acid sequences in T-cell subsets from healthy control subjects and patients were so-called private sequences that were not shared with other subjects. There is very limited sequence sharing between healthy control subjects and patients within a given T-cell subset (Fig E5, A-E) or between T cells of the same subset from control subjects and patients (Fig E5, F-I). No dominant sequence was found to be exclusively expressed in the patients, indicating that no candidate sequence is relevant to the pathogenesis of the disease. In both healthy control subjects and patients, different T-cell subsets within each cohort shared some public sequences (ie, present in >1 subject; Fig E5, F-I).

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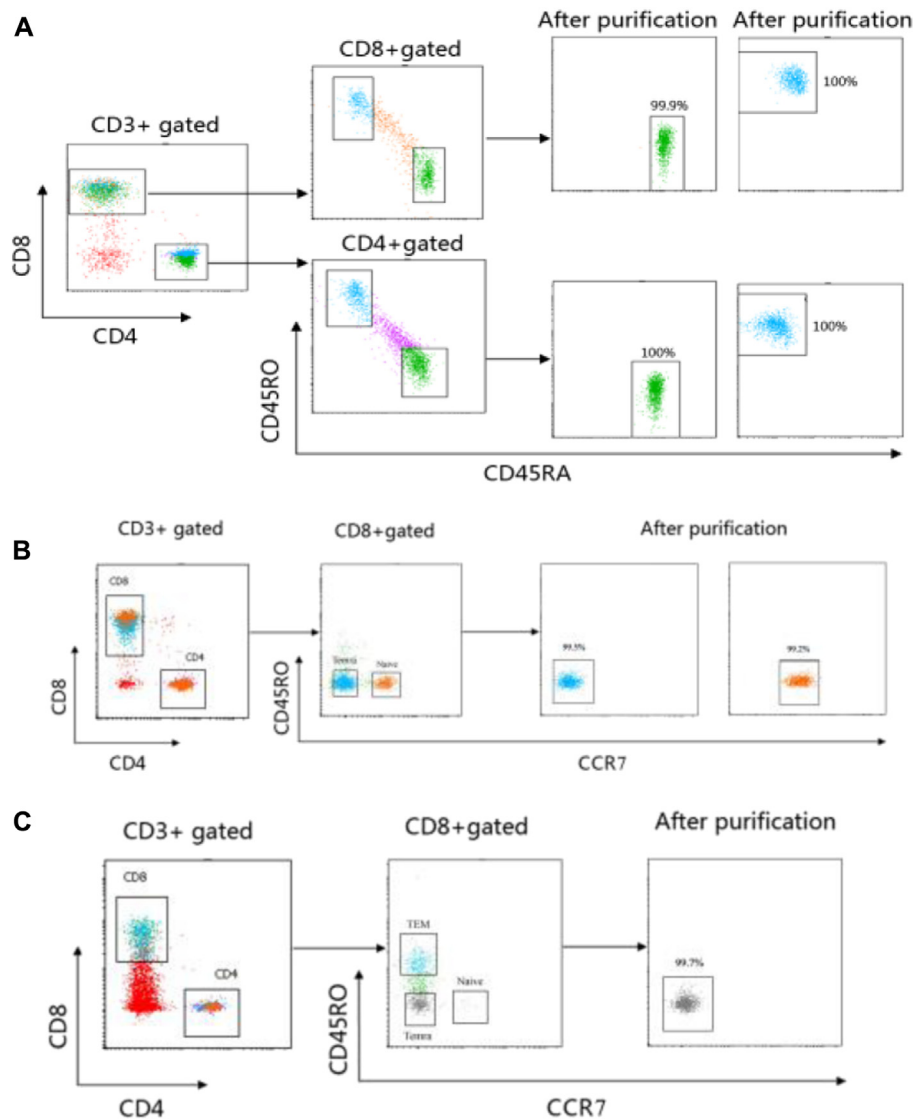


FIG E1. Purity of sorted cell populations. $CD4^{+}$ and $CD8^{+}$ T cells ($CD3^{+}$, left-hand dot plot) were gated and sorted by expression of CD45RA or CD45RO to purify naive (green) and memory (blue) subsets in **A**, respectively. Expression of CD45RO or CCR7 was used to purify the $CD8^{+}$ Temra cells (blue dots in **B** and gray dots in **C**) and naive $CD8^{+}$ T-cell (orange dots in **B**) subsets, respectively. Three representative patients are shown.

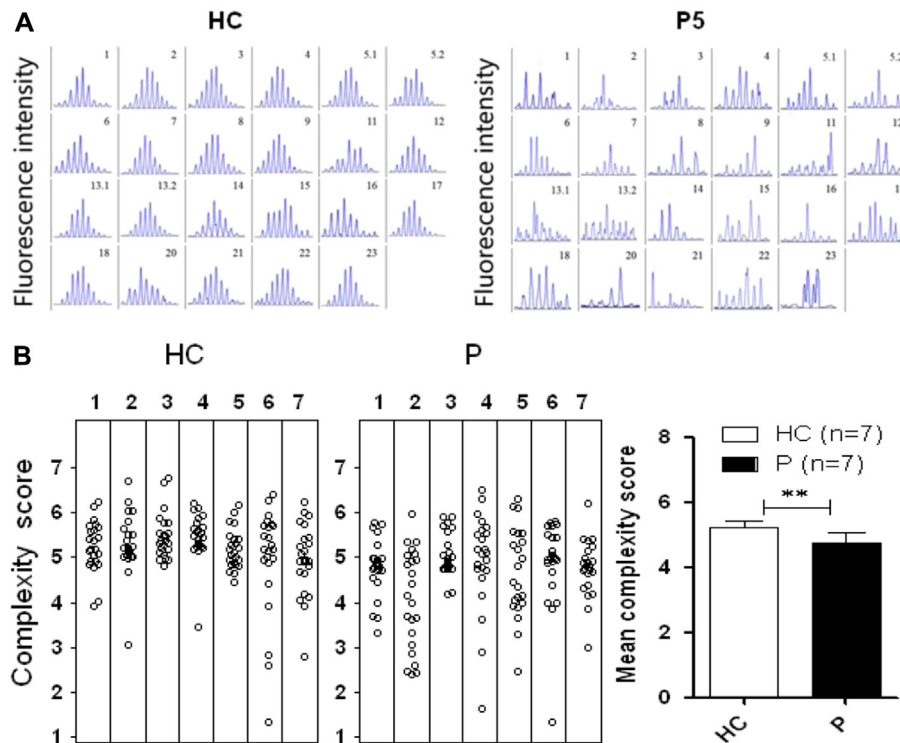


FIG E2. Analysis of T-cell repertoires in total peripheral T cells. **A**, CDR3 size distribution of TCR V β subfamilies. The CDR3 size distribution in total peripheral T cells from a control subject and a patient with WAS (*P5*) are shown. **B**, TCR V β complexity scores. The complexity scores for each healthy control subject (*left*) and patient (*middle*) are shown. The mean (with range) complexity scores of the patient and control groups are shown in the histogram (*right*). HC, Healthy control subjects; P, patients. ** $P < .01$.

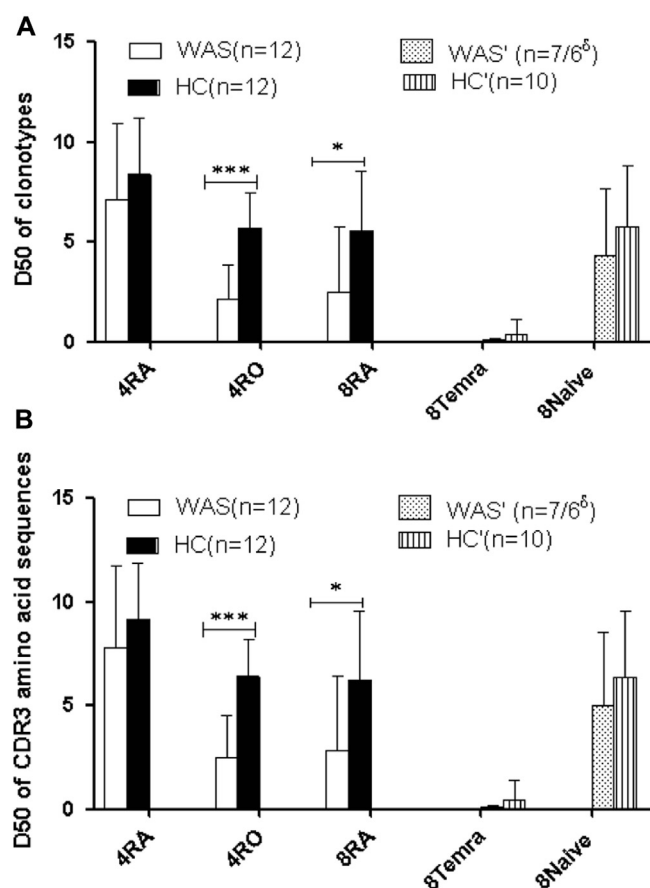


FIG E3. HTS of TCR diversity analysis. TCR diversity was measured based on the D50 value of clonotypes (**A**) and the D50 value of CDR3 amino acid sequences (**B**) in the indicated group. HC, Healthy control subjects; $n = 7/6^{\delta}$, $n = 7$ in 8Temra cells but $n = 6$ in 8 naive cells; 8Naive, naive CD8⁺ T cells (CD8⁺CD45RO⁻CCR7⁺); P, patients; 4RA, CD4⁺CD45RA⁺ T cells; 8RA, CD8⁺CD45RA⁺ T cells; 4RO, CD4⁺CD45RO⁺ T cells; 8RO, CD8⁺CD45RO⁺ T cells; 8Temra, CD8⁺ terminally differentiated effector memory T cells (CD8⁺CD45RO⁻CCR7⁻). * $P < .05$ and ** $P < .01$.

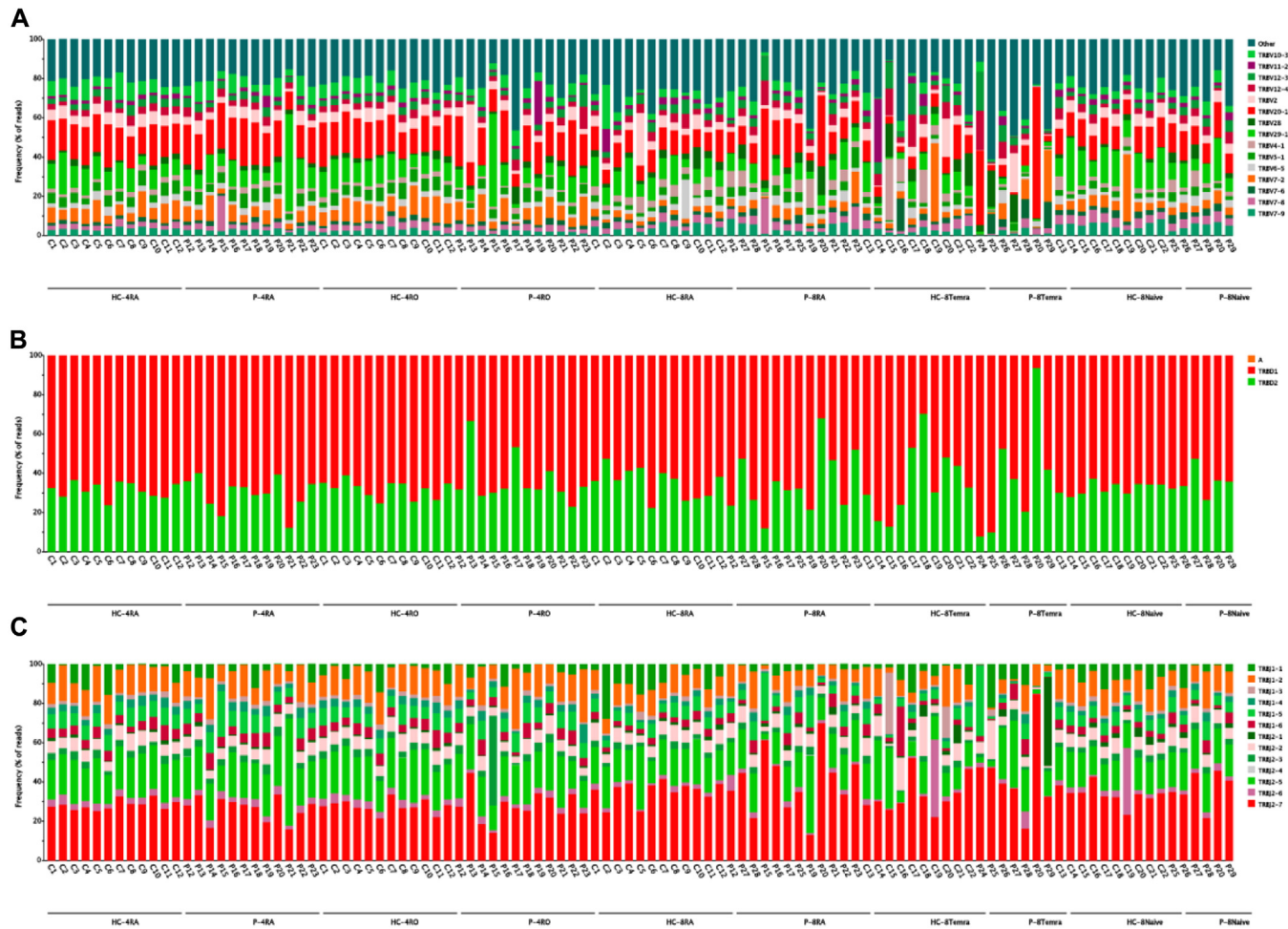


FIG E4. Analysis of V, D, and J gene use in the analyzed cohorts by using HTS. **A**, V β gene use of TCRs in different T-cell subsets from 25 healthy control subjects and 15 patients with WAS. The 15 most frequent V β genes are indicated in *color*, and the remaining 33 V β genes are grouped together in *dark green*. **B** and **C**, D β (Fig E4, *B*) and J β (Fig E4, *C*) gene use of TCRs in different T-cell subsets from patients with WAS and healthy control subjects. *HC*, Healthy control subjects; *P*, patients; *4RA*, CD4⁺CD45RA⁺ T cells; *8RA*, CD8⁺CD45RA⁺ T cells; *4RO*, CD4⁺CD45RO⁺ T cells; *8Naive*, CD8⁺CD45RO⁻CCR7⁺ T cells; *8Temra*, CD8⁺CD45RO⁻CCR7⁻ T cells.

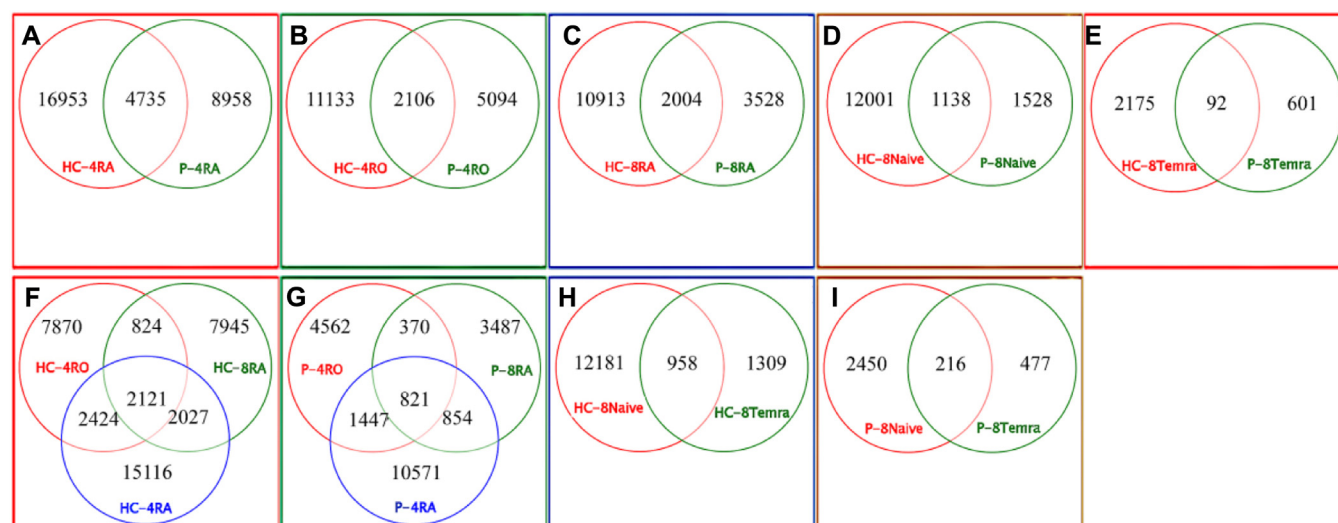


FIG E5. Expansion of public CDR3 amino acid sequences in healthy control subjects and patients with WAS. The *Venn diagrams* present the total number of CDR3 amino acid sequences that were shared between healthy control subjects (*red*) and patients (*green*) for a given T-cell subset (**A-E**) or between different T-cell subsets within the same donor population (patients or control subjects; **F** and **G**: *red*, 4RO; *green*, 8RA; and *blue*, 4RA; **H** and **I**: *red*, 8Naive; and *green*, 8Temra). Numbers of CDR3 sequences that are unique to each subset are shown in the nonoverlapping sections. Numbers of CDR3 sequences that are common to any 2 and 3 of these subsets are indicated in the relevant overlapping areas. *HC*, Healthy control subjects; *8Naive*, CD8⁺CD45RO⁻CCR7⁺ T cells; *P*, patients; *4RA*, CD4⁺CD45RA⁺ T cells; *8RA*, CD8⁺CD45RA⁺ T cells; *4RO*, CD4⁺CD45RO⁺ T cells; *8Temra*, CD8⁺CD45RO⁻CCR7⁻ T cells.

TABLE E1. Proportions of the indicated T-cell subsets in patients with WAS

Patient ID	Percentage of 4RA cells	Percentage of 4RO cells	Percentage of 8RO cells	Percentage of CD8 naive cells	Percentage of CD8 Temra cells
	In CD4 ⁺ T cells	In CD4 ⁺ T cells	In CD8 ⁺ T cells	In CD8 ⁺ T cells	In CD8 ⁺ T cells
P12	22.5	70	18.5		
P13	58.5	41.5	14	1.5	84.6
P14	77.6	22.4	9.3	54.8	36
P15	55	44	33.9		
P16	58.5	32.6	60.3		
P17	59.7	35.6	29.5		
P18	90.8	6.4	3.7	51.4	45
P19	70.4	26.9	12.5		
P20	89.9	6.7	6.5	28.7	67.6
P21	81.5	14	23.5		
P22	47.8	42.8	52.7		
P23	35.1	56.5	57.5		
P24				1	56.8
P25				61.1	29.6
P26				3	87
HC, median (range)	65.2 (46-79.4)	24.4 (12.3-48.8)	15.05 (3.6-33.2)	61.8 (45.5-82.4)	22.8 (11.2-41.5)

HC, Healthy control; CD8 naive, CD8⁺ naive T cells (CD8⁺CD45RO⁻CCR7⁺); CD8 Temra, CD8⁺ terminally differentiated effector memory T cells (CD8⁺CD45RO⁻CCR7⁻); 4RA, CD45RA⁺CD4⁺ T cells (CD4⁺CD45RA⁺RO⁻); 4RO, CD45RO⁺CD4⁺ T cells (CD4⁺CD45RA⁻RO⁺); 8RO, CD45RO⁺CD8⁺ T cells (CD8⁺CD45RA⁻RO⁺).

TABLE E2. CDR3 sequence reads for different T-cell subsets

Sample	Total reads	Effective reads*	Productive reads†	Total clonotypes	Unique peptides
4RA-P	18,780,000 ± 4,908,000	16,630,000 ± 4,360,000	11,330,000 ± 3,238,000	139,368 ± 36,577	109,613 ± 30,439
4RA-C	17,710,000 ± 3,550,000	15,350,000 ± 3,393,000	10,280,000 ± 2,391,000	148,800 ± 37,457	121,312 ± 37,391
4RO-P	19,810,000 ± 3,020,000	17,060,000 ± 2,573,000	12,690,000 ± 2,319,000	131,527 ± 36,565	98,035 ± 31,458
4RO-C	17,870,000 ± 2,367,000	15,340,000 ± 2,893,000	10,670,000 ± 1,992,000	146,493 ± 39,410	114,205 ± 34,780
8RA-P	20,550,000 ± 4,180,000	17,460,000 ± 3,890,000	13,000,000 ± 3,411,000	115,676 ± 43,616	84,476 ± 32,101
8RA-C	19,380,000 ± 2,766,000	17,210,000 ± 2,042,000	11,770,000 ± 1,883,000	144,709 ± 23,390	111,430 ± 18,465
8Temra-P	22,990,000 ± 7,522,000	18,450,000 ± 4,210,000	14,260,000 ± 3,076,000	53,993 ± 35,341	35,643 ± 25,466
8Temra-C	19,680,000 ± 7,616,000	16,000,000 ± 4,440,000	12,360,000 ± 3,652,000	87,197 ± 27,178	60,104 ± 19,163
8Naive-P	20,730,000 ± 6,420,000	16,380,000 ± 5,222,000	11,350,000 ± 3,616,000	133,315 ± 43,914	98,283 ± 35,309
8Naive-C	20,750,000 ± 6,329,000	16,530,000 ± 5,551,000	9,956,000 ± 4,145,000	149,787 ± 46,941	117,066 ± 40,619

C, Control; 8Naive, CD8⁺ naive T cells (CD8⁺CD45RO⁻CCR7⁺); P, patient; 4RA, CD45RA⁺CD4⁺ T cells (CD4⁺CD45RA⁺RO⁻); 8RA, CD45RA⁺CD8⁺ T cells (CD8⁺CD45RA⁺RO⁻); 4RO, CD45RO⁺CD4⁺ T cells (CD4⁺CD45RA⁻RO⁺); 8Temra, CD8⁺ terminally differentiated effector memory T cells (CD8⁺CD45RO⁻CCR7⁻).

*Effective reads are reads that can be mapped with both V and J germline segments.

†Productive reads are those with complete CDR3β sequences in the correct reading frame.