

ORIGINAL ARTICLE

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 γ_2 (PLC γ_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 γ_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 $\text{C}\gamma_2$ FUNCTION

We assayed the enzymatic activity of phospholipase $\text{C}\gamma_2$ (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

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.

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).

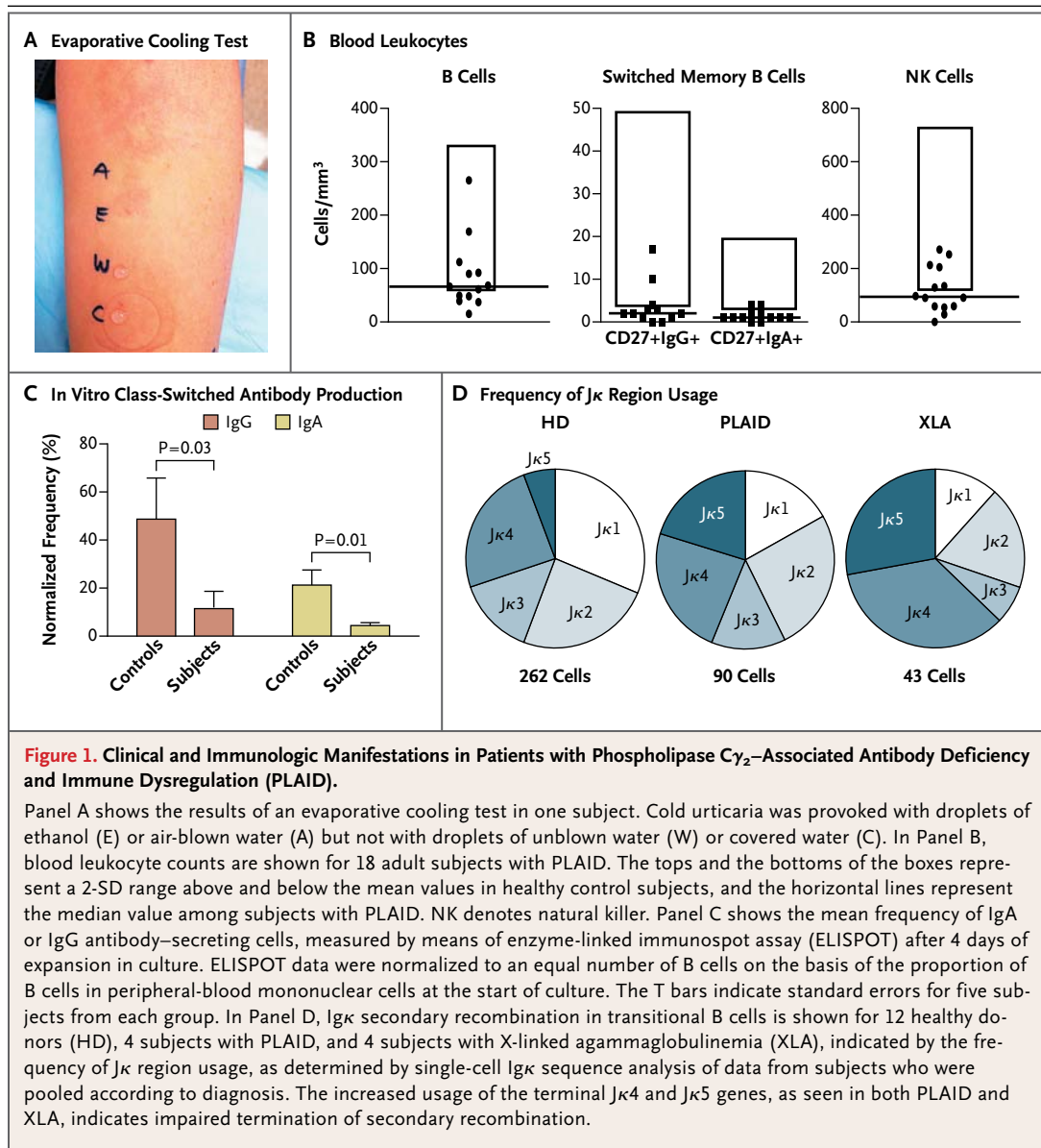
Common laboratory findings in affected subjects included low levels of serum IgM and IgA, depressed levels of circulating CD19+ B cells and IgA+ and IgG+ class-switched memory B cells, and low levels of natural killer cells, whereas IgE levels were elevated in a majority of the subjects (Fig. 1B, and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

phil, 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 class-switched-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[–] 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,

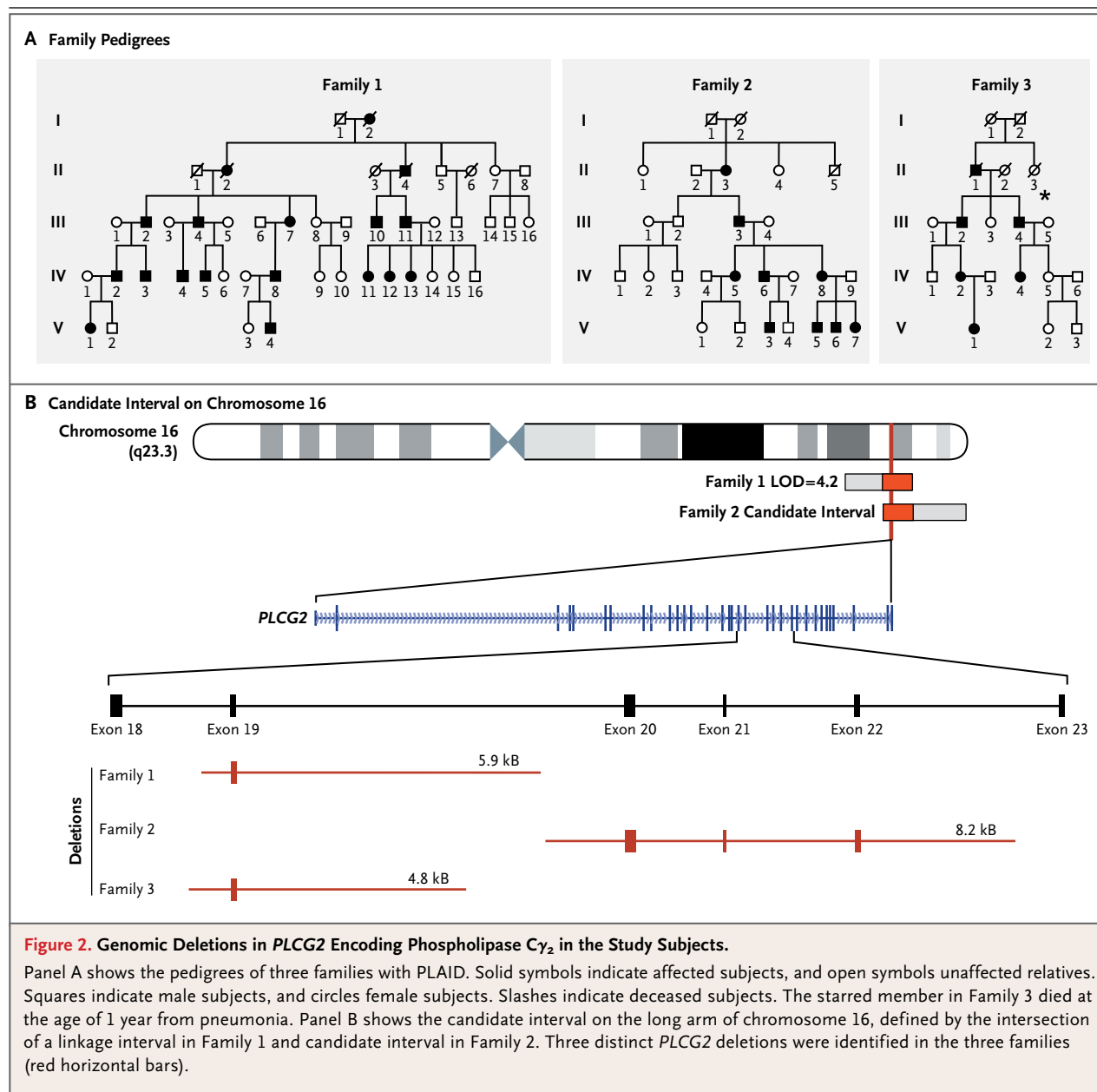


phosphatidylinositol 4,5-bisphosphate (PIP₂).¹⁸ The IP₃ that is produced is responsible for calcium release from the endoplasmic reticulum, a critical event in cellular activation. There are two members of the PLC γ family, PLC γ_1 and PLC γ_2 . Although PLC γ_1 is expressed in many cell types, the Rac-responsive 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 Appen-



dix). 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 se-

quencing (see the Methods section in the Supplementary Appendix). We propose the term PLAID ($PLC\gamma_2$ -associated antibody deficiency and immune dysregulation) to describe this unique constellation of clinical, genetic, and functional findings.

FUNCTIONAL STUDIES OF MUTANT $PLC\gamma_2$

Each of the three deletions that were identified in these families involved the C-terminal Src-homology 2 (cSH2) domain of $PLC\gamma_2$ (Fig. 3A), which is

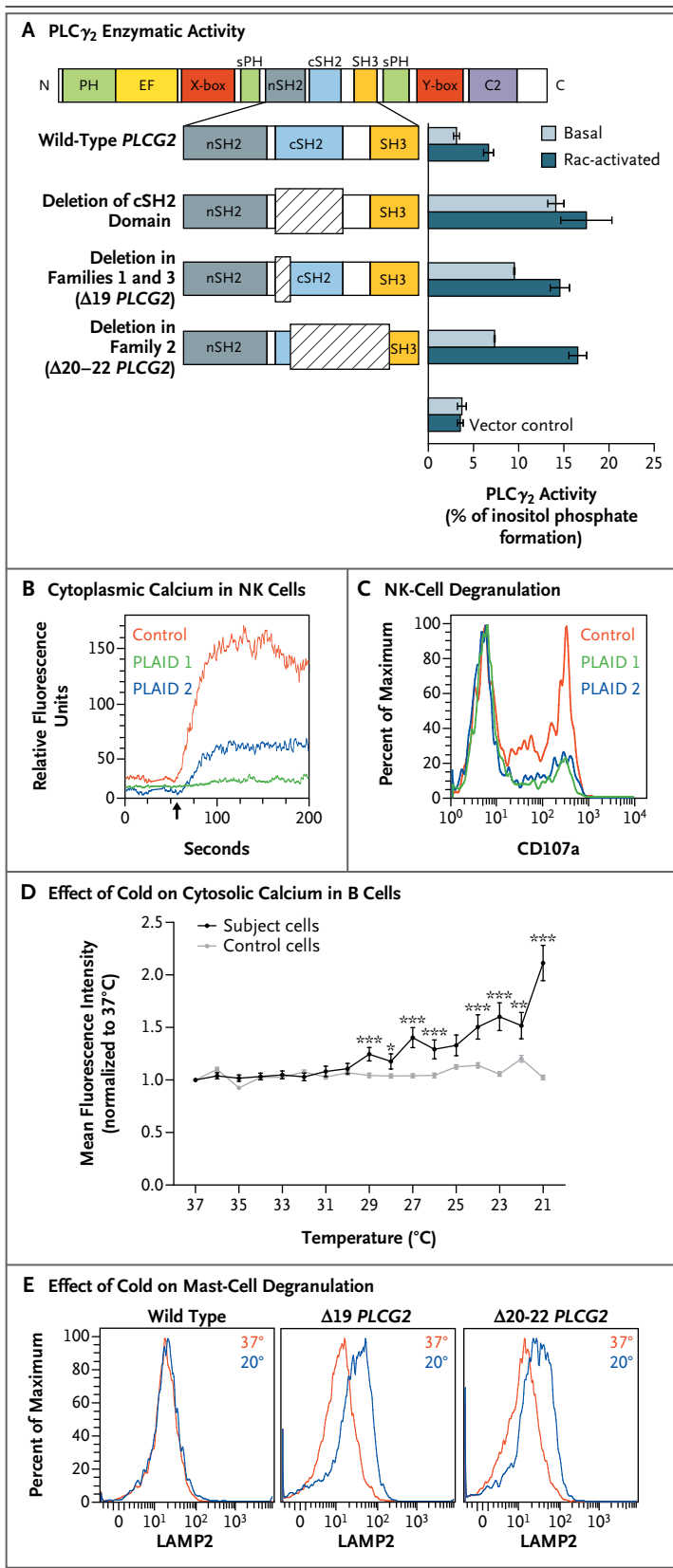


Figure 3. Effects of PLAID-Associated *PLCG2* Deletions, Including Increased Phospholipase Activity, Decreased Cellular Activation at Physiologic Temperatures, and Enhanced Cellular Activation at Subphysiologic Temperatures.

Panel A shows enzymatic activity of phospholipase $C\gamma_2$ (*PLC γ_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 γ_2* activity was assayed by quantification of [^{3} H] 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 X 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*.

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 $PLC\gamma_2$ -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 $PLC\gamma_2$, 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 $PLC\gamma_2$.

The PLAID-associated *PLCG2* deletions affected the regulatory region, including the autoinhibitory cSH2 domain, which normally couples the enzymatic activity of $PLC\gamma_2$ 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 $PLC\gamma_2$ catalytic site and that this inhibitory interaction is relieved by phosphorylation of critical tyrosine residues, which facilitates a conformational change in $PLC\gamma_2$, thereby exposing the catalytic site.^{21–23}

Nonetheless, B cells and natural killer cells from subjects with PLAID clearly had decreased $PLC\gamma_2$ -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 $PLC\gamma_2$ -mediated signal transduction remain to be elucidated, there are several possible explanations. Increased phospholipase activity may lead to depletion of the substrate PIP_2 proximate to $PLC\gamma_2$, resulting in impaired IP_3 production and IP_3 -mediated calcium release.²⁷ Alternatively, excessively high concentrations of the products of $PLC\gamma_2$ 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 $PLC\gamma_2$ -mediated signaling, in addition to the phospholipase activity.³⁰

Cold urticaria in patients with PLAID is characterized by mast-cell degranulation after cold stim-

ulation, 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.

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APPENDIX

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Supplementary Appendix

This appendix has been provided by the authors to give readers additional information about their work.

Supplement to: Ombrello MJ, Remmers EF, Sun G, et al. Cold urticaria, immunodeficiency, and autoimmunity related to *PLCG2* deletions. N Engl J Med 2012. DOI: 10.1056/NEJMoa1102140.

SUPPLEMENTARY APPENDIX

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

Ombrello MJ, Remmers EF, Sun G, et al.

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I. Supplementary Methods:

Patients

In addition to their enrollment in clinical studies at the National Institutes of Health Clinical Center, some patients were also enrolled in an IRB approved study protocol by the University of California San Diego¹.

Cold Urticaria Provocative Testing

A subset of members of Family 1 were challenge tested for cold induced-urticaria. They underwent 1) “ice-cube testing” for 10 minutes using a 50 ml beaker of ice water placed on the volar surface of their forearm, 2) generalized cold room exposure (4°C) while partially clothed for 10 minutes, and 3) evaporative cooling with 0.5 ml droplets of water alone (W), water covered from the environment (C), water with air flow (A), and ethanol (E) for 10 minutes. Positive tests included erythema, swelling and pruritis of the exposed area.

Skin Biopsies and Immunohistochemistry

Punch biopsy specimens of dry unmoistened skin were performed in sterile fashion on 2 affected subjects from family 2 after exposure to room temperature for 3 hours. The right upper back was prepared and anesthetized with a 0.2-mL lidocaine HCl 1% and epinephrine 1:100,000 USP subcutaneous injection. One 4-mm punch biopsy specimen was taken from each subject followed by the placement of a single 4-0 nylon suture. The patients were then cold challenged for 15 minutes in a dry outdoor environment at temperatures of approximately 4°C with their upper backs exposed. A second biopsy was performed immediately over an erythematous site within 2 cm of the first biopsy in the same fashion. Skin biopsy specimens were fixed in formalin, embedded in paraffin, sectioned, mounted on slides, deparaffinized, and rehydrated before analysis. Sections were subjected to staining with hematoxylin and

eosin dyes. Tissue mast cell staining was done with primary mouse monoclonal IgG against tryptase (Abcam, Inc, Cambridge, Mass) at 1:150 dilution, secondary biotinylated antibody to mouse IgG at 1:200 dilution, peroxidase-labeled avidin detection (Vector ABC PK-6102; Vector Laboratories, Burlingame, Calif), and DAB chromagen (DAB Substrate Kit for Peroxidase SK-4100, Vector Laboratories). Hematoxylin was used for counterstaining. Slides were dehydrated and mounted with Cytoseal. Staining was also performed on 3 normal control skin biopsy samples. Normal control samples obtained after a 30-minute cold challenge at 4°C did not show any tryptase release.

Cells

Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood by density gradient centrifugation (Hypaque-Ficoll; Pharmacia). PBMCs were maintained in RPMI 1640 medium supplemented with 10% FBS, 2 mM L-glutamine, and 1% penicillin/streptomycin.

SNP Genotyping, Linkage Analysis and Haplotype Analysis

Genomic DNA was extracted from whole blood of members of Families 1 and 2, and single nucleotide polymorphism (SNP) genotypes were generated using Human Linkage 12 beadchips and an iScan Array Reader (Illumina), or Human Mapping 50K Array (Affymetrix), respectively. Linkage and haplotype analysis were performed independently on Families 1 and 2 using MERLIN linkage software assuming an autosomal dominant model of inheritance, an allele frequency of 0.0001, and complete penetrance.

Whole Genome Sequencing

Whole genome sequencing was performed on genomic DNA from one affected member of Family 1, as previously described². Briefly, sequencing was performed using an

Illumina Genome Analyzer II. The median insert size was 269 bases, and the sequence reads were mostly paired-end at 65, 80, 90 or 100 bases in length. There were 114 billion bases from paired-end reads that passed the Illumina analysis filter and 24 billion bases that were unpaired.

DNA was aligned to the reference genome (NCBI Build 36 Ensembl release 50) using the BWA software (version 0.4.9)³. SAMtools (version 0.1.5c) was used to remove potential PCR duplicates via the rmdup command and to identify variants using the pileup command with the *-c* option and default settings⁴. All single nucleotide variants were screened for quality by only keeping those with a consensus score of at least 20, a quality score of at least 20, and at least 3 reads supporting the variant. Indels were only included if they had a consensus score of at least 20, a quality score of at least 50, and at least 20% of the reads supporting the variant (minimum of 3 supporting reads). The average coverage, excluding gaps in the reference and potential PCR duplicates, was 39x.

Mutational Analysis of *PLCG2*

RNA was isolated from peripheral blood mononuclear cells using the RNeasy kit (Qiagen), and complementary DNA (cDNA) was generated from 1 µg RNA samples using the SuperScript II Reverse Transcriptase kit (Invitrogen). *PLCG2* cDNA was analyzed by PCR amplification of overlapping segments with direct sequencing of the products using Big Dye terminator chemistry (v1.1, Applied Biosystems) and a capillary sequencer (ABI PRISM 3130xl Genetic Analyzer, Applied Biosystems). Coding exons and intron-exon junctions of *PLCG2* were amplified using PCR assay of 50 ng of genomic DNA, 1 µM of target-specific oligonucleotides, and 12.5 µl of AmpliTaqGold Master Mix (Applied Biosystems) in a final volume of 25 µl. Genomic deletional boundaries were mapped with long-range genomic PCR assays using TaKaRa LA kit (TaKaRa) with direct sequencing of the products. Long-range

PCR assays designed to screen for each deletion were performed on all subjects, as well as 200 healthy individuals of Northern European Ancestry (panel HD200CAU, Coriell Cell Repositories). Primer sequences for all PCR assays are available in the Supplementary Table 3.

Calling Structural Variants Using ERDS

Structural variants were called from the whole-genome sequence using Estimated Read Depth with SNVs (ERDS) v.1.02 (<http://www.duke.edu/~mz34/erds.htm>), which uses a sliding window size of 2 kb. This parameter makes identification of structural variants below 10 kb in size difficult, and the causal deletion was not identified either prospectively or retrospectively. A *post hoc* analysis of the whole-genome sequence data was performed with the newly-developed ERDS v.1.03 software (<http://www.duke.edu/~mz34/erds.htm>) was able to identify the 5928 bp deletion using a sliding window size of 1 kb.

Quantification of Degranulation in NK Cells

Degranulation of NK cells was determined by cell-surface expression of CD107a, as described⁵. Briefly, peripheral blood lymphocytes (PBL) (2×10^5) were mixed with an equal number of K562 cells or 721.221 cells. Cells were spun down for 3 min at $20 \times g$ and incubated for 2 h at 37°C. Thereafter, cells were spun down, and cell pellets were resuspended in FACS buffer (PBS with 2% FBS) and stained with CD3-PerCP, CD56-PE, and CD107a-FITC mAbs. Lymphocytes were gated on forward scatter/side scatter, and CD107a expression of CD3⁺CD56^{dim} NK cells was analyzed by flow cytometry.

Flow Cytometric Measurement of Ca²⁺ Flux

Intracellular Ca²⁺ flux was measured by flow cytometry after labeling with FLUO-4 AM (Invitrogen), as described with slight modifications⁶. Briefly, PBLs were labeled for 30 min at 30°C with dye-loading buffer (HBSS with 1% FBS, 2 µg/ml FLUO-4 AM, and 4 mM probenecid), washed once, and resuspended in HBSS with 1% FBS. Cells were stained with CD3-PerCP and either CD56-APC or CD19-APC in the presence of 10 µg/ml of the indicated stimulating mAbs for 30 min on ice. Cells were washed twice and resuspended in HBSS with 1% FBS, and transferred to FACS tubes. Cells were warmed for 5 min at 37°C in a water bath, and placed on the flow cytometer. After 30 s of data acquisition, tubes were removed, 10 µg/ml anti-IgM F(ab')₂ (for B-cells) or 4 µg/ml cross-linking goat anti-mouse F(ab')₂ (for T cells and NK cells) was added. Cells were vortexed, placed back on the flow cytometer, and events were acquired for 5 min. Data were analyzed by FlowJo software with gating on CD3⁻CD56⁺ cells, CD3⁻CD19⁺ cells, and CD3⁺CD19⁻ cells for analysis of NK, B, and T cell populations, respectively.

Culture of B Cells and Analysis of Class Switching

PBMC from subjects with PLAID and control subjects were cultured for four days with SAC and CpG, and the frequencies of antibody-secreting cells (ASCs) were measured by ELISPOT, as previously described⁷. The percentage of B cells among input or cultured PBMCs was measured by flow cytometry.

Measurement of B-cell Receptor Editing

Peripheral B cells were purified from the blood of subjects with PLAID by positive selection with anti-human CD20 magnetic microbeads (Miltenyi Biotec). CD20-enriched peripheral B cells were stained with anti-human CD27-FITC, anti-human CD10-PE, anti-human IgM- Biotin,

and allophycocyanin (APC) anti-human CD19 (Pharmingen, Becton Dickinson). Biotinylated antibodies were revealed using PE-cy7 conjugated streptavidin (Pharmingen, Becton Dickinson). Single CD19⁺CD10⁺IgM^{hi}CD27⁻ transitional B-cells were sorted on a FACSVantage (Becton Dickinson) into 96- well PCR plates containing 4µl lysis solution (0.5X PBS containing 10 mM DTT, 8U RNasin (Promega), 0.4U 5'-3' RNase Inhibitor (Eppendorf), and immediately frozen on dry ice. All samples were stored at -70°C. RNA from single cells was reverse-transcribed in the original 96 well plate in 12.5µl reactions containing 100U of Superscript II RT (Gibco BRL) for 45 minutes at 42°C. RT-PCR reactions and primer sequences were as described⁸. Igk sequences were analyzed by Ig BLAST comparison with GenBank.

Culture, Transfection and Analysis of Inositol Phosphate Formation in Intact COS-7

Cells

The method was essentially as described previously⁹⁻¹¹. COS-7 cells were maintained at 37°C in a humidified atmosphere of 95 % air and 5 % CO₂ in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen) supplemented with 10 % (v/v) fetal bovine serum (Invitrogen) and 2.5 mM glutamine. Prior to transfection, cells were seeded into 6- well plates at a density of 2.5x10⁵ cells/well and grown for 16 h in 2 ml/well of the same medium. For transfection, 0.5-2 µg of plasmid (pTriEx4) DNA encoding human PLC γ ₂ variants (the wild type, deletion of the cSH2 domain and deletions corresponding to those found in patients), without or with 1 µg of plasmid (pcDNA3.1) DNA encoding human Rac2^{G12V}, was mixed with 1 µl PlusReagentTM and 7 µl LipofectamineTM (Invitrogen) and the mixture added to COS-7 cells in 0.8 ml DMEM without serum. The cells were incubated for 3.5 h at 37 °C, 5% CO₂.

For analysis of inositol phosphates, 24 h post-transfection cells were labeled with 1.5µCi/ml *myo*-[2-³H]inositol. After a further 24 h the cells were incubated for 1 hr in 1.2 ml inositol

free DMEM without serum containing 20 mM LiCl. The cells were lysed by addition of 1.2 ml 4.5 % perchloric acid and supernatants and pellets were separated. Inositol phosphates were collected using AG1-X8 200–400 columns (BioRad). Levels of inositol phosphates were quantified by liquid scintillation counting using Ultima-Flo scintillation fluid (Perkin Elmer). We also analyzed the lipid fraction. The pellets from the lysates were treated with chloroform:methanol:HCl to separate aqueous and lipid phases. An aliquot of the lipid phase was used for scintillation counting with Ultima-Flo scintillation fluid. This value was used for normalization of PLC activity so that PLC activity is expressed as the total inositol phosphates formed relative to the amount of [^3H]myoinositol in the phospholipid pool. Because the differences in steady state labeling of inositol-lipids are small (within 20 %) this normalized PLC activity corresponds closely to PLC values expressed as total inositol phosphates; however, the error bars between the duplicates are generally smaller. The PLC activity derived from these measurements was expressed as “PLC activity”. Data shown are the mean $\pm$ SD of duplicate samples and are representative of three or more independent experiments.

Measurement of Cytosolic Ca^{2+} by Confocal Microscopy

Peripheral B cells were purified from PBMC of subjects with PLAID or control individuals by the human B cell isolation kit (Miltenyi Biotec), an indirect magnetically labeling system for the isolation of B cells from PBMC. Coverglass slides for imaging were coated with 100 $\mu\text{g}/\text{ml}$ poly-L-lysine (Sigma) for 1hr, and washed with PBS followed by distilled water. Isolated B cells were labeled with 2 μM FLUO-4 AM (Invitrogen) and seeded onto pre-coated 4-chambered coverglass slides (NUNC) at a density of 0.2×10^6 cells per well in phenol red-free RPMI (Lonza). Cells were imaged in phenol red-free RPMI with 1% FCS at different temperatures (starting at 37°C and decreasing progressively to 19°C) on a Leica SP5

Confocal Imaging System. Temperature was regulated by a heating/cooling stage adapter (Harvard Apparatus); additionally temperature in the dish was monitored using a temperature probe (World Precision Instruments). FLUO-4 was excited at 488 nm and emission in the range of 500-580 nm was collected. Images were acquired in stacks with a z-step size of 1 μ m to cover the whole cell. Images were analyzed using Imaris 7.2 software by creating surfaces to encompass the volume of each cell. Serial mean fluorescence intensity (MFI) measurements were taken for individual cells at different temperatures, and data were represented as the average MFI of all cells (25-30 cells per field).

To measure ligand-mediated calcium flux, purified B cells were labeled with 2 μ M FLUO-4-AM, plated on poly-L-lysine coated plates in HBSS supplemented with Ca^{2+} and Mg^{2+} and imaged with a confocal microscope with a 488 laser. Anti-IgM F(ab')₂ (12 μ g/ml) (Jackson Immuno Research) was added and 15 minute movies were taken by making a Z stack to include the entire cell each time, every 20 seconds. The data were then analyzed with Imaris, creating surfaces for each cell and tracking the cells over time. Mean fluorescence was extracted from these data and plotted as a function of time.

Flow Cytometric Measurement of ERK Phosphorylation in PBMC

Human PBMCs (1×10^6 cells in RPMI 1640 medium supplemented with 10% FBS, 2mM L-glutamine and 1% penicillin/streptomycin) were stimulated with 12 μ g/ml cross-linking goat anti-human IgM (Jackson ImmunoResearch) for 5 minutes in a heating block at the designated temperatures. Unstimulated cells were incubated at the designated temperature for 5 minutes without cross-linking goat anti-human IgM. All tubes were placed in the heating block at the same time and the temperature was decreased one degree every five minutes. After 5 minutes at the appropriate temperature, cells were fixed with 1.6% paraformaldehyde for 10 minutes at room temperature and then centrifuged. Cells were resuspended in cold

100% methanol and incubated at -20⁰ overnight. The next day, cells were washed twice with PBS/0.5% BSA. Cells were stained with pERK FITC, CD19 APC and CD4 PeCy7 (all from BD Biosciences) at room temperature for one hour in the dark. Cells were washed once with PBS/0.5% BSA then run on a BD LSRFortessa. Data were analyzed using FlowJo software by gating on CD19⁺ CD4⁻ B cells.

Transfection and Measurement of ERK Phosphorylation in A20 Cells

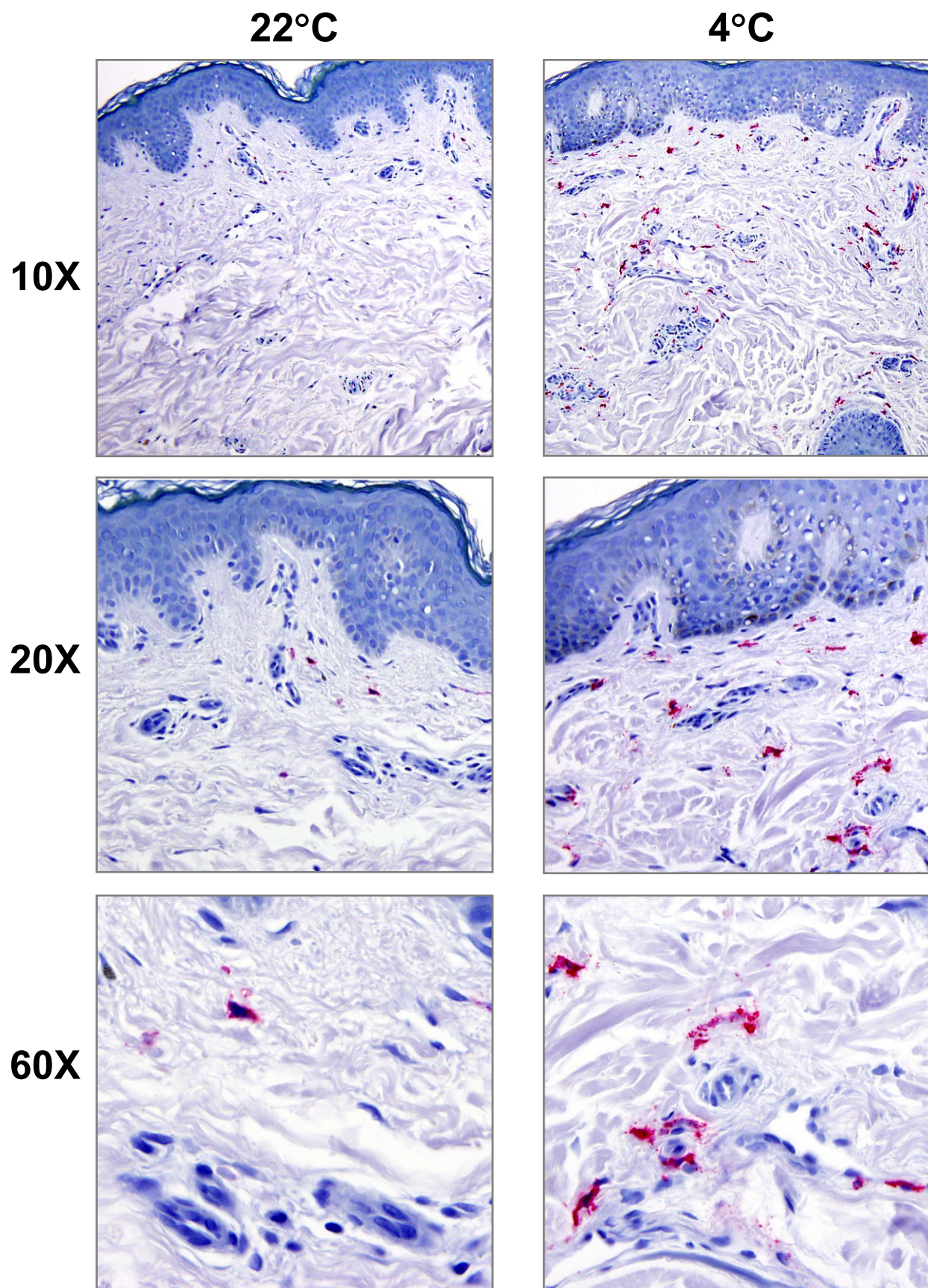
The murine A20 B cell lymphoma cell line was transfected using the Amaxa Cell Line Nucleofection V kit (Lonza) according to the manufacturer's optimized protocol for A20 cells. After 24 h, A20 cells were incubated at either 37°C or 20°C for 20 min. Cells were then stimulated at their designated temperatures with 5 µg of goat anti-mouse IgG (Jackson ImmunoResearch) for 4 minutes, while unstimulated cells were incubated at their designated temperatures without cross-linking antibodies. Cells were fixed with 1.6% paraformaldehyde for 10 minutes at room temperature and then centrifuged. Cells were resuspended in cold 100% methanol and incubated at -20°C overnight. The next day, cells were washed twice with PBS/0.5% BSA. Cells were stained with Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) (D13.14.4E) XP[®] Rabbit mAb (PE Conjugate) (Cell Signaling Technology, Inc.) at room temperature for one hour in the dark. Cells were washed once with PBS/0.5% BSA and then run on a BD LSRFortessa. Data were analyzed using FlowJo software by gating on GFP⁺, PE⁺ cells.

Transfection and Measurement of Degranulation in LAD2 Cells

The LAD2 human mast cell line was transfected using the Amaxa Nucleofector as described¹². After 24h, 2 x 10⁵ LAD2 cells were incubated at either 20°C or 37°C for 30 min, and then placed on ice. LAD2 cells stimulated with 1 µM thapsigargin at 37°C for 30 min were

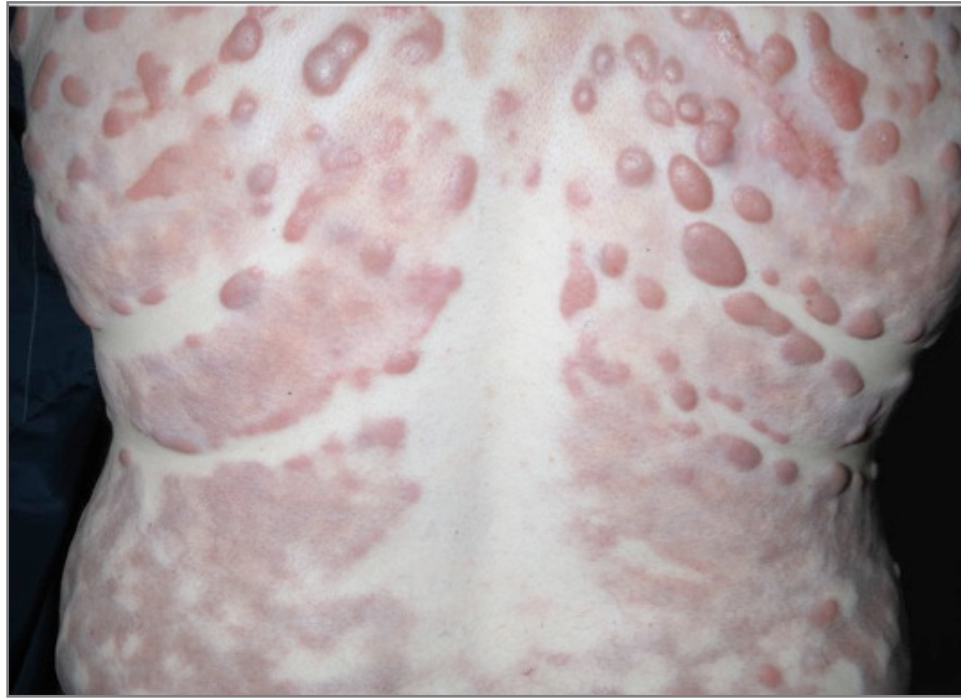
used to induce maximal degranulation and were then also placed on ice. Cells were washed twice in PBS 0.1% BSA and stained with 1 µg/ml PE conjugated mouse anti-human LAMP2 (Clone H4B4) (Abcam, Cambridge, UK) for 45 min on ice. LAD2 cells were then washed twice in PBS 0.1% BSA. Flow cytometry was carried out on the FACSCalibur machine (BD Biosciences) and analyzed for LAMP2 staining in the GFP⁺ populations using FlowJo software (version 9.2).

II. Supplementary Figures

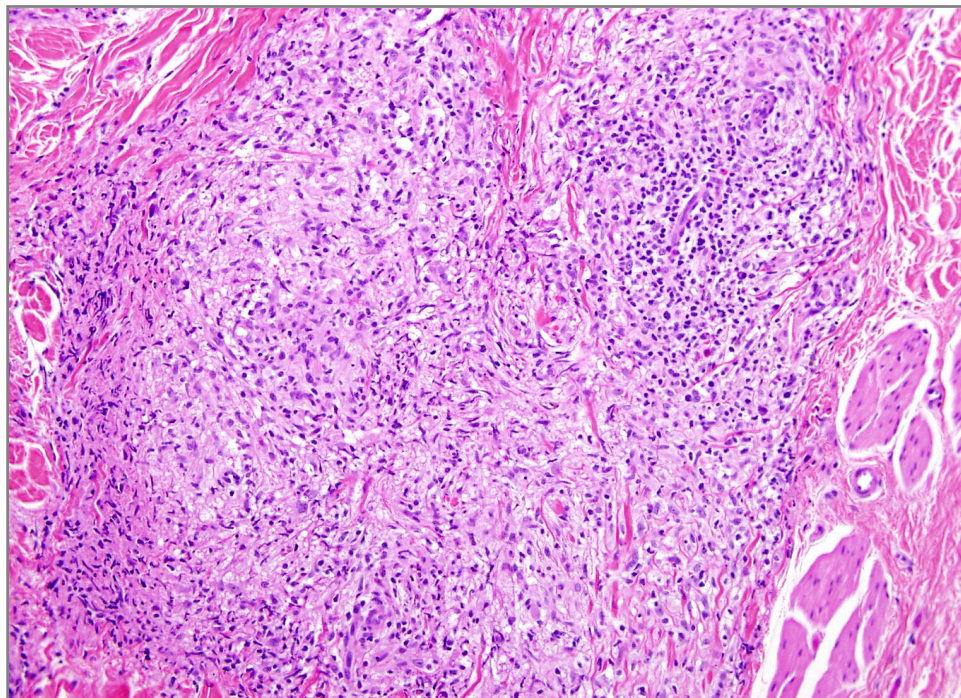


Supplementary Figure 1. Mast Cell Degranulation in Skin Biopsies of Subjects with PLAID Following Exposure to Ambient Cold Air. Mast cell tryptase (red) staining was performed on 4-mm skin biopsy specimens obtained from subjects with PLAID following exposure to 22°C (left column) and 4°C (right column) ambient air for 15 minutes (10X, 20X, and 60X magnification).

A

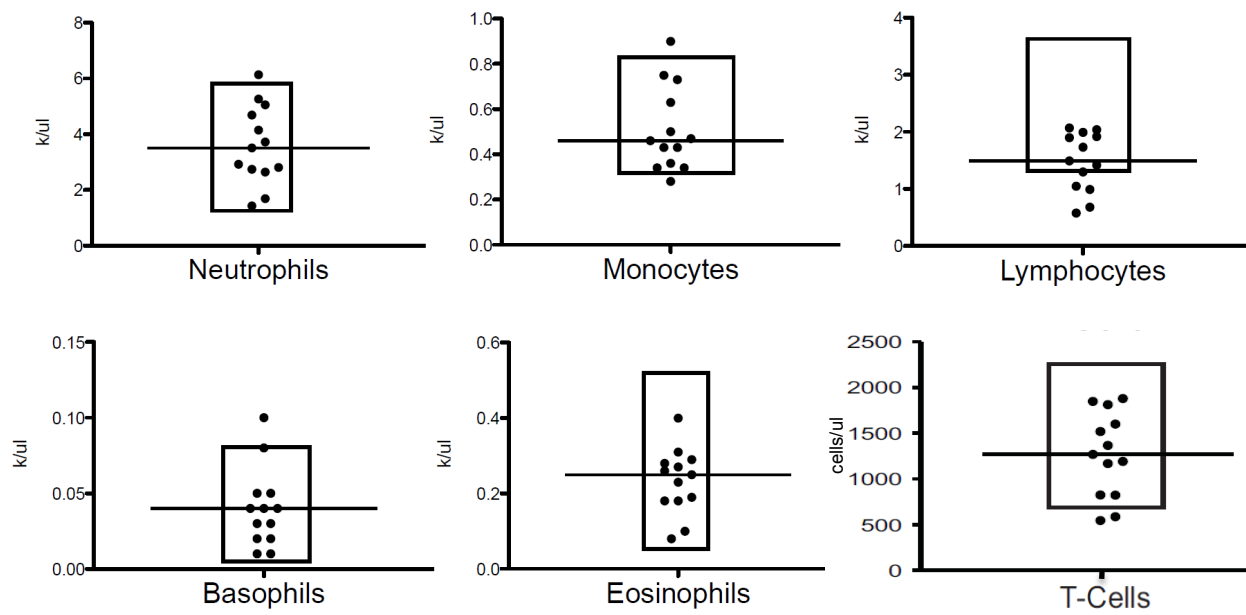


B

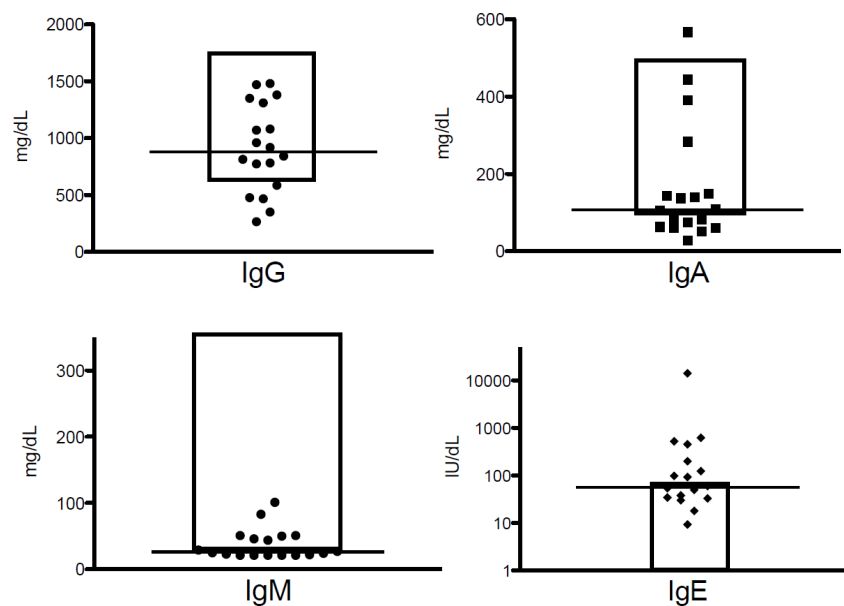


Supplementary Figure 2. Severe Granulomatous Skin Disease in a Subject with PLAID. Panel A depicts nodular skin lesions present in an affected member of Family 1. H&E staining of the lesions demonstrate granulomatous inflammation with multiple foci of epithelioid histiocytes and multinucleated giant cells surrounded by lymphocytes, Langerhans cells, and mast cells (Panel B). These granulomata were temperature sensitive and worsened in cold environmental conditions.

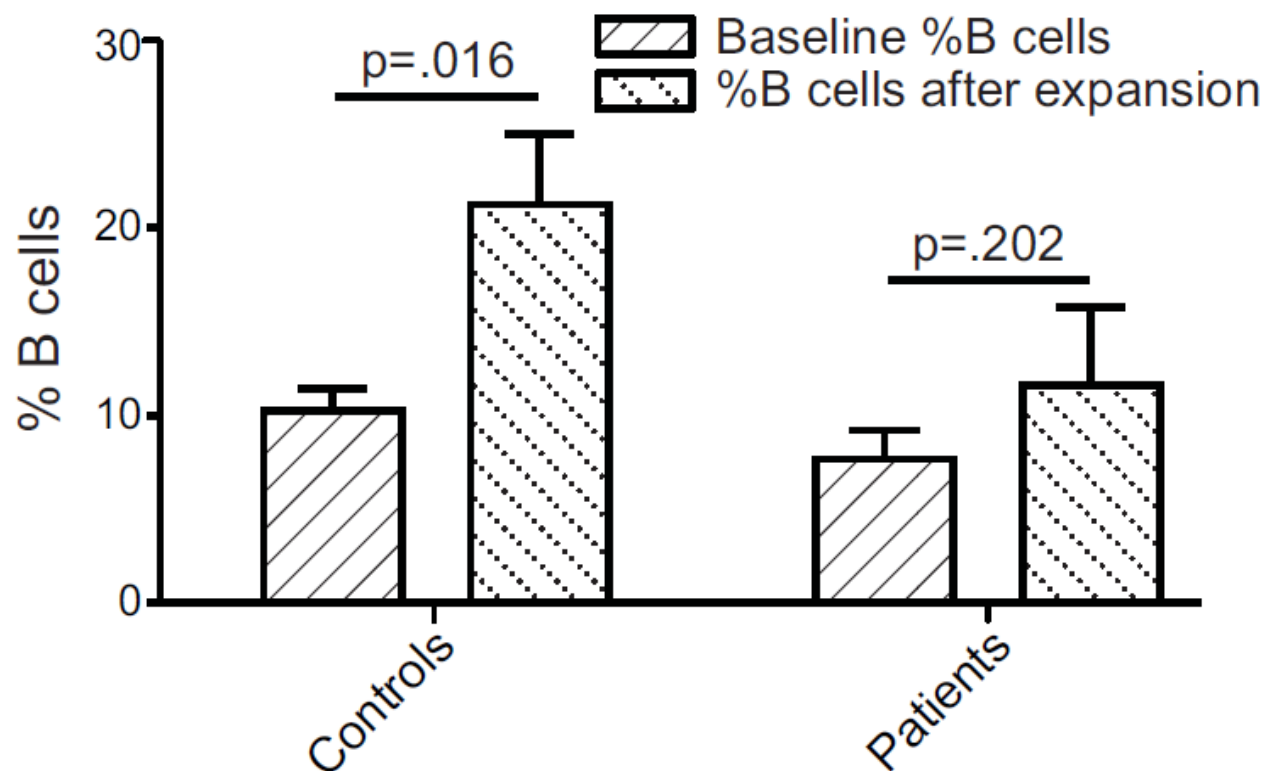
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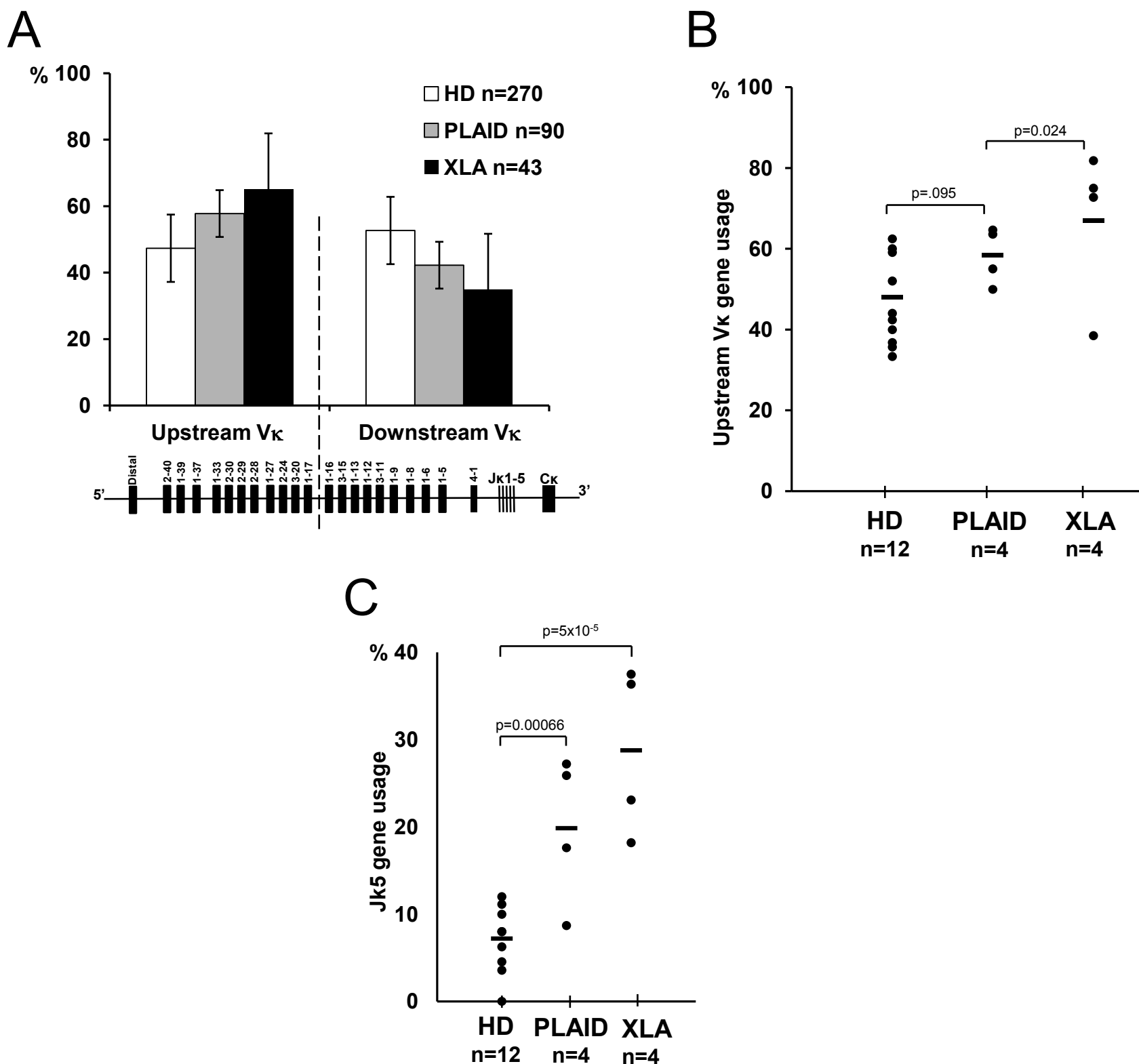
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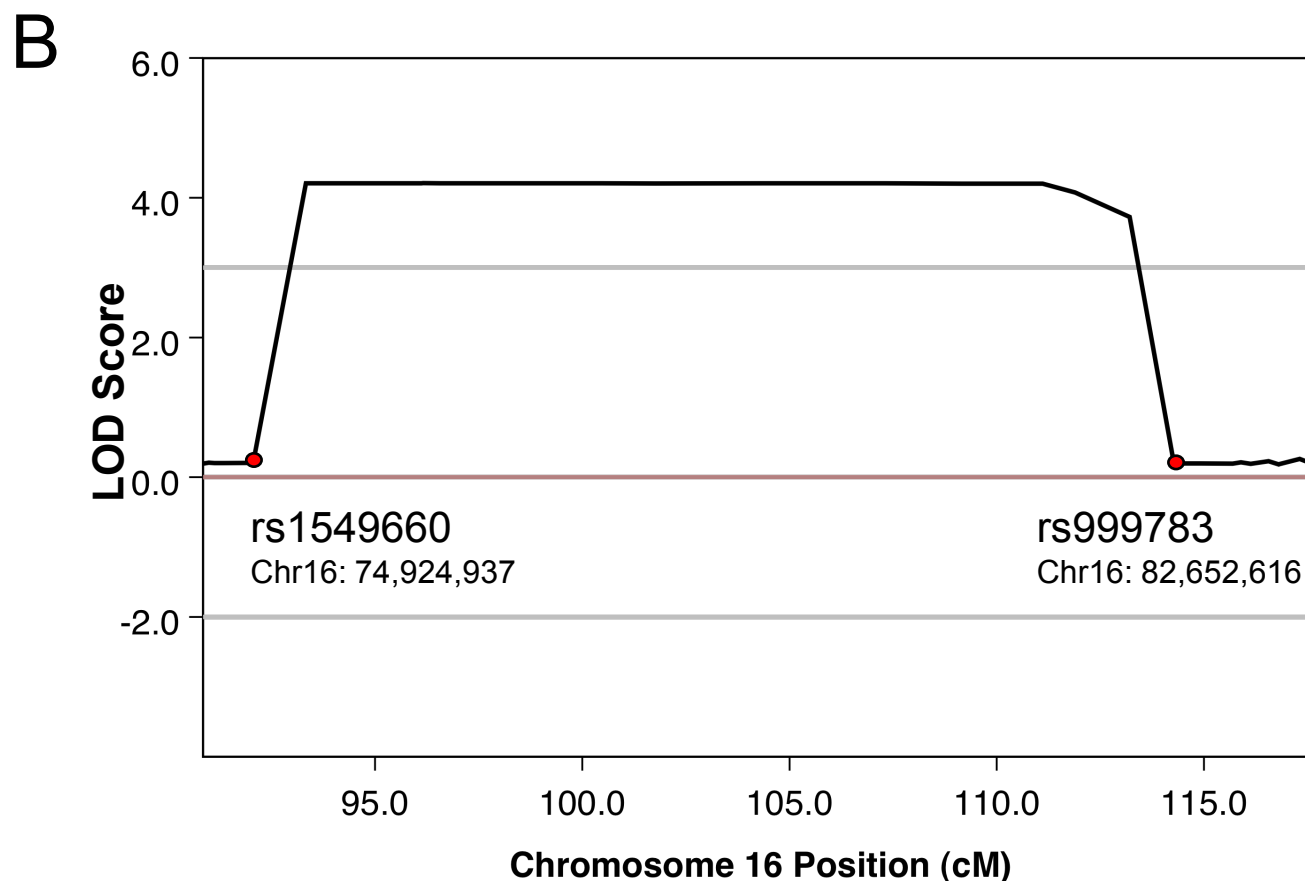
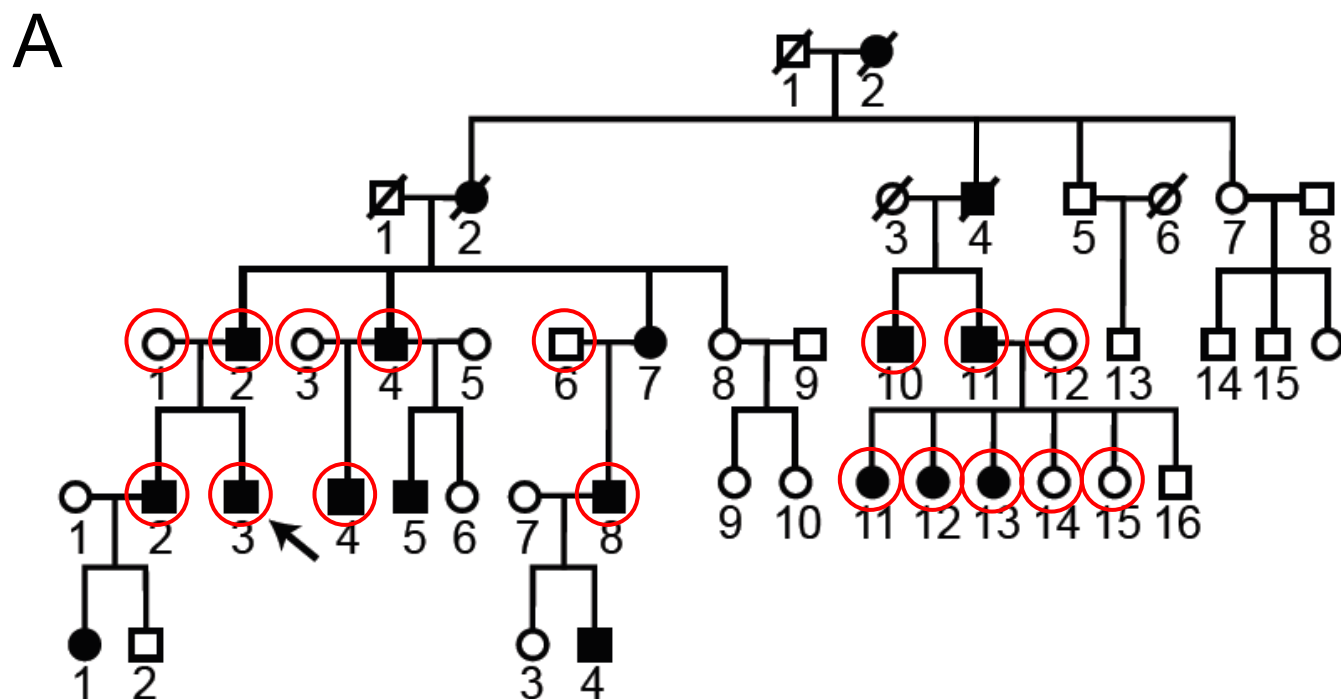
Supplementary Figure 3. Serum Immunoglobulins and White Blood Cell Differential Cell Counts from Adult Subjects with PLAID. Panel A demonstrates peripheral blood leukocyte counts, while Panel B shows serum immunoglobulin measurements. The rectangles are 2 SD above and below the test mean determined in healthy control subjects, and the horizontal black line represents the median value among tested subjects.



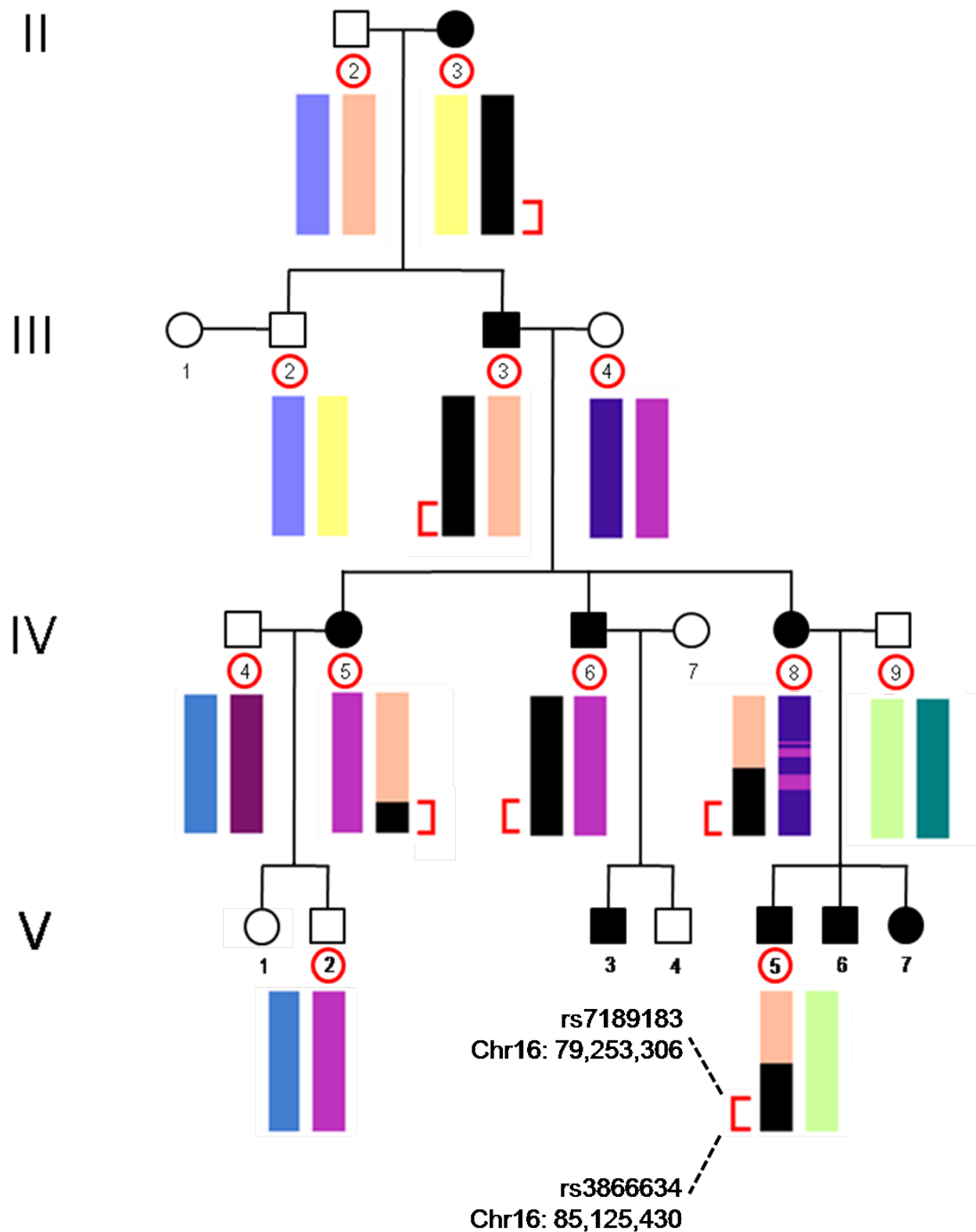
Supplementary Figure 4. Reduced Proliferative Capacity and Expansion of B Cells from Subjects with PLAID. Bar graphs demonstrate the percentage of B cells in PBMCs from subjects with PLAID and control subjects before and after four days of expansion in culture. Data shown are means and SEM for five individuals from each group.



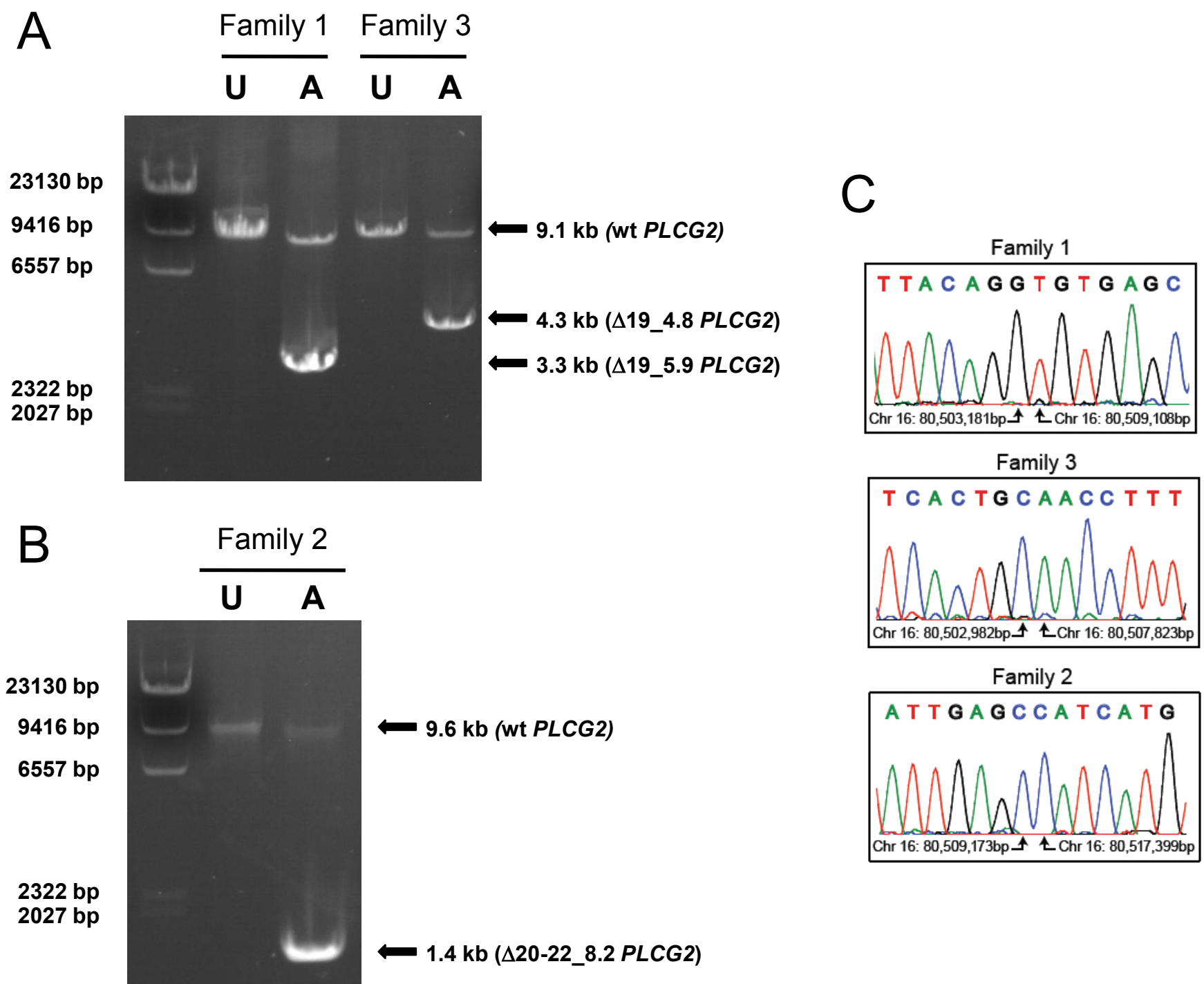
Supplementary Figure 5. Ig κ Secondary Recombination in Transitional B Cells from Subjects with PLAID, Healthy Donors (HD), and Patients with X-Linked Agammaglobulinemia (XLA). Bar graphs (A) show the frequency of V κ region use determined by single cell Ig κ sequence analysis from 12 healthy donors, 4 PLAID patients, and 4 XLA patients pooled by disease (n=number of cells evaluated). The scatter plots (B and C) illustrate the upstream V κ and J κ 5 usage (percent of total), respectively, of individual subjects (n = number of individuals). The horizontal black line represents the mean.



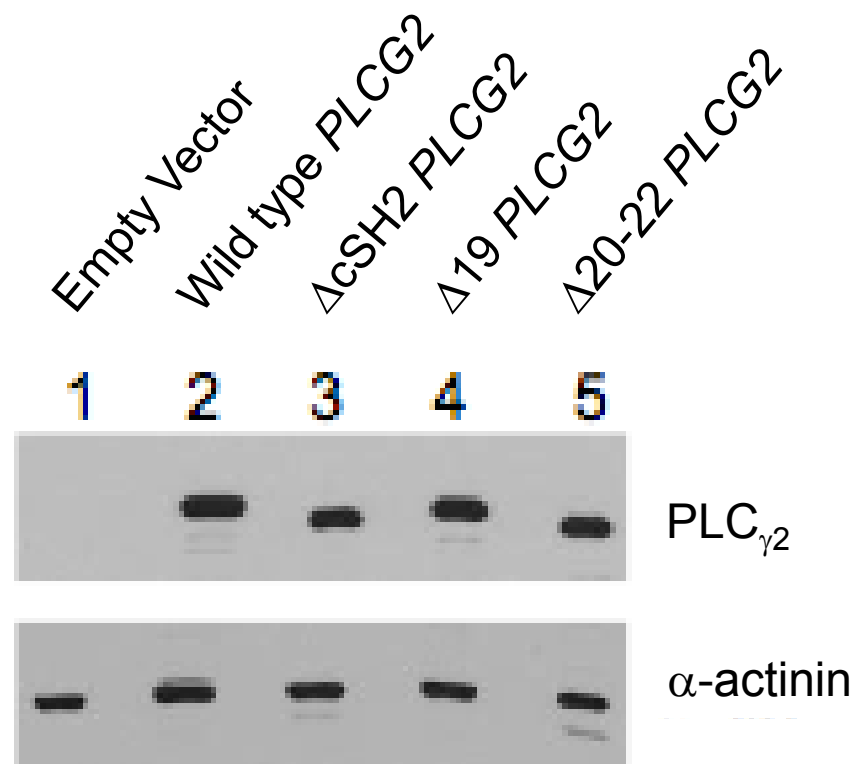
Supplementary Figure 6. Results of Linkage Analysis in Family 1. In the pedigree of Family 1 (panel A), individuals included in the linkage analysis are highlighted with red circles. Panel B demonstrates the single linkage interval on chromosome 16 identified through linkage analysis of the individuals in Panel A.



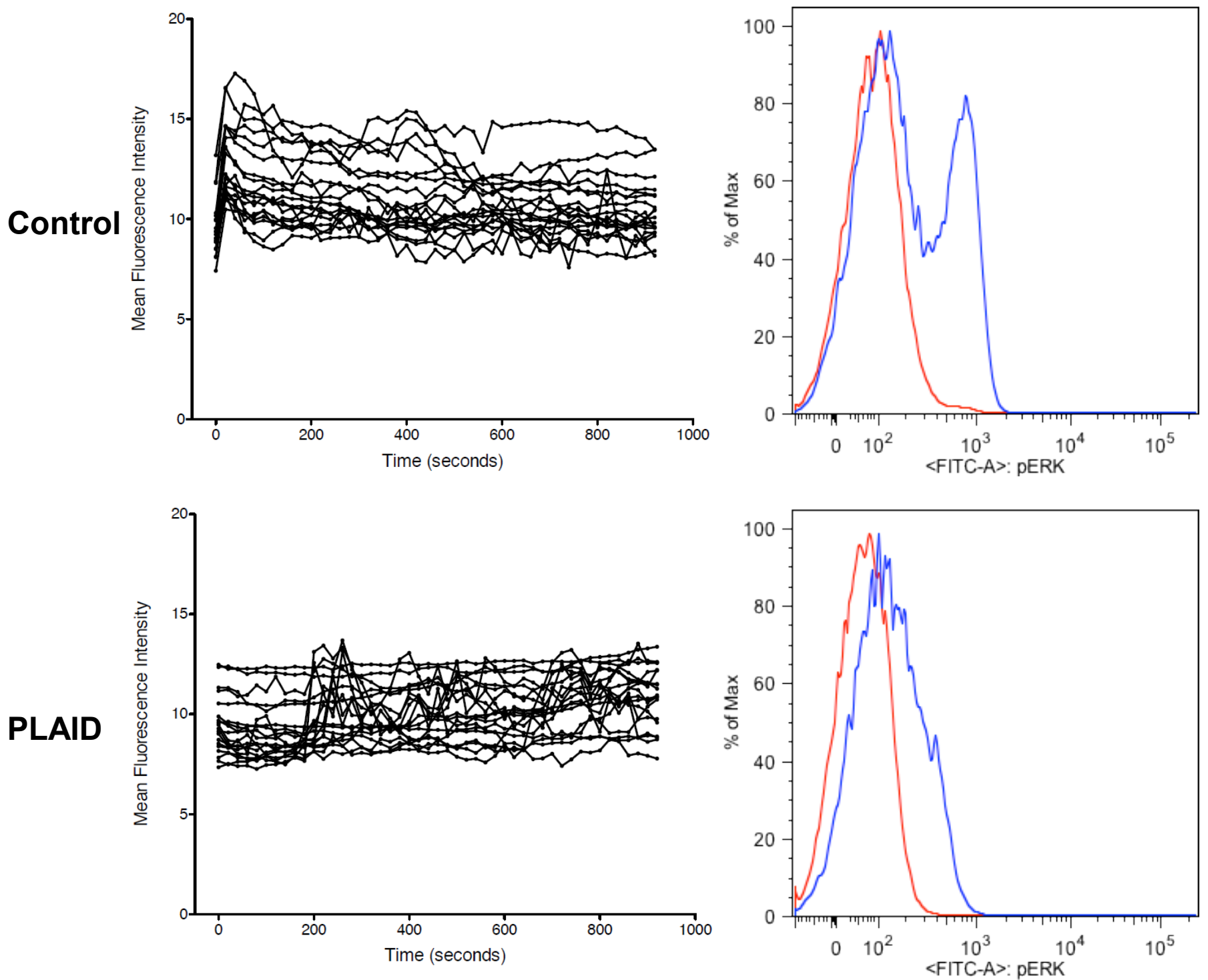
Supplementary Figure 7. Results of Linkage and Haplotype Analysis in Family 2. Haplotype analysis was performed on the individuals in Family 2 highlighted with red circles, and a chromosome 16 haplotype was identified that segregated perfectly with cold urticaria. Red brackets indicate the location of the disease-associated haplotype.



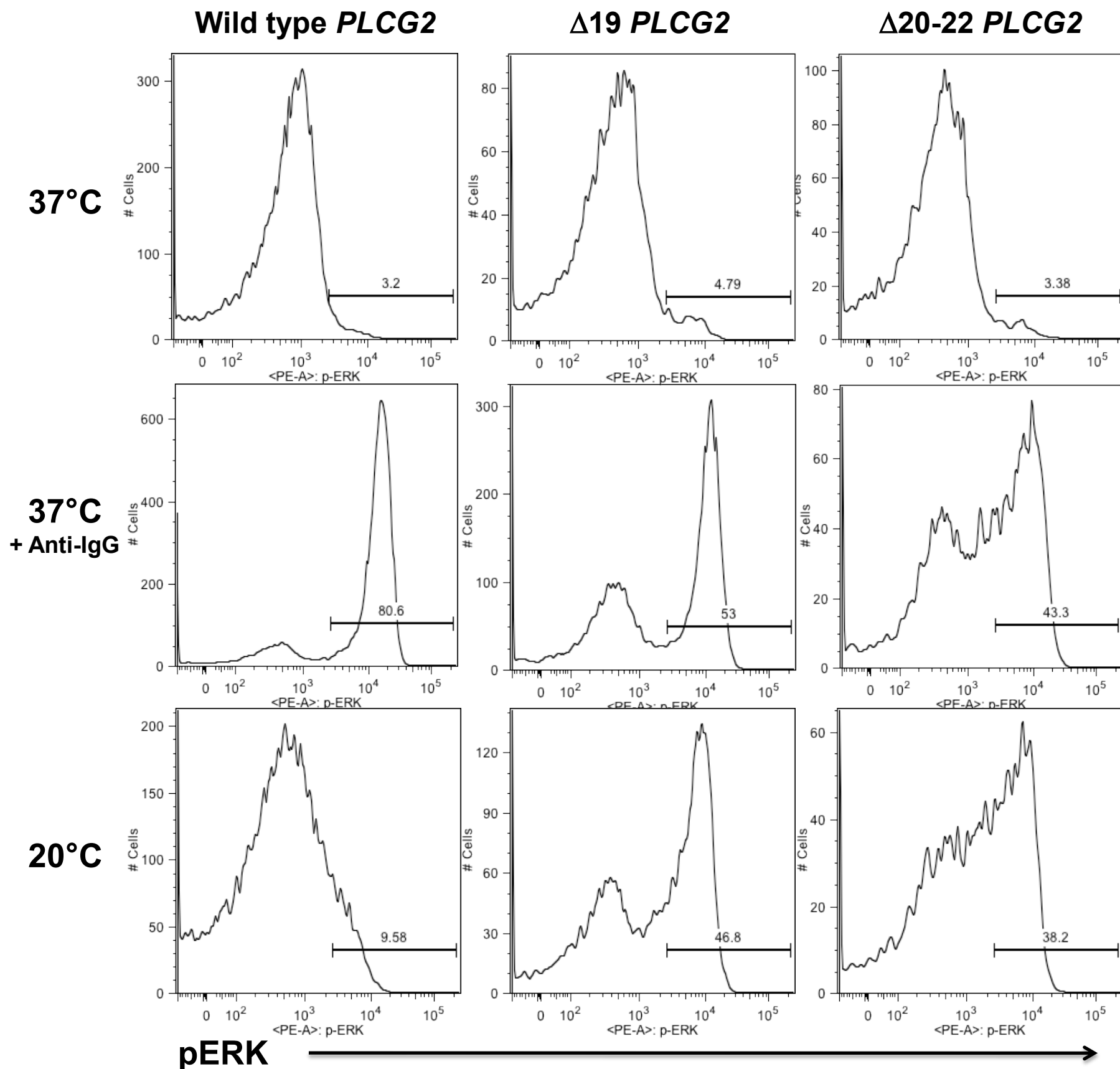
Supplementary Figure 8. Genomic DNA Skip Assays Identify Novel Deletions in Each PLAID Family. Panel A demonstrates PCR products from unaffected (U) and affected (A) members of Families 1 and 3 obtained from the Exon 19 Deletion Genomic Screening Assay (Supplementary Table 3) which amplifies the region between exon 18 and exon 20 of *PLCG2*. Panel B demonstrates the PCR products from an unaffected (U) and an affected (A) member of Family 2, obtained from the Exon 20-22 Deletion Genomic Screening Assay (Supplementary Table 3) which amplifies the region between exon 19 and exon 23 of *PLCG2*. Panel C displays electropherograms that contain the deletional breakpoint in each Family. Genomic coordinates are based on human genome build 18 (March 2006, NCBI36/hg18).



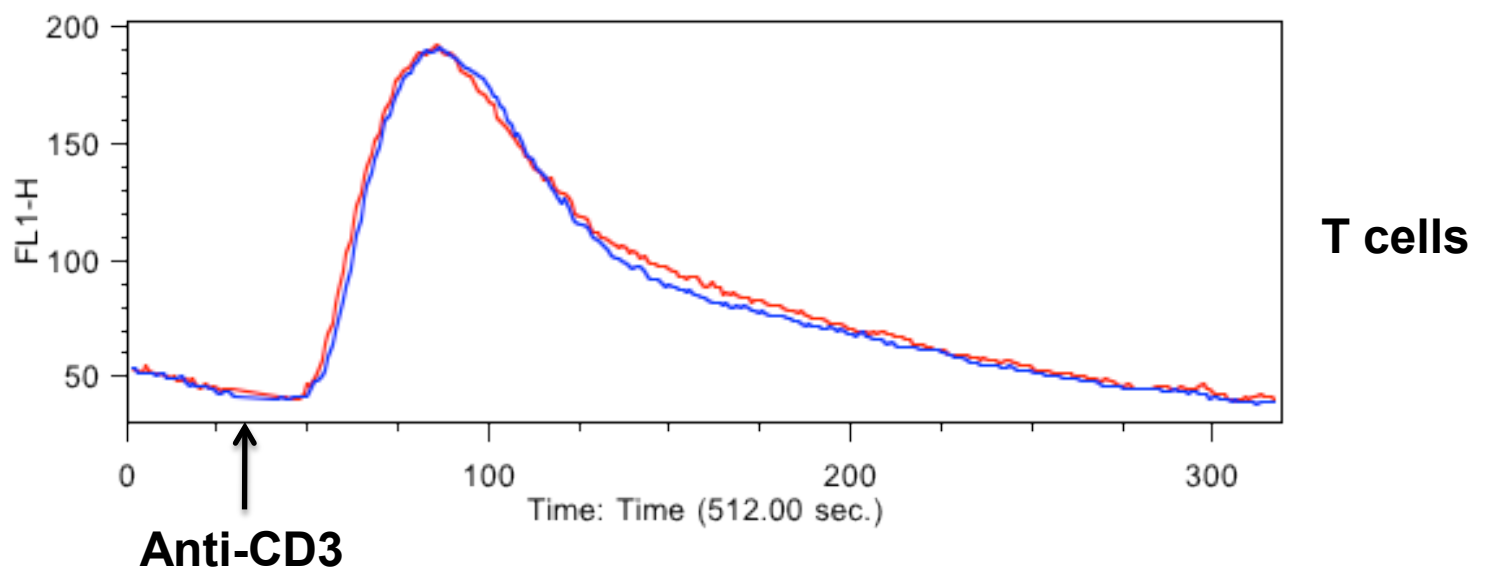
Supplementary Figure 9. Detection of PLC γ_2 in Transfected COS-7 Cells by Western Blotting. Western blot of whole cell protein extracts from COS-7 cells transfected with an empty vector, wt *PLCG2*, Δ cSH2 *PLCG2*, Δ 19 *PLCG2*, and Δ 20-22 *PLCG2* demonstrating the presence of PLC γ_2 and actin from each condition. Extracts were generated from cells that were transfected in parallel with those assayed in Figure 3A, however without radiolabelling.



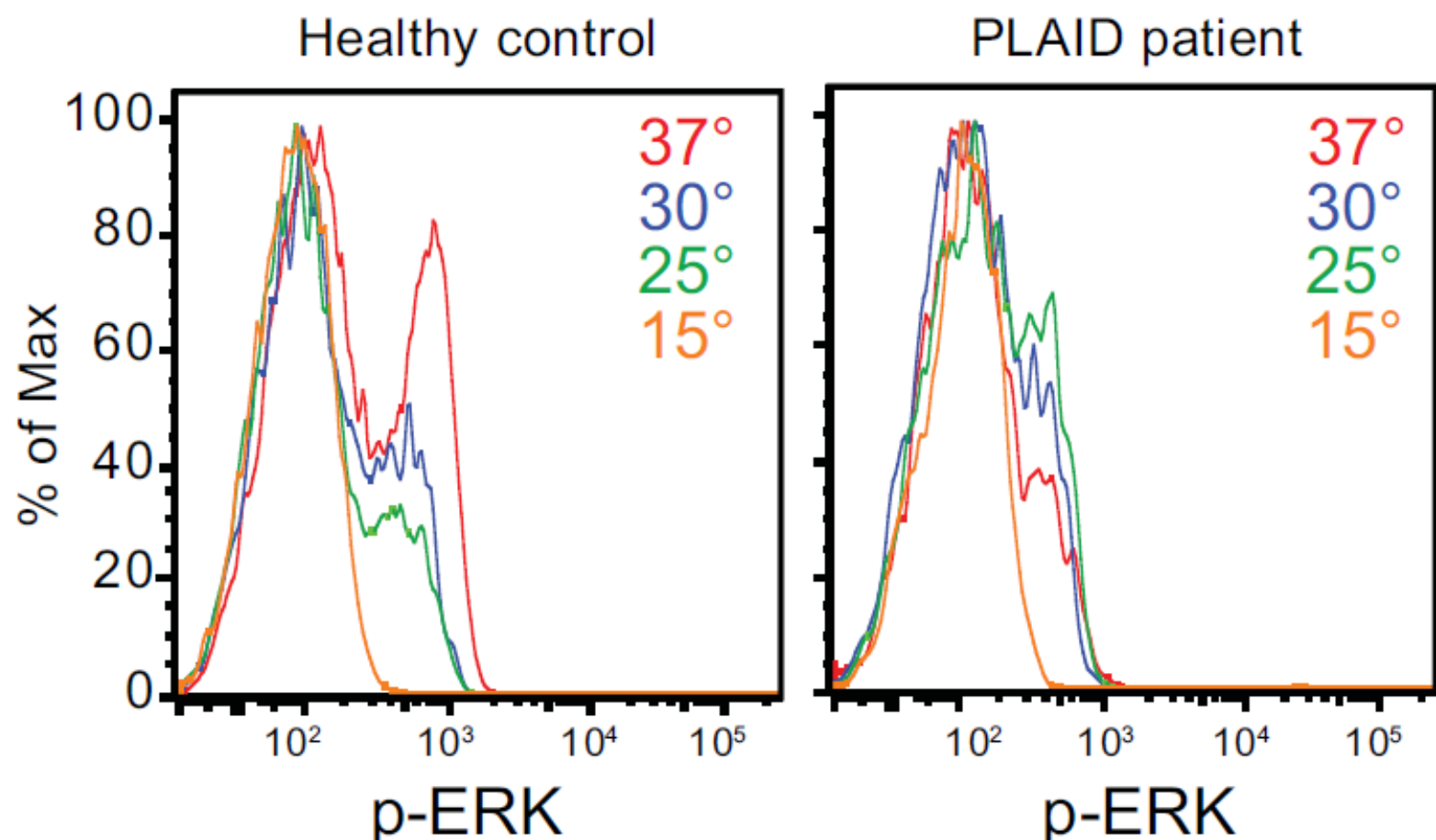
Supplementary Figure 10. Single Cell Analysis of Calcium Flux in Primary B Cells by Confocal Microscopy and ERK Phosphorylation by Flow Cytometry. Calcium flux was measured in single cells by quantifying FLUO-4 content via confocal microscopy over time, following addition of anti-IgM F(ab)[']₂ for surface cross-linking (left panel). Each line shows the fluorescence from a single cell followed over time. Data are representative of two independent experiments, assaying two cases and two controls. The right panels show flow cytometric measurement of ERK phosphorylation 5 minutes after surface anti-IgM cross-linking (blue line), and with no stimulation (red line). These data are representative of three independent experiments, assaying three cases and three controls.



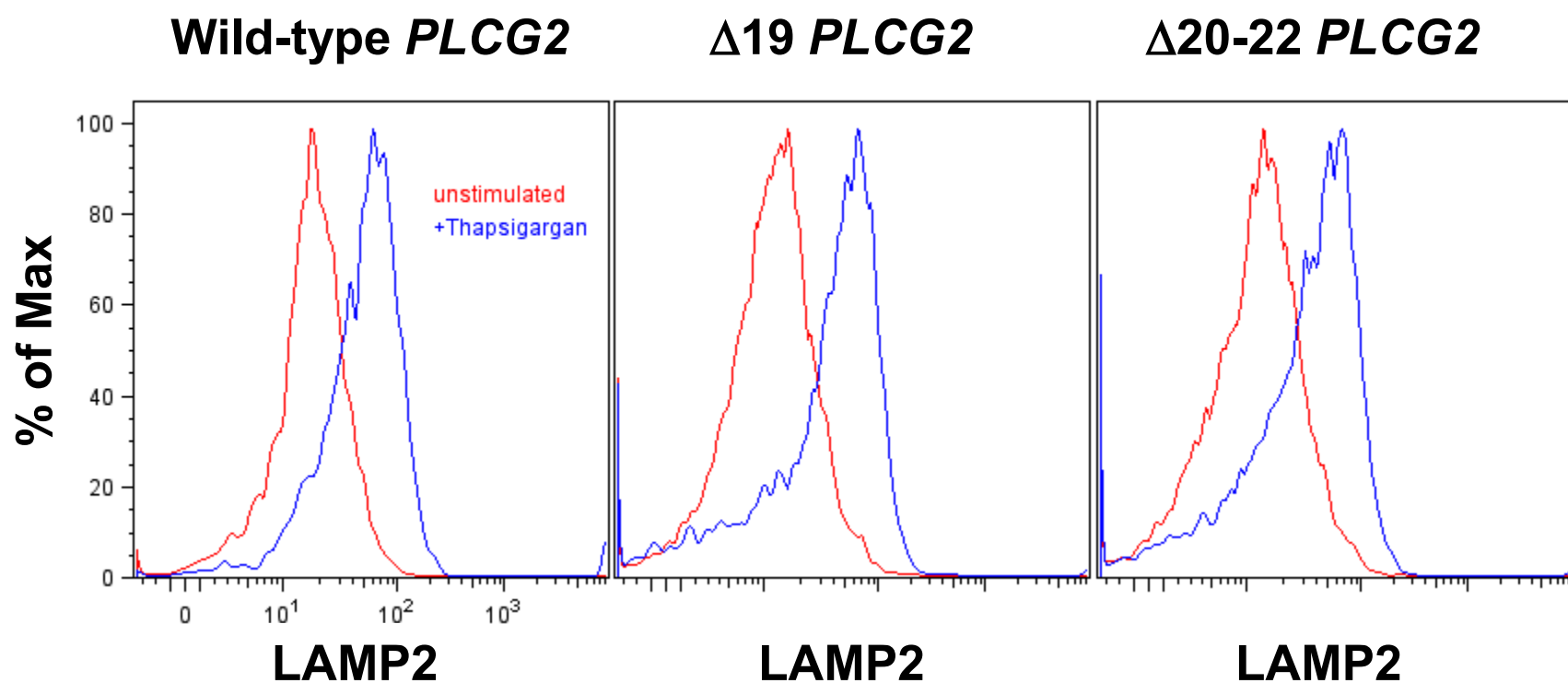
Supplementary Figure 11. Effect of Subphysiologic Temperature on Mutant *PLCG2*-Transfected A20 Cells. Flow cytometric measurement of ERK phosphorylation in murine A20 B cell lymphoma cells transfected with wild-type *PLCG2*, $\Delta 19$ *PLCG2*, or $\Delta 20-22$ *PLCG2* following incubation at either 37°C or 20°C, with or without goat anti-IgM stimulation, gated on GFP⁺ cells. These data are representative of three independent experiments.



Supplementary Figure 12. Analysis of Calcium Flux in T cells by Flow Cytometry. Flow cytometric measurement of calcium flux using FLUO-4 following surface CD3 cross-linking on T-cells. Patient cells are in blue, control cells in red. Arrow indicates time of addition of cross-linking agent.



Supplementary Figure 13. Exposure to Subphysiologic Temperature Restores Capacity for Ligand-Induced Activation of PLAID B Cells. Following stimulation of PBMC with anti-IgM F(ab')₂ for 5 minutes at the indicated temperature, ERK phosphorylation was measured by flow cytometry in gated CD19⁺ B cells. Data reflect three independent experiments, including three distinct patients and three distinct controls.



Supplementary Figure 14. Mast Cell Degranulation in *PLCG2*-Transfected Human LAD2 Mast Cells in Response to Thapsigargin. LAMP2 surface expression, an indicator of mast cell degranulation, was measured by flow cytometry in human LAD2 mast cells transfected with wt *PLCG2*, Δ19 *PLCG2*, or Δ20-22 *PLCG2* following treatment with thapsigargin (blue) or without thapsigargin (red).

III. Supplementary Tables

Supplementary Table 1. Extended Clinical Information of Three Families with PLAID

Subject	Age/Sex	Cold Urticaria	Allergy	Granulomatous Disease	Autoimmunity	Infectious History	IgG 642-1730 mg/dl	IgM 34-342 mg/dl	IgA 91-499 mg/dl	IgE 0-90 IU/dl
1 III-2	46/M	+	Asthma, Eczema	Skin granuloma of nose, fingers and ears from birth (Resolved by age 1)	Positive ANA		1350	27	444	93
1 III-4	53/M	+	Asthma, A.R., Food: fish	Erosive granulomas of nasal septum, invasive sarcoidosis-- symptoms worsen in cold climate		Recurrent pneumoniae, bronchitis	1480	101	567	620
1 III-7	57/F	+	A.R., Drug: PCN, Food: shellfish and chocolate	Granulomatous dermatitis-- NLD vs. sarcoid	Positive ANA	Recurrent sinusitis	1310	51	390	33
1 III-10	51/M	+	None			Onychomycosis, Recurrent varicella	265	<21	60	60
1 III-11	55/M	+	None			Onychomycosis	325	<21	43	18
1 IV-2	22/M	+	None	Skin granuloma of nose, fingers and ears from birth (Resolved by age 1)	Positive ANA		843	24	148	199
1 IV-3	21/M	+	Food: fish	Diffuse granulomatous dermatitis and histiocytic granulomata with skin, palatal and pharyngeal involvement, Sarcoid-like	Seronegative inflammatory arthritis, worsens in cold climate	Recurrent sinusitis and pneumoniae, Treated with IVIG	350	25	27	14146
1 IV-4	24/M	+	Asthma	Skin granuloma of nose (Resolved by age 1)	Vitiligo Positive ANA		1380	44	283	54
1 IV-5		+	None			Recurrent O.M.				
1 IV-8	34/M	+	Asthma, A.R., Drug: PCN		Vitiligo (ANA not measured)					50
1 IV-11	24/F	+	None				478	<21	63	98
1 IV-12	18/F	+	None				468	<21	51	454

1 IV-13	15/F	+	A.R., A.C.				1080	23	139	525
1 V-1	3/F	+	None		Positive ANA					43
2 III-3	60/M	+	Asthma, A.R.		Positive ANA, Positive anti-TPO antibody	Recurrent varicella zoster starting at age 40, Recurrent post- herpetic neuralgia	814	50	143	38
2 IV-5	28/F	+	None	Granuloma annulare	Hashimoto's thyroiditis, Vitiligo, Positive ANA	Recurrent pneumonia during childhood	1470	83	82	34.5
2 IV-6	31/M	+	Food: dark alcohol		Positive ANA	Recurrent Group A Strep pharyngitis	1120	65	84	152
2 IV-8	29/F	+	None		Hashimotos thyroiditis, Positive ANA	Recurrent pneumonia as a teenager	774	46	80	9.3
2 V-3	2/M	+	None							
2 V-5	3/M	+	None			Recurrent O.M., Pneumonia				
2 V-6	5/M	+	Asthma, Eczema		Strongly positive ANA	Recurrent O.M., Pneumonia	675 (Normal 504- 1465 mg/dl)	22 (Normal 24- 210 mg/dl)	61 (Normal 27- 195 mg/dl)	12.3
2 V-7	19mo/F	+	Eczema			Recurrent O.M.				
3 II-3	1/F	unknown	None			Died at one year of pneumonia				
3 III-2	64/M	+	Food: wheat Asthma A.R.			Recurrent O.M. and sinusitis, Osteomyelitis at age 29	919	<21	75	0
3 III-4	65/M	+	Asthma, A.R.		Positive anti-ENA Positive ANA	Recurrent sinusitis and pneumoniae, Failure to respond to pneumococcal vaccine, Treated with IVIG	585	10	60	124

3 IV-2	42/F	+	Asthma, A.R.	Unspecified connective tissue disorder, Positive ANA	Recurrent O.M. and pharyngitis, Recurrent sinopulmonary infections unresolved without antibiotics, Pyelonephritis, Treated with IVIG	783	51	108	30
3 IV-4	34/F	+	A.R. Eczema	Vitiligo, Positive ANA, Positive anti-TPO antibody		1070	22	105	50
3 V-1	16/F	+	None						

Notes: Autoantibodies were identified in 14 out of 21 individuals tested, while symptomatic autoimmune disease was present in 7 out of 27 individuals evaluated, one of whom did not have detectable autoantibodies. As a result, the presence of either autoantibodies or symptomatic autoimmune disease occurred in 15 out of 27 individuals. A.R. = allergic rhinitis, A.C. = allergic conjunctivitis; ANA = antinuclear antibodies; ENA = extractable nuclear antigens; IVIG = intravenous immunoglobulin; NLD = necrobiosis lipoidica diabetorum; O.M. = otitis media; PCN = penicillin; TPO = thyroid peroxidase

Supplementary Table 2. Candidate Genes within the Intersection of the Family 1 Linkage Interval and the Family 2 Candidate Interval

MIR548H4
DYNLRB2
CDYL2
C16orf61
CENPN
ATMIN
C16orf46
GCSH
PKDIL2
BCMO1
GAN
CMIP
PLCG2
SDR42E1
HSD17B2
MPHOSH6
CDH13
HSBP1
MLYCD
OSGIN1
NECAB2
EFCBP2
SLC38A8
MBTPS1

Supplementary Table 3. Summary of PCR Conditions and Primers

Primer target	Forward Primer Sequence	Reverse Primer Sequence	Extension Time
cDNA seg 1	GCCAGCTTCCTGATTTCTCC	TTTAGAGTCAGCTGCCAAGC	1 min
cDNA seg 2	AAAGATTTTCGAGCGAGCAAA	AGATGGAACTGTTCAAAGCTGA	1 min
cDNA seg 3	CATCTTGCCCCTGATCAACT	GTCCACCGCGTCATACTTCT	1 min
cDNA seg 4	GATGACACCATGCGTGAAAC	CACTGGGAAGCTCGAGGTAA	1 min
cDNA seg 5	ATGGGAAGCCGGTCATCTAC	GGGTATATCCTGGGGCACTT	1 min
cDNA seg 6	ATGGAGGACAAGAAGGACGA	AGTGCTGGATGAGGGCATAG	1 min
cDNA seg 7	CCCCAATGACTACACCCTGT	ACGAGCTCCACCAGACTCTC	1 min
cDNA seg 8	CGACTCCTATGCCATCACCT	GGATGGGAAGTACTGCTGGA	1 min
cDNA seg 9	AGCGATGAGCTGAGCTTCTG	CTTGTTTTTGCTGGTTGGTT	1 min
cDNA seg 10	GGTTTCAGAGCATCCGAGAG	GA CTGTCAGCGTCATCAGGA	1 min
cDNA seg 11	GCAGATGAATCACGCATTGT	GCCGCATCTCACAGAAAAC	1 min
cDNA seg 12	CGATCCCAACTTTCTTGCTC	GATGGCAGGCTTGAAGAAAA	1 min
Exon 1	CGGGTTGCCTCAGTTTCTT	CTGAGCCAGGACGCAGAC	1 min
Exon 2	TGCCTTGCCACTAGAACCTT	TGACAAGACAGGAAGGTGGA	1 min
Exon 3	GATCCTGTTGGGGAAGGAAG	TCAAAACCATGACCCCAAAT	1 min
Exon 4	CAGCATAGGCTTCTCCCATC	G TAGAGCCCACACCGTACT	1 min
Exon 5	TGCACATTCCGTAGGACTCA	CTCCCGACAAGTCATGCTC	1 min
Exon 6	GGA ACTGATGGGAGCAGCTA	ACGGA ACTCAAAGGCAAGC	1 min
Exon 7	GCATGCCATTTCTGAAACAA	TGGGTTTCTTAGGAACATGC	1 min
Exon 8	CCTTTTGCTTCTTAAGTTTCTGTT	ATCTCTATCCACGCCACAGG	1 min
Exon 9	TGGCCAACCTGGTACCTAAC	TCGTTCCATGAAGACAGGTG	1 min
Exon 10	GTCTTCGATTGCGACTGGAT	CAGCAAAGTTCTCCAAGGAA	1 min
Exon 11	CGTGGGTA ACTGAGGTCTGG	CTCCACCTGCTT CACAACAG	1 min
Exon 12	ACAACACCCTGAGGTGCAG	TCGAAAGAAAACATGGAGCA	1 min
Exon 13	TCTAGTAACTGAACTGGTGTGTGG	TTTGCCTGTCTGTCCATCTG	1 min
Exon 14	GCAGATGTGGGGGTTGTG	AGGACATAGAACTTGGTGAACG	1 min
Exon 15	GGGAAGAGGCAGATGAAGG	TGGGAACAAGACAGGCGTAT	1 min
Exon 16	AACTGGGAAAAGCACATCCA	CAGAGAAGCCACATGGGAAG	1 min
Exon 17	TGAGGCTGGCCTCTCTATGT	CCTGCCTGAGCTAGAACCAC	1 min
Exon 18	AGGAGCAGAGGGAAGGTTGT	ACCACCAGGCCAGCTTCT	1 min
Exon 19	TGGTGCCATTATCTTGTCCTC	GTGAGCCTCCACCTGAAGTT	1 min
Exon 20	CCCACTTCTCTAAGGCTGGA	TGCTGAATTCATGAACAGATGA	1 min
Exon 21	AAGGAAGCCTAGAACCCTTGTT	AAGTCTTCCCTTGCCCTGTT	1 min
Exon 22	GGCCTGACCTTTTCTTCTT	GACAAAGGGGGTCAGACTTG	1 min
Exon 23	CATCAGAATTGAGCCAGCAG	GCAGCACATGGAAAAATTCA	1 min
Exon 24	AAACGGTGTGCTTTGGAAC	AGCCACCTCCCTGTGTAGG	1 min
Exon 25	CCATGAGAGAAACAGCTCAGG	TCTGAATCCACCTGGTCTCC	1 min
Exon 26	TGTGCAAGAAAGCAAAGTGG	CTCTGCCCCCTCTGAAAATA	1 min

Exon 27	AATCTGAGCATCCAGCCATT	CACCACATGGTATCGCTGAC	1 min
Exon 28	TTGAACAGCTGCCTCACATT	CAAGACAACCAGCCTCCCTA	1 min
Exon 29	TCATCCAGTGTCACCTCTAGAACC	CAAAATCCCCCACGAAGA	1 min
Exon 30	GGTTGCTAAGGGGATTCACA	CCACCCAATCGGTTTTACAG	1 min
Exon 31	CCTATGATCCCGAGGTAGCC	CATGCTTGGGCTAGACAATTC	1 min
Exon 32	CTTGTGAGATGCCAGGTTCA	CAACTCAGAGGGGCTTTCCAG	1 min
Exon 33	TGGAGTCTGCCTCTCTGACA	TCATCCTCCATGGACATCAG	1 min
Ex 18 to Ex 20	ATGCCCTCATCCAGCACTAC	TGCGACAATGCTTTACCTTG	9 min 12 sec
Ex 19 to Ex 23	AGCGACTCCTATGCCATCAC	GGATGGGAAGTACTGCTGGA	9 min 36 sec
Exon 19 Deletion Genomic Screening Assay	ATGCCCTCATCCAGCACTAC	TGCGACAATGCTTTACCTTG	9 min 12 sec
Exon 20-22 Deletion Genomic Screening Assay	CCCACCAGCACTTCTCCTT	GGATGGGAAGTACTGCTGGA	9 min 36 sec
Exon 20-22 Deletion cDNA Screening Assay	CGACTCCTATGCCATCACCT	CTTGGTTTTGCTGGTTGGTT	1 min

IV. Supplementary References

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