

Defective actin accumulation impairs human natural killer cell function in patients with dedicator of cytokinesis 8 deficiency

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Background: Dedicator of cytokinesis 8 (DOCK8) mutations are responsible for a rare primary combined immunodeficiency syndrome associated with severe cutaneous viral infections, increased IgE levels, autoimmunity, and malignancy. Natural killer (NK) cells are essential for tumor surveillance and defense against virally infected cells. NK cell function relies on Wiskott-Aldrich syndrome protein for filamentous actin (F-actin) accumulation at the lytic NK cell immunologic synapse. DOCK8 activates cell division cycle 42, which, together with Wiskott-Aldrich syndrome protein, coordinates F-actin reorganization. Although abnormalities in T- and B-cell function have been described in DOCK8-

deficient patients, the role of NK cells in this disease is unclear.

Objectives: We sought to understand the role of DOCK8 in NK cell function to determine whether NK cell abnormalities explain the pathogenesis of the clinical syndrome of DOCK8 deficiency.

Methods: A cohort of DOCK8-deficient patients was assembled, and patients' NK cells, as well as NK cell lines with stably reduced DOCK8 expression, were studied. NK cell cytotoxicity, F-actin content, and lytic immunologic synapse formation were measured.

Results: DOCK8-deficient patients' NK cells and DOCK8 knockdown cell lines all had decreased NK cell cytotoxicity, which could not be restored after IL-2 stimulation. Importantly, DOCK8 deficiency impaired F-actin accumulation at the lytic immunologic synapse without affecting overall NK cell F-actin content.

Conclusions: DOCK8 deficiency results in severely impaired NK cell function because of an inability to form a mature lytic immunologic synapse through targeted synaptic F-actin accumulation. This defect might underlie and explain important attributes of the DOCK8 deficiency clinical syndrome, including the unusual susceptibility to viral infection and malignancy. (*J Allergy Clin Immunol* 2013;■■■:■■■-■■■.)

Key words: DOCK8 deficiency, natural killer cells, actin, cytotoxicity, immunologic synapse

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Patients with autosomal recessive hyper-IgE syndrome (HIES) can be affected by recalcitrant cutaneous herpes virus and papillomavirus infections, as well as immune dysregulation.^{1,2} The major genetic abnormality found in these patients is deletion or loss-of-function mutations in the gene encoding dedicator of cytokinesis 8 (DOCK8).^{3,4} Mutagenized mice with an autoimmune phenotype were also identified as possessing DOCK8 mutations.⁵ Both DOCK8-deficient patients and mice are documented to have combined immunodeficiency of the B- and T-cell compartments.⁵⁻⁸ However, the high incidence of recurrent cutaneous viral infections distinguishes them from other patients with combined immunodeficiencies, and a mechanistic explanation for this phenotype is lacking. Currently, the only definitive therapy for patients with DOCK8 deficiency is hematopoietic stem cell transplantation.⁹⁻¹¹

One immunologic characteristic found in patients with susceptibility to herpesviruses and papillomaviruses is abnormalities

Abbreviations used

Cdc42: Cell division cycle 42
 DOCK8: Dedicator of cytokinesis 8
 F-actin: Filamentous actin
 HIES: Hyper-IgE syndrome
 IS: Immunologic synapse
 MTOC: Microtubule organizing center
 NK: Natural killer
 shRNA: Short-hairpin RNA
 STAT3: Signal transducer and activator of transcription 3
 WAS: Wiskott-Aldrich syndrome
 WASp: Wiskott-Aldrich syndrome protein

of natural killer (NK) cells.^{12,13} This is illustrated by a number of human NK cell deficiency states, NK cell-depleted murine models, and some of the specific aspects of virology.¹³⁻¹⁵ NK cells are lymphocytes of the innate immune system that can inherently recognize many virally infected or malignantly transformed cells, especially those that escape the adaptive T-cell response caused by downregulated MHC (as in herpesvirus infections). NK cells have not been previously evaluated in patients with DOCK8 deficiency other than absolute numbers, which were normal.^{1,3,4} Specifically, functional assessments of NK cells in patients with DOCK8 deficiency have not been reported.

DOCK8 is a member of the DOCK180 superfamily of atypical guanine-nucleotide exchange factors^{16,17} and activates cell division cycle 42 (Cdc42), a Rho GTPase critical for reorganization of the filamentous actin (F-actin) cytoskeleton in NK and dendritic cells.^{18,19} Cdc42 functions along with Wiskott-Aldrich syndrome (WAS) protein (WASp) to regulate NK cell function.²⁰ Defects in WASp are associated with the well-known primary immunodeficiency WAS, and these patients exhibit several characteristics shared with patients with DOCK8 deficiency, including susceptibility to cutaneous viral infections and malignancy. NK cells isolated from patients with WAS are functionally deficient.²⁰⁻²² Given the clinical phenotype of patients with DOCK8 and its integral role in Cdc42 activation, we hypothesized that there was a substantive defect in NK cell function in patients with DOCK8 deficiency.

METHODS**Patients and human subjects**

Ten patients with DOCK8 mutations were given diagnoses based on either targeted gene sequence or genomic approaches. Patients DOCK8-4, DOCK8-5, DOCK8-8, and DOCK8-9 have previously been reported.^{4,23,24} There were 2 sibling pairs, patients DOCK8-4 and DOCK8-5 and patients DOCK8-3 and DOCK8-6, respectively. Three patients with signal transducer and activator of transcription 3 (STAT3) deficiency were included as disease control subjects. Blood samples from patients and healthy donors were obtained after patient-provided or parental informed consent under the approval of the local Institutional Review Board for the Protection of Human Subjects of the Children's Hospital of Philadelphia, Baylor College of Medicine, or Ludwig-Maximilians-Universität, Munich, Germany.

NK cells and cell lines

PBMCs or *ex vivo* NK cells were isolated from whole blood by means of centrifugation through Ficoll-Paque Plus (Amersham Biosciences, Piscataway, NJ) with or without negative selection by using the RosetteSep human NK cell enrichment cocktail (STEMCELL Technologies, Vancouver, British Columbia, Canada), as previously described.^{25,26} *Ex vivo* NK cells and

PBMCs were used immediately after preparation or cryopreserved for later use, and purity was determined by means of flow cytometry. The human NK cell line YTS was used to establish the *in vitro* DOCK8 knockdown model. Briefly, HEK293T cells were cotransfected with lentiviral packaging plasmids (pPACK; System Biosciences, Mountain View, Calif) and pLKO.1 lentiviral vector with puromycin resistance and either DOCK8 or scrambled DOCK8 sequence short-hairpin RNA (shRNA; MissionRNAi; Sigma-Aldrich, St Louis, Mo) to produce replication-incompetent viral particles, which were used to infect YTS cells. 721.221 B-lymphoblastoid, K562 erythroleukemia, and K562 cells stably expressing CD86 (KT86)²⁷ were used as target cells and maintained as previously described.

Cytotoxicity

Standard ⁵¹Cr-release assays were used to measure the cytolytic activity of PBMCs isolated from human samples and NK cell lines, as previously described.²¹ Patients DOCK8-1 and DOCK8-4 were assessed 3 distinct times. Controls included both shipping and local donor control blood samples. Higher effector/target cell ratios were used for PBMCs than for YTS cell lines. Lytic units were calculated, as previously described.²¹

Flow cytometry

NK cells among PBMCs were quantified by means of flow cytometry, as previously described,²¹ with fluorophore-conjugated mAbs (BD Biosciences, San Jose, Calif) for CD56 and CD3. NK cell F-actin content was also measured by means of flow cytometry using our published methods.^{22,27} Patients DOCK8-1 and DOCK8-4 were assessed 3 distinct times.

Microscopy and image analysis

NK cells from patients and control donors, as well as YTS cells, were prepared for evaluation of fixed effector/target cell conjugates by using immunofluorescence microscopy, as previously described,^{22,27,28} and images were acquired with a Zeiss-Z1 microscope outfitted with a Yokogawa CSU10 spinning disc and 63× 1.45 NA objective. Images were acquired and quantified by using Velocity software (PerkinElmer, Waltham, Mas), and data were exported to GraphPad Prism software (GraphPad Software, La Jolla, Calif). Previously published quantitative algorithms were applied to measure F-actin and CD18 accumulation²⁸ and pericentrin distance from the immunologic synapse (IS).²⁷

Quantitative PCR

RNA was harvested with the Qiagen RNeasy Mini kit (Qiagen, Hilden, Germany) and reverse transcribed into cDNA by using the TaqMan Reverse Transcription kit (Applied Biosystems, Foster City, Calif). DOCK8 expression was analyzed by using quantitative PCR (7900HT Analyzer, Applied Biosystems) with forward (5'-ACGCGCCGTGTAAGTGTGAA-3') and reverse (5'-CCCCGAGCTCCTGGGCAA-3') primers, as previously reported,³ with each assay performed in triplicate. Expression of glyceraldehyde-3-phosphate dehydrogenase (forward 5'-CTCATTTCCTGGTATGACAA CG-3', reverse 5'-TTACTCCTTGAGGCCATGT-3') was used as a control for normalization.

Statistics

Data were compared by using unpaired Student 2-tailed *t* tests or exact Wilcoxon-Mann-Whitney *U* tests, with significance defined as a *P* value of less than .05.

RESULTS**DOCK8-deficient patients have impaired NK cell cytotoxicity that is not rescued by IL-2 stimulation**

We collected an international cohort of 10 DOCK8-deficient patients to examine NK cell function. Our patients ranged from

1.5 to 26 years of age at evaluation, and 90% had a history of herpesvirus infection, papillomavirus infection, or molluscum contagiosum. Specific DOCK8 mutations varied, except for 2 sets of siblings who shared the same homozygous splice site mutation or homozygous deletion as indicated (Table I). Other clinical findings, including increased IgE levels and eosinophil counts, presence of asthma, autoimmunity, eczema, and allergies are consistent with previously reported DOCK8-deficient patients.^{3,4,23} Patients DOCK8-4, DOCK8-5, DOCK8-8, and DOCK8-9 have previously been reported,^{4,23,24} and all others are unique to this series. CD4⁺ and CD8⁺ T-cell percentages were within normal ranges, but absolute values were decreased in 7 of 10 and 5 of 10 patients, respectively. B-cell numbers were normal in 8 of 10 of our DOCK8-deficient patients. In those patients in which mitogen-induced (9 patients) and/or antigen-induced (6 patients) lymphocyte proliferation assays were performed, only cells derived from patients DOCK8-2 and DOCK8-5 responded normally. Only DOCK8-2 maintained normal pneumococcal and tetanus antibodies; however, patients DOCK8-1 and DOCK8-6 also produced sustained tetanus antibodies.

PBMCs isolated from individual patients were used in ⁵¹Cr-release assays against K562 erythroleukemia target cells to assess NK cell cytolytic function. All patients assessed had decreased killing, as measured by using lytic units per NK cell (Fig 1, A). The deficit in NK cell cytotoxicity relative to control levels could not be rescued by short-term stimulation of PBMCs with IL-2 (Fig 1, B), a known potent activator of human NK cells.²⁹ Three patients with autosomal dominant HIES caused by STAT3 mutation were studied to ensure that the deficiency in NK cell cytotoxicity was not simply a feature of the HIES clinical presentation.³⁰ In contrast to cells from patients with DOCK8 deficiency, those from the STAT3-deficient patients had normal NK cell cytotoxicity when evaluated as lytic units per NK cell (Fig 1, A). Cell from the STAT3-deficient patients also robustly responded to IL-2 with increased K562 cell killing (Fig 1, B). Thus NK cell cytotoxicity was deficient in *ex vivo* cells from patients with DOCK8 deficiency and could not be corrected by cytokine simulation.

NK cell numbers and F-actin content are normal in DOCK8-deficient patients

Because our DOCK8-deficient patients had reduced NK cell cytotoxicity, we evaluated the presence of NK cells among PBMCs because reduction in activity could result from an overall decrease in NK cell numbers. Historically, reports of DOCK8 deficiency did not identify quantitative deficits in NK cells.^{1,3,4} CD56⁺CD3⁺ lymphocytes were measured in *ex vivo* blood samples to more accurately evaluate the patients under study in this cohort. Although the percentage of NK cells within patients' PBMCs was variable, in aggregate, they were statistically indistinguishable from our control subjects (Fig 2) and fell within the range of age-specific published normal values.³¹ The STAT3 disease control cohort was also similar to the DOCK8-deficient patients with regard to the size of the NK cell population. Thus it is unlikely that the decrease in NK cell cytotoxicity we observed in DOCK8-deficient patient cells was a pure feature of quantitative abnormalities in NK cells.

Given that NK cells were present in DOCK8-deficient patients, we next considered other activities of DOCK8 downstream targets. We focused on Cdc42, which is required for the induction

of WASp and is essential for the maintenance of F-actin content, as well as focused F-actin accumulation at the lytic synapse in NK cells.^{22,32} In this light, patients with WAS are defective in NK cell cytotoxicity but have normal NK cell percentages.²¹ To determine whether DOCK8 deficiency might represent a functional phenocopy of WAS, we first evaluated total NK cell F-actin content using flow cytometry. Surprisingly, F-actin content in NK cells from DOCK8-deficient patients was statistically indistinguishable from that seen in control donors, as well as from that in NK cells from STAT3-deficient patients (Fig 3). There was some variability between individual patients, but again, this is in contrast to what has been observed in NK cells from patients with WAS.^{21,33} Thus total F-actin content in NK cell from DOCK8-deficient patients was normal.

Lytic NK cell immunologic synapse is abnormal in DOCK8-deficient patients because of impaired synaptic F-actin accumulation and granule polarization

Although WASp is needed for maintenance of F-actin content, as well as its reorganization in NK cells, the role of Cdc42 is more specific to activation-induced WASp function.²⁰ If DOCK8 functions as a Cdc42 activator in NK cells, its deficiency might result in an inability to target F-actin accumulation in a WASp-dependent manner. Thus we evaluated the NK cell lytic IS because F-actin is known to accumulate at this site after activation. To obtain the most direct correlate to the patients, we isolated NK cells from *ex vivo* PBMCs using negative selection and used them immediately in experiments. NK cells were allowed to conjugate with K562 target cells for 30 minutes and then fixed and evaluated for the presence of F-actin by means of confocal fluorescence microscopy. We also evaluated conjugates for the presence of β_2 -integrin (CD18); perforin, the pore-forming component of NK cell lytic granules; and pericentrin, the epicenter of the microtubule-organizing center (MTOC). CD18 was studied because it typically clusters at the lytic IS but is dependent on WASp function for redistribution.²⁰ Perforin and MTOC localization were considered because by 30 minutes of activation, they typically polarize toward the target cell in a WASp-dependent manner.³²

Control donor NK cells demonstrated robust accumulation of F-actin at the lytic IS, as well as clustering of CD18 and polarization of both perforin and pericentrin (Fig 4, A). The IS formed similarly in NK cells isolated from a STAT3-deficient patient with noted F-actin accumulation, CD18 clustering, and perforin/pericentrin polarization. However, NK cells from DOCK8-deficient patients appeared quite different. Although they demonstrated clear F-actin in the cell cortex, they did not show accumulation of F-actin at the IS. Similar to what we had observed in NK cells from a patient with WAS or NK cells in which F-actin had been inhibited,^{22,32} NK cells from DOCK8-deficient patients also did not cluster CD18 or polarize perforin and the MTOC to the IS.

Multiple conjugates were measured from control donors, 3 individual STAT3-deficient patients and 4 DOCK8-deficient patients, to quantitatively evaluate these differences. Using a previously developed quantitative algorithm for F-actin accumulation,²⁸ we found that NK cells from DOCK8-deficient patients had statistically decreased synaptic F-actin relative to that in NK cells from control donors or STAT3-deficient patients (Fig 4, B). Furthermore NK cells from DOCK8-deficient patients did not

TABLE I. DOCK8-deficient patient cohort

	DOCK8-1	DOCK8-2	DOCK8-3	DOCK8-4	DOCK8-5
Age at evaluation	13 y	12 y	6 y	17 y	18 y
Sex	F	M	M	F	F
Mutation	Homozygous deletion upstream of exon 1 including exon 26	Homozygous deletion upstream of exon 1 including exon 2	Homozygous deletion (2 bp) in exon 8: c850_851 delCT; pL284fsX293	Homozygous splice donor site mutation: c3120+1 g>t (exon 25+1 GTA>TTA; on cDNA: exon 25 skipped)	Homozygous splice donor site mutation: c3120+1 g>t (exon 25+1 GTA>TTA; on cDNA: exon 25 skipped)
IgE levels (IU/mL)	10,970	35,720	2,303	25,987	62,429
Eosinophils (cells/ μ L)	8,968	4,365	2,635	11,033	693
Eczema	Yes	Yes	Yes	Yes	Yes
Viral infections	HSV, MCV	HSV	HSV, MCV	VZV, HSV	none
Bacterial infections	Recurrent pneumonia, skin abscesses	Pneumonia, skin abscesses	Recurrent pneumonia, skin abscesses	Recurrent pneumonia, skin abscesses	Pneumonia, skin abscesses
Fungal infections	No	Skin	CMC	CMC	Skin
Autoimmunity	No	No	No	Hemolytic anemia	No
Asthma	Yes	Yes	No	Yes	No
Food allergies	Yes	Yes	Yes	Yes	Yes
Malignancy	No	No	No	No	No
Previously published	No	No	No	Yes	Yes

BKV, BK virus; CMC, cutaneous mucosal candidiasis; F, female; HPV, human papillomavirus; HSV, herpes simplex virus; M, male; MCV, molluscum contagiosum virus; NA, not available; SCC, squamous cell carcinoma; VZV, varicella zoster virus.

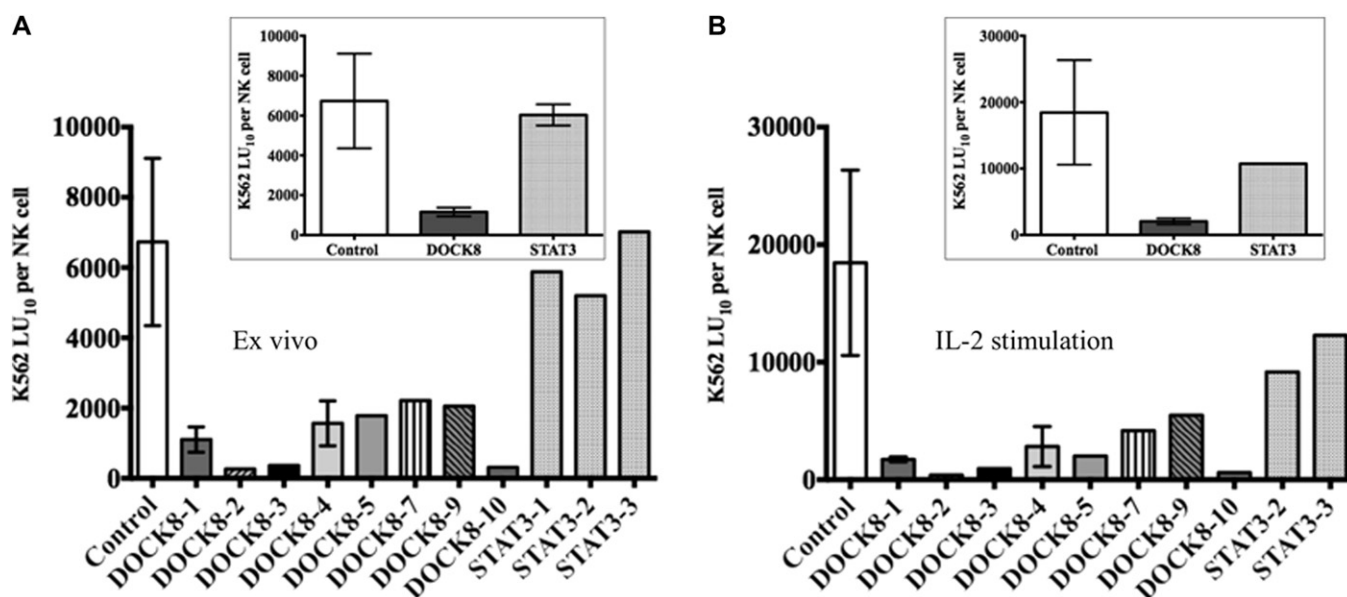


FIG 1. DOCK8 deficiency impairs NK cell cytotoxicity, which is not restored by IL-2. Control donor PBMCs ($n = 15$) and PBMCs from DOCK8-deficient and STAT3 mutant patients were used in chromium-release assays *ex vivo* without (**A**) or with (**B**) 4 hours of stimulation with 1000 U of IL-2. Cytotoxic function was expressed as lytic units (LU)₁₀/NK cell. Error bars represent means $\pm$ SEMs for patients analyzed 3 times.

demonstrate any statistically significant synaptic F-actin accumulation. The same algorithm for F-actin accumulation was also applied to CD18 accumulation and similarly demonstrated that NK cells from DOCK8-deficient patients, but not those from control donors or STAT3-deficient patients, had decreased synaptic CD18 levels (Fig 4, C). The distance of pericentrin to the synapse was measured directly and was significantly greater, on average,

in NK cells from DOCK8-deficient patients compared with those from control donors or STAT3-deficient patients (Fig 4, D). This demonstrates that cells from the DOCK8-deficient patients did not polarize their lytic machinery, which is a prerequisite for cytotoxicity. Overall, these quantitative analyses define abnormal focal F-actin accumulation at the NK cell synapse and aberrant overall synapse maturity in cells from DOCK8-deficient patients.

TABLE I. (Continued)

DOCK8-6	DOCK8-7	DOCK8-8	DOCK8-9	DOCK8-10
3 y F	8 y F	26 y M	17 mo F	12 y F
Homozygous deletion (2 bp) in exon 8: c850_851 delCT; pL284fsX293	Homozygous deletions 9p24.3 323,819 324,708	Heterozygous deletion g(371, 489_380, 404)_ (462,145_468,814) del plus 1266delC	c.(1-?_404+?del)+(1-?_404+?del) (deletion of exons 1-5)	Homozygous deletion of exons 22-25; heterozygous deletion of exon 3-21 and 26-32
38,908 12,096	NA NA	1,162 260	1,143 430	6,270 1,100
Yes	Yes	No	Yes	Yes
MCV	CMV, BKV	HSV, HPV	HSV, VZV, MCV	HPV, MCV
Recurrent pneumonia, skin abscesses	Chronic salmonella and recurrent sinopulmonary infections, skin abscesses	Recurrent sinopulmonary infections	Recurrent sinopulmonary infections	Meningitis, bacteremia
Skin	No	No	No	Skin
No	Sclerosing cholangitis	No	No	Vasculopathy
No	Yes	No	No	Yes
Yes	Yes	Yes	Yes	Yes
No	No	SCC	No	No
No	No	Yes	Yes	No

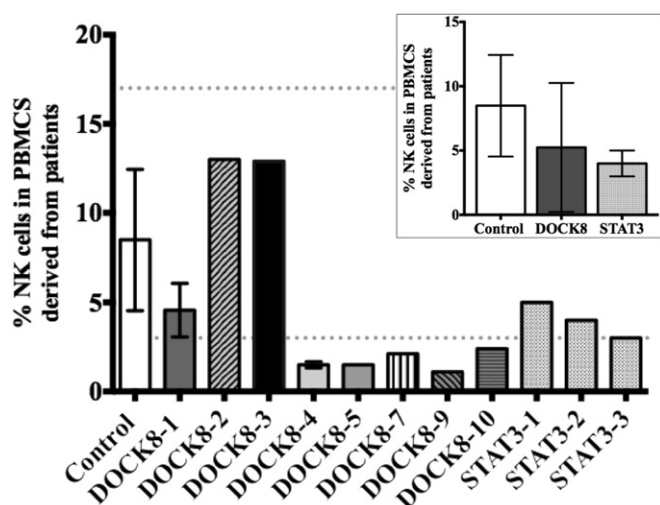


FIG 2. DOCK8-deficient patients have NK cells. Results of flow cytometric analysis of the percentage of CD56⁺CD3⁺ cells in PBMCs are shown. Dotted lines display ranges for the control donors ($n = 15$). Error bars represent means $\pm$ SDs for patients analyzed 3 times. The inset displays the mean NK cell percentages for DOCK8-deficient patients compared with control donors and STAT3-deficient patients.

Stable knockdown of DOCK8 with shRNA in NK cells recapitulates the patient's phenotype

The observations in NK cells from DOCK8-deficient patients suggest a role for DOCK8 in normal cytolytic function through the promotion of F-actin accumulation at the IS in NK cells. To address this more directly, we modeled DOCK8 deficiency in human NK cells *in vitro*. Specifically, we used the human YTS NK cell line and transduced shRNA expression constructs targeting DOCK8 packaged in lentiviruses. As controls, we used a

scrambled DOCK8 shRNA sequence, as well as a vector containing no shRNA sequence at all (empty vector). Transduced cells were selected through puromycin resistance and evaluated in cytotoxicity assays against the 721.221 lymphoblastoid cell line. Parental unmanipulated YTS cells killed 721.221 target cells effectively and similarly to those containing empty vector or scrambled shRNA. In contrast, YTS cells stably expressing the DOCK8 shRNA had drastically reduced cytotoxicity at all effector/target cell ratios (Fig 5, A). Real-time PCR was performed and demonstrated that YTS cells containing the specific shRNA had a greater than 50% reduction in DOCK8 mRNA compared with all control cells to confirm the effectiveness of the DOCK8 shRNA (Fig 5, B). Thus DOCK8 is required for NK cell cytotoxicity.

DOCK8 knockdown cell line conjugates between YTS and susceptible KT86 target cells were evaluated to further elucidate the proposed mechanism for DOCK8 in enabling NK cytotoxicity raised by our study of NK cells from DOCK8-deficient patients. In these experiments KT86 target cells were used because of a lower cortical actin content in this cell line. As is characteristic for a mature lytic synapse, YTS cells expressing the scrambled shRNA accumulated F-actin at the synapse after 30 minutes of conjugation with targets (Fig 6). These control cells also demonstrated accumulation of CD18 at and polarization of perforin to the IS. In contrast, cells containing the DOCK8 shRNA did not accumulate F-actin at the IS and, similar to cells from DOCK8-deficient patients, did not appear to have reduced overall cortical F-actin content. The decrease in F-actin accumulation in the DOCK8 shRNA-containing cells relative to those containing the scrambled shRNA was statistically significant across multiple synapses (DOCK8 shRNA mean actin accumulation = -77 ± 668 , scrambled mean actin accumulation = 1438 ± 603 , $P < .05$). DOCK8 shRNA-expressing YTS cells also did not accumulate CD18 at or polarize perforin to the lytic synapse. Therefore through specific and

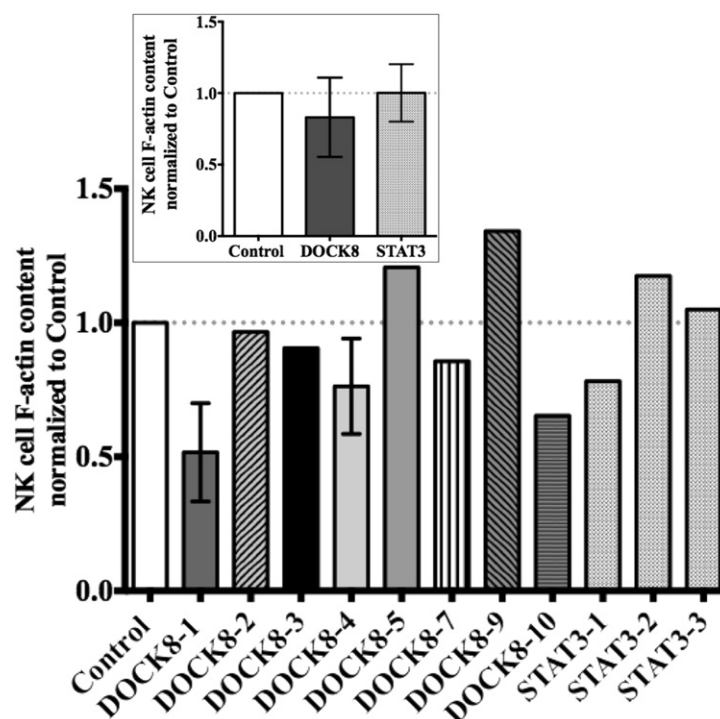


FIG 3. F-actin content is normal in DOCK8-deficient patients. Flow cytometry was performed for F-actin content within CD56⁺CD3⁺ PBMCs from patients and control subjects ($n = 15$). Values were normalized to each specific control used. Error bars represent means $\pm$ SDs for patients analyzed 3 times. The inset displays the mean NK cell F-actin content for DOCK8-deficient patients compared with control subjects and STAT3-deficient patients.

targeted DOCK8 knockdown, we recapitulated the mechanistic observations derived from cell from DOCK8-deficient patients. Specifically, DOCK8 is required in NK cells for synaptic activation-induced F-actin accumulation and subsequent intracellular events needed in access to cytolytic function. This suggests that DOCK8 is an upstream regulator of the activation-induced actin reorganization machinery in NK cells, which is required for their ability to effectively participate in host defense functions.

DISCUSSION

Human DOCK8 deficiency is a relatively severe primary immunodeficiency characterized by susceptibility to infections and immune dysregulation. The immunologic effect of DOCK8 deficiency has been documented in both the innate and adaptive arms of the immune system.^{19,34} Given the susceptibility to herpesviruses and papillomaviruses in affected patients, we extended previous assessment of DOCK8-deficient patients to include NK cell function.^{1,3,4} The reason for this is that the primary immunodeficiencies known to affect NK cell function share susceptibility to these infectious agents as a common feature.^{12,13} Genetic immunodeficiencies resulting in NK cell defects are also associated with malignancies, presumably because NK cells are held to serve an important role in tumor surveillance.^{14,15} Aside from the occurrence of recalcitrant viral infections, DOCK8-deficient patients also have susceptibility to cancers, which in this case has been hypothesized to be linked to viral infections.²³

Initial evaluations of NK cells in human DOCK8 deficiency have been limited to flow cytometric quantification.^{3,4} In DOCK8-deficient mice NK cells have similarly only been

quantified.⁸ Thus we decided to approach NK cells in DOCK8-deficient patients from a functional perspective. Because there are numerous differences between murine and human NK cells, including lack of *ex vivo* cytotoxicity in mouse NK cells,³⁵ we opted to focus our efforts on patients and *in vitro* human NK cell modeling. Our results agreed with those previously published in that NK cells were present in patients' peripheral blood,^{3,4} although at variable percentages, but herein we identify a pervasive functional deficiency regarding cytotoxicity.

There are a variety of potential explanations for functional NK cell deficiency in DOCK8-deficient patients. In other immune cells, cell-cycle, proliferative, and even migratory defects have been identified.^{5-7,19} The presence of mature NK cells in the peripheral blood of our cohort of DOCK8-deficient patients that are CD56^{dim} and express perforin, however, suggests that NK cell development and proliferation are at least occurring. Thus we focused on the previously reported role of DOCK8 as a Cdc42 activator.¹⁹ Cdc42 has been described to serve a number of functions in lymphocytes and NK cells. For example, it is required for actin organization and MTOC polarization in both T cells and NK cells.^{18,27,36,37} Mature NK cells use Cdc42 to activate WASp and direct activation-induced actin reorganization to enable their spontaneous cytotoxic activity.²⁰ Although we identified an inability of DOCK8-deficient cells to accumulate F-actin at the IS, we were surprised to find normal overall F-actin content, thus distinguishing our patients from those with WAS. Thus an ability to generate actin filaments must be intact in NK cells from DOCK8-deficient patients, but the induction of actin reorganization after receptor signaling is likely to be deficient. This is consistent with our observed defect in focused actin accumulation in both NK cells

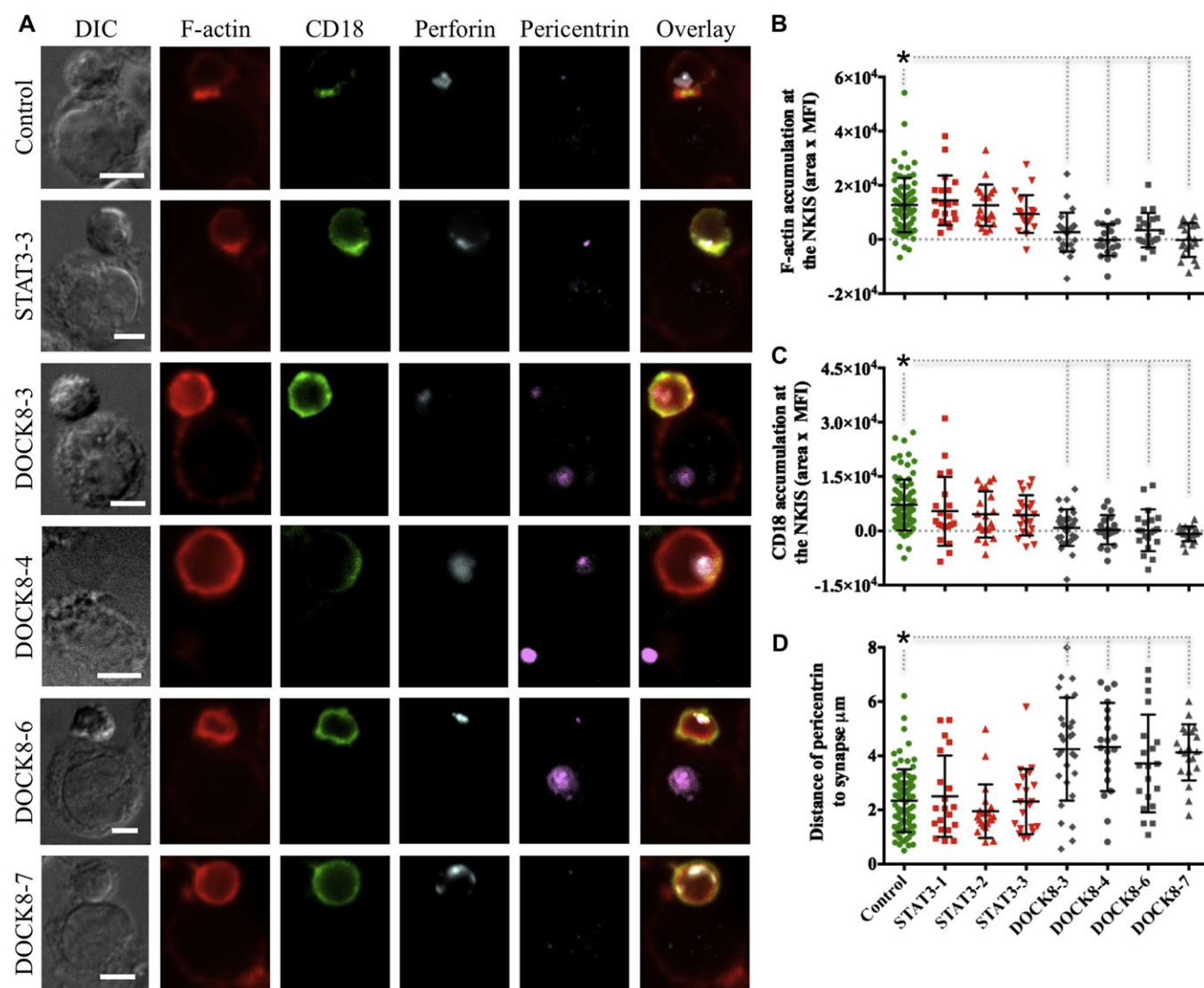


FIG 4. Abnormal F-actin accumulation at the DOCK8-deficient NK cell IS. **A**, Representative confocal immunofluorescent micrographs of *ex vivo* natural killer (eNK):K562 cell conjugates showing differential interference contrast (DIC) F-actin (red), CD18 (green), perforin (blue), pericentrin (pink), and an overlay. **B-D**, Measurement of F-actin accumulation (Fig 4, B) at, CD18 accumulation (Fig 4, C) at, and MTOC polarization (Fig 4, D) to the IS. Points represent single cell values. Error bars represent means $\pm$ SDs. * $P < .05$.

from patients and DOCK8-knockdown NK cells. This suggests that DOCK8 is not required for maintenance of the filamentous actin network but for its activation-induced reorganization.

Unlike NK cells from patients with WAS, we found that the cytolytic activity of cells from DOCK8-deficient patients could not be restored by IL-2 stimulation. We also attempted classical human NK cell bulk culture and expansion according to established protocols (which rely on IL-2)²⁵ with NK cells derived from DOCK8-deficient patients on 3 separate occasions. Unlike patients with WAS, we were unable to expand NK cells from the 3 DOCK8-deficient patients for whom this was attempted. Because we have previously defined that IL-2 can bypass WASp and use the homologous actin effector WAVE2 to productively reorganize actin,²² the lack of IL-2 response in NK cells from patients can be telling. Specifically, this observation suggests that DOCK8 might serve as a more general and upstream

activator of actin reorganization machinery. This could be due to multiple pathways dependent on Cdc42 but might also suggest that DOCK8 lies upstream of other Rho GTPases necessary for inducing actin branching complexes. Further experiments will be needed to define a potential role for DOCK8 as a master regulator of activation-induced actin function.

The present observations also provide further insight into the overall regulation of the NK cell lytic IS and subsequent access to cytotoxicity. Previously, F-actin was shown to be required for lytic granule polarization and integrin clustering after synapse formation.^{21,22} Specifically, the use of actin-depolymerizing agents that deplete the cells of cortical filaments altogether blocks both. NK cells from WASp-deficient patients had a defect akin to NK cells treated with chemical inhibitors but could not be used to distinguish any role for actin accumulation because they have an overall reduced F-actin content. However, DOCK8 deficiency

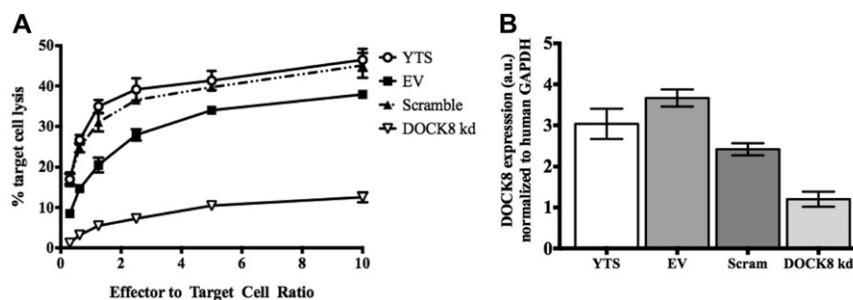


FIG 5. Stable knockdown of DOCK8 inhibits NK cell cytotoxicity. **A**, NK cell lines (YTS) transduced with empty vector (EV), shRNA targeting DOCK8 (*DOCK8 kd*), or a scramble sequence were used in chromium-release cytotoxicity assays. **B**, DOCK8 expression measured by using real-time PCR normalized to human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression. Experiments were performed in triplicate, and representative graphs are depicted.

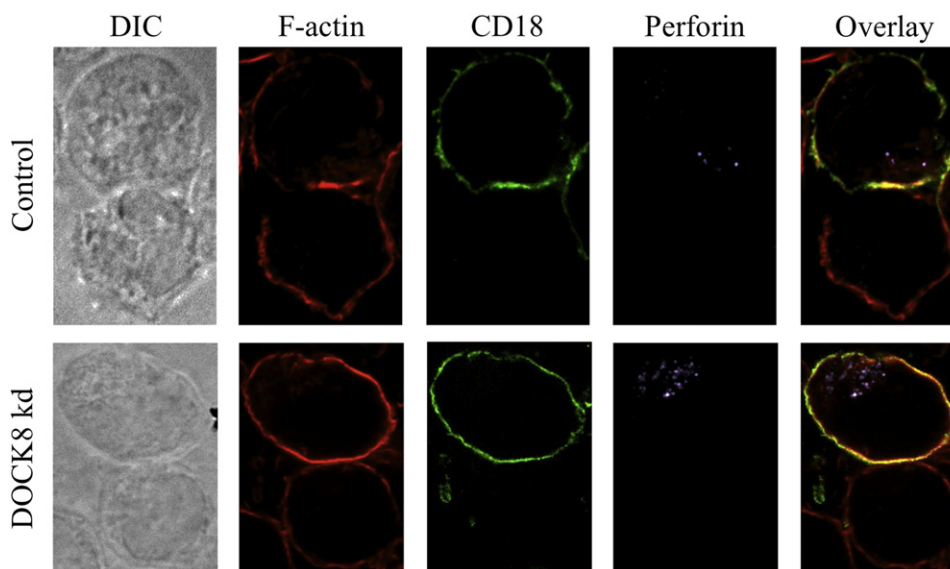


FIG 6. DOCK8 knockdown abrogates synaptic F-actin accumulation and granule polarization. Representative confocal immunofluorescence micrographs of conjugates between YTS cells containing DOCK8-targeting or control scrambled shRNA and KT86 target cells are shown. Differential interference contrast (DIC) with localization of F-actin (red), CD18 (green), perforin (pink), and an overlay are shown.

suggests that it is the targeted reorganization of F-actin at the IS that is needed for both CD18 clustering and, ultimately, lytic granule polarization.^{32,38} This is likely to be a feature of the physical movement of the receptor in the case of CD18 and resulting amplification of cell signaling in the case of granule polarization. The study of DOCK8-deficient cells therefore advances these basic immunologic mechanisms as likely reliant on synaptic actin accumulation.

Our observations add a pervasive deficiency in NK cell cytotoxicity to the clinical syndrome resulting from DOCK8 deficiency. Although IL-2 does not induce function, other potentially therapeutic strategies for accessing NK cell function from patients' cells should be explored. This would be of potential value to patients given the severe consequences of viral infections in affected subjects. However, we hypothesize that NK cell cytotoxicity will be difficult to access because of the upstream role of DOCK8. This suggests that other therapeutic options would more likely be appropriate in patients with DOCK8 deficiency. It is unclear whether gene-corrected NK cells will

have a proliferative advantage, but if there is at least some parallel to WAS, DOCK8 gene therapy might be advantageous. Because DOCK8 is expressed outside of the hematopoietic system, it is unclear whether immune reconstitution will solve all clinical issues that have been observed in patients. Regardless, our results underscore the extensive immunodeficiency in DOCK8 deficiency and reiterate a clinical need and rationale for hematopoietic transplantation. Further experience will determine whether the reconstitution of NK cell defenses in DOCK8-deficient patients can provide increased resistance against cancers and viral infections. Overall, our work defines an essential role for DOCK8 in human NK cell cytotoxic function and demonstrates a specific mechanism by which it enables activation-induced immune function.

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Key messages

- NK cells are functionally impaired in patients with DOCK8 deficiency, which is consistent with patients' infectious phenotypes.
- DOCK8 deficiency prevents F-actin accumulation at the NK cell lytic immunologic synapse without reducing overall F-actin content.

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