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;366:330-8. 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 Diego[1].

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 described[2]. 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 settings [4]. 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 described[5]. 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 x *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 Ca^{2+} Flux

Intracellular Ca^{2+} flux was measured by flow cytometry after labeling with FLUO-4 AM (Invitrogen), as described with slight modifications[6]. 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 described[7]. 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 described[8]. Igκ 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 previously[9-11]. COS-7 cells were maintained at 37°C in a humidified atmosphere of 95 % air and 5 % CO_2 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 $2.5x10^5$ 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 PLCγ_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 PlusReagent™ and 7 µl Lipofectamine™ (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% CO_2.

For analysis of inositol phosphates, 24 h post-transfection cells were labeled with 1.5µ Ci/ml *myo*-[2-^{3}H]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 [^{3}H]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 ± 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 μg/ml poly-L-lysine (Sigma) for 1hr, and washed with PBS followed by distilled water. Isolated B cells were labeled with 2μM FLUO-4 AM (Invitrogen) and seeded onto pre-coated 4-chambered coverglass sildes (NUNC) at a density of 0.2 x 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 1μm 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 2μM 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 μg/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 x 10^6 cells in RPMI 1640 medium supplemented with 10% FBS, 2mM L-glutamine and 1% penicillin/streptomycin) were stimulated with 12 μg/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 -20^0 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

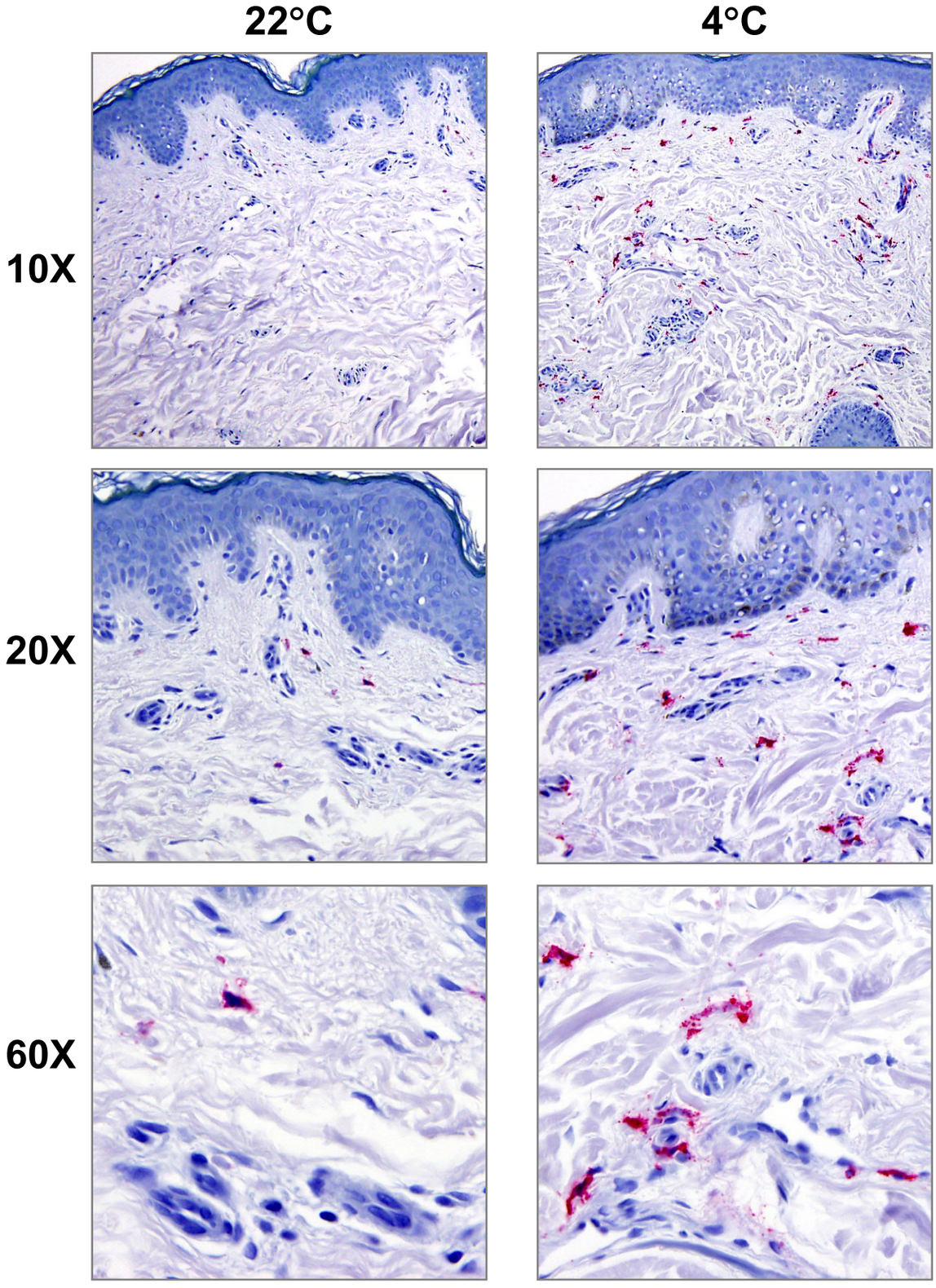
The murine A20 B cell lymphoma cell line was transfected using the Amaxa Cell Line Nucleofection V kit (Lonza) according to the manufacturer's optimized protocol for A20 cells. After 24 h, A20 cells were incubated at either 37°C or 20°C for 20 min. Cells were then stimulated at their designated temperatures 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 described[12]. After 24h, 2 x 10^5 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

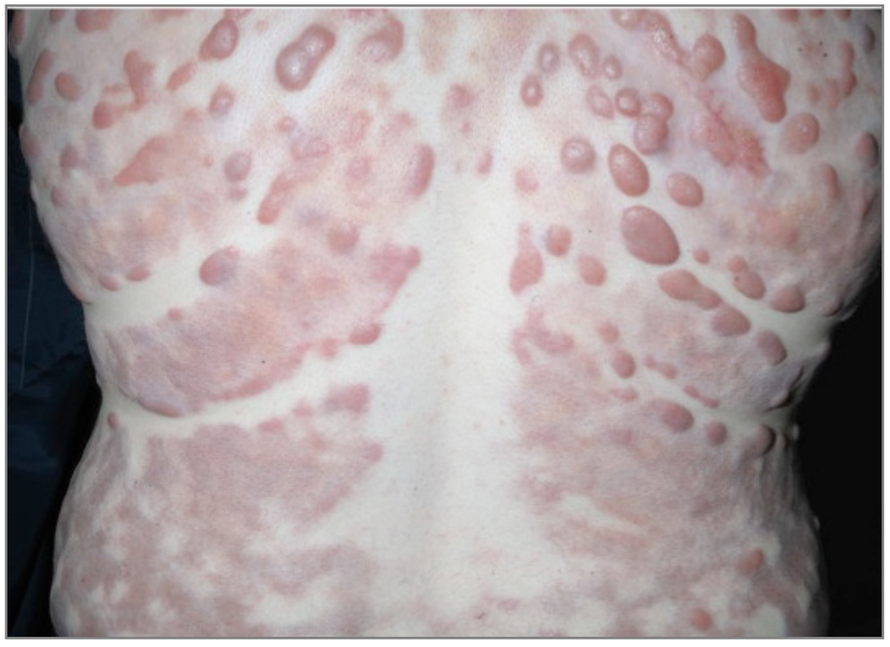
used to induce maximal degranulation and were then also placed on ice. Cells were washed twice in PBS 0.1% BSA and stained with 1 µg/ml PE conjugated mouse anti-human LAMP2 (Clone H4B4) (Abcam, Cambridge, UK) for 45 min on ice. LAD2 cells were then washed twice in PBS 0.1% BSA. Flow cytometry was carried 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



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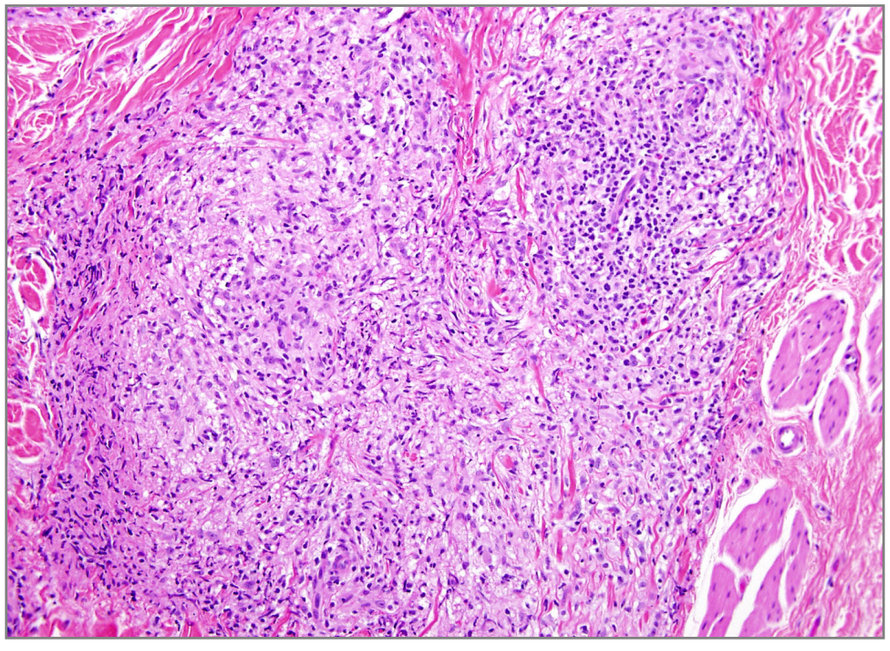
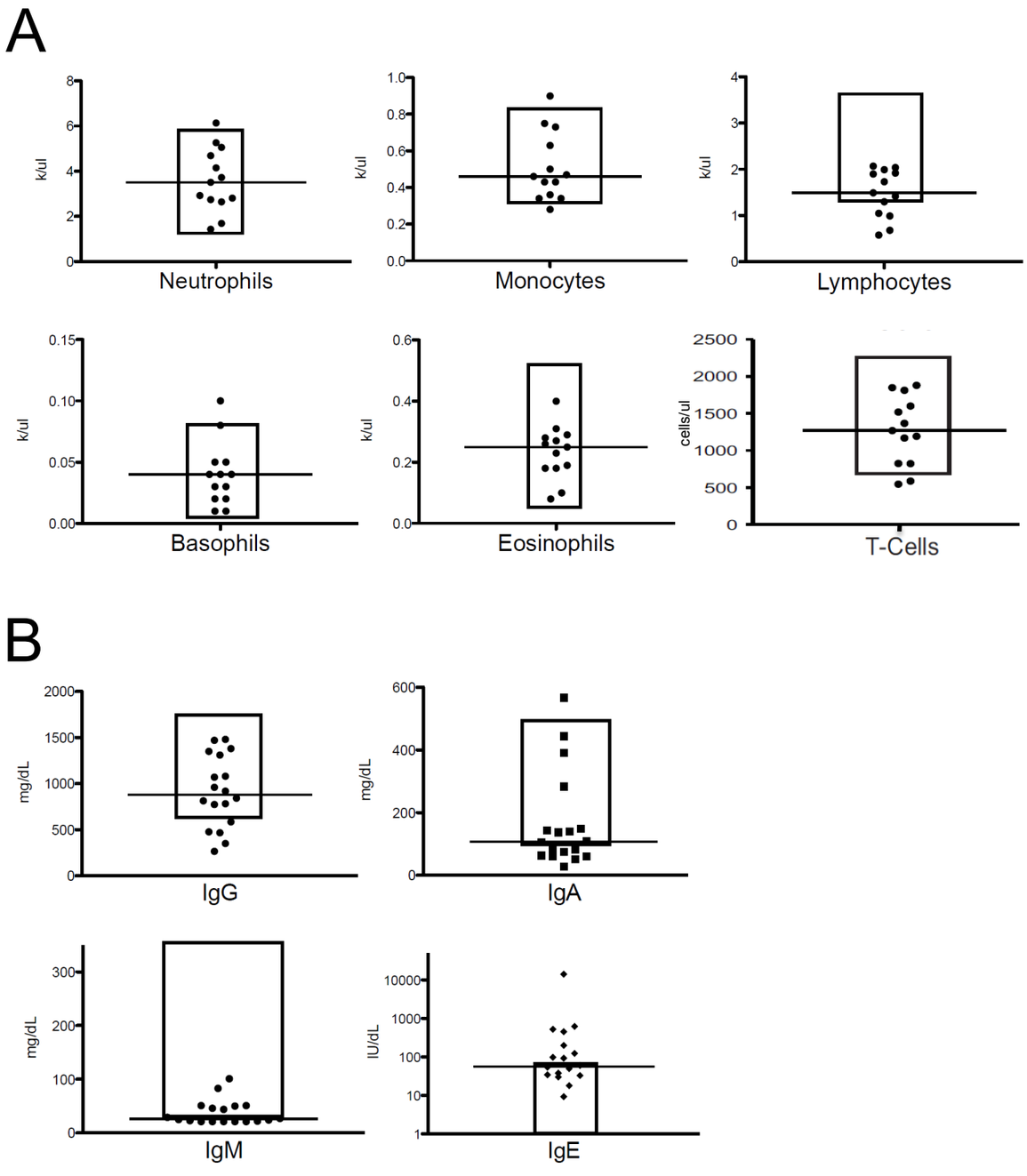
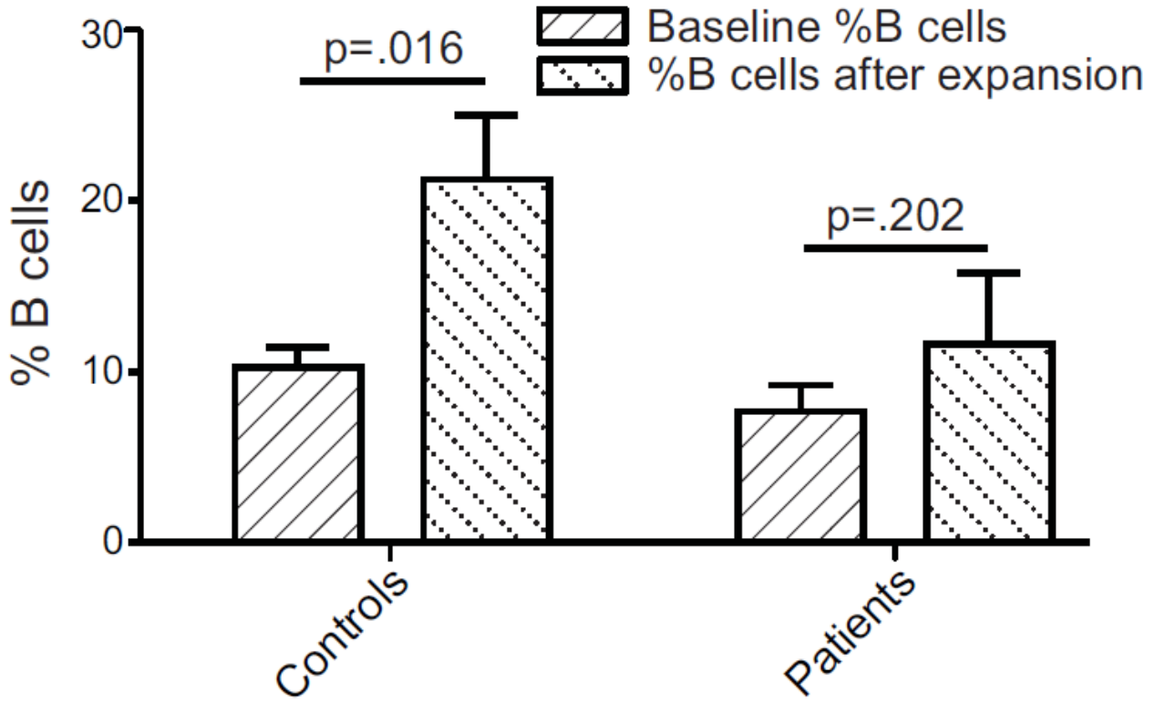


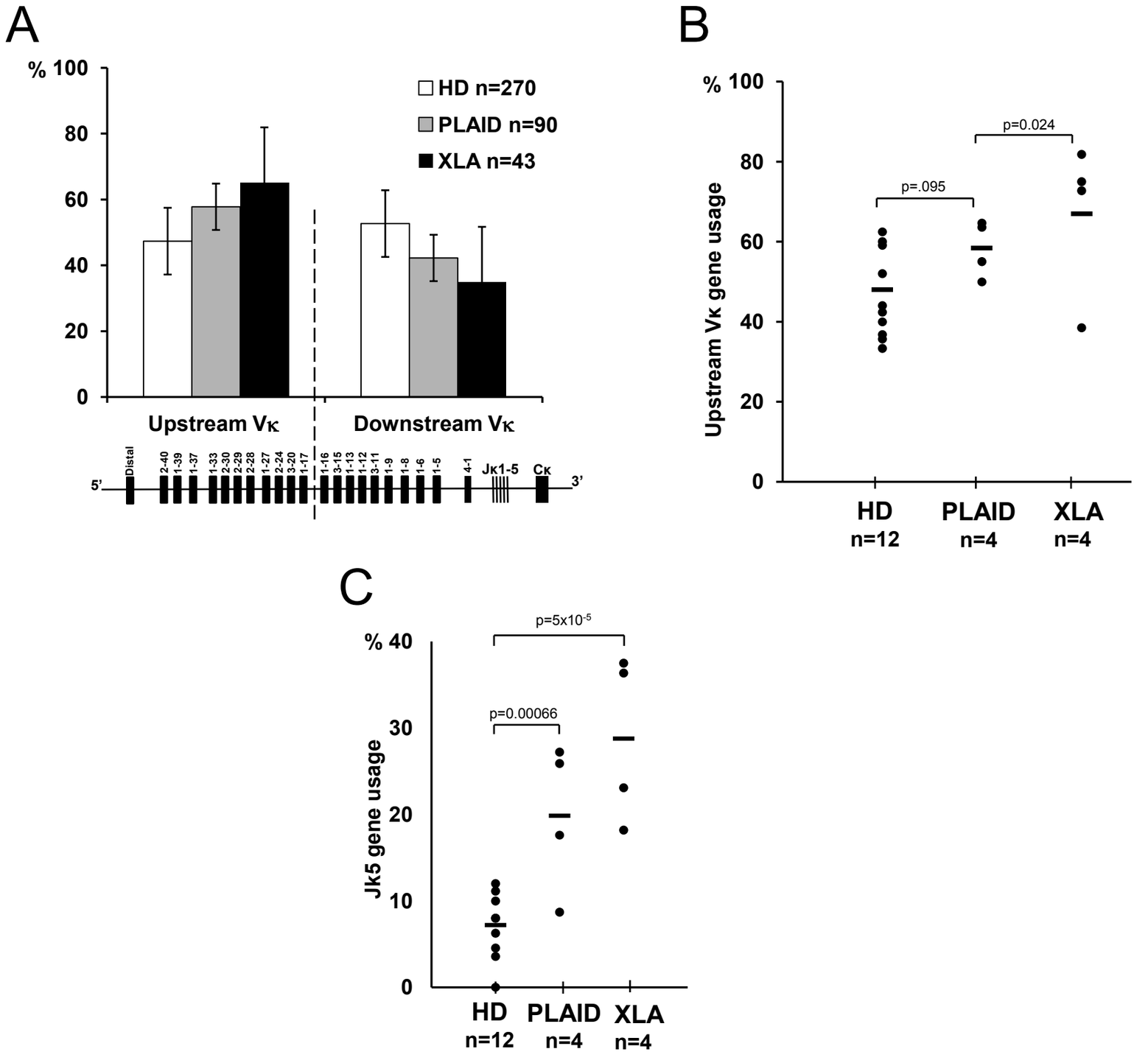
Supplementary Figure 2. Severe Granulomatous Skin Disease in a Subject with PLAID. Panel A depicts nodular skin lesions present in an affected member of Family 1. H&E staining of the lesions demonstrate granulomatous inflammation with multiple foci of epithelioid histiocytes and multinucleated giant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



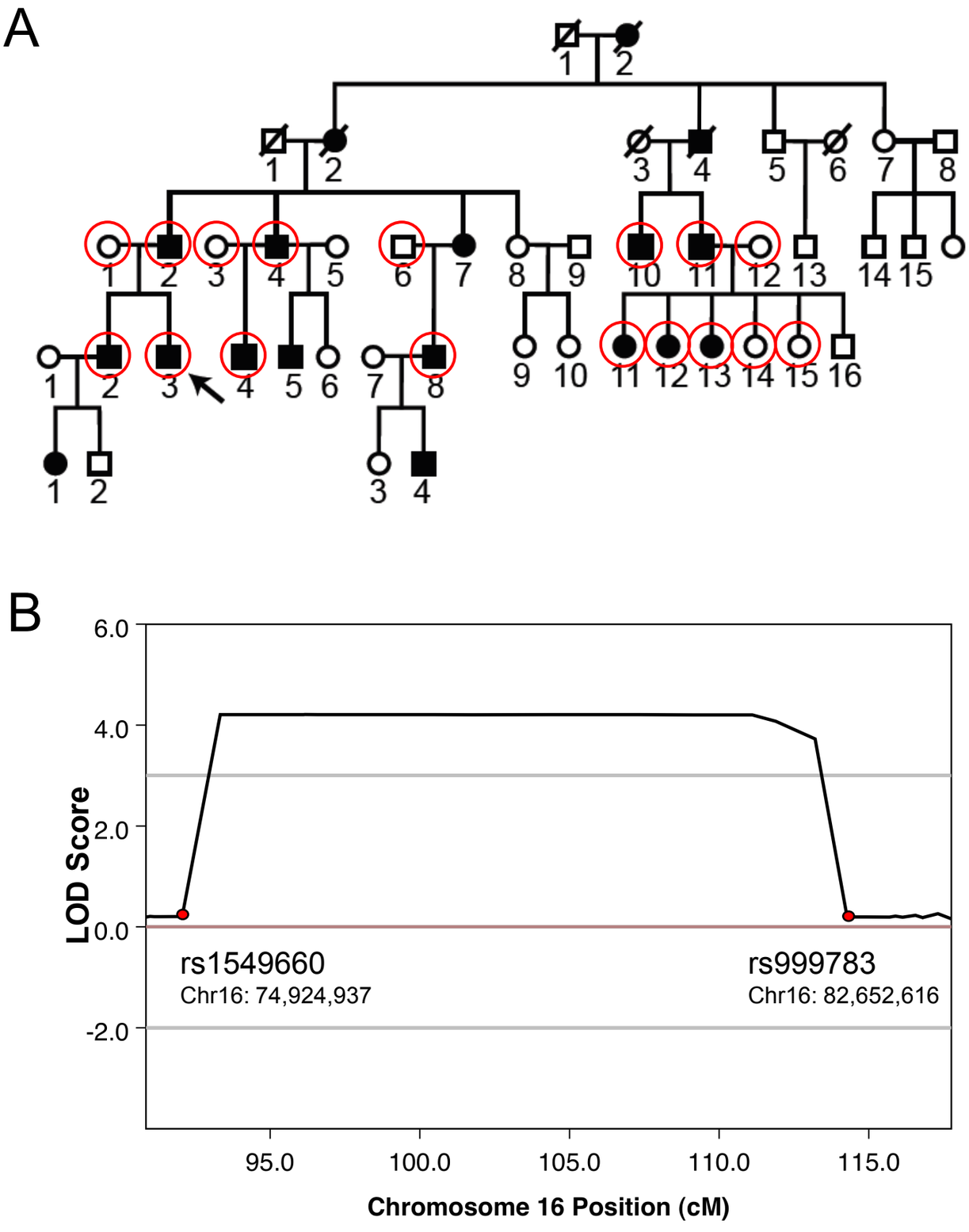
Supplementary Figure 3. Serum Immunoglobulins and White Blood Cell Differential Cell Counts from Adult Subjects with PLAID. Panel A demonstrates peripheral blood leukocyte counts, while Panel B shows serum immunoglobulin measurements. The rectangles are 2 SD above and below the test mean determined in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



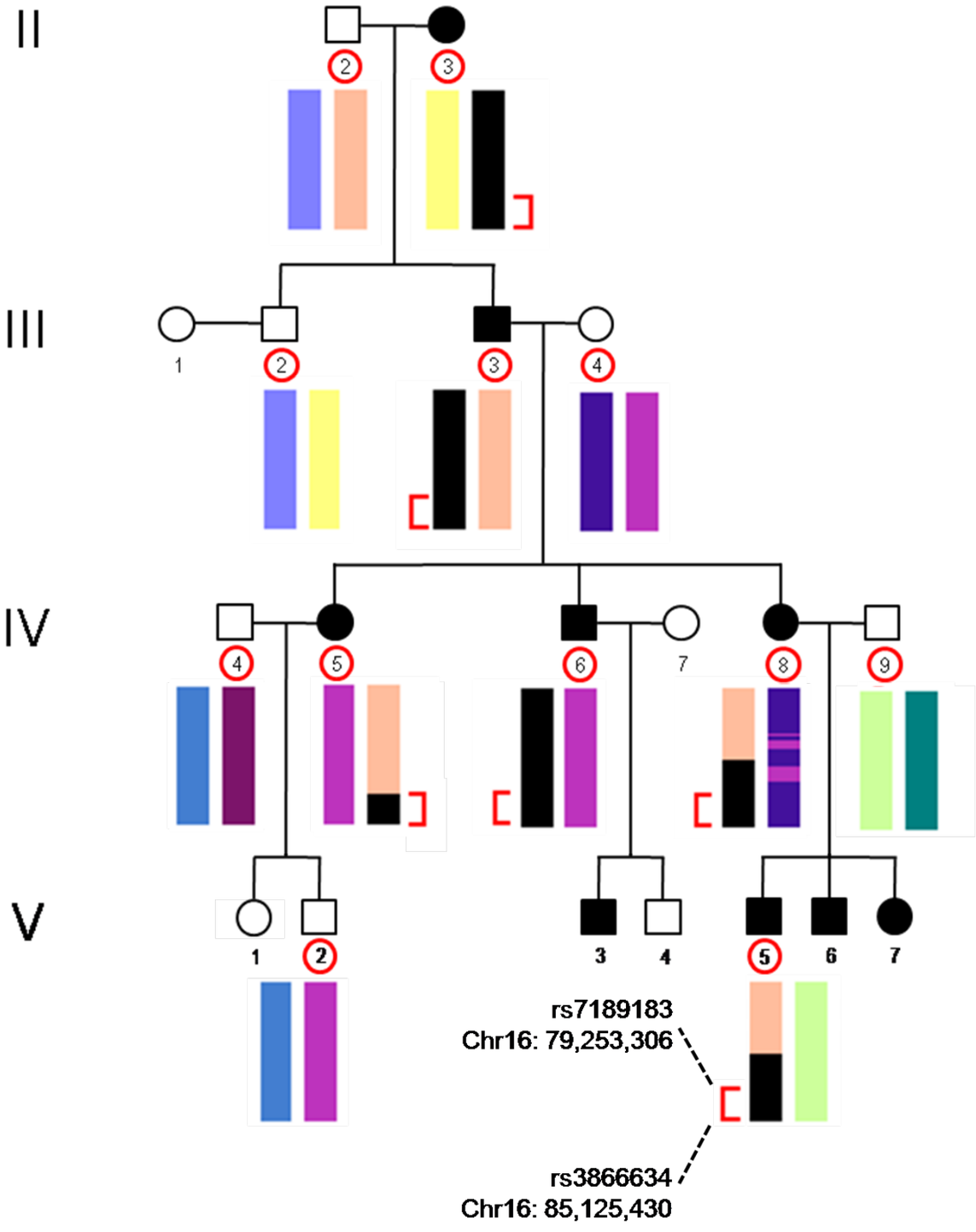
Supplementary Figure 4. Reduced Proliferative Capacity and Expansion of B Cells from Subjects with PLAID. Bar graphs demonstrate the percentage of B cells in PBMCs from subjects with PLAID and control subjects before and after four days of expansion in culture. Data shown are means and SEM for five individuals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



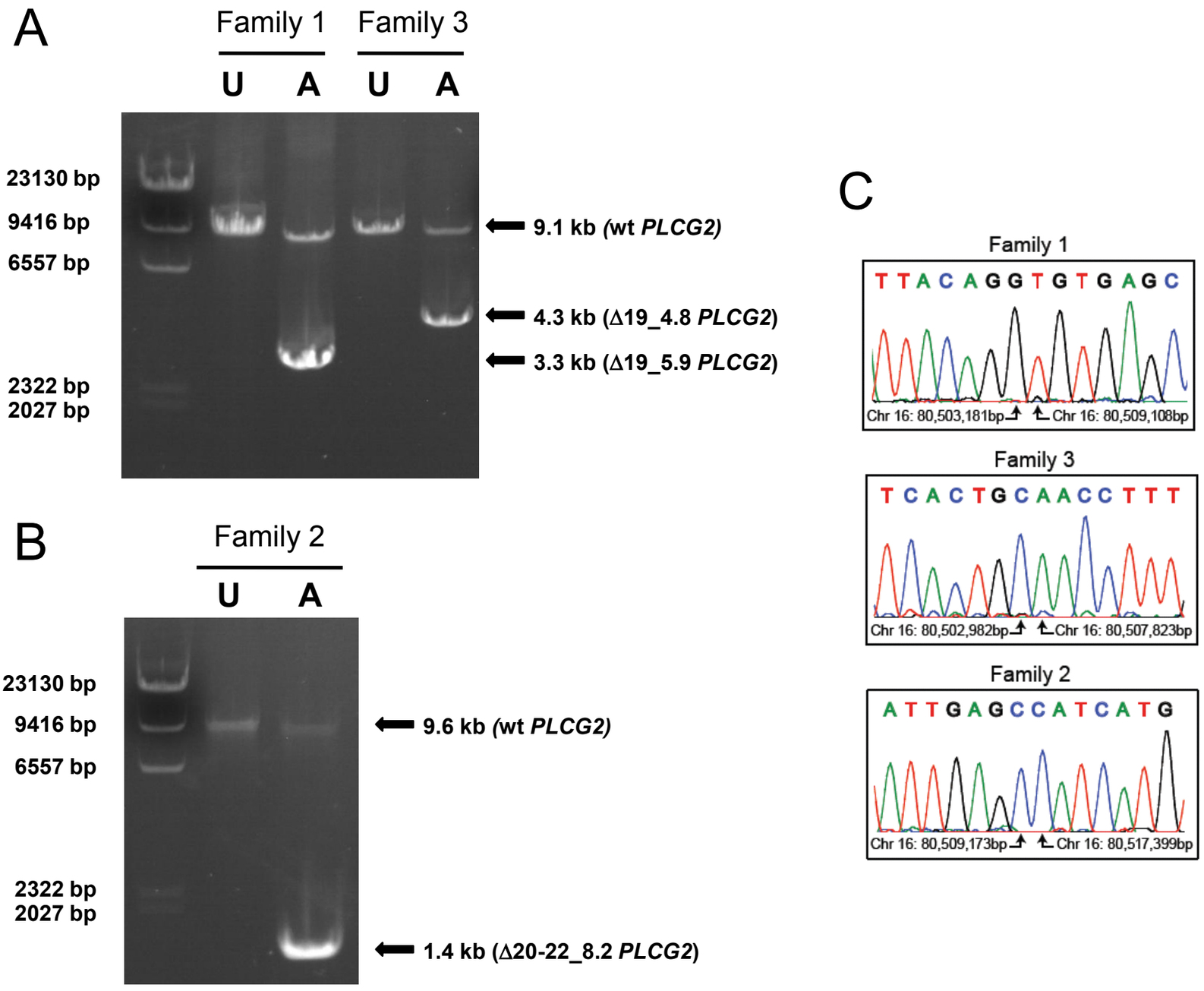
Supplementary Figure 5. Igκ Secondary Recombination in Transitional B Cells from Subjects with PLAID, Healthy Donors (HD), and Patients with X-Linked Agammaglobulinemia (XLA). Bar graphs (A) show the frequency of Vκ region use determined by single cell Igκ sequence analysis from 12 healthy donors, 4 PLAID patients, and 4 XLA patients pooled by disease (n=number of cells evaluated). The scatter plots (B and C) illustrate the upstream Vκ and Jκ5 usage (percent of total), respectively, of individual subjects (n = number of individuals). The horizontal black line represents the mean.



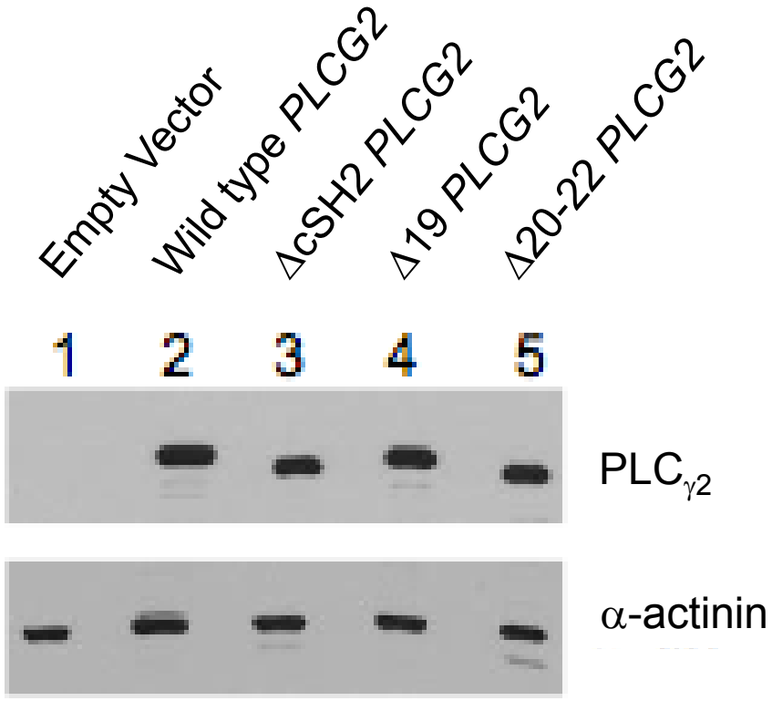
Supplementary Figure 6. Results of Linkage Analysis in Family 1. In the pedigree of Family 1 (panel A), individuals included in the linkage analysis are highlighted with red circles. Panel B demonstrates the single linkage interval on chromosome 16 identified through linkage analysis of the individuals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



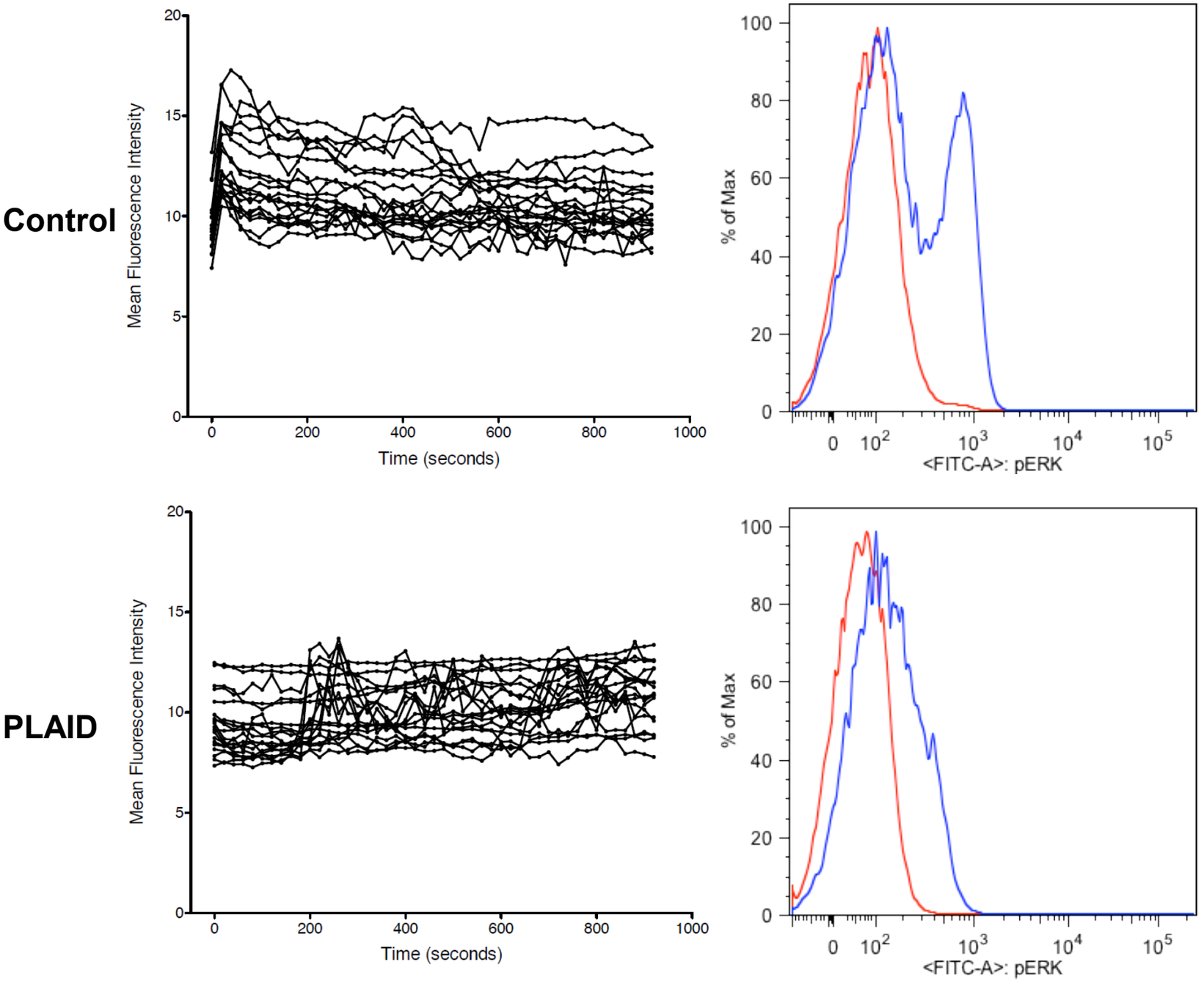
Supplementary Figure 7. Results of Linkage and Haplotype Analysis in Family 2. Haplotype analysis was performed on the individuals in Family 2 highlighted with red circles, and a chromosome 16 haplotype was identified that segregated perfectly with cold urticaria. Red brackets indicate the location of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



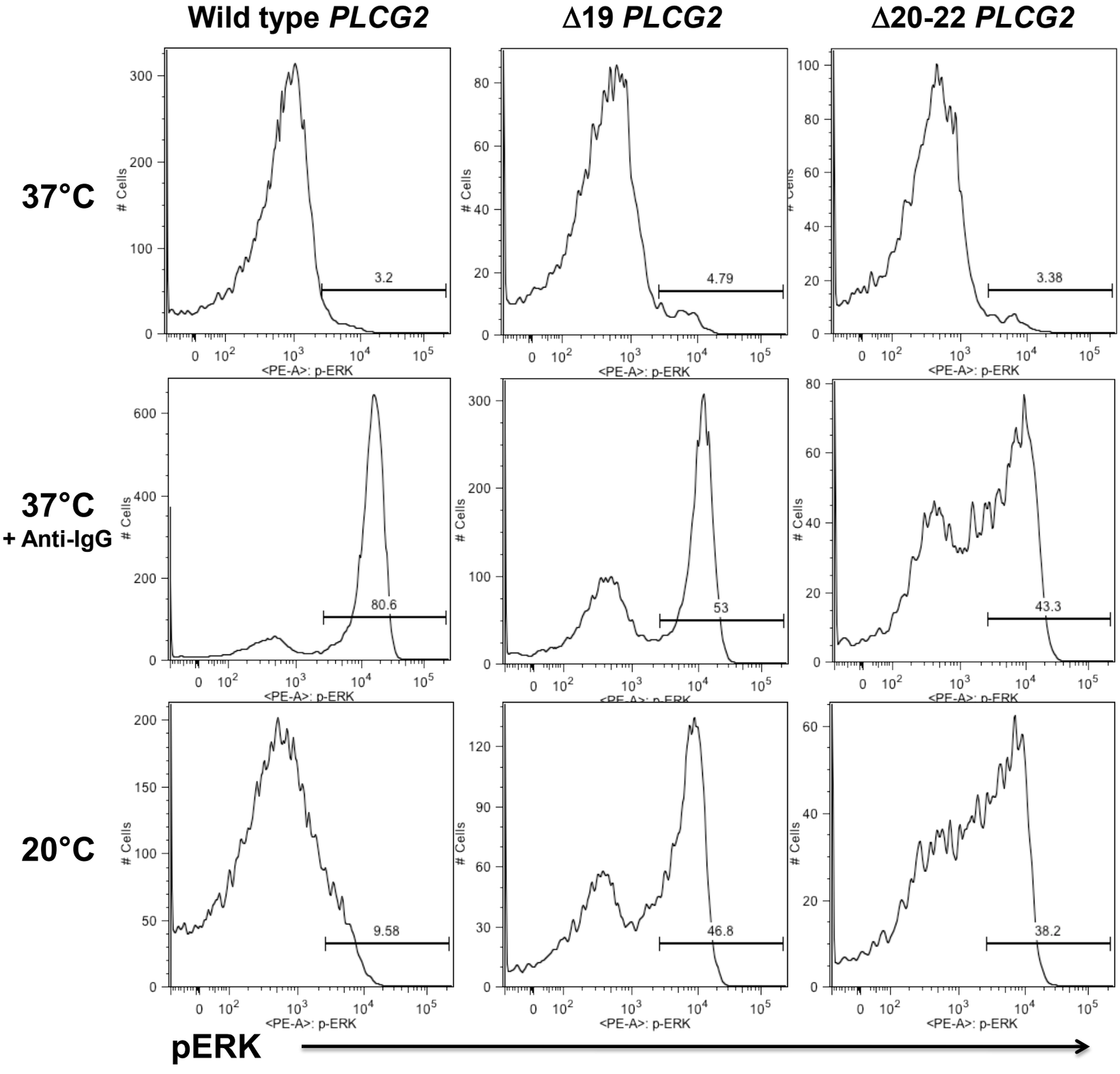
Supplementary Figure 8. Genomic DNA Skip Assays Identify Novel Deletions in Each PLAID Family. Panel A demonstrates PCR products from unaffected (U) and affected (A) members of Families 1 and 3 obtained from the Exon 19 Deletion Genomic Screening Assay (Supplementary Table 3) which amplifies the region 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).



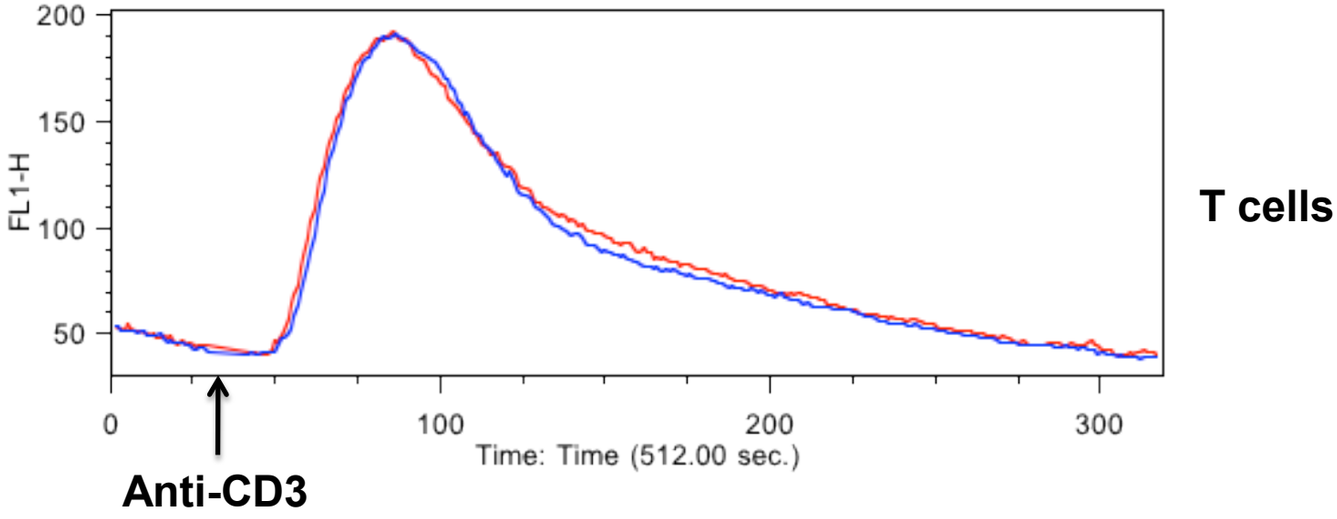
Supplementary Figure 9. Detection of PLCγ_2 in Transfected COS-7 Cells by Western Blotting. Western blot of whole cell protein extracts from COS-7 cells transfected with an empty vector, wt *PLCG2*, ΔcSH2 *PLCG2*, Δ19 *PLCG2*, and Δ20-22 *PLCG2* demonstrating the presence of PLCγ_2 and actin from each condition. Extracts were generated from cells that were transfected in parallel with those assayed in Figure 3A, however without radiolabelling.



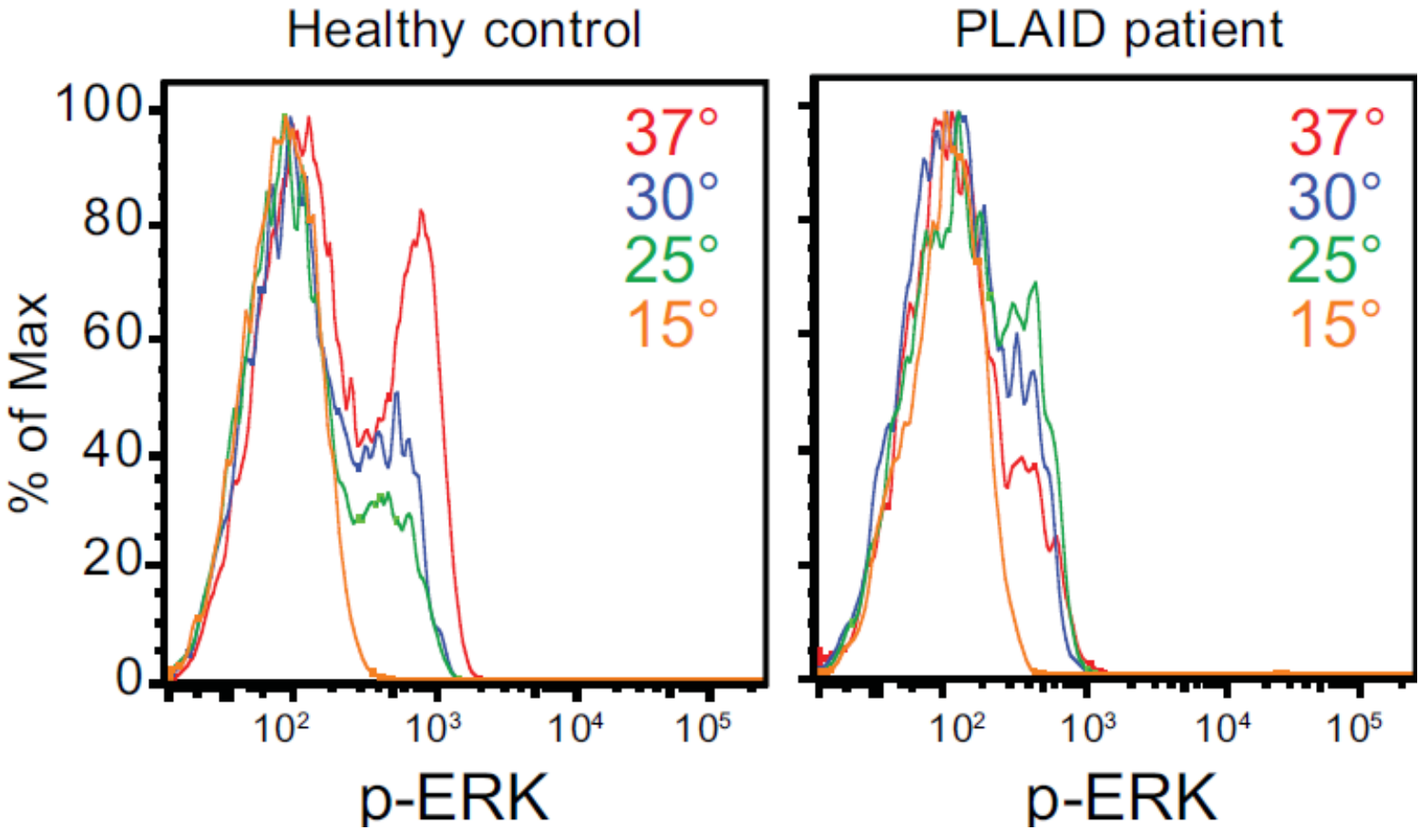
Supplementary Figure 10. Single Cell Analysis of Calcium Flux in Primary B Cells by Confocal Microscopy and ERK Phosphorylation by Flow Cytometry. Calcium flux was measured in single cells by quantifying FLUO-4 content via confocal microscopy over time, following addition of anti-IgM F(ab)'2 for surface 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.



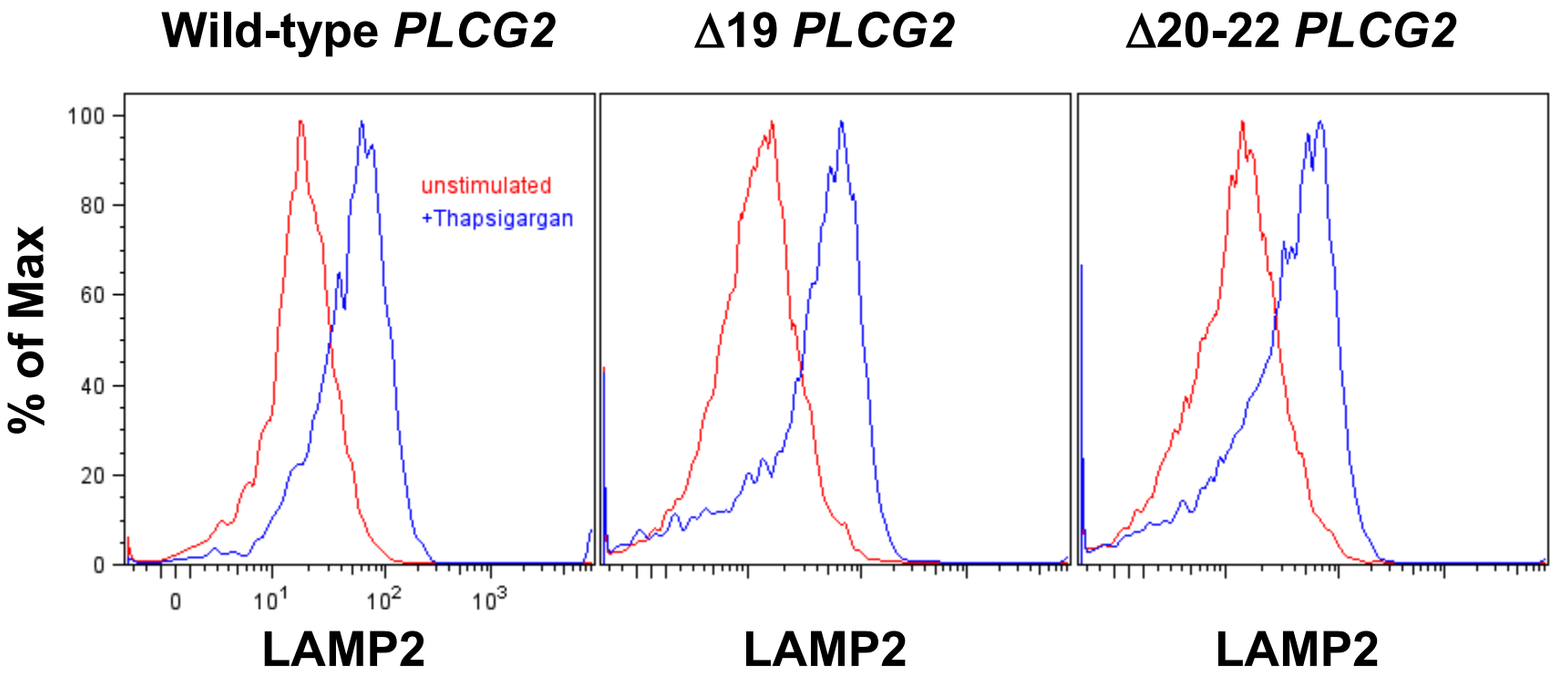
Supplementary Figure 11. Effect of Subphysiologic Temperature on Mutant *PLCG2*-Transfected A20 Cells. Flow cytometric measurement of ERK phosphorylation in murine A20 B cell lymphoma cells transfected with wild-type *PLCG2*, Δ19 *PLCG2*, or Δ20-22 *PLCG2* following incubation at either 37°C or 20°C, 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 Thapsigargan. 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 thapsigargan (blue) or without thapsigargan (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, bronchitits	1480	101	567	620
1 III-7	57/F	+	A.R., Drug: PCN, Food: shellfish and chocolate	Granuomatous 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 diabeticorum; 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	AAAGATTTCGAGCGAGCAAA	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	CTTGGTTTTGCTGGTTGGTT	1 min
cDNA seg 10	GGTTTCAGAGCATCCGAGAG	GACTGTCAGCGTCATCAGGA	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	GTAGAGCCCACACCGCTACT	1 min
Exon 5	TGCACATTCCGTAGGACTCA	CTCCCGACAAGTCATGCTC	1 min
Exon 6	GGAACTGATGGGAGCAGCTA	ACGGAACTCAAAGGCAAGC	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	CGTGGGTAACTGAGGTCTGG	CTCCACCTGCTTCACAACAG	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	GGCCTGACCTTTTCCTTCTT	GACAAAGGGGGTCAGACTTG	1 min
Exon 23	CATCAGAATTGAGCCAGCAG	GCAGCACATGGAAAAATTCA	1 min
Exon 24	AAACGGTGTGCTTTGGAAAC	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	TCATCCAGTGTCACTCTAGAACC	CAAAATCCCCCACGAAGA	1 min
Exon 30	GGTTGCTAAGGGGATTCACA	CCACCCAATCGGTTTTACAG	1 min
Exon 31	CCTATGATCCCGAGGTAGCC	CATGCTTGGGCTAGACAATTC	1 min
Exon 32	CTTGTGAGATGCCAGGTTCA	CAACTCAGAGGGCTTTCCAG	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

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