

# Differential impact of high and low penetrance *TNFRSF1A* gene mutations on conventional and regulatory CD4<sup>+</sup> T cell functions in TNFR1-associated periodic syndrome

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## ABSTRACT

TNFR-associated periodic syndrome is an autoinflammatory disorder caused by autosomal-dominant mutations in *TNFRSF1A*, the gene encoding for TNFR superfamily 1A. The lack of knowledge in the field of TNFR-associated periodic syndrome biology is clear, particularly in the context of control of immune self-tolerance. We investigated how TNF- $\alpha$ /TNFR superfamily 1A signaling can affect T cell biology, focusing on conventional CD4<sup>+</sup>CD25<sup>-</sup> and regulatory CD4<sup>+</sup>CD25<sup>+</sup> T cell functions in patients with TNFR-associated periodic syndrome carrying either high or low penetrance *TNFRSF1A* mutations. Specifically, we observed that in high penetrance TNFR-associated periodic syndrome, at the molecular level, these alterations were secondary to a hyperactivation of the ERK1/2, STAT1/3/5, mammalian target of rapamycin, and NF- $\kappa$ B pathways in conventional T cells. In addition, these patients had a lower frequency of peripheral regulatory T cells, which also displayed a defective suppressive phenotype. These alterations were partially found in low penetrance TNFR-associated periodic syndrome, suggesting a specific link between the penetrance of the *TNFRSF1A* mutation and the observed T cell phenotype. Taken together, our data envision a novel role for adaptive

immunity in the pathogenesis of TNFR-associated periodic syndrome involving both CD4<sup>+</sup> conventional T cells and T<sub>regs</sub>, suggesting a novel mechanism of inflammation in the context of autoinflammatory disorders. *J. Leukoc. Biol.* 99: 761–769; 2016.

## Introduction

TNF- $\alpha$  is produced by epithelial and immune cells [1–4] and, through the binding to TNFRs, TNFRSF1A (or TNFR1A p55/p60-TNFR) and TNFR1B (or TNFR2, p75/80-TNFR), regulates several biologic processes, including immunity, inflammation, apoptosis, and cell differentiation and proliferation [5, 6]. TNFRSF1A, constitutively expressed in many cell types, including lymphocytes, mediates TNF- $\alpha$  signaling through the recruitment of various signaling proteins, resulting in the activation of transcription factors such as NF- $\kappa$ B and AP-1, apoptosis pathways, and MAPK signaling [7–9]. Mutations in the

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*TNFRSF1A* gene that encode for TNFRSF1A are known to be associated with an autosomal dominant autoinflammatory syndrome known as TRAPS [10, 11]. The autoinflammatory syndromes are an increasing group of conditions characterized by an impaired innate immune response associated with generalized systemic inflammation without involvement of autoantibodies or autoreactive T cells [12]. TRAPS is characterized clinically by recurrent fever attacks, periorbital edema, conjunctivitis, a migratory skin rash, myalgia, arthralgia and/or arthritis, polyserositis, and amyloidosis as a more severe complication [13–19]. More than 50 *TNFRSF1A* mutations (<http://fmf.igh.cnrs.fr/infefers/>) have been associated with TRAPS. Most of them are heterozygous missense mutations, localized in the extracellular portion of the receptor [20]. TRAPS-associated mutations can be separated into high penetrance, also termed “structural mutations,” because they mainly affect the cysteine residues involved in receptor folding and related to a more severe phenotype, and low penetrance, defined as “nonstructural mutations or variants of unknown significance” that seem to be associated with a milder TRAPS phenotype [13, 14, 21–23]. The precise pathogenic mechanism of TRAPS is not well understood. Moreover, it has been observed that TRAPS-associated mutations can alter TNFRSF1A cell surface expression, shedding, TNF- $\alpha$  binding affinity, TNFRSF1A intracellular trafficking, and TNF- $\alpha$ /TNFRSF1A signaling and autophagy [21, 24–29]. Recently, it has been demonstrated that peripheral blood leukocytes, either from patients with TRAPS or mice harboring TRAPS-derived *TNFRSF1A* mutations, showed intracellular receptor accumulation, which sensitized these cells to LPS stimulation through increased MAPK activation [30]. These cells also exhibited increased mitochondrial respiration and an unconventional unfolded protein response, leading to reactive oxygen species production, events known to potentiate MAPK signaling and secretion of several proinflammatory cytokines [31–33].

Because little is known about the involvement of adaptive immunity in the pathogenic events leading to TRAPS symptoms, we investigated the effect of *TNFRSF1A* mutations on different T cell subsets. Specifically, we delineated how multiple variants of TNFR1 mutations are able to affect the  $T_{conv}/T_{reg}$  balance, thus providing novel insights into the detailed molecular mechanisms regulating immune self-tolerance in TRAPS.

## MATERIALS AND METHODS

### Patients

Serum and blood samples were obtained from 35 patients: 20 with HP-TRAPS (C43R in 2; C43T in 1; T50M in 7; C52Y in 2; C125R in 2; C55Y in 1; S59N in 1; S59P in 1; C114W in 1; Del 103-104 in 1; and L167-175del in 1 patient), 15 with LP-TRAPS (R92Q in 12; D12E in 1; V95M in 1; and R104Q in 1 patient). Serum and blood samples were also obtained from 32 age-, gender- and BMI-matched HCs without inflammatory disease (17 males, 15 females; median age 42 y, range 9–65) attending the outpatient clinic at the Rheumatology Unit of

the University of Siena for fibromyalgia and/or carpal tunnel syndrome and who tested negative for TRAPS mutations. These subjects underwent detailed clinical and laboratory evaluations to rule out any other inflammatory, metabolic, and neoplastic disorders. The laboratory assessment included ESR, CRP, and SAA. ESR was measured using the Westergren method (mm/h) and was considered normal if  $>15$  mm/h for males and  $<20$  mm/h for females. The serum CRP concentrations were measured using a nephelometric immunoassay; 0.5 mg/dl was considered normal. SAA levels were measured using particle-enhanced nephelometry (BNII autoanalyzer; Dade Behring, Marburg, Germany), and the reference value was 6.4 mg/L. All blood samples were collected from patients during a remission phase of the disease. Patients exhibited a symptom-free interval during the routine follow-up visit, although some displayed elevated acute phase reactants (i.e., ESR, CRP, SAA).

Both patients and HCs provided written informed consent. The University of Siena institutional ethics committee, in accordance with the Declaration of Helsinki, reviewed and approved the study protocol. The clinical and demographic characteristics of the patients with HP- and LP-TRAPS, at the time of blood collection, are summarized in **Table 1**.

### Immunophenotype

Immune cell profiling of the cells from the patients with TRAPS and HCs was done at the time of the blood sampling. Before flow cytometry to determine the lymphocyte subsets, whole blood cells were analyzed with a clinical grade hemocytometer to determine the absolute lymphocyte numbers in each sample. For the HCs and patients with TRAPS, 100  $\mu$ l of blood was incubated for 30 min at room temperature with the specific antibody combinations. The erythrocytes were lysed using BD FACS Lysing Solution 2 (BD Biosciences, San Diego, CA, USA) for 10 min and subsequently washed and resuspended in 300  $\mu$ l of PBS. Flow cytometry was performed on cells gated on CD45<sup>+</sup> side scatter. Immunophenotypic analysis was performed using an EPICS XL flow cytometer (Beckman Coulter, Fullerton, CA, USA) using the Beckman Coulter XL System II software program. Triple combinations of different human monoclonal antibodies (i.e., FITC- and PE-anti-CD3, PE- and PC5-anti-CD4, PC5-anti-CD8, PE-anti-CD16, PC5-anti-CD19, PE-anti-CD25, FITC-anti-CD45, and PE-anti-CD56; all from Immunotech, Beckman Coulter) were used to identify the different cell populations.

### Cell cultures and T cell proliferation assays

Human PBMCs were isolated by stratifying 15 ml of heparinized whole blood on 5 ml of Ficoll-Paque PREMIUM (GE Healthcare Life Sciences, Pittsburgh, PA, USA) and centrifuging the solution at 1.2 g for 20 min. PBMCs ( $2 \times 10^5$  per well) were cultured, in triplicate, in 96-well round-bottomed plates (BD Falcon; BD Biosciences) in medium supplemented with 5% (vol/vol) AS or 5% (vol/vol) commercial pooled AB HS (Sigma-Aldrich, St. Louis, MO, USA) and were stimulated, or not, in parallel with 0.1  $\mu$ g/ml anti-CD3 monoclonal antibody (OKT3 Orthoclone; Janssen-Cilag SpA, Milan, Italy). Supernatants were collected from 48-h TCR-stimulated PBMCs from patients with TRAPS and HCs. The human Th1/Th2 11plex kit, FlowCytomix (Bender Medsystems, eBioscience, San Diego, CA, USA), was used for quantitative detection of human IFN- $\gamma$ , TNF- $\alpha$ , TNF- $\beta$ , IL-1 $\beta$ , IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, and IL-12p70 by flow cytometry (Becton-Dickinson, Franklin Lakes, NJ, USA), according to the manufacturer's instructions.

Human CD4<sup>+</sup>CD25<sup>+</sup> ( $T_{reg}$ ) and CD4<sup>+</sup>CD25<sup>−</sup> ( $T_{conv}$ ) T cells were purified from PBMCs from HCs and patients with TRAPS using magnetic cell separation with the DynalCD4<sup>+</sup>CD25<sup>+</sup>  $T_{reg}$  Kit (Dynal Biotech, Invitrogen, Carlsbad, CA, USA) and were rapidly cleaned with the Detach reagent (Dynal Biotech) from surface-bound CD25 mAbs. The magnetic bead-based purification technique yielded a highly expressing CD25<sup>+</sup> population (98% pure by FACS analysis), 90% of which expressed FoxP3. The  $T_{reg}:T_{conv}$  ratio in the suppression experiments was 1:1. We cultured cells ( $1 \times 10^4$  cells per well) in round-bottom 96-well plates (BD Falcon; BD Biosciences) with RPMI 1640 medium supplemented with 100 UI/ml penicillin and 100  $\mu$ g/ml streptomycin (all from Life Technologies, Carlsbad, CA, USA) and supplemented with either 5% AS or 5% AB HS. The cells were stimulated for 48 h in the presence of anti-CD3/anti-CD28-coated Dynabeads (0.5 bead per cell; Invitrogen). On the last day, [<sup>3</sup>H]thymidine (0.5  $\mu$ Ci per well; Amersham Pharmacia Biotech, Piscataway, NJ, USA) was added to the cultures, and the

Abbreviations: AS = autologous subject serum, BMI = body mass index, CRP = C-reactive protein, DMARDs = disease-modifying antirheumatic drugs, ESR = erythrocyte sedimentation rate, HC = healthy control, HP-TRAPS = high penetrance TRAPS, HS = human heterologous serum, LP-TRAPS = low penetrance TRAPS, mTOR = mammalian target of rapamycin, SAA = serum amyloid A,  $T_{reg}$  = regulatory T cell,  $T_{conv}$  = conventional T cells, TNFRSF1A = TNF receptor superfamily 1A, TRAPS = TNFR-associated periodic syndrome

**TABLE 1. Clinical and demographic characteristics of patients with TRAPS with high- and low-penetrance *TNFRSF1A* mutations (at sample collection)**

Characteristic	HP-TRAPS ( <i>n</i> = 20)	LP-TRAPS ( <i>n</i> = 15)
Age (y)		
Median	44	36
Range	9–71	8–56
Gender ( <i>n</i> )		
Male	8	7
Female	12	8
Disease onset (y)		
Median	6	15
Range	1–49	3–55
Fever attacks annually ( <i>n</i> )		
Median	5	2
Range	0–14	0–15
Duration of fever attacks (d)		
Median	8	8
Range	0–20	0–25
ESR (mm/h)		
Median	6	12
Range	1–45	1–31
CRP (mg/dl)		
Median	0.65	0.31
Range	0–11.4	0–9
SAA (mg/L)		
Median	5.70	18.5
Range	0.05–1734	17.8–224.8
BMI (kg/m <sup>2</sup> )		
Median	23.6	23.4
Range	19.0–28.0	21.3–28.7
Amyloidosis ( <i>n</i> )	5/20	0/15
Patients with chronic course ( <i>n</i> )	4/20	6/15
Patients treated with anti-IL-1 ( <i>Canakinumab</i> or <i>Anakinra</i> ) <sup>a</sup>	15/20	5/15
Patients naïve to anti-IL-1 (NSAIDs or corticosteroids on demand)	5/20	10/15

NSAIDs = nonsteroidal anti-inflammatory drugs. <sup>a</sup>All samples were collected during a remission phase of the disease at a routine follow-up visit. For patients treated with *Canakinumab* (anti-IL-1 $\beta$ , half-life 26 d), the blood sample was taken 8 wk after biologic drug administration. For patients treated with *Anakinra* (anti-IL-1 receptor A, half-life range 4–6 h), the blood sample was taken 12 h after biologic drug administration. No patient was taking steroids at the time of blood sampling.

cells were harvested after 12 h. Radioactivity was measured using a  $\beta$ -cell plate scintillation counter (Wallac; PerkinElmer, Turku, Finland).

## Flow cytometry

On freshly isolated PBMCs from patients with TRAPS and HCs, we performed FACS analysis (BD FACS-Canto). To stain isolated T<sub>reg</sub> cells, we used, in a 1: 50–100 dilution, APC-H7-labeled anti-CD4 (BD Biosciences). Thereafter, we washed, fixed, and permeabilized cells (fixation-permeabilization buffer; eBioscience) and stained with PE-labeled anti-FoxP3 (eBioscience) and FITC-labeled anti-Ki67 (BD Biosciences) monoclonal antibodies. We performed the analyses with Diva software from BD Biosciences and FlowJo software (Tree Star, Ashland, OR, USA).

## Molecular signaling and Western blot analyses

T<sub>conv</sub>s ( $3 \times 10^5$ ) were lysed in cold radio-immuno-precipitation assay buffer (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS, and protease and phosphatase inhibitors) for 15 min at 4°C. The lysate was centrifuged (10,000 *g* for 10 min). The supernatant was collected, quantified, and resuspended in electrophoresis sample buffer, heated to 95°C for 5 min and resolved on a SDS 8% polyacrylamide gel. Western blot with specific antibodies was used according to the protocol

provided by the supplier. For all Western blots shown, 8  $\mu$ g of total protein extracts were loaded. The antibodies used were anti-phospho-STAT1 and anti-STAT1, anti-phospho-STAT3, anti-STAT3, anti-phospho-STAT5, anti-STAT5, anti-phospho-S6, and anti-S6 (Cell Signaling Technology, Beverly, MA, USA); and anti-ERK1/2, anti-phospho-ERK1/2, anti-NF- $\kappa$ B p65, and anti-actin (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA).

## Statistical analysis

We used nonparametric Mann-Whitney *U* or Student's *t* tests. Correlation analyses were performed using Spearman's correlation coefficients. The statistical software used was GraphPad InStat3, version 4.0 (GraphPad Software, Inc., San Diego, CA, USA). The results are expressed as the mean  $\pm$  SEM. *P*  $\leq$  .05 was considered statistically significant.

## RESULTS

### Selective increase of CD4<sup>+</sup> T cells in patients with HP-TRAPS

To evaluate the immunologic profile of the patients with TRAPS, we analyzed several immune cell subpopulations in the

peripheral blood from HP-TRAPS ( $n = 17$ ), LP-TRAPS ( $n = 13$ ), and HC ( $n = 32$ ) samples. The HP-TRAPS samples had a significantly higher number of total lymphocytes, CD3<sup>+</sup> T cells, and CD4<sup>+</sup> T cells and a significantly higher frequency of CD3<sup>+</sup> T cells with a memory phenotype (CD45RO<sup>+</sup>). In addition, CD4<sup>+</sup>CD45RO<sup>+</sup> and CD8<sup>+</sup>CD45RA<sup>+</sup> T cells were higher in the HP-TRAPS samples than in the HC samples. Furthermore, significant differences were observed for the absolute number of circulating CD4<sup>+</sup>CD28<sup>+</sup> and CD4<sup>+</sup>CD25<sup>+</sup> cells, with increases in the HP-TRAPS samples compared with the HC samples (Fig. 1 and Supplemental Table 1). In contrast, the LP-TRAPS samples had a higher number of CD3<sup>+</sup>, CD4<sup>+</sup>, and CD8<sup>+</sup> T cells with a naïve phenotype (CD45RA<sup>+</sup>) compared with the HC samples (Fig. 1 and Supplemental Table 1). Finally, differences between the patients with HP- and LP-TRAPS were found in the absolute number of total CD4<sup>+</sup> T cells and CD3<sup>+</sup> and CD4<sup>+</sup> T cells with a memory phenotype (CD45RO<sup>+</sup>) and in circulating CD4<sup>+</sup>CD28<sup>+</sup> T cells (Fig. 1 and Supplemental Table 1). Finally, the frequency of total CD4<sup>+</sup>CD45RO<sup>+</sup> T cells and total CD3<sup>+</sup>CD45RO<sup>+</sup> T cells did not correlate with the SAA levels in both HP- and LP-TRAPS (Supplemental Fig. 1).

### Impaired T cell proliferation and cytokine secretion after TCR stimulation in patients with HP-TRAPS

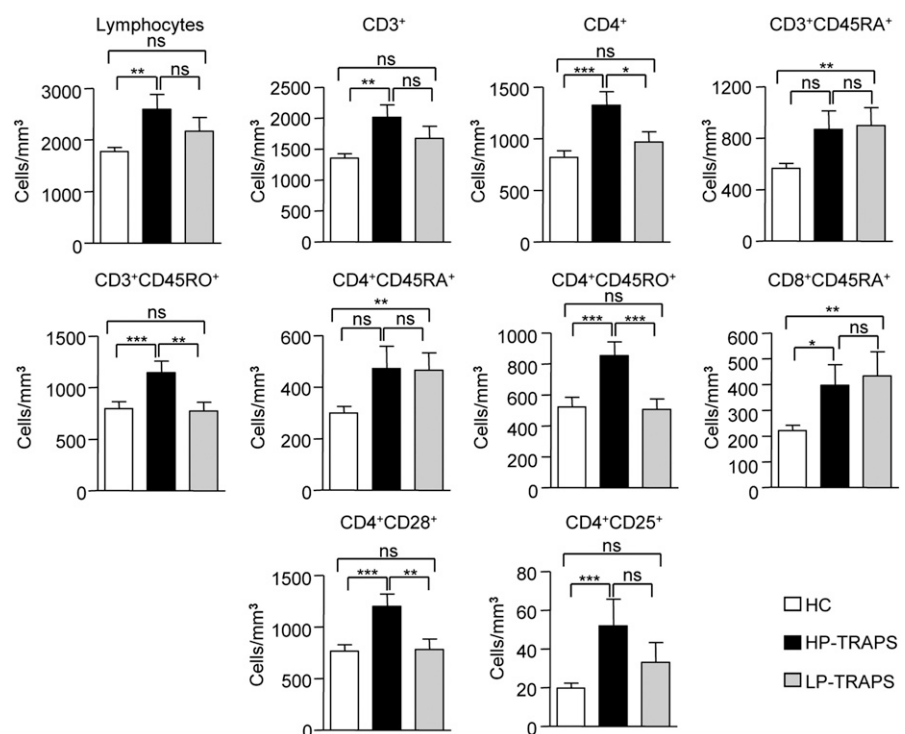
We evaluated the T cell proliferative profile in PBMCs from HP-TRAPS, LP-TRAPS, and HC in culture medium supplemented with either 5% AS or 5% pooled HS (Fig. 2A) to understand whether soluble serum factors (either an endogenous factor or related to the drug treatment received by the patients) could affect the T cell responses in vitro. PBMCs isolated from TRAPS and HC samples were stimulated by physiologic TCR specific

stimuli (anti-CD3–OKT3 mAbs). We found a reduced proliferation in HP-TRAPS samples compared with HC samples in both HS- and AS-supplemented culture medium (Fig. 2A). No significant difference was found between LP-TRAPS and HC samples or between HP-TRAPS and LP-TRAPS samples (Fig. 2A).

We next measured the amount of several pro- and anti-inflammatory cytokines in T cell-derived supernatants. The HP-TRAPS samples showed lower levels of IL-5 and IFN- $\gamma$  in both HS- and AS-supplemented culture conditions. Lower levels of TNF- $\alpha$ , IL-1 $\beta$ , IL-6, and IL-12p70 were observed only in the AS-supplemented medium. In addition, a significantly higher concentration of IL-8 was observed in the HP-TRAPS samples compared with the HC samples in both HS- and AS-supplemented medium. Finally, HP-TRAPS showed decreased levels of TNF- $\alpha$ , IL-1 $\beta$ , IL-5, and IL-12p70 in AS-supplement medium, a lower amount of IFN- $\gamma$  in HS-supplemented medium, and reduced IL-5 in both HS- and AS-supplemented media compared with LP-TRAPS. No significant difference was observed in cytokine production between LP-TRAPS and HC samples in both HS- and AS-supplemented media (Fig. 2B–J).

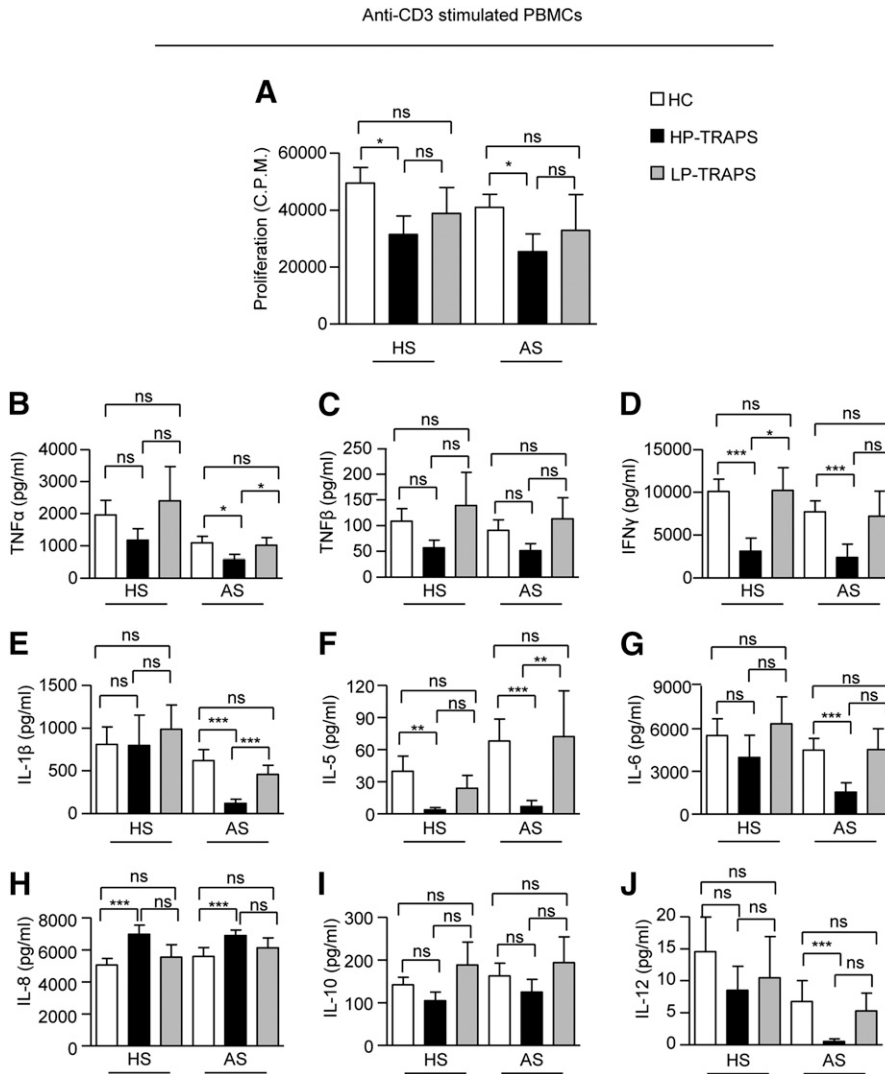
### Increased T<sub>conv</sub> proliferation in patients with HP-TRAPS

To better understand the effect of *TNFRSF1A* mutation on different CD4<sup>+</sup> T cell subsets, we focused our attention on T<sub>conv</sub> (CD4<sup>+</sup>CD25<sup>−</sup>) cells, which are increased in several autoimmune and inflammatory disorders [34]. Specifically, we evaluated TRAPS T<sub>conv</sub> proliferation during anti-CD3/anti-CD28 stimulation and observed that the HP-TRAPS T<sub>conv</sub> proliferative rate was higher than that of the HC and LP-TRAPS (Fig. 3A). In addition, we found that HP-TRAPS T<sub>conv</sub>s showed higher expression of



**Figure 1. Immunophenotype of patients with TRAPS with low and high penetrance *TNFRSF1A* variants.** HP-TRAPS ( $n = 17$ ; 2 with C43R, 1 with C43T, 7 with T50M, 2 with C52Y, 2 with C125R, 1 with C55Y, 1 with S59P, and 1 with Del 103-104); LP-TRAPS ( $n = 13$ ; 10 with R92Q, 1 with R104Q, 1 with D12E, and 1 with V50M); and HCs ( $n = 32$ ). Data are expressed as mean  $\pm$  SEM. Patients with HP-TRAPS treated with anti-IL-1,  $n = 13$ ; patients with LP-TRAPS treated with anti-IL-1,  $n = 5$ . Statistical significance was determined using the Mann-Whitney nonparametric  $U$  test. \* $P \leq 0.05$ , \*\* $P \leq 0.03$ , \*\*\* $P \leq 0.01$ .





**Figure 2. PBMC proliferative profile and cytokine release in patients with TRAPS and HCs during anti-CD3 stimulation.** (A) Proliferation (counts per minute [C.P.M.]-[<sup>3</sup>H] incorporation) and (B–J) cytokine production (pg/ml) of PBMCs isolated from patients with TRAPS with high ( $n = 10$ ) and low ( $n = 7$ ) penetrance *TNFRSF1A* mutation and HCs ( $n = 16$ ) after TCR-mediated stimulation (anti-CD3) in medium supplemented with HS or AS. Patients with HP-TRAPS treated with anti-IL-1,  $n = 9$ ; patients with LP-TRAPS treated with anti-IL-1,  $n = 4$ . Data are expressed as mean  $\pm$  SEM. Statistical significance was determined using the Mann-Whitney nonparametric *U* test. \* $P \leq 0.05$ , \*\* $P \leq 0.03$ , \*\*\* $P \leq 0.01$ .

several intracellular pathways related to proliferation and activation, such as ERK1/2, STAT1, STAT3, STAT5, and NF- $\kappa$ B p65 compared with HC  $T_{conv}$ s. In contrast, in the LP-TRAPS samples, we found that only the expression of STAT1 was higher (Fig. 3B). Finally, we analyzed S6 activation, a downstream target of the mTOR. Its phosphorylation was significantly higher in HP-TRAPS than in HC samples, although no significant difference was observed between the LP-TRAPS and HC samples (Fig. 3B).

### Decreased $T_{reg}$ frequency and suppressive function in patients with HP-TRAPS

To obtain further insights into the mechanisms involved in a possible break of self-tolerance in TRAPS, we also analyzed the  $T_{reg}$  compartment. It has been well delineated that  $T_{reg}$ s display a central role in the regulation of immune homeostasis by reducing the inflammatory responses and suppressing  $T_{conv}$  functions [35, 36]. We found that those with HP-TRAPS had a lower frequency of CD4<sup>+</sup>FoxP3<sup>+</sup>  $T_{reg}$ s than that of the HCs, although no difference was found between those with HP-TRAPS and those with LP-TRAPS or between those with LP-TRAPS and

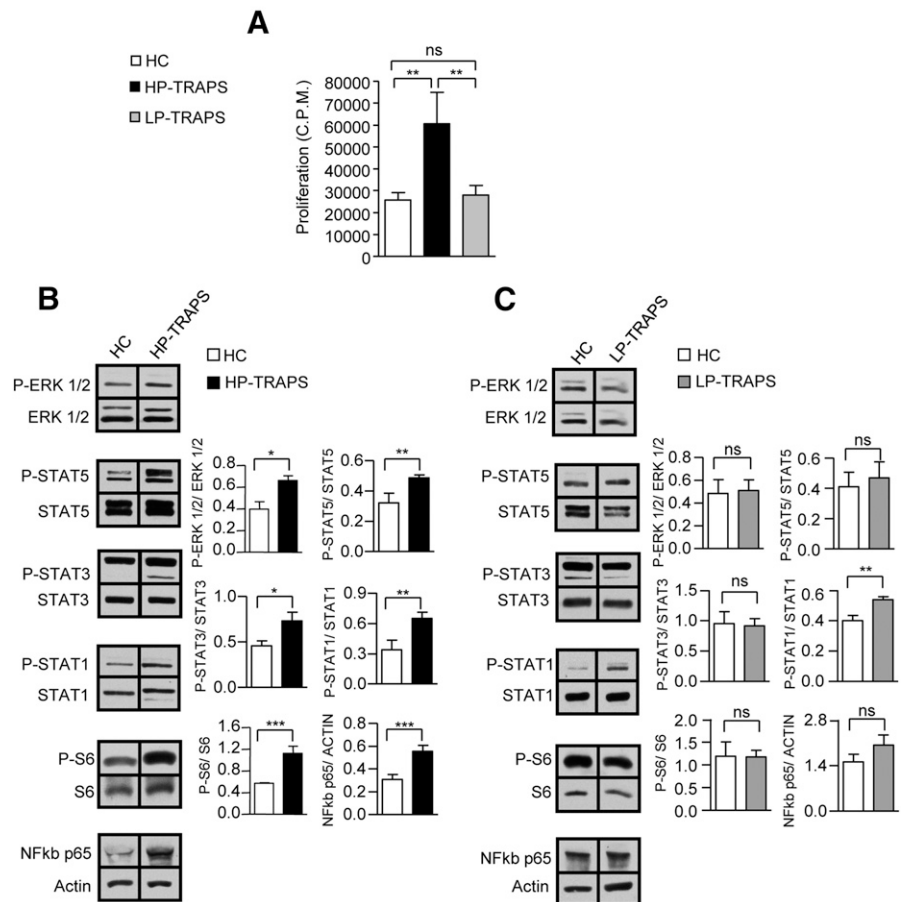
HCs (Fig. 4A and B). In addition, HP-TRAPS  $T_{reg}$ s showed a reduced ex vivo proliferation rate, suggested by the lower expression of Ki67 compared with HC and LP-TRAPS  $T_{reg}$ s. No difference was found between LP-TRAPS and HC  $T_{reg}$ s (Fig. 4C and D). In contrast, HP-TRAPS  $T_{reg}$ s exhibited a higher proliferative rate in vitro during anti-CD3/anti-CD28 stimulation compared with TRAPS-LP and HC  $T_{reg}$ s, although the difference did not reach statistical significance ( $P = 0.06$ ; Fig. 4E).

Finally, we analyzed the suppressive capacity of  $T_{reg}$ s isolated from HP-TRAPS, LP-TRAPS, and HC samples by evaluating  $T_{conv}$  proliferation when cocultured with  $T_{reg}$ s. HP-TRAPS  $T_{reg}$ s showed reduced suppressive capacity compared with LP-TRAPS and HC  $T_{reg}$ s. In contrast, no significant difference was observed in LP-TRAPS  $T_{reg}$ s compared with HC  $T_{reg}$ s (Fig. 4E and F). A similar trend was also detected in HS medium, although the difference was not significant (Supplemental Fig. 2).

## DISCUSSION

In the present study, we found that the *TNFRSF1A* mutation strongly affects the adaptive immune compartment, suggesting a

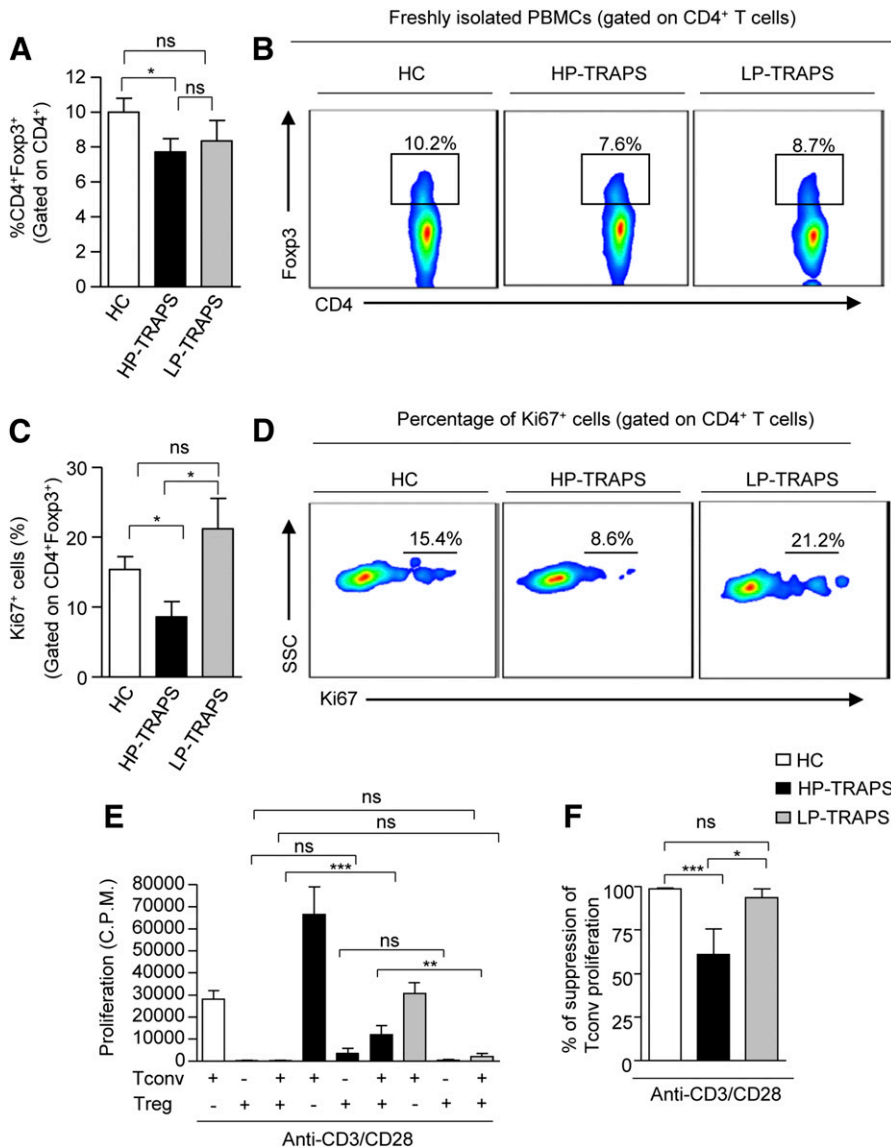
**Figure 3. Enhanced activation and proliferation of T<sub>conv</sub>s in HP-TRAPS associated with increased STAT-mTOR signaling.** (A) Proliferation (counts per minute [C.P.M.]-[<sup>3</sup>H] incorporation) of anti-CD3/CD28-stimulated T<sub>conv</sub>s from HP-TRAPS ( $n = 5$ ; 2 with C52Y, 1 with T50M, 1 with DEL103-104, and 1 with S59P), LP-TRAPS ( $n = 5$ ; 3 with R92Q, 1 with D12E, and 1 with R104Q) and HC ( $n = 7$ ). Cells were cultured in 5% AS medium and stimulated with 0.5 bead per cell for 48 h. (B and C) Western blot analysis of p-ERK1/2, p-STAT1, p-STAT3, p-STAT5, NF- $\kappa$ B p65, and p-S6 in 24-h anti-CD3/CD28-stimulated T<sub>conv</sub>s in HP-TRAPS, LP-TRAPS, and HCs. Cells were cultured in 5% AS-supplemented medium and stimulated with 0.2 bead per cell. Data are representative of  $\geq 3$  independent experiments and are expressed as mean  $\pm$  SEM. Patients with HP-TRAPS treated with anti-IL-1,  $n = 4$ ; patients with LP-TRAPS treated with anti-IL-1,  $n = 2$ . Statistical significance was determined using unpaired Student's  $t$  test. \* $P \leq 0.05$ , \*\* $P \leq 0.03$ .



key role for T cells in the pathogenesis of TRAPS. Patients with HP-TRAPS had a selective increase in CD4<sup>+</sup> T cells that displayed a memory (CD4<sup>+</sup>CD45RO<sup>+</sup>) and an effector phenotype, with overexpressed markers of T cell activation such as the CD25 (known as the IL-2R $\alpha$  chain) and CD28. This is a classic picture of autoimmune disorders, which has been described as a peripheral conversion of naïve CD4<sup>+</sup> T cells into a memory phenotype. HP-TRAPS showed this peripheral conversion, suggesting the involvement of these cells in the pathophysiology of this autoinflammatory syndrome. Furthermore, the frequency of peripheral memory CD3<sup>+</sup>CD45RO<sup>+</sup> and CD4<sup>+</sup>CD45RO<sup>+</sup> T cells did not correlate with either serum SAA levels (Supplemental Fig. 1) or ESR and CRP levels (data not shown) in both HP- and LP-TRAPS, suggesting that the observed immunologic profile was likely to be ascribed to an altered TNFR signaling pathway, irrespective of the inflammatory milieu and disease activity [34]. In line with this evidence, all patients were in a remission phase of the disease when were enrolled in the present study and undergoing blood sampling.

We next evaluated TRAPS PBMC function during TCR-dependent stimulation, which selectively stimulates the whole T cell compartment. However, HP-TRAPS T cells displayed a reduced proliferative rate compared with HC T cells in either HS- or AS-supplemented culture medium. This result probably could have resulted from an impairment of T cell antigen

presentation/costimulation in the context of PBMCs, secondary to the TNFRSF1A signaling. Our hypothesis is related to the finding that anti-CD3-mediated T cell proliferation of PBMCs needs the presence of abundant APCs, which must carry on their surface MHC-II and costimulatory molecules, whose expression is linked to TNFR signaling [37]. Clearly, further studies are needed to better explain this phenomenon. In addition, in line with this result, we found that anti-CD3-stimulated HP-TRAPS PBMCs secreted lower amounts of several cytokines, indicating impaired activation. Surprisingly, the only cytokine increased in HP-TRAPS anti-CD3-stimulated PBMC supernatants was IL-8, a well-known cytokine involved in neutrophil activation and chemotaxis, confirming the involvement of these cells in the pathogenesis of TRAPS, as previously reported [21, 38]. In line with this evidence, we found in HP-TRAPS serum samples, high levels of myeloperoxidase (data not shown). Finally, we ruled out that the medications present in TRAPS serum could have altered the T cell-mediated responses, because no differences were observed in the T cell proliferation or cytokine profiles between the culture media supplemented with either AS or HS. Moreover, we did not observe specific relationships with therapy in all performed experiments. Also, no significant difference was observed in T cell proliferation, cytokine profile, or signaling pathways between the patients naïve to treatment and the patients with anti-IL-1-treated HP- and LP-TRAPS (data not shown).



**Figure 4. Reduced T<sub>reg</sub> number and suppressive function in patients with HP-TRAPS.** (A) Foxp3 expression in freshly isolated PBMCs from HP-TRAPS ( $n = 15$ ; 2 with C43R, 1 with C43T, 2 with C52Y, 2 with C125R, 6 with T50M, 1 with DEL103-104, and 1 with S59P), LP-TRAPS ( $n = 12$ ; 9 with R92Q, 1 with R104Q, 1 with D12E, and 1 with V95M), and HCs ( $n = 15$ ). PBMCs were stained for CD4 and Foxp3 expression. (B) Representative flow cytometry plots of Foxp3 expression in freshly isolated PBMCs from HP-TRAPS, LP-TRAPS, and HC samples. Patients with HP-TRAPS treated with anti-IL-1,  $n = 12$ ; patients with LP-TRAPS treated with anti-IL-1,  $n = 5$ . (C) Ki67 expression in freshly isolated T<sub>regs</sub> defined by gating CD4<sup>+</sup>Foxp3<sup>+</sup> cells from HCs ( $n = 11$ ), those with HP-TRAPS ( $n = 8$ ; 2 with C125R, 4 with T50M, 1 with DEL103-104, and 1 with S59P), and those with LP-TRAPS ( $n = 5$ ; 4 with R92Q and 1 with R104Q). (D) Representative flow cytometry plots of Ki67 expression in freshly isolated PBMCs gated on CD4<sup>+</sup>Foxp3<sup>+</sup> from HP-TRAPS, LP-TRAPS, and HC samples. Patients with HP-TRAPS treated with anti-IL-1,  $n = 5$ ; patients with LP-TRAPS treated with anti-IL-1,  $n = 2$ . (E) Proliferation of anti-CD3/CD28 stimulated T<sub>conv</sub>s and T<sub>regs</sub> from HP-TRAPS ( $n = 5$ ; 2 with C52Y, 1 with T50M, 1 with DEL103-104, and 1 with S59P), LP-TRAPS ( $n = 5$ ; 4 with R92Q and 1 with D12E), and HC ( $n = 7$ ) samples, alone or in coculture. (F) Percentage of suppression of T<sub>conv</sub> proliferation when in coculture with T<sub>conv</sub>s. The T<sub>reg</sub>:T<sub>conv</sub> ratio in the suppression experiments was 1:1. Cells were cultured in 5% AS-supplemented medium and stimulated with anti-CD3/CD28 (0.5 bead per cell) for 48 h. Comparisons were evaluated using the nonparametric Mann-Whitney  $U$  test. Patients with HP-TRAPS treated with anti-IL-1,  $n = 4$ ; patients with LP-TRAPS treated with anti-IL-1,  $n = 1$ . Data are reported as the mean  $\pm$  SEM. \* $P \leq 0.05$ , \*\* $P \leq 0.03$ .

To provide a novel mechanism of inflammation as the basis of TRAPS, we also analyzed the functional aspects of isolated T<sub>conv</sub>s and T<sub>regs</sub>. We found an enhanced T<sub>conv</sub> proliferative response in those with HP-TRAPS associated with a high expression of multiple inflammatory signaling pathways, such as STAT1, STAT5, STAT3, ERK1/2, NF- $\kappa$ B p65, and S6, irrespective of the proinflammatory milieu, as previously described in TRAPS PBMCs [39]. These results could be explained by a “constitutive activation,” induced by mutant TNFR1, of these inflammatory intracellular signaling pathways. This evidence could, in part, justify the diverse pathophysiology of TRAPS and the limited efficacy of cytokine-blocking biologic agents. Furthermore, LP-TRAPS T<sub>conv</sub>s had an intermediate/mild phenotype, because they behaved very similarly to HC T<sub>conv</sub>s, showing only elevated expression of STAT1. This latter result is of high interest, because it has been reported that STAT1 signaling plays a central role in IL-1 $\beta$  activation [40] and helps to clarify why patients with symptomatic LP-TRAPS benefit from anti-IL-1 treatment. In

summary, although the whole T cell population of PBMCs showed a reduced proliferative rate during anti-CD3 stimulation, when CD4<sup>+</sup> T<sub>conv</sub>s were isolated from PBMCs, they showed a higher proliferative phenotype secondary to enhanced mTOR-STAT-NF- $\kappa$ B signaling.

On the side of immune tolerance, the patients with HP-TRAPS showed a reduced frequency of peripheral CD4<sup>+</sup>FoxP3<sup>+</sup> T<sub>regs</sub>, which expressed a defective suppressive capacity compared with LP-TRAPS and HC T<sub>regs</sub>. One of the immune abnormalities observed in autoimmune disorders is a reduction in the suppressive function and/or number of natural T<sub>regs</sub> [41–43]. Under normal circumstances, T<sub>regs</sub> are typically hyporesponsive to TCR stimulation in vitro and highly proliferative in vivo. Moreover, it has been well delineated that in vitro T<sub>reg</sub> anergy (a state of hyporesponsiveness to antigenic stimulation) is crucial for an optimal suppressive function [35]. In our study, we observed that HP-TRAPS T<sub>regs</sub> had reduced anergy in vitro (higher proliferation after TCR stimulation) and a lower

expression of Ki67<sup>+</sup> T<sub>regs</sub> ex vivo that could explain their impaired suppressive function and higher T<sub>conv</sub> proliferative response. These alterations were not found in patients with LP-TRAPS. In this context, it has recently been shown that TNFR1 is crucial for T<sub>reg</sub> functions; thus, alterations involving the expression of this receptor on the T cell surface might promote inflammatory responses [43], and our data are in agreement with this recent finding.

Taken together, our report has unveiled a novel mechanism of inflammation in TRAPS involving CD4<sup>+</sup> T cells (both T<sub>conv</sub>s and T<sub>regs</sub>) and reveals a novel scenario in which a dysfunction in the adaptive immune cell compartment is present in the context of autoinflammatory disorders, previously ascribed mainly to an altered innate immune response. Thus, it is possible to speculate that autoinflammatory syndromes and autoimmune diseases share a series of common features never reported before.

## AUTHORSHIP

V.P., O.M.L., L.C., and G. Matarese conceived and designed the study; L.O., G. Merlini, M.C., F.L.T., R.T., M.C.M., F.M., and O.M.L. enrolled the subjects and patients and collected the clinical data; V.P., O.M.L., M.T.L., V.D.R., F.P., and M. Galgani performed the experiments, analysis, and interpretation of the data; G. Marone and M. Galeazzi performed critical reading of the manuscript; V.P. and G. Matarese wrote the manuscript; L.C. and G. Matarese revised and approved the final manuscript.

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## DISCLOSURES

The authors declare no conflicts of interest.

## REFERENCES

- Beutler, B., Cerami, A. (1989) The biology of cachectin/TNF—a primary mediator of the host response. *Annu. Rev. Immunol.* **7**, 625–655.
- Ware, C. F., Crowe, P. D., Grayson, M. H., Androlewicz, M. J., Browning, J. L. (1992) Expression of surface lymphotoxin and tumor necrosis factor on activated T, B, and natural killer cells. *J. Immunol.* **149**, 3881–3888.
- Vassalli, P. (1992) The pathophysiology of tumor necrosis factors. *Annu. Rev. Immunol.* **10**, 411–452.
- Gordon, J. R., Galli, S. J. (1990) Mast cells as a source of both preformed and immunologically inducible TNF- $\alpha$ /cachectin. *Nature* **346**, 274–276.
- Bazzoni, F., Beutler, B. (1996) The tumor necrosis factor ligand and receptor families. *N. Engl. J. Med.* **334**, 1717–1725.
- Hehlgans, T., Pfeffer, K. (2005) The intriguing biology of the tumour necrosis factor/tumour necrosis factor receptor superfamily: players, rules and the games. *Immunology* **115**, 1–20.
- Micheau, O., Tschopp, J. (2003) Induction of TNF receptor I-mediated apoptosis via two sequential signaling complexes. *Cell* **114**, 181–190.
- Muppidi, J. R., Tschopp, J., Siegel, R. M. (2004) Life and death decisions: secondary complexes and lipid rafts in TNF receptor family signal transduction. *Immunity* **21**, 461–465.
- Wajant, H., Scheurich, P. (2011) TNFR1-induced activation of the classical NF- $\kappa$ B pathway. *FEBS J.* **278**, 862–876.
- McDermott, M. F., Aksentjevich, I., Galon, J., McDermott, E. M., Ogunkolade, B. W., Centola, M., Mansfield, E., Gadina, M., Karenko, L., Pettersson, T., McCarthy, J., Frucht, D. M., Aringer, M., Torosyan, Y., Teppo, A. M., Wilson, M., Karaarslan, H. M., Wan, Y., Todd, I., Wood, G., Schlimgen, J. R., Kumarajew, T. R., Cooper, S. M., Vella, J. P., Amos, C. L., Mulley, J., Quane, K. A., Molloy, M. G., Ranki, A., Powell, R. J., Hitman, G. A., O'Shea, J. J., Kastner, D. L. (1999) Germline mutations in the extracellular domains of the 55 kDa TNF receptor, TNFR1, define a family of dominantly inherited autoinflammatory syndromes. *Cell* **97**, 133–144.
- Williamson, L. M., Hull, D., Mehta, R., Reeves, W. G., Robinson, B. H., Toghill, P. J. (1982) Familial Hibernian fever. *Q. J. Med.* **51**, 469–480.
- Masters, S. L., Simon, A., Aksentjevich, I., Kastner, D. L. (2009) Horror autoinflammaticus: the molecular pathophysiology of autoinflammatory disease (\*). *Annu. Rev. Immunol.* **27**, 621–668.
- Aganna, E., Hammond, L., Hawkins, P. N., Aldea, A., McKee, S. A., van Amstel, H. K., Mischung, C., Kusuhara, K., Saulsbury, F. T., Lachmann, H. J., Bybee, A., McDermott, E. M., La Regina, M., Arostegui, J. I., Campistol, J. M., Worthington, S., High, K. P., Molloy, M. G., Baker, N., Bidwell, J. L., Castañer, J. L., Whiteford, M. L., Janssens-Korpola, P. L., Manna, R., Powell, R. J., Woo, P., Solis, P., Minden, K., Frenkel, J., Yagüe, J., Mirakian, R. M., Hitman, G. A., McDermott, M. F. (2003) Heterogeneity among patients with tumor necrosis factor receptor-associated periodic syndrome phenotypes. *Arthritis Rheum.* **48**, 2632–2644.
- Aksentjevich, I., Galon, J., Soares, M., Mansfield, E., Hull, K., Oh, H. H., Goldbach-Mansky, R., Dean, J., Athreya, B., Reginato, A. J., Henrickson, M., Pons-Estel, B., O'Shea, J. J., Kastner, D. L. (2001) The tumor-necrosis-factor receptor-associated periodic syndrome: new mutations in TNFRSF1A, ancestral origins, genotype-phenotype studies, and evidence for further genetic heterogeneity of periodic fevers. *Am. J. Hum. Genet.* **69**, 301–314.
- Hull, K. M., Drewe, E., Aksentjevich, I., Singh, H. K., Wong, K., McDermott, E. M., Dean, J., Powell, R. J., Kastner, D. L. (2002) The TNF receptor-associated periodic syndrome (TRAPS): emerging concepts of an autoinflammatory disorder. *Medicine (Baltimore)* **81**, 349–368.
- Dodé, C., André, M., Bienvenu, T., Hausfater, P., Pêcheux, C., Bienvenu, J., Lecron, J. C., Reinert, P., Cattani, D., Piette, J. C., Szajnert, M. F., Delpech, M., Grateau, G.; French Hereditary Recurrent Inflammatory Disorder Study Group. (2002) The enlarging clinical, genetic, and population spectrum of tumor necrosis factor receptor-associated periodic syndrome. *Arthritis Rheum.* **46**, 2181–2188.
- Cantarini, L., Lucherini, O. M., Baldari, C. T., Laghi Pasini, F., Galeazzi, M. (2010) Familial clustering of recurrent pericarditis may disclose tumour necrosis factor receptor-associated periodic syndrome. *Clin. Exp. Rheumatol.* **28**, 405–407.
- Cantarini, L., Lucherini, O. M., Brucato, A., Barone, L., Cumetti, D., Iacoponi, F., Rigante, D., Brambilla, G., Penco, S., Brizi, M. G., Patrosso, M. C., Valesini, G., Frediani, B., Galeazzi, M., Cimaz, R., Paolazzi, G., Vitale, A., Imazio, M. (2012) Clues to detect tumor necrosis factor receptor-associated periodic syndrome (TRAPS) among patients with idiopathic recurrent acute pericarditis: results of a multicentre study. *Clin. Res. Cardiol.* **101**, 525–531.
- Cantarini, L., Lucherini, O. M., Cimaz, R., Galeazzi, M. (2010) Recurrent pericarditis caused by a rare mutation in the TNFRSF1A gene and with excellent response to anakinra treatment. *Clin. Exp. Rheumatol.* **28**, 802.
- Touitou, I., Lesage, S., McDermott, M., Cuisset, L., Hoffman, H., Dode, C., Shoham, N., Aganna, E., Hugot, J. P., Wise, C., Waterham, H., Pugnere, D., Demaille, J., Sarraute de Menthiere, C. (2004) Infervers: an evolving mutation database for auto-inflammatory syndromes. *Hum. Mutat.* **24**, 194–198.
- D'Osualdo, A., Ferlito, F., Prigione, I., Obici, L., Meini, A., Zulian, F., Pontillo, A., Corona, F., Barcellona, R., Di Duca, M., Santamaria, G., Traverso, F., Picco, P., Baldi, M., Plebani, A., Ravazzolo, R., Ceccherini, I., Martini, A., Gattorno, M. (2006) Neutrophils from patients with TNFRSF1A mutations display resistance to tumor necrosis factor-induced apoptosis: pathogenetic and clinical implications. *Arthritis Rheum.* **54**, 998–1008.
- Obici, L., Merlini, G. (2012) Amyloidosis in autoinflammatory syndromes. *Autoimmun. Rev.* **12**, 14–17.
- Ravet, N., Rouaghe, S., Dodé, C., Bienvenu, J., Stirnemann, J., Lévy, P., Delpech, M., Grateau, G. (2006) Clinical significance of P46L and R92Q



- substitutions in the tumour necrosis factor superfamily 1A gene. *Ann. Rheum. Dis.* **65**, 1158–1162.
24. Lobito, A. A., Kimberley, F. C., Muppidi, J. R., Komarow, H., Jackson, A. J., Hull, K. M., Kastner, D. L., Sreaton, G. R., Siegel, R. M. (2006) Abnormal disulfide-linked oligomerization results in ER retention and altered signaling by TNFR1 mutants in TNFR1-associated periodic fever syndrome (TRAPS). *Blood* **108**, 1320–1327.
  25. Nedjai, B., Hitman, G. A., Yousaf, N., Chernajovsky, Y., Stjernberg-Salmela, S., Pettersson, T., Ranki, A., Hawkins, P. N., Arkwright, P. D., McDermott, M. F., Turner, M. D. (2008) Abnormal tumor necrosis factor receptor I cell surface expression and NF-kappaB activation in tumor necrosis factor receptor-associated periodic syndrome. *Arthritis Rheum.* **58**, 273–283.
  26. Yousaf, N., Gould, D. J., Aganna, E., Hammond, L., Mirakian, R. M., Turner, M. D., Hitman, G. A., McDermott, M. F., Chernajovsky, Y. (2005) Tumor necrosis factor receptor I from patients with tumor necrosis factor receptor-associated periodic syndrome interacts with wild-type tumor necrosis factor receptor I and induces ligand-independent NF-kappaB activation. *Arthritis Rheum.* **52**, 2906–2916.
  27. Nedjai, B., Hitman, G. A., Quillinan, N., Coughlan, R. J., Church, L., McDermott, M. F., Turner, M. D. (2009) Proinflammatory action of the antiinflammatory drug infliximab in tumor necrosis factor receptor-associated periodic syndrome. *Arthritis Rheum.* **60**, 619–625.
  28. Churchman, S. M., Church, L. D., Savic, S., Coulthard, L. R., Hayward, B., Nedjai, B., Turner, M. D., Mathews, R. J., Baguley, E., Hitman, G. A., Gooi, H. C., Wood, P. M., Emery, P., McDermott, M. F. (2008) A novel TNFRSF1A splice mutation associated with increased nuclear factor kappaB (NF-kappaB) transcription factor activation in patients with tumour necrosis factor receptor associated periodic syndrome (TRAPS). *Ann. Rheum. Dis.* **67**, 1589–1595.
  29. Bachetti, T., Chiesa, S., Castagnola, P., Bani, D., Di Zanni, E., Omenetti, A., D'Osualdo, A., Fraldi, A., Ballabio, A., Ravazzolo, R., Martini, A., Gattorno, M., Ceccherini, I. (2013) Autophagy contributes to inflammation in patients with TNFR-associated periodic syndrome (TRAPS). *Ann. Rheum. Dis.* **72**, 1044–1052.
  30. Simon, A., Park, H., Maddipati, R., Lobito, A. A., Bulua, A. C., Jackson, A. J., Chae, J. J., Ettinger, R., de Koning, H. D., Cruz, A. C., Kastner, D. L., Komarow, H., Siegel, R. M. (2010) Concerted action of wild-type and mutant TNF receptors enhances inflammation in TNF receptor 1-associated periodic fever syndrome. *Proc. Natl. Acad. Sci. USA* **107**, 9801–9806.
  31. Bulua, A. C., Simon, A., Maddipati, R., Pelletier, M., Park, H., Kim, K. Y., Sack, M. N., Kastner, D. L., Siegel, R. M. (2011) Mitochondrial reactive oxygen species promote production of proinflammatory cytokines and are elevated in TNFR1-associated periodic syndrome (TRAPS). *J. Exp. Med.* **208**, 519–533.
  32. Dickie, L. J., Aziz, A. M., Savic, S., Lucherini, O. M., Cantarini, L., Geiler, J., Wong, C. H., Coughlan, R., Lane, T., Lachmann, H. J., Hawkins, P. N., Robinson, P. A., Emery, P., McGonagle, D., McDermott, M. F. (2012) Involvement of X-box binding protein 1 and reactive oxygen species pathways in the pathogenesis of tumour necrosis factor receptor-associated periodic syndrome. *Ann. Rheum. Dis.* **71**, 2035–2043.
  33. Rebelo, S. L., Bainbridge, S. E., Amel-Kashipaz, M. R., Radford, P. M., Powell, R. J., Todd, I., Tighe, P. J. (2006) Modeling of tumor necrosis factor receptor superfamily 1A mutants associated with tumor necrosis factor receptor-associated periodic syndrome indicates misfolding consistent with abnormal function. *Arthritis Rheum.* **54**, 2674–2687.
  34. Cosmi, L., Maggi, L., Santarlasci, V., Liotta, F., Annunziato, F. (2014) T helper cells plasticity in inflammation. *Cytometry A* **85**, 36–42.
  35. Miyara, M., Sakaguchi, S. (2007) Natural regulatory T cells: mechanisms of suppression. *Trends Mol. Med.* **13**, 108–116.
  36. Carbone, F., De Rosa, V., Carrieri, P. B., Montella, S., Bruzzese, D., Porcellini, A., Procaccini, C., La Cava, A., Matarese, G. (2014) Regulatory T cell proliferative potential is impaired in human autoimmune disease. *Nat. Med.* **20**, 69–74.
  37. Watts, T. H. (2005) TNF/TNFR family members in costimulation of T cell responses. *Annu. Rev. Immunol.* **23**, 23–68.
  38. Nowlan, M. L., Drewe, E., Bulsara, H., Esposito, N., Robins, R. A., Tighe, P. J., Powell, R. J., Todd, I. (2006) Systemic cytokine levels and the effects of etanercept in TNF receptor-associated periodic syndrome (TRAPS) involving a C33Y mutation in TNFRSF1A. *Rheumatology (Oxford)* **45**, 31–37.
  39. Negm, O. H., Mannsperger, H. A., McDermott, E. M., Drewe, E., Powell, R. J., Todd, I., Fairclough, L. C., Tighe, P. J. (2014) A pro-inflammatory signalome is constitutively activated by C33Y mutant TNF receptor 1 in TNF receptor-associated periodic syndrome (TRAPS). *Eur. J. Immunol.* **44**, 2096–2110.
  40. Joshi, V. D., Kalvakolanu, D. V., Chen, W., Zhang, L., Kang, T. J., Thomas, K. E., Vogel, S. N., Cross, A. S. (2006) A role for Stat1 in the regulation of lipopolysaccharide-induced interleukin-1beta expression. *J. Interferon Cytokine Res.* **26**, 739–747.
  41. Procaccini, C., De Rosa, V., Galgani, M., Carbone, F., Cassano, S., Greco, D., Qian, K., Auvinen, P., Cali, G., Stallone, G., Formisano, L., La Cava, A., Matarese, G. (2012) Leptin-induced mTOR activation defines a specific molecular and transcriptional signature controlling CD4+ effector T cell responses. *J. Immunol.* **189**, 2941–2953.
  42. Hori, S., Takahashi, T., Sakaguchi, S. (2003) Control of autoimmunity by naturally arising regulatory CD4+ T cells. *Adv. Immunol.* **81**, 331–371.
  43. McCann, F. E., Perocheau, D. P., Ruspi, G., Blazek, K., Davies, M. L., Feldmann, M., Dean, J. L., Stoop, A. A., Williams, R. O. (2014) Selective tumor necrosis factor receptor I blockade is antiinflammatory and reveals immunoregulatory role of tumor necrosis factor receptor II in collagen-induced arthritis. *Arthritis Rheumatol.* **66**, 2728–2738.

# KEY WORDS:

TRAPS · T<sub>regs</sub> · T<sub>conv</sub>s · autoimmunity · immune tolerance