Impaired respiratory burst contributes to infections in PKCδ-deficient patients

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Patients with autosomal recessive protein kinase C δ (PKCδ) deficiency suffer from childhood-onset autoimmunity, including systemic lupus erythematosus. They also suffer from recurrent infections that overlap with those seen in patients with chronic granulomatous disease (CGD), a disease caused by defects of the phagocyte NADPH oxidase and a lack of reactive oxygen species (ROS) production. We studied an international cohort of 17 PKCδ-deficient patients and found that their EBV-B cells and monocyte-derived phagocytes produced only small amounts of ROS and did not phosphorylate p40phox normally after PMA or opsonized Staphylococcus aureus stimulation. Moreover, the patients’ circulating phagocytes displayed abnormally low levels of ROS production and markedly reduced neutrophil extracellular trap formation, altogether suggesting a role for PKCδ in activation of the NADPH oxidase complex. Our findings thus show that patients with PKCδ deficiency have impaired NADPH oxidase activity in various myeloid subsets, which may contribute to their CGD-like infectious phenotype.

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Introduction

Protein kinase C δ (PKCδ) is a cytosolic kinase of the novel serine/threonine PKC group (diacylglycerol [DAG]-dependent, Ca2+-independent). It is ubiquitously expressed and is an essential regulator of immune homeostasis and B cell development in humans and mice (Guo et al., 2004; Griner and Kazanietz, 2007). It can be activated in response to various stimuli, leading to individual patterns of PKCδ phosphorylation and, thus, to the differential activation of various downstream targets. PKCδ is involved in regulating apoptosis, proliferation, and cell survival in various cells, including lymphocytes and phagocytes (Salzer et al., 2016; Reyland et al., 1999; Wu-Zhang et al., 2012; Gemel et al., 2007; DeVries-Seimon et al., 2007). It is activated by stimulation with DAG and phospholipids produced by the phospholipase Cγ (PLCγ)-mediated hydrolysis of membrane inositol and phorbol esters (Nishizuka, 1995; Duquesnes et al., 2011). In addition to the conformational changes induced by binding to DAG, PKCδ requires autophosphorylation and phosphorylation by phosphoinositide-dependent kinase-1 (PDK-1) for activation (Durgan et al., 2007). Its enzymatic activity is modulated by members of the Src kinase family, protein tyrosine kinase 2 (PYK2), mechanistic target of rapamycin, and phosphoinositide 3-kinase (PI3K; Duquesnes et al., 2011; Li et al., 1994). PKCδ activity is regulated via its regulatory domain, which contains a pseudosubstrate domain and two conserved domains (C1 and C2-like), with hydrophobic residues within the C1 motif responsible for the binding of PKCδ to DAG and phorbol esters (e.g., PMA; Zhang et al., 1995; Kazanietz et al., 1995). The pseudosubstrate domain, which is located between the C1 and C2-like domains, blocks access to the substrate-binding pocket, keeping PKCδ in an inactive state. The enzymatic activity of PKCδ is dependent on the catalytic domain, composed of the C3 and C4 domains, containing the ATP- and substrate-binding sequences, respectively (Duquesnes et al., 2011; Pappa et al., 1998; Cho, 2001). Several proteins, including STAT1 and -3, nuclear factor of IL-6 (NF-IL6), and ERK1 and -2, have been identified as substrates of PKCδ, implicating this protein in diverse pathways (Salzer et al., 2016; Yang et al., 2019; Nishikawa et al., 1997).

Studies of Pkcd knockout mice have revealed an essential role for PKCδ in B cell homeostasis and tolerance, by demonstrating an accumulation of autoreactive B cells due to defective negative selection in germinal centers and autonomous B cell hyperproliferation (Mecklenbräuker et al., 2002; Limander et al., 2011). Pkcd-knockout mice thus develop systemic lupus erythematosus (SLE) occurring before the age of 10 yr. These patients had small numbers of memory B cells (CD19+CD27+), very large numbers of naïve (CD19+CD27-) and immature (CD19+CD27-) B cells, and an impaired Ig class switch (Kuehn et al., 2013; Belot et al., 2013). High counts of circulating CD8+ T cells and double-negative (CD4-/CD8+) T cells were observed in some cases, together with slightly lower levels of T cell proliferation in vitro (Kuehn et al., 2013; Sharifinejad et al., 2020; Kiykim et al., 2015). Intriguingly, these patients suffer not only from autoimmune disease, but also from recurrent and/or severe infections (Kuehn et al., 2013; Belot et al., 2013; Kiykim et al., 2015; Salzer et al., 2015; Lei et al., 2018; Nanthapisal et al., 2017; Meyts et al., 2021; Sharifinejad et al., 2020). They manifest with a monogenic form of systemic lupus erythematosus (SLE) occurring before the age of 10 yr. These patients had small numbers of memory B cells (CD19+CD27+), very large numbers of naïve (CD19+CD27-) and immature (CD19+CD27-) B cells, and an impaired Ig class switch (Kuehn et al., 2013; Belot et al., 2013). High counts of circulating CD8+ T cells and double-negative (CD4-/CD8+) T cells were observed in some cases, together with slightly lower levels of T cell proliferation in vitro (Kuehn et al., 2013; Sharifinejad et al., 2020; Kiykim et al., 2015). Intriguingly, these patients suffer not only from autoimmune disease, but also from recurrent and/or severe infections (Kuehn et al., 2013; Belot et al., 2013; Kiykim et al., 2015; Salzer et al., 2015; Lei et al., 2018; Nanthapisal et al., 2017; Meyts et al., 2021; Sharifinejad et al., 2020). In particular, PKCδ-deficient patients present viral, bacterial, and fungal infections, mostly affecting the lung, gastrointestinal tract, and lymph nodes, reminiscent of those seen in patients with chronic granulomatous disease (CGD). CGD is an inborn error of immunity caused by loss-of-function (LOF) or hypomorphic mutations of any of six genes encoding components of the NADPH oxidase complex (Thomas et al., 2019; Anjani et al., 2019). Like patients with CGD, PKCδ-deficient individuals have been reported to suffer from recurrent, and even invasive, bacterial infections, including in particular infections with Staphylococcus spp. and Pseudomonas spp. (Kuehn et al., 2013; Belot et al., 2013; Kiykim et al., 2015; Salzer et al., 2013; Lei et al., 2018; Nanthapisal et al., 2017; Sharifinejad et al., 2020). The immunosuppressive treatment of SLE is a potential confounding factor, but we hypothesized that these patients might display an intrinsic susceptibility to infections. Based on the role of PKCδ in NADPH oxidase activation, and the overlap between the infections seen in patients with CGD and those with PKCδ deficiency, we tested the hypothesis that AR PKCδ deficiency impairs ROS production in phagocytic cells.
thereby underlying susceptibility to CGD-like infectious diseases. We therefore analyzed NADPH oxidase expression and activity in various subsets of myeloid cells, and in EBV-immortalized B cells (EBV-B cells) from all 11 previously-reported patients and 6 newly identified patients with AR PKCδ deficiency.

**Results**

**Clinical description of 17 patients with PKCδ deficiency**

We studied 17 patients from 10 families originating from Mexico (kindred A), Turkey (kindreds B, D, H, and I), France (kindred C), Pakistan (kindred E), the UK (kindred F), and Iran (kindreds G and J; Table 1 and Fig. 1 A). The 10 families were unrelated, and 6 were known to be consanguineous. 11 patients (P1–P10, P12) from 7 kindreds (A–G) have already been reported to have PKCδ deficiency (Kuehn et al., 2013; Belot et al., 2013; Kiykim et al., 2015; Salzer et al., 2015; Lei et al., 2018; Nanthapisal et al., 2017; Meyts et al., 2021; Sharifinejad et al., 2020). We identified five new patients by whole-exome sequencing (WES). The detailed clinical data of one patient (P11) will be reported elsewhere (unpublished data). Most patients (P1–P15) developed clinical manifestations before the age of 10 yr and displayed hepatosplenomegaly and various features of SLE, including photosensitivity, malar rash, alopecia, and oral ulcers, whereas P16 and P17 displayed no SLE-like symptoms. Furthermore, vasculitis and glomerulonephritis were documented in four (P1, P4, P7, and P8) and autoimmune hemolytic anemia in six (P1, P4, P7, P14, and P15) patients. Most patients displayed B lymphoproliferation and/or high levels of autoreactive antibody aggregates (ANAs; P1–P15) or anti–double-stranded DNA (anti-dsDNA) antibodies, as found in nine patients (P3–P9, P12, and P13). In addition to SLE, the patients suffered from recurrent and/or severe infections (Table 1). All patients were vaccinated with Bacillus Calmette-Guérin (BCG) against tuberculosis, but only four patients (P13–P16) developed BCG-related disease. In addition, P13 also suffered from Salmonella bacteremia, recurrent gingivitis, and inguinal lymphadenitis due to Staphylococcus aureus. One patient suffered from severe Legionella pneumonia (P3); one from Streptococcus pneumoniae pneumonia (P12); one from Listeria meningitis, sepsis due to Enterococcus faecium, systemic Candida infection, and recurrent Achromobacter xyloswitcher cervical lymphadenitis (P10); and one from sepsis caused by Haemophilus influenzae (P11). P4 died at the age of 13 yr, from septic shock due to Pseudomonas aeruginosa (Table 1). Five patients suffered from viral infections, most caused by herpesviruses, namely EBV (P1), CMV (P2), and varicella–zoster virus (P15), or other viruses, including papillomavirus (P3) and severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2; P10). Six patients suffered from recurrent pneumonia (P1, P2, P3, P5, P6, and P12), four from recurrent otitis media (P1, P6, P10, and P12), and six from recurrent gastroenteritis (P2, P6, P11, P12, P14, and P15), although no causal microbe could be identified in any of these cases. Most patients were treated with corticosteroids (P1, P3–P9, and P12–P15), in combination with anti–CD20 antibodies in some cases (P4 and P6–P11), whereas one patient (P2) was successfully treated with hydroxychloroquine alone. Three patients received mycophenolate mofetil (MMF; P4, P5, and P11), two received azathioprine (P7 and P8), and one was treated with sirolimus (P10). Most of the patients have received intravenous immunoglobulin (IVIG; P2, P5–P11, P13, and P15) and/or antibiotics (P2, P6, P12, and 13) as prophylaxis (Table 1).

**Bi-allelic PRKCD variants in 17 individuals from 10 families**

Eight disease-causing PRKCD variants in 12 patients have been found: 10 patients (P1–P9 and P12) were found to be homozygous for rare PRKCD variants, including four missense variants (c.1840C>T [p.R614W], c.742G>A [p.G248S], c.1525G>A [p.G510S], and c.1294G>T [p.G432W]), one small insertion (c.1293_1294insA, predicted p.G432Rfs*15), and one essential splice site substitution (c.1352 + 1G>A; Table 1 and Fig. 1 A; Kuehn et al., 2013; Belot et al., 2013; Kiykim et al., 2015; Salzer et al., 2015; Lei et al., 2018; Nanthapisal et al., 2017; Meyts et al., 2021; Sharifinejad et al., 2020). Two patients, P10 and P11, were compound heterozygous for a nonsense variant (c.57IC>T [p.Q191*]) and a variant located at the essential splice acceptor site at the end of intron 9 (c.788-2A>G; Meyts et al., 2021; unpublished data). The newly identified patients, P13–P17, were found to carry previously unknown homozygous PRKCD variants: P13 carried an essential splice site variant (c.57I + 2dup) located in intron 7, P14 and P15 carried a nonsense variant (c.1384C>T [p.Q462*]), and P16 and P17 carried a small homozygous deletion of 1 bp (c.642del) predicted to lead to a premature stop codon (p.N214Kfs*26; Fig. 1, A and B; and Table 1). All variants, in all patients, were confirmed by Sanger sequencing, and when available, familial segregation analysis was performed, confirming that the disease followed an AR pattern of inheritance (Fig. 1 A).

Surprisingly, one individual was homozygous for a variant but was asymptomatic at the age of 6 yr (kindred J, P17). 8 of the 11 variants had already been reported, but we completed the study in silico. 6 of the 11 variants studied were private and were absent from public databases (p.G432W, c.571 + 2dup, c.642del, c.788-2A>G, c.1293_1294insA, and c.1352 + 1G>A), whereas 5 were reported in the heterozygous state in the Genome Aggregation Database (gnomAD v2.1.1; p.Q191*, p.G248S, p.Q462*, p.G510S, and p.R614W), with a minor allele frequency <10−4 (Fig. 1 C). No homozygotes for any of these variants were found in public databases. All missense variants affected sites known to have been conserved over evolution and had combined annotation-dependent depletion (CADD) scores well above the mutation significance cutoff (MSC) of 26.5, whereas the variants predicted to affect splicing had a CADD score close to or slightly below the MSC (Figs. 1 C and S1 A). Only six other non-synonymous variants of PRKCD were found in the homozygous state in the gnomAD database (v2.1.1) and our in-house cohort (Laboratory of Human Genetics of Infectious Diseases) of >9,500 patients with various infectious diseases. They were missense variants with CADD scores below the MSC and were, therefore, not predicted to be deleterious. These data strongly suggest that the 17 patients studied have AR PKCδ deficiency, consistent with previous reports (Kuehn et al., 2013; Belot et al., 2013; Kiykim et al., 2015; Salzer et al., 2013; Lei et al., 2018; Nanthapisal et al., 2017; Sharifinejad et al., 2020).

**PRKCD splice site variants are predicted to encode truncated proteins**

Essential splice site variants have been reported for PRKCD, but nothing is known about the consequences of these variants for
| Kindred | Patient | Mutation (predicted protein variant) | Origin | Sex | Follow-up | Age at onset of symptoms | Autoimmune manifestations | BCG status; clinical infectious phenotype (infections, microbiology, pathology results); treatment, prophylaxis | Reference |
|---------|---------|-------------------------------------|--------|-----|----------|--------------------------|--------------------------|---------------------------------------------------------------------------------------------------------------------------------|-----------|
| A       | P1      | c.1840C>T/c.1840C>T (p.R614W/p.R614W) | Mexico, living in USA | M   | Alive    | 3 yr                     | Autoimmune hemolytic anemia, SLE ANA\(^+\), anti-RNP\(^+\), anti-smith\(^+\), anti-SSA\(^+\) | BCG-vaccine: no AE; recurrent pneumonia, EBV infection, recurrent otitis media; corticosteroids, rapamycin; prophylaxis: NR | Kuehn et al., 2013 |
| B       | P2      | c.742G>A/c.742G>A (p.G248S/p.G248S) | Turkey | M   | Alive    | 1 yr                     | SLE ANA\(^+\)            | BCG-vaccine: no AE; recurrent pneumonia, CMV infection, recurrent gastroenteritis; hydroxychloroquine; prophylaxis: IVIG, antibiotics | Kiykim et al., 2015 |
| C       | P3      | c.1528G>A/c.1528G>A (p.G510S/p.G510S) | France | F   | Alive    | 10 yr                    | SLE ANA\(^+\), anti-dsDNA\(^+\) | BCG-vaccine: no AE; severe Legionella pneumonia, recurrent pneumonia, severe papillomatosis; corticosteroids, hydroxychloroquine; prophylaxis: NR | Belot et al., 2013 |
|         | P4      | c.1528G>A/c.1528G>A (p.G510S/p.G510S) |        | F   | Dead     | 3 yr                     | Autoimmune hemolytic anemia, SLE ANA\(^+\), anti-dsDNA\(^+\) | BCG-vaccine: no AE; died at age 13 yr from septic shock due to *P. aeruginosa*; corticosteroids, MMF, anti-CD20 (rituximab); prophylaxis: NR | |
|         | P5      | c.1528G>A/c.1528G>A (p.G510S/p.G510S) |        | M   | Alive    | 6 yr                     | SLE ANA\(^+\), anti-dsDNA\(^+\) | BCG-vaccine: no AE; pleuritis and recurrent pneumonia; corticosteroids, MMF; prophylaxis: IVIG | |
| D       | P6      | c.1352+1G>A/c.1352+1G>A (??) | Turkey | M   | Alive    | 1 yr                     | SLE ANA\(^+\), anti-dsDNA\(^+\), anti-cardiolipin\(^+\) | BCG-vaccine: no AE; recurrent pneumonia, urinary tract infections, recurrent gastroenteritis, recurrent otitis media; corticosteroids, anti-CD20 (rituximab); prophylaxis: IVIG, antibiotics | Salzer et al., 2013 |
| E       | P7      | c.1294G>T/c.1294G>T (p.G432W/p.G432W) | Pakistan, living in UK | F   | Alive    | 1 yr                     | Autoimmune hemolytic anemia, SLE ANA\(^+\), anti-dsDNA\(^+\) | BCG-vaccine: no AE; corticosteroids, anti-CD20 (rituximab), azathioprine; prophylaxis: IVIG | Lei et al., 2018; Nanthapisal et al., 2017 |
|         | P8      | c.1294G>T/c.1294G>T (p.G432W/p.G432W) |        | F   | Alive    | 1 yr                     | Autoimmune hemolytic anemia, SLE ANA\(^+\), anti-dsDNA\(^+\) | BCG-vaccine: no AE; cervical and inguinal lymphadenopathy; corticosteroids, anti-CD20 (rituximab), azathioprine; prophylaxis: IVIG | |
|         | P9      | c.1294G>T/c.1294G>T (p.G432W/p.G432W) |        | M   | Alive    | 2 yr                     | SLE ANA\(^+\), anti-dsDNA\(^+\) | BCG-vaccine: no AE; corticosteroids, anti-CD20 (ofatumumab); prophylaxis: IVIG | |
Table 1. The clinical spectrum of patients with PKCδ deficiency (Continued)

| Kindred | Patient | Mutation (predicted protein variant) | Origin | Sex | Follow-up | Age at onset of symptoms | Autoimmune manifestations | BCG status; clinical infectious phenotype (infections, microbiology, pathology results; treatment, prophylaxis) | Reference |
|---------|---------|--------------------------------------|--------|-----|-----------|--------------------------|---------------------------|--------------------------------------------------------------------------------------------------------------------------------|----------|
| F       | P10     | c.788-2A>G/c.571C>T                  | UK     | M   | Alive     | 9 mo                     | SLE ANA*                  | BCG-vaccine; no AE; Listeria meningitis, recurrent otitis media, early-onset enterocolitis, systemic Candida infection, sepsis due to E. faecium, recurrent A. xylosoxidans cervical lymphadenitis, SARS-CoV-2 infection; sirolimus, anti-CD20 (rituximab); prophylaxis: IVIG | Meyts et al., 2021 |
|         | P11     | c.788-2A>G/c.571C>T                  |        | F   | Alive     | 6 mo                     | SLE ANA*                  | BCG-vaccine; no AE; H. influenzae sepsis, recurrent gastroenteritis, early-onset enterocolitis; MMF, anti-CD20 (rituximab); prophylaxis: IVIG | Unpublished data; |
| G       | P12     | c.1293_1294insA/ c.1293_1294insA (p.G432Rfs*15/p.G432Rfs*15) | Afghanistan, living in Iran | M   | Alive     | 1 yr                     | SLE ANA*, anti-dsDNA*     | BCG-vaccine: no AE; S. pneumoniae pneumonia, H. influenzae otitis media, recurrent oral candidiasis, recurrent pneumonia, recurrent gastroenteritis; corticosteroids, NSAIDs, azathioprine; prophylaxis: cyclic rotation of sulfamethoxazole-trimethoprim/amoxicillin and azithromycin | Sharifnejad et al., 2020 |
| H       | P13     | c.571+2dup/c.571+2dup (?)            | Turkey | F   | Alive     | 1 yr                     | SLE ANA*, anti-dsDNA*     | BCG-vaccine: at 2 mo of age, BCG-osis; Salmonella bacteremia; S. aureus inguinal lymphadenitis, recurrent gingivitis; antimycobacterial treatment, ceftriaxone; corticosteroids; prophylaxis: amoxicillin, IVIG | This paper |
| I       | P14     | c.1384C>T/c.1384C>T                  | Turkey | M   | Alive     | 7 mo                     | Autoimmune hemolytic anemia, ANA* | BCG-vaccine: BCG-itis; recurrent gastroenteritis; isoniazid, rifampicin; corticosteroids; prophylaxis: none at present | This paper |
|         | P15     | c.1384C>T/c.1384C>T                  |        | M   | Alive     | 7 mo                     | Autoimmune hemolytic anemia, ANA* | BCG-vaccine: BCG-itis; recurrent gastroenteritis, shingles, isoniazid, rifampicin; corticosteroids; prophylaxis: IVIG | This paper |
| J       | P16     | c.642del/c.642del                    | Iran   | F   | Alive     | 1 yr                     | No SLE, ANA*, anti-dsDNA* | BCG vaccine: BCG-itis; isoniazid, rifampicin, azithromycin; prophylaxis: none at present | This paper |
|         | P17     | c.642del/c.642del                    |        | M   | Alive     |                           | No SLE, ANA*, anti-dsDNA* | BCG vaccine: no AE; asymptomatic; prophylaxis: none at present | |

Family code, patient number, and mutations are as in Fig. 1. Predicted protein variations are indicated in parenthesis. ? indicates unknown prediction of splice site variants. Patient origin, sex, current vital status, age at onset of symptoms, and clinical phenotypes are shown. AE, adverse effect; F, female; M, male; NR, not reported; NSAID, nonsteroidal anti-inflammatory drug.
Figure 1. AR PKCδ deficiency in 10 families. (A) Pedigree of the 10 unrelated kindreds showing familial segregation of the different PRKCD alleles. Generations are indicated by Roman numerals (I–II), and each individual is indicated by an Arabic numeral (1–4). Male and female individuals are represented by squares and circles, respectively. Double horizontal lines indicate consanguinity. Affected patients are represented by closed black symbols, the index cases are indicated by an arrow, and asymptomatic carriers are indicated by a black vertical line. Individuals of unknown genotype are indicated by “E?” Symbols crossed with a black diagonal line indicate deceased individuals. (B) Schematic representation of the PRKCD gene. Coding exons are numbered (3–19). The PKCδ protein is represented with four domains: C2-like, without Ca2+ binding motif; C1, binding to diacylglycerol and phorbol esters; C3, ATP-binding; and C4, substrate-binding domain. The positions of the variants observed in the patients are indicated by arrows. (C) Minor allele frequency (MAF) and CADD score for all PRKCD variants studied (colored symbols) or found in the homozygous state in gnomAD v2.1.1 (white circles; https://gnomad.broadinstitute.org/) or our in-house cohort (Laboratory of Human Genetics of Infectious Diseases [HGID], white triangles). The missense and nonsense mutations of the patients are represented as blue lozenges, and essential splice site mutations are indicated by closed red circles. The dotted line corresponds to the MSC of 26.5, with its 95% confidence interval.
splicing (Salzer et al., 2013; Meyts et al., 2021). We assessed the impact of all the essential splice site variants identified, by performing topoisomerase-based cloning (TOPO-TA) on full-length cDNA and sequencing 100 transcripts for patients and controls (Fig. S1 B). We detected two aberrantly spliced variants for c.1352 + 1G>A (P6), leading to the skipping of exon 14 (22%; c.1261_1352del; p.D421Vfs*5) or to a skipping of four exons (6%; c.788_825del; p.D263Gfs*14), the skipping of exon 10 (8%; c.788_888del; p.D263Efs*101), or the retention of 124 bp (16%; c.787_788ins124; p.D263Efs*9) or 140 bp (18%; c.787_788ins140; p.D263Efs*34) of intron 9, respectively. Finally, the c.571 + 2dup (16%; c.787_788ins124; p.D263Efs*9) or 140 bp (18%; c.787_788ins140; p.D263Efs*34) of intron 9, respectively. For c.1133A>T [p.K378M], which was previously described LOF mutant (c.1133A>T [p.K378M]), which served as negative control (Belot et al., 2013). All PRKCD mRNAs were produced in similar amounts, as shown by real-time quantitative PCR (RT-qPCR; Fig. 2 A). Protein expression and activity in the basal state were analyzed by Western blotting with antibodies against specific phosphorylation sites of PKCδ known to be required for protein activity (Fig. 2 B; Newton, 2001). The p.G248S, p.G432W, p.G510S, and p.R614W PKCδ variants were expressed at the same molecular weight as the WT protein, whereas transient transfection with cDNAs encoding nonsense and frameshift PRKCD variants led to the expression of truncated proteins of lower molecular weight, consistent with in silico predictions (Figs. 2 B and S1 B). No reinitiation of translation downstream from the premature stop codons was observed, in analyses with an anti-DDK antibody (not depicted). All nonsense and frameshift variants were LOF for the autophosphorylation of PKCδ at the T505 and S643 residues, whereas p.G432W, p.G510S, and p.R614W were hypomorphic (Fig. 2, B and C). The p.G248S variant displayed no impairment of autophosphorylation. Taken together, these results indicate that the nonsense and frameshift mutations strongly impair PKCδ protein expression and abolish PKCδ autophosphorylation, and that all but one of the missense mutations strongly impair autophosphorylation, suggesting that these mutations are responsible for AR PKCδ deficiency.

Altered PKCδ expression and function in the EBV-B cells of eight PKCδ-deficient patients

PKCδ is strongly expressed in human peripheral B lymphocytes and EBV-B cells (Mecklenbräuker et al., 2004; Kuehn et al., 2013; Salzer et al., 2013). We used EBV-B cells to assess the impact of the various mutations on mRNA and protein levels in an endogenous system. We evaluated PRKCD transcript levels in cells from patients and healthy controls by RT-qPCR, with two different probes spanning the junctions of exons 3 and 4 and of exons 17 and 18 (Fig. 3 A). No EBV-B cells were available for P4, P7–P9, P12, or P14–P17. Low levels of PRKCD transcripts were detected in EBV-B cells from P6, P10, P11, and P13; these transcripts carried nonsense or essential splice site mutations and were shown to encode truncated proteins (Figs. 3 A and S1 B). However, for P1–P3 and P5, PRKCD transcript levels were within the range of controls (Fig. 3 A). We further investigated PKCδ protein levels by Western blotting with whole-cell lysates. A total loss of PKCδ protein was observed in the EBV-B cells of P6, P10, P11, and P13 (Fig. 3 B). The essential splice site and nonsense mutations of these patients were predicted to result in truncated proteins, which were detected in overexpression experiments in HEK293T cells, but not in the patients’ cells. However, low (P1, P3, and P5) or high (P2) PKCδ protein levels were detected in EBV-B cells carrying nonsense mutations (Fig. 3 B). We also assessed the functional consequences of PKCδ deficiency for autophosphorylation and PMA-induced apoptosis, as PKCδ is involved in proapoptotic signaling after phorbol ester stimulation or DNA damage (Fig. 3, C and D; Basu and Pal, 2010). Consistent with our previous overexpression findings, autophosphorylation was abolished (P1, P6, P10, P11, and P13) or impaired (P3 and P5) in the patients’ EBV-B cells, except for those of P2, which displayed normal autophosphorylation (Fig. 3 C). However, the EBV-B cells of P2 displayed impaired myristoylated alanine-rich C-kinase substrate (MARCKS) phosphorylation in response to PMA stimulation (Fig. S2). The EBV-B cells of all the patients (including P2) were resistant to PMA-induced cell death but remained as sensitive as the EBV-B cells of healthy controls to alternative apoptosis inducers, such as FAS (Fig. 3 D; Kuehn et al., 2013). Overall, these findings suggest that PRKCD mRNAs carrying nonsense or frameshift mutations undergo nonsense-mediated mRNA decay, resulting in a loss of...
expression of the PKCδ protein, whereas at least two of the missense mutations are associated with the production of smaller amounts of PKCδ protein. Strikingly, the EBV-B cells of all patients tested presented a defect of PMA-induced apoptosis, indicating a loss of PKCδ function.

**Impaired NADPH oxidase activity and complementation in the patients’ EBV-B cells**

Members of the PKC family are known to be involved in NADPH oxidase activity in murine phagocytes, and PKCδ, in particular, has been shown to regulate the activation of NADPH oxidase by phosphorylating p40phox (Li et al., 2016; Someya et al., 1999). However, the role of PKCδ in human phagocytes and EBV-B cells has been less studied (Li et al., 2016; Szilagyi et al., 2015). We therefore evaluated NADPH oxidase activity in EBV-B cells from patients with PKCδ deficiency, comparing the results with those for healthy controls, and cells from patients with CGD (X-linked recessive complete gp91phox deficiency and AR p40phox deficiency). The PMA-induced production of intracellular superoxide (O2·−), measured in a luminol chemiluminescence assay, was

\[ \text{Figure 2. In vitro characterization of the various PRKCD alleles in an overexpression system.} \]

(A) RT-qPCR of cDNA from HEK293T cells non-transfected (NT) or transfected with an empty vector (EV), WT PRKCD, or mutated PRKCD. GUSB was used for normalization (n = 2; mean ± SD).

(B) Western blot of total protein extracts from HEK293T cells either NT or transfected with EV, WT PRKCD, or mutated PRKCD, all inserted into pCMV6 with a C-terminal DDK tag. PKCδ was detected with a polyclonal anti-PKCδ antibody and an antibody directed against the C-terminal DDK tag. PKCδ phosphorylation was detected with polyclonal antibodies against the T505 (upper panel) and S643 (lower panel) phosphorylation sites. An antibody against GAPDH was used as a loading control. The results shown are representative of three independent experiments. Transcripts identified by TOPO-TA cloning are indicated in blue. (C) Quantification of phosphorylated PKCδ expression compared with the amount of total PKCδ. All values were normalized to the WT transfected cells (n = 3; mean ± SD).

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strongly impaired in the EBV-B cells of all PKCδ-deficient patients tested relative to healthy controls, whereas the EBV-B cells of p40phox- and gp91phox-deficient patients had impaired and abolished O$_2^-$ production, respectively (Fig. 4 A, left). Similarly, a strong decrease in O$_2^-$ production by EBV-B cells was observed for all PKCδ-deficient patients tested following stimulation with heat-killed S. aureus cells (Pansorbin), for receptor-mediated NADPH oxidase activation (Fig. 4 A, right). In addition, analyses of Amplex Red oxidation after PMA stimulation showed that much less hydrogen peroxide (H$_2$O$_2$) was released by the EBV-B cells of all PKCδ-deficient patients tested than by the EBV-B cells of healthy controls, with the amount of H$_2$O$_2$ released by the patients’ cells being similar to that released by EBV-B cells from patients with p40phox deficiency (Fig. 4 B). H$_2$O$_2$ production in response to PMA was restored in the EBV-B cells of all patients by retroviral transduction with the WT PRKCD cDNA, as shown by Amplex Red oxidation (Figs. 4 C and S3). We also evaluated the impact of all the PRKCD alleles identified on ROS production by EBV-B cells, by transducing EBV-B cells from P13 (PRKCD−/−) with WT and all mutant-PRKCD cDNAs. In PRKCD−/− EBV-B cells, H$_2$O$_2$ production was restored only by the overexpression of the PRKCD WT allele. H$_2$O$_2$ production was not increased by transduction with any of the mutant alleles (Fig. 4 D). These findings demonstrate that PKCδ plays a nonredundant role in the activation of the NADPH oxidase in EBV-B cells.

Interaction of PKCδ with p40phox/p47phox and impaired p40phox phosphorylation in the patients’ EBV-B cells

We investigated the mechanism underlying the impaired NADPH oxidase activity, by first evaluating the expression of all known NADPH oxidase subunits in the patients’ EBV-B cells. Western blotting showed that all subunits of the NADPH oxidase (gp91phox, p67phox, p47phox, p40phox, p22phox, and EROS) were produced in normal amounts in EBV-B cells from all the PKCδ-deficient patients studied (Fig. 5 A). Flow cytometry confirmed the normal levels of gp91phox, p67phox, p47phox, p22phox, and cytochrome b$_{558}$, the membrane complex formed by gp91phox and p22phox (Fig. S4 A). We then investigated possible interactions between PKCδ and all known subunits of the NADPH oxidase, by performing coimmunoprecipitation experiments in an overexpression system based on HEK293T cells. Immunoprecipitation and immunoblotting confirmed an interaction between p40phox and PKCδ and between p47phox and PKCδ (Fig. 5 B and Fig. S4, B and C). However, PKCδ did not interact with the other cytosolic (p67phox and RAC2) or membrane-bound (gp91phox, p22phox, and EROS) subunits (Fig. 5, C–E; and Fig. S4, D and E). Overexpression of PKCδ with p40phox or p47phox in HEK293T cells showed that PKCδ was able to phosphorylate both proteins when coexpressed (Fig. 5, F and G). Interestingly, the level of p40phox phosphorylation at the T154 residue was much lower in the EBV-B cells of the PKCδ-deficient patients than in those of the controls after PMA and S. aureus stimulation, whereas the phosphorylation of p47phox at two known PKCδ target residues (S304 and S315) remained similar to that in control EBV-B cells (Fig. 6, A–D; Fontayne et al., 2002). Phosphorylation at other known PKCδ-target sites of p47phox (S230, S238, and S379) could not be evaluated due to a lack of specific antibodies. The transduction of EBV-B cells with PRKCD WT cDNA restored p40phox phosphorylation, for all the patients studied (Fig. 6 E). In conclusion, the EBV-B cells of patients with PKCδ deficiency have impaired ROS production, associated with lower levels of phosphorylation of the cytosolic NADPH oxidase subunit p40phox by PKCδ.

Impact of PKCδ deficiency on NADPH oxidase activity in the patients’ phagocytic cells

Because the EBV-B cells of PKCδ-deficient patients had very low levels of ROS production, we investigated NADPH oxidase activity in response to various stimuli in the primary cells of the patients. In particular, we assessed dihydrorhodamine 123 (DHR) oxidation, which detects intracellular H$_2$O$_2$ production, in neutrophils and monocytes following stimulation with PMA or serum-opsonized Escherichia coli. The patients’ neutrophils and monocytes displayed impaired but not abolished DHR oxidation in response to both stimuli, like the cells of patients with inherited p40phox deficiency (Fig. 7, A and B; and Fig. S5, A and B). In addition, neutrophils and monocytes of P3 and P13 showed low or abolished PKCδ expression, respectively (Fig. S5 C). We also evaluated the formation of NETs by neutrophils from three patients (P3, P16, and P17) from two unrelated kindreds following stimulation with PMA. NET formation was strongly impaired in the patients’ cells, whereas it was abolished in cells from a gp91phox-deficient patient (Figs. 7 C and S5 D). We also explored NADPH oxidase activity in monocyte-derived macrophages (MDMs). MDMs from all the patients tested displayed impaired H$_2$O$_2$ release following stimulation with IFN-γ, IFN-γ plus PMA, or PMA alone, relative to healthy controls or patients with p40phox deficiency (Figs. 7 D and S5 E). Similarly, the NADPH oxidase activity of monocyte-derived dendritic cells (MDDCs), evaluated by assessing H$_2$O$_2$ release after stimulation with LPS, LPS plus PMA, or PMA, was weaker than that of cells from healthy controls and p40phox-deficient patients (Fig. 7 E). As observed for EBV-B cells, the patients’ MDMs displayed reduced or abolished PKCδ expression as well as impaired p40phox phosphorylation at the T154 residue, but normal p47phox phosphorylation at two other residues (S304 and S315) tested (Figs. 7 F and S5 F). The primary phagocytes of heterozygous relatives of PKCδ-deficient patients displayed no defects of ROS production (not depicted). Overall, these data suggest that PKCδ is essential for NADPH oxidase activation and NET formation in primary phagocytic cells, possibly through the phosphorylation of p40phox.

Discussion

We report an impairment of NADPH oxidase activity in 17 patients from 10 unrelated families with AR PKCδ deficiency. ROS production was severely impaired in the EBV-B cells, primary phagocytes (neutrophils and monocytes), MDMs, and MDDCs of all patients studied (Table 2). The transduction of the patients’ EBV-B cells with WT PRKCD cDNA restored not only ROS production, but also the phosphorylation of p40phox on the T154 residue after PMA stimulation, strongly suggesting a role for PKCδ in activation of the NADPH oxidase complex. The
Figure 3. **PKCδ deficiency in patients’ EBV-B cells.** (A) RT-qPCR for PRKCD with a probe spanning the junction between exons 3 and 4 (left) and a probe spanning the junction between exons 17 and 18 (right), in EBV-B cells from healthy controls (Ctrl; n = 10) and patients. GUSB was used for normalization (n = 3; mean ± SD). (B) Western blot of total protein extracts from EBV-B cells from healthy controls (C1, C2) and patients (left). PKCδ was detected with a polyclonal anti-PKCδ antibody. The asterisk indicates nonspecific bands. Quantification of the PKCδ protein normalized against GAPDH (right; n = 3; mean ± SD). The results shown are representative of three independent experiments. (C) Western blot of total protein extract from the EBV-B cells of controls or patients. PKCδ was detected with a polyclonal anti-PKCδ antibody. The autophosphorylation of PKCδ was detected with antibodies against the T505 (upper panel) and S643 (lower panel) phosphorylation sites. The results shown are representative of three independent experiments. (D) Apoptosis of EBV-B cells from healthy controls (Ctrl; n = 10) and patients (n = 8) after 24 h of stimulation with APO-1-1 (APO; 1 µg/ml) or PMA (100 ng/ml). The percentages of live cells were normalized against the number of nonstimulated (NS) cells, after acquisition by flow cytometry over a constant time (n = 3; mean ± SD). A linear mixed model was used to determine whether survival rates were the same for PKCδ-deficient patients and controls after APO or PMA stimulation (n.s., not significant; ***, P < 0.001).
Phosphorylation of the T154 residue of p40phox is a key regulatory step in neutrophils and B lymphocytes, leading to assembly of the soluble cytosolic NADPH oxidase subunits with the membrane cytochrome b558 (Someya et al., 1999; Chessa et al., 2010; Grandvaux et al., 2001; van de Geer et al., 2018; Matute et al., 2005). Indeed, the MDMs of the PKCδ-deficient patients displayed impaired p40phox phosphorylation and very low levels of ROS production. However, the MDMs of p40phox-deficient patients displayed normal ROS production, indicating that these cells are not dependent on p40phox, and therefore on p40phox phosphorylation, for PMA-induced ROS production.

These findings suggest the requirement of another PKCδ substrate for normal ROS production.

Figure 4. NADPH oxidase activity and retroviral transduction of the patients’ EBV-B cells. (A) Production of O$_2^-$ by EBV-B cells from healthy controls (Ctrl; n = 3), PKCδ-deficient patients (n = 8), p40phox-deficient patients (n = 2), and gp91phox-deficient patients (n = 1) after stimulation with PMA stimulation (left; 400 ng/ml) or Pansorbin (right; 2 mg/ml), as assessed by luminol bioluminescence. LU, luminescence units. The results shown are representative of two or three independent experiments. (B) Extracellular H$_2$O$_2$ production by EBV-B cells from healthy controls (n = 3), PKCδ-deficient patients (n = 8), p40phox-deficient patients (n = 2), and gp91phox-deficient patients (n = 1) after PMA stimulation (400 ng/ml), as assessed with the Amplex Red test. The results shown are representative of three independent experiments. (C) Extracellular H$_2$O$_2$ production by EBV-B cells from a healthy control (C1), a gp91phox-deficient patient, and PKCδ-deficient patients (n = 8) transduced with an empty vector (EV) or PRKCD WT cDNA, after PMA stimulation (400 ng/ml), as assessed with the Amplex Red test (mean ± SD). The results shown are representative of two independent experiments. (D) Production of H$_2$O$_2$ by healthy control (C1), and PRKCD−/− EBV-B cells transduced with EV, WT, or the various mutant PRKCD cDNAs, upon PMA stimulation (400 ng/ml), as assessed with the Amplex Red test. The results shown are representative of two independent experiments.
Figure 5. **NADPH oxidase subunit expression and interaction with PKCδ.** (A) Western blot of total protein extracts from the EBV-B cells of healthy controls (C1, C2), PKCδ-deficient patients and CGD patients. Antibodies against gp91phox, p67phox, p47phox, p40phox, p22phox, and EROS were used. An antibody against GAPDH was used as a loading control. (B) Coimmunoprecipitation (IP) of protein lysates from HEK293T cells transfected with empty vector (EV) or plasmids encoding PKCδ, p40phox, or p47phox. Pulldowns with anti-DDK (PKCδ) and immunoblots (IB) with anti-DDK or specific antibodies (p40phox, upper panel; p47phox, lower panel) are shown. (C) IP on protein lysates from HEK293T cells transfected with EV, CYBB, or PRKCD cDNAs. The pulldown of PKCδ (left) and gp91phox (right) was performed with an anti-DDK antibody. (D) IP on protein lysates from HEK293T cells transfected with the EV, CYBA, or PRKCD cDNAs. The pulldown of PKCδ (left) and p22phox (right) was performed with an anti-DDK antibody. (E) IP on protein lysates from HEK293T cells transfected with EV, CYBC1, or PRKCD cDNAs. The pulldown of PKCδ (left) and EROS (right) was performed with an anti-DDK antibody. (F and G) Detection of p40phox (F) and p47phox (G) phosphorylation by Western blot on cell lysates of HEK293T cells transfected with EV, NCF4, NCF1, and PRKCD cDNAs. Representative images of three independent experiments.
Figure 6. \( p^{40\text{phox}} \) and \( p^{47\text{phox}} \) phosphorylation in PKC\( \delta \)-deficient EBV-B cells. (A) Phosphorylation of \( p^{40\text{phox}} \) in total protein extracts from EBV-B cells of healthy controls (C1, C2), PKC\( \delta \)-deficient patients, and a \( p^{40\text{phox}} \)-deficient patient, before (–) and after (+) 30 min of PMA stimulation (400 ng/ml), measured by Western blot. (B) Phosphorylation of \( p^{40\text{phox}} \) in total protein extracts from EBV-B cells of healthy controls, PKC\( \delta \)-deficient patients, and a \( p^{40\text{phox}} \)-deficient patient, before (–) and after (+) 30 min of Pansorbin stimulation (2 mg/ml), measured by Western blot. (C) Phosphorylation of \( p^{47\text{phox}} \) in EBV-B cells of healthy controls, PKC\( \delta \)-deficient patients, and a \( p^{47\text{phox}} \)-deficient patient before (–) and after (+) 30 min of PMA stimulation (400 ng/ml), measured by Western blot. (D) Phosphorylation of \( p^{47\text{phox}} \) in total protein extracts from the EBV-B cells of healthy controls, PKC\( \delta \)-deficient patients, and a \( p^{47\text{phox}} \)-deficient patient, before (–) and after (+) 30 min of PMA stimulation (400 ng/ml), measured by Western blot. (E) Phosphorylation of \( p^{40\text{phox}} \) in total protein extracts from EBV-B cells of a healthy control, and PKC\( \delta \)-deficient patients either nontransduced (NT) or transduced with an EV or PRKCD WT cDNA, before (–) and after (+) 30 min of PMA stimulation (400 ng/ml). Total protein extracts from EBV-B cells of a \( p^{40\text{phox}} \)-deficient patient were used as controls. All results shown are representative of two to three independent experiments.
neutrophils, this suggests that PKCδ is redundant for p47phox phosphorylation at these residues (Bey et al., 2004; Fontayne et al., 2002; Segal et al., 2000). However, p47phox phosphorylation at S379 appears to be the most relevant for its activation and was shown to be a PKCδ target site (Fontayne et al., 2002; Meijles et al., 2014). Unfortunately, no antibodies are yet available for the evaluation of p47phox phosphorylation at S379, and further studies are required to evaluate the exact contribution of PKCδ to p47phox phosphorylation at various sites.

In all subsets of primary (neutrophils and monocytes) or monocye-derived (MDMs and MDDCs) phagocytic cells tested after stimulation with PMA or E. coli, we observed no difference in ROS production or p40phox phosphorylation between patients with and without corticosteroid treatment. In addition to the low level of ROS production in neutrophils and monocytes after stimulation with PMA or E. coli, we observed a strong impairment of NET formation in PKCδ-deficient neutrophils, potentially contributing further to the impairment of bactericidal and fungicidal activity. In addition, a previous study reported a neutrophil killing defect in response to E. coli and C. albicans in PKCδ-deficient neutrophils from three of our patients (P3, P5, and P6; Szlągjy et al., 2018). These data are consistent with those obtained for PKCδ-deficient mice, which display poor ROS production by bone marrow neutrophils, impaired NET formation, and impaired killing of C. albicans (Soroush et al., 2019; Li et al., 2016). Further studies are required to determine the contributions of PKCδ to the assembly and activity of the NADPH oxidase and pathogen killing. In this respect, patient-derived induced pluripotent stem cells could be used to generate various subsets of myeloid cells. A humanized mouse model could also be used to dissect the development of different subsets of phagocytic cells and their contribution to ROS production (Neehus et al., 2018; Sontag et al., 2017; Lachmann et al., 2015; Evren et al., 2020). Overall, our data suggest that PKCδ is important for host innate immunity through its role in NADPH oxidase activation.

Our studies of NADPH oxidase activity in PKCδ-deficient cells revealed a cellular phenotype related to, but different from, that seen in patients with classic CGD, more closely resembling that seen in patients with p40phox deficiency (van de Geer et al., 2018; Wright et al., 2019; Matute et al., 2009). The defect of ROS production in PKCδ-deficient patients probably at least partly accounts for the recurrent and/or severe infections observed in these patients. The patients studied had recurrent bacterial infections, mostly affecting the lungs, lymph nodes, and gastrointestinal tract (Kuehn et al., 2013; Belot et al., 2013; Kiykim et al., 2015; Salzer et al., 2013; Lei et al., 2018; Nanthapisal et al., 2017; Meyts et al., 2021; Sharifinejad et al., 2020; unpublished data). Two patients had invasive infections, one (P16, who survived infection) with Candida and another (P4, who died) with P. aeruginosa. Recurrent pneumonia and infections with S. aureus are also frequently seen in patients with CGD (Anjani et al., 2019; Winkelstein et al., 2000; de Oliveira-Junior et al., 2015; Song et al., 2011). Nevertheless, patients with classic CGD typically suffer from invasive, life-threatening, recurrent infections with specific bacteria and fungi, such as Burkholderia cepacia, Staphylococcus spp., and Aspergillus spp., due to defective ROS production in all phagocytic cells (de Oliveira-Junior et al., 2015; Marciano et al., 2015; Winkelstein et al., 2000).

The infectious phenotype observed in our cohort of PKCδ-deficient patients was milder than that observed in classic CGD. Like patients with CGD or X-linked recessive Mendelian susceptibility to mycobacterial disease (MSMD) due to hypomorphic mutations of CYBB, PKCδ-deficient patients can suffer from BCG disease, with incomplete penetrance (Conti et al., 2016; Norouzi et al., 2012; Bustamante et al., 2011). However, whereas patients with classic CGD or p40phox deficiency do not seem to have any particular predisposition to severe viral illnesses, five PKCδ-deficient patients (P1, P2, P3, P10, and P15) presented with EBV, CMV, papillomavirus, SARS-CoV-2, and varicella-zoster virus infections, respectively (Meyts et al., 2021; Kuehn et al., 2013; Omarjee et al., 2019). Furthermore, none of the reported p40phox-deficient patients suffered from any severe invasive bacterial or fungal infections, whereas PKCδ-deficient patients did not present with any of the cutaneous infections typically seen in patients with p40phox deficiency (Li et al., 2016; van de Geer et al., 2018; Wright et al., 2019; Matute et al., 2009). Interestingly, p40phox-deficient patients, who have low to normal levels of ROS production in neutrophils and monocytes following phorbol ester stimulation, have no detectable impairment of ROS production by MDMs and MDDCs (van de Geer et al., 2018; Wright et al., 2019; Matute et al., 2009). Unlike patients with classic CGD, PKCδ-deficient patients displayed residual ROS production by circulating and monocyte-derived phagocytes, possibly accounting for the milder clinical phenotype of these patients than of patients with CGD (van de Geer et al., 2018; Matute et al., 2009; Song et al., 2011). The differences in clinical presentation between patients with PKCδ deficiency, p40phox deficiency, and classic CGD can be also explained by the mechanisms proposed here, according to which the PKCδ-dependent phosphorylation not only of p40phox, but also of p47phox, is required for ROS production. Finally, the greater susceptibility to viral illnesses observed in PKCδ-deficient patients may result from low levels of NK cell cytolytic activity, as previously shown (Kuehn et al., 2013; Kiykim et al., 2015).

One of the limitations of our study is the small size of the cohort of PKCδ-deficient patients followed over a short period. Studies of a larger number of patients are required to improve comparisons of the immunological and clinical features of PKCδ deficiency, p40phox deficiency, and classic and variant CGD (Royer-Pokora et al., 1986; Dinauer et al., 1987). Nevertheless, our data indicate that PRKCD mutations underlie a new, “syndromic form” of CGD, characterized by susceptibility to some, but not all, of the infections typical of CGD, associated with early-onset SLE. Indeed, the most striking phenotype observed in PKCδ-deficient patients is the early onset of SLE, which was diagnosed in 13 patients of this cohort (P1–P13), with two others presenting signs of autoimmune (P14 and P15) and two displaying no autoimmune phenotype at the ages of 3 (P16) and 5 (P17) yr (Kuehn et al., 2013; Belot et al., 2013; Kiykim et al., 2015; Salzer et al., 2013; Lei et al., 2018; Nanthapisal et al., 2017; Meyts et al., 2021; Sharifinejad et al., 2020; unpublished data). Several monogenic disorders underlying SLE have been reported.

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Figure 7. NADPH oxidase activity in primary phagocytic cells from PKCδ-deficient patients. (A) Neutrophil intracellular ROS production, as measured by DHR, upon PMA stimulation, for travel controls (ctl; n = 11), PKCδ-deficient patients (n = 11), p40phox-deficient patients (n = 5), and gp91phox-deficient patients (n = 2), or upon E. coli stimulation, for travel controls (n = 5), PKCδ-deficient patients (n = 5), p40phox-deficient patients (n = 1), and gp91phox-deficient patients (n = 2). (B) Monocyte intracellular ROS production, measured by DHR, upon PMA stimulation, for travel controls (n = 11), PKCδ-deficient patients (n = 11), p40phox-deficient patients (n = 5), and gp91phox-deficient patients (n = 2), or upon E. coli stimulation, for travel controls (n = 5), PKCδ-deficient patients (n = 5), p40phox-deficient patients (n = 1), and gp91phox-deficient patients (n = 1). All values are expressed as a percentage of DHR oxidation normalized against travel controls.

(C) Left: Quantification of NET formation for healthy controls (n = 6), PKCδ-deficient patients (n = 3), and a gp91phox-deficient patient. All values are expressed as a percentage of NET-forming cells. Right: Representative images of PMA-induced NET formation by neutrophils of local and travel controls and a PKCδ-deficient patient (P17). Green represents myeloperoxidase, and blue, DNA (DAPI). Scale bar = 60 µm.

(D) Extracellular H2O2 production in response to stimulation with IFN-γ, PMA, or both (mean ± SD) for MDMs from local controls (n = 10), travel controls (n = 10), PKCδ-deficient patients (n = 12), p40phox-deficient patients (n = 3), and gp91phox-deficient patients (n = 2).

(E) Extracellular H2O2 production in response to stimulation with LPS, PMA, or both (mean ± SD) in MDDCs from local controls (n = 10), travel controls (n = 10), PKCδ-deficient patients (n = 8), p40phox-deficient patients (n = 2), and gp91phox-deficient patients (n = 2).
affecting the complement system, type I IFN immunity, nucleic acid metabolism, or B cell development checkpoints (Costa-Reis and Sullivan, 2017; Rahman and Isenberg, 2008; Omarjee et al., 2019). B cell development is tightly regulated to prevent self-reactivity, as B cell receptor diversification inevitably results in some B cell receptors recognizing self-antigens (Meffre and Wardemann, 2008). Autoreactive B cells are eliminated through multiple steps during B cell development. PKCδ plays a key role in regulating B cell survival, differentiation, and apoptosis (Guo et al., 2004; Griner and Kazanietz, 2007). As previously shown in mice, human PKCδ deficiency may underlie autoimmunity due to defective proapoptotic signaling during B cell development (Mecklenbräuker et al., 2002, 2004; Limnander et al., 2014).

Intriguingly, some SLE manifestations are also observed in some patients with p40δ deficiency or classic CGD (van de Geer et al., 2018; Xie et al., 2016; Farvaneh et al., 2014). Despite the overlap between the cellular phenotypes of these conditions and that of PKCδ deficiency in terms of ROS production, the SLE-like manifestations in these other conditions may be unrelated to the role of PKCδ in B cell checkpoints. SLE is often associated with an increase in type I IFN activity and a dysregulation of type I IFN metabolism (Crow, 2011; Picard and Belot, 2017; Rönnblom et al., 1990). In the absence of NADPH oxidase activity, the mitochondrial ROS-dependent NET-osis of low-density granulocytes leads to the externalization of proinflammatory oxidized mitochondrial DNA and the subsequent activation of type I IFN synthesis (Lood et al., 2016). Enhanced type I IFN activity may, therefore, contribute to the SLE-like phenotype observed in patients with impaired NADPH oxidase activity. Overall, the autoimmunity observed in PKCδ-deficient patients is probably mostly due to the defective selection of autoreactive B cells, although defective ROS production may also be involved.

Materials and methods
Clinical information for the newly identified patients
P13 (kindred H, II.1) was born in 2007 to nonconsanguineous parents of Turkish origin. She was vaccinated with BCG, on the left shoulder, at the age of 2 mo and developed BCG-itis 3 mo later. An excisional biopsy of the left axillary lymph node was performed, and M. tuberculosis PCR and culture were negative. At the age of 11 mo, P13 presented with fever and left axillary, retroauricular, and cervical lymphadenopathies. Salmonella sp. was documented in blood cultures and treated with ceftriaxone for 21 d. The axillary lymph node was removed; histological examination revealed necrotizing granulomatous inflammation, and Mycobacterium bovis–BCG was identified by culture. Serological tests for HIV were negative. The patient was treated with triple antimycobacterial therapy and was diagnosed with MSMD. Whole-blood activation tests showed that the production of IFN-γ and IL-12p40 in response to stimulation with BCG + IL-12 and BCG + IFN-γ was normal. At 18 mo of age, P13 presented with a skin rash, joint pain, and high ANA levels. Drug-induced lupus erythematosus was suspected on clinical grounds, and isoniazid was discontinued. However, physical examination showed a worsening of her condition, with the appearance of lupus-like symptoms (arthralgia, morning stiffness, and limitation of movement). At the age of 24 mo, antimycobacterial treatment was discontinued. Leukocyte, absolute neutrophil, and absolute lymphocyte counts were normal. Serum IgG levels were low, whereas serum IgA, IgM, and IgE levels were within normal ranges. The immunophenotyping of B cell subsets showed predominantly high naïve B cell and low memory B cell counts, with markedly high levels of CD21low and transitional B cells. In summary, this patient received multiagent antituberculosis treatment for 33 mo, without relapse or the use of other antimycobacterial treatments. The patient has had recurrent gingivitis since the age of 6 yr and, at the age of 7 yr, presented with inguinal lymphadenitis, leading to the detection of S. aureus by culture. The patient is now 14 yr old and remains free from mycobacterial and other infectious diseases. However, SLE and the high levels of ANA persist.

P14 and P15 (kindred I, II.1 and II.2) were born in 2010 and 2013, respectively, to nonconsanguineous parents of Turkish origin. Both were vaccinated with BCG at 1 mo of age and developed BCG-itis 6 mo later. P14 presented with fever, diarrhea, hepatosplenomegaly, failure to thrive, and left axillary lymphadenopathy with purulent discharge. Lymph node biopsy showed granulomatous inflammation with no microbial cause identified upon culture. Similarly, P15 developed left axillary lymphadenopathy and hepatosplenoomegaly after BCG vaccination. Both patients were treated with isoniazid and rifampicin for 4 (P14) and 5 (P15) yr; P15 also received IVIG treatment. Prophylaxis was not indicated in P14. Both patients had high levels of ANA and developed autoimmune anemia, with no other lupus-like symptoms. Both patients are currently well.

P16 and P17 (kindred J, II.2 and II.1) were born in 2017 and 2014, respectively, to consanguineous parents of Iranian origin. Both were vaccinated with BCG at birth, but only P16 developed BCG-itis; her brother (P17) remained asymptomatic. P16 presented at 14 mo of age with bilateral anterior and posterior cervical adenopathies and mild hepatosplenoomegaly. Lymph node biopsy showed chronic granulomatous inflammation with caseating necrosis. No microbe was identified, but a purified protein derivative skin test was positive. The patient received triple antimycobacterial therapy (isoniazid, rifampicin, and azithromycin) for 6 mo until the adenopathies disappeared. Her serum IgG, IgA, and IgM levels were within the normal range, but her antibody responses to tetanus and diphtheria vaccines were nonprotective (anti-tetanus antibodies, 0.04 mg/dl; anti-diphtheria antibodies, 0.04 mg/dl [normal >0.1 mg/dl]). P16 and P17 both tested negative for autoantibodies and have not yet developed any lupus-like symptoms. Both patients are well, without prophylaxis.
**Table 2. Summary of the functional studies in the patients’ cells**

| Patient (kindred) | Mutation | PKCδ expression | p40<sup>Phox</sup> phosphorylation | ROS production in circulating phagocytes | ROS production in monocyte-derived cells |
|-------------------|----------|-----------------|------------------------------------|----------------------------------------|---------------------------------------|
| P1 (A)            | p.R614W/p.R614W | Impaired (EBV-B cells) | Impaired after PMA and Pansorbin (EBV-B cells) | NT | Impaired after PMA, PMA + IFN-γ and PMA + LPS (MDMs and MDDCs) |
| P2 (B)            | p.G248S/p.G248S | Normal (EBV-B cells and MDMs) | Impaired after PMA and Pansorbin (EBV-B cells and MDMs) | Impaired after PMA (neutrophils and monocytes) | Impaired after PMA, PMA + IFN-γ and PMA + LPS (MDMs and MDDCs) |
| P3 (C)            | p.G510S/p.G510S | Impaired (EBV-B cells, neutrophils, and MDMs) | Impaired after PMA and Pansorbin (EBV-B cells and MDMs) | Impaired after PMA and E. coli (neutrophils and monocytes) | Impaired after PMA, PMA + IFN-γ and PMA + LPS (MDMs and MDDCs) |
| P4 (C)            | p.G510S/p.G510S | NT | NT | NT | NT |
| P5 (C)            | p.G510S/p.G510S | Impaired (EBV-B cells) | Impaired after PMA and Pansorbin (EBV-B cells) | Impaired after PMA (neutrophils and monocytes) | Impaired after PMA, PMA + IFN-γ and PMA + LPS (MDMs and MDDCs) |
| P6 (D)            | c.1352 + 1G>A/c.1352 + 1G>A | Abolished (EBV-B cells) | Impaired (EBV-B cells) | Impaired after PMA (neutrophils and monocytes) | Impaired after PMA, PMA + IFN-γ and PMA + LPS (MDMs and MDDCs) |
| P7 (E)            | p.G432W | NT | NT | NT | NT |
| P8 (E)            | p.G432W | NT | NT | NT | NT |
| P9 (E)            | p.G432W | NT | NT | NT | NT |
| P10 (F)           | c.788-2A>G/p.Q191* | Abolished (EBV-B cells) | Impaired after PMA and Pansorbin (EBV-B cells) | Impaired after PMA (neutrophils and monocytes) | Impaired after PMA, PMA + IFN-γ and PMA + LPS (MDMs and MDDCs) |
| P11 (F)           | c.788-2A>G/p.Q191* | Abolished (EBV-B cells) | Impaired after PMA and Pansorbin (EBV-B cells) | Impaired after PMA (neutrophils and monocytes) | Impaired after PMA, PMA + IFN-γ and PMA + LPS (MDMs and MDDCs) |
| P12 (G)           | c.1293_1294insA/c.1293_1294insA | NT | NT | NT | NT |
| P13 (H)           | c.571 + 2dup/c.571 + 2dup | Abolished (EBV-B cells, neutrophils, monocytes, and MDMs) | Impaired after PMA and Pansorbin (EBV-B cells) | Impaired after PMA (neutrophils and monocytes) | Impaired after PMA, PMA + IFN-γ and PMA + LPS (MDMs and MDDCs) |
| P14 (I)           | c.1384C>T/c.1384C>T | Abolished (MDMs) | Impaired (MDMs) | Impaired after PMA and E. coli (neutrophils and monocytes) | Impaired after PMA and PMA + IFN-γ (MDMs) |
| P15 (I)           | c.1384C>T/c.1384C>T | NT | NT | Impaired after PMA and E. coli (neutrophils and monocytes) | Impaired after PMA and PMA + IFN-γ (MDMs) |
| P16 (J)           | c.642del/c.642del | Abolished (MDMs) | Impaired (MDMs) | Impaired after PMA and E. coli (neutrophils and monocytes) | Impaired after PMA and PMA + IFN-γ (MDMs) |
| P17 (J)           | c.642del/c.642del | Abolished (MDMs) | Impaired (MDMs) | Impaired after PMA and E. coli (neutrophils and monocytes) | Impaired after PMA and PMA + IFN-γ (MDMs) |

Functional results obtained in cell lines (EBV-B cells) or primary cells (neutrophils, monocytes, MDMs, and MDDCs). EBV-B cells, EBV-immortalized B cells; NT, not tested.

**Blood collection**

Blood samples were collected from all patients and relatives, at the local institutions, after written informed consent had been obtained. The study was approved by the institutional review boards of the Institut National de la Santé et de la Recherche Médicale (INSERM), Rockefeller University, and Necker Hospital for Sick Children. All these procedures were conducted in accordance with the 1975 Declaration of Helsinki, as revised in 2013.

**Molecular genetics**

For P13, genomic DNA (gDNA) was isolated from whole blood with the iPrep PureLink gDNA Blood Kit and iPrep Instruments (Life Technologies, Thermo Fisher Scientific). Exome capture was performed with the SureSelect Human All Exon 71 Mb kit (Agilent Technologies), with 3 µg gDNA. Single-end sequencing was performed on an Illumina Genome Analyzer IIX (Illumina). For P14 and P15, gDNA was obtained from the patients’ peripheral blood.
samples with the QIAamp DNA Blood Mini QIAcube Kit (Qiagen). The library was constructed with a 200-amplitude primary immunodeficiency panel kit from Sistemas Genomicos. Samples and index reading primers were loaded into a 300-cycle MiSeq Reagent Kit v2 300 cartridge (Illumina), and parallel sequencing was performed in MiSeq v2 Flowcell (Illumina). The variants of all the other patients were identified by WES (P1, P2, P3, P6, P7, P12, P16, and P17), Sanger sequencing (P4, P5, P8, and P9), or focused WES with 1 µg of the plasmid in the presence of X-tremeGENE 9 DNA transfection reagent (Merck) for 24 h. Protein extraction and Western blotting were performed as described above.

Expression of mutated PKCδ proteins in transfected HEK293T cells

Site-directed mutagenesis with specific primers (Table S2) was performed to generate the PRKCD variants in the pCMV6-PRKCD-WT-DDK plasmid (aRC221652; OriGene). HEK293T cells were transfected with 1 µg of the plasmid in the presence of X-tremeGENE 9 DNA transfection reagent (Merck) for 24 h. Protein extraction and Western blotting were performed as described above.

TOPO-TA cloning

RNA was extracted from the EBV-B cells of P2, P6, P10, P11, P13, and a healthy control with the Quick-RNA Microprep Kit (Zymo Research), and RNA was transcribed to generate cDNA with the High-Capacity RNA-to-cDNA Kit (Thermo Fisher Scientific). Full-length PRKCD cDNA was amplified by PCR with the following primers: 5′-CTATGAGCTGGGCTCCCTG-3′ and 5′-CCAGGAGGTTGCTGAAATTTGG-3′. The purified PCR products were inserted into a pCR4-TOPO vector (Thermo Fisher Scientific) and used to transform competent NEB 10-β E. coli cells (New England Biolabs). We picked 100 single colonies per reaction, and the inserted cDNA was amplified with M13 forward and reverse primers.

RT-qPCR

Gene expression was analyzed by RT-qPCR with the TaqMan Universal PCR Master Mix (Thermo Fisher Scientific) and TaqMan probes for PRKCD (Hs01090047_m1 and Hs01090051_m1) and GUSB (4326320E; all from Thermo Fisher Scientific). Gene expression was normalized against GUSB as an endogenous control for each sample and then according to the mean value for controls in one experiment.

Western blot analysis

EBV-B cells were treated with 400 ng/ml PMA (Sigma-Aldrich) or 2 mg/ml Pansorbin (Sigma-Aldrich) in HBSS for 30 min or were left untreated, and total protein extracts were prepared with insoluble protein buffer (20 mM Tris-HCl, pH 8, 150 mM NaCl, 1 mM EDTA, and 1% Triton X-100, supplemented with 1 mM 4-[2-aminoethyl] benzene sulfonyl fluoride hydrochloride, 20 ng/ml chymostatin, 10 nM leupeptin, and 2 mM PMSF; all from Sigma-Aldrich). Protein extracts (30 µg protein per lane) were resolved by electrophoresis in a 10% Criterion TGX precast gel, and the bands obtained were transferred onto a nitrocellulose membrane by standard transfer methods, in a Transblot turbo system (Bio-Rad). Membranes were probed by overnight incubation with the appropriate primary antibodies (Table S3) at 4°C. After incubation with the corresponding secondary antibody (goat anti-rabbit IgG (H+L)-HRP and goat anti-mouse IgG (H+L)-HRP; Bio-Rad), proteins were detected by chemiluminescence. For the quantification of PKCδ protein, membranes were probed with anti-PKCδ and anti-GAPDH antibodies, and binding was visualized with the Odyssey CLx Imaging system (LI-COR) after incubation with IRDye 800CW goat anti-mouse IgG (H+L) and IRDye 680RD goat anti-rabbit IgG (H+L; both LI-COR) secondary antibodies.

Flow cytometry

EBV-B cells were incubated with LIVE/DEAD Fixable Aqua Dead Cell Stain (Thermo Fisher Scientific) for 10 min at 37°C. Cells were fixed and permeabilized in intracellular fixation and permeabilization buffer (eBioscience, Thermo Fisher Scientific) for 30 min. Intracellular staining was performed for 30 min at 4°C with the appropriate antibodies (Table S3). Cells were analyzed by flow cytometry with a Gallios FACS Analyzer.

Coimmunoprecipitation

Coimmunoprecipitation was performed as previously described (Béziat et al., 2018). Briefly, HEK293T cells were cotransfected with constructs encoding Myc-tagged, DDK-tagged, or V5-tagged proteins or the empty pCMV6 or pCDNA3.1 plasmid in the presence of X-tremeGENE 9 DNA transfection reagent. After 24 h, cells were lysed with lysis buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 2 mM EDTA, and 0.5% Triton X-100) supplemented with aprotinin (10 µg/ml; Sigma Aldrich), PMSF (1 mM), and leupeptin (10 µg/ml) for 30 min at 4°C and centrifuged for 5 min at 16,000 ×g. The supernatant was collected for immunoprecipitation. Immunoprecipitation was performed with anti-V5 (aR96025; Thermo Fisher Scientific) or anti-DDK (aOTI4C5; OriGene) antibodies and agarose-A/G beads (Santa Cruz Biotechnology), after a background clearing step with a normal mouse IgG antibody (wsc-2025; Santa Cruz Biotechnology) and agarose-A/G beads. Total and immunoprecipitated proteins were then analyzed by Western blotting, as described above.

Apoptosis measurement

We used 1 × 10⁵ EBV-B cells to inoculate RPMI 1640 containing 10% FCS in the presence or absence of PMA (100 ng/ml) or APO-1-1 (1 µg/ml; Enzo Life Science), in triplicate, in 96-well plates. The cells were cultured for 24 h at 37°C. Cells were stained with LIVE/DEAD Fixable Aqua Dead Cell Stain, and live cells were counted by flow cytometry with constant time acquisition on a Gallios FACS Analyzer. The percentage of live cells was calculated as previously described (Kuehn et al., 2013).

Retroviral transduction of EBV-B cells

Retrovirus-mediated transduction was performed as previously described (Martínnez-Barricarte et al., 2016). In brief, the coding
sequences of the PRKCD WT and PRKCD mutant alleles were inserted into the plLZS-IRE-S-ΔNGFR plasmid. Retroviruses were produced by transfecting Phoenix A cells with the newly generated plasmids, and EBV-B cells from PKCδ-deficient patients were transduced with the viral concentrate obtained. The transduced cells were subjected to magnetic activated cell sorting purification with anti-CD271 MicroBeads (Miltenyi Biotec), and purified cells were used in subsequent experiments. Transduction was confirmed by flow cytometry with anti-CD271-PE antibody (BD Bioscience).

**Differentiation of MDMs and MDDCs**

MDMs and MDDCs were differentiated as previously described (van de Geer et al., 2018; Bustamante et al., 2011; Conti et al., 2015). In brief, CD14+ cells were isolated from peripheral blood mononuclear cells by positive selection with anti-CD14 MicroBeads (Miltenyi Biotec). For MDM differentiation, cells were cultured in RPMI 1640 containing 10% FCS and M-CSF (50 ng/ml). On day 7, IL-4 (50 ng/ml) was added, and the cells were incubated for a further 7 d for the completion of differentiation. MDDCs were obtained by incubation in the presence of GM-CSF (50 ng/ml) and IL-13 (20 ng/ml; all R&D Systems) for 7 d.

**NADPH oxidase functional assays**

The intracellular production of O2− in EBV-B cells was assessed by chemiluminescence (Sigma-Aldrich; van de Geer et al., 2018). We stimulated 5 × 10⁵ cells with PMA (400 ng/ml) or heat-killed S. aureus cells (Pansorbin, 2 mg/ml; Sigma-Aldrich) and light production was monitored every 5 min with a Victor X4 plate reader. NADPH oxidase activity was assessed in EBV-B cells, MDMs, and MDDCs by measuring extracellular H2O2 release with an Amplex Red Kit (Thermo Fisher Scientific), as previously described (van de Geer et al., 2018; Bustamante et al., 2011; Conti et al., 2015). In brief, 3 × 10⁴ cells were stimulated with PMA (400 ng/ml), and H2O2 release was quantified with a Victor X4 plate reader (PerkinElmer). MDMs or MDDCs were cultured for 16 to 18 h before the experiment, in the presence of 5 × 10⁵ IU/ml IFN-γ (Imukin, Boehringer Ingelheim) or 1 μg/ml LPS (Salmonella minnesota; Sigma-Aldrich), respectively. ROS production by neutrophils and monocytes was quantified with the Bürtest (Phagoburst kit; BD) containing PMA and opsonized E. coli bacteria. The assay was performed according to the manufacturer’s instructions, and the amount of rhodamine 123 (mean fluorescence intensity) was assessed by flow cytometry with a Gallios FACS Analyzer. DHR oxidation is expressed as a percentage of the rhodamine 123 produced by the corresponding travel controls.

**NET formation**

Neutrophils were isolated from whole blood with density gradient centrifugation and Polymorphprep (Progen). We used 1 × 10⁷ neutrophils to seed 8-well microslides (Ibidi), which were then incubated at 37°C for 15 min. PMA (100 ng/ml) was then added, and the cells were incubated for 3 h at 37°C. The cells were fixed by incubation in 4% paraformaldehyde for 15 min, blocked and permeabilized by incubation with 5% BSA in PBS-Tween for 1 h, and incubated overnight with an anti-myeloperoxidase antibody (ab45977; Abcam). Secondary staining was performed by incubation for 1 h with a goat anti-rabbit Alexa Fluor 488 antibody (Thermo Fisher Scientific). Cells were mounted in ProLong Gold antifade reagent containing DAPI (Thermo Fisher Scientific), and images were acquired at the Necker Institute Imaging Facility with a Leica SP8 gSTED confocal microscope (Leica). Percentage NET formation was quantified by counting cells with decondensed nuclei and extracellular DAPI-positive fibers in at least five image fields selected at random.

**Statistics**

A linear mixed model was used to analyze the data presented in Fig. 3 D, to take repeated measurements into account. The null hypothesis was that the distribution of EBV-B cell survival rates after PMA or APO stimulation was similar in patients and controls. The null hypothesis was rejected if the P value was <0.05.

Statistical analysis and visualization of results for Fig. 7, A–E, were performed using GraphPad Prism 8.4.3. For Fig. 7, A, B, D, and E, two-way ANOVA with Tukey’s multiple comparison was performed. One-way ANOVA was performed for Fig. 7 C. A P value <0.05 was considered statistically significant.

**Online supplemental material**

Fig. S1 shows evolutionary conservation of the missense variants and splicing consequences of the variants affecting slice sites. Fig. S2 shows decreased phosphorylation of MARCKS in the patients’ EBV-B cells. Fig. S3 presents the complementation of the patients’ EBV-B cells with WT PRKCD. Fig. S4 provides additional information about the NADPH oxidase subunit expression and their interaction with PKCδ. Fig. S5 presents additional evidence of impaired ROS production in the patients’ primary cells including ROS production in neutrophils and monocytes, NET formation and phosphorylation of p47phox. Table S1, Table S2, and Table S3 provide primer sequences for sequencing and site-directed mutagenesis, and antibody references.

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References

Anjani, G., P. Vignesh, V. Joshi, J.K. Shandilya, D. Bhattacharjya, J. Sharma, and A. Rawat. 2009. Recent advances in chronic granulomatous disease. Genes Dis. 7:84–92. https://doi.org/10.1016/j.gendis.2009.07.010

Basu, A., and D. Pal. 2010. Two faces of protein kinase Cδ: the contrasting roles of PKCδ in cell survival and cell death. ScientifitWorldJournal. 10: 2272–2284. https://doi.org/10.1002/js.2010.214

Belambi, S.A., L. Rolas, H. Raad, M. Hurtado-Nedelec, P.M.-C. Dang, and J. El-Benna. 2018. NADPH oxidase activation in neutrophils: Role of the phosphorylation of its cytosolic partner. Eur. J. Clin. Invest. 48(Suppl 2):e12951. https://doi.org/10.1111/eci.12951

Belot, A., P.R. Kasher, E.W. Trotter, A.-P. Foray, A.-L. Debaud, G.J. Rice, M. Szyndzielowiec, M.-T. Zabot, I. Rouvet, S.S. Bhaskar, et al. 2013. Protein kinase Cδ deficiency causes mendelian systemic lupus erythematosus with S cell-defective apoptosis and hyperproliferation. Arthritis Rheum. 65:2161–2171. https://doi.org/10.1002/art.38008

Bey, E.A., B. Xu, A. Bhattacharjee, C.M. Oldfield, X. Zhao, Q. Li, V. Subulakshmi, G.M. Feldman, F.B. Wientjes, and M.K. Cathcart. 2004. Protein kinase C δ is required for p47phox phosphorylation and translocation in activated human monocytes. J. Immunol. 173:5730–5738. https://doi.org/10.4049/jimmunol.173.9.5730

Bézat, V., J. Li, J.-X. Lin, C.S. Ma, P. Li, A. Bousiflha, I. Pellerin, S. Zoghli, S. Bazal, S. Keles, et al. 2018. A recessive form of hyper-IgE syndrome by disruption of ZNF341-dependent STAT3 transcription and activity. Sci. Immunol. 3:eaa4956. https://doi.org/10.1126/sciimmunol.aat4956

Bouin, A.P., N. Grandvaux, P.V. Vignais, and A. Fuchs. 1998. p40(phox) is phosphorylated on threonine 155 and serine 315 during activation of the phagocyte NADPH oxidase. Implication of a protein kinase c-type kinase in the phosphorylation process. J. Biol. Chem. 273:30097–30103. https://doi.org/10.1074/jbc.273.46.30097

Brown, G.E., M.Q. Stewart, H. Liu, V.-L. Ha, and M.B. Yaffe. 2003. A novel assay system implicates PtdIns(3,4)P(2), PtdIns(3)P, and PKC δ in intracellular production of reactive oxygen species by the NADPH oxidase. Mol. Cell. 11:35–47. https://doi.org/10.1016/S1097-2765(03)00005-4

Bustamante, J., A.A. Arias, G. Voge, C. Picard, L.B. Galicia, C. Prando, A.V. Gromani, C.C. Marchal, M. Hubeau, A. Chappet, et al. 2011. Germline CYBB mutations that selectively affect macrophages in X-linked predisposition to tuberculous mycobacterial disease. Nat. Immunol. 12:213–221. https://doi.org/10.1038/ni.1992

Chessa, T.A.M., K.E. Anderson, Y. Hu, Q. Xu, O. Rausch, L.R. Stephens, and P.T. Hawkins. 2010. Phosphorylation of threonine 154 in p40phox is an important physiological signal for activation of the neutrophil NADPH oxidase. Blood. 116:6027–6036. https://doi.org/10.1182/blood-2010-08-300889

Cho, W. 2001. Membrane targeting by C1 and C2 domains. J. Biol. Chem. 276:32410–32414. https://doi.org/10.1074/jbc.R100007200

Conti, F., W.C. Aragão Filho, C. Prando, C. Deswarte, M. Hubeau, P.E. Newburger, J.-L. Casanova, J. Bustamante, and A. Condino-Neto. 2015. Phagocyte nicotinamide adenine dinucleotide phosphate oxidase activity with B cell-defective apoptosis and hyperproliferation. Curr. Opin. Immunol. 49:87–95. https://doi.org/10.1016/j.coi.2017.10.008

Crow, Y.J. 2011. Type I interferonopathies: a novel set of inborn errors of immunity. Ann. N.Y. Acad. Sci. 1238:91–98. https://doi.org/10.1111/j.1749-6632.2011.06220.x

de Oliveira-Junior, E.B., N.B. Zurro, C. Prando, O. Cabral-Mareque, P.V.S. Pereira, L.-F. Schimke, S. Klaver, B. Buclin, L. Blancas-Galicia, L. Santos-Argumedo, et al. 2015. Clinical and Genotypic Spectrum of Chronic Granulomatous Disease in 71 Latin American Patients: First Report from the LASID Registry. Pediatr. Blood Cancer. 62:2101–2107. https://doi.org/10.1002/pbc.25674

DeVries-Seimon, T.A., A.M. Ohm, M.J. Humphries, and M.E. Reyland. 2007. Induction of apoptosis is driven by nuclear retention of protein kinase C delta. J. Biol. Chem. 282:23207–23214. https://doi.org/10.1074/jbc.M703661200

Dinauer, M.C., S.H. Orkin, R. Brown, A.J. Jesaitis, and C.A. Parkos. 1987. The locus is a component of the neutrophil cytochrome b complex. Nature. 327:717–720. https://doi.org/10.1038/327717a0

Duquesnes, N., F. Lezoualc’h, and B. Crozatier. 2011. PKC-delta and PKC-epsilon: foes of the same family or strangers? J. Mol. Cell. Cardiol. 51: 665–673. https://doi.org/10.1016/j.yjmcc.2011.07.013

Durgan, J., N. Michael, N. Totey, and P.J. Parker. 2007. Novel phosphorylation site markers of protein kinase C delta activation. FEBS Lett. 581: 3377–3381. https://doi.org/10.1016/j.febslet.2007.06.035

El-Benna, J., P.-M.-C. Dang, M.-A. Gougerot-Pocidalo, J.-C. Marie, and F. Braut-Boucher. 2009. p47phox, the phagocyte NADPH oxidase/NOX2 organizer: structure, phosphorylation and implication in diseases. Exp. Mol. Med. 41:217–225. https://doi.org/10.3858/emm.2009.41.4.058
Kazanietz, M.G., S. Wang, G.W. Milne, N.E. Lewin, H.L. Liu, and P.M. Humphries, M.J., A.M. Ohm, J. Schaack, T.S. Adwan, and M.E. Reyland. 2008. Impaired respiratory burst in PKC epsilon relevant to phorbol ester binding as revealed by site-directed mutagenesis. J. Biol. Chem. 272:9521–9527. 

Kiykim, A., I. Ogulur, S. Baris, E. Salzer, E. Karakoc-Aydiner, A.O. Ozen, W. Evren, E., E. Ringqvist, and T. Willinger. 2020. Origin and ontogeny of lung NADPH oxidase activity. Blood 144:3309–3315. https://doi.org/10.1182/blood-2009-07-231498

Mecklenbrauker, I., S.L. Kalled, M. Leitges, F. Mackay, A. Tarakhovsky. 2004. Regulation of B-cell survival by BAF-dependent PKCe-mediated nuclear signalling. Nature. 431:456–461. https://doi.org/10.1038/nature02955

Mecklenbrauker, I., K. Saijo, N.-Y. Zheng, M. Leitges, and A. Tarakhovsky. 2002. Protein kinase Cdelta controls self-antigen-induced B-cell tolerance. Nature 416:860–865. https://doi.org/10.1038/416860a0

Meffre, E., and H. Wardemann. 2008. B-cell tolerance checkpoints in health and autoimmunity. Curr. Opin. Immunol. 20:632–638. https://doi.org/10.1016/j.coi.2008.09.001

Meijles, D.N., L.M. Fan, B.J. Howlin, and J.-M. Li. 2014. Molecular insights of p47phox phosphorylation dynamics in the regulation of NADPH oxidase activation and superoxide production. J. Biol. Chem. 289:22759–22770. https://doi.org/10.1074/jbc.M114.561519

Miyamoto, A., K. Nakayama, H. Imaoki, S. Hirose, Y. Jiang, M. Abe, T. Tsukiyama, H. Nagahama, S. Ohno, S. Hatakeyama, and K.I. Nakayama. 2002. Increased proliferation of B cells and autoimmunity in mice lacking protein kinase Cdelta. Nature 416:865–869. https://doi.org/10.1038/416865a0

Nanthapalis, S., E. Omonynnj, C. Murphy, A. Standing, M. Eisenhut, D. Eleftheriou, and P.A. Brogan. 2017. Early-Onset Juvenile SLE Associated With a Novel Mutation in Protein Kinase Cdelta. Pediatrics. 139:e20160781. https://doi.org/10.1542/peds.2016-0781

Neehus, A.-L., J. Lam, K. Haake, S. Merkert, N. Schmidt, A. Mucci, M. Ackermann, M. Schubert, C. Happe, M.P. Kuhnel, et al. 2018. Impaired IFNy-Signaling and Mycobacterial Clearance in IFNRI-Deficient Human iPSC-Derived Macrophages. Stem Cell Reports. 10:7–16. https://doi.org/10.1016/j.stemcr.2017.12.011

Newton, A.C. 2001. Protein kinase C: structural and spatial regulation by phosphorylation, cofactors, and macromolecular interactions. Chem. Rev. 101:2353–2364. https://doi.org/10.1021/cr0002801

Nishikawa, K., A. Toker, F.-J. Johannes, Z. Songyang, and L.C. Cantley. 1997. Determination of the specific substrate sequence motifs of protein kinase C isozymes. J. Biol. Chem. 272:952–960. https://doi.org/10.1074/jbc.272.2.952

Nishizuka, Y. 1995. Protein kinase C and lipid signaling for sustained cellular responses. FASEB J. 9:484–496. https://doi.org/10.1096/fasebj.9.7.773745

Nordouzi, S., A. Aghamohammadi, S. Mamishi, S.D. Rosenzweig, and N. Rezaei. 2012. Bacillus Calmette-Guérin (BCG) complications associated with primary immunodeficiency diseases. J. Allergy Clin. Immunol. 885–897. https://doi.org/10.1016/j.jaci.2012.05.008

Omejec, O., C. Picard, C. Prachett, M. Moreews, F. Rieux-Laucat, P. Soulas-Srauel, S. Viel, J.-C. Lega, B. Bader-Meunier, T. Walzer, et al. 2019. Monogenic lupus: Dissecting heterogeneity. Autoimmun. Rev. 18:102361. https://doi.org/10.1016/j.autrev.2019.102361

Pappa, H., J. Murray-Rust, L.V. Dekker, P.J. Parker, and N.Q. McDonald. 1998. Crystal structure of the C2 domain from protein kinase C-S. Structure. 6:885–894. https://doi.org/10.1016/S0969-4990(98)00090-2

Parvaneh, V.J., R. Shiari, L. Mahbobi, and D. Babaei. 2014. Chronic granulomatous disease associated with systemic lupus erythematosus and systemic onset juvenile idiopathic arthritis. Pediatr. Rheumatol. 12(S1):P169. https://doi.org/10.1186/1541-7786-12-S1-P169

Picard, C., and A. Belot. 2017. Does type-I interferon drive systemic autoimmunity? Autoimmun. Rev. 16:897–902. https://doi.org/10.1016/j.autrev.2017.07.001

Neehus et al. Impaired respiratory burst in PKCdelta deficiency. Journal of Experimental Medicine. 2018. https://doi.org/10.1084/jem.2012501
Impaired respiratory burst in PKCδ deficiency

Soroosh, F., Y. Tang, K. Guglielmo, A. Engelmann, E. Liverani, A. Patel, J. Langston, S. Sun, K. Konapuli, M.F. Kiani, and L.E. Kilpatrick. 2019. Protein Kinase C-Delta (PKCδ) Tyrosine Phosphorylation is a Critical Regulator of Neutrophil-Endothelial Cell Interaction in Inflammation. Shock. 51:538–547. https://doi.org/10.1097/SHK.0000000000001247

Szilagyi, K., R.P. Gazendam, J.L. van Hamme, A.T.J. Tool, M. van Houdt, W.A.J.W. Vos, P. Verkuiljen, H. Janssen, A. Belot, L. Juillard, et al. 2015. Impaired microbial killing by neutrophils from patients with protein kinase C delta deficiency. J. Allergy Clin. Immunol. 136:1404–7.e10: 10. https://doi.org/10.1016/j.jaci.2015.06.016

Thomas, D.C.-L. Mar, Charbonnier, A. Schejtmann, H. Aldehkeri, E.L. Coomber, E.R. Dufficy, A.E. Beenken, J.C. Lee, S. Clare, A.O. Speak, et al. 2019. EROS/CYBC1 mutations: Decreased NADPH oxidase function and chronic granulomatous disease. J. Allergy Clin. Immunol. 143:782–785.e1. https://doi.org/10.1016/j.jaci.2019.09.019

van de Geer, A., A. Nieto-Patrán, D.B. Kuhns, A.T.J. Tool, A.A. Arias, M. Bouaziz, M. de Boer, J.L. Franco, R.P. Gazendam, J.L. van Hamme, et al. 2018. Inherited p40phox deficiency differs from classic chronic granulomatous disease. J. Clin. Invest. 128:3957–3975. https://doi.org/10.1172/JCI97116

Winkelstein, J.A., M.C. Marino, R.B. Johnston Jr., J. Boyle, J. Curnutte, J.I. Gallin, H.L. Malech, S.M. Holland, H. Ochs, P. Quie, et al. 2000. Chronic granulomatous disease. Report on a national registry of 368 patients. Medicine (Baltimore). 79:155–169. https://doi.org/10.1097/00005792-200005000-00003

Wu-Zhang, A.X., A.N. Murphy, M. Bachman, and A.C. Newton. 2012. Isozyme-specific interaction of protein kinase Cδ with mitochondria dissected using live cell fluorescence imaging. J. Biol. Chem. 287:37891–37906. https://doi.org/10.1074/jbc.M112.412635

Xie, C., T. Cole, C. McLean, and J.C. Su. 2016. Association Between DiscoID Lupus Erythematosus and Chronic Granulomatous Disease—Report of Two Cases and Review of the Literature. Pediatr. Dermatol. 33:e114–e120. https://doi.org/10.1111/pde.12826

Yang, Q., J.C. Langston, Y. Tang, M.F. Kiani, and L.E. Kilpatrick. 2019. The Role of Tyrosine Phosphorylation of Protein Kinase C Delta in Infection and Inflammation. Int. J. Mol. Sci. 20:1498. https://doi.org/10.3390/ijms20061498

Zhang, G., M.G. Kazanietz, P.M. Blumberg, and J.H. Hurley. 1995. Crystal structure of the cys2 activator-binding domain of protein kinase C delta in complex with phorbol ester. Cell. 81:917–924. https://doi.org/10.1016/0092-8674(95)90311-X

Zhao, X., B. Xu, A. Bhattacharjee, C.M. Oldfield, F.B. Wienntjes, G.M. Feldman, and M.K. Cathcart. 2005. Protein kinase Cδelta regulates p67phox phosphorylation in human monocytes. J. Leukoc. Biol. 77:414–420. https://doi.org/10.1189/jlb.0504284

Rahman, A., and D.A. Isenberg. 2008. Systemic lupus erythematosus. N. Engl. J. Med. 358:929–939. https://doi.org/10.1056/NEJMr072197

Reyland, M.E., S.M. Anderson, A.A. Matassa, K.A. Barzen, and D.O. Quissell. 1999. Protein kinase Cδ is essential for apoptosis in salivary gland acinar cells. J. Biol. Chem. 274:19115–19123. https://doi.org/10.1074/jbc.274.27.19115

Rönnblom, L.E., G.V. Alm, and K.E. Oberg. 1990. Possible induction of systemic lupus erythematosus by interferon-alpha treatment in a patient with a malignant carcinoid tumour. J. Intern. Med. 227:207–210. https://doi.org/10.1111/j.1365-2796.1990.tb0144.x

Royer-Pokora, B., L.M. Kunkel, A.P. Monaco, S.C. Goff, P.E. Newburger, R.L. Baehner, F.S. Cole, J.T. Curnutte, and S.H. Orkin. 1986. Cloning the gene for an inherited human disorder—chronic granulomatous disease—on the basis of its chromosomal location. Nature. 322:32–38. https://doi.org/10.1038/322032a0

Salzer, L., E. Santos-Valente, B. Keller, K. Warnatz, and K. Bostug. 2016. Protein Kinase Cδ: a Gatekeeper of Immune Homeostasis. J. Clin. Immunol. 36:631–640. https://doi.org/10.1007/s10875-016-0323-0

Schwegmann, A., R. Guler, A.J. Cutler, B. Arendse, W.G.C. Horsnell, A. Segal, B.H., T.L. Leto, J.I. Gallin, H.L. Malech, and S.M. Holland. 2000. Ge-

Sharifinejad, N., G. Azizi, N. Behniafard, M. Zaki-Dizaji, M. Jamee, R. Yaz-

dani, H. Abolhassani, and A. Aghamohammadi. 2020. Protein Kinase C-Delta Defect in Autoimmune Lymphoproliferative Syndrome-Like Disease: First Case from the National Iranian Registry and Review of the Literature. Immunol. Invest.:1–12. https://doi.org/10.1080/08820199 .2020.1829638

Someya, A., H. Nuno, T. Hasebe, and I. Nagaoka. 1999. Phosphorylation of p40-phox during activation of neutrophil NADPH oxidase. J. Leukoc. Biol. 66:851–857. https://doi.org/10.1002/jlb.66.5.851

Song, E., G.B. Jaishankar, H. Saleh, W. Jithpratuck, R. Sahni, and G. Krishnaswamy. 2001. Chronic granulomatous disease: a review of the infectious and inflammatory complications. Clin. Mol. Allergy. 9:10. https://doi.org/10.1186/1476-7961-9-10

Sontag, S., M. Förster, J. Qin, P. Wanek, S. Mitzka, H.M. Schüler, S. Korschmieder, S. Rose-John, K. Seré, and M. Zenke. 2017. Modelling IFNβ Deficient Human Hematopoiesis and Dendritic Cell Development with Engineered iPS Cells. Stem Cells. 35:898–908. https://doi.org/10.1002/ stem.2565

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https://doi.org/10.1084/jem.20210501
Figure S1. Evolutionary conservation of PKCδ and PRKCD transcripts in cells carrying essential splice site variants. (A) Amino-acid residue conservation in various species, for the four PKCδ missense mutations (indicated by red arrows) found in kindreds A–D. (B) Schematic diagram and proportions of the different splice variants produced in cells from homozygous carriers of the c.1352 + 1G>A or c.517 + 2dup variants and compound heterozygous carriers of the p.Q191* and c.788-2A>G variants. Positions of affected amino acids are indicated by red triangles.
Figure S2. Phosphorylation of MARCKS in the patients’ EBV-B cells. Phosphorylation of MARCKS was detected by Western blot using whole-cell protein lysates of EBV-B cells of two healthy controls (C1, C2) and PKCδ-deficient patients before (−) and after (+) 30-min PMA activation (upper panel; 400 ng/ml). Antibodies against phospho-MARCKS (p-MARCKS), MARCKS, and PKCδ were used. An antibody against GAPDH was used as loading control. Quantification of pMARCKS expression compared with the amount of total MARCKS (lower panel; n = 2; mean ± SD). The results shown are representative of two independent experiments.
Figure S3. **Complementation of the patients’ EBV-B cells with WT PRKCD.** (A) Surface expression of CD271 on EBV-B cells of a healthy control (C1) and PKCδ-deficient patients after retroviral transduction with the empty pLZRS vector (EV) and WT PRKCD cDNA (gray, isotype; black, surface staining). (B) Extracellular H$_2$O$_2$ production by the EBV-B cells of a healthy control (C1), a gp91$^{	ext{phox}}$-deficient patient, and PKCδ-deficient patients either nontransduced (NT) or transduced with the empty pLZRS plasmid (EV) or WT-PRKCD, at various time points after PMA stimulation (400 ng/ml), as assessed with the Amplex Red test. Representative results of two independent experiments (duplicates, mean ± SD).
Figure S4. Expression of NADPH oxidase components in PKCδ-deficient EBV-B cells and their interaction with PKCδ. (A) Extracellular detection of cytochrome b$_{558}$ (cytB) and intracellular staining for gp91$^{phox}$, p67$^{phox}$, p47$^{phox}$, and p22$^{phox}$ in EBV-B cells from healthy controls (C1, C2), PKCδ-deficient patients, and CGD patients (gray, isotype; black, surface staining). Representative results are shown for three independent experiments. (B) Coimmunoprecipitation (IP) on protein lysates from HEK293T cells transfected with the empty vector (EV), NCF4, or PRKCD cDNAs. The pulldown of p40$^{phox}$ was performed with an anti-DDK antibody. (C) IP on protein lysates from HEK293T cells transfected with EV, NCF1, or PRKCD cDNAs. The pulldown of p47$^{phox}$ was performed with an anti-DDK antibody. (D) Co-IP on protein lysates from HEK293T cells transfected with EV, NCF2, or PRKCD cDNAs. The pulldown of PKCδ (upper panel) and p67$^{phox}$ (lower panel) was performed with an anti-DDK or an anti-V5 antibody, respectively. (E) IP on protein lysates from HEK293T cells transfected with EV, RAC2, or PRKCD cDNAs. The pulldown of PKCδ (upper panel) and Rac2 (lower panel) was performed with an anti-DDK or an anti-V5 antibody, respectively. All results shown are representative of two independent experiments.
Figure S5. **NADPH oxidase activity and p47phox phosphorylation in primary cells of PKCδ-deficient patients.** (A and B) Representative flow cytometry images of intracellular ROS production measured by DHR in neutrophils (A) and monocytes (B) of a healthy control and a PKCδ-deficient patient (P14) are shown on the right (gray filled, nonstimulated; black solid line, PMA stimulation; black dotted line, E. coli stimulation). (C) PKCδ expression in neutrophils and monocytes of healthy controls and two PKCδ-deficient patients, measured by Western blot. (D) Representative images of PMA-induced NET formation by neutrophils of controls, three PKCδ-deficient patients, and a gp91phox-deficient patient. Green represents myeloperoxidase (MPO) and blue DNA (DAPI). The merged images of the local and travel controls and of P17 are the same as displayed in Fig. 7 C. Scale bars, 60 µm. (E) Representative kinetic of extracellular H2O2 production after PMA plus IFN-γ stimulation by MDMs of patients (P14, P15) and controls. (F) Western Blot of MDMs of healthy controls (n = 4) and PKCδ-deficient patients (n = 5) before (−) and after (+) 30 min of PMA stimulation (400 ng/ml). Antibodies against PKCδ, phospho5304-p47phox, phospho5315-p47phox, and p47phox were used. Anti-GAPDH and anti-Vinculin antibodies served as a loading controls. The control marked with an asterisk is the same as in Fig. 7 D because proteins of P2 (Fig. 7 D) and P13 were blotted on the same membrane. Solid bars between two images indicate different regions of the same membrane.
Provided online are three tables. Table S1 lists primer sequences and conditions for genomic amplification of the PRKCD gene. Table S2 lists primer sequences for site-directed mutagenesis using the pCMV6-PRKCD-WT-DDK plasmid. Table S3 lists antibodies used.