

A peptide derived from the Wiskott-Aldrich syndrome (WAS) protein-interacting protein (WIP) restores WAS protein level and actin cytoskeleton reorganization in lymphocytes from patients with WAS mutations that disrupt WIP binding

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Background: The Wiskott-Aldrich syndrome (WAS) and X-linked thrombocytopenia (XLT) are caused by mutations in WAS, which encodes for WAS protein (WASP). The WASP-interacting protein (WIP) stabilizes WASP, as evidenced by severely decreased WASP levels in T cells from WIP-deficient mice. The majority of missense mutations in patients with WAS/XLT are located in the WIP-binding domain of WASP and might result in dissociation of the WASP-WIP complex and WASP degradation.

Objective: To restore WASP levels and correct T-cell function in WAS/XLT patients with mutations in the WIP-binding domain of WASP.

Methods: WIP, and a WIP-derived 41-amino acid-long peptide, which interacts with WASP and was designated nanoWIP (nWIP), were fused to enhanced green fluorescent protein and introduced by electroporation into EBV-transformed B cells, and by retroviral transduction into purified blood T cells from patients with WAS. WASP levels were measured by intracellular fluorescence-activated cell sorting staining. The actin cytoskeleton was visualized by intracellular phalloidin staining. **Results:** Introduction of WIP and nWIP restored WASP levels to normal in EBV-transformed B-cell lines from XLT patients with missense mutations in the WIP-binding domain of WASP and residual WASP levels, and corrected the defective spreading and pseudopodia formation of their T cells in response to immobilized anti-CD3.

Conclusion: A WASP-binding WIP-derived peptide stabilizes WASP in cells from XLT patients with missense mutations that

disrupt WIP binding, and corrects their T-cell actin cytoskeleton defect. This may provide a novel therapeutic strategy for these patients. (*J Allergy Clin Immunol* 2011;■■■:■■■-■■■.)

Key words: Wiskott-Aldrich syndrome, WASP, WIP, T cells, actin cytoskeleton

Wiskott-Aldrich syndrome (WAS) is an X-linked immunodeficiency characterized by recurrent infections, eczema, thrombocytopenia, and an increased risk of autoimmunity and malignancy.¹ A milder form of the disease, known as X-linked thrombocytopenia (XLT), is characterized by thrombocytopenia with small platelet size.²⁻⁴ WAS and XLT are caused by mutations in the WAS gene, which encodes a multidomain protein (WASP) expressed only in hematopoietic cells.⁵⁻⁷

WASP links the T-cell receptor (TCR)/CD3 complex to the actin cytoskeleton. WASP-deficient T cells fail to spread, extend pseudopodia, and increase their F-actin content upon stimulation with immobilized anti-CD3.^{8,9} Defective cytoskeletal organization in WASP-deficient T cells may underlie their impaired chemotaxis to stromal cell-derived factor-1 α , transcellular diapedesis, and homing to lymphoid organs.¹⁰⁻¹² Because F-actin polymerization is important for IL-2 secretion after TCR/CD3 ligation,¹³ defective TCR-driven actin polymerization may also underlie the failure of WASP-deficient T cells to proliferate and secrete IL-2 in response to immobilized anti-CD3 stimulation.^{9,14-17}

In T cells, close to 80% of WASP is in complex with the WASP-interacting protein (WIP). WIP stabilizes WASP, as evidenced by the observation that WASP levels are severely reduced in T cells from WIP-deficient mice and are restored by the reintroduction of WIP.¹⁸ The interaction of WASP and WIP is mediated by the N-terminal WASP homology domain 1 (WH1)/Ena-VASP homology domain 1 (EVH1) of WASP, and a C-terminal region of WIP that spans amino acids (aa) 451 to 485.^{19,20} On the basis of sequence alignment, the WH1/EVH1 domain of human WASP has been proposed to span aa 47 to 137.²¹ Experimentally, aa 26 to 147 of rat neuronal WASP, which correspond to aa 34 to 149 of human WASP, have been shown to be important for WIP binding.²⁰

The majority of missense mutations in WAS involve exons 1 and 2, which encode the WH1/EVH1 domain of WASP. These mutations are most often associated with decreased WASP expression and typically result in XLT phenotype.^{1,22,23} Two of

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Abbreviations used

aa:	Amino acid
EGFP:	Enhanced green fluorescent protein
EBV-B:	EBV-transformed B
Ena/VASP:	Enabled/vasodilator-stimulated phosphoprotein
EVH1:	Ena-VASP homology domain 1
MFI:	Mean fluorescence intensity
nWIP:	nano—Wiskott-Aldrich syndrome protein—interacting protein
TCR:	T-cell receptor
WAS:	Wiskott-Aldrich syndrome
WASP:	Wiskott-Aldrich syndrome protein
WH1:	Wiskott-Aldrich syndrome protein homology domain 1
WIP:	Wiskott-Aldrich syndrome protein—interacting protein
WT:	Wild type
XLT:	X-linked thrombocytopenia

these mutations, R86H and A134T, affect residues predicted by nuclear magnetic resonance studies to be contact points for WIP, and have been shown to virtually abolish the interaction of WASP and WIP.^{20,23} Mutagenesis of 40 aa in the WH1/EVH1 domain of WASP has identified 13 residues that are important for WASP-WIP interaction in a yeast 2-hybrid assay.²⁴

Allogeneic hematopoietic stem cell transplantation from matched donors provides effective treatment for WAS. However, its results are less successful when haploidentical donors are used.¹ Furthermore, treatment of XLT is highly controversial because the majority of these patients survive into adulthood,²⁵ making hematopoietic stem cell transplantation less compelling. However, infections, autoimmunity, and malignancies represent severe, potentially fatal complications for patients with XLT. In this work, we demonstrate that overexpression of a 41-aa peptide that spans the WASP-binding region of WIP restores the levels of WASP to normal in lymphocytes from patients with WAS mutations that disrupt WIP binding, and corrects their defective T-cell actin cytoskeleton reorganization. Introduction of this peptide into autologous hematopoietic stem cell may provide a novel therapeutic strategy for patients with WAS/XLT.

METHODS**Subjects**

Peripheral blood was obtained from patients and healthy controls after securing informed consent forms approved by the Institutional Review Board of the Children's Hospital, Boston, and according to the Declaration of Helsinki. The patient samples are referred to by the position and nature of the mutated aa in the WASP sequence. XLT patients with mutations E31K, L39P, P58R, D77G, D77H, L105P, and A236G have a clinical score of 1 to 3, whereas the WAS patient with mutation N204fs has a clinical score of 4.²² The patient with mutation R86H is a previously unreported XLT patient with a clinical score of 2. The patient with the A134T mutation is a previously unreported WAS patient with a clinical score of 5.

Mice

WASP^{-/-} and WIP^{-/-} mice have been described.^{17,26} Wild type (WT) littermates were used as controls. Mice were housed under pathogen-free conditions, and studies were performed in accordance with Children's Hospital policies and procedures.

Plasmid construction

Mouse WIP, or 123 nucleotides from the 3' end of WIP that code for the WASP-binding domain (aa 435-475; termed nanoWIP [nWIP]; Fig 1, A), were amplified by PCR and cloned in pEGFP-c2 (Clontech, Mountain View, Calif). For the transduction of primary human T cells, enhanced green fluorescent protein (EGFP), EGFP-WIP, and EGFP-nWIP were cloned in the lentivirus expression vector pHAGE-fullefla-MCS-IZsGreen. HIV-based lentivirus vectors were packaged in the vesicular stomatitis virus G (VSV-G) envelope at the Harvard Gene Therapy Initiative.

Flow-cytometric analysis of WASP in mouse T cells

Mouse T cells were purified using the Pan T cell isolation kit (Miltenyi, Auburn, Calif), stimulated for 96 hours with 5 µg/mL phytohemagglutinin P (Sigma-Aldrich, St Louis, Mo), then nucleofected with plasmid DNA using the Mouse T cell Nucleofector Kit and Nucleofector II device from Amaxa (Lonza Group Ltd, Basel, Switzerland). Cells were then cultured for 48 hours, processed for intracellular WASP staining using Fix & Perm cell permeabilization reagent (Invitrogen, Carlsbad, Calif), and analyzed on FACSCanto flow cytometer (BD Biosciences, San Jose, Calif) and FlowJo software (Tree Star Inc, Ashland, Ore). WASP was detected with rabbit polyclonal anti-WASP²⁷ and allophycocyanin-conjugated antirabbit IgG F(ab')₂ (Jackson ImmunoResearch, West Grove, Penn). WASP levels were calculated by subtracting the background WASP staining in the EGFP⁺ population of WASP^{-/-} T cells from the EGFP⁺ population of each sample in the same experiment, and expressing the results as a percentage of WASP in EGFP⁺ WT cells.

Jurkat cell transfection, immunoprecipitation, and Western blot analysis

Jurkat cells were electroporated using Gene PulserII (Bio-Rad, Hercules, Calif) at 380 V and 975 microFarad (µF) and cultured for 24 hours in complemented RPMI 1640. Cells were lysed (1% Triton X-100, 100 mmol/L TRIS-Cl pH 7.5, and 50 mmol/L NaCl) and lysates incubated with Living Colors A.v. Polyclonal Antibody (Clontech). Immune complexes were captured with Protein-G Sepharose (GE Healthcare, Piscataway, NJ), denatured by boiling in sample buffer, separated on 8% acrylamide gels, and subjected to Western blot. EGFP was detected with Living Colors A.v. mouse mAb JL-8 (Clontech). WASP was detected with rabbit polyclonal antibody K374.⁸ Actin was detected with antiactin mouse mAb (Chemicon, Billerica, Mass).

Quantitative real-time PCR

RNA was extracted from B cells immortalized with EBV using RNAqueous (Applied Biosystems, Austin, Tex), and then cDNA was synthesized using iScript cDNA synthesis reagent (Bio-Rad). WAS mRNA was quantified on ABI7700 real-time PCR system using TaqMan primers/probe Hs00166001_m1 (Applied Biosystems) and normalized with human β2-microglobulin. WAS mRNA levels for all patients were determined in triplicate, and compared with the WAS mRNA levels averaged from 4 controls, each performed in triplicate.

EBV-transformed B-cell Western blot analysis

Lysates from 1 million cells were used for Western blot. WIP was detected with mAb 3D10.²⁸ WASP and actin were detected as described. The levels of WASP are determined by dividing the density of the WASP band by the density of actin used as a loading control, and then comparing the ratios of WASP from each patient to the average of WASP ratios in 3 controls.

EBV-transformed B-cell transfection and flow cytometry analysis

Five million EBV-transformed B (EBV-B) cells were electroporated with 10 µg plasmid DNA, cultured for 48 hours at 37°C in complete RPMI 1640 supplemented with 10 µg/mL insulin, and processed for intracellular WASP

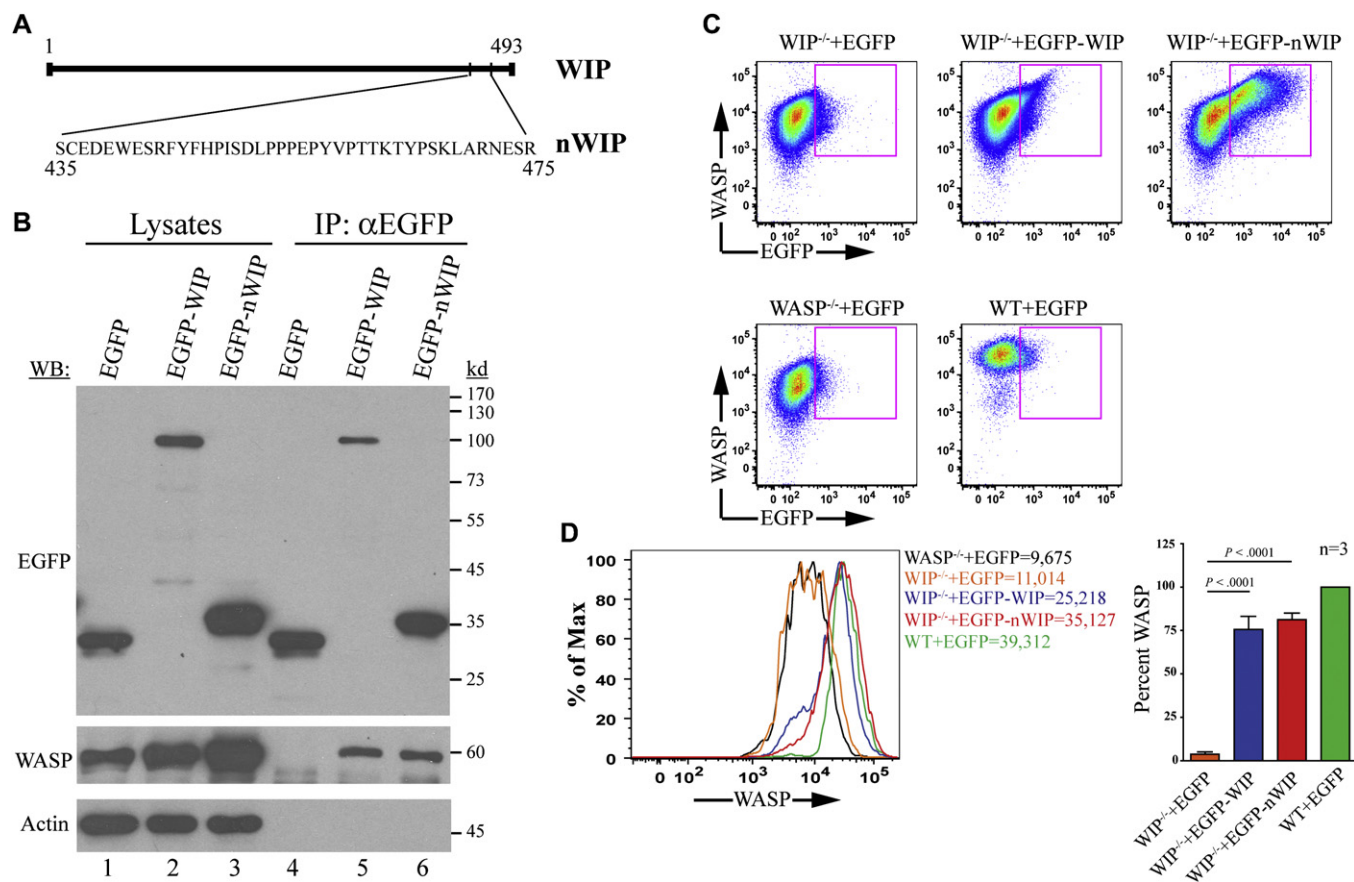


FIG 1. nWIP interacts with and stabilizes WASP. **A**, Sequence and position of nWIP. **B**, Co-immunoprecipitation of WASP with WIP and nWIP in Jurkat T cells. Membranes were Western-blotted with antibodies to EGFP, WASP, and actin. The experiment is representative of 3 independent experiments with similar results. **IP**, Immunoprecipitation; **WB**, Western blot. **C**, Representative intracellular WASP staining in mouse T cells expressing EGFP, EGFP-WIP, or EGFP-nWIP. **D**, Histogram overlay showing the level of WASP in the gated EGFP⁺ populations. **Bar graph** shows the means $\pm$ SEMs of WASP levels from 3 independent experiments. **Max**, Maximum.

staining as described. The average WASP level was calculated by subtracting the background WASP staining in N204fs cells expressing EGFP from each patient's cells expressing EGFP, EGFP-WIP, or EGFP-nWIP, and presenting the result as percent WASP compared with the control.

Lentivirus infection of primary human T cells

PBMCs were isolated on a Ficoll-Paque PLUS gradient (GE Healthcare). T cells were purified using the Pan T cell isolation kit II (Miltenyi), then transduced with lentiviruses at a multiplicity of infection of 5 in complemented RPMI 1640 containing 8 μ g/mL polybrene (Sigma-Aldrich) and 3 μ g/mL phytohemagglutinin P (Sigma-Aldrich), 12 hours at 37°C. Cells were then washed and rested in medium, and the experiment was performed 24 hours after transduction.

Spreading of primary human T cells on immobilized anti-CD3 antibodies

T cells were sedimented onto glass coverslips coated with 1 mg/mL polylysine (Sigma-Aldrich) or polylysine and 10 μ g/mL anti-CD3 (EMD/Calbiochem, Gibbstown, NJ). Cells were stimulated for 15 minutes at 37°C, fixed with paraformaldehyde, and permeabilized with 0.5% Triton X-100 in PBS, and F-actin was stained with tetramethyl rhodamine isothiocyanate-labeled phalloidin. Three controls and 2 siblings with the same mutation in the WAS gene were used in the study.

Fluorescence microscopy

Slides were viewed with a Nikon Eclipse E800 microscope (Nikon Inc, Melville, NY) using a Plan Apo lens at 60X oil. Images were acquired by using a CoolSNAP EZ camera (Photometrics, Tucson, Ariz) and NIS-Elements BR 2.30 software (Nikon Inc). Pictures were processed using Adobe Photoshop and Adobe Illustrator (Adobe Systems, Waltham, Mass).

Statistical analysis

A 2-tailed Student *t* test was used to compare the differences between groups by using GraphPad PRISM software (GraphPad Software, La Jolla, Calif).

RESULTS

A 41-aa peptide that spans the WASP-binding domain of WIP interacts with WASP in T cells

Nuclear magnetic resonance studies and Glutathione S-transferase pulldown assays have demonstrated that a 35-aa-long sequence in the C-terminal region of human WIP (aa 451-485) is critical for optimal interaction with the isolated WH1/EVH1 domain of rat neuronal WASP.^{20,29} This region is highly conserved between human and mouse WIP (94% homology) and corresponds to aa 441 to 475 in mouse WIP. To

determine whether this region of WIP can bind WASP in T cells, we generated a construct encoding a fusion protein consisting of aa 435 to 475 of mouse WIP (Fig 1, A), which we designated as nWIP, fused to the C-terminal end of EGFP (EGFP-nWIP). Transfection of EGFP-nWIP-encoding, EGFP-WIP-encoding, or EGFP-encoding constructs in Jurkat T cells resulted in the expression of all 3 proteins as determined by Western blotting of cell lysates and EGFP immunoprecipitations with anti-EGFP (Fig 1, B, lanes 1-6). As expected, probing of EGFP immunoprecipitates with anti-WASP revealed that WASP coprecipitated with EGFP-WIP, but not with EGFP (Fig 1, B, lanes 4-5). WASP also coprecipitated with EGFP-nWIP (Fig 1, B, lane 6), indicating that nWIP interacts with WASP in T cells. The amount of WASP in lysates of Jurkat cells transfected with EGFP-WIP and EGFP-nWIP was higher than the amount of WASP in lysates of Jurkat cells transfected with EGFP (Fig 1, B, lanes 1-3). This was confirmed by intracellular fluorescence-activated cell sorting staining (see this article's Fig E1 in the Online Repository at www.jacionline.org).

To demonstrate directly that nWIP stabilizes WASP, T cells from WIP^{-/-} mice were transfected with EGFP-nWIP, EGFP-WIP, or EGFP, and their WASP content was determined by intracellular staining (Fig 1, C). EGFP-transfected T cells from WT and WASP^{-/-} T cells were used as positive and negative controls, respectively. The number of EGFP⁺ cells and the level of EGFP expression were higher in cells transfected with EGFP-nWIP than in cells transfected with EGFP-WIP or EGFP, consistent with the higher expression levels of EGFP-nWIP in Jurkat T cells. Expression of EGFP-WIP, but not EGFP, increased WASP levels in WIP^{-/-} T cells (Fig 1, C and D), as previously reported.¹⁸ More importantly, expression of EGFP-nWIP significantly increased WASP levels in WIP^{-/-} T cells. Fig 1, C, shows that the increase in the mean fluorescence intensity (MFI) of WASP (shown in the ordinate) in the gated EGFP⁺ population of cells was proportional to the level of EGFP-nWIP and EGFP-WIP expression (shown in the abscissa). The level of WASP in the WIP^{-/-} T-cell population transfected with EGFP-nWIP and EGFP-WIP was comparable to the level of WASP in the population of WT T cells transfected with EGFP (Fig 1, D). These findings demonstrate that, similar to full-length WIP, nWIP stabilizes WASP in T cells.

Expression of nWIP restores WASP levels in B cells from patients with missense mutations in the WH1/EVH1 domain of WASP

We next examined whether overexpression of nWIP in lymphocytes from patients with missense mutations in the WIP-binding WH1/EVH1 domain of WASP might overcome the effect of the low affinity of mutant WASP to WIP and restore WASP levels. We established EBV-B-cell lines from 7 such patients with the L39P, P58R, D77G, D77H, R86H, L105P, and A134T mutations, as well as from a patient with a missense mutation N-terminal to WH1/EVH1 domain (E31K) and 2 patients with mutations C-terminal to that domain (A236G and N204fs). WAS mRNA levels were normal in the EBV-B cells from all the patients except the last 2, who had virtually absent WAS mRNA (Fig 2, A). Western blot analysis of cell lysates revealed significantly decreased WASP levels (15% to 65% of normal) in B cells from 5 (L39P, P58R, D77G, D77H, and L105P) of the 7 patients with mutations in the WH1/EVH1 domain (Fig 2, B and C). WASP was virtually undetectable (<5% of normal) in B cells

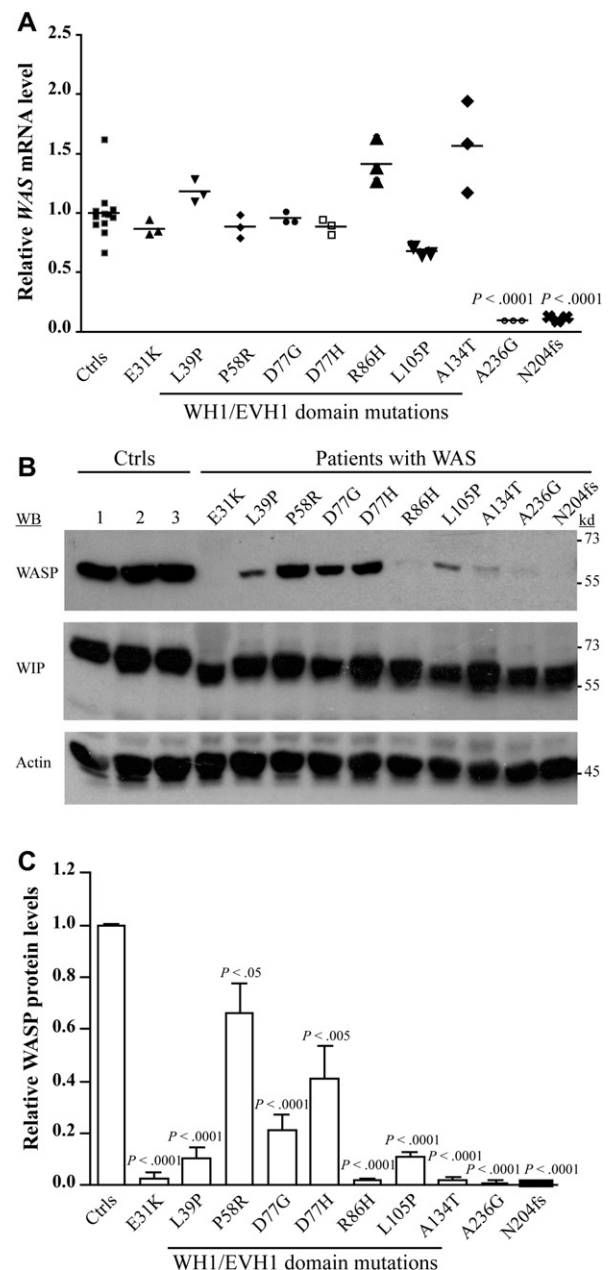


FIG 2. WAS mRNA and protein levels in patient cells. **A**, Quantitative real-time PCR of WAS mRNA from EBV-B cells of patients with WAS/XLT and controls. Results are expressed as fold change from the average of WAS mRNA levels in controls. **B**, Representative Western blot of WASP and WIP in lysates of EBV-B cells from patients with WAS/XLT and 3 controls. **C**, Bar graph represents the means $\pm$ SEMs of WASP levels from 3 different experiments, expressed as fold change compared with the controls. *Ctrl*s, Controls.

from the other 5 patients. The level of WIP in B cells from all patients was comparable to that in normal control B cells (Fig 2, B).

The effect of overexpression of EGFP-nWIP and EGFP-WIP on WASP levels in EBV-B cells from patients with WAS/XLT was examined by intracellular fluorescence-activated cell sorting analysis of WASP in EGFP⁺ cells. EGFP-transfected normal EBV-B cells and EBV-B cells from the patient with the N204fs mutation, which expressed no detectable WASP, were used as positive and negative controls, respectively. Fig 3 shows that

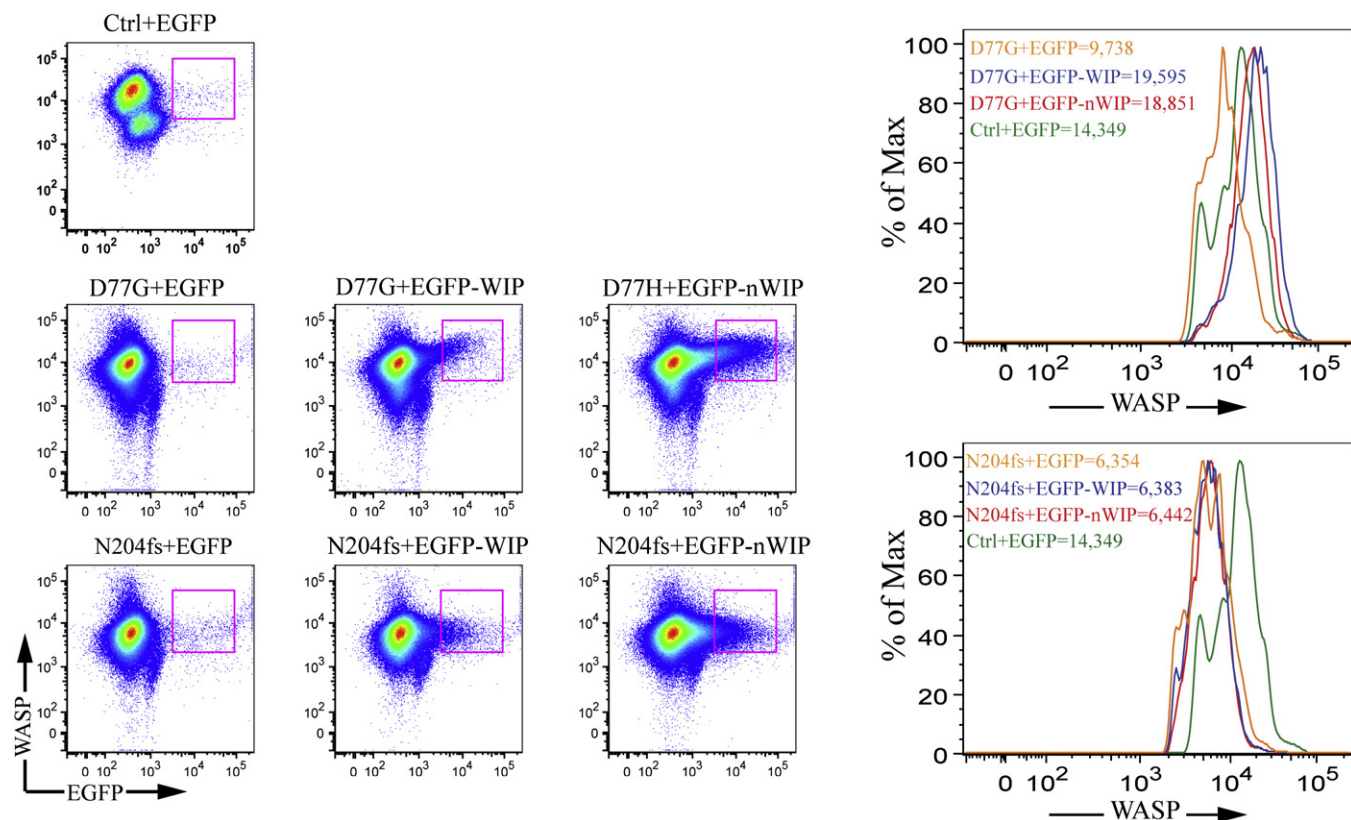


FIG 3. Stabilization of WASP in EBV-B cells from WAS/XLT patients with the D77G and N204fs mutations. Representative WASP staining in EBV-B cells from patients D77G and N204fs expressing EGFP, EGFP-WIP, or EGFP-nWIP. Shown are the dot plots and the histogram overlay of WASP MFI in their gated EGFP⁺ populations. *Ctrl*, Control; *Max*, maximum.

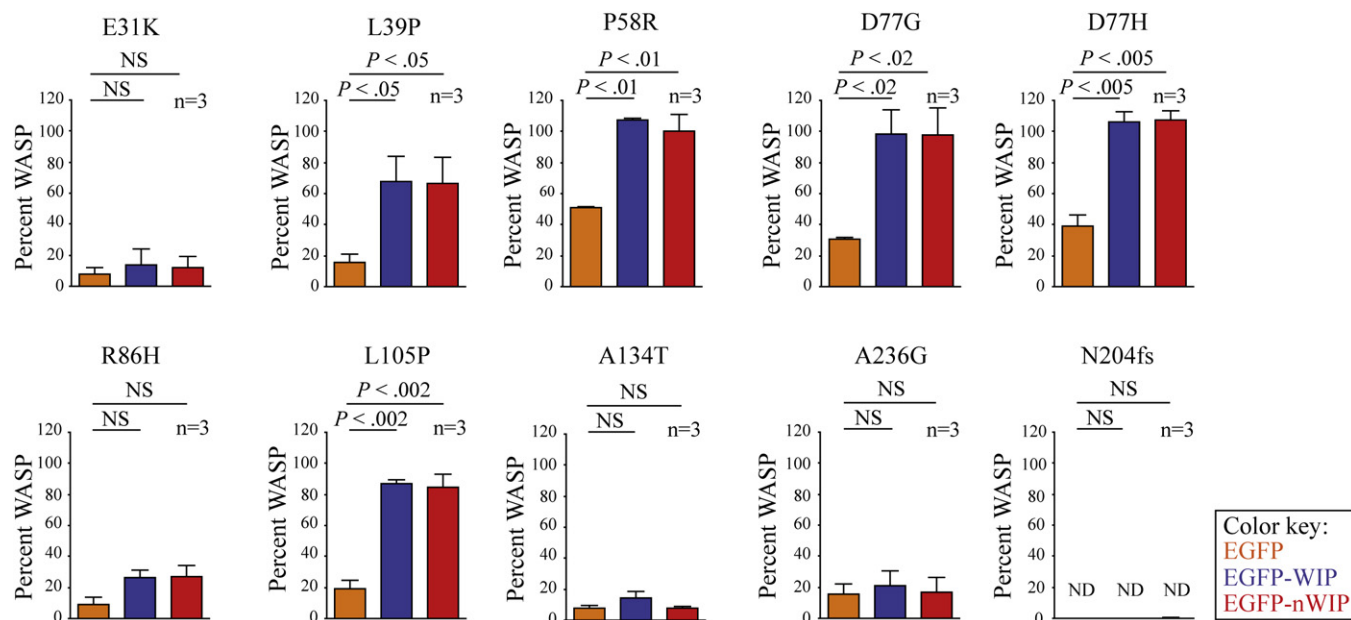


FIG 4. Average WASP increase in EBV-B cells from WAS/XLT patients. *Bar graphs* represent the means $\pm$ SEMs of WASP levels in the EGFP⁺ populations from 3 independent experiments for each patient. *ND*, Not detected; *NS*, not significant.

expression of EGFP-nWIP and EGFP-WIP but not EGFP caused an increase in WASP levels in cells from the patient with the D77G mutation, as evidenced by the increase in the MFI of WASP (shown in the ordinate) in the gated EGFP⁺ population of cells, which was proportional to the level of EGFP-nWIP and EGFP-WIP expression (shown in the abscissa). In contrast, no increase in WASP levels was detected in cells from the patient with the N204fs mutation upon expression of EGFP-nWIP or EGFP-WIP. Fig 4 depicts the results of 3 independent experiments on EBV-B cells from each of the 10 patients studied. Expression of EGFP-nWIP and EGFP-WIP, but not EGFP, significantly increased WASP levels in EBV-B cells from all 5 patients with missense mutations in the WH1/EVH1 domain that exhibited residual WASP levels (L39P, P58R, D77G, D77H, and L105P). WASP increase in EGFP-nWIP-expressing and EGFP-WIP-expressing cells from these patients reached 70% to 100% of WASP levels in control EBV-B cells. In contrast, expression of EGFP-nWIP or EGFP-WIP caused no significant increase in WASP levels in cells from the 5 patients with near absent WASP levels (E31K, R86H, A134T, A236G, and N204fs).

Expression of nWIP corrects the actin cytoskeleton defect of T cells from patients with WASP mutations that partially disrupt WIP binding

T cells were freshly purified from the blood of the patients with the L39P and D77G mutations, as well as from their untransplanted 2 siblings who carried the same mutations, and from 3 controls. The T cells were stimulated with immobilized anti-CD3, permeabilized, and stained with phalloidin-tetramethyl rhodamine isothiocyanate, and their actin cytoskeleton-dependent projections at the mobile edge of the cells were visualized by fluorescence microscopy. Cells that exhibited increased area of contact with the substrate and/or developed 2 or more pseudopodia were considered spread. Virtually all T cells from controls and patients with WAS/XLT that adhered to polylysine-coated glass coverslips displayed a round morphology characteristic of unstimulated T cells (Fig 5, A), with less than 8% of the cells spread (Fig 5, B). The majority of T cells from controls (77% + 6%) spread and developed pseudopodia after plating over immobilized anti-CD3 (Fig 5, A and B). In contrast, only 12% + 7% of T cells that carried the L39P mutation spread and developed pseudopodia when plated on immobilized anti-CD3 (Fig 5, A and B). Furthermore, the magnitude of the spread of these T cells was notably less than that of control T cells (Fig 5, A, arrow). TCR-driven actin cytoskeleton reorganization was also significantly impaired in the 2 patients with the D77G mutation, albeit to a lesser extent than in the 2 patients with the L39P mutation (Fig 5, A and B). Because WASP levels were higher in cells with the D77G mutation than in cells with the L39P mutation (Fig 2, B and C), these findings suggest that the residual levels of WASP could determine the severity of the defect in T-cell actin cytoskeletal reorganization.

To determine whether expression of nWIP corrects the spreading defect of T cells from the patients with the L39P and D77G mutations, T cells from the patients and controls were transduced with lentiviruses that express EGFP-nWIP, EGFP-WIP, or EGFP. The percentage of EGFP⁺ cells always exceeded 60% (see this article's Fig E2 in the Online Repository at www.jacionline.org). Expression of EGFP-nWIP, EGFP-WIP, or EGFP in control T cells had no effect on their spreading as assessed by comparing

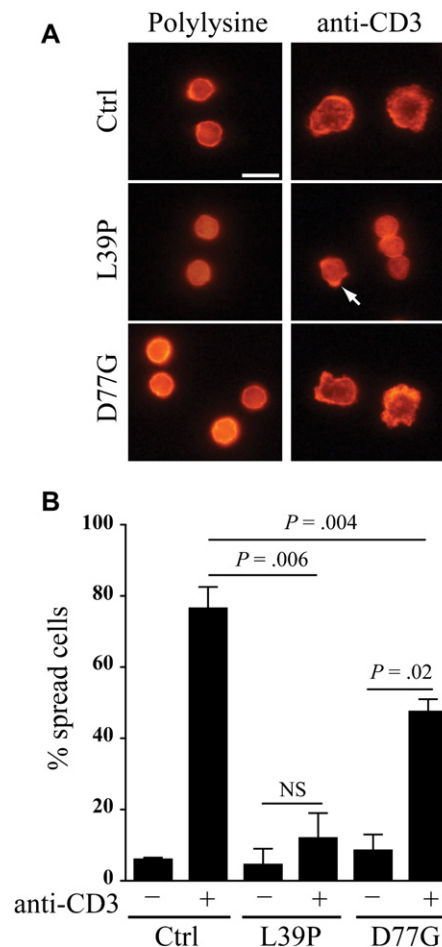


FIG 5. Spreading of T cells from patients with mutations in WASP. **A**, Phalloidin staining of the actin cytoskeleton of T cells from control (Ctrl), and patients with L39P and D77G mutations (2 each) stimulated with immobilized anti-CD3. Bar = 10 μ m. **B**, Pooled results represent the means $\pm$ SEMs of the percentage of spread cells from 3 controls and 2 patients (2 for each mutation). NS, not significant.

EGFP⁺ and EGFP⁻ populations (data not shown). Expression of EGFP alone had no effect on the percentage of spread patients T cells, or on the magnitude of their spread (Fig 6, A and B). In contrast, expression of EGFP-nWIP or EGFP-WIP in T cells that carry the L39P mutation led to a significant increase in the percentage of spread cells, and in the magnitude of spreading in response to immobilized anti-CD3, compared with expression of EGFP (Fig 6, A and B). The percentage of spread EGFP⁺ cells was 63% + 3% for EGFP-nWIP, 73% + 2% for EGFP-WIP, and 16% + 2% for EGFP. Expression of EGFP-WIP and EGFP-nWIP also significantly increased the percentage of spread T cells that carry the D77G mutation (Fig 6, A and B). The percentage of spread EGFP⁺ cells was 76% + 6% for EGFP-nWIP, 74% + 5% for EGFP-WIP, and 44% + 4% for EGFP.

DISCUSSION

We have demonstrated that a peptide that encodes the WASP-binding sequence of WIP restores WASP levels in lymphocytes from patients with mutations in the WIP-binding domain of WASP, and corrects the defects in reorganization of actin cytoskeleton-dependent structures in T cells from these patients.

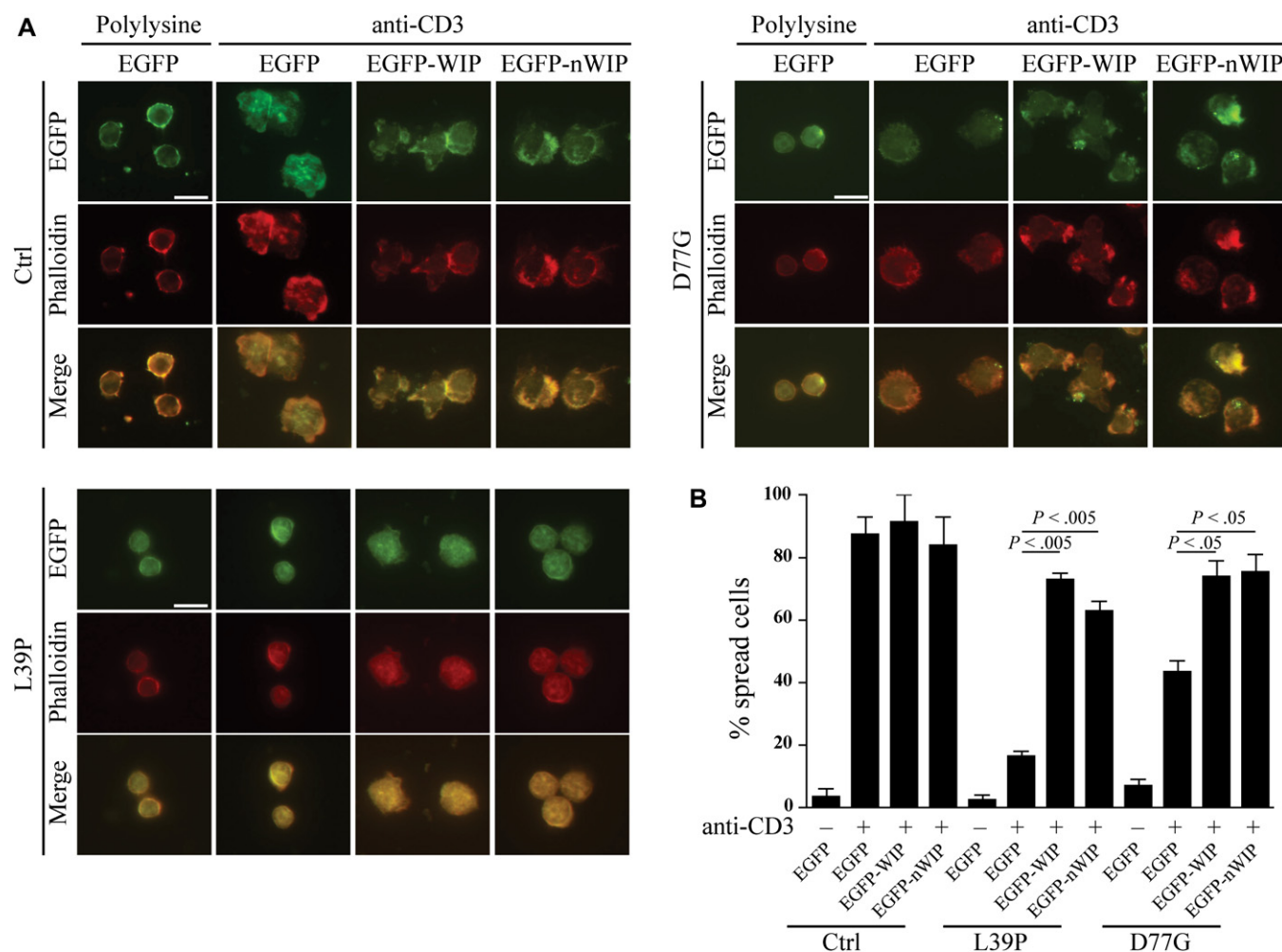


FIG 6. Correction of T-cell spreading by expression of nWIP and WIP. **A**, Intracellular fluorescence for EGFP (top row), phalloidin (middle row), and merge (bottom row) of T cells from control (Ctrl), and patients with L39P and D77G mutations transduced with lentiviruses expressing EGFP, EGFP-WIP, or EGFP-nWIP, then stimulated with immobilized anti-CD3. Bar = 10 μ m. **B**, Pooled results represent the means $\pm$ SEMs of the percentage of spread cells from 3 controls and 2 patients (2 for each mutation).

We demonstrated that nWIP, a 41-aa-long peptide that spans the WASP-binding region of WIP (aa 451-485), binds WASP when introduced in Jurkat cells as an EGFP-nWIP fusion protein (Fig 1, B). Although EGFP-nWIP was expressed at higher levels than EGFP-WIP in the Jurkat cells, the amounts of WASP that co-precipitated with EGFP-nWIP and EGFP-WIP were comparable, suggesting that nWIP might have a lower affinity for WASP than full-length WIP. Overexpression of both EGFP-nWIP and EGFP-WIP in Jurkat cells was accompanied by an increase in WASP levels, suggesting that WASP is normally expressed in excess of WIP, and that the portion of WASP that is not bound to WIP is degraded.

Introduction of WIP and nWIP in B cells from patients with the L39P, P58R, D77G, D77H, and L105P mutations, which expressed residual levels of WASP, restored WASP levels to normal (Fig 4). This suggests that the decreased affinity of mutant WASP to WIP could be overcome by overexpression of nWIP and WIP. The failure of nWIP and WIP to increase WASP levels in B cells with the R86H and A134T mutations, which expressed negligible levels of WASP, is consistent with the previous demonstration that

these 2 mutations result in complete loss of WIP binding,²³ and suggests that they severely disrupt the structure of the WH1/EVH1 domain. In fact, R86H plays a central role in coordinating and orienting an otherwise repulsive cluster of 3 acidic residues (D77, E98, and E100) that are important for the interaction of WIP with WASP.²⁰ The failure of nWIP and WIP overexpression to elevate WASP levels in cells with the E31K mutation suggests that, although aa E31 is located N-terminal to the WH1/EVH1 domain, substitution of a positively charged residue for a negatively charged one at this position severely interferes with WIP binding through electrostatic effects. The failure of nWIP and WIP overexpression to increase WASP levels in B cells from the 2 patients with mutations that resulted in virtually absent WAS mRNA (A236G and N204fs) is not unexpected.

The ability of nWIP to interact with, and stabilize WASP was biochemically tested in several cell types that include Jurkat cells, primary mouse T cells, and EBV-B cells derived from patients with WAS/XLT. We strongly believe that the ability of nWIP to increase WASP levels is not restricted to these cell types but extends to natural killer cells and platelets/megakaryocytes.

A transgenic animal model in which knock-in mice that harbor a point mutation in the WIP-binding domain of WASP are crossed to mice that overexpress nWIP through all stages of myeloid cell differentiation will address this important issue and will help determine whether overexpression of nWIP *in vivo* results in adverse side effects.

Introduction of nWIP and WIP in primary T cells isolated from 4 XLT patients with the L39P and D77G mutations (2 siblings each) resulted in the correction of their actin cytoskeleton reorganization defect. The correction of this WASP-mediated function⁸ by the WASP-binding domain of WIP suggests that the role of WASP in actin cytoskeleton rearrangement might not be dependent on the presence of other domains of WIP in the WASP/WIP complex. However, we cannot rule out the possibility that WASP and WIP may both be independently required for normal actin cytoskeleton rearrangement.

Our findings suggest that cell-permeable designer drugs that are mimetics of nWIP may provide a novel therapeutic strategy for the treatment of patients with WAS/XLT with mutations that partially disrupt WASP binding to WIP.

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Clinical implications: A peptide derived from the WASP-binding domain of WIP stabilizes mutant WASP in lymphocytes from patients with mutations in the WIP-binding domain of WASP and corrects the defective actin cytoskeletal reorganization in their T cells.

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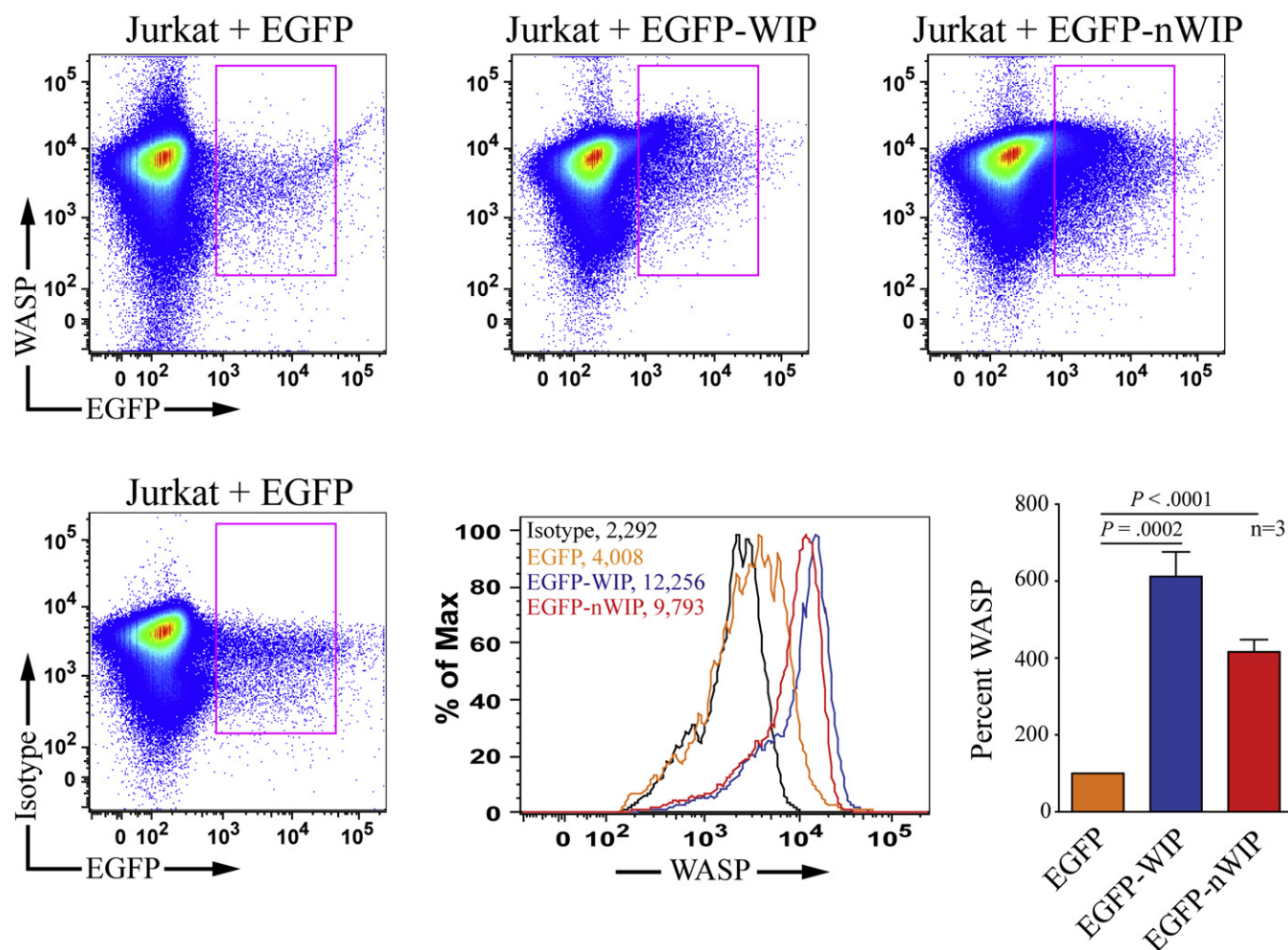


FIG E1. Expression of EGFP-WIP and EGFP-nWIP increases WASP levels in Jurkat T cells. Intracellular WASP staining was performed in Jurkat cells expressing EGFP-nWIP, EGFP-WIP, or EGFP. The gate indicates the population of cells used in the analysis. The histogram overlay shows the levels of WASP in the gated EGFP⁺ populations. The bar graph shows the means $\pm$ SEMs of WASP levels from 3 independent experiments. The respective samples, their MFIs, and bars are indicated using the same color as the histograms. WASP level was calculated by subtracting the background isotype staining from the samples' MFIs and expressing it as a percentage of WASP in EGFP⁺ cells. *Max*, Maximum.

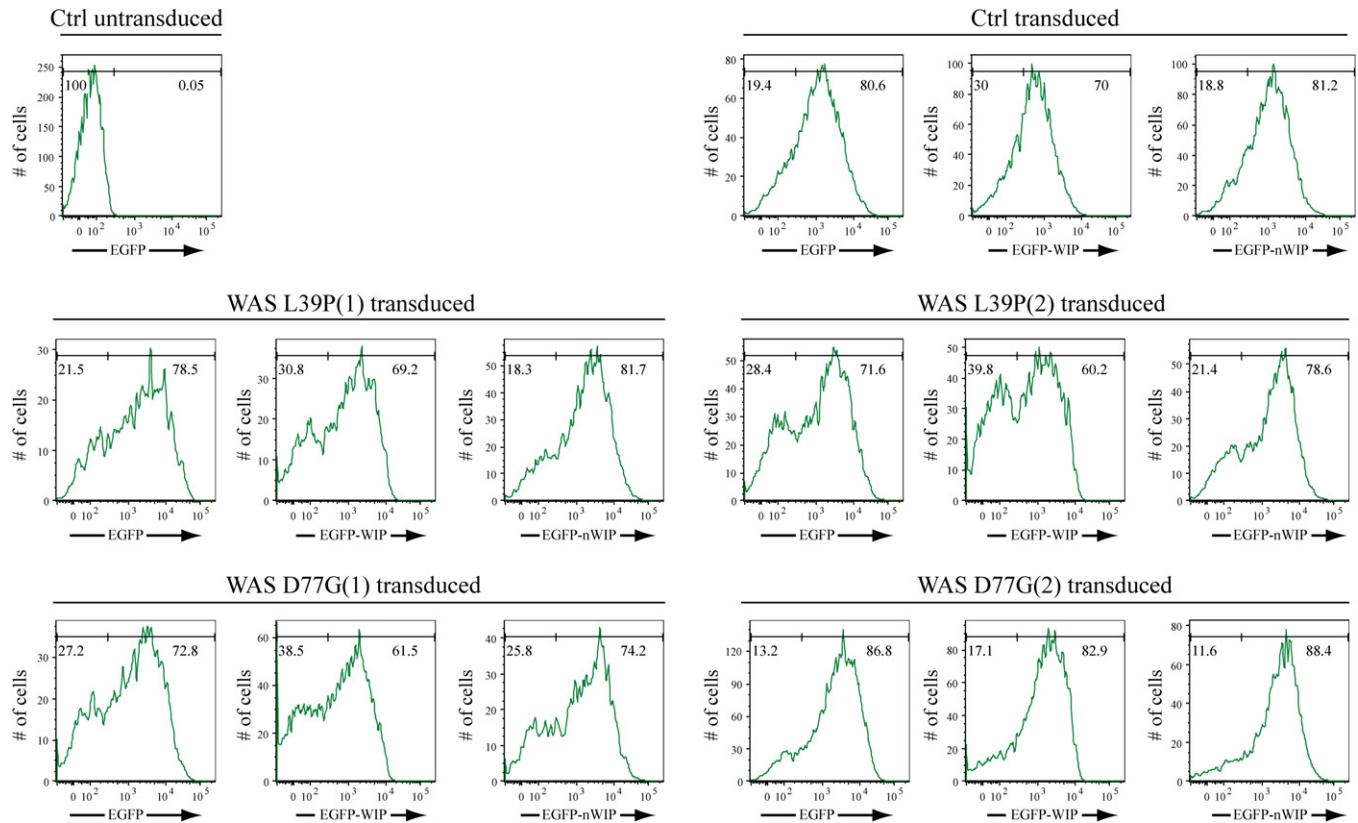


FIG E2. Efficiency of expression of EGFP, EGFP-WIP, and EGFP-nWIP in purified primary T cells. T cells purified from 3 controls (*Ctrl*) and 4 patients (2 siblings each) with the mutations L39P and D77G in the WH1/EVH1 domain of WASP were transduced with lentiviruses expressing EGFP, EGFP-WIP, and EGFP-nWIP. The expression of the fusion proteins was determined 24 hours posttransduction. Shown are representative histograms from 1 control and the 2 affected siblings.