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Cold Urticaria, Immunodeficiency, and Autoimmunity Related to *PLCG2* Deletions

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Abstract

Background—Mendelian analysis of disorders of immune regulation can provide insight into molecular pathways associated with host defense and immune tolerance.

Methods—We identified three families with a dominantly inherited complex of cold-induced urticaria, antibody deficiency, and susceptibility to infection and autoimmunity. Immunophenotyping methods included flow cytometry, analysis of serum immunoglobulins and autoantibodies, lymphocyte stimulation, and enzymatic assays. Genetic studies included linkage analysis, targeted Sanger sequencing, and next-generation whole-genome sequencing.

Results—Cold urticaria occurred in all affected subjects. Other, variable manifestations included atopy, granulomatous rash, autoimmune thyroiditis, the presence of antinuclear antibodies, sinopulmonary infections, and common variable immunodeficiency. Levels of serum IgM and IgA and circulating natural killer cells and class-switched memory B cells were reduced. Linkage analysis showed a 7-Mb candidate interval on chromosome 16q in one family, overlapping by 3.5 Mb a disease-associated haplotype in a smaller family. This interval includes *PLCG2*, encoding phospholipase $C\gamma_2$ ($PLC\gamma_2$), a signaling molecule expressed in B cells, natural killer cells, and mast cells. Sequencing of complementary DNA revealed heterozygous transcripts lacking exon 19 in two families and lacking exons 20 through 22 in a third family. Genomic sequencing identified three distinct in-frame deletions that cosegregated with disease. These deletions, located within a region encoding an autoinhibitory domain, result in protein products with constitutive phospholipase activity. *PLCG2*-expressing cells had diminished cellular signaling at 37°C but enhanced signaling at subphysiologic temperatures.

Conclusions—Genomic deletions in *PLCG2* cause gain of $PLC\gamma_2$ function, leading to signaling abnormalities in multiple leukocyte subsets and a phenotype encompassing both excessive and deficient immune function. (Funded by the National Institutes of Health Intramural Research Programs and others.)

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The genetic dissection of unique inflammatory phenotypes can identify and elucidate immunologic pathways and mechanisms. Such investigations have ultimately led to findings whose significance extends beyond the monogenic diseases harboring the mutations. Examples include the recognition that *FOXP3* is essential for the differentiation of regulatory T cells in *Scurfy* mice and in patients with profound immune dysregulation,¹⁻⁴ the demonstration of a critical role for *AIRE* in thymic negative selection of T cells in patients with a specific autoimmune polyendocrinopathy,⁵ and the identification of *NLRP3* as a critical regulator of interleukin-1 in families with cold-induced inflammation.⁶

Cold-induced urticaria is a unique inflammatory phenotype that is characterized by mast-cell degranulation and triggered by exposure to cold stimuli, a condition that can culminate in life-threatening anaphylaxis.^{7,8} There are various forms of cold urticaria that differ with respect to the mode of inheritance, ages at onset and resolution, and responses to a range of cold provocative testing.⁷ Although mast cells have been clearly implicated in the pathogenesis of cold urticaria,⁹ the processes leading to the degranulation of such cells are poorly understood. We describe three families with cold urticaria, the genetic causes of their disease, and the implications of these findings for the understanding of disease mechanisms.

Methods

Study Subjects

Subjects were admitted to the National Institutes of Health Clinical Center, where they were enrolled in clinical protocols approved by the institutional review board of the National Institute of Allergy and Infectious Diseases. All subjects provided written informed consent.

Genetic Analysis

We isolated genomic DNA from whole blood of members of two families and genotyped single-nucleotide polymorphisms (SNPs). We performed parametric multipoint linkage and haplotype analysis independently in each family, using Merlin software.¹⁰ We carried out whole-genome sequencing in one affected member of Family 1, as described previously.¹¹ We screened the coding exons, splice junctions, and complementary DNA (cDNA) of *PLCG2* for mutations by means of polymerase-chain-reaction (PCR) amplification of overlapping segments and then sequencing of the products using Sanger's method. We mapped genomic deletions using long-range PCR amplification and Sanger sequencing. Additional data on assays, reagents, and primer sequences are provided in the Supplementary Appendix, available with the full text of this article at NEJM.org.

Evaluation of Phospholipase $C\gamma_2$ Function

We assayed the enzymatic activity of phospholipase $C\gamma_2$ (PLC γ_2) in a COS-7-cell transfection system.¹² We measured intracellular calcium flux in peripheral-blood lymphocytes¹³ and determined degranulation of natural killer cells¹⁴ using flow cytometry. We quantified B-cell class switching in cultured peripheral-blood mononuclear cells *ex vivo*.¹⁵ Secondary recombination in B cells was measured by sequencing the *Ig κ* transcript of single CD19⁺CD10⁺IgM^{hi}CD27⁻ transitional B cells obtained by fluorescence-activated cell sorting.¹⁶ We measured the effect of temperature on the intracellular calcium concentration in negatively selected, column-sorted, FLUO-4-stained, unstimulated B cells from the study subjects, using confocal microscopy. Laboratory of Allergic Diseases 2 (LAD2) human mast cells were transfected with mutant *PLCG2* cDNA fused to green fluorescent protein, and transfected cells were then assayed for degranulation by means of flow cytometry. Additional information about assays, reagents, and statistical techniques is provided in the Supplementary Appendix.

Results

Disease Manifestations

We identified three independent families of European ancestry with cold urticaria and variable immune defects (Table 1, and Table S1 in the Supplementary Appendix). The cold urticaria developed at a very young age and was lifelong; the cold-related symptoms in one of these families have been described previously.⁷ The study subjects differed from patients with typical cold urticaria in that they had negative results on skin testing with ice-cube and cold-water immersion. However, they had positive results on skin testing for evaporative cooling (Fig. 1A, and Fig. S1 in the Supplementary Appendix) and generalized exposure to cold air, findings that are consistent with their reports that symptoms were often elicited by cold wind rather than contact with cold objects.

Of the 27 subjects who were tested, 26 had immunologic abnormalities in addition to cold urticaria (Table S1 in the Supplementary Appendix). We detected antibody deficiency in 75% of subjects who were tested, with 56% reporting a history of recurrent infection; 3 subjects had received treatment with intravenous immune globulin for severe recurrent infection and hypogammaglobulinemia. Autoantibodies or autoimmune disease was present in 56% of subjects. Granulomatous disease was observed in 7 subjects from 2 unrelated families; 3 of these subjects were born with cutaneous lesions on the fingers, nose, and ears that disappeared during infancy. The remaining 4 subjects had persistent granulomatous skin disease, and 1 of these subjects had disfiguring granulomata with soft-palate and laryngeal involvement (Fig. S2 in the Supplementary Appendix).

Common laboratory findings in affected subjects included low levels of serum IgM and IgA, depressed levels of circulating CD19+ B cells and IgA+ and IgG+ class-switched memory B cells, and low levels of natural killer cells, whereas IgE levels were elevated in a majority of the subjects (Fig. 1B, and Table S1 and Fig. S3 in the Supplementary Appendix). Neutrophil, monocyte, eosinophil, and basophil counts were normal, as were numbers of naive and memory T cells (Fig. S3 in the Supplementary Appendix).

Of the 21 subjects who were tested for the presence of antinuclear antibodies, 13 (62%) had positive results (Table 1, and Table S1 in the Supplementary Appendix). B cells were characterized by poor in vitro expansion and reduced classswitched-antibody production after stimulation with *Staphylococcus aureus* Cowan I (SAC) and unmethylated cytosine-guanine dinucleotide (CpG)-containing oligonucleotide (Fig. 1C, and Fig. S4 in the Supplementary Appendix). Secondary recombination in CD19+CD10^{high}IgM^{high}CD27^{low} transitional B cells was increased, as shown by the skewing of light-chain gene usage toward the terminal elements, a characteristic that is also observed in Bruton's tyrosine kinase deficiency in patients with X-linked agammaglobulinemia,¹⁷ a disease that is associated with severe impairment in B-cell signaling (Fig. 1D, and Fig. S5 in the Supplementary Appendix). Unlike patients with X-linked agammaglobulinemia, who have few peripheral-blood mature B cells, subjects with *PLCG2* mutations have a substantial mature B-cell compartment, which may lead to an increased occurrence of autoimmunity.

Linkage and Mutational Analysis

Linkage analysis based on the cold urticaria phenotype in Family 1 identified a single linkage interval of 7.7 Mb on chromosome 16q21 with a logarithm of odds (LOD) score of 4.2 (Fig. 2, and Fig. S6 in the Supplementary Appendix). Whole-genome sequencing of one affected member of Family 1 did not identify any novel coding or splice-site single-nucleotide variants, insertions, or deletions within this interval. The linkage interval was narrowed by an independent analysis of Family 2 (Fig. S7 in the Supplementary Appendix), which identified a 12.6-Mb haplotype that segregated with disease status and overlapped the

linkage interval in Family 1 by 3.5 Mb (Fig. 2). Among the 24 genes contained within the narrowed candidate interval, we identified *PLCG2* as the primary candidate gene (Table S2 in the Supplementary Appendix). The encoded protein, PLC γ_2 , is a member of the phosphoinositide-specific phospholipase C family. Members of this family propagate a wide range of extracellular signals by producing diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP₃) through hydrolysis of a phospholipid, phosphatidylinositol 4,5-bisphosphate (PIP₂).¹⁸ The IP₃ that is produced is responsible for calcium release from the endoplasmic reticulum, a critical event in cellular activation. There are two members of the PLC γ family, PLC γ_1 and PLC γ_2 . Although PLC γ_1 is expressed in many cell types, the Rac-responsive PLC γ_2 isoform predominates and appears to have nonredundant functions in B cells¹⁹ and natural killer cells,²⁰ both of which had abnormalities in the three families in our study.

Direct sequencing of cDNA from peripheral-blood mononuclear cells from members of Family 1 identified *PLCG2* transcripts lacking exon 19 on one allele, a mutation that was present only in affected family members. Amplification of the genomic segment of *PLCG2* that is flanked by exons 18 and 20 revealed a heterozygous deletion that cosegregated with cold urticaria (Fig. S8 in the Supplementary Appendix). By directly sequencing PCR products from overlapping segments flanking exon 19, we identified a deletion of 5.9 kb in *PLCG2* (Fig. S8 in the Supplementary Appendix).

Using this same approach, we identified additional heterozygous deletions of *PLCG2* in Families 2 and 3 (Fig. S8 in the Supplementary Appendix). In Family 2, we identified *PLCG2* transcripts that lacked exons 20 through 22, caused by an 8.2-kb deletion. In Family 3, we identified *PLCG2* transcripts that also lacked exon 19 but were caused by a 4.8-kb deletion with breakpoints distinct from those found in Family 1. These family-specific deletions were not detected in 200 healthy subjects of Northern European ancestry. A post hoc analysis of the whole-genome sequence from 1 member of Family 1 confirmed the 5.9-kb deletion that was initially discovered on Sanger sequencing (see the Methods section in the Supplementary Appendix). We propose the term PLAID (PLC γ_2 -associated antibody deficiency and immune dysregulation) to describe this unique constellation of clinical, genetic, and functional findings.

Functional Studies of Mutant PLC γ_2

Each of the three deletions that were identified in these families involved the C-terminal Src-homology 2 (cSH2) domain of PLC γ_2 (Fig. 3A), which is autoinhibitory and normally prevents constitutive enzymatic function.^{21,22} Transfection of COS-7 cells with *PLCG2* expression constructs with a deletion of the full cSH2 domain, a deletion of exon 19 ($\Delta 19$), and a deletion of exons 20 through 22 ($\Delta 20-22$) produced equivalent PLC γ_2 protein expression (Fig. S9 in the Supplementary Appendix) but elevated basal and Rac-activated phospholipase activity, as compared with a nonmutant PLC γ_2 expression construct (Fig. 3A).

Functional Lymphocyte Studies

Despite the gain of enzymatic function caused by disruption of the autoinhibitory domain, distal signaling and PLC γ_2 -dependent functions were diminished in the affected family members. B cells showed defective calcium flux and phosphorylation of extracellular signal-regulated kinase (ERK) in response to surface IgM cross-linking (Fig. S10 in the Supplementary Appendix). Transfection of A20 cells with $\Delta 19$ or $\Delta 20-22$ *PLCG2* resulted in reduced ERK phosphorylation stimulated by B-cell receptor ligation, as compared with untransfected or nonmutant *PLCG2*-transfected cells, indicating a dominant effect of these mutations on B-cell receptor signaling (Fig. S11 in the Supplementary Appendix). In

addition, natural killer cells from affected patients showed reduced calcium flux after cross-linking of activating receptors NKG2D and 2B4 (Fig. 3B) and reduced degranulation after incubation with sensitive target cells (Fig. 3C). In contrast, T-cell calcium flux triggered by CD3 cross-linking was normal (Fig. S12 in the Supplementary Appendix).

Effect of Cold Exposure on Mutant Cells

Cytosolic calcium levels in unstimulated, purified primary B cells from subjects with PLAID steadily rose with decreasing temperature but were unchanged with cooling in nonmutant B cells (Fig. 3D). Likewise, PLAID B cells that were stimulated by B-cell-receptor cross-linking had increased ERK phosphorylation with decreasing temperatures, as compared with nonmutant B cells (Fig. S13 in the Supplementary Appendix). Mutant *PLCG2*-transfected A20 cells had spontaneous ERK phosphorylation at 20°C but not at 37°C (Fig. S11 in the Supplementary Appendix). Moreover, transfection with mutated *PLCG2* into LAD2 mast cells, which endogenously express PLC γ ₂, led to spontaneous degranulation at 20°C, which was not observed in LAD2 cells transfected with nonmutated *PLCG2*. The degranulation was equivalent to that seen after treatment with thapsigargin (Fig. S14 in the Supplementary Appendix) but not in LAD2 cells transfected with nonmutated *PLCG2* (Fig. 3E).

Discussion

In three families with a dominantly inherited syndrome of cold urticaria and pleiotropic immune dysregulation, we found that genomic deletions in *PLCG2* were responsible for this unique phenotype. Diminished receptor-mediated signaling in PLAID B cells resulted in abnormal activation, class-switch recombination, and receptor editing, which have been associated with antibody deficiency, recurrent infection, and impaired central tolerance. Furthermore, the signaling defect in PLAID was temperature-sensitive, as shown by the enhanced signaling and cellular activation observed at subphysiologic temperatures, even in the absence of surface-receptor ligation in B cells and mast cells expressing mutant PLC γ ₂.

The PLAID-associated *PLCG2* deletions affected the regulatory region, including the autoinhibitory cSH2 domain, which normally couples the enzymatic activity of PLC γ ₂ to upstream pathways.²¹ PLAID-associated deletions resulted in the failure of autoinhibition and constitutive phospholipase activity, as measured by standard enzymatic assays. These data are consistent with the hypothesis that, at rest, cSH2 obstructs the PLC γ ₂ catalytic site and that this inhibitory interaction is relieved by phosphorylation of critical tyrosine residues, which facilitates a conformational change in PLC γ ₂, thereby exposing the catalytic site.²¹⁻²³

Nonetheless, B cells and natural killer cells from subjects with PLAID clearly had decreased PLC γ ₂-dependent signaling and function, with responses similar to those observed in *Plcg2*-deficient mice.¹⁹ This apparently paradoxical loss of downstream function may be the direct result of chronic signaling, just as chronic B-cell-receptor stimulation results in calcium currents of diminished amplitude, alteration of signaling cascades, and ultimately, proliferative anergy.²⁴⁻²⁶ Although the specific mechanisms that underlie the reduction in PLC γ ₂-mediated signal transduction remain to be elucidated, there are several possible explanations. Increased phospholipase activity may lead to depletion of the substrate PIP₂ proximate to PLC γ ₂, resulting in impaired IP₃ production and IP₃-mediated calcium release.²⁷ Alternatively, excessively high concentrations of the products of PLC γ ₂ may induce feedback-mediated down-regulation of the distal signaling pathways.²⁸ Both these mechanisms suggest that phospholipase inhibitors²⁹ might be used to treat PLAID. It is also possible that the deleted regions have other functions that are critical for PLC γ ₂-mediated signaling, in addition to the phospholipase activity.³⁰

Cold urticaria in patients with PLAID is characterized by mast-cell degranulation after cold stimulation, which is similar to the lesions reported in other types of cold urticaria.^{7,9} Previous serum transfer experiments have suggested that causative autoantibodies may underlie some cases of cold urticaria.^{9,31} However, in our study, B cells and mast cells expressing the mutant forms of PLC γ ₂ had increased cellular activation and effector function at subphysiologic temperatures without receptor stimulation, indicating that the altered function of mutant PLC γ ₂ was responsible for cold urticaria in the subjects in a dominant, cell-intrinsic fashion.

There are two gain-of-function *Plcg2* mouse models, but they differ from PLAID in that these mice lack constitutively activated PLC γ ₂. In addition, the mouse cells are subject to abnormally high calcium flux after surface-receptor ligation, and all the mice have severe inflammatory arthritis.^{32,33} Furthermore, the autoimmunity in subjects with PLAID and in one of these mouse models (*Plcg2*^{Ali5} mice) appears to develop through different mechanisms, given that the induction of intracellular signaling by receptor stimulation is reduced in PLAID B cells but increased in *Plcg2*^{Ali5} B cells. Although cold urticaria is not manifested in either of these mouse models, *Plcg2*^{Ali5} mice have inflammatory dermatitis on the coolest body parts (the ears and distal extremities), resembling the distribution of cutaneous granulomatous lesions in several subjects with PLAID in our study.

Common variable immunodeficiency (CVID) is a prototypic antibody-deficiency disorder, which is also associated with autoimmunity. Although CVID was present in only three subjects in our study, there is substantial phenotypic overlap between PLAID and CVID. In addition to antibody deficiency and autoimmunity, common features of the two disorders include granulomatous disease,¹² diminished class-switched memory B cells,¹⁷ impaired B-cell calcium flux,³⁴ and low numbers of natural killer cells.³⁵ Like PLAID, CVID is associated with mutations that affect proteins involved in B-cell activation.³⁶⁻⁴⁰ Given the overlap of pathways and phenotypes between the two diseases, it seems likely that an understanding of PLAID will provide mechanistic insights into the pathogenesis of CVID and CVID-associated symptoms.

The deletions that we discovered in the three families occur in a region of *PLCG2* that is rich in repetitive elements known to facilitate aberrant recombination events. Indeed, five of the six deletion breakpoints occurred within repetitive elements, suggesting that sporadic cases of PLAID may be caused by similar de novo or even somatic mutations in *PLCG2*.

PLAID-associated *PLCG2* mutations and the resulting phenotypes provide a rare example of a monogenic disease defined by an atopic phenotype. The effects of the mutant forms of PLC γ ₂ in this complex disorder illuminate vital elements of phospholipase-mediated signaling, including its role in leukocyte function, host defense, and self-tolerance. In addition, such effects illuminate how novel genetic variants can cause atypical temperature responses by altering the balance among complex biologic pathways.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Appendix

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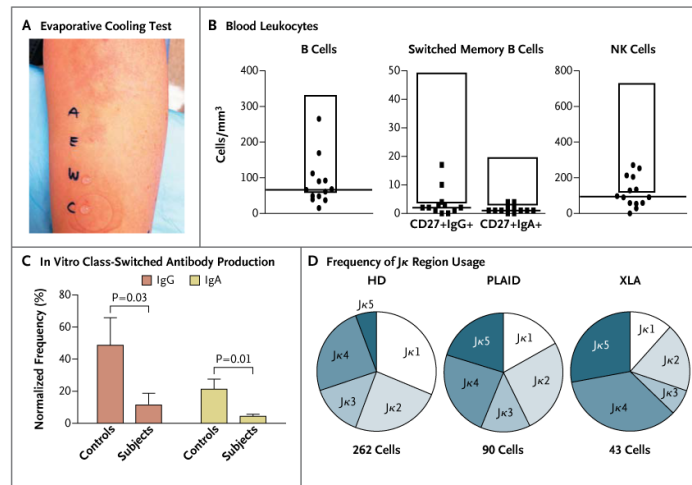


Figure 1. Clinical and Immunologic Manifestations in Patients with Phospholipase $C\gamma 2$ -Associated Antibody Deficiency and Immune Dysregulation (PLAID)

Panel A shows the results of an evaporative cooling test in one subject. Cold urticaria was provoked with droplets of ethanol (E) or air-blown water (A) but not with droplets of unblown water (W) or covered water (C). In Panel B, blood leukocyte counts are shown for 18 adult subjects with PLAID. The tops and the bottoms of the boxes represent a 2-SD range above and below the mean values in healthy control subjects, and the horizontal lines represent the median value among subjects with PLAID. NK denotes natural killer. Panel C shows the mean frequency of IgA or IgG antibody-secreting cells, measured by means of enzyme-linked immunospot assay (ELISPOT) after 4 days of expansion in culture. ELISPOT data were normalized to an equal number of B cells on the basis of the proportion of B cells in peripheral-blood mononuclear cells at the start of culture. The T bars indicate standard errors for five subjects from each group. In Panel D, Ig κ secondary recombination in transitional B cells is shown for 12 healthy donors (HD), 4 subjects with PLAID, and 4 subjects with X-linked agammaglobulinemia (XLA), indicated by the frequency of J κ region usage, as determined by single-cell Ig κ sequence analysis of data from subjects who were pooled according to diagnosis. The increased usage of the terminal J $\kappa 4$ and J $\kappa 5$ genes, as seen in both PLAID and XLA, indicates impaired termination of secondary recombination.

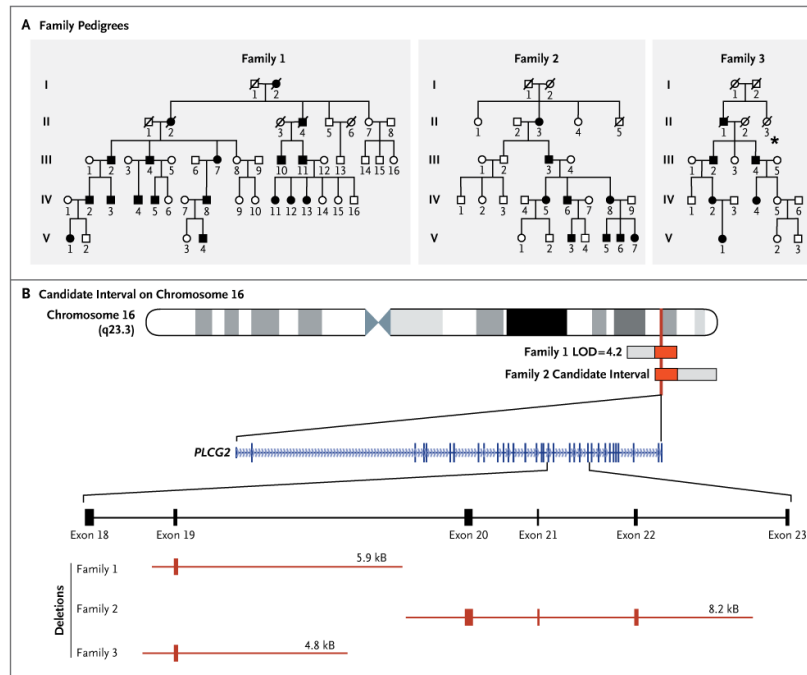


Figure 2. Genomic Deletions in *PLCG2* Encoding Phospholipase $C\gamma_2$ in the Study Subjects
 Panel A shows the pedigrees of three families with PLAID. Solid symbols indicate affected subjects, and open symbols unaffected relatives. Squares indicate male subjects, and circles female subjects. Slashes indicate deceased subjects. The starred member in Family 3 died at the age of 1 year from pneumonia. Panel B shows the candidate interval on the long arm of chromosome 16, defined by the intersection of a linkage interval in Family 1 and candidate interval in Family 2. Three distinct *PLCG2* deletions were identified in the three families (red horizontal bars).

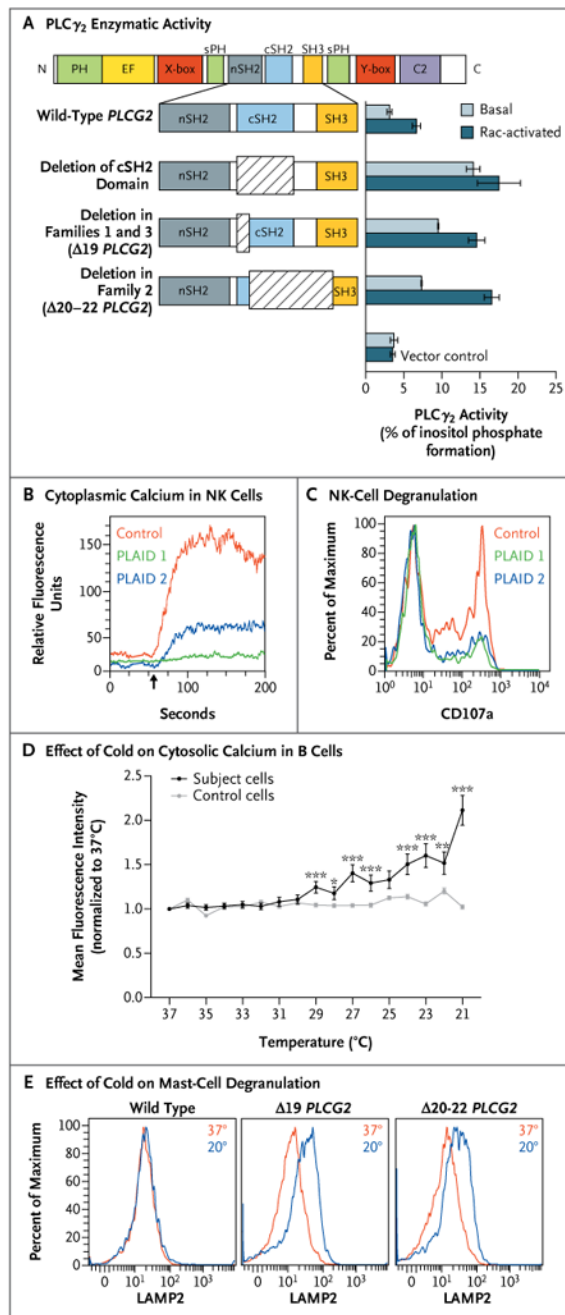


Figure 3. Effects of PLAID-Associated *PLCG2* Deletions, Including Increased Phospholipase Activity, Deceased Cellular Activation at Physiologic Temperatures, and Enhanced Cellular Activation at Subphysiologic Temperatures

Panel A shows enzymatic activity of phospholipase $C_{\gamma 2}$ ($PLC_{\gamma 2}$) mutants. COS-7 cells were transfected with DNA encoding either wild-type *PLCG2* or *PLCG2* with deletions of the C-terminal Src homology 2 (cSH2) domain, exon 19 ($\Delta 19$), and exons 20 through 22 ($\Delta 20-22$) (at left). $PLC_{\gamma 2}$ activity was assayed by quantification of [3H] inositol phosphates generated by the respective enzymes (at right). Basal activity was quantified in COS-7 cells transfected with *PLCG2* alone, whereas the Rac-activated condition included cotransfection with a Rac2 V12 expression construct. The I bars indicate 1 SD above and below the mean release of

inositol phosphate for each condition. Data are representative of three independent experiments. C2 denotes calcium-binding C2 domain, EF EF hand motif, nSH2 N-terminal SH2 domain, PH pleckstrin homology domain, SH3 SH3 domain, sPH split pleckstrin homology domain, X-box × catalytic domain, and Y-box Y catalytic domain. Panel B shows cytoplasmic calcium content (as measured by FLUO-4 staining) in natural killer (NK) cells before and after surface-receptor cross-linking (arrow indicates time of administration) in two subjects with PLAID and one control subject. Panel C shows degranulation of NK cells after incubation with sensitive target cells, as measured by cell-surface expression of CD107a as a percent of the maximum CD107a expression observed in control NK cells. Panel D shows the effect of temperature reduction, in the absence of surface stimulation, on cytosolic calcium content in B cells sorted from a case subject and a control subject, as measured on confocal microscopy. Data points reflect the average mean fluorescence intensity of 100 individually imaged cells, and I bars indicate standard errors. Data are representative of three independent experiments. One asterisk indicates a P value of 0.01 to 0.05, two asterisks indicate a P value of 0.001 to less than 0.01, and three asterisks indicate a P value of less than 0.001. Panel E shows mast-cell degranulation, as indicated by the surface expression of lysosomal-associated membrane protein 2 (LAMP2) and measured by means of flow cytometry at 37°C (red) and 20°C (blue) in Laboratory of Allergic Diseases 2 (LAD2) human mast cells transfected with wild-type and deleted forms of *PLCG2*.

Table 1
Summary of the Clinical Manifestations of Phospholipase C γ_2 -Associated Antibody Deficiency and Immune Dysregulation in the Subjects

Clinical Manifestation	Frequency no./total no. (%)
Cold urticaria	27/27 (100)
Recurrent sinopulmonary infection	12/27 (44)
Antibody deficiency*	15/20 (75)
Common variable immunodeficiency	3/27 (11)
Symptomatic autoimmune disease [†]	7/27 (26)
Positive test for antinuclear antibodies [‡]	13/21 (62)
Symptomatic allergic disease	15/27 (56)

* Antibody deficiency was defined as a serum IgM, IgG, or IgA level that was more than 2 SD below the age-specific mean.

[†] Symptomatic autoimmune disease was defined as the presence of any autoimmune disease, including vitiligo, autoimmune thyroiditis, inflammatory arthritis, and undifferentiated connective-tissue disease.

[‡] The test was weakly positive (1 to 3 enzyme-linked immunosorbent assay [ELISA] units) in 12 subjects and strongly positive (>3 ELISA units) in 1 subject.