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

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Abbreviations used: CFSE, carboxyfluorescein succinimidyl ester; hWIP, human WIP; IP10, C-X-C motif chemokine immune protein-10; rIL-2, recombinant IL-2; WAS, Wiskott-Aldrich syndrome; WASP, WAS protein; WIP, WASP interacting protein.

The Wiskott-Aldrich syndrome (WASOMIM 301000) is an X-linked disorder characterized by thrombocytopenia, eczema, and immunodeficiency (Thrasher and Burns, 2010). T lymphocytes from WAS patients fail to proliferate and secrete IL-2 in response to cross-linking of the TCR–CD3 complex. Moreover, they exhibit actin cytoskeletal defects illustrated by defective F-actin polymerization after TCR–CD3 cross-linking, reduced ability to spread upon contact with anti-CD3–coated surfaces, impaired chemotaxis in vitro, and poor homing in vivo to lymphoid organs (Thrasher and Burns, 2010). NK cell function and immune synapse formation with target cells is also impaired in WAS (Orange et al., 2002; Gismondi et al., 2004; Stabile et al., 2010).

In T lymphocytes, WASP is almost totally complexed with the WASP-interacting protein (WIP; de la Fuente et al., 2007). The function of WIP is to stabilize WASP and prevent its degradation. WASP protein levels, but not mRNA levels, are severely reduced in T cells from WIP-deficient mice. Introduction of full-length WIP, but not of WIP that lacks the WASP binding domain, restores WASP levels in these cells (de la Fuente et al., 2007). We describe for the first time a patient who presented in early infancy with a phenotype of WAS who was found to have a homozygous mutation 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.
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. 1 A and Table S1). 90% of

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**Figure 1. Functional characterization of WIP deficiency.** (A) Evaluation of the proportion of T, B, and NK cells on gated lymphocytes in whole blood (top and middle row), and of CD4+ and CD8-expressing cells on the CD3+ gated cells (bottom row) of a control (Ctrl), the patient (Pt), and a WAS-null patient. Data are representative of three independent analyses. (B) T cell proliferation to PHA and anti-hCD3 with or without rIL-2, as assessed by CFSE dilution. PBMCs were used and FACS analysis was performed on CD3+ gated cells. The solid gray histogram represents exemplificative profile of unstimulated cells. An overlapping profile was obtained from unstimulated cells of control and WAS patients (not depicted). The red, gray, and blue profiles represent CFSE content in stimulated cells from patient, control, and a WAS-null patient, respectively. Data are representative of two independent experiments. (C) PBMCs were stimulated with IL-2, and gated CD3+ cells were analyzed by FACS for intracellular pSTAT5 using anti-pY694STAT5. The solid line represents the signal in unstimulated patient’s cells, which was comparable to that of unstimulated control and WAS-null patient cells (not depicted). The pSTAT5 signal in the rIL-2 stimulated cells from patient, control, and WAS patient are indicated in red, gray, and blue, respectively. Data are representative of three independent experiments. (D) Migration of PHA blasts from a healthy control, the patient and from a WAS-null patient toward the chemokine IP-10. The gray, red, and blue histograms represent migration of cells from control, patient, and WAS-null patient, respectively. Data are representative of two independent experiments. (E) Expression of the IP-10 receptor CXCR3 on PHA blasts from patient, control, and WAS-null patient. Isotype control staining of patient PHA blasts is shown by the filled histogram and was comparable to that of control T cells and WAS patient (not depicted). The CXCR3 signals in cells from patient, control, and WAS patient are indicated in red, gray, and blue, respectively. Data are representative of two independent experiments. (F) Expression of CD56, NKp30, NKGD2, and NKp46 on NK cells from patient (red) and control (gray). The filled histogram represents isotype control staining of patient NK cells, and was comparable to that of control NK cells (not depicted). Data are representative of two analyses. (G) Cytolytic activity by patient and control NK cell lines against the LCL 721.221 target cells, measured as the percentage of net 51Cr release at effector (E) to target (T) ratios of 3:1 and 6:1. Data are representative of two independent experiments on the same expanded NK cells.
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 \textit{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 upstream 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, introduction 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. 3A). More importantly, introduction of EGFP-hWIP, but not EGFP, in the patient's T cells resulted in increased WASP expression by EGFP$^+$ cells (Fig. 3B). WASP expression in the EGFP-hWIP-transfected cells from the patient correlated with WIP expression (Fig. 3C). 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 patient's T cells would restore their function or examine the cortical 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 characteristics and laboratory findings in the patient resemble those found in WAS. They include recurrent infections, eczematous skin rash, thrombocytopenia, T cell lymphopenia affecting...
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 mice, 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 WASP 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 correspondingly 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 (Cainor-Novartis) in RPMI medium containing 10% FCS, 1% -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-PE 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 (ENSNG0000015285) and WIPF1 (ENSG0000013935) 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 RNeasy Mini kit (QIAGEN) and transcribed 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 5AS (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 × 10⁶) resuspended in 100 µl of the appropriate medium. To assess WASP and WIP 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 (5AS; 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-NKG2D2), and 289 (IgG2a, anti-CD3). For cytometric 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 (Proleneum-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-PECy7, or with isotype-matched mAb PE (BD). Acquisition was performed using a FACSCalibur (BD), and data were analyzed with FlowJo Software v7.5 (Tree Star).

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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 × 10⁵ PHA T blasts were added to the upper chamber of a plate 0.45 µm 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 BglII–PstI cloning sites of the pAC-EGFP vector. Confirmation of the WT WIP sequence and its in-frame insertion in the vector was obtained by direct sequencing. PHA T blasts were transfected using the Amaxa nucleofection technology (Lonza) according to the manufacturer’s protocol, which is optimized for stimulated human T cells. In brief, 2–3 × 10⁶ cell suspension was mixed with 1 µg/10⁶ cell of both mock and hWIP-containing plasmid DNA, transfected using the T-020 program, then cultured for 48 h in the growth medium. Mock- and hWIP-transfected cells were treated with the Fix/Perm kit (Invitrogen) for FACS analysis. Cells were stained with mouse IgG1 anti WIP (3D10) and mouse IgG2a anti WASP (5A5), followed by anti–IgG1-PE and biotinylated anti–IgG2a-streptavidin-PECys, 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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