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Research In-Press Preview **Immunology** **Infectious disease**

Bi-allelic inactivating mutations in *DOCK8* cause a combined immunodeficiency characterised by severe pathogen infections, eczema, allergies, malignancy and impaired humoral responses. These clinical features result from functional defects in most lymphocyte lineages. Thus, DOCK8 plays a key role in immune cell function. Hematopoietic stem cell transplantation (HSCT) is curative for DOCK8 deficiency. While previous reports have described clinical outcomes for DOCK8 deficiency following HSCT, the effect on lymphocyte reconstitution and function has not been investigated. Our study determined whether defects in lymphocyte differentiation and function in DOCK8-deficient patients were restored following HSCT. DOCK8-deficient T and B lymphocytes exhibited aberrant activation and effector function *in vivo* and *in vitro*. Frequencies of αβ T and MAIT cells were reduced while γδT cells were increased in DOCK8-deficient patients. HSCT improved, abnormal lymphocyte function in DOCK8-deficient patients. Elevated total and allergen-specific IgE in DOCK8-deficient patients decreased over time following HSCT. Our results document the extensive catalogue of cellular defects in DOCK8-deficient patients, and the efficacy of HSCT to correct these defects, concurrent with improvements in clinical phenotypes. Overall, our findings provide mechanisms at a functional cellular level for improvements in clinical features of DOCK8 deficiency post-HSCT, identify biomarkers that correlate with improved clinical outcomes, and inform the general dynamics of immune reconstitution in patients with monogenic immune disorders following HSCT.

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Bethany A Pillay[1,2], Danielle T Avery[1], Joanne M Smart[3], Theresa Cole[3], Sharon Choo[3], Damien Chan[4], Paul E Gray[5,6,7], Katie Frith[5,6], Richard Mitchell[5,8], Tri Giang Phan[1,2,7] Melanie Wong[7,9], Dianne E. Campbell[7,9], Peter Hsu[7,9], John B Ziegler[5,6,7], Jane Peake[10], Frank Alvaro[11], Capucine Picard[12,13,14], Jacinta Bustamante[,12,15,16], Benedicte Neven[17], Andrew J Cant[18,19], Gulbu Uzel[20], Peter D Arkwright[21], Jean-Laurent Casanova[14,15,16,22], Helen C Su[20], Alexandra Freeman[20], Nirali Shah[23], Dennis D Hickstein[24], Stuart G Tangye[1,2,7*], Cindy S Ma,[1,2,7*]

[1]Garvan Institute of Medical Research, Sydney, NSW, Australia
[2]St Vincent's Clinical School, Faculty of Medicine, UNSW Sydney, NSW, Australia
[3]Royal Children's Hospital, Melbourne, VIC, Australia
[4]Women and Children's Hospital, Adelaide, SA, Australia
[5]Department of Immunology and Infectious Diseases, Sydney Children's Hospital, Australia
[6]School of Women's and Children's Health, UNSW Sydney, NSW, Australia
[7]Clinical Immunogenomics Research Consortia of Australia, Sydney, NSW, Australia
[8]Kids Cancer Centre, Sydney Children's Hospital, Randwick NSW Australia
[9]Children's Hospital at Westmead, NSW, Australia
[10]Lady Cilento Children's Hospital, Brisbane, QLD, Australia
[11]Pediatric Hematology, John Hunter Hospital, New Lambton, NSW, Australia
[12]Laboratory of Lymphocyte Activation and Susceptibility to EBV infection, Institut National de la Santé et de la Recherche Médicale (INSERM) UMR 1163, Imagine institut, Paris, France
[13]Study Center for Primary Immunodeficiencies, Assistance Publique-Hôpitaux de Paris (AP-HP), Necker Hospital for Sick Children, Paris, France
[14]Pediatric Hematology and Immunology Unit, Necker Hospital for Sick Children, AP-HP, Paris, France
[15]Laboratory of Human Genetics of Infectious Diseases, Necker Branch, INSERM U1163, Institut IMAGINE, Necker Medical School, University Paris Descartes Paris, France
[16]St Giles Laboratory of Human Genetics of Infectious Diseases, Rockefeller Branch, The Rockefeller University, New York, USA
[17]Pediatric Hematology-Immunology Unit, Necker Hospital for Sick Children, AP-HP, 75015 Paris, France
[18]Great North Children's Hospital, Newcastle upon Tyne Hospitals, NHS Foundation Trust, Newcastle upon Tyne, UK
[19]Primary Immunodeficiency Group, Institute of Cellular Medicine, Newcastle upon Tyne University, Newcastle upon Tyne, UK
[20]Laboratory of Clinical Immunology and Microbiology, National Institutes of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland, USA
[21]University of Manchester, Royal Manchester Children's Hospital, Manchester M13 9WL, UK
[22]Howard Hughes Medical Institute, NY, USA
[23]Pediatric Oncology Branch, National Cancer Institute, Bethesda, Maryland, National Institutes of Health, Bethesda, MD, USA

[24]Experimental Transplantation and Immunology Branch, National Cancer Institute, Bethesda, MD, USA

* equal contribution

Corresponding authors

Cindy Ma, Stuart Tangye

Garvan Institute of Medical Research

384 Victoria St, Darlinghurst. NSW. 2010 Australia

Phone: +61 2 9295 8455; Fax: +61 2 9295 8404

e-mail: c.ma@garvan.org.au ; s.tangye@garvan.org.au

Running Title: HSCT corrects lymphocyte defects of DOCK8 deficiency

Abstract

Bi-allelic inactivating mutations in *DOCK8* cause a combined immunodeficiency characterised by severe pathogen infections, eczema, allergies, malignancy and impaired humoral responses. These clinical features result from functional defects in most lymphocyte lineages. Thus, DOCK8 plays a key role in immune cell function. Hematopoietic stem cell transplantation (HSCT) is curative for DOCK8 deficiency. While previous reports have described clinical outcomes for DOCK8 deficiency following HSCT, the effect on lymphocyte reconstitution and function has not been investigated. Our study determined whether defects in lymphocyte differentiation and function in DOCK8-deficient patients were restored following HSCT. DOCK8-deficient T and B lymphocytes exhibited aberrant activation and effector function *in vivo* and *in vitro*. Frequencies of αβ T and MAIT cells were reduced while γδT cells were increased in DOCK8-deficient patients. HSCT improved, abnormal lymphocyte function in DOCK8-deficient patients. Elevated total and allergen-specific IgE in DOCK8-deficient patients decreased over time following HSCT. Our results document the extensive catalogue of cellular defects in DOCK8-deficient patients, and the efficacy of HSCT to correct these defects, concurrent with improvements in clinical phenotypes. Overall, our findings provide mechanisms at a functional cellular level for improvements in clinical features of DOCK8 deficiency post-HSCT, identify biomarkers that correlate with improved clinical outcomes, and inform the general dynamics of immune reconstitution in patients with monogenic immune disorders following HSCT.

Introduction

Primary immunodeficiencies (PIDs) are rare conditions caused by mutations in a single gene that cripples the development and/or function of immune cells (1, 2). Currently, >350 genes have been identified that, when mutated, can result in immune dysregulation (1, 3). Although these inborn errors of immunity have been typically associated with heightened susceptibility to disease due to recurrent pathogen infections, the phenotype of PIDs is much broader and can include autoimmunity, autoinflammation, allergy and malignancy (1, 4-6). While PIDs due to a specific gene defect are rare, the rapid discovery of the molecular causes of novel PIDs, the ongoing appreciation of the diversity of clinical presentations of these conditions, and the application of newborn screening across several countries are revealing that collectively the incidence of PIDs is much greater than typically reported (2, 3, 7, 8). For these reasons, it is important to understand the biology and pathogenesis of individual PIDs, and have a thorough knowledge of the optimal treatments and subsequent outcomes for PIDs resulting from mutations in specific pathways.

Severe combined immunodeficiencies (SCID), due to mutations in *IL2RG*, *JAK3*, *ADA*, *RAG*, or *IL7R*, or combined immunodeficiencies (CIDs), due to mutations in eg *DOCK2*, *STK4*, *MALT1*, *CARD11*, *IKBKB*, are usually fatal unless early therapeutic intervention such as hematopoietic stem cell transplant (HSCT), gene therapy or enzyme replacement is applied (2). The first HSCTs for PID were performed in 1968 (9, 10). While results from these initial transplants for PID were disappointing (9, 10), remarkable advances have been made over the past 50 years such that the overall survival of SCID/CID patients following HSCT can exceed 95% (11-16). However, depending on the age at time of transplant, incidence of infection, and nature and source of the donor, mortality post-HSCT can remain significant, with 5-10 year survival ranging from <40 to ~80% (11-13, 15, 16). Thus, in order to improve therapeutic outcomes, it is critical to identify correlates or biomarkers of successful immune cell reconstitution in PID patients following HSCT.

DOCK8 is a guanine nucleotide exchange factor with key roles in regulating cytoskeletal rearrangement, cell activation, migration and survival (17, 18). Despite its broad expression, DOCK8 has a critical and non-redundant role in immunity, as revealed by the discovery that bi-allelic *DOCK8* mutations cause a CID characterised by recurrent mucocutaneous viral, bacterial and fungal infections (80-90% of cases), severe eczema (>95%), allergies (~70%), hyper-IgE (98%), and increased susceptibility to malignancy (HPV-induced carcinoma, EBV-lymphoma) and autoimmunity (17-22).

Numerous studies have investigated cellular defects in DOCK8 deficiency to understand both the non-redundant roles of DOCK8 in lymphocyte biology and mechanisms of disease in DOCK8-deficient patients. These investigations revealed dysregulated survival, proliferation, differentiation, migration and senescence/exhaustion of $CD4^+$ and $CD8^+$ T cells (19, 23-27), decreased regulatory T cell (Treg) function (28), NK cell cytotoxicity (29, 30) and NKT cell development (31), and reduced B-cell activation *in vitro* and memory B cell generation *in vivo* (32, 33).

Similar to other CIDs, outcomes for DOCK8 deficiency are poor with >95% mortality by 40 years (median survival ~10-20 years), and the incidence of life-threatening infections and malignancy increasing every decade (21, 22). Consequently, HSCT is the standard of care for the life-threatening infections and related immune complications associated with DOCK8 deficiency (22). Several studies have examined outcomes of HSCT in DOCK8 deficiency, with generally positive results (~80% survival), but varying degrees of clinical improvement. Eczema, cutaneous viral and bacterial infections, responses to vaccines and levels of serum IgM, IgG and IgA all markedly improved post-HSCT (34-45). In contrast, allergic disease following HSCT is highly variable, either resolving (32, 40, 46), improving (32, 34, 35, 37) or persisting (32, 41, 47). Clinical improvements in transplanted DOCK8-deficient patients have been associated with both mixed (40, 44, 47) or complete (34, 36, 41, 42) donor chimerism.

In this study, we have used DOCK8 deficiency as a model to delineate mechanisms underlying disease pathogenesis pre-HSCT and improvement of clinical features of PID post-HSCT and identify correlates of immune reconstitution and function following HSCT. This allowed us to extensively catalogue cellular defects due to DOCK8 deficiency and investigate quantitative and qualitative improvement of these defects post-HSCT. Cellular improvements correlated with reconstitution of DOCK8 protein expression and clinical outcomes in these patients. To date, this is the largest study of this kind and provides important insights into the functional changes that may predict successful immune reconstitution and guide ongoing treatments and management of DOCK8-deficient patients following HSCT. Furthermore, our study provides proof-of principle for performing high-dimensional multi-functional cellular analyses pre- and post-therapy in other PIDs to understand treatment-induced alternations in cellular behaviour and clinical outcomes and guide implementation of optimal treatments for these conditions.

Results

DOCK8 is constitutively expressed by lymphocytes in healthy donors and DOCK8-deficient patients post-HSCT.

To gain insight into the role of DOCK8 in immune function, we first determined DOCK8 expression in the major lymphocyte subsets in peripheral blood mononuclear cells (PBMCs) of healthy volunteers. DOCK8 was highly and comparably expressed in total T cells, $CD4^+$ and $CD8^+$ T cells, B cells and NK cells (Fig 1A)(48, 49). We also established that DOCK8 is constitutively expressed in NKT and MAIT cells (Fig 1A). Next, we confirmed lack of expression in patients with *DOCK8* mutations and assessed restoration of DOCK8 expression following HSCT. Patients studied here exhibited near-undetectable levels of DOCK8 protein, with expression in lymphocytes (Fig 1B), $CD4^+$ T, $CD8^+$ T and $CD20^+$ B cells (Fig 1C) being drastically reduced compared to healthy volunteers. Importantly, DOCK8 expression in these lymphocyte populations from transplanted patients was restored to similar levels as lymphocytes from healthy volunteers (Fig 1B, 1C).

Clinical Characteristics of DOCK8 deficient patients – impact of HSCT

We studied an international cohort of DOCK8-deficient patients (Table 1) who had either confirmed bi-allelic mutations in *DOCK8* (n=18) or lacked DOCK8 protein in their leukocytes (n=2). In total, immune cells were examined in 18 DOCK8-deficient patients post-HSCT; matched PBMC samples were available from 7 patients pre- and post-HSCT, and 2 patients at two time-points post-HSCT. The source of transplant was haploidentical (n=6), matched unrelated (n=7) or matched related (n=6) donors. Consistent with a recent study of HSCT for a large cohort of DOCK8-deficient patients (32)., no correlations were observed between the source of the transplant and the overall clinical outcome of the patients' post-HSCT (Table 2), All DOCK8-deficient patients studied here suffered recurrent viral and bacterial infections (Table 1). Candidiasis was reported in 30%, allergies in 80%, and impaired vaccine responses in >90% of patients (Table 1). Following HSCT, infections were reduced and vaccines responses improved in all DOCK8-deficient patients (Table 2). Allergies improved in only 1/11 patients tested (Table 2) however this is an underestimate as many of the patients did not undergo

formal clinical allergy testing post-HSCT. Consistent with flow cytometric analysis of DOCK8 expression, which revealed comparable expression in patients post-HSCT and healthy donors (Fig 1B, 1C), donor engraftment following transplant was >90% in all patients (Table 2).

DOCK8-deficient lymphocytes exhibit a unique phenotype typical of aberrant *in vivo* differentiation

To elucidate effects of DOCK8 deficiency on lymphocytes and the impact of HSCT on these defects, we undertook extensive phenotypic analysis. Consistent with previous observations (20, 26), we found reductions in proportions of $CD3^+$ T cells in DOCK8-deficient patients (Fig 2A), largely due to reduced frequencies of $CD4^+$ T cells (Fig 2A). Analysis of T cell subsets confirmed skewing of DOCK8-deficient $CD8^+$ T cells to effector memory (T_{EM}) and effector memory $CD45RA^+$ (T_{EMRA}) cells at the expense of naïve and central memory (T_{CM}) cells (Fig 2B). In contrast, proportions of naïve $CD4^+$ T cells were comparable to healthy controls, while $CD4^+$ T_{CM} cells were decreased and $CD4^+$ T_{EM} cells were increased (Fig 2C). Tregs were proportionally increased in DOCK8-deficient patients compared to controls (not shown). DOCK8 deficiency also affected αβ and γδ T cells, with reductions and increases, respectively, in these subsets in patients compared to controls, resulting in a skewed αβ/γδ T cell ratio (Fig 2D). Furthermore, DOCK8-deficient patients had ~10-fold fewer MAIT cells than healthy controls (Fig 2E), frequencies of NK cells and NK cell subsets were normal (Fig 2F, not shown, refs (20, 29)) and NKT cells were reduced (Fig 2G)(31). In contrast, proportions of total (Fig 2A) and naïve (Fig 2H) B cells were significantly increased in patients compared to healthy donors, however DOCK8-deficient patients have significantly decreased proportions of total memory B cells (Fig 2H). Interestingly, frequencies of Ig class switched memory B cells were unaffected by DOCK8 deficiency (Fig 2I).

Impact of HSCT on lymphocyte differentiation *in vivo* in DOCK8-deficient patients

Detailed analysis of immune cells in 18 DOCK8-deficient patients 6 to 43 months post-HSCT (mean: 15 months) revealed most of these defects in lymphocyte differentiation were improved. Specifically,

$CD3^+$ T cell proportions were significantly increased due to the recovery of total $CD4^+$ T cells, although overall remained reduced compared to controls (Fig 2A). However, proportions of $CD4^+$ T cells tended to reached normal levels in patients ≥12 months post-HSCT (Supp Fig 1A). The loss of naïve $CD8^+$ T cells and skewing to a $CD8^+$ T_{EM} phenotype in untransplanted patients improved following HSCT (Fig 2B). While at the level of the total cohort these values were not significantly different to healthy controls (Fig 2B), they normalised in patients ≥12 months post HSCT (Supp Fig 1C). $CD4^+$ T_{CM} cells and Tregs were normalised after HSCT, but $CD4^+$ T_{EM} cells persisted at an increased frequency at the expense of naïve $CD4^+$ T cells (Fig 2C; not shown) until ≥23 months post HSCT (Supp Fig 1D). Proportions of αβ and γδ T cells were promptly re-established by HSCT (Fig 2D; Supp Fig 1E); MAIT cells in transplanted DOCK8-deficient patients were significantly increased compared to pre-HSCT levels, but remained significantly reduced relative to healthy donors in all patients irrespective of time of analysis post-HSCT (Fig 2E; Supp Fig 1F). Strikingly, proportions of NKT cells remained unchanged in transplanted DOCK8-deficient, being significantly decreased at all times post-HCST (Fig 2G, Supp Fig 1H).

HSCT corrected the increased frequencies of total (Fig 2A) and naïve (Fig 2H) B cells in DOCK8-deficient patients. Importantly, memory B cells were significantly increased post-transplant compared to untransplanted patients, but remained decreased compared to healthy donors (Fig 2H). The kinetics of the concurrent decline in proportions of transitional and increases in naïve and memory B cells in transplanted DOCK8-deficient patients (Supp Fig 1B) is reminiscent of the temporal reappearance of these B-cell subsets in individuals undergoing HSCT for haematological malignancies (50, 51). Thus, DOCK8-deficiency affects the generation and differentiation of a wide range of lymphocytes and HSCT improves most defects, but some improvements are likely to be more time-dependent.

Memory T cells in DOCK8-deficient patients exhibit signs of exhaustion, some of which persist after HSCT

Memory T cells from DOCK8-deficient patients exhibit phenotypic features of chronic activation or exhaustion/senescence (23, 26), which likely impedes their effector function (52, 53). Thus, frequencies of DOCK8-deficient memory $CD4^+$ and $CD8^+$ T_{EM} cells expressing CD57 and PD-1 were significantly increased compared to healthy volunteers (Fig 3A, B). This was coupled with significantly decreased expression of CD127, CD27 and/or CD28 on DOCK8-deficient $CD4^+$ memory and $CD8^+$ T_{EM} cells (Fig 3A, B). Proportions of patient memory $CD4^+$ T cells expressing PD1 and CD127 normalised post-HSCT and CD57 was significantly decreased following HSCT, but continued to exceed that of healthy volunteers (Fig 3A). CD27 expression on patient memory $CD4^+$ T cells improved post-HSCT but remained less than that on memory $CD4^+$ T cells from healthy volunteers (Fig 3A). Similar phenotypic changes were observed for $CD8^+$ T_{EM} cells, with HSCT normalising PD-1, CD28, and CD127 and partially correcting CD57 expression (Fig 3B). Interestingly, amelioration of the senescent phenotype of $CD4^+$ and $CD8^+$ T cells did not appear to further improve over time (Supp Fig 1I, J). Taken together, HSCT partially restores the exhausted/senescent phenotype of $CD4^+$ and $CD8^+$ T cells in DOCK8-deficient patients.

Defects in proliferation, acquisition of cytotoxic effector function and cytokine secretion by $CD8^+$ T cells in DOCK8-deficient patients are improved by HSCT

While proliferative, cytoskeletal and survival defects of $CD8^+$ T cells have been proposed to underlie impaired anti-viral immunity in DOCK8 deficiency (23, 24), the role of DOCK8 in $CD8^+$ T cell responses has only been assessed in a few patients (19). To investigate $CD8^+$ T cell dysfunction due to DOCK8 deficiency, and the effect of HSCT on these functions, $CD8^+$ T cells were labelled with CFSE and cultured for five days with anti-CD2/CD3/CD28 mAbs (TAE beads). Analysis of CFSE dilution revealed reduced proliferation of DOCK8-deficient $CD8^+$ T cells (Fig 4A, left panels) (23). While exogenous IL-2 improved proliferation, the extent of division of DOCK8-deficient $CD8^+$ T cells remained diminished compared to healthy control $CD8^+$ T cells (Fig 4A, right panels).

We next measured acquisition of a cytotoxic phenotype following *in vitro* activation. CD107a expression, an indicator of degranulation in $CD8^+$ T cells (54), and Granzyme A/B secretion (Fig 4B, C) were significantly decreased for DOCK8-deficient $CD8^+$ T cells compared to healthy volunteers. DOCK8-deficient $CD8^+$ T cells also exhibited generalised activation defects, with significantly reduced expression of CD25 and CD95 following *in vitro* stimulation (Fig 4D, E). IL-2, IFNγ and TNFα secretion by DOCK8-deficient $CD8^+$ T cells was also significantly less than healthy controls (Fig 4F). While exogenous IL-2 increased secretion of granzymes (Fig 4C), TNFα and IFNγ (Fig 4G) and CD25 expression (Fig 4D) by $CD8^+$ T cells, the overall response of IL-2 treated DOCK8-deficient $CD8^+$ T cells remained significantly less than healthy control $CD8^+$ T cells.

Post-HSCT, $CD8^+$ T cells from DOCK8-deficient patients proliferated as well as those from healthy controls even without addition of IL-2 (Fig 4A). Furthermore, degranulation (Fig 4B), Granzyme B expression (Fig 4C), CD25 induction (Fig 4D), and IFNγ, TNFα, and IL-2 secretion (Fig 4F) were all restored to normal levels. However, Granzyme A secretion (Fig 4C) and CD95 expression (Fig 4E) remained significantly decreased. Thus, multiple effector functions of $CD8^+$ T cells are severely compromised in DOCK8-deficient patients, but HSCT largely restores functionality to levels similar to healthy controls.

Dysregulated cytokine production by $CD4^+$ T cells in DOCK8-deficient patients is normalised by HSCT

Cytokine production by *in vitro* activated memory $CD4^+$ T cells provides information about their differentiation *in vivo* (55). Memory $CD4^+$ T cells from DOCK8-deficient patients showed significantly reduced production of Th1 (IFNγ/TNFα) and Th17 (IL-17A/IL-17F), but increased Th2 (IL-4, IL-5, IL-13), cytokines compared to healthy controls (Fig 5A, Suppl Fig 2A)(25, 26).

The defect in generating Th17-type cells *in vivo* in DOCK8-deficient patients was intrinsic, revealed by impaired induction of IL-17A/F by DOCK8-deficient naïve $CD4^+$ T cells cultured under Th17-

polarising conditions (Fig 5B). Th2 cytokines (IL-5, IL-13) were normal (Fig 5B). IFNγ expression by DOCK8-deficient naïve $CD4^+$ T cells under Th1 conditions was intact (Supp Fig 2B), despite reduced secretion (Fig 5B), thus suggesting an extrinsic defect underlies poor generation of Th1 cells in DOCK8-deficient patients *in vivo* (26).

Consistent with our previous work, both DOCK8-deficient naïve (Fig 5B) and memory (Fig 5A) $CD4^+$ T cells showed defects in proliferation compared to normal healthy controls cells (23, 26). Since this could impact cytokine production (56), we investigated IFNγ expression by memory and Th1-stimulated naïve $CD4^+$ T cells in the context of cell division. IFNγ production was decreased across all divisions and hence did not result from proliferative defects (Supp Fig 2C, D).

Given the implication of the Th1/Th2 axis in allergy (57), we further explored Th1 and Th2 cytokine production by memory $CD4^+$ T cells at the single cell level. We used intracellular staining to calculate the ratio of cells producing Th2 (IL-4/IL-13) vs Th1 (IFNγ/TNFα) cytokines. DOCK8-deficient memory $CD4^+$ T cells showed a significantly increased Th2:Th1 ratio compared to controls (Fig 5C). By representing each donor and patient on a plot showing proportions of Th2 vs Th1 cells, DOCK8-deficient memory $CD4^+$ T cells formed a distinct cluster away from control memory cells (Fig 5D). This also revealed that the perturbed Th2:Th1 ratio in each patient resulted from increased Th2 and corresponding decreased Th1 cytokine production, further supporting skewed differentiation *in vivo*.

HSCT greatly improved $CD4^+$ T cell function *in vivo* in DOCK8-deficient patients. First, proliferation of naïve and memory $CD4^+$ T cells from transplanted patients was comparable to controls (Fig 5A, 5B). Second, Th1 cytokine production (Fig 5A) by memory $CD4^+$ T cells was recovered, while there was significantly increased Th17 and decreased Th2 cytokines produced by these cells (Fig 5A, Supp Fig 2A). $CD4^+CD45RA^-CXCR3^-CCR6^+$ Th17 cells (55) also increased post-HSCT (pre: 4.3 ± 2.1% [n=8]; post: 10.4 ± 6.1% [n=17]). Third, naïve $CD4^+$ T cells from DOCK8-deficient patients post-HSCT produced normal levels of Th1, Th2, and Th17 cytokines following appropriate polarization

(Fig 5B, Supp Fig 2A). Fourth, the Th2:Th1 cytokine ratio was normalised post-HSCT (Fig 5C). This was due to co-incident decreases in Th2 and increases in Th1 cells, evidenced as a population clustered between DOCK8-deficient patients and controls (Fig 5D). Thus, $CD4^+$ T cell differentiation defects in DOCK8-deficient patients are restored to normal or near-normal levels by HSCT.

Defective production of IL-21 by DOCK8-deficient $CD4^+$ T cells

IL-21 potently induces B cell activation, differentiation and Ab production (58). IL-21 production by DOCK8-deficient memory $CD4^+$ T cells was significantly decreased compared to healthy donors (Fig 5E). This defect was also cell intrinsic since IL-21 induction in naïve DOCK8-deficient $CD4^+$ T cells *in vitro* was also significantly impaired (Fig 5F). Reduced IL-21 production by DOCK8-deficient naïve $CD4^+$ T cells was not due to diminished proliferation as fewer IL-21^+ cells were detected across all divisions measured compared to those from healthy donors (Supp Fig 1D). Strikingly, following HSCT, production of IL-21 (Fig 5E) by memory $CD4^+$ T cells in DOCK8-deficient patients was restored, while IL-21 induction in naïve $CD4^+$ T cells was significantly increased (Fig 5F).

HSCT overcomes the B-cell intrinsic impairment in survival, proliferation and differentiation due to DOCK8 deficiency

Many DOCK8-deficient patients have impaired antibody responses to vaccines (19, 21, 33) (Table 1). *Dock8* is required in murine B cells to generate germinal centres and long-lived humoral immunity (59), and studies of DOCK8-deficient humans reported reductions in memory B cells (33), compromised *in vitro* activation of naïve B cells and identified DOCK8 as an adaptor for TLR signalling (33, 60, 61). To extend these findings, we examined the impact of DOCK8 deficiency on naïve B cell function by assessing Ig secretion *in vitro*. When stimulated with mimics of T-cell help (CD40L/IL-21), TLR ligands (CpG) and BCR agonists, naïve B cells from healthy volunteers secrete IgM (Fig 6A). CD40L/IL-21 or CD40L/CpG/BCR stimulation also induced switching to IgG and IgA (Fig 6A), with CD40L/CpG/BCR being less efficient than CD40L/IL-21 (Fig 6A). DOCK8-deficient naïve B cells secreted significantly lower levels of IgM, with a trend towards decreased IgG and IgA

under most *in vitro* conditions (Fig 6A). DOCK8-deficient naïve B cells also exhibited significantly compromised survival and proliferation *in vitro* compared to control naïve B cells (Fig 6B,C). These data demonstrate that DOCK8-deficient B cells are defective in responding to not only TLR- (33, 61) and BCR-mediated (60) signals, but also those delivered via CD40 and cytokines. Survival, proliferation and secretion of IgM and IgG by naïve B cells isolated from transplanted DOCK8-deficient patients were largely restored (Fig 6B,C). In contrast, IgA secretion by naïve B cells from transplanted patients remained significantly lower than controls (Fig 6D). These findings reveal DOCK8-deficiency intrinsically impairs naïve B cell survival, proliferation and differentiation, establishing that DOCK8-dependent signals are elicited in B cells downstream of numerous stimulatory receptors. However, these key functions are almost completely regained following HSCT, thus explaining improved humoral immune responses in DOCK8 deficiency post-HSCT (Table 2).

Elevated serum IgE and allergen specific IgE levels decrease in a time-dependent manner following HSCT of DOCK8-deficient patients.

Most DOCK8-deficient patients have extremely high levels of total and allergen-specific IgE, consistent with severe allergies (19, 20, 26). Indeed, total and food allergen-specific IgE were increased 50-1000 fold in the DOCK8-deficient patients studied here compared to healthy controls (Table 1, Fig 7A). However dust mite-specific IgE was normal or moderately increased in DOCK8-deficiency (Fig 7A). After HSCT, all patients showed decreased total and food allergen-specific IgE compared to pre-HSCT levels (Table 1, 2, Fig 7A). One patient had moderately positive dust mite allergen-specific IgE levels pre-HSCT, which was also reduced following HSCT (Fig 7A).

HSCT has been reported to have mixed outcomes on allergy in DOCK8 deficiency (32, 46, 47). We also noted variability in reductions in total and allergen-specific IgE, ranging from 2 to >1000-fold (Fig 7A). To investigate this further, we expressed IgE levels following HSCT as a percentage of pre-transplant levels for each patient and as a function of time post-HSCT. For this, we could also study 2 patients longitudinally. This revealed that the magnitude of the reduction in total and specific IgE

levels in DOCK8-deficient patients was time-dependent post-transplant (Fig 7B). In general, food specific IgE levels declined at a slower rate than total IgE levels (Figure 7B). Hence, elevated IgE levels in DOCK8-deficient patients are decreased with HSCT and tend to normalise over time.

Impact of HSCT on lymphocyte reconstitution in other PID patients

It was possible that some of the impairments in lymphocyte differentiation in transplanted DOCK8-deficient patients were unique to DOCK8 deficiency or a general consequence of HSCT in PID. To differentiate between these possibilities, we examined lymphocytes in 8 additional PID patients who underwent HSCT due to loss-of-function mutations in *UNC13D* (n=2), *STK4* (n=2), *CYBB* (n=2), *CD40LG* (n=1) or *SH2D1A* (n=1) (Table 3)(62-64). Time post HSCT ranged from 5-84 (mean 44.5) months (Table 3). Proportions of $CD3^+$, $CD4^+$ and $CD8^+$ T cells in these transplanted PID patients were comparable to healthy controls, while B cells were increased (Fig 8A). Memory B cells were reduced following HSCT (Fig 8B) but differentiation of $CD4^+$ and $CD8^+$ T cells was normal (Fig 8C, 8D). Expression of exhaustion/senescence markers on $CD4^+$ memory T cells in these transplanted PID patients was comparable to $CD4^+$ memory T cells from healthy donors (Fig 8E) however patient $CD8^+$ T_{EM} cells showed decreased expression of CD28 and CD127 relative to controls (Fig 8F). Interestingly, these PID patients exhibited decreased frequencies of MAIT (Fig 8G) and NKT cells (Fig 8H) post-HSCT.

We investigated STK4-deficient patients in more detail, as STK4 deficiency shares clinical features with DOCK8 deficiency, including susceptibility to viral infections, elevated serum IgE, naïve T cell lymphopenia and reduced memory B cells (18, 62). Naïve B cells from STK4-deficient patients exhibit impaired production of IgM, IgG and IgA following *in vitro* stimulation with CD40L/IL21 (62)(Fig8I). However, Ig production by naïve B cells isolated from these patients and activated *in vitro* was normalised following HSCT (Fig 8I). Thus, analysis of patients with DOCK8 deficiency or other inborn errors of immunity allowed us to identify specific and general outcomes for reconstitution of lymphocyte development, differentiation and function following HSCT.

Discussion

The study of cellular defects due by DOCK8 deficiency is essential to reveal mechanisms underlying the constellation of clinical features in these patients. Previous studies showed DOCK8 deficiency compromises memory B cell generation, NK cell cytotoxicity, NKT cell development, $CD8^+$ T cell differentiation, $CD4^+$ T cell cytokine production and Treg function (23, 25-31, 33). We have confirmed and substantially extended these findings to produce a comprehensive catalogue of phenotypic and functional defects in DOCK8-deficient lymphocytes, including impaired $CD8^+$ T cell cytotoxicity, reduced B-cell survival and proliferation, poor induction of Tfh-type cells, and defective generation of MAIT cells. Collectively, these defects explain poor control of pathogen infections, impaired humoral immunity and severe atopic disease in individuals with *DOCK8* mutations.

HSCT is the only treatment capable of curing DOCK8 deficiency. Cellular mechanisms underlying clinical improvements post-HSCT have not previously been established. DOCK8-deficient patients assessed in our study reported poor vaccine responses prior to HSCT (Table 1), but normal responses post-HSCT (Table 2). This correlated with functional improvements in naïve B cell survival, proliferation and differentiation *in vitro* and a significant, albeit incomplete, increase in memory B cell formation *in vivo*. CD19 expression is reduced on DOCK8-deficient human B cells, and it has been proposed that this contributes to poor B-cell responses *in vivo* (60). We confirmed this reduction in CD19 expression and noted that HSCT increased CD19 on patient B cells (not shown); this may also facilitate improved B-cell behaviour in DOCK8-deficient patients post-HSCT. Due to the kinetics of memory B cell generation post-HSCT (50, 51) and the increases in serum IgG and IgA during the first years of life (65), reconstitution of the memory compartment in DOCK8-deficient patients is expected to increase with time following HSCT. Indeed, we observed a normal frequency of memory B cells is achieved in patients who are ≥23 months post transplant (Suppl Fig 2B). This was also observed for the non-DOCK8 PID patients examined inasmuch that the patients who were tested ≥60 months post-HSCT had the highest proportions of memory B cells in peripheral blood (Fig 8B). It is likely that the

increased ability of naïve and memory $CD4^+$ T cells from HSCT- DOCK8-deficient patients to produce IL-21 would also contribute to improved B-cell function, given the potency of IL-21 in inducing human B-cell proliferation and differentiation into plasma cells (PCs)(58).

The prevalence and severity of infections in DOCK8-deficient patients pre-transplant was dramatically reduced following HSCT. Many cellular changes are likely responsible for improved host defence, including increased frequencies, proliferation and normalised differentiation of $CD4^+$ T cells. Increased Th1 and Th17 cytokines would contribute to effective control of bacterial, viral and fungal infections in transplanted DOCK8-deficient patients. Furthermore, restored differentiation and production of cytokines and cytolytic mediators by DOCK8-deficient $CD8^+$ T cells would reduce viral infections post-HSCT. The mild recovery of MAIT cells may also be important in host defence in DOCK8 deficiency post-HSCT (66). Lastly, resolution of severe skin inflammation post-HSCT could in part be attributed to decreased Th2 skewing and concordant reductions in total and allergen-specific IgE. IL-21R-deficient mice and humans have increased serum IgE (67, 68), and administering IL-21 to *Dock8*-deficient mice alleviates disease in an allergic asthma model (69). Thus, increased IL-21 production by $CD4^+$ T cells in transplanted DOCK8-deficient patients may not only contribute to improved humoral immunity, but also mediate improvements in allergy and reductions in serum IgE. Similarly, since CpG activation suppresses IgE production by CD40L/IL-4-stimulated human naïve B cells (61), restored TLR signaling in B cells post-HSCT may also contribute to reductions in serum IgE in DOCK8-deficient patients.

Malignancies occur in 10-15% of DOCK8-deficient patients. Indeed, ~30% of patients studied here reported malignancies prior to HSCT. Incidence of malignancies in transplanted patients has not been described, likely due to insufficient follow-up times. However, the lack of malignancy amongst patients in our study and other reports (37, 43, 44) suggests that, by restoring $CD8^+$ T cell cytotoxic function, HSCT also protects against tumorigenesis in DOCK8 deficiency.

Despite effective control of infections due to improved cellular function in patients' post-HSCT, memory T cells still exhibited modest signs of chronic activation/exhaustion. This could reflect reactivation of latent viruses. Indeed, EBV reactivation occurs in ~20% of HSCT patients (70), while CMV reactivation following HSCT is associated with exhausted $CD8^+$ T cells compared to patients who did not experience viral reactivation post-HSCT (71). Interestingly, one patient we studied had CMV reactivation post-HSCT (DOCK8 #15), while another had an episode of shingles due to VZV reactivation (DOCK8 #18) following transplantation, but no further complications. Additionally, exhaustion of $CD4^+$ and $CD8^+$ memory T cells in DOCK8-deficient patients was reduced ≥12 months post-HSCT (Suppl Fig 2I, 2J), suggesting the effects of viral reactivation are short-lived due to functional reconstitution of immune cells.

HSCT has been reported to have variable outcomes for allergic disease in DOCK8-deficient patients. Thus, it has been reported that HSCT lead to resolution (34, 37, 72), improvement (35, 44) or persistence (41, 47, 73) of allergies in these patients. However, a recent study of 56 transplanted DOCK8-deficient patients found that food allergies resolved or improved in 61% of cases (32). Furthermore, as a substantial proportion of this cohort avoided allergen exposure post-HSCT, the actual level of allergy improvement was nearly 80% (34/43 patients)(32), suggesting HSCT positively impacts allergic disease in DOCK8 deficiency. Our findings of decreased production of Th2 cytokines by memory $CD4^+$ T cells, and corresponding reductions in serum levels of allergen-specific IgE in DOCK8-deficient patients with time are consistent with decreased allergic disease in cohorts of transplanted DOCK8 deficient patients. Furthermore, the kinetics of reduction in total and allergen-specific IgE could explain conflicting results regarding the impact of HSCT on allergic disease in DOCK8-deficiency. Long-lived host PCs in bone marrow may produce IgE in DOCK8-deficient patients post-HSCT (40, 44, 47). They may survive conditioning but be displaced from survival niches by newly-generated PCs, resulting in apoptosis (74). Hence, the time-dependent decrease of allergen-specific IgE in transplanted patients could result from the gradual turnover of IgE^+ PCs by the

reconstituted donor immune system. Continued longitudinal assessment of transplanted patients will establish the long-term efficacy of HSCT on allergic disease in DOCK8-deficient patients.

Successful immune reconstitution following HSCT often depends on high donor chimerism (75). Previous reports detected >95% chimerism in $CD4^+$ and $CD8^+$ T cells (34, 36, 40, 41), but varying (0-100%) levels for B cells (38, 41), in HSCT DOCK8-deficient patients. Despite this, DOCK8-deficient patients with mixed B cell chimerism showed improvements comparable to patients with complete donor chimerism (40), indicating that high donor chimerism is not the sole determinant of restored immune function following HSCT of these patients. Interestingly, some DOCK8-deficient patients undergo somatic reversion and re-express functional DOCK8 protein in leukocytes (48). Interestingly, DOCK8 was highly expressed in memory T cells from somatically reverted patients, but in low proportions of naïve $CD4^+$ T cells, B cells and NK cells (48). Despite having $DOCK8^+$ lymphocytes, clinical improvements in DOCK8-revertant patients were mild, with all continuing to have significant disease and some undergoing HSCT (48). This may be because the revertant population continues to express an exhausted/senescent phenotype (48), which may compromise their response to antigenic stimuli. These data also suggest that DOCK8 is required in most lymphocyte populations, including naïve T and B cells, to achieve significant clinical improvements. Indeed, the finding that the TCR repertoire of DOCK8-revertant cells is dominated by a few Vβ clonotypes (48) infers that a restricted repertoire is not conducive with eliciting robust immune responses in DOCK8-revertant patients. We detected >90% donor chimerism and normal levels of DOCK8 expression in all transplanted patients. This likely explains dramatic differences in disease outcome in DOCK8-deficient patients who have undergone HSCT and those with somatic reversion in only some lymphoid cells.

A caveat of our study was that we could not analyse pre- and post-HSCT samples from all DOCK8-deficient patients. However, there were several matched patients. Examination of these individual patients pre- and post-transplant revealed identical results to those from all untransplanted and

transplanted DOCK8-deficient patients (Suppl Fig 3). This indicates that our collective results are representative of analysis derived from matched patients.

Comparison of our results with data from other PID patients who underwent HSCT highlights outcomes that are a general consequence of the transplant process and those that are unique to DOCK8-deficiency. The shared characteristic of reduced frequencies of memory B cells across both cohorts of patients was consistent with kinetics of B cell reconstitution post-HSCT in other clinical settings (50). The lack of complete reconstitution of MAIT and NKT cells post-HSCT in both DOCK8-deficient and the other PID patients post-HSCT has similarly been reported in patients undergoing HSCT for hematological malignancies as late as 12-24 months following transplant (76, 77). This may result from increased sensitivity of MAIT cells to GVHD prophylactic immunosuppressive drugs (77). These observations for MAIT and NKT cells in DOCK8-deficiency and the other PIDs examined are reminiscent of the persistent deficiency of other populations of innate lymphocytes, including NK cells and ILCs, in X-SCID and JAK/SCID patients decades post-HSCT (78). Thus, an alterative explanation is an inability of precursors of different innate lymphoid cell populations to adequately seed developmental niches following HSCT to enable the reconstitution of these immune cell lineages. In contrast to these findings, DOCK8-deficient patients continued to exhibit greater signs of T-cell exhaustion/senescence post-HSCT, with $CD8^+$ T cells in transplanted DOCK8-deficient patients remaining skewed toward T_{EM} and T_{EMRA} and being enriched for $CD57^+CD127^{dim}$ cells.

In conclusion, we detail the numerous adverse effects of DOCK8 deficiency on the differentiation and function of $CD8^+$ and $CD4^+$ T, MAIT, NK and B cells which underlie clinical features of patients with *DOCK8* mutations. By demonstrating that effective restoration of key functional defects in adaptive immune cells following HSCT corresponds to improved clinical features, we have identified cellular mechanisms for the clinical efficacy of HSCT as a treatment for DOCK8 deficiency. Comparison to other PID patients who have also undergone HSCT identified unique and general consequences of

HSCT in PIDs. Combining our defined readouts of cellular function with clinical features post-HSCT may facilitate predicting long-term outcomes for DOCK8-deficient patients undergoing potentially curative HSCT. Such an approach has been applied to SCID patients, with several clinical, cellular and functional improvements being established as predictors of successful outcomes of HSCT (12, 79, 80). Collectively, our study underscores the value and importance of determining the impact of monogenic mutations on immune cell function and applying these findings following therapeutic interventions in order to ensure optimal patient management and outcomes.

Materials and Methods

Human Samples

PBMCs and plasma were from healthy volunteers (Australian Red Cross) and DOCK8-deficient patients pre- and/or post-HSCT (Table 1, 2). HSCT conditioning is detailed in Table 2. PBMCs were available from 7 patients pre- and post-HSCT, and one patient at 2 time-points and another patient at 3 time-points post-HSCT. Plasma was analyzed for total and allergen-specific IgE (food or mite mix) (26). PBMCs were also collected from patients with recessive mutations in *CYBB*, *STK4*, *UNC13D*, or hemizygous *CD40LG* or *SH2D1A* mutations who had previously undergone HSCT (Table 3)(62-64).

Phenotyping

Proportions of $CD3^+$, $CD4^+$ T ($CD3^+CD4^+$), $CD8^+$ T ($CD3^+CD8^+$), B cells ($CD20^+$); naïve (N; $CD45RA^+CCR7^+$), central memory (T_{CM}; $CD45RA^-CCR7^+$), effector memory (T_{EM}; $CD45RA^-CCR7^-$), $CD45RA^+$ revertant memory (T_{EMRA}; $CD45RA^+CCR7^-$) cells; αβ ($CD3^+TCR\alpha\beta^+$) and γδ ($CD3^+TCR\gamma\delta^+$) T cells; mucosal associated invariant T (MAIT; $CD3^+TCRV\alpha7.2^+CD161^+$), NK ($CD3^-CD56^+$) and NKT cells ($CD3^+TCRV\alpha24^+$ $V\beta11^+$); transitional ($CD20^+CD10^+CD27^-$), naive ($CD20^+CD10^-CD27^-$), memory ($CD20^+CD10^-CD27^+$) and class-switched ($CD20^+CD27^+$ IgD^-IgM^-) B cell subsets were determined by flow cytometry (23, 51, 53, 55, 66). PD1, CD57, CD27, CD28 and CD127 expression were examined on T cells to examine exhaustion (23, 53).

Analysis of DOCK8 expression

PBMCs were labelled with CD3, CD4, CD8, CD20, CD56, TCRVα24, TCRVβ11, CD161 and Vα7.2 mAbs, fixed, permeabilised, and stained with anti-DOCK8. Alternatively, PBMCs were labelled with mouse IgG2 mAbs to CD4, CD8 and CD20, fixed, permeabilised, and stained with anti-DOCK8 or isotype control IgG1, then with PE-anti-mouse IgG1.

Lymphocyte isolation and functional analysis

Naive and memory $CD4^+$ T cells were isolated after excluding Tregs ($CD25^{hi}CD127^{lo}$), and sorting $CD4^+CD45RA^+CCR7^+$ and $CD4^+CD45RA^-$ cells respectively (55). $CD8^+$ T and naive B cells were isolated as $CD8^+CD4^-$ and $CD20^+CD10^-CD27^-IgG^-$ cells (23, 51), respectively. B cells were cultured with CD40L (200ng/ml), 50ng/ml IL-21, 1μg/ml CpG 2006 and/or 0.05% *Staphylococcus aureus* Cowan (SAC) to crosslink the BCR (51, 81). Cells were harvested, stained with Zombie dye to determine viability and enumerated with calibrate beads. Ig secretion was determined as described (51). $CD8^+$ T cells were cultured as previous (23, 53, 54). $CD4^+$ T cells were cultured under Th0, Th1-, Th2-, or Th17 polarising conditions and cytokine production determined (26, 55). Cells were labelled with CFSE (23, 26); proliferation was determined by assessing CFSE dilution (26).

Statistics

All statistically analysis was performed with GraphPad Prism using unpaired t-test with Welch's correction. *p <0.05, **p <0.01, ***p <0.005, ****p <0.001.

Study approval

This study was approved by the Sydney Local Health District RPAH Zone Human Research Ethics Committee and Research Governance Office, Royal Prince Alfred Hospital, Camperdown, NSW, Australia (Protocol X16-0210/LNR/16/RPAH/257); the South East Sydney Local Health District Human Research Ethics Committee, Prince of Wales/Sydney Children's Hospital, Randwick, NSW, Australia (Protocol HREC/11/POWH/152); the Royal Children's Hospital Melbourne Human Research Ethics Committee, Parkville, Victoria, Australia (Protocol 33146A) and the National Institute of Allergy and Infectious Diseases Institutional Review Board, Bethesda, MD, USA (Protocol 95-I-0066). Written informed consent was obtained from participants or their guardians.

Authorship contributions: BAP designed the research, performed experiments, analyzed and interpreted results, and wrote the manuscript; DTA performed experiments and analyzed results; JSM, TC, SC, DC, PEG, KF, RM, TGP, MW, DEC, PH, JBZ, JP, AJC, CP, JB, GU, PDA, JLC, HCS, AF provided patient samples, clinical details and managed patient care; SGT and CSM designed the research, analyzed and interpreted results, wrote the manuscript, and provided funding for the project; all authors commented on the manuscript.

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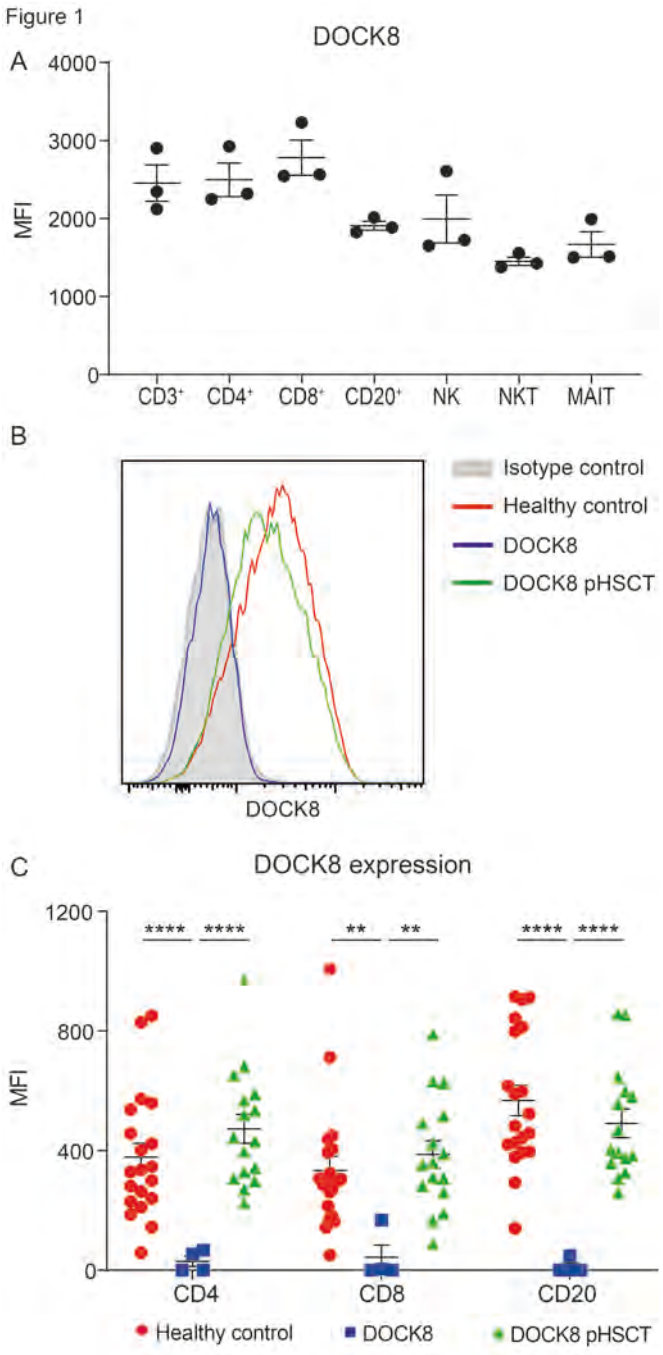

Figure 1: DOCK8 is highly expressed in lymphocyte subsets, absent in DOCK8-deficient patients and restored following HSCT

(A) PBMCs from healthy donors (n=3) were stained with Abs against CD3, CD4, CD8, CD20, CD56, CD161 and TCR Vα24, Vβ11, and Vα7.2. The cells were then fixed, permeabilised and stained with anti-DOCK8 mAb. Expression of intracellular DOCK8 in total T cells ($CD3^+$), $CD4^+$ T cells ($CD3^+CD4^+CD8^-$), $CD8^+$ T cells ($CD3^+CD4^-CD8^+$), B cells ($CD20^+CD3^-$), NK cells ($CD3^-CD56^+$), NKT cells ($CD3^+TCRV\alpha24^+V\beta11^+$) and MAIT cells ($CD3^+CD161^+TCRV\alpha7.2^+$) was then

determined. Data represent the average geometric MFI ± SEM of different lymphocyte subsets from four unrelated donors labelled with anti-DOCK8 mAb less the MFI of cells labelled with isotype control mAb.

(B, C). PBMC from healthy donors (n=20) or DOCK8-deficient patients before (n=4) or following HSCT (n=16) were stained with Abs against CD4, CD8 and CD20 before fixing, permeabilisation and staining for DOCK8. DOCK8 expression was determined in total lymphocytes (B), as well as in $CD4^+$ T cells, $CD8^+$ T cells and $CD20^+$ B cells (C). The histogram in (B) depicts DOCK8 expression in total lymphocytes from one representative healthy donor, and lymphocytes from the same DOCK8-deficient patient pre- and post-transplant as well as an isotype control. The graph in (C) represents the mean MFI ± SEM of DOCK8 expression (minus MFI of isotype control mAb). Statistics were performed using unpaired t-test with Welch's correction *<0.05, **<0.01, ***<0.005, ****<0.001.

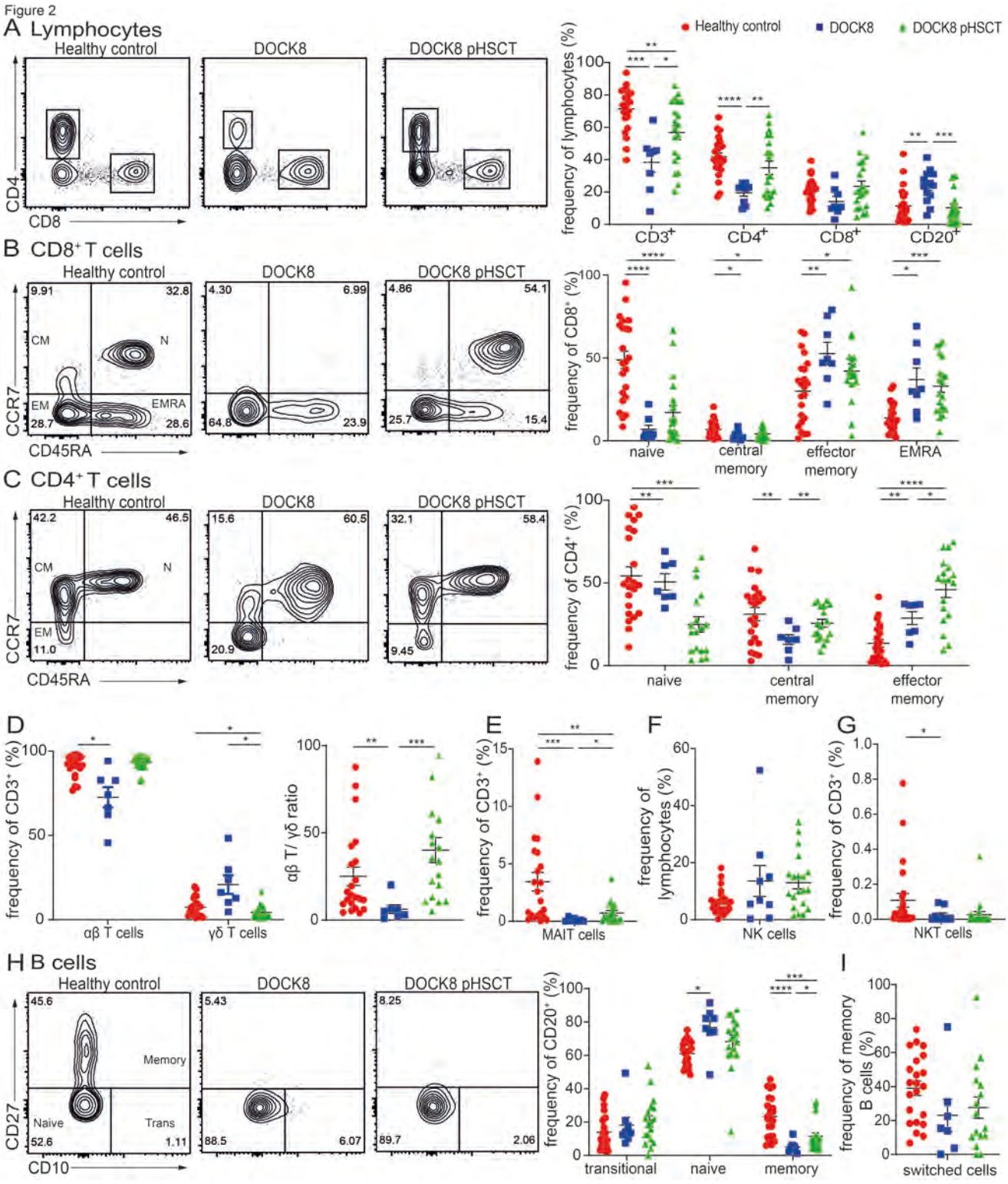

Figure 2: Effect of HSCT on lymphocyte phenotype and differentiation in DOCK8-deficient patients

PBMCs from healthy donors (n=22-24), untransplanted DOCK8-deficient patients (n=7-9), or DOCK8-deficient patients following HSCT (DOCK8 pHSCT) (n=18-20) were labelled with mAbs

against CD3, CD4, CD8, CD20, CD56, CD45RA, CCR7, CD10, CD27, IgG, IgA, TCRαβ, TCRγδ, TCRVα24, TCRVβ11, CD161 and TCR Vα7.2. Proportions of (A) $CD3^+$ cells, $CD4^+$ T cells ($CD3^+CD4^+$), $CD8^+$ T cells ($CD3^+CD8^+$), B cells ($CD20^+$); (B) $CD8^+$ naïve ($CD45RA^+CCR7^+$), central memory (T_{CM}; $CD45RA^-CCR7^+$), effector memory (T_{EM}; $CD45RA^-CCR7^-$) and $CD45RA^+$ revertant memory (T_{EMRA}; $CD45RA^+CCR7^-$) cells; (C) $CD4^+$ naïve, T_{CM} and T_{EM} cell subsets; (D) αβ and γδ TCR^+ T cells; (E) MAIT cells ($CD3^+TCRV\alpha7.2^+CD161^+$); (F) NK cells ($CD3^-CD56^+$); (G) NKT cells ($CD3^+TCRV\alpha24^+$ $V\beta11^+$); (H) transitional ($CD20^+CD10^+CD27^-$), naive ($CD20^+CD10^-CD27^-$) and memory ($CD20^+CD10^-CD27^+$) B cell subsets; and (I) Ig class-switched memory ($CD20^+CD27^+$ IgD^-IgM^-) B cells were then determined by flow cytometric analysis. Contour plots depict one representative normal donor and one DOCK8-deficient patient pre- and post-HSCT. Data are the mean ± SEM. Statistics performed using Prism unpaired t-test with Welch's correction *<0.05, **<0.01, ***<0.005, ****<0.001.

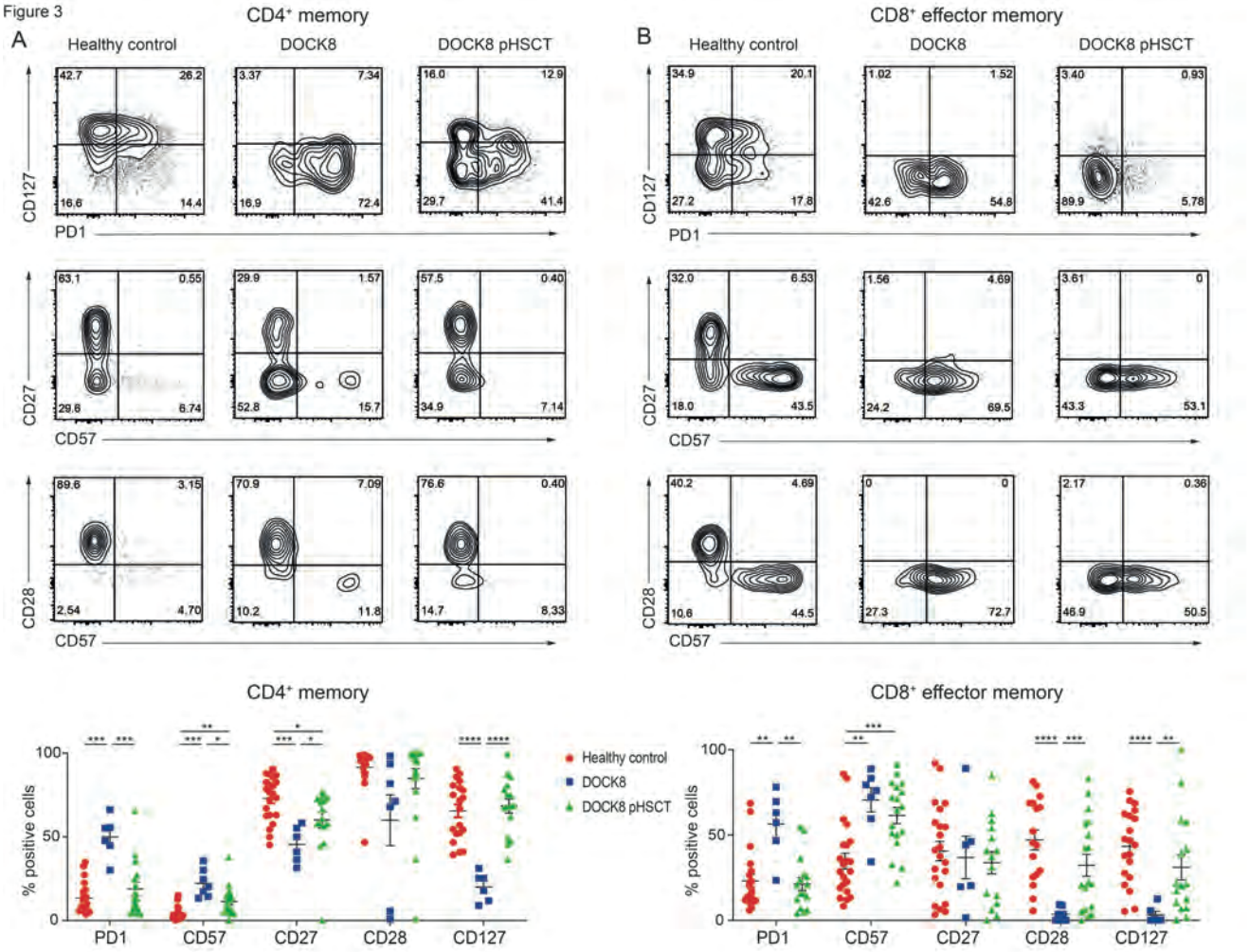

Figure 3: DOCK8-deficient memory CD4$^+$ and CD8$^+$ T cells exhibit signs of exhaustion which decline after HSCT

PBMCs from healthy donors (n=17-22) and DOCK8-deficient patients either before (n=6-7) or after (n=16-19) HSCT were labelled with mAbs against CD4, CD8, CD45RA, CCR7, CD127, CD27, CD28, PD1 and CD57. Co-expression of CD127 and PD1, CD27 and CD57, and CD28 and CD57 by (A) memory CD4$^+$ T cells (CD4$^+$CD8$^-$CD45RA$^-$CCR7$^\pm$) or (B) T_{EM} CD8$^+$ T cells (CD8$^+$CD4$^-$ CD45RA$^-$CCR7$^-$) was determined. Contour plots are representative of one healthy donor and the same DOCK8-deficient patient assessed before and 9-months following HSCT. The graphs show the mean ± SEM. Statistics performed using Prism unpaired t-test with Welch's correction *<0.05, **<0.01, ***<0.005, ****<0.001.

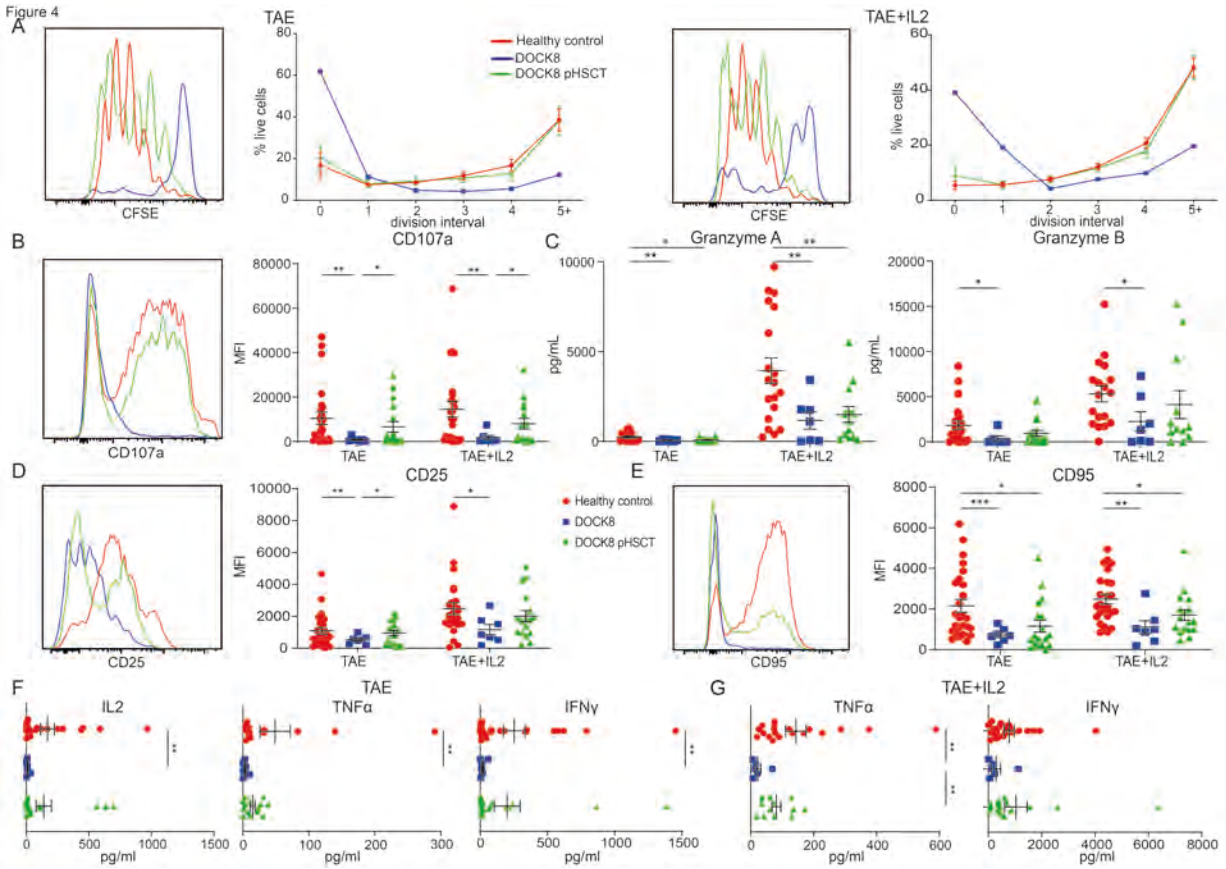

Figure 4: HSCT overcomes CD8$^+$ T cell functional defects due to DOCK8-deficiency

CD8$^+$ T cells were sorted from the peripheral blood of healthy donors (n=18-26), untransplanted DOCK8-deficient patients (n=2-7), or DOCK8-deficient patients following HSCT (DOCK8 pHSCT) (n=13-18), labelled with CFSE and then cultured with TAE (anti-CD2/CD3/CD28) beads in the absence or presence of IL-2. After five days, culture supernatants were collected, cells were harvested and then restimulated with PMA/ionomycin for six hours with Brefeldin A, monensin and anti-CD107a mAb being added after 1 hr. (A) The frequency of cells in each division was determined by dilution of CFSE. (B) Expression of CD107a and (C) secretion of Granzyme A and Granzyme B were determined by flow cytometry and cytometric bead arrays, respectively. (D) Surface expression of CD25 and (E) CD95 was determined by flow cytometry. (F, G) Secretion of IFNγ, TNFα and IL-2 were determined by cytometric bead arrays. Histograms in (A), (B), (D) and (E) are representative of one healthy donor and one paired DOCK8-defcient patient pre- and post-HSCT. Data represent the mean ± SEM. Statistics performed using Prism unpaired t-test with Welch's correction *<0.05, **<0.01, ***<0.005, ****<0.001.

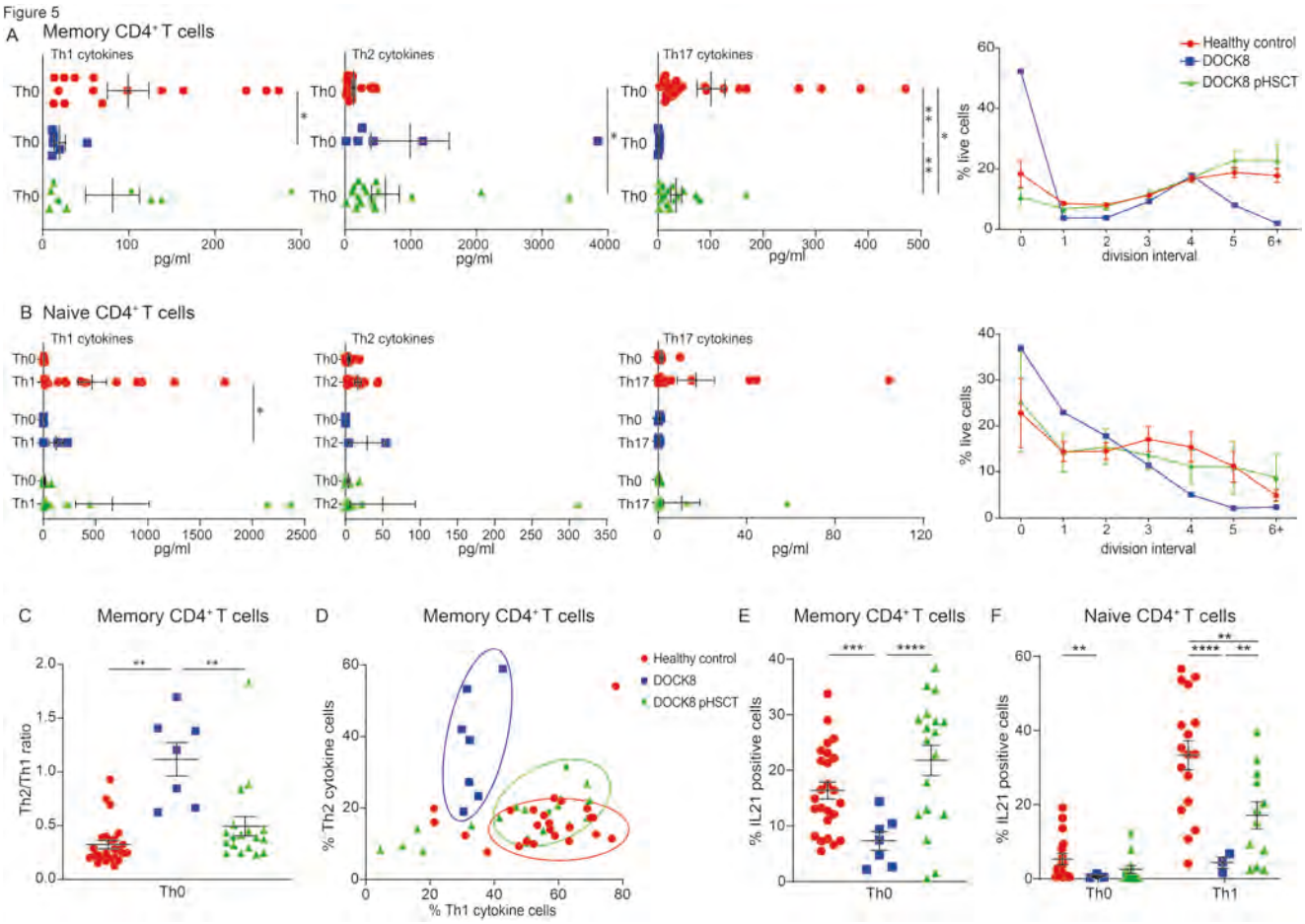

Figure 5: Dysregulated cytokine production by DOCK8-deficient CD4$^+$ T cells is greatly improved following HSCT

Naïve and memory CD4$^+$ T cells were sort-purified from the peripheral blood of healthy donors (n=7-25), untransplanted DOCK8-deficient patients (n=2-7), or DOCK8-deficient patients following HSCT (DOCK8 pHSCT) (n=6-18). The cells were labelled with CFSE and then cultured under Th0 conditions (TAE beads; naïve and memory), or Th1 (+IL-12), Th2 (+ IL-4) or Th17 (IL-1β, IL-6, IL-21, IL-23, TGF-β, prostaglandin E2) polarising conditions (naïve only) for five days.

(A, B) Cells and culture supernatants were harvested to assess proliferation of (CFSE dilution) and cytokine secretion by (A) memory CD4$^+$ T cells of IFNγ, Th2 cytokines (IL4/IL5/IL13) or Th17 cytokines (IL17A/IL17F) and (B) naïve CD4$^+$ T cells of IFNγ, Th2 cytokines (IL5/IL13) or Th17 cytokines (IL17A/IL17F).

(C, D) Cells were restimulated with PMA/ionomycin before permeabilization and intracellular staining to determine proportions of cells expressing Th1 (IFNγ, TNFα) and Th2 (IL-4, IL-13)

cytokines. Data are depicted as (C) the ratio of cells expressing Th2 vs Th1 cytokines and (D) the combined % cells from individual donors and patients expressing Th1 (i.e. %IFNγ^+/TNFα^+/ IFNγ^+TNFα^+ cells) vs Th2 (i.e. %IL-4$^+$/IL-13$^+$/IL-4$^+$IL-13$^+$ cells) cytokines.

(E, F) Intracellular expression of IL-21 by memory CD4$^+$ T cells (E) and naive CD4$^+$ T cells cultured under Th0 or Th1-polarising conditions (F) cells was measured.

Graphs depict mean ± SEM. Statistics performed using Prism unpaired t-test with Welch's correction *<0.05, **<0.01, ***<0.005, ****<0.001.

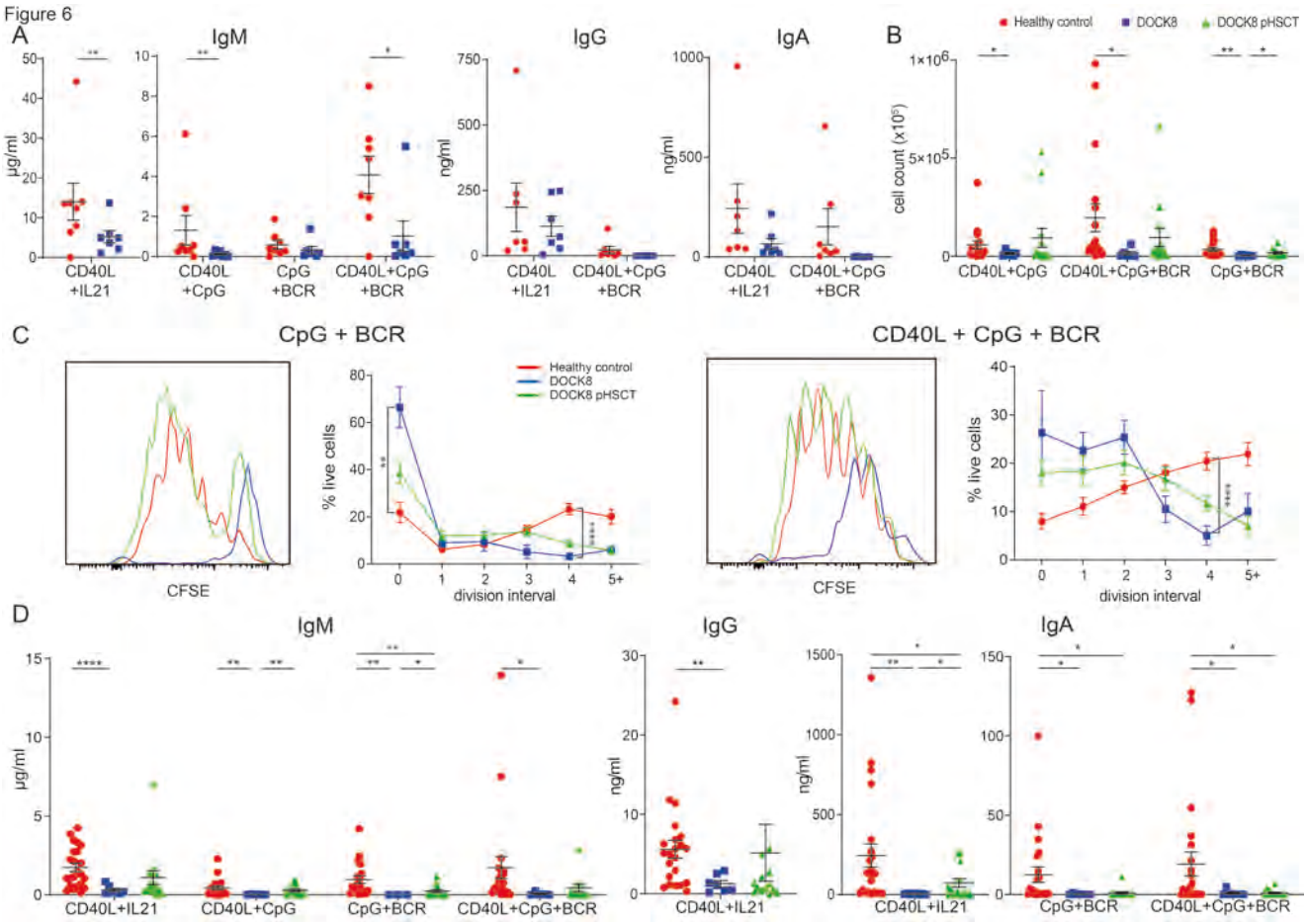

Figure 6: B cell functional defects due to DOCK8-deficiency are improved following HSCT.

(A) Naïve B cells were sort-purified from healthy donors and DOCK8-deficient patients (n=7) and then cultured with CD40L/IL-21, CD40L/CpG, CpG/BCR agonist (*Staphylococcus aureus* Cowan I), or CD40L/CpG/BCR. After 11 days, culture supernatants were harvested and levels of secreted IgM, IgG and IgA then determined. Data represent the mean ± SEM.

(B-D) Naïve B cells were sort-purified from healthy donors (n=18-23), untransplanted DOCK8-deficient patients (n=5-7), or DOCK8-deficient patients following HSCT (DOCK8 pHSCT) (n=12-15), labelled with CFSE and then cultured with combinations of CD40L, IL-21, CpG and BCR stimulus for five days. After this time, cells and culture supernatants were harvested. (B) Cell number was determined using Calibrite beads. (C) Frequency of cells in each division was determined by CFSE dilution. Histogram plots show CFSE dilution from one representative healthy donor and one paired DOCK8-deficient patient pre and post-HSCT. (D) Ig secretion of IgM, IgG and IgA was determined by ELISAs. Data in each graph represents the mean ± SEM. Statistics performed using Prism unpaired t-test with Welch's correction *<0.05, **<0.01, ***<0.005, ****<0.001.

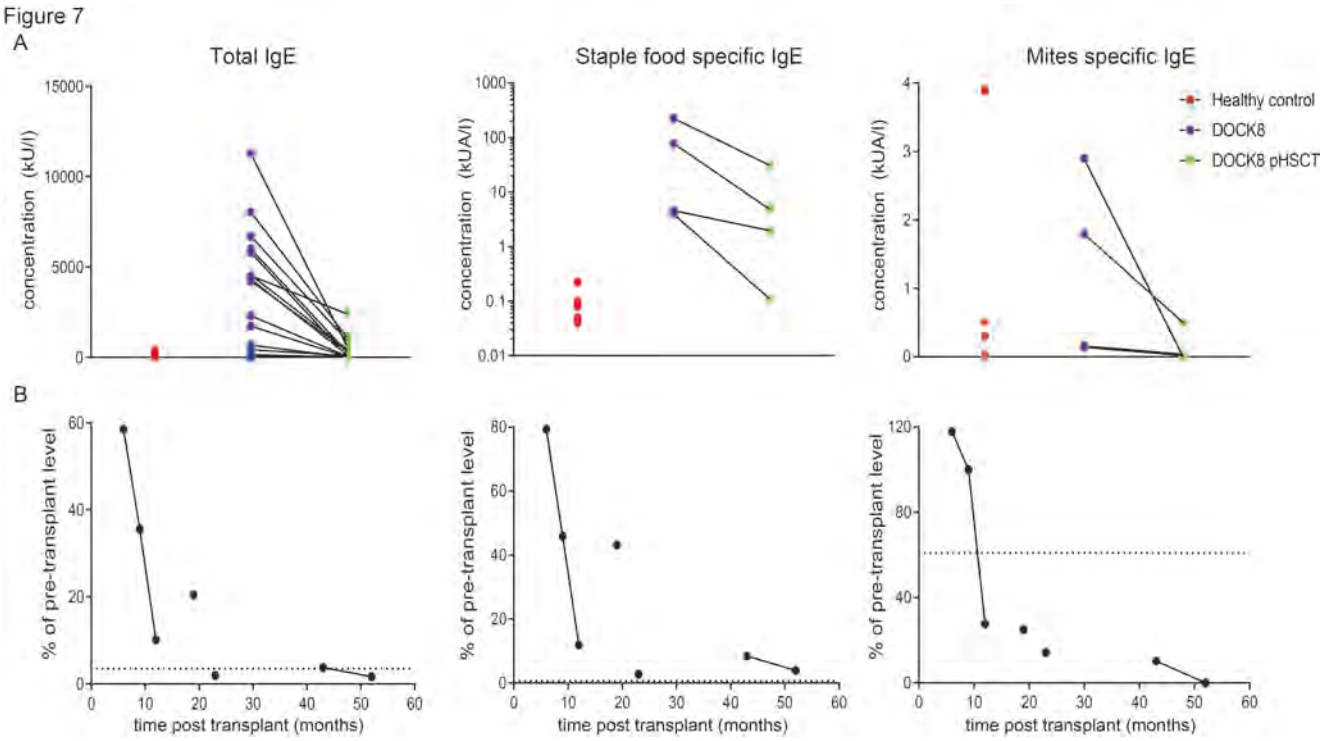

Figure 7: Elevated total and allergen specific serum IgE levels decrease in a time-dependent manner in DOCK8-deficient patients following HSCT.

(A) Serum from healthy donors (n=5) and DOCK8-deficient patients (n=4-18) collected before and after HSCT were analysed for concentrations of total IgE and IgE specific for staple foods and dust mites. (B) Data in (A) expressed as a percentage of pre-transplant levels of total and allergen-specific IgE for each patient and plotted against the time post-HSCT. Points joined by a line are from the same patients (#16, #18; Table 1) assayed at different times post-HSCT. Dotted line indicates average of healthy control values as % of average of patient pre-transplant values.

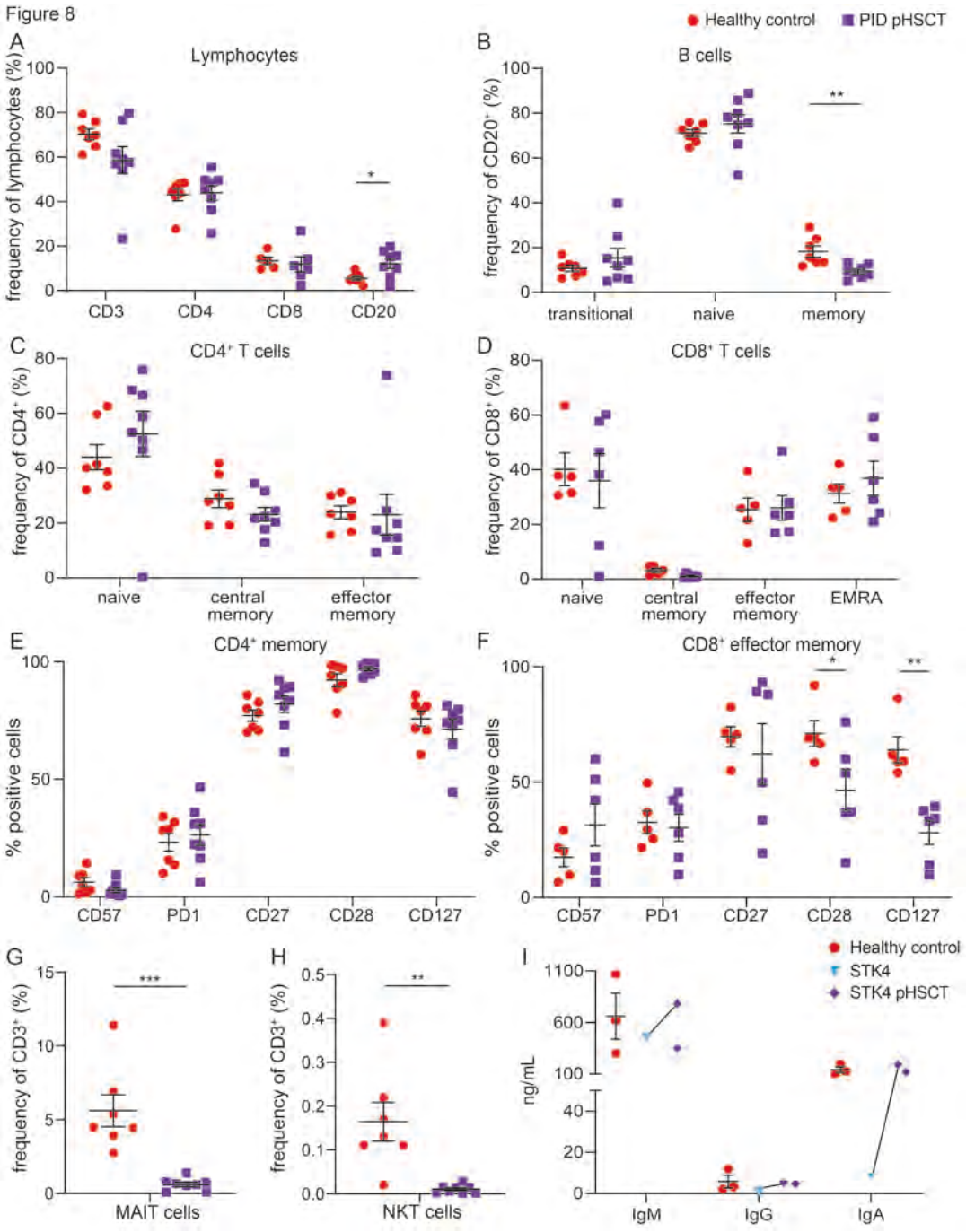

Figure 8: Lymphocyte phenotype and differentiation in PID patients pHSCT

PBMCs from healthy donors (n=5-7) or patients with mutations in *UNC13D* (n=2), *STK4* (n=2), *CYBB* (n=2), *CD40LG* (n=1) or *SH2D1A* (n=1) who had previously undergone HSCT (PID pHSCT) (n=6-8) were labelled with mAbs against CD3, CD4, CD8, CD20, CD45RA, CCR7, CD10, CD27, CD28, CD57, CD127, PD1, TCRVα24, TCRVβ11, CD161 and TCR Vα7.2 (see Figure 1 legend). Proportions of (A) $CD3^+$ cells, $CD4^+$ T cells ($CD3^+CD4^+$), $CD8^+$ T cells ($CD3^+CD8^+$), B cells

($CD20^+$); (B) transitional, naïve and memory B cell subsets; (C) $CD4^+$ naïve, T_{CM} and T_{EM} cell subsets; (D) $CD8^+$ naïve, T_{CM}, T_{EM} and T_{EMRA} cell subsets; (E) $CD4^+$ memory and (F) $CD8^+$ T_{EM} cells expressing PD1, CD57, CD27, CD27 and CD127; (G) MAIT cells and (H) NKT cells were then determined by flow cytometric analysis.

(I) Naive B cells were sorted from healthy donors (n=3), STK4-deficient patients (n=1), or STK4-deficient patients following HSCT (STK4 pHSCT) (n=2) and cultured with CD40L+IL21 for five days. After this time, culture supernatants were harvested and Ig secretion of IgM, IgG and IgA was determined by ELISAs. Points joined by lines represent data from the same patient before and after HSCT. Data are the mean ± SEM. Statistics performed using Prism unpaired t-test *<0.05, **<0.01, ***<0.005, ****<0.001.

Table 1: Clinical details of DOCK8-deficient patients pre-HSCT

Patient	Gender	mutation	bacterial infection		viral infections	
			skin	other	cutaneous	other
1	F	Homozygous Del exon 37	yes	sinopulmonary	HPV , HSV	EBV viremia
2	M	Compound het; c.1895G>A, p.W602*; c.4540delG, p.E1514Kfs8	yes	sinopulmonary	HPV, Molluscum, VZV	EBV viremia
3	F	Compound het; Del exons 1-13. R249*	yes	sinopulmonary	HPV, Molluscum	
4	F	Homozygous Del exons 4-13	yes	sinopulmonary	HPV	
5	F	Homozygous Del exons 28-35	yes	sinopulmonary	HSV, zoster, HPV	EBV viremia
6	F	Compound het; c.1266delC (p.Try423Thrfs*18), Del exons 2-47	no	sinopulmonary	HPV, Molluscum	EBV viremia
7	M	Compound het; c.538-18 C>G; Del exons 14-26	yes	sinopulmonary, osteomyelitis	HPV	
8	M	Compound het; c.1266delC (p.Try423Thrfs*18), Del exons 2-47	no	sinopulmonary	HPV, HSV	EBV viremia
9	F	Homozygous C.2044G>T (E682*)	yes	sinopulmonary	HPV, HSV	EBV viremia
10	M	Compound het; c.1153G>T(E385*); deletion of DOCK8	yes	sinopulmonary, disseminated Neisseria	HPV, Molluscum, VZV	
11	M	Compound het; c.3194delC, p.T1065fs	yes	sinopulmonary	varicella	EBV viremia
12	F	Compound het; c.1805G>A (p.W602S) C. 4540delG (p.E1514Kfs*8)	no	sinopulmonary	HPV, EBV malignancy	EBV viremia
13	M	Compound het: C.403IdupT; p.Asp1345Argfs*2; Del exons 1-14	yes	sinopulmonary	HSV	EBV viremia
14	F	Homozygous Del exons 15-48	no	salmonella, severe dental caries	adenovirus, Molluscum, CMV	RSV, rhinovirus, adenovirus, CMV
15	M	unknown but DOCK8 protein deficient	no	Streptococcus pyogenes, Haemophilus Influenzae	HSV, CMV, VZV	HHV6, EBV, norovirus, adenovirus
16	F	unknown but DOCK8 protein deficient	no	recurrent lower respiratory tract infection	HSV (ocular)	chronic ear infections.
17	M	c3733_3734delAG pR1245Efs*5	no	Staphylococcus aureus (MRSA); recurrent OM	Molluscum	CMV viraemia
18	F	Homozygous c.3803delT p.Phe1268SERfs*3	Severe staph aureus pustulosis	recurrent otitis externa with pseudomonas	no	adenovirus; low grade CMV viraemia
19	F	A->T; K271*	no	pneumonia, Pneumococcal meningitis	Molluscum	
20	M	Homozygous Del exons 6-14 included	unknown	pneumonia, OM	unknown	CMV viremia (asymptomatic)

CMV=Cytomegalovirus; DLBCL=Diffuse Large B-Cell Lymphoma; EBV=Epstein-Barr Virus; HSCT=Haematopoietic Stem Cell Transplantation; HSV=Herpes Simplex Virus; HHV6=Human Her
RSV=Respiratory Syncytial Virus; SCC=Squamous Cell Carcinoma; VZV=Varicella Zoster Virus.

fungal infections	other infections	allergies	eczema	malignancy	vaccine response	IgE	IV Ig treatment
no		food	severe	vulvar SCC	poor	>6000	yes
no		dust mites	moderate	no	variable (poor pneumococcal)	4223	no
vaginal candidiasis		food	mild	DLBCL	poor	5801	yes
no		antibiotics	mild	no	variable	2	no
mucocutaneous candidiasis		food	moderate	no	poor	8031	yes
no		no	Mild (resolved pre-HSCT)	no	poor	38.5	yes
no		food	moderate	no	variable (poor pneumococcal)	4550	no
no		food	mild	yes-SCC	poor	118	yes
yes		food	severe	no	variable (poor pneumococcal)	6690	no
no		no	moderate	no	poor	11,279	yes
oral candidiasis		latex	moderate	no	variable	679	yes
no		no	mild	EBV lymphoma	poor	143	yes
no		environmental	mild	no	poor	21.5	yes
cutaneous candidiasis	Cryptosporidia, Giardia	food, drug	mild	no	Good response to pneumococcal polysaccharide vaccine - pneumovax23	~2300	no
candidiasis, Aspergillis		food	yes	no	unknown	2586	yes
no		Food (egg, milk, macadamia) Environmental (house dust mites) Asthma, Allergic rhinitis	yes	no	unknown	8100	yes
no		food, environmental	moderate	no	unknown	~1500	yes
no		food, drug, dust mite	severe	no	unknown	4868	yes
no		no	yes	no	poor Penumococcal Ab responses	10000	no
unknown		food allergy (egg, milk, arachide), asthma	yes	no	not tested	not done	yes

rpes Virus 6; HPV=Human Papilloma Virus; IV Ig=Intravenous Immunoglobulin; MRSA= Methicillin-Resistant Staphylococcus aureus; OM=Otitis Media;

Table 2: Clinical details of DOCK8-deficient patients post-HSCT

Patient	age at HSCT	Time post-HSCT	Transplant type	Transplant source	Transplant source (PBSCs/BM)	Conditioning regime	Dose of Conditioning regime	GvHD prophylaxis	Donor chimerism at time of analysis (whole blood)	bacterial infection		viral infections		fungal infections	allergies	eczema	vaccine response	IgE	IV Ig treatment	GVHD	Immunosupression at time of analysis
										skin	other	cutaneous	other								
1	19	6 m	Haplo-identical (T cell replete with post-transplant Cy)	Mother	BM	Bu/Flu	myeloablative	TAC/Cy/MMF	100% (T, B, myeloid)	no	sinus	no		no	yes - has not been re-challenged	no	unknown	314 - 3 years post	no	no	tacrolimus
2	20	6 m	Haplo-identical (T cell replete with post-transplant Cy)	Father	BM	Bu/Flu	myeloablative	TAC /Cy/MMF	100% (T, B, myeloid)	no		no		no	unknown	no	normal	389 - 3 years post	no	acute; grade 3 GI	tacrolimus and low dose prednisone
3	20	6 m	Haplo-identical (T cell replete with post-transplant Cy)	Brother	BM	Bu/Flu	myeloablative	TAC /Cy/MMF	100% (T, B, myeloid)	no		no		no	yes	no	normal	88 - 3 years post	no	acute; grade 1 skin	tacrolimus
4	9	12 m	MUD	MUD	BM	Bu/Flu	myeloablative	TAC/MTX	100% T and myeloid; B not enough cells	no		no		no	yes - medications	no	unknown	64 - 3 years post	Yes - for cGVHD	chronic, skin and myositis	prednisone, MMF, tacrolimus, rituximab
5	18	12 m	MRD	MRD	BM	Bu/Flu	myeloablative	TAC/MTX	100% (T, B, myeloid)	no		no	resolved- oral severe HSV	no	yes - same	no	unknown	1117 - 3 years post	no	acute; grade 2 skin	cyclosporine
6	25	6 m	MUD	MUD	BM	Bu/Flu	myeloablative	Cys/MTX	100% (T, B, myeloid)	no	pulmonary (bronchiectasis persisted)	no		no	yes - shellfish (developed post-HSCT)	no	normal	6.4 - 3 years post	no	no	cyclosporine
7	19	6 m	MUD	MUD	BM	Bu/Flu	myeloablative	TAC/MTX	100% (T, B, myeloid)	no	sinus	no		no	yes - has not been re-challenged	no	normal	453 - 3 years post	no	no	tacrolimus
8	27	18 m	MUD	MUD	PBSCs	Bu/Flu	myeloablative	TAC/MTX	100% (T, B, myeloid)	no		no		no	yes - has not been re-challenged	no	unknown	17.4 - 3 years post	no	no	none
9	10	6 m	MRD	Relative	BM	Bu/Flu	myeloablative	TAC	100% B, myeloid; 99% CD3 T	no		no	HSV keratitis vs immune reconstitution	no	yes - has not been re-challenged	no	normal	946 - 3 years post	no	no	tacrolimus
10	23	23 m	MRD	MRD	BM	Bu/Flu	myeloablative	TAC/MTX	100% (T, B, myeloid)	no		no		no	no	no	normal	644 - 3 years post	no	acute; grade 3 skin	none
11	7	6 m	Haplo-identical (T cell replete with post-transplant Cy)	Father	BM	Bu/Flu	myeloablative	TAC/Cy/MMF	100% (T, B, myeloid)	no		no		no	unknown	no	normal	15.7 - 2 years post	no	no	tacrolimus
12	16	6 m + 12 m	MUD	MUD	PBSCs	Bu/Flu	myeloablative	TAC	100% (T, B, myeloid)	no	sinus	no		no	no	no	normal	12.6 - 3 years post	no	no	6 mos-cyclosporine ; 12 months none
13	7	39 m	MRD	brother	BM	Bu/Flu	myeloablative	TAC	100% (T, B, myeloid)	no		no		no	no	mild	unknown	12.8 - 2.5 years post	no	yes	none
14	3	23 m	MUD	MUD	BM	Bu/Flu/ATG	submyeloablative	MMF/Cys	100%	no		no	no	no	yes - pistachio, tree nut	no	protective measles and varicella IgG post vaccination; Responded to prevanar 13	64 - 14 mths post	no	chronic	none

15	5	40, 50 m	Haplo-identical (CD3 TCRαβ/CD19 depleted)	father	PBSCs	Flu/Thio/ATG	N/A	MMF/Cys	100%	no	recurrent OM; CVL infections - Staphylococcus aureus, Corynebacterium urealyticum	no	Immediate post-HSCT CMV reactivation, low level adenoviraemia, post-HSCT trivirus (CMV EBV, adenovirus) specific CTLs	no	yes - dairy, egg, nuts	no	tetanus IgG 0.22 IU/mL, diptheria IgG 0.37 IU/mL	67	yes	no	none
16	N/A	N/A	N/A	N/A		N/A	N/A		N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
17	16	19 m	Haplo-identical (CD3 TCRαβ/CD19 depleted)	mother	PBSCs	Treo/Flu/Thio /ATG	N/A	MMF	89.3% (99% T cells)	MRSA skin lesions	Streptococcus mitis bacteraemia	no	CMV viraemia	no	yes - skin prick positive to milk at 2 years post-HSCT	no	unknown	ND	yes - stopped 20 m post-HSCT	post-transplant haemolytic anaemia	none
18	2	6, 9, 12 mo	MUD	MUD	BM	Bu/Flu/ATG	submyeloablative	MMF/Cys	100%	no		no	post-HSCT CMV reactivation and CMV retinitis	no	unknown	no	unknown	ND	no	no	none
19	11	11mo	MRD	sibling	BM	Treo/Flu/Cam	N/A	MMF/Cys	100%	no		no		no	dairy	no	normal	89.4	no	no	none
20	2	12mo	MRD	sister	PBSCs	Bu/Flu/Cam	myeloablative	MMF/Cys	100%	no		no		no	Persistent allergies to eggs and milk	no	yes - T cell proliferation in response to tetanus	4.5	stop 1 year post-HSCT	acute; grade 2 GI	none

ATG=Anti-Thymocyte Globulin; BM=Bone Marrow; Bu=Busulfan; Cam=Campath; CVL=Catheter Venous Line; cGVHD=chronic Graft Verses Host Disease; Cy=Cyclophosphamide; Cys=cyclosporin; CMV=Cytomegalovirus; CTL=Cytotoxic T Lymphocytes; EBV=Epstein-Barr Virus; Flu=Fludarabine; GI=Gastrointestinal; HSCT=Haematopoietic Stem Cell Transplantation; HSV=Herpes Simplex Virus; IV Ig=Intravenous Immunoglobulin; MRSA= Methicillin-Resistant Staphylococcus aureus; MTX=Methotrexate; MRD=Matched Related Donor; MUD=Matched Unrelated Donor; MMF=Mycophenylate Mofetil; N/A=Not Applicable; ND=Not Done; OM=Otitis Media; TAC=Tacrolimus; Thio=Thiotepa; Treo=Treosulphan.

Table 3: Clinical details of other post-HSCT

Patient	Gender	age at HSCT	Time post-HSCT	Transplant type	Transplant source	Transplant source (PBSCs/BM/CB)	Conditioning regime	Dose of Conditioning regime	GvHD prophylaxis	Donor Chimerism at time of analysis (whole blood)	GVHD	Immunosupression at time of analysis
1. *CGD*	M	1 y	52 m	MUD	MUD	BM	Bu/Flu/Cam	submyeloablative	Cys/MMF	75%	post-transplant haemolytic anaemia	no
2. *CGD*	M	2 y	60 m	MUD	MUD	BM	Bu/Flu/Cam	submyeloablative	Cys/MMF	72%	acute; grade 2 skin	no
3. *STK4*	F	10	5 m	MUD	MUD	BM	Bu/Flu/Cy	myeloablative	Cys/CD34 sel	100%	Skin stage 3, Grade II	Pred/Cys
4. *STK4*	M	10	84 m	MUD	MUD	PBSCs	Bu/Flu/Cy	myeloablative	Cys/CD34 sel	100%	no	no
5. *UNC13D*	F	9 y	24 m	MRD	sibling	BM	Flu/Mel/Cam	submyeloablative	Cys/MMF	100%	yes	no
6. *UNC13D*	F	15 y	37 m	MUD	MUD	BM	Flu/Mel/Cam	submyeloablative	Cys/MMF	100%	no	no
7. *CD40LG*	M	1 y	27 m	MUD	MUD	BM	Treo/Flu/Cam	submyeloablative	Cys/MMF	≥95%	no	no
8. *SH2D1A*	M	9 y	67 m	MUD	MUD	CB	Bu/Flu/ATG	myeloablative	Cys/MMF	≥95%	no	no

ATG=Anti-Thymocyte Globulin; BM=bone marrow; Bu=Busulfan; Cam=Campath; CD34 sel=CD34 selection; CB=cord blood; Cy=cyclophosphamide; Cys=cyclosporin; Flu=Fludarabine; MRD=Matched Related Donor; Mel=Melphalan; MUD=Matched Unrelated Donor; MMF=mycophenylate mofetil; PBSCs=peripheral blood stem cells; Pred=Prednisone; Treo=Treosulphan.