A novel primary human immunodeficiency due to deficiency in the WASP-interacting protein WIP

Gaetana Lanzi,1 Daniele Moratto,1 Donatella Vairo,1 Stefania Masneri,1 Ottavia Delmonte,2 Tiziana Paganini,1 Silvia Parolini,3 Giovanna Tabellini,3 Cinzia Mazza,1 Gianfranco Savoldi,1 Davide Montin,2 Silvana Martino,2 Pierangelo Tovo,2 Itai M. Pessach,4 Michel J. Massaad,4 Narayanaswamy Ramesh,4 Fulvio Porta,6 Alessandro Plebani,1 Luigi D. Notarangelo,4,5 Raif S. Geha,4 and Silvia Giliani1

RESULTS AND DISCUSSION

Clinical characteristics

The index patient was the second female child of consanguineous Moroccan parents. She was referred at 11 d of age with poor weight gain, recurrent infections, eczema, thrombocytopenia, defective T cell proliferation and chemotaxis, and impaired natural killer cell function. Cells from this patient had undetectable WASP protein (WASP), but normal WAS sequence and messenger RNA levels. WASP interacting protein (WIP), which stabilizes WASP, was also undetectable. A homozygous c.1301C>G stop codon mutation was found in the WIPF1 gene, which encodes WIP. Introduction of WIP into the patient’s T cells restored WASP expression. These findings indicate that WIP deficiency should be suspected in patients with features of WAS in whom WAS sequence and mRNA levels are normal.

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an eczematous rash, papulovesicular lesions on the scalp, and ulcerative lesions on the hard palate and tongue. A previous female sibling suffered from ulcerative and vesicular skin lesions and died of sepsis at 4 mo of age.

Laboratory findings included thrombocytopenia (59 × 10^3/µl platelets) with normal platelet volume and elevated levels of C reactive protein (10.3 mg/dl; Table S1). Blood and urine cultures were negative. Stool cultures revealed no pathogenic organisms. *S. epidermidis* and *K. pneumoniae* grew from the skin vesicular lesions. The patient developed respiratory distress and required oxygen supplementation. A tracheal aspirate was positive for respiratory syncytial virus by PCR. She was placed on wide-spectrum antibiotic therapy, fungal and viral prophylaxis, immunoglobulin replacement, and platelet and red cell transfusions as needed. She developed rotavirus enteritis at 2 mo of age, and acute hepatitis of unknown etiology at 3 mo of age. No clinical manifestations of autoimmunity or bleeding tendency were noted. Because of persistent deterioration, failure to gain weight, and poor T cell function, at the age of 4.5 mo she underwent unrelated cord blood transplantation. 16 mo after the procedure, she is alive and well with >98% of T cells, >98% of B cells, 94% of NK cells, 50% of monocytes, and 41% of granulocytes of donor origin. The oral ulcerations resolved after bone marrow transplant, suggesting that they were secondary to deficient immune function.

**Immunological analysis**

Analysis of peripheral blood revealed low percentages and numbers of CD3+ cells (809 cells/µl), with CD8+ cells more affected than CD4+ cells (Fig. 1A and Table S1). 90% of
Expression of the IP-10 receptor CXCR3 was lower in the patient’s T cells (Fig. 1 E), and may have contributed to their failure to migrate toward IP-10. Analysis of cytolytic activity of freshly isolated NK cells from the patient was precluded by the amount of blood that could be drawn. To circumvent this limitation, NK cells were expanded by stimulating T cell–depleted PBMCs from patient and a control with PHA and rIL-2 in the presence of feeder cells (Castriconi et al., 2007). The resulting cell lines contained >90% CD56+ NK cells, which expressed comparable levels of CD56 on their surface. However, expression of NKP30, NKP46, and NKG2D, all of which are implicated in NK cytotoxicity, were reduced on the patient’s cells (Fig. 1 F). Analysis of cytolytic activity against LCL 721.221 target cells demonstrated a drastic reduction in the functional activity of the patient’s NK cell line compared with that from control (Fig. 1 G). Previous studies have shown impaired cytotoxicity of freshly isolated NK cells from WAS patients (Gismondi et al., 2004). We have been unable to derive NK cell lines from the WAS patient for direct comparison with the NK cell line from our patient.

WIPF1 is mutated in the patient

Although the WAS gene is located on the X-chromosome, several cases of WAS in females caused by extreme lyonization or biallelic WAS mutations have been described.
had a homozygous point mutation c.1301C>G in exon 6 of
the WIPF1 gene located on chromosome 2 (Fig. 2, E and F).
This mutation results in a change from serine to premature
termination in codon 434 (S434X), situated immediately up-
stream of the region encoding the WASP-binding domain of
WIP (451–485 aa; Ramesh et al., 1997). Both parents were
heterozygous for the c.1301C>G substitution, confirming
the autosomal recessive inheritance of the mutation.

**Expression of WIP in the patient’s cells corrects the defect in WASP expression**

We examined whether expression of human WIP (hWIP) in
the patient’s cells corrects their defective WASP expression.
PHA T blasts from the patient and a control were transfected
with vectors expressing EGFP-hWIP fusion protein or EGFP
alone, and their content of WIP and WASP was determined
by FACS analysis of intracellular staining. As expected, intro-
duction of EGFP-hWIP, but not EGFP, in the patient’s T cells
resulted in WIP expression, as indicated by the detection
of WIP+ cells in the EGFP+ gate (Fig. 3 A). More impor-
tantly, introduction of EGFP-hWIP, but not EGFP, in the
patient’s T cells resulted in increased WASP expression by
EGFP+ cells (Fig. 3 B). WASP expression in the EGFP-
hWIP–transfected cells from the patient correlated with WIP
expression (Fig. 3 C). These findings indicate that the
absence of WIP in the patient’s T cells resulted in WASP
instability and degradation. Because the patient was transplanted at 4.5 mo
of age and because of the limited
amounts of blood we could obtain
pretransplant, we were not able to
examine whether transduction of WIP
in the patients T cells would restore
their function or examine the corti-
scular actin meshwork in the patient’s
T cells, which is severely attenuated
in WIP−/− but not WASP−/− mice
(Antón et al., 2002).

Several of the clinical characteris-
tics and laboratory findings in the
patient resemble those found in WAS.
They include recurrent infections,
eczematous skin rash, thrombocyto-
penia, T cell lymphopenia affecting

![Figure 3.](https://jem.rupress.org/)
CD8+ lymphocytes more severely, impaired T cell proliferation to immobilized anti-CD3, defective T cell chemotaxis, and increased NK cell number but decreased NK cell function. In addition, the patient displayed immune abnormalities that are observed in WIP-deficient patients, but not in WAS patients or WASP-deficient mice. They include complete failure to proliferate to TCR ligation with anti-CD3, impaired response of T cells to IL-2, and complete abrogation of T cell chemotaxis (Fig. 1; Haddad et al., 2001; Gallego et al., 2006; Le Bras et al., 2009). In contrast to WAS patients, platelet volume was normal in the patient, like in WIP-deficient mice (Curcio et al., 2007). However, we hesitate to draw a firm conclusion on platelet size in human WIP deficiency based on a single patient.

Despite undetectable WAS in the patient, no mutations were detected in the coding region of the WAS gene, and WAS mRNA levels were normal, findings strongly indicative of WASP instability. WIP, which is critical for the stability of WASP, was undetectable in the patient’s cells, and a homozygous mutation that introduces a premature stop codon in the WIPF1 coding sequence was identified in the patient. The same mutation was found in the heterozygous state in both parents, indicating that WIP deficiency was inherited as an autosomal recessive trait. This is consistent with an older sibling having died from a similar condition. The fact that both parents showed reduced levels of WIP, to approximately half of normal, suggests a gene dose effect. The corresponding reduced WASP level in the parents suggests that WIP tightly regulates WASP levels. We have observed a similar reduction of WASP levels in mice heterozygous for a WIP-null allele (unpublished data). Importantly, we demonstrated that reconstitution of the patient’s T cells with WIP restores WASP levels, indicating that loss of WIP expression was the cause of WASP instability in the patient’s cells.

Collectively, the data indicate that WIP deficiency is responsible for the immune dysfunction in the patient. Based on our findings, WAS cannot be diagnosed solely on the basis of lack of WASP expression, but requires sequence analysis of WAS. WIP deficiency should be suspected in patients with features of WAS in whom WAS sequence and mRNA levels are normal, and the diagnosis should be confirmed by sequencing WIPF1.

MATERIALS AND METHODS
Cell purification and culture. Patient’s samples were obtained upon informed consent by the local Institution Review Board and in respect to the Helsinki declaration. Human studies were approved by the Ethical Committee of Azienda Ospedaliera Spedali Civili, Brescia. Isolation of peripheral blood mononuclear cells (PBMCs) and of specific cell populations and establishment of cell lines were performed as follow: to obtain PHA T blasts, PBMCs were stimulated with 5 µg/ml PHA (Sigma-Aldrich) and 600 U/ml rIL-2 (Cairon-Novartis) in RPMI medium containing 10% FCS, 1% l-glutamine, and 1X antibiotics (EuroClone). To generate NK cell lines, NK cells were purified by NK cell separation cocktail (Rosette Sep; StemCell Technologies). The purity of NK cells was >96% as assessed by flow cytometric analysis of cells stained with a mixture of CD56-PC5 and CD3-FITC (Beckman Coulter). CD3 contamination was <1%. Purified NK cells were cultured on irradiated feeder cells in the presence of 100 U/ml of rIL-2 and 5 µg/ml of PHA (Invitrogen) to obtain activated polyclonal NK cell populations. Activated polyclonal NK cells were maintained in complete RPMI medium containing 1,200 U/ml of rIL-2.

Molecular genetic analyses. Genomic DNA was isolated using the automatic DNA extractor Maxwell 16 (Promega). Sequencing of genomic DNA corresponding to the coding regions of WAS (ENS00000105285) and WIPF1 (ENSG00000115935) genes was performed by direct sequencing after PCR amplification of exons with flanking intronic regions. Primers and conditions are reported in Table S2.

Total RNA was isolated from PBMCs using the RNaseasy Mini kit (QIAGEN) and transcription into complementary DNA (cDNA). Quantitative PCR experiments were performed by reverse transcription of 200 ng of DNase-treated total RNA to synthesize the first strand of cDNA by the GeneAmp RNA PCR kit (Applied Biosystems). Analysis of WASP, WIPF1, and GAPDH gene expression was assessed by RealTime PCR using Assays-on-Demand products and TaqMan Master Mix from Applied Biosystems. The level of expression was normalized using GAPDH as a reference.

Immunoblotting. Cell lysates from PHA T blasts were prepared and 10 µg protein were loaded on a 10% SDS-PAGE and transferred onto a polyvinylidene fluoride membrane (GE Healthcare). Specific proteins were detected using mouse IgG1 anti-WIP mAb (3D10; Koduru et al., 2007), mouse IgG2a anti-WASP mAb 5A5 (BD), and mouse anti-actin mAb (US Biological).

Flow cytometry. Flow cytometry for surface and intracellular proteins was performed on 100 µl of whole blood or isolated cells (1.5 × 10⁸) resuspended in 100 µl of the appropriate medium. To assess WIP and WAS expression, cells were treated with the Fix and Perm kit (Invitrogen) for FACS analysis. Cells were stained with mouse IgG1 anti WIP (3D10; Koduru et al., 2007) and mouse IgG2a anti-WASP (5A5; BD), followed by anti IgG1PE and biotinylated anti-IgG2a-streptavidinPECy5, respectively. Acquisition was performed using a FACSCalibur (BD), and data were analyzed with FlowJo Software v7.5 (Tree Star).

Standard flow cytometric methods were also used for CFSE assay. CFSE labeling (100 nM) was performed in PBS for 6 min at 37°C. Cells were then washed twice in RPMI 10% FCS. Cells were cultured for 96 h in the absence of stimulation or stimulated with anti-CD3 (clone OKT3, 100 ng/ml) and anti-human CD28 (1 µg/ml) or PHA (5 µg/ml) and then analyzed for CFSE dilution.

For the characterization of NK cells, the following mAbs, which were produced in our laboratories, were used in this study: BAB281 (IgG1anti-NKp46), AZ20 (IgG1, anti-NKp30), c218 (IgG1, anti-CD56), BAT221 (IgG1, anti-NKG2D), and 289 (IgG2a, anti-CD3). For cytokine/fluorometric analysis, cells were stained with mAb PE-conjugated isotype-specific goat anti-mouse secondary antibody (SouthernBiotech). Cell acquisition was performed on a FACSScan flow cytometer (BD), and data were analyzed using the CellQuest software (BD).

Lymphocyte functional analysis. Analysis of STAT5 phosphorylation in T cells in response to IL-2 was assessed by flow cytometry: 100 µl of whole blood was incubated with or without 100 ng/ml rIL-2 (Proluemkin-Chiron) for 10 min at 37°C. Activation was stopped using Lyse/Fix buffer (BD). After washing, the samples were permeabilized by PBII Buffer (BD) for 30 min on ice and incubated with anti-phospho-STAT5 (Y694)-PE and CD3-FITC, or with isotype-matched mAb PE (BD) for 30 min on ice and incubated with anti-CD3 (clone OKT3, 100 ng/ml) and anti-human CD28 (1 µg/ml) or PHA (5 µg/ml) and then analyzed for CFSE dilution.

Brief Definitive Report

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**Chemotaxis assay.** In vitro chemotaxis of PHA T blasts toward IP-10 was examined using a standard Transwell chamber assay. A total of 3 × 10^5 PHA T blasts were added to the upper chamber of a plate 6.5 mm in diameter with a pore size of 5 µm (Costar). 600 µl of RPMI 1640 with 0.1% FCS was added to the bottom chamber with or without IP10 (250, 500, and 1,000 ng/ml; PeproTech). After 2 h at 37°C, cells that migrated to the lower chamber were collected and counted. The experiment was performed in duplicates. Expression of the IP-10 receptor CXCR3 was examined by FACS using a mouse anti–CXCR3-FITC mAb (R&D Systems).

**WIP reconstitution experiment.** hWIP cDNA was generated from normal PBMCs by RT-PCR and cloned into the pAC-EGFP vector. Confirmation of the WT WIP sequence and its expression level gating on EGFP+ cells. Transfected PHA-T cells were evaluated for WASP and WIP expression using a mouse anti–WASP and anti–IgG2a-streptavidin-PECy5, respectively. Acquisition was performed using a FACSCalibur (BD), and analysis was performed by FlowJo software (Tree Star). Transfected PHA-T cells were evaluated for WASP and WIP expression level gating on EGFP+ cells.

**Online supplemental material.** Table S1 provides the Laboratory data of the patient at 3 wk of age. Table S2 provides the list of the primers used for the amplification of the WIPF1 gene. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20110896/DC1.

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**REFERENCES**


