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Exogenous interleukin-2 can rescue *in-vitro* T cell activation and proliferation in patients with a novel capping protein regulator and myosin 1 linker 2 mutation

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Summary

Capping protein regulator and myosin 1 linker 2 (CARMIL2) deficiency is characterized by impaired T cell activation, which is attributed to defective CD28-mediated co-signaling. Herein, we aimed to analyze the effect of exogenous interleukin (IL)-2 on *in-vitro* T cell activation and proliferation in a family with CARMIL2 deficiency. This study included four children (one male and three females; aged 2·5–10 years at presentation). The patients presented with inflammatory bowel disease and recurrent viral infections. Genetic analysis revealed a novel homozygous 25-base pairs deletion in *CARMIL2*. Immunoblotting demonstrated the absence of CARMIL2 protein in all four patients and confirmed the diagnosis of CARMIL2 deficiency. T cells were activated *in-vitro* with the addition of IL-2 in different concentrations. CD25 and interferon (IFN)-γ levels were measured after 48 h and 5 days of activation. CD25 surface expression on activated $CD8^+$ and $CD4^+$ T cells was significantly diminished in all patients compared to healthy controls. Additionally, $CD8^+$ T cells from all patients demonstrated significantly reduced IFN-γ production. When cells derived from CARMIL2-deficient patients were treated with IL-2, CD25 and IFN-γ production increased in a dose-dependent manner. T cell proliferation, as measured by Cell Trace Violet, was impaired in one patient and it was also rescued with IL-2. In conclusion, we found that IL-2 rescued T cell activation and proliferation in CARMIL2-deficient patients. Thus, IL-2 should be further studied as a potential therapeutic modality for these patients.

Keywords: activation, CARMIL2, primary immune deficiency, proliferation, T cell rescue

Introduction

Capping protein regulator and myosin 1 linker 2 (CARMIL2) is a cell membrane cytoskeleton-associated protein with known roles in T cell migration, polarity, protrusion formation and actin polymerization regulation [1,2]. It binds to actin capping protein (CP) and diminishes its activity via CP- and CARMIL-specific interaction motifs [3]. CARMIL2 also plays a critical role in CD28-mediated co-stimulation and activation of T cells [3,4]. When the proline-rich domain in CARMIL2 binds to the growth factor receptor-bound protein 2 adaptor, the CARMIL2 protein forms a bridge between CD28 and caspase recruitment domain family member (CARD)11 which, in turn, induces intracellular signaling by nuclear factor kappa-light-chain-enhancer B (NF-κB) [3,5].

The fundamental value of CARMIL2 for immune defense was also recently proven as patients with bi-allelic CARMIL2 mutations were diagnosed. Although rare, reports of affected patients have recently been accumulating [1,4,6-12]. CARMIL2 deficiency is characterized by the reduced regulatory T cell (T_{reg}) counts and impaired T cell activation, which is attributed to a defect in CD28-mediated co-stimulation [4,7-9]. This attribution was confirmed in murine models with a mutated CARMIL2 protein [13]. Hence, patients with CARMIL2 deficiencies manifest with immune dysregulation and increased rates of infections, including specific susceptibility to Epstein–Barr virus (EBV) infection.

Interleukin (IL)-2 is a pleiotropic cytokine with an important role in modulating the activity of cytotoxic T lymphocytes (CTL), natural killer (NK) cells and T_{regs}. IL-2 has a dual mechanism of action: it can induce either immune suppression or immune stimulation, depending on the target cell [14]. IL-2 was explored as an add-on treatment in cancer immunotherapy for the purpose of potentiating tumor-reactive $CD8^+$ T cells [15] .It was shown that IL-2 had potentiation activity in various malignancies, in addition to its supportive effect in chimeric antigen receptor-modified T cell therapy [16].

Co-stimulation through CD28 is critical for up-regulating IL-2 production; thus, CD28 serves as a survival factor for T cells [17]. IL-2 signaling alone cannot substitute for CD28 co-stimulation; thus, it cannot rescue the transcriptional phenotype of cells stimulated via T cell receptor (TCR) signaling [18]. However, it was previously shown that adding IL-2 could completely restore the generation and function of mature effector CTLs in mice that lacked B7 molecules or CD28 [19]. Those studies suggested that at least part of CD28 co-stimulation-mediated T cell expansion was due to IL-2-dependent regulation of cell cycle progression [17]. Indeed, in one study, IL-2 was shown to rescue NK and CTL degranulation in patients with CARMIL2 deficiency [4].

Herein, we describe four patients with a novel mutation causing CARMIL2 deficiency. We aimed to evaluate whether impairments in T cell activation and proliferation in these patients can be rescued by IL-2 treatment.

Materials and methods

Patients

Patients were treated at the pediatric primary immune deficiency (PID) clinic, Shaare Zedek Medical Center (Jerusalem, Israel) during 2016–19. All patients were diagnosed based on genetic analyses. We accessed computerized medical records to acquire data, including disease presentation, course, outcome, laboratory test results and preliminary immunological findings.

Immunological findings

T cell maturation based on immune phenotyping with flow cytometry. We isolated peripheral blood mononuclear cells (PBMCs) from patients with Lymphoprep™ purification, according to the manufacturer's protocol [20]. PBMCs were surface-labeled with the following antibodies (all purchased from BioLegend, San Diego, CA, USA): anti-CD45RA (clone: H1100), anti-CD45RO (UCHL1), anti-CD127 (A019D5), anti-CD56 (HCD56), anti-CD62L (DREG-56), anti-CD4 (OKT4), anti-CD3 (SK7), anti-CD8 (SK1), anti-CD28 (CD28.2) and anti-CD19 (HIB19). The cells were then washed with fluorescence-activated cell sorting buffer, which contained phosphate-buffered saline (PBS) supplemented with 2% bovine serum albumin (BSA) and 2 mM ethylenediamine tetraacetic acid (EDTA). Samples were subjected to flow cytometry analysis to estimate the ratios of the different subpopulations of lymphocytes and their maturation states.

Counting regulatory T cells. T_{regs} [$CD4^+CD25^+$forkhead box protein 3 ($FoxP3^+$)] were measured with a standard BioLegend kit. PBMCs purified with Lymphoprep™ were surface-labeled with anti-CD4 (BioLegend; OKT4) and anti-CD25 (BioLegend; BC96) antibodies. Next, PBMCs were fixed, permeabilized, labeled with an intranuclear anti-FoxP3 antibody (eBioscience; PCH101), then counted with flow cytometry.

Testing T cell proliferation capacity, survival and effector functions. PBMCs purified with Lymphoprep™ were labeled with Cell Trace Violet (Invitrogen, Carlsbad, CA, USA). Labeled cells were activated in 96-well (flat-shaped) plates pre-coated with anti-CD3 (BioLegend; OKT3; 3 µl/0·5 ml) and anti-CD28 (BioLegend; CD28.2; 3 µl/0·5 ml) antibodies in 50 µl of 0·1 M borate buffer (pH = 8·5) for 24 h at 4°C. Recombinant human IL-2 was added to the medium at different doses (0, 80 and 400 U/ml).

To assess T cell effector functions, CD25 (BioLegend antibody: BC96) and intracellular interferon (IFN)-γ; BD antibody: 4S.B3) expression levels were measured with flow cytometry at 48 h post-activation. Prior to flow cytometry reading, IFN-γ staining was conducted using a BioLegend standard kit (BioLegend).

T cell proliferation capacity was evaluated 5 days post-activation by analyzing the Cell Trace Violet fluorescence intensity with flow cytometry. Furthermore, patients' PBMCs were stimulated with phytohemagglutinin (6 and 25 μg/ml) and anti-CD3 antibodies. Thereafter, T cell DNA replication was evaluated with a [^{3}H]-thymidine incorporation assay, as previously described [21].

Immunoblot analysis. Proteins were separated by lysing effector T cells with radioimmunoprecipitation assay (RIPA) buffer containing a protease inhibitor (APExBIO; catalog number: K1007). Samples were then subjected to sonication (2 min, 70% amplitude) and blotted into the membrane. Thereafter, non-specific binding sites were blocked with 5% skimmed milk (diluted in Tris-buffered saline buffer with Tween 20). Anti-CARMIL2 (Abcam, Cambridge, MA, USA; ab122717) antibodies were then added to detect CARMIL2 protein, and anti-heat shock protein (HSP)90 (Cell Signaling; 4874S) antibodies were added to detect the loading control.

T cell receptor repertoire study and TREC analysis. T cell receptors (TCR) and the TCR excision circles (TREC) were analyzed in the PID laboratory of Sheba Medical Center, Ramat-Gan, Israel, as previously detailed [21].

Genetics-based diagnosis

For whole-exome sequencing (WES), we extracted genomic DNA from the patients' peripheral blood with standard protocols. Exomes were captured with the Illumina Next (Illumina, San Diego, CA, USA) era DNA sample preparation kit. We performed high-throughput sequencing on the Illumina Hiseq 2500 with paired-end reads of 100 base pairs (bp) fragments analyzed in duplicate. Overall, ~50 M sequence reads were produced for each sample. We applied the BWA-mem program to determine alignments with the hg19 version of the human genome. The median coverage was approximately 40 reads per base. GATK version 2.4.7 was applied with the Unified Genotyper algorithm for variant calling, including all steps mentioned in the best practice pipeline. KGG-seq was used for annotating the detected variants and for comparing them with allele frequency population databases. We analyzed autosomal recessive changes and applied in-house scripts for filtering, based on family pedigrees and intersections [10]. To confirm the results and validate segregation, we completed Sanger sequencing for the identified CARMIL2 mutations in samples from all the patients and in samples from their first-degree relatives.

Statistical analysis

Results were analyzed with GraphPad Prism version 6 for Windows. Unpaired *t*-tests were used to compare groups. A P-value ≤ 0·05 was considered statistically significant.

Ethical review of the study

This study was approved by the Shaare Zedek Institutional Review Board.

Results

Clinical characteristics of the patients

The clinical characteristics of the patients are detailed in Table 1. We identified four patients (P1–P4) born to two related consanguineous Arab families (Fig. 1a). The mean age at clinical presentation was 5·37 (range = 2·5–10) years. Lymphocytic esophagitis was found in P1 and P3 (Fig. 1b). P1, P2 and P4 presented with inflammatory bowel disease (IBD; P1 and P4 with Crohn's disease and P2 with ulcerative pancolitis). P1 had an atypical presentation of IBD, meeting qualifications for Crohn's disease with extensive extra-colonic inflammation, colonic mucosa showing patchy chronic colitis with small ill-defined epithelioid granulomas. However, the main feature was pancolitis (Fig. 1c). In all patients, inflammatory findings seen in biopsies taken from the colon or esophagus were not enriched with eosinophils, the presence of which suggests an allergic to hypersensitivity-type reaction. However, allergic disorders were evident in two patients and consisted of asthma (P1 and P2) and allergic rhinitis (P1).

Recurrent cytomegalovirus (CMV) and human papillomavirus (HPV) infections with warts were observed in all four patients (Fig. 1f,g). P1 presented with severe *Pneumocystis jirovecii* pneumonia and CMV viremia after initiating infliximab for his colitis. EBV viremia was noted in P3. Dermatological manifestations consisted of eczematous eruptions. In addition, P2 presented with the unusual appearance of multiple seborrheic keratosis (Leser–Trelat sign) at age 15 years (Fig. 1d,e). Of note, a family history of sarcoidosis was reported for the father of P2 and P4.

All patients were treated with preventive co-trimoxazole and all are currently alive. P1 was treated with intravenous immunoglobulins and valganciclovir. His colitis is managed without immune-modulating agents, while P4 was treated with azathioprine for her IBD. Both P1 and P3 were treated with oral (topical) budesonide for lymphocytic esophagitis. P2 was vaccinated for HPV, which was followed by an improvement of her viral warts.

Table 1. Clinical characteristics of the patients

Patient*	Family history of immune-mediated diseases	Ethnicity/ Gender	Clinical presentation				Age at presentation/ current age (years)	Treatment /outcome
			Autoimmune	Infectious	Allergic	Other		
1	None	A/M	Crohn's disease; lymphocytic esophagitis	CMV viremia, retinitis and pneumonia; HPV warts; PjP	Asthma and allergic rhinitis	FTT; left retinal detachment	4/8	IVIG, valgancyclovir, cotrimoxazole, budesonide metronidazole/alive
2	Father with sarcoidosis	A/F	Ulcerative pancolitis	HPV warts; CMV and EBV viremia; recurrent pneumonia; *E. coli* UTI	Asthma	Seborrheic keratosis; hearing loss; thalassemia minor; ocular–macular scarring	5/16	HPV vaccination/cotrimoxazole/alive
3	None	A/F	Lymphocytic esophagitis	CMV and EBV viremia; HPV warts; esophageal candidiasis	–	–	10/14	Cotrimoxazole, budesonide/ alive
4	Father with sarcoidosis	A/F	Crohn's disease	CMV colitis and retinitis; CDT colitis; recurrent pneumonia; HPV warts	–	FTT	2.5/9	Cotrimoxazole, infliximab, azathioprine, GCS/alive

A = Arab; F = female; M = male; IVIG = intravenous immunoglobulins; CMV = cytomegalovirus; EBV = Epstein–Barr virus; HPV = human papilloma virus; PjP = *Pneumocystis jiroveci* pneumonia; FTT = failure to thrive; CDT = *Clostridium difficile* toxin; *E. coli* = *Escherichia coli*; UTI = urinary tract infections; GCS = glucocorticosteroids.

*All patients were born to consanguineous families and are homozygous to the CARMIL2 gene mutation (Hg19): c.A689del.GCCTTGAGGTCTCAGAACAGATTCT (25 base pairs); p.S230SdelX2 exon 10: 67681202.

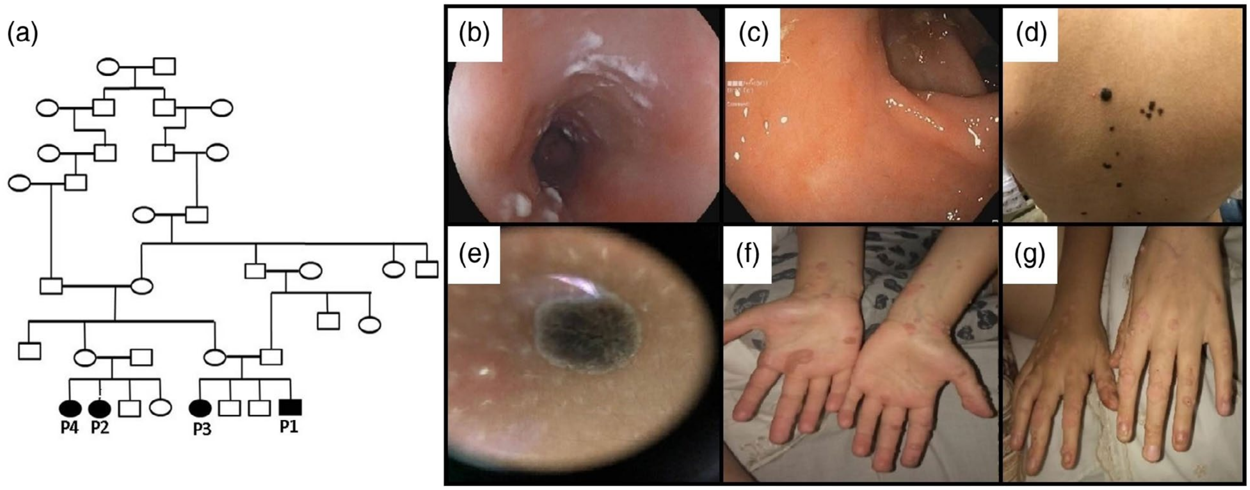

Fig. 1. Capping protein regulator and myosin 1 linker 2 (CARMIL2)-deficient patients from a consanguineous family demonstrate recurrent viral infections and immune dysregulation. (a) Family pedigree of CARMIL2-deficient patients (P) 1–4, notable for consanguinity. (b) Upper endoscopy demonstrating lymphocytic esophagitis in P1. (c) Inflammation of the sigmoid colon of P1 compatible with pancolitis, as seen in colonoscopy. (d,e) Seborrheic keratosis in P2 seen in physical examination and dermatoscopy, respectively. (f,g) Diffused viral warts in P4.

The patients were suspected to have a PID because of the familial consanguinity and clinical characteristics. Therefore, WES was performed

Genetic work-up and confirmation of CARMIL2 deficiency

In all reported patients, the WES revealed a novel homozygous, 25 bp deletion in exon 10 of *CARMIL2* at genomic position (Hg19): 67681202; c.A689del GCCTTGAGG TCTCAGAACAGATTCT, p.S230del-fs*2. This genetic deletion was confirmed with Sanger sequencing in all patients (Fig. 2a). Heterozygous mutation in *CARMIL2* was identified in the mother, father and two brothers of P1; all were asymptomatic carriers.

To confirm that the deletion led to a CARMIL2 deficiency at the protein level, we isolated effector T cells from healthy controls (HC) and patient samples, extracted the proteins and subjected them to an immunoblot analysis with an anti-CARMIL2 specific antibody probe (Fig. 2b). The CARMIL2 protein was absent from all the patient samples and was identified in all the HC samples. This finding demonstrated that the genetic deletion led to diminished CARMIL2 protein expression.

Immune phenotyping

Following the genetic analysis, we performed an in-depth characterization of the immune status of our patients. We found that the TREC levels were within the normal range in all patients (Table 2) [22,23]. T$_{reg}$ (CD4$^+$CD25$^+$FoxP3$^+$) counts were available for P1–P3. We found that the T$_{reg}$ ratios were reduced in these patients compared to HC (Fig. 3a). In addition, all four patients had reduced ratios of effector memory and central memory CD8$^+$ T cells and low percentages of CD56$^+$ NK cells (Table 2). Analyses of TCR variable beta-chains repertoire were available for three patients; these analyses demonstrated normal/polyclonal repertoires in P1 and P3 and a skewed/restricted repertoire in P4 (Supporting information, Fig. S1). CD19$^+$ B cell numbers and immunoglobulin levels were within normal ranges in all four patients. However, P1 demonstrated a lack of specific IgG antibodies to all vaccines, except rubella. IgE and anti-nuclear antibody titers were available for two patients, and both showed normal titers (Table 2).

T cell activation is impaired in patients with CARMIL2 deficiency

Previous studies demonstrated impaired T cell activation and effector functions in patients with CARMIL2 deficiencies [4,6]. Indeed, our patients presented with recurrent viral infections. Therefore, we aimed to elucidate the functional fitness of T cells in these patients. For this purpose, we evaluated *in-vitro* changes in T cell activation markers and IFN-γ production upon stimulation. Stimuli-induced IL-2 receptor α (CD25) surface expression was significantly diminished in both CD4$^+$ and CD8$^+$ T cells from all patients compared to HC T cells (Fig. 3b,c; $P = 0·022$ and $P = 0·004$, respectively). Additionally, CD8$^+$ T cells from all patients demonstrated reduced intracellular IFN-γ staining upon activation (Fig. 3d; $P = 0·006$), which suggested that patient T cells showed impaired cytokine production in response to stimulation.

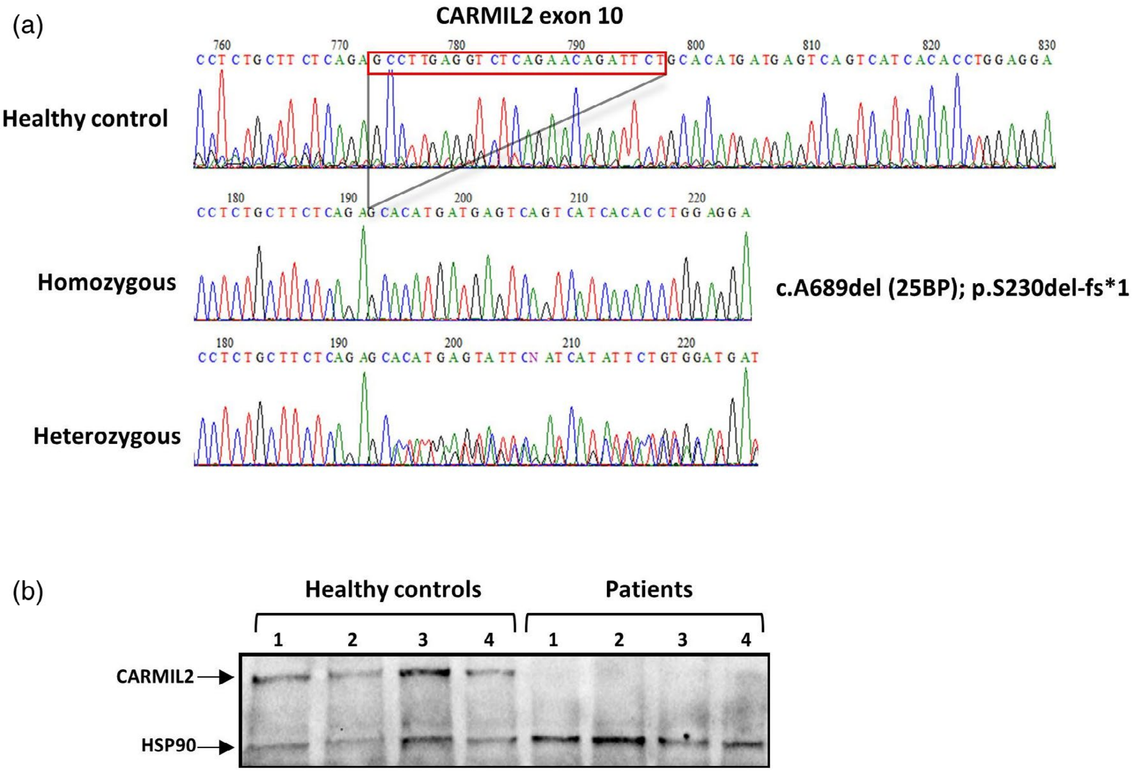

Fig. 2. Capping protein regulator and myosin 1 linker 2 (*CARMIL2*) deletion mutation leads to absent protein expression. (a) Chromatogram showing a novel homozygous 25 base pairs (bp) deletion in exon 10 of *CARMIL2* [genomic position (Hg19): 67681202; c.A689del GCCTTGAGGTCTCAG AACAGATTCT, p.S230del-fs*2]. Presented are a healthy control (HC; upper row), homozygous patient (P1; middle row) and heterozygous carrier (P1's father; lower row). (b) Immunoblot analysis using anti-CARMIL2 antibody. Anti-heat-shock protein (HSP) 90 was used as a loading control.

CARMIL2 is required for CD28-mediated co-stimulation, which is required for the activation of naive T cells, their maturation into T memory cells and their differentiation into T helper and T_{reg} cells [4,24]. Consistent with this characteristic, we found that CD28 expression on T cell membranes was significantly reduced in all four patients compared to HC (Fig. 3e,f; $P = 0·0013$ and $P = 0·0011$ for $CD8^+$ and $CD4^+$ T cells, respectively). This finding could at least partly explain the impairment in T cell activation observed in our patients.

IL-2 rescues T cell activation in patients with a CARMIL2 deficiency

Consistent with previously published data [4,6], our results supported the finding that impaired T cell activation was due to diminished co-stimulation through CD28. IL-2 is known to be a key player in the co-stimulation required for T cell activation [25]. In addition, CARMIL2-deficient patients were found to have a decreased endogenous IL-2 secretion upon T cell activation [4]. Therefore, we investigated whether IL-2 treatment might restore T cell activation in our cohort.

We examined T cell activation in the presence of different IL-2 concentrations. Consistent with our hypothesis, upon IL-2 treatment we observed a substantial increase in CD25 surface expression at 48 h post-stimulation (Fig. 4a,b). Notably, this rescue of T cell activation by IL-2 was dose-dependent. Significant rescue was observed in $CD8^+$ T cells ($P = 0·009$; IL-2 at 400 *versus* 0 U/ml), and a substantial (but not significant) trend was observed in the rescue of $CD4^+$ T cells (Fig. 4b; $P = 0·532$, $P = 0·137$ and $P = 0·265$ for IL-2 at 80 *versus* 0, at 400 *versus* 0 and at 400 *versus* 80 U/ml, respectively). Similarly, IFN-γ production was also significantly increased in an IL-2 dose-dependent manner (Fig. 4C; $P = 0·034$ for IL-2 at 400 *versus* 0 U/ml), which suggested that IL-2 rescued the function of CARMIL2-deficient $CD8^+$ T cells. However, IL-2 treatment did not completely restore T cell activation (Fig. 4a,b).

After observing the rescue of T cell activation following 48-h stimulation, we explored the possibility that the rescue might not be sustained. To that end, we repeated the above experiments, but with 5-day stimulations at different IL-2 doses. Consistent with the 48-h stimulation results, IL-2

Table 2. Immune work-up of CARMIL-2-deficient patients

Parameter					P1 (8 years)	P2 (16 years)	P3 (14 years)	P4 (9 years)	Normal range^{a,b}		
									7–8 years (Ig: 6–8 years)	9–13 years (Ig: 9–10 years)	14–18 years (Ig: > 10 years)
Absolute leukocyte count (10^9/l)					7.6	6.50	7.70	7.20	4.50–13.50	4.50–13.50	4.50–13.00
Absolute lymphocyte count (10^9/l)					3.59	2.88	3.77	3.73	1.50–6.80	1.50–6.50	1.20–5.20
	T cells	CD3$^+$ (% of lymphocytes)			81.05	**91.66**	**86.59**	67.45	61.00–84.00	60.00–79.00	62.00–81.00
Lymphocyte subpopulations (%)	CD4	Total (% of lymphocytes)		CD4$^+$	29.29	37.51	33.74	42.10	26.00–53.00	29.00–48.00	31.00–53.00
		Naive		CD45RA$^+$CD27$^+$	**77.45**	**87.27**	**87.89**	**87.75**	32.00–68.00	25.00–63.00	31.00–57.00
		Memory	T$_{cm}$	CD45RA$^-$CD45RO$^+$CD62L$^+$CD127$^+$	**60.33**	**45.77**	**51.06**	**37.62**	9.00–24.00	11.00–25.00	10.00–27.00
			T$_{em}$	CD45RA–CD45RO$^+$CD62L– CD127$^+$	26.79	15.67	24.47	**32.16**	9.00–32.00	12.00–30.00	12.00–44.00
		T$_{emra}$		CD45RA$^+$CD27$^-$	*1.15*	*2.31*	*1.20*	*0.94*	4.00–15.00	4.00–24.00	4.00–12.00
		T$_{regs}$		CD25$^+$FoxP3$^+$	*0.30*	*0.31*	*0.46*	n.a.	0.90–3.20	1.00–3.70	0.80–4.30
	CD8	Total (% lymphocytes)		CD8$^+$	31.50	19.57	27.92	30.70	19.00–35.00	18.00–33.00	19.00–30.00
		Naive		CD54RA$^+$CD27$^+$	**74.85**	42.58	56.91	55.48	30.00–61.00	22.00–58.00	18.00–61.00
		Memory	T$_{cm}$	CD45RA–CD45RO$^+$CD62L$^+$ CD127$^+$	*6.02*	*1.47*	*0.00*	*0.88*	2.00–12.00	2.00–15.00	3.00–12.00
			T$_{em}$	CD45RA–CD45RO$^+$CD62L– CD127$^+$	*4.05*	*2.73*	*19.61*	*1.70*	20.00–45.00	24.00–58.00	25.00–58.00
		T$_{emra}$		CD45RA$^+$CD27$^-$	17.17	**31.04**	**37.98**	**30.49**	7.00–26.00	7.00–26.00	5.00–20.00
	NK cells			CD56$^+$ (% of lymphocytes)	*4.34*	*0.44*	*5.21*	*4.67*	6.00–21.00	8.00–22.00	6.00–23.00
	B cells			CD19$^+$ (% of lymphocytes)	13.39	6.42	6.17	8.29	8.00–23.00	8.00–21.00	6.00–21.00
TREC (copies)					1277.00	1387.00	2765.00	5209.00	> 400.00	> 400.00	> 400.00
TCR V–beta					Normal/ polyclonal	n.a.	Normal/ polyclonal	Restricted/ skewed	–	–	–
Lymphocyte mitogenic responses (cpm; patient/ control)		No mitogen			1190/2031	260/223	496/467	*320/2167*	> 50% of control		
		PHA 6 µl/ml			*35 855/86 286*	7061/5982	13 688/25 129	68 320/123 757			
		PHA 25 µl/ml			80 838/100 561	10 146/7632	11 695/14 668	128 192/166 609			
		Anti–CD3			*28 444/70 959*	*1003/4501*	*666/12 529*	*11 413/83 758*			
Serum Ig		IgG (mg/dl)			1050.00	**1710.00**	1370.00	800.00	633.00–1280.00	608.00–1572.00	639.00–1349.00
		IgA (mg/dl)			*<26.00*	**314.00**	442.00	206.00	33.00–202.00	45.00–236.00	70.00–312.00
		IgM (mg/dl)			**431.00**	350.00	159.00	197.90	48.00–207.00	52.00–242.00	56.00–352.00
		IgE (IU/ml)			7.80	3.00	7.00	n.a.	1.03–161.3	0.98–570.60	2.06–195.20
Specific IgG antibodies		*Streptococcus pneumoniae* (mg%)			**2.00**	n.a.	n.a.	n.a.	2.00–9999.00	2.00–9999.00	2.00–9999.00
		Isohemagglutinin			n.a.	**Anti–A 1 : 14**	n.a.	n.a.	–	–	–
					n.a.	**Anti–B 1 : 8**	n.a.	n.a.	–	–	–
		Tetanus (U/ml)			0.00	n.a.	n.a.	n.a.	< 0.10	< 0.10	< 0.10
		Diphtheria (U/ml)			0.00	n.a.	n.a.	n.a.	< 0.10	< 0.10	< 0.10
		Rubella (U/ml)			**122.00**	**> 500.00**	n.a.	**146.00**	0.00–30.00	0.00–30.00	0.00–30.00
		Varicella zoster virus (U/ml)			n.a.	**179.00**	n.a.	12.00	< 135.00	< 135.00	< 135.00
		Mumps (U/ml)			Negative*	Negative*	n.a.	Negative*	< 8.90	< 8.90	< 8.90
		Pertussis (U/ml)			n.a.	Negative*	n.a.	n.a.	< 40.00	< 40.00	< 40.00

Table 2. (Continued)

Parameter		P1 (8 years)	P2 (16 years)	P3 (14 years)	P4 (9 years)	Normal range^{a,b} 7–8 years (Ig: 6–8 years)		
						7–8 years (Ig: 6–8 years)	9–13 years (Ig: 9–10 years)	14–18 years (Ig: > 10 years)
	Measles (U/ml)	Negative*	**16.40**	n.a.	n.a.	≤ 13.50	≤ 13.50	≤ 13.50
	HBV surface (U/ml)	Negative*	1.30	n.a.	Negative*	0.00–9.90	0.00–9.90	0.00–9.90
	CMV (U/ml)	**100.00**	**> 250.00**	Negative*	**89.00**	0.00–6.00	0.00–6.00	0.00–6.00
	EBV VCA (S/CO)	**> 750.00**	**60.93**	**Positive***	**> 750.00**	0–0.75	0–0.75	0–0.75
	EBV EBNA (S/CO)	< 3.00	0.01	Negative*	< 3.00	< 0.50	< 0.50	< 0.50
Autoantibodies	ANA	n.a.	Negative	n.a.	Negative	> 1 : 100	>1 : 100	>1 : 100

CBC = complete blood count; T_{cm} = central memory T cells; T_{em} = effector memory T cells; T_{emra} = T effector-memory cells with reacquired RA; n.a. = data not available; T_{regs} = regulatory T cells; NK = natural killer; PHA = phytohemagglutinin; cpm = counts per minute; FoxP3 = forkhead box P3; TREC = T cell receptor excision circles; TCR = T cell receptor; Ig = immunoglobulins; ANA = anti-nuclear antibodies; HBV = hepatitis B virus; EBV = Epstein–Barr virus; VCA = viral capsid antigen; EBNA = EBV nuclear antigen; CMV = cytomegalovirus. Bold type and italics = values are above and below normal reference range, respectively.

*Specific IgG antibody titer is not available.

[a] Age-matched reference ranges for T cell subsets are taken from Garcia-Prat *et al.*[22];

[b] age-matched IgG, IgM and IgA reference ranges are taken from Jolliff *et al.*[23].

stimulation induced dose-dependent CD25 surface expression, which significantly increased in both CD4$^+$ and CD8$^+$ T cells (Fig. 4d,e; P = 0·013 and P = 0·024 for IL-2 at 400 *versus* 0 U/ml, respectively). This finding supports the notion that IL-2 treatment promoted the sustained rescue of T cell activation in patients with a CARMIL2 deficiency.

IL-2 shows potential in rescuing T cell proliferation in patients with CARMIL2 deficiency

Previous studies found that T cell proliferation was also impaired in patients with CARMIL2 deficiencies [6,8,11]. Based on those results, we evaluated T cell proliferation in our cohort. A [^{3}H]-thymidine incorporation assay indicated that T cell DNA replication was reduced in response to an anti-CD3 antibody stimulus in all four patients (Table 2). To explore T cell division directly, T cells were labeled with Cell Trace Violet and activated with anti-CD3 and anti-CD28 antibodies. We analyzed cellular division with flow cytometry. Interestingly, we found that T cell proliferation was not impaired in P1 and P2, but it was markedly reduced in P3 (Fig. 5a,b). Finally, we tested the compelling possibility that IL-2 might rescue T cell proliferation. Indeed, we noticed that in P3, whose T cell proliferation was impaired, IL-2 treatment resulted in a substantial increase in T cell divisions. However, in P1 and P2, who were not found to have decreased T cell proliferation, exogenous IL-2 did not markedly influence T cell divisions (Fig. 5c,d). Furthermore, we analyzed activation-induced cell death (AICD) in IL-2-treated CD8$^+$ and CD4$^+$ T cells of the patients and HC. In P3, IL-2 treatment appeared to rescue T cell proliferation without causing AICD (Supporting information, Fig. S2).

Discussion

In this study, we have described four patients from two related families with a novel mutation in CARMIL2, which resulted in the loss of CARMIL2 protein expression, diminished T_{reg} counts and impaired T cell activation. Our results suggested that IL-2 could rescue T cell activation *in vitro*. Thus, IL-2 treatment could potentially benefit T cell effector functions in these patients.

Consistent with these findings, other mutations that led to CARMIL2 deficiencies were reported to confer a similar immune phenotype [4,6–12,26]. In our study, we demonstrated that CARMIL2-deficient naive T cells expressed diminished levels of surface CD28. Notably, in a previous study describing another family with *CARMIL2* mutation that induced protein absence, activated T cells (not naive) were found to have normal CD28 surface expression [4]. These results can be explained by the up-regulation of CD28 surface expression following T cell activation. The reduction in CD28

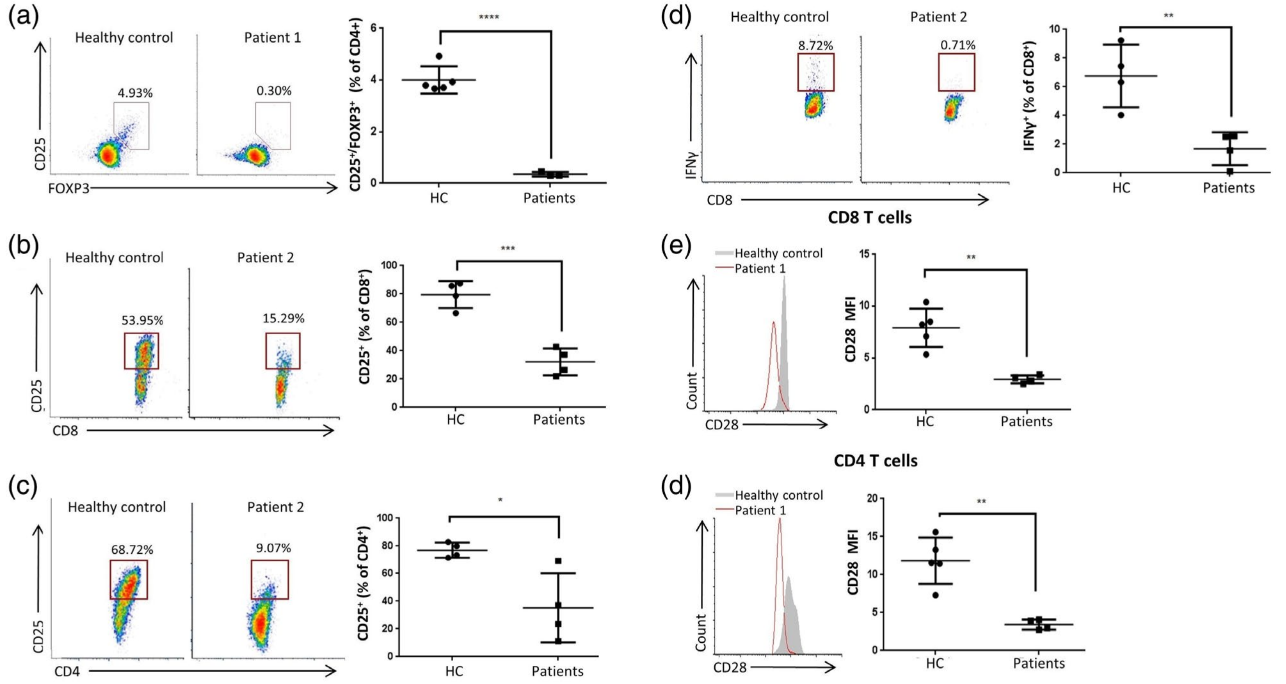

Fig. 3. Decreased regulatory T cells and impaired T cell activation in capping protein regulator and myosin 1 linker 2 (CARMIL2)-deficient patients. (a) Flow cytometry analysis of forkhead box protein 3 (FoxP3) *versus* CD25 gated on CD4$^+$ T cells (regulatory T cells). The left panel shows representative density-plot (P1) and the right summarizes comparison between P1–P3 and healthy controls (HC). (b) Flow cytometry analysis of CD8 *versus* CD25 gated on CD8$^+$ T cells. The left panels show representative density-plots (P2) and the right summarizes comparison between P1–P4 and HC. (c) Similar to (b) (P2), CD4 *versus* CD25 gated on CD4$^+$ T cells. (d) Peripheral blood mononuclear cells (PBMCs) were activated for 48 h using a plate-bound anti-CD3 and anti-CD28 antibodies. Cells were treated with brefeldin A and were subjected to intracellular staining. The figure presents flow cytometry analysis of CD8 *versus* interferon (IFN)-γ gated on CD8$^+$ T cells. The left panels show representative density-plots (P2) and the right summarizes comparison between P1–P4 and HC. (e,f) CD28 surface expression on CD8$^+$ (e) and CD4$^+$ (f) T cells from PBMCs of all patients and HC. The left panels show representative histogram plots (P1) and right panels summarize all patients *versus* HC. $^*P = 0.0221$; $^{**}P \leq 0{\cdot}0062$; $^{***}P = 0{\cdot}0004$; $^{****}P < 0{\cdot}0001$; two-tailed *t*-test, standard error of the mean (s.e.m.).

expression in our cohort expanded the understanding of the role of CARMIL2 in bridging CD28 and CARD11 activities [3,5]. This mechanism of action might also underlie the defective peripheral T_{reg} count observed in our patients. Previous data supported this hypothesis, as CD28$^{(-/-)}$ mouse models also exhibited low T_{reg} numbers and increased susceptibility to autoimmune thyroiditis [27].

Colitis and various skin manifestations have been described in patients with CARMIL2 deficiencies [8,10]. In the present study, P2 developed multiple seborrheic keratosis over her trunk and proximal limbs at age 15 years. A polymerase chain reaction (PCR) assay for HPV-typing showed negative results. Seborrheic keratosis is a common benign epidermal tumor that occurs exclusively in adults. To the best of our knowledge, it has not been described previously in children or adolescents, and is thought to be associated with skin aging. Eruptive seborrheic keratosis might be a paraneoplastic condition, also known as the Leser–Trélat sign [28]. Therefore, P2 underwent a comprehensive work-up to rule out malignancy with negative results. The mechanism responsible for the appearance of seborrheic keratosis in patients with a CARMIL2 deficiency remains to be defined.

P1 presented with severe pneumonia caused by a *P. jirovecii* infection and CMV-induced pneumonitis and retinitis, which caused the retinal detachment. CMV-induced retinitis was also noted in P4. To date, these two pathogens have not been reported in patients with CARMIL2 deficiencies. Based on our results, infections with these pathogens should be considered in patients with respiratory and visual complaints.

Therapeutic modalities are limited in treating CARMIL2 deficiencies. Currently, there is no specific treatment that directly targets the impaired underlying immune pathway. Moreover, no published studies have described hematopoietic stem cell transplantations in patients with CARMIL2 deficiencies, and success rates

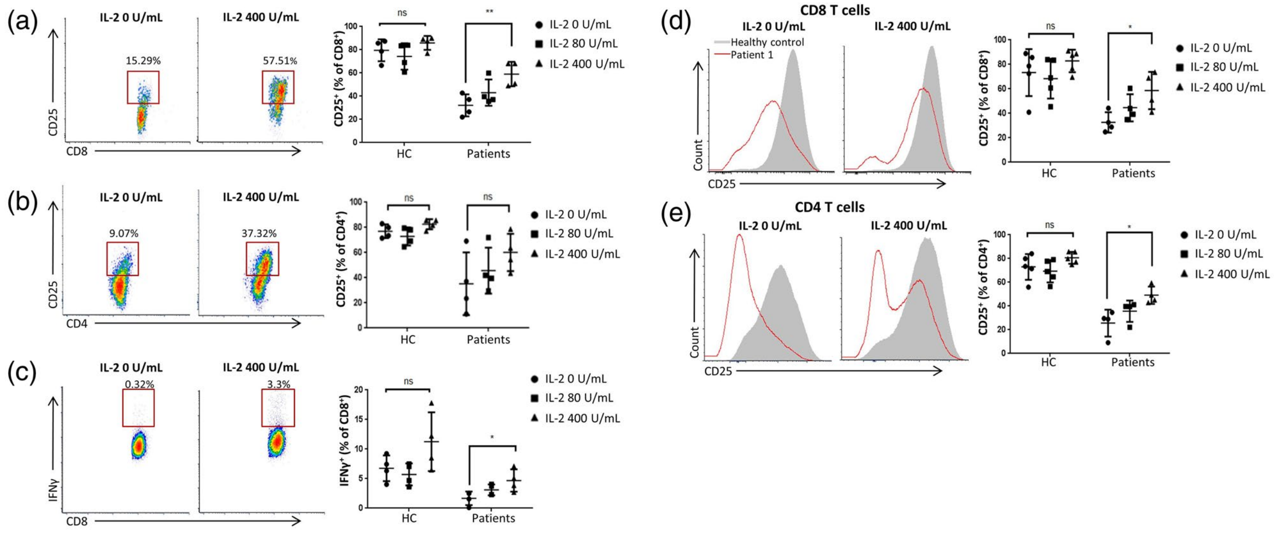

Fig. 4. Interleukin (IL)-2 rescues *in-vitro* T cell activation of capping protein regulator and myosin 1 linker 2 (CARMIL2)-deficient patients. (a–c) Peripheral blood mononuclear cells (PBMCs) were activated for 48 h using anti-CD3 and anti-CD28 antibodies in the presence of IL-2 at the indicated doses. (a) Flow cytometry analysis of CD8 *versus* CD25 gated on $CD8^+$ T cells. The left panels are representative of density-plots (P2) and the right panel summarizes comparison between P1–P4 and healthy controls (HC). (b) Similar to (a) (P2) for CD4 *versus* CD25 gated on $CD4^+$ T cells. (c) Cells were treated with brefeldin A and were subjected to intracellular staining. The figure presents flow cytometry analysis of CD8 *versus* interferon (IFN)-γ gated on $CD8^+$ T cells. The left panels show representative density-plots (P3) and the right summarizes comparison between P1–P4 *versus* HC. (d,e) PBMCs were activated for 5 days using anti-CD3 and anti-CD28 antibodies in the presence of IL-2 at the indicated doses. (d) Left panels show representative histogram plots (P1) of CD25 expression on $CD8^+$ T cells. The right panel compares all patients to HC. (e) Similar to (d) for $CD4^+$ T cells. $^*P < 0{\cdot}05$; $^{**}P = 0{\cdot}009$; two-tailed *t*-test, standard error of the mean (s.e.m.).

remain unknown. There is some rationale for treating the autoimmunity and T_{reg} dysfunction in these patients with rapamycin. This approach is commonly used for treating patients with immune dysregulation, polyendocrinopathy, enteropathy and X-linked syndrome (IPEX) [29]. However, in our patients, this treatment might exacerbate T cell dysfunction and worsen recurrent infections and EBV/CMV viremia.

CD28 co-stimulation is partially mediated by IL-2 [30]. Indeed, our findings supported this notion, because treating patient T cells *in vitro* with IL-2 could partly rescue T cell activation. Moreover, in one patient IL-2 treatment also improved T cell proliferation. Notably, P1 and P2 exhibited nearly normal T cell proliferation; in these patients, IL-2 had no significant effect.

The observed IL-2-mediated effects might also influence the reduced T_{reg} counts in patients with CARMIL2 deficiencies. The surface expression of IL-2 receptor α and its signaling pathway is critical for T_{reg} survival and function [16] Indeed, knocking out IL-2-related genes in mice led to T_{reg} dysfunction and an autoimmune phenotype [31]. Low-dose IL-2 is currently being evaluated for several autoimmune diseases. A prospective Phase I–IIa clinical trial found that IL-2 treatment could effectively attenuate symptoms in 11 different autoimmune diseases, including ulcerative colitis and Crohn's disease. A correlative study showed that IL-2 treatment induced the T_{reg} expansion without $CD8^+$ effector T cell activation [32]. Therefore, IL-2 treatment showed potential as a therapeutic modality in patients with CARMIL2 deficiencies.

Several issues must be considered in using IL-2 for treating patients with CARMIL2 deficiencies. On one hand, the IL-2 induction of T_{reg} expansion might benefit autoimmunity, and the IL-2 rescue of T cell activation may assist in resolving severe and recurrent infections. These effects would be particularly important in our patients, who had both colitis and EBV/CMV viremia. However, on the other hand, IL-2 activation of effector $CD8^+$ T cells might, in fact, exacerbate autoimmunity, due to the expansion of a specific autoreactive T cell clone. In that regard, our findings were limited, because investigations of T_{reg} expansion and the TCR repertoire in response to different doses of IL-2 were beyond the scope of this study.

Our study had several limitations. The data were limited to a single family, and analyses were restricted to *in-vitro* human T cell cultures. Therefore, further comparative studies are warranted that focus on IL-2 effects in other patients with different mutations. In addition, studies with CARMIL2-deficient murine models are

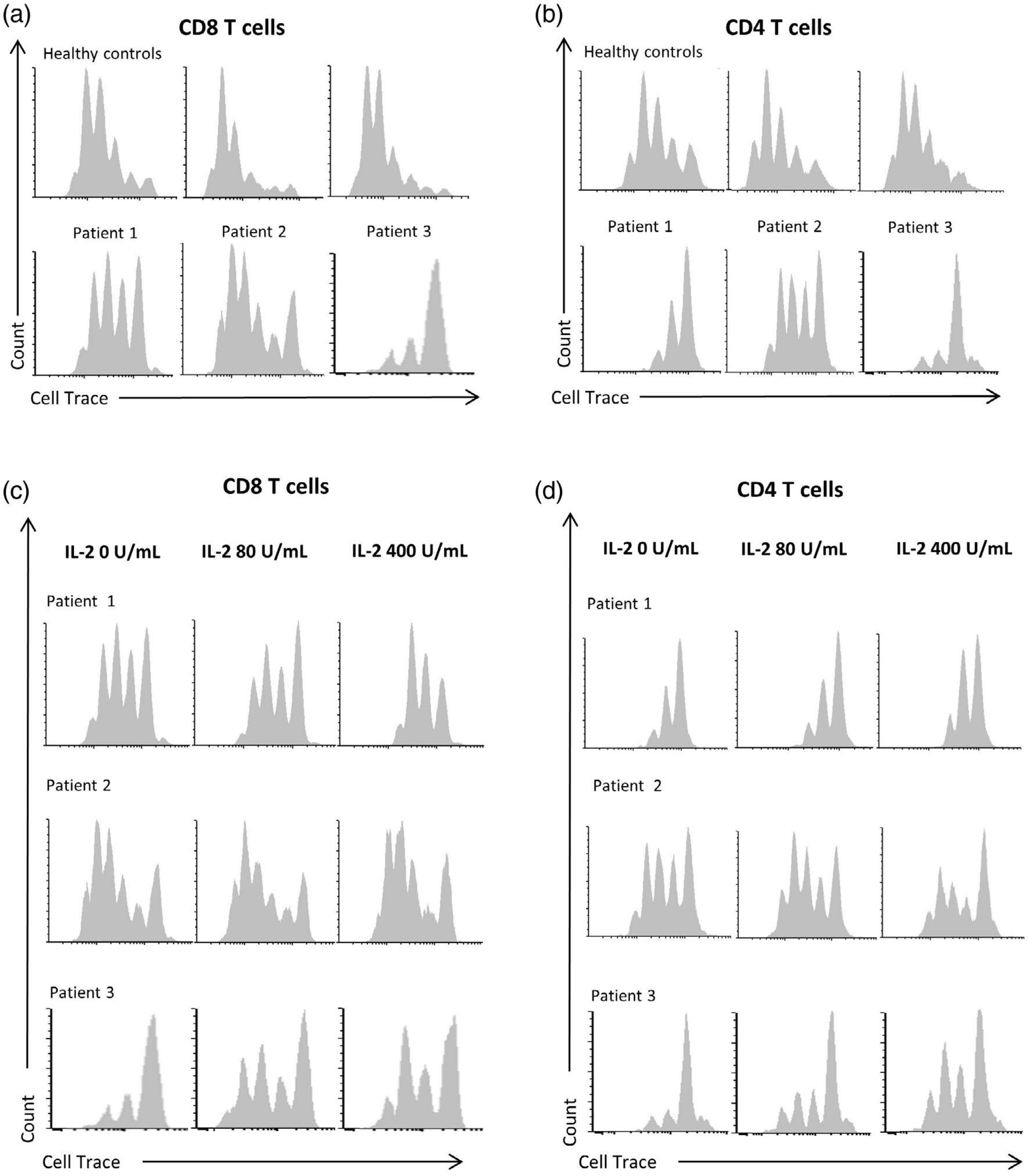

Fig. 5. Interleukin (IL)-2 has the potential to rescue *in-vitro* proliferation of T cells in capping protein regulator and myosin 1 linker 2 (CARMIL2)-deficient patients. (a) Histogram plots of Cell Trace intensity gated on $CD8^+$ T cells that were activated for 5 days with anti-CD3 and anti-CD28 antibodies. Presented are P1–3 and three healthy controls (HC). (b) Similar to (a) gated on $CD4^+$ T cells. (c,d) Peripheral blood mononuclear cells (PBMCs) of P1–P3 were activated as above in the presence of IL-2 at the indicated doses. Histogram plots of Cell Trace Violet intensity gated on $CD8^+$ (C) and $CD4^+$ (D) T cells.

particularly important, because they could lead the way to clinical trials.

Conclusions

This study has demonstrated that IL-2 could be beneficial in treating CARMIL2 deficiencies. This finding has extended the current knowledge on this rare PID. In the future, our results might be useful in improving patient care.

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Disclosures

The authors declare no conflicts of interest related to this paper.

Author contributions

O. S., study design, immune laboratory work-up and writing of the manuscript; A. L., immune and genetic work-up; A. J. S., genetic consultation; R. Z., immune consultation, immune and genetic work-up, revision of the manuscript and study supervision; O. M., infectious disease consultation and patient treatment; E. P. and J. L., pulmonological consultation and patient treatment; O. L., P. M. and M. S., gastrointestonal consultation and patient treatment; V. M. P., dermatological consultation and patient treatment; Y. T., immune consultation; O. T., patient treatment, immune work-up, manuscript revision and study supervision; and M. B., study design, revision of the manuscript, immune work-up and study supervision.

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Supporting Information

Additional supporting information may be found in the online version of this article at the publisher's web site:

Fig. S1. T cell receptor (TCR) repertoire in CARMIL2-deficient patients. (a,b) TCR repertoire demonstrates polyclonal/normal distribution in P1 and P3, respectively. (c) TCR repertoire is skewed/ restrictive in P4.

Fig. S2. Activation-induced cell death in CARMIL2-deficient patients.