



Diabetes With Multiple Autoimmune and Inflammatory Conditions Linked to an Activating SKAP2 Mutation

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OBJECTIVE

Multiple genome-wide association studies have identified a strong genetic linkage between the *SKAP2* locus and type 1 diabetes (T1D), but how this leads to disease remains obscure. Here, we characterized the functional consequence of a novel *SKAP2* coding mutation in a patient with T1D to gain further insight into how this impacts immune tolerance.

RESEARCH DESIGN AND METHODS

We identified a 24-year-old individual with T1D and other autoimmune and inflammatory conditions. The proband and first-degree relatives were recruited for whole-exome sequencing. Functional studies of the protein variant were performed using a cell line and primary myeloid immune cells collected from family members.

RESULTS

Sequencing identified a *de novo* *SKAP2* variant (c.457G>A, p.Gly153Arg) in the proband. Assays using monocyte-derived macrophages from the individual revealed enhanced activity of integrin pathways and a migratory phenotype in the absence of chemokine stimulation, consistent with *SKAP2* p.Gly153Arg being constitutively active. The p.Gly153Arg variant, located in the well-conserved lipid-binding loop, induced similar phenotypes when expressed in a human macrophage cell line. *SKAP2* p.Gly153Arg is a gain-of-function, pathogenic mutation that disrupts myeloid immune cell function, likely resulting in a break in immune tolerance and T1D.

CONCLUSIONS

SKAP2 plays a key role in myeloid cell activation and migration. This particular mutation in a patient with T1D and multiple autoimmune conditions implicates a role for activating *SKAP2* variants in autoimmune T1D.

Recent insights into type 1 diabetes (T1D) have come from genome-wide association studies (GWAS) and studies of rare monogenic disorders that have implicated a host of genes involving immune cell function (1–12). Multiple GWAS have identified a strong genetic linkage between the Src kinase-associated phosphoprotein 2 (*SKAP2*) locus and T1D (6,13,14). *SKAP2* is a signaling adaptor protein that plays a key role in mediating integrin responses in monocyte-derived cell populations like macrophages (15,16), but linking how common noncoding risk variants near *SKAP2*

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alter its activity has remained obscure. Rapid advances in next-generation sequencing have now allowed for a deeper exploration and discovery of rare variants that are linked to disease with techniques like whole-exome sequencing (WES) (17). Here, we used a similar approach to identify a de novo *SKAP2* variant (p.Gly153Arg) in a single patient with T1D and other autoimmune and inflammatory features, and we leveraged this finding to identify a functional alteration in *SKAP2*. Monocyte-derived macrophages from the individual displayed enhanced activity of integrin pathways and a migratory phenotype in the absence of chemokine stimulation, consistent with *SKAP2* p.Gly153Arg being constitutively active. The p.Gly153Arg variant, located in the well-conserved lipid-binding loop, induced similar phenotypes when expressed in a human macrophage cell line. When combined with previous GWAS, our findings suggest that activating *SKAP2* variants result in T1D with comorbid autoimmune and inflammatory disease by promoting increased activity and migration of macrophages.

RESEARCH DESIGN AND METHODS

Study Approval

This study was approved by the institutional review boards at the University of California, San Francisco (UCSF), and The University of Chicago. The proband and her family members were consented for participation through The University of Chicago Monogenic Diabetes Registry (<https://monogenicdiabetes.uchicago.edu/registry>), which collects longitudinal information regarding diagnosis and treatment of diabetes, other medical problems or complications, family history of diabetes, and results of genetic testing through surveys and medical records.

WES and Analysis

Genomic DNA extraction, targeted capture, and massive parallel sequencing were performed using standard procedures. Briefly, genomic DNA was sheared by Covaris S2 (Covaris Inc., Woburn, MA) to a target size of 200–300 base pairs and assembled into a library with TruSeq adapters containing indexes that differentiate libraries in a capture reaction as well as a sequencing run (Kapa Biosystems, Wilmington, MA).

Libraries were pooled into a capture reaction that contains biotinylated DNA oligonucleotides (called “baits”) from SeqCap EZ Human Exome Library v3.0 (Roche-Nimblegen, Madison, WI) for 72 h. The DNA–bait–DNA hybrids were then pulled out of the complex mixture by incubation with streptavidin-labeled magnetic beads and captured onto a strong magnet. After washing, the targeted DNA of interest was eluted and subjected to 18 cycles of DNA amplification. Captured DNA libraries were sequenced with the HiSeq 2500 System (Illumina, Hayward, CA), yielding 150 (2 × 75) base pairs from the final library fragments. Sequence data were mapped to the GRCh37 reference genome and analyzed using the Ingenuity Variant Analysis platform (QIAGEN, Venlo, the Netherlands). Variants identified by WES were filtered using criteria in Supplementary Fig. 6. Sanger sequencing of *SKAP2* variant was performed using standard methods with the following primers: forward: ATGGCTATGATAATTGGGAG; reverse: CTGGTATTGGAGCATTTGTC.

Culture and Differentiation of THP-1 Monocytes

THP-1 cells (TIB-202; ATCC) were cultured in RPMI medium with 1× L-glutamine (Corning), 10% heat-inactivated FBS (Gibco), and 1× penicillin/streptomycin (Gibco). Cells were maintained at a density between 0.2 and 1 × 10⁶ cells/mL. To differentiate the cells into a macrophage-like cell for experimentation, THP-1 cells were added to fresh culture media containing 10 ng/mL phorbol 12-myristate 13-acetate (#534400; Calbiochem) and plated at a density of 1 × 10⁶ cells/mL on nontissue culture–treated dishes. The differentiation media was removed after 72 h, and the adherent cells were rested in normal culture media for 24 h before experimentation. If the cells were to be used for analysis of phosphorylation status, the media in the wells was changed to culture media with 2% heat-inactivated FBS to reduce background kinase signaling. If used for immunofluorescence microscopy, cells were differentiated at a density of 10⁵ cells/well in eight-well–chambered slides (#154534; LabTek).

Generation of *SKAP2*-Null THP-1 Monocytes Using CRISPR-Cas9

Generation of *SKAP2*-null THP-1 monocytes using CRISPR-Cas9 was performed

by following the protocol outlined by Hultquist et al. (18) using the Amaxa 4D-Nucleofector system (#AAF-1002X; Lonza). Guide RNAs were assembled by mixing trans-activating crRNA (crRNA) (#1072533; Integrated DNA Technologies) with predesigned crRNA (design identifier Hs.Cas9.SKAP2.1.AD; Integrated DNA Technologies) and incubating for 30 min at 37°C. Purified Cas9 endonuclease (Berkeley QB3 MacroLab) was added to the guide RNA and incubated for 15 min at 37°C. Five hundred thousand undifferentiated THP-1 cells suspended in Amaxa nucleoporation solution (#V4XC-3024; Lonza) were added to 96-well Nucleocuvette Plates and nucleofected using the Amaxa 4D-Nucleofector X Unit 96-well Shuttle system with the program FF-100. Nucleofected cells were rested for 4 days in 96-well plates in RPMI medium with 1× L-glutamine (Corning), 10% heat-inactivated FBS (Gibco), and 1× penicillin/streptomycin (Gibco). After resting, the edited cells were counted and seeded at one cell per well, and clones were expanded. Clones were assessed for biallelic *SKAP2* editing by DNA sequencing around the expected editing site followed by Tracking of Indels by Decomposition (TIDE) analysis using the TIDE webtool (<https://tide.nki.nl>). Western blot analysis with an antibody against Skap2 (#12926-1-AP; Proteintech) was also used to confirm the loss the protein expression. Cell lines that developed insertions and deletions causing premature stop codons on both alleles of *SKAP2* and that showed no expression of the Skap2 protein were considered *Skap2*-null. Sequences used were as follows: *SKAP2* crRNA (Hs.Cas9.SKAP2.1.AD), 5'-CCTCTCATCATAATCTC-3'; *SKAP2* TIDE PCR forward primer, 5'-AGATGCTGAAGAATGGGTACAG-3'; and *SKAP2* TIDE PCR reverse primer, 5'-GCCACAAGTCCCAAGAGATTA-3'.

Retroviral Transduction of THP-1 Cells

The wild-type and mutant *SKAP2* genes were cloned into the multiple cloning sites of pMIG-W transfer plasmids using standard restriction cloning methods. Amphotropic retroviruses were then produced using third-generation methods by the UCSF ViraCore (<https://viracore.ucsf.edu>). *SKAP2*-null THP-1 cells were transduced by resuspending 1 × 10⁶ cells in 1 mL of the retroviral

supernatant supplemented with 8 $\mu\text{g}/\text{mL}$ protamine sulfate (#20FK79; W.W. Grainger, Inc.) and centrifuging the suspension in a tissue culture–treated six-well plate at 2,000 rpm for 1 h at room temperature (RT). Following centrifugation, an additional 1 mL of culture media was added to each well, and the cells rested for 1 day in a 37°C incubator. After 1 day, the cell media containing the retroviral supernatant were removed, and the cells were resuspended in 3 mL culture media. The cells were sorted on a FACSAria machine on the basis of GFP expression 3 days following transduction. Western blot analysis using a Skap2 antibody (#12926-1-AP; Proteintech) was performed to confirm the expression of the transduced *SKAP2* gene.

Western Blotting and Integrin Stimulation

Wells of nontissue culture–treated six-well plates (#CLS3736; Corning) were coated for 1 h at RT with 1 mL of 2 $\mu\text{g}/\text{mL}$ fibronectin-like engineered peptide (#F8141; Sigma) in Hanks' balanced salt solution (HBSS) with 20 mmol/L HEPES or 5% nonfat dry milk (Bio-Rad) in HBSS with 20 mmol/L HEPES. Wells were washed once with deionized water before seeding cells. THP-1 cells or primary monocytes derived from the patient were gently detached from their culture wells using 5 mmol/L warm EDTA in Dulbecco's PBS. Cells were then seeded into the coated wells at a density of 1.2×10^6 cells/well. The plates were centrifuged at 500 rpm at 4°C for 3 min to allow for simultaneous integrin activation and then incubated for 45 min at 37°C. Both adherent and suspended cells were then lysed in the wells using 200 μL ice-cold radioimmunoprecipitation assay buffer for 10 min and centrifuged at 13,000 rpm for 10 min at 4°C. The supernatant was transferred to cold $1 \times$ NuPage LDS Sample Buffer (Invitrogen) containing $1 \times$ NuPage Sample Reducing Agent (Invitrogen). Samples were loaded in a 4–12% gradient NuPage (Invitrogen) gel and blotted using semidry transfer onto a polyvinylidene fluoride membrane. Membranes were blocked with 5% nonfat dry milk or 5% BSA (Sigma) in Tris-buffered saline with Tween (TBS-T) and were stained and incubated with primary antibodies (see Supplementary Table 4) in 5%

BSA and TBS-T at 4°C overnight. After washing with TBS-T, the secondary IR-Dye antibodies (see Supplementary Table 5) were added to the membrane and incubated for 30 min at RT in TBS-T containing 5% BSA and 0.1% SDS. The membrane was imaged and quantified with the LI-COR Odyssey CLx and Image Studio (LI-COR) software. Antibodies are listed in Supplementary Tables 4 and 5.

Isolation of Primary Human Monocytes Through Density Gradient Centrifugation

Blood was collected in 10-mL heparinized tubes and brought to RT. In equal amounts, Histopaque 1077 (Sigma) was layered on top of Histopaque 1119 (Sigma) in a sterile 15-mL reaction tube (Falcon). The final layer was an equal amount of blood. The tube was centrifuged at 900g for 30 min at RT, using the lowest settings on brake and acceleration. The isolation yields three major layers which, from top to bottom, are the thrombocyte/monocyte/T-cell layer, the neutrophil granulocytes, and the erythrocyte pellet. The top layer was collected and washed in 20 mmol/L HEPES-buffered HBSS. Residual erythrocytes were then lysed for 90 s in 5.25 mL distilled water and 1.25 mL HBSS/20 mmol/L HEPES, after which isotonicity was restored by addition of 2.2 mL of 3% NaCl before centrifugation. Cells were resuspended in HBSS/20 mmol/L HEPES and counted. The top layer monocytes were resuspended in 1 mL heat-inactivated FBS (Gibco) containing 10% DMSO (Sigma) and stored in liquid nitrogen until further use.

Culture of Primary Human Monocyte-Derived Macrophages

Monocytes were cultured using RPMI medium with $1 \times$ L-glutamine (Corning), 10% heat-inactivated FBS (Gibco), $1 \times$ penicillin/streptomycin (Gibco), and 50 ng/mL human recombinant granulocyte-monocyte colony-stimulating factor (215-GM-010; R&D Systems). The whole supernatant was discarded and replenished with fresh media after 3 days. Experiments and staining were performed following day 7 of the differentiation period. For immunofluorescence microscopy, monocytes were cultured at a density of 10^5 cells/well in eight-

well-chambered slides (#154534; LabTek). For Western blotting, cells were differentiated in 6-cm culture dishes (Corning).

Single-Cell RNA Sequencing of Patient Neutrophils

Blood samples from the SKAP2 patient and control subject were collected in heparinated tubes. Then, red blood cells were lysed (BD Pharm Lyse lysing buffer, Cat #555899; BD Biosciences), and samples were washed with sterile FACS buffer (HBSS [Gibco], 2 mmol/L EDTA, and 2% heat-inactivated FBS [Gibco]). After blocking for 5 min (Human BD Fc Block, Cat #555899; BD Pharmingen), specimens were stained with aqua dye (#L34957, 1:100; Invitrogen) and anti-CD45 (Cat #555485, RRID:AB_398600, 1:20; BD Biosciences) for 20 min at 37°C and 5% CO₂ in a humidified incubator. Finally, samples were washed again and filtrated into sterile flow cytometry tubes (#352058; Corning). One hundred fifty thousand cells were sorted using a BD Fusion SORP with a 70- μm nozzle in a biosafety cabinet. Fifty thousand cells were transferred to 0.04% BSA (Sigma) in DPBS (Gibco) also containing 1 unit/ μL RNase inhibitor (PN-3335399001; Sigma-Aldrich). Sample processing was performed using Chromium Technology (10 \times Genomics) to the manufacturer's instructions with 5'-primed and T-cell receptor sequencing. Sample barcoding and demultiplexing and single-cell counting were performed using the 10 \times Genomics Cell Ranger Single Cell Software Suite. The reads were aligned to GRCh38 (the 3.0.0 version from the Cell Ranger website) with Cell Ranger 3.0.2 using the Cell Ranger count command with the default settings. Reads were quantified and filtered using Cell Ranger count. The Seurat package (19) in R was used for the following downstream analysis (<https://www.rstudio.com>). First, cells with mitochondrial gene expression >7.5%, RNA count <200 and >25,000, and feature RNA <100 and >2,500 were excluded. Samples were then scaled with factor 10,000, and the two samples (SKAP2 p.Gly153Arg patient and SKAP2 wild-type control) were integrated with the 2,000 most variable features. Principal component analysis was performed next, and dimensionality for clustering was determined using an elbow plot. Uniform manifold approximation

and projection clustering was performed on the first 21 principal components. To identify the marker genes for each cluster, the differential gene expression of that cluster was compared with all the other clusters. Using the packages EnhancedVolcano (<https://github.com/kevinblighe/EnhancedVolcano>) and Pathview (20) in the Bioconductor Project Package Repository (<https://CRAN.R-project.org/package=BiocManager>) in R, further integrated downstream analysis was performed, testing the SKAP2 G153R sample against the SKAP2 wild-type control sample. Pathway analysis was based on the “org.HS.eg.db” pathway gene library with a *P* value cutoff of 0.05.

Immunofluorescence Microscopy

For stimulation assays, monocyte-derived macrophages and THP-1 cells were stimulated with monocyte differentiation media or THP-1 culture media containing either 100 ng/mL C-C motif chemokine 2 (CCL2) (#Z02829; GenScript) or 100 ng/mL recombinant human C-X-C motif chemokine ligand 1 (CXCL1) (#275-GR-010; R&D Systems) and were stained directly in their wells. Cells were fixed in 4% paraformaldehyde (Sigma) in DPBS (Gibco) containing 2 mmol/L MgCl₂ and 1 mmol/L CaCl₂ (PBS^{+/-}) for 15 min at RT. After washing with PBS^{+/+}, cells were permeabilized with 0.1% Triton X-100 in PBS^{+/+} for 10 min at RT. Followed by another wash, cells were blocked with 5% BSA in PBS^{+/+} for 1 h at RT and then stained with primary antibodies against SKAP2 (Cat #12926-1-AP, RRID:AB_2189317, 1:200; Proteintech) and Wiskott-Aldrich syndrome protein (WASp) (sc-5300, RRID:AB_628446, 1:50; Santa Cruz Biotechnology) in 1% BSA/PBS^{+/+} and incubated overnight at 4°C. Secondary staining was then performed with Alexa Fluor 488 donkey anti-rabbit IgG (H&L) (A-21206, RRID:AB_2535792, 1:1,000; Thermo Fisher Scientific), Alexa Fluor 647 goat anti-mouse IgG (H&L) (ab150115, RRID:AB_2687948, 1:1,000; Abcam), and Alexa Fluor 546 Phalloidin (A-22283, RRID:AB_2632953, 1:40; Thermo Fisher Scientific) in 1% BSA in PBS^{+/+} for 1 h at RT. Slides were mounted using ProLong Gold Antifade Mountant with DAPI (P36934; Thermo Fisher Scientific) and sealed with nail polish (#72180; Electron Microscopy Science).

Immunofluorescence microscopy of fixed and phalloidin-stained cells was performed using a Zeiss Axio Imager M2 with Apotome 2 configuration using ZEN Blue Pro software (Zeiss). Confocal microscopy was performed with the Nikon A1R using NIS Elements software, and Imaris 9.5 (Oxford Instruments) was used for image processing. Colocalization analysis was done using confocal images of fixed cells stained for SKAP2 and WASp proteins and then for calculating the ratio of positive cells to total cells per condition. Scoring of positive cells was performed using three blinded, independent scorers. Cell shape analysis was performed using ImageJ and an ImageJ macro (Supplementary Fig. 7).

Statistics

For quantification of colocalization and shape change analyses, data sets were compared by two-way ANOVA, and *P* values were adjusted with Tukey multiple comparisons test using R Studio software (<https://www.Rstudio.com>). pERK/ERK quantification data were compared by Student *t* test using Excel software (Microsoft Corporation). Quantification of colocalization and pERK/ERK data is displayed as mean ± SE. Quantification of shape change data is displayed as box blots with centered median.

RESULTS

Clinical Characteristics of the Patient With SKAP2 Variant

In an effort to identify people with T1D with a strong underlying genetic cause, we examined individuals from The University of Chicago Monogenic Diabetes Registry (<https://monogenicdiabetes.uchicago.edu/registry>) who had been diagnosed with T1D but did not have one of the known forms of monogenic diabetes (e.g., mutations in the insulin gene). Out of 10 families with T1D from the registry selected for WES, we identified a 24-year-old individual with multiple autoimmune conditions (Supplementary Tables 1 and 2) and no known family history of any of these conditions. She initially presented with symptoms of hypothyroidism at the age of 14 years and was incidentally found to have glucosuria, hyperglycemia, and a hemoglobin A_{1c} of 8.4%. Her BMI at that time was near the 50th percentile for age. Her T1D genetic risk score falls

within the 25–50th percentile (21), and she is heterozygous for HLA-DR3. She tested positive for autoantibodies against the pancreatic islet β-cell proteins GAD and islet cell antigen 512 and autoantibodies against thyroid proteins thyroglobulin and thyroid peroxidase. Additional medical history included other forms of autoimmunity, including eczema, intermittent hemolytic anemia, undifferentiated connective tissue disease, and Raynaud syndrome. Subsequent testing detected additional autoantibodies, including antinuclear antibody and antitopoisomerase 1 antibody.

In Silico Identification and Characterization of the SKAP2 p.Gly153Arg Variant

WES on DNA samples from the proband (Fig. 1A, member II-1), unaffected brother (II-2), and unaffected parents (I-1 and I-2) revealed a novel de novo germline coding variant in the SKAP2 gene (c.457G>A, p.Gly153Arg) (Supplementary Table 3) that encodes for SKAP2 protein. Sanger sequencing confirmed that the SKAP2 variant was present in the affected family member and absent in unaffected family members (Fig. 1B). Parenthood was confirmed with rare single nucleotide polymorphisms identified by WES. The variant was not found in >270,000 alleles in the Genome Aggregation Database (22). No SKAP2 mutations are known to cause human disease, although GWAS have linked single nucleotide polymorphisms in the SKAP2 locus to autoimmune diseases, including T1D (6,23–25,28).

Species alignment showed that Gly153 is highly conserved in SKAP2 from species as distant as *Xenopus tropicalis* and *Danio rerio* (Fig. 1C). SKAP2 forms a homodimer using an N-terminal four-helix bundle dimerization domain (DM), against which its two pleckstrin homology (PH) domains arrange in a conformation incompatible with phosphoinositide binding (Fig. 1D). Biochemical, cell biological, and crystallographic studies of SKAP2 have demonstrated that the DM and PH domains form a lipid-responsive molecular switch (26). Lipid binding induces a conformational change in SKAP2, allowing it to bind and activate the WASp/Arp2/3 protein complex, which can then initiate actin polymerization at the cell

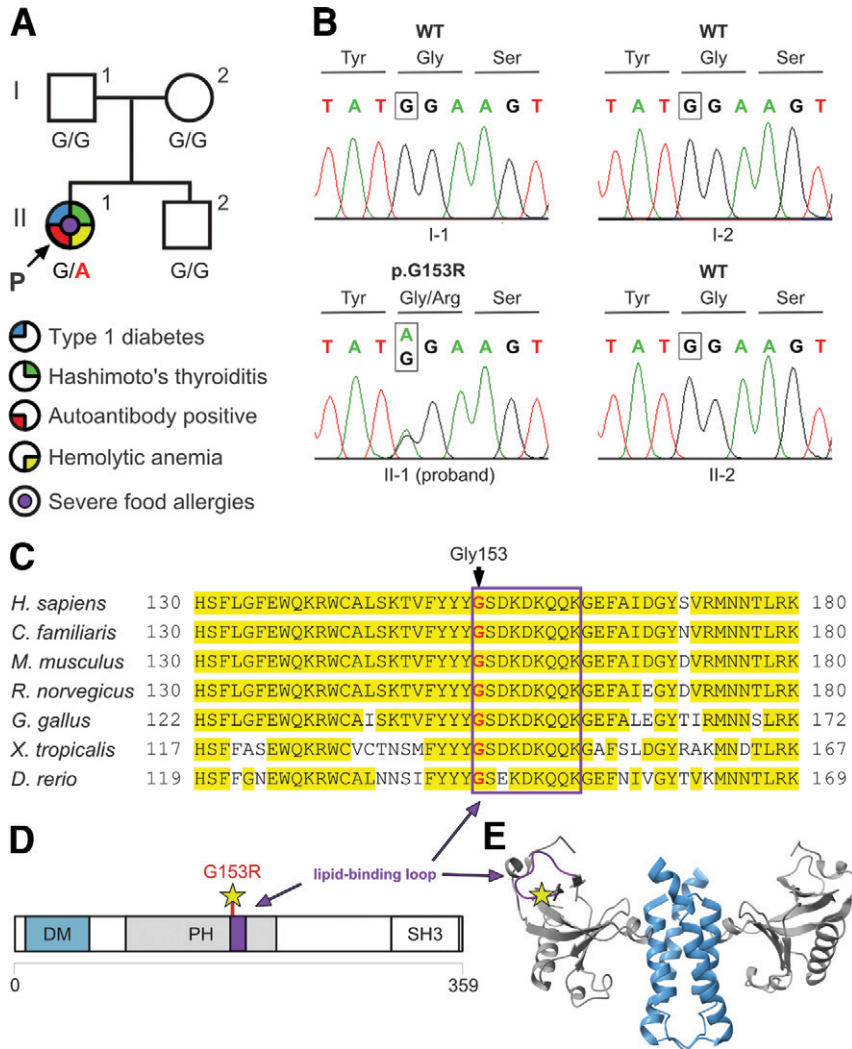


Figure 1—A de novo SKAP2 mutation in the 3'-phosphoinositide binding loop. **A:** Pedigree of kindred. The participant with T1D, Hashimoto thyroiditis, and Raynaud syndrome is shown with a filled symbol, and nonaffected participants are shown with unfilled symbols. **B:** Sanger sequencing chromatograms showing the wild-type (WT) SKAP2 sequence of the unaffected parents (I-1 and I-2) and brother (II-2) and the SKAP2 variant c.457G>A (p.Gly153Arg) identified in the affected proband (II-1). **C:** Alignment and conservation of residues encoded by SKAP2 orthologs. Residues that are conserved in three or more species are highlighted in yellow. p.Gly153Arg is located in a highly conserved lipid-binding loop (box). Several deletions and mutations mapping to this region result in dominant active or negative SKAP2 activity (26). **D:** Schematic of the SKAP2 protein. **E:** Homodimeric 3D structure of the murine SKAP2 DM and PH domains (Protein Data Bank identifier: 2OTX) visualized with ChimeraX (27).

membrane (15,16). Several deletions and mutations mapping to this region result in dominant active or negative SKAP2 activity (26). The p.Gly153Arg variant is located within the highly conserved lipid-binding loop (Fig. 1E), suggesting a possible functional consequence.

In Vitro Functional Assessment of the SKAP2 p.Gly153Arg Variant

Genetic studies in mice have shown that SKAP2 is a critical mediator of the “inside-out” and “outside-in” integrin intracellular signaling pathways that

regulate cytoskeletal changes in myeloid immune cells (15,16,29). Consistent with these studies, SKAP2 is expressed in immune tissues and is enriched in myeloid cell types (Supplementary Fig. 1). To evaluate the p.Gly153Arg variant, we generated SKAP2-deficient (SKAP2^{-/-}) macrophages from the human THP-1 monocyte cell line (30) and reintroduced either wild-type or p.Gly153Arg SKAP2 using retroviral transduction. Western blot analysis of SKAP2^{-/-} THP-1 macrophages confirmed loss of endogenous SKAP2

expression and similar levels of retrovirally expressed wild-type or p.Gly153Arg SKAP2 (Supplementary Fig. 2). SKAP2^{-/-} THP-1 macrophages had reduced integrin-induced phosphorylation of extracellular signal-regulated kinases 1 and 2 (ERK) and did not change shape in response to CCL2 (Supplementary Fig. 2), consistent with the phenotype of murine Skap2^{-/-} macrophages (15).

SKAP2 moves from the cytosol to the cellular membrane in response to chemokine signals, then recruits WASp to drive localized actin polymerization (15,16,31). We therefore assayed for SKAP2 and WASp localization by immunostaining. Both SKAP2 and WASp were detected by immunostaining in the cytoplasm of resting SKAP2^{-/-} THP-1 macrophages infected with lentivirus expressing wild-type SKAP2 (Fig. 2A). Nuclear localization of WASp, but not SKAP2, was also observed, consistent with previous reports (32). Treatment with CXCL1, a proinflammatory macrophage chemokine (33), induced significant colocalization of SKAP2 and WASp at the plasma membrane (Supplementary Fig. 3), consistent with the activation of these proteins. In contrast, we found significant colocalization of SKAP2 and WASp at the plasma membrane in resting THP-1 macrophages with lentivirus-expressed SKAP2 p.Gly153Arg (Fig. 2A and B, arrows), indicating that SKAP2 p.Gly153Arg is active in the absence of chemokine treatment.

SKAP2 also mediates chemokine-stimulated integrin activation, which induces the extracellular region of integrins on the surface of myeloid cells to adopt an open conformation that increases integrin ligand avidity (inside-out signaling). This change increases the cellular response to extracellular integrin ligands (outside-in signaling) (34,35). To assess these integrin pathways, we assayed for the phosphorylation of ERKs following plating of resting THP-1 macrophages on poly-Arg-Gly-Asp (pRGD)-coated surfaces. ERK phosphorylation in resting, untreated cells expressing SKAP2 p.Gly153Arg was significantly higher than in cells expressing wild-type SKAP2 (Fig. 2C and D and Supplementary Fig. 3), further demonstrating that SKAP2 p.Gly153Arg acts in a dominant active fashion (34,35).

Chemokine activation of actin polymerization and integrin signaling induces shape changes during cellular migration (36). Consistent with this induction, resting

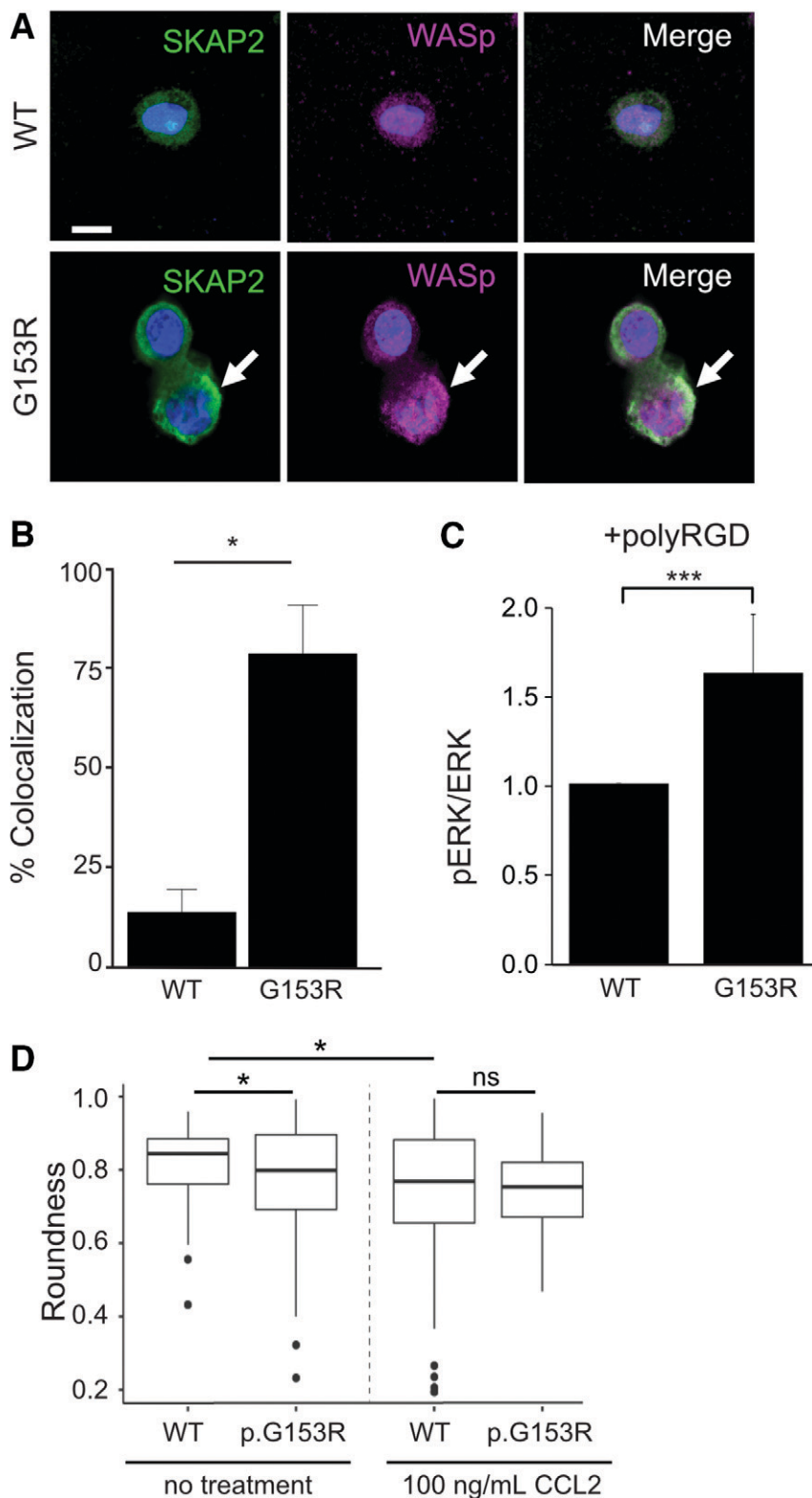


Figure 2—Functional evaluation of the SKAP2 p.Gly153Arg variant in a human macrophage cell line. **A:** Immunostaining for SKAP2 (green) and WASp (purple) in untreated SKAP2^{-/-} THP-1 macrophages expressing either wild-type (WT) or p.Gly153Arg SKAP2 by retroviral transduction. DNA marker DAPI is in blue. Scale bar = 15 μm. **B:** Percent colocalization of SKAP2 and WASp (mean ± SE, two-way ANOVA, *n* = 3). **P* < 0.05. **C:** Quantification of pERK/ERK in pRGD-plated SKAP2^{-/-} THP-1 macrophages expressing either WT or p.Gly153Arg SKAP2 by retroviral transduction (mean ± SE, Student *t* test, *n* = 6). ****P* < 0.001. **D:** Cell shape analysis of indicated THP-1 macrophages with and without treatment with macrophage chemokine CCL2 (median-centered box plot, two-way ANOVA, *n* = 3). **P* < 0.05.

THP-1 macrophages expressing wild-type SKAP2 acquired a less rounded, migratory phenotype after treatment with the potent chemokine CCL2 (Fig. 2E). In contrast, resting THP-1 macrophages expressing SKAP2 p.Gly153Arg displayed a migratory phenotype in the absence of CCL2 treatment, and their shape did not change further after the addition of CCL2 (Fig. 2E).

Next, we evaluated whether primary macrophages carrying the SKAP2 p.Gly153Arg heterozygous allele displayed similar phenotypes by harvesting monocytes from the proband (Fig. 1A, I-1, “patient”) and unaffected mother (I-2, “control”) and then differentiated these cells into macrophages *ex vivo*. Western blot analysis of patient and control macrophages revealed that the p.Gly153Arg variant did not alter SKAP2 protein expression (Supplementary Fig. 4). In resting control macrophages, wild-type SKAP2 and WASp were largely cytosolic (Fig. 3A), while treatment with CXCL1 resulted in significant colocalization of SKAP2 and WASp at the plasma membrane (Supplementary Fig. 5). Strikingly, we found significant colocalization of SKAP2 p.Gly153Arg and WASp at the plasma membrane in resting patient macrophages (Fig. 3A, arrows), similar to what was observed in THP-1 macrophages expressing the p.Gly153Arg SKAP2 protein (Fig. 2A). Patient macrophages also had higher levels of integrin-activated ERK phosphorylation compared with control cells and assumed a less rounded, more migratory phenotype in the absence of CCL2 treatment (Fig. 3C and D and Supplementary Fig. 5), similar to observations in THP-1 cells expressing the pGly153Arg SKAP2 variant. Finally, we performed single-cell RNA sequencing on FACS-sorted patient and control neutrophils, another myeloid immune cell population that depends on SKAP2 function (16). Consistent with SKAP2 p.Gly153Arg myeloid cell activation, patient neutrophils showed upregulation of key signaling molecules, including RIPK2, TLR2, IL3RA, and IL1RN, and broad activation of secretory and kinase signaling pathways (Supplementary Fig. 8).

CONCLUSIONS

We identified the first monogenic, functional variant of the SKAP2 gene linked to

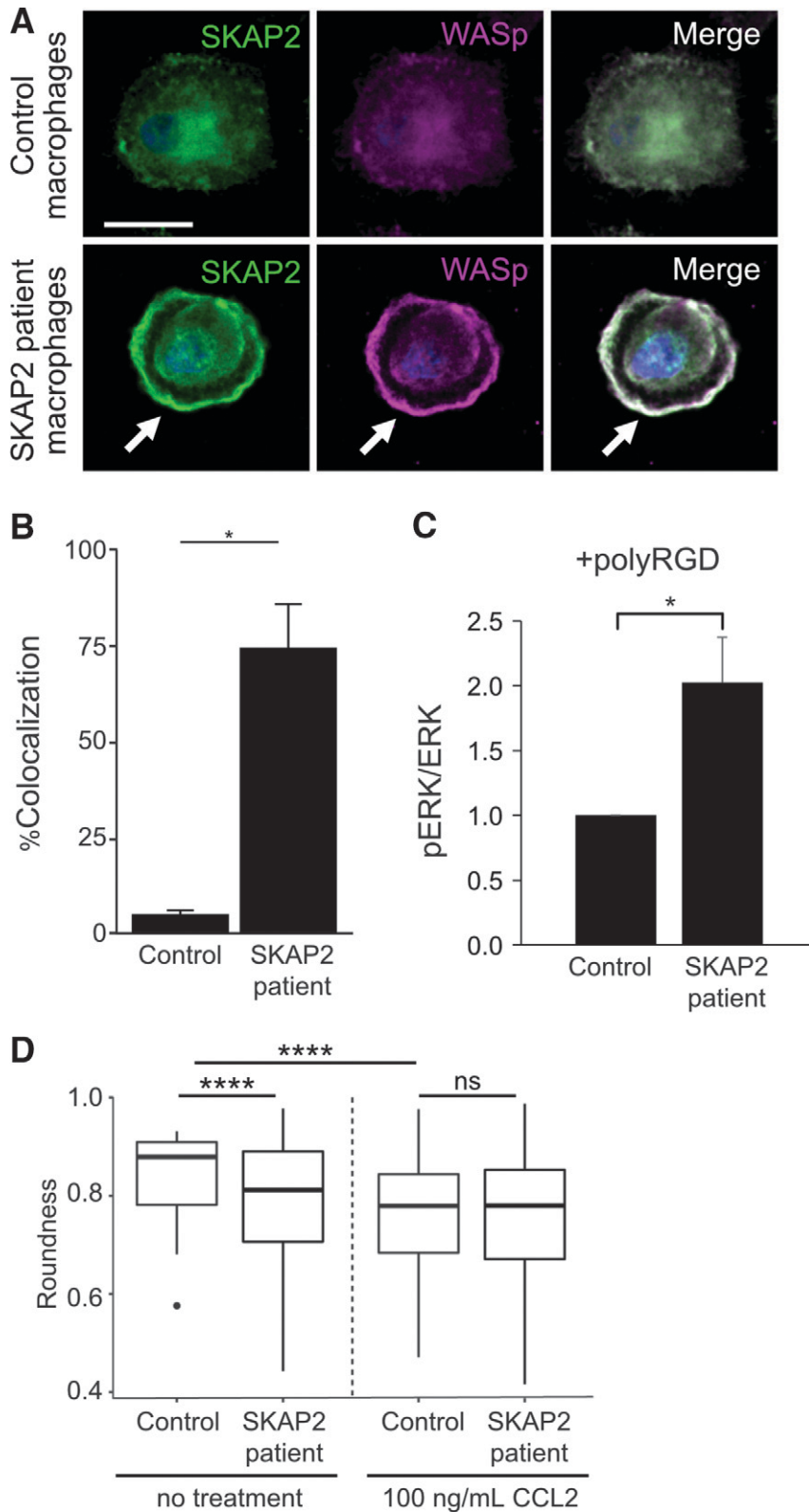


Figure 3—Phenotypes of SKAP2 patient macrophages. **A:** Immunostaining for SKAP2 (green) and WASp (purple) in resting SKAP2 patient and control macrophages. DNA marker DAPI is in blue. Scale bar = 15 μ m. **B:** Percent colocalization of SKAP2 and WASp (mean \pm SE, two-way ANOVA, $n = 3$). * $P < 0.05$. **C:** Quantification of pERK/ERK in SKAP2 patient and control macrophages plated on pRGD (mean \pm SE, Student t test, $n = 3$). * $P < 0.05$. **D:** Cell shape analysis of SKAP2 patient and control macrophages with and without treatment with macrophage chemokine CCL2 (median-centered box plot, $n = 3$). **** $P < 0.0001$.

a patient with T1D. Our data show that the SKAP2 p.Gly153Arg protein acts in a dominant active fashion. Because SKAP2 mediates both inside-out and outside-in integrin signaling, we propose that SKAP2 p.Gly153Arg drives the constitutive activation of these pathways in the absence of chemokine signaling, which likely results in the chronic hyperactivity of myeloid immune cell populations that express SKAP2 and contributes to the inflammation underlying autoimmune diabetes and other immune conditions in the patient (Fig. 4).

The development of T1D involves dysregulation of the innate and adaptive arms of the immune system, which is influenced by genetic and environmental factors (37,38). Insights into this dysregulation have come from studies of rare monogenic disorders (12). For example, patients carrying mutations in *AIRE* or *FOXP3* frequently develop T1D, along with other autoimmune disorders. Dissecting the consequences of these mutations has improved our understanding of the role of central T-cell tolerance and T regulatory cells in preventing organ-specific autoimmune diseases like T1D (1,3–12).

SKAP2 is a key regulator of integrin function in myeloid cells of the innate immune system, and numerous studies in humans and mice have observed the persistent activation of these cells during both the initiation and the development of T1D (39–45). In addition, several examples demonstrate that loss-of-function mutations in proteins that act as negative regulators of myeloid cell integrin signaling can lead to autoimmunity (46,47). Loss of these integrin regulators leads to hyperactive myeloid cells, in particular dendritic cells, which drive development of self-reactive T and B cells. In these models, treatments that target integrin signaling components disrupt this circuit and provide some therapeutic benefit (34). Further, hyperactivation of nuclear factor- κ B signaling pathways in dendritic cells in mice leads to constitutive inflammatory cytokine production that can also drive autoimmunity (48). Also important to note is that the overactive form of SKAP2 in this patient case is associated with other autoimmune conditions beyond T1D, including thyroiditis and autoimmune hemolytic anemia, revealing a major break in

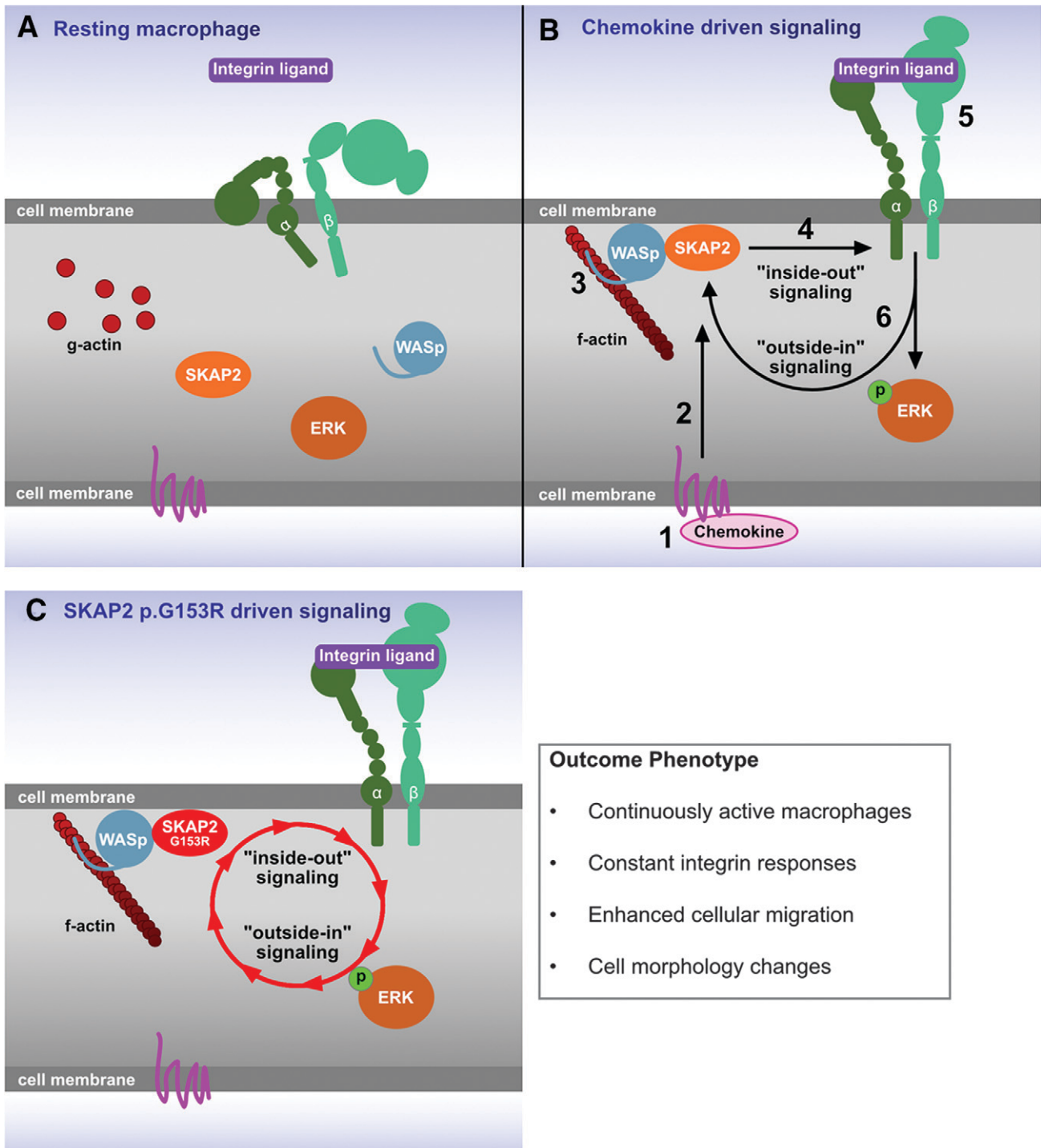


Figure 4—Proposed model for the constitutive activation of macrophages with the SKAP2 p.Gly153Arg mutation. **A:** In the absence of inflammation, macrophages are resting, SKAP2 and WASp are cytosolic, and integrins are in a bent conformation with reduced affinity for extracellular ligands. **B:** In response to inflammatory chemokines (1), SKAP2 is activated (2) and recruits WASp to the cell membrane to initiate actin polymerization (3). Activated SKAP2 also initiates inside-out integrin signaling (4) that results in conformational changes in the extracellular region of the integrins to increase ligand affinity (5). This initiates outside-in integrin signaling (6), which activates multiple intracellular targets such as ERK and SKAP2. **C:** SKAP2 p.Gly153Arg is constitutively active, creating an endless cycle of integrin signaling in the absence of inflammatory chemokines.

immune tolerance in this patient. These findings are consistent with broad overactivity of monocytes and neutrophils, which could promote activation of multiple autoreactive T- and B-lymphocyte

populations, resulting in a spectrum of autoimmunity beyond T1D.

Together, our data indicate that mutations in SKAP2, which result in gain-of-function constitutive signaling in

myeloid lineage cells, can lead to T1D with comorbid autoimmune and inflammatory disease. The precise mechanism for how the inflammation induced by SKAP2 p.Gly153Arg leads to a break in tolerance

and autoimmune diabetes remains to be determined.

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