CARMIL2 deficiency disrupts activation-induced metabolic reprogramming in T cells and is partially rescued by glutamine supplementation

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Background: T-cell activation requires signaling through the T-cell receptor and costimulatory molecules, including CD28, triggering metabolic reprogramming to support growth and proliferation of the activating T cell. CARMIL2, a scaffold protein, facilitates CD28-mediated signaling. Individuals with *CARMIL2* mutations experience inborn errors of immunity, leading to T-cell dysfunction and severe infectious and inflammatory comorbidities. However, how CARMIL2 deficiency impacts T-cell metabolic reprogramming remains unknown.

Objective: We sought to investigate how CARMIL2 deficiency affects activation-induced metabolic reprogramming in T cells.

Methods: $CD4^+$ T cells were isolated from patients with CARMIL2 deficiency and matched healthy controls.

A transcriptomic profile was analyzed by bulk RNA sequencing and whole-cell metabolomics by LC-MS/MS. Activation markers and signaling pathways were measured by flow cytometry. These approaches informed identification of specific amino acids for rescue experiments.

Results: Nine patients with CARMIL2 deficiency and 16 age- and sex-matched healthy controls were recruited. RNA sequencing of $CD4^+$ T cells revealed decreased expression of genes associated with metabolic activity, including mTOR signaling, glycolysis, 1-carbon metabolism, and glutamine metabolism. Whole-cell metabolomics reinforced these results and highlighted glutamine deficiency as a potential driver of the observed metabolic phenotype. Glutamine supplementation restored NF-κB and mTOR activity, as measured by p-65 and RPS6 phosphorylation, respectively, and upregulated the expression of IL17A in CARMIL2-mutated $CD4^+$ T cells.

Conclusion: CARMIL2 deficiency disrupts T-cell metabolic reprogramming and was partially rescued *ex vivo* with glutamine supplementation. These findings highlight a potential therapeutic approach targeting metabolism to improve immune function in individuals with CARMIL2 deficiency. (J Allergy Clin Immunol 2025;156:1390-400.)

Key words: *CARMIL2, T cell, metabolism, mTOR, glutamine*

Inborn errors of immunity (IEIs) comprise a heterogeneous group of diseases caused by mutations in immune-related genes[1,2] and are associated with significant morbidity and mortality. Hematopoietic stem cell transplantation (HSCT) is the only curative option for many of these diseases and is often associated with significant side effects.[3,4] Deficiency in CARMIL2, caused by biallelic mutations in the *CARMIL2* gene, is a known cause of IEIs.[5,6] In healthy individuals, CARMIL2 interacts with CARD11,[7,8] a component of the CARD11-BCL10-MALT1 (CBM) complex,[3,4,9,10] which is assembled downstream of CD28 during T-cell activation. CARMIL2 deficiency disrupts T-cell function, resulting in a reduced activation rate and inability to differentiate into T_H1 and T_H17 effector cells.[10] In addition, patients with CARMIL2 deficiency have a reduced frequency of regulatory T cells, natural killer cells, and memory T and B lymphocytes. As a result, affected individuals experience recurrent bacterial, fungal, and viral infections as well as dysregulated inflammation of the skin and gastrointestinal tract.[5,6,10-15] In particular, patients show increased susceptibility to EBV-associated malignancies.[5,6,10] Given the limited therapeutic options for infectious and inflammatory diseases in patients with CARMIL2 deficiency, we sought to uncover novel mechanisms of immunodeficiency in these patients and hypothesized that CARMIL2 loss interfered with activation-induced metabolic reprogramming in patients' T cells.

Metabolic demands of T cells increase on stimulation as they upregulate expression of transporters and boost the uptake of metabolites including glucose and amino acids.[16] Metabolic reprogramming is triggered by signaling downstream to the T-cell receptor (TCR) and CD28 and promotes energy production and

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Abbreviations used
CBM: CARD11-BCL10-MALT1
FTT: Failure to thrive
HC: Healthy control
HSCT: Hematopoietic stem cell transplantation
IBD: Inflammatory bowel disease
IEI: Inborn error of immunity
IPA: Ingenuity pathway analysis
PMA: Phorbol 12-myristate 13-acetate
RNA-Seq: RNA sequencing
TCA: Tricarboxylic acid
TCR: T-cell receptor

biosynthesis to support cell growth, proliferation, effector molecule production, and differentiation.[16] When T cells are deprived of necessary fuels during activation, metabolic pathways are restricted and nutrient-sensing signaling pathways are altered, affecting fate and function of T cells.[16-24] The role of CARMIL2 in CD28-mediated signaling suggests that its deficiency could interfere with metabolic activity of T cells.

Nine patients with CARMIL2 deficiency and 16 healthy controls (HCs) were recruited for this study. RNA sequencing (RNA-Seq) analysis revealed downregulation of genes associated with activation-induced metabolic reprogramming in T cells. Liquid chromatography–mass spectrometry metabolomics identified decreased levels of metabolites in key metabolic pathways associated with T-cell activation, including glycolysis and the tricarboxylic acid (TCA) cycle. Finally, supplementation of CARMIL2-mutated T cells with selected metabolites upregulated activation-induced signaling and cytokine expression.

METHODS

Participants

Patients and HCs were recruited from the Department of Dermatology of Emek Medical Center, the Department of Hematopoietic Stem Cell Transplantation of Hadassah Medical Center, and the immunology service of Sheba Medical Center. Patients, HCs and/or their legal guardians provided informed consent before their inclusion in the study according to a protocol approved by the 3 medical centers. Patients or their legal guardians signed informed consent forms for publication of clinical photos.

$CD4^+$ T-cell isolation and culture

PBMCs were isolated by Ficoll-Hypaque density centrifugation (cat. no. 10771; Sigma-Aldrich, St Louis, Mo). The cells were then cultured in RPMI-1640 medium supplemented with 10% FCS. $CD4^+$ cells were isolated using the EasySep Human $CD4^+$ T-Cell Isolation Kit (cat. no. 17952; Stem Cell Technologies, Cambridge, UK) according to the manufacturer's protocol.

RNA-Seq

Total RNA was extracted from 7 $CD4^+$ cell pellet samples (CARMIL2-mutated cells: n = 4; HC cells: n = 3; 200,000 cells each) using the RNeasy Micro Kit (cat. no. 74004; Qiagen, Germantown, Md). After homogenization, samples were extracted automatically using the QIAcube Connect (Qiagen). Seven RNA-Seq libraries were constructed simultaneously using SMART-Seq Total RNA Single Cell library preparation kit (cat. no. 634360; Takara, San Jose, Calif) with ribosomal RNA removal, according to the manufacturer's protocol; 1.5 ng total RNA was used as starting material. RNA-Seq library quality control was performed by measuring library concentration using Qubit (Invitrogen) with the Equalbit RNA HS Assay Kit (cat. no. EQ211; Vazyme, Nanjing, China) and size determination using the TapeStation 4200 with the High Sensitivity D1000 kit (cat. no. 5067-5584; Agilent, Santa Clara, Calif). All libraries were mixed into a single tube with equal molarity. The RNA-Seq data were generated on Illumina NextSeq2000, using P2 XLEAP-SBS 100 cycles (Read1-100; Index1-8; Index2-8) (cat. no. 20100987; Illumina) sequencing kit. Quality control was assessed on 100-bp single reads using Fastqc (v0.12.1) and MultiQC (v1.14). Reads were trimmed for adapters, minimum quality Phred score of 20, and minimum length of 35 using CUTADAPT (v4.4). Reads were aligned to *Homo sapiens* (GRCh38) reference genome and annotation file using STAR (v2.7.10b) with mismatch ratio allowed < 0.2; the minimum and maximum intron sizes were set to 21 and 1,000,000, respectively. The number of reads per gene was counted using Htseq-count (v2.0.3) with reverse mode and gene feature type. Normalization and differential expression analyses were conducted using DESeq2 R package (v1.44.0). The threshold for significantly differentially expressed genes is determined by 2 factors: adjusted $P \leq .05$ and base-mean independent filtering threshold, which is calculated by the DESeq2 algorithm for each comparison. False discovery rate was calculated using the default approach of DESeq2, the Benjamini-Hochberg procedure. GSEA (v4.3.3) graphical user interface for Windows was used for gene set enrichment analyses. Normalized count table derived from DESeq2 analyses was used as input.

Metabolomics

CARMIL2-mutated cells (n = 6) and HC $CD4^+$ cells (n = 8) were thawed and grown in culture using RPMI-1640 medium supplemented with 10% FCS and stimulated with anti CD3 (7.5 μg/mL)/CD28 (3.75 μg/mL) for 48 hours.[25,26] Then, metabolites were extracted from cells and media for analysis using a Q Exactive Plus Hybrid Quadrupole-Orbitrap Mass Spectrometer (Thermo Fisher Scientific). The analysis combined hydrophilic interaction liquid chromatography for polar compounds and reversed-phase ion-pairing chromatography to cover a broad range of metabolites. The instrument operated in negative ion mode to enhance the detection of negatively charged metabolites. Data were normalized according to the protein amount in the samples and processed using Maven software (https://maven.apache.org/). A heatmap displaying all metabolites with a significant P value ($P < .05$) was generated using the Morpheus platform (https://software.broadinstitute.org/morpheus/).

Flow cytometry

For cell surface staining, $CD4^+$ T cells from HCs or patients with CARMIL2 deficiency were activated for 72 hours with anti-CD3 (7.5 μg/mL) (Ultra-LEAF Purified anti-human CD3, cat. no. 317326; BioLegend, San Diego, Calif) and anti-CD28 (3.75 μg/mL) (Ultra-LEAF Purified anti-human CD28, cat. no. 302934; BioLegend) antibodies. Cells were then suspended in separation buffer (PBS containing 2% FBS and 2 mM EDTA) and incubated for 20 minutes on ice with anti-CD25 (Brilliant Violet 650 anti-human CD25, cat. no. 302633; BioLegend) or ASCT2 (Anti-Human ASCT2/SLC1A5 [extracellular]-FITC, ANT-083-F) antibodies. After incubation, cells

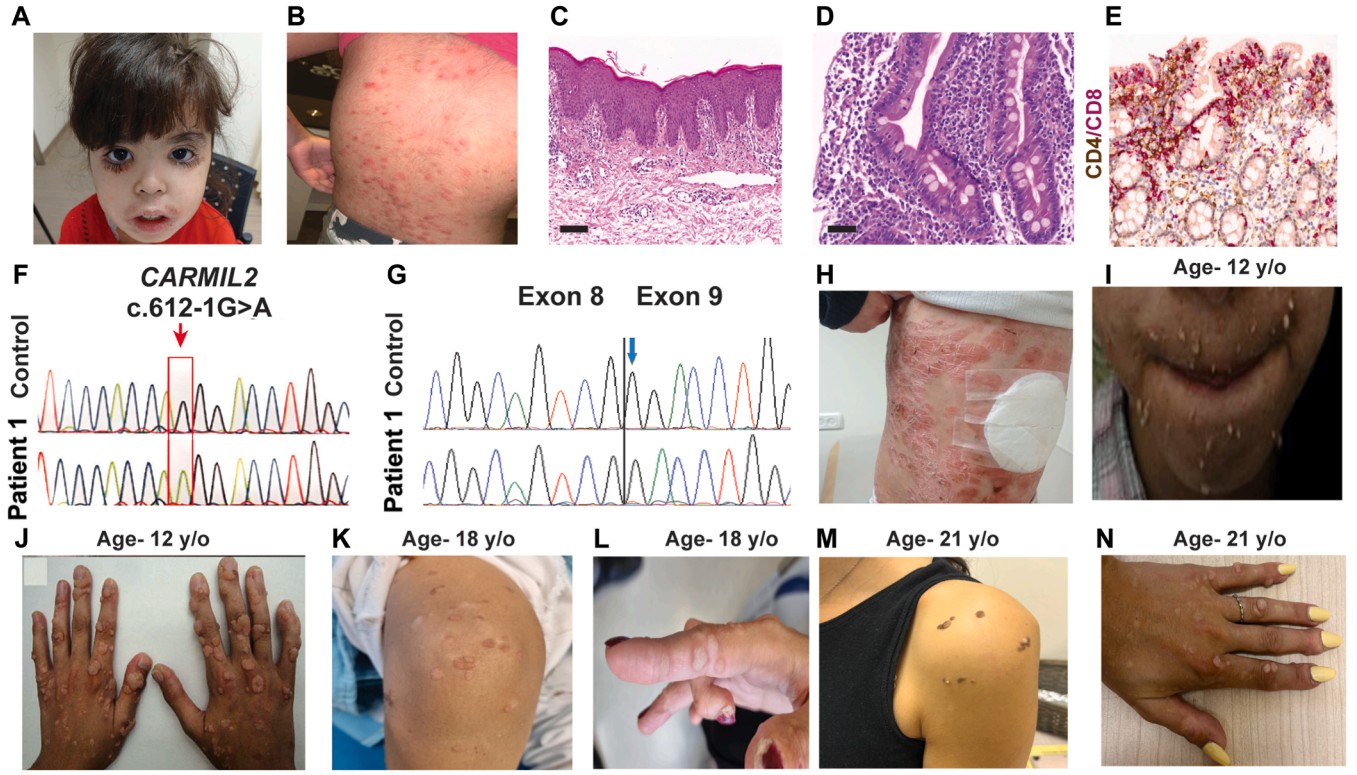

FIG 1. Clinical characteristics of the patients with CARMIL2- deficiency cohort. **(A** and **B)** Patient 1 presented with infantile-onset skin rash, with features of severe atopic dermatitis. **(C)** Skin biopsy of patient 1 shows acanthosis, mild spongiosis, and perivascular lymphocytic infiltration admixed with eosinophils (magnification bar = 100 μM). **(D** and **E)** Duodenal biopsy from patient 1 revealed villi atrophy and intraepithelial infiltration of small and medium-sized T lymphocytes (magnification bar = 50 μM). **(F)** Sanger sequencing of patient 1 DNA verified a homozygous variant *CARMIL2* c.612-1G>A. **(G)** Sequencing of patient 1 cDNA demonstrates omission of single nucleotide in exon 8–exon 9 junction *(blue arrow).* **(H)** Deterioration of skin rash of patient 1 after 3 months of treatment with dupilumab. **(I-N)** Early-onset seborrheic keratosis and recalcitrant human papillomavirus warts, along clinical follow-up, in patient 4 with homozygous *CARMIL2* c.A689del.GCCTTGAGGTCTCAGAACAGATTCT variant.

were centrifuged, washed, resuspended in separation buffer, and immediately analyzed using an Attune flow cytometer (Thermo Fisher Scientific). For intracellular staining, RPS6 phosphorylation staining was performed following stimulation with anti-CD3/CD28 antibodies for 15 minutes. Cells were then permeabilized using the True-Nuclear Transcription Factor Buffer Set (cat. no. 424401; BioLegend) and stained with anti-phospho-RPS6 (Brilliant Violet 421 anti-RPS6 Phospho Ser235/Ser236, cat. no. 608609; BioLegend). For phospho-NF-κB p65 staining, cells were stimulated with a phorbol 12-myristate 13-acetate (PMA) activation cocktail containing brefeldin A (cat. no. 423304; BioLegend) for 5 minutes, then fixed with 4% formaldehyde and permeabilized with 100% methanol. Staining was performed using anti-phospho-NF-κB p65 (Ser536) (93H1 Rabbit mAb Alexa Fluor 488, cat. no. 4886; Cell Signaling Technology, Danvers, Mass). After staining, cells were washed, resuspended in separation buffer, and analyzed using FlowJo v10 software (https://www.flowjo.com/flowjo10/overview).

Rescue experiment

$CD4^+$ T cells were incubated for 2 hours with L-glutamine (5 mM); with a mixture of L-glutamine (5 mM), L-leucine (5 mM), and methionine (250 μM); or without metabolite supplementation. Cells were then stimulated with either anti-CD3 (7.5 μg/mL) and anti-CD28 (3.75 μg/mL) antibodies for 15 minutes or with PMA/brefeldin A for 5 minutes, followed by staining for phospho-RPS6 and phospho-NF-kB, respectively. The impact of glutamine supplementation on CARMIL2-mutated $CD4^+$ cell proliferation and cytokine expression was tested through flow cytometry, RT-PCR, and ELISA (detailed in this article's Methods section in the Online Repository available at www.jacionline.org).

RESULTS

Study cohort

Patient 1 is a girl born to consanguineous relatives. Since birth, she has presented with a disseminated pruritic rash consisting of erythematous plaques covered with scale crust (Fig 1, *A* and *B*; Fig E1, *A* and *B*, in the Online Repository at www.jacionline.org). Skin biopsy revealed spongiotic dermatitis (Fig 1, *C*). She also had failure to thrive (FTT) and experienced multiple episodes of diarrhea and vomiting. Duodenal biopsy showed villi effacement, intraepithelial infiltration of small and medium-sized T lymphocytes (Fig 1, *D* and *E*), and a low number of regulatory T cells (Fig E1, *C*). Given the combined clinical manifestations of severe dermatitis, FTT, and chronic enteritis, an IEI was suspected. Whole-exome sequencing identified a homozygous variant c.612-1G>A in the *CARMIL2* gene. The variant was verified by Sanger sequencing and cosegregated with the phenotype (Fig 1, *F*). The variant, which was not identified in genetic databases (eg, gnomAD, TOPmed Bravo, GME

Variome), is classified as deleterious owing to a splice site alteration, as confirmed by cDNA sequencing showing the omission of a single nucleotide in the exon 8–exon 9 junction, resulting in a frameshift and premature stop codon (c.612delG, p.Asp205Thrfs*4) (Fig 1, *G*). Patient 1 was treated for her skin disease with topical corticosteroids, phototherapy, and methotrexate with no improvement. Based on previous reports of successful dupilumab treatment of dermatitis in patients with IEIs[27] and an increased ratio of IL-13/IL-17 gene expression measured in her skin biopsy (Fig E1, *D*), treatment with dupilumab 300 mg/month was initiated, resulting in a marked worsening of her skin disease after 3 months of therapy (Fig 1, *H*).

Given the limited therapeutic options for this and other patients, and because CARMIL2 interacting proteins (CD28, CARD11) are involved in metabolic regulation of $CD4^+$ T cells, we investigated how CARMIL2 deficiency affected T-cell metabolism. To this end, 8 additional patients with CARMIL2 deficiency were recruited for this study (patients 2-9). Some of these patients were previously reported,[28,29] and their current clinical status is shown in Table I. In brief, patient 2 showed a predominant clinical phenotype of early-onset inflammatory bowel disease (IBD) that improved after subtotal colectomy. The early-onset psoriasiform rash disappeared spontaneously at 6 years of age and has not recurred. Patients 3 through 6 were family members who demonstrated a combined phenotype of recurrent infections, early-onset seborrheic keratosis, disseminated viral warts (Fig 1, *I-N*), and early-onset IBD. Patients 3 and 6 underwent successful HSCT and were completely cured. Patient 7 presented with a psoriasis-like rash, recurrent skin infections, and recurrent mastoiditis. Immune workup showed mild hypogammaglobulinemia and negative tetanus, diphtheria, hepatitis A, and hepatitis B vaccines serology. He had normal T- and B-cell counts and normal TCR repertoire and TCR excision circle levels. Genetic workup revealed a homozygous variant in *CARMIL-2* c.1973C>T p.Ala658Val. Patients 8 and 9 presented with dermatitis, FTT, recurrent skin infections, recurrent mastoiditis and pneumonia, persistent EBV viremia, and recalcitrant viral warts. Immune workup revealed normal immunoglobulins, normal T- and B-cell counts, and normal TCR repertoire and TCR excision circle levels. Genetic workup revealed a homozygous variant in *CARMIL2* c.1865C>T; p.Ala622Val.

Cells were isolated from all patients. Importantly, patients included in this study were not treated with immunosuppressive drugs for at least 6 months before sample collection. Samples from patients 3 and 6 were cryopreserved and collected before chemotherapy and HSCT.

$CD4^+$ T cells derived from CARMIL2-deficient patients show impaired activation

Impaired $CD4^+$ T-cell immunity is a key driver of pathogenicity in patients with CARMIL2 deficiency. To investigate the functionality of $CD4^+$ T cells in our cohort, $CD4^+$ T cells were purified from blood samples of 4 patients with CARMIL2 deficiency (patients 1, 2, 4, and 5) and 6 age- and sex-matched HCs and stimulated *ex vivo* using anti-CD3 (7.5 μg/mL) and anti-CD28 (3.75 μg/mL). CARMIL2-mutated T cells exhibited low cell growth (Fig 2, *A* and *B*) and low expression levels of CD25 (Fig 2, *C-E*), indicators of improper activation. Thus, in agreement with previous studies,[3,4,10] our analysis showed impaired activation in CARMIL2-mutated $CD4^+$ T cells.

To search for mechanisms underlying T-cell dysfunction, we performed bulk RNA-Seq on $CD4^+$ T cells from CARMIL2-deficient patients (n = 4 [patients 1, 2, 4, and 5]) and compared them with age- and sex-matched HCs (n = 3). $CD4^+$ T cells were stimulated for 72 hours before collection and RNA purification.[30,31] Principal component analysis revealed separate clustering of HC and CARMIL2-mutated T cells with the exception of patient 2 (Fig 2, *F*). This may reflect the milder clinical phenotype of patient 2 compared with the multiorgan phenotype of the other patients included in this analysis (Table I). A Euclidean distance heat map similarly showed a difference between the T cells of patients with CARMIL2 and HCs (Fig E2, *A*, in the Online Repository at www.jacionline.org). Consistent with this, large differences in gene expression were identified, as 3155 genes were significantly upregulated and 3045 genes were significantly downregulated in CARMIL2-mutated T cells compared with HCs (adjusted $P \leq .05$ and $\log_2$ fold change ≥ 1) (Fig 2, *G*). Analysis of downregulated genes in CARMIL2-mutated T cells revealed enrichment of pathways involved in DNA replication, nucleotide biosynthesis, protein translation, and metabolism (Fig 2, *H*). Ingenuity pathway analysis (IPA) further identified downregulation of several canonical pathways, including those related to immune regulation (Fig 2, *I*). In particular, we found enrichment of genes associated with signaling pathways that regulate T-cell activation, including NF-κB, PI3K/Akt, and mTOR (Fig 2, *I*). Analysis of upstream regulators revealed impairment of MYC, CD3, CD28, CD40, and IL-2 signaling in CARMIL2 deficiency and corroborated the inhibition of mTOR and mitogen-activated protein kinase pathways (Fig E2). To further investigate the metabolic status of CARMIL2-mutated T cells, we focused on canonical metabolic pathways and found enrichment of genes related to central carbon metabolism and ATP synthesis, including glycolysis, folate metabolism, and glutamine metabolism, all were underrepresented in patient T cells (Fig 2, *J*). In agreement, IPA analysis of genes upregulated in CARMIL2-mutated $CD4^+$ T cells identified AMP-activated protein kinase signaling indicative of nutrient deprivation and low energy (Fig 2, *K*).[24]

Activation-induced metabolic activity is altered in CARMIL2-mutated $CD4^+$ T cells

The enrichment of metabolic pathways among differentially expressed genes, together with the fact that CARMIL2 is part of the CD28 signaling cascade, prompted us to test whether loss of CARMIL2 impairs activation-induced metabolic reprogramming in patients' T cells. $CD4^+$ T cells (collected from patients 1-6) and HC T cells (n=8) were activated for 48 hours before analysis by liquid chromatography–mass spectrometry for targeted whole-cell metabolomics of 96 metabolites (Table E1 in the Online Repository at www.jacionline.org).

Our analysis detected 62 metabolites. The concentrations of 20 metabolites differed significantly between the 2 groups: all were downregulated in CARMIL2-mutated $CD4^+$ T cells (Table E2 in the Online Repository at www.jacionline.org) and clustered into a few metabolic pathways, including glycolysis, TCA cycle, pentose phosphate pathway, methionine cycle and transsulfuration, purines, and pyrimidines (Fig 3, *A*). These results were consistent with some of the metabolic pathways identified in the RNA-Seq analysis (Fig 2, *J*). Stratifying deficient metabolites to metabolic pathways pointed to glutamine deficiency as a potential contributor to the observed metabolic phenotype (Fig 3, *B*). In activated T cells, glutamine is fed into the TCA cycle to replenish carbon consumed for lipid biosynthesis and is used for *de novo* purine and pyrimidine biosynthesis.[32] In addition, low S-adenosylmethionine and S-adenosylhomocysteine levels (Fig 3, *A*) could indicate a low uptake of methionine. Consistent with this, RNA-Seq data showed reduced

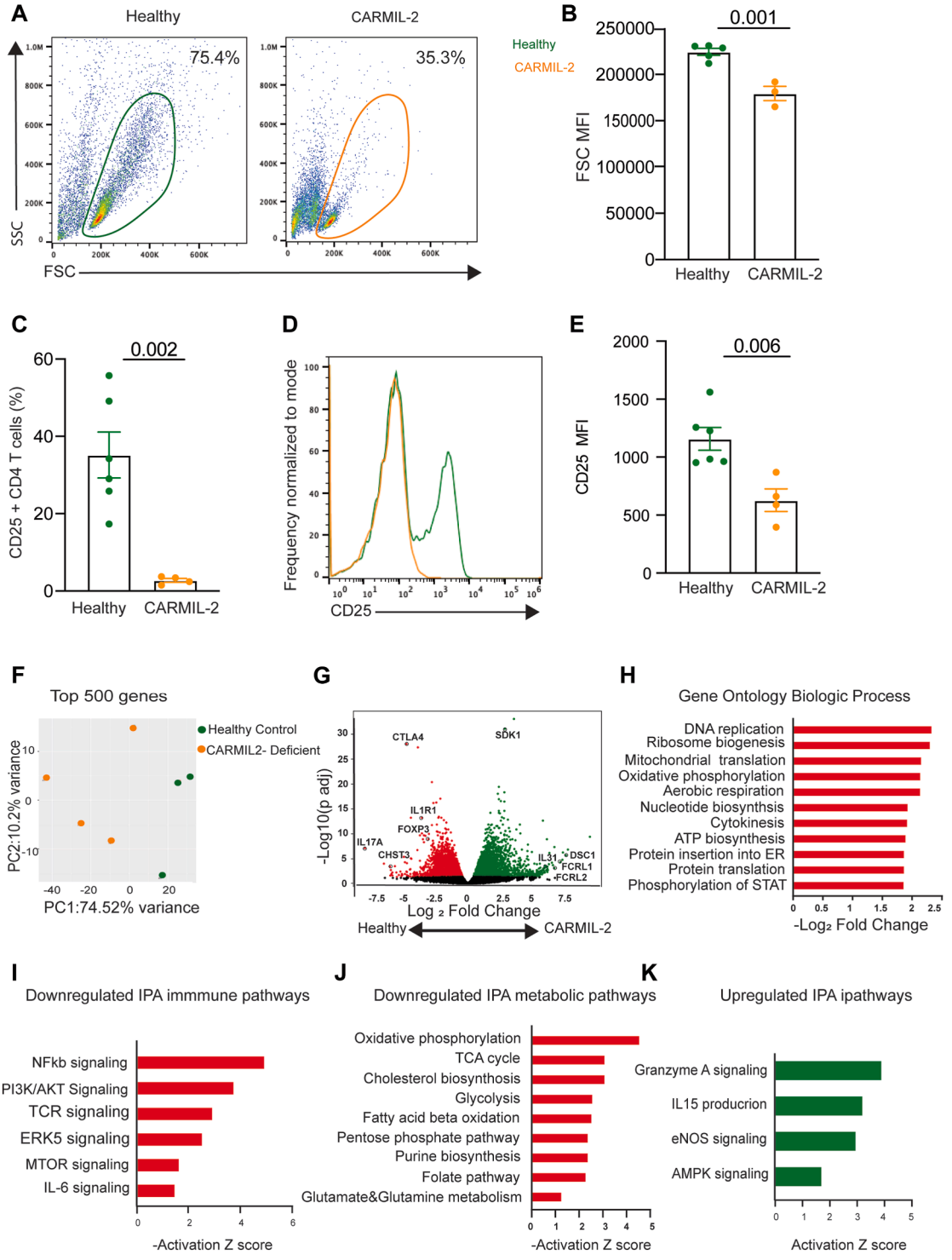

FIG 2. CARMIL2 deficiency is associated with impaired immunity and metabolism. **(A)** Scatter plot of forward and side parameters obtained from flow cytometry of stimulated $CD4^+$ cells of patient 1 and HC 1. **(B)** Bar plot of activation induced cell growth of CARMIL2-mutated and HC $CD4^+$ cells. *P* value indicated by 2-tailed Student *t* test. **(C)** CD25 expressing $CD4^+$ cells in CARMIL2-mutated and HC groups (*P* value indicated by 2-tailed Student *t* test). **(D)** Histogram of CD25 expression obtained from flow cytometry of stimulated $CD4^+$ cells of patient 2 and HC 2. **(E)** CD25 mean fluorescence intensity in CARMIL2-mutated and HC cells. *P* value indicated by 2-tailed Student *t* test. **(F)** Principal component analysis plot for CARMIL2-mutated and HC $CD4^+$ cells. **(G)** Volcano plot of upregulated and downregulated genes in bulk RNA-Seq data from CARMIL2-mutated and HC $CD4^+$ cells. **(H)** Gene Ontology biologic process terms significantly overrepresented in downregulated genes in CARMIL2-mutated $CD4^+$ cells. False discovery rate < 0.1, $P < .01$. **(I)** IPA of downregulated differentially expressed genes involved in canonical immune response in CARMIL2-mutated and HC $CD4^+$ cells. The x-axis indicates the z score, and the y-axis indicates the corresponding canonical pathways. $P < .01$, percentage overlap >20%. **(J)** IPA of

expression of genes encoding glutamine and methionine transporters in CARMIL2-mutated T cells compared with controls (Fig 3, *C*).[17,33-35] Reduced expression of ASCT2, the major glutamine transporter in T cells, was further confirmed by flow cytometry (Fig 3, *D*).

Glutamine enhances activation-induced signaling and cytokine expression in CARMIL2-deficient CD4$^+$ T cells

CARMIL2 deficiency impaired T-cell activation and metabolic reprogramming. Considering the role of glutamine in multiple affected pathways identified by both RNA-Seq analysis and metabolomics and the low expression of ASCT2 in CARMIL2-deficient T cells, we hypothesized that glutamine supplementation would improve T-cell activation under CARMIL2 deficiency. T cells derived from patients with CARMIL2 deficiency and HCs were stimulated *ex vivo* and supplemented with either glutamine alone or in combination with methionine and leucine for 2 hours before analysis (Fig 4, *A*). Methionine was added to replenish methionine cycle and 1-carbon metabolism (Fig 3, *B*) and leucine for its ability to restore mTOR signaling even in the absence of glutamine.[17] Phosphorylation of p65-NF-κB and RPS6 were quantified to assess activation of the NF-κB and mTOR signaling cascades (Fig 4, *B*). In the absence of metabolite supplementation, levels of p65 NF-κB phosphorylation were significantly lower in CARMIL2-mutated versus healthy T cells. Strikingly, increasing glutamine concentration in growth media restored p65-NF-κB phosphorylation (Fig 4, *C* and *D*). Similarly, RPS6 phosphorylation was reduced in CARMIL2-mutated T cells and elevated by addition of the 3 amino acids or glutamine alone (Fig 4, *E* and *F*).

Restoration of NF-κB and mTOR suggests that increasing glutamine availability enhances activation responses in CARMIL2-mutated T cells. To investigate the functional outcomes of glutamine supplementation, we examined cytokine gene expression in CARMIL2-mutated CD4$^+$ T cells with or without high-dose glutamine supplementation for 36 hours. Cells were stimulated only with anti-CD3/CD28, and no polarizing cytokines were added. We found a significant increase in IL-17A mRNA (Fig 4, *G*), consistent with previous findings linking glutamine metabolism to T_H17 differentiation via mTOR- and α-ketoglutarate-dependent signaling.[36] No significant differences were observed in the expression of IFN-γ, IL-2, or IL-13 (Fig E3, *A*, in the Online Repository at www.jacionline.org). A complementary analysis of cytokine secretion showed elevation in IL-4 and IL-22 secretion by CARMIL2-mutated cells on glutamine supplementation (Fig E3, *B*). Values for IL-2, IFN-γ, IL-13, and IL-17 were increased on glutamine supplementation, not reaching statistical significance (Fig E3, *B*).

NF-κB and mTOR signaling play an important role in T-cell proliferation. Consistent with previous studies, the proliferation rate was lower in CARMIL2-mutated CD4$^+$ T cells compared with HC cells. Importantly, the effect on proliferation varied across different patients (Fig E3, *C*). Glutamine supplementation improved proliferation only in T cells in which proliferative capacity was completely abolished before the treatment (patients 1 and 4) (Fig 4, *H*). T cells derived from 2 other patients (patients 8 and 9) maintained partial proliferative capacity, which was not improved by glutamine supplementation (Fig 4, *H*; Fig E3, *D*).

DISCUSSION

CARMIL2 deficiency impairs multiple arms of the immune response. In this study, we conducted a comprehensive analysis of the transcriptomic and metabolic profiles of activated CD4$^+$ cells from patients with CARMIL2 deficiency, comparing them with those of healthy subjects. Our cohort included patients with 5 distinct homozygous mutations and a range of phenotypic presentations including skin and gastrointestinal inflammation as well as recurrent bacterial and viral infections, all characteristic of this condition.[3,4,10-16] Whereas all patients with CARMIL2 deficiency have CARMIL2 loss of function, the residual activity of the mutated protein might determine the degree of immunologic defect (Fig 4, *H*; Fig E3, *A* and *B*) and the consequent clinical manifestation (Table I). In line with this, we identified differences in proliferation between T cells from patients with frameshift or large in-frame deletion variants and negligible CARMIL2 expression[28] compared with T cells obtained from patients with missense variant and residual CARMIL2 activity.[10] Whereas the former showed almost no proliferation, T cells bearing a missense variant, with residual activity,[10] retained some degree of proliferative capacity.

Consistent with prior reports, our analysis confirmed decreased activation of CD4$^+$ T cells and aberrant NF-κB signaling in CARMIL2 deficiency. Notably, we further identified impaired mTOR signaling and skewed metabolic reprogramming in T cells derived from CARMIL2 deficient patients with CARMIL2 deficiency. PI3K/Akt, the upstream regulator of mTOR, upregulates the glucose transporter GLUT1 and glucose uptake in response to T-cell receptor ligation,[37,38] whereas mTORC1 signaling is essential for the proper function of 1-carbon metabolism, fatty acid activation, lipid biosynthesis, and the electron transport chain that enables T-cell activation.[39-43]

Metabolomic and transcriptomic analyses revealed altered glutamine metabolism in CARMIL2-mutated CD4$^+$ T cells. Glutamine replenishes the mitochondrial TCA cycle through α-ketoglutarate production and incorporates into nucleotides both critical for T-cell proliferation.[44-46] CARMIL2-mutated CD4$^+$ T cells showed decreased membrane expression of ASCT2, the primary glutamine transporter in T cells, compared with HC cells. The CBM complex regulates ASCT2 expression and its membrane localization after CD28 costimulation and is required for downstream mTORC1 and NF-κB signaling.[17,47,48]

Mutations in genes encoding components of the CBM complex such as *CARD11* gene[49,50] also impair NF-κB and mTOR signaling, leading to combined immunodeficiency that shares clinical features with CARMIL2 deficiency, including severe skin dermatitis, early-onset IBD, and recurrent infections.[49,50] These similarities may stem from the interaction between CARMIL2 and CARD11[7]; in CARMIL2- mutated T cells, CARD11

differentially expressed genes involved in metabolic processes in CARMIL2-mutated and HC CD4$^+$ cells. $P < .05$, percentage overlap >20%. **(K)** IPA of upregulated differentially expressed genes involved in canonical immune processes in CARMIL2-mutated and HC CD4$^+$ cells. $P < .01$, percentage overlap >20%. *AMPK,* AMP-activated protein kinase; *eNOS,* endothelial nitric oxide synthase; *ER,* endoplasmic reticulum; *FSC,* forward scatter; *MFI,* mean fluorescence intensity; *PC1, PC2,* principal component 1, 2; *SSC,* side scatter.

TABLE I. Clinical characteristics of patient cohort

Patient	Age/ Sex	Infectious	Skin	Allergic	Gastrointestinal	Treatment history	Genetic variant in *CARMIL2* gene
1	8/F	Viral warts	Severe dermatitis	—	Lymphocytic esophagitis Chronic lymphocytic intestinal inflammation FTT	Phototherapy Methotrexate Dupilumab	c.612-1G>A
2	18/M	No	Dermatitis—resolved	No	Eosinophilic esophagitis Duodenitis Colitis	Topical steroids Colectomy Sirolimus Dupilumab	c.1590C>A
3	13/M	CMV viremia, CMV retinitis Pneumonia HPV warts PJP	—	Asthma Allergic rhinitis	Crohn disease Lymphocytic esophagitis FTT	Azathioprine Infliximab Steroids Prophylactic antibiotics IVIG HSCT	c.689_713del.GCCTTGAGGTCTCAGAACAGATTCT
4	21/F	HPV warts CMV viremia CMV colitis EBV viremia Pneumonia Left ear deafness	—	Asthma Allergic rhinitis	Ulcerative colitis CMV	Steroids Prophylactic antibiotics Valganciclovir IVIG	c.689_713del.GCCTTGAGGTCTCAGAACAGATTCT
5	19/F	HPV warts CMV and EBV viremia Esophageal candidiasis	—	—	Lymphocytic esophagitis	Steroids Prophylactic antibiotics	c.689_713del.GCCTTGAGGTCTCAGAACAGATTCT
6	13/F	CMV colitis, CMV retinitis Recurrent pneumonia HPV warts	—	—	Crohn disease FTT	Azathioprine Infliximab Steroids Prophylactic antibiotics IVIG HSCT	c.689_713del.GCCTTGAGGTCTCAGAACAGATTCT
7	15/M	Recurrent ear, skin, and eye infections including mastoiditis, blepharitis	Recurrent skin eczema	—	—	Azithromycin (prophylactic) Itraconazole Topicals (Diprosalic, Protopic, Sebosel shampoo) IVIG	c.1973C>T (p.Ala658Val)
8	17/F	Recurrent ear and skin infections Persistent EBV viremia (1.1×10^5 copies/mL)	Scalp dermatitis, warts	—	FTT	Resprim Fluconazole Monthly IVIG	c.1865C>T (p.Ala622Val)
9	14/M	Recurrent pneumonia and mastoiditis Mild EBV viremia (5.5×10^3 copies/mL)	Recurrent dermatitis, warts	—	FTT Nodular gastritis Eosinophilic esophagitis	Resprim Fluconazole Monthly IVIG	c.1865C>T (p.Ala622Val)

CMV, Cytomegalovirus; *F,* female; *HPV,* human papillomavirus; *IVIG,* intravenous immunoglobulin; *M,* male; *PJP, Pneumocystis jirovecii* pneumonia.

fails to accumulate in the central supramolecular activation complex, impairing NF-κB signaling.[8]

The interplay between immunologic activation and metabolic reprogramming is driven by 2 intersecting processes: top-down signaling, where TCR/CD28 activation drives metabolic adaptation through c-MYC and mTOR, and bottom-up regulation, where amino acids modulate immune signaling, including mTOR activation.[24,51,52] Disruption of top-down signaling in CARMIL2 deficiency may be compensated by bottom-up interventions. For example, glutamine supplementation restored mTOR signaling in ASCT2 null $CD4^+$ T cells,[4,17] whereas leucine-enriched growth media rescued mTOR activation and T_H17 differentiation in ASCT2-null and in CBM-deficient $CD4^+$ T cells.[4,17]

Notably, metabolic dysregulation may underlie many of the broader T-cell functional abnormalities observed in CARMIL2 deficiency. Glutamine metabolism and mTOR signaling are essential for T-cell polarization, cytotoxic granule production, and migration, and their disruption could explain the impaired effector function and chemotaxis reported in this condition.[10,36,53]

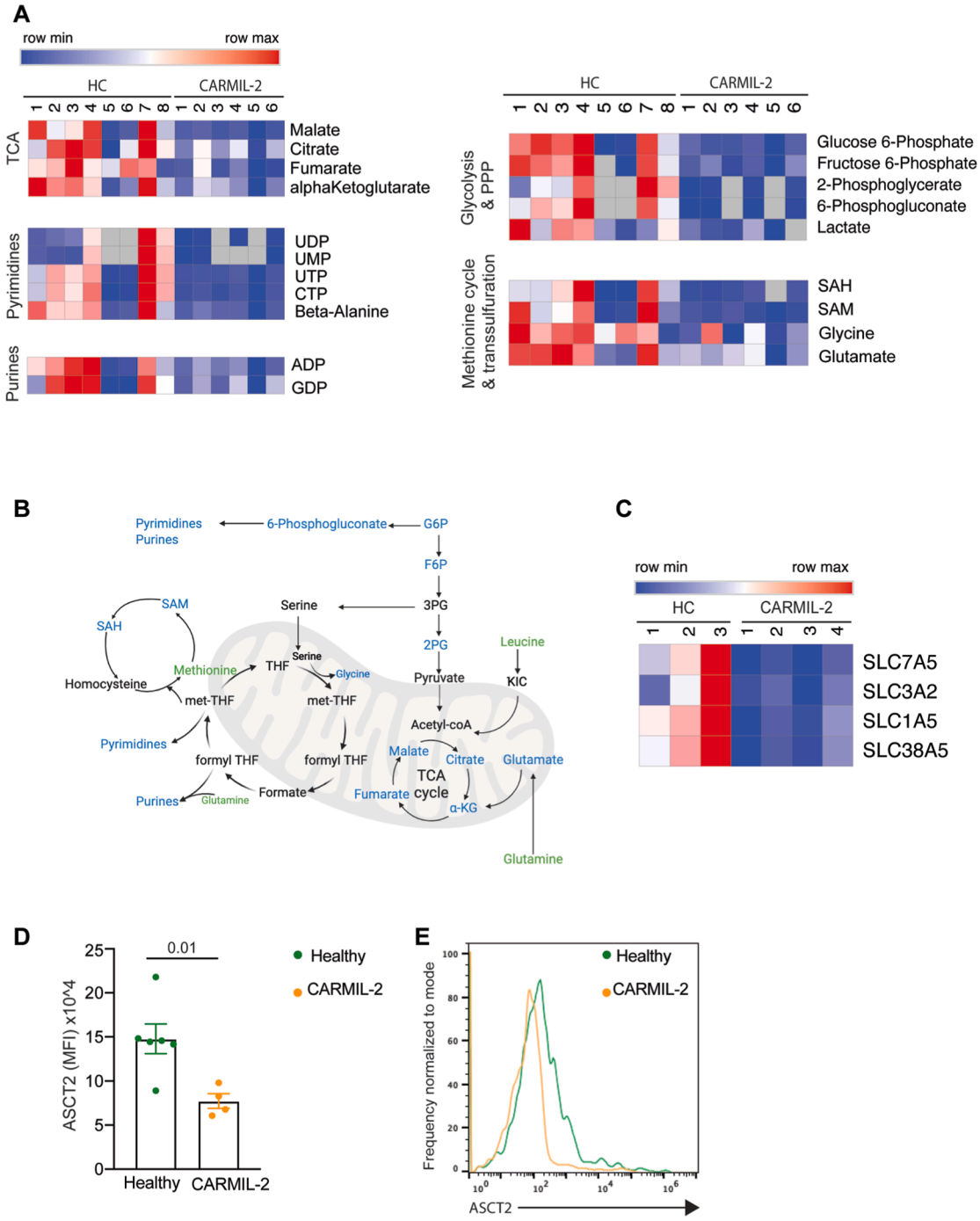

FIG 3. CARMIL2-mutated CD4$^+$ cells show dysregulation of multiple metabolic pathways. **(A)** Heat map of significantly different intracellular metabolites in CARMIL2-mutated and HC CD4$^+$ cells following 48 hours of stimulation. **(B)** Illustration of dysregulated metabolic pathways in CARMIL2-mutated CD4$^+$ cells. *Blue labels* denote significantly reduced levels in CARMIL2 cells compared with HC cells. **(C)** Expression of glutamine and methionine transporter genes. Expression levels obtained from RNA-Seq data. All differences were significant with $P < .01$. **(D)** Protein expression levels of main glutamine transporter, ASCT2, in CARMIL2-mutated (n = 4) and HC CD4$^+$ (n = 6) cells 72 hours after stimulation with anti-CD3/CD28. P value indicated by 2-tailed Student *t* test. **(E)** Flow cytometry histogram of membrane ASCT2 transporter in CD4$^+$ cells of patient 1 and HC 1 72 hours after stimulation with anti-CD3/CD28. *MFI,* Mean fluorescence intensity; *PPP,* pentose phosphate pathway; *SAH, S*-adenosylhomocysteine; *SAM, S*-adenosylmethionine.

In our study, supplementation with glutamine or a mixture of glutamine, leucine, and methionine restored mTOR activation in CARMIL2-mutated lymphocytes. Such intervention was similarly effective in lymphocytes from a patient with hypomorphic *CARD11* mutations.[49] Interestingly, glutamine further enhanced the activation of the NF-κB pathway in our study. This finding, which to our knowledge has not been reported previously in IEIs, may be related to the interaction of glutamine dehydrogenase with the IκB kinase

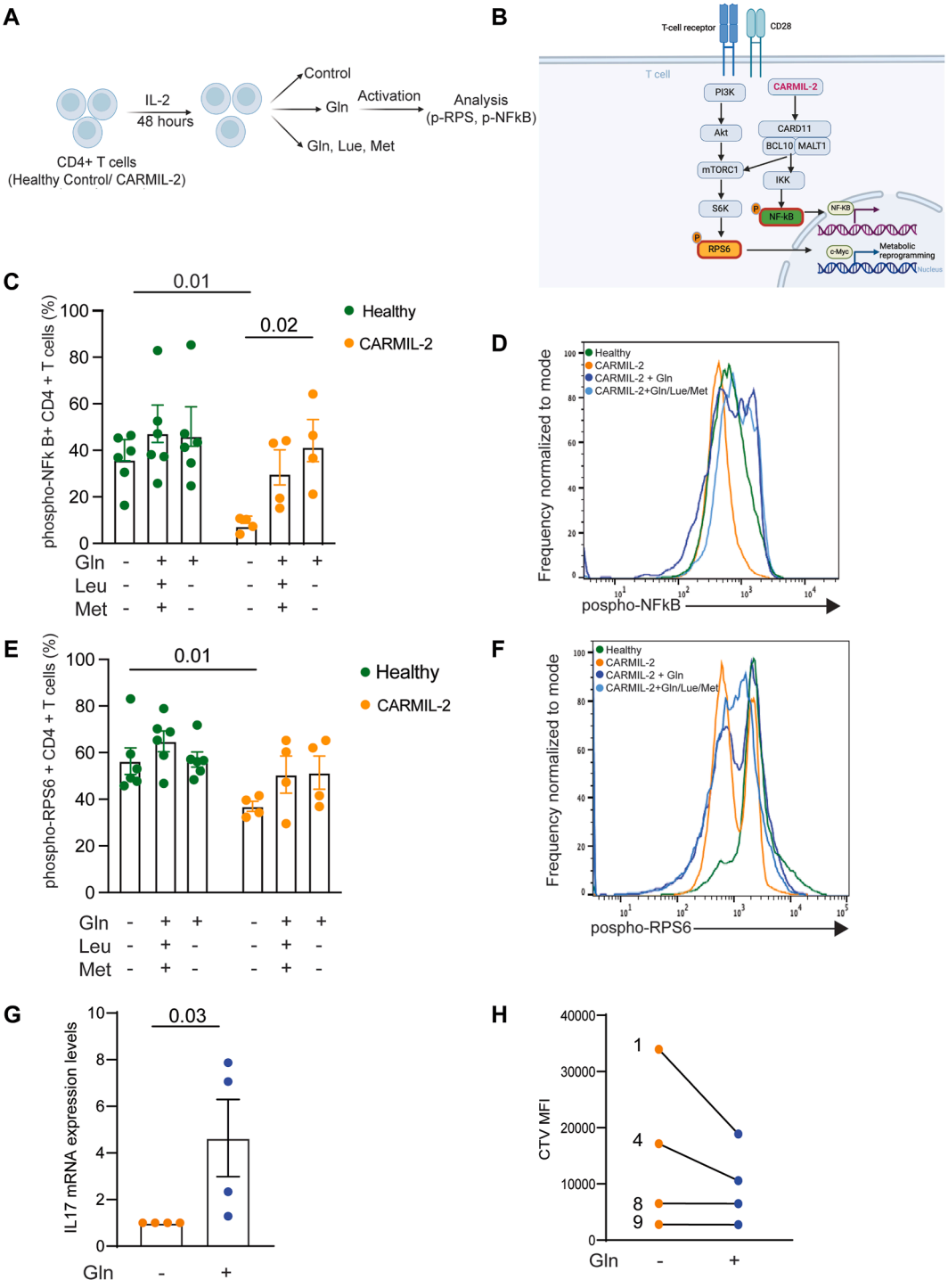

FIG 4. Supplementation of amino acids enhances signaling pathways and cytokine expression in CARMIL2-mutated CD4$^+$ cells. **(A)** Illustration of experimental rescue workflow. **(B)** Model of rescue. **(C)** Flow cytometry histogram of phospho-p65 NF-κB in patient 1 and HC 1 CD4$^+$ cells 5 minutes after stimulation with PMA without or with glutamine or glutamine, leucine, and methionine supplementation. **(D)** Quantification of percentage of phospho-p-65-NF-κB$^+$ cells within CD4$^+$ cells of patient with CARMIL2 mutation (n = 4) or HC (n = 6) without or with glutamine or glutamine, leucine, and methionine supplementation. *P* value indicated by 2-way ANOVA. **(E)** Flow cytometry histogram of phospho-RPS6 in patient 1 and HC 1 CD4$^+$ cells 15 minutes after stimulation with anti-CD3/CD28 without or with glutamine or glutamine, leucine, and methionine supplementation. **(F)** Quantification of percentage of phospho-RPS6$^+$ cells within CD4$^+$ cells of patient with CARMIL2 mutation (n = 4) or HC (n = 6) without or with glutamine or glutamine, leucine, and methionine supplementation. *P* value indicated by 2-way ANOVA. **(G)** mRNA expression of IL17A in CARMIL2-mutated CD4$^+$ T cells with or without glutamine for 36 hours (n = 4, normalized to GAPDH, significance indicated by 2-tailed Student *t* test). **(H)** Mean fluorescence intensity of cell trace violet in CARMIL2-mutated CD4$^+$ T cells with or without glutamine for 5 days. *CTV,* Cell trace violet; *MFI,* mean fluorescence intensity.

complex, which promotes the production of α-ketoglutarate and the activation of NF-κB in cancer cells.[54] Alternatively, p-65 has been shown to enhance glutaminolysis by repressing miR-23a, thereby increasing glutaminase expression.[55]

Additionally, glutamine supplementation improved CARMIL2-mutated T-cell functionality, such as enhancement of cytokine expression, cytokine secretion, and CARMIL2-mutated T-cell proliferation (Fig 4, *G* and *H*; Fig E3). These observations support the hypothesis that metabolic dysfunctions associated with CARMIL2 deficiency are directly linked to disease pathogenesis.

Parallels can be drawn between CARMIL2-related immune dysfunction and glutamine deprivation observed in the tumor microenvironment. In both contexts, T-cell function is compromised owing to limited glutamine availability—whether secondary to intrinsic genetic defects or extrinsic nutrient competition. Similar to our findings in CARMIL2-mutated T cells, tumor cells have been shown to reprogram their environment to deplete glutamine, thereby suppressing T-cell effector function through mTOR and NF-κB signaling impairment.[45,47,56]

In summary, CARMIL2 deficiency disrupts T-cell metabolic reprogramming, impairing mTOR and NF-κB signaling, as well as cytokine expression. Our study focused on $CD4^+$ T cells, which mediate many of the main disease phenotypes, such as severe dermatitis, enteropathy, and immune dysregulation. However, further investigation of $CD8^+$ T cells would be beneficial given the persistent viral infections experienced by patients, as well as other immune cells involved in the disease. Despite some key limitations, including the small number of patients included in this study and technical limitations arising from the use of primary cells, our findings suggest that metabolic modulation may represent a potential therapeutic strategy for patients with IEIs.

DISCLOSURE STATEMENT

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Key Messages

- **CARMIL2 deficiency is associated with impaired immunity and reduced activity of NF-κB and mTOR signaling.**
- **Activation-induced reprogramming of cell metabolism is skewed in CARMIL2 mutated T cells and is partially restored by glutamine supplementation.**
- **Understanding how mutations that cause IEI interfere with activation-induced metabolic reprogramming in T cells may reveal new therapeutic targets.**

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