The formation of podosomes and phagocytic cups is severely affected in macrophages from WAS patients (3, 12, 42), suggesting that WASP is involved in the formation of these structures. However, the detailed molecular mechanisms of their formation remain unknown. WASP is complexed with a cellular WASP-interacting partner, WASP-interacting protein (WIP) (13, 14). Recently, two groups (including us) have demonstrated that WASP and WIP form a complex and that the WASP-WIP complex is required for the formation of podosomes (4, 15) and phagocytic cups (16). Here, we identified formin-binding protein 17 (FBP17) as a protein interacting with the WASP-WIP complex and examined the role of FBP17 in the formation of podosomes and phagocytic cups.

EXPERIMENTAL PROCEDURES

Reagents and Antibodies—Recombinant human macrophage-colony stimulating factor-1 (M-CSF-1) was purchased from R&D Systems (Minneapolis, MN). Phenylmethylsulfonyl fluoride, leupeptin, pepstain A, aprotinin, IGEPAL CA-630, paraformaldehyde, saponin, bovine serum albumin, 3-methyladenine, latex beads (3 μm in diameter), phorbol 12-myristate 13-acetate (PMA), human IgG, glycerol, Triton X-100, anti-FLAG monoclonal antibody (M2), and anti-β-actin antibody were purchased from Sigma-Aldrich. The anti-WASP monoclonal antibody, anti-WIP polyclonal antibody, and anti-Myc monoclonal antibody (9E10) were obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). The anti-dynamin-2 antibody was purchased from BD Biosciences. The rat anti-hemagglutinin (HA) monoclonal antibody (3F10) was purchased from Boehringer Ingelheim (Ridgefield, CT). The Cy2-labeled anti-rat IgG was obtained from Jackson ImmunoResearch Laboratories (West Grove, PA).

Yeast Two-hybrid Screening—We screened a human lymphocyte cDNA library (Origene Technology Inc., Rockville, MD) using a full-length WIP as bait. A cDNA encoding full-length WIP was cloned into pGilda (BD Biosciences Clontech). The EGY48 yeast strain was transformed with pGilda-WIP, the human lymphocyte cDNA library, and pSH18-34, a reporter plasmid for the β-galactosidase assay. Transformants were assayed for Leu prototrophy, and a filter assay was performed for β-galactosidase measurement (17).

Cells and Transfection—THP-1 and human embryonic kidney (HEK) 293 cells were purchased from the American Type Culture Collection (Manassas, VA) and cultured in RPMI1640 and Dulbecco's modified Eagle's high glucose medium (Invitrogen), respectively, both supplemented with 10% fetal bovine serum. For human primary monocyte isolation, 10–30 ml of peripheral blood was drawn from healthy volunteers and WAS patients after informed consent was obtained. Monocytes were prepared from peripheral blood samples (10–30 ml) using a monocyte isolation kit II (Miltenyi Biotech Inc., Auburn, CA). Transfection of THP-1 cells and monocytes was performed with a Nucleofector device using a cell line Nucleofector kit V and a human monocyte Nucleofector kit, respectively, according to the manufacturer's instructions (Amaxa Biosystems, Gaithersburg, MD). Transfection of HEK293 cells was performed using SuperFect transfection reagent (Qiagen, Valencia, CA). THP-1 cells and monocytes were co-transfected with the FBP17 constructs and a GFP-expressing plasmid, pmaxGFP (Amaxa Biosystems Inc.), as a transfection marker. The transfection efficiency measured using pmaxGFP was 40–50% for THP-1 cells and 10–20% for monocytes.

RNA Interference—A short interfering RNA (siRNA) for FBP17 and its scrambled control siRNA was synthesized by Dharmacon (Lafayette, CO). The targeting sequence was 5′-CCCACTTCATATGTCGAAGTCTGTT-3′ (18). THP-1 cells and monocytes were transfected with siRNA using a cell line Nucleofector kit V and a human monocyte Nucleofector kit, respectively, and a Nucleofector device. Cells were co-transfected with an fluorescein isothiocyanate (FITC)-conjugated control siRNA, BLOCK-IT (Invitrogen), as a transfection marker. The transfection efficiency measured using BLOCK-IT was 40–50% for THP-1 cells and 10–20% for monocytes.

Immunoprecipitation—For immunoprecipitation of WASP from THP-1 cells, 2×10^7 cells were lysed in buffer A (50 mM Tris-HCl, pH 7.5, 75 mM NaCl, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, 1 μg/ml pepstatin A, 1 μg/ml aprotinin). Lysates were centrifuged at 10,000 $\times$ *g* at 4 °C for 15 min. The supernatant was incubated with 2 μg/ml anti-WASP monoclonal antibody (Santa Cruz Biotechnology) at 4 °C for 2 h and then incubated with anti-mouse IgG agarose (Sigma). The resin binding the immune complex was washed three times with 0.5 ml of buffer B (50 mM Tris-HCl, pH 7.5, 10% glycerol, 0.1% Triton X-100), and the complex was eluted with 1× Laemmli's SDS-PAGE sample buffer. Eluted proteins were subjected to SDS-PAGE and analyzed by immunoblotting for WASP, WIP, and FBP17.

GST Pull-down Assay—Glutathione *S*-transferase (GST) and a fusion protein of GST and the src homology 3 (SH3) domain of FBP17 (548–609 amino acids) (GST-FSH3) were purified from *Escherichia coli* (XL-1B) extracts using glutathione-Sepharose-4B. HEK293 cells were transfected with the cDNAs of Myc- or FLAG-tagged protein and lysed in buffer A. Lysates from the transfected cells were incubated with the affinity matrices of GST alone or GST-FSH3 at 4 °C for 1 h. After a 1-h incubation, the matrices were washed five times with buffer A, and pull-down samples were analyzed by immunoblotting using anti-Myc or anti-FLAG antibody.

Immunofluorescence Microscopy—THP-1 cells and monocytes grown on coverslips were differentiated into macrophages by incubation with 12.5 ng/ml PMA (Sigma) and 20 ng/ml M-CSF-1 (R&D Systems), respectively, for 72 h. HEK293 cells were transfected with various cDNA constructs and then cultured on coverslips for 48 h. Cells were fixed with 4% (w/v) paraformaldehyde, permeabilized with 0.1% (w/v) saponin, and blocked with 1% (w/v) bovine serum albumin. Cells were stained with primary antibodies and Alexa Fluor 488- or Alexa Fluor 564-labeled secondary antibodies (Invitrogen). Cells were also stained with Alexa Fluor 568-labeled phalloidin (Invitrogen). Cell staining was examined under a fluorescence microscope (Zeiss Axioplan AR) or an MRC 1024 SP laser point scanning confocal microscope (Bio-Rad).

Assays for the Formation of Podosomes and Phagocytic Cups—The formation of podosomes and phagocytic cups was assayed by visualizing these actin-based membrane structures by F-actin staining as described previously (4, 16). Briefly, podosomes

in differentiated THP-1 cells or macrophages were visualized by F-actin staining with Alexa Fluor 568-phalloidin. To form phagocytic cups in differentiated THP-1 cells or macrophages, latex beads (3 μm, Sigma) were opsonized with 0.5 mg/ml human IgG (Sigma), and cells grown on coverslips were incubated with the IgG-opsonized latex beads at 37 °C for 10 min in the presence of 10 mM 3-methyladenine (Sigma) to stabilize the phagocytic cups (16). The phagocytic cups were then also visualized with Alexa Fluor 568-phalloidin. Cells were examined under a fluorescence microscope (Zeiss Axioplan AR).

Assays for Macrophage Migration and Phagocytosis—For the macrophage migration assay, human macrophages (2×10^5 cells) were plated onto chemotaxis membranes with 5-μm pores (Corning, Acton, MA) coated with 0.15% gelatin/phosphate-buffered saline placed within Boyden chamber inserts. M-CSF-1 was used as a chemoattractant and diluted in serum-containing RPMI 1640 medium in lower chambers. After a 4-h incubation, non-migrating cells were removed by gently wiping the upper surface of the filter. The filter was removed from the inserts using a razor blade and mounted onto glass plates, and the number of migrating cells was counted under a fluorescence microscope. For the phagocytosis assay, human macrophages (1×10^6 cells) were seeded on coverslips and incubated with 0.5 ml of RPMI 1640 medium containing IgG-opsonized latex beads (3 μm) at 4 °C for 10 min, allowing the beads to attach to cells. Phagocytosis was initiated by adding 1.5 ml of preheated RPMI 1640 medium, and the cells were incubated with the beads at 37 °C for 30 min. Control plates were incubated at 4 °C to estimate nonspecific binding of latex beads to the cells. After incubation, the cells were vigorously washed with phosphate-buffered saline, and the number of intracellular latex beads was determined by counting beads within cells under a fluorescence microscope. The percentage of phagocytosis was calculated as the total number of cells with at least one bead as a percentage of the total number of cells counted. At least 100 cells were examined.

Cell Fractionation—To prepare the cytoplasmic and membrane fractions, macrophages (1×10^6 cells) were washed with ice-cold phosphate-buffered saline and suspended in 50 mM Tris-HCl buffer, pH 7.5, containing 1 mM EDTA and proteinase inhibitors as described above. The cell suspensions were sonicated four times on ice for 5 s each using a bath-type sonicator followed by ultracentrifugation at 265,000 × *g* at 4 °C for 2 h. The supernatant was used as the cytosolic fraction, and the pellet was resuspended in 50 mM Tris-HCl, pH 7.5, containing 1 mM EDTA and used as the membrane fraction. Anti-Caspase-3 (Santa Cruz Biotechnology) and anti-sodium potassium ATPase antibodies (AbCam, Inc., Cambridge, MA) were used to determine the purity of the cytosolic and membrane fractions, respectively.

Statistics—Statistically significant differences were determined using the Student's *t* test. Differences were considered significant if $p < 0.05$.

RESULTS

FBP17 Binds to the WASP-WIP Complex and Dynamin-2 in Macrophages—To explore the detailed molecular mechanisms of the formation of podosomes and phagocytic cups, we searched for a protein interacting with the WASP-WIP complex. We identified FBP17 as a WIP-binding protein in a yeast two-hybrid screen using the full-length WIP as bait. FBP17 was originally identified as a protein binding to formin, a protein that regulates the actin cytoskeleton (19). FBP17 is a member of the *Schizosaccharomyces pombe* Cdc15 homology (PCH) protein family (20) and contains an N-terminal extended FER-CIP4 homology (EFC) domain (also known as the FER-CIP4 homology and Bin-amphiphysin-Rvs (F-BAR) domain), protein kinase C-related kinase homology region 1 (HR1), and an SH3 domain (Fig. 1*E*). The EFC/F-BAR domain has membrane binding and deformation activities, and FBP17 is involved in endocytosis in transfected COS-7 cells (18, 21, 22).

To confirm that FBP17 directly interacts with WIP or WASP, we performed GST pull-down assays using a fusion protein of GST and the SH3 domain of FBP17 (GST-FBPSH3). Purified GST and the GST-FSH3 fusion protein were subjected to SDS-PAGE (Fig. 1*F*, *lanes 1* and *2*). The HEK293 transfected cells express the Myc- and FLAG-tagged proteins (Fig. 1*F*, *lanes 3–6*). The results from the GST pull-down assays were shown (Fig. 1*F*, *lanes 7–14*). Both WASP and WIP were pulled down by GST-FSH3 (Fig. 1, *lanes 10* and *14*), indicating that the SH3 domain of FBP17 directly interacts with both proteins.

It has previously been shown that FBP17 binds to N-WASP and dynamin in transfected cells (18, 21). We examined whether FBP17 binds to WASP, WIP, and dynamin-2 in macrophages. THP-1 (human monocyte cell line) cells closely resemble monocyte-derived macrophages when differentiated by stimulation with PMA (23) and form podosomes and phagocytic cups that are morphologically and functionally indistinguishable from those in primary macrophages (supplemental Fig. 1) (4, 16, 23). WASP was immunoprecipitated from the lysates of PMA-differentiated THP-1 cells with an anti-WASP monoclonal antibody (Fig. 1*G*, *lanes 2*, *5*, and *8*) followed by immunoblotting using antibodies to FBP17 (21), WASP, and WIP. Both WIP and FBP17 co-immunoprecipitated with WASP (Fig. 1*G*, *lanes 5* and *8*). FBP17 also co-immunoprecipitated with dynamin-2 (Fig. 1*G*, *lanes 14*). These results, taken together with the results in Fig. 1*F*, suggest that FBP17 binds to the WASP-WIP complex and dynamin-2 in macrophages.

We next used immunofluorescence to examine whether FBP17 localizes at podosomes and phagocytic cups because the WASP-WIP complex is an essential component of podosomes (4, 15) and phagocytic cups (16). THP-1 cells transfected with FLAG-tagged FBP17 (FLAG-FBP17) and differentiated by stimulation with PMA were stained with an anti-FLAG monoclonal antibody to visualize FBP17 and with phalloidin to visualize the F-actin in podosomes and phagocytic cups (Fig. 1, *H* and *I*, *left* and *middle panels*). Merged images revealed that both F-actin and FBP17 are present in podosomes and phagocytic cups (Fig. 1, *H* and *I*, *right panels*), indicating that FBP17 localizes at podosomes and phagocytic cups.

Importance of FBP17 in the Formation of Podosomes and Phagocytic Cups—To determine the importance of FBP17 in the formation of podosomes and phagocytic cups, we knocked down FBP17 in THP-1 cells with siRNAs. To confirm that the expression of FBP17 was knocked down in cells, we transfected THP-1 cells with siRNAs, prepared lysates from the total

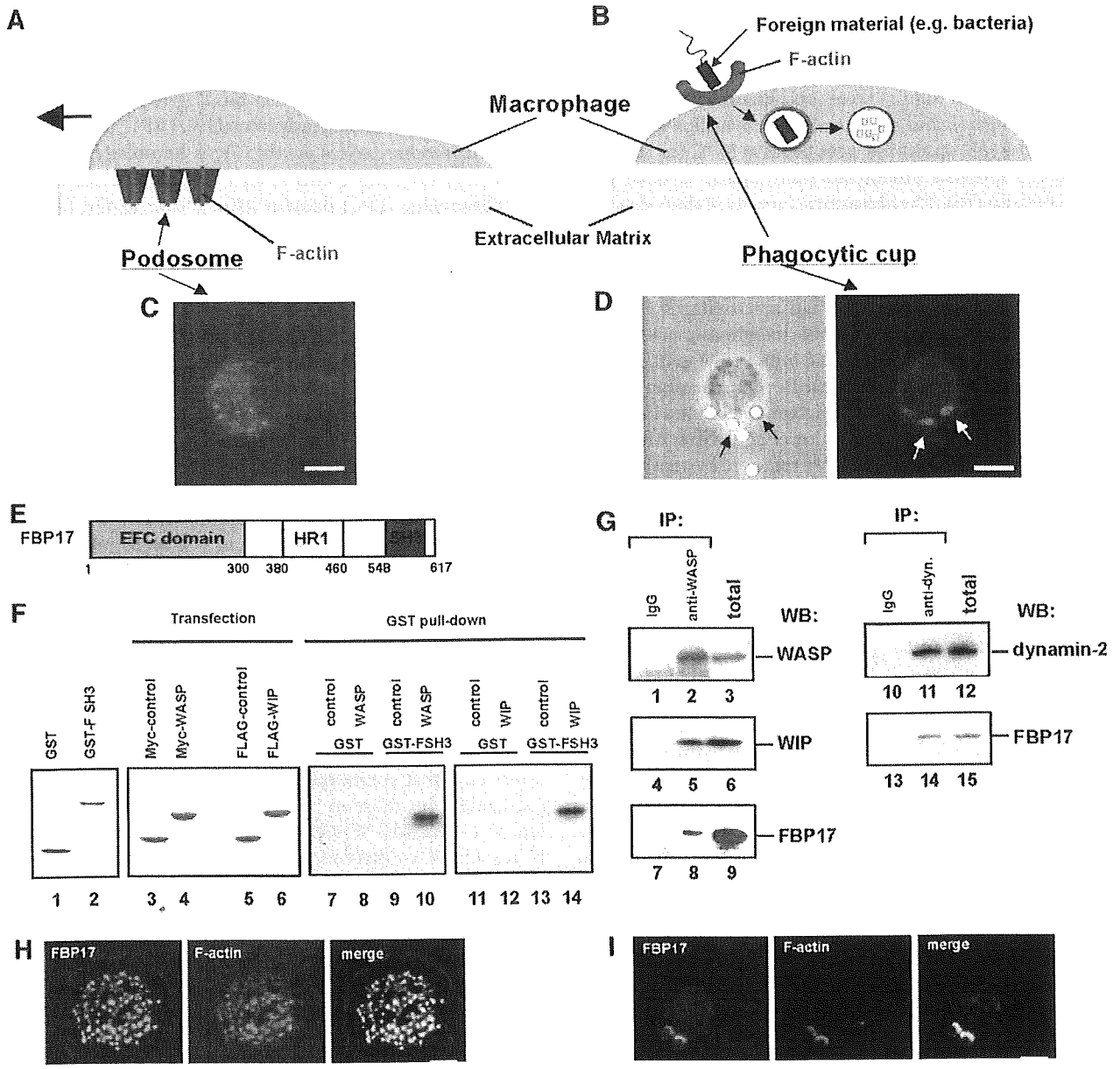

FIGURE 1. **FBP17 is a component of podosomes and phagocytic cups.** *A* and *B*, schematic drawings of podosomes (*A*) and a phagocytic cup (*B*) in macrophages. *C*, podosomes in macrophages were visualized by F-actin staining using Alexa Fluor 568-phalloidin. *D*, macrophages incubated with IgG-opsonized latex beads formed phagocytic cups to ingest the beads. A phase contrast image of a macrophage forming phagocytic cups (*left panel*). *Black arrows* indicate the latex beads ingested by the macrophage. Phagocytic cups were visualized by F-actin staining using Alexa Fluor 568-phalloidin (*right panel*). *White arrows* indicate the phagocytic cups. The *bar* is 10 μm. *E*, the domain organization of FBP17. *HR1*, protein kinase C-related kinase homology region 1. *F*, FBP17 interacts directly with WASP and WIP via its SH3 domain. GST and the GST-FBP17 SH3 domain fusion protein (GST-FSH3) were purified from bacteria extracts. Purified proteins were subjected to SDS-PAGE and stained with Coomassie Brilliant Blue (*lanes 1* and *2*). HEK293 cells were transfected with the cDNAs of Myc-tagged control protein (Myc-PDZ-GEF), Myc-WASP, FLAG-PDZ-GEF, or FLAG-WIP, and the expression of those proteins were analyzed by immunoblotting (*lanes 3–6*). Lysates from the HEK293 transfected cells were incubated with the affinity matrices of GST alone or GST-FSH3. Pull-down samples were analyzed by immunoblotting using anti-Myc antibody (*lanes 7–10*) and anti-FLAG antibody (*lanes 11–14*). *G*, FBP17 binds WASP, WIP, and dynamin-2. WASP was immunoprecipitated (*IP*) from the lysates of PMA-differentiated THP-1 cells with anti-WASP or a control IgG (*left panel*, *lanes 1–9*). The WASP immunoprecipitates and total lysates were analyzed by immunoblotting (*WB*) for WASP (*lanes 1–3*), WIP (*lanes 4–6*), and FBP17 (*lanes 7–9*). Dynamin was also immunoprecipitated from the THP-1 cell lysates with an anti-dynamin polyclonal antibody. The dynamin immunoprecipitates and total lysates were analyzed by immunoblotting for dynamin-2 (*lanes 10–12*) and FBP17 (*lanes 13–15*). *H* and *I*, confocal laser scanning micrographs of PMA-differentiated THP-1 cells. *H*, THP-1 cells transfected with FLAG-tagged FBP17 cDNA (FBP17) were double-stained with an anti-FLAG monoclonal antibody (*left panel*) and phalloidin (*center panel*) to visualize the F-actin in podosomes. *Yellow* indicates co-localization of FBP17 (*green*) and F-actin, podosomes (*red*) (*right panel*). *I*, THP-1 cells transfected with FLAG-FBP17 cDNA were incubated with IgG-opsonized latex beads and double-stained with anti-FLAG antibody and phalloidin. Phagocytic cups were visualized by F-actin staining (*center panel*). *Yellow* indicates co-localization of FBP17 (*green*) and F-actin, phagocytic cups (*red*) (*right panel*). The *bar* is 10 μm.

siRNAs-transfected cells, and analyzed the expression level of FBP17 by immunoblotting. THP-1 cells transfected with the siRNA for FBP17 expressed ~40% less FBP17 than cells transfected with a scrambled control siRNA based on the immunoblots (Fig. 2*A*, *lanes 1* and *2*) but expressed the same level of β-actin (Fig. 2*A*, *lanes 3* and *4*). The transfection efficiency of

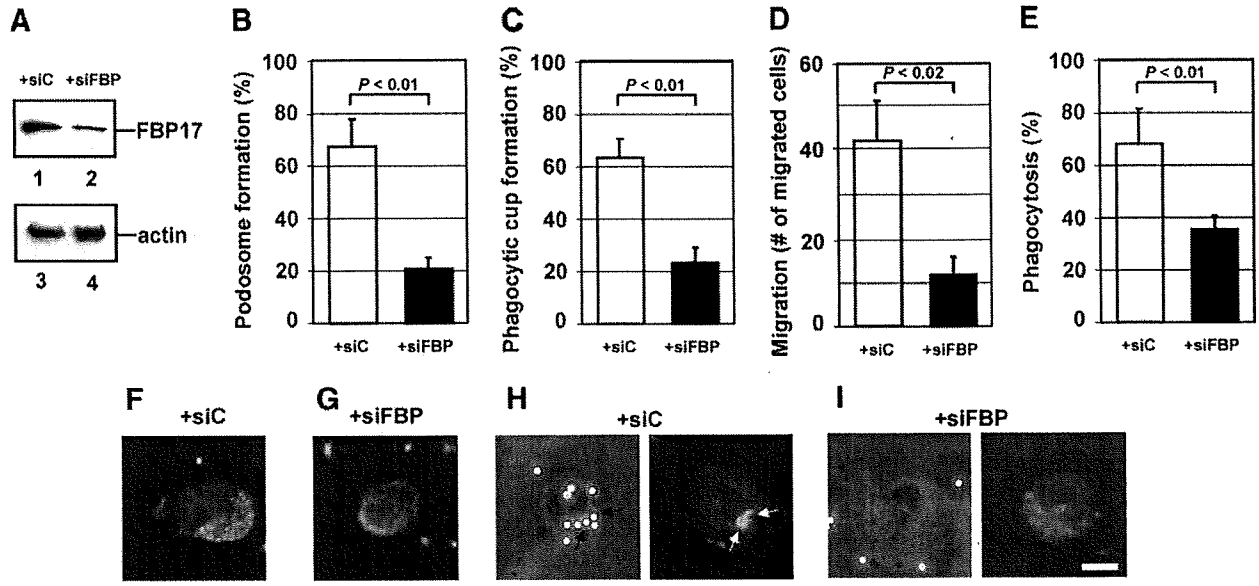

FIGURE 2. **The importance of FBP17 in the formation of podosomes and phagocytic cups.** *A*, expression of FBP17 was reduced by transfection of siRNA. THP-1 cells were transfected with siRNA for FBP17 (siFBP; *lanes 2* and *4*) or its scrambled control siRNA (siC; *lanes 1* and *3*). Lysates prepared from total transfected cells were analyzed by immunoblotting for FBP17 (*lanes 1* and *2*) and β-actin (*lanes 3* and *4*). *B* and *C*, effects of FBP17 siRNA on the formation of podosomes and phagocytic cups in macrophages. Human primary monocytes were co-transfected with siFBP (*closed bars*) or siC (*open bars*) and an FITC-conjugated control siRNA and then differentiated into macrophages with M-CSF-1. FITC-positive transfected cells were examined for the formation of podosomes (*B*) or phagocytic cups (*C*), and the percentage of cells with podosomes or phagocytic cups was scored. *D* and *E*, effects of FBP17 siRNA on the functions of podosomes and phagocytic cups. Macrophages co-transfected with siFBP (*closed bars*) or siC (*open bars*) and the FITC-conjugated control siRNA were assayed for macrophage migration (*D*) or phagocytosis of IgG-opsonized latex beads (*E*). Data represent the mean ± S.D. of triplicate experiments. *F–I*, immunofluorescence micrographs of a representative cell from each experiment. Cells transfected with siC (*F*) and siFBP (*G*) were stained with Alexa Fluor 568-phalloidin. Cells transfected with siC (*H*) or siFBP (*I*) were incubated with IgG-opsonized latex beads and then stained with phalloidin. The *left* and *right panels* are phase contrast and immunofluorescence micrographs, respectively. The *bar* is 10 μm.

THP-1 cells was estimated to be 40–50% from the expression of green fluorescent protein (GFP) used as a transfection control. Therefore, the decrease in FBP17 expression indicates that FBP17 was efficiently knocked down in most transfected cells.

Human primary monocytes were co-transfected with the FBP17 siRNAs and a FITC-conjugated control siRNA as a transfection marker. After differentiation of the monocytes into macrophages with M-CSF-1, FITC-positive cells were examined for the formation of podosomes and phagocytic cups. To quantify their formation, we scored the percentage of cells with podosomes or phagocytic cups among FITC-positive cells. When the expression of FBP17 was knocked down, the formation of both podosomes and phagocytic cups in macrophages was significantly reduced ($p < 0.01$; Fig. 2, *B* and *C*). These results suggest that FBP17 is necessary for the formation of podosomes and phagocytic cups. A representative cell from each experiment is shown in Fig. 2, *F* and *G*, for podosomes and in Fig. 2, *H* and *I*, for phagocytic cups. We then assayed macrophage migration as a podosome function and phagocytosis as a phagocytic cup function. When expression of FBP17 was knocked down, macrophage migration through a gelatin filter toward a chemoattractant was significantly reduced in cells transfected with FBP17 siRNA ($p < 0.02$; Fig. 2*D*). Phagocytosis of IgG-opsonized latex beads was also reduced (Fig. 2*E*). These results suggest that FBP17 is essential for chemotaxis and phagocytosis because of its role in forming podosomes and phagocytic cups, respectively.

FBP17 Recruits the WASP-WIP Complex to the Plasma Membrane—Recent biochemical analyses revealed that FBP17 binds to a membrane phospholipid, phosphatidylinositol 4,5-bisphosphate ($PI(4,5)P_2$), through its EFC/F-BAR domain and to N-WASP and dynamin via its SH3 domain (18, 21, 24). We have shown that although WASP and WIP are cytosolic proteins, the WASP-WIP complex localizes at podosomes and phagocytic cups (4, 16). We then examined whether FBP17 recruits the WASP-WIP complex to the plasma membrane in macrophages. We focused on the roles of the EFC and SH3 domains of FBP17 and constructed three FBP17 mutants for the recruitment experiments: a Lys-33 to Glu (K33E) substitution, a Lys-166 to Ala (K166A) substitution, and an SH3 domain deletion (dSH3). Both substitution mutations in the EFC domain (K33E and K166A) significantly reduce membrane binding and deformation (22), and the dSH3 mutant does not bind to WASP and WIP because the SH3 domain is the binding site of WASP and WIP (Fig. 1*F*). We co-transfected HEK293 cells with the FLAG-tagged FBP17 constructs, WASP, and WIP. A C-terminal fragment (1146–1429 amino acids) of PDZ-GDP exchange factor (PDZ-GEF) was used as a negative control for FBP17 because this fragment is stable in the cytosol and does not interact with any WASP-related proteins (4, 16, 25). We confirmed the expression of FBP17 and its mutants in cells by immunoblotting (supplemental Fig. 2) and immunoprecipitated FLAG-tagged proteins from lysates of the transfected cells with anti-FLAG antibody (Fig. 3*A*, *lanes 1–5*). WASP and WIP were detected in the immunoprecipitates from cells

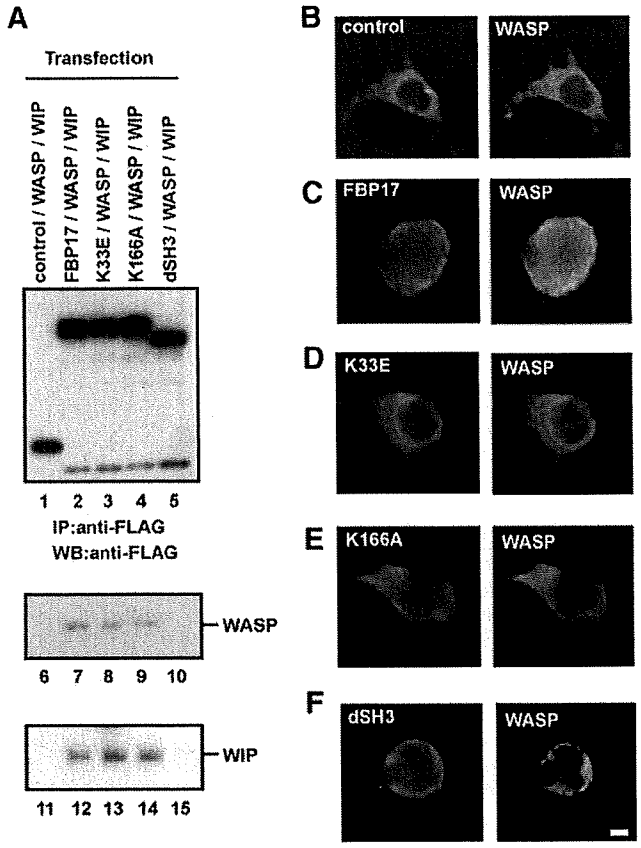

FIGURE 3. **FBP17 recruits WASP, WIP, and dynamin-2 to the plasma membrane.** *A*, HEK293 cells were co-transfected with cDNAs of the indicated FLAG-tagged proteins, Myc-tagged WASP, and HA-tagged WIP. The FLAG-tagged proteins were immunoprecipitated (*IP*) from lysates of the transfected cells with an anti-FLAG antibody followed by immunoblotting (*WB*) using antibodies to FLAG (*lanes 1–5*), WASP (*lanes 6–10*), and WIP (*lanes 11–15*). *B–F*, transfected HEK293 cells expressing FLAG-tagged proteins, Myc-WASP, and HA-WIP were double-stained with an anti-FLAG antibody and anti-WASP antibody. *B–F*, cells expressing FLAG-PDZ-GEF (*B*), FLAG-FBP17 (*C*), the FLAG-tagged FBP17 mutant with the K33E missense mutation (*D*), K166A (*E*), and the SH3-deleted FBP17 mutant dSH3 (*F*). The *bar* is 10 μm.

expressing the FLAG-tagged FBP17, K33E, and K166A constructs (Fig. 3*A*, *lanes 7–9* and *12–14*) but not the FLAG-tagged PDZ-GEF and dSH3 constructs (Fig. 3*A*, *lanes 6*, *10*, *11*, and *15*), indicating that FBP17 and its mutants K33E and K166A form a complex with WASP and WIP but that dSH3 not.

Next, cells expressing the FLAG-tagged proteins, WASP, and WIP were examined under the immunofluorescence microscope for the localization of the FLAG-tagged proteins and WASP. WASP and WIP were localized in the cytosol in cells transfected with only the WASP cDNA and only the WIP cDNA, respectively, as well as in cells expressing both WASP and WIP (supplemental Fig. 3). In cells co-expressing FLAG-PDZ-GEF (control) with WASP and WIP, both FLAG-PDZ-GEF and WASP were cytosolic (Fig. 3*B*). In cells co-expressing FLAG-FBP17 with WASP and WIP, FLAG-FBP17 localized at the plasma membrane because its EFC domain binds to the plasma membrane (Fig. 3*C*, *left panel*). In those cells, WASP also localized at the plasma membrane (Fig. 3*C*, *right panel*), indicating that FBP17 shifted the localization of WASP from the cytosol to the plasma membrane (Fig. 3, *B* and *C*). To confirm that the WASP-WIP complex was recruited to the plasma membrane, cells co-expressing FLAG-FBP17 with WASP and HA-tagged WIP were stained with an anti-FLAG monoclonal antibody and an anti-WASP polyclonal antibody or an anti-HA rat monoclonal antibody. Double staining revealed that both WASP and WIP co-localized with FLAG-FBP17 at the plasma membrane (supplemental Fig. 4, *A* and *B*). To further confirm the localization of the FBP17 mutants, cells co-expressing the FLAG-tagged FBP mutants, WASP, and WIP were stained with anti-FLAG monoclonal antibody. The K33E and K166A mutants were cytosolic and the SH3-deleted FBP17 mutant localized at the plasma membrane (supplemental Fig. 4*C*).

To determine the roles of the EFC and SH3 domains of FBP17 in this recruitment, we examined the localization of the FBP17 mutants and WASP in cells co-expressing the FBP mutants with WASP and WIP. Membrane tubulation in cells transfected with an FBP17 cDNA is an indicator of the membrane binding and deformation activities of FBP17 (18, 22). We detected *in vivo* membrane tubulation in cells expressing FBP17 and dSH3 but not in cells expressing K33E and K166A (supplemental Fig. 5). In cells co-expressing either FBP17 mutant (K33E or K166A) with WASP and WIP, both K33E and K166A were cytosolic (Fig. 3, *D* and *E*, *left panels*), and WASP was also cytosolic (Fig. 3, *D* and *E*, *right panels*). These results indicate that K33E and K166A are unable to recruit WASP to the plasma membrane, consistent with the inability of K33E and K166A to bind and deform the plasma membrane (supplemental Fig. 5).

The SH3-deleted FBP17 mutant, dSH3, localized at the plasma membrane (Fig. 3*F*, *left panel*) because its EFC domain is intact. However, WASP was cytosolic in cells co-expressing dSH3 with WASP and WIP (Fig. 3*F*, *right panel*), consistent with the inability of the dSH3 mutant to bind to WASP and WIP (Fig. 3*A*, *lanes 5*, *10*, and *15*).

To quantify the recruitment, we scored the percentage of cells in which WASP and WIP were localized at the plasma membrane. Cells expressing the FBP17 mutants (K33E, K166A, or dSH3) exhibited significantly lower plasma membrane localization of WASP and WIP than cells expressing FBP17 ($p < 0.05$; supplemental Fig. 6, *A* and *B*). FBP17 also recruited dynamin-2 to the plasma membrane, and both EFC and SH3 domains are necessary for this recruitment (supplemental Fig. 6*C*), as reported previously (18, 21). To confirm the localization of FBP17 and its mutants in cells co-expressing FBP17 with WASP, WIP, and dynamin-2, the transfected cells were stained with anti-FLAG monoclonal antibody. The wild-type FBP17 and dSH3 localized at the plasma membrane and the FLAG-PDZ-GEF (control) and the FBP mutants (K33E and K166A) were cytosolic (supplemental Fig. 6*D*).

Subcellular Localization of FBP17, WASP, WIP, and Dynamin-2 in Macrophages—To determine whether WASP, WIP, and dynamin-2 are recruited to the plasma membrane in macrophages when podosomes and phagocytic cups are formed, we examined the subcellular localization of FBP17, WASP, WIP, and dynamin-2 in macrophages forming podosomes or phagocytic cups. The cytosolic and membrane fractions were prepared from macrophages and analyzed by immunoblotting. Caspase-3 is a cytosolic marker, and sodium

potassium ATPase is a plasma membrane marker (26). FBP17 was detected in the membrane fraction from macrophages forming podosomes (Fig. 4*A*, *lane 9*). WASP, WIP, and dynamin-2 were also detected in the membrane fraction, although they are cytosolic proteins (Fig. 4*A*, *lanes 12, 15,* and *18*). FBP17 was detected in the membrane fraction from macrophages forming phagocytic cups (Fig. 4*B*, *lane 9*). WASP, WIP, and dynamin-2 were also detected in the membrane fractions from macrophages forming phagocytic cups (Fig. 4*B*, *lanes 12, 15,* and *18*). These results, taken together with Fig. 3, suggest that FBP17 recruits the WASP-WIP complex and dynamin-2 to the plasma membrane in macrophages and that both the EFC and the SH3 domains are necessary for this recruitment.

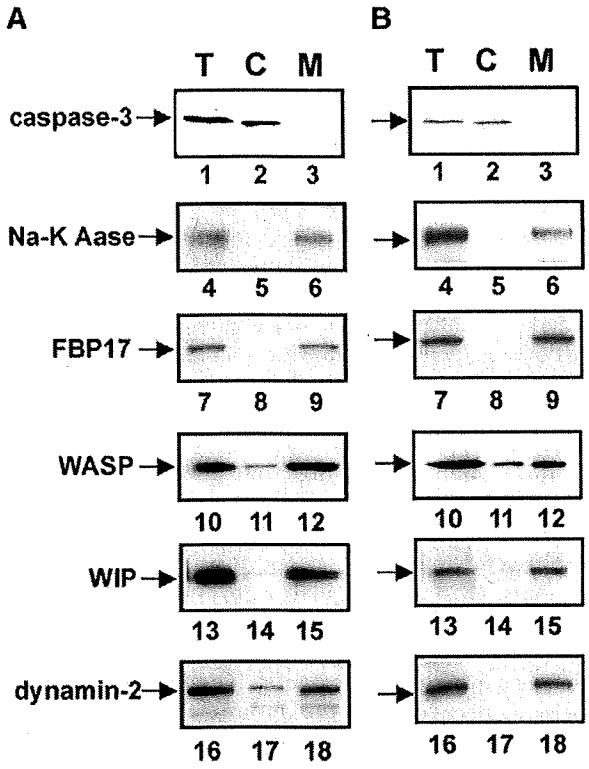

FIGURE 4. **Subcellular localization of FBP17, WASP, WIP, and dynamin-2 in macrophages.** *A*, macrophages forming podosomes. *B*, macrophages forming phagocytic cups. Total lysates (*T*), the cytosolic fraction (C), and the membrane fraction (*M*) prepared from macrophages forming podosomes (*A*) or phagocytic cups (*B*) were analyzed by immunoblotting for caspase-3 (*lanes 1–3*), sodium potassium ATPase (*Na-K Aase; lanes 4–6*), FBP17 (*lanes 7–9*), WASP (*lanes 10–12*), WIP (*lanes 13–15*), and dynamin-2 (*lanes 16–18*). Caspase-3 and sodium potassium ATPase (Na-K ATPase) are markers for the cytosol and plasma membrane, respectively.

The Role of Each Domain of FBP17 in the Formation of Podosomes and Phagocytic Cups—To determine the roles of the EFC and SH3 domains in the formation of podosomes and phagocytic cups, we examined whether overexpression of the FBP17 mutants affects the formation of these structures. We transfected THP-1 cells with the FBP17 constructs and confirmed the expression of FBP17 or the FBP17 mutants in transfected THP-1 cells by immunoblotting (Fig. 5*A*). When THP-1 cells were differentiated to obtain macrophage phenotypes with PMA, podosome formation was significantly reduced in cells overexpressing the K33E, K166A, and dSH3 FBP17 mutants when compared with the FBP17 wild type ($p < 0.01$; Fig. 5*B*). Phagocytic cup formation was also reduced in cells overexpressing the FBP17 mutants (Fig. 5*C*). These results indicate that the EFC domain and SH3 domain are essential for the formation of podosomes and phagocytic cups in macrophages.

Defects in Macrophages from WAS Patients—Our results suggest that the complex formation of FBP17 with WASP, WIP, and dynamin-2 at the plasma membrane is a critical step in the formation of podosomes and phagocytic cups (Figs. 1*G*, 3, and 4). In macrophages from WASP-deficient WAS patients, the complex does not form properly due to a lack of WASP expression. We examined macrophages from WASP-deficient WAS patients for the formation of podosomes and phagocytic cups. Two genetically independent WAS patients (WAS1, 211delT; and WAS2, 41–45delG) (27, 28) were assayed for the formation of those structures. Podosomes were completely absent (Fig. 6, *A–C*), and phagocytic cup formation was severely impaired (Fig. 6, *D–F*) in macrophages from both WAS patients, although FBP17, WIP, and dynamin-2 were expressed at the same level in patients as in normal individuals (Fig. 6*G*). In fact, the formation of podosomes and phagocytic cups was impaired in macrophages when the expression of WASP was reduced by siRNA transfection (4, 16). This is the first result showing that both podosome and phagocytic cup formations are defective in macrophages from WASP-deficient patients. These results are consistent with the previous observations (3, 12).

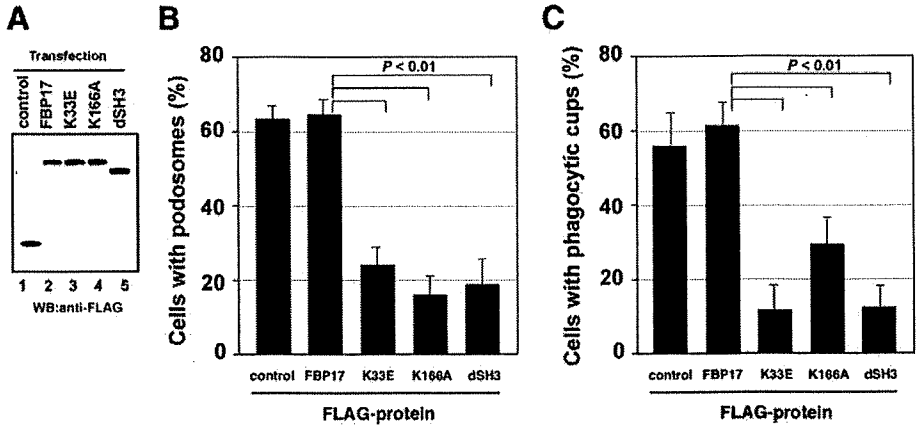

FIGURE 5. **The role of the EFC and SH3 domains of FBP17 in the formation of podosomes and phagocytic cups.** *A*, expression of FLAG-tagged proteins in transfected THP-1 cells. Total lysates prepared from transfected THP-1 cells were analyzed by immunoblotting (*WB*) using an anti-FLAG antibody. All of the FLAG-tagged proteins, FLAG-PDZ-GEF (control, *lane 1*), FLAG-FBP17 (*lane 2*), and the FBP17 mutants, K33E, K166A, and dSH3 (*lanes 3–5*) were expressed in THP-1 cells at similar levels. *B* and *C*, THP-1 cells co-transfected with cDNAs for the FLAG-tagged proteins and pmaxGFP were differentiated with PMA and then assayed for the formation of podosomes (*B*) and phagocytic cups (*C*). The percentage of cells with podosomes or phagocytic cups among all GFP-positive cells was scored. Data represent the mean ± S.D. of triplicate experiments.

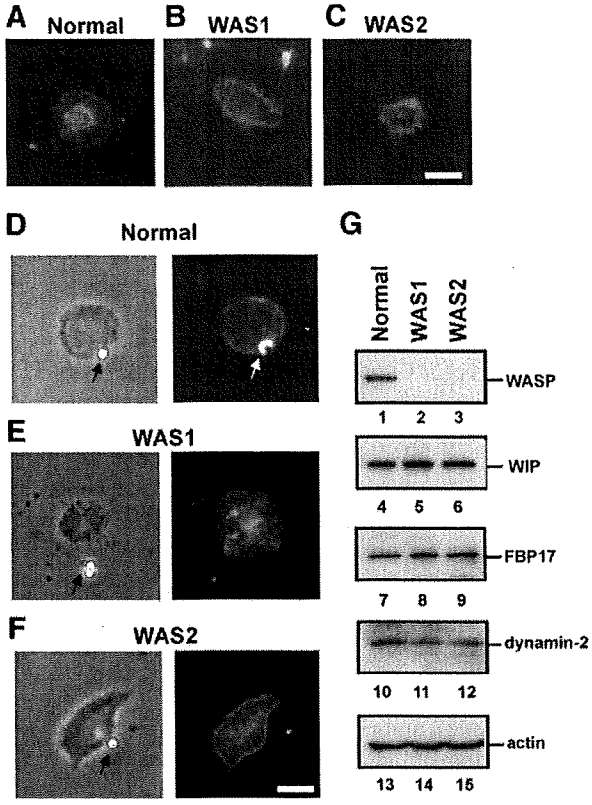

FIGURE 6. **Defective formation of podosomes and phagocytic cups in macrophages from WAS patients.** *A–F*, macrophages from a normal control and two genetically independent WAS patients (WAS1 and WAS2) were examined for the formation of podosomes (*A–C*) and phagocytic cups (*D–F*). The patients, WAS1 and WAS2, have the deletion mutations 211delT and 41–45delG, respectively, in their genomic DNAs. The *bars* are 10 μm. *G*, expression levels of WASP, WIP, FBP17, dynamin-2, and β-actin in WAS patients. Lysates prepared from macrophages from a normal control and two WAS patients (WAS1 and WAS2) were subjected to immunoblotting. WASP was not detected in the lysates from these WAS patients (*lanes 2* and *3*). Podosomes were completely absent (*A–C*) and phagocytic cup formation was severely impaired (*D–F*) in macrophages from both WAS patients, although FBP17, WIP, and dynamin-2 were expressed at the same level in patients as in normal individuals (*G*) (*lanes 4–12*).

These results give us a natural example that supports the importance of the complex formation of FBP17 with WASP, WIP, and dynamin-2 for the formation of podosomes and phagocytic cups.

DISCUSSION

Cell biological and structural analyses of the EFC domain of FBP17 have shown that the EFC domain binds to and deforms the plasma membrane (18, 22). It has previously been shown that the SH3 domain of FBP17 binds to N-WASP and dynamin in transfected cells (18, 21). However, physiologically important processes to which those activities of FBP17 contribute were unknown. Here, we have demonstrated that FBP17 recruits the WASP-WIP complex from the cytosol to the plasma membrane and that this recruitment is necessary for the formation of podosomes and phagocytic cups in macrophages. Our results suggest that FBP17 facilitates membrane deformation and actin polymerization induced by the WASP-WIP complex to occur simultaneously at the same membrane sites and that both are required for the formation of podosomes and phagocytic cups. This is supported by the observations that regulated actin polymerization is an essential process for the formation of podosomes (3) and phagocytic cups (29). Thus, FBP17 mediates a common molecular step in the formation of podosomes and phagocytic cups.

Macrophages have the ability to form both podosomes and phagocytic cups (Fig. 1, *A–D*). When macrophages having podosomes are stimulated with IgG-opsonized latex beads, the podosomes immediately disappear, and the phagocytic cups are formed at the site that the IgG beads attach. This observation indicates that the transition of the membrane structures occurs from podosomes to phagocytic cups. Macrophages migrate to sites of inflammation where they phagocytose pathogenic microbes and damaged tissue compounds and mediate local effector functions. Once macrophages encounter those materials at the site of inflammation, they stop migrating and phagocytose those materials. The transition of the macrophage functions occur from migration to phagocytosis. Podosomes and phagocytic cups are the essential membrane structures for migration and phagocytosis, respectively. Thus, the transition of the membrane structures from podosomes to phagocytic cups is essential and significant for the transition of the macrophage functions. Recently, two reports suggest that macrophage migration and phagocytosis include a common molecular mechanism to regulate actin cytoskeleton (40, 41). In this study, we identified a critical common molecular step mediated by FBP17 for the formation of podosomes and phagocytic cups, which are essential for migration and phagocytosis, respectively. In the future, elucidation of the molecular mechanisms underlying the transition would be intriguing.

It has been reported that dynamin-2 is also required for the formation of podosomes in transformed cells and osteoclasts (30–32) and phagocytic cups in a mouse macrophage cell line (33, 34) and that the FBP17-dynamin complex regulates the plasma membrane invagination (35). Our results suggest that FBP17 recruits dynamin-2 to the same site as membrane deformation and that this recruitment is also necessary for the formation of these structures (Figs. 3–5 and supplemental Fig. 6*C*). The formation of podosomes and phagocytic cups involves the process of the membrane protrusion (Fig. 1, *A–D*). The membrane protrusion requires the delivery of new membrane material (2). Our results, taken together with the above observations, suggest that dynamin-2 recruited by FBP17 to the plasma membrane probably plays an essential role in the formation of podosomes and phagocytic cups by regulating the recruitment of vesicles to the plasma membrane as new membrane material in macrophages.

Recently, the EFC domain of FBP17 was shown to bind strongly to the $PI(4,5)P_2$ (18, 22). On the other hand, it has been shown that $PI(4,5)P_2$ localizes at the podosomes in osteoclasts (36) and phagocytic cups (37, 38). These observations suggest that $PI(4,5)P_2$ is synthesized upon stimulation at the plasma membrane and plays an important role in the recruitment of FBP17 to the plasma membrane. Presumably, the $PI(4,5)P_2$ binding activity of the EFC domain is necessary for the localization of FBP17, and therefore, of the WASP-WIP complex and dynamin-2, at the sites where podosomes and phagocytic cups will form.

We suggest that the complex formation of FBP17 with WASP, WIP, and dynamin-2 at the plasma membrane is critical for the formation of podosomes and phagocytic cups (Figs. 1*G* and 3–5). In macrophages from WASP-deficient WAS patients, defects in the complex formation of FBP17 with WASP, WIP, and dynamin-2 impair the formation of podosomes and phagocytic cups (WAS1:. 211delT (27); WAS2: 41–45delG(28) in Fig. 6), thereby reducing chemotaxis and phagocytosis by macrophages, which in turn would decrease the ability of host defense. The severity of WAS-associated symptoms was estimated and expressed as a score of 1–5. A score of 1 was assigned to patients with only thrombocytopenia and small platelets, and a score of 2 was assigned to patients with additional findings of mild, transient eczema or minor infections. Those with treatment-resistant eczema and recurrent infections despite optimal treatment received a score of 3 (mild WAS) or 4 (severe WAS). Regardless of the original score, if any patients then had autoimmune disease or malignancy, the score was changed to 5. The patients, WAS1 and WAS2, receive scores of 5 and 4, respectively. Both patients have the recurrent infections. We suggest that defective formation of podosomes and phagocytic cups in their macrophages (Fig. 6, *A–F*) reduces chemotaxis and phagocytosis, which are the critical processes to protect the body against infection, resulting in the recurrent infections. In addition, defective phagocytosis reduces the clearance of self-antigens such as apoptotic cells. This may cause the autoimmune diseases seen in WAS patients. In fact, Cohen *et al.* (39) recently reported that reduced clearance of apoptotic cells resulted in development of autoimmunity. Our findings therefore provide a potential mechanism for the recurrent infections and autoimmune diseases seen in WAS patients.

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Lack of association between E148Q *MEFV* variant and Kawasaki disease

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ABSTRACT

We investigated a possible association between Kawasaki disease (KD), a systemic vasculitis of unknown etiology, or its coronary artery lesions (CAL) and *MEFV* gene variants including E148Q, the most common and mild mutation in the *MEFV* gene for familial Mediterranean fever or vasculitis-related disorders. The study population comprised a total of 138 Japanese patients with KD, including 45 patients with CAL and 93 patients without CAL and 170 normal controls. Sequence variations for the *MEFV* gene were detected by direct sequencing, followed by the TaqMan SNP genotyping assay. The genotype and allele frequencies of *MEFV* gene variants (E148Q, L110P, R202Q, P369S, R408Q) were compared between KD patients with and without CAL or between KD patients with CAL and controls. E148Q heterozygotes and homozygotes were observed in 37.1 and 5.5% of healthy controls, 33.3 and 5.1% of KD patients, and 37.8 and 4.4% of KD patients with CAL. No significant differences were observed in the genotype and allele frequencies of other *MEFV* gene variants (L110P, R202Q, P369S, R408Q) between KD patients with and without CAL or between KD patients with CAL and controls. No associations were detected between the *MEFV* gene variants and the development of KD or CAL formation in KD.

1. Introduction

Kawasaki disease (KD) is an acute, self-limited systemic vasculitis that occurs predominantly in infants and young children. Coronary artery aneurysm or ectasia develops in <5 to 25% of untreated children with the disease [1,2]. Its etiology remains unknown; however, clinical and epidemiological features strongly indicate that it is caused by one or several widely distributed infectious agents [2]. It is likely that KD results from an abnormal immunologic response to certain microbial agents in genetically susceptible individuals. The higher rate of KD in the siblings of KD patients and the racial difference in its incidence support this consideration [2]. Recently, several host genetic factors have been identified in the development of KD and coronary artery lesions (CAL) [3–6].

Familial Mediterranean fever (FMF) is an inherited inflammatory disease that is common in Arabs, non-Ashkenazi Jews, Armenians, and Turks, whereas it is uncommon in east Asia, including Japan. FMF is characterized by self-limited periodic fever and various symptoms such as peritonitis, arthritis, rash, pleurisy, and pericarditis. The *MEFV* gene is responsible for FMF [7,8]. Among the *MEFV* mutations, the role of E148Q (c.442 G>C) is still controversial. Although some reports indicated that E148Q was only one of the gene polymorphisms, other reports indicated that E148Q was associated with the mildest disease with a low penetrance or usually required another additional *MEFV* mutation to cause the classical manifestation of FMF [9,10]. Although FMF is an uncommon disorder in Japan, the frequency of E148Q is higher in Japanese than in European or Arab populations [11–13]. *MEFV* was predominantly expressed in granulocytes and monocytes [7], both of which play major roles in the pathophysiology of KD at the acute phase [2]. Several reports revealed that *MEFV* mutations were associated with vasculitis-related disorders such as Behçet's disease, Henoch–Schönlein purpura, and polyarteritis nodosa [14–16], suggesting that *MEFV* gene mutations contribute to the development of a broader spectrum of vasculitis. Furthermore, it was reported that *MEFV* mutations might increase the baseline of inflammation, induce the development of rheumatic diseases, and affect the clinical course of inflammatory disorders [17].

To clarify the role of the *MEFV* gene in the development of KD as one of the host genetic factors, we investigated the associations between KD and *MEFV* gene variants, particularly E148Q, which is common in Japanese populations.

2. Subjects and methods

One hundred thirty-eight KD patients who were treated with oral aspirin plus intravenous immunoglobulin (IVIG:1–2 g/kg/total in CAL^- patients and 3–4 g/kg/total in CAL^+ patients) at Kyushu University Hospital or its affiliated hospitals from 1991 through 2003 were enrolled. Informed consent was obtained from their parents, and the Ethical Committees of Kyushu University approved the study. All patients were Japanese and met the appropriate diagnostic criteria for KD [18]. The study population consisted of 92 boys and 46 girls; the median age at diagnosis was 19 months

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ORIGINAL PAPER

Fulminant sepsis/meningitis due to *Haemophilus influenzae* in a protein C-deficient heterozygote treated with activated protein C therapy

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Abstract A 13-month-old Japanese female with *Haemophilus influenzae* type b meningitis presented with unusually severe septic shock and cerebral infarction in half a day of fever. The initial therapy of plasma-derived activated protein C (Anact C®) led to an impressive effect on the aggressive condition. However, purpura fulminans and the consistent decline of plasma protein C activity (<20%) required prolonged activated protein C therapy and gene analysis. The patient carried a novel heterozygous mutation of *PROC* (exon 4; 335 GAC>TAC, Asp46Tyr). This is the first report of infectious purpura fulminans in a protein C-deficient heterozygote. The clinical onset and treatment course adequately corroborated the aggravated immune/hemostatic reactions and the cytoprotective effects of activated protein C replacement in human heterozygous protein C deficiency. The monitoring of plasma protein C activity and sufficient administration of activated protein C product could improve the outcome of severe sepsis in children.

Keywords Purpura fulminans · Sepsis · Heterozygous protein C deficiency · Activated protein C therapy

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Abbreviations

aPC	Activated protein C
APTT	Activated partial thromboplastin time
CSF	Cerebrospinal fluid
CRP	C-reactive protein
CT	Computed tomography
DIC	Disseminated intravascular coagulopathy
FVL	Factor V Leiden
FDP	Fibrinogen and fibrin degradation products
Hib	*Haemophilus influenzae* type b
PC	Protein C
PIC	Plasmin α2–antiplasmin complex
PS	Protein S
PT	Prothrombin time
SIRS	Systemic inflammatory response syndrome
TAT	Thrombin–antithrombin complex

Introduction

Sepsis/systemic inflammatory response syndrome (SIRS) involves the hemostatic system. Excessive activation of procoagulant and anti-fibrinolytic processes, along with endothelial damage, leads to the formation of microthrombi in the microvasculature and disseminated intravascular coagulopathy (DIC). Purpura fulminans is a life-threatening complication in sepsis children, presenting with petechiae, ecchymosis, hemorrhagic bullae, and acral necrosis that may require amputation [2, 3, 8]. It occurs spontaneously in the neonates with homozygous protein C (PC)/protein S (PS) deficiency. Young or middle-aged adults with the heterozygous PC/PS mutation of are at risk of stroke, pulmonary embolism, and deep vein thrombosis. Activated

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ORIGINAL ARTICLE

Cardiovascular Complications Associated with Chronic Active Epstein–Barr Virus Infection

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Abstract This study aimed to assess the outcome of cardiovascular diseases for patients with chronic active Epstein–Barr virus infection (CAEBV). The study enrolled 15 patients (7 boys and 8 girls) who fulfilled the diagnostic criteria for CAEBV, including 10 patients with T-cell type and 3 patients with natural killer (NK)-cell type. The median age at the CAEBV onset was 6.3 years (range, 1.2–17.8 years). Regular cardiologic studies were performed during the median follow-up period of 8 years (range, 2–20 years). Nine patients (60%) had cardiac diseases including coronary artery lesion (CAL) ($n = 4$, 44%), decreased left ventricular ejection fraction and pericardial effusion in ($n = 3$, 33%), complete atrioventricular block ($n = 1$), and sudden arrest ($n = 1$). The frequency of fever (78%, $p = 0.04$) or cytopenias (100%, $p = 0.01$), as the major symptom among patients with cardiac complications, was higher than among those without complications. The median time from disease onset to detection of CAL was 3.4 years (range, 1.8–8.6 years). The mean z-score increased to 3.98. Seven patients (78%) with cardiac complications died of disease progression, hematopoietic stem cell transplantation–related events, or both. In two patients, CAL regressed after allogeneic cord blood transplantation. Among CAEBV patients, CAL was the most common cardiac complication and could not be controlled without the eradication of EBV-infected T- and NK-cells.

Keywords Cardiac complication · Coronary artery lesion · Epstein–Barr virus

Abbreviations

BMT	Bone marrow transplantation
CAL	Coronary artery lesion
CBT	Cord blood transplantation
CAEBV	Chronic active Epstein–Barr virus infection
HSCT	Hematopoietic stem cell transplantation
LVEF	Left ventricular ejection fraction

Introduction

Epstein–Barr virus (EBV) infects human B-cells and persists for the person's lifetime. Humans with primary EBV infection are asymptomatic or experience acute infectious mononucleosis. Chronic active EBV infection (CAEBV) is a rare mononucleosis syndrome characterized by fever, liver dysfunction, hepatosplenomegaly, and the unique presentation of coronary artery lesions (CALs), interstitial pneumonitis, chorioretinitis, sicca, hypersensitivity to mosquito bites, and hydroa vacciniforme [17, 27]. Occurring in previously healthy children with no underlying diseases, CAEBV has been recognized as an EBV-associated T-cell and natural killer (NK)-cell lymphoproliferative disease based on the detection of clonally proliferating EBV-infected T-cells (T-cell type) or NK-cells (NK-cell type).

Neither type of CAEBV is curable without successful allogeneic hematopoietic stem cell transplantation (HSCT) [29]. Cardiac, neurologic, and enteral complications may

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Unique activation status of peripheral blood mononuclear cells at acute phase of Kawasaki disease

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Summary

Although Kawasaki disease (KD) is characterized by a marked activation of the immune system with elevations of serum proinflammatory cytokines and chemokines at acute phase, the major sources for these chemical mediators remain controversial. We analysed the activation status of peripheral blood mononuclear cells (PBMCs) by flow cytometry, DNA microarray and quantitative reverse transcription–polymerase chain reaction. The proportions of $CD69^+$ cells in both natural killer cells and γδT cells at acute-phase KD were significantly higher than those at convalescent-phase KD. Microarray analysis revealed that five genes such as *NAIP*, *IPAF*, *S100A9*, *FCGR1A* and *GCA* up-regulated in acute-phase KD and the pathways involved in acute phase KD were related closely to the innate immune system. The relative expression levels of damage-associated molecular pattern molecule (DAMP) (*S100A9* and *S100A12*) genes in PBMCs at acute-phase KD were significantly higher than those at convalescent-phase KD, while those of *TNFA*, *IL1B* and *IL6* genes were not significantly different between KD patients and healthy controls. Intracellular production of tumour necrosis factor-α, interlaukin-10 and interferon-γ in PBMCs was not observed in KD patients. The present data have indicated that PBMCs showed a unique activation status with high expression of DAMP genes but low expression of proinflammatory cytokine genes, and that the innate immune system appears to play a role in the pathogenesis and pathophysiology of KD.

Keywords: acquired immunity, cytokines, innate immunity, Kawasaki disease, peripheral blood mononuclear cells

Introduction

Kawasaki disease (KD) is an acute febrile illness of childhood with systemic vasculitis characterized by the occurrence of coronary arteritis. Although KD is characterized by a marked activation of the immune system with elevations of serum proinflammatory cytokines and chemokines at acute phase [1–3], no previous studies have demonstrated that peripheral blood mononuclear cells (PBMCs) serve as the major sources for these chemical mediators. Although the activation of monocytes/macrophages has been reported to have an important role at acute phase of KD [4], there were no significant differences in the expression levels of *IL6*, *IL8* and *TNFA* genes in separated monocytes before and after high-dose gammaglobulin therapy [5].

Activation status of PBMCs, especially T cells, at acute phase of KD is also controversial. In a previous report, it has been thought that most activated T cells moved to local tissues from peripheral blood at acute phase and returned from there at convalescent phase [3]. Although numerous immunological studies on T cells have been reported, no previous studies analysed T cells by separating them into two distinct populations, αβT cells and γδT cells, which are involved mainly in acquired and innate immunity, respectively.

To clarify the pathophysiology of KD, we analysed the activation status of PBMCs including αβT cells, γδT cells, natural killer (NK) cells and B cells by flow cytometry, DNA microarray and quantitative reverse transcription–polymerase chain reaction (RT–PCR). These analyses have shown consistently that the innate immune system might be involved in the pathogenesis and pathophysiology of KD, and that PBMCs were not a major source for proinflammatory cytokines such as interleukin (IL)-6 and tumour necrosis factor (TNF) in acute-phase KD sera.

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FULL-LENGTH ORIGINAL RESEARCH

Interleukin-10 is associated with resistance to febrile seizures: Genetic association and experimental animal studies

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SUMMARY

Purpose: Febrile seizures (FS) are the most common form of childhood convulsions. Many reports have shown that a proinflammatory cytokine, interleukin-1 (IL-1) β, may have a facilitatory effect on the development of FS. We have previously shown that the *IL1B* -511C/T single nucleotide polymorphism (SNP) is associated with simple FS of sporadic occurrence. The balance between pro- and antiinflammatory cytokines influences the regulation of infections and could, therefore, play a role in the pathogenesis of FS. Here, to determine whether pro- and antiinflammatory cytokine genes are responsible for the susceptibility to FS, we have performed an association study on functional SNPs of cytokine genes in FS patients and controls.

Methods: The promoter SNPs of four inflammatory cytokine genes (*IL6* -572C/G, *IL8* -251A/T, *IL10* -592A/C and *TNFA* -1037C/T) were examined in 249 patients with FS (186 simple and 63 complex FS) and 225 controls. Because the IL10 -592 SNP showed a positive association with FS, two additional SNPs (*IL10* -1082A/G and -819T/C) were subjected to haplotype analysis. Furthermore, we examined the in vivo role of IL-10 in hyperthermia-induced seizures using immature animal models.

Results: The frequencies of the *IL10* -592C allele and -1082A/-819C/-592C haplotype were significantly decreased in FS as compared with in controls (p = 0.014 and 0.013, respectively). The seizure threshold temperature in the IL-10–administered rats was significantly higher than that in the saline-treated control ones (p = 0.027).

Conclusions: The present study suggests that IL-10 is genetically associated with FS and, contrary to IL-1β, confers resistance to FS.

KEY WORDS: Febrile seizures, Polymorphism, Cytokines, Interleukin-10, Animal model.

Febrile seizures (FS) are the most common form of convulsions, occurring in 2–5% of infants in Europe and North America, and 6–9% in Japan (Tsuboi, 1984; Stafstrom, 2002). The etiology of FS is considered to be multifactorial and heterogenous. The importance of genetic factors in the occurrence of FS has long been recognized, on the basis of family studies, twin studies, and complex segregation analysis (Hauser et al., 1985; Rich et al., 1987; Tsuboi, 1987; Corey et al., 1991). In large families or families of probands with multiple FS, the febrile seizure susceptibility trait is inherited in an autosomal dominant manner with reduced penetrance. In contrast, the inheritance appears to be polygenic or multifactorial in small families, sporadic cases, or families of probands with a single febrile seizure, which account for a large proportion of FS (Rich et al., 1987; Kugler & Johnson, 1998). Linkage analysis, mainly of large families, revealed seven putative febrile seizure loci, chromosomes 8q (Wallace et al., 1996), 19p (Johnson et al., 1998), 2q (Peiffer et al., 1999), 5q (Nakayama et al., 2000), 6q (Nabbout et al., 2002), 18p (Nakayama et al., 2004), and 3p (Nabbout et al., 2007), which are at least partly associated with epilepsy or afebrile seizures. Mutations of the sodium channel (*SCN1A*, 2q24.3; *SCN1B*, 19q13.1) and

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gamma-aminobutyric acid receptor (*GABRG2*, 5q31.1–33.1) genes, which cause fever-related epileptic syndromes (e.g., generalized epilepsy with FS and severe myoclonic epilepsy of infancy), were identified in only a small number of patients with FS (Wallace et al., 1998; Malacarne et al., 2002; Audenaert et al., 2003).

FS are mostly provoked by infections. A proinflammatory cytokine, interleukin (IL)-1β, plays an important role in host-defense responses during infections and acts as an endogenous pyrogen. Therefore, there have been many reports on the possible contribution of IL-1β to the pathogenesis or pathophysiology of FS with respect to the plasma or cerebrospinal fluid level (Tutuncuoglu et al., 2001; Haspolat et al., 2002), IL-1β production by peripheral blood mononuclear cells (Helminen & Vesikari, 1990; Matsuo et al., 2006), genetic polymorphic markers (Virta et al., 2002a), and experimental FS with an immature rodent model (Dube et al., 2005). Our previous case–control study of 229 Japanese FS patients demonstrated that the -511C/T polymorphism of the *IL1B* gene was associated with the development of simple FS of sporadic occurrence (Kira et al., 2005). Consequently, it is possible that IL-1β might have a facilitatory effect on the development of FS.

The balance between proinflammatory [IL-1, tumor necrosis factor (TNF)-α, IL-8, and IL-6] and antiinflammatory (IL-1 receptor antagonist and IL-10) cytokines influences the regulation of infections and could, therefore, play a role in the pathogenesis of FS. However, there are few general views about the contribution of these cytokines to the pathogenesis of FS based on investigations using clinical samples (Straussberg et al., 2001; Virta et al., 2002b).

In the present study, to determine whether pro- and antiinflammatory cytokine genes are responsible for susceptibility to FS, we have performed an association study on functional single-nucleotide polymorphisms (SNPs) of promoter regions of the *IL6* (Ferrari et al., 2003), *IL8* (Hull et al., 2000), *IL10* (Kurreeman et al., 2004), and *TNFA* (Higuchi et al., 1998) genes in patients with FS and controls. Because the haplotype of the *IL10* gene showed a significant and positive association with FS, we further examined the in vivo role of IL-10 in hyperthermia-induced seizure models.

Subjects, Materials, and Methods

Association study

Subjects

The study population consisted of 249 unrelated patients with FS, comprising 186 simple (105 males and 81 females) and 63 complex FS (34 males and 29 females) patients, living in the northern Kyushu area of Japan, and 225 normal 6- to 9-year-old children (114 males and 111 females) in the same area. All 229 patients and 158 controls in our previous study (Kira et al., 2005) were included. The patients visited the Kyushu University Hospital and related hospitals and clinics from January 1999 to October 2000. Most of the precipitating infectious diseases were upper respiratory tract infections and influenza.

Febrile seizure was defined as a convulsion associated with a temperature of higher than 38.0°C not caused by an intracranial infection. We used the following criteria for the simple type of FS (Fukuyama et al., 1979), which are frequently employed in Japan: (1) negative family history of epilepsy, (2) negative past history of any disease with potential to cause brain damage (e.g., birth injury), (3) age at the first FS between 6 months and 6 years of age, (4) duration of convulsions less than 20 min, (5) pattern of convulsions: generalized, bilateral symmetrical, or lacking focal symptoms, (6) no clustering of frequent convulsions within a short period, (7) postictal phase; uneventful and complete recovery without sequelae (e.g., long-lasting disturbance of consciousness, hemiplegia, aphasia, and dementia), and (8) interictally, neither obvious neurologic nor mental defects. FS not in accord with any one of the above eight items were considered as being of the complex type. Family histories were obtained through pediatricians' interviews with the parents. Sixty-eight simple FS patients had a positive family history of FS in near relatives, whereas 118 patients with simple FS were sporadic. Most patients with complex FS exhibited one or more of the following criteria: prolonged FS (n = 22), repetitive FS (n = 24), or a positive family history of epilepsy (n = 8). The ages at the initial seizure and blood sampling for all FS, simple FS, and complex FS are shown in Table 1.

Informed consent was obtained from the patients and/or their parents. The present study was approved by the ethics committee of Kyushu University, Japan (No. 136).

Table 1. Clinical data for patients with all FS, simple FS, and complex FS

	All FS (N = 249)	Simple FS (N = 186)	Complex FS (N = 63)
Sex (M/F)	139/110	105/81	34/29
Age at initial FS (months)			
Median	21	23	17
Range	5–81	6–60	5–81
Age at blood sampling (months)			
Median	33	34	31
Range	6–192	6–146	7–192

DNA extraction

Genomic DNA was extracted from peripheral blood using a MagExtractor (Toyobo, Osaka, Japan).

Selection of SNPs

We selected functional and disease-associated SNPs in the promoter region of the following inflammatory cytokine genes; *IL6* -572C/G (rs1800796) (Ferrari et al., 2003), *IL8* -251A/T (rs4073) (Hull et al., 2000), *IL10* -592A/C (rs1800872) (Kurreeman et al., 2004), and *TNFA* -1037T/C (rs1799724) (Higuchi et al., 1998). For haplotype analysis, we selected two additional SNPs in the promoter region of the *IL10* gene; *IL10* -1082A/G (rs 1800896) and -819T/C (rs 1800871) (Turner et al., 1997).

Genotype analysis of SNPs

Genotype analysis of these SNPs was carried out by the TaqMan method with an ABI PRISM 7700 Sequence Detection System, using TaqMan SNP Genotyping Assays (Applied Biosystems, Foster City, CA, U.S.A.). Polymerase chain reaction (PCR) was carried out with mixes consisting of 8 ng of genomic DNA, 5 μl of Taqman master mix, 0.5 μl of 20× assay mix and double dissolved H_2O up to 10 μl in final volume. The following amplification protocol was used: denaturation at 95°C for 10 min, followed by 40 cycles of denaturation at 92°C for 15 s, and annealing and extension at 60°C for 1 min. After PCR, the genotype of each sample was determined automatically by measuring the allelic-specific fluorescence with the ABI PRISM 7700 Sequence Detection Systems using the SDS 2.2.2 software for allelic discrimination (Applied Biosystems).

Data analysis

The p-values of genotype and haplotype distributions were calculated with a 3 × 2 table and the chi-square test for trend or Fisher's exact test (when the cell number was less than 5). The p-values of allele or haplotype frequencies were calculated by means of chi-square analysis with a 2 × 2 contingency table. The calculation of haplotype frequencies and the chi-square test for linkage disequilibrium among pairs of alleles were performed with use of the Estimating Haplotype-frequencies (EH) program from Rockefeller University (ftp://linkage.rockefeller.edu/software/eh/).

A p-value of <0.05 was considered to be statistically significant except for subgroup comparison, for which a p-value of $<0.05/2$ or $<0.05/3$ was considered to be significant, taking the results of multiple tests into consideration.

Hyperthermia-induced seizures in developing rats

Because we found a significant and positive association between the *IL10* gene and FS, we next examined the in vivo role of IL-10 in hyperthermia-induced seizure models (Fukuda et al., 2007).

Sixteen male Lewis rats (21–24 days of age), soon after weaning, were used. They were kept under a standard schedule of 12 h light–12 h dark, controlled temperature, and free food and water in quiet facilities during all experimental periods. After the head of a rat had been fixed to a stereotaxic holder (Narishige, Tokyo, Japan), two holes were made in the skull over the right frontal and occipital cortex, and silver electrodes for two channel electroencephalography (EEG) were placed in them. Another hole was made over the left central cortex for the thermometer for measuring the brain temperature. These manipulations were performed under anesthesia with pentobarbital sodium (Dainippon Pharma, Osaka, Japan), 30 mg/kg intraperitoneally. In addition, cefotaxime sodium (Aventis Pharma, Tokyo, Japan), 500 mg/kg, was injected to prevent bacterial infections intraperitoneally.

The rats were divided into two groups (n = 8 for each): control and IL-10 groups at 72 h after the surgery. Recombinant human IL-10 (Bender MedSystems, Vienna, Austria) dissolved in 0.9% saline, with a total volume of 20 μl, was given intranasally (500 ng/rat) to each rat in the IL-10 group, whereas only saline with an equal volume to each one in the control group. At 1 h after intranasal administration of saline or recombinant human IL-10, each rat was placed in a special plastic cage and hyperthermic seizures were induced with moist warm air (45°C) with monitoring by EEG. The threshold temperature was defined as the brain temperature measured at the onset of continuous seizure discharges on EEG. The seizure latency was defined as the time from hyperthermia onset until the appearance of continuous seizure discharges on EEG.

Another eight male Lewis rats (24 days of age) were used for measurement of the brain IL-10 concentration. The rats were intranasally given either saline (n = 4) or recombinant human IL-10 (500 ng/rat) dissolved in the saline (n = 4). At 1 h after intranasal administration of IL-10 or the control saline, each rat was anesthetized with diethyl ether (Kanto Chemical, Tokyo, Japan), and its brain was removed quickly. The frontal lobes (anterior to the optic chiasma) were isolated and stored at −80°C until the time of assay. Samples were weighed and homogenized in 50 mM phosphate-buffered saline (PBS) (pH = 7.4). Thereafter, they were centrifuged for 15 min (2,400 × g, 5,000 rpm), and the supernatants were removed and stored. An enzyme-linked immunosorbent assay (ELISA) was performed using IL-10 Human Easy ELISA (Amersham Biosciences, Buckinghamshire, UK) to determine the IL-10 level in the frontal lobe of the brain. The unpaired Student's *t* test was performed for statistical analysis. A p-value of <0.05 was considered to be statistically significant.

All experimental procedures conformed to the guidelines of the Ministry of Education of Japan and were approved by the animal experimental committee of Ehime University School of Medicine (No. TE-17-2).

Results

SNP analysis

The frequencies of each genotype in all FS, simple FS, and complex FS patients and controls are shown in Table 2. The distribution of the genotypes in the control population was in Hardy-Weinberg equilibrium. The *IL10* -819T/C polymorphism was excluded from analysis because it was in absolute linkage disequilibrium with the *IL10* -592A/C polymorphism. The distributions of the *IL10* -592 genotypes in all FS patients ($p = 0.015$) and simple FS patients ($p = 0.012$) were significantly different from that in control subjects. There were no significant differences in the genotype and allele frequencies of the *IL6* -572, *IL8* -251, and *TNFA* -1037 SNPs between controls and all FS, simple FS, and complex FS patients.

Haplotype analysis of the *IL10* gene promoter

Table 3 shows the estimated haplotype frequencies of the *IL10* SNPs. The frequency of the -1082A/-819C/-592C (ACC) haplotype was significantly decreased in all FS ($p = 0.013$) and simple FS patients ($p = 0.013$) versus controls.

Hyperthermia-induced seizures in developing rats

All rats in the experiments exhibited seizures while febrile, and recovered well soon after the seizures. Fig. 1 shows that the threshold temperatures in the IL-10 group (mean ± SEM, 43.2 ± 0.2°C) were significantly higher than those in the control group (42.8 ± 0.3°C) ($p = 0.027$). The seizure latencies were not significantly different between the IL-10 group (mean±SEM, 393 ± 71 s) and the control group (335 ± 62 s). The levels of IL-10 in the frontal lobe of the brain were significantly increased in the IL-10–administered rats (mean±SEM, 147 ± 16.6 pg/ml) compared with in the saline-treated control rats (41.5 ± 15.6 pg/ml) ($p = 0.0002$).

Table 2. Genotype and allele frequencies of *TNFA*, *IL6*, *IL8*, and *IL10* promoter polymorphisms in patients with FS and controls

Genotype/allele	Control subjects (N = 225)	FS			p-value		
		All (N = 249)	Simple (N = 186)	Complex (N = 63)	All versus controls	Simple versus controls	Complex versus controls
TNFA -1037T/C							
TT	138	161	127	34	0.701	0.265	0.387
TC	82	79	53	26			
CC	5	9	6	3			
MAF	0.20	0.20	0.18	0.25	0.710	0.281	0.232
IL6 -572C/G							
CC	140	130	99	31	0.082	0.123	0.059
CG	75	110	79	31			
GG	10	9	8	1			
MAF	0.21	0.26	0.26	0.26	0.096	0.134	0.225
IL8 -251A/T							
AA	104	110	83	27	0.943	0.886	0.905
AT	93	112	81	31			
TT	28	27	22	5			
MAF	0.33	0.33	0.33	0.33	0.942	0.882	0.904
IL10 -592A/C							
AA	89	131	98	33	0.015	0.012	0.275
AC	109	93	72	21			
CC	27	25	16	9			
MAF	0.36	0.29	0.28	0.31	0.014[a]	0.012[b]	0.273

N, number of patients; MAF, minor allele frequency.

p-values for comparison of genotypes were calculated by means of the chi-square test for trend or Fisher's exact test with a 3 × 2 table.

p- values for comparison of MAF were calculated by means of the chi-square test with a 2 × 2 contingency table.

p-values of less than 0.05/2 were considered to be significant for simple and complex FS versus controls.

[a]Odds ratio (OR) 0.71, 95% confidence interval (CI) 0.54–0.93.

[b]OR 0.68, 95% CI 0.51–0.92.

Table 3. Haplotype analysis of the *IL10* promoter region

Haplotype for assigned -1082, -819, -592	Control subjects (N = 225)	FS All (N = 249)	FS Simple (N = 186)	FS Complex (N = 63)	p-value All versus controls	p-value Simple versus controls	p-value Complex versus controls
A-C-C/A-C-C	22	19	14	5	0.016	0.016	0.239
A-C-C/–	95	80	58	22			
–/–	108	150	114	36			
A-C-C frequency	0.31	0.24	0.23	0.25	0.013[a]	0.013[b]	0.233

N, number of patients.
p-values for comparison of diplotypes were calculated by means of a 3 × 2 contingency table and the chi-square test for trend.
p-values for comparison of ACC frequency were calculated by means of the chi-square test with a 2 × 2 contingency table.
p-values 0.05/2 were considered to be significant for simple and complex FS versus controls.
[a]Odds ratio (OR) 0.69, 95% confidence interval (CI) 0.52–0.93.
[b]OR 0.67, 95% CI 0.49–0.92.

Discussion

In the present study, we first demonstrated an association between the *IL10* gene and resistance to FS. The current data suggest that the *IL10* -592C allele and -1082A/-819C/-592C (ACC) haplotype, which have been reported to be associated with increased production of IL-10 (Kurreeman et al., 2004), may confer resistance to FS. In addition, our hyperthermia-induced seizure models showed that administration of IL-10 increased the seizure threshold temperature. Taken together, these results imply that IL-10 is genetically associated with FS and acts as an attenuating factor for them.

IL-10 is a multifunctional antiinflammatory cytokine produced by monocytes, macrophages, B cells, T cells, and microglias (Williams et al., 1996), and inhibits the production of proinflammatory cytokines including TNF-α, IL-1, IL-6, and IL-8 (Moore et al., 2001). Two studies have provided conflicting results as to the relationship between FS and IL-10. Straussberg et al. (2001) have shown increased lipopolysaccharide-induced IL-10 production by peripheral blood mononuclear cells from children with a history of FS. Virta et al. (2002b) have reported that no difference in plasma IL-10 level was found between children with and without FS. Therefore, little is known about the involvement of IL-10 in the pathogenesis of FS.

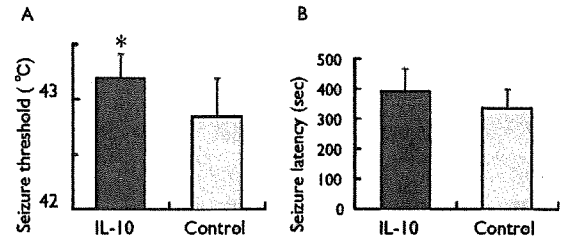

Figure 1.
Intranasal administration of 500-ng interleukin-10 (IL-10) increased the threshold for hyperthermia-induced seizures. (**A**) Threshold temperature for the onset of these seizures was significantly higher in rats with IL-10 administrated: 43.2 ± 0.3°C (mean ± SEM) versus 42.8 ± 0.4°C in control rats given saline. (**B**) Administration of IL-10 did not affect seizure latencies. *$p < 0.05$.
Epilepsia © ILAE

A rapid increase in fever and/or high body temperature during an infectious disease can trigger seizures in individuals who are prone to FS (Aicardi, 1994; Berg et al., 1995). These clinical observations lead to the following speculations as to the effect of IL-10 on FS. First, since the antipyretic role of IL-10 has been indicated in humans and mice (Pajkrt et al., 1997; Leon et al., 1999), IL-10 may suppress the fever response during acute infections, resulting in inhibition of temperature elevation and subsequent decreased susceptibility to FS. Therefore, overproduction of IL-10 may be related to genetic resistance factors reacting with environmental agents. Second, considering the results of our animal experiment under high-temperature conditions, it is possible that IL-10 may decrease the sensitivity to seizures, leading to reduced neuronal hyperexcitability and an increased seizure threshold. In fact, Levin and Godukhin (2007) reported the protective effect of IL-10 on the development of epileptiform activity induced by brief hypoxic episodes in the CA1 area of rat hippocampal slices.

Hyperthermia-induced seizure models are widely used to study the pathophysiology of FS (Baram, 2002). Recently, Dube et al. (2005) demonstrated that IL-1β reduced the seizure threshold in such models. Mice deficient in the IL-1 receptor showed a significantly increased threshold temperature for the generation of experimental FS as compared with wild-type mice. In addition,

exogenous IL-1β reduced the seizure threshold in receptor-expressing but not receptor-deficient mice. We also confirmed that exogenous IL-1β, as opposed to IL-10, decreased the seizure threshold temperature by using the same seizure models (Fukuda and Ishizaki, unpublished data), although the hyperthermic seizure paradigm in the present experiment was different from that used by Dube et al. (Morimoto et al., 1991; Fukuda et al., 1997; Dube et al., 2000, 2005).

Simple FS are clinically defined as brief generalized seizures that occur only once during a 24-h period in febrile children who do not have intracranial infections or severe metabolic disturbances (Baumann, 1999). Patients with simple FS show a lower risk of subsequent development of epilepsy than those with complex FS (prolonged, focal, or recurrent in 24 h) (Nelson & Ellenberg, 1978). In our subgroup analysis, we found that the *IL10* promoter genotype/haplotype was significantly related to simple FS but not complex FS, as in the case of the *IL1B* promoter genotype (Kira et al., 2005). Simple FS may be more homogenous not only in their clinical manifestations but also in the etiology compared with complex FS, thereby showing a positive association with these genotypes. On the other hand, it is suggested that IL-1β contributes to long-lasting hyperexcitability and excitotoxity underlying prolonged and repetitive FS, which have been closely linked to the development of hippocampal epilepsy (Dube et al., 2000, 2005). Because IL-10 has a neuroprotective effect against glutamate-induced or hypoxic–ischemic neuronal cell death (Grilli et al., 2000; Mesples et al., 2003), further investigation is needed to determine whether IL-10 could play a role in the pathophysiology of complex FS, as represented by prolonged and repetitive FS.

Genetic association studies offer a powerful approach to identify the multiple variants of small effects that modulate susceptibility to common, complex disease. On the other hand, they have several methodologic limitations, including small sample sizes, inappropriate selection of controls, population stratification, and inappropriate stringent significance threshold (Tan et al., 2004). In the present genetic study, potential bias caused by population stratification can be minimized by sampling and matching cases and controls from the same source population and geographic region. However, we cannot exclude the possibility of false-positive results caused by the limitation of statistical power, since the association detected between *IL10* gene and FS is only weakly significant ($p = 0.014$ for the C allele and 0.013 for the ACC haplotype). Studies with thousands of samples are needed to validate this finding (Tan et al., 2004).

In conclusion, the -592C allele and ACC haplotype in the promoter region of the *IL10* gene are significantly associated with resistance to FS. In experimental hyperthermic seizures in immature rodent models, IL-10 plays an anticonvulsant role. It is possible that IL-1β contributes to the susceptibility to FS, whereas IL-10 confers resistance to FS.

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We confirm that we have read the Journal's position on issues involved in ethical publication and affirm that this report is consistent with those guidelines.

Conflict of interest: There is no conflict of interest in the work reported in the present manuscript.

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Association of toll-like receptor 3 gene polymorphism with subacute sclerosing panencephalitis

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Innate immunity plays an important role in measles virus (MV) infection. MV-derived double-stranded RNA is recognized by toll-like receptor 3 (TLR3), retinoic acid–inducible protein I (RIG-I) and melanoma differentiation–associated gene 5 (MDA5). We investigated whether genes encoding these molecules contributed to the development of subacute sclerosing panencephalitis (SSPE) in Japanese individuals. Four single nucleotide polymorphisms (SNPs) of the three genes (*TLR3* rs3775291:Leu412Phe, *RIG1* rs277729 and rs9695310, and *MDA5* rs4664463) were assessed in 40 SSPE patients and 84 controls. Because the *TLR3* SNP showed a positive association with SSPE, three additional SNPs were subjected to haplotype analysis. The frequency of 412Phe allele of *TLR3* rs3775291 in SSPE patients was significantly higher than that in controls ($P = .03$). In haplotype analysis of four SNPs in the *TLR3* gene, the frequency of −7C/IVS3+71C/Phe412/c.1377C haplotype was significantly increased in SSPE patients ($P = .006$, odds ration [OR]: 2.2). *TLR3* gene may confer host genetic susceptibility to SSPE in Japanese individuals. - *Journal of NeuroVirology* (2008) **14**, 486–491.

Keywords: measles; polymorphism; TLR

Introduction

Subacute sclerosing panencephalitis (SSPE) is a fatal degenerative neuronal disease caused by persistent infection with a measles virus (MV), the nature of which is quite different from that of common wild types. The viruses isolated from SSPE brains, called SSPE viruses, are not able to form infective particles owing to extensive mutations in their envelope-associated proteins, especially the M protein. Sequence analysis, however, has revealed that most of the mutations considered to be common and specific for SSPE viruses are also found in recently isolated wild-type strains (Jin *et al*, 2002; Kai *et al*, 1996)

In addition to viral factors, host factors seem to contribute to the development of SSPE (Gascon, 1996; Schneider-Schaulies *et al*, 1999). Epidemiologic studies have shown that contraction of measles before 2 years of age increases the risk of SSPE, suggesting that immaturity of the host immune system and central nervous system (CNS) plays an important role in host susceptibility factors for the development of SSPE. Our previous study demonstrated that the combination of TT genotype of

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