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PLCG2 associated immune dysregulation (PLAID) comprises broad and distinct clinical presentations related to functional classes of genetic variants

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Abstract

Background: Pathogenic variants of *PLCG2* cause 2 related forms of autosomal dominant immune dysregulation, PLCγ2-associated antibody deficiency and immune dysregulation

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Conflict of Interest Statement.

IIC is on the advisory board to Enzyvant, is a consultant for Pharming, and is a medical writer for UpToDate. KC is on the advisory board to Takeda Pharmaceuticals and the speaker's bureau for Horizon Pharma. SCG is a consultant for Janssen. AAG is a co-investigator in studies with Innate Pharma, CRISPR Therapeutics, StemLine Therapeutics, and Kyowa Kyrin (Co-Investigator), and is a consultant to StemLine Therapeutics and BluePrints Medicine. EH is an adviser to Jasper Therapeutics, Octapharma, CSL-Behring and Takeda.EJ is a consultant for Vivet Therapeutics and laboratoire CTRS, France.IM is on an advisory board to Boehringer Ingelheim and receives research funding (paid to UZ Leuven) from CSL Behring. SBS declares the following interests: Scientific advisory board participation for Pfizer, Pandion, Celgene, Lilly, Takeda, Cosmo Pharmaceuticals, Merck, Sonoma Biotherapeutics, and EcoR1; grant support from Pfizer, Amgen Takeda, and Novartis. Consulting for Amgen, Kyverna, BMS, Merck, Third Rock, 89bio, GentiBio and Apple Tree Life Sciences. TT is a consultant for Takeda, Horizon, X4 Pharma, and Pharming Healthcare; her serves on the Data Safety and Monitoring Board for Takeda (formerly Baxalta); and he receives research funding from the Paul G. Allen Family Foundation, NIH, and Eli Lilly. JEW receives grant/research/clinical trial support from Takeda, Janssen, Chiesi, MustangBio, ADMA Biologicals, Octapharma, X4- Pharmaceuticals, Novartis, Regeneron and Bristol-Myers Squibb; is a consultant or advisory board member for Takeda, X4- Pharmaceuticals, CSL-Behring, Grifols, ADMA Biologicals, Enzyvant and Regeneron; is on the Speaker's Bureau for Takeda; and is a medical write for UptoDate. BRW receives research support from Merck and Swedish Orphan Biovitrum; clinical trial support from AstraZeneca and Blueprint Medicines; speakers fees from Takeda Pharmaceuticals, and serves on the DSMB for REDHART2 clinical trial. All other co-authors have reported that they have no conflicts of interest to disclose.

(PLAID) and autoinflammatory PLAID (APLAID). Since describing these conditions, many *PLCG2* variants of uncertain significance (VUS) have been identified by clinical sequencing of patients with diverse features of immune dysregulation.

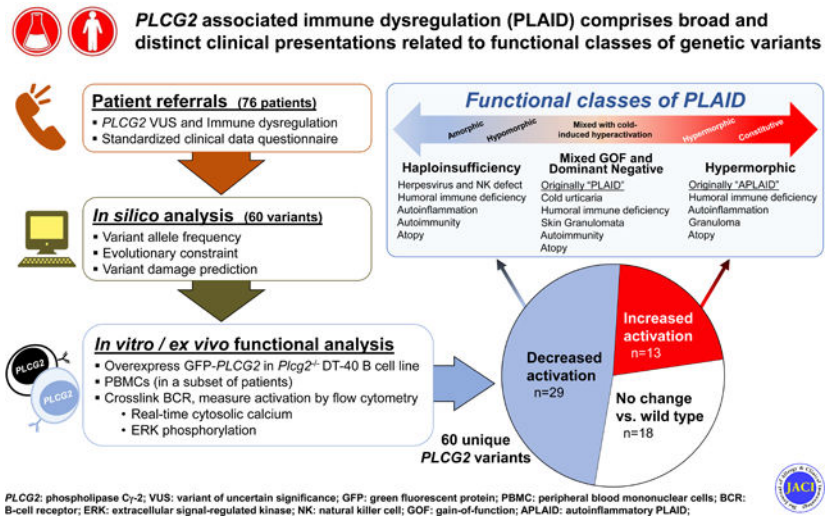
Objective: To functionally classify *PLCG2* variants and explore known and novel genotypefunction-phenotype relationships.

Methods: Clinical data from patients with *PLCG2* variants were obtained via standardized questionnaire. *PLCG2* variants were generated by mutagenesis of e*GFP-PLCG2* plasmid, which was overexpressed in *Plcg2*-deficient DT-40 B-cells. BCR-induced calcium flux and ERK phosphorylation were assayed by flow cytometry. In some cases, stimulation-induced calcium flux was also measured in primary patient cells.

Results: Three-fourths of *PLCG2* variants produced functional alteration of B-cell activation, *in vitro.* Thirteen variants led to gain-of-function (GOF), however most functional variants defined a new class of *PLCG2* mutation, monoallelic loss-of-function (LOF). Susceptibilty to infection and autoinflammation were common with both GOF and LOF variants, while a new phenotypic cluster consisting of humoral immune deficiency, autoinflammation, susceptibility to herpesviral infection and natural killer (NK) cell dysfunction were observed in association with multiple heterozygous LOF variants detected in both familial and sporadic cases. In some cases, *PLCG2* variants produced greater effects in NK cells than B-cells.

Conclusion: This work expands the genotypic and phenotypic associations with functional variation in *PLCG2,* including a novel form of immune dysregulation in carriers of heterozygous loss of *PLCG2* function. It also demonstrates the need for more diverse assays for assessing the impact of *PLCG2* variants on human disease.

Graphical Abstract



Keywords

Phospholipase C gamma 2; Immune dysregulation; Primary immune deficiency; Variants of Uncertain Significance; Autoinflammation; Antibody deficiency

Introduction

Primary immune regulation disorders (PIRDs) are caused by high-impact genetic variants that are transmitted within families leading to immune dysregulation (ID). They provide opportunities to gain insights into immune signaling pathways and to dissect combinatorial effects of genetic variation in complex or polygenic diseases. Furthermore, the varied penetrance and expressivity of symptoms in these and similar disorders points to the need to re-evaluate classical genoty-pephenotype relationships.

PLCγ2 is a pleiotropic signaling molecule that controls cellular responses in many hematopoietic cells, including B lymphocytes and natural killer (NK) cells[1]. Activated by sequential phosphorylation by Bruton's tyrosine kinase (BTK) and other kinases[2], PLCγ2 undergoes an allosteric conformational change, unveiling its active site and bringing it into proximity of its substrate, phosphatidylinositol 4,5-bisphosphate (PIP2), which is then rapidly hydrolyzed into the second messengers, diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP3)[3]. This leads to rapid movement of calcium into the cytoplasm from endoplasmic reticular stores and the extracellular space, resulting in cellular activation.

Dominant variants of *PLCG2* in humans lead to aberrant leukocyte signaling with highly penentrant but variably expressed features that include hypogammaglobulinemia, recurrent sinopulmonary infections, autoimmunity cold urticaria and sterile inflammation. *PLCG2* associated antibody deficiency and immune dysregulation (PLAID) is caused by large in-frame genomic deletions that disrupt the autoinhibitory domain of PLCγ2, leading to cold-induced activation, but poor signaling at normal physiologic temperatures, likely the result of disruptions of the signaling complex[4,5]. Autoinflammatory PLAID (APLAID) is so-named because of the observation that patients with more classical gain-of-function (GOF) mutations presented with significant systemic autoinflammation, interstitial lung disease (ILD) and enterocolitis, in addition to humoral defects similar to those seen in PLAID[6]. APLAID associated mutations did not lead to temperature sensitivity or clinical phenotypes of cold urticaria. To date, seven mutations of *PLCG2* have been reported to cause APLAID in 11 individuals[6–12]. *PLCG2* variants also associate with common diseases which are associated with immune dysregulation including age related macular degeneration[13], multiple sclerosis[14], vasculitis[15], inflammatory bowel disease[16], nephrotic syndrome[17] and Alzheimer's disease[18]. Together, these associations suggest more broad roles for *PLCG2* in human immune disorders, and a spectrum of penetrance and expressivity which may depend on the qualitative and quantitative impact of *PLCG2* variants on function.

Here, we describe 76 individuals with ID in whom variants of uncertain significance (VUS) of *PLCG2* were identified. Although none exhibited cold-induced urticaria, the hallmark of PLAID, many had features reminiscent of PLAID and/or APLAID, including humoral immune deficiency, fungal infection, atopy, autoimmunity and autoinflammation. In addition, novel phenotypes of mycobacterial infection, severe viral infection and cytopenias were observed. Using a B-cell overexpression system, we functionally characterized the effect of each variant on intracellular calcium and ERK phosphorylation following B-cell antigen receptor (BCR) stimulation. In addition to multiple GOF mutations which appear on the spectrum of what has been described as APLAID, we discovered that *PLCG2* loss-of-

function (LOF) mutations are associated with antibody defects, infection, autoinflammation and immune dysregulation, suggesting that the term PLAID would be better used to broadly describe immune deficiency and dysregulation which occurs in association with multiple types of functional *PLCG2* mutations.

Methods

Study subjects and *PLCG2* variants.

Patients with ID and protein-altering variants of *PLCG2* were identified by their treating physicians using clinically-obtained whole exome or candidate gene panel sequencing. The cases reported here are the result of the referring providers contacting us for clarification of the potential role of the encountered *PLCG2* variant in their patient. When other known or likely disease-causing variants were found in the clinical analysis, cases were referred if there was concern that the *PLCG2* variant might modify disease, and we have noted those cases to avoid confounding phenotypic associations with *PLCG2* variants. *PLCG2* variant information and clinical features were collected through a standardized form. Clinical immunophenotyping data (e.g. flow cytometry, immunoglobulin levels, NK function) were obtained from referring clinicians who sent the assays based on their clinical judgement and availability of clinical assays to the referring provider. In some cases, results were reported as normal/abnormal. Deeper phenotyping was performed on primary cells obtained from a small subset of patients through institutional review board approved protocols at the National Institutes of Health.

In silico assessment of *PLCG2* variants

Degree of evolutionary constraint and predicted deleteriousness of variants were assessed with SiPhy and CADD algorithms, respectively[19,20]. Characteristics of *PLCG2*-ID variants were compared to those of *PLCG2* variants observed in the Genome Aggregation Database (gnomAD, v2.1.1).

Construction of plasmids.

GFP-tagged PLCγ2 was generated by cloning the open-reading frame of human *PLCG2* into pEGFP-C1 (Takara Bio USA, San Jose, CA). mCherry (Takara Bio USA) was subcloned in place of EGFP in pEGFP-*hPLCG2*. Variant constructs were generated by site-directed mutagenesis by Bioinnovitase (Rockville, MD).

Cell Culture and Transfection.

Wild-type (WT) and *Plcg2*-deficient DT-40 chicken B-cell lines were a generous gift from Dr. Matilda Katan. Five million cells were transfected with 5ug of endotoxin-free pEGFP-*hPLCG2* plasmid constructs (Kit T; Program A30; Lonza, Walkersville, MD) and plated in antibiotic-free media. Transfected cells were collected for assays 16–18 hours post-transfection. For co-transfection, 2.5ug of mCherry-*hPLCG2* DNA was simultaneously transfected with 2.5ug pEGFP-*hPLCG2* variant. Detailed methods are available in the online supplement.

Calcium Flux Analysis.

Transfected cells were stained with 2mM Indo-1 (I2251, Life Technologies, Carlsbad, CA), a calcium-binding fluorophore. Viability of transfected DT-40 cells was evaluated with 7-Aminoactimomycin D (Life Technologies). Using a BD Fortessa cytometer, viable GFP-positive cells were analyzed for calcium-bound (violet) and unbound (blue) Indo-1 fluorescence prior to, and for 3 minutes following BCR cross-linking with 2ug/ml anti-chicken IgM antibody (Southern Biotech, Birmingham,AL). FlowJo software (Ashland, OR) was used to calculate the Indo-1 violet-to-blue ratio over time and the 3-minute area under the curve (AUC), which was normalized to that of WT PLCγ2. Peripheral blood mononuclear cells (PBMC) were stained with Indo-1, together with antibodies to distinguish T, B and NK cell populations. Stained PBMC were treated with T, B and NK activators and the real-time ratio of Indo-1 blue/violet was measured simultaneously in T, B and NK cells. The detailed protocol is provided in the online supplement.

Intracellular ERK Phosphorylation Analysis.

Transfected cells were incubated for 5 minutes at 37°C with or without BCR-stimulation. Cells were stained with rabbit anti-Phospho-ERK1/2 (p44-p42-Tyr/Thr; Cell Signaling Technology, Danvers, MA) primary antibody, followed by APC-conjugated anti-rabbit secondary antibody (Cell Signaling Technology). ERK phosphorylation was quantified in fixed, GFP-positive cells with a BD Celesta cytometer and expressed as mean fluorescence intensity (MFI) of APC, relative to WT. The detailed protocol is provided in the online supplement.

Western blot analysis.

Transfected cells were lysed with ice cold RIPA lysis buffer. Equal amounts of protein were separated on a 4–20% SDS-PAGE gel and transferred to a PVDF membrane (BioRad, Hercules, CA). Blots were probed with antibodies against GFP (Santa Cruz Biotechnology, Dallas, TX), PLCγ2 (Cell Signaling Technology), B-actin (Cell Signaling Technology) and GAPDH (Cell Signaling Technology) and developed with chemiluminescent substrate (Biorad).

Quantitative RT-PCR.

RNA was extracted from tranfected cells using RNAeasy (Qiagen, Germantown, MD). cDNA was synthesized with SuperScript III (Life Technologies). *PLCG2* expression was assessed using Taqman probe sets (Life Technologies). *EGFP* and *PLCG2* expression were normalized to expression levels of glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

NK cell cytotoxicity assay.

NK cell cytotoxicity was measured by Cr^{51} release assay using PBMC and K562 target cells for 4 hours at 37°C, as previously described[21]. The detailed protocol is provided in the online supplement.

Statistical Analysis.

All analyses were performed using Prism (GraphPad, LaJolla, CA). Flow cytometric data were expressed as median with interquartile range. Differences between groups were analyzed using the Mann-Whitney U nonparametric test or the Student's T-test. Correlations between groups were assessed with Spearman's rank correlation. Statistical significance was defined as p-value < 0.05.

Results

Novel and ultra-rare *PLCG2* variants identified in people with ID.

Seventy-six subjects were referred to our study for consideration of whether a *PLCG2* variant may define a novel genotype-phenotype relationship, or for suspicion of a known *PLCG2*-associated disorder. All of them displayed clinical features characteristic of ID, including elements of immune deficiency, autoimmunity, autoinflammation and atopy/allergy (Table 1, Supplementary Tables 1 and 2). The most prevalent phenotype, observed in nearly three-quarters of patients, was recurrent or atypical infections, including sinopulmonary, viral, fungal and mycobacterial infections. Evaporative cooling induced urticaria, such as that seen in PLAID, was not observed in any of the referred cases. Immunologically, antibody deficiency (low IgG and/or IgA) and B-cell abnormalities (reduced numbers of absolute and/or memory B-cells) were the most common feature, but CD4+ lymphopenia and NK cell abnormalities (reduced NK cell numbers and/or impaired NK cell function *ex vivo*) were also observed.

In silico examination of *PLCG2* variants.

Sixty distinct, protein-altering variants of *PLCG2* were identified among this group of 76 people (Supplementary Table 3). These variants spanned the protein coding sequence of *PLCG2* and included 55 missense substitutions, 2 in-frame deletions and 3 truncating mutations (1 frameshift deletion-early termination mutation, 2 nonsense mutations; Fig 1A). Three of the 60 variants have been reported previously in association with ID[7,9,11]. All variants were rare (f < 0.001) in the gnomAD population, and 40% were not observed in any gnomAD participant (Fig 1B). All 60 variants occurred at evolutionarily conserved positions (SiPhy>3) and 80% localized to highly constrained residues (SiPhy>10; Fig 1C). Most of the 60 variants were predicted to be highly damaging, with 85% of variants scoring in the top 1% of predicted deleteriousness (phred-scaled CADD>20) and 13% in the top 0.1% (phred-CADD>30; Fig 1D). When compared with protein-altering *PLCG2* variants from gnomAD, the ID variants had a greater degree of conservation and predicted deleteriousness (p<2.2E-16; Supplementary Figure 1; Supplementary Tables 4 - 6). These *in silico* findings support the potential pathogenicity of these *PLCG2* variants.

Majority of observed *PLCG2* variants alter B-cell activation.

To evaluate the functional effects of *PLCG2* variants *in vitro*, we transiently expressed EGFP-tagged WT or mutated forms of *PLCG2* in a *Plcg2*-deficient DT-40 B-cell line (Supplementary Figure 2). Intracellular calcium levels and pERK were quantified by flow cytometry before and after BCR cross-linking to quantify B-cell activation in real time.

Using this approach, we determined that 42 of the 60 *PLCG2* variants led to altered calcium flux and/or ERK phosphorylation following stimulation, including 29 variants with reduced function and 13 variants with increased function, relative to WT *PLCG2* (Figure 1). The effects of the variants on calcium flux and ERK phosphorylation were strongly correlated (Spearman ρ = 0.800 [0.646, 0.890], Supplementary Figure 3).

Phenotypes of *PLCG2* variants associated with reduced B-cell activation.

Twenty-nine of the *PLCG2* variants produced weaker calcium flux and/or less ERK phosphorylation than WT *PLCG2* following BCR ligation (Figure 2, Supplementary Figure 4A). These LOF variants were distributed across the protein (Figure 2A). The size of the reductions ranged from modest to profound, and when stratified by relative stimulation-induced ERK phosphorylation, 17 variants displayed modest reduction (fold change 0.75–1.0), 6 had moderate reduction (fold change 0.5–0.749), 3 had severe reduction (fold change 0.25–0.499) and 3 variants led to a profound reduction of ERK phosphorylation (null variants; fold change <0.25; Figure 2). The effect of the 3 null variants on calcium flux was even more dramatic than the reduction of pERK, with all 3 variants failing to generate even a minimal increase in cytoplasmic calcium following stimulation.

Among 37 participants with hypomorphic mutations, recurrent or atypical infection was the most common feature (84%, Table 2). Beyond recurrent sinopulmonary infections, 10 people had recurrent or severe viral infections, including 8 with systemic herpesvirus infections (Epstein-Barr virus, cytomegalovirus and/or varicella zoster virus). Fungal infection was observed in 5 patients, including diffuse mediastinal coccidioidomycosis, chronic mucocutaneous candidiasis and recurrent fungal skin infections. There were also 3 cases of mycobacterial infection, including 1 case of *M. abscessus* infection and 2 cases with infection of mycobacterium avium complex. Autoinflammatory features were present in 68% of people with hypomorphic variants, including periodic fever (12), enteritis (9), ILD (4) and granulomatous disease (3). Other common features of ID in people with hypomorphic variants included antibody deficiency (51%), atopy (38%), low NK cell numbers (16%) and lymphoproliferation or malignancy (14%). Spearman correlation analysis revealed a weak correlation between LOF variants and periodic fever (ρ=0.24); Supplementary Table 7). When the clinical features of the 6 patients with extreme hypomorphic variants (severe or null) were compared to those of the 24 patients with modest hypomorphisms, no significant differences in their manifestations were observed. This suggests that clinical ID caused by hypomorphic variants of *PLCG2* does not vary based on the degree of LOF (Supplementary Table 8).

Two hypomorphic variants were observed in multiple unrelated subjects. The V735M hypomorphic variant was observed in 3 independent probands with ID, all of whom manifested recurrent infections (2 fungal, 1 mycobacterial) and autoinflammation, and 2 of whom also had atopic disease. Similarly, the hypomorphic R89C variant was identified in 2 unrelated probands from Finland, one a heterozygote and the other a homozygote, both of whom manifested severe lymphoproliferation that ultimately evolved into lymphoma that was not associated with EBV or CMV viremia.

Autosomal dominant transmission of haploinsufficiency of PLCG2 featuring autoinflammation, bacterial and viral infection, and NK dysfunction.

Among the 60 probands were four multigenerational families in whom autosomal dominant ID co-segregated perfectly with *PLCG2* variants (Figure 3). Family 1 (T292M) included a father and son who both experienced autoinflammation, recurrent sinopulmonary and viral infections, low levels of NK cells and low class-switched peripheral B-cells. Family 2 (N1097del) was composed of a mother, son and daughter, all of whom had CVID and recurrent herpesviral infections. The children both experienced severe enteritis and recurrent febrile episodes with oral ulcers, eczema and defective NK cell killing (Figure 3). Family 3 (L170F) consisted of a father and son who together bore striking phenotypic similarity to family 2, with recurrent fevers, oral ulcers, recurrent herpesvirus infections and low numbers of circulating NK cells. Family 4 (E565D) included a mother and daughter who both experienced recurrent sinopulmonary infections, antibody deficiency and recurrent inflammatory episodes that featured fever, myalgia and arthritis. E565D was also observed in an unrelated proband whose features were very similar to those of family 4. The mutations from families 1, 2 and 4 were each modestly hypomorphic, producing ~15% reductions in pERK *in vitro*. Despite the fact that it did not reach statistical significance, the variant in Family 3 actually led to a 10% reduction in calcium flux, relative to WT.

Similar to families 1–3, recurrent invasive viral infections and NK cell defects were observed in two singleton probands with modestly hypomorphic variants (L183F and H193Q), one of whom was investigated in greater detail in a paralell study[22]. Given the observed phenotypes of severe viral infections in many of these cases and reduced NK cell numbers in some, we hypothesized that heterozygous loss of PLCγ2 signaling could lead to a focal immune regulation disorder. We therefore sought to examine whether NK functional impairment might be more pronounced than the B-cell defect in primary patients cells, thus representing an additional mechanistic explanation for a number of the phenotypes seen in this cohort. When primary NK cells from families 2 and 3 were isolated and stimulated, *ex vivo*, they had a significantly reduced cytotoxic capacity that was not restored by stimulation with IL-2 (Figure 4). NK maturation, as indicated by the ratio of $CD56^{bright}$ to $CD56^{dim}$ NK cells, was also evaluated in members of families 2 and 3. This revealed an accumulation of immature CD56bright NK cells in patients carrying N1097del (Supplementary Figure 5), a finding that was not shared by the carriers of L170F. This suggests that *PLCG2* variation may have a partially penetrant effect on NK cell maturation, and that the maturation phenotype is not required for the NK cell killing defect. Importantly, we noted that in 4 individuals from 2 families, NK cell calcium flux, degranulation and/or killing was impaired (Figure 3 and 4; Supplementary Figure 6) even when B-cell function was impacted only modestly, if at all, arguing that there are cell-type specific effects of these LOF variants.

To further evaluate the LOF mutations, DT-40 cells were co-transfected with equimolar quantities of mCherry-fused WT *PLCG2* and GFP-fused LOF variants of *PLCG2*. Unlike the mutations that cause PLAID, which exert a complex dominant-negative effect[4], the activation of cells bearing the LOF variants could be reconstituted, at least in part, by co-expression of WT *PLCG2* (Supplementary Figure 4). This result argues that these LOF variants act in a haploinsufficient manner. Furthermore, we observed that a subset of LOF

variants led to reduced expression of PLCγ2 protein in the DT-40 overexpression system (Supplementary Figure 4), as well as in primary PBMC *ex vivo* (Supplementary Figure 7). Consistent with this, studies of predicted LOF (pLOF) variation in the gnomADv2.1.1 dataset show that loss of a single copy of *PLCG2* is not tolerated, with the highest possible probability of LOF intolerance (pLI) score of 1.0[23,24].

A common *PLCG2* variant already links reduced expression of *PLCG2* with immunopathology. R268W, which is associated with both inflammatory bowel disease[16] and coccidioidomycosis[25], correlates with reduced expression of *PLCG2* in several cell and tissue types (Supplementary Figure 8). Primary PBMC from R268W heterozygotes demonstrated reduced inflammatory cytokine production in response to stimulation with the PLCγ2-dependent beta glucan, dectin-1[25]. In the DT-40 overexpression system, we observed that R268W produces reduced stimulation-induced calcium flux, similar to P317L variant that we identified in a patient with severe coccidiodiomycosis (Supplementary Figure 8).

Novel GOF and constitutive activation variants in PLCG2.

Thirteen *PLCG2* variants led to stronger calcium flux and/or increased ERK phosphorylation than WT *PLCG2* following BCR ligation. These included 11 previously unreported variants and 2 GOF mutations known to cause APLAID (A708P and L848P; Figure 5)[7,9]. Most hypermorphic variants clustered in the X-Y linker region of the protein, however 4 variants were situated in the Y-box and C2 domains (Figure 5A). Six of the 13 variants also led to increased ERK phosphorylation following IgM cross-linking (Figure 5C), while one paradoxically led to a slight reduction. In addition to exaggerated responses to stimulation, 6 of 13 hypermorphic mutations exhibited constitutive cellular activation, *in vitro*, as indicated by elevated cytoplasmic calcium concentration in unstimulated/resting cells (Figure 5B). Consistent with the *in vitro* findings, each constitutively activating mutation is located at or near residues where activating somatic mutations have been shown to facilitate mutational escape of BTK inhibition by chronic lymphocytic leukemia (CLL; Figure 5; Table 2)[26–28]. Such escape mutations have been shown to involve the cSH2, sPH, Y-Box and C2 domains[28–30].

Clinically, autoinflammatory features characteristic of APLAID were present in 77% of individuals with hypermorphic mutations, including 5 with granulomatous disease and 5 with inflammatory bowel disease. There were 3 subjects with interstitial lung disease, 1 of whom required lung transplantation (G699S) and another who required lobectomy (L845S) (Tables 2 and 3). Atypical or recurrent infections were also observed in 77% of participants, including 8 with recurrent sinopulmonary infections, 4 with recurrent viral infections, and 4 with fungal infections. Mucocutaneous candidiasis was present in 3 people, including one with candidal esophagitis and another who succumbed to mixed candidal and CMV colitis. A third person died after developing disseminated aspergillus fumigatus infection. Other features of ID in people with hypermorphic variants included atopy (62%), B-cell lymphopenia (31%), low memory/class-switched memory B-cells (46%), autoimmunity (38%) and low IgG, IgA, and in some cases IgM (31%). Spearman correlation analysis revealed moderate correlations between GOF variants and B-cell defect (ρ=0.47) and

granulomatous disease (ρ=0.41; Supplementary Table 8). No one carrying a hypermorphic variant knew of an affected family member. The R742C mutation was paternally transmitted to our proband from a father with no known history of immunologic disease.

Discussion

Our data show that different functional variations in *PLCG2* can lead to a spectrum of presentations which appear to be variations on the theme of *PLCG2*-associated antibody deficiency and immune dysregulation (PLAID). The interpretation of VUS is a major obstacle to the incorporation of molecular genetics data into the immunology-allied subspecialty clinics. Faced with a large collection of monoallelic *PLCG2* VUS in individuals with ID, many with clinical presentations distinct from the known *PLCG2*-associated monogenic diseases, we classified the functional effects of the variants and revealed the complexity and limitations of *PLCG2* variant evaluation. The majority of *PLCG2* VUS (72%) produced a functional effect on *PLCG2*-dependent activation, *in vitro*, and we sought to define genotype-function-phenotype relationships and identify novel discrete clinical entities. First, we found that the majority of functional *PLCG2* variants lead to LOF of varying degrees in our system. Familial segregation studies uncovered a discrete *PLCG2*-associated entity composed of humoral immune deficiency, autoinflammation, susceptibility to herpesviral infection and NK cell dysfunction that is linked to heterozygous loss of PLCγ2 function (Figure 3). Recurrent variants observed in singleton probands reinforced the associations between LOF variants and recurrent infection and autoinflammatory features (V735M) and lymphoproliferation/lymphoma (R89C). Second, we found that one-third of the functional variants led to increased signaling in our system and were largely associated with clinical presentations seen in APLAID though with more broad phenotypic variability, reflecting in part the variability in the degree of GOF activity. Some GOF variants associated with distinct and/or novel phenotypes suggesting either an expansion of the clinical presentations associated with APLAID, or novel signaling consequences leading to separate phenotypes which require additional mechanistic investigation. Finally, we observed enrichment of other phenotypes, including atopic diseases, fungal infections, mycobacterial infections and cytopenias, however Spearman correlation analyses failed to identify any significant correlations with LOF variants. This may be in part related to the heterogeneous landscape of clinical features in people with LOF mutations, which largely overlapped with those in people with GOF mutations, suggesting that gain and loss of PLCG2 function as measured in our B-cell transfection assay can lead to similar outcomes. Additional cell-specific functional analyses and larger association studies are needed to a) determine whether a variant leads to functional changes or lack thereof in non-B-cells and b) determine whether there are true causal relationships between a number of the observed variants and clinical outcomes.

Although 7 out of 10 *PLCG2* mutations previously reported to cause ID were reported to be GOF mutations[4,6–12] 29 of the 42 functional *PLCG2* variants identified in this study led to LOF. Co-expression of wt *PLCG2* with each of the 6 most severe hypomorphic variants partially restored downstream signaling, indicating that these variants likely act through relative haploinsufficiency of *PLCG2*. This is mechanistically distinct from PLAID, where in-frame deletions of the X-Y linker region result in dominant negative LOF at

physiologic temperature and unbridled cellular activation at sub-physiologic conditions[4]. We did not note temperature sensitivity in the variants we evaluated here. The individual and familial cohort studies showed that even modest loss of *PLCG2* function can be associated with increased susceptibility to bacterial and herpesviral infection, autoimmunity, atopy, inflammation and malignancy. Importantly, when primary cells were available, we saw that several mutations (N1097del, L170F) appear to impact NK cells more profoundly than B-cells, suggesting that the impact of the mutations may differ between cell types. If this is the case, then it is possible that a B-cell overexpression system may not capture the full impact of some *PLCG2* mutations.

The variability of *PLCG2* variant impact from cell type to cell type may reflect differences in the cell-intrinsic expression levels of *PLCG2*, *PLCG1* or other associated signaling molecules. It is noteworthy that both NK cells and monocytes express significantly less *PLCG2* than B-cells (Supplementary Figure 9)[30]. The P522R variant in *PLCG2*, which has been identified as a protective factor against the development of Alzheimer's disease, leads to modest hypermorphic activity in myeloid-derived oligodendrocytes, however in the DT-40 B-cell overexpression system, this variant did not lead to alteration in either calcium flux or ERK (data not shown). If confirmed, the presence of cell-type specific effects of *PLCG2* variation will add an additional layer of complexity to efforts to molecularly classify *PLCG2* variants.

The phenotypic overlap of subjects with GOF and LOF mutations in *PLCG2* suggests that the clinical presentations of PLAID and APLAID may well represent a continuum of impact of the variants as opposed to clean GOF vs. LOF. The temperature sensitivity in PLAID is unique, and as such the data here are relevant only for consequences of variants at physiologic temperatures. Further illustrating this point, we observed that the H193Q mutation, which was previously reported to cause APLAID and assumed to be a GOF mutation[11], is in fact hypomorphic, *in vitro* (Figure 4). The published patient's clinical features included fever, enterocolitis and recurrent vesiculopustular eruptions, which were consistent with the features that we observed in our proband with H193Q and numerous other individuals/families with LOF variants in our study (L170F, T292M, N1097del, L183F).

Although there is substantial phenotypic overlap between individuals with GOF and LOF mutations, as evidenced by the comparably high burdens of infectious and inflammatory complications in both groups (Table 2), a closer inspection reveals some differences in the types of inflammatory features and infections that they develop. For example, ILD (10–15%) and enteriocolitis (25–30%) were observed at similar frequencies in subjects with GOF or LOF variants, whereas periodic fever was more commonly observed with LOF mutations (32% vs. 8%) and granulomatous disease was more prevalent with GOF mutations (38% vs. 8%). Similarly, recurrent viral infections were present at similar levels in both groups, whereas invasive viral infections (often with herpesviruses) were more common in people with LOF mutations (22% vs. 8%), in whom they often co-existed with NK cell dysfunction. Mycobacterial infections were only observed in subjects with LOF mutations (n=3).

Rare but severe fungal infections were also present in subsets of people with either GOF or LOF mutations, which was not unexpected given the dependence of key fungal pattern recognition sensors (dectin-1 and dectin-2) on *PLCG2* signaling[31–33]. Disseminated coccidioidomycosis was present in a person with P317L, a severely hypomorphic mutation. Consistent with this, Hsu et al. described a significant association between common germline dectin-1/PLCγ2 pathway variation and disseminated coccidioidomycosis[34], further observing that PBMC of patients with *PLCG2* variants displayed reduced dectin-1 signaling.

We have previously reported that beyond cold urticaria, atopic disease and/or IgE elevation is present in more than half of cases of PLAID [21], but it has not been described in APLAID. In addition, heterozygous loss of *Plcg2* function leads to enhanced antigen-specific IgE responses in mice[35]. Here, we report increased susceptibility to an array of atopic diseases among people with either GOF (62%) or LOF (38%) mutations of *PLCG2*. While atopic dermatitis was present in around a quarter of cases with both LOF and GOF mutations, other atopic features were observed slightly more commonly with GOF than LOF mutations.

Non-allergic cutaneous manifestations are also commonly observed in both PLAID and APLAID, including granulomatous and vesiculopustular eruptions. Here, we identified 4 individuals with *PLCG2* LOF mutations and lichenoid skin conditions, including oral lichen planus (LP) in 2 unrelated females (R50W, V735M) and lichen sclerosis (LS) in a mother and daughter with NK cytotoxicity defects (N1097del). Although the causes are LP/LS are unknown, defective cytotoxic killing by peripheral NK cells[36] and familial clustering suggestive of genetic susceptibility[37] have both been described. We also observed a patient with hidradenitis suppurativa and a *PLCG2* GOF mutation (T706I). Of note, an existing case report describes a man with hidradenitis suppurativa in whom a rare missense mutation of *PLCG2* (T983I) was found. Based on these findings, an examination of *PLCG2* variation in population-based studies of each of these conditions is warranted.

Lymphopenia and other forms of cytopenia, as well as lymphoproliferation seen in association with GOF and LOF variants requires additional mechanistic examination to determine whether lineage-intrinsic effects—such as reported roles for PLCγ2 in thymic T cell selection[38]—or extrinsic effects—such as B-cell tolerance defects leading to anti-hematopoetic cell antibody formation[39], enhanced NK-mediated ADCC reported in certain patients with idiopathic CD4 T-cell lymphopenia[37], and impaired NK cell-mediated tumor surveillance or immune dysregulation[40,41].

Based on our observations, the penetrance and expressivity of LOF and GOF *PLCG2* variants are variable. Multi-generational examinations were not undertaken for most probands, however we clearly observed cases of penetrant familial variants and recurrent variants, together with a few instances of incomplete penetrance (Supplementary Table 2). Without larger family cohorts and information on the phenotypes of other carriers of variants reported here, it is not possible to fully assess the penetrance of individual *PLCG2* variants.

There are important consequences to understanding the specific pathophysiologic mechanisms of *PLCG2* variants, given that a precision therapy might aim to suppress or augment PLCγ2 enzymatic activity depending on the defect. Specific variants or functional

classes of variants might have important prognostic implications, such as conferring protection against (or risk of) developing Alzheimer's disease[18], further highlighting the importance of understanding the pathophysiology of those variants. Recently G-CSF was identified as a putative pathogenic biomarker In APLAID[42], and diminished G-CSF is associated with increased risk for Alzheimer's dementia[43,44]. As such, measurement of G-CSF levels in the serum of carriers of these variants would be an important correlate to the functional assays. In order to best determine the complete cell-specific and pathway roles for these variants, future studies are required, such as CRISPR knock-in of variants into primary T, B, NK, and myeloid lineage cells, and via dissection of multiple downstream signaling and cellular functions beyond beyond phospholipase activity, calcium flux, MAP Kinase activation, and NK cell killing.

In summary, this study expands the spectrum of genotypic and phenotypic associations with functional variation in *PLCG2* and defines a novel immune disorder associated with heterozygous loss of *PLCG2* function. Surprisingly, autoinflammatory and infectious phenotypes transcended functional classes, suggesting that PLAID and APLAID may represent a false dichotomy. To address this, we propose to use the name *PLCG2* associated immune dysregulation (PLAID) to refer to all subjects with immune dysregulation caused by *PLCG2* variation, with specification of molecular effect, such as dominant negative PLAID, hypermorphic PLAID, or haploinsufficient PLAID. More diverse assays are required to assess the specific impact(s) of *PLCG2* variants on human disease and one can foresee revisions of the sub-classifications as additional molecular insights are gained.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

APLAID	autoinflammatory PLCG2-associated antibody deficiency and immune dysregulation
BCR	B-cell receptor
BTK	Bruton's tyrosine kinase
CADD	combined annotation dependent depletion
DAG	diacylglycerol gnomAD, Genome Aggregation Database
GOF	gain-of-funciton
ID	immune dysregulation
IP3	inositol 1,4,5-triphosphate
LOF	loss-of-function
NK	natural killer
PIRD	primary immune regulation disorder
PIP2	phosphatidylinositol 4,5-bisphosphate
PLAID	PLCG2-associated antibody deficiency and immune dysregulation
PLCG1	phospholipase C gamma 1
PLCG2	phospholipase C gamma 2
SiPhy	Site-specific phylogenetic analysis
WT	Wild type

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Capsule Summary

This study of people with immune dysregulation and *PLCG2* variants reveals that *PLCG2* variation leads to a spectrum of functional changes which may differentially impact cell types and risk for a broad set of immune phenotypes.

Clinical Implications

PLCG2 variants can be frequently encountered in patients with immune dysregulation. While variants which cause PLAID and APLAID have been identified, other classes of variation suggest a haploinsufficient mechanism with variable penetrance, necessitating functional evaluation of variants, as well as further genotyping and phenotyping of carriers to best determine their pathogenicity.

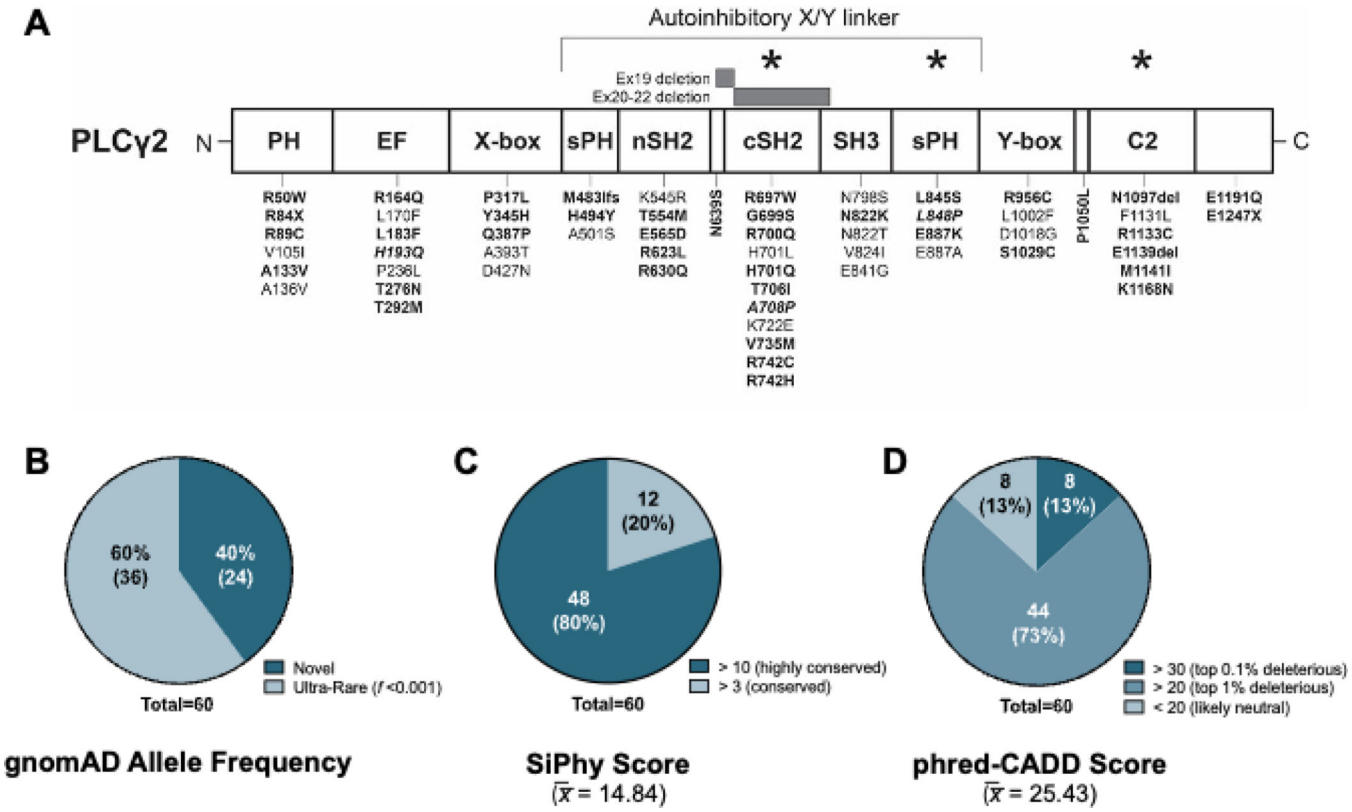


Figure 1. Summary of *PLCG2* variants identified in subjects with immune dysregulation.

A linear schematic of PLCγ2 displays the domain localization of *PLCG2* variants identified in subjects with immune dysregulation in this study (A). PLAID-causing deletions (grey bars) and gain-of-function hotspots (asterisks) are noted above the schematic; variants that alter *in vitro* activation in this study are shown in bold; previously reported variants are italicized. In panels B-D, pie charts illustrate distributions of variant characteristics, including variant allele frequencies from the Genome Aggregation Database (gnomAD) v2.1.1 population (B), degree of evolutionary conservation as indicated by SiPhy scores (C), and damage prediction as indicated by phred-scaled CADD scores (D) for the 60 *PLCG2* variants examined in the study.

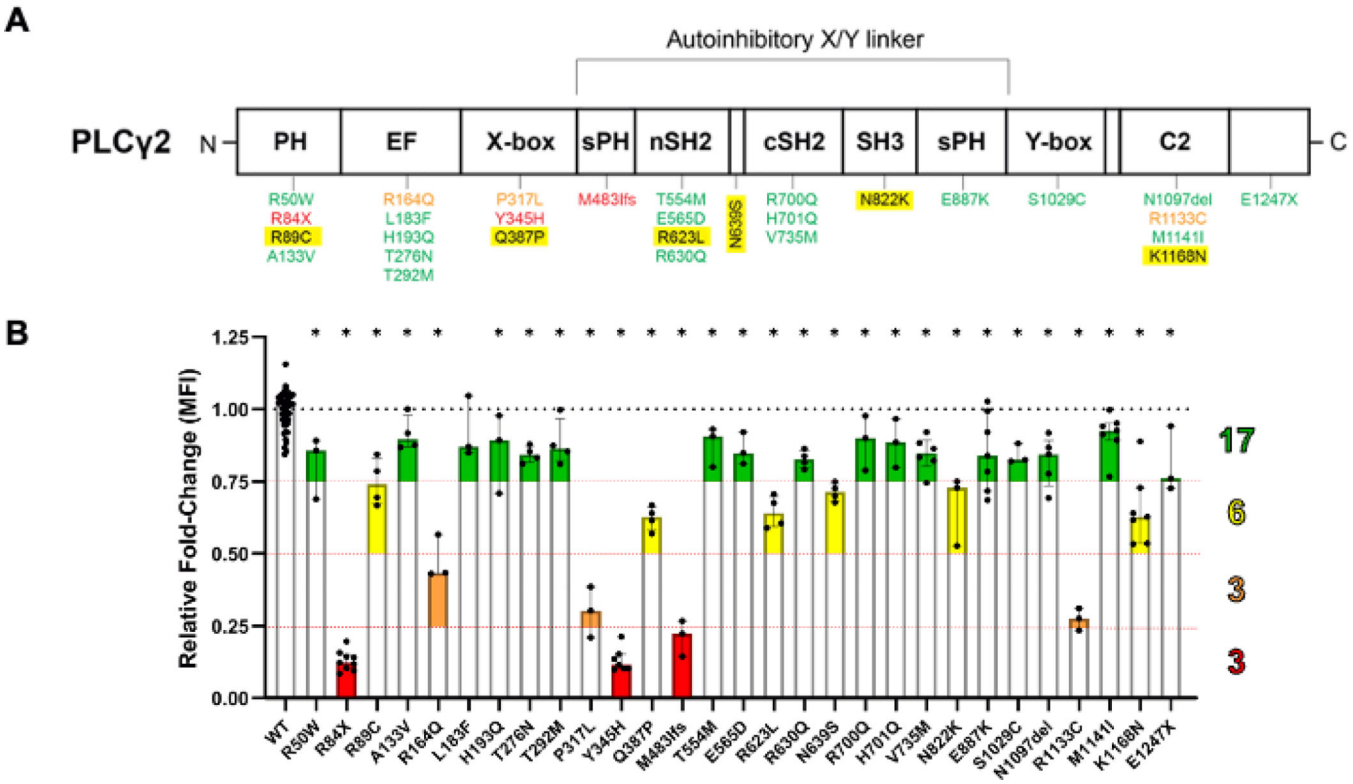
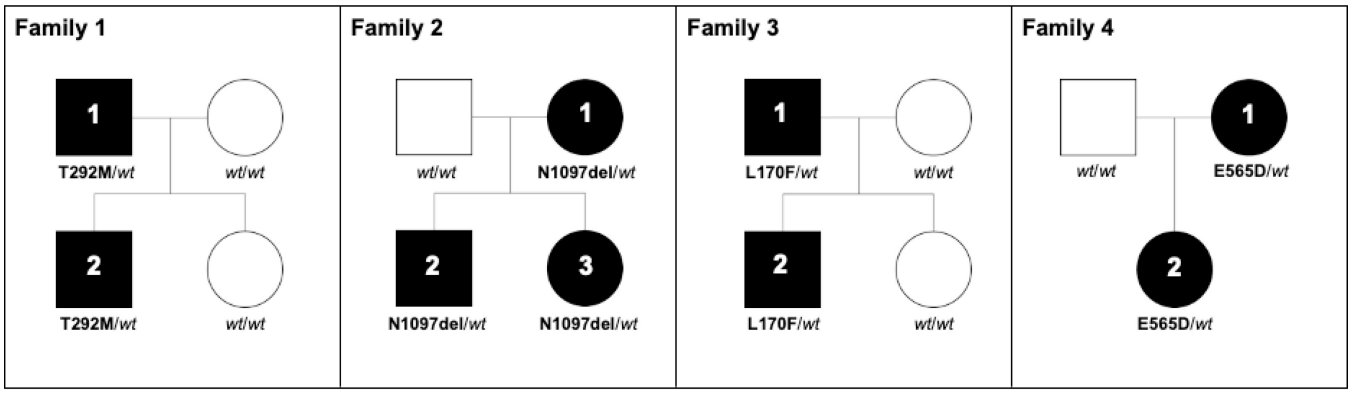


Figure 2. Loss of function variation is the largest functional class of *PLCG2* variation in subjects with immune dysregulation.

A linear schematic of PLCγ2 displays the domain localization of the 29 hypomorphic variants identified in subjects with immune dysregulation in this study **(A)**. In **Panel B**, bar graphs display relative fold-change (FC) of ERK phosphorylation by flow cytometry in *Plcg2*-deficienct DT-40 cells overexpressing either wild type or mutant *PLCG2* constructs 5 minutes after BCR cross-linking **(B).** The bars indicate the median of 3 or more independent experiments, error bars indicate interquartile range, and asterisks indicate statistical significance ($p < 0.05$) by Mann-Whitney nonparametric test. Variant labels **(A)** and bar plots **(B)** are colored to indicate the degree of hypomorphism (FC>0.75, green; FC 0.75–0.51, orange; FC 0.49–0.25, yellow; FC<0.25, red).



Family-Subject	Autoinflammatory	Autoimmune	Infectious	Atopic/Allergic	Immunologic
1-1	IBD		Recurrent sinopulmonary and viral infections		Low IgG, low B and NK cells, low memory B cells
1-2	ILD, uveitis		Recurrent sinopulmonary infections		Low B cells
2-1		Lichen sclerosus	Recurrent herpesvirus infections		Low IgG/IgA
2-2	Recurrent fever, oral ulcers, IBD	Sjogren's disease, autoimmune thyroiditis	Recurrent sinopulmonary and herpesvirus infections	Eczema	Low IgA/IgM, Low NK cells, NK killing defect, NK maturation defect
2-3	Recurrent fever, oral ulcers, IBD	Lichen sclerosus	Recurrent sinopulmonary and herpesvirus infections	Eczema, urticaria	Low IgG/IgA, Low NK cell cells, NK killing defect, NK maturation defect
3-1	Recurrent fever, oral ulcers		Recurrent herpesvirus infections		Low NK cells, NK killing defect
3-2	Recurrent fever, oral ulcers		Recurrent sinopulmonary and herpesvirus infections	Eosinophilic esophagitis	Low IgG/IgA/IgM, Low NK cells, NK killing defect
4-1	Recurrent fever	Arthritis	Recurrent sinopulmonary infections		Low IgG/IgA
4-2	Recurrent fever	Arthritis	Recurrent sinopulmonary infections		Low IgG/IgA

Figure 3. Autosomal dominant familial immune dysregulation co-segregates with variants of uncertain significance of *PLCG2*.

Pedigrees are displayed for 4 families in whom *PLCG2* variants co-segregated with multi-generational immune dysregulation. The *PLCG2* variant status is indicated beneath each subject. The table displays a summary of clinical features of immune dysregulation observed in the affected members of the 4 families. *wt,* wild type; IBD, inflammatory bowel disease; NK, natural killer; ILD, interstitial lung disease.

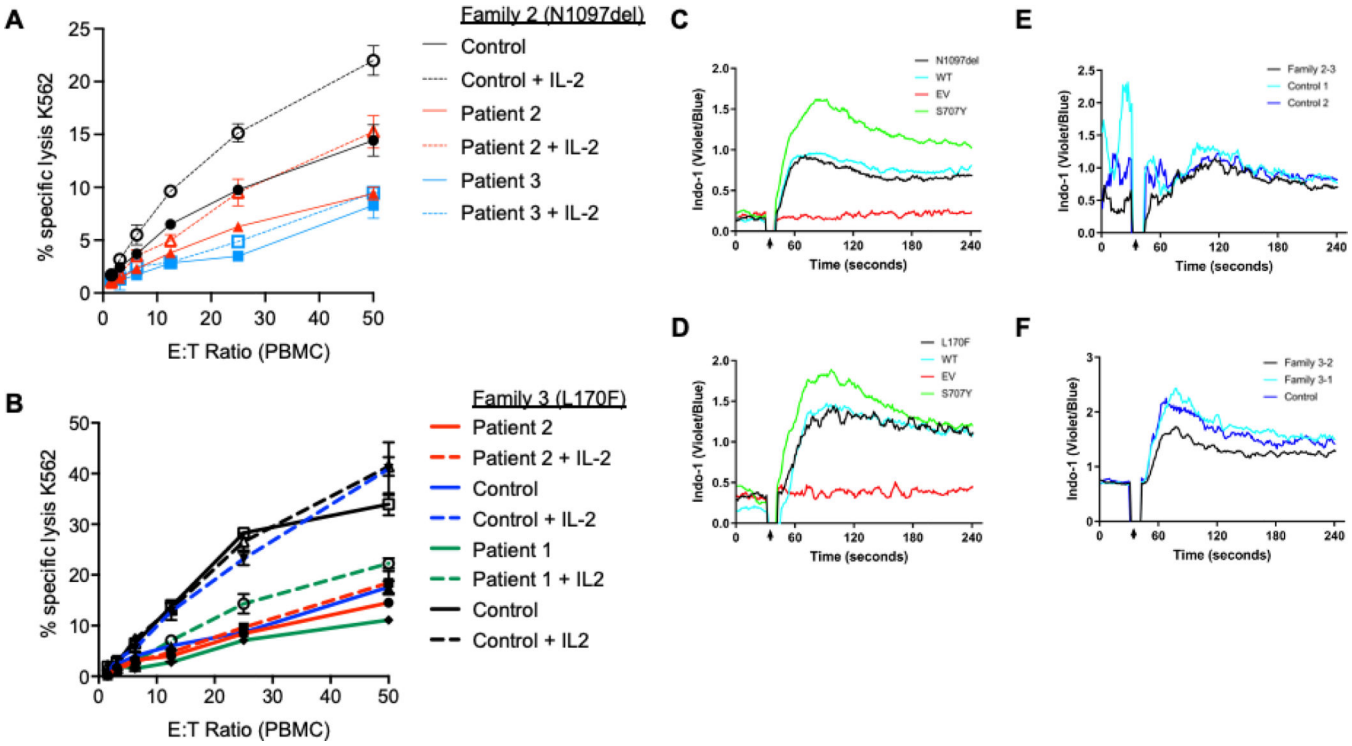


Figure 4. Deficient natural killer cell activity in 2 families with *PLCG2* variants, autoinflammation and recurrent herpesvirus infections.

NK *c*ytotoxic killing, expressed as % specific lysis of K562 cells in ^{51}CR release *ex vivo* assay, is plotted in 2 affected subjects from Family 2 and an unrelated control subject **(A)** and 2 affected subjects and an unaffected family member from Family 3 **(B).** Representative tracings of *in vitro* stimulated calcium flux in *Plcg2*$^{-/-}$ DT-40 cells expressing N1097del **(C)** and L170F **(D)** variants are paired with tracings of *ex vivo* stimulated calcium flux in primary B-cells from Family 2–2 and 2 unrelated control subjects **(E)** and 1 unaffected and 2 affected members of Family 3 **(F)**. *In vitro* tracings are representative of 3 or more independent experiments. IL-2, interleukin-2.

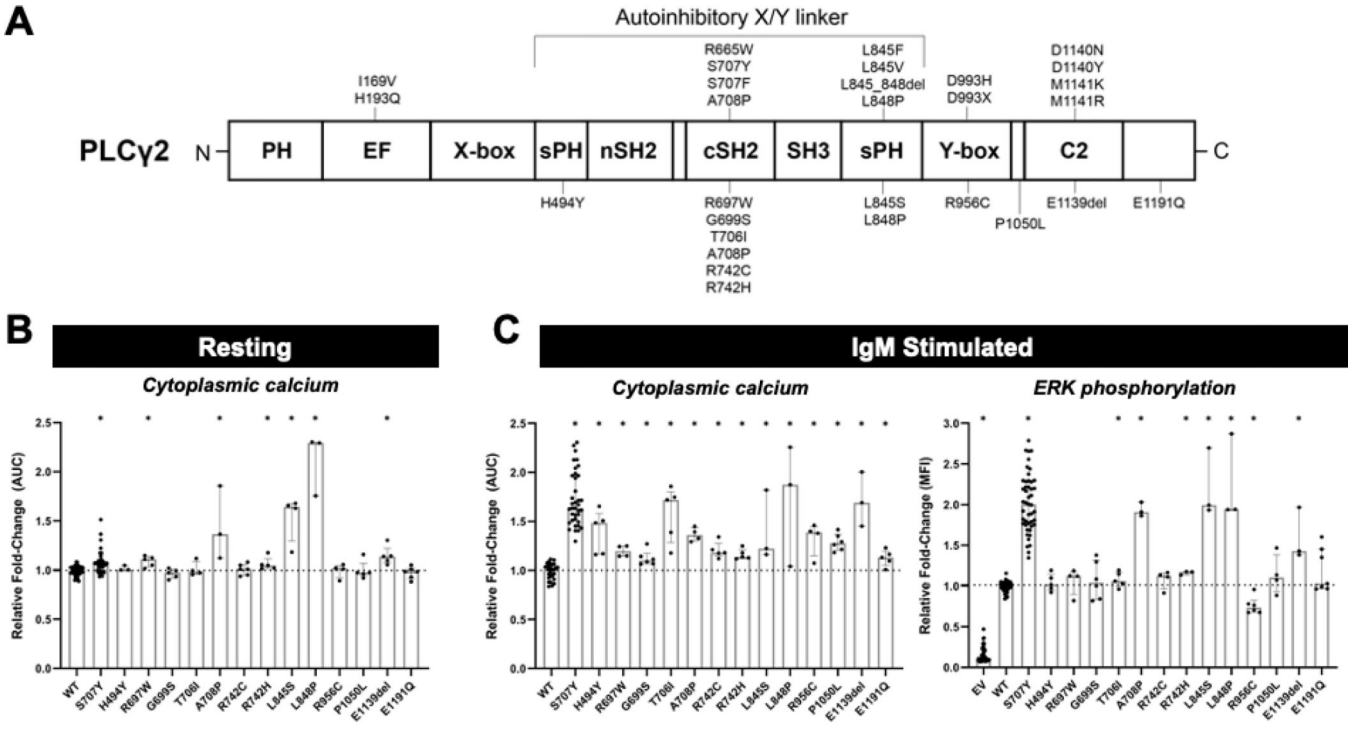


Figure 5. A subset of *PLCG2* variants from subjects with immune dysregulation are hypermorphic following stimulation and/or constitutively active.

A linear schematic of PLCg2 displays the domain localization of reported hypermorphic variants of *PLCG2* **(above),** together with the hypermorphic variation identified in subjects with immune dysregulation in this study **(bottom, A).** In **Panels B and C,** wild-type (WT) or mutagenized *PLCG2* plasmids were overexpressed in a *Plcg2*-deficienct DT-40 B-cell line and transfected cells were stimulated with IgM. Cytoplasmic calcium was measured in resting cells by flow cytometry for 5 seconds and displayed as fold change of the area under the curve (AUC) for that interval, relative to WT *PLCG2* **(B).** Cytoplasmic calcium was also measured by flow cytometry for 240 seconds following stimulation with IgM and ERK phosphorylation was measured by flow cytometry 10 minutes after stimulation **(C).** Calcium data are displayed as fold change of the area under the curve (AUC) for the interval, relative to WT *PLCG2*. ERK phosphorylation is displayed as fold-change of mean fluorescence intensity (MFI), relative to WT *PLCG2*. Bars indicate the median of 3 or more independent experiments, error bars display the interquartile range. Statistical comparisons were performed using the Mann-Whitney U Test. *, $p < 0.05$. EV, empty vector. WT, wild type.

Table 1.

Clinical Features of Immune Dysregulation among 76 Subjects with PLCG2 Variants of Uncertain Significance

Clinical Phenotypes	n	%
Recurrent and/or Atypical Infection	**58**	**76%**
Recurrent Sinopulmonary	42	55%
Viral	22	29%
Fungal (Candida, Coccidiodes, Aspergillus)	11	14%
Mycobacterial (Abscessus, Avium)	4	5%
Autoinflammation	**46**	**61%**
Enterocolitis	18	24%
Periodic Fevers	18	24%
Interstitial Lung Disease	10	13%
Granulomatous Disease	9	12%
Lymphocyte Abnormality	**40**	**53%**
B Lymphocyte Deficiency	27	36%
B-cell Lymphopenia	16	21%
B-cell Defect (low class-switch/memory) *	16 (of 24)	21%
CD4/CD8 Lymphopenia	14	18%
NK Cell Abnormality	13	17%
Low NK cell number	11	15%
Functional NK cell defect (degranulation/killing) **	5 (of 5)	7%
Cutaneous Inflammation	**39**	**51%**
Non-atopic Cutaneous Inflammation	19	25%
Atopy (Food Allergy, AD, Elevated IgE, Eosinophilia)	**34**	**46%**
Antibody Deficiency ***	**33**	**43%**
Autoimmunity	**21**	**28%**
Malignancy	**5**	**7%**
Lymphoproliferative Disease	**5**	**7%**

* B lymphocyte subset data was available in 24 subjects;

** Natural killer cell functional testing (killing, degranulation) was performed in 5 subjects;

*** Antibody deficiency indicates IgG and/or IgA levels were below the lower limit of normal; NK, natural killer; AD, atopic dermatitis.

Table 2.

Comparison of Phenotypes Observed in Subjects with hypermorphic and hypomorphic variants of *PLCG2*

Clinical Phenotypes	GOF (N=13) n (%)	LOF (N=37) n (%)	P value
Recurrent and/or Atypical Infection	10 (77)	31 (84)	
Recurrent Sinopulmonary	8 (62)	21 (57)	
Viral	4 (31)	10 (27)	
Fungal (Candida, Coccidiodes, Aspergillus)	4 (31)	5 (14)	
Mycobacterial (Abscessus, Avium)	0 (0)	3 (8)	
Autoinflammation	10 (77)	25 (68)	
Gastrointestinal Disease (IBD)	5 (38)	9 (24)	
Periodic Fevers	1 (8)	12 (32)	
Interstitial Lung Disease	3 (23)	4 (11)	
Granulomatous Disease	5 (38)	3 (8)	**0.02**
Autoimmunity	5 (38)	11 (30)	
Peripheral Blood Abnormality	9 (69)	26 (70)	
B Lymphocyte Abnormality	7 (54)	14 (38)	
B-cell Lymphopenia	4 (31)	8 (22)	
B-cell Defect (low class-switch/memory) *	6 (46)	7 (19)	**0.02**
CD4/CD8 Lymphopenia	3 (23)	5 (14)	
NK Cell Abnormality	2 (15)	8 (22)	
Low NK Cell Number	2 (15)	6 (16)	
Functional NK Cell Defect (degranulation/killing) **	NE	3 (8)	
Antibody Deficiency ***	4 (31)	19 (51)	
Atopy (Food Allergy, AD, Elevated IgE, Eosinophilia)	8 (62)	14 (38)	
Cutaneous Inflammation	8 (62)	19 (51)	
Atopic Dermatitis / Eczema	3 (23)	9 (24)	
Non-Allergic Cutaneous Inflammation	5 (38)	12 (32)	
Lymphoproliferative Disease	0 (0)	3 (8)	
Malignancy	0 (0)	4 (11)	

* B lymphocyte subset data was available in 7 GOF and 12 LOF subjects

** Natural killer cell functional testing (killing, degranulation) was performed in 0 GOF subjects and 3 LOF subjects

*** Antibody deficiency indicates IgG and/or IgA levels were below the lower limit of normal; NK, NK cell GOF, gain of function variants; N, number of subjects per class; n, number of subjects per phenotype; LOF, loss of function variants; P value, Fisher exact P value, IBD, inflammatory bowel

Table 3.

Characteristics of hypermorphic variants of *PLCG2* are consistent with established APLAID mutations

Variant	Domain	Autoinflammatory features	Atypical or recurrent infections	Increased pERK	Constitutive	Ibrutinib Resistance
H494Y	sPH	IBD, granulomas, ILD, uveitis				
R697W	cSH2		Recurrent sinopulmonary infections		X	X
G699S	cSH2	ILD (lung transplant)	Chronic mucocutaneous candidaisis			
T706I	cSH2	IBD, granulomas, arthritis, hidradenitis suppurativa		X		
S707Y	cSH2	IBD, granulomas, ILD, uveitis	Recurrent viral infections Recurrent sinopulmonary infections Pulmonary aspergillosis	X	X	X
A708P	cSH2	IBD, granulomas	Severe viral infections Recurrent sinopulmonary infections (ICU)	X	X	X
R742C	cSH2	IBD	Recurrent viral infections Recurrent sinopulmonary infections			X
R742H	cSH2	IBD	Recurrent sinopulmonary infections CMV and candidal colitis (died)	X	X	X
L845S	sPH	ILD (lobectomy), blistering skin rash		X	X	X
L848P	sPH	Granulomas	Recurrent sinopulmonary infections	X	X	X
R956C	Y-Box		Pulmonary aspergillosis (died)	*Reduced*		
P1050L	Y-Box/C2	Periodic fever				
E1139del	C2	Granulomas	Recurrent viral infections	X	X	X
M1141K	C2	IBD, ILD, blistering skin rash	Recurrent viral infections Recurrent sinopulmonary infections	X	X	X
E1191Q	Post-C2		Recurrent sinopulmonary infections			

Prototypic APLAID mutations are shaded in gray[6,10]; pERK, phosphorylated ERK; sPH, split pleckstrin homology domain; cSH2, c-terminal Src homology domain 2; Y-Box, Y-box catalytic domain; C2, C2 domain; ILD, interstitial lung disease; IBD, inflammatory bowel disease