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DOCK8-DEFICIENT CD4+ T CELLS ARE BIASED TO A TH2 EFFECTOR FATE AT THE EXPENSE OF TH1 AND TH17 CELLS

Stuart G Tangye, PhD[1,2], **Bethany Pillay, BSc Hons**[1,2], **Katrina L Randall, MBBS, PhD, FRACP, FRCPA**[3,4], **Danielle T Avery, BApplSci**[1], **Tri Giang Phan, MBBS, PhD, FRACP, FRCPA**[1,2], **Paul Gray, FRACP**[5], **John B Ziegler, MD, FRACP**[5], **Joanne M Smart, MBBS, FRACP**[6], **Jane Peake, MBBS**[7], **Peter D Arkwright, FRCPCH, DPhil**[8], **Sophie Hambleton, DPhil**[9], **Jordan Orange, MD, PhD**[10], **Christopher C. Goodnow, PhD**[1,2], **Gulbu Uzel, MD**[11], **Jean-Laurent Casanova, MD, PhD**[12,13,14,15], **Saul Oswaldo Lugo Reyes, MD**[16], **Alexandra F. Freeman, MD**[11], **Helen C Su, MD, PhD**[17], **Cindy S Ma, PhD**[1,2]

[1]Immunology Division, Garvan Institute of Medical Research, Darlinghurst, NSW, Australia

[2]St Vincent's Clinical School, UNSW Australia

[3]Department of Immunology, The John Curtin School of Medical Research, Acton, Australian Capital Territory, Australia

[4]Australian National University Medical School, Australian National University, Acton, Australian Capital Territory, Australia

[5]University of New South Wales School of Women's and Children's Health, NSW, Australia

[6]Department of Allergy and Immunology, Royal Children's Hospital Melbourne, VIC, Australia

[7]University of Queensland and Lady Cilento Children's Hospital, Brisbane, QLD 4006, Australia

[8]University of Manchester, Royal Manchester Children's Hospital, Manchester M13 9WL, UK

[9]Institute of Cellular Medicine, Newcastle University and Great North Children's Hospital, Newcastle upon Tyne, NE4 6BE, UK

[10]Texas Children's Hospital Center for Human Immunobiology, Houston, TX, USA

[11]Laboratory of Clinical Infectious Diseases, NIAID, NIH, Bethesda, Maryland, USA

[12]Laboratory of Human Genetics of Infectious Diseases, Necker Branch, INSERM U1163, Institut IMAGINE, Necker Medical School, University Paris Descartes, Paris, France

[13]Pediatric Hematology and Immunology Unit, Necker Hospital for Sick Children, AP-HP, Paris, France

[14]St Giles Laboratory of Human Genetics of Infectious Diseases, Rockefeller Branch, The Rockefeller University, New York, USA

[15]Howard Hughes Medical Institute, NY, USA

Corresponding authors Professor Stuart Tangye; Dr Cindy Ma, Immunology Division, Garvan Institute of Medical Research, 384 Victoria St, Darlinghurst. NSW. 2010 Australia, Phone: +61 2 9295 8455; Fax: +61 2 9295 8404, s.tangye@garvan.org.au; c.ma@garvan.org.au.

[16]Immunodeficiencies Research Unit, National Institute of Pediatrics, Mexico City 04530, Mexico

[17]Laboratory of Host Defenses, NIAID, NIH, Bethesda, Maryland, USA

Abstract

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

Objective: To investigate the phenotype and function of the peripheral $CD4^+$ T cell compartment in DOCK8-deficient patients and compare it to normal healthy donors to determine (1) intrinsic and extrinsic defects in $CD4^+$ T cells and (2) how these defects account for the characteristic clinical features of DOCK8 deficiency.

Methods: We performed indepth analysis of the $CD4^+$ T cell compartment of DOCK8-deficient patients and normal healthy controls. We enumerated naïve, central memory and effector memory $CD4^+$ T cells as well as regulatory T cells, T follicular helper cells, Th1, Th2 and Th17 subsets, and assessed cytokine production and transcription factor expression by $CD4^+$ T cell subsets *in vitro* following non-polarising as well as Th1-, Th2- and Th17-polarising conditions. Finally, using the ImmunoCAP assay, plasma from DOCK8-deficient patients and normal healthy controls was investigated for IgE specific for staple food (egg white, milk, codfish, wheat, peanut, soyabean) and house dust mite allergens.

Results: DOCK8-deficient memory $CD4^+$ T cells were biased towards a Th2 type, characterised by high production of IL-4, IL-5 and IL-13. This was at the expense of other subsets as evidenced by defects in IFNγ-expressing Th1 cells and IL-17A-, IL-17F-, and IL-22 expressing Th17 cells when compared to normal memory $CD4^+$ T cells. Examination of allergen specific IgE revealed that plasma IgE from DOCK8-deficient patients was directed against staple food antigens, but not house dust mites.

Conclusion: Investigations into the $CD4^+$ T cell compartment in DOCK8 deficient patients provided an explanation for some of the clinical signs of this disorder. On one hand, the Th2 bias is likely to contribute to atopic disease, such as eczema and food allergies, which are characteristic to DOCK8 deficiency. On the other hand, defects in Th1 and Th17 cells compromise anti-viral and anti-fungal immunity, respectively explaining the characteristic infectious susceptibility of DOCK8-deficient patients.

CAPSULE SUMMARY

DOCK8-deficient $CD4^+$ T cells exhibit dysregulated cytokine responses, with exaggerated production of Th2 cytokines, and impaired production of Th1 and Th17 cytokines. Collectively these findings provide explanations for some of the clinical features of DOCK8 deficiency, such as eczema and food allergies, and recurrent viral and microbial infections.

Keywords

Dedicator of cytokinesis 8; CD4$^+$ T cell differentiation; Th2 skewing; allergy; atopic disease; chronic mucocutaneous candidiasis; viral immunity

INTRODUCTION

Bi-allelic loss-of-function mutations in dedicator of cytokinesis 8 (*DOCK8*) cause a combined immunodeficiency also known as an autosomal recessive form of hyper IgE syndrome (AR-HIES)[1, 2]. Affected patients typically present with recurrent *Staphylococcus aureus* skin infections, recurrent and severe cutaneous viral infections (herpes simplex virus, human papillomavirus, *Molluscum contagiosum* virus), elevated 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 following natural infection or vaccination. Strikingly, DOCK8 deficiency predisposes most affected patients to developing 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 in turn mediate downstream events including cell activation, division, survival, differentiation, adhesion, and migration[6–8]. Despite this, it is not immediately clear how mutations in *DOCK8* result in the devastating immune abnormalities characteristic of patients with AR-HIES. However, as DOCK8 is predominantly expressed by hematopoietic cells, it is likely to play critical lymphocyte-intrinsic roles in generating appropriate cellular and humoral immune responses against infectious diseases. Consistent with this hypothesis, allogeneic hematopoietic stem cell transplant (HSCT) of DOCK8-deficient patients overcomes recurrent cutaneous viral infections, eczematous rash, and reduces IgE levels and eosinophilia[9–14]. In regards to food allergies in DOCK8 deficiency, some reports have documented improvement post-HSCT[10, 11, 14], while others reported amelioration to symptoms[13] or no change[9, 15].

Ex vivo and *in vitro* analyses of lymphocyte subsets 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 further analysis revealed a decrease in circulating memory (CD27$^+$) B cells[5, 16]. Functionally, compared with normal B cells, DOCK8-deficient B cells exhibit poor responses to the TLR9 ligand CpG, although responses following CD40 engagement are largely intact[5]. In B cells, DOCK8 acts as an adaptor protein connecting the TLR9-MYD88 pathway to STAT3 signalling, 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 are likely to underlie poor humoral immunity in DOCK8-deficiency. Paradoxically, an increase in autoantibodies directed against nuclear, cytoplasmic and extracellular matrix antigens has been detected in DOCK8-deficient patients, possibly due to decreased regulatory T cells (Tregs) in these patients[21].

Previously, when we examined the T cell compartment in DOCK8-deficient individuals, we found a severe reduction in naïve, central memory ($CD45RA^{-}CCR7^{+}$) and effector memory ($CD45RA^{-}CCR7^{-}$) $CD8^{+}$ T cells but a marked accumulation of $CD45RA^{+}CCR7^{-}$ terminally differentiated (i.e. "exhausted") effector memory cells[22]. Strikingly, central and effector memory $CD8^{+}$ T cells from DOCK8-deficient individuals displayed phenotypic features of T cell exhaustion, with increased expression of CD57, 2B4 and CD95, and accelerated loss of CD28 and CD127 (IL-7Rα)[22]. Furthermore, DOCK8-deficient naïve and memory $CD8^{+}$ T cells failed to 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 cell survival, morphology and trafficking[23]. These defects prevented the generation of long-lived resident memory $CD8^{+}$ T cells in the skin and subsequently impaired the immune response to herpes virus infection at this site[23]. Taken together, these defects in the $CD8^{+}$ T cell compartment provide a plausible explanation for viral susceptibility in DOCK8-deficient patients. DOCK8-deficient patients have also been found to have defects in the development of NKT cells and function of NK cells[24, 25]. These defects may contribute to clinical features of DOCK8-deficeint individuals, such as increased susceptibility to viral infections and malignancies.

In contrast to these established defects in B cells, Tregs, $CD8^{+}$ T cells, NK cells and NKT cells, much less is known about the consequences of *DOCK8* mutations in other human $CD4^{+}$ T helper cells. While it has been reported that the frequencies of naïve and memory $CD4^{+}$ T cells in DOCK8-deficient patients are normal, DOCK8-deficient naïve and memory $CD4^{+}$ T cells do have a defect in TCR-induced proliferation; however this is less severe than that observed for DOCK8-deficient $CD8^{+}$ T cells[22]. Consequently, this deficit is unlikely to cause clinical features such as atopic disease (dermatitis, severe food allergies) and increased IgE production in 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 towards Th2 cytokine expression (ie IL-4, IL-5, IL-13) and concomitant defective production of Th1 (IFNγ) and Th17 (IL-17A, IL-17F, IL-22) cytokines. Furthermore, this Th2 cytokine bias, as well as impaired Th17 immunity, in the absence of DOCK8 expression was T cell intrinsic and independent of defects in proliferation. This intrinsic Th2 bias by DOCK8-deficient $CD4^{+}$ T cells may underlie atopic disease and hyper-IgE displayed by DOCK8-deficient patients. Additionally, impaired Th1 and Th17 cell responses are likely to account for impaired viral immunity and fungal infections such as chronic mucocutaneous candidiasis, respectively in DOCK8-deficient patients.

METHODS

Human samples

PBMCs and/or plasma were isolated from normal healthy donors (Australian Red Cross) and patients with DOCK8 deficiency (Table 1). The genotype of some of these patients have been previously reported[1, 2, 15, 22, 24]. DOCK8 expression in PBMCs from controls and patients was determined by flow cytometry, as previously described (Supplementary Fig

1)[26]. All studies were approved by Institutional Human Research Ethics Committees and written informed consent was obtained from patients.

Antibodies and Reagents

Alexa488-conjugated anti-GATA3, Alexa647-conjugated anti-CXCR5, APC-Cy7-conjugated anti-CD4, BUV395-conjugated anti-IFNγ, BV711-conjugated anti-IL-2, Pe-Cy7-conjugated anti-CD25, PE-conjugated anti-CCR6 and anti-mouse IgG1, and PerCpCy5.5-conjugated anti-CD127 and anti-Tbet were purchased from Becton Dickinson. Alexa488-conjugated anti-IL-10, eFluor660-conjugated anti-IL-21, FITC-conjugated anti-CD45RA, PE-conjugated IL-22, Pe-Cy7-conjugated anti-IL-4 and mouse IgG1 were purchased from eBiosciences. APC-Cy7-conjugated anti-IL-17A, BV421-conjugated anti-CXCR3, and BV605-conjugated anti-TNFα was purchased from Biolegend. FITC-conjugated anti-CCR7 and recombinant human IL-12 was purchased from R&D Systems. Anti-DOCK8 mAb was purchased from Santa Cruz Biotechnology. Recombinant human TGFβ, IL-1β, IL-6, IL-21 and IL-23 were from Peprotech. Prostaglandin E2, PMA, calcium ionophore (ionomycin), Brefeldin A, and saponin were purchased from Sigma-Aldrich and recombinant human IL-4 was provided by Dr Rene de Waal Malefyt (DNAX Research Institute, Palo Alto, CA). T cell activation and expansion (TAE) beads (anti-CD2/CD3/CD28) were purchased from Miltenyi Biotec and CFSE was purchased from Invitrogen.

$CD4^+$ T cell phenotyping

To identify naïve, central memory (T_{CM}) and effector memory (T_{EM}) $CD4^+$ T cell populations, PBMCs were incubated with mAbs to CD4, CCR7 and CD45RA and the frequency of $CD4^+CCR7^+CD45RA^+$ (naïve), $CD4^+CCR7^+CD45RA^-$ (T_{CM}), and $CD4^+CCR7^-CD45RA^-$ (T_{EM}) populations determined by flow cytometry. To identify $CD4^+$ T helper cell populations, PBMCs were incubated with mAbs to CD4, CD25, CD127, CXCR5, CD45RA, CCR6 and CXCR3, and the frequency of Tregs ($CD4^+CD25^{hi}CD127^{lo}$), Tfh ($CD4^+CD25^{lo}CD127^{hi}CD45RA^-CXCR5^+$), Th1 ($CD4^+CD25^{lo}CD127^{hi}CD45RA^-CXCR5^-CXCR3^+CCR6^-$), Th2 ($CD4^+$ $CD25^{lo}CD127^{hi}CD45RA^-CXCR5^-CXCR3^-CCR6^-$) and Th17 ($CD4^+CD25^{lo}CD127^{hi}CD45RA^-CXCR5^-CXCR3^-CCR6^+$) subsets determined as previously described[20].

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

Naive and memory $CD4^+$ T cells or naïve, memory and T_{EMRA} $CD8^+$ T cells[22] were isolated by cell sorting on a FACS ARIA (Becton Dickinson; > 98% purity) and cultured with T cell activation and expansion (TAE) beads (anti-CD2/CD3/CD28; Miltenyi Biotech) in 96 well round bottomed well 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, IFNγ and TNFα determined by cytometric bead arrays (CBA; Becton Dickinson). For cytokine expression, activated T cells were re-stimulated 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 IFNγ, IL-4, IL-17A, IL-22, IL-21, IL-10, TNFα and IL-2 detected by intracellular staining[20, 27–29].

Analysis of transcription factor expression by CD4+ T cells

Expression of TBET and GATA3 protein was assessed by intracellular staining using a Fix/Perm kit from eBioscience. Expression of *RORC* was determined by QPCR as previously described[29].

Analysis of DOCK8 expression

To determine intracellular DOCK8 expression, PBMCs were fixed with formaldehyde and stained with an unconjugated DOCK8 or an isotype control mouse IgG1 mAb. A secondary PE conjugated rat anti-mouse IgG1 was then used with saponin as the permeablising agent.

Analysis of CD4+ T cell proliferation

To investigate proliferation, naïve and memory CD4+ T cells were isolated by cell sorting and then labeled with CFSE. Their proliferation status was determined by assessing dilution of CFSE after 5 days of *in vitro* culture[28, 29].

In vitro Th1, Th2, Th17 cell differentiation

Naive and memory $CD4^+$ T cells were isolated by cell sorting and cultured under neutral/Th0 (TAE beads alone), or under Th1 (50 ng/ml IL-12), Th2 (100 U/ml, IL-4) or Th17 (2.5 ng/mL TGFβ, 50 ng/mL IL-1β, 50 ng/mL IL-6, 50 ng/mL IL-21, 50 ng/mL IL-23, 50 ng/mL PGE2) polarising conditions. After 5 days cytokine secretion was analysed by CBA and intracellular staining[27, 29, 30].

ImmunoCAP assay

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

Statistical analysis

Significant differences were determined using either a Students *t*-test, multiple t-tests, one-way or two-way ANOVA (Prism; GraphPad Software).

RESULTS

Effects of DOCK8 deficiency on the generation of effector CD4+ T cell subsets in vivo.

As an initial step in investigating $CD4^+$ Th cell function in the absence of DOCK8, we assessed the $CD4^+$ T cell compartment in DOCK8-deficient patients to determine whether the generation and differentiation of $CD4^+$ T cells was affected and whether this could contribute to the combined immunodeficiency typical of these individuals. 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 individuals 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). Lack of

DOCK8 expression in lymphocytes and monocytes from a representative healthy control, one unaffected sibling and 4 DOCK8-deficient patients is depicted in Supplementary Fig 1. Analysis of this larger cohort of DOCK8-deficient patients confirmed a statistically significant reduction in $CD4^+$ T cells compared to normal donors (Fig 1A, normal: 42 ± 2%, n = 25; DOCK8: 31 ± 3.5%, n = 18; p = 0.0045). Naïve, central memory (T_{CM}) and effector memory (T_{EM}) $CD4^+$ T cells can be resolved according to the differential expression of CD45RA and CCR7[31] (Fig 1B). This analysis revealed that the naïve and T_{CM} compartments in DOCK8-deficient patients are comparable to normal individuals, but T_{EM} $CD4^+$ T cells were significantly increased in DOCK8-deficient patients (Fig 1C). Hence, despite the reduction in total $CD4^+$ T cells, DOCK8-deficient $CD4^+$ T cells differentiate normally into naïve and T_{CM} cells; this is accompanied by a mild increase in T_{EM} cells.

Using a recently described gating strategy[20, 32], we next examined the $CD4^+$ T cell compartment for additional effector subsets, namely $CD25^{hi}CD127^{lo}$ Tregs (Fig 1D, G)[33], $CXCR5^+CD45RA^-$ T follicular helper (Tfh) cells (Fig 1E, G), $CD45RA^-CXCR5^-CXCR3^+CCR6^-$ Th1 (Fig 1F, G), $CD45RA^-CXCR5^-$ $CXCR3^-CCR6^-$ Th2 (Fig 1F, G), and $CD45RA^-CXCR5^-$ $CXCR3^-CCR6^+$ Th17 (Fig 1F, G) cells. DOCK8-deficient patients had an increased frequency of Tregs (Fig 1D, G, normal: 5.3 ± 0.43%, n = 16; DOCK8: 10 ± 2%, n = 11; p = 0.011) but decreased frequency of Th17 cells (Fig 1F, G, normal: 6.8 ± 1.4%, n = 15; DOCK8: 2.8 ± 0.7%, n = 10; p = 0.037), while frequencies of Tfh, Th1 and Th2 cells according to this phenotypic delineation in DOCK8-deficient patients were similar to normal donors (Fig 1D – G). Thus, there appears to be selective paucity of Th17 cells due to DOCK8 mutations.

Assessment of expression of additional surface markers associated with $CD4^+$ T cell differentiation indicated that the naïve, 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 normal donors (Fig 1H–M). Specifically, the loss of expression of CD27 (Fig 1H), CD28 (Fig 1I) and CD127 (Fig 1J) and acquisition of CD57 (Fig 1K), CD95 (Fig 1L) and PD-1 (Fig 1M) by $CD4^+$ T_{CM} and T_{EM} cells was exaggerated for DOCK8-deficient patients compared to controls. Collectively, DOCK8 deficiency compromises the generation of Th17 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 towards Th2 cytokines.

Given the decrease in $CCR6^+CXCR3^-$ cells – which are enriched for Th17-cytokine producing cells in healthy donors[20, 34–36] – in DOCK8-deficient patients, we investigated cytokine expression by naïve and memory $CD4^+$ T cells (Fig 2). Naïve ($CD45RA^+CCR7^+$) and total memory ($CD45RA^-CCR7^{+/-}$) $CD4^+$ T cells were sort-purified from normal donors and DOCK8-deficient patients and then cultured with T cell activation and expansion (TAE) beads conjugated to mAbs specific for CD3, CD28 and CD2 for 5 days. After this time cells were restimulated with PMA and ionomycin in the presence of Brefeldin A and intracellular expression of IFNγ, IL-4, IL-17A, IL-22, IL-21, IL-10, TNFα and IL-2 determined (Fig 2). Apart from IL-2 (Fig 2A) and TNFα (Fig 2B), which are expressed by 40–80% of normal naïve cells, only a small proportion of naïve cells (ie <5%) expressed

any of the other cytokines examined. DOCK8-deficient naïve $CD4^+$ T cells expressed a comparable level of IL-2 (Fig 2A) and TNFα (Fig 2B) to that of normal naïve $CD4^+$ 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 to normal memory $CD4^+$ T cells (Fig 2C), suggesting a skewing to the Th2 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 memory $CD4^+$ T cell compartment, but a comparable amount of IL-4 is produced on a per cell basis. Furthermore, this increase in IL-4^+ cells in DOCK8-deficient memory $CD4^+$ T cells was accompanied by significant reductions in expression of Th1 cytokines IFNγ (Fig 2D) and TNFα (Fig 2B), the Th17 cytokines IL-17A (Fig 2E) and IL-22 (Fig 2F), and the Tfh cytokine IL-21 (Fig 2G). In contrast to these defects, expression of IL-10 (Fig 2H) and IL-2 (Fig 2A) by memory $CD4^+$ T cells was unaffected by DOCK8 deficiency.

The Th2 skewing by DOCK8-deficient memory $CD4^+$ T cells was also assessed by measuring cytokine secretion during the 5-day activation period (Fig 3). This indicated concordance between expression and secretion of cytokines when assessed by intracellular staining and flow cytometry or 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 Th2 cytokines IL-5 and IL-13 (Fig 3A–C) and less Th1 (IFNγ and TNFα; Fig 3D, E) and Th17 (IL-17A and IL-17F; Fig 3F, G) cytokines. Production of IL-6 (Fig 3H) by DOCK8-deficient memory $CD4^+$ T cells was also significantly less than normal memory cells. There were trends for reduced production of IL-10 and IL-2 by DOCK8-deficient memory $CD4^+$ T cells, however these values were not significantly less than controls (Fig 3I, J). Lastly, production of TNFα and IL-2 by DOCK8-deficient naïve $CD4^+$ T cells was normal (Fig 3E, J). Taken together, these results indicate that memory $CD4^+$ T cells from DOCK8-deficient patients display a Th2 bias in that they primarily express IL-4, IL-5 and IL-13 and notably lower levels of cytokines characteristic of other T helper subsets.

Th2 cytokine bias by DOCK8-deficient memory $CD4^+$ T cells is independent of defects in cell proliferation.

Previous work has shown that several features of lymphocyte differentiation such as Ig class switching and antibody secretion by naïve B cells, and cytokine production and cell surface phenotype expression by naïve T cells are regulated by cell division[28, 37–39]. DOCK8-deficient naïve (Fig 3K) and memory (Fig 3L) $CD4^+$ T cells were found to have impaired cell division *in vitro*, consistent with our previous findings[22]. Thus, it was possible that the perturbed cytokine profile reflected reduced proliferation by DOCK8-deficient memory $CD4^+$ T cells. However, the Th2 bias of DOCK8-deficient memory $CD4^+$ T cells was not due to a proliferative defect as evidenced by two 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 3M). Similarly, the

poor production of Th1 and Th17 cytokines by DOCK8-deficient memory $CD4^+$ T cells did not result from impaired proliferation because reductions in expression of IFNγ (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 divisions *in vitro* revealed that the decrease in IFNγ (Fig 3N) and increase in IL-4 (Fig 3O) expression 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 Th2, but not Th1, cytokines is independent of any proliferative defects in these cells.

Naive DOCK8-deficient $CD4^+$ T cells can differentiate into effector cells producing Th1 and Th2, but not Th17, cytokines in vitro.

To determine if the defects in cytokine production displayed by DOCK8-deficient memory $CD4^+$ T cells are cell-intrinsic or due to extrinsic factors, we isolated naïve $CD4^+$ T cells from normal donors and DOCK8-deficient patients and subjected them to *in vitro* culture under Th0, Th1, Th2 or Th17 polarising conditions. Interestingly, DOCK8-deficient naïve $CD4^+$ T cells were able to differentiate into Th1 cells (IFNγ and TNFα) to the same extent as normal naïve $CD4^+$ T cells (Fig 4A, **left panel**). Consistent with the data for memory $CD4^+$ T cells *ex vivo*, DOCK8-deficient naïve $CD4^+$ T cells produced greater amounts of Th2 (IL-5 and IL-13) cytokines than control naïve $CD4^+$ T cells under Th2-polarising conditions (Fig 4A, **middle panels**). Similarly, DOCK8-deficient naïve $CD4^+$ T cells failed to differentiate into IL-17A- and IL-17F-secreting cells when subjected to Th17 polarising conditions *in vitro* (Fig 4A, **right panels**). Notably, DOCK8-deficient naïve $CD4^+$ T cells were capable of responding to the Th17 culture as shown by the reduction in basal levels of secretion of IL-5 and IL-13 compared to the Th0 culture (data not shown). When we examined memory $CD4^+$ T cells from healthy donors, we found that production of IFNγ and IL-17A/F could be increased approximately 2–4 fold by Th1 and Th17 culture conditions, respectively, compared to Th0 conditions (Fig 4B). The net increase in production of these cytokines by DOCK8-deficient memory $CD4^+$ T cells under Th1 and Th17 conditions compared to Th0 conditions was also ~2–6 fold. Despite this, the levels of IFNγ and IL-17A/F secreted by Th1- and Th17-stimulated DOCK8-deficient memory $CD4^+$ T cells were substantially less than not only Th1- and Th17-stimulated normal memory $CD4^+$ T cells, but also Th0-stimulated normal memory $CD4^+$ T cells (Fig 4B). This likely reflects expansion of the few Th1 and Th17 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 naïve $CD4^+$ T cells that were polarised towards Th1 and Th2 fates upregulated TBET (Fig 4C) and GATA3 (Fig 4D), respectively, to the same extent as normal naïve $CD4^+$ T cells. Furthermore, compared to the Th0 culture, TBET and GATA3 expression was decreased in DOCK8-deficient naïve $CD4^+$ T cells cultured under Th17 polarising conditions, demonstrating responsive of these cells to this culture (Fig 4C, D). In our hands, detection of RORγt expression by flow cytometry was not particularly sensitive, as we found that only a small proportion of naïve $CD4^+$ T cells (~5%) expressed RORγt in Th17 compared to Th0 activated cultures (data not shown). To overcome this, *RORC* expression was determined by QPCR. When *RORC*

expression was examined, it was not expressed by naive $CD4^+$ T cells activated under Th0 conditions, but was up-regulated in both normal and DOCK8-deficient naïve $CD4^+$ T cells cultured under Th17 polarising conditions (Fig 4E). Taken together, these data indicate the Th17 cytokine defect in DOCK8 deficiency is T cell intrinsic, and cannot be restored by Th17 polarising conditions for either naïve or memory cells. Furthermore, the ability of Th17 culture conditions to induce *RORC* expression in the absence of DOCK8 indicates that the defect in Th17 differentiation is downstream of *RORC*. In contrast, DOCK8-deficient naïve $CD4^+$ T cells can differentiate normally into Th1 cells, and exhibit exaggerated Th2 differentiation, when provided with the appropriate stimuli *in vitro*.

Specific sensitisation of DOCK8-deficient patients to food allergens

Exaggerated Th2 immune responses have traditionally been associated with allergy and atopic disease[40]. It was thus intriguing to note that $CD4^+$ T cells from DOCK8-deficient patients were biased towards production of Th2 cytokines, and that these patients have severe allergies. To determine if the Th2 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 (i.e. egg white, milk, codfish, wheat, peanut, soyabean), as well as to non-food allergens such as house dust mites. We found that a comparable frequency of normal individuals and DOCK8-deficient patients had IgE specific to house dust mites (Fig 5A). Strikingly, the majority of plasma samples from DOCK8-deficient patients (80%; 12/15), but none of the normal controls tested, had IgE that was specific for the staple food mix (Fig 5B). Thus, DOCK8-deficient patients have a Th2 bias that manifest clinically as specific sensitisation to oral allergens and this may explain the marked propensity of these immunodeficient patients to develop food allergies.

DISCUSSION

Identifying defects in lymphocyte development or function in primary immunodeficiencies provides the opportunity to elucidate the cellular and molecular basis for the clinical features of the disease. Studies of DOCK8-deficient humans and mice have indeed revealed critical cell-intrinsic roles for DOCK8 in the generation of B-cell memory and long-lived humoral immunity[5, 41], $CD8^+$ T cell differentiation and anti-viral responses[22, 23, 42, 43], NK cell cytotoxic function[24] and NKT cell development[25]. Collectively, these defects underlie poor Ab responses to T-dependent and T-independent Ags, and impaired cell-mediated immunity to pathogens including herpes viruses, human papilloma virus (HPV) and *Molluscum contagiosum* virus. We have now investigated $CD4^+$ T cell differentiation in DOCK8-deficient patients in an attempt to understand other aspects of AR-HIES, such as susceptibility to bacterial and fungal infections, atopic disease, food allergies and hyper-IgE.

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, CMV, VZV), HPV and *Molluscum contagiosum* virus, akin to what has been described for $CD8^+$ T cells in not only DOCK8 deficiency[22], but other primary immunodeficiencies such as XLP[44, 45], autosomal

dominant HIES (STAT3 deficiency[46]) and *PIK3CD* gain of function mutations[47], which are characterised by chronic exposure to infectious pathogens. Furthermore, in the absence of DOCK8, memory $CD4^+$ T cells are polarised towards a Th2 cytokine phenotype at the expense of Th1 and Th17 cytokines. The reduction in Th17 cells was apparent not only from the lack of cells producing IL-17A, IL-17F and IL-22, but also the reduction in $CCR6^+$ memory $CD4^+$ T cells. This is consistent with our previous studies which revealed parallel reductions in cells secreting IL-17A/IL-17F and $CCR6^+$ $CD4^+$ T cells in patients with *STAT3* loss-of function or *STAT1*gain-of function mutations[17, 20, 29], indicating that flow cytometric analysis of $CCR6^+$ memory $CD4^+$ T cells is a reliable and rapid means of quantifying Th17 cells. Interestingly, DOCK8-deficient naïve $CD4^+$ T cells were able to differentiate into TBET-expressing and Th1-cytokine secreting cells when provided with exogenous signals *in vitro*. This suggests that defects in IFNγ production by DOCK8-deficient memory $CD4^+$ T cells *ex vivo* are extrinsic, and possibly result from suboptimal priming by Ag-presenting cells and provision of IL-12 *in* vivo. Consistent with this, DOCK8-deficient murine dendritic cells failed to accumulate in the parenchyma of lymph nodes where they are required for T cell priming during immune responses[48]. This defect was attributed to compromised Cdc42 function in the absence of DOCK8 expression[48]. Another possibility is that excessive production of IL-4, which restrains differentiation of human naïve $CD4^+$ T cells into Th1 cells[49], is responsible for impairing IFNγ production by DOCK8-deficient memory $CD4^+$ T cells. This would be consistent with our recent observations of heightened production of Th2 cytokines and corresponding reductions in IFNγ production *ex vivo* by memory $CD4^+$ T cells from individuals with loss-of function mutations in *STAT3*, *IL21R*, *IL12RB1*, *TYK2* or *RORC*[20, 50]. While DOCK8-deficient naïve $CD4^+$ T cells could express *RORC in vitro* following activation under Th17-polarisng conditions, IL-17A/F cytokine secretion remained greatly impaired. Thus, an intrinsic defect distal to induction of *RORC* expression underlies the inability of DOCK8-deficient $CD4^+$ T cells to become Th17 cells. Although Th1- and Th17-polarising conditions did increase IFNγ and IL-17A/F production by DOCK8-deficient memory $CD4^+$ T cells, these cells continued to produce lower levels of these cytokines than normal cells under similar culture conditions. Interestingly, $CD4^+$ T cells from DOCK8-deficient mice expressed normal levels of TBET and GATA3 when activated under Th1 and Th2 polarising conditions, respectively, *in vitro*[42]. Furthermore, while IFNγ expression by *in vitro*-derived murine DOCK8-deficient Th1 cells was normal, DOCK8-deficient $CD4^+$ T cells cultured under Th2 conditions showed an increase in IL-4-expressing cells[42], suggesting that consistent with human DOCK8 deficiency, murine DOCK8 deficient $CD4^+$ T cells also display a Th2 bias under certain settings.

These findings provide a potential explanation for some of the clinical features of DOCK8 deficiency, such as infectious susceptibility, severe food allergies, hyper IgE and eosinophilia. First, the lack of Th17 cells would predispose DOCK8-deficient individuals to mucocutaneous infections with *Candida albicans*. This is reminiscent of other monogenic primary immunodeficiencies characterised by impaired Th17/IL-17-mediated immunity and the high incidence of chronic mucocutaneous candidiasis (CMC) in affected individuals ie loss-of-function mutations in *STAT3*, *IL17RA*, *IL17RC*, *IL17F*, *ACT1* and *RORC*, and gain-of-function mutations in *STAT1*[20, 29, 50–55]. Compared to other primary immunodeficiencies

with defects in Th17 cytokines, IL-17A/IL-17F production by DOCK8-deficient memory CD4$^+$ T was less than that observed for *RORC*- or *STAT3*-deficient memory CD4$^+$ T cells[20, 50]. Remarkably, the quantitative impact of specific gene mutations on the generation of Th17 cells correlates with, or predicts, the incidence of fungal infections such as CMC in these individuals. Thus, ~85% of patients with mutations in *STAT3* or *RORC* develop CMC [50, 56], but fungal infections is observed in only ~40–60% of DOCK8-deficient patients, as shown for the cohort studied here (Table 1), and in a larger study of 57 patients[57]. Thus, it is likely that there is a direct association between levels of IL-17A/IL-17F production in different PID patients 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 pathophysiological Th2 features of AR-HIES: severe allergy, eosinophilia and hyper-IgE[58]. This exaggerated Th2 response may also reduce Th17 differentiation[59], thereby further compromising Th17-mediated anti-fungal immune responses. Although memory CD4$^+$ T cells displayed reduced IFNγ production *ex vivo*, DOCK8-deficient naïve CD4$^+$ T cells could differentiate into Th1 cells *in vitro*. Thus, Th1-mediated immunity, while reduced, may be sufficient in these individuals to elicit protective immunity. Indeed, this would be consistent with a lack of disease caused by poorly virulent mycobacteria, such as BCG vaccines and environmental species - which require IFNγ-mediated immunity for protection[60] - in DOCK8 deficiency. In the scenario of anti-viral immunity, the increased Th2-cytokine environment within the memory CD4$^+$ T cell compartment may also inhibit IFNγ production by CD8$^+$ T cells. Consistent with this, analysis of DOCK8-deficient memory CD8$^+$ T cells *ex vivo* revealed a defect in IFNγ expression and secretion compared to memory cells from healthy donors (Supplementary Fig 2A, B)[1]. Thus, by diminishing Th1 responses, a Th2 bias could contribute to persistent viral infections displayed by DOCK8-deficient patients. Third, beyond Th1, Th2 and Th17 cytokines, we also noted reduced production of IL-6 by DOCK8-deficient memory CD4+ T cells. While there have been no genetic studies linking impaired IL-6 production with infection with specific pathogens, autoantibodies against IL-6 were reported in an individual with recurrent staphylococcal infection (Puel et al J Immunol 2008). Thus it is possible that poor IL-6-mediated immunity in DOCK8 deficiency underlies staphylococcal infection in affected patients. Fourth, while 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 may also contribute to impaired humoral immune responses in AR-HIES, as these cytokines are the main drivers of human B cell activation, proliferation and differentiation[61]. This is supported by our observation that DOCK8-deficient memory CD4$^+$ T cells present with defects in IL-21 expression *ex vivo* (Figure 2) and that naïve DOCK8-deficient CD4$^+$ T cells failed to differentiate into IL-21-expressing cells as efficiently as normal naïve CD4$^+$ T cells when cultured under Tfh cell polarising conditions (Supplementary Fig 2C).

A characteristic and perhaps unique feature of DOCK8 deficiency compared to other primary immunodeficiencies (including primary immunodeficiencies in which there are high levels of IgE such as dominant negative mutations in *STAT3*) is the very high incidence of food allergies[1–5]. When we examined allergen-specific IgE from DOCK8-deficient patients we found it was mostly directed towards staple foods rather than non-food allergens such

as house dust mites. This is consistent with a recent report which also showed that this pattern of allergen-specific IgE is unique to DOCK8 deficiency[62], inasmuch that DOCK8 deficient patients had IgE directed towards food Ags, while patients with atopic dermatitis tended to have IgE specific for aeroallergens, yet the reactivity of IgE in STAT3-deficient individuals against specific allergens was comparable to that of normal donors[62]. Since food allergies are more common in children who often outgrow them once they reach adolescence, IgE sensitisation to food Ags and not house dust mites in DOCK8 deficiency could be attributable to the younger age of our DOCK8 cohort compared to our normal controls. However, this is unlikely as 9 of the 12 DOCK8 deficient patients that still had IgE specific to food Ags were adolescents or adults. In the scenario of STAT3 deficiency, the reduced level of IgE specific for food allergens when compared to 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[63]. This is interesting because although patients with mutations in *DOCK8* or *STAT3*, or individuals with atopic dermatitis, all display increased serum IgE, 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 Th2 bias of DOCK8-deficient memory $CD4^+$ T cells. While Th2 skewing has been reported in DOCK8-deficient mice following Th2 polarisation *in vitro*[42], to our knowledge, IgE responses following exposure to food allergens has not been investigated in mice, but may 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 patient cells and highlight the need to be cognisant of species-specific differences that impact translation of murine studies to humans.

The underlying cause for the biased Th2 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 normal 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 towards specific subtypes. Specifically, low doses of Ag/low level TCR signalling favour humoral or IL-4-mediated Th2 immune responses while high doses of Ag/strong TCR signalling favour cellular or IFNγ-mediated Th1 immune responses[64–66]. This is also supported genetically, with the finding that murine $CD4^+$ T cells with a hypomorphic mutation in *Card11* reduces TCR-mediated signal strength resulting in exaggerated Th2 differentiation, allergic disease, dermatitis and hyper-IgE[67]. Based on this, we hypothesise that in the absence of DOCK8 $CD4^+$ T cells receive a qualitatively weaker TCR signal, potential due to defective immunological synapse formation[41], which favors their preferential differentiation into Th2 cells at the expense of other Th cell subsets. The original studies on strength of TCR signals influencing murine Th cell differentiation predated the discovery of Th17 cells. However, studies in mice and humans have since demonstrated a requirement for sustained and robust TCR signalling in naïve $CD4^+$ T cells for commitment to a Th17 functional phenotype *in vitro* and *in vivo*[68, 69]. Thus, we would also hypothesise that reduced TCR signal strength in DOCK8-deficient $CD4^+$ T cells impairs their differentiation into Th17 cells.

In conclusion we reveal that the $CD4^+$ T cell compartment is greatly altered in the absence of DOCK8. Specifically, DOCK8-deficient patients present with an increase in Th2 cells and defects in Th1 and Th17 cell differentiation. This skewing of $CD4^+$ T helper subsets is likely to account for some of the clinical manifestations in DOCK8-deficient individuals. On one hand, defects in Th17 cells explain susceptibility of DOCK8-deficient patients to mucocutaneous candidiasis. On the other hand, increased Th2 cells may contribute to the allergic phenotype and hyper-IgE displayed by AR-HIES patients. Strikingly, within our DOCK8 cohort, all the patients investigated had IgE that was specific for at least one of the following foods - egg white, milk, codfish, wheat, peanut and soyabean-, but not non-food allergens. These results indicate that the detection of high titers of IgE specific for food but not to other allergens is predictive of DOCK8 deficiency. Thus, future studies to identify signalling 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 individuals, but also patients with atopic disease.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS USED:

AR-HIES	autosomal recessive hyper IgE syndrome
BCG	Bacille Calmette-Guerin
CMC	chronic mucocutaneous candidiasis
CMV	cytomegalovirus
DOCK8	Dedicator of cytokinesis 8
EBV	Epstein-Barr virus
HHV6	human herpes virus 6
HPV	human papilloma virus
HSCT	Hematopoietic stem cell transplant
HSV	herpes simplex virus
STAT	signal transducer and activator of transcription

TAE	T cell activation and expansion
T_{CM}	central memory T cell
TCR	T cell receptor
T_{EM}	effector memory T cell
Tfh	T follicular helper
Tregs	regulatory T cells
VZV	Varicella-zoster virus
XLP	X-liked lymphoproliferative disease

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KEY MESSAGES

- DOCK8-deficient $CD4^+$ T cells present with a Th2 cytokine bias, but also defects in Th1 and Th17 cells
- The Th2 cytokine bias by DOCK8-deficient Th2 cells contributes to atopic disease such as eczema and food allergies in DOCK8 deficiency
- Th17 cell defect is T cell intrinsic and contributes to compromised anti-fungal immunity in DOCK8-deficient patients.

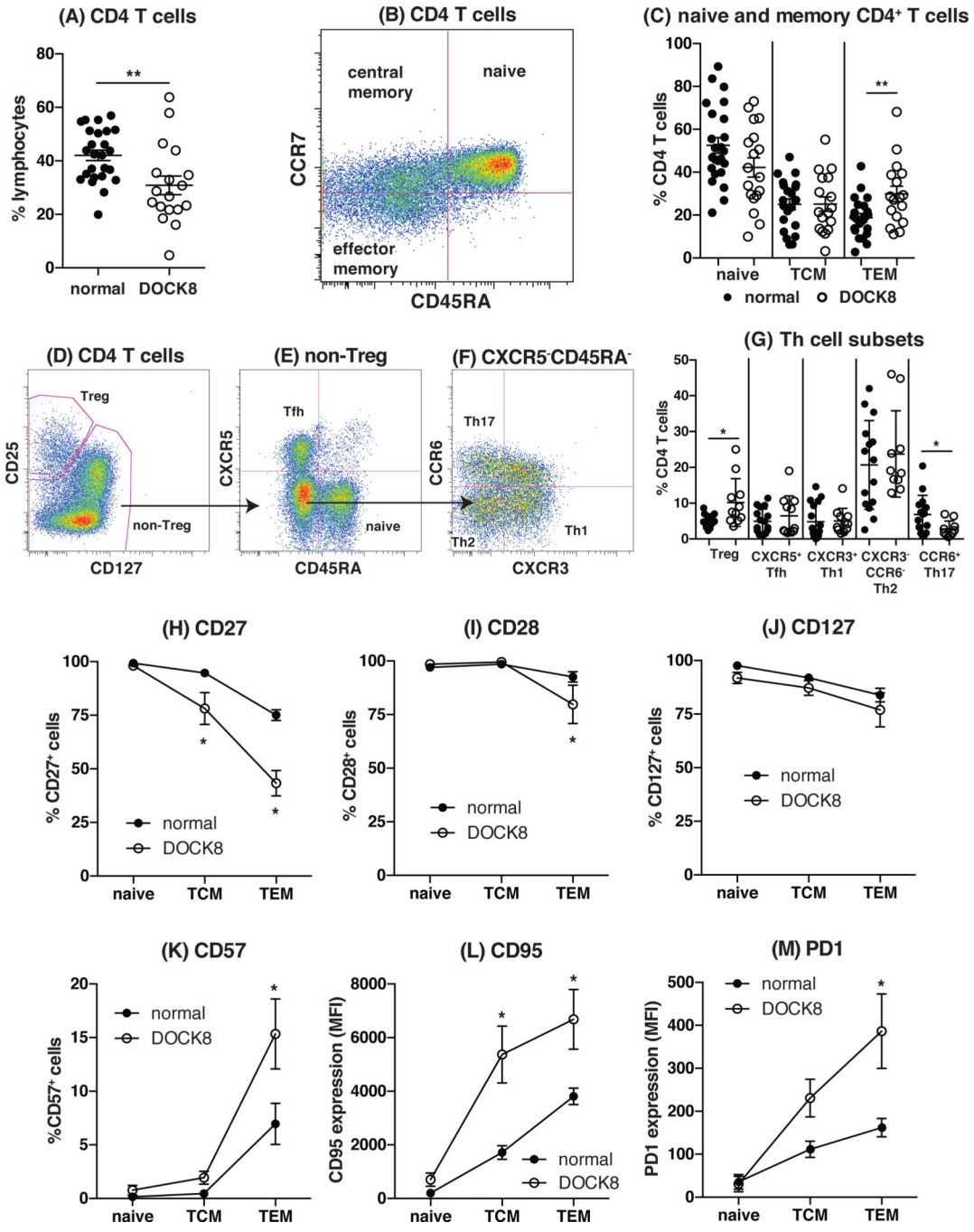

Figure 1: Phenotype of the peripheral CD4$^+$ T cell compartment in DOCK8-deficient patients.

(A) The frequency of CD4$^+$ T cells in normal donors and DOCK8-deficient patients. (B, C) Naïve (CD45RA$^+$CCR7$^+$), central memory (T_{CM}; CD45RA$^-$CCR7$^+$) and effector memory (T_{EM}; CD45RA$^-$CCR7$^-$) populations in normal donors (closed symbol; n = 25) and DOCK8-deficient patients (open symbol; n = 18) were enumerated based on expression of CD45RA and CCR7. (D-G) PBMCs were labelled with mAbs against CD4, CD45RA, CD25, CD127, CXCR5, CXCR3 and CCR6. (D) Treg cells were identified as CD25^{hi}CD127^{lo}. (E) Amongst the non-Treg population naïve and Tfh cells were

identified as $CXCR5^{-}CD45RA^{+}$ and $CXCR5^{+}CD45RA^{-}$, respectively. (F) Th1, Th2 and Th17 populations were identified within the population of $CXCR5^{-}CD45RA^{-}$ memory $CD4^{+}$ T cells as $CXCR3^{+}$ $CCR6^{-}$, $CCR6^{-}CXCR3^{-}$ and $CCR6^{+}CXCR3^{-}$ cells, respectively. (G) Using this gating the frequency of Tregs, Tfh, Th1, Th2 and Th17 cells within the $CD4^{+}$ T cell compartment was determined in normal individuals (closed symbol; n = 15 or 16) and in DOCK8-deficient patients (open symbol; n = 10 or 11). Each point represents an individual donor or patient. Statistics performed with Prism using Student t-test. (H-M) Naïve ($CD45RA^{+}CCR7^{+}$), central memory (T_{CM}; $CD45RA^{-}CCR7^{+}$) and effector memory (T_{EM}; $CD45RA^{-}CCR7^{-}$) populations in normal donors (closed symbol) and DOCK8-deficient patients (open symbol) were identified and assessed for expression of (H) CD27, (I) CD28, (J) CD127, (K) CD57, (L) CD95 and (M) PD1. Each point corresponds to the mean ± SEM % of cells expressing the indicated surface receptor, or MFI (mean fluorescence intensity) of expression (n = 4 – 12 normal donors or DOCK8-deficient individuals). Statistics performed with Prism using t-test.

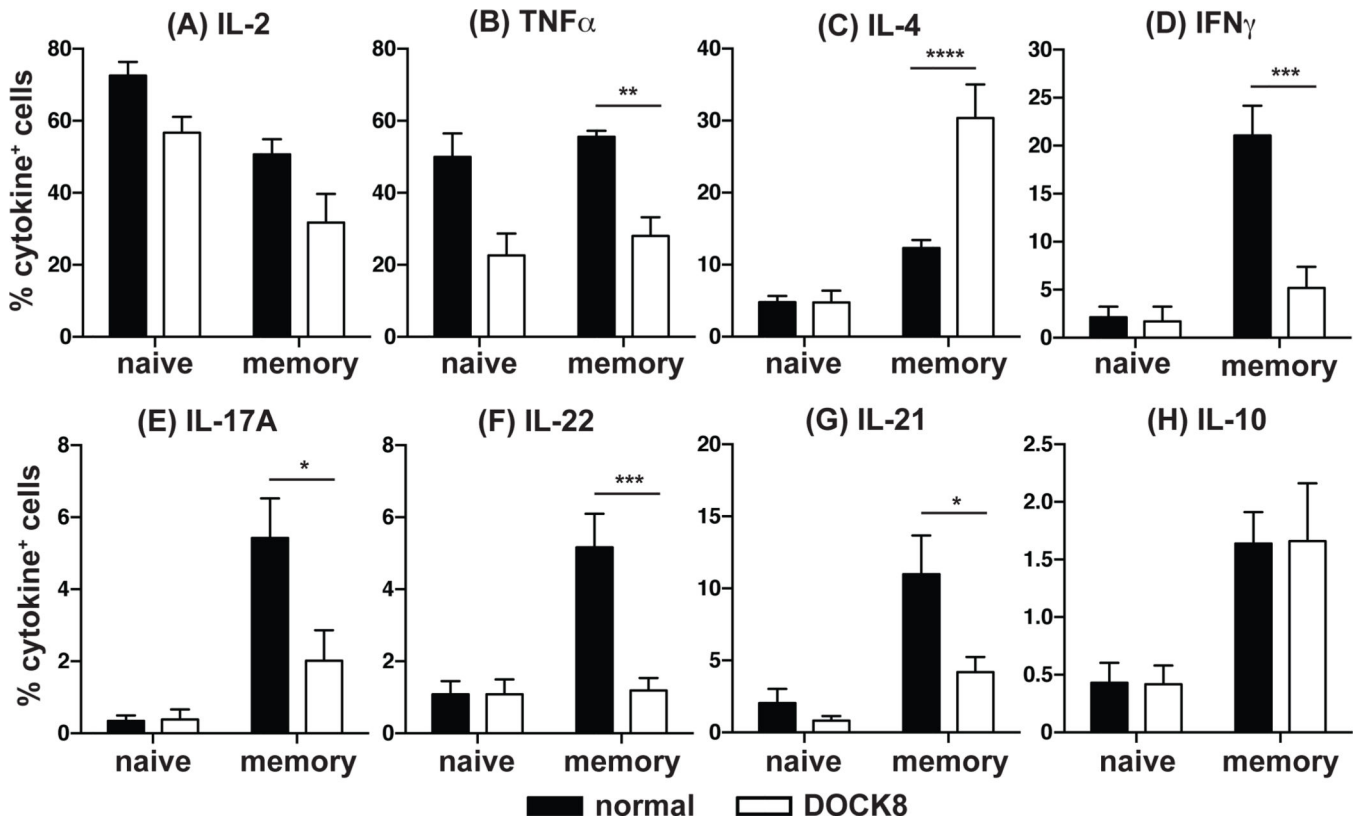

Figure 2: DOCK8-deficient memory CD4$^+$ T cells display a Th2 cytokine expression bias.

Naïve (CD45RA$^+$CCR7$^+$) and memory (CD45RA$^-$CCR7$^{+/-}$) CD4$^+$ T cells were isolated from normal donors and DOCK8-deficient patients and cultured with TAE beads for 5 days. Cells were then re-stimulated with PMA/ionomycin for 6 hours in the presence of Brefeldin A for the last 4 hours. Intracellular expression of (A) IL-2, (B) TNFα, (C) IL-4, (D) IFNγ, (E) IL-17A, (F) IL-22, (G) IL-21 and (H) IL-10 was determined using saponin as the permeabilising agent followed by flow cytometric analysis. Data represent the mean ± SEM of 8 normal donors or 8 DOCK8-deficient patients. Statistics performed with Prism using One-way ANOVA.

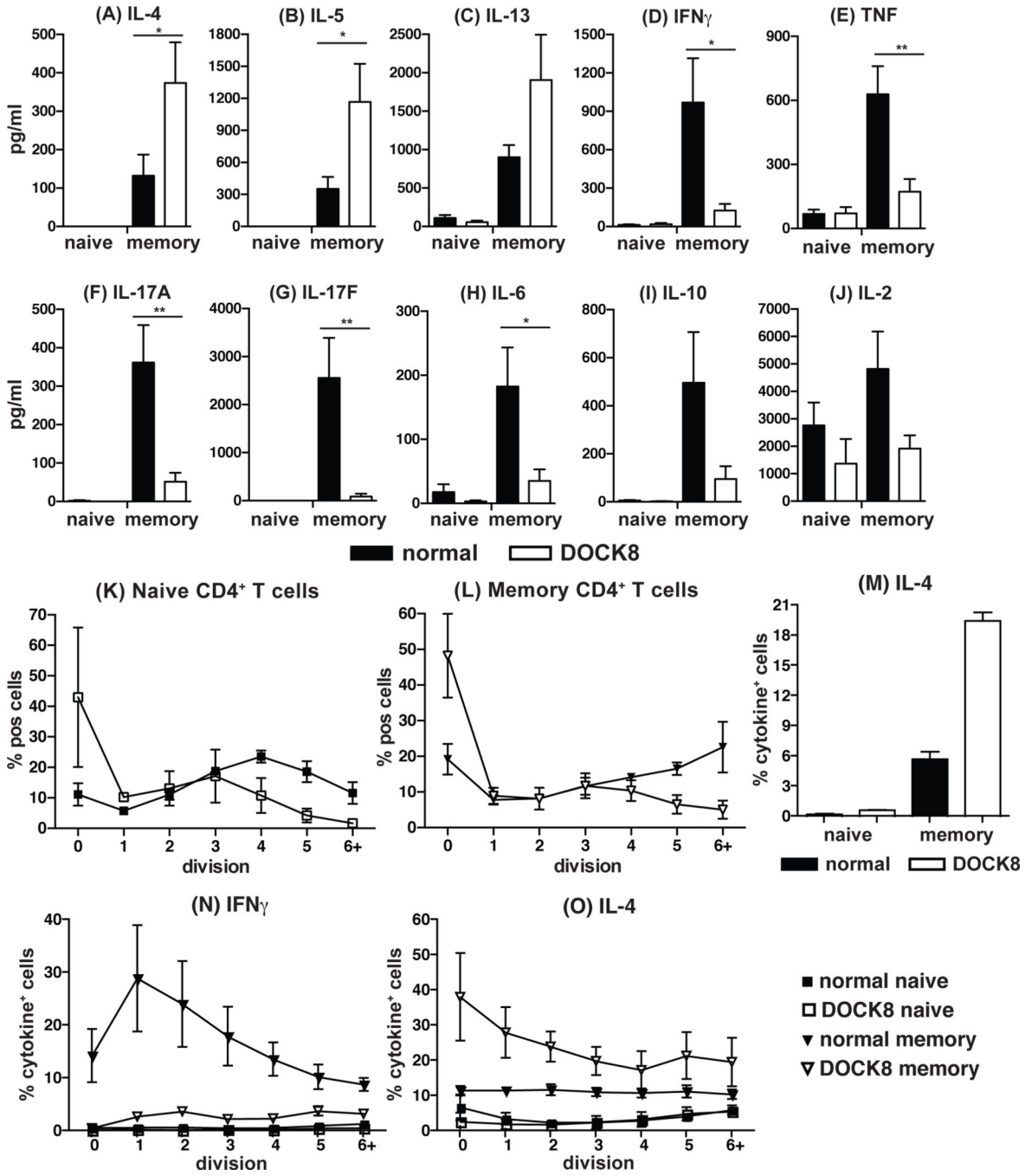

Figure 3: DOCK8-deficient memory CD4$^+$ T cells secrete elevated quantities of the Th2 cytokines IL-4, IL-5 and IL-13 independently of differences in cell proliferation.

Naïve and memory CD4$^+$ T cells were sorted from normal donors and DOCK8-deficient patients and cultured with TAE beads for 5 days. After this time, culture supernatants were examined for secretion of (A) IL-4 (B) IL-5, (C) IL-13, (D) IFNγ, (E) TNF, (F) IL-17A, (G) IL-17F, (H) IL-6, (I) IL-10, (J) IL-2, using a custom designed cytometric bead array (CBA; BD biosciences). Data represent the mean ± SEM of experiments using cells from 9 normal donors or DOCK8-deficient patients. Statistics performed with Prism using One-way ANOVA. (K-L) Naive (K) and memory (L) CD4$^+$ T cells were isolated from normal donors

(n = 4) and DOCK8-deficient patients (n = 4), labelled with CFSE and cultured with TAE beads for 5 days. After this time, the frequency of cells in each division was determined by dilution of CFSE. (M) Sorted naïve and memory $CD4^+$ were immediately restimulated with PMA/ionomycin for 6 hours in the presence of Brefeldin A and IL-4 expression determined by intracellular staining and flow cytometry. (N, O) Naive and memory $CD4^+$ T cells were labelled with CFSE, cultured with TAE beads for 5 days, and the proportion of cells expressing (L) IFNγ or (M) IL-4 was determined for each division interval by dilution of CFSE. Data represent the mean ± SEM of 2 – 4 normal donors and DOCK8-deficient patients.

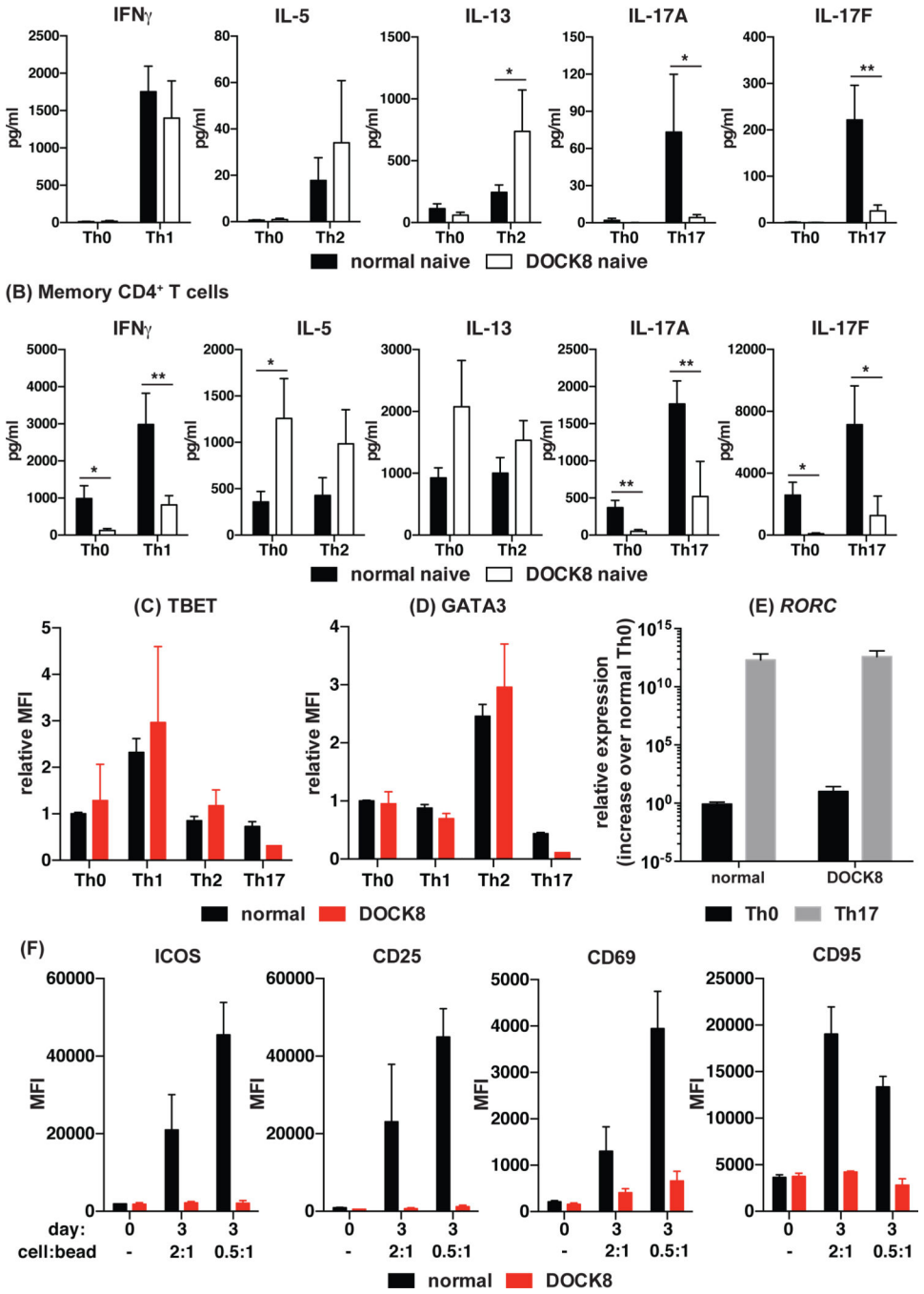

Figure 4: Intrinsic defects in CD4$^+$ T cell cytokine secretion due to *DOCK8* mutations.

(A) Naïve and (B) memory CD4$^+$ T cells were isolated from normal donors and DOCK8-deficient patients and activated under neutral conditions (Th0; TAE only), or Th1- (+ IL-12), Th2- (+ IL-4), or Th17- (+ IL-1β, IL-6, IL-21, IL-23, TGFβ, PG) polarising conditions. After 5 days, secretion of Th1 (IFNγ), Th2 (IL-5, IL-13) and Th17 (IL-17A, IL-17F) cytokines was determined by CBA. The data represent the mean ± SEM of experiments using cells from 5 – 9 normal donors and DOCK8-deficient patients. Expression of (C) TBET and (D) GATA3 was determined by flow cytometry; the data represent the fold

change (mean ± sem) in expression of the indicated transcription factor relative to Th0 culture of the normal control. (E) expression of *RORC* was determined by QPCR. Data represent the mean and SEM of 2 – 3 normal donors and DOCK8-deficient patients. Statistics performed with Prism using two-way ANOVA

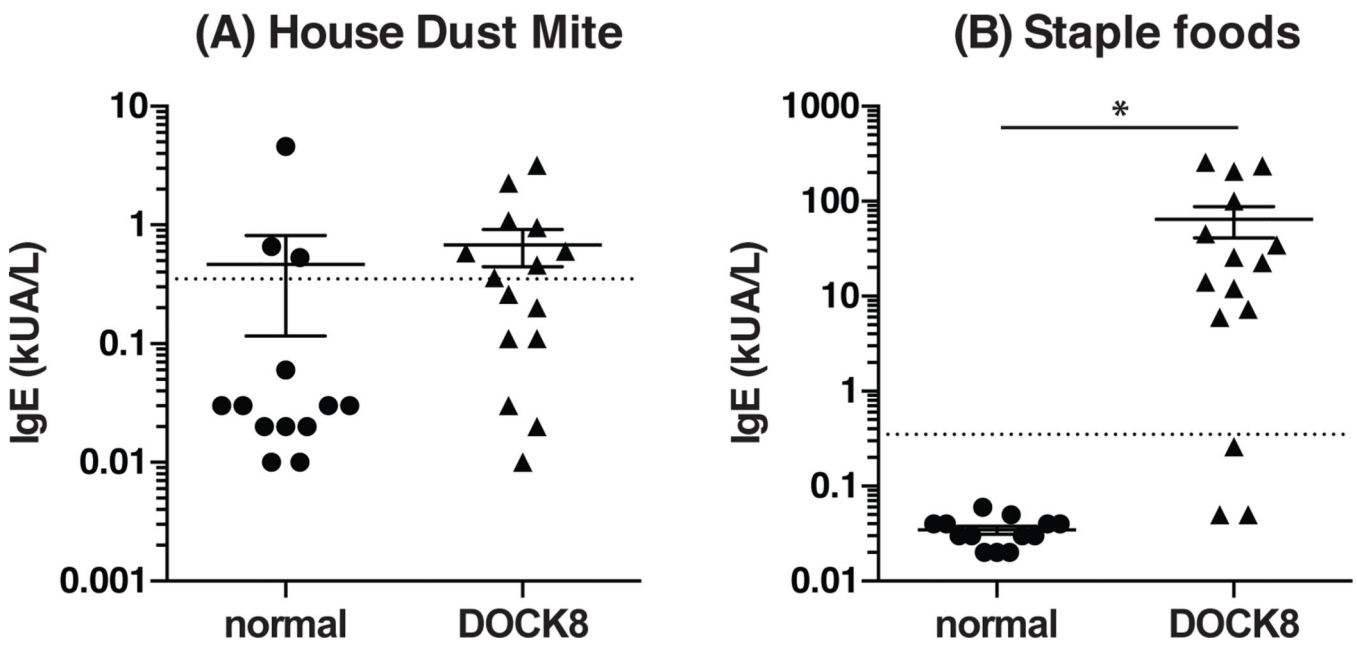

Figure 5: IgE in DOCK8 deficient patients is specific for staple foods and not other Ags such as house dust mites.

Plasma from normal donors and DOCK8-deficient patients was analysed for IgE specific for (A) a staple food mix (egg white, milk, codfish, wheat, peanut and soyabean) and (B) a house dust mite mix by ImmunoCAP. The data represent the mean ± SEM of 13 normal donors and 15 DOCK8-deficient patients. The dotted line refers to the upper limit of the negative reference interval (0.35 kUA/L).

Table 1:

DOCK8 deficient patients

DOCK8-deficient patients	Mutation	Gender	Age at study	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	Homoz 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/mm3)	Stomatitis, *M. contagiosum*, respiratory syncytial virus, HSV1, *Candida sp, H. influenza, P. 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/mm3).	Recurrent otitis media, herpes labialis, HPV, disseminated plain warts, onychomycosis, *Salmonella sp*.	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/mm3).	Recurrent upper respiratory tract infection, HPV, flat warts, herpetic stomatitis, *Giardia lamblia, Salmonella enterica, E. coli*.	Uncomplicated chickenpox. Inflammatory bowel disease, abdominal vasculitis, thrombocytosis. Tolerated BCG vaccine. Deceased.
#6	c.3733_3734del AG; p.R1245EfsX5	male	12	1,500	- Multiple food allergies (egg, cow's milk) - Peanut sensitised (tolerant) - Environmental allergies (house dust mite, rye grass, bermuda grass). Previous allergic rhinitis - Infrequent episodic asthma (viral 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*, 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 transplant.

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DOCK8-deficient patients	Mutation	Gender	Age at study	IgE (IU/ml)	Allergies/atopic disease	Infections	Other
#9	• Large heterozygous. deletion (~200kb) • 2bp heterozygous deletion in exon 41 (c.5307–5308 del AC, pL1770fsX1783	female	7	>6,000	- No known allergies - Severe eczema (lichenification) - Eosinophilia (>3,000/mm3)	Skin abscesses, *M. contagiosum*, recurrent respiratory tract infection, chronic otitis, maxillary sinusitis, bronchiectasis, HPV warts, HSV, *H. influenza, Salmonella spp.*	↑ IgG, ↓ IgM, ↑IgA, $CD4^+$ lymphopenia.
#10	• Large heterozygous. deletion (~200kb) • 2bp 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, S. pyrogenes.*	↑ IgG, ↓ IgM, ↑ IgA, $CD4^+$ lymphopenia.
#11	heterozygous large deletions one deletion involving the two gene copies of 80kb in 5' part of the gene and a deletion of one copy of 320kb encompassing the 2/3rd 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/mm3)	Chronic otitis, clavicle osteomyelitis, bronchitis, pneumonia, bronchiectasis, *Morganella spp., P. aeruginosa, Proteus mirabillis, H. influenza, Giardia intestinalis.*	Sclerosing cholangitis, ↑ IgA, ↓ IgM, lymphopenia. Died of post-HSCT complications.
#12	splice site mutation (exon 11) > frame shift, 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 etiology. Calficied 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, onchycomycosis, 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 DOCK8 protein; see Supplementary Fig 1)	male	5	17,300	- Food allergies (milk, egg, cashew, pistachio, almond, beef, lamb) - Eczema - Asthma - Bronchiectasis	HSV, S. *pyrogenes, H. influenzae, C. albicans*, Adenovirus, Norovirus, HHV6, EBV, CMV, VZV, *Aspergillus Niger, Cladosporium.*	
#16	Unknown (lack DOCK8 protein; see Supplementary Fig 1)	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.
#17	Homozygous deletion spanning exon 15–48	female	4	2,294	- Food allergies (peanut cashew, pistachio, sesame) - Sensitization to walnut and egg	Cryptosporidial cholangitis, chronic adenoviral carriage, mild *M. contagiosum, Giardia*, non-typhi	

DOCK8-deficient patients	Mutation	Gender	Age at study	IgE (IU/ml)	Allergies/atopic disease	Infections	Other
					- Drug allergy (Propofol) - Mild Eczema	*Salmonella*, low level CMV viraemia, 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. influenzae*, 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 Exon19: 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 pre-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-B cell lymphoma.
#24	Large deletion (exons 13 to 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's lymphoma (EBV negative), vasculopathy of mid-aorta with bilateral renal artery stenosis, heart failure, improved post HSCT.
#26	Homozygous deletion of at least exons 4–13	female	9	2	- Asthma, - Mild eczema	Sinopulmonary infections, warts.	
#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 of more than 174 kb affecting most of *DOCK8* (260876_43519 0) from intron 1 to exon 39	female	12	1,855-8,460	- Food allergies (egg and lentils) - Eczema - Eosinophilia (1,532/mm3)	Diarrhea, upper respiratory infections, recurrent meningoencephalitis, chronic otitis media, esophageal candidiasis, lower urinary tract infection, pyelonephritis (twice), *Pseudomonas sp* (ear), *E. coli*.	Failure to thrive (short stature), mild scoliosis, seronegative hepatitis, liver steatosis, mild hepatosplenomegaly, extensive abdominal vasculitis, elevated liver

DOCK8-deficient patients	Mutation	Gender	Age at study	IgE (IU/ml)	Allergies/atopic disease	Infections	Other
							enzymes, ↑IgA ↑ IgG, ↑IgM, $CD3^+$ lymphopenia (600/ml).

The following patients were used in these experiments:

• phenotyping (#1–18);

• *ex vivo* cytokine and *in vitro* differentiation (#1, #2, #6, #7, #9, #10, #15, #17, #18);

• plasma IgE (#6, #12, #14, #15, #17, #19–28)