Dedicator of cytokinesis 8–deficient CD4$^+$ T cells are biased to a T_H2 effector fate at the expense of T_H1 and T_H17 cells

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Background: Dedicator of cytokinesis 8 (DOCK8) deficiency is a combined immunodeficiency caused by autosomal recessive loss-of-function mutations in *DOCK8*. This disorder is characterized by recurrent cutaneous infections, increased serum IgE levels, and severe atopic disease, including food-induced anaphylaxis. However, the contribution of defects in CD4$^+$ T cells to disease pathogenesis in these patients has not been thoroughly investigated.

Objective: We sought to investigate the phenotype and function of DOCK8-deficient CD4$^+$ T cells to determine (1) intrinsic and extrinsic CD4$^+$ T-cell defects and (2) how defects account for the clinical features of DOCK8 deficiency.

Methods: We performed in-depth analysis of the CD4$^+$ T-cell compartment of DOCK8-deficient patients. We enumerated subsets of CD4$^+$ T helper cells and assessed cytokine production and transcription factor expression. Finally, we determined the levels of IgE specific for staple foods and house dust mite allergens in DOCK8-deficient patients and healthy control subjects.

Results: DOCK8-deficient memory CD4$^+$ T cells were biased toward a T_H2 type, and this was at the expense of T_H1 and T_H17 cells. *In vitro* polarization of DOCK8-deficient naive CD4$^+$ T cells revealed the T_H2 bias and T_H17 defect to be T-cell intrinsic. Examination of allergen-specific IgE revealed plasma IgE from DOCK8-deficient patients is directed against staple food antigens but not house dust mites.

Conclusion: Investigations into the DOCK8-deficient CD4$^+$ T cells provided an explanation for some of the clinical features of this disorder: the T_H2 bias is likely to contribute to atopic disease, whereas defects in T_H1 and T_H17 cells compromise antiviral and antifungal immunity, respectively, explaining the infectious susceptibility of DOCK8-deficient patients. (J Allergy Clin Immunol 2016;■■■:■■■-■■■.)

Key words: *Dedicator of cytokinesis 8, CD4$^+$ T-cell differentiation, T_H2 skewing, allergy, atopic disease, chronic mucocutaneous candidiasis, viral immunity*

Biallelic loss-of-function mutations in dedicator of cytokinesis 8 *(DOCK8)* cause a combined immunodeficiency also known as autosomal recessive hyper-IgE syndrome (AR-HIES).[1,2] Affected

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Abbreviations used

APC:	Allophycocyanin
AR-HIES:	Autosomal recessive hyper-IgE syndrome
CFSE:	Carboxyfluorescein succinimidyl ester
CMC:	Chronic mucocutaneous candidiasis
DOCK8:	Dedicator of cytokinesis 8
HPV:	Human papilloma virus
HSV:	Herpes simplex virus
ICOS:	Inducible costimulator
NK:	Natural killer
PD-1:	Programmed cell death protein 1
PE:	Phycoerythrin
PID:	Primary immunodeficiency
PMA:	Phorbol 12-myristate 13-acetate
RORC:	RAR-related orphan receptor C
STAT:	Signal transducer and activator of transcription
TAE:	T-cell activation and expansion
T-bet:	T-box transcription factor
T_{CM}:	Central memory T
TCR:	T-cell receptor
T_{EM}:	Effector memory T
T_{FH}:	Follicular helper T
Treg:	Regulatory T

patients typically present with recurrent *Staphylococcus aureus* skin infections, recurrent and severe cutaneous viral infections (herpes simplex virus [HSV], human papilloma virus [HPV], and *Molluscum contagiosum* virus), increased serum IgE levels, lymphopenia, eosinophilia, and an increased risk of malignancy.[1-3] DOCK8-deficient patients also exhibit impaired humoral immune responses against protein and polysaccharide antigens after natural infection or vaccination. Strikingly, DOCK8 deficiency predisposes most affected patients to asthma and severe allergies against food and environmental antigens.[1-5] However, the mechanisms underlying severe allergy are currently unknown.

DOCK8 functions as a guanine nucleotide exchange factor to activate Rho-family GTPases, such as CDC42, which mediate events, including cell activation, division, survival, differentiation, adhesion, and migration.[6-8] Despite this, it is not immediately clear how *DOCK8* mutations result in the devastating immune abnormalities characteristic of patients with AR-HIES. However, because DOCK8 is predominantly expressed by hematopoietic cells, it is likely to play critical lymphocyte-intrinsic roles in cellular and humoral immune responses against infectious diseases. Consistent with this, allogeneic hematopoietic stem cell transplantation overcomes recurrent cutaneous viral infections and eczematous rash and reduces IgE levels and eosinophilia.[9-14] In regard to food allergies in patients with DOCK8 deficiency, some reports have documented improvement after hematopoietic stem cell transplantation,[10,11,14] whereas others reported amelioration of symptoms[13] or no change.[9,15]

Ex vivo and *in vitro* analyses of lymphocytes from DOCK8-deficient patients have shed some light on disease pathogenesis. For instance, DOCK8-deficient patients have normal to increased numbers of total B cells but decreased circulating memory ($CD27^+$) B cells.[5,16] Functionally, compared with normal B cells, DOCK8-deficient B cells exhibit poor responses to the Toll-like receptor 9 ligand CpG, whereas CD40-mediated responses were largely intact.[5] In B cells DOCK8 acts as an adaptor protein connecting the Toll-like receptor 9–myeloid differentiation primary response gene–88 pathway to signal transducer and activator of transcription (STAT) 3 signaling, which is required for B-cell proliferation and differentiation, as evidenced by defective function of STAT3-deficient human B cells *in vivo* and *in vitro*.[17-20] These defects underlie poor humoral immunity in patients with DOCK8 deficiency. Paradoxically, an increase in autoantibodies directed against nuclear, cytoplasmic, and extracellular matrix antigens has been detected in DOCK8-deficient patients, possibly because of decreased regulatory T (Treg) cell numbers in these patients.[21]

Our previous study of T cells in DOCK8-deficient patients revealed a severe reduction in naive, central memory ($CD45RA^-CCR7^+$), and effector memory ($CD45RA^-CCR7^-$) $CD8^+$ T cells but a marked accumulation of $CD45RA^+CCR7^-$ terminally differentiated (ie, "exhausted") effector memory cells.[22] Strikingly, central and effector memory $CD8^+$ T cells from DOCK8-deficient subjects displayed phenotypic features of exhaustion, with increased expression of CD57, 2B4, and CD95 and accelerated loss of CD28 and CD127 (IL-7 receptor α).[22] Furthermore, DOCK8-deficient naive and memory $CD8^+$ T cells did not proliferate *in vitro* in response to T-cell receptor (TCR) stimulation.[22] More recently, DOCK8-deficient $CD8^+$ T cells were reported to undergo "cytothripsis," a form of cell death associated with defects in morphology and trafficking that prevented the generation of long-lived resident memory $CD8^+$ T cells in the skin and subsequently impaired immune responses to herpes virus infection at this site.[23] Taken together, these defects in $CD8^+$ T cells provide a plausible explanation for viral susceptibility in DOCK8-deficient patients. DOCK8-deficient patients also have defects in the development of invariant natural killer (NKT) cells and NK cell function,[24,25] which might contribute to increased susceptibility to viral infections and malignancies.

In contrast to these established defects in B cells, Treg cells, $CD8^+$ T cells, NK cells, and NKT cells, much less is known about the consequences of *DOCK8* mutations on other human $CD4^+$ T helper cells. Although it has been reported that the frequencies of naive and memory $CD4^+$ T cells in DOCK8-deficient patients are normal, DOCK8-deficient naive and memory $CD4^+$ T cells do have a defect in TCR-induced proliferation, although less severe than that seen in DOCK8-deficient $CD8^+$ T cells.[22] Consequently, this deficit is unlikely to cause clinical features, such as atopic disease (dermatitis and severe food allergies) and increased IgE levels in patients with DOCK8 deficiency. For this reason, we have undertaken a detailed analysis of the $CD4^+$ T-cell compartment in DOCK8-deficient patients.

We found that DOCK8-deficient memory $CD4^+$ T cells have a bias toward T_H2 cytokine expression (ie, IL-4, IL-5, and IL-13) and concomitant defective production of T_H1 (IFN-γ) and T_H17 (IL-17A, IL-17F, and IL-22) cytokines. Furthermore, the T_H2 cytokine bias and impaired T_H17 immunity in the absence of DOCK8 were T cell intrinsic and independent of defects in proliferation. This intrinsic T_H2 bias of DOCK8-deficient $CD4^+$ T cells might underlie atopic disease and hyper-IgE displayed by DOCK8-deficient patients. Additionally, impaired T_H1 and T_H17 responses likely account for impaired viral immunity and fungal infections, such as chronic mucocutaneous candidiasis (CMC), respectively, in DOCK8-deficient patients.

TABLE I. DOCK8-deficient patients

DOCK8-deficient patients	Mutation	Sex	Age at study (y)	IgE (IU/mL)	Allergies/atopic disease	Infections	Other
1	Homozygous 114-kb deletion spanning exons 4-26	Female	14	4,864-10,000	● No known allergies ● Eczema ● Hypereosinophilia without lymphopenia	Pneumonia, cutaneous lesions and abscesses, fungal infections, lymphadenitis, cheilitis, *Chrysosporium parvum*	Chronic diarrhea, rectal prolapse, bronchiectasis, tolerated BCG vaccine; deceased
2	Homozygous A->T; position 70 exon 7; K271X	Female	12	10,000	● No known allergies ● Eczema	Severe *M contagiosum*, pneumonia, meningitis	
3	Homozygous 400-kb deletion (totality of DOCK8 + 5′ of KANK1)	Female	12	>5,000	● Multiple food, environmental, and drug allergies ● Severe eczema (lichenification) ● Hypereosinophilia (>3000/mm^3)	Stomatitis, *M contagiosum*, respiratory syncytial virus, HSV1, *Candida* species, *Haemophilus influenzae*, *Pneumocystis jirovecii*	Abdominal vasculitis, lymphadenopathy, splenomegaly, CD3$^+$ lymphopenia; successful HSCT
4	Homozygous 114-kb deletion spanning exons 4-26	Male	10	1,552	● No known allergies ● Eczema ● Hypereosinophilia (7800/mm^3)	Recurrent otitis media, herpes labialis, HPV, disseminated plain warts, onychomycosis, *Salmonella* species	Arthritis, uveitis, interstitial lung disease, inflammatory bowel disease, mesenteric vasculitis; tolerated BCG vaccine; deceased
5	Homozygous 114-kb deletion spanning exons 4-26	Female	12	19,302	● No known allergies ● Eosinophilia (5,000/mm^3)	Recurrent upper respiratory tract infection, HPV, flat warts, herpetic stomatitis, *Giardia lamblia*, *Salmonella enterica*, *Escherichia coli*	Uncomplicated chickenpox; inflammatory bowel disease, abdominal vasculitis, thrombocytosis; tolerated BCG vaccine; deceased
6	c.3733_3734delAG; p.R1245EfsX5	Male	12	1,500	● Multiple food allergies (egg, cow's milk) ● Peanut sensitized (tolerant) ● Environmental allergies (house dust mite, rye grass, Bermuda grass); previous allergic rhinitis ● Infrequent episodic asthma (virus induced) in childhood ● Eczema	Methicillin-resistant *S aureus* infection, *M contagiosum*, recurrent otitis media	
7	Homozygous deletion 9p24.3 323,819-324,708	Female	8	9,196	● Food allergies ● Diffuse colonic and esophageal eosinophilia ● Eczema ● Asthma	CMV, BK virus, chronic *Salmonella* species, recurrent sinopulmonary infections, skin abscesses	Sclerosing cholangitis
8	Heterozygous deletions involving exons 22-25 and 3-32	Female	14	6,270	● Food allergies ● Environmental allergies ● Rhinitis ● Asthma ● Allergic conjunctivitis ● Eczema	HPV, *M contagiosum*, meningitis, bacteremia, fungal skin infections	Vasculopathy; allergic symptoms improved after transplantation

(Continued)

TABLE I. *(Continued)*

DOCK8-deficient patients	Mutation	Sex	Age at study (y)	IgE (IU/mL)	Allergies/atopic disease	Infections	Other
9	● Large heterozygous deletion (~200 kb) ● 2-bp Heterozygous deletion in exon 41 (c.5307-5308 del AC, pL1770fsX1783	Female	7	>6,000	● No known allergies ● Severe eczema (lichenification) ● Eosinophilia (>3,000/mm^3)	Skin abscesses, *M contagiosum*, recurrent respiratory tract infection, chronic otitis, maxillary sinusitis, bronchiectasis, HPV warts, HSV, *H influenzae*, *Salmonella* species	↑ IgG, ↓ IgM, ↑ IgA, CD4$^+$ lymphopenia
10	● Large heterozygous deletion (~200 kb) ● 2-bp Heterozygous deletion in exon 41 (c.5307-5308 del AC, pL1770fsX1783	Male	10	>4,400	● No known allergies ● Moderate eczema ● Eosinophilia	Skin abscess, *M contagiosum*, recurrent upper respiratory tract infection, HPV disseminated warts, HSV stomatitis, *S aureus*, *Streptococcus pyogenes*	↑ IgG, ↓ IgM, ↑ IgA, CD4$^+$ lymphopenia
11	Heterozygous large deletions: 1 deletion involving the 2 gene copies of 80 kb in 5′ part of the gene and a deletion of 1 copy of 320 kb encompassing two thirds of the 3′ region of DOCK8 gene and the 5′ part of the KANK1 gene	Male	13	>1,100	● No known allergies ● Severe eczema (lichenification) ● Eosinophilia (>700/mm^3)	Chronic otitis, clavicle osteomyelitis, bronchitis, pneumonia, bronchiectasis, *Morganella* species, *Pseudomonas aeruginosa*, *Proteus mirabilis*, *H influenza*, *Giardia intestinalis*	Sclerosing cholangitis, ↑ IgA, ↓ IgM, lymphopenia; died of post-HSCT complications
12	Splice site mutation (exon 11) > frameshift, homozygous	Male	17	17,045	● Food allergies (pork, peanut, chocolate, dairy, egg) ● Severe eczema	Chronic cutaneous and ocular HSV, *M contagiosum*, warts, *S aureus* skin infections, cutaneous dermatophyte infection	Chronic liver disease with vanishing bile ducts on biopsy of unclear cause; calcified dilated aorta
13	Exon 41: c5182C>T homozygous p.R1728X	Male	3	24,893	● Food allergies (milk, egg, tree nuts, peanut) ● Severe eczema ● Asthma	*S aureus* skin infections, herpetic keratitis, warts, onychomycosis, bacterial, viral and pneumocystis pneumonia	
14	Large deletion + stop codon (exon 11)	Male	16	51,010	● Eczema ● Asthma	Sinopulmonary infections, *Neisseria meningitides* arthritis, *M contagiosum*, and warts	
15	Unknown (lack of DOCK8 protein; see Fig E1)	Male	5	17,300	● Food allergies (milk, egg, cashew, pistachio, almond, beef, lamb) ● Eczema ● Asthma ● Bronchiectasis	HSV, *S pyogenes*, *H influenzae*, *Candida albicans*, adenovirus, norovirus, HHV6, EBV, CMV, VZV, *Aspergillus niger*, *Cladosporium* species	
16	Unknown (lack of DOCK8 protein; see Fig E1)	Female	4	8,100	● Food allergies (egg, milk, macadamia) ● Environmental allergies (house dust mites) ● Eczema ● Asthma ● Allergic rhinitis	Ocular herpes, recurrent lower respiratory tract infection, chronic ear infections	Bell's palsy

(Continued)

TABLE I. *(Continued)*

DOCK8-deficient patients	Mutation	Sex	Age at study (y)	IgE (IU/mL)	Allergies/atopic disease	Infections	Other
17	Homozygous deletion spanning exons 15-48	Female	4	2,294	● Food allergies (peanut cashew, pistachio, sesame) ● Sensitization to walnut and egg ● Drug allergy (Propofol) ● Mild eczema	Cryptosporidial cholangitis, chronic adenoviral carriage, mild *M contagiosum*, *Giardia* species, nontyphi *Salmonella* species, low-level CMV viremia, otitis externa	
18	c.12114A>G: p. K405R	Female	18	>10,000	● Food allergies (beans, beef, chicken, cow's milk, egg, fish, peanut, pork, tree nuts, tomato) ● Environmental allergies (dust, dog, grasses, mold) ● Drug allergies (cefipime, Lactinex, propofol) ● Eczema (herpeticum)	*S aureus*, *H influenza*e, cryptococcal meningitis, *Acinetobacter baumannii* sepsis, HSV keratitis, herpes zoster virus	Delayed puberty; deceased
19	Homozygous for a deletion of exons 28-35	Female	17	8,031	● Food allergies (lentils) ● Severe eczema	Chronic oral HSV, sinopulmonary infections, onychomycosis and thrush, *S aureus* skin infections	
20	Homozygous nonsense mutations exon 19: c.2044G>T, p.E682X	Female	11	6,690	● Food allergies eggs, milk, nuts, soy, wheat) ● Severe eczema	*S aureus* skin infections, HSV keratitis	
21	Large deletion (exon 21 to end of gene) + small indel with frameshift mutation (exon 12)	Male	25	1,162	● Food allergies (nuts) ● Eczema	HSV keratitis, sinopulmonary infections, extensive warts	Squamous cell carcinoma before HSCT
22	Large deletion (exon 21 to end of gene) + small indel with frameshift mutation (exon 12)	Female	22	39	● No known allergies	Extensive warts, sinopulmonary infections	Severe bronchiectasis
23	Nonsense mutation (exon 17) + small indel with frameshift mutation (exon 36)	Female	16	180	● Mild eczema	*M contagiosum*, warts, sinopulmonary infections	EBV-associated B-cell lymphoma
24	Large deletion (exons 13-26) + splicing mutation (intron 5)	Male	12	1,563	● Food allergies (tree nuts) ● Mild eczema	Extensive warts, sinopulmonary infections, *S aureus* osteomyelitis	
25	Large deletion (promoter to exon 17) + nonsense mutation (exon 8)	Female	19	5,604	● Food allergies (milk, egg, wheat, nuts) ● Asthma ● Moderate eczema	Sinopulmonary infections, warts and *M contagiosum*, *Pneumocystis* pneumonia, *S aureus* skin infections, mucosal candidiasis	Burkitt lymphoma (EBV negative), vasculopathy of the midaorta with bilateral renal artery stenosis, heart failure; improved after HSCT
26	Homozygous deletion of at least exons 4-13	Female	9	2	● Asthma ● Mild eczema	Sinopulmonary infections, warts	

(Continued)

TABLE I. *(Continued)*

DOCK8-deficient patients	Mutation	Sex	Age at study (y)	IgE (IU/mL)	Allergies/atopic disease	Infections	Other
27	Homozygous deletion of exon 36	Female	20	>6,000	● Food allergies (milk, kiwi) ● Asthma ● Moderate eczema	Sinopulmonary infections, warts, chronic cutaneous HSV	Cerebral vasculopathy with stroke and aortic vasculopathy
28	large homozygous deletion >174 kb affecting most of *DOCK8* (260876_435190) from intron 1 to exon 39	Female	12	1,855-8,460	● Food allergies (egg and lentils) ● Eczema ● Eosinophilia (1,532/mm^3)	Diarrhea, upper respiratory tract infections, recurrent meningoencephalitis, chronic otitis media, esophageal candidiasis, lower urinary tract infection, pyelonephritis (twice), *Pseudomonas* species (ear), *E coli*	Failure to thrive (short stature), mild scoliosis, seronegative hepatitis, liver steatosis, mild hepatosplenomegaly, extensive abdominal vasculitis, increased liver enzyme levels, ↑ IgA, ↑ IgG, ↑ IgM, CD3$^+$ lymphopenia (600/mL)

The following patients were used in these experiments: phenotyping (patients 1-18), *ex vivo* cytokine and *in vitro* differentiation (patients 1, 2, 6, 7, 9, 10, 15, 17, and 18), and plasma IgE (patients 6, 12, 14, 15, 17, and 19-28).
CMV, Cytomegalovirus; *HHV6*, human herpes virus 6; *HSCT*, hematopoietic stem cell transplant; *VZV*, varicella-zoster virus.

METHODS

Human samples

PBMCs, plasma, or both were isolated from healthy donors (Australian Red Cross) and patients with DOCK8 deficiency (Table I). The genotype of some of these patients has been previously reported.[1,2,15,22,24] All studies were approved by institutional human research ethics committees, and written informed consent was obtained from patients.

Antibodies and reagents

Alexa Fluor 488–anti–GATA-3, Alexa Fluor 647–anti-CXCR5, allophycocyanin (APC)-Cy7–anti-CD4, BUV395–anti–IFN-γ, BV711–anti-CD69, BV711–anti–IL-2, phycoerythrin (PE)–anti-CCR6, PE–anti-CD95, Pe-Cy7–anti-CD25, anti-mouse IgG$_1$, PerCpCy5.5–anti-CD127, and anti–T-box transcription factor (T-bet) were from Becton Dickinson (Mountain View, Calif). Alexa Fluor 488–anti–IL-10, APC–anti–inducible costimulator (ICOS), eFluor660–anti–IL-21, fluorescein isothiocyanate–anti-CD45RA, PE–IL-22, Pe-Cy7–anti–IL-4, and mouse IgG$_1$ were from eBioscience (San Diego, Calif). APC-Cy7–anti–IL-17A, BV421–anti-CXCR3, and BV605–anti–TNF-α were from BioLegend (San Diego, Calif). Fluorescein isothiocyanate–anti-CCR7 and recombinant human IL-12 were from R&D Systems (Minneapolis, Minn). Anti-DOCK8 mAb was from Santa Cruz Biotechnology (Dallas, Tex). Recombinant human TGF-β, IL-1β, IL-6, IL-21, and IL-23 were from PeproTech (Rocky Hills, NJ). Prostaglandin E$_2$, phorbol 12-myristate 13-acetate (PMA), calcium ionophore (ionomycin), Brefeldin A, and saponin were purchased from Sigma-Aldrich (St Louis, Mo). Recombinant human IL-4 was provided by Dr Rene de Waal Malefyt (DNAX Research Institute, Palo Alto, Calif). T-cell activation and expansion (TAE) beads (anti-CD2/CD3/CD28) were purchased from Miltenyi Biotec (Bergisch Gladbach, Germany), and carboxyfluorescein succinimidyl ester (CFSE) was purchased from Invitrogen (Carlsbad, Calif).

CD4$^+$ T-cell phenotyping

To identify naive, central memory T (T$_{CM}$) cell, and effector memory T (T$_{EM}$) cell CD4$^+$ populations, PBMCs were incubated with mAbs to CD4, CCR7, and CD45RA, and the frequency of CD4$^+$CCR7$^+$CD45RA$^+$ (naive), CD4$^+$CCR7$^+$CD45RA$^-$ (T$_{CM}$), and CD4$^+$CCR7$^-$CD45RA$^-$ (T$_{EM}$) populations were determined by using flow cytometry. To identify CD4$^+$ T-cell populations, PBMCs were incubated with mAbs to CD4, CD25, CD127, CXCR5, CD45RA, CCR6, and CXCR3, and the frequency of Treg (CD4$^+$CD25^{hi}CD127^{lo}), follicular helper T (T$_{FH}$; CD4$^+$CD25^{lo}CD127^{hi}CD45RA$^-$CXCR5$^+$), T$_H$1 (CD4$^+$CD25^{lo}CD127^{hi}CD45RA$^-$CXCR5$^-$CXCR3$^+$CCR6$^-$), T$_H$2 (CD4$^+$CD25^{lo}CD127^{hi}CD45RA$^-$CXCR5$^-$CXCR3$^-$CCR6$^-$), and T$_H$17 (CD4$^+$CD25^{lo}CD127^{hi}CD45RA$^-$CXCR5$^-$CXCR3$^-$CCR6$^+$) subsets were determined.[20]

Analysis of cytokine expression/secretion by CD4$^+$ and CD8$^+$ T cells

Naive and memory CD4$^+$ T cells or naive, memory, and T$_{EM}$ cells expressing CD45RA (T$_{EMRA}$) CD8$^+$ T cells[22] were isolated by sorting on a FACSAria (Becton Dickinson; >98% purity) and cultured with TAE beads (anti-CD2/CD3/CD28) in 96-well, round-bottomed plates. After 5 days, supernatants were harvested, and production of IL-2, IL-4, IL-5, IL-6, IL-10, IL-13, IL-17A, IL-17F, IFN-γ, and TNF-α was determined by using the cytometric bead array (Becton Dickinson). For cytokine expression, activated T cells were restimulated with PMA (100 ng/mL) and ionomycin (750 ng/mL) for 6 hours, with Brefeldin A (10 μg/mL) added after 2 hours. Cells were then fixed with formaldehyde, and expression of IFN-γ, IL-4, IL-17A, IL-22, IL-21, IL-10, TNF-α, and IL-2 was detected by means of intracellular staining.[20,26-28]

Analysis of transcription factor expression by CD4$^+$ T cells

Expression of T-bet and GATA-3 protein was assessed by means of intracellular staining with a Fix/Perm kit from eBioscience. Expression of RAR-related orphan receptor C *(RORC)* was determined by using quantitative PCR.[28]

Analysis of DOCK8 expression

PBMCs were fixed with formaldehyde and stained with an unconjugated DOCK8 or an isotype control IgG$_1$ mAb to determine intracellular DOCK8 expression. PE-rat anti-mouse IgG$_1$ was then used with saponin as the permeabilizing agent.[29]

Analysis of CD4$^+$ T-cell proliferation

Naive and memory CD4$^+$ T cells were isolated by means of sorting and then labeled with CFSE. Their proliferation status was determined by assessing dilution of CFSE after 5 days of *in vitro* culture.[27,28]

In vitro T_H1, T_H2, and T_H17 cell differentiation

Naive and memory $CD4^+$ T cells were isolated by means of sorting and cultured under T_H0 (TAE beads alone), T_H1-polarizing (50 ng/mL IL-12), T_H2-polarizing (100 U/mL IL-4), or T_H17-polarizing (2.5 ng/mL TGF-β, 50 ng/mL IL-1β, 50 ng/mL IL-6, 50 ng/mL IL-21, 50 ng/mL IL-23, and 50 ng/mL prostaglandin E_2) conditions. After 5 days, cytokine secretion was analyzed (cytometric bead array and intracellular staining).[26,28,30]

ImmunoCAP assay

Plasma from healthy donors and DOCK8-deficient patients was analyzed for allergen-specific IgE antibodies by the Sydney South West Pathology Service (Royal Prince Alfred Hospital, Sydney, Australia) using the Phadia 250 ImmunoCAP platform (Thermo Scientific, Waltham, Mass). IgE levels specific for a staple food mix (FX5; egg white, milk, codfish, wheat, peanut, and soybean) or house dust mite mix was determined.

Statistical analysis

Significant differences were determined by using either the Student *t* test, multiple *t* tests, or 1- or 2-way ANOVA (Prism software; GraphPad Software, La Jolla, Calif).

RESULTS

Effects of DOCK8 deficiency on the generation of effector $CD4^+$ T-cell subsets *in vivo*

As an initial step in investigating $CD4^+$ T-cell function in the absence of DOCK8, we assessed the $CD4^+$ T-cell compartment to determine whether the generation and differentiation of $CD4^+$ T cells was affected by DOCK8 deficiency and whether this could contribute to the combined immunodeficiency typical of these subjects. We previously investigated the peripheral T-cell compartment in a small cohort (n = 6) of DOCK8-deficient patients.[22] We have now increased our cohort to comprise 18 subjects from 15 unrelated families and have extended our analysis to include additional surface markers to further distinguish different subsets within the $CD4^+$ T-cell population (Fig 1). DOCK8 expression in lymphocytes and monocytes from a representative healthy control subject, 1 unaffected sibling, and 4 DOCK8-deficient patients is depicted in Fig E1 in this article's Online Repository at www.jacionline.org. Analysis of this larger cohort of DOCK8-deficient patients confirmed a statistically significant reduction in numbers of $CD4^+$ T cells compared with those seen in healthy donors (Fig 1, *A*). Naive, T_{CM}, and T_{EM} $CD4^+$ T cells can be resolved according to the differential expression of CD45RA and CCR7 (Fig 1, *B*).[31] This analysis revealed that the naive and T_{CM} compartments in DOCK8-deficient patients are comparable with those of healthy subjects but that T_{EM} $CD4^+$ T-cell numbers were significantly increased in DOCK8-deficient patients (Fig 1, *C*). Hence despite the reduction in total $CD4^+$ T-cell numbers, DOCK8-deficient $CD4^+$ T cells differentiate normally into naive and T_{CM} cells; this is accompanied by a mild increase in T_{EM} cell numbers.

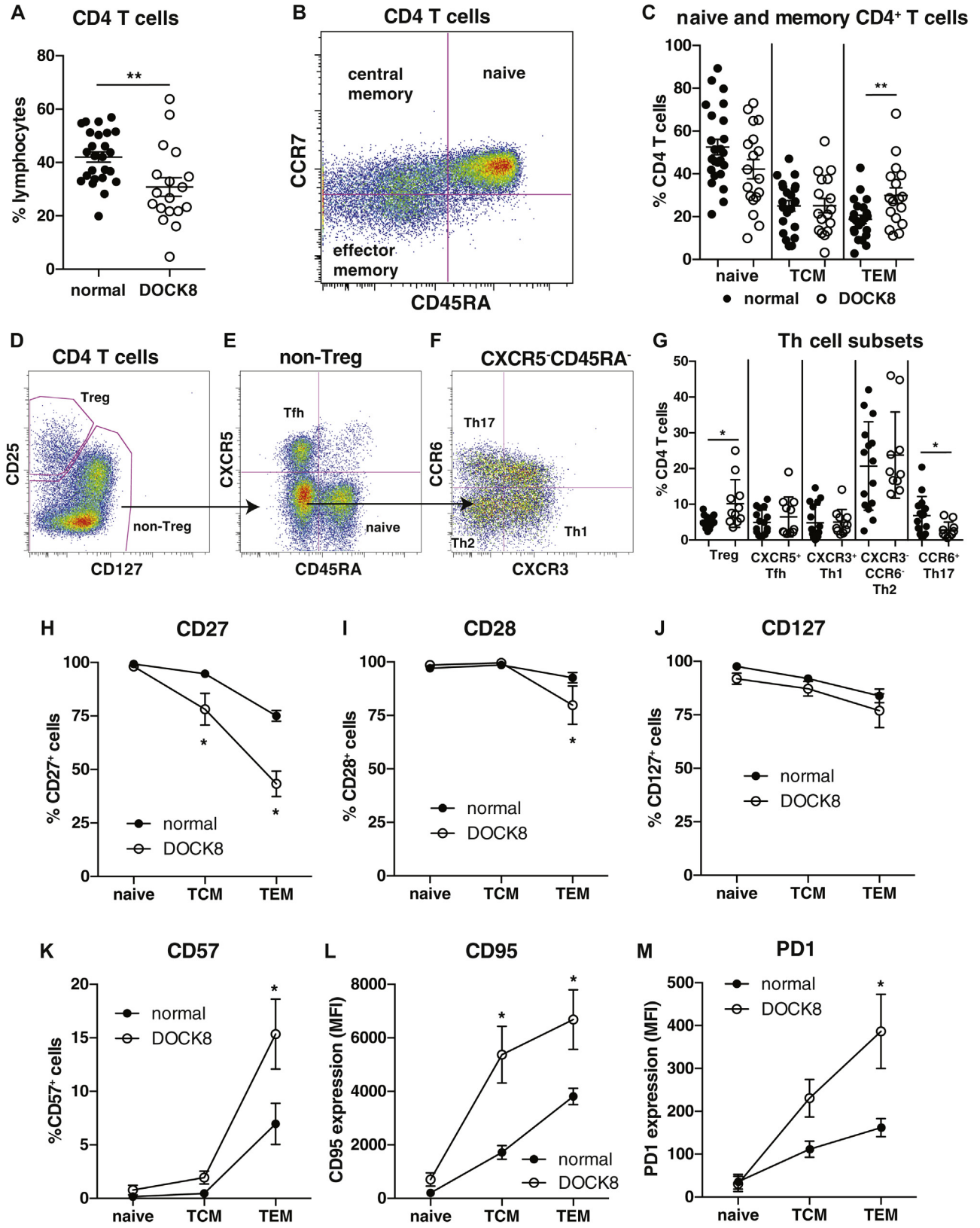
Using a recently described gating strategy,[20,32] we next examined the $CD4^+$ T-cell compartment for additional effector subsets: $CD25^{hi}CD127^{lo}$ Treg (Fig 1, *D* and *G*),[33] $CXCR5^+$ $CD45RA^-$ T_{FH} (Fig 1, *E* and *G*), $CD45RA^-CXCR5^-$ $CXCR3^+CCR6^-$ T_H1 (Fig 1, *F* and *G*), $CD45RA^-CXCR5^-$ $CXCR3^-CCR6^-$ T_H2 (Fig 1, *F* and *G*), and $CD45RA^-CXCR5^-CXCR3^-CCR6^+$ T_H17 (Fig 1, *F* and *G*) cells. DOCK8-deficient patients had an increased frequency of Treg cells (Fig 1, *D* and *G*) but decreased frequency of T_H17 cells (Fig 1, *F* and *G*), whereas frequencies of T_{FH}, T_H1, and T_H2 cells according to this phenotypic delineation in patients were similar to those of healthy donors (Fig 1, *D-G*). Thus there is a selective paucity of T_H17 cells due to *DOCK8* mutations.

Assessment of expression of additional surface markers associated with $CD4^+$ T-cell differentiation indicated that the naive, T_{CM}, and T_{EM} $CD4^+$ T-cell populations from *DOCK8*-deficient patients had undergone greater activation and terminal differentiation than corresponding $CD4^+$ T-cell subsets isolated from healthy donors (Fig 1, *H-M*). Specifically, loss of expression of CD27 (Fig 1, *H*), CD28 (Fig 1, *I*), and CD127 (Fig 1, *J*) and acquisition of CD57 (Fig 1, *K*), CD95 (Fig 1, *L*), and programmed cell death protein 1 (PD-1; Fig 1, *M*) by $CD4^+$ T_{CM} and T_{EM} cells was exaggerated for DOCK8-deficient patients compared with that seen in control subjects. Collectively, DOCK8 deficiency compromises the generation of T_H17 cells and results in the premature terminal differentiation of memory cells, such that they acquire a senescent/exhausted phenotype.

DOCK8-deficient memory $CD4^+$ T cells are biased toward T_H2 cytokines

Given the decrease in numbers of $CCR6^+CXCR3^-$ cells, which are enriched for T_H17 cytokine–producing cells in healthy donors,[20,34-36] in DOCK8-deficient patients, we investigated cytokine expression by naive and memory $CD4^+$ T cells (Fig 2). Naive and total memory ($CD45RA^-CCR7^{+/-}$) $CD4^+$ T cells were sort purified from normal donors and DOCK8-deficient patients and then cultured with TAE beads conjugated to anti-CD2/CD3/CD28 mAbs for 5 days. After this time, cells were restimulated with PMA/ionomycin, and intracellular expression of IFN-γ, IL-4, IL-17A, IL-22, IL-21, IL-10, TNF-α, and IL-2 was determined (Fig 2). Apart from IL-2 (Fig 2, *A*) and TNF-α (Fig 2, *B*), which are expressed by 40% to 80% of normal naive cells, only a small proportion of naive cells (ie, <5%) expressed any of the other cytokines examined. DOCK8-deficient naive $CD4^+$ T cells expressed comparable levels of IL-2 (Fig 2, *A*) and TNF-α (Fig 2, *B*) to those of normal naive $CD4^+$ T cells. However, analysis of the memory $CD4^+$ T-cell compartment in DOCK8-deficient patients revealed marked perturbations in differentiation *in vivo*. A significantly greater proportion of DOCK8-deficient memory $CD4^+$ T cells expressed IL-4 compared with normal memory $CD4^+$ T cells (Fig 2, *C*), suggesting a skewing to the T_H2 effector lineage. Examination of mean fluorescence intensity of IL-4^+ cells in DOCK8-deficient and normal memory $CD4^+$ T cells revealed no significant differences (data not shown), suggesting there is an increase in the frequency of IL-4–expressing cells in the DOCK8-deficient memory $CD4^+$ T-cell compartment but that a comparable amount of IL-4 is produced per cell. The increase in numbers of IL-4^+ cells in *DOCK8*-deficient memory $CD4^+$ T cells was accompanied by significant reductions in expression of the T_H1 cytokines IFN-γ (Fig 2, *D*) and TNF-α (Fig 2, *B*), the T_H17 cytokines IL-17A (Fig 2, *E*) and IL-22 (Fig 2, *F*), and the T_{FH} cytokine IL-21 (Fig 2, *G*). Expression of IL-10 (Fig 2, *H*) and IL-2 (Fig 2, *A*) by memory $CD4^+$ T cells was unaffected by DOCK8 deficiency.

The T_H2 skewing by DOCK8-deficient memory $CD4^+$ T cells was also assessed by measuring cytokine secretion during the 5-day culture (Fig 3). This indicated concordance between the


A
CD4 T cells
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**
normal
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DOCK8
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CD25
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E
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Tfh
naive
CXCR5
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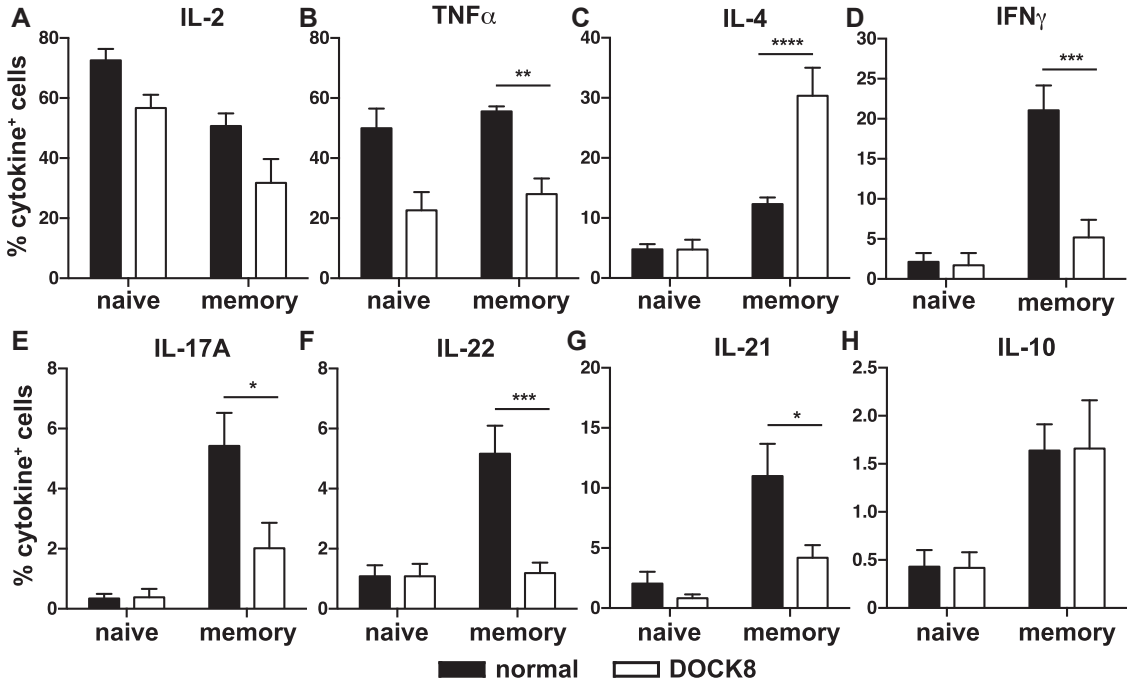


FIG 2. DOCK8-deficient memory CD4$^+$ T cells display a T_H2 cytokine expression bias. Naive (CD45RA$^+$CCR7$^+$) and memory (CD45RA$^-$CCR7$^{+/-}$) CD4$^+$ T cells were isolated from healthy donors and DOCK8-deficient patients and cultured with TAE beads for 5 days. Cells were then restimulated with PMA/ionomycin for 6 hours in the presence of Brefeldin A for the last 4 hours. Intracellular expression of IL-2 **(A)**, TNF-α **(B)**, IL-4 **(C)**, IFN-γ **(D)**, IL-17A **(E)**, IL-22 **(F)**, IL-21 **(G)**, and IL-10 **(H)** was determined by using saponin as the permeabilizing agent, followed by flow cytometric analysis. Data represent means ± SEMs of 8 healthy donors or 8 DOCK8-deficient patients. Statistics were performed with Prism software by using 1-way ANOVA. *$P < .05$, **$P < .01$, ***$P < .005$, and ****$P < .001$.

expression and secretion of cytokines when assessed by using intracellular staining and flow cytometry or the cytometric bead array, respectively. Analysis of an extended panel of cytokines showed that DOCK8-deficient memory T cells secreted not only more IL-4 than normal memory CD4$^+$ T cells but also more of the T_H2 cytokines IL-5 and IL-13 (Fig 3, *A-C*) and less of the T_H1 (IFN-γ and TNF-α; Fig 3, *D* and *E*) and T_H17 (IL-17A and IL-17F; Fig 3, *F* and *G*) cytokines. Production of IL-6 (Fig 3, *H*) was also significantly reduced. There were trends for less production of IL-10 and IL-2 by DOCK8-deficient memory CD4$^+$ T cells; however, these reduced values were not significant (Fig 3, *I* and *J*). Production of TNF-α and IL-2 by DOCK8-deficient naive CD4$^+$ T cells was normal (Fig 3, *E* and *J*). Taken together, memory CD4$^+$ T cells from DOCK8-deficient patients display a T_H2 bias, primarily expressing IL-4, IL-5, and IL-13 and notably lower levels of cytokines characteristic of other T helper subsets.

T_H2 cytokine bias by DOCK8-deficient memory CD4$^+$ T cells is independent of defects in cell proliferation

Previous work showed that lymphocyte differentiation, such as immunoglobulin class-switching and antibody secretion by naive

FIG 1. Phenotype of the peripheral CD4$^+$ T-cell compartment in DOCK8-deficient patients. **A,** Frequency of CD4$^+$ T cells in healthy donors and DOCK8-deficient patients. **B** and **C,** Naive (CD45RA$^+$CCR7$^+$), T_{CM} (CD45RA$^-$CCR7$^+$), and T_{EM} (CD45RA$^-$CCR7$^-$) cell populations in healthy donors (*solid symbols*; n = 25) and DOCK8-deficient patients (*open symbols*; n = 18) were enumerated based on expression of CD45RA and CCR7. **D-G,** PBMCs were labeled with mAbs against CD4, CD45RA, CD25, CD127, CXCR5, CXCR3, and CCR6. Fig 1, *D*, Treg cells were identified as CD25^{hi}CD127^{lo}. Fig 1, *E*, Among the non-Treg cell population, naive and T_{FH} cells were identified as CXCR5$^-$CD45RA$^+$ and CXCR5$^+$CD45RA$^-$, respectively. Fig 1, *F*, T_H1, T_H2, and T_H17 populations were identified within the population of CXCR5$^-$CD45RA$^-$ memory CD4$^+$ T cells as CXCR3$^+$CCR6$^-$, CCR6$^-$CXCR3$^-$, and CCR6$^+$CXCR3$^-$ cells, respectively. Fig 1, *G*, By using this gating, the frequency of Treg, T_{FH}, T_H1, T_H2, and T_H17 cells within the CD4$^+$ T-cell compartment was determined in healthy subjects (*solid symbols*; n = 15 or 16) and in DOCK8-deficient patients (*open symbols*; n = 10 or 11). Each *point* represents an individual donor or patient. Statistics were performed with Prism software by using the Student *t* test. **H-M,** Naive (CD45RA$^+$CCR7$^+$), T_{CM} (CD45RA$^-$CCR7$^+$), and T_{EM} (CD45RA$^-$CCR7$^-$) cell populations in healthy donors *(solid symbols)* and DOCK8-deficient patients *(open symbols)* were identified and assessed for expression of CD27 (Fig 1, *H*), CD28 (Fig 1, *I*), CD127 (Fig 1, *J*), CD57 (Fig 1, *K*), CD95 (Fig 1, *L*), and PD-1 (Fig 1, *M*). Each *point* corresponds to the mean ± SEM percentage of cells expressing the indicated surface receptor or the mean fluorescence intensity *(MFI)* of expression (n = 4-12 healthy donors or DOCK8-deficient patients). Statistics were performed with Prism software by using the *t* test. *$P < .05$ and **$P < .01$.

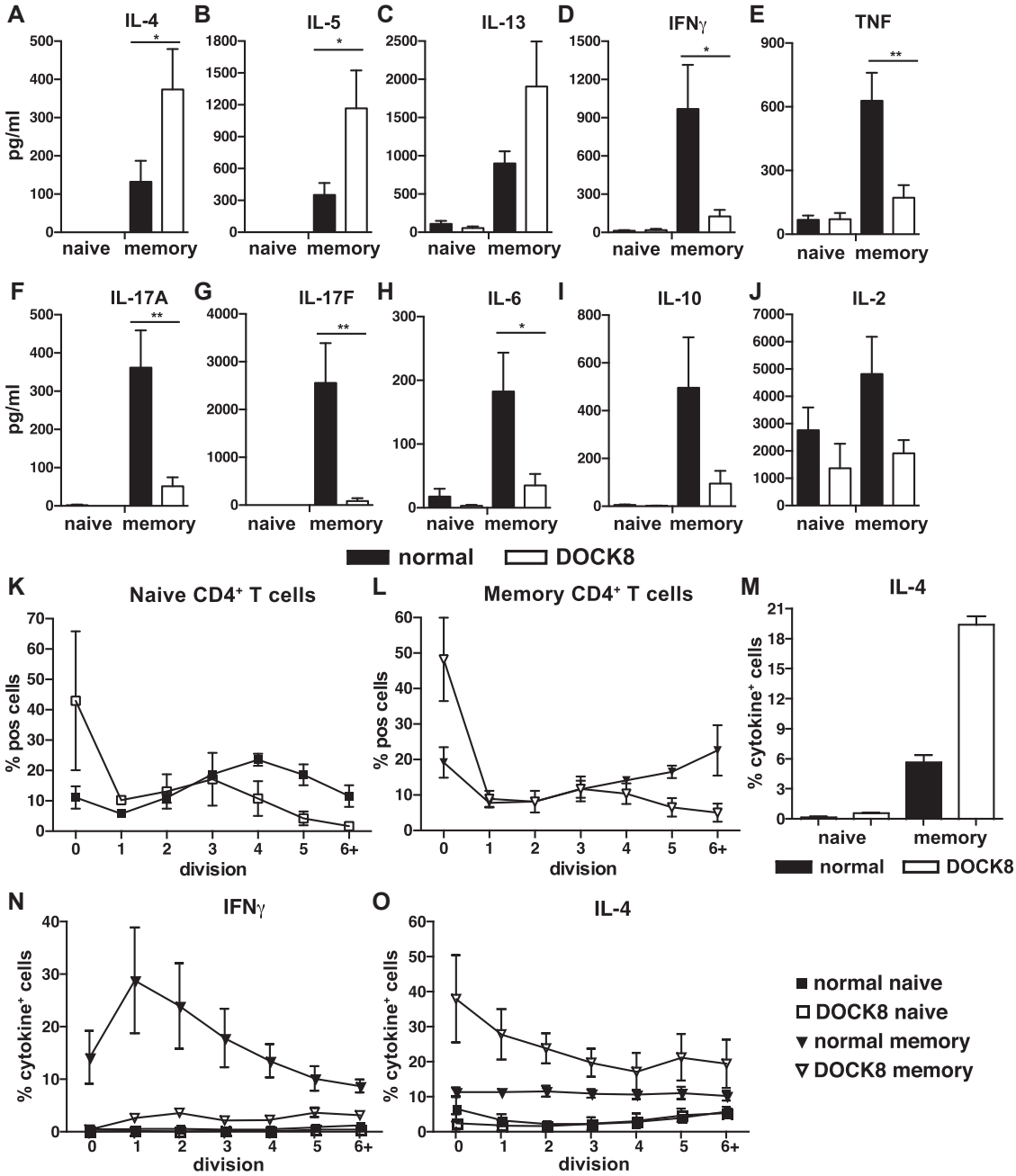
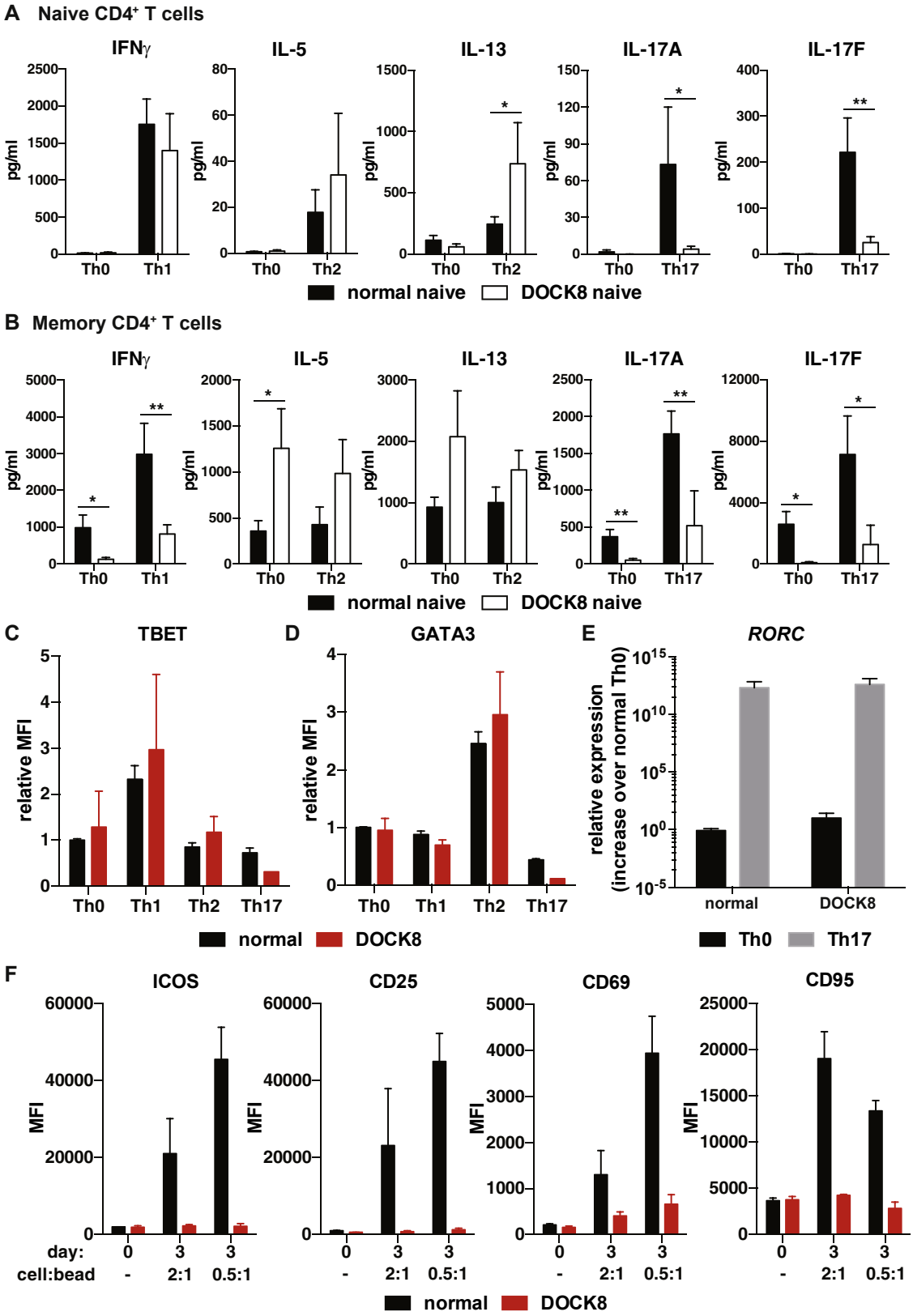


FIG 3. DOCK8-deficient memory CD4$^+$ T cells secrete increased quantities of the T_H2 cytokines IL-4, IL-5, and IL-13 independently of differences in cell proliferation. Naive and memory CD4$^+$ T cells were sorted from healthy donors and DOCK8-deficient patients and cultured with TAE beads for 5 days. **A-J,** After this time, culture supernatants were examined for secretion of IL-4 (Fig 3, *A*), IL-5 (Fig 3, *B*), IL-13 (Fig 3, *C*), IFN-γ (Fig 3, *D*), TNF (Fig 3, *E*), IL-17A (Fig 3, *F*), IL-17F (Fig 3, *G*), IL-6 (Fig 3, *H*), IL-10 (Fig 3, *I*), and IL-2 (Fig 3, *J*) by using a custom-designed cytometric bead array (BD Biosciences). Data represent means ± SEMs of experiments by using cells from 9 healthy donors or DOCK8-deficient patients. Statistics were performed with Prism software by using 1-way ANOVA. **K** and **L,** Naive (Fig 3, *K*) and memory (Fig 3, *L*) CD4$^+$ T cells were isolated from healthy donors (n = 4) and DOCK8-deficient patients (n = 4), labeled with CFSE, and cultured with TAE beads for 5 days. After this time, the frequency of cells in each division was determined by means of dilution of CFSE. **M,** Sorted naive and memory CD4$^+$ cells were immediately restimulated with PMA/ionomycin for 6 hours in the presence of Brefeldin A and IL-4 expression determined by using intracellular staining and flow cytometry. **N** and **O,** Naive and memory CD4$^+$ T cells were labeled with CFSE and cultured with TAE beads for 5 days, and the proportion of cells expressing IFN-γ (Fig 3, *L*) or IL-4 (Fig 3, *M*) in each division interval was determined by means of dilution of CFSE. Data represent means ± SEMs of 2 to 4 healthy donors and DOCK8-deficient patients. *$P < .05$ and **$P < .01$.


A Naive CD4+ T cells
IFNγ
IL-5
IL-13
IL-17A
IL-17F
pg/ml
Th0
Th1
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Th17
normal naive
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B Memory CD4+ T cells
C
TBET
relative MFI
D
GATA3
E
RORC
relative expression (increase over normal Th0)
normal
DOCK8
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day:
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B cells and cytokine production and cell-surface phenotype expression by naive T cells, is regulated by cell division.[27,37-39] DOCK8-deficient naive (Fig 3, *K*) and memory (Fig 3, *L*) $CD4^+$ T cells were found to have impaired cell division *in vitro*, which is consistent with previous findings.[22] Thus it was possible that the perturbed cytokine profile reflected reduced proliferation by DOCK8-deficient memory $CD4^+$ T cells. However, the T_H2 bias of DOCK8-deficient memory $CD4^+$ T cells was not due to a proliferative defect, as evidenced by 2 important and related findings.

First, when memory cells were isolated and restimulated immediately for analysis of cytokine expression, the preferential production of IL-4 by DOCK8-deficient over normal memory $CD4^+$ T cells was still observed in the absence of cell proliferation (Fig 3, *M*). Similarly, the poor production of T_H1 and T_H17 cytokines by DOCK8-deficient memory $CD4^+$ T cells did not result from impaired proliferation because reductions in expression of IFN-γ (normal, 17.7%; DOCK8, 6.9%) and IL-22 (normal, 3.7%; DOCK8, 1.8%), respectively, were also observed when assessed under these *ex vivo* stimulatory conditions.

Second, analysis of cells that had undergone different rounds of division *in vitro* revealed that the decrease in IFN-γ (Fig 3, *N*) and increase in IL-4 (Fig 3, *O*) levels displayed by DOCK8-deficient versus normal memory $CD4^+$ T cells was evident for all division intervals examined. Thus the preference of DOCK8-deficient memory $CD4^+$ T cells to produce T_H2, but not T_H1, cytokines is independent of any proliferative defects in these cells.

Naive DOCK8-deficient $CD4^+$ T cells can differentiate into effector cells producing T_H1 and T_H2, but not T_H17, cytokines *in vitro*

To determine whether defects in cytokine production by DOCK8-deficient memory $CD4^+$ T cells are cell intrinsic or due to extrinsic factors, we isolated naive $CD4^+$ T cells from healthy donors and DOCK8-deficient patients and subjected them to *in vitro* culture under T_H0 and T_H1-, T_H2-, or T_H17-polarizing conditions. Interestingly, DOCK8-deficient naive $CD4^+$ T cells differentiated into T_H1 cells (IFN-γ and TNF-α) to the same extent as normal naive $CD4^+$ T cells (Fig 4, *A*, left panel). Consistent with the data for memory $CD4^+$ T cells *ex vivo*, DOCK8-deficient naive $CD4^+$ T cells produced significantly greater amounts of the T_H2 cytokine IL-13 than control naive $CD4^+$ T cells under T_H2-polarizing conditions (3-fold increase; Fig 4, *A*, middle panels). We also analyzed T_H2 differentiation by assessing cytokine expression in naive $CD4^+$ T cells by means of intracellular staining and flow cytometry after *in vitro* T_H2 polarization. This confirmed a preferential differentiation of DOCK8-deficient cells toward a T_H2 fate, with increased proportions of DOCK8-deficient naive $CD4^+$ T cells expressing IL-4 (9.9% DOCK8-deficient vs 5.5% control $CD4^+$ T cells) and IL-13 (5.9% DOCK8-deficient vs 1.7% control $CD4^+$ T cells). Together, these data provide evidence of a predominant intrinsic bias of DOCK8-deficient naive $CD4^+$ T cells differentiating toward a T_H2 effector fate. DOCK8-deficient naive $CD4^+$ T cells did not differentiate into IL-17A– and IL-17F–secreting cells when subjected to T_H17-polarizing conditions *in vitro* (Fig 4, *A*, right panels). Notably, DOCK8-deficient naive $CD4^+$ T cells responded to the T_H17 culture, as shown by reductions in basal levels of IL-5 and IL-13 secretion compared with the T_H0 culture (data not shown).

When we examined memory $CD4^+$ T cells from healthy donors, production of IFN-γ and IL-17A/F could be increased approximately 2- to 4-fold by T_H1 and T_H17 culture conditions, respectively, compared with T_H0 conditions (Fig 4, *B*). The net increase in production of these cytokines by DOCK8-deficient memory $CD4^+$ T cells under T_H1 and T_H17 conditions compared with T_H0 conditions was also approximately 2- to 6-fold. Despite this, levels of IFN-γ and IL-17A/F secreted by T_H1- and T_H17-stimulated DOCK8-deficient memory $CD4^+$ T cells were substantially less than not only those secreted by T_H1- and T_H17-stimulated normal memory $CD4^+$ T cells but also T_H0-stimulated normal memory $CD4^+$ T cells (Fig 4, *B*). This likely reflects expansion of the few T_H1 and T_H17 cells present in the DOCK8 memory $CD4^+$ T-cell compartment rather than *de novo* differentiation into these effector subsets *in vitro*.

Consistent with the data for cytokine secretion, DOCK8-deficient naive $CD4^+$ T cells that were polarized toward T_H1 and T_H2 fates upregulated T-bet (Fig 4, *C*) and GATA-3 (Fig 4, *D*), respectively, to the same extent as normal naive $CD4^+$ T cells. In our hands detection of RORγt expression by using flow cytometry was not particularly sensitive because we found that only a small proportion of naive $CD4^+$ T cells (approximately 5%) expressed RORγt in T_H17- compared with T_H0-activated cultures.[40] To overcome this, *RORC* expression was determined by using quantitative PCR. *RORC* was not expressed by naive $CD4^+$ T cells activated under T_H0 conditions but was upregulated in normal and DOCK8-deficient naive $CD4^+$ T cells cultured under T_H17-polarizing conditions (Fig 4, *E*). Taken together, these data indicate the T_H17 cytokine defect in patients with DOCK8 deficiency is T cell intrinsic and cannot

FIG 4. Intrinsic defects in $CD4^+$ T-cell cytokine secretion caused by *DOCK8* mutations. **A** and **B,** Naive (Fig 4, *A*) and memory (Fig 4, *B*) $CD4^+$ T cells were isolated from healthy donors and DOCK8-deficient patients and activated under neutral conditions (T_H0; TAE only), or T_H1-polarizing (+IL-12), T_H2-polarizing (+IL-4), or T_H17-polarizing (+IL-1β, IL-6, IL-21, IL-23, TGF-β, and prostaglandin) conditions. After 5 days, secretion of T_H1 (IFN-γ), T_H2 (IL-5 and IL-13), and T_H17 (IL-17A and IL-17F) cytokines was determined by using the cytometric bead array. Data represent means ± SEMs of experiments with cells from 12 healthy donors and 8 DOCK8-deficient patients. **C** and **D,** Expression of T-bet (Fig 4, *C*) and GATA-3 (Fig 4, *D*) was determined by means of flow cytometry; data represent the fold change (mean ± SEM) in expression of the indicated transcription factor relative to T_H0 culture of the healthy control subject. **E,** expression of *RORC* was determined by using quantitative PCR. Data represent means and SEMs of 2 to 3 healthy donors and DOCK8-deficient patients. **F,** Memory $CD4^+$ T cells from healthy donors or DOCK8-deficient patients (n = 2) were cultured with TAE beads at a cell/bead ratio of 2:1 and 0.5:1, and expression of ICOS, CD25, CD69, and CD95 was determined before culture (day 0) and 3 days after activation. Values represent means ± SEMs of the mean fluorescence intensity *(MFI)* of each of the indicated surface receptors. Statistics were performed with Prism software by using 2-way ANOVA. $*P < .05$ and $**P < .01$.

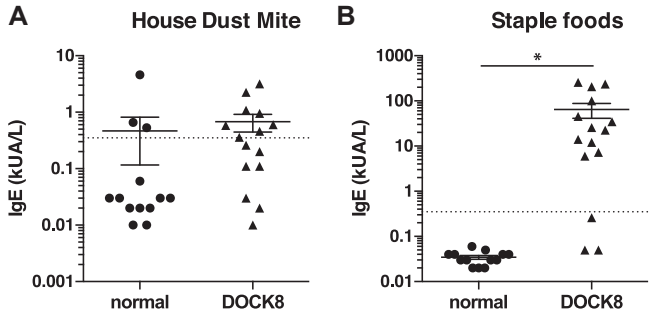

FIG 5. IgE in DOCK8-deficient patients is specific for staple foods but not other antigens, such as house dust mites. Plasma from healthy donors and DOCK8-deficient patients was analyzed for IgE specific for a staple food mix (egg white, milk, codfish, wheat, peanut, and soybean; **A**) and a house dust mite mix **(B)** by using ImmunoCAP. Data represent means ± SEMs of 13 healthy donors and 15 *DOCK8*-deficient patients. The *dotted line* refers to the upper limit of the negative reference interval (0.35 kU_A/L). $*P < .05$.

be restored by T_H17-polarizing conditions for either naive or memory cells. Furthermore, the ability of T_H17 culture conditions to induce *RORC* in the absence of DOCK8 indicates the defect in T_H17 differentiation is downstream of *RORC*. In contrast, DOCK8-deficient naive $CD4^+$ T cells differentiate normally into T_H1 cells and exhibit exaggerated T_H2 differentiation when provided with the appropriate stimuli *in vitro*.

Preferential production of T_H2 cytokines by DOCK8-deficient $CD4^+$ T cells correlates with reduced TCR-mediated activation

The strength of signal provided to $CD4^+$ T cells through the TCR greatly influences their differentiation to cytokine-producing effector cells. For instance, reduced signal strength favors T_H2 cells,[41-44] whereas differentiation to T_H17 cells requires stronger or sustained TCR signaling.[45,46] Our findings of heightened production of T_H2 cytokines by DOCK8-deficient naive and memory $CD4^+$ T cells led us to hypothesize that mutations in DOCK8 compromised TCR signal strength. To assess this, we cultured DOCK8-deficient $CD4^+$ T cells with differing doses of anti-CD2/CD3/CD28 beads for 3 days and then measured levels of expression of the activation molecules ICOS, CD25, CD69, and CD95. The rationale here is that decreasing the dose of the beads results in a qualitatively weaker signal. Although $CD4^+$ T cells from healthy control subjects exhibited heightened expression of ICOS, CD69, and CD25 at the 2 different doses of anti-CD2/CD3/CD28 beads tested, induction of these same molecules on DOCK8-deficient $CD4^+$ T cells was severely blunted (Fig 4, *F*). Thus mutations in *DOCK8* compromise T-cell activation by reducing the strength of signal delivered through the TCR and costimulatory receptor signaling pathways. In the case of T-cell differentiation, this results in skewing of the cells toward a T_H2 phenotype.

Specific sensitization of DOCK8-deficient patients to food allergens

Exaggerated T_H2 immune responses have traditionally been associated with allergy and atopic disease.[47] Thus it was intriguing to note that $CD4^+$ T cells from DOCK8-deficient patients were biased toward production of T_H2 cytokines and that these patients have severe allergies. To determine whether the T_H2 bias in DOCK8-deficient human $CD4^+$ T cells is related to their increased susceptibility to food allergies, we examined the specificity of IgE in serum samples from DOCK8-deficient patients and normal healthy donors to staple foods (ie, egg white, milk, codfish, wheat, peanut, and soybean), as well as to nonfood allergens, such as house dust mites. We found that a comparable frequency of healthy subjects and DOCK8-deficient patients had IgE specific to house dust mites (Fig 5, *A*). Strikingly, the majority of plasma samples from DOCK8-deficient patients (80% [12/15]) but none of the healthy control subjects tested had IgE that was specific for the staple food mix (Fig 5, *B*). Thus DOCK8-deficient patients have a T_H2 bias that manifests clinically as specific sensitization to oral allergens, and this might explain the marked propensity of these immunodeficient patients to have food allergies.

DISCUSSION

Identifying defects in lymphocyte development or function in patients with primary immunodeficiencies (PIDs) provides the opportunity to elucidate the cellular and molecular basis for the clinical features of the disease. Indeed, studies of DOCK8-deficient human subjects and mice have revealed critical cell-intrinsic roles for DOCK8 in generating B-cell memory and long-lived humoral immunity,[5,48] $CD8^+$ T-cell differentiation and antiviral responses,[22,23,49,50] NK cell cytotoxicity,[24] and NKT cell development.[25] Collectively, these defects underlie poor antibody responses to specific antigens and impaired cell-mediated immunity to pathogens, including HSV, HPV, and *M contagiosum* virus. We have now investigated $CD4^+$ T-cell differentiation in DOCK8-deficient patients to understand other aspects of AR-HIES, such as susceptibility to bacterial and fungal infections, atopic disease, food allergies, and hyper-IgE syndrome.

Our data revealed that DOCK8-deficient $CD4^+$ T cells have dysregulated expression of surface molecules, including CD27, CD57, CD95, and PD-1. This likely results from chronic infection with pathogens, such as herpes viruses (HSV, cytomegalovirus, and varicella-zoster virus), HPV, and *M contagiosum* virus, akin to what has been described for $CD8^+$ T cells in not only DOCK8 deficiency[22] but also other PIDs, such as X-liked lymphoproliferative disease,[51,52] STAT3 deficiency,[53] and individuals with *PIK3CD* gain-of-function mutations,[54] which are characterized by chronic exposure to infectious pathogens. In the absence of DOCK8, memory $CD4^+$ T cells are polarized to a T_H2 cytokine phenotype at the expense of T_H1 and T_H17 cytokines. The reduction in T_H17 cell numbers was apparent not only from the lack of cells producing IL-17A, IL-17F, and IL-22 but also the reduction in numbers of $CCR6^+$ memory $CD4^+$ T cells. This is consistent with our previous studies that revealed parallel reductions in numbers of $CD4^+$ T cells secreting IL-17A/IL-17F and expressing $CCR6^+$ in patients with *STAT3* loss-of-function or *STAT1* gain-of-function mutations,[17,20,28] indicating that flow cytometric analysis of $CCR6^+$ memory $CD4^+$ T cells can be a reliable and rapid means of quantifying T_H17 cells.

Interestingly, DOCK8-deficient naive $CD4^+$ T cells differentiated into T-bet–expressing and T_H1 cytokine–secreting cells when provided with exogenous signals *in vitro*. This suggests that defects in IFN-γ production by DOCK8-deficient memory $CD4^+$ T cells *ex vivo* are extrinsic, possibly resulting from suboptimal priming by antigen-presenting cells and provision of

IL-12 *in vivo*. Consistent with this, DOCK8-deficient murine dendritic cells did not accumulate in the lymph node parenchyma, where they are required for T-cell priming during immune responses.[55] This defect was attributed to compromised Cdc42 function in the absence of DOCK8.[55] Another possibility is that excessive production of IL-4, which restrains differentiation of human $CD4^+$ T cells into T_H1 cells,[56] impairs IFN-γ production by DOCK8-deficient memory $CD4^+$ T cells. This is consistent with our recent observations of heightened production of T_H2 cytokines and corresponding reductions in IFN-γ production *ex vivo* by memory $CD4^+$ T cells from subjects with loss-of-function mutations in *STAT3*, *IL21R*, *IL12RB1*, *TYK2*, or *RORC*.[20,57] Although DOCK8-deficient naive $CD4^+$ T cells could express *RORC in vitro* after activation under T_H17-polarizing conditions, IL-17A/F cytokine secretion remained greatly impaired. Thus an intrinsic defect distal to inducing *RORC* expression underlies the inability of DOCK8-deficient $CD4^+$ T cells to become T_H17 cells. Although T_H1- and T_H17-polarizing conditions increased IFN-γ and IL-17A/F production by DOCK8-deficient memory $CD4^+$ T cells, these cells produced lower levels of these cytokines than normal cells under similar culture conditions. Interestingly, $CD4^+$ T cells from DOCK8-deficient mice expressed normal levels of T-bet and GATA-3 when activated under T_H1- and T_H2-polarizing conditions, respectively, *in vitro*.[49] Interestingly, although IFN-γ expression by *in vitro*–derived murine DOCK8-deficient T_H1 cells was normal, T_H2-polarized DOCK8-deficient $CD4^+$ T cells showed increases in IL-4–expressing cell numbers,[4] suggesting that murine DOCK8-deficient $CD4^+$ T cells also display a T_H2 bias.

These findings provide potential explanations for some of the clinical features of DOCK8 deficiency. First, lack of T_H17 cells would predispose DOCK8-deficient patients to infections with *Candida albicans*. This is akin to other monogenic PIDs characterized by impaired T_H17/IL-17–mediated immunity and the high incidence in CMCs in affected subjects (ie, loss-of-function mutations in *STAT3*, *IL17RA*, *IL17RC*, *IL17F*, *ACT1*, and *RORC* and gain-of-function mutations in *STAT1*).[20,28,57-62] Compared with other PIDs with defects in T_H17 cytokines, IL-17A/IL-17F production by DOCK8-deficient memory $CD4^+$ T cells was less impaired than that observed for *RORC*- or *STAT3*-deficient memory $CD4^+$ T cells.[20,57] Remarkably, the quantitative effect of specific gene mutations on generating T_H17 cells correlates with or predicts the incidence of fungal infections in these subjects. Thus approximately 85% of patients with mutations in *STAT3* or *RORC* have CMC,[57,63] but fungal infections are observed in only approximately 40% to 60% of DOCK8-deficient patients, as shown for the cohort studied here (Table I) and in a larger study of 57 patients.[64] Thus there is likely a direct association between IL-17A/IL-17F production in patients with different PIDs and incidence of CMC.

Second, the predominance of memory $CD4^+$ T cells producing high levels of IL-4, IL-5, and IL-13 could contribute to the characteristic pathophysiologic T_H2 features of AR-HIES: severe allergy, eosinophilia, and hyper-IgE.[65] This exaggerated T_H2 response might also reduce T_H17 differentiation,[66] further compromising T_H17-mediated antifungal immune responses. Although memory $CD4^+$ T cells displayed reduced IFN-γ production *ex vivo*, DOCK8-deficient naive $CD4^+$ T cells could differentiate into T_H1 cells *in vitro*. Thus T_H1-mediated immunity, although reduced, might be sufficient in these subjects to elicit protective immunity. Indeed, this is consistent with a lack of disease caused by poorly virulent mycobacteria, such as BCG vaccines and environmental species, which require IFN-γ–mediated immunity for protection,[67] in patients with DOCK8 deficiency. In the scenario of antiviral immunity, the increased T_H2 cytokine environment within the memory $CD4^+$ T-cell compartment might inhibit IFN-γ production by $CD8^+$ T cells. Indeed, analysis of DOCK8-deficient memory $CD8^+$ T cells *ex vivo* revealed defective IFN-γ expression and secretion compared with that seen in healthy donors (see Fig E2, *A* and *B*, in this article's Online Repository at www.jacionline.org).[1] Thus by diminishing T_H1 responses, a T_H2 bias could contribute to persistent viral infections in DOCK8-deficient patients.

Third, beyond T_H1, T_H2, and T_H17 cytokines, we also noted reduced production of IL-6 by DOCK8-deficient memory $CD4^+$ T cells. Although there have been no genetic studies linking impaired IL-6 production with infection with specific pathogens, autoantibodies against IL-6 were reported in a patient with recurrent staphylococcal infection.[68] Thus it is possible that poor IL-6–mediated immunity in patients with DOCK8 deficiency underlies staphylococcal infection in affected patients.

Fourth, although previous work demonstrated that DOCK8 functions intrinsically in B cells to regulate differentiation, reduced production of IL-21 (and potentially IL-10) by DOCK8-deficient memory $CD4^+$ T cells might also contribute to impaired humoral immune responses in patients with AR-HIES because these cytokines are the main drivers of human B-cell activation, proliferation, and differentiation.[69] This is supported by our observation that DOCK8-deficient memory $CD4^+$ T cells present with defects in IL-21 expression *ex vivo* (Fig 2) and naive DOCK8-deficient $CD4^+$ T cells did not differentiate into IL-21+ cells as efficiently as normal naive $CD4^+$ T cells when cultured under T_{FH} cell–polarizing conditions (see Fig E2, *C*).

A characteristic and perhaps unique feature of DOCK8 deficiency compared with other PIDs (including those in which there are high levels of IgE, such as mutations in *STAT3*) is the very high incidence of food allergies.[1-5] The allergen-specific IgE from DOCK8-deficient patients was directed mostly toward staple foods rather than nonfood allergens, such as house dust mites. This is consistent with a recent report that showed that this pattern of allergen-specific IgE is unique to DOCK8 deficiency,[70] inasmuch that DOCK8-deficient patients had IgE directed toward food antigens, whereas patients with atopic dermatitis have IgE specific for aeroallergens, yet the reactivity of IgE in STAT3-deficient subjects against specific allergens was comparable with that seen in healthy donors.[70] Because food allergies are more common in children, who often outgrow them once they reach adolescence, IgE sensitization to food antigens and not house dust mites in patients with DOCK8 deficiency could be attributable to the younger age of our DOCK8 cohort compared with our healthy control subjects. However, this is unlikely because 9 of the 12 DOCK8-deficient patients who still had IgE specific to food antigens were adolescents or adults. In the scenario of STAT3 deficiency, the reduced level of IgE specific for food allergens when compared with that seen in patients with atopic dermatitis has been attributed to a defect in basophil activation and mast cell degranulation, with the latter process found to be STAT3 dependent.[71] This is interesting because although patients with mutations in *DOCK8* or *STAT3* or patients with atopic dermatitis all display increased serum IgE levels, eczema, and atopic

disease, DOCK8 deficiency specifically predisposes to food allergies. The mechanism whereby this occurs is unclear, but it is tempting to speculate that it is related to the T_H2 bias of DOCK8-deficient memory $CD4^+$ T cells. Although T_H2 skewing has been reported in DOCK8-deficient mice *in vitro*,[49] to our knowledge, IgE responses after exposure to food allergens have not been investigated in mice but might provide invaluable insights into whether exposure to food allergens is the driver of IgE production in DOCK8 deficiency. Nevertheless, our findings reinforce the value of direct interrogation of patients' cells and highlight the need to be cognizant of species-specific differences that affect translation of murine studies to human subjects.

The underlying cause for the biased T_H2 nature of memory $CD4^+$ T cells in DOCK8-deficient patients remains to be determined. Examination of the TCR Vβ repertoire in the $CD4^+$ T-cell compartment of DOCK8-deficient patients and healthy donors did not reveal any substantial differences (data not shown). However, there is evidence showing that the strength of the signal received through the TCR greatly influences differentiation of $CD4^+$ T cells. Specifically, low doses of antigen/low-level TCR signaling favor humoral or IL-4–mediated T_H2 immune responses, whereas high doses of antigen/strong TCR signaling favor cellular or IFN-γ–mediated T_H1 immune responses.[41-43] This is also supported genetically because murine $CD4^+$ T cells with a hypomorphic *Card11* mutation reduce TCR-mediated signal strength, resulting in exaggerated T_H2 differentiation, allergic disease, dermatitis, and hyper-IgE.[44] Based on this, we hypothesize that DOCK8-deficient $CD4^+$ T cells receive a qualitatively weaker TCR signal, potentially because of defective immunologic synapse formation,[48] which favors their preferential differentiation into T_H2 cells at the expense of other T_H cell subsets. Our data demonstrating reduced induction of expression of activation markers on DOCK8-deficient $CD4^+$ T cells in response to increasing doses of anti-CD2/CD3/CD28 bead stimulation supports this hypothesis.

The original studies on the strength of TCR signals influencing murine T_H cell differentiation predated the discovery of T_H17 cells. However, studies in mice and human subjects have since demonstrated a requirement for sustained TCR signaling in naive T cells for commitment to a T_H17 phenotype *in vitro* and *in vivo*.[45,46] Thus we would predict that reduced TCR signal strength in DOCK8-deficient $CD4^+$ T cells impairs their differentiation into T_H17 cells.

In conclusion, we reveal that the $CD4^+$ T-cell compartment is greatly altered in the absence of DOCK8. Specifically, DOCK8-deficient patients have increased T_H2 cells and defects in T_H1 and T_H17 cell differentiation. This skewing of $CD4^+$ T-cell subsets likely accounts for some of the clinical manifestations in DOCK8-deficient patients. Strikingly, within our DOCK8 cohort, all the patients investigated had IgE specific for at least 1 of the food allergens (egg white, milk, codfish, wheat, peanut, and soybean) but not for nonfood allergens. These results indicate that the detection of high titers of IgE specific for food but not other allergens is predictive of DOCK8 deficiency. Thus future studies to identify signaling pathways and cellular processes affected by DOCK8 deficiency in $CD4^+$ T cells will not only improve our understanding of disease pathogenesis in affected DOCK8-deficient patients but also that in patients with atopic disease.

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Key messages

- **DOCK8-deficient $CD4^+$ T cells present with a T_H2 cytokine bias but also defects in T_H1 and T_H17 cells.**
- **The T_H2 cytokine bias by DOCK8-deficient cells contributes to atopic disease, such as eczema and food allergies, in patients with DOCK8 deficiency.**
- **The T_H17 cell defect is T cell intrinsic and contributes to compromised antifungal immunity in DOCK8-deficient patients.**

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SUPPLEMENTARY FIGURES LEGENDS

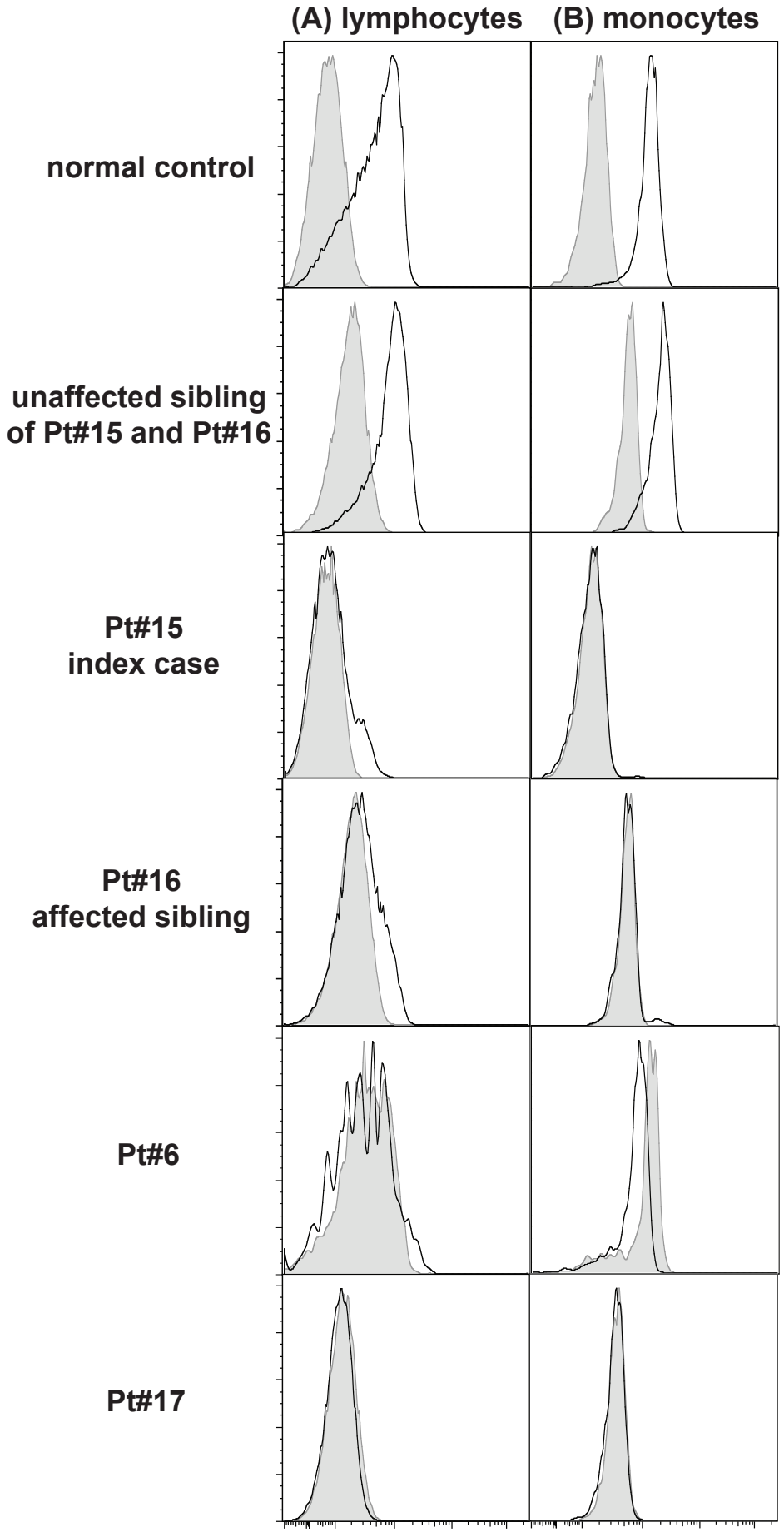
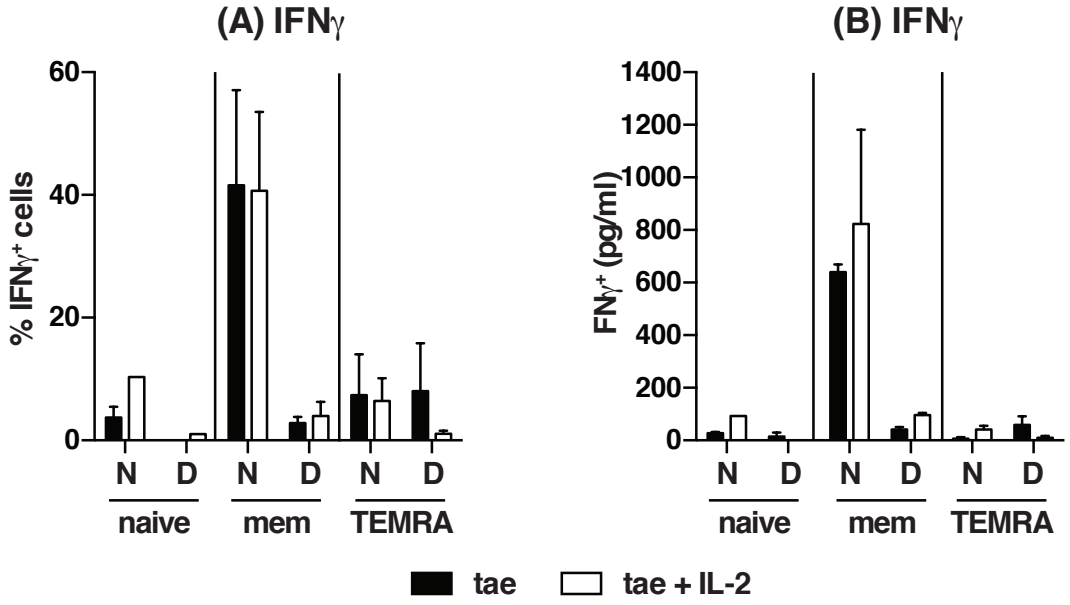
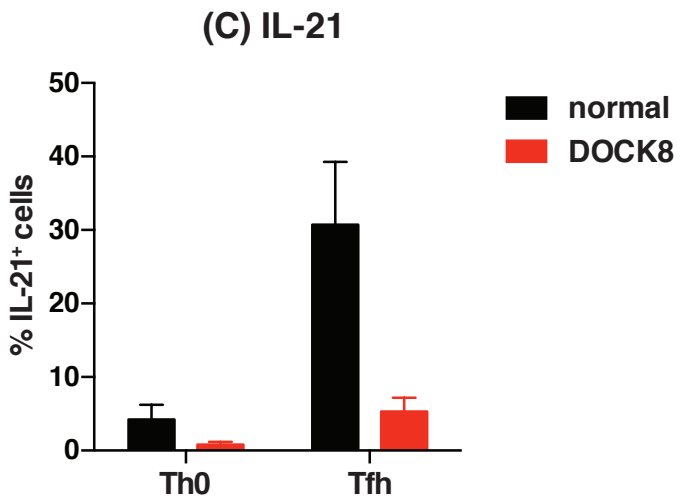
Supplementary Figure 1: Absence in DOCK8 expression in 3 families.

Representative FACS plots of DOCK8 expression in (A) lymphocytes and (B) monocytes from a normal donor as well as 3 DOCK8 deficient families. DOCK8 expression (open histogram) isotype control mAb (tinted histogram).

Supplementary Figure 2: Defects in IFN¥ expression by DOCK8 deficient $CD8^+$ T cells and induction of IL-21 by DOCK8 deficient $CD4^+$ T cells.

Naïve, memory and T_{EMRA} $CD8^+$ T cells were sorted from normal donors and DOCK8-deficient patients and cultured with TAE beads in the absence (solid columns) or presence of IL-2 (open columns) for 5 days. After this time (A) cells were re-stimulated with PMA/ionomycin in the presence of Brefeldin A intracellular expression of IFNγ determined or (B) culture supernatants were harvested and examined for secretion of IFNγ. Data represents the mean and SEM of 2 normal donors and DOCK8 deficient patients. (C) Naïve $CD4^+$ T cells were isolated from normal donors (black columns) and DOCK8-deficient patients (red columns) and activated under neutral conditions (Th0; TAE only), or Tfh-polarising conditions (+ IL-12) for 5 days. Cells were then re-stimulated with PMA/ionomycin in the presence of Brefeldin A intracellular IL-21 expression determined. Data represents the mean and SEM of 4-7 normal donors and DOCK8 deficient patients.

Supplementary Figure 1



Supplementary Figure 2