

ORIGINAL ARTICLE

Autoimmunity and Clinical Immunology

Quantitative profiling of cytokines and chemokines in DOCK8-deficient and atopic dermatitis patients

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Abstract

Background: Hyper-IgE syndromes (HIES) are a clinically overlapping, heterogeneous group of inborn errors of immunity characterized by elevated serum IgE level, eosinophilia, atopy, and immune dysregulation. Deficiency of DOCK8 protein is potentially a life-threatening autosomal recessive HIES and only curable with bone marrow transplantation. Hence, the diagnosis of DOCK8 deficiency is critical and should be sought at an early stage to initiate definitive therapy.

Methods: Serum samples from patients with DOCK8 deficiency and atopic dermatitis were profiled on a cytokine/chemokine panel for potential differential expression.

Results: CXCL10 and TNF- α were upregulated in DOCK8 patients when compared to AD, possibly contributing toward increased susceptibility to infections and cancer. In contrast, epidermal growth factor (EGF) was significantly downregulated in a subgroup of DOCK8-deficient and AD patients, while IL-31 expression was comparable between both DOCK8-deficient and AD cohorts, possibly contributing toward pruritus seen in both groups.

Conclusion: This comprehensive cytokine profile in HIES patients reveals distinctive biomarkers that differentiate between the DOCK8-deficient and AD patients. The unique expression profile of various inflammatory cytokines in patients with DOCK8 deficiency vs atopic dermatitis likely reflects disease-specific perturbations in multiple cellular processes and pathways leading to a predisposition to infections and allergies seen in these patients. These data agree with the role for EGF replacement therapy in EGF-deficient individuals with AD as well as DOCK8 deficiency through a potential shared pathway. In addition, these novel biomarkers may be potentially useful in distinguishing DOCK8 deficiency from AD allowing early-targeted treatment options.

KEYWORDS

atopic dermatitis, CXCL10, DOCK8, epidermal growth factor, hyper-IgE syndrome

Abbreviations: EGF, epidermal growth factor; EPAS1, endothelial PAS domain protein 1; GCSF, CSF3 is colony-stimulating factor3; HIES, hyper-IgE syndrome; IL-31, interleukin-31; MDC, macrophage-derived chemokine; OPLS-DA, orthogonal projections to latent structures discriminant analysis; PGM3, phosphoglucomutase 3; STAT3, signal transducer activator of transcription 3; TNF, tumor necrosis factor.

1 | INTRODUCTION

Hyper-IgE syndromes (HIES) are primary immunodeficiency disorders, characterized by elevated IgE levels, recurrent staphylococcal skin abscesses, eczema, and pulmonary infections.¹ They are genetically heterogeneous inherited syndromes, first described by Davis et al.(1966) and linked mainly to mutations in signal transducer activator of transcription 3 (*STAT3*) or dedicator of cytokinesis 8 (*DOCK8*), either in an autosomal dominant or recessive pattern, respectively.²⁻⁴ Recently, additional mutations in phosphoglucomutase 3 (*PGM3*), involved in the glycosylation pathway, and *IL6ST* have been identified in the autosomal recessive forms of HIES.^{5,6} In contrast to *STAT3* mutations, *DOCK8*-deficient patients have severe T-cell dysfunction associated with severe inflammation and allergic manifestations.^{7,8} Common pathogens include herpes simplex virus (HSV), human papillomavirus (HPV), molluscum contagiosum virus (MCV), and varicella-zoster virus (VZV). Susceptibility to recurrent sinopulmonary infections is also caused by a wide variety of pathogens including streptococcus pneumoniae, haemophilus influenzae, pneumocystis jirovecii, histoplasma capsulatum, and legionella pneumophila. Cancers related to cutaneous viral infections and T-cell lymphoma had been described in *DOCK8* deficiency which is thought to be related to impaired CD8 T-cell functions suggesting that *DOCK8* has a tumor suppressor function.⁹

DOCK8 is mainly expressed in cells of the immune system and controls many cellular processes including migration, phagocytosis, and adhesions.¹⁰ A study by Su Hu et al. (2010) emphasized the regulatory role of *DOCK8* on lymphocytes, where *DOCK8* is essential for the activity of these immune cells, and its absence leading to susceptibility to infection.⁸

Atopic dermatitis (AD) is a pervasive pediatric, chronic, highly pruritic inflammatory skin disorder, commonly associated with high IgE level. Eczematous skin lesions and pruritus are common overlapping clinical features in all subtypes of HIES and AD suggesting immune dysregulation. Through EPAS1-dependent activation of the IL-31 promoter, CD4+ T cells from *DOCK8*-deficient mice were recently shown to produce large quantities of IL-31, a significant pruritogen associated with AD.¹¹ It is speculated that the severity of infection and the skin hypersensitivity could be due to an altered cytokine production pattern in *DOCK8*-deficient and AD patients.

The clinical management of patients with moderate-severe AD and *DOCK8* deficiency can be challenging and quite complex. Patient and family education, avoidance of known contact, food and aeroallergens, controlling pruritis (using antihistamines) and proper skin hygiene and hydration using emollients and moisturizers are important first steps. Topical therapy include using corticosteroids of varying potencies, calcineurin inhibitors, phosphodiesterase 4 (PDE4) inhibitor (crisaborole). Acute exacerbations of chronic AD may require systemic corticosteroids. Phototherapy as well as immunosuppression (such as oral cyclosporin) is more frequently and regularly used in adults compared to children. More recently, systemic (subcutaneous) immunomodulation using dupilumab, an (IgG4) anti-IL-4 and IL-13 receptor blocker, became available as an alternative

option for patients with very difficult to control moderate to severe atopic dermatitis. Viral and bacterial cutaneous infections also require special attention and prompt management. Because *DOCK8* deficiency is a complex systemic disorder, these patients require additional supportive therapies and ultimately a definitive curative treatment with hematopoietic stem cell therapy (HSCT). Adequate nutritional support (to prevent failure to thrive and secondary nutritional deficiencies), pulmonary and liver function assessments and monitoring, prophylactic antibacterial, antiviral as well as antifungal (cryptosporidial infections) and immunoglobulin prophylaxis are frequently required. Due to the high morbidity and mortality associated with *DOCK8* deficiency, and as several clinical reports have demonstrated high survival, robust cellular and humoral immune reconstitution and improvements in both infections and atopy post-transplant, HSCT became the preferred definitive therapy for this disorder.¹²

Cytokines and their receptors play an essential role in the development, homeostasis as well as pathogenesis of tumors in HIES. High levels of IgE seen in these patients signal cytokine dysregulation and an imbalance in Th1/Th2 cytokine profile contributing toward the pathogenesis of the disease.¹³ Altered or dysregulated cytokine expression leads to pathologies such as inflammation, tumorigenesis, and autoimmunity. Patients with *DOCK8* deficiency have increased Th2 cells (expression of IL-4, IL-5, IL-6, IL-10, IL-13), which indicate its role in T-cell regulation of allergic disease.¹⁴ IL-31 produced by Th2 cells is involved in promoting dermatitis and epithelial responses that characterize allergic and nonallergic diseases.¹⁵ Over the past decade, several studies have led to the hypothesis that HIES might be a disease of impaired inflammatory response rather than a disease of pathologic IgE production. Elevated IgE levels, eczema, asthma, food, and environmental allergies are common to HIES and AD, although warts, severe bacterial and viral infections, and pneumonia are exclusively seen in *DOCK8*-deficient patients. Therefore, measurement of immune-related proteins in serum may help in characterizing the local inflammatory responses that develop during HIES pathogenesis.

Differentiating *DOCK8*-deficient patients from those with atopic diseases at an early stage is significant in a child's development, as treatment modalities differ considerably. In the current study, we generated a comprehensive cytokine/chemokine profile in both patient cohorts to identify a set of differentiating biomarkers which may reveal insight, linking these disorders with their clinical phenotypes thus facilitating improved understanding of disease pathogenesis and progression.

2 | METHODS

2.1 | Study design and ethics

Symptomatic patients with confirmed pathogenic *DOCK8* mutations ($n = 10$), moderate to severe AD ($n = 9$) diagnosed based on the Hanifin and Rajka clinical criteria,¹⁶ and healthy controls ($n = 15$) were enrolled in this study (Table 1). All recruited subjects attended the Allergy/Immunology Clinics at King Faisal Specialist Hospital and

TABLE 1 Laboratory findings in DOCK8 and AD patients

Diagnosis	Mutation	Age (Y)	Sex	IgE levels (KU/L)	RBC 10 ¹² /L	WBC 10 ⁹ /L	Eosinophils 10 ⁹ /L	CD4/CD8 Ratio	VAS	SCORAD
DOCK8										
P1	NM_203447.3:c.[2606-1G>A; c.405_827del]	21	F	25000	0.01	9.2	24.8	2.1	15	68.7
P2	NM_203447.3:c.[2606-1G>A; c.405_827del]	16	M	9140	4.17	3.72	35	0.8	13	67
P3	NM_203447.3:c.5625T>G;p.Y1875	16	F	1690	4.2	4.63	50.3	1	14	59.8
P4	NM_203447.3:c.827 + 6T>C	16	F	11240	5.9	4.9	11.4	0.73	15	77.4
P5	NM_203447.3:c.5962_6068del	7	M	10170	5.29	9.93	26.4	0.7	17	79.5
P6	NM_001193536:c.3949 + 1G>T	14	M	15550	6.82	4.46	11.7	1.3	15	62.5
P7	NM_001193536:c.1593 + 1G>T	4	M	86940	5.3	24.9	31.4	9	17	70.6
P8	NM_001193536:c.1593 + 1G>T	9	F	44630	5.26	12.49	34.8	8.1	9	58.2
P9	NM_001190458:c.1905_1905 + 1delGG	8	F	26340	3.81	21.42	35.1	3.2	14	58.8
P10	NM_203447.3:c.5625T>G;p.Y1875	21	M	265	4.7	9.7	12	1.1	12	70.1
Average ± SEM		13.2 ± 5.9	5/5 (M/F)	19817.30 ± 4772.6	4.5 ± 0.57	10.53 ± 2.3	27.29 ± 4.02	2.8 ± 0.99	14.1 ± 0.75	67.26 ± 2.37
Atopic dermatitis										
P1	Negative DOCK8 mutation	6	M	6540	5.2	10.6	16.5	0.9	16	64.4
P2		15	M	16500	6.39	3.72	15.6	1.4	14	71.3
P3		10	M	1612	5.48	8.01	13.9	1.2	16	73.3
P4		3	M	839	5.3	6.59	6.7	-	13	56.1
P5		8	M	1221	4.9	5.09	13	1.5	9	40.8
P6		13	M	659	5.2	5.15	13	1.2	10	54.9
P7		15	F	1387	4.71	9.63	12.3	1.4	15	75
P8		16	M	365	5.15	2.71	7.9	2.3	14	65.1
P9		12	M	4587	5.66	9.2	5.1	1.5	16	70.6
Average ± SEM		10.8 ± 1.4	8/1(M/F)	5288.20 ± 1736.3	5.33 ± 0.16	6.74 ± 0.92	27.29 ± 1.3	1.43 ± 0.14	13.6 ± 0.86	63.5 ± 3.702

F, female; M, male; VAS, visual analogue score; NA, not applicable.

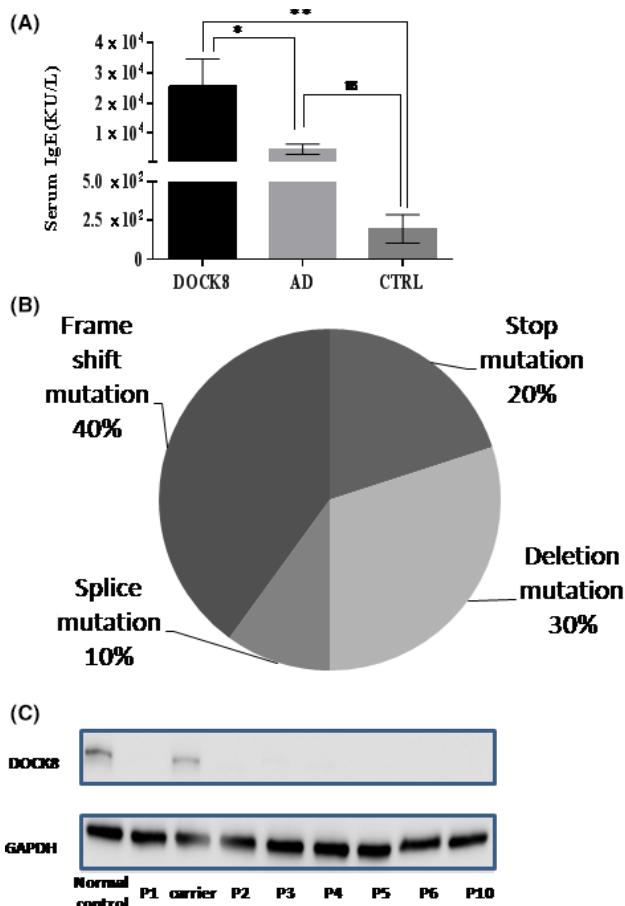


FIGURE 1 DOCK8 mutations and protein expression. (A) Serum IgE levels in patients with DOCK8 deficiency, atopic dermatitis (AD), and healthy controls (Ctrl) measured at collection point (one-way ANOVA, post hoc Tukey's **P*-value <0.05, ***P*-value <0.001). (B) Distribution of DOCK8 gene mutations in hyper-IgE syndrome (HIES) patients with DOCK8 deficiency. (C) Western blot analysis of DOCK8 protein in human lymphoblastoid cells of patients with DOCK8 deficiency (P1-P6 and P10) [Colour figure can be viewed at wileyonlinelibrary.com]

Research Centre (KFSHRC). Samples from the study participants were collected after signed informed consent was provided. Clinical and laboratory findings, as well as a comprehensive baseline questionnaire, were completed for each patient by the recruiting clinician. The consent form and baseline questionnaire were reviewed and approved by the Institutional Review Board's (IRB) ethics committee at KFSHRC (RAC No. 2160 015). The Severity Scoring of AD (SCORAD) and the visual analogue scale (VAS) pruritis scores were determined for the DOCK8-deficient and AD groups.¹⁷

2.2 | Cell lines

The lymphoblastoid cell lines were established by transformation of peripheral blood mononuclear cells (PBMCs) with Epstein-Barr virus as per standard protocols. PBMCs were isolated by Ficoll density gradient centrifugation and grown by re-suspension in Roswell Park Memorial Institute (RPMI)1640 media supplemented with 15% fetal bovine serum (FBS), 50 units/mL penicillin, and a mixture of 1%

glutamine (Gibco, Life Technologies, Saint-Aubin, France) in a 5% CO₂-humidified tissue culture incubator.

2.3 | Multiplex cytokine/chemokines analysis

The human magnetic bead panel array was used to quantify the cytokine/chemokine levels in patients' sera. The MILLIPLEX MAP kit (Millipore Darmstadt, Germany) was used according to the manufacturer's instructions. Color code microspheres coated with a specific capture antibody were incubated with 100 μ L of serum. The phycoerythrin-conjugated detection was used to form sandwich complexes, and the fluorescence data were acquired on the Luminex appliance, FLEXMAP 3D where one laser excites the phycoerythrin and measures the analytes, whereas the other laser detects the fluorophores inside the bead and measures its expression in the sample. The targeted cytokines (*n* = 39) are listed in Table S1. Serum samples were assayed in duplicates, and analyte concentrations were calculated by constructing a standard curve to convert the optical density values into concentrations (pg/mL) using the Milliplex Analyst software, version 5.1 (Vigene Tech Massachusetts, USA).

2.4 | IL-31 measurement using ELISA

An ELISA kit (R&D Systems Minneapolis, USA) was used to quantify IL-31 in human sera. The samples were incubated with an anti-human IL-31 primary antibody and then with the biotinylated secondary antibody. A streptavidin-labeled horseradish peroxidase (HRP) was coupled to the sandwich complex for color development. Fluorescence intensity was measured at 450 nm and 570 nm wavelengths of excitation and emission, respectively, using Varioskan Flash multi-mode reader (Thermo Fisher Scientific Pandan crescent, Singapore).

2.5 | Immunoblotting

Cell lysate was prepared using Radio Immuno Precipitation Assay (RIPA) buffer and loaded to SDS-PAGE (National Diagnostics, Atlanta, USA) for separation. The protein bands were transferred onto a PVDF membrane (GE Healthcare Waukesha, WI, USA) and incubated with HRP-conjugated antibodies, anti-DOCK8 (Santa Cruz Biotechnology Dallas, TX, USA) and mouse anti-beta actin (Abcam Cambridge, MA, USA). The complex was detected using the SuperSignal West Pico Chemiluminescent (Thermo Fisher Scientific) and visualized on an ImageQuant LAS 4000 (GE Healthcare Life Sciences).

2.6 | DOCK8 mutation analysis

Sanger sequencing of gDNA isolated from peripheral blood leukocytes using standard methods as previously described.¹⁸

2.7 | Data and statistical analysis

The cytokines/chemokines global data analysis was performed using MetaboAnalyst version 3.0 (McGill University). The raw data were normalized to the sample total median to ensure all samples were

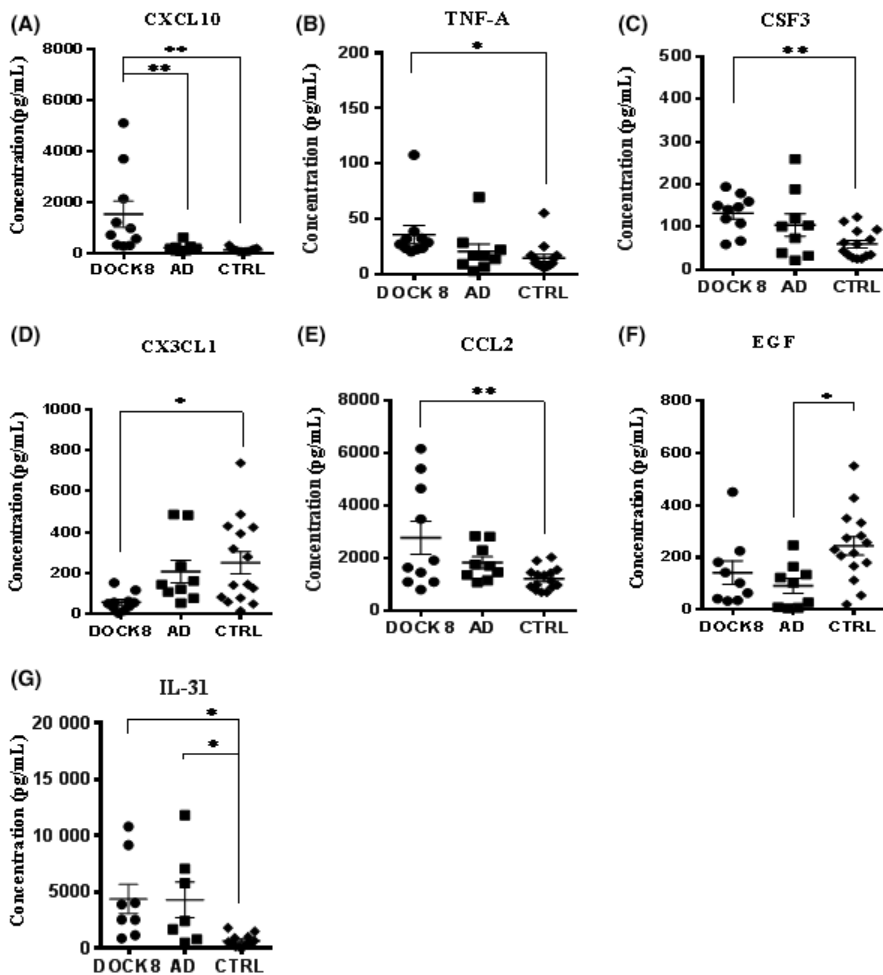


FIGURE 2 Significant cytokines in serum of individuals with DOCK8 deficiency, atopic dermatitis, and healthy control. (A) CXCL10, (B) tumor necrosis factor alpha (TNF-A), (C) granulocyte colony-stimulating factor, GCSF (CSF3), (D) CX3CL1 (fractalkine), (E) macrophage-derived chemokine, MDC (CCL2), (F) epidermal growth factor (EGF), and (G) interleukin-31(IL-31) DOCK8 ($n = 8$) and AD ($n = 7$). The statistical analysis was performed using one-way ANOVA, post hoc Tukey's method where * indicates significance with P -value < 0.05 , ** P -value < 0.001 , and otherwise are not significant (ns)

normally distributed. To visualize the difference among the study groups and make individual features more comparable, data were log-transformed, and Pareto scaled, respectively. As the data were Gaussian distributed, the unpaired two-tailed Student's t test was used for binary comparison between any two study groups, where the significance levels for cytokines data were considered at an FDR-corrected P -value < 0.05 , and values were presented as mean $\pm$ SEM.

The targeted statistical analysis of multiple study groups (data in Figures 1 and 2) was carried out using GraphPad Prism (version 6.0, GraphPad software LA Jolla, CA). Analysis of variance (ANOVA) was performed by choosing column mean type analysis. Post hoc Tukey's method of analysis was used for more binary analysis and information such as adjusted P -values for each comparison. These differences between the groups contributed to the significance of an ANOVA test. In all experiments, a calculated P -value < 0.05 was considered to be statistically significant.

3 | RESULTS

3.1 | Clinical profile of HIES patient

DOCK8 deficiency and AD patients were recruited through the allergy and immunology clinics at KFSHRC. In DOCK8-deficient

patient group, 50% were females and the mean age was 13.2 ± 5.9 years, whereas for the AD group (8 males and 1 female), the mean age was 10.8 ± 1.4 years (Table 1), and for the Ctrl group 23 ± 1.03 years (8M/7F). Eosinophilia was noticeable in both groups, whereas the median RBCs and WBCs were 1.93 ± 0.6 ($10^{12}/L$) and 11.29 ± 2.44 ($10^9/L$), respectively. The median of the CD4/CD8 ratio in DOCK8 patients was 2.8 ± 0.99 , compared to 1.43 ± 0.14 in the AD (Table 1). DOCK8 mutations were confirmed by standard Sanger sequencing of peripheral blood gDNA.

Dermatitis and food allergies were the most common clinical manifestations in both groups, followed by bronchial asthma. Staphylococcus infections and pneumonia were the most common clinical features seen in the DOCK8 patients, while four patients presented with thrush candidiasis and warts (Table 2). In conjunction with severe skin infections, itching, and rashes, DOCK8 patients (50%) developed cutaneous abscesses. In addition, four DOCK8-deficient patients acquired viral infections caused by molluscum contagiosum, and two had sclerosing cholangitis, whereas AD patients had fungal infections of the scalp (22%) and rhinitis (50%). Furthermore, three AD patients developed ear and chest infections (Table 2). Sinusitis was seen in four DOCK8-deficient patients. Owing to recurrent infections, six DOCK8-deficient patients showed retardation in their physical growth. Additional features of DOCK8 deficiency include

TABLE 2 Clinical manifestations in DOCK8 and AD patients

Findings	DOCK8 (n = 10) (%)	AD (n = 9) (%)
Dry skin/itching	100	100
Atopic dermatitis/eczema	100	100
Food allergies	100	100
Pneumonia	100	0
Staphylococcal infection	100	11
Bronchial asthma	90	44
Bronchiectasis	50	0
Molluscum contagiosum	40	0
Sclerosing cholangitis	20	0
Thrush candidiasis	40	0
Warts	40	0
Abscesses	50	0
Otitis media	30	0
Sinusitis	40	0
Rhinitis	0	50
Hodgkin lymphoma	9	0
Failure to thrive	60	0
Seizures	20	0
Cataract	20	0
Congestive heart failure	10	0
Deceased (Hodgkin lymphoma)	10	0

cataract and seizures in two patients each, congestive heart failure in one patient and one who developed fatal Hodgkin lymphoma.

Both cohorts demonstrated elevated serum IgE levels compared to Ctrl. The IgE levels were significantly higher in DOCK8 patients (P -value <0.05) than AD ranging from 5 to 500 KU/L (IU/mL) (Figure 1A). In DOCK8, splicing mutations were the most common (70%), followed by stop gain (20%) and deletion (10%) (Figure 1B). Mutations in DOCK8 were excluded in all AD patients except one. Immunoblot-based DOCK8 expression analysis of cell line extracts using an N-terminus specific antibody (Figure 1C) showed near complete loss of DOCK8 protein expression in all tested patients ($n = 7$) regardless of their mutation type. In three patients (P7,8,9), immunoblot analysis could not be performed due to inadequate sample.

3.2 | Changes in serum cytokines

In this study, a full analysis of the 39 analytes revealed only seven that were differentially expressed among the three comparative groups at P -value <0.05 . As shown in Figure 2A, CXCL10 was upregulated in DOCK8-deficient patients compared to AD and Ctrl groups (P -value <0.001). TNF- α , GCSF, and MDC were significantly upregulated only in DOCK8-deficient patients when compared to controls (Figure 2B,C,E). CX3CL1 was significantly downregulated in DOCK8-deficient patients (P -value <0.05) compared to Ctrl groups (Figure 2D), whereas EGF was downregulated in AD (P -value <0.05) and not significant when DOCK8 group was compared to Ctrl

(Figure 2F). However, IL-31 was significantly upregulated in both patient groups compared to controls (Figure 2G).

3.3 | Biomarker evaluation

For evaluating these cytokines for potential biomarker discovery, the data were combined and globally analyzed using MetaboAnalyst software version 3.0 (<http://www.metaboanalyst.ca>). The raw data were normalized, transformed, and scaled by a median, log, and Pareto, respectively, to make sure all the data are visualized under Gaussian distribution. For paired analysis, a combination of the t test and fold change analyses is represented in a volcano plot (Figure S1A). The comparison between DOCK8-deficient and Ctrl groups was demonstrated in an orthogonal projections to latent structures discriminant analysis (OPLS-DA); the separation between the two groups represents the degree of random relative expression in the whole panel due to DOCK8 deficiency ($Q^2 = 0.864$) (Figure 3A). Analytes are ordered in Figure S1B based on their contribution in separating the two groups. Among CXCL10, TNF- α , CSF3, CCL22, CX3CL1, IFNG, and TGFA that were above the volcano plot's cutoff values, CXCL10 was evaluated as a potential biomarker using the support vector machine (SVM) model to generate a receiver operating characteristic (ROC) curve, where the area under the curve (AUC) amounted to 1 (Figure 3B). Another OPLS-DA-based binary comparison between Ctrl and AD groups showed a Q^2 of 0.32 only (Figure S2C). The relatively narrow spacing between the two groups indicates the lower ability of this profile to distinguish between the AD and control individuals. As suggested by the volcano plot, the detected unique cytokine, EGF, which was downregulated in AD patients, had a ROC and AUC of 0.859 (Figure S2D).

Since the main aim of this study was to find disease-specific biomarker(s) able to differentiate between DOCK8 deficiency and AD, the binary comparison revealed clear cluster separation in OPLS-DA with a Q^2 value of 0.693 (Figure 4A). Comparison between all three groups also showed comparable separation but some overlapping was observed between the AD group and controls (Figure 4B) may be due to mild eczema seen in the control group. Interestingly, CXCL10 and TNF- α were the only signature cytokines, which were above the volcano plot's cutoff values and were significantly upregulated in DOCK8-deficient patients. The typified linear combination of these cytokines will be a potentially robust differentiating tool between DOCK8 and AD with AUC 0.978 (Figure 4C,D).

4 | DISCUSSION

While significant progress has been made, the molecular mechanisms underlying specific cytokine functions remain largely unknown. The last decade has witnessed accumulating evidence supporting the central role of cytokines in immune surveillance as it relates to autoimmune diseases. It is essential to identify DOCK8 deficiencies before serious life-threatening complications arise. Diagnosis can be difficult in infants and young children because of coinciding

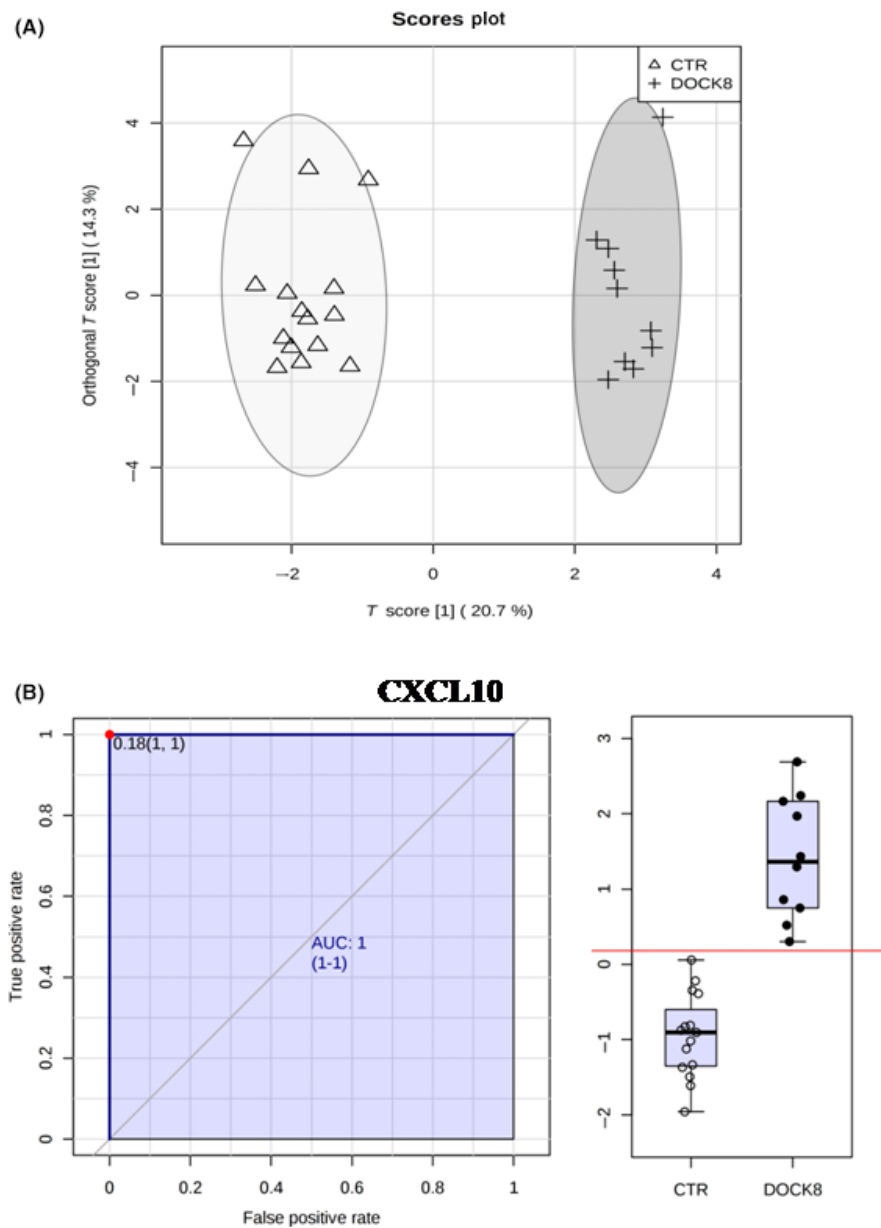


FIGURE 3 Orthogonal projections to latent structures discriminant analysis (OPLS-DA) for binary comparisons and area under the curve (AUC) in DOCK8-deficient patients. (A) Comparison between DOCK8-deficient patients and healthy controls with a calculated space $Q^2 = 0.864$ that represents the level of differential expression of the panel analytes (B) CXCL10 is upregulated with AUC:1 in patients with DOCK8 deficiency [Colour figure can be viewed at wileyonlinelibrary.com]

laboratory findings and clinical profiles with AD and other types of HIES. There is global interest in identifying biomarkers that are disease-specific for the various clinical and molecular subtypes of HIES. The combination of recurrent viral infections, atopy, food allergies, consanguinity, and bronchial hyperactivity strongly suggests DOCK8 deficiency. Supportive treatments including prophylactic antibiotics, nutritional support, and immunomodulatory agents remain unsatisfactory, and patients usually have very short life expectancy. Due to high morbidity and mortality associated with DOCK8 deficiency, curative treatment using hematopoietic stem cell transplantation is becoming the last preferred choice, resulting in successful immune reconstitution.¹⁹

In this study, among the 39 cytokines, seven (CXCL10, TNF- α , GCSF, CX3CL1, MDC, EGF, and IL-31) were differentially expressed (Figure 2A-G). CXCL10 is a chemokine which is known to be highly expressed in various autoimmune diseases,²⁰

pneumonia triggered by viral and bacterial infection²¹ as well as in asthma.^{22,23} Upregulation of this cytokine in the DOCK8-deficient cohort seems to be associated with the commonly seen asthma, infections, allergies, and pneumonia phenotypes seen in these patients (Figure 2A). In contrast, TNF- α is a multifunctional proinflammatory cytokine, known to mediate tumor initiation, psoriasis, AD, and autoimmune diseases, and participates in cell survival/death and cancer.²⁴ Upregulation of TNF- α (Figure 2B) seems to be contributing toward the pruritus and immune dysregulation observed in DOCK8 patients.

Furthermore, CSF3 is a glycoprotein linked with proliferation and maturation of neutrophils.²⁵ The role of CSF3 is evident in various cancers.^{26,27} Dehqanzada et al. in 2006 demonstrated that CSF3 was significantly overexpressed in breast cancer patients.²⁸ Higher expression of CSF3 (Figure 2C) demonstrated in those studies supports the role of immune responses, favoring tumor growth and

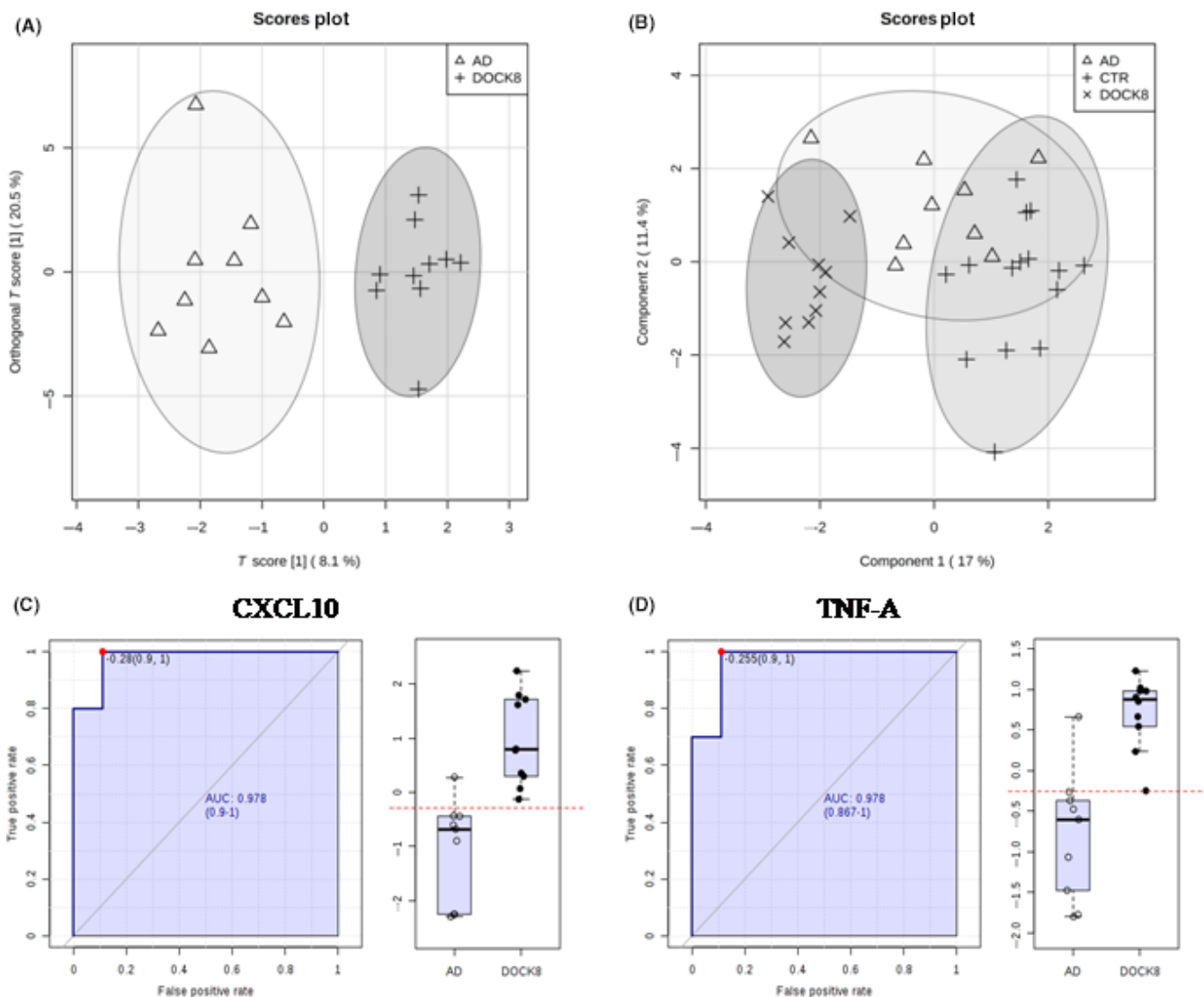


FIGURE 4 Orthogonal projections to latent structures discriminant analysis (OPLS-DA) for binary comparison and area under the curve (AUC) in patients with DOCK8 deficiency and atopic dermatitis. (A) Score plot comparison between AD patients and DOCK8 patients. (B) Overall comparison between AD patients, DOCK8-deficient patients, and controls. (C) CXCL10 is upregulated in DOCK8-deficient patients compared to AD patients, with AUC 0.978. (D) Tumor necrosis factor alpha (TNF-A) is upregulated in DOCK8 patients compared to AD patients, AUC- 0.978 [Colour figure can be viewed at wileyonlinelibrary.com]

progression which is in agreement with disease phenotypes seen in the DOCK8 cohort. Moreover, CX3CL1 is known to be associated with various inflammatory conditions like AD²⁹ and allergic asthma.³⁰ It was downregulated in DOCK8 patients when compared to Ctrls (Figure 2D). MDC was shown to be involved in various immune responses and elimination of pathogens,³¹ allergic reactions,³² and inflammation in the lungs.³³ Hence, upregulation of MDC (Figure 2E) is consistent with the clinical features (infections, allergies, and pneumonia) seen in the DOCK8-deficient patients.

Atopic dermatitis is a chronic inflammatory skin disease with frequent recurrences, and cytokines appear to play a significant role in its complex pathogenesis.^{34,35} EGF is reported to regulate fundamental functions in mammalian cells including survival, migration, proliferation, and homeostasis. This cytokine is expressed at the site

of infection, and its primary function is the protection of the skin against bacterial colonization mediated through the EGFR signaling.³⁶⁻³⁸ In this study, EGF shows to be significantly downregulated in AD patients, but not in DOCK8-deficient patients, when compared with Ctrls (Figure 2F); hence, low levels of EGF may impair proliferation and anti-inflammatory immune response in the skin. IL-31 is a recently discovered cytokine produced by Th2 cells, and elevated levels of IL-31 are correlated with increased serum IgE levels and disease severity and are responsible for the induction of pruritus in the AD patients.³⁴ Recently, it has been shown that topical administration of EGF suppresses immune response and protects skin barrier in DNCB-induced AD in NC/Nga mice. This effect appeared to be the result of EGF upregulation of epidermal proteins and downregulation of TSLP (thymic stromal lymphopoietin)-mediated mast cell

and Th2 cell activation.³⁹ Therefore, we propose that in subsets of patients with EGF deficiency, replacement therapy with topical or systemically administered EGF may be of clinical value.

CXCL10 and TNF-A were identified as potential biomarkers differentiating DOCK8-deficient and AD patients (Figure 4). The reliability of these cytokines as diagnostic markers should be confirmed by questioning more massive datasets, to assess whether these cytokines are specific to DOCK8 gene expression or resulting from secondary disease complications. A binary analysis was performed in DOCK8-deficient patients with and without one of the following clinical manifestations: asthma, bronchiectasis, molluscum contagiosum infection, sclerosing cholangitis, thrush candidiasis, warts, sinusitis, rhinitis, and malignancy. For instance, CSF3 was upregulated in DOCK8 patients with warts and molluscum contagiosum, while CX3CL1 and IFNG were downregulated in the DOCK8 patient with malignancy. Moreover, Figure S2, which represents volcano plots for binary comparisons between AD vs control and DOCK8 vs AD, also demonstrates the potential biomarkers EGF, CXCL10, and TNF-A. As shown in Figure S3, neither of the potential biomarkers, CXCL10 and TNF, was overexpressed in any of these binary analyses. Instead, they were downregulated in association with bronchiectasis and malignancy, suggesting that these biomarkers are very likely to be related mainly to the disease mechanism caused by DOCK8 deficiency. These findings should be further studied in other HIES to determine their specificity.

CXCL10 is one of the CXCR3 ligands secreted by CD4+, CD8+ T cells, natural killer (NK), or NK-T cells in response to INFG. The average CD4+/CD8+ ratio in DOCK8 patients was higher (2.8 ± 0.99) than AD groups (1.43 ± 0.14). The C-terminal of CXCL10 has a high affinity for cell surface heparan sulfate glycosaminoglycan (HS-GAG), which is expressed on epithelial, endothelial, and hematopoietic cells. The higher circulated level of CXCL10 in blood is due to either a higher competitive binding with a viral protein inflammation,⁴⁰ (as it attracts the activated T lymphocytes, which are the only inflammatory cells expressing the chemokine receptor CXCR3), or DOCK8 inhibition of HS-GAG synthesis. From the clinical data collected for this study (Table 2), the spectrum of infections observed in DOCK8-deficient patients such as pneumonia (100%), staphylococcal infections (100%), and bronchiectasis (50%) suggests a connection between pathogens binding to HS-GAG, and the degree of inflammation, which increases the circulated CXCL10.⁴¹

In summary, significant differential expression of cytokines CXCL10, CSF3, CCL22, CX3CL1, and TNF-A was identified in DOCK8 deficiency, which possibly contributes to increased susceptibility to infection and cancer. CXCL10 and TNF-A seem to be important biomarkers in differentiating between DOCK8-deficient and AD patients, whereas EGF was found to be a useful biomarker in AD patients. Cytokine IL-31 expression was comparable in both cohorts thus contributing toward pruritus, which is common to both groups. The unique expression profiles including CXCL10, CSF3, CCL22, CX3CL1, and TNF-A that we detected in patients with DOCK8 deficiency probably reflect disease-specific perturbations in multiple cellular processes and pathways leading to a predisposition to infections,

allergy, and malignancy seen in these patients. The linear combination of three “CXCL10, TNF-A, and EGF” typified biomarkers signature could lead to a unique scoring system for early effective diagnosis and distinction of DOCK8 deficiency vs atopic dermatitis.

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CONFLICT OF INTERESTS

The authors declare that they have no conflict of interests.

AUTHOR CONTRIBUTIONS

MJ collected the samples, performed the cytokine experiment and ELISA tests, analyzed the data, compiled the summary tables, and drafted the manuscript. DBF provided technical support for FLEX-MAP 3D. SA and AMA helped with the immunoblots. RA, BAS, and HAM cared for patients and recruited them for the study. MD helped with the study design, data analysis, and manuscript critical revisions. ALL critically revised the article and approved its final version to be published. AMAR designed the study, supervised experiments, analyzed the data, and finalized the paper. All authors approved the final version of the manuscript.

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SUPPORTING INFORMATION

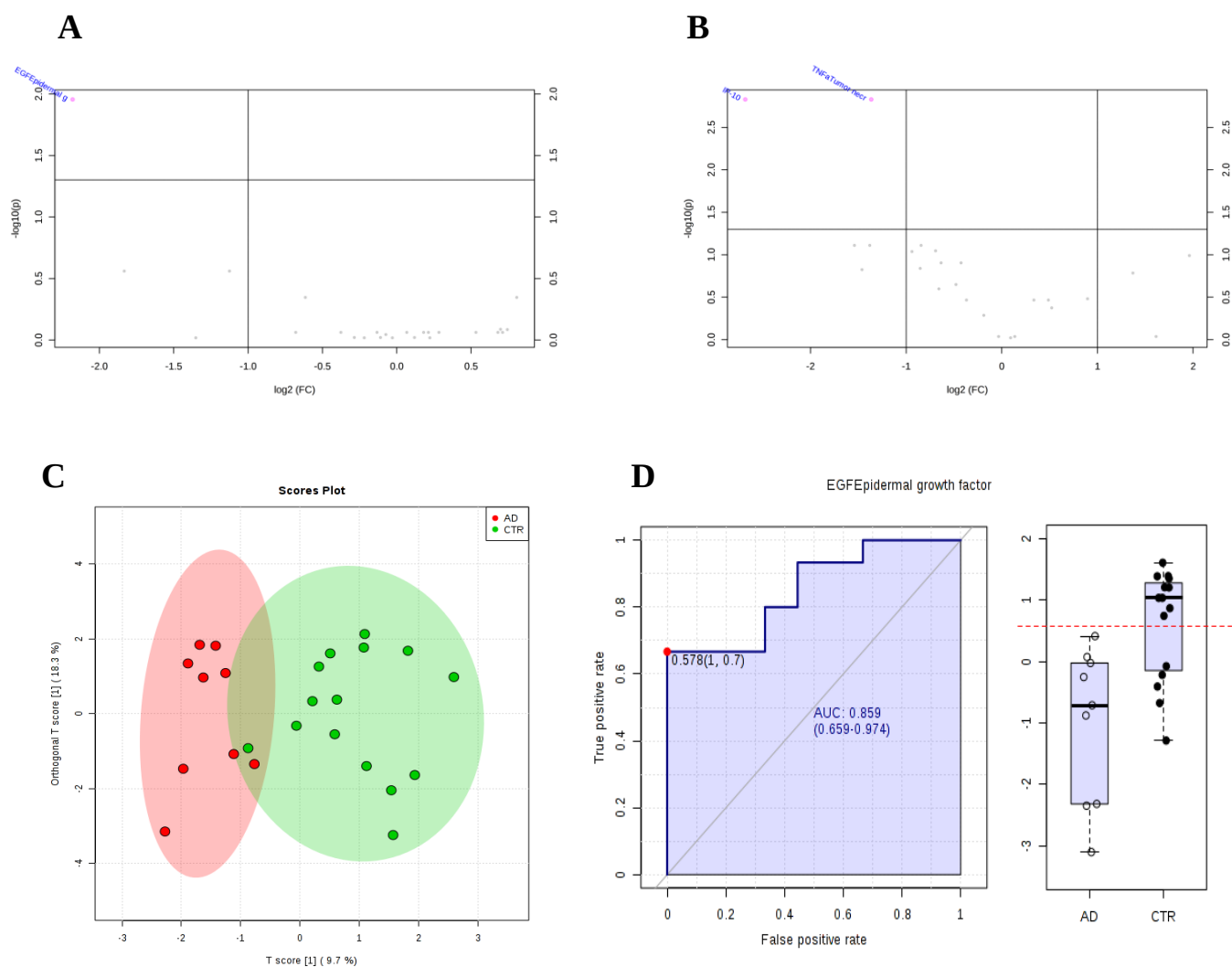
Additional supporting information may be found online in the Supporting Information section at the end of the article.

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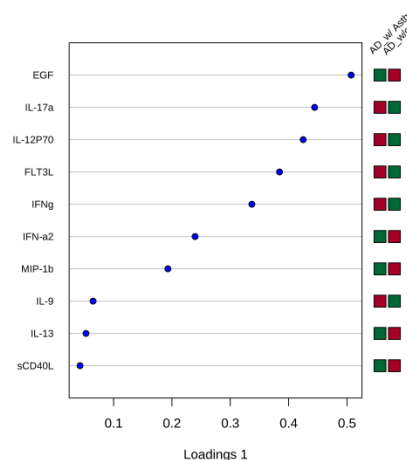
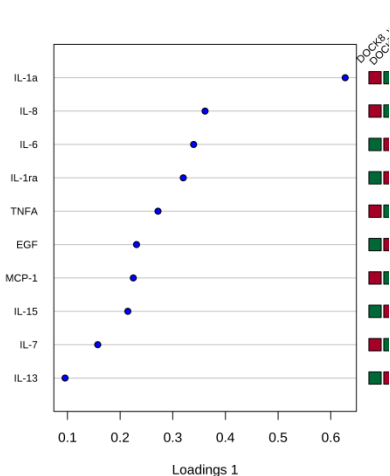
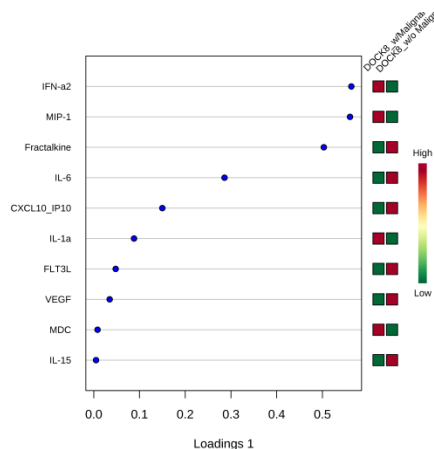
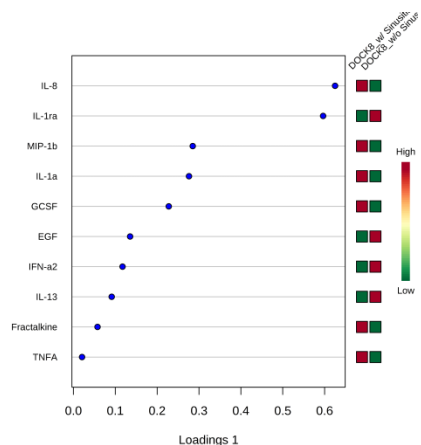
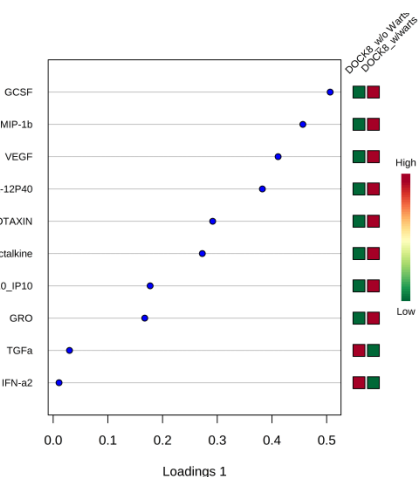
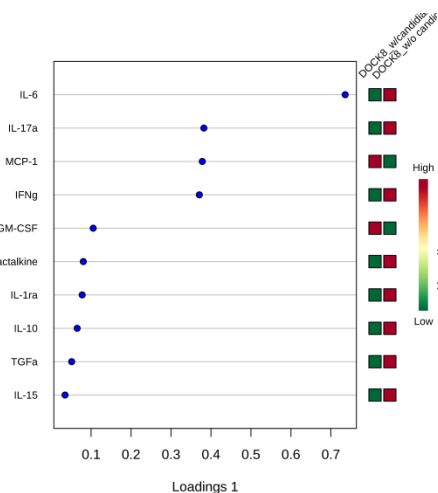
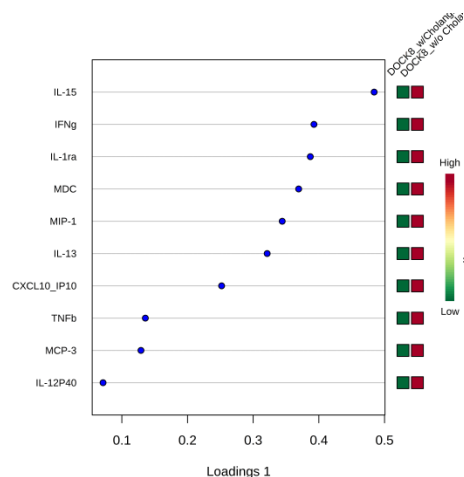
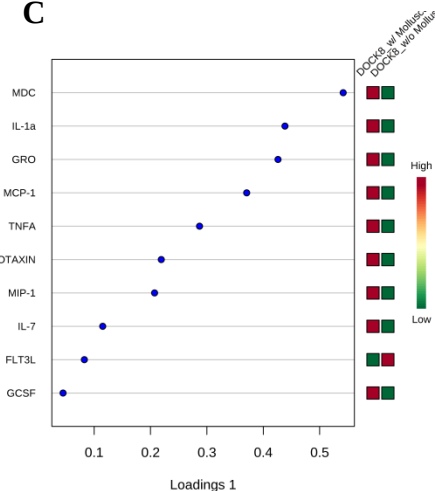
Scatter plot showing Loadings 1 for various cytokines and chemokines. The x-axis represents Loadings 1, ranging from 0 to 0.5. The y-axis lists the factors: CXCL10_IP10, TNFA, GCSF, MDC, IFNg, Fractalkine, TGFa, GRO, GM-CSF, and IL-8. A color scale on the right indicates the magnitude of the loading, from Low (green) to High (red). Each factor has a corresponding dot and a color-coded bar.

Factor	Loadings 1 (approx.)	Color (Low to High)
CXCL10_IP10	0.53	Red
TNFA	0.53	Red
GCSF	0.46	Red
MDC	0.27	Red
IFNg	0.20	Red
Fractalkine	0.20	Red
TGFa	0.14	Red
GRO	0.08	Red
GM-CSF	0.08	Red
IL-8	0.04	Red

(A) Volcano plots show upregulation of CXCL10 and TNFA in DOCK8 deficient patients compared to healthy controls. **(B)** Loading plots for DOCK8 vs control.



Supplemental Figure 2:Volcano plots and Binary comparison: **(A)** AD vs control, **(B)** DOCK8 vs AD **(C)** Comparison between AD patients and healthy controls ($Q2=0.32$) where $Q2$ represents the level of differential expression of the panel analytes **(D)** Epidermal growth factor (EGF) is down regulated in the AD patients when compared to healthy controls, AUC: 0.859.

A**B****C**

Supplemental Figure 3: PLS-DA loading plots based on binary comparisons in AD and DOCK8 deficient patients with/without various clinical phenotypes including (A) asthma, (B) bronchiectasis, (C) molluscum contagiosum, (D) sclerosing cholangitis, (E) candidiasis, (F) warts, (G) sinusitis, (H) malignancy.

Supplemental Table 1: List of 39 Cytokine/Chemokines/Growth factors

Cytokines (n=22)
FLT3LG(Flt-3L),IFNA1 (IFN-a2), IFNG (IFNg), IL-10, IL12B(IL12p40),IL12p70,IL-13, IL-15, (IL17A)IL-17A, IL-1A, IL-1B, IL-1RA,IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, TNF-A(TNFa),TNF-B (TNFb)
Chemokines (n=9)
CCL-2(MCP-1),CCL-3(MIP-1a),CCL-4(MIP-1b),CCR3(MCP-3),CCL-11(Eotaxin),CCL22 (MDC), CX3CL1 (Fractalkine),CXCL2(GRO),CXCL10(IP-10)
Growth factor/ others (n=7)
EGF, FGF-2, TGF-A, CSF3(GCSF), CSF2 (GMCSF),CD40LG(sCd40l), VEGA
IL-31(n=1)
Human IL-31 DUOSet Elisa kit