

Revertant T lymphocytes in a patient with Wiskott-Aldrich syndrome: Analysis of function and distribution in lymphoid organs

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Background: The Wiskott-Aldrich syndrome (WAS) is a rare genetic disease characterized by thrombocytopenia, immunodeficiency, autoimmunity, and hematologic malignancies. Secondary mutations leading to re-expression of WAS protein (WASP) are relatively frequent in patients with WAS.

Objective: The tissue distribution and function of revertant cells were investigated in a novel case of WAS gene secondary mutation.

Methods: A vast combination of approaches was used to characterize the second-site mutation, to investigate revertant cell function, and to track their distribution over a 18-year clinical follow-up.

Results: The WAS gene secondary mutation was a 4-nucleotide insertion, 4 nucleotides downstream of the original deletion. This somatic mutation allowed the T-cell-restricted expression of a stable, full-length WASP with a 3-amino acid change compared with the wild-type protein. WASP⁺ T cells appeared early in the spleen (age 10 years) and were highly enriched in a mesenteric lymph node at a later time (age 23 years). Revertant T cells had a diversified T-cell-receptor repertoire and displayed *in vitro* and *in vivo* selective advantage. They proliferated and produced cytokines normally on T-cell-

receptor stimulation. Consistently, the revertant WASP correctly localized to the immunologic synapse and to the leading edge of migrating T cells.

Conclusion: Despite the high proportion of functional revertant T cells, the patient still has severe infections and autoimmune disorders, suggesting that re-expression of WASP in T cells is not sufficient to normalize immune functions fully in patients with WAS. (J Allergy Clin Immunol 2010;125:439-48.)

Key words: Primary immunodeficiency, Wiskott-Aldrich syndrome, secondary mutation

The Wiskott-Aldrich syndrome (WAS) is a rare X-linked immunodeficiency, characterized by thrombocytopenia, eczema, and a high frequency of infections, autoimmunity, and hematologic malignancies.¹ WAS is a result of mutations in the gene encoding the WAS protein (WASP), which is expressed in hematopoietic cells.^{2,3} WASP controls actin cytoskeleton remodeling by promoting Arp2/3-dependent actin nucleation in response to extracellular stimuli. In resting cells, WASP is stabilized by WASP-interacting protein and adopts an autoinhibited conformation.⁴ On stimulation, Cdc42 binds to WASP, which releases the intramolecular interaction, thereby allowing actin nucleation.⁵ WASP controls several immune cell functions, including adhesion and chemotaxis,⁶⁻¹¹ assembly of immunologic synapses,^{12,13} production of cytokines by T cells,¹⁴ regulatory T-cell activity,¹⁵ natural killer (NK)-cell lytic activity,¹⁶ and B-cell homeostasis.^{17,18} Consequently, clinical manifestations associated with WAS arise from the combination of impaired hematopoietic cell migration, defects in intercellular communication, and lineage-specific developmental defects.

Somatic revertant mosaicism has been reported in approximately 10% of patients with WAS,¹⁹ without preferences of age or clinical score. The mechanisms responsible for this high incidence of reversion remain unclear.²⁰ Expression of revertant WASP has been reported mainly in T cells,²¹⁻²⁷ but also in B cells^{28,29} and NK cells.²⁹⁻³¹ The frequency and diversity of these secondary genetic events is most likely underestimated, as illustrated by multiple revertant genotypes within individual patients with WAS.^{27,29,32}

We report here a novel case of somatic mosaicism in a patient with WAS leading to the expression of a functional, point-mutated WASP in T cells. The function of revertant T cells and their localization in secondary lymphoid organs were investigated.

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Abbreviations used

aa:	Amino acid
HD:	Healthy donor
NK:	Natural killer
TCR:	T-cell receptor
WAS:	Wiskott-Aldrich syndrome
WASP:	Wiskott-Aldrich syndrome protein
wt:	Wild-type

These findings are discussed in the context of the clinical status of the patient.

METHODS**Patients and cell lines**

Patient W4 is a 28-year old man with a full-blown WAS (clinical score 5). His clinical evolution is detailed in Table I. No HLA-matched sibling or unrelated donor was available, and he was not a candidate for HLA-mismatched related donor transplant. Patients W1, W2, and W6 have been previously described,¹⁴ as well as patient W31.³³ Patient W10 carries a 5-nucleotide deletion in exon 10 (positions 1115-1119) resulting in a stop codon (codon 445). Blood samples from patients and age-matched healthy donors (HDs) were obtained following standard ethical procedures (Helsinki protocol) and with the approval of the concerned internal review boards. Spleen, bone marrow, and lymph node samples were collected for therapeutic or diagnostic purposes.

Untransformed T-cell lines were generated by stimulating PBMCs with 1 μ g/mL phytohemagglutinin and 100 IU/mL recombinant human IL-2 (Chiron, Uxbridge, UK). T-cell clones were generated from the phytohemagglutinin/IL-2-stimulated cells by limiting dilution. A feeder mixture consisting of irradiated allogenic PBMCs (1×10^6 /mL), EBV-transformed B cells (JY cell line, 1×10^7 /mL), and 1 μ g/mL phytohemagglutinin was used to expand the cells. At day three, 50 IU/mL recombinant human IL-2 was added to the cultures. T-cell lines and clones were restimulated every 2 weeks.

DNA analysis

For the mutational status analysis of the WAS gene, Genomic DNA was extracted from PBMCs, primary T cells, and EBV cell lines by using QIAmp DNA blood mini kit (Qiagen, Hilden, Germany). For T-cell clones, DNA was extracted from a pellet of 0.5 to 1×10^6 cells. Exon 6 was amplified by PCR using Taq DNA Polymerase (Roche, Basel, Switzerland) and the following primers: forward 5'-CTAGAAAAGTCCCTCTCATG-3'; reverse, 5'-CCAACTCTCATTCCTCCATC-3'. The PCR product was visualized on 1.5% agarose gels, purified, and cloned in a TA vector (Invitrogen, Carlsbad, Calif). Positive clones were subsequently selected and sequenced.

For the size distribution analysis of T-cell receptor (TCR) rearrangements, freshly isolated PBMCs were stained with WASP and CD3 as described. WASP⁺ and WASP⁻ T cells were sorted with a MoFlo cell sorter (Beckman Coulter, Brea, Calif). DNA was prepared by using the QIAmp DNA FFPE Tissue Kit (Qiagen). As described in detail in this article's Online Repository (Methods section) at www.jacionline.org, PCR amplifications were performed by using the IdentiClone TM TCR β Gene Clonality Assay (InVivoScribe Technologies, San Diego, Calif) applying the BIOMED-2 multiplex PCR protocols³⁴ that amplify all TCR β CDR3 regions.

Flow-cytometry analysis of WASP expression

Staining of WASP on whole blood was performed after fixation/permeabilization (Fix&Perm kit; BD Pharmingen, San Diego, Calif) by incubation with anti-WASP 5A5 mAbs (BD Pharmingen) conjugated to AlexaFluor-488 (Microscale Protein Labeling kit; Invitrogen). Surface staining was performed with anti-CD3-APC (tandem conjugates of allophycocyanin), anti-CD19-APC,

anti-CD27-phycoerythrin, anti-CD16-phycoerythrin, anti-CD56-APC, and anti-CD14-phycoerythrin mAbs (all from BD Pharmingen). Staining of WASP on cultured T cells was performed with AlexaFluor-488-conjugated anti-WASP 5A5 mAbs. Alternatively, unconjugated anti-WASP 5A5 mAbs followed by anti-IgG_{2a}-phycoerythrin mAbs (Southern Biotechnology, Birmingham, Ala) were used. Samples were analyzed on a FACSCanto or FACSCalibur cytometer (Becton Dickinson, Franklin Lakes, NJ) and processed with the FCSexpress or FlowJo softwares.

Western blot analysis of WASP expression

T lymphocytes were lysed in a RIPA buffer supplemented with a protease inhibitor cocktail (Sigma Aldrich, Saint Louis, Mo). Lysates were boiled, separated through 7.5% SDS-polyacrylamide, and transferred to nitrocellulose membranes. Membranes were blocked and incubated with anti-WASP (H250; Biotechnology, Santa Cruz, Calif) and anti- β -actin (AC15; Sigma) antibodies, followed by goat antirabbit IgG-IR Dye 800CW (LI-COR Biosciences, Lincoln, Neb) and goat antimouse-AlexaFluor-680 mAbs (Molecular Probes, Eugene, Ore). WASP and β -actin expression was recorded on an Odyssey Infrared Imaging System (LI-COR Biosciences).

Analysis of WASP localization by confocal microscopy

To induce a migratory phenotype, CD4⁺ T cells were incubated 20 minutes at 37°C onto poly-L-lysine treated slides coated with 100 ng/mL CXCL12 (Preprotech, London, UK). Cells were fixed/permeabilized and stained with phalloidin-AlexaFluor-488 (Molecular Probes) and anti-WASP Abs (H-250; Santa Cruz Biotechnology) followed by donkey antirabbit-AlexaFluor-555 Abs (Invitrogen), and anti- α -tubulin IgG₁ mAb (Sigma) followed by goat antimouse IgG₁-AlexaFluor-633 Abs (Invitrogen). After mounting (Vectashield; Vector Laboratories, Burlingame, Calif), samples were examined on a Zeiss LSM510 confocal microscope (x63-1.4 oil immersion objective). To induce immunologic synapse formation, CD4⁺ T cells were incubated 20 minutes at 37°C with JY cells (ratio 2:1) pulsed with superantigens (100 ng/mL Staphylococcal Enterotoxin B, Staphylococcal Enterotoxin E and Toxic Shock Syndrome Toxin-1). Cell conjugates were transferred onto poly-L-lysine-coated slides and processed as described, with the exception that the α -tubulin staining was replaced by a CD2 staining (anti-CD2 IgG₁ mAbs from BD followed by goat antimouse IgG₁-AlexaFluor-633 Abs).

Analysis of WASP expression in tissue biopsies

Formalin-fixed or Bouin-fixed specimens (spleen and lymph node or bone marrow, respectively) were paraffin-embedded. Four-micrometer-thick sections were cut, deparaffinized, and stained with hematoxylin-eosin for routine histopathological examination. In parallel, tissue sections were stained with anti-CD3, anti-paired box gene 5 (PAX5) mAbs (Dako) and anti-WASP mAbs (clone D1; Santa Cruz Biotechnology) after antigen retrieval with TRIS-EDTA. Reactions were developed with horseradish peroxidase polymer (Lab Vision, Ipswich, Mass), and sections were counterstained with hematoxylin.

Proliferation and cytokine production

Proliferation was assessed by liquid scintillation counting of ³H-thymidine incorporation in T-cell lines and clones stimulated for 72 hours with antibody-coated latex beads, as previously described.¹⁷

Intracytoplasmic cytokines were measured in PBMCs stimulated with beads coated with 10 μ g/mL anti-CD3 and anti-CD28 mAbs. After 3 hours, 10 μ g/mL Brefeldin A (Calbiochem-Merck, Nottingham, UK) was added. After an additional 3 hours, cells were stained with anti-CD4-phycoerythrin-cyanine5 and anti-CD8-APC mAbs (BD). Cells were then fixed/permeabilized and stained with anti-WASP-AlexaFluor-488 plus anti-human IL-2-phycoerythrin or anti-human IFN- γ -phycoerythrin mAbs (BD). Analysis was performed on a FACSCanto cytometer, and data were processed with the FCSexpress software (De Novo Software, Los Angeles, Calif).

TABLE I. Clinical follow-up of patient W4 with WAS

Age	Clinical events	Laboratory data	Treatments
Birth	Hematoma Eczematous skin areas	Hyperbilirubinemia Thrombocytopenia (30,000/cubic millimeter) with small platelets Decreased lymphocyte counts Decreased IgM/IgG Increased IgA/IgE	Intravenous Immunoglobulins (IV Ig) Hydrocortisone
1½ y*	Eczematous skin areas	Thrombocytopenia (30,000- 40,000/cubic millimeter)	IV Ig Hydrocortisone
8 y	Bronchopneumonia Varicella zoster infection	Antismooth muscle reactivity Positive direct and indirect Coombs tests	
9 y	Autoimmune hemolytic anemia		Steroid treatment†
10 y	Autoimmune hemolytic anemia	Persistent thrombocytopenia	Splenectomy‡
12 y	Hemorrhagic vasculitis Migratory joint pain and swelling	Decreased platelet counts (10,000/cubic millimeter)	Prednisone therapy
13 y	Hemorrhagic vasculitis Migratory joint pain and swelling	Autoimmune work-up negative except for mildly positive direct Coombs test	Nonsteroidal anti- inflammatory drugs alternated with steroid therapy (transitory clinical improvement)
20 y	Increased incidence of viral and bacterial infectious complications	<i>Molluscum contagiosum</i> and pyodermitis	IV Ig Treatment of infectious episodes
23 y	Severe pyodermitis with satellite inguinal lymphadenitis	Multiple abdominal lymphadenopathy Reactive adenitis positive for <i>Staphylococcus aureus</i> § Bone marrow dyshematopoiesis	IV Ig Aggressive intravenous antibiotics Transient steroid treatment
27 y	Severe pyodermitis associated with subcutaneous adenitis	Positivity for <i>Staphylococcus aureus</i>	IV Ig Antibacterial prophylaxis Steroid treatment

*Diagnosis of WAS established.

†The patient has been steroid-dependent since then.

‡Splenectomy led to transient resolution of the hemolysis and normalization of platelet counts.

§Mesenteric lymph node laparoscopic biopsy failed to show lymphoproliferative disorder.

||Bone marrow biopsy failed to show either lymphoid or myeloid proliferation.

RESULTS

Revertant WASP expression in T lymphocytes of a patient with WAS

The patient with WAS studied here (W4) was known to carry a single nucleotide deletion introducing a premature stop codon (Jin et al,³⁵ patient 126-B). However, flow-cytometry analysis revealed expression of WASP in a fraction of peripheral blood T cells (Fig 1, A). In a sample collected when the patient was 25 years old, WASP expression was detected in approximately 50% of peripheral blood T cells (both in CD4⁺ and CD8⁺ T cells; see this article's Fig E1 in the Online Repository at www.jacionline.org), but not in B cells, NK cells, or monocytes. Complementary data also failed to show WASP expression in megakaryocytes (see this article's Fig E2 in the Online Repository at www.jacionline.org). HLA typing confirmed that WASP⁺ T cells did not derive from the engraftment of cells from the mother or others (data not shown). The region containing the germline mutation in the WAS gene was sequenced, confirming the t570 point deletion in exon 6, leading to frame shift and premature stop at codon 260 (sequence W4 in Fig 1, B). A secondary 4-nucleotide (gcgc) insertion was also detected, 4 nucleotides downstream of the original deletion (sequence W4R in Fig 1, B). This insertion was predicted to lead to restoration of the reading frame with a LP→RPR amino acid (aa) substitution. The revertant W4R sequence was found in DNA clones established from both a peripheral blood-derived T-cell line and from an abdominal lymph node biopsy, but not from an EBV B-cell line (data not shown). Flow-cytometry analysis of a T-cell line showed that revertant WASP expression level was comparable to that of wild-type (wt) WASP in HD T cells (Fig

1, C). This is in contrast with the reduced expression of WASP observed in W6 patient, a patient with X-linked thrombocytopenia carrying a single aa substitution. In agreement with the predicted W4R sequence, Western blot analysis showed normal molecular weight for the revertant WASP (Fig 1, D). A modeling analysis predicted that the revertant protein would adopt a conformation closely related to that of the wt one (see this article's Fig E3 in the Online Repository at www.jacionline.org). Taken together, these data indicate that patient W4 is a novel case of secondary WAS gene mutation and suggest that the stability of the revertant protein may be preserved.

Correct subcellular localization of revertant WASP

Subcellular localization of revertant WASP was analyzed by confocal microscopy. As shown in Fig 2, A, WASP staining of W4 CD4⁺ T cells clearly discriminated WASP⁺ (empty arrows) from WASP⁺ revertant T cells. Similarly to wt WASP, revertant WASP distributed beneath the plasma membrane and colocalized with cortical actin. In the revertant W4 T cells that polarized in response to CXCL12, revertant WASP localized normally at the F-actin-rich leading edge lamellipodia (Fig 2, A, lower panels, filled arrows). As indicated by the α-tubulin costaining, both wt and revertant WASP were also enriched around the microtubule-organizing center, at the basis of the uropod. As shown in Fig 2, B (upper panels), revertant WASP colocalized normally with cortical actin in T cells at the contact with unpulsed APC. On contact with superantigen-pulsed APC, revertant WASP and actin redistributed to the immunologic synapse, as revealed by the polarization of CD2 (Fig 2, B, lower panels, white arrows).

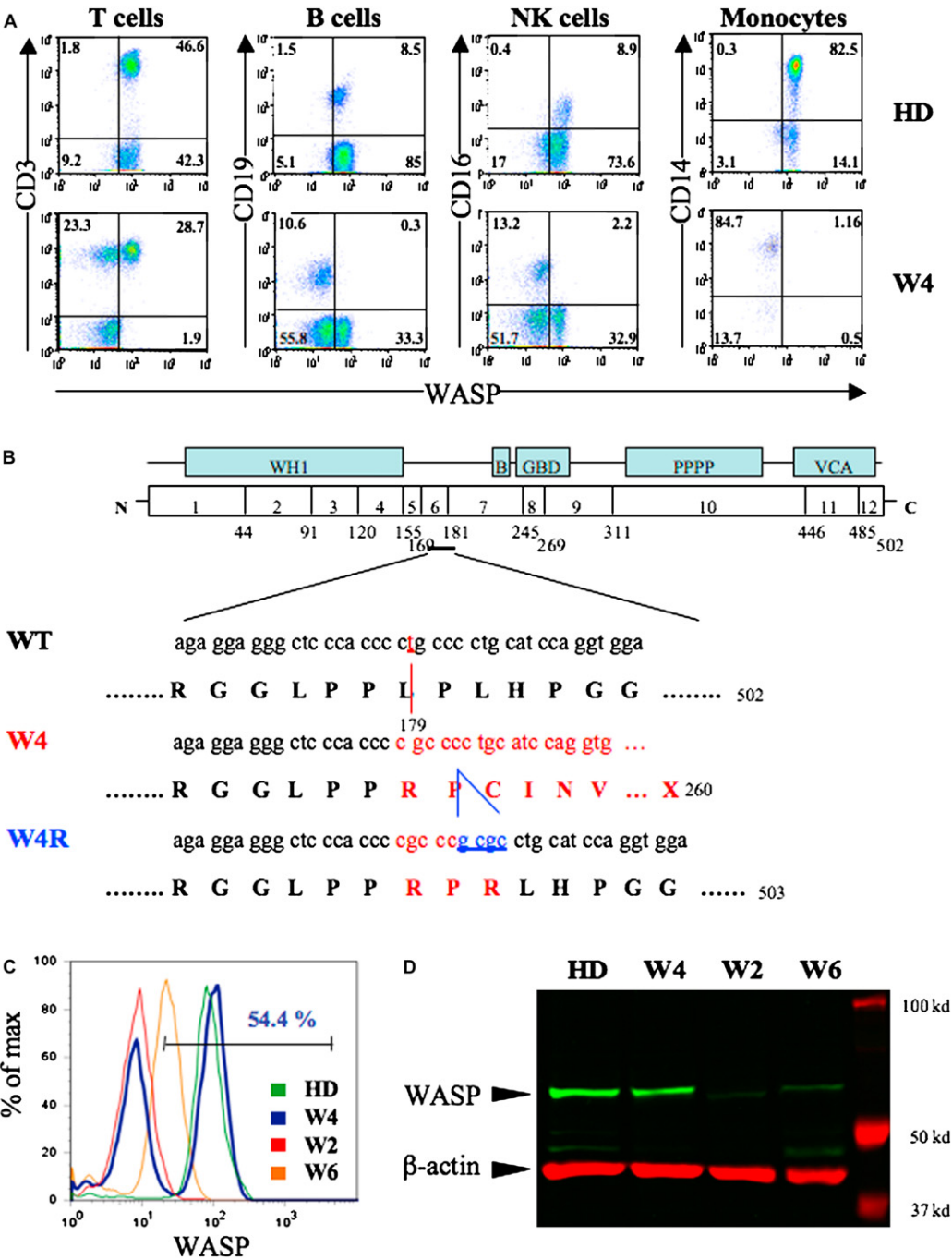


FIG 1. Characterization of revertant WASP expression caused by secondary site mutation. **A**, Flow-cytometry analysis of WASP expression in patient W4 (age 25 years) and HD PBMCs, in combination with CD3, CD19, CD16, and CD14 stainings. **B**, Nucleotide and aa sequences of W4 germ-line (W4) and secondary (W4R) mutations. Flow-cytometry (**C**) and Western blot (**D**) analysis of WASP expression in CD4⁺ T-cell lines from 1 HD and patients W4, W2, and W6.

Together, these data indicate that revertant WASP displays a normal subcellular localization, at resting state and on stimulation.

Selective advantage and TCR diversity of revertant T cells

From the age 20 to 28 years, the fraction of WASP⁺ T cells (both CD4⁺ and CD8⁺) in the blood of patient W4 progressively increased from approximately 10% to 70% (Fig 3, A). This

indicates that WASP⁺ revertant T cells had a selective growth advantage *in vivo* over WASP⁻ T cells. Interestingly, on T-cell culture, a further rapid increase of the percentage of WASP⁺ cells was observed (Fig 3, A). To study revertant T-cell TCR repertoire diversity, WASP⁺ and WASP⁻ peripheral blood T cells were sorted to analyze separately the size distribution of the major TCR V β rearrangements. Similarly to T cells isolated from a HD, WASP⁺ T cells exhibited a diversified TCR size distribution.

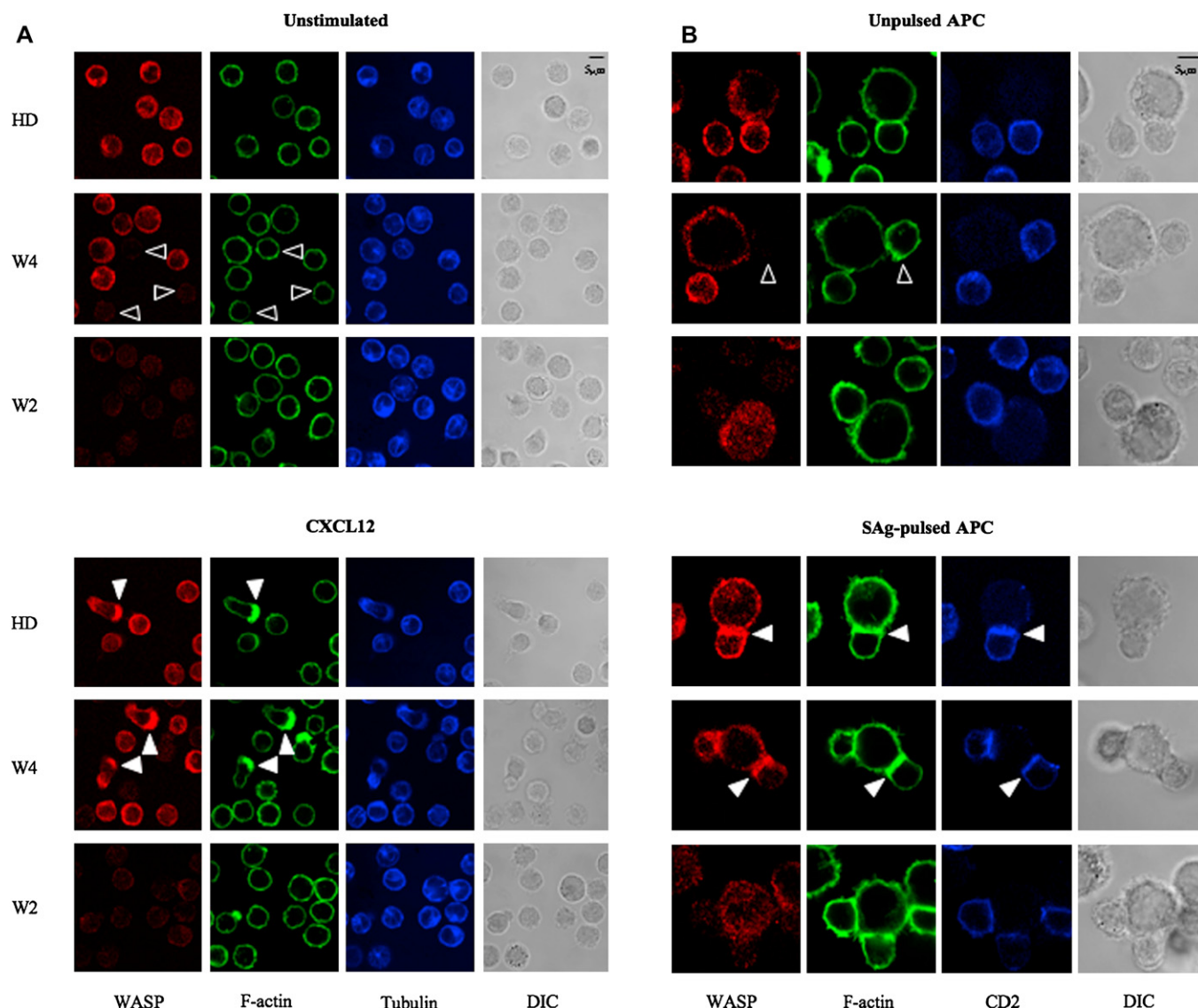


FIG 2. WASP subcellular localization. HD, patient W4, and patient W2 $CD4^+$ T cells stimulated with CXCL12 (A) or superantigen (SAg)-pulsed APC (B) and costained for WASP, F-actin, α -tubulin, or CD2. Differential interference contrast (DIC) is shown in parallel. Empty arrows indicate WASP-negative cells. Filled arrows indicate CXCL12-induced lamellipodial protrusion enriched in WASP and F-actin (A) or immunologic synapses enriched in WASP, F-actin, and CD2 (B). Results representative of 3 independent experiments.

In contrast, WASP⁻ T cells appeared to have a more restricted pattern, suggesting reduced TCR repertoire diversity (Fig 3, B).

Normal response to TCR stimulation in revertant T cells

To assess the TCR-driven proliferation of W4 revertant T cells, a T-cell line enriched in WASP⁺ T cells (70%) was stimulated with anti-CD3 mAbs and compared with T-cell lines from 2 WASP⁻ patients (W2 and W10) and 4 HDs (1-4). As shown in Fig 4, A, W4 T cells proliferated at low concentrations of anti-CD3 mAbs (0.005-0.1 μ g/mL), similarly to control T cells. This was in contrast with the WASP⁻ T cells that failed to proliferate at these concentrations. At higher concentrations, W4 T cells reached a proliferation plateau lower than that of HD T cells,

probably because of residual WASP⁻ T cells. The production of IL-2 and IFN- γ was then tested in the peripheral $CD4^+$ T cells of the patient. On anti-CD3/CD28 mAb stimulation, approximately one third of HD and WASP⁺ W4 T cells produced IL-2 (Fig 4, B). Conversely, only a very small proportion of WASP⁻ W4 T cells produced IL-2, similarly to WASP⁻ W31 T cells. W4 revertant T cells also produced IFN- γ on stimulation, in a proportion and at levels similar to those of HD T cells (Fig 4, B). To assess more precisely the impact of the secondary mutation on WASP expression and on TCR-driven proliferation, $CD4^+$ T-cell clones were generated from patient W4 ($n = 34$). In the W4 T-cell clones, WASP expression was either negative (as in representative clone W4 3.19) or positive (as in representative clone W4 03.2) and correlated with the absence or presence of the W4R secondary mutation, respectively (Fig 5, A).

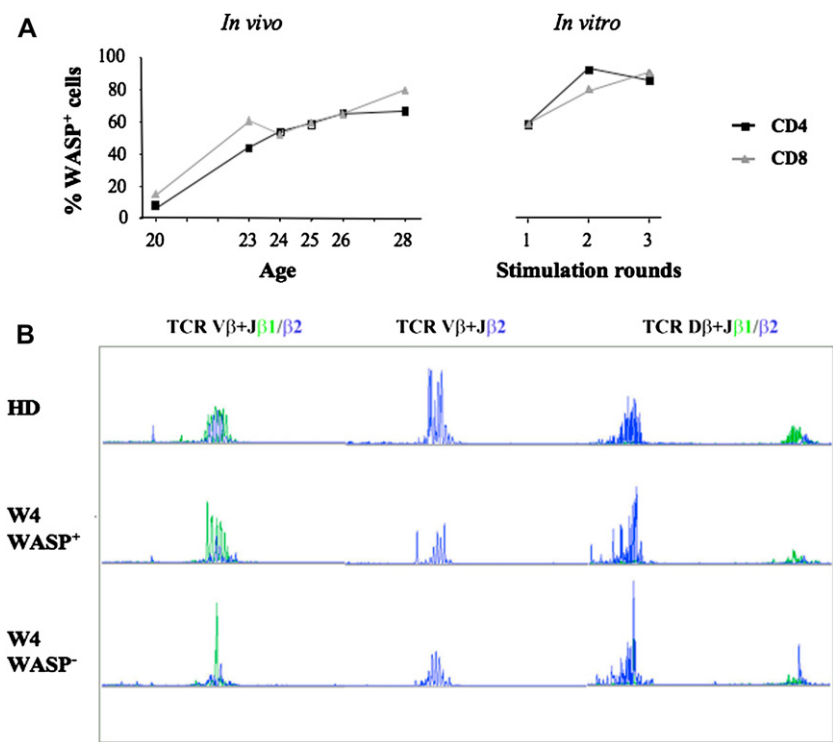


FIG 3. Expansion and TCR diversity of revertant T cells. **A**, Flow-cytometry analysis of WASP expression in patient W4 peripheral blood CD4⁺ and CD8⁺ T cells along time (age 20-28 years) and in a T-cell line stimulated and expanded *in vitro* (from PBMCs obtained at age 25 years). **B**, Size distribution of the major TCRβ rearrangements (Vβ-Jβ1/2.2/2.6/2.7; Vβ-Jβ2.1/2.3/2.4/2.5; Dβ-Jβ, as indicated by green and blue) analyzed on peripheral blood T cells from a HD and on sorted WASP⁺ and WASP⁻ peripheral blood T cells from patient W4.

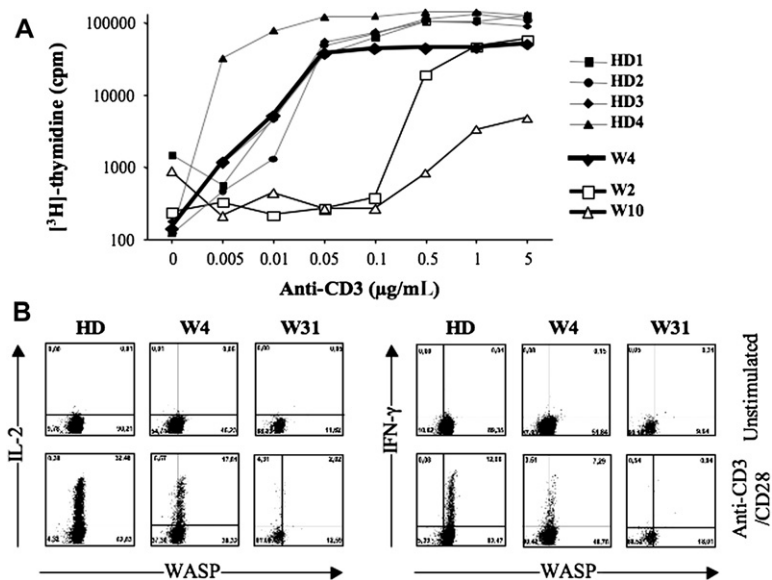


FIG 4. Proliferation and cytokine production. **A**, Anti-CD3/CD28 mAb-driven proliferation in counts per minute (cpm) of T cell lines from HDs and patients W4, W2, and W10. **B**, Anti-CD3/CD28 mAb-driven production of IL-2 and IFN-γ in peripheral blood CD4⁺ T cells from a HD and patients W4 and W31.

Importantly, all WASP⁺ clones analyzed (n = 11) carried the same secondary mutation, and WASP expression levels were comparable in W4 WASP⁺ and HD clones (mean fluorescence intensity, 68.8 ± 10.0 and 71.3 ± 13.8, respectively). Analysis of

TCR diversity in the WASP⁺ clones showed diversified Vβ use (see this article's Table E1 in the Online Repository at www.jacionline.org), confirming the TCR repertoire diversity observed in fresh revertant T cells. On stimulation with anti-CD3/CD28

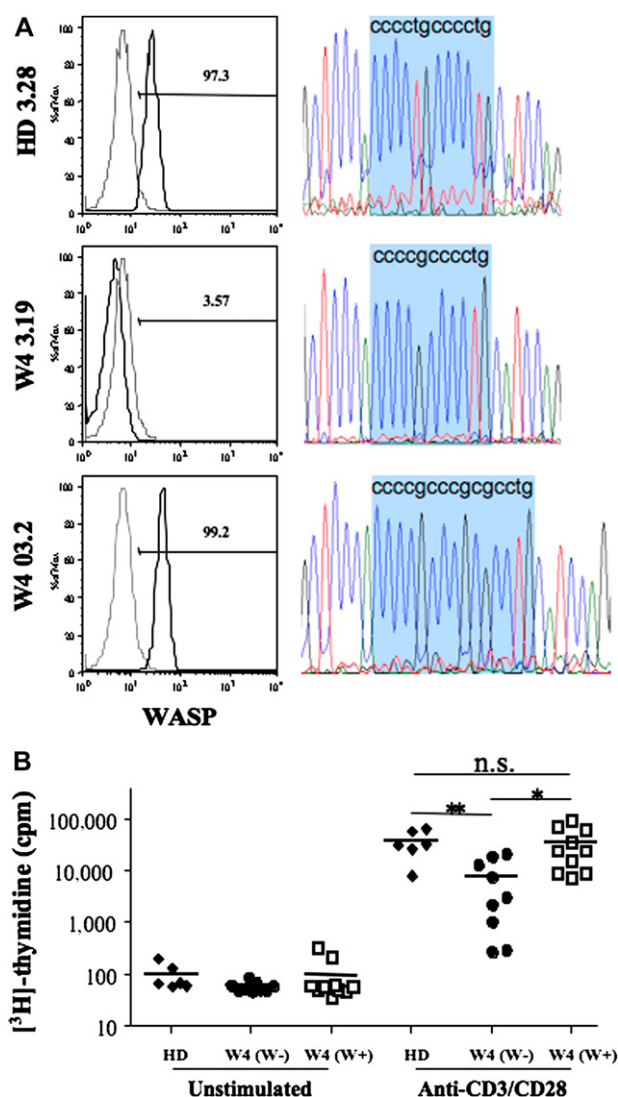


FIG 5. Analysis of T-cell clones. **A**, Flow-cytometry analysis of WASP expression in representative CD4⁺ T-cell clones with corresponding sequence (1 clone from a HD; 1 WASP⁻ and 1 WASP⁺ clone from patient W4, age 23 years). **B**, Anti-CD3/CD28 mAb-driven proliferation in counts per minute (cpm) of CD4⁺ T-cell clones from 1 HD and patient W4 (divided according to WASP expression). The average of 3 different experiments is shown (* $P < .05$; ** $P < .01$; n.s., not significant).

mAbs, the WASP⁺ revertant T-cell clones proliferated at levels similar to those of control HD T-cell clones, whereas the WASP⁻ T-cell clones proliferated more heterogeneously and to lower levels (Fig 5, B). These results demonstrate that only 1 type of reverse mutation was likely to be responsible for the expression of WASP in revertant T cells, which showed normal proliferation and cytokine production.

Early accumulation of revertant T cells in T-cell zones of lymphoid tissues

We then developed an immunohistologic analysis of WASP to track the distribution of revertant cells within different hematopoietic tissues. WASP staining was first validated in control tissues (see this article's Fig E4 in the Online Repository at

www.jacionline.org). The analysis of the spleen from patient W4, removed at the age of 10 years, revealed that WASP was already expressed by a large amount of cells in the white pulp, mainly in periarteriolar sheets (Fig 6, A). A parallel CD3 staining suggests that WASP⁺ cells corresponded to T cells and that the majority of T cells were indeed WASP⁺ (Fig 6, B). This finding shows that the expansion of revertant T cells likely started more than a decade before their detection in the blood. A mesenteric lymph node biopsy (age 23 years) was highly enriched in WASP⁺ lymphocytes (Fig 6, C), identified as CD3⁺ T cells belonging to paracortical areas and B-cell follicles (Fig 6, D). This was indirectly confirmed by the absence of colocalization between WASP and the B-cell marker Pax5 (Fig 6, E). In the bone marrow (age 23 years), most of the analyzed area showed rare WASP⁺ lymphoid cells (Fig 6, F). Loose aggregates of WASP⁺ lymphoid cells corresponding to mature T-cell aggregates were observed (Fig 6, G), as confirmed by CD3 staining on parallel sections (data not shown). Together, these data suggest that WASP⁺ T cells have a strong selective advantage *in vivo* and localize correctly to secondary lymphoid organs.

DISCUSSION

Cases of somatic revertant mosaicisms have been reported in different inherited disorders,³⁶ including primary immunodeficiencies.³⁷⁻³⁹ In WAS, the frequency of spontaneous revertant mutations is estimated to be 10%.^{19,20} Remarkably, multiple reverse mutations occurring at distinct sites in the WAS gene were found in single patients.^{27,29,32} These reversion cases are unique in that they provide relevant information about the role of WASP in the development, function, and homeostasis of hematopoietic cells. We report here the case of a patient with WAS expressing a functional revertant WASP mutant in T cells. For the first time in such a case, revertant WASP expression was tracked over time in various hematopoietic organs.

The original mutation was a single-base deletion causing a frame shift and a premature stop codon, leading to the loss of WASP expression. We identified a novel 4-nucleotide insertion, 4 nucleotides downstream the original mutation. This region of the WAS gene has not been described as a mutational hotspot. However, the high CG content and the presence of 2 cytosine tracts surrounding the original mutation may have favored secondary insertion. This event restored the reading frame and allowed mutant protein expression with a predicted LP→RPR aa change. Modeling analysis indicated that the overall conformation of the mutant protein would be preserved. Because the mutation lies in exon 6, which does not encode any known function or binding domain of the protein, the function of the revertant protein may also be preserved.

Revertant WASP displayed a normal colocalization with F-actin both in resting and activated conditions, suggesting that revertant WASP is functional during chemotaxis and antigen stimulation. Consistently, the presence of revertant T cells in the T-cell zone of the spleen and of a mesenteric lymph node indicates that revertant T cells migrated and distributed normally to secondary lymphoid organs. In addition, revertant T cells displayed normal TCR-driven proliferation even at low-dose anti-CD3 mAbs, and normal IL-2 and IFN- γ production, suggesting that they could respond to physiological doses of antigen. Together, these data indicate that revertant WASP is functional, the best evidence being the selective advantage of revertant T cells *in vivo*.

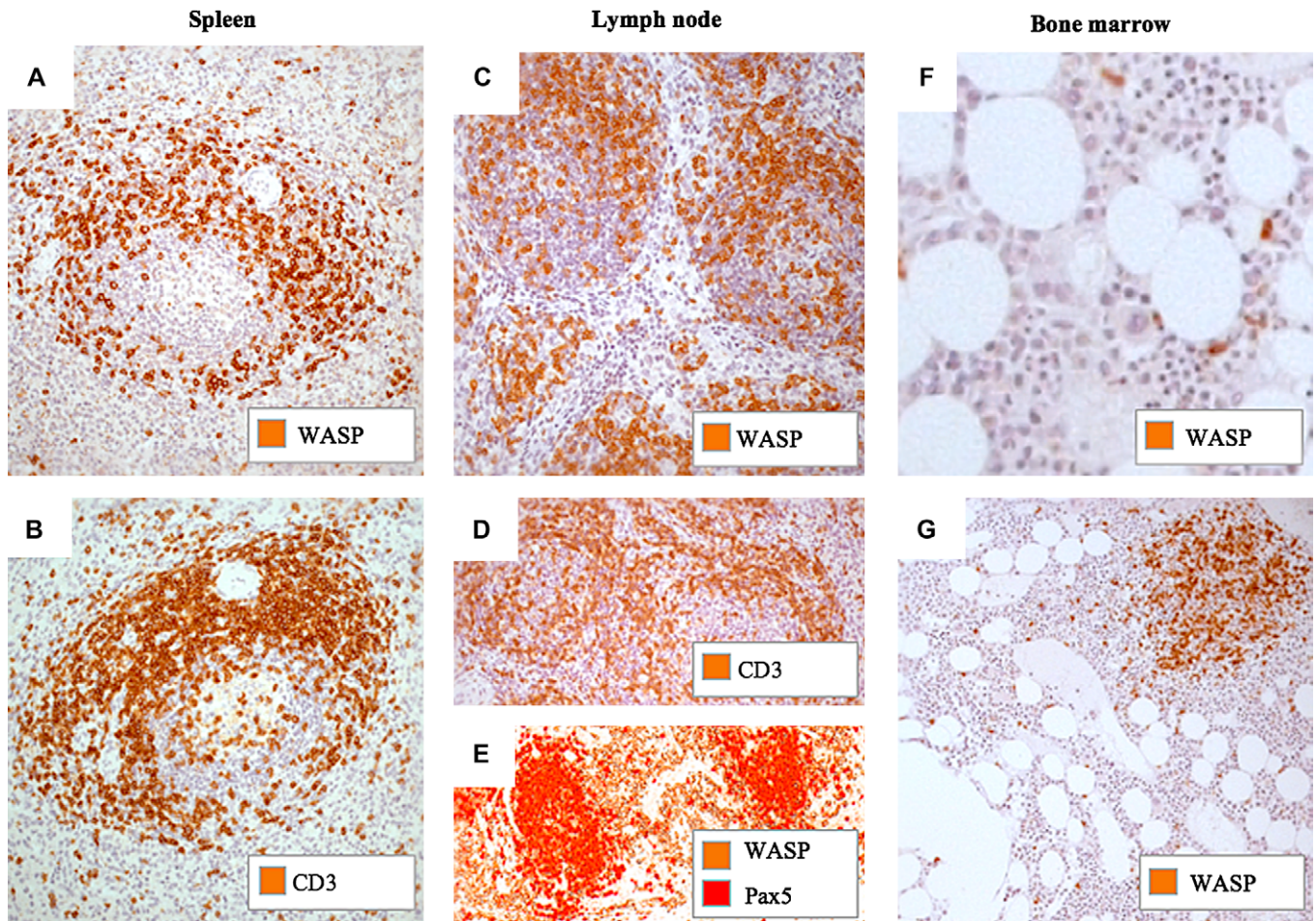


FIG 6. Lymphoid tissue distribution of revertant WASP expression. Histologic analysis of WASP expression in lymphoid tissues from patient W4, including spleen (**A and B**) collected at age 10 years, and lymph node (**C-E**) and bone marrow (**F and G**) collected at age 23 years. Stainings for WASP (**A, C, E, F, and G**) and CD3 (**B and D**) on adjacent sections or Pax5 as costaining (**E**). Pictures taken with a $\times 10$ (**G**) or $\times 20$ (**A-F**) objective show representative areas of each tissue.

The detection of a high proportion of WASP⁺ cells in the spleen (age 10 years) suggests that the reverse mutation event occurred early in life. Accumulation of WASP⁺ cells may have been favored by white-pulp hypoplasia (data not shown). Given the apparently higher enrichment of revertant T cells in the lymph node compared with the blood, it can be speculated that revertant T cells may have used secondary lymphoid organs as reservoirs. This observation is reminiscent of the preferential enrichment within the spleen of genetically corrected T cells that developed after gene therapy in WAS-knockout mice.^{40,41} Revertant T-cell chimerism in the blood may depend on the properties of the revertant protein but also on the nature and strength of the stimuli encountered. Interestingly, sorted WASP⁺ revertant peripheral blood T cells had a normally diversified TCR repertoire, whereas the WASP⁺ T-cell counterpart displayed reduced diversity, as expected for adult patients with WAS.⁴² This suggests that revertant WASP expression occurred as early as in a T-cell progenitor and that it helped maintaining a diversified TCR repertoire in the progeny. Alternatively, the secondary mutation event may have occurred in a more primitive hematopoietic progenitor, although our approaches failed to reveal WASP expression in B cells, NK cells, megakaryocytes, or myeloid cells. We found higher

percentages of revertant T cells within the memory, compared with the naive, subset (**Fig E1**), suggesting that part of the selective pressure may have occurred on antigen stimulation. Possibly, the few WASP⁺ cells detected in the bone marrow could be resident central memory T cells.⁴³ Together, the detection of revertant T cells in multiple lymphoid organs points toward a normal homing and survival of WASP⁺ T cells.

Our follow-up study indicates that although apparently functional revertant T cells started to expand early in life, the patient presented a high incidence of viral and bacterial infections and developed autoimmunity. Several explanations may account for the observed clinical course. Progressive restoration of WASP expression restricted to the T-cell compartment is probably not sufficient to ameliorate significantly the immune control of infectious episodes, in particular in the context of immunosuppressive therapy. In comparison, patients with X-linked thrombocytopenia, expressing from birth residual WASP in all hematopoietic lineages, appear not to have recurrent infections.⁴⁴ The autoimmune manifestations may also be caused by the presence of residual WASP-negative autoreactive lymphocytes, as suggested by the striking observation that a majority of patients with WAS with mixed chimerism after hematopoietic stem cell

transplantation develop autoimmunity.⁴⁵ Moreover, revertant WASP expression may have variable outcomes in restoring functions in distinct CD4⁺ T-cell subpopulations involved in the control versus triggering of autoimmunity. We previously reported defective suppressive activity in regulatory T cells isolated from the patient,¹⁵ although we could not determine whether they expressed revertant WASP. On the other hand, preliminary data indicate enrichment of WASP⁺ cells within the CD4⁺CD161⁺ T cells (Fig E5), suggesting a biased selective advantage in T_H17 cells.⁴⁶

In summary, clinical improvement after genetic reversion most probably depends on the size, diversity, and function of the revertant cell pool. In the context of WAS gene therapy strategies, optimal therapeutic effects will also depend on those parameters. Current strategies target gene delivery to hematopoietic stem cells potentially to correct all hematopoietic cell lineages.^{47,48} In addition, the high incidence of reversion cases in patients with WAS should be taken into consideration during the evaluation of the best therapeutic options, including gene therapy.

We are grateful to all the patients and their families, especially to patient W4 and his family, for their strong involvement in the study. We thank Robert Bredius, Silvana Martino, and Luigi Notarangelo for providing patient samples for this study. We thank Katharina Fleischhauer for the HLA typing study. We thank Sophie Allard from the IFR150 microscopy platform (Toulouse). We thank Grazia Andolfi and Massimiliano Mirolo for excellent technical assistance. We thank Marina Brambilla for the immunohistology analysis.

Clinical implications: Given the high rate of secondary mutations in patients with WAS, this study provides key information relevant to the clinical follow-up of these patients.

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METHODS

Size distribution analysis of TCR β rearrangements

Three PCR reactions targeting the large majority of complete and incomplete TCR β rearrangements (TCR β A: V β -J β 1/2.2/2.6/2.7; TCR β B: V β -J β 2.1/2.3/2.4/2.5; TCR β C: D β -J β) were performed. Negative controls (without template) and polyclonal and monoclonal controls were included for each experiment. Two microliters of PCR product was diluted 10-fold in formamide-containing 500 ROX DNA size standard (Applied Biosystems, Foster City, Calif) and denatured (95°C for 5 minutes) before separation in a high-resolution denaturing polyacrylamide gel (POP7 polymer; Applied Biosystems) and detection via a scanning laser using an automated ABI 3130 Genetic Analyzer. Analysis of PCR products was performed by GeneMapper software (Applied Biosystems) analysis.

Modeling method used to study the polypeptide fragment mapping the secondary mutation

Models of the secondary structures of the sequences spanning residues 163-192 of the wt protein (WT) and 163-193 of the revertant W4 patient protein (W4R) were initially predicted by using 3 different programs: PSIPRED,^{E1} SSPPRO/3DPRO,^{E2} and APSP2.^{E3} Molecular Dynamics (MD) simulations were then performed on shorter peptides excluding the unstructured C terminal regions. Each polypeptide chain was put in a cubic box ($6.8 \times 6.8 \times 6.8$ nm³), solvated, and neutralized by adding a sodium ion for each negative charge. The systems energy was minimized by using the steepest descent method. Thirty-nanosecond simulations were performed (pressure = 1 bar, Temperature = 300 Kelvin degrees, Particle Mesh Ewald electrostatics) using the LINear Constraint Solver algorithm for bond length constraint.^{E4,E5} To obtain a more reliable structure, Replica Exchange Molecular Dynamics (REMD)^{E6,E7} simulations were performed in the canonical ensemble (constant number of particles, constant volume and constant temperature) using the simulation parameters previously adopted by means of 16 replicas (total simulation time, 32 ns for each system) ranging from 300 to 345 K.^{E8} An exchange was attempted every 500 steps, with the acceptance ratio computed according to the Metropolis criterion.^{E6} The average exchange probability was $9.3\% \pm 2.2\%$, a level ensuring an efficient exploration of the conformational space for polypeptide chains.^{E6} The MD and REMD calculations were performed by using the GRONingen Machine for Chemical Simulation 3.2.1 system and an IBM eServer Blade-Center equipped with 28 double-processor JS20 Blades (56 PPC64 2.8 GHz processors) and Myrinet communication system. Secondary structure analysis was performed on the resulting trajectories using the DSSP program. Images were obtained using the Visual Molecular Dynamics software (Beckman Institute, Urbana, Ill).

RESULTS

Revertant WASP is expressed equally in peripheral CD4⁺ and CD8⁺ T cells and preferentially in memory T cells

As the W4 patient reached 24 years of age, revertant WASP appeared to be expressed in approximately 50% of peripheral blood T cells, with comparable percentages in CD4⁺ and CD8⁺ T cells (Fig E1). Higher percentages of WASP⁺ revertant T cells were present within the memory T cell subset (CD45RA⁻) than the naive T-cell subset (CD45RA⁺; Fig E1).

Revertant WASP is not detected in megakaryocytes

We investigated by confocal microscopy whether WASP would be expressed in megakaryocytes. For that purpose, megakaryocytes were differentiated from CD34⁺ cells by stimulation with human *thrombopoietin* (10 ng/mL), human FMS-like tyrosine kinase 3 ligand (20 ng/mL), and human stem cell factor (50 ng/mL). After 11 days of differentiation, cells were seeded on fibrinogen-coated glass slides (1 mg/mL), fixed

with 4% paraformaldehyde, and permeabilized with 0.1% Triton X-100 in PBS. Nonspecific binding was blocked in PBS-BSA 2%, and cells were incubated with phycoerythrin-conjugated anti-CD41 and with anti-WASP Abs revealed with goat antirabbit Alexa-488 Abs. 4'-6-Diamidino-2-phenylindole, dihydrochloride was applied for nucleus staining and cells were analyzed by laser scanning microscopy (Leica confocal microscope TSC SP2) with a 63 $\times$ oil objective. The representative images shown in Fig E2 clearly indicate that, in contrast with the control, no WASP expression could be detected in the megakaryocytes derived from the CD34⁺ cells of the patient W4.

Structure modeling of the WASP fraction surrounding the reversion event predicts preserved stability

To investigate further the impact of the predicted LP $\rightarrow$ RPR aa change on the folding of the revertant WASP, we modeled the corresponding fractions (30-31 aa long) of the wt and revertant proteins. MD simulations showed that both sequences folded as an extended random coil, with the exception of a short region exhibiting an α -helical/turn structure (Fig E3, A). REMD simulations (done on 19-20 aa polypeptides) indicated that the structural configurations were energetically stable and located in a deep potential energy minimum, increasing the reliability of the initial computational model. The secondary structure content, sampled during the whole MD simulation, showed that the helical content of the WT peptide was overall less stable than the one present in the W4R peptide (Fig E3, B). The WT peptide presented a mixed population of helical and hydrogen bonded turn states during the whole simulation time, whereas a net separation between helix and turn was detected in the W4R peptide, indicating an increased flexibility of residues 11 to 14. However, the presence of an arginine in the C-terminus part of the W4R sequence would allow the formation of a β -bridge between residues 3 and 4 and residues 17 and 18. As a result, the W4R peptide was predicted to be slightly more rigid than the WT peptide. Altogether, this model shows that although the revertant RPR sequence is expected to increase local rigidity, the overall structure and stability of the revertant protein should be preserved.

Immunohistologic analysis of WASP in control hematopoietic tissues

We developed a WASP staining on control tissues including spleen, lymph node, and bone marrow from control donors (not affected by WAS). In control spleen and lymph node, WASP was expressed in the cytoplasm (mainly beneath the plasma membrane) of B and T lymphocytes as well as in monocytes and macrophages, but not in erythroid cells (Fig E4, A and B), whereas in normal bone marrow (Fig E4, C), WASP was absent from erythroid precursors but clearly expressed by myeloid precursors and megakaryocytes.

Clonal analysis confirms diversified V β use in revertant W4 CD4⁺ T cells

CD4⁺ T-cell clones were generated from a blood sample collected as patient W4 was 23 years of age. After analysis of WASP expression by flow cytometry, the clones were classified as WASP-negative or WASP-positive (revertant; Table E1).

Analysis of TCR V β expression by flow cytometry (TCR V β Repertoire Kit including the determination of 24 TCR V β ; Beckman Coulter-Immunotech, Marseille, France) clearly shows a highly diversified V β usage in the W4 WASP⁺ clones, similar to that of W4 WASP⁻ clones and control clones generated from an healthy donor.

Preferential enrichment of revertant WASP⁺ cells within the CD4⁺CD161⁺ T-cell subset

To investigate the proportion of WASP⁺ revertant cells within the T_H17 subset, freshly isolated PBMCs were stained for CD4, CD161, and WASP. CD4⁺CD161⁺ T cells have been shown to correspond to the T_H17-cell subset. These cells were found in apparently reduced proportions in W4 CD4⁺ T cells (Fig E5). Further analysis of WASP expression clearly showed a higher enrichment in WASP⁺ cells within the CD4⁺CD161⁺ T cells (85%) compared with CD4⁺CD161⁻ T cells (63%). This suggests that CD4⁺CD161⁺ T_H17 T cells

underwent a stronger selective advantage than the rest of the CD4⁺ T cells.

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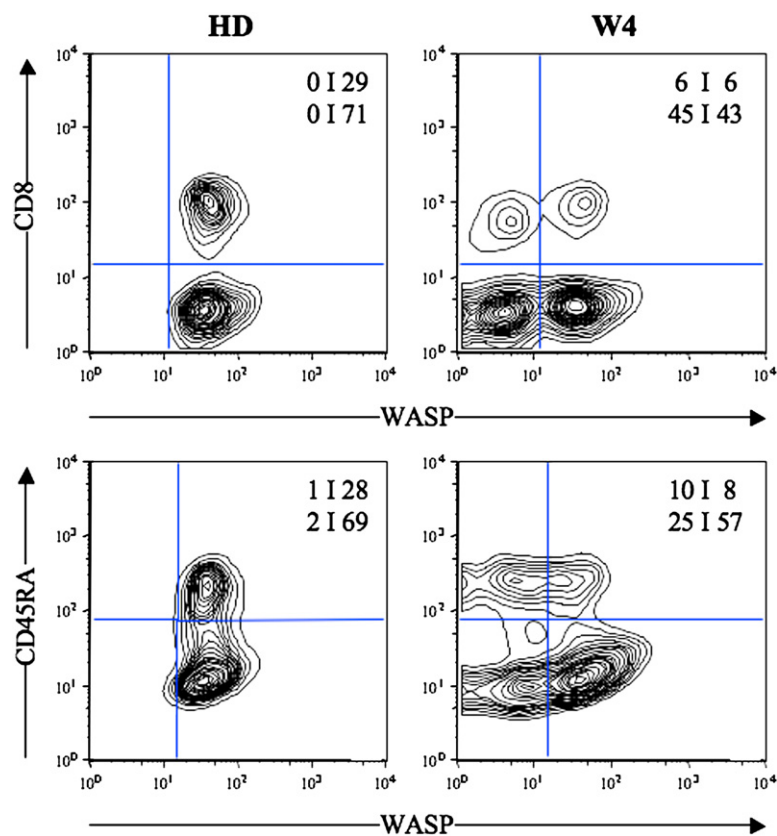


FIG E1. Revertant WASP expression in CD4⁺ and CD8⁺ T-cell subsets from patient W4. PBMCs were purified from a blood sample collected when the patient was 24 years of age. Expression of CD8 and WASP (*upper panels*) or CD45RA and WASP (*lower panels*) was analyzed by flow cytometry in T cells gated on the basis of CD3 expression.

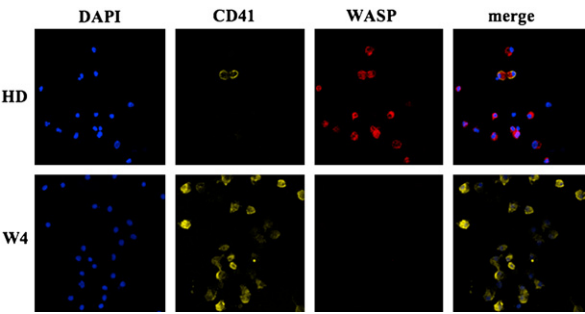


FIG E2. Analysis of WASP expression in megakaryocytes. Confocal microscopy analysis of megakaryocytes differentiated from CD34⁺ cells of a HD and the patient W4. Cells were colabelled with 4'-6-diamidino-2-phenylindole, dihydrochloride (DAPI; blue) to identify nuclei, the specific megakaryocyte marker CD41 (yellow), and WASP (red).

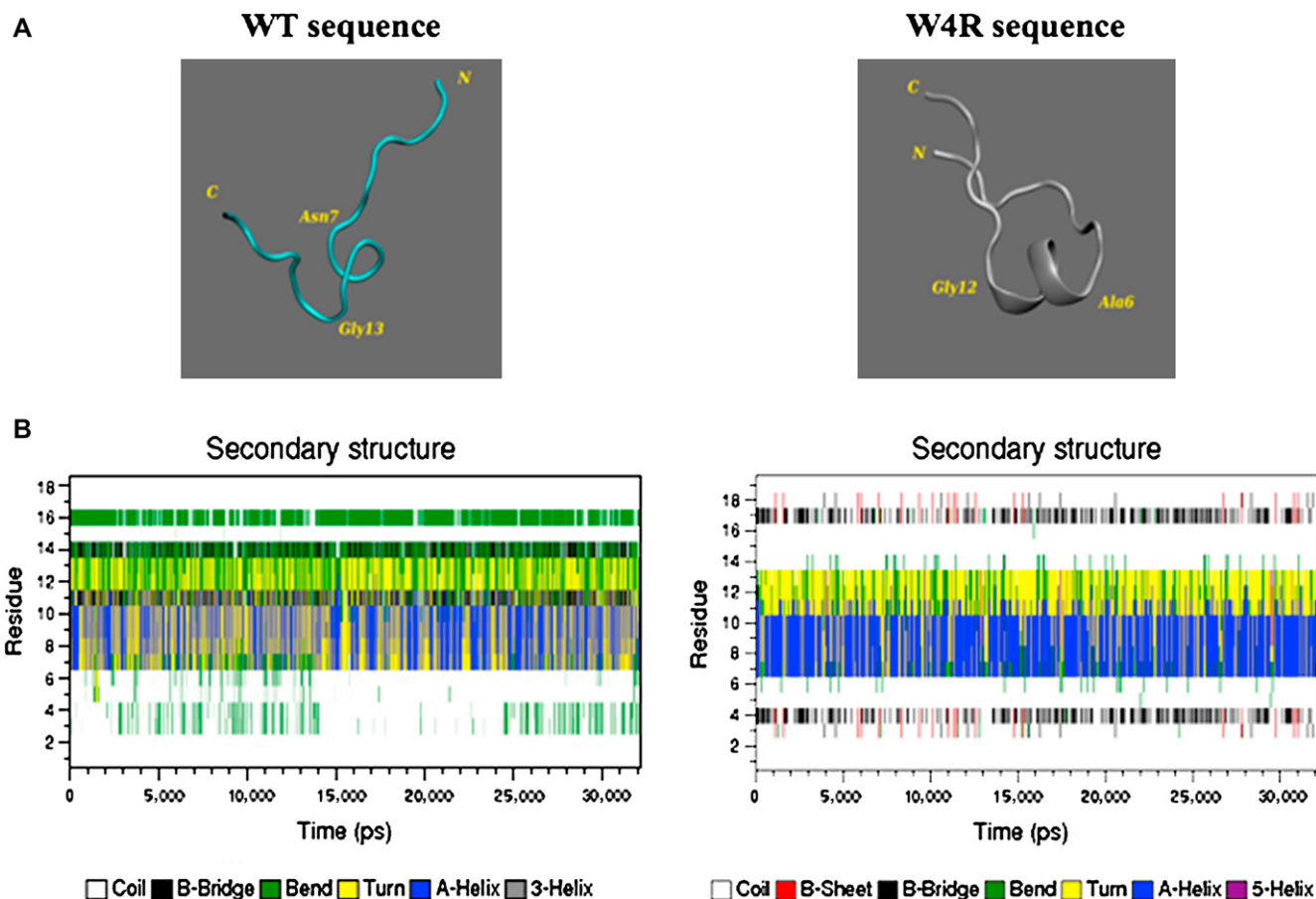


FIG E3. Structure modeling of the protein fraction surrounding the reversion event. **A**, Schematic representation of the average structure of MD simulations for the WT and for the W4R peptide. The residues are numbered according to internal enumeration of the model peptides (163→1). **B**, Secondary structure content of WT and W4R peptides computed during the 32,000-picosecond (ps) MD simulations using the DSSP algorithm as implemented in the GROningen Machine for Chemical Simulation suite.

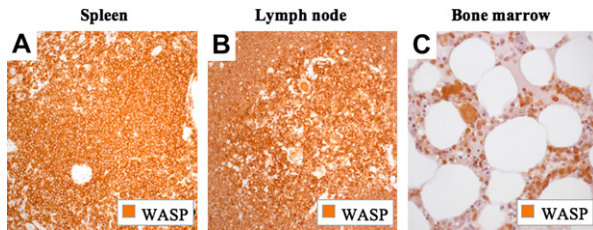


FIG E4. Physiological lymphoid tissue distribution of WASP expression. Histologic analysis of WASP expression in normal human lymphoid tissues, including spleen (**A**), lymph node (**B**), and bone marrow (**C**). WASP-specific staining appears in *brown*, whereas hematoxylin counterstaining appears in *blue*. Pictures taken with a $\times 20$ objective show representative areas of each tissue.

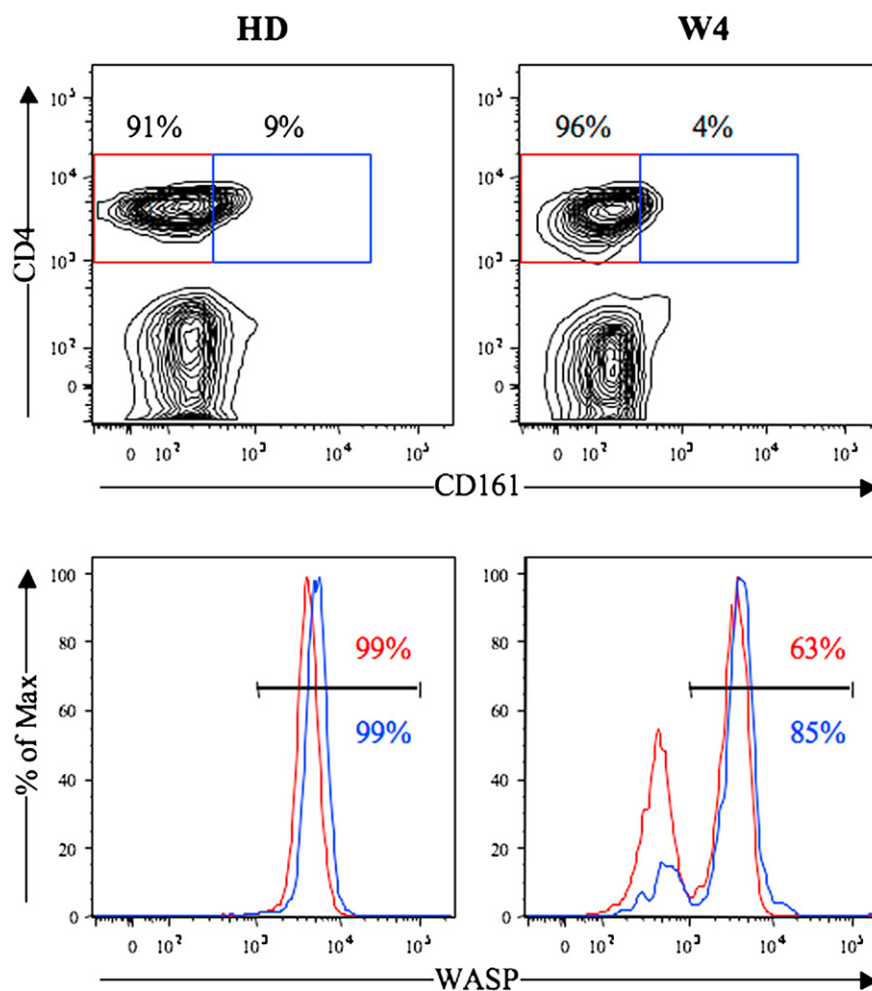


FIG E5. Revertant WASP expression in CD4⁺CD161⁺ and CD4⁺CD161⁻ T cells. PBMCs from a healthy donor and from patient W4 (age 27 years) were analyzed by flow cytometry for the expression of CD4, CD161, and WASP. *Upper panels* show the distribution of CD161 expression in CD4⁺ gated lymphocytes, and *lower panels* show the expression of WASP in the CD161⁻ (red) and CD161⁺ (blue) subpopulations. *Max*, Maximum.

TABLE E1. Distribution of WASP and Vβ expression in T-cell clones

HD	WASP	Vβ	W4	WASP	Vβ	W4	WASP	Vβ
1.10	+	1	03.6	-	1	03.1	+	1
1.38	+	1	1.4	-	2	1.47	+	1
1.13	+	3	1.78	-	4	1.48	+	1
03.2	+	3	3.31	-	4	03.18	+	2
03.1	+	5.1	1.87	-	5.1	1.53	+	3
03.12	+	5.3	3.19	-	5.1	1.7	+	3
03.4	+	13.2	1.34	-	7.1	1.84	+	4
1.4	+	13.6	3.29	-	8	03.2	+	5.1
1.40	+	14	3.13	-	13.6	1.58	+	7.1
3.28	+	17	03.3	-	14	1.16	+	8
1.14	+	18	3.73	-	14	03.10	+	9
						03.12	+	9
						3.23	+	13.1
						3.40	+	13.1
						1.14	+	13.6
						1.41	+	17
						3.32	+	21.3
						3.5	+	22
						3.39	+	22
						3.47	+	22
n = 11*		9/24†	n = 11*		8/24†	n = 20*		13/24†

*Number of clones analyzed.
†Number of different Vβ T-cell receptors found in the analyzed clones (out of 24 Vβ T-cell receptors tested).