Dedicator of cytokinesis 8 regulates signal transducer and activator of transcription 3 activation and promotes T_H17 cell differentiation

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Background: The autosomal recessive hyper-IgE syndrome (HIES) caused by dedicator of cytokinesis 8 (DOCK8) deficiency shares clinical features with autosomal dominant HIES because of signal transducer and activator of transcription 3 *(STAT3)* mutations, including recurrent infections and mucocutaneous candidiasis, which are suggestive of T_H17 cell dysfunction. The mechanisms underlying this phenotypic overlap are unclear.
Objective: We sought to elucidate common mechanisms operating in the different forms of HIES.
Methods: We analyzed the differentiation of $CD4^+$ T_H cell subsets in control and DOCK8-deficient subjects. We also examined the role of DOCK8 in regulating STAT3 activation in T cells. T_H cell differentiation was analyzed by ELISA, flow cytometry, and real-time PCR measurements of cytokines and T_H cell transcription factors. The interaction of DOCK8 and STAT3 signaling pathways was examined by using flow cytometry, immunofluorescence, coimmunoprecipitation, and gene expression analysis.
Results: There was a profound block in the differentiation of DOCK8-deficient naive $CD4^+$ T cells into T_H17 cells. A missense mutation that disrupts DOCK8 guanine nucleotide exchange factor (GEF) activity while sparing protein expression also impaired T_H17 cell differentiation. DOCK8 constitutively associated with STAT3 independent of GEF activity, whereas it regulated STAT3 phosphorylation in a GEF activity–dependent manner. DOCK8 also promoted STAT3 translocation to the nucleus and induction of STAT3-dependent gene expression.
Conclusion: DOCK8 interacts with STAT3 and regulates its activation and the outcome of STAT3-dependent T_H17 differentiation. These findings might explain the phenotypic overlap between DOCK8 deficiency and autosomal dominant HIES. (J Allergy Clin Immunol 2016;138:1384-94.)

Key words: *Cell division cycle 42, dedicator of cytokinesis 8, guanine nucleotide exchange factor, hyper-IgE syndrome, mucocutaneous candidiasis, suppressor of cytokine signaling 3, signal transducer and activator of transcription 3, T_H17*

The autosomal recessive (AR) form of hyper-IgE syndrome (HIES) is a combined primary immunodeficiency disease characterized by susceptibility to viral and bacterial infections, mucocutaneous candidiasis, atopic dermatitis and food allergy, virus-induced malignancies, and autoimmunity.[1-4] Most subjects with this disorder have loss-of-function lesions in the gene encoding dedicator of cytokinesis 8 *(DOCK8)*, a guanine nucleotide exchange factor (GEF) member of the DOCK180 superfamily of GEFs.[5] DOCK8 coordinates the actin cytoskeleton response to mitogenic and chemokine signals through the reversible activation of small G proteins, most notably cell division cycle 42 (Cdc42).[6-9] Patients with DOCK8 deficiency carry deletions, splice junction mutations, premature stop codons, and, very rarely, missense mutations in *DOCK8*, leading to absent or trace amounts of expressed DOCK8 protein.[2,3,10-16] Somatic reversions of some mutations can lead to partial expression of DOCK8 protein in some cell lineages but not others.[13]

Several features of DOCK8 deficiency overlap with those of the autosomal dominant (AD) form of HIES caused by loss-of-function mutations in the gene encoding signal transducer and activator of transcription 3 *(STAT3)*.[1,17-20] Subjects with both disorders exhibit high serum IgE levels, eczema, recurrent staphylococcal skin abscesses, frequent upper and lower respiratory tract infections, candidiasis, and hypereosinophilia. In particular, patients with AD-HIES manifest a profound block in T_H17 differentiation, reflecting the requirement for STAT3 in this process.[21-24] Those with AR-HIES were also shown to exhibit defective T_H17 differentiation.[18] However, the subjects studied were very few in number and included both DOCK8-deficient and sufficient subjects, making it difficult to definitively attribute

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Abbreviations used
AD: Autosomal dominant
AR: Autosomal recessive
Cdc42: Cell division cycle 42
ChIP: Chromatin immunoprecipitation
DOCK8: Dedicator of cytokinesis 8
GAPDH: Glyceraldehyde-3-phosphate dehydrogenase
GEF: Guanine nucleotide exchange factor
HIES: Hyper-IgE syndrome
IL-6R: IL-6 receptor
Na_3VO_4: Sodium orthovanadate
pSTAT3: Phospho-STAT3
Rac1: Ras-related C3 botulinum toxin substrate 1
RORγt: Retinoic acid–related orphan receptor γt
SOCS: Suppressor of cytokine signaling
STAT: Signal transducer and activator of transcription
WT: Wild-type

T_H17 deficiency to the absence of DOCK8. Furthermore, the status of STAT3 activation in DOCK8 deficiency remains poorly defined. In this study we have sought to specifically examine the effect of DOCK8 on STAT3 activation and STAT3-dependent T_H17 cell differentiation. Our studies identify a novel function for DOCK8 in amplifying STAT3 activation in a GEF-dependent manner.

METHODS

Subjects

A child with functional DOCK8 deficiency (patient 1; see Table E1 in this article's Online Repository at www.jacionline.org) originally presented at 4 years of age with a history of recurrent upper and lower respiratory tract infections, mucocutaneous candidiasis, recurrent oral herpes infection, and hepatomegaly. She had increased serum IgE and decreased IgM concentrations, peripheral blood eosinophilia, and CD4 T-cell lymphopenia (see Table E1). Her liver enzymes levels were mildly increased (70-100 U/L), but her liver biopsy specimen was diagnostic of cirrhosis. Sanger sequencing analysis of *DOCK8* revealed a missense mutation in exon 45 (c.5956 A>T), resulting in a N1986Y substitution in DOCK8 (see the Results section). She was given a diagnosis of functional DOCK8 deficiency and started on antibacterial and antifungal prophylaxis and monthly intravenous immunoglobulin infusions. On follow-up, her liver and spleen were found to be enlarged after portal hypertension, and endoscopic evaluation revealed portal hypertensive gastropathy and esophageal varicoses. She successfully underwent bone morrow transplantation at the age of 7 years, with full engraftment. However, she passed away 4 months after her transplantation of decompensated liver disease and encephalopathy triggered by infection.

Sixteen other patients with DOCK8 deficiency (1-9 years old, 6 male and 10 female subjects, followed up over 6 months to 6 years) were included in the study. Diagnosis was established based on clinical history, corroborated by flow cytometric analysis of DOCK8 expression in lymphocytes, and confirmed by mutational analysis of *DOCK8* (see Table E1). Patient recruitment and the studies reported herein were approved by the Institutional Review Board at Boston Children's Hospital and by the local ethics committees at the institutions of the respective referring physicians. Written informed consent was obtained from participating families. Healthy parents of patients or healthy control subjects were included as control subjects.

Protein modeling

The N1986Y mutation was generated from the crystal structure of DOCK8[7] seen in Coot[25] by using the same side chain torsion angle in residue N1986. The molecular representation was displayed in Pymol.[26]

STAT3 phosphoflow assay

Cytokine (IL-6 or IL-21)–induced STAT3 phosphorylation was evaluated by using flow cytometry.[18] Briefly, T-cell blasts were expanded from PBMCs by treatment with CD2/CD3/CD28 mAbs for 5 days in the presence of IL-2 (100 ng/mL). T-cell blasts were treated with different concentrations of the indicated cytokines and time intervals. The cells were then fixed in 4% paraformaldehyde; permeabilized by treatment with 90% methanol; stained with anti-CD3, anti-CD4 (BioLegend, San Diego, Calif), and anti–phospho-STAT3 (pSTAT3) (Y705) mAbs (BD Biosciences, San Jose, Calif); and evaluated for STAT3 phosphorylation by using the FACS FORTESSA cell analyzer (BD Biosciences).[18]

pSTAT3 imaging

Subcellular localization of pSTAT3 was determined by using confocal microscopy, as previously described.[14] Briefly, T-cell blasts were spun down over cover slips coated with poly-D-lysine (50 μg/mL; Sigma, St Louis, Mo) and either sham coated (PBS) or coated with anti-CD3 mAb (2 μg/mL in PBS overnight) and incubated for 30 minutes at 37°C in the absence or presence of added IL-6 (20 ng/mL). The cover slips were then washed with PBS, and the cells were fixed with 4% paraformaldehyde, permeabilized with 0.2% saponin, and blocked with 4% BSA. They were stained with anti-CD4 mAb, anti-pSTAT3 mAb, and Prolong Gold Antifade Reagent with 4′-6-diamidino-2-phenylindole dihydrochloride (DAPI; Invitrogen, Carlsbad, Calif) and evaluated by confocal microscopy.[14]

Ras-related C3 botulinum toxin substrate 1 and cdc42 pulldown assays

Ras-related C3 botulinum toxin substrate 1 (Rac1) and cdc42 pulldown assays were carried out with Pierce Thermo Scientific pulldown assay kits (Pierce, Rockford, Ill). Briefly, T cells from control subjects and *DOCK8*^{N1986Y} and *DOCK8*^{null} patients were either left untreated or treated with 1 μg/mL anti-CD3 mAb for 30 minutes. Cell lysates were derived and precipitated with a chimeric protein composed of glutathione-S-transferase fused with the GTPase-binding domain of p21-activated kinase (GST1-PAK1). The precipitates and aliquots of the total cell extracts were subjected to immunoblot analysis by using anti-Rac1 or anti-Cdc42 mAbs.[14,27]

Transient transfection assays

cDNA encoding Flag-tagged human DOCK8 isoform 1 and hemagglutinin-tagged human *STAT3* isoform 1 were obtained from OriGene (Rockville, Md) and GeneCopoeia (Rockville, Md), respectively. Mutagenesis of the respective cDNA clone was carried out with QuikChange II and Q5 site-directed mutagenesis kits (Agilent Technologies, Santa Clara, Calif, and New England Biolabs, Ipswich, Mass, respectively). Plasmids were transfected into Jurkat or HEK293 cells by electroporation. At 36 hours after transfection, the cells were lysed with buffer containing 0.75% NP-40. Immunoprecipitation and immunoblotting was carried out, as described in the Results section.

Autoantibody array

Plasma aliquots from patients and control subjects were analyzed by using microarrays spotted with 84 autoantigens (University of Texas Southwestern Medical Center, Genomic and Microarray Core Facility), as previously described.[28] Data were normalized to healthy control subjects. A value of 1 (white) is equal to the control average + 1 SD. A value of greater than 1 (red) or less than 1 (blue) is more or less than 1 SD greater than or less than the healthy control mean, respectively.

Immunoprecipitation

Cellular lysates were derived and precleared by incubation with Protein G Dynabeads (Life Technologies, Grand Island, NY), followed by immunoprecipitation with Protein G Dynabeads and the indicated antibodies. MOPC21 mAb was used as an IgG isotype control in immunoprecipitation studies. Immunoprecipitates were resolved by SDS-PAGE, transferred to

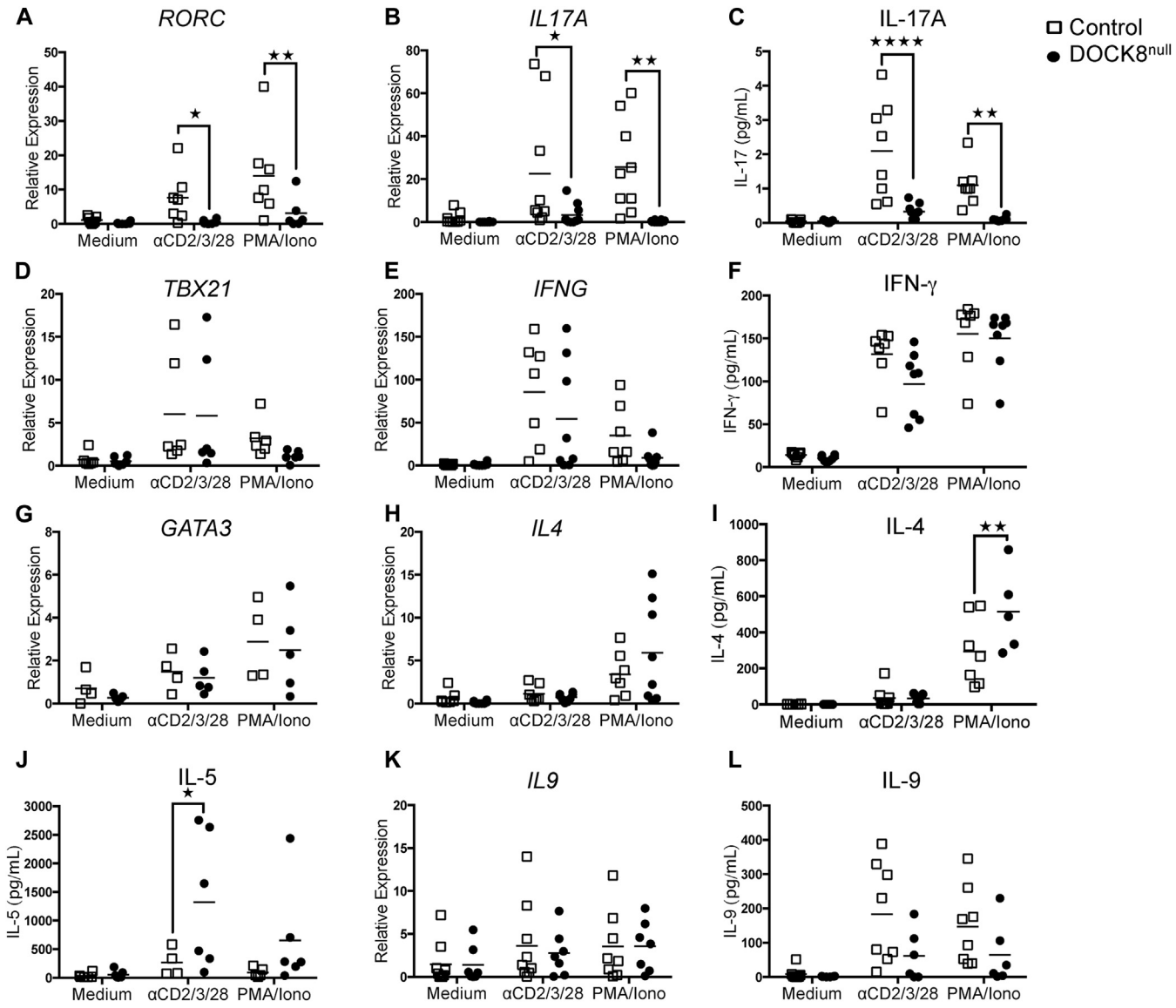

FIG 1. DOCK8 deficiency impairs T_H17 cell differentiation. **A-C,** Expression of *RORC* and *IL17A* transcripts (Fig 1, *A* and *B*) and IL-17A (Fig 1, *C*) by naive $CD4^+$ T cells from control and DOCK8-deficient subjects differentiated under polarizing T_H17 cell conditions and then were either left unstimulated *(Medium)* or stimulated with anti(α)-CD2/CD3/C28 mAbs or phorbol 12-myristate 13-acetate plus ionomycin *(Io)*. **D-F,** Expression of *TBX21* and *IFNG* transcripts (Fig 1, *D* and *E*) and IFN-γ (Fig 1, *F*) in naive T cells differentiated under T_H1 cell–polarizing conditions and stimulated as in Fig 1, *A-C*. **G-L,** Expression of *GATA3* and *IL4* transcripts (Fig 1, *G* and *H*), IL-4 and IL-5 (Fig 1, *I* and *J*), *IL9* transcripts (Fig 1, *K*), and IL-9 (Fig 1, *L*) in naive $CD4^+$ T cells differentiated under T_H2 cell–polarizing conditions and stimulated as in Fig 1, *A-C*. Results are representative of at least 2 independent experiments. $*P < .05$, $**P < .01$, and $****P < .0001$, 1-way ANOVA and posttest analysis.

polyvinylidene difluoride membranes, and immunoblotted with the indicated antibodies, as previously described.[3]

Statistical analysis

Significance was calculated by using both parametric and nonparametric methods (1- and 2-way ANOVA, Student *t* test, and Mann-Whitney *U* test). A *P* value of less than .05 was considered significant.

Other methods

Information on additional real-time PCR analysis, flow cytometry and intracellular staining reagents, antibodies, ELISA, T_H cell differentiation, immunoblotting, and chromatin immunoprecipitation (ChIP) assays is provided in the Methods section in this article's Online Repository at www.jacionline.org.

RESULTS

DOCK8 deficiency profoundly impairs T_H17 cell differentiation

Our previous studies demonstrated defective differentiation of AR-HIES T cells into T_H17 cells, but the genotype of those patients was undetermined.[18] To determine the effect of DOCK8 deficiency on T_H17 cell differentiation, we examined the capacity

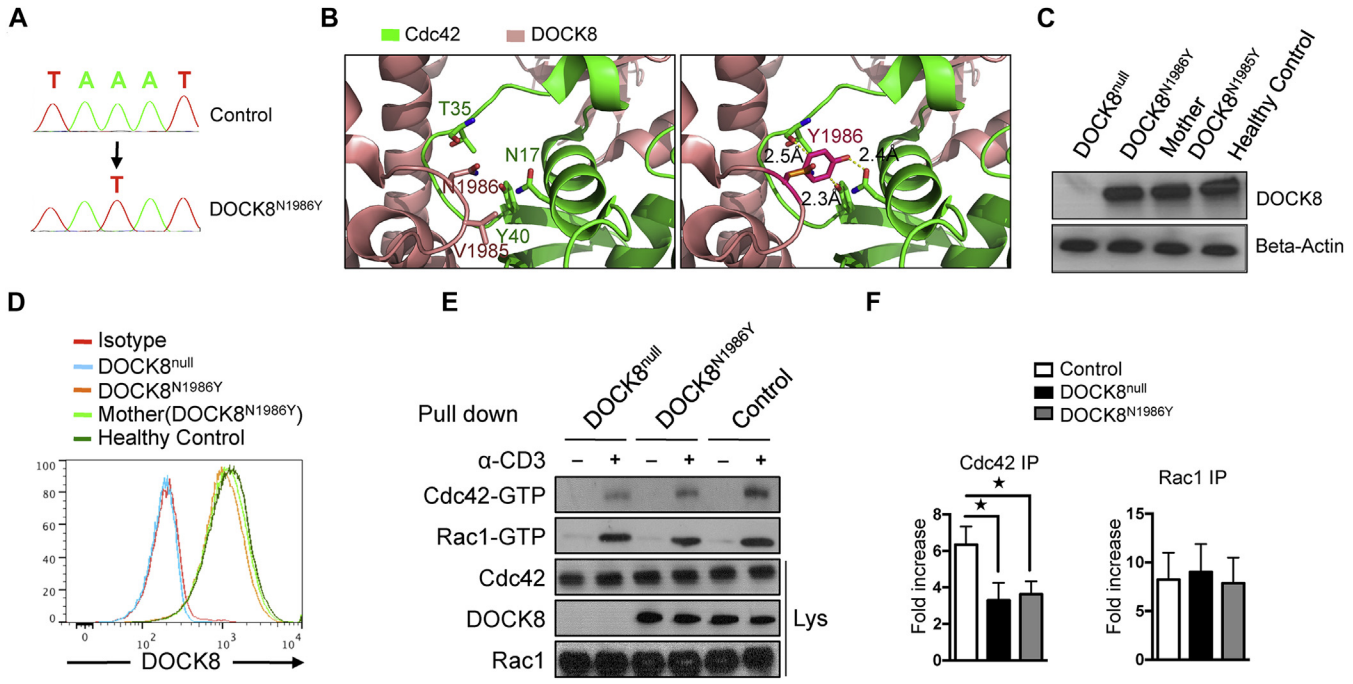

FIG 2. DOCK8^{N1986Y} mutation disrupts GEF catalytic activity and impairs T_H17 cell response. **A,** Sanger sequencing fluorograms showing genomic DNA mutation in patient P1 compared with a healthy control subject *(Ctrl)*. **B,** Ribbon diagram, produced by using PyMOL, of the DOCK8 DHR2-Cdc42 interface, showing the effects of N1986Y substitution. **C** and **D,** Immunoblotting (Fig 2, *C*) and flow cytometric analysis (Fig 2, *D*) of DOCK8 expression in T-cell blasts of a DOCK8-deficient subject (DOCK^{null}), a patient with DOCK8^{N1986Y}, her mother, and a healthy control subject. **E,** GTP-bound Cdc42 and Rac1 pulldown assay carried out on control, DOCK8^{null}, and DOCK8^{N1986Y} T cells at baseline and after activation with anti-CD3 mAbs. **F** and **G,** Densitometric analysis of Rac1 and Cdc42 precipitates (n = 3 per group). *$P < .05$, 1-way ANOVA and posttest analysis.

of naive CD4$^+$ T cells isolated from patients with complete DOCK8 deficiency (DOCK8^{null}) and control subjects to differentiate *in vitro* into T_H17 cells. DOCK8-sufficient naive CD4$^+$ T cells effectively differentiated into T_H17 cells when stimulated with anti-CD2/CD3/CD28 mAbs in the presence of pro-T_H17–polarizing cytokines. In contrast, DOCK8^{null} naive CD4$^+$ T cells did not differentiate into T_H17 cells, as revealed by profoundly decreased retinoic acid–related orphan receptor γt *(RORC)* transcripts encoding the T_H17 lineage–specifying transcription factor retinoic acid–related orphan receptor γt (RORγt), as well as *IL17* transcripts and IL-17 protein in T cells from patients compared with control subjects (Fig 1, *A-C*).[18,29] Ineffective T_H17 cell differentiation was not corrected by using a combination of phorbol 12-myristate 13-acetate and ionomycin to bypass surface receptor activation, indicating that the effect of DOCK8 on T_H17 differentiation extended downstream of T-cell receptor stimulation.

Naive CD4$^+$ T cells of patients and control subjects were analyzed for T_H1, T_H2, and T_H9 differentiation in the presence of T-cell mitogens and the respective polarizing cytokines to determine whether the defect in T_H17 differentiation reflected a generalized abnormality in T_H cell differentiation. Results revealed that T-box–specific transcription factor *(TBX21)* transcripts, which encode the master T_H1 cell differentiation factor T-bet; *IFNG* transcripts; and IFN-γ protein were similarly expressed in T_H1-differentiated T cells from DOCK8-deficient patients compared with those from control subjects (Fig 1, *D-F*). Although expression of transcripts encoding the master T_H2 cytokine GATA3 was similar in T_H2-differentiated T cells from patients and control subjects, there was a trend toward increased *IL4* transcripts, as well as increased IL-4 and IL-5 expression, by DOCK8-deficient T_H2-differentiated T cells. In contrast, T_H9 differentiation was similar in DOCK8-deficient and sufficient T cells (Fig 1, *G-L*).

A missense mutation in the DOCK8 GEF catalytic center recapitulates the phenotype of DOCK8 deficiency and T_H17 differentiation defect

As indicated above, the overwhelming majority of patients with DOCK8 deficiency carry deletions, splice junction mutations or nonsense mutations in *DOCK8* that severely compromise or abolish protein expression.[11] Uniquely, 1 (patient 1) of 61 patients who we have identified with verified *DOCK8* mutations carried a missense mutation (c.5956 A>T), resulting in an N1986Y amino acid substitution in the GEF catalytic loop immediately next to the invariant V1985 nucleotide sensor (Fig 2, *A*).[30,31] The mutant tyrosine residue was determined to be deleterious by using POLYPHEN2 (score, 1.0) and SIFT (score, 0.05) analyses.[32,33] The human N1986Y mutation was modeled onto the mouse DOCK8 structure in complex with Cdc42.[7] The Y1986 side chain was predicted by protein modeling onto the mouse DOCK8 structure in complex with Cdc42 to create steric clash with Cdc42 at several residues, including residue T35 at the switch 1 region, N17, and Y40 (Fig 2, *B*).[7] Expression of the mutant protein was preserved, as revealed by immunoblotting and flow cytometric analysis (Fig 2, *C* and *D*).[34] However, Cdc42 activation was defective in DOCK8^{N1986Y}-expressing T cells, whereas Rac1 activation was normal (Fig 2, *E* and *F*). The N1986Y mutation recapitulated the clinical and immunologic features of DOCK8 deficiency,

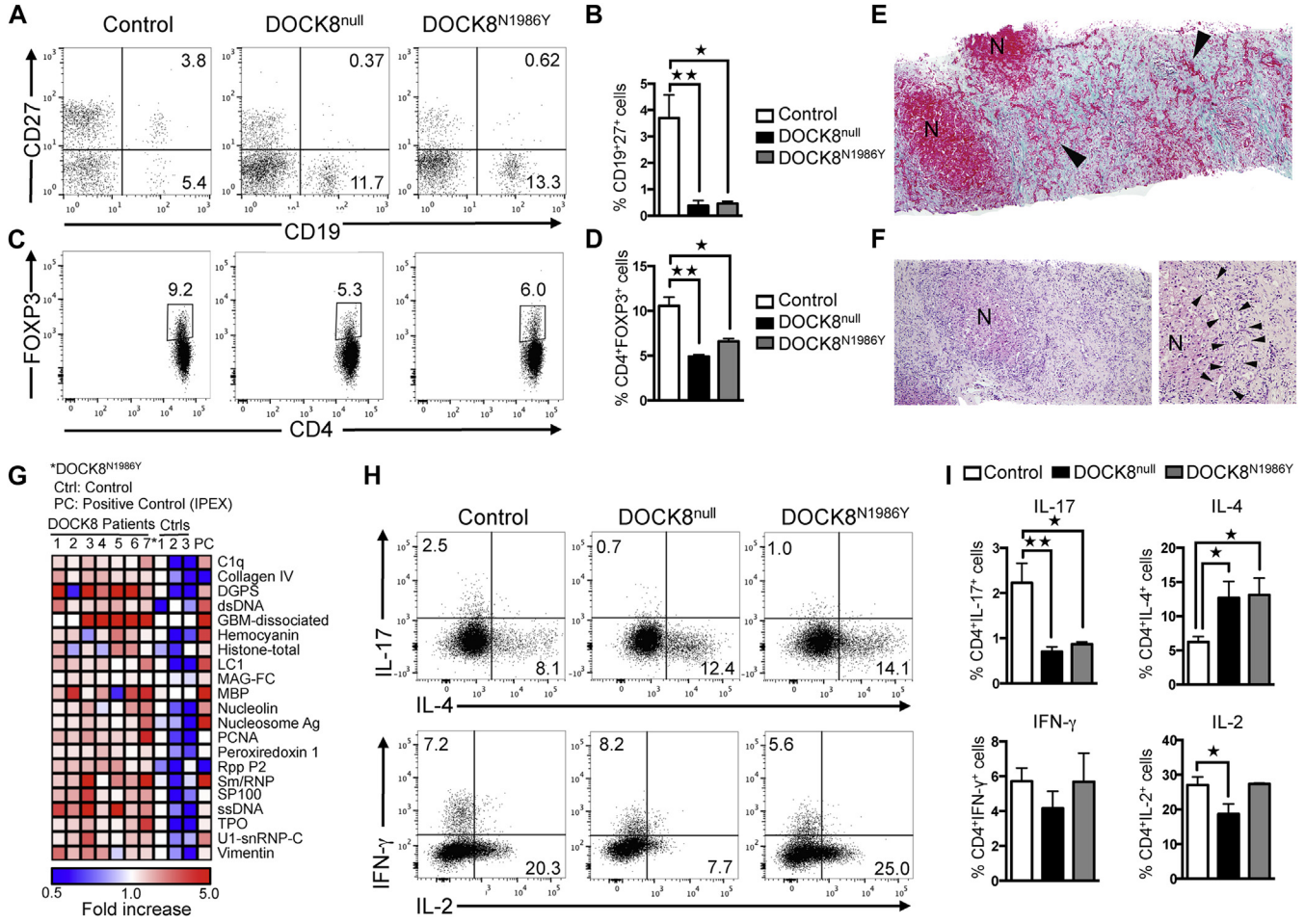

FIG 3. DOCK8^{N1986Y} mutation recapitulates the phenotype of DOCK8 deficiency and impairs T_H17 cell responses. **A** and **B,** Flow cytometric analysis of CD19 and CD27 expression (Fig 3, *A*) and percentage of CD27$^+$CD19$^+$ memory B cells (Fig 3, *B*) in PBMCs of control subjects, DOCK8^{null} subjects, and the DOCK8^{N1986Y} patient. **C** and **D,** Flow cytometric analysis (Fig 3, *C*) and frequencies (Fig 3, *D*) of CD4$^+$ forkhead box P3 (Foxp3)$^+$ cells in PBMCs of control and DOCK8^{null} subjects and those of the DOCK8^{N1986Y} patient (2 separate observations) gated on CD4$^+$ cells. **E,** Liver histology of the DOCK8^{N1986Y} patient, showing bile duct proliferation *(arrowheads)* and nodular regeneration *(N)*; collagen is stained *green* with trichrome stain. **F,** Left regenerative nodule *(N)* with irregular outlines caused by cholangiolar damage and hypertrophy hematoxylin and eosin stain. *Right,* Cholangioles along the limiting plate (between *arrowheads*) show inflammation, with scattered lymphocytes and neutrophils (hematoxylin and eosin). **G,** Heat map display of autoantibody reactivity against self-proteins in 6 DOCK8-deficient patients (1-6), the DOCK8^{N1986Y} patient (7*), control subjects *(Ctrls)*, and a positive control subject (*PC,* patient with forkhead box P3 deficiency). **H** and **I,** Flow cytometric analysis (Fig 3, *H*) and frequencies (Fig 3, *I*) of IL-17, IL-4, IFN-γ, and IL-2 expression in peripheral blood CD4$^+$ T cells of control subjects, DOCK8^{null} subjects, and the DOCK8^{N1986Y} patient stimulated *ex vivo* with phorbol 12-myristate 13-acetate plus ionomycin and then stained for respective cytokines. Results are representative of at least 2 to 3 independent experiments. *P < .05 and **P < .01, 1-way ANOVA and posttest analysis.

including eczema, persistent viral infections and recurrent sinopulmonary infections, failure of memory B-cell differentiation, and regulatory T-cell deficiency (Fig 3, *A-D*).[12,14] The patient also had a well-established biliary-type cirrhosis. The pathology was consistent with advanced primary sclerosing cholangitis with no identified microsporidial or cryptosporidial organisms, which is similar to what has been previously described for some DOCK8-deficient patients and suggestive of an autoimmune process (Fig 3, *E* and *F*).[4] Consistent with autoimmunity, the patient demonstrated evidence of active autoantibody production, as previously described for other patients with DOCK8 deficiency (Fig 3, *G*).[12] Importantly, flow cytometric analysis revealed decreased IL-17$^+$ T-cell numbers in PBMCs of patient compared with control subjects, indicating a requirement for DOCK8 GEF activity in T_H17 cell differentiation (Fig 3, *H* and *I*).

DOCK8 deficiency results in defective STAT3 phosphorylation and nuclear translocation

T_H17 cell differentiation is orchestrated by STAT3-activating cytokines, including IL-6 and IL-21, acting in synergy with T-cell receptor/CD3 signals and TGF-β.[35,36] Both antigen and cytokine receptor signaling are associated with STAT3 phosphorylation, and the 2 pathways synergistically induce

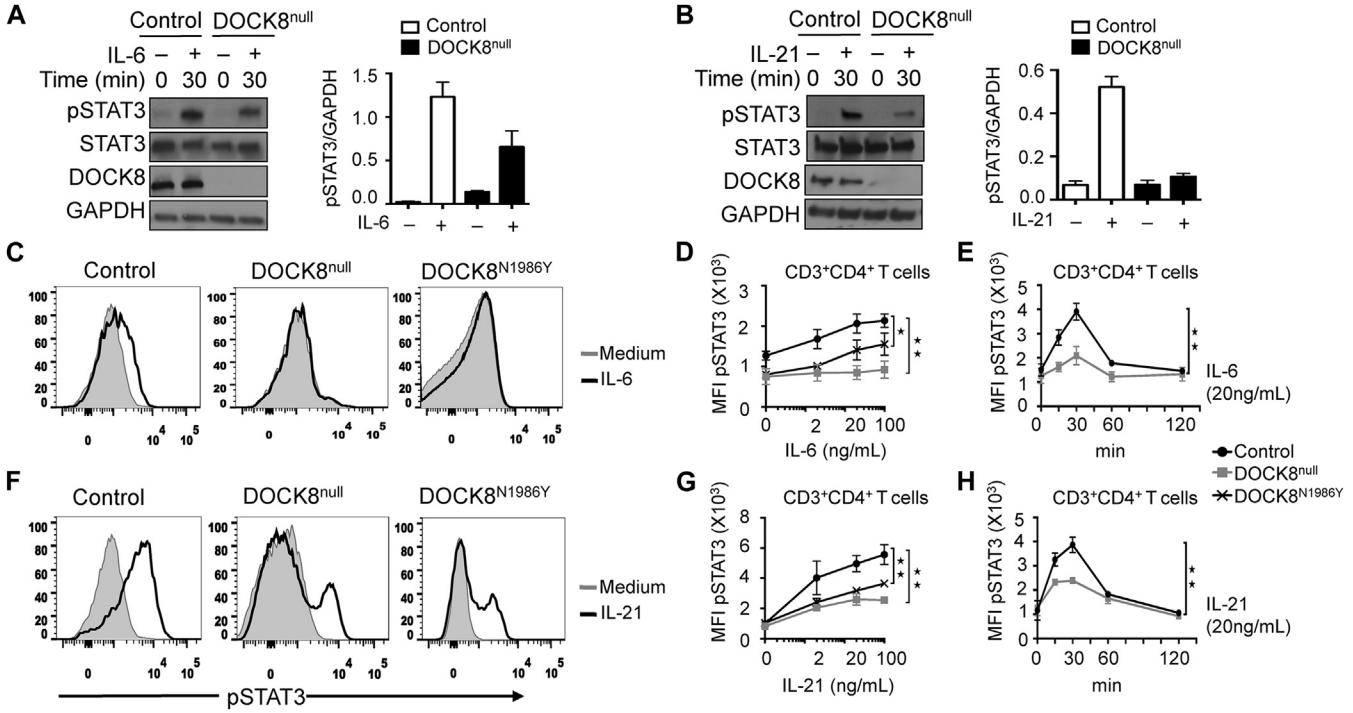

FIG 4. Defective STAT3 activation in primary DOCK8 mutant T cells. **A** and **B,** *Left panels:* immunoblot analysis of pY705-STAT3 present in lysates of control and DOCK8^{null} T cells at baseline and after treatment for 30 minutes with IL-6 (Fig 4, *A*) or IL-21 (Fig 4, *B*). *Right panels:* densitometric quantitation of pSTAT3 in the respective lanes of Fig 4, *A* and Fig 4, *B*, normalized for total GAPDH content in the lysates. Results are shown as means of 2 experiments. *Error bars* represent SEM. **C-H,** Flow cytometric analysis and mean fluorescence intensity quantitation of pY705-STAT3 in control, DOCK8^{null}, and DOCK8^{N1986Y} T cells treated with increasing concentrations of IL-6 (Fig 4, *C* and *D*) or IL-21 (Fig 4, *F* and *G*) for 30 minutes or 20 ng/mL IL-6 or IL-21 for the indicated time periods (Fig 4, *E* and *H*). Results are representative of at least 2 to 3 independent experiments (n = 2 per group for Fig 4, *A* and *B*, and n = 3 per group for Fig 4, *D*, *E*, *G*, and *H*). **P* < .05; ***P* < .01, repeated-measures 2-way ANOVA.

STAT3 activation and translocation to the nucleus. To determine whether DOCK8 deficiency in T cells affected the activation of STAT3 by T_H17-polarizing cytokines, we examined STAT3 phosphorylation at the regulatory Y705 residue (pSTAT3) in T cells after IL-6 or IL-21 treatment. Patients with DOCK8 deficiency exhibited a profound defect in pSTAT3 phosphorylation in response to stimulation with both cytokines, as revealed by using immunoblotting (Fig 4, *A* and *B*). The role of DOCK8 GEF activity on STAT3 activation was further deduced from studies on pSTAT3 induction in primary DOCK8^{null} and DOCK8^{N1986Y} T cells stimulated with IL-6 or IL-21, as determined by flow cytometry. Results showed that DOCK8^{null} and DOCK8^{N1986Y} CD4$^+$ T cells both exhibited a profound decrease in pSTAT3 induction in response to both IL-6 and IL-21 in a cytokine concentration– and treatment time–dependent manner compared with control CD4$^+$ T cells (Fig 4, *C-H*). Expression of IL-6 receptor (IL-6R) α chain, IL-6R signal transducer (gp130), and IL-21 receptor α chain was normal, indicating that the defect in STAT3 phosphorylation was not due to deficiency of cytokine-binding and signal-transducing receptor subunits (data not shown).

The role of DOCK8 in amplifying cytokine-induced pSTAT3 formation was confirmed by using Jurkat human leukemic T cells, which lack DOCK8 expression. Transfection of Jurkat cells with a construct encoding DOCK8, but not empty construct, resulted in a marked increase in pSTAT3 formation in response to IL-21 treatment in association with DOCK8 expression (Fig 5, *A* and *B*). We further compared the activity of DOCK8^{N1986Y} with wild-type (WT) DOCK8 protein (DOCK8^{WT}) using the Jurkat cell reconstitution system. Results showed that although DOCK8^{WT} enabled cytokine-induced pSTAT3 formation, DOCK8^{N1986Y} did not (Fig 5, *C* and *D*). These results established that DOCK8 acts to amplify STAT3 phosphorylation in response to different activating cytokines in a GEF activity–dependent manner.

The defect in cytokine-induced STAT3 activation by IL-6 in DOCK8-deficienct T cells was associated with a decreased presence of pSTAT3 into the nucleus, as detected by immunofluorescence analysis with anti-pSTAT3 antibody. Stimulation of control T cells with anti-CD3 mAb, and pronouncedly more so with IL-6, resulted in induction of pSTAT3 and its translocation to the nucleus, and the combination of the 2 stimuli was additive (Fig 6, *A-C*). In contrast, pSTAT3 induction and nuclear translocation by these 2 stimuli, either alone or in combination, was profoundly decreased in DOCK8-deficient T cells. DOCK8^{N1986Y} T cells also demonstrated defective pSTAT3 induction and nuclear translocation, which is consistent with a requirement for intact GEF activity for STAT3 activation (Fig 6, *A-C*).

To examine the consequences of decreased STAT3 activation in DOCK8 mutant cells on gene expression, we analyzed the capacity of IL-6 treatment to induce expression of transcripts encoding suppressor of cytokine signaling 3 (SOCS3), which is

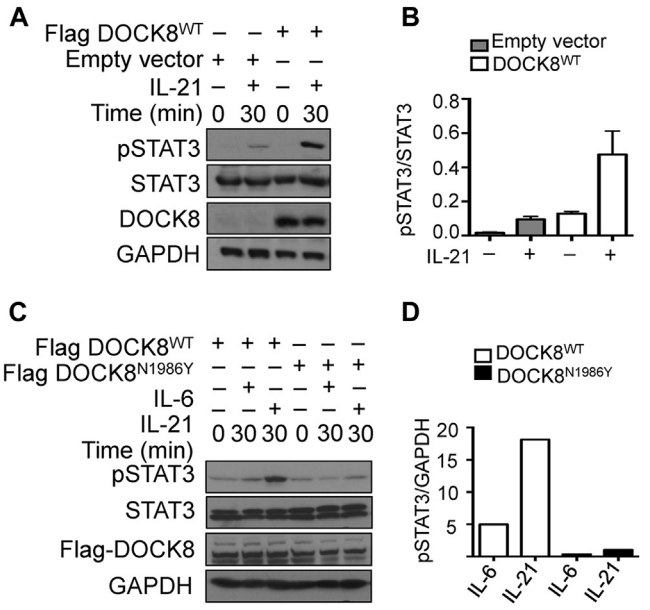

FIG 5. DOCK8 amplifies STAT3 activation in Jurkat T cells through a GEF-dependent mechanism. **A,** Immunoblot analysis of pY705-STAT3 in lysates of Jurkat cells transfected with empty vector or encoding DOCK8^{WT} and either left untreated or treated with IL-21. **B,** Densitometric quantitation of pSTAT3 in the respective lanes of Fig 5, *A*, normalized for total STAT3 protein content (n = 2 experiments per group). **C,** Immunoblot analysis of pY705-STAT3 in lysates of Jurkat cells transfected with empty vector or ones encoding Flag DOCK8^{WT} or DOCK8^{N1986Y} and then left untreated or treated with IL-6 or IL-21. **D,** Densitometric quantitation of pSTAT3 in the respective lanes of Fig 5, *C*, normalized for total GAPDH protein content.

dependent on STAT3 activation.[37] Results revealed that *SOCS3* transcripts were markedly decreased in DOCK8-deficient T cells. In contrast, transcripts encoding SOCS1 and SOCS2, expression of which is not regulated by STAT3 signaling, were unaffected (Fig 6, *D*). Furthermore, ChIP assays revealed decreased binding of pSTAT3 to the *SOCS3* promoter in DOCK8-deficient T cells treated with IL-6, which is consistent with the decreased *SOCS3* transcription in these cells (Fig 6, *E*).

DOCK8 associates with STAT3 in a nucleotide sensor–independent manner

To determine whether DOCK8 and STAT3 associate in a complex, we examined their coimmunoprecipitation with antibodies to the respective protein. Immunoprecipitation of DOCK8 from lysates of control T cells with an anti-DOCK8 antibody resulted in the coimmunoprecipitation of STAT3, as detected by immunoblotting with an anti-STAT3 antibody (Fig 7, *A*, left panel). Treatment with IL-6 resulted in no significant changes in the amounts of STAT3 protein coprecipitating with DOCK8 despite the increase in pSTAT3 expression, indicating that the interaction is independent of Y705-STAT3 phosphorylation (Fig 7, *A*, left panel). In reciprocal experiments immunoprecipitation with anti-STAT3 antibodies resulted in coimmunoprecipitation of DOCK8, as detected by immunoblotting with an anti-DOCK8 antibody (Fig 7, *A*, right panel).

We also examined the effect of the DOCK8^{N1986Y} mutation on the association of DOCK8 with STAT3 by using patients' T-cell blasts. Results revealed that STAT3 also coimmunoprecipitated with DOCK8^{N1986Y} (Fig 7, *B*). To confirm the association of DOCK8 with STAT3, we transfected Jurkat human leukemic T cells with constructs encoding Flag-tagged DOCK8^{WT} and DOCK^{N1986Y} and hemagglutinin-tagged STAT3. Immunoprecipitation studies with an anti-Flag mAb revealed that a hemagglutinin-reactive 100-kDa protein, which is consistent with recombinant STAT3, associated equally well with both Flag-tagged DOCK8^{WT} and DOCK^{N1986Y} proteins. Together, these results confirmed that STAT3 and DOCK8 associate independent of DOCK8 GEF activity (Fig 7, *C*).

To determine whether other STAT proteins interact with DOCK8, we carried out immunoprecipitation studies on T-cell blasts using antibodies specific for STAT1, STAT3, STAT4, STAT5, and STAT6. Results revealed that whereas DOCK8 coimmunoprecipitated with STAT3, it did not do so with other STAT proteins, indicating that DOCK8 specifically interacted with STAT3 (Fig 7, *D*).

DOCK8 directly interacts with STAT3 independent of Y705-STAT3 phosphorylation

Y705 phosphorylation regulates the transcriptional activity of STAT3.[38,39] To determine whether Y705 phosphorylation was important for the association of STAT3 with DOCK8, we generated cDNA encoding an hemagglutinin-tagged STAT3 mutant in which the Y705 residue was substituted with phenylalanine (F705). Jurkat cells were transfected with cDNA encoding Flag-tagged DOCK8 together with hemagglutinin-tagged WT STAT3 protein (STAT3^{WT}) or hemagglutinin-tagged STAT3^{F705}. Coprecipitation experiments revealed that both hemagglutinin-tagged STAT3^{WT} and hemagglutinin-tagged STAT3^{F705} were recovered in equal amounts on precipitation of recombinant DOCK8 with anti-Flag mAb (Fig 8, *A*). pSTAT3 was detected in immunoprecipitates of WT but not hemagglutinin-tagged STAT3^{F705} transfected cells, which is consistent with the STAT3^{F705} mutation acting in a dominant negative fashion similar to what is seen in patients with HIES.[18] These results indicated that the coassociation of STAT3 and DOCK8 was independent of the phosphorylation status of STAT3.

STAT3 phosphorylation at Y705 is regulated by tyrosine phosphatases.[40,41] To determine whether decreased pSTAT3 formation caused by DOCK8 deficiency or DOCK8^{N1986Y} mutation reflected enhanced dephosphorylation of STAT3, we analyzed pY705-STAT3 formation in DOCK8-deficient T cells treated with IL-6 in the absence of presence of the phosphotyrosine phosphatase inhibitor sodium orthovanadate (Na_3VO_4). Whereas treatment with Na_3VO_4 on its own induced no discernible increase in pY705-STAT3 levels, it potentiated IL-6–induced pY705-STAT3 formation in both WT and DOCK8-deficient T cells, with the latter reaching levels close to those observed in WT T cells (Fig 8, *B* and *C*). These results suggest that DOCK8-STAT3 association might protect the pY705 residue of the latter from dephosphorylation.

DISCUSSION

Our results showed that DOCK8-deficient patients have a profound defect in T_H17 differentiation related to decreased STAT3 phosphorylation, translocation to the nucleus, and transcriptional activity. DOCK8 was found to constitutively and specifically associate with STAT3 but not other STAT proteins and to enable activation-induced STAT3 translocation to the nucleus. Furthermore, DOCK8 GEF activity was necessary for

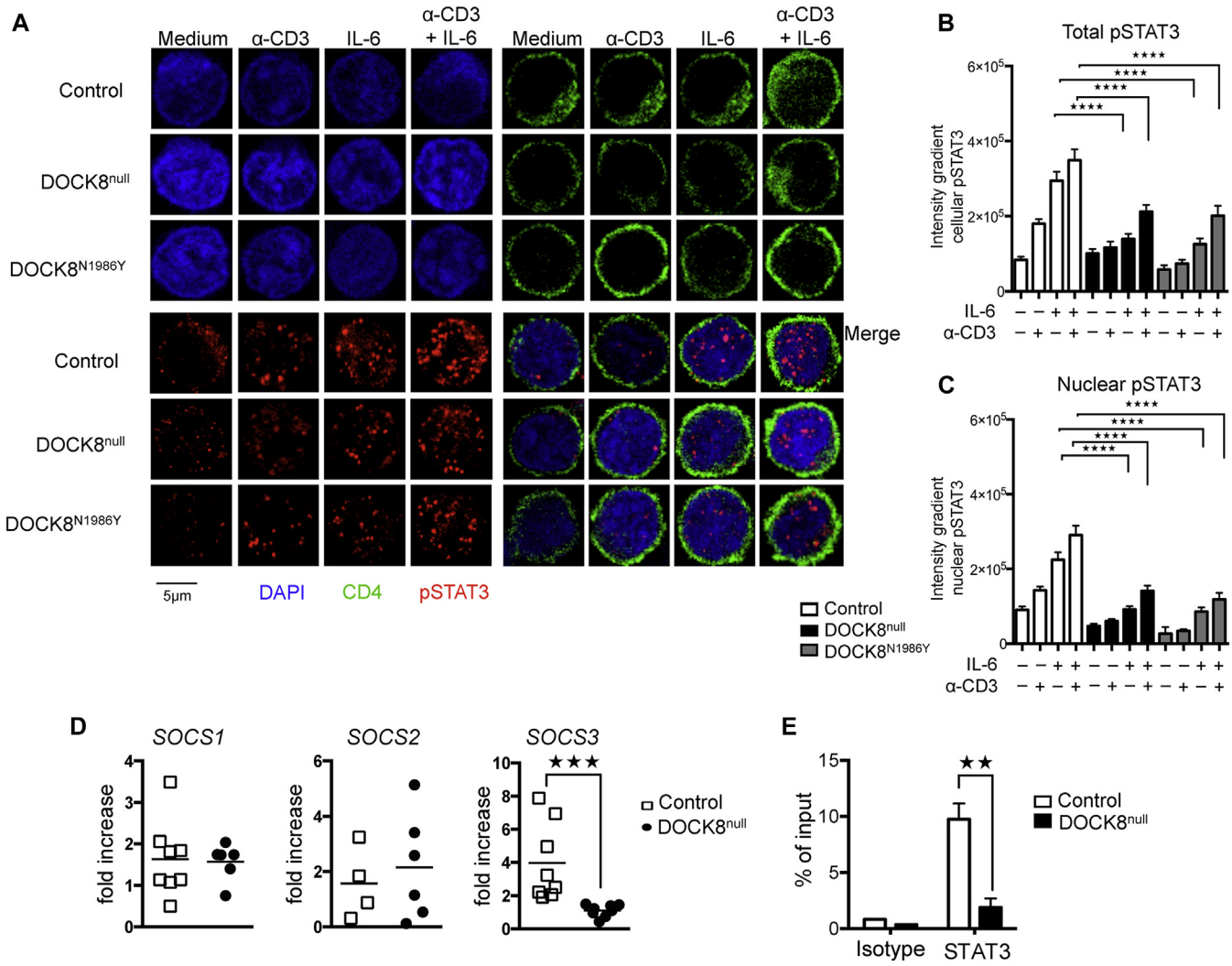

FIG 6. DOCK8 deficiency impairs pSTAT3 translocation to the nucleus and induction of STAT3-dependent gene expression. **A,** DOCK8 deficiency impairs activation-induced pSTAT3 translocation to the nucleus. Cells were treated with anti-CD3 mAb, IL-6, or both and stained for nuclear DNA (DAPI) and pSTAT3 and examined by confocal microscopy for total cellular versus nuclear pSTAT3 (n = 30-50 cells per group). *Scale bar* = 5 μm. **B** and **C,** Quantitation of total cellular versus nuclear pSTAT3 staining. **D,** Real-time PCR analysis of *SOCS1*, *SOCS2*, and *SOCS3* transcript expression expressed as the fold increase over baseline in T-cell blasts of DOCK8-deficient versus control subjects treated with IL-6 (100 ng/mL; 30 minutes). **E,** ChIP analysis of STAT3 binding at the SOCS3 promoter in T-cell blasts from patients and control subjects either untreated or stimulated with IL-6 (n = 5 per group). Results are representative of at least 2 independent experiments. $^{**}P < .01$, $^{***}P < .001$, and $^{****}P < .0001$, 1- and 2-way ANOVA with posttest analysis.

optimal STAT3 phosphorylation and T_H17 differentiation but not STAT3-DOCK8 interaction. These findings establish a mechanistic basis for the phenotypic convergence of the AD and AR forms of HIES centered on DOCK8 regulation of STAT3.

DOCK8 interacts with the small GTPase Cdc42 through the DOCK homology region 2 domain and mediates its activation by stimulating the GTP-GDP exchange reaction.[7] DOCK8 deficiency did not affect Rac1 activation, which is compromised in patients with DOCK2 deficiency.[27] Importantly, a mutation in the DOCK8 nucleotide sensor loop, $DOCK8^{N1986Y}$, which disrupted GEF activity, recapitulated the phenotype of DOCK8 deficiency, including impaired STAT3 Y705 phosphorylation and T_H17 cell deficiency, indicating an essential role for DOCK8 GEF function in STAT3 activation and disease pathogenesis.

Although the promotion of STAT3 phosphorylation by DOCK8 was dependent on the latter's GEF activity, the physical association of the 2 proteins was not, as evidenced by normal association of STAT3 with $DOCK8^{N1986Y}$. The association was also independent of STAT3 Y705 phosphorylation status. Nevertheless, the $DOCK8^{N1986Y}$ mutation abolished the enhancement of STAT3 phosphorylation induced by DOCK8, indicating a requirement for the GEF activity for this effect. Cdc42 activates downstream kinases, including activated kinase, which have been implicated in STAT3 activation, suggesting a role for such kinases in mediating DOCK8-dependent upregulation of STAT3 phosphorylation.[42]

Previous studies have shown that DOCK8 links Toll-like receptor 9–myeloid differentiation primary response gene 88 signaling in B cells to STAT3 activation through a Src-Syk kinase

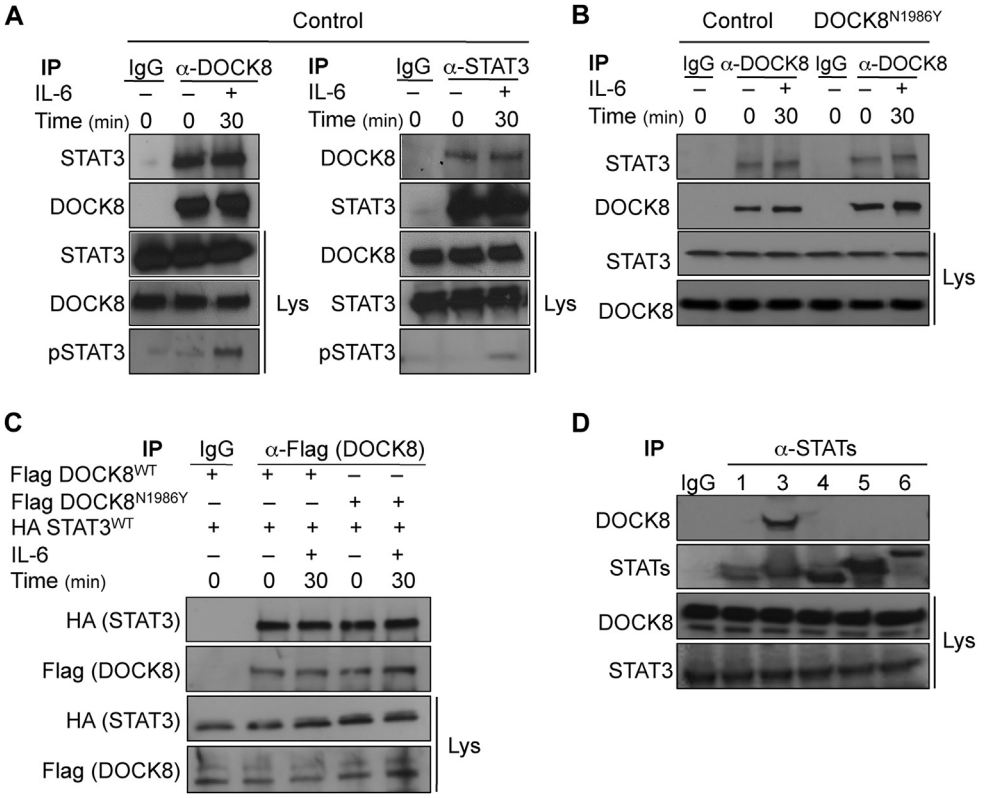

FIG 7. DOCK8 associates with STAT3 independent of GEF activity. **A,** DOCK8 and STAT3 are constitutively associated in primary T cells. DOCK8 *(left)* and STAT3 *(right)* immunoprecipitates *(IP)* derived from T-cell lysates were blotted with anti-STAT3 or anti-DOCK8 antibodies, respectively. The immunoblots were reprobed with anti-STAT3 *(left)* or DOCK8 *(right)*. Lysates were also probed for STAT3, DOCK8, and pSTAT3. **B,** $DOCK8^{N1986Y}$ associates with STAT3 in primary T cells. DOCK8 immunoprecipitates were derived from control and $DOCK8^{N1986Y}$ T-cell lysates. The immunoprecipitates and total cellular lysates were probed for STAT3 and DOCK8 by immunoblotting. **C,** Flag-tagged $DOCK8^{WT}$ and $DOCK8^{N1986Y}$ proteins expressed in Jurkat human leukemic T-cell coimmunoprecipitate with hemagglutinin-tagged STAT3. Cells were transfected with indicated constructs and anti-Flag immunoprecipitates derived and probed with the indicated antibodies. **D,** DOCK8 specifically associates with STAT3 but not other STAT proteins. STAT proteins were immunoprecipitated with the respective STAT protein antibody, and the immunoprecipitates were probed with anti-STAT3 mAb. Results are representative of at least 3 independent experiments.

cascade, whereas cytokine-induced STAT3 phosphorylation appeared normal.[14] DOCK8 has also been implicated in the generation of $ROR\gamma t^{+}$ type 3 innate lymphoid cells and in promoting IL-23–dependent STAT3 activation.[43] The failure in earlier studies to detect marked differences in cytokine-induced STAT3 phosphorylation in DOCK8-deficient lymphocytes probably reflects the low concentrations and delayed time courses of cytokine treatment in those studies, which masked the function of DOCK8 in amplifying STAT3 phosphorylation that is more readily detected at higher concentrations and earlier time points (Fig 4, *E* and *H*).[14]

Our present studies expand on these observations in several ways. First, we have demonstrated that DOCK8 amplifies cytokine-dependent STAT3 activation in a GEF activity–dependent manner. We have also demonstrated that DOCK8 and STAT3 physically associate as a signaling module independent of the former's GEF activity, thus allowing more focused channeling of STAT3 activation through DOCK8. Finally, DOCK8 promotes STAT3 nuclear translocation, an event that might contribute to sustained STAT3 activation in the nuclear compartment.

Although both STAT3 mutations in patients with AD-HIES and DOCK8 deficiency impair STAT3 activation and, consequently, T_H17 cell differentiation, the 2 gene defects differ in their effect. AD-HIES is associated with STAT3-dependent extrahematopoietic manifestations that are either lacking or uncommon in patients with DOCK8 deficiency.[44] DOCK8's function as a signal amplifier of STAT3 activation is particularly relevant in immune cells, where DOCK8 is highly expressed, but not in other tissues, where alternative compensatory mechanisms for amplifying STAT3 activation might be operative.[45] Other phenotypes of DOCK8 deficiency not shared with AD-HIES, such as autoimmunity and susceptibility to viral infections, can also reflect distinct functions of DOCK8 beyond STAT3 activation, such as its regulation of the actin cytoskeleton, chemotaxis, and antiviral cytokine production.[7,12,16,46]

In conclusion, our studies identify an essential and specific function for DOCK8 in mediating STAT3 activation and nuclear translocation. DOCK8-STAT3 interaction illustrates a novel mechanism for the regulation of STAT signaling by DOCK proteins. Putative interactions between different members of the DOCK and STAT family proteins might play an important role in

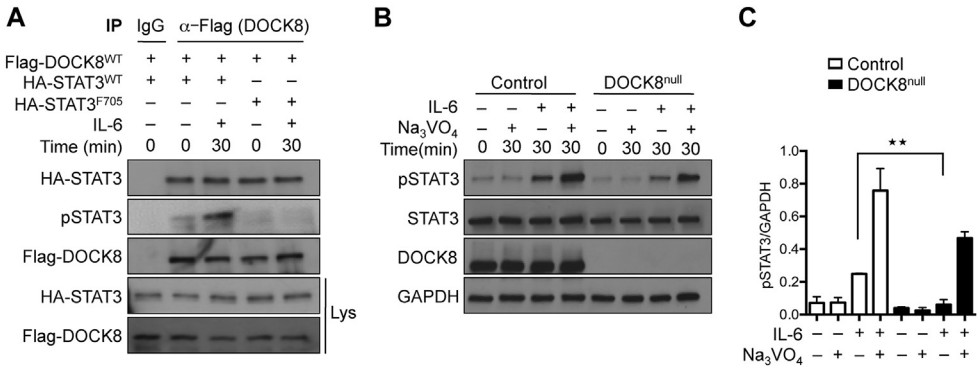

FIG 8. DOCK8 and STAT3 directly associate independent of STAT3-Y705 phosphorylation status. **A,** DOCK8 coimmunoprecipitates with $STAT3^{F705}$. Immunoprecipitates *(IP)* were derived from Jurkat cells expressing Flag-DOCK8 and hemagglutinin-tagged $STAT3^{WT}$ or hemagglutinin-tagged $STAT3^{F705}$ proteins by using anti-Flag mAbs and probed with anti-hemagglutinin, anti-Flag, and anti-p705-STAT3 mAb. The lysates were probed with anti-Flag and anti-hemagglutinin mAbs. **B,** Rescue of defective STAT3 phosphorylation in DOCK8-deficient cells with Na_3VO_4 treatment. Primary T-cell blasts from control and DOCK8-deficient subjects were either left untreated or treated with $Na3VO_4$ for 30 minutes and then stimulated with IL-6, as indicated. Cellular lysates were immunoblotted with pSTAT3, STAT3, DOCK8, and GAPDH mAbs, as indicated. **C,** Densitometric quantitation of pSTAT3 formation in experiments carried out as in Fig 8, *B* (n = 3). Results are representative of at least 3 independent experiments. $^{**}P < .01$, 1- and 2-way ANOVA with posttest analysis.

the regulation of STAT signaling, including the differentiation of T_H cell subsets.

This article is dedicated to the memory of Professor Dr Işil Berat Barlan (1958-2015). We thank Drs Michel Massad and Luigi Notarangelo for critical review of the manuscript.

Key messages

- **DOCK8 regulates T_H17 differentiation by promoting STAT3 phosphorylation and translocation to the nucleus.**
- **DOCK8 constitutively associates with STAT3 in a GEF activity–independent manner but amplifies STAT3 activation in a GEF activity–dependent manner.**
- **T_H17 deficiency can contribute to the heightened susceptibility of DOCK8-deficient subjects to *Candida* species and bacterial infections.**

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METHODS

Antibodies, cytokines, and reagents

The following anti-human mAbs to the indicated cell markers were used in flow cytometric analyses: CD3 (UCHT1), CD4 (RPA-T4), CD8 (RPA-T8), CD127 (A019D5), CD25 (BC96), CD45RO (UCHL1), CD45RA (HI100), CCR7 (G043H7; BioLegend), forkhead box P3 (PCH101; eBioscience, San Diego, Calif), CD19 (HIB19), and CD27 (M-T271) (BD Biosciences). The following antibodies were used for immunoblot analysis: anti-DOCK8 (HPA003218), anti–Flag M2 (F-3165; Sigma-Aldrich), anti-DOCK8 (sc376911), anti-STAT1 (p84/p91, M-22, sc-592), anti-STAT3 (sc-482), anti-STAT4 (sc-486), anti-STAT5 (sc-835), anti-STAT6 (sc-621), anti-hemagglutinin (sc-7392; Santa Cruz Biotechnology, Dallas, Tex), anti-STAT3 (124H6), anti-pY705-STAT3 (9131S), glyceraldehyde-3-phosphate dehydrogenase (GAPDH; 2118S), β-actin (4970S; Cell Signaling, Danvers, Mass), and purified mouse IgG_1 (MOPC21; BioLegend). IL-1β, IL-2, IL-6, IL-21, IL-23, TGF-β1, and IFN-γ were from PeproTech (Rocky Hill, NJ). Anti–IL-6Rα (551850), anti–IL-21Rα (560264), and anti-gp130 (555757) mAbs were from BD Biosciences.

T_H cell differentiation

Naive T cells were isolated from PBMCs by negative selection with magnetic bead sorting (Miltenyi Biotec, Bergisch Gladbach, Germany). Cells were then differentiated by stimulation with bead-immobilized anti-CD2/CD3/CD28 mAbs (Miltenyi Biotec) under T_H17-polarizing conditions (20 ng/mL each of IL-1β, IL-6, IL-21, and IL-23 and 10 ng/mL TGF-β1), T_H1 (20 ng/mL IL-12 and 10 μg/mL anti–IL-4 mAb), or T_H2 (20 ng/mL IL-4 and 10 μg/mL anti–IFN-γ mAb), as previously described.[E1,E2] Polarized T cells were resuspended at 1 to 2×10^6 cells/mL and either left untreated, stimulated with a combination of 20 ng/mL phorbol 12-myristate 13-acetate and 1 μg/mL ionomycin (Sigma-Aldrich), or stimulated with anti-CD2/CD3/CD28 mAbs for 48 hours.

ELISA for T_H cell cytokines

Culture supernatants were assayed for IFN-γ, IL-4, IL-5, IL-9, and IL-17A by ELISA (eBioscience).[E2]

Immunoblotting

T-cell lines ($1\text{-}2 \times 10^6$ cells per condition) were left unstimulated or stimulated with recombinant IL-6 (100 ng/mL) or IL-21 (100 ng/mL) for the indicated time periods. In some experiments cells were treated with the phosphotyrosine phosphatase inhibitor Na_3VO_4 (1 mmol/L, Sigma-Aldrich) for 30 minutes before cytokine stimulation. Cells were lysed with buffer containing 0.75% NP-40, as previously described. Total cell lysates were separated by SDS-PAGE and transferred to polyvinylidene difluoride membranes. Immunoblotting was carried out, as described, by using the indicated antibodies.

Real-time PCR

RNA was isolated from unpolarized cells and T_H1, T_H2, and T_H17 cells polarized from naive T cells after 48 hours of stimulation with or without anti-CD2, anti-CD3, and soluble CD28 mAbs by using the RNeasy kit (Qiagen, Hilden, Germany). Reverse transcription was performed with Superscript III and oligo dT, dNTP mix, RNase Out (Invitrogen). *IFNG*, *IL4*, *IL9*, *IL17A*, *TBX21*, GATA-binding protein 3 *(GATA3)*, and *RORC* real-time PCR assays were carried out with TaqMan Universal Fast Master Mix (Applied Biosystems, Foster City, Calif) and run on a Step-One-Plus PCR machine (Applied Biosystems). Expression of cytokine and transcription factor mRNA transcripts was normalized with *GAPDH* transcript levels (Applied Biosystems). Results were normalized to average levels of those cytokines in subjects with DOCK8 mutations. To evaluate *SOCS1*, *SOCS2*, and *SOCS3* expression (Applied Biosystems) on T cells, mRNA was isolated from T-cell cultures at baseline and at 4 hours after IL-6 treatment (100 ng/mL), and transcripts levels of the respective genes were determined by using real-time PCR.

ChIP assays

T-cell blasts were rested overnight in serum-free culture medium (Lonza, Basel, Switzerland) and then stimulated with IL-6 (60 ng/mL) for 30 minutes. The cells were then washed and cross-linked with 1% formaldehyde for 10 minutes. Subsequent steps were conducted with the Magnetic ChIP kit, according to the manufacturer's instructions (Life Technologies). Briefly, cross-linked cells were lysed and sonicated, followed by overnight immunoprecipitation with anti-STAT3 mAb (Cell Signaling) or normal rabbit IgG. Eluted, reverse, cross-linked protein-DNA complexes were digested with proteinase K for 90 minutes. Digested samples were then purified with a DNA cleanup column from the same kit, according to the manufacturer's instructions. PCR amplification was subsequently carried out with primers specific to *SOCS3*. The primer pairs used for qPCR amplification were as follows: *SOCS3*, 5′-GCGCTCAGCCTT-TCTCTG-3′ and 5′ GGAGCAGG GAGTCCAAGTC-3′.[E3]

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E6. Keles S, Jabara HH, Reisli I, McDonald DR, Barlan I, Hanna-Wakim R, et al. Plasmacytoid dendritic cell depletion in DOCK8 deficiency: rescue of severe herpetic infections with IFN-alpha 2b therapy. J Allergy Clin Immunol 2014;133:1753-5.e3.

TABLE E1. Genotypic and immunologic features of DOCK8-deficient patients

Patient no.	Age (y)/ sex	IgE (IU/mL)	Eosinophils (cells/μL)	*DOCK8* mutation	IgM (mg/dL)	IgA (mg/dL)	IgG (mg/dL)	CD3 (%)	CD4 (%)	CD8 (%)	References
1	4.5/F	1,000	13,900	Homozygous amino acid substitution in exon 45: c.[5956 A>T]; [5956 A>T]*	42.7	177	2,450	54.5	19.4	34	This report
2	8F	10,500	5,100	Homozygous IVS16 splice acceptor site mutation: c.[1869 –1 G>C]; [1869 –1 G>C]	62	239	1,500	46	32		ARH008[E4,E5]
3	1.5/F†	5,570	970	Homozygous IVS16 splice acceptor site mutation: c.[1869 –1 G>C]; [1869 –1 G>C]	61	223	1,910	37	10	21	ARH043.1[E4]
4	2/M†	1,060	2,910	Homozygous IVS16 splice acceptor site mutation: c.[1869 –1 G>C]; [1869 –1 G>C]	70	186	1,600	65	19	28	ARH043.2[E4]
5	5/F	36,000	29,000	Homozygous IVS16 splice acceptor site mutation: c.[1869 –1 G>C]; [1869 –1 G>C]	37	94	1,280	54	18	34	Patient 1[E6] ARH043.3
6	4/F	14,000	8,000	Homozygous deletion encompassing exons 26-48: c.[3121 ?__7340+?del]; [3121 –?__7340+?del]	81	174	1,740	56	35	21	Patient 2[E6]
7	9/F	10,900	3,180	Homozygous deletion encompassing exons 26-48: c.[3121 ?__7340+?del]; [3121 –?__7340+?del]	50.4	144	1,360	68	28	27	Patient 4[E6]
8	1/F	489	43,100	Homozygous deletion encompassing exons 1-23: c.[1 –?__2874+?del]; [1 –?__2874+?del]	56.7	234	1,460	33	17	17	Patient 3[E6]
9	8/M	5,000	1,400	Homozygous large deletion encompassing exons 1-22: c.[1–?_ 2778 +?del]; [1–?_ 2778 +?del]	29	484	2,460	53	19	31	ARH004[E5]
10	4/M	3,103	8,100	Homozygous deletion encompassing exons 1-27: c.[1 –?_3390+?del]; [1 –?_3390+?del]	46	108	1,260	45	22	19	This report
11	7.5/M	2,782	2,782	Homozygous deletion encompassing exons 1-27: c.[1 –?_3390+?del]; [1 –?_3390+?del]	30.1	445	1,610	69.9	22.2	26.7	This report
12	6/M	1,533	33,000	Homozygous deletion encompassing exons 1-27: c.[1 –?_3390+?del]; [1 –?_3390+?del]	121	118	1,290	47	17	27	This report
13	1.5/F	347	4,600	Homozygous deletion encompassing exons 1-27: c.[1 –?_3390+?del]; [1 –?_3390+?del]	22.1	148	1,250	65.9	32.3	32.7	This report
14	5/F	780	29,504	Premature stop codon at the tail end of exon 21: c.[2603 C > G]; [2603 C > G]	99.6	248	1,340	36.4	19.8	17.7	This report
15	4/M	116	3,500	Premature stop codon at the tail end of exon 21: c.[2603 C > G]; [2603 C > G]	136	680	2,580	57	11	33	This report
16	3/F	4,527	500	Homozygous deletion encompassing exons 1-9 c.[1 ?_1044+?del]; [1 ?_1044+?del]	58	63	1,520	33	19	9	This report

CD3/CD4/CD8, $CD3^+/CD4^+/CD8^+$ T cells (in peripheral blood); *F*, female; *IVS16*, Intervening sequence 16; *M*, male.

*The numbering of exons and nucleotides is according to sequence NM_203447.3.

†Patients 3 and 4 are siblings. All other patients are not immediate relatives, even though some share common alleles.