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BRIEF COMMUNICATION

Expansion of CCR4+ activated T cells is associated with memory B cell reduction in DOCK8-deficient patients



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Abstract Hyper-IgE syndrome (HIES) is a genetic disorder characterized by elevated IgE serum levels, mostly due to mutations in *STAT3* or *DOCK8*. Despite clinical heterogeneity between the two forms of the disease, clinical manifestations may not be conclusive for diagnosis and immunological differences are still unclear.

Herein, we performed a detailed characterization of the T- and B-cell compartments by flow cytometry in seven HIES patients with homozygous *DOCK8* mutations and six patients presenting heterozygous *STAT3* mutations. We observed that *DOCK8*-deficient patients showed a marked reduction of naive and recent thymic emigrant (RTE) T lymphocytes together with a relative increase of activated T cells, most of which co-expressed the chemokine receptor CCR4, a marker of Th2

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polarization. Moreover, an extreme reduction of memory B cells was detected, despite a normal/increased proportion of immunoglobulin-secreting cells.

These observations indicate that *DOCK8*-deficient patients display a distinctive immunophenotype which is characteristic of this form of HIES.

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1. Introduction

Hyper-immunoglobulin E syndrome (HIES) is a rare primary immunodeficiency characterized by high IgE serum levels. This disorder is mostly due to the autosomal dominant form of Hyper IgE syndrome (AD-HIES) caused by *de novo* dominant-negative mutations in the signal transducer and activator of transcription 3 (*STAT3*) [1–5]. A recessive form of the disease (AR-HIES) has also been characterized and associated with mutations in the loci encoding dedicator of cytokinesis 8 (*DOCK8*) or, in a very limited number of patients, tyrosine kinase 2 (*TYK2*) [6–9]. AD-HIES patients are often characterized by staphylococcal cold abscesses and skeletal or dental abnormalities [6,10], while AR-HIES usually features T-cell lymphopenia and viral infections [11,12]. But, HIES patients do not necessarily present with typical clinical manifestations, especially in childhood.

In the present study we analyzed the T- and B-cell immunophenotype of seven *DOCK8* deficient patients. Our observations demonstrate both a distinctive expansion of CCR4 activated T cells and a profound reduction of memory B cells, despite a normal or even increased proportion of immunoglobulin secreting cells. This suggests a possible role of activated T cells in the pathogenesis of the B cell dysfunction that is observed in *DOCK8*-deficient patients.

2. Methods

EDTA-treated blood samples were collected from thirteen HIES patients, in keeping with the Declaration of Helsinki and according to protocols approved by the Hospital Ethical Committee.

Immunophenotypes were determined using whole blood and standard protocols with mixtures of FITC/PE/PerCp-Cy5.5/APC fluorochrome-conjugated commercial mAbs (Suppl. Methods). Quantification of regulatory T cells (CD4+, CD25^{hi}, FOXP3+) was performed on Ficoll-Paque-isolated PBMC (Suppl. Methods). Determination of Th17 cells was performed by quantitation of cells expressing IL17A but not IFN γ , after stimulation of CD4+ lymphocytes with anti-CD3 and anti-CD28 monoclonal antibodies plus IL-2 (Suppl. Methods).

The patients' ages ranged from 3 to 36 years (see Table 1), with six *STAT3*- (PS1–PS6) and seven *DOCK8*-deficient patients (PD1–PD7) having a median of 12.2 and 11.9 years, respectively. Healthy age-matched controls were nine adults and 30 children (range 2–37 years; median age 13.0 years). Statistical comparisons of groups were performed using the Kruskal–Wallis nonparametric test, followed by Dunn's multiple comparison test. The level of significance was $p < 0.05$.

At least 5 μ g of genomic DNA were isolated from blood samples and used for genetic analyses (Suppl. Methods). PHA T-cell blasts were generated and cultured for patients PD1–

PD7. RNA and protein extracts were isolated from lymphoblasts and used to examine *DOCK8* transcripts and *DOCK8* protein expression, respectively (Suppl. Methods).

3. Results and discussion

Thirteen individuals were enrolled, from ten unrelated families, with elevated serum IgE, eczema, pulmonary and mucocutaneous infections and staphylococcal abscesses. Six of them (PS1–PS6) displayed heterozygous mutations of *STAT3* (Table 1) [3,4]. In the remaining patients, exome sequencing (PD4–PD6) [9] and dideoxy sequencing (PD1–PD7) revealed homozygous mutations in *DOCK8* (Table 1) that prevented the generation of the normal primary transcript, thus causing absence or marked reduction of protein expression (Supplemental Figure).

Absolute leukocyte counts showed several abnormalities in *DOCK8*-deficient patients. Neutrophil counts were increased in patients PD1–PD3, PD5, while PD1–PD3, PD5 and PD7 had eosinophilia, and PD4–PD5 and PD7 had lymphopenia (Table 1) [13].

Evaluation of CD4+ T-cell maturation profile by flow cytometry revealed a significant decrease ($p < 0.01$) of naïve cells (CD45RA+, CCR7+) [14] and recent thymic emigrants (RTE) (CD45RA+, CCR7+, CD31+) [15] in *DOCK8*-deficient patients, compared to the control group and to *STAT3*-mutated patients (mean $16.5 \pm 15.7\%$ vs. $54.3 \pm 14.3\%$ and $62.0 \pm 14.8\%$, respectively, for naïve cells; $13.6 \pm 13.5\%$ vs. $44.4 \pm 13.6\%$ and $50.5 \pm 12.4\%$, respectively, for RTE) (Fig. 1A columns 1–6 and Supplemental Table). Among CD4+ memory subsets a significantly increased proportion of effector T cells (CD45RA-, CCR7-) [14] was noticed (mean $56.5 \pm 22.0\%$ vs. $17.2 \pm 7.7\%$, $p < 0.001$, for healthy controls, and 14.0 ± 7.0 , $p < 0.01$, for *STAT3*-mutated patients) (Fig. 1A columns 10–12 and Supplemental Table). A similarly altered ratio between naïve and memory cells was also observed in CD8+ lymphocytes of *DOCK8*-deficient patients, which displayed a mean of $4.9 \pm 4.2\%$ for naïve cells vs. $45.5 \pm 19.1\%$ ($p < 0.001$) and $48.2 \pm 28.5\%$ ($p < 0.05$) for healthy controls and *STAT3*-mutated patients, respectively (Fig. 1B columns 1–3 and Supplemental Table); a mean of $52.4 \pm 21.3\%$ for effector cells vs. $26.5 \pm 11.6\%$ ($p < 0.05$) and $23.7 \pm 13.2\%$ (N.S.) for healthy controls and *STAT3*-mutated patients, respectively (Fig. 1B columns 7–9 and Supplemental Table).

Comparison of absolute cell counts confirmed a severe deficiency of thymic output in *DOCK8*-deficient patients; in fact, both CD4+ RTE and naïve CD8+ cells of *DOCK8*-deficient patients were markedly reduced compared to the age-matched control group and *STAT3* patients (mean $81 \pm 101c/mm^3$ vs. $540 \pm 318c/mm^3$, $p < 0.001$, and $978 \pm 828c/mm^3$,

Table 1 Clinical and immunological features of DOCK8- and STAT3-deficient patients at diagnosis.

	DOCK8							STAT3					
	PD1	PD2	PD3	PD4	PD5	PD6	PD7	PS1	PS2	PS3	PS4	PS5	PS6
Mutation in genomic DNA	c.325-?_1475+? del	c.1-?_624+?del	Exon30 c.3804_3805delAT	Exon26 c.3193delA	Exon26 c.3193delA	Exon26 c.3193delA	c.325_1664 del	Exon 21 c.1909G/A ^a	Exon 21 c.1979T/G ^a	Exon 21 c.1909G/A ^a	Exon 10 c.1025G/A ^b	Exon15 c.1294G/C ^b	Exon 13 c.1144C/T ^b
Amino acid change	p.exon 5-13del Arg492 LysfsX35	p.0	p.Leu1268 LeufsX2	p.Ser1065 AlafsX17 ^c	p.Ser1065 AlafsX17 ^c	p.Ser1065 AlafsX17 ^c	p.exon 5-15del	p. Val637Met ^d	p. Met660Arg ^e	p. Val637Met ^d	p. Gly342Asp ^d	p. Val432Leu ^e	p. Arg382Trp ^d
Sex	F	F	F	M	M	M	M	F	F	F	M	M	F
Age at diagnosis (y)	7	3	7	13	16	27	10	36	15	3	25	3	5
Status	Alive	Deceased ^f	Alive	Alive	Deceased ^g	Alive	Alive	Alive	Alive	Alive	Alive	Alive	Alive
Consanguinity	—	—	+	+	+	+	?	—	—	—	—	—	—
IgE U/ml	6510	35200	>5000	>5000	171	NA	>5000	1290	>5000	1590	15420	>5000	3130
IgG U/ml	1169	672	1110	2010	1110	NA	1560	1060	1810	282	1404	819	741
IgA U/ml	157	59	120	930	310	NA	216	101	77	29	151	44	30
IgM U/ml	89	86	32	25	43	NA	18	75	259	68	67	86	89
WBC ^h (cells/ μ l)	14860	21580	15600	6880	12820	7900	8760	5750	5020	10260	5820	21150	6134
Neutrophils (cells/ μ l)	9496 ^{high}	9926 ^{high}	8736 ^{high}	5194	8204 ^{high}	5767	3540	2900	2400	2400	2677	8690 ^{high}	2067
Eosinophils (cells/ μ l)	1980 ^{high}	8157 ^{high}	2810 ^{high}	250	1666 ^{high}	395	2820 ^{high}	300	540	400	524	2517 ^{high}	200
Lymphocytes (cells/ μ l) ⁹	2348	2156	3120	600 ^{low}	740 ^{low}	1438	1430 ^{low}	2000	4040	4220	1920	7650 ^{high}	3570
CD3 (cells/ μ l) ⁱ	1256	1423	1894	160 ^{low}	448 ^{low}	1140	970 ^{low}	1348	2606	3207	1574	5760 ^{high}	3052
CD4 (cells/ μ l) ⁱ	657	939	740	89 ^{low}	312	249 ^{low}	165 ^{low}	732	886	2101	1299	3923 ^{high}	1999
CD8 (cells/ μ l) ⁱ	383	370	678	46 ^{low}	48 ^{low}	335	600	450	852	709	222	1215	687
CD19 (cells/ μ l) ⁱ	533	582	484	173 ^{low}	121 ^{low}	101	257	119	945 ^{high}	755	257	1377	336

^a Mutation resides in the SH2 domain of STAT3.^b Mutation resides in the DNA-binding domain of STAT3.^c Mutation described in Ref. [9].^d Mutation described in Ref. [10].^e Mutation described in Ref. [4].^f Anaphylactic shock.^g During chemotherapeutic treatment of a T-cell lymphoma, as described in Ref. [9].^h White blood cells.ⁱ Reference values in Ref. [13].

$p < 0.001$, respectively, for RTE cells, and $12 \pm 12\text{c/mm}^3$ vs. $302 \pm 209\text{c/mm}^3$, $p < 0.001$, and $336 \pm 385\text{c/mm}^3$, $p < 0.001$, respectively, for CD8+ naïve cells (data not shown). Despite the increased mean percentage and absolute cell count of RTE and naïve T lymphocytes, and, consequently, a decrease of effector subsets, no significant differences between STAT3-mutated patients and healthy controls were observed in the T-cell subsets as evaluated in the present study (Figs. 1A–B and Supplemental Table).

We observed that T-cell activation markers, such as HLA-DR and CD25, were expressed by a larger proportion of CD4+ T cells in all DOCK8-deficient patients, as compared to control subjects and STAT3-deficient patients (mean $45.3 \pm 12.7\%$ vs. $7.4 \pm 5.0\%$ $p < 0.001$, and $9.9 \pm 5.0\%$ $p < 0.05$, respectively, for HLA-DR; $43.5 \pm 10.2\%$ vs. $17.1 \pm 7.8\%$ $p < 0.001$, and $17.0 \pm 8.8\%$ $p < 0.05$, respectively, for CD25) (Fig. 1C columns 1–6 and Supplemental Table). A similar increase was noticed for CD4+ cells expressing CCR4+, a chemokine receptor that is associated with Th2 commitment [16] (mean $59.1 \pm 14.1\%$ in DOCK8-deficient patients vs. $23.0 \pm 8.7\%$ in the healthy control group and $14.8 \pm 4.6\%$ in STAT3-deficient patients, respectively, $p < 0.001$) (Fig. 1C columns 7–9). Most of the CD25+ activated T cells in DOCK8-deficient patients co-expressed CCR4 (mean $36.9 \pm 8.8\%$ on total CD4+ cells in four patients vs. $10.3 \pm 4.5\%$ $p < 0.05$ in ten healthy controls and $3.1 \pm 2.2\%$ $p < 0.01$ in three STAT3 patients, respectively) (data not shown). Expression profile of DOCK8-deficient patients was similar to the one observed in Omenn syndrome, a primary immunodeficiency characterized by marked Th2 polarization (Fig. 1D) [17]. Quantification of the proportion of CD4+ cells expressing the Th1-associated chemokine receptor CXCR3 [18] did not reveal any statistically significant difference between the three groups (data not shown), thus suggesting that the T-cell hyper-activation observed in DOCK8-deficient patients is mainly aimed at Th2 differentiation.

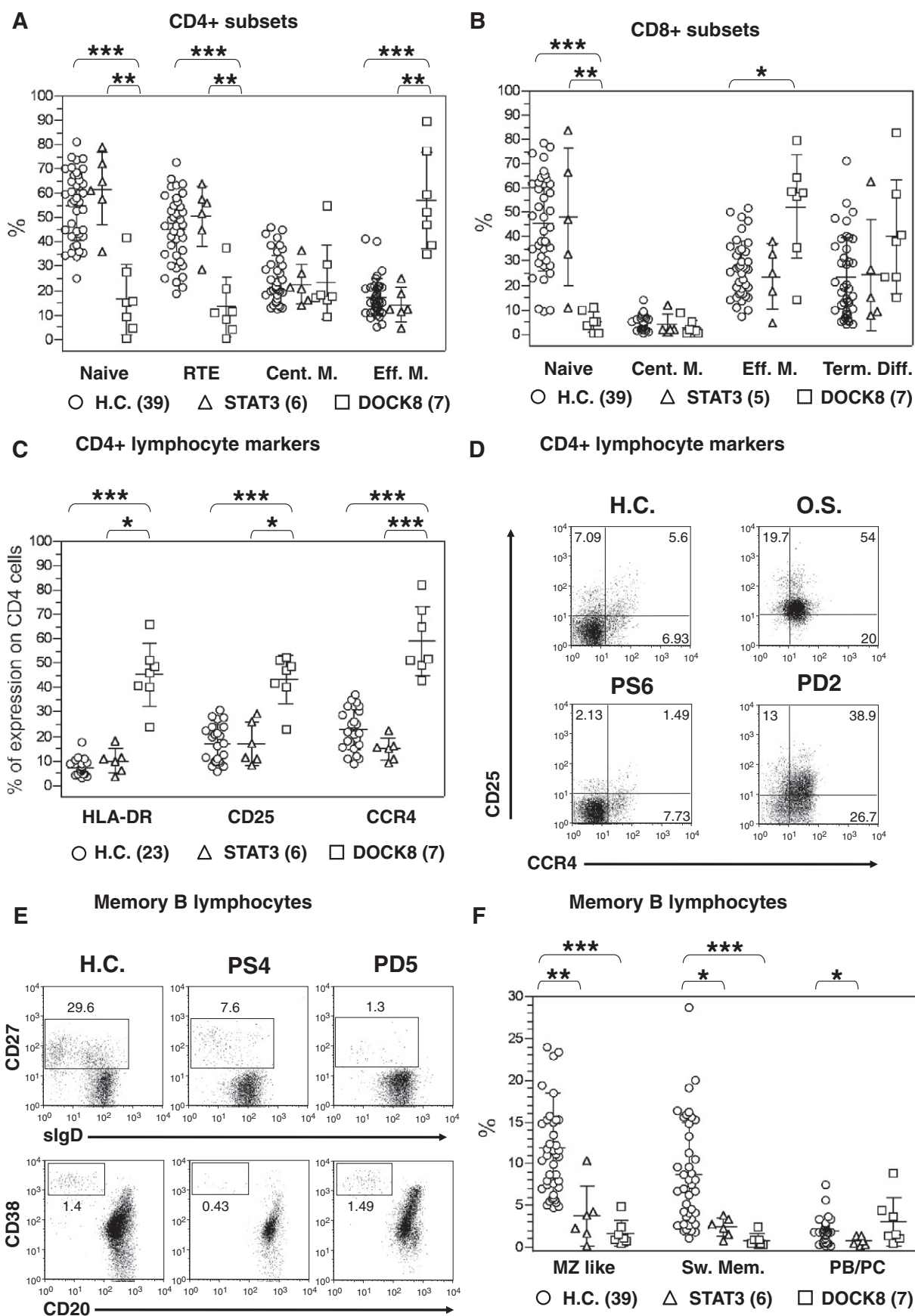
Quantification of regulatory T cells (Tregs) in patients PD1 and PD4–6 did not reveal any significant difference of Treg numbers compared to the age-matched healthy donor group and to four STAT3 patients (mean $4.4 \pm 0.83\%$ vs. $6.2 \pm 2.8\%$ and $5.6 \pm 0.98\%$, respectively) (see also Supplemental Table). Analysis of FOXP3 expression intensity and Treg suppressive ability were also tested in patients PD4–6, and found to be normal as already reported [9]. Quantification of Th17 cells in patients PD2–3 and PD7 showed that the proportion of cells expressing interleukin-17A, but not IFN-gamma, by activated CD4+ cells was reduced, although not significantly, in DOCK8 patients ($0.12 \pm 0.09\%$) as compared to the healthy donor group ($0.68 \pm 0.34\%$); by contrast, Th17 values in DOCK8 patients were more similar to the ones observed in the six STAT3 patients ($0.05 \pm 0.04\%$) (Supplemental Table).

Comparison of the B-cell compartment showed a significant reduction of CD27+ memory B cells between both AR- and AD-HIES patients and healthy controls, as previously reported [19–21] (Fig. 1E upper row). This defect affected both marginal zone (MZ) like as well as switched memory cells, identified as IgD+, IgM+ and IgD-, IgM-cells, respectively, in the gate of CD27+ memory cells [22,23] (Fig. 1F columns 1–6). In DOCK8-deficient patients reduction of memory B cells was particularly severe as compared to values observed in healthy controls (mean percentage of MZ-like and switched cells were $1.70 \pm 1.51\%$ and $0.82 \pm 0.78\%$, respectively, vs. $11.8 \pm 6.6\%$ and $8.7 \pm 6.3\%$ for the

healthy donor group, $p < 0.001$) (Supplemental Table). A marked reduction of these subsets was also observed by the analysis of absolute cell counts (mean $6.4 \pm 7.1\text{c/mm}^3$ vs. $36.6 \pm 18.6\text{c/mm}^3$, $p < 0.001$ for MZ-like cells and $2.0 \pm 1.4\text{c/mm}^3$ vs. $23.8 \pm 12.4\text{c/mm}^3$, $p < 0.001$ for switched cells) (data not shown). STAT3-mutated patients displayed a less severe reduction of memory B cells ($3.75 \pm 3.57\%$ and $2.43 \pm 1.13\%$ for MZ-like, $p < 0.01$, and switched cells, $p < 0.05$, respectively) (Fig. 1F columns 1–6) together with a significantly lower percentage of plasmablasts/plasmacells (CD20- CD38+++ CD27+++ [24,25], as compared to the control group (mean of $0.73 \pm 0.56\%$ vs. $1.96 \pm 1.42\%$ on total B cells, $p < 0.05$) (Fig. 1E lower row, 1F columns 7–9 and Supplemental Table). Instead, the mean percentage of DOCK8-mutated patients was even higher ($3.01 \pm 2.91\%$).

Herein, we report the T and B immunophenotypic analysis of thirteen patients affected by the two most common forms of HIES. Only three patients among them displayed a reduction of total lymphocyte and T cell counts, implying that the use of these parameters to identify potential DOCK8-deficient patients may be misleading. Both the two forms of the disease are characterized by an impaired memory B-cell maturation, but DOCK8-deficient patients displayed a more severe reduction of the memory B cell compartment ($p < 0.001$ for both MZ-like and switched cells) and maintained the ability to generate a normal or even increased number of effector B cells (plasmablasts and plasmacells). Recognition of this B cell phenotype in patients with elevated IgE will probably be useful not only to distinguish DOCK8-deficient patients from normal subjects with atopic disease [19], who present a normal number of memory B cells, but also from AD-HIES patients, in which dysregulation of B-cell maturation seems more permissive for memory B cell differentiation, but considerably affects also the generation of effector cells. It is likely that the severe reduction of switched memory B cells in DOCK8-deficient patients could be associated with a higher frequency and severity of infections as compared to AD-Hyper IgE syndrome patients, but this hypothesis was not evaluated in this study due to the limited number of patients. In contrast to the observation reported in a recent paper by Alsum et al. [26] of an increased occurrence of autoimmunity in DOCK8-deficient patients, our cohort of patients did not show any clinical sign of autoimmunity over the course of the study, despite the high number of effector B cells detected. On the other hand, neither *in vitro* test nor serum analyses were assessed to evaluate autoantibody production. The high number of effector B cells and the adequate IgG serum levels observed in our cohort of AR-HIES patients may also suggest a normal antibody production, although assessment of functional antibody response (vaccine-specific titers) was not available. Of note, impaired antibody responses against both viral and bacterial antigens have been reported in DOCK8-deficient patients [12].

Although B cell immunophenotyping revealed some differences between AD-HIES and DOCK8-deficient patients, the peculiar distribution of the T-lymphocyte subsets observed in the latter group of patients provided an even more unambiguous analytical strategy to differentiate between the two groups. In fact, a detailed characterization of T-cell subsets demonstrates that the presence of a poor naïve and RTE compartment is a unique trait of patients with



DOCK8 mutations already detectable in infancy. This observation is even more intriguing after the remark that memory T lymphocytes are reduced in AD-HIES patients, as shown in our study and previously reported by Siegel et al. [27]. Finally, we have shown that patients with DOCK8 deficiency display a marked relative increase of chronically-activated T lymphocytes that co-express the Th2 marker CCR4. This observation is in accord with the high frequency of allergic manifestations observed in our patients, including a case of fatal anaphylactic shock in PD2. The same T cell immune-phenotype can be identified in other primary immunodeficiencies, such as Omenn and Wiskott–Aldrich syndromes, in which impaired T-cell activity and proliferative response are also present together with increased IgE serum levels [28]. The expansion of activated T cells that is observed in DOCK8-deficient patients apparently is not due to a reduction of Tregs, because these cells are present in normal number and display normal suppressive activity (Supplemental Table and Ref. [9]. In contrast, the increased proportion of activated T cells is probably related to expansion of Th2-committed cells. In conclusion, our study demonstrates that both the increased percentage of activated T cells and the Th2 polarization are peculiar features of DOCK8-deficient patients that can be instrumental for prompt recognition and treatment of these patients.

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.clim.2014.03.008>.

Conflict of interest statement

The author(s) declare that there are no conflicts of interest.

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References

- [1] S.M. Holland, F.R. DeLeo, H.Z. Elloumi, A.P. Hsu, G. Uzel, N. Brodsky, A.F. Freeman, A. Demidowich, J. Davis, M.L. Turner,

- V.L. Anderson, D.N. Darnell, P.A. Welch, D.B. Kuhns, D.M. Frucht, H.L. Malech, J.I. Gallin, S.D. Kobayashi, A.R. Whitney, J.M. Voyich, J.M. Musser, C. Woellner, A.A. Schäffer, J.M. Puck, B. Grimbacher, STAT3 mutations in the hyper-IgE syndrome, *N. Engl. J. Med.* 357 (16) (2007) 1608–1619 (18).
- [2] Y. Minegishi, M. Saito, S. Tsuchiya, I. Tsuge, H. Takada, T. Hara, N. Kawamura, T. Ariga, S. Pasic, O. Stojkovic, A. Metin, H. Karasuyama, Dominant-negative mutations in the DNA-binding domain of STAT3 cause hyper-IgE syndrome, *Nature* 448 (7157) (2007) 1058–1062.
- [3] H. Jiao, B. Tóth, M. Erdos, I. Fransson, E. Rákóczi, I. Balogh, Z. Magyarics, B. Dérfalvi, G. Csorba, A. Szaflarska, A. Megarbane, C. Akatcherian, G. Dbaibo, E. Rajnavölgyi, L. Hammarström, J. Kere, G. Lefranc, L. Maródi, Novel and recurrent STAT3 mutations in hyper-IgE syndrome patients from different ethnic groups, *Mol. Immunol.* 46 (1) (2008) 202–206.
- [4] C. Woellner, E.M. Gertz, A.A. Schäffer, M. Lagos, M. Perro, E.O. Glocker, M.C. Pietrogrande, F. Cossu, J.L. Franco, N. Matamoros, B. Pietrucha, E. Heropolitańska-Pliszka, M. Yeganeh, M. Moin, T. Español, S. Ehl, A.R. Gennery, M. Abinun, A. Breborowicz, T. Niehues, S.S. Kilic, A. Junker, S.E. Turvey, A. Plebani, B. Sánchez, B.Z. Garty, C. Pignata, C. Cancrini, J. Litzman, O. Sanal, U. Baumann, R. Bacchetta, A.P. Hsu, J.N. Davis, L. Hammarström, E.G. Davies, E. Eren, P.D. Arkwright, J.S. Moilanen, D. Viemann, S. Khan, L. Maródi, A.J. Cant, A.F. Freeman, J.M. Puck, S.M. Holland, B. Grimbacher, Mutations in STAT3 and diagnostic guidelines for hyper-IgE syndrome, *J. Allergy Clin. Immunol.* 125 (2) (2010) 424–432 (e8).
- [5] M. Giacomelli, N. Tamassia, D. Moratto, P. Bertolini, G. Ricci, C. Bertulli, A. Plebani, M. Cassatella, F. Bazzoni, R. Badolato, SH2 domain mutations of STAT3 gene result in impairment of IL-10 function in hyper-IgE syndrome patients, *Eur. J. Immunol.* 41 (10) (2011) 3075–3084.
- [6] L.F. Schimke, J. Sawalle-Belohradsky, J. Roesler, A. Wollenberg, A. Rack, M. Borte, N. Rieber, R. Cremer, E. Maass, R. Dopfer, J. Reichenbach, V. Wahn, M. Hoenig, A.F. Jansson, A. Roesen-Wolff, B. Schaub, R. Seger, H.R. Hill, H.D. Ochs, T.R. Torgerson, B.H. Belohradsky, E.D. Renner, Diagnostic approach to the hyper-IgE syndromes: immunologic and clinical key findings to differentiate hyper-IgE syndromes from atopic dermatitis, *J. Allergy Clin. Immunol.* 126 (3) (2010) 611–617 (e1); Erratum in: *J. Allergy Clin. Immunol.* 126 (5) (2010) 1015.
- [7] Y. Minegishi, M. Saito, T. Morio, K. Agematsu, S. Tsuchiya, H. Takada, T. Hara, N. Kawamura, T. Ariga, H. Kaneko, N. Kondo, I. Tsuge, A. Yachie, Y. Sakiyama, T. Iwata, F. Bessho, T. Ohishi, K. Joh, K. Imai, K. Kogawa, M. Shinohara, M. Fujieda, H. Wakiguchi, S. Pasic, M. Abinun, H.D. Ochs, E.D. Renner, A. Jansson, B.H. Belohradsky, A. Metin, N. Shimizu, S. Mizutani,

Figure 1 Peripheral blood T- and B-cell immunophenotyping in two groups of patients affected by hyper-IgE syndrome due to genetic alterations in DOCK8 or STAT3 genes. Panels A–B: comparison of T-cell subsets between STAT3 and DOCK8 patients and 39 age-matched healthy controls. Percentage of naïve, recent thymic emigrants (RTE), central and effector memory cells are taken into account for CD4+ lymphocytes (panel A), while percentages of naïve, central and effector memory, and terminally differentiated cells are reported for CD8+ lymphocytes (panel B). Healthy controls (○), STAT3 patients (Δ) and DOCK8 patients (□). Panel C–D: comparison of T-cell activation and Th2 commitment between STAT3, DOCK8 and 23 healthy controls (age range: 8–35 years). Percentage of CD4+ lymphocytes expressing activation markers such as HLA-DR (left) and CD25 (center) or the Th2-associated chemokine receptor CCR4 (right) are presented for healthy controls (○), STAT3 patients (Δ) and DOCK8 patients (□) (panel C). Exemplificative dot plot panels of CD4+ gated cells that account for the level of activation in Th2-committed cells (upper right quadrant) in STAT3 and DOCK8 patients compared to a normal individual and a patient suffering from Omenn syndrome (panel D). Panels E–F: Representative dot plot panels showing the percentage of CD20+ gated B cells displaying a memory immunophenotype (CD27+) (upper row), and the percentage of CD19+ gated cells displaying a plasmablast/plasmacell immunophenotype (CD20-CD38+++ (lower row) in a healthy control, one STAT3 and one DOCK8 patient (panel E). Comparison of memory and terminally differentiated B-cell subsets between STAT3 and DOCK8 patients and 39 age-matched healthy controls. Percentages of MZ-like and switched memory cells as well as plasmablasts/plasmacells are presented for healthy controls (○), STAT3 patients (Δ) and DOCK8 patients (□) (panel F). Numbers of subjects analyzed for each group are indicated in parenthesis. **p* < 0.05, ***p* < 0.01, ****p* < 0.001.

- T. Miyawaki, S. Nonoyama, H. Karasuyama, Human tyrosine kinase 2 deficiency reveals its requisite roles in multiple cytokine signals involved in innate and acquired immunity, *Immunity* 25 (5) (2006) 745–755.
- [8] K.R. Engelhardt, S. McGhee, S. Winkler, A. Sassi, C. Woellner, G. Lopez-Herrera, A. Chen, H.S. Kim, M.G. Lloret, I. Schulze, S. Ehl, J. Thiel, D. Pfeifer, H. Veelken, T. Niehues, K. Siepermann, S. Weinspach, I. Reisli, S. Keles, F. Genel, N. Kutukculer, Y. Camcioglu, A. Somer, E. Karakoc-Aydiner, I. Barlan, A. Gennery, A. Metin, A. Degerliyurt, M.C. Pietrogrande, M. Yeganeh, Z. Baz, S. Al-Tamemi, C. Klein, J.M. Puck, S.M. Hollandi, E.R. McCabe, B. Grimbacher, T.A. Chatila, Large deletions and point mutations involving the dedicator of cytokinesis 8 (DOCK8) in the autosomal-recessive form of hyper-IgE syndrome, *J. Allergy Clin. Immunol.* 124 (6) (2009) 1289–1302 e4; Erratum in: *J. Allergy Clin. Immunol.* 125 (3) (2010) 743 (Kutuculer, Necil [corrected to Kutukculer, Necil]).
- [9] D.L. Dinwiddie, S.F. Kingsmore, S. Caracciolo, G. Rossi, D. Moratto, C. Mazza, C. Sabelli, R. Bacchetta, L. Passerini, C. Magri, C.J. Bell, N.A. Miller, S.L. Hateley, C.J. Saunders, L. Zhang, G.P. Schroth, S. Barlati, R. Badolato, DOCK8 and CLEC7A mutations causing immunodeficiency in three brothers with diarrhea, eczema and infections, *J. Allergy Clin. Immunol.* 131 (2) (2013) 594–597.
- [10] B. Grimbacher, S.M. Holland, J.I. Gallin, F. Greenberg, S.C. Hill, H.L. Malech, J.A. Miller, A.C. O'Connell, J.M. Puck, Hyper IgE syndrome with recurrent infections an autosomal dominant multisystem disorder, *N. Engl. J. Med.* 340 (1999) 692–702.
- [11] W. Al-Herz, R. Ragupathy, M.J. Massaad, R. Al-Attayah, A. Nanda, K.R. Engelhardt, B. Grimbacher, L. Notarangelo, T. Chatila, R.S. Geha, Clinical, immunologic and genetic profiles of DOCK8-deficient patients in Kuwait, *Clin Immunol* 143 (3) (2012) 266–272.
- [12] Q. Zhang, J.C. Davis, I.T. Lamborn, A.F. Freeman, H. Jing, A.J. Favreau, H.F. Matthews, J. Davis, M.L. Turner, G. Uzel, S.M. Holland, H.C. Su, Combined immunodeficiency associated with DOCK8 mutations, *N. Engl. J. Med.* 361 (21) (2009) 2046–2055.
- [13] W.M. Comans-Bitter, R. de Groot, R. van den Beemd, H.J. Neijens, W.C. Hop, K. Groeneveld, H. Hooijkaas, J.J. van Dongen, Immunophenotyping of blood lymphocytes in childhood. Reference values for lymphocyte subpopulations, *J. Pediatr.* 130 (3) (1997) 388–393.
- [14] F. Sallusto, D. Lenig, R. Förster, M. Lipp, A. Lanzavecchia, Two subsets of memory T lymphocytes with distinct homing potentials and effector functions, *Nature* 401 (6754) (1999) 708–712.
- [15] S. Junge, B. Kloeckener-Gruissem, R. Zufferey, A. Keisker, B. Salgo, J.C. Fauchere, F. Scherer, T. Shalaby, M. Grotzer, U. Siler, R. Seger, T. Güngör, Correlation between recent thymic emigrants and CD31+ (PECAM-1) CD4+ T cells in normal individuals during aging and in lymphopenic children, *Eur. J. Immunol.* 37 (11) (2007) 3270–3280.
- [16] R. Bonecchi, G. Bianchi, P.P. Bordignon, D. D'Ambrosio, R. Lang, A. Borsatti, S. Sozzani, P. Allavena, P.A. Gray, A. Mantovani, F. Sinigaglia, Differential expression of chemokine receptors and chemotactic responsiveness of type 1 T helper cells (Th1s) and Th2s, *J. Exp. Med.* 187 (1) (1998) 129–134.
- [17] D. Brugnani, P. Airò, F. Facchetti, L. Blanzuoli, A.G. Ugazio, R. Cattaneo, L.D. Notarangelo, *In vitro* cell death of activated lymphocytes in Omenn's syndrome, *Eur. J. Immunol.* 27 (11) (1997) 2765–2773.
- [18] S. Qin, J.B. Rottman, P. Myers, N. Kassam, M. Weinblatt, M. Loetscher, A.E. Koch, B. Moser, C.R. Mackay, The chemokine receptors CXCR3 and CCR5 mark subsets of T cells associated with certain inflammatory reactions, *J. Clin. Invest.* 101 (4) (1998) 746–754.
- [19] C. Speckmann, A. Enders, C. Woellner, D. Thiel, A. Rensing-Ehl, M. Schlesier, J. Rohr, T. Jakob, E. Oswald, M.V. Kopp, O. Sanal, J. Litzman, A. Plebani, M.C. Pietrogrande, J.L. Franco, T. Espanol, B. Grimbacher, S. Ehl, Reduced memory B cells in patients with hyper IgE syndrome, *Clin. Immunol.* 129 (3) (2008) 448–454.
- [20] D.T. Avery, E.K. Deenick, C.S. Ma, S. Suryani, N. Simpson, G.Y. Chew, T.D. Chan, U. Palendira, J. Bustamante, S. Boisson-Dupuis, S. Choo, K.E. Bleasel, J. Peake, C. King, M.A. French, D. Engelhard, S. Al-Hajjar, S. Al-Muhsen, K. Magdorf, J. Roester, P.D. Arkwright, P. Hissaria, D.S. Riminton, M. Wong, R. Brink, D.A. Fulcher, J.L. Casanova, M.C. Cook, S.G. Tangye, B cell-intrinsic signaling through IL-21 receptor and STAT3 is required for establishing long-lived antibody responses in humans, *J. Exp. Med.* 207 (1) (2010) 155–171.
- [21] H.H. Jabara, D.R. McDonald, E. Janssen, M.J. Massaad, N. Ramesh, A. Borzutzky, I. Rauter, H. Benson, L. Schneider, S. Baxi, M. Recher, L.D. Notarangelo, R. Wakim, G. Dbaiibo, M. Dasouki, W. Al-Herz, I. Barlan, S. Baris, N. Kutukculer, H.D. Ochs, A. Plebani, M. Kanariou, G. Lefranc, I. Reisli, K.A. Fitzgerald, D. Golenbock, J. Manis, S. Keles, R. Ceja, T.A. Chatila, R.S. Geha, DOCK8 functions as an adaptor that links TLR-MyD88 signaling to B cell activation, *Nat. Immunol.* 13 (6) (2012) 612–620.
- [22] C. Wehr, T. Kivioja, C. Schmitt, B. Ferry, T. Witte, E. Eren, M. Vlkova, M. Hernandez, D. Detkova, P.R. Bos, G. Poerksen, H. von Bernuth, U. Baumann, S. Goldacker, S. Gutenberger, M. Schlesier, F. Bergeron-van der Cruyssen, M. Le Garff, P. Debré, R. Jacobs, J. Jones, E. Bateman, J. Litzman, P.M. van Hagen, A. Plebani, R.E. Schmidt, V. Thon, I. Quinti, T. Espanol, A.D. Webster, H. Chapel, M. Vihinen, E. Oksenhendler, H.H. Peter, K. Warnatz, The EUROclass trial: defining subgroups in common variable immunodeficiency, *Blood* 111 (1) (2008) 77–85.
- [23] G.J. Driessen, M.C. van Zelm, P.M. van Hagen, N.G. Hartwig, M. Trip, A. Warris, E. de Vries, B.H. Barendregt, I. Pico, W. Hop, J.J. van Dongen, M. van der Burg, B-cell replication history and somatic hypermutation status identify distinct pathophysiologic backgrounds in common variable immunodeficiency, *Blood* 118 (26) (2011) 6814–6823.
- [24] E. Arce, D.G. Jackson, M.A. Gill, L.B. Bennett, J. Bancheureau, V. Pascual, Increased frequency of pre-germinal center B cells and plasma cell precursors in the blood of children with systemic lupus erythematosus, *J. Immunol.* 167 (4) (2001) 2361–2369.
- [25] L.W. Terstappen, S. Johnsen, I.M. Segers-Nolten, M.R. Loken, Identification and characterization of plasma cells in normal human bone marrow by high-resolution flow cytometry, *Blood* 76 (9) (1990) 1739–1747.
- [26] Z. Alsum, A. Hawwari, O. Alsmadi, S. Al-Hissi, E. Borrero, A. Abu-Staiteh, H.G. Khalak, S. Wakil, A.M. Eldali, R. Arnaout, A. Al-Ghoniaim, S. Al-Muhsen, H. Al-Dhekri, B. Al-Saud, H. Al-Mousa, Clinical, immunological and molecular characterization of DOCK8 and DOCK8-like deficient patients: single center experience of twenty-five patients, *J. Clin. Immunol.* 33 (1) (2013) 55–67.
- [27] A.M. Siegel, J. Heimall, A.F. Freeman, A.P. Hsu, E. Brittain, J. M. Brechley, D.C. Douek, G.H. Fahle, J.I. Cohen, S.M. Holland, J.D. Milner, A critical role for STAT3 transcription factor signaling in the development and maintenance of human T cell memory, *Immunity* 35 (5) (2011) 806–818.
- [28] E. Ozcan, L.D. Notarangelo, R.S. Geha, Primary immune deficiencies with aberrant IgE production, *J. Allergy Clin. Immunol.* 122 (6) (2008) 1054–1062.

Supplemental methods

DOCK8 mutations in patients PD1, PD2, PD3, PD7 were identified by Sanger sequencing as described¹¹. Direct sequencing of DOCK8 exons was performed using the BigDye Terminator Kit (Applied Biosystems, Foster City, CA) and the sequences were analyzed on a BioEdit software (BioEdit sequence alignment editor). Patients PD4, PD5, PD6 were identified by exome sequencing (Ref 9) and confirmed by Sanger sequencing as described above.

Total RNA was extracted using the RNeasy Mini Kit (Qiagen, Milan, Italy) according to the manufacturer's instructions. 1 µg of DNase-treated total RNA was used to synthesize the first strand of cDNA by the GeneAmp®RNA PCR kit (Applied Biosystems). The *DOCK8* gene was amplified by PCR by using 14 paired primers (conditions and primers available upon request).

Cells were lysed in RIPA buffer and complete protease inhibitor (Roche). Cytosolic Lysates was prepared according to standard procedures, separated on 4-12% TRIS-acetate gels (Invitrogen) and transfer onto nitrocellulose. Nitrocellulose membranes were first blocked for 1h at room temperature in TBST containing 5% dry milk and then incubated for 1h in the presence of specific primary Abs. Polyclonal rabbit anti-DOCK8 and anti-actin were purchased from Sigma-Aldrich. Detection was carried out using HRP-conjugated anti-rabbit IgG (Amersham Biosciences) and revealed using the LiteAblot–EuroCloneECL (Euroclone) according to the manufacturer's instructions.

Immunophenotypical analyses were performed staining 100-200 µl of whole blood with appropriate mixtures of FITC/PE/PerCp-Cy5.5/APC fluorochrome-conjugated commercial monoclonal antibodies (mAbs). The following combinations of mAbs were used for T lymphocyte subset analyses: anti-CD45RA FITC (eBioscience), CCR7 PE (eBioscience), CD4 or CD8 PerCP-Cy5.5 (Becton Dickinson) and CD31 APC (Miltenyi) were used for quantification of CD4⁺ and CD8⁺ subsets; specifically, CD4⁺ or CD8⁺ T lymphocytes were isolated with a specific gating strategy, then CD45⁺CCR7⁺ naïve, CD45RA⁺CCR7⁺ central memory, CD45RA⁺CCR7⁻ effector memory and CD45RA⁺CCR7⁻ terminally differentiated cells were separated by a quadrant gate; finally the percentage of CD31⁺ RTE cells were determined on the CD4⁺ naïve population.

The following combinations of mAbs were used for T lymphocyte activation/commitment determinations: anti-CD3 FITC (Becton Dickinson), HLA-DR PE (Becton Dickinson) or CD25 PE (BioLegend), CD4 PerCP-Cy5.5 (Becton Dickinson) and CD8 APC (Becton Dickinson), were used to determine the percentage of HLA-DR or CD25 positive cells on both CD4⁺ and CD8⁺ gated T cells; anti-CXCR3 or CCR4 FITC (R&D systems), CD25 PE (BioLegend), CD4 PerCP-Cy5.5 (Becton Dickinson) and CD3 APC (Becton Dickinson), were used to determine the percentage of

CD25-CCR4 or CD25-CXCR3 double positive cells after isolation of CD4⁺ T lymphocytes using a proper gating strategy.

The following combinations of mAbs were used for B lymphocyte subset analyses: anti-IgD FITC (Serotec), CD27 (Becton Dickinson), CD20 PerCP-Cy5.5 (Becton Dickinson), and IgM APC (Becton Dickinson) were used for quantification of MZ like (IgD⁺IgM⁺) and switched memory (IgD⁺IgM⁻) cells after isolation of CD27⁺ cells (Fig. 1F upper panel) on the B lymphocyte (CD20⁺) gate; CD20 FITC (Becton Dickinson), CD138 PE (Miltenyi), CD38 PerCP-Cy5.5 (Becton Dickinson) and CD19 APC (Becton Dickinson) were used for quantification of effector cells which are gated as CD20-CD38^{hi} (Fig. 1F lower panel) on the B lymphocyte (CD19⁺) gate, and are further separated in plasmacells based on CD138 positivity.

Analyses of regulatory T cells were performed on PBMC obtained from 1 ml of peripheral blood after Ficoll-Paque density gradient centrifugation. Firstly, PBMC were stained with CD25 PE (BioLegend), CD4 PerCP-Cy5.5 (Becton Dickinson) and CD127 APC (eBioscience). Then, half of the cells were used for intracellular staining of Foxp3 mAb and half for staining the appropriate isotype control (both Biolegend). The intracellular staining was performed according to the manufacturer's protocol using a specific Fix/Perm buffer set (Biolegend).

Analyses of Th17 cells were performed on activated CD4⁺ isolated cells. PBMC were initially isolated with a Ficoll-Paque density gradient centrifugation from 5 ml of peripheral blood, then, CD4⁺ cells were isolated with a positive selection using magnetic beads (Miltenyi). Isolated cells were cultured for 4 days in a 24 well plate at a density of 5x10⁵ cells per well with interleukin-2 (20U/ml) (Peprotech) plus coated anti-CD3 mAb (5 µg/ml) and anti-CD28 mAb at the concentration of 750 ng/ml (both Becton Dickinson), directly in the culture medium. After 84 hours of culture, phorbol myristate acetate (*PMA*) (100 ng/ml) and ionomycin (750 ng/ml) (both Sigma) were added to the culture medium. Finally, brefeldin A (Sigma) at a final concentration of 10 ng/ml was added to the culture medium four hour before the cell staining. Stimulated cells were collected, washed and surface stained with a CD4 PerCP-Cy5.5 (Becton Dickinson) mAb. The intracellular staining was performed using fix&perm cell permeabilization reagents (Caltag) according to the manufacturer's protocol, incubating with interleukin-17A Alexa 488 and interferon gamma PE mAbs (both eBioscience).

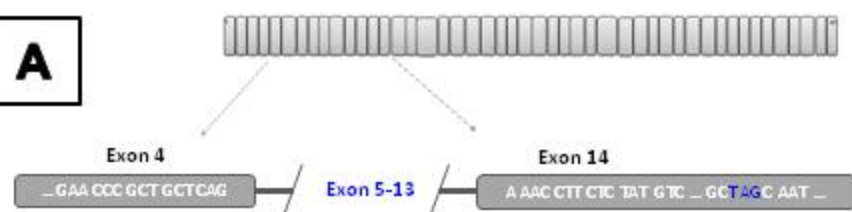
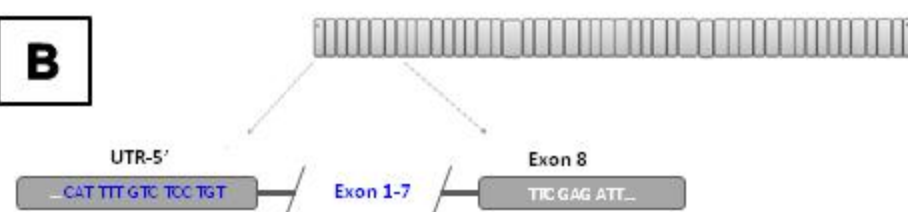
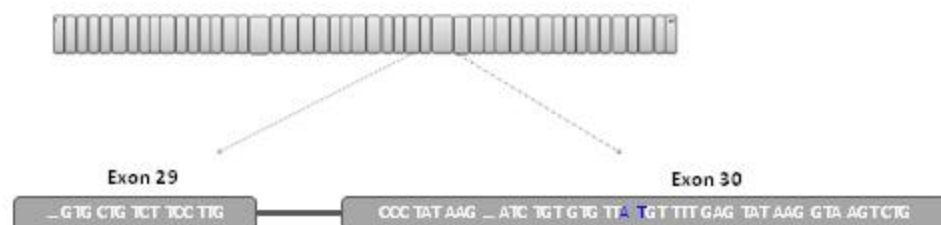
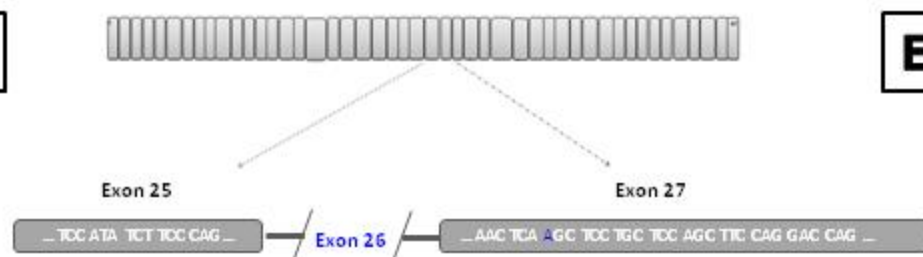
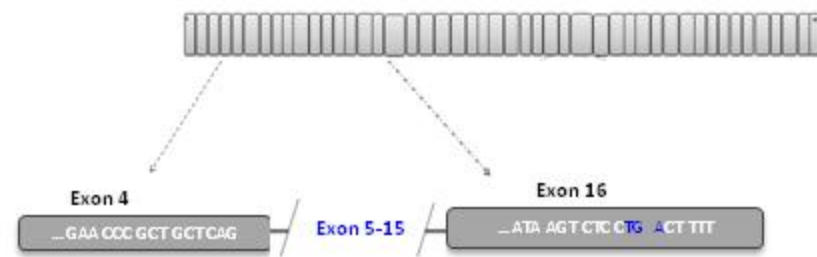
After staining blood samples were lysed and fixed. Then, they were acquired on a FACScalibur flow cytometer (Becton Dickinson) and analyzed using the FlowJo software version 8.8.6 (TreeStar).

Supplemental figure: schematic representation of DOCK8 mutations and their effect on protein expression in patients PD1-PD7.

(A) Frameshift deletion of exons 5-13 of DOCK8 in PD1. (B) Deletion from UTR5' to exon 7 of DOCK8 in PD2. (C) AG deletion in exon 30 in PD3. (D) Adenosine deletion in exon 26 in PD4-PD6. (E) Frameshift deletion of exons 5-15 of DOCK8 in PD7. (F) Immunoblot of PHA-blasts lysates of two healthy controls (lanes 1 and 6) and PD1-PD3, PD7 patients blotted with an anti-DOCK8 (upper panel) and an anti-actin (lower panel) polyclonal antibody.

Supplemental table. Percentage of T- and B-cell subsets of DOCK8- and STAT3-deficient patients compared to the range of the healthy donor group.

	CD4+ subsets				CD8+ subsets				CD4+ markers			T reg	Th17	B cell subsets		
	Na+ve	RTE	Cent. M.	Eff. M.	Na+ve	Cent. M.	Eff. M.	Term. Diff.	HLA-DR+	CD25+	CCR4+			MZ like	Sw. Mem.	PB/PC
Patients																
PD1	15.0	11.1	30.6	52.2	4.8	1.9	35.4	57.6	45.8	48.7	65.0	4.4	n.a.	2.37	0.24	8.76
PD2	27.7	20.8	17.0	47.0	3.3	0.1	14.1	82.5	51.5	52.4	49.0	n.a.	0.06	4.82	0.97	4.35
PD3	42.0	37.4	17.9	38.7	5.3	0.3	56.5	38.0	41.0	40.4	42.8	n.a.	0.22	0.89	0.48	1.34
PD4	4.6	3.9	16.0	77.5	10.8	1.4	64.3	23.4	48.5	42.0	71.8	5.3	n.a.	0.98	0.87	1.04
PD5	9.3	8.2	55.0	35.0	9.6	8.8	58.0	23.6	40.2	46.9	82.0	3.3	n.a.	0.50	0.41	1.49
PD6	0.4	0.4	9.1	89.5	0.1	5.2	79.4	15.3	66.0	22.7	51.5	4.6	n.a.	0.79	2.48	3.66
PD7	15.7	10.9	18.2	59.5	0.3	0.3	58.8	40.6	24.0	51.1	51.8	n.a.	0.07	1.56	0.30	0.46
Mean	16.5	13.6	23.4	56.5	4.9	2.6	52.4	40.1	45.3	43.5	59.1	4.40	0.12	1.70	0.82	3.01
SD	15.7	13.5	15.3	22.0	4.2	3.3	21.3	23.4	12.7	10.2	14.1	0.83	0.09	1.51	0.78	2.91
PS1	61.4	56.2	26.9	11.4	47.1	12.1	32.9	7.9	9.9	16.9	16.3	3.7	0.02	10.4	3.80	1.43
PS2	36.3	28.6	36.6	25.1	10.6	1.8	24.9	62.8	18.2	26.0	10.9	10.0	0.08	4.29	1.53	1.39
PS3	57.7	45.0	21.2	18.5	n.a.	n.a.	n.a.	n.a.	6.3	10.6	15.5	n.a.	0.02	2.30	2.80	0.70
PS4	72.5	51.7	13.8	12.3	66.5	1.1	17.6	14.9	11.1	29.4	9.2	6.3	0.04	3.73	3.36	0.43
PS5	79.1	63.8	16.3	4.3	83.7	2.0	4.7	9.5	3.3	8.0	22.3	n.a.	0.01	1.60	2.32	0.34
PS6	64.8	57.8	21.0	12.6	33.0	2.0	38.3	26.7	10.7	11.3	14.9	4.7	0.10	0.20	0.80	0.10
Mean	62.0	50.5	22.6	14.0	48.2	3.8	23.7	24.4	9.9	17.3	14.8	5.60	0.05	3.75	2.43	0.73
SD	14.8	12.4	8.2	7.0	28.5	4.7	13.2	22.7	5.0	8.8	4.6	0.98	0.04	3.57	1.13	0.56
CTRLs																
CTRL ÅÚ	39	39	39	39	39	39	39	39	23	23	23	14	15	39	39	39
Mean	54.3	44.4	24.7	17.2	45.5	4.4	26.5	23.2	7.4	17.1	23.0	6.20	0.68	11.8	8.68	1.96
SD	14.3	13.6	9.9	7.7	19.1	2.9	11.6	16.7	5.0	7.8	8.7	2.80	0.34	6.60	6.31	1.42

A**B****C****D****E****F**