Identification and Functional Analysis of Novel DOCK8 Mutations

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This Thesis, The Identification and Functional Analysis of Novel DOCK8 Mutations, presented by Dansu Yuan, and Submitted to the Faculty of The Harvard Medical School in Partial Fulfillment of the Requirements for the Degree of Master of Medical Sciences in Immunology has been read and approved by:

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Date: Apr 10, 2019
Identification and Functional Analysis of Novel DOCK8 Mutations

Dansu Yuan

A Thesis Submitted to the Faculty of

The Harvard Medical School

in Partial Fulfillment of the Requirements

for the Degree of Master of Medical Sciences in Immunology

Harvard University

Boston, Massachusetts.

May, 2019
DOCK8, the gene encoding the Dedicator of Cytokinesis 8, was first identified in 2004. DOCK8 is a member of the DOCK family of proteins, found most abundantly in cells of the immune system. DOCK8 has pleiotropic functions, including role in lymphocyte migration, cytoskeleton organization, helper T cells differentiation and regulatory T cell suppression. DOCK8 deficiency is an autosomal-recessive combined immunodeficiency associated with recurrent infections, hyper IgE syndrome, autoimmunity, eczema and food allergy.

Most patients with DOCK8 deficiency lack protein expression (null protein). Here, we identified two patients with missense point mutations on their DOCK8 gene (c.2882 A>G, p.H962R; c.3460 C>T, p.R1154C) that gave rise to typical phenotypes of DOCK8 null deficiency but normal level of DOCK8 protein expression. Using these two mutations, we sought to explore more aspects and features of DOCK8’s functions in the immune system.

In recent years, DOCK8 has been reported presenting in nucleus. As DOCK8 is conventionally described as a cytosolic protein, it is meaningful to explore the specific functions that DOCK8 is executing in nucleus as well as the mechanism of this nuclear translocation. We speculated that a sequence on DOCK8 protein might be responsible for this transporting process which we named as nuclear localization signal (NLS), we generated the vector expressing mutant DOCK8 carrying NLS deletion to test this speculation and study the specific influence that NLS deletion might bring to the normal functionality of DOCK8 protein.
In this project, we first need to confirm the relevance of these two mutations using site-directed mutagenesis on full-length DOCK8 plasmids. Second, we would like to determine the mechanisms by which these mutations (the two missense mutations and NLS deletion) act to disrupt DOCK8 functions.

To achieve these goal, we generated expression vectors encoding the human DOCK8 proteins, either fused with GFP or Myc tag at their C-termini. Vectors encoding either wild type or mutant DOCK8 proteins would be transfected into DOCK8-deficient Jurkat cells (leukemia T cells) or HEK293 cells (human embryonic kidney cells). Using the transfected cells, we performed in vitro experiments to observe the changes brought by these mutations on DOCK8 functionality. GFP-fused DOCK8 is used for immunofluorescent assays, including confocal microscopy to see DOCK8’s location. Myc-fused DOCK8 is used for blotting assays, including co-immunoprecipitation to see the proteins interacting with DOCK8.

Our results suggest that the mutation p.H962R impairs the nuclear translocation of DOCK8 protein. In nucleus, DOCK8 is associated with the gene of SOCS3, which is an inhibitor of STAT3 activation. There is no obvious difference between the STAT3 phosphorylation facilitated by DOCK8^{WT} and DOCK8^{H962R}. The patient carrying p.H962R mutation expresses comparable level of IL-17 comparing to the shipping healthy control. Overall, it is clear that the nuclear translocation of DOCK8 is impacted by the mutation p.H962R. However, the effects of this mutation on STAT3 function and Th17 cell differentiation remain unclear. The mechanism of DOCK8^{H962R} causing disease still needs further investigation to be elucidated.

As reflected by the severe abnormal phenotypes displayed by deficient patients, DOCK8 plays a role in multiple important immune processes relevant to allergic and autoimmune diseases.
Nevertheless, the full spectrum of signaling pathways that DOCK8 links to still remains largely unknown. Through this research, we sought to map the structural and functional features of DOCK8 in regulating the immune response. Our study could help establish the structural and functional attributes of DOCK8 relevant to different clinical phenotypes of DOCK8-deficient patients, also enable precision therapeutic approaches in the treatment of relevant allergic and autoimmune diseases.

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Acknowledgements

I would first like to thank my thesis advisor, Dr. Talal Chatila, for consistently mentoring and supporting me on my way of pursuing science as professional career. Dr. Chatila has always let me have enough chance and freedom to become an independent researcher, meanwhile he is more than willingly to give me constructive advice and kindly offer any help that I need to get the works in paper moved forward.

I very much appreciate the guidance from my advising postdoctoral fellows, Dr. Louis-Marie Charbonnier, Dr. Chiaki Iwamura and Dr. Ye Cui. They mentored me with great patience and efficiency on troubleshooting, practical skill learning and assay design. The aspects that they have given me helps in span over from the most general context of the whole project to the slightest details of a single experiment. The assays in this paper would not be successfully conducted without their careful thoughts and professional supports.

I am also thankful to the other young researcher working on the DOCK8 project in the Chatila lab, Ms. Helin Tercan. I gained a considerable amount of helpful suggestions from her highly professional opinions during our collaboration and communication. The mental supports she kindly provided me with is another significant factor that has facilitated me to move on.

At last I want to express my gratitude to the MMSc Immunology program. Dr. Shiv Pillai and Dr. Michael Carroll consistently offered me kind instruction through my research period. I received a lot of practical guidance and warm support from the previous curriculum fellow Dr. Diane Lam. The program manager, Mrs. Selina Sarmiento, provided me with countless help on both my study
and my life, as well as my classmates. Having been a member in this program will always be a
great pleasure to me.
Chapter 1, Background

1.1 Dedicator of cytokinesis (DOCK) protein family

Dedicator of cytokinesis is a large protein family that closely related to intracellular signaling (Fig.1). Generally, they function as a guanine exchange factor to exchange the GDP into GTP, thus activate their targeting/interacting molecules including small GTPase such as Rac and CDC42\textsuperscript{1,2,3}. Among all the protein members, the deficiency of DOCK2 and DOCK8 have been reported as severely disease-causing and lethal, though both rare, there have been much more cases of DOCK8 deficiency comparing to DOCK2 deficiency\textsuperscript{1,4,5}.

![Figure 1: Dedicator of cytokinesis protein family](image-url)
1.2 Major clinical manifestations of DOCK8 deficiency

DOCK8 is a protein mostly expressed in immune cells and involved in multiple functional signaling pathways, this protein was first isolated and characterized in 2004\(^6\). DOCK8 deficiency is an autosomal-recessive disease which is relatively prevalent in middle-eastern area, the majority of DOCK8-deficient patients are of Turkish or Arabic descent, where the populations have higher rate of consanguineous family\(^1,4,7,8\). Due to the impaired immune functions, DOCK8 deficient patients have weak defense to environmental microbes, thus usually present with recurrent cutaneous infections, especially viral infections\(^1,4,9,10,11\). Sinopulmonary and bronchial infections have also been seen in patients with DOCK8 deficiency, possibly causing permanent damages to the respiratory system due to the infections themselves or long-term intubation/ventilation treatments\(^9\).

Another major clinical feature of DOCK8 deficiency is elevated level of immunoglobulin E, often combined with eosinophilia\(^7,9,12\). Literatures have shown that the differentiations of naïve T cells in DOCK8 deficient patients are commonly disrupted. These described patients tend to have abnormally more Th2 cell differentiation and present with severe type 2 immune response, also express higher level of IL-4 and IL-13\(^12,13,14\). In accordance to these, DOCK8 deficiency is also characterized with allergy, atopy, eczema and autoimmunity\(^15,16,17,18\). Lots of DOCK8 patients present with lymphopenia as well due to the imbalance of lymphocyte differentiation/development.
1.3 The genetics of DOCK8

![Chromosome 9 - NC_000009.12](image)

**Figure 2: The location of DOCK8 on chromosome**

The gene *DOCK8* was first identified in 2004\(^6\), it is a large gene located on chromosome 9, containing 48 exons that span over 200 kilobases\(^{1,7,11,13}\) (Fig.2). The major type of mutations happen on *DOCK8* are deletions varying from a size of small number of base pair to the large ones spanning the entire locus. Nonsense and splice site mutations have also been described. The majority of these mutations result in an absent expression of the protein, the patients either completely lack DOCK8 or only express severely truncated DOCK8 which is dysfunctional. Missense mutations rarely happen\(^{1,7,9}\). Some patients have somatic alterations that recover the expression of DOCK8 in T cell types but not the others and B cells. These somatic alterations could possibly be misleading during the molecular diagnosis of DOCK8 deficient patients\(^9\). Though some of the patients carrying them suffered less allergic syndromes, these somatic reversions do not ameliorate the risk and manifestations of viral infections, hematopoietic stem cell transplantation is still necessarily required for a long-term cure\(^{12,15}\).
1.4 Structure and the major discovered functions of DOCK8

1.4.1 DOCK8 and cytoskeleton rearrangement

DOCK8 is a large protein approximately 190 kDa in size. All members of DOCK family have 2 commonly distinguished domains known as DOCK homology regions (DHR)\(^2,8,10\). DHR-1 has been characterized as locating DOCK8 to cell membrane during its involved signaling pathways, while DHR-2 contains the catalytic subunit that actually execute the guanine exchange factor function of DOCK protein changing GDP into GTP. In this way, DOCK proteins activate the members of Ras homolog (Rho) gene family of guanine triphosphate binding proteins (GTPases).
Figure 4: DOCK8 protein structure

For DOCK8, the interacting member of Rho family is cell division cycle 42 (CDC42). Activated CDC42 subsequently integrates signaling from cell membrane upon stimulation to control the pathways involved in actin polymerization and cytoskeleton rearrangement, which are critical for immune cell migration towards secondary lymphoid organs and immuno-surveillance\textsuperscript{19,20,21}. CDC42 is not the only molecule that DOCK8 involved with during the signaling of actin polymerization and cytoskeleton rearrangement. It has been observed that the clinical phenotypes of Wiskott-Aldrich syndrome (WAS) overlap with the ones of DOCK8 deficiency, including susceptibility to viral, bacterial and fungal infections, allergic manifestations, autoimmunity and malignancy. WAS is associated with the mutations in WAS protein (WASp). The overlapped manifestations suggested that a common pathogenic mechanism of WAS and DOCK8 deficiency might be shared and that DOCK8 and WASp might be associated during specific signaling pathways. Literatures have shown that DOCK8 is bridged with WASp via WAS interacting protein (WIP) as a molecular complex upstream the occurrence of cytoskeleton rearrangement upon TCR ligation\textsuperscript{20,21}.

1.4.2 DOCK8 and helper T cell differentiation
The autosomal-recessive hyper IgE syndrome (AR-HIES) caused by the mutations in DOCK8 shares clinical phenotypes with the autosomal-dominant hyper IgE syndrome (AD-HIES) caused by the mutations in the gene of signal transducer and activator of transcription 3 (STAT3), demonstrating another important function of DOCK8 in intracellular signaling, activating STAT3. The STAT3 signaling upstream RAR-related orphan receptor gamma (RORgt) activation is known as significant for the expression of IL-17 and naïve T cell differentiation towards Th17 cells. Literatures have shown that DOCK8 deficient patients expressed conspicuously less IL-17 and showed lower count of Th17 cells compared to healthy controls. Upon the stimulation of IL-6 or IL-21, the T cell blasts from DOCK8 deficient patients presented with lower level of Y705-phosphor-STAT3 (pSTAT3). Also, the Jurkat T cells transfected with DOCK8-encoding vector showed higher level of STAT3 phosphorylation compared to the ones transfected with empty vector. DOCK8 facilitating STAT3 phosphorylation is a GEF-dependent process. This has been supported by the STAT3 phosphorylation analysis from a patient carrying rare missense mutation in DOCK8 (c. 5956A>T). The mutation does not affect the expression of DOCK8 protein, the patient showed comparable level of DOCK8 expression with healthy control. This mutation results in an amino acid change p.N1986Y, which is right in the DHR-2 domain where the catalytic subunit that executes DOCK8’s GEF function is included. While keeping the normal expression level of DOCK8 protein, this patient presented with phenotypes of DOCK8 null deficiency, she had recurrent upper and lower respiratory tract infections, mucocutaneous candidiasis, recurrent oral herpes infection and hematomegaly. Her isolated CD4⁺ T cells showed conspicuously lower level of pSTAT3 compared to healthy control upon IL-6 stimulation, indicating the requirement of GEF activity of DOCK8 in the process of facilitating STAT3 phosphorylation. When stimulating primary T cells from healthy subjects by IL-6, the level of pSTAT3 was elevated, but
the co-immunoprecipitated STAT3 with DOCK8 did not show obvious change, suggesting that the physical association of DOCK8 and STAT3 molecules is not necessarily involved in the process of STAT3 phosphorylation\(^\text{12}\).

1.4.3 DOCK8 in B cells

DOCK8 plays an important role in B cell differentiation and the formation of serological memory. Patients with DOCK8 deficiency presented with impacted antibody response to infections and had considerably lower count of CD19\(^+\)CD27\(^+\) memory B cells. It was then proved that DOCK8 functions as an adaptor linking TLR9-MyD88 signaling to B cell proliferation and differentiation via Pyk2-Src-Syk-STAT3 signaling cascade\(^\text{25}\). Upon CpG oligodeoxynucleotide (CpG ODN, later indicated as CpG, a ligand of Toll-like receptor 9) stimulation, the DOCK8-deficient patients showed conspicuously lower ability of B cell proliferation and immunoglobulin (IgM and IgG) production compared to healthy controls. STAT3-deficient patients with AD-HIES also showed impaired B cell proliferation and IgM, IgG production upon CpG stimulation, overlapping with the results obtained from DOCK8-deficient patients. Furthermore, the DOCK8-deficient patients showed undetectable pSTAT3 level in PBMCs upon CpG stimulation, and this impaired STAT3 phosphorylation could not be rescued by exogenous IL-6 added up to CpG. In another aspect, DOCK8-deficient patients had impaired CpG-driven tyrosine phosphorylation of protein Pyk2 and Src, indicated by the lower level of p-Pyk2 and p-Src upon CpG stimulation. Furthermore, TLR9-driven Pyk2 and Src phosphorylation was MyD88-dependent. Thus, it could be concluded that DOCK8 bridges the TLR9-MyD88 membrane signaling to Pyk2-Src-Syk-STAT3 cascade that subsequently leads to the occurrence of B cell proliferation and immunoglobulin production.
1.4.4 DOCK8 and regulatory T cells

The number and suppressive functionality of regulatory T cells are dependent on DOCK8. DOCK8-deficient patients have lower frequency of CD25^+CD127^{lo}FoxP3^+ T_{reg} cells comparing to healthy donors\textsuperscript{16}. Furthermore, the regulatory T cells isolated from DOCK8-deficient patients present with lower expression level of CD45RO comparing to healthy controls. Normally, central and effector memory T cells would gain the expression of CD45RO after antigen experience. Besides the lower cell number, DOCK8-deficient regulatory T cells also show impacted ability to suppress the proliferation of effector T cells from both DOCK8-deficient patients and healthy donor subjects. Furthermore, T_{eff} cells from DOCK8-deficient patients are normally suppressed by T_{reg} from healthy donors, indicating that DOCK8-deficient T_{eff} cells are not resistant to this suppression. These results suggest that the deficiency of DOCK8 severely impair the normal functionality of regulatory T cells.

1.5 Lacunae of DOCK8 structure, function and cellular distribution

1.5.1 DOCK8 in nucleus

Conventionally, DOCK8 has always been identified as a cytosolic functional protein, the majority of DOCK8’s revealed or elucidated roles and its relevant intracellular signaling exist and process in cytosplasm, especially closely involved with the change and activity of membrane. In recent years, it has been observed that DOCK8 also appears in nucleus with detectable amount. The
nuclear role of DOCK8 and its mechanism of nuclear translocation remain undetermined. We speculate that DOCK8’s nuclear distribution plays a regulatory role of gene expressions, also may serve as a promoting factor to the nuclear translocation of other functional molecules that interact with DOCK8, most possibly STAT3. Another speculation is that nuclear DOCK8 could interact with transcriptional factors or involved in some transcriptional mechanisms to control the differentiation of T lymphocytes. DOCK8-deficient T cells potentially have impaired differentiation to Th17 cells and abnormally polarized differentiation to Th2 cells, considering that the immediate determining process of T cell differentiation is on transcriptional level, nuclear DOCK8 may serve as an important factor to determine the fate of naïve T cells, especially in the commitment to Th17 and Th2 cells. The elucidation of DOCK8’s nuclear function and its nuclear translocation may contribute to reveal novel mechanisms of DOCK8 deficiency causing disease.

1.5.2 The mechanism of DOCK8-STAT3 interaction

Previous literature has shown that DOCK8 promotes STAT3 phosphorylation in a guanine exchange factor (GEF)-dependent manner, but physically associates with STAT3 in a GEF-independent manner\(^{12}\). To delineate the specific domain of DOCK8 interacting with STAT3 and the mechanism of this interaction is an important part of the future research of DOCK8 unknown function, as well as the relevance between this interaction and the downstream signaling that consequently lead to the final cytokine expression and T cell differentiation.
Chapter 2, Data and Methods

2.1 Short introduction

The protein decicator of cytokinesis 8 (DOCK8) is involved in multiple immune processes, including T cell migration, T helper (Th) cell differentiation and regulatory T cell suppression. DOCK8 promotes STAT3-dependent Th17 cell differentiation while restraining Th2 cell polarization \(^{23,24}\). Consequently, DOCK8 deficiency is associated with Th17 cell deficiency and an exaggerated Th2 cell response. DOCK8 deficient patients present with a hyper IgE syndrome-like phenotype with eosinophilia and severe type 2 immune response \(^{26}\). DOCK8 also plays an important role in immune synapse formation and actin cytoskeleton organization \(^{27}\). Accordingly, DOCK8-deficient patients have impaired lymphocyte activation and adhesion, and defective tissue migration \(^{28,29,30}\). These defects contribute to a clinical phenotype of recurrent infections, especially persistent cutaneous viral infections, as well as dysregulated immune responses.

Most patients with DOCK8 deficiency lack protein expression or express only severely truncated DOCK8 protein. Here, we analyzed 2 DOCK8-deficient patients with missense mutations (c.2882 A>G, p.H962R; c.3460 A>T, p.R1154C) that gave rise to classical DOCK8 deficient phenotypes in the face of normal protein expression (Fig. 5). Patient 1 carrying mutation p.H962R has lymphopenia; low count of CD19\(^+\)CD27\(^+\)IgM\(^+\)IgD\(^-\) memory B cells; human papilloma virus (HPV) infection; elevated IgE level. Patient 2 carrying mutation p.R1154C has cytomegalovirus (CMV) infection; recurrent eye cellulitis and herpes keratitis; elevated IgE level and eosinophilia. These rare missense point mutations offer an opportunity to specifically look into the unknown
acting pattern of DOCK8 protein and that new structural/functional domains on DOCK8 molecule might be discovered. We seek to confirm the pathogenicity of these 2 mutations, and explore the mechanisms by which these mutations act to disrupt the normal functionality of DOCK8 protein.

Figure 5: The information of the two DOCK8-deficient patients. a, the identification of 2 missense mutations on DOCK8 gene and DOCK8 protein. b, the expression of mutant DOCK8 in the two patients

To achieve these goals, we generated vectors carrying wild type DOCK8 cDNA, and used directed mutagenesis technique to create the same mutations with the 2 patients in DOCK8 cDNA. By reconstituting the WT and mutant DOCK8 cDNA into DOCK8-deficient cell lines, we obtain the relevant readouts that reflect the functions and signaling disturbed by these mutations (Fig. 6).
DOCK8 cDNA is tagged with either GFP or Myc-DDK expressing gene for different further experiments and analysis.

In recent years, DOCK8 has been found in nucleus. The potential roles of DOCK8 in nucleus and the mechanism of its nuclear translocation remain unclear. Our previous data showed that the deletion of a nucleus localization sequence (NLS) p.2029–2099 on DOCK8 abolish its nuclear presence. Apart from the plasmid of DOCK8<sup>H962R</sup> and DOCK8<sup>R1154C</sup>, we also generated the plasmid of DOCK8<sup>ΔNLS</sup>, on which we deleted the NLS sequence.

Figure 6: Generating mutant DOCK8 cDNA and transfection into DOCK8-deficient cell lines

2.2 Materials and Methods

2.2.1 Directed mutagenesis
We used DOCK8 (NM203447) human tagged ORF clone from Origene as the template for directed mutagenesis PCR and the expression plasmid for transfection. Directed mutagenesis was performed with Q5 Site-directed Mutagenesis Kit from New England Biolabs. Polymerase chain reaction is programmed as follow: $98^\circ C \text{ 2mins} > (98^\circ C \text{ 10 secs} > 72^\circ C \text{ 20 secs} > 72^\circ C \text{ 14 mins})_{25 \text{ cycles}} > 72^\circ C \text{ 2mins} > 4^\circ C \infty$. PCR product was transformed into competent *E.coli* C2987 (New England Biolabs) via heat shock at $52^\circ C$ for 40 secs. Transformed bacteria was then selected with lysogeny broth medium (LB) containing Kanamycin. After 12-16h overnight incubation, picked up 10 clones from each medium plate and isolated plasmid (QIAprep Spin Miniprep Kit, Qiagen) for sanger sequencing (performer: Eton Bioscience) to select the real positive clone carrying the desired point mutation. Overnight amplified the selected clone by shaking incubation at $37^\circ C$, then isolate plasmid (PureLink™ HiPure Plasmid Maxiprep Kit, Invitrogen) from the amplified culture to obtain high-concentration plasmid for subsequent transfection.

2.2.2.1 HEK293 cell transfection

For HEK293 cells, we perform the following steps to transfecxt with Lipofectamine 3000 reagent:

1) Seed HEK293 cells in 6-well plate one day before the transfection, 1~1.5M per well with DMEM medium containing 10% fetal bovine serum. The cells are supposed to be about 90% confluent before transferring.

2) Dilute 7.5uL of lipofectamine reagent in 125uL of Opti-MEM medium.
3) Dilute 5uL of P3000 reagent in 125uL of Opti-MEM and add 3ug plasmid.

4) Add the P3000-plasmid mixture in step 3) into diluted lipofectamine 3000 in step 2). Pipet to homogenize, incubate for 15 mins at room temperature.

5) Add the mixture of step 4) into previously seeded HEK293 cells, incubate for 20~24 hours for further experiments.

2.2.2.2 Jurkat cell transfection

For Jurkat cells, we have performed the several methods as the following steps to transfect WT/mutant DOCK8 plasmid.

2.2.2.2.1 Bio-Rad Gene Pulser Xcell™ Electroporation System:

1) Count 1M Jurkat cells and re-suspend in 250uL complete RPMI medium (1% non-essential amino acid, 1% sodium pyruvate, 1% penicillin/streptomycin, 10% FBS).

2) Add the cells and 30ug of plasmid into a Bio-Rad gene-pulser expression cuvette.

3) Electroporation at 250V, 960mA, 4mm.

4) Immediately transfer the cell-plasmid mix into complete RPMI medium.

5) Incubate for 24~36 hours before proceeding to further experiments.

2.2.2.2.2 Lipofectamine 3000 reagent transfection: same with 2.2.1.

2.2.2.2.3 Neon transfection system:

1) Pre-warm non-antibiotics complete RPMI medium to 37 ° C in 48-well plate (250uL medium/well).
2) Count and transfer 1M Jurkat cells into Eppendorf. Cells are supposed to be 70~90% confluent before use.

3) Wash the cells with phosphate-buffered saline (PBS).

4) Re-suspend the cells in proper amount of buffer R from Neon Transfection System Kit (Thermo Fisher Scientific), the final cell density is supposed to be $2 \times 10^7$/mL.

5) Electroporation at 1350 V, 3 pulses (each lasts for 10ms).

6) Immediately pipet the cells into pre-warmed non-antibiotics complete RPMI medium.

7) Incubate at 37°C for 24 hours before further analysis.

2.2.3 Cell lysis and immuno-blotting assays

2.2.3.1 HEK293 cell lysis

1) Aspirate and discard the medium.

2) Detach and wash the cells twice with PBS.

3) Re-suspend the cells in mix of 10uL 10× protease inhibitor and 90uL lysis/binding/wash buffer (Thermo Fisher Scientific). Vortex for 10 secs then incubate on ice for 10 mins.

4) Spin at 14000g × 10mins at 4°C. Transfer the supernatant into a clean pre-chilled Eppendorf (samples for protein concentration test).

5) Test the protein concentration of each sample using Bradford assay. Calculate and dilute the samples to the same concentration. Proceed to western blot or immunoprecipitation.
2.2.3.2 Jurkat cell lysis and PBMC/T cell blast lysis

1) Spin to pellet the cells

2) Wash twice with PBS.

3)~5) Same with 2.3.1

2.2.3.3 Western blotting

1) Cast a 7.5% SDS-PAGE gel. Prepare 1L running buffer (diluted from 5× stock: 15.1g Tris-base, 94g glycine, 50mL of 10% SDS in a total volume of 1L), 1L transfer membrane buffer (diluted from 10× stock: 58.2g Tris-base, 144g glycine in a total volume of 1L). Pre-chill the transfer membrane buffer in 4°C fridge before use.

2) Apply the samples into gel, 15uL samples + 5uL 4× loading buffer per well. First run at 100V constantly, after the samples are out of the wells, increase the voltage to 180V and constantly run until the blue dye is just out of the gel.

3) Transfer the proteins from SDS-PAGE gel to a nitro-cellulose membrane in transfer membrane buffer, constantly run at 400mA for 2 hours.

4) Blocking the membrane in 4% bovine serum albumin (BSA)/TBST for 1 hour.

5) Properly dilute the primary antibody in 2% BSA/TBST and incubate with the membrane for proper time at proper temperature (time and temperature are dependent on the specific used antibody).
6) 5~10-min wash the membrane with TBST for 3 times.

7) Bind the secondary antibody on membrane as the same step in 5)

8) Treat the membrane with proper amount of HRP substrate and develop to see the results.

2.2.3.4 Nuclear & cytoplasmic fraction

NE-PER Nuclear and Cytoplasmic Extraction Reagents, Thermo Fisher Scientific

2.2.3.5 Immunoprecipitation

Dynabeads Protein G Immunoprecipitation Kit, Thermo Fisher Scientific

2.2.4 Intracellular cytokine staining

1) Stimulate the cells with PMA/Ionomycin/Golgi-plug at 37° C for 4 hours.

2) Wash the cells with FACS buffer (0.5% FBS/PBS).

3) Stain the surface molecules in FACS buffer for 30 mins at 4° C.

4) Wash the cells with cold FACS buffer.

5) Re-suspend the cells and incubate with fix/perm buffer for 30 mins at 4° C.

6) Wash the cells twice with cold permeabilization buffer.
7) Stain the intracellular molecules in permeabilization buffer overnight at 4°C.

8) Wash the cells with cold permeabilization buffer, then wash again with FACS buffer.

9) Re-suspend the cells in FACS buffer and analyze with flow-cytometry.

2.3 Results

2.3.1 Confirm the normal DOCK8 expression level in the patients

![Figure 7: DOCK8 expression in the 2 patients carrying DOCK8 missense mutation](image)

We assessed the DOCK8 expression in the two patients with missense point mutations and the shipping healthy controls. The result shows that these 2 patients express comparable levels of DOCK8 with shipping healthy controls, confirming that the mutations p.H962R and p.R1154C do not affect the normal expression of DOCK8.

2.3.2 Generating missense mutations on DOCK8 cDNA
We have successfully generated the mutation c.2882 A>G on both GFP- and Myc-tagged DOCK8 cDNA, the mutation c.3460 C>T on GFP-tagged DOCK8 cDNA.

2.3.3 Jurkat cell transfection with GFP-tagged DOCK8 cDNA

![Sanger sequencing result of the directed mutagenesis products](image)

**Figure 8: Sanger sequencing result of the directed mutagenesis products**
Since Jurkat cells are difficult to transfect and DOCK8 cDNA is of especially large size, we have tried several methods to get decent transfection efficiency usable for further experiments. So far, we got the relatively highest transfection efficiency using Neon electroporation system, but the problem is that the electroporation causes a high rate of cell death, we presently have not yet obtained enough cell survival to perform immuno-blotting assays. We are currently in the process of electroporation protocol optimization in search of a stably usable transfection efficiency.

2.3.4 The mutation p.H962R impairs the nuclear translocation of DOCK8 expressed in HEK293 cells

We used lipofectamine 3000 reagents to transfect HEK293 cells DOCK8 plasmids then performed cytosolic/nuclear fraction. The result shows that DOCK8<sup>H962R</sup> presents less in nucleus comparing to wild type DOCK8, suggesting that the mutation p.H962R impairs the nuclear translocation of DOCK8 protein.
2.3.5 The mutation p.H962R does not affect the Y705-phosphorylation of STAT3 in DOCK8-transfected HEK293 cells

![Image of Western Blot](image.png)

**Figure 11: The phosphorylation of STAT3 in DOCK8-transfected HEK293 cells**

We stimulated the DOCK8-transfected HEK293 cells with IL-6 (30min-stimulation), the result shows that the Y705-phosphorylation of STAT3 is not impaired by the p.H962R mutant DOCK8. However, HEK293 cells are originally sufficient to phosphorylate STAT3 upon the stimulation of IL-6, the negative control sample (cells transfected with no plasmid) shows strong signal of pSTAT3. Therefore, we are planning to repeat this experiment in STAT3-KO Jurkat cells co-transfected with DOCK8 and STAT3 plasmids after modifying the Neon electroporation protocol.

2.3.6 DOCK8\(^{H962R}\) associates with more STAT3 comparing to wild type DOCK8
We co-transfected HEK293 cells with HA-STAT3 (hemagglutinin-tagged STAT3) and DOCK8 plasmids then pulled down DOCK8 and assessed the STAT3 associating with DOCK8. The result shows that the DOCK8<sup>H962R</sup> associates with more STAT3 comparing to the wild type DOCK8, indicating that the mutation p.H962R increases the association of DOCK8 with STAT3.

2.3.7 The patient carrying DOCK8<sup>H962R</sup> produces abnormally high IL-17A
We stimulated the T cell blast of patient 1 (DOCK8$^{H962R}$) and patient 2 (DOCK8$^{R1154C}$) via PMA/ionomycin and stained intracellular IL-4, IL-17. The result shows that there is no obvious change on IL-4 and IL-17A expressions between patients and their matching healthy controls.

2.3.8 Nuclear DOCK8 binds to the gene of SOCS3 which is an inhibitor of STAT3 activation

![Figure 14: The ChIP result of DOCK8 pull-down with anti-Myc antibody (HEK293 cells transfected with Myc-tagged DOCK8 plasmid)](image)

In summary, our results suggest the following pathway: DOCK8 protein translocates into nucleus and binds to SOCS3, up-regulate the expression of SOCS3, which subsequently inhibits the activation of STAT3, leading to a restriction of IL-17A expression and Th17 cell differentiation.
The mutation p.H962R impairs the nuclear translocation of DOCK8, yet it does not have obvious effects on the phosphorylation of STAT3, the expression of IL-17A and Th17 cell differentiation. The mechanism of this missense mutation causing disease still needs further investigation to be convincingly elucidated.

2.4 Brief discussion

In this project, we confirmed the normal expression of DOCK8 in the 2 patients carrying missense point mutations. We currently have generated the mutation c.2882 A>G (p.H962R) on both GFP- and Myc-tagged DOCK8 cDNA, and the mutation c.3460 A>T (p.R1154C) on GFP-tagged DOCK8 cDNA. We showed that the mutation p.H962R has the following effects on DOCK8: 1) impairing the nuclear translocation of DOCK8; 2) increasing the association of DOCK8 with STAT3.

Overall, these results suggest that nuclear DOCK8 has the function of inhibiting the activity of STAT3 via binding and up-regulating the expression of SOCS3. The mutation p.H962R impairs the nuclear translocation of DOCK8, thus disrupts the up-regulation of SOCS3, yet the effects of this impaired nuclear translocation on STAT3 phosphorylation and the subsequent IL-17A expression remain unclear and need further study to be elucidated, as well as the mechanism of this mutation causing disease.
Chapter 3, Discussion and Perspectives

3.1 Limitations

In this project, the basic foundation to ensure the solidity of functional study results is the efficiency of plasmid transfection. All results presented in this paper was processed in HEK293 cells transfected via lipofectamine 3000 reagents. The advantage of using HEK293 cell lipofection is the stable good transfection efficiency. The major disadvantage of this method is that HEK293 cells are not immune cells. DOCK8 protein most importantly functions in the immune system and is mainly expressed in immune cells. The results of functional and phenotypic study obtained from HEK293 cells may not be absolutely reliable to reflect the real circumstance of DOCK8 in the immune system. This limitation should be considered in the STAT3 Y705 phosphorylation assay (2.3.6), it is apparent that HEK293 cells themselves are sufficient to phosphorylate STAT3 upon IL-6 stimulation without any DOCK8 protein (The negative control group went through the whole transfection process with no DOCK8 plasmid in the system, yet it presented with strong signal of Y705-phosphorylated STAT3). Ideally, this experiment is supposed to be done in STAT3-knock out Jurkat cells co-transfected with DOCK8 and STAT3 plasmids. First, this could avoid the disturbance of endogenous STAT3 phosphorylation; second, Jurkat cells are immune cells (leukemic T cells), they could better recapitulate the cellular environment of DOCK8 functioning in the immune system. Third, HEK293 cells are unavailable to study the molecular pathways involved with TCR signaling. As it was mentioned in the background chapter, one significant function of DOCK8 is activating CDC42 to initiate the CDC42-WasP-WIP signaling leading to actin polarization and cytoskeleton rearrangement, this whole pathway is triggered by TCR
stimulation via αCD3/CD28. This TCR involvement makes only Jurkat cells usable for the transfection assays for functional analysis.

One major hurdle of this project is the low cell survival through electroporation process while electroporation is the only method that could efficiently deliver the DOCK8 plasmid (15-kilobase in size) into Jurkat cells. We did get transfected living cells detectable by FACS, however, the cell number was not enough for the immune-blotting assays which took the majority part of this work. We speculate that the following factors might play a role in the high-rate cell death of electroporation. First, our plasmid is of an unusually large size, indicating that there is higher toxicity than common occasions. Thus, the pre-optimized parameter setting in the commercial manual may not be fitting to this specific transfection and needs to be further adjusted. Second, the Neon electroporation system is located in another building, the transporting process before and after the electroporation may cause extra distress to the cells, including the extra longer time that the cells have to be kept in the electroporation buffer which is slightly toxic, and the cold weather that the cells have to suffer when they are at an especially fragile stage after electroporation.

Second, since the electroporation is a transient transfection that the plasmids could only be shortly contained in the cells, thus it would be significant to generate a stable cell line containing the plasmids in nucleus. The Neon electroporation system is able to open the nuclear membrane thus occasionally enables the plasmids to get into nucleus, but the percentage of this nuclear delivery among all the plasmid individuals is uncertain. Thus, we have planned to achieve this stable cell line by consistently selecting transfected cells. We have tried two methods to process this selection:
antibiotic selection using G418 and FACS sorting. Neither of them worked. G418 (concentration ranged from 0 to 10mg/ml) failed to kill the non-transfected WT Jurkat cells; FACS selected cells were too few to be able to proliferate. Since it was not effective to obtain a stable cell line by electroporation and selection culture, we are planning to try lentiviral transduction, which has been considered as an efficient way to deliver large plasmid into nucleus. We have already purchased the commercial lentiviral vector inserted with human DOCK8 cDNA, we are currently in the process of generating the two missense mutations in this new plasmid. 

Third, due to the unsuccessful Jurkat cell transfection, the project currently lacks the study of mutant DOCK8 affecting actin polarization and cytoskeleton rearrangement, which is assessed by phalloidin staining followed with confocal microscopy analysis.

Fourth, before processing into any experiment using reconstitute model, more functional studies need to be done with the primary cells isolated from the patient to obtain the immediate evidence of pathogenic phenotype and integrate the characterization of manifestation. Before STAT3-relevant analysis, we should sort CD4 naïve T cells by FACS and treat the cells with Th17 differentiation cytokines including IL-6, IL-21 and TGF-β1, to see if the two patients really have affected Th17 development potential.

All blotting assays should be repeated for three times and have signal intensity quantified via software. This is another flaw of this work. Furthermore, more healthy control data is required in the cytokine expression experiment to convincingly indicate whether the DOCK8-deficient patients’ cytokine expressions are disrupted.
3.2 Future research

3.2.1 The influence on helper T cell differentiation caused by the mutations p.H962R and p.R1154C on DOCK8

We seek to study whether these two mutations would affect the potential of naïve T cells to differentiate into different helper T cell population. We plan to isolate the naïve T cells from the patients’ T cell blast via fluorescence-activated cell sorting (FACS), then respectively treat with different inducing cytokines, including IFNγ, IL-4 and IL-6/21, followed by assessing the expression of effector cytokines after the stimulation of αCD3/CD28.

3.2.2 Jurkat cell electroporation/transfection protocol modification

We plan to adapt the Jurkat cell electroporation in the following aspects. First, play around with the parameter settings. It has been demonstrated that the transfection efficiency of 15-kb plasmid into cancer cells could be significantly elevated by adjusting to a single high voltage pulse (1000V/cm, 100us) followed by a lower voltage (between 25~100V/cm) and long pulse duration (e.g. 10ms). We would first start from this setting as the adjustment. Second, we need to enhance the transportation condition as much as possible, including processing the assay when the outdoor temperature is proper and using insulated container for carry.

If the adjustment of electroporation fails to obtain stable and usable transfection efficiency and cell survival, another potential solution is switching to lentiviral transduction. We have purchased
the lentiviral vector inserted with DOCK8 cDNA. Currently, we are in the process of generating the two missense point mutations on the lentiviral DOCK8 plasmid.

3.2.3 Molecular experiments in Jurkat cells

First, all the molecular assays processed in HEK293 cells should be repeated in Jurkat cells after 3.2.2 is done, including DOCK8-STAT3 nuclear translocation and the interaction between them tested by immunoprecipitation.

Second, DOCK8-transfected Jurkat cells enable us to study the pathways stimulated upon TCR signaling, including the cytoskeleton rearrangement. After it is doable to stably and effectively transfect Jurkat cells, phalloidin staining should be processed to observe whether/how the actin polarization triggered by TCR ligation is disrupted with the presence of mutant DOCK8 protein.
Bibliography


