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ARTICLE

Immunodeficiency associated with a novel functionally defective variant of *SLC19A1* benefits from folinic acid treatment

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Insufficient dietary folate intake, hereditary malabsorption, or defects in folate transport may lead to combined immunodeficiency (CID). Although loss of function mutations in the major intestinal folate transporter *PCFT/SLC46A1* was shown to be associated with CID, the evidence for pathogenic variants of *RFC/SLC19A1* resulting in immunodeficiency was lacking. We report two cousins carrying a homozygous pathogenic variant c.1042 G > A, resulting in p.G348R substitution who showed symptoms of immunodeficiency associated with defects of folate transport. *SLC19A1* expression by peripheral blood mononuclear cells (PBMC) was quantified by real-time qPCR and immunostaining. T cell proliferation, methotrexate resistance, NK cell cytotoxicity, Treg cells and cytokine production by T cells were examined by flow cytometric assays. Patients were treated with and benefited from folinic acid. Studies revealed normal NK cell cytotoxicity, Treg cell counts, and naive-memory T cell percentages. Although *SLC19A1* mRNA and protein expression were unaltered, remarkably, mitogen induced-T cell proliferation was significantly reduced at suboptimal folic acid and supraoptimal folinic acid concentrations. In addition, patients' PBMCs were resistant to methotrexate-induced apoptosis supporting a functionally defective SLC19A1. This study presents the second pathogenic *SLC19A1* variant in the literature, providing the first experimental evidence that functionally defective variants of *SLC19A1* may present with symptoms of immunodeficiency.

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INTRODUCTION

Folates play a critical role in both DNA and RNA synthesis in cells [1–6]. Insufficient dietary intake of folates or intestinal absorption, or defective folate transport disrupt purine and pyrimidine nucleotide synthesis, especially for rapidly proliferating tissues such as hematopoietic and immune systems [7–9]. Folate derivatives are anionic in physiological pH and require predominantly active transporters to pass through cell membranes [10–14]. Mammalians have three important membrane folate and folate derivative transporters, proton-coupled folate transporter (PCFT, encoded by *SLC46A1*), reduced folate carrier (RFC, encoded by *SLC19A1*) and folate receptors (FR) α, β, γ (encoded by *FOLR1*, *2* and *3*) [10–15]. PCFT expression is restricted to the duodenum and jejunum, liver, thus enabling folates to be absorbed from the intestine [10–14]. Loss of function (LOF) mutations in the *SLC46A1* gene have been reported to decrease folate reabsorption from the intestinal lumen leading to a condition called hereditary folate malabsorption (HFM) [13, 16]. Hematological and neurological symptoms are frequently observed in HFM, and the patients suffer from combined immunodeficiency and are treated with folate supplementation [15–17]. Folate receptors are also expressed in a tissue-specific manner [11]. FRα is mostly expressed in the epithelia of the choroid plexus, proximal kidney tubules, uterus, placenta, and retina [11, 18–21]. FRβ expression was shown in the placenta as well as hematopoietic cells and tissues. FRγ on the other hand is a secretable receptor that can be shed from leukemic hematopoietic tissues [11]. Both FRα and β can bind folic acid and reduced folate products with high affinity at neutral pH and facilitate their transport via receptor-mediated endocytosis [11]. Defects in FRα were associated with renal and cerebral folate deficiency in humans [11, 21, 22]. Additionally, *Folr1*-deficient mice also have developmental problems and embryonic lethality [11, 23, 24].

RFC, encoded by the *SLC19A1* gene, has lower affinity for 5-methyl tetra hydrofolate (THF) and other reduced folate

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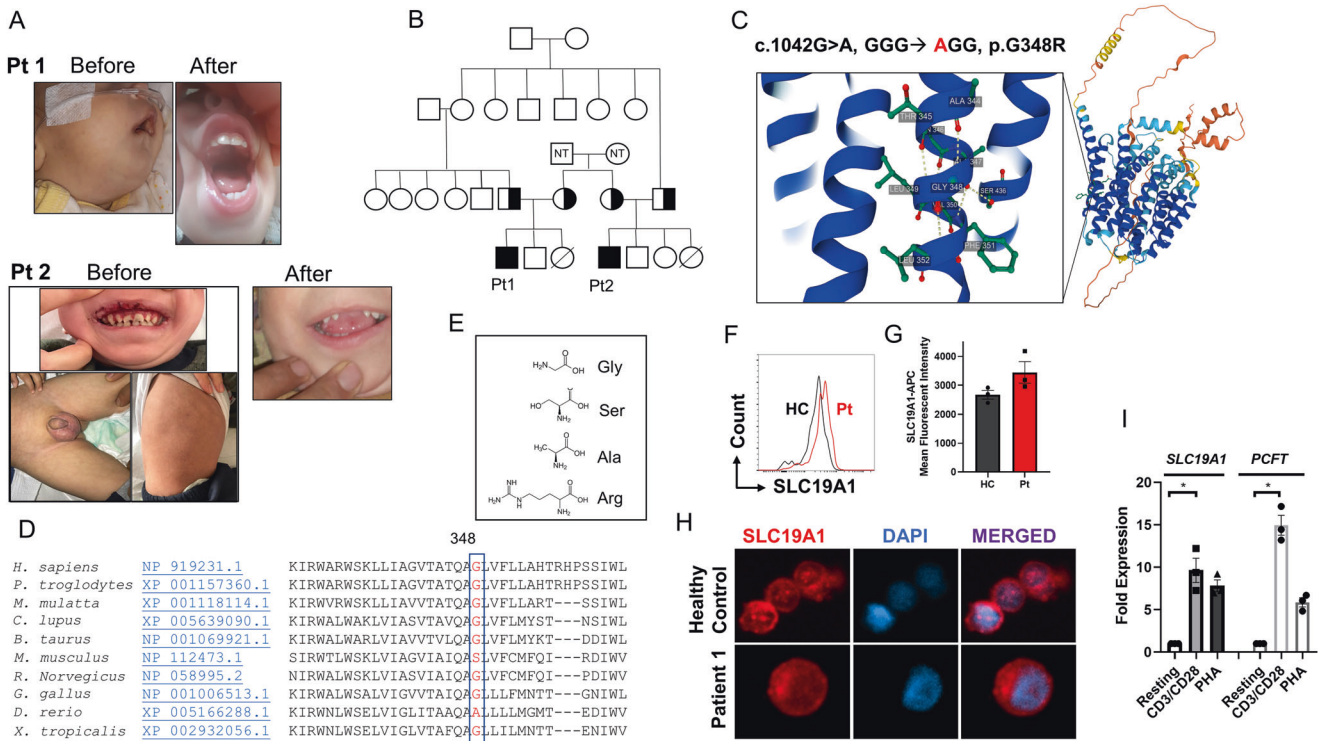
Fig. 1 G348R substitution does not alter SLC19A1 protein expression. A Pictures of patient 1 (Pt1) and Pt2 before and after folinic acid treatment. **B** Pedigree of the patients. **C** Structure of SLC19A1, position of G348R substitution (**D**) alignment of SLC19A proteins from different species shows the degree of conservation of G348, (**E**) and the structures of amino acids found in that position and arginine. **F** PHA-activated T cells after 7 days culture were stained with anti-SLCA19A1 after fixation and permeabilization and examined on FACSAria III. Histogram and (**G**) mean fluorescent intensity (MFI) graphs were shown. The staining was performed three times on different occasions. **H** Additionally, after SLC19A1 and DAPI staining, T cells of the healthy control and Pt 1 were also examined with confocal microscopy, 40x magnification, and a representative focus was shown. **i** The gene expression of *SLC19A1, PCFT* in bead-sorted CD4 + T cells was quantified by real-time qPCR in three conditions: resting, anti-CD3/CD28 (1 µg/mL each) or Phytohemagglutinin (PHA) (5 µg/mL) activated for 3 days (n = 3). *Indicates $P < 0.05$. The error bars show ± standard error of means (SEM). NT Not tested.

products (Km = 2–7 µM) and for folic acid (Km = ~200 µM) compared with FRs [11, 25–27]. However, RFC is ubiquitously expressed in the mammalian cells and tissues [10, 11]. Murine *SLC19A1* knockout models are embryonic lethal unless supplemented with folic acid at implantation [28, 29]. LOF mutations in *SLC19A1* disrupt the entry of folate derivatives as well as antifolates (methotrexate (MTX) etc.) into cells [11, 25–29]. In Leiden Open Variation Database (LOVD), ~57 unique variants of *SLC19A1* have been identified, almost all are polymorphisms and have been associated with drug resistance, neurological disorders, and cardiovascular diseases. In cancer treatment, antifolates such as MTX, which are structurally similar to folates, use the same membrane carriers (specifically RFC) [26, 27]. Polymorphisms in the *SLC19A1* are the most important cause of drug resistance or excessively increased toxicity in cancer treatment [26, 27].

To this date, only one case of LOF mutation in *SLC19A1* gene has been reported with severe megaloblastic anemia [30]. In addition, unlike the *SLC46A1* [31], that variant of *SLC19A1* was not investigated in detail with regard to features of immunodeficiency. In the current report, we describe the second novel pathogenic variant of *SLC19A1*, which had a G348R substitution and provide evidence that this novel variant leads to a reduced function and impairs T cell proliferation response in non-optimal folate conditions, and thus may lead to immunodeficiency that could be alleviated with folic/folinic acid supplementation.

METHODS
See detailed Supplemental Methods.

RESULTS
Case history of patient 1
The patient was born at the 40th week of pregnancy and was hospitalized for 15 days due to respiratory distress. The patient was treated with intravenous antibiotics for one week due to lower respiratory tract infection (LRTI), bleeding in the mouth, and mucositis (Fig. 1A). Cytopenia, high triglyceride, low fibrinogen, and high ferritin levels were found. The patient had a sibling who died due to an uncontrolled pneumonia infection at 3.5 months of age (Fig. 1B). The patient was referred to our hospital for metabolic disease and immunodeficiency. Physical examination revealed fever (38.2 °C), height <10p, weight <10p, head circumference <10p, bleeding aphthous ulcers in the mouth, 2*1 cm swelling in the right sternocleidomastoid muscle. The liver and spleen were palpable below the costal margin 2 and 3 cm, respectively. Laboratory revealed WBC: $4.48 \times 10^3/mm^3$, Hb: 8gr/dl, Plt: $37 \times 10^3/mm^3$, MCV: 86.2 fl, Absolute Neutrophil Count (ANC): $1.03 \times 10^3/mm3$, Absolute Lymphocyte Count (ALC): $3.13 \times 10^3/mm^3$. AST: 38 U/L, ALT: 21 U/L, Triglyceride: 194 mg/dl, fibrinogen: 135 mg/dl, ferritin: 849 ng/ml, vitamin B12: 1095 pg/ml, folate: 11.4 ng/ml. The patient's immunological tests revealed slightly lower (B cells and immunoglobulins, borderline CD3 + T cells) than the normal range (Table 1). Hemophagocytic lymphohistiocytosis was considered. Megaloblastic changes were observed in the bone marrow aspiration smear. Ceftriaxone (80 mg/kg/day) was started and 1 gr/kg/day IVIG was given for 2 days. Whole-exome sequencing (WES) was performed to assess the underlying genetic defect. CMV PCR test resulted in 2349 copies/ml, ganciclovir treatment was initiated. CMV PCR was negative at the 2nd week of treatment. IgG level was as low as

Table 1. Summary of the clinical and laboratory results of patient 1.

Period (age)	CBC						Lymphocyte subgroups					Immunoglobulins (Ig)		
	WBC X10³/mm³	Hb gr/dl	Plt X10³/mm³	MCV fl	ANC X10³/mm³	ALC /mm³	CD3 % (#)	CD4 % (#)	CD8 % (#)	CD 16 + 56 % (#)	CD19 % (#)	IgA (mg/dl)	IgM (mg/dl)	IgG (mg/dl)
TI^{lo}-TI^{up} (40 d-6 mo)						2416–9694	50.4-79.6 (1492–6385)	31.6-57.9 (909–4523)	10.7-28.2 (254–2123)	1.8-27.4 (101-1633)	10.2-36 (237-2564)	13-72	33-154	294-1165
(40 days, at diagnosis)	4.48	8	37	86.2	1.03	3130	77.8 (2435)	57.4 (1796)	20.3 (635)	10.6 (331)	9.2 (287)	40	55	1053 (IVIG)
(2 months)	6.92	7.2	27	95.7	4.5	1900	79.9 (1518)	53.7 (1020)	24 (456)	8.7 (165)	11.2 (212)	16	33	218
2nd month of treatment	16.22	12.1	411	77	3.39	11000	45.3 (4983)	23.3 (2563)	19.4 (2134)	31.2 (3432)	21.9 (2409)	27.4	64	574
TI^{lo}-TI^{up} (1–2 y)						1829-10242	51-81.8 (1338-6611)	27.6-55.6 (820-4138)	12.7-30.9 (540-2812)	2-26.3 (101-1741)	11-34.2 (516-3083)	30-107	66-228	605-1430
8th month of treatment	16.86	13.7	457	74	2.95	12300	58.5 (7195)	31.7 (3899)	22.8 (2804)	22.9 (2.816)	18.4 (2.263)	34.4	78.2	735
10st month of treatment	10.75	12	405	71.7	3.84	5510	59.4 (3272)	36.9 (2033)	19 (1046)	10.2 (562)	30.4 (1675)	29.4	35.4	685
12th month of treatment	16.53	12.4	272	73.6	6.02	8570	61.2 (5220)	29.9 (2570)	26.1 (2.228)	12.9 (1114)	25.8 (2211)	44.3	86.1	1004
18th month of treatment	12.29	12.8	360	73.5	3.87	6830	61.4 (4190)	30.6 (2089)	27.4 (1870)	12.9 (881)	25.4 (1734)	38	46.6	798
24th month of treatment	16.5	12.5	440	75.6	6.78	7670	59.9 (4600)	30.5 (2300)	22.5 (1725)	17.6 (1340)	22.4 (1718)	36.7	71.1	902

Lower and upper tolerance interval limits were taken from the article of Besci et al. [42].
ALC absolute lymphocyte count, *ANC* absolute neutrophil count, *CBC* complete blood count, *WBC* white blood cell, *Hb* hemoglobin, *Plt* platelet, *MCV* mean corpuscular volume, *TI^{lo}-TI^{up}* lower and upper tolerance interval limits, *d* day, *mo* month, *Ig* immunoglobulins.

Table 2. Summary of the clinical and laboratory results of patient 2.

Period (age)	CBC						Lymphocyte subgroups					Immunoglobulins (Ig)		
	WBC X10^3/mm^3	Hb gr/dl	Plt X10^3/mm^3	MCV fl	ANC X10^3/mm^3	ALC /mm^3	CD3 % (#)	CD4 % (#)	CD8 % (#)	CD 16+56 % (#)	CD19 % (#)	IgA (mg/dl)	IgM (mg/dl)	IgG (mg/dl)
Tl^{lo}–Tl^{up} (2–5 y)						1703–6738	57.6–81.2 (1200–4706)	23.6–52.5 (458–2755)	12.1–35.7 (165–1878)	3.5–22.2 (205–1341)	8.4–28.5 (88–1393)	26–296	71–235	604–1941
(2 y, at diagnosis)	12.84	10.6	51	86.2	6.35	4940	58.5 (2880)	28.9 (1427)	26.6 (1314)	12.1 (597)	29.2 (1442)	112	112	1362
2nd month of treatment	17.39	11.8	296	85.3	6.66	7210	63.5 (4578)	27.8 (2004)	30.4 (2191)	17.8 (1283)	18.8 (1355)	160	139	1600
6th month of treatment	10.89	12	360	72	3.67	5870	59.3 (3480)	29.1 (1702)	29.9 (1755)	13.2 (774)	27.1 (1590)	103	80.9	1169
12th month of treatment	15.26	13	366	72.9	6.56	5930	55.8 (3308)	23.6 (1399)	27.5 (1630)	16.0 (948)	28.1 (1666)	96.2	99.4	1072

Lower and upper tolerance interval limits were taken from the article of Besci et al. [42].

ALC absolute lymphocyte count, ANC absolute neutrophil count, CBC complete blood count, WBC white blood cell, Hb hemoglobin, Plt platelet, MCV mean corpuscular volume, Tl^{lo}–Tl^{up} lower and upper tolerance interval limits, y year, Ig immunoglobulins.

200 mg/dl before the IVIG. In WES, a homozygous variant in the *SLC19A1* gene (c.1042 G > A p.G348R), and a homozygous variant (c.*353dupAA) in *ELK4* gene were reported. The patient's clinical and laboratory results were consistent with the defect in the *SLC19A1*. The patient's symptoms ameliorated, and hematological and immunological tests normalized in the 2nd month of the folinic acid supplementation (Table 1). In the 2nd year of the treatment, neuromotor development is normal, and mouth sores and severe infections do not occur anymore (Fig. 1A). Upon his successful treatment, patient 1's cousin (Patient 2) sought treatment presenting with neurological and immunological complaints. The detailed history of patient 2 is given in Supplemental Methods. The sores of patient 2 are shown in (Fig. 1A), and hematological and immunological tests are given in (Table 2), whose WES results returned the same mutation in *SLC19A1*. Both patients had the homozygous *SLC19A1* variant (c.1042 G > A p.G348R), which was confirmed by Sanger sequencing (Fig. S1a).

G348R substitution does not alter SLC19A1 protein expression or stability

SLC19A1 is made up of 12 alpha-helices which are transmembrane domains [32]. The missense mutation (G348R) resides in 9th alpha helix (Fig. 1C). In the predicted model (UniProtKB- P41440), glycine in that position interacts with leucine 352, alanine 344 through hydrogen bonds (Fig. 1C). G348 has been conserved in several species from bats, humans to frogs (Fig. 1D) and has been replaced with alanine in *Danio rerio*, and serine in *B. taurus*. Compared with alanine and serine, which are similar in structure to glycine, arginine is bulkier, with more potential to impact the structure and/or function of SLC19A1 in the G348R variant (Fig. 1E). Antibody staining of SLC19A1 (which recognizes an epitope between 407 and 591 residues) has revealed that the protein is produced within the cells at comparable levels (Fig. 1F and G). The microscopical examination also revealed that mutant SLC19A1 can correctly shuttle to the plasma membrane (Fig. 1h). Importantly, when T cells are activated with mitogens such as anti-CD3/CD28 or PHA, mRNA expression of *SLC19A1* and *PCFT* increased up to 10-fold in accord with the increased need for DNA synthesis (Fig. 1I). However, this increase in folate transporters and receptors was not unique to *SLC19A1*, and was observed also for *PCFT*, *FR1,2* and *3* mRNA as well as the dihydrofolate reductase (*DHFR*) (Fig. S1b-c). These results collectively suggest that the protein expression and stability are unaffected by the G348R substitution and that proliferating T cells upregulate *SLC19A1* expression.

SCL19A1 mutant T cells have proliferation defects in non-optimal folic/folinic acid concentrations and are resistant to MTX-induced cell death

To assess whether G48R substitution leads to a functional defect, lymphocyte proliferation assays were performed with different T cell mitogens. When the T cells were cultured in the complete medium containing 1 µg/mL RPMI 1640 medium, no detectable differences in proliferation between healthy controls and the two patient's lymphocytes were observed in response to activation with CD3 + CD28, CD3 + CD28 + IL-2, PHA (Figs. 2A, S2a). On the other hand, when the medium was diluted 1:1 with PBS which halved the folic acid concentration, (without reducing L-glutamine, antibiotics, fetal bovine serum amounts by adding them back) T cell proliferation was dramatically reduced, suggesting that the functional impact of the mutation may become visible in limiting folic acid conditions (Figs. 2B, S2b). Additionally, when folinic acid was added into the regular medium which increased its levels to 10 µg/mL, T cells from healthy controls proliferated more robustly compared with patient-derived T cells (Fig. 2C), collectively suggesting that the functional consequences of the mutation are observed under suboptimal and supraoptimal concentrations of folic/folinic acid. Although high level of folic acid recently has been shown to inhibit the proliferation of a cell
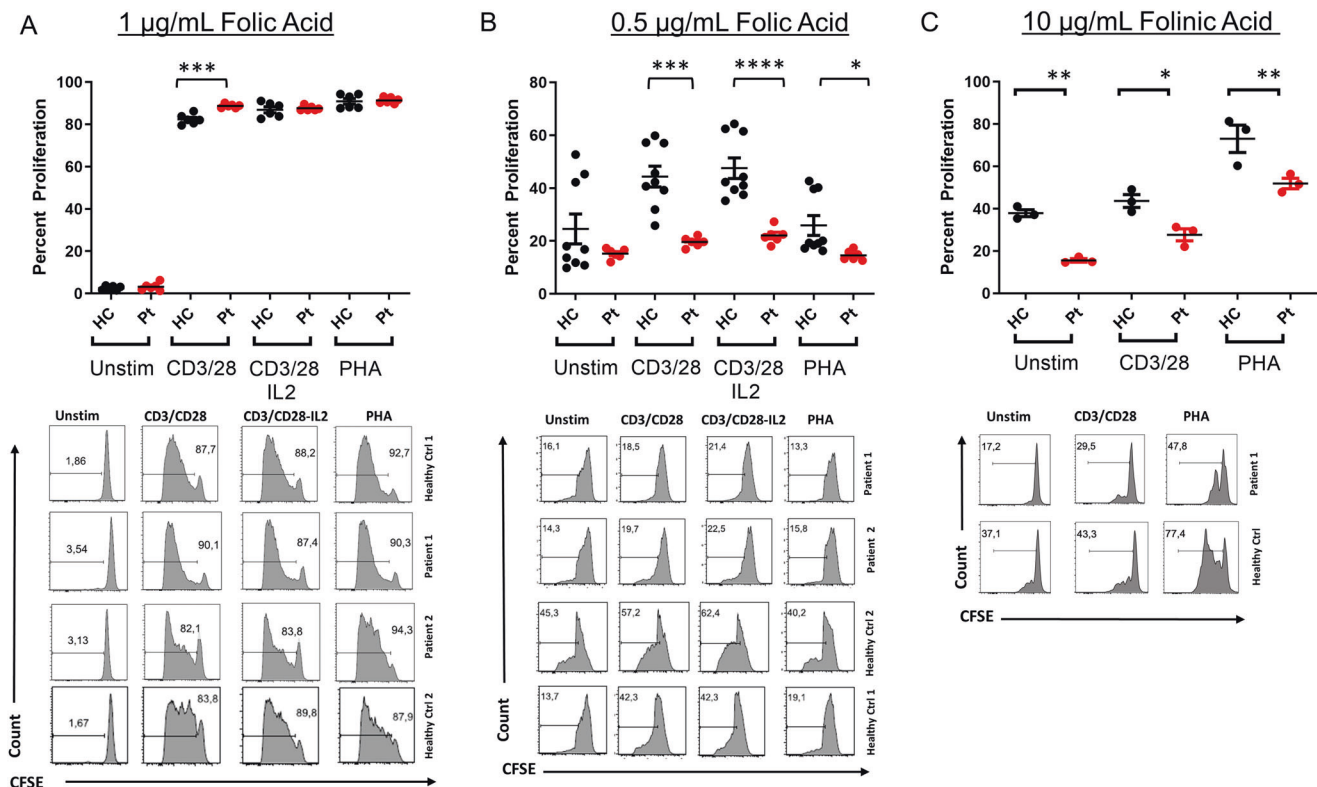
Fig. 2 G348R substitution in SLC19A1 reduces T cell proliferation in nonoptimal folate concentrations. A PBMC's from Pt1 and 2 were labeled with CFSE and activated with CD3/CD28, CD3/CD28/IL-2 or PHA for 4 days in complete medium, cell proliferation was quantified in the top panel, a representative plot for each condition was shown in the lower panel ($n = 3$ for Healthy Controls (run in duplicates/donor), $n = 2$ for patients (run in triplicates/patient)). **B** The same experiment was repeated in culture medium diluted twofold with PBS to reduce folic acid by half. FBS, L-glutamine, and antibiotics were kept constant by adding back. The cell proliferation was quantified in the top panel, a representative plot for each condition was shown in the lower panel, ($n = 3$ for Healthy Controls (run in triplicates/donor), $n = 2$ for patients (run in triplicates/patient)). **C** PBMC's from Pt1 were labeled with CFSE and activated with CD3/CD28, or PHA for 4 days in complete medium supplemented with folinic acid (10 μg/mL final concentration), the cell proliferation was quantified in the top panel, a representative plot for each condition was shown in the lower panel, ($n = 3$ healthy controls, $n = 3$ technical replicates for Pt1 (run in triplicates)). *Indicates $P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$. The error bars show ± SEM.

line [33], others have shown increased survival and functions [34], therefore our data show that supraphysiological levels of folinic acid do not appear to be toxic or inhibitory for primary T cells.

MTX is an antifolate and blocks DHFR enzyme and is transported across cell membrane by SLC19A1 [12, 13, 26]. MTX reduces the activation of the immune system in autoimmune diseases and ceases cell proliferation in cancer and leads to apoptosis. Thus, to assess the functional defects in SLC19A1 with G348R substitution in a different assay, we further compared the import of MTX to cells by SLC19A1 indirectly by measuring the MTX-induced apoptosis (Fig. 3A and B). Indeed, when the two patient's cells were exposed to increasing doses of MTX, compared with healthy controls, they were more resistant to apoptosis, suggesting an impaired transporter activity due to G348R substitution (Fig. 3A and B). Collectively, these data support that the G348R substitution impairs the function of SLC19A1.

Normal Treg number, NK cell cytotoxicity but altered cytokine production by T cells in SLC19A1 deficiency
Because the patient was initially suspected of HLH, and a heterozygous mutation in RAB27A was identified in patient 1, we compared the cytotoxic ability of patient-derived (Pt1 and Pt2) PBMCs to healthy controls to determine the cytotoxic potential of NK cells [35]. Comparable cytotoxicity of patients and healthy donors suggests normal NK cell function, and that the phenotype of both patients may be related to the mutation in SLC19A1 (Fig. 4A).

T and B cells are particularly sensitive to folate deficiency [7, 12, 36]. To the best of our knowledge, the number of Treg cells has not yet been studied in patients with SLC19A1 defects. We found that the FOXP3 + Treg cell frequency among CD4 + T cells was comparable between SLC19A1-deficient patients and the age-matched controls (Fig. 4B). As shown in Fig. S3a, there was also no significant difference in the naive/memory ratio of CD4 + or CD4-CD3 + T cells between the patient's pre/post-treatment samples and age-matched controls. Examination of T helper-associated cytokines production by freshly isolated PBMCs after stimulation with PMA/Ionomycin in normal folic acid concentrations (1 μg/mL) revealed that both patient 1 and patient 2 had a significantly increased IL-10 and GM-CSF percent and number compared to control (Figs. 4C top, S3b). In addition, no significant differences in IL-17A, IL-22, and IFN- γ production between the patients and controls were observed (Fig. 4C bottom). More recently, two independent studies demonstrated that SLC19A1 was critical in the transport of cyclic dinucleotides into the cytosol in vitro and that clustered regularly interspaced short palindromic repeats (CRISPR)-generated knockouts of SLC19A1 led to a reduced activation of cyclic GMP–AMP synthase (cGAS)–stimulator of interferon genes (STING) pathway, ultimately leading to impaired production of antiviral interferons [37, 38]. To test whether patient-derived T cells had a bias towards the production of certain cytokines, we cultured microbead-selected CD4+ T cells in reduced folic acid conditions (0.5 μg/mL) in the presence of soluble anti-CD3/28 (1 μg/mL) for 4 days. Real-time qPCR analyses

A
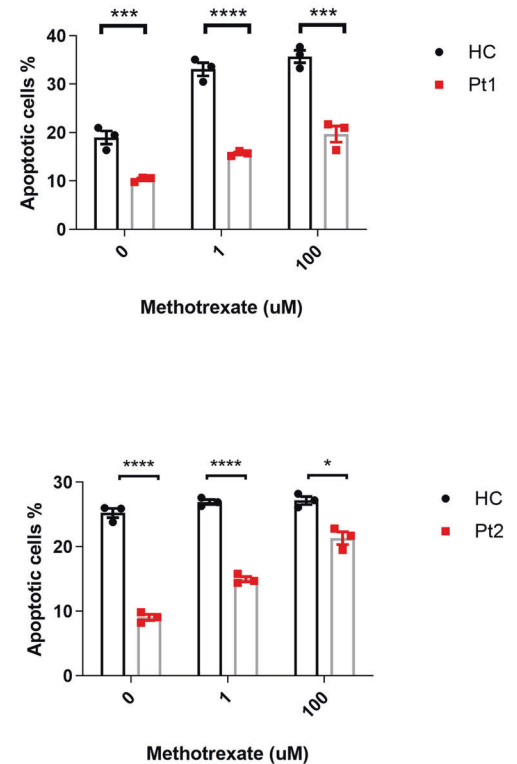
Methotrexate µM: 0   1   100
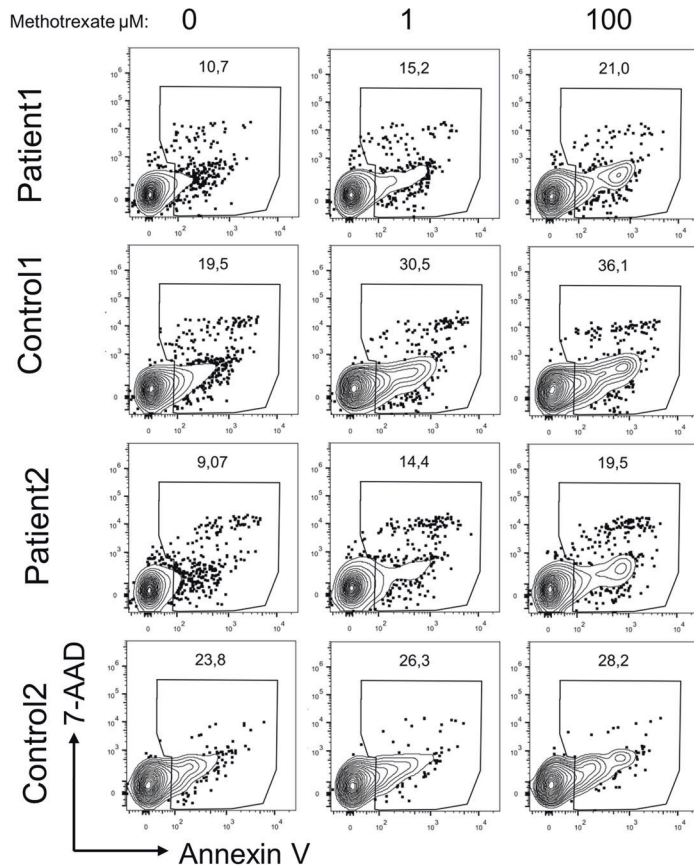
B

Fig. 3 G348R substitution in SLC19A1 leads to methotrexate resistance. A PBMCs from Pt1 and Pt2 were cultured for 24 hours with Methotrexate (0 µM, 1 µM, 100 µM) at 37 °C. Then the cells were stained with Annexin V-FITC and 7-AAD, a representative plot for each condition was shown, and apoptosis was quantified in (**B**), ($n = 3$ for Healthy Controls, $n = 3$ technical replicates for Pt1 or Pt2 separately (patients run in triplicates)). *Indicates $P < 0.05$, ***$P < 0.001$, ****$P < 0.0001$. The error bars show ± SEM.

of several cytokines revealed reduced *IFNA, IFNG, TNFA*, and *IL6* gene expression by CD4 + T cells of the patients compared with healthy controls (Fig. 4D). These data collectively suggest that helper T cell cytokine production may be skewed to the production of GM-CSF and that antiviral immunity may be compromised due to reduced function of SLC19A1.

DISCUSSION
Folate deficiency either due to insufficient dietary intake, or SLC46A1 deficiency (HFM) results in reduced systemic folate levels and leads to combined immunodeficiency which is classified under Table IIIb of IUIS 2019 classification [1, 3, 10, 15, 31]. Hematologic defects in SLC46A1 deficiency are mostly reversible when folate levels are restored by parenteral route [16]. On the other hand, a reduction in serum folate levels in SLC19A1 deficiency is not expected unless dietary intake is limited. Indeed, in Pt1 and Pt2, folate levels were normal at the time of diagnosis. Despite normal intestinal absorption of folates, *SLC19A1* deficiency has potential to affect the generation and function of immune cells because cellular uptake will be impaired. Indeed, in the murine model, SLC19A1 deficiency is embryonic lethal, and only partially could be rescued by folate supplementation of the pregnant mice, even so hematopoiesis could not be rescued due to impaired cellular uptake [28, 29]. The clinical history of both of our patients corroborates an immunodeficiency: LRTI and mucositis at the 1st week of birth, cytopenia (low IgG, reduced lymphocyte counts, borderline CD3 + T cells, reduced B cells),

death of a sibling at 3.5 months due to infection, sores in the mouth in the Pt1 (40 days old at diagnosis); recurrent sores in the mouth, skin, and diaper area since 1 month old, rifts on the lip and tooth abnormality, erosive dermatitis in the scrotal area, growth and mental retardation, also a sibling death at 2 years of age (unknown cause) in the Pt2 (2 years old at diagnosis). The observation that the immunological symptoms of Pt1 (Table 1), sores, and general health of both patients greatly benefited from the folinic acid prescription supports that G348R substitution leads to reduced function. Additionally, the neurological symptoms of Pt2, which are consistent with folic acid transport defect, provide additional support for the pathogenicity of this newly described *SLC19A1* variant and underline the importance of folinic acid supplementation for the younger patient, Pt1.

The experimental evidence for the pathogenicity of the G348R substitution variant of SLC19A1 was provided by two different assays. Remarkably, mitogen-induced T cell proliferation experiments revealed a significant reduction in T cell proliferation only when the folic/folinic acid levels were reduced or very high, but not in normal media conditions. This observation suggests that the patients will be vulnerable to infection when folate intake is insufficient. This variant may present a problem during infections, especially when B and T cells need to undergo clonal expansion, and their folate demands are high. Indeed, our data show that during proliferation T cells increase SLC19A1 levels dramatically to accommodate that demand. It is also noteworthy that the increase in the expression of folate transporter and receptors is not unique to *SLC19A1* but also applies to *PCFT, FR1,2* and *3* mRNA as well as
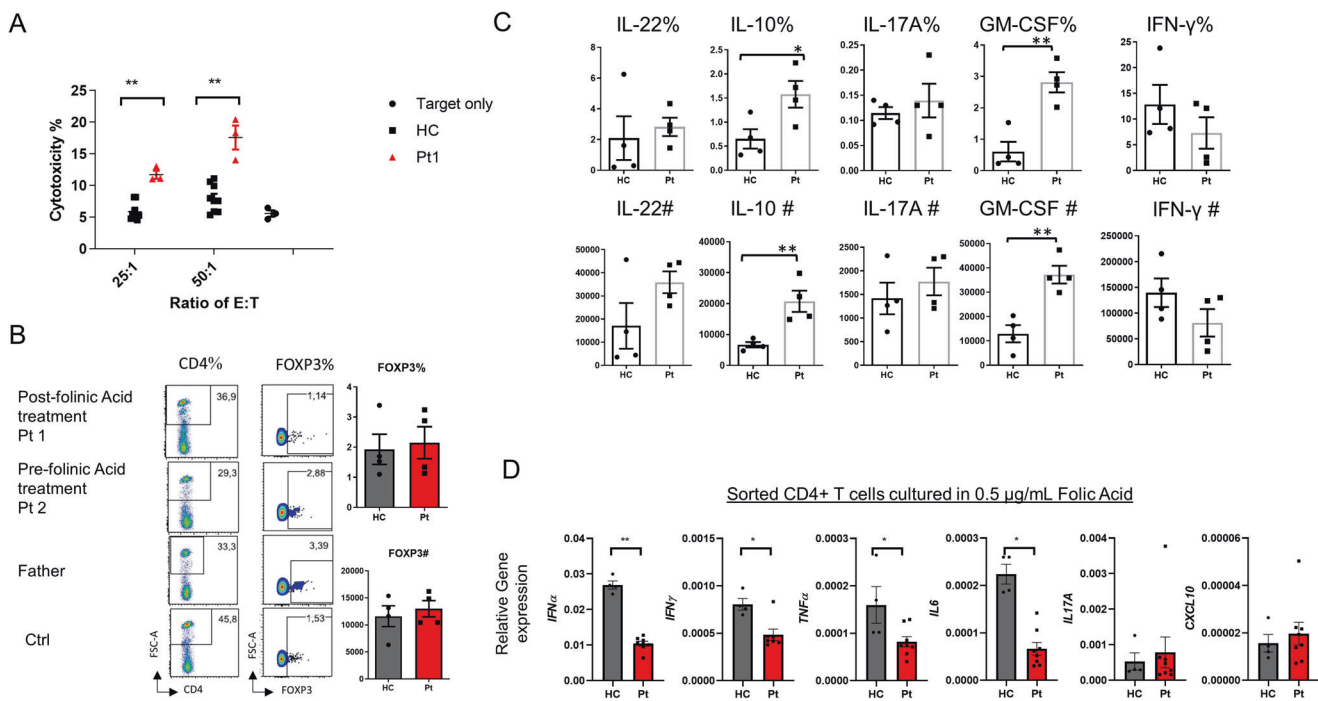
Fig. 4 Normal NK cell cytotoxicity, normal Treg cell numbers, but increased GM-CSF and IL-17A producing T cells in both patients. **A** PBMCs from Pt1 ($n = 3$ technical replicates), and healthy controls, ($n = 3$) donors (triplicates/donor), were cocultured with Tag-it-violet labeled K562 cells (1:25, 1:50 effector: target ratios) for 4 h and apoptosis of target cells was quantified. **B** CD4 + FOXP3 + Treg cells were quantified among PBMCs obtained from Pt1, Pt2, the father, and healthy controls. Pt1's sample was obtained after folinic acid treatment. ($n = 2$ patients, two technical replicates/patient), and ($n = 2$) healthy donors (two technical replicates/donor). **C** PBMCs from Pt1, Pt2 (before folinic acid treatment) and healthy controls were stimulated with PMA/ionomycin and Golgi Plug for 4 h and cytokines IL-22, IL-10, IL-17A, GM-CSF and IFN-γ production by TCRαβ + T cells (percentage and absolute numbers) were quantified. ($n = 2$ patients, three technical replicates/patient), and ($n = 2$) healthy donors (two technical replicates/donor). **D** CD4 + T cells were selected with microbeads from PBMCs isolated from both patients and healthy control and expanded in complete media with PHA for 5 days. Then, rested for 2 days in the presence of IL-2, IL-15, and IL-7 (20 ng/mL each) for 2 days. Then T cells were transferred to the reduced folic acid medium (0.5 µg/mL) and cultured in the presence of soluble anti-CD3/28(1 µg/mL) and the cytokines at same concentrations for 4 days. Real-time qPCR was performed with cells for *IFNA, IFNG, TNFA, IL6, IL17A, CXCL10*. ($n = 2$) patients, four technical replicates/patient), and ($n = 2$) healthy donors (two technical replicates/donor). *Indicates $P < 0.05$, **$P < 0.01$. The error bars show ± SEM.

the DHFR. Such increase, in other transporters and receptors, does not appear to be compensating for the defect. Given that basal T and B cell numbers in Pt2 are within the reference range, frequent infections in those patients may be due to defective proliferation of T and B cells because of impaired cellular transport at limited/or normal folate concentrations in the environment. Folate analogs such as MTX are also transported by SLC19A1 [1, 10, 11, 30, 37, 38]. Loss of *SLC19A1* function mutations creates MTX-resistant cancer cells [26, 27, 30, 39, 40]. Svaton *et al.* showed that gene-edited K562 cells carrying the c.634_636delTTC (p.Phe212del) mutation in *SLC19A1*, also became resistant to MTX [30]. The T cells of both patients in this study also demonstrated resistance to MTX-induced apoptosis further supporting that G348R leads to reduced transporter function.

While there are studies showing a decrease in T cell proliferation in folate deficiency, no detailed research has been conducted on CD4 + T cell subsets [7, 36, 41]. In the current study, we evaluated the CD4 + FOXP3 + Treg cell frequency of SLC19A1 deficient patients for the first time. Treg levels were normal in patients compared with healthy controls, although further functional studies are necessary to address potential functional defects, and if any, whether they are reversible. Analyses of cytokine profiles of T cells showed elevated GM-CSF + , IL-10 + T cell percentage, and increased absolute numbers of GM-CSF + , IL-10+ collectively pointing to a bias towards the production of GM-CSF in two patients. Folate deficient diet in mice was shown to reduce Th1-derived IFN-γ production by CD4 + T cells [41]. GM-CSF levels were not assessed in that study. It is yet unclear, how

in vivo folate-deficient diet impacts type 3 immunity and particularly, Th17 cells, and IL-17A/F, IL-22, and GM-CSF cytokines. Further studies with murine conditional knockouts of *SLC19A1* and SLC46A1 will shed more light on the nature of inflammation observed in oral mucosa and skin of SLC19A1 deficient patients. The final information revealed by our study is the reduced mRNA expression of *IFNA, IFNG, TNFA*, and *IL6* by *SLC19A1*-deficient CD4 + T cells in culture, in the reduced folic acid environment. These data corroborate earlier findings that SLC19A1 may be important for antiviral immunity by transporting ligands of the cGAS-STING pathway and that these patients may have further disadvantages against viral infections due to curbed type I interferon response [37, 38].

Our study has also limitations. Although the neurological symptoms of the patients, MTX resistance of patients-derived PBMCs, and reduced proliferative response in non-optimal folic/folinic acid concentrations are convincing findings arguing for a reduced SLC19A1 function, however, they do not prove that the variant is a complete LOF, which requires further studies involving the expression of the variant in other cell lines. Given that the complete RFC deficiency in mice is embryonic lethal, the variant described in our current study appears to have reduced function rather than to be a complete LOF. Measurement of cellular levels of total folate, folic acid, and reduced folates will also provide invaluable information as to the pathogenicity of this variant in the future.

In summary, in the current study, we identified a novel pathogenic mutation (c.1042 G > A, p.G348R) in the *SLC19A1* gene in two related

children and provide the first experimental evidence that a reduced function variant of *SLC19A1* may present with symptoms of immunodeficiency, and that immunological defect of those patients could benefit from folinic acid supplementation. Additionally, our data also suggest that genotyping patients suffering from hematological and neurological symptoms due to HFM for his novel RFC variant or other RFC variants might prove important.

DATA AVAILABILITY

All the raw data are available upon reasonable request from the corresponding authors.

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AUTHOR CONTRIBUTIONS

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COMPETING INTERESTS
The authors declare no competing interests.

ETHICS APPROVAL
All experimental procedures were approved by Erciyes University institutional review board (#2021/17) and conducted according to regulations. Parents of the patients signed the informed consents for the immunological study and provided consent for the publication of data. Controls were selected randomly from amongst the age and sex-matched healthy donors.

CONSENT TO PARTICIPATE
Written informed consent was obtained from the parents.

CONSENT TO PUBLISH
The authors affirm that human research participants provided informed consent for publication of the images in (Fig. 1).

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