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SPECIFIC INVOLVEMENT OF CONVENTIONAL AND REGULATORY CD4+ T CELLS IN TUMOR NECROSIS FACTOR RECEPTOR-ASSOCIATED PERIODIC SYNDROME (TRAPS)

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Abstract

Tumor necrosis factor-receptor associated periodic syndrome (TRAPS) is a dominantly inherited auto-inflammatory disorder caused by mutations in *TNFRSF1A,* the gene encoding for tumour necrosis factor receptor superfamily 1A. The mechanism of inflammation in TRAPS is still unknown. In particular the involvement of adaptive immunity in auto-inflammatory disorders hasn't been investigated yet. In this project we investigated how TNFα/TNRSF1A signalling network regulates T cell responses. In particular, we focused on conventional $CD4^+CD25^-$ (Tconv) and regulatory $CD4^+CD25^+$ (Treg) T cell functions in TRAPS patients carrying either high or low penetrance mutation in TNFRSF1A gene (HP-TRAPS and LP-TRAPS, respectively). HP-TRAPS showed an up-regulation of several inflammation-related molecular signalling pathways in Tconv cells. In addition, these patients had a lower frequency of peripheral Treg cells which also displayed a defective suppressive phenotype. These alterations were partially found in LP-TRAPS who also carried a milder symptomatology thus suggesting suggest a specific link between the penetrance of the TNFRSF1A mutation and the T cell phenotype. Taken together, these data envision a novel role for adaptive immunity in the pathogenesis of TRAPS involving both $CD4^+$ Tconv and Treg cells raising a novel mechanism of inflammation in the context of auto-inflammatory disorders.

1. Introduction

1.1 Plasticity of adaptive immunity

A tight cross-talk between the innate and adaptive immune systems is necessary in order to achieve an efficient defense against invading pathogens.

Presenting cells such as dendritic cells (DCs), upon antigen recognization, help $CD4^+$ T cells to differentiate into a variety of effector subsets, Th1, Th2, Th17, follicular helper T (Tfh) cells, and induced Treg (iTreg) cells. Cytokines in the microenvironment and the strength of the interaction of the T cell antigen receptor with antigen orchestrate the differentiation pathway (1). Th1 cells are characterized by IFN-γ production and are involved in cellular immunity against intracellular microorganisms. The signal transducer and activator of transcription 4 (Stat4), Stat1, and T box transcription factor T-bet are activated during Th1 polarization.

Th2 cells produce IL-4, IL-5, and IL-13 and are required for humoral immunity to control helminths and other extracellular pathogens. GATA3 and Stat6 are needed for the differentiation of Th2 cells. Th17 cells produce IL-17A, IL-17F, and IL-22 and play important roles in clearance of extracellular bacteria and fungi, especially at mucosal surfaces. Th17 cell differentiation requires retinoidrelated orphan receptor (ROR)γt, a transcription factor that is induced by TGF-β in

combination with the pro-inflammatory cytokines IL-6, IL-21, and IL-23, all of which activate Stat3 phosphorylation (2). Tfh cells are a subset of helper T cells that regulate the maturation of B cell responses. Differentiation of these cells requires the cytokine IL-21 (3, 4) and the expression of the transcription factor Bcl-6 (5).

Regulation of effector T cell responses is required for effective control of infections and autoimmune diseases. Aberrant Th1 and Th17 cell responses play critical roles in organ-specific autoimmunity, whereas Th2 cells are mainly involved in allergy and asthma. Treg cells have essential roles in maintenance of immune homeostasis, regulating these effector T cell responses and thus preventing their potentially pathogenic effects through a variety of mechanisms (6, 7).

1.2 Treg cells

Self-tolerance is maintained by $CD4^{+}CD25^{+}$ regulatory T cells. These cells were firstly identified in mice (8) and later in humans (9). Treg cells are characterized by the expression of the forkhead transcription factor Foxp3 which plays a critical role in maintaining their functional program and generation (10, 11). Of note, patients affected by immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX), were found to have mutations in the Foxp3 gene thus

supporting the hypothesis of a pivotal role of Treg cells in the regulation of immune tolerance (12).

Two major categories of $Foxp3^+$ Treg cells have been identified so far: the naturally occurring $CD4^+CD25^+$ Treg (nTreg) cells that arise in the thymus and the TGF-β-induced iTreg cells produced in the periphery from Tconv cells (13, 14). In humans, $CD45RA^+CD25^+Foxp3^+$ cells represent thymus-derived Tregs, while $CD45RA^-$ Tregs are a mixed population including antigen-experienced thymic and peripheral Tregs (15). The stability of $Foxp3^+$ Tregs is debated (16). Foxp3 regulates distinct cell surface and signalling molecules, interacting with several transcription factors, inducing miRNAs and modulating epigenetic machinery to maintain Treg identity, function and stability in response to diverse environmental cues.

Tissue microenvironment influences the functional specialization of $Foxp3^+$ Treg. Indeed, Treg can lose the capability to express Foxp3 with the acquisition of an effector phenotype in presence of an abundance of pro-inflammatory stimuli (17, 18).

In mice, only very small fractions of $Foxp3^+$ Tregs were found to lose Foxp3 and regulatory functions (18). In humans, $CD45RA^+$ but not $CD45RA^-$ Tregs could be stably expanded *in vitro* (19), suggesting different stabilities of thymic and peripheric Tregs.

Tregs also inhibit anti-tumor CTL responses (20), and interestingly they can acquire cytotoxic properties in tumor-draining lymphnodes in mice (21) and *in vitro* in humans (22, 23). Similar to helper T cells, Tregs that secrete different types of effector cytokines, and these Treg subsets might specifically suppress different types of immune responses (24).

IL-17 or IFN-γ producing human $Foxp3^+$ T cells can be isolated (25, 26), but while IL-17 producing Treg cells were normally suppressive (27), IFN-γ producing Tregs showed reduced suppressive functions (26).

Both natural and induced Treg cells participate in maintenance of peripheral tolerance and prevention of autoimmunity (28). Alteration of their number and/or their suppressive function has been identified in many autoimmune disorders (29) but the underlying mechanism is still unknown. Many studies have highlighted how immune cells rely upon bioenergetic pathways to support their growth, proliferation, migration and differentiation. Alteration of these metabolic pathways can lead to chronic inflammation and autoimmunity. In this context *De Rosa et al* have recently shown that glycolysis is important for Foxp3 expression. Patients with multiple sclerosis and type 1 diabetes display impaired rates of glycolysis, which in turn determines altered peripheral Treg generation and functions (30).

Tfr cells are a newly identified CD4 Foxp3$^+$ Treg subset located in the germinal centres (GCs) of lymphoid organs, which downregulates the GC response by suppressing Tfh and B cells functions. Tfh and Tfr cells are defined by expression of the CXC-chemokine receptor 5 (CXCR5) which directs them to B cell follicles in GCs via gradients of the CXC-chemokine ligand 13 (CXCL13). Defects of Tfr number and functions have been associated to alteration of the homeostasis of B cells in germinal centers favoring the production of autoreactive antibodies and the onset of autoimmunity (31).

1.3 Auto-inflammatory disorders

The field of autoinflammatory disorders (AD) has expanded significantly in the last few years. Recent advances in basic sciences have provided new insights into the biology of the inflammatory responses in AD.

The term autoinflammatory was coined to differentiate AD from autoimmune disorders. Indeed, AD are characterised by the absence of pathogens, circulating autoantibodies, or self-reactive T cells (32). To date, nearly all mutations linked to AD have been associated with inflammatory signalling pathways regulating innate immune responses. This is following by the activation of the inflammasome, a multimeric cytosolic protein complex regulating the proteolytic processing of proIL-1β into

the biologically active IL-1β thus inducing a vast spectrum of systemic inflammatory signs (33).

The most common AD are: (a) familial Mediterranean fever (FMF) associated with mutation in the MEFV gene located on the chromosome 16p13.3 and encoding a protein named pyrin, a natural repressor of proinflammatory molecules. Ths results in uncontrolled relapsing one-site or multi-site inflammation, increased leukocyte migration to serosal membranes or joints, and inappropriate response to inflammatory stimuli (34); (b) tumor necrosis factor receptor-associated periodic syndrome (TRAPS) related to mutations in the soluble TNF receptor superfamily 1A gene (35); (c) cryopyrin-associated periodic syndrome (CAPS), caused by mutations in the NLRP3 gene located on the chromosome 1. This group includes familial cold urticaria syndrome (FCAS), Muckle-Wells syndrome, and chronic infantile neurological cutaneous articular syndrome (CINCAs), respectively, ranging from the least to the most severe (36); (d) mevalonate kinase deficiency (MKD), also known as hypergammaglobulinemia D syndrome, caused by homozygosity or compound heterozygosity for disease-causing mutations in the MVK gene, which has been localized in the chromosome 12q24 and encodes for mevalonate kinase, the first committed enzyme of cholesterol biosynthesis (37); (e) NLRP12-associated autoinflammatory disorder (NLRP12-ad), or FCAS type 2, caused by mutations in the NLRP12 gene,

which encodes for the monarch-1 protein, working as a regulator of NF-κB activation (38); (f) Blau syndrome (BS), genetic form of a condition previously known as "early onset sarcoidosis", due to mutations of the NACHT domain of the gene CARD15 (also renamed NOD2) (39).

Some of these diseases such as FMF and MKD are transmitted by autosomal recessive inheritance, while the others such as TRAPS, CAPS, NLRP12-ad, and BS are autosomal dominant. **Table 1** summarizes the main clinical features of AD.

In addition to the monogenic AD, many other syndromes, sharing similar overlapping manifestations with autoinflammatory conditions, such as systemic juvenile idiopathic arthritis, Still's disease, gout, idiopathic recurrent pericarditis, generalized pustular psoriasis, Schnitzler's syndrome, Behçet's disease, periodic fever aphthous stomatitis, pharyngitis and cervical adenitis (PFAPA) syndrome, and many other rare or even frequent conditions, have been included in the group of polygenic multi-factorial AD (40).

Most of the monogenic AD has a general clinical onset during the pediatric age (41, 42). However, a substantial number of patients may have a disease onset during adulthood.

The main clinical features are recurrent brief episodes of fever, serositis, joint symptoms, and erysipelas-like erythema in the lower limbs. Of note, long-term exposure to uncontrolled

inflammation can lead to development of amyloidosis. A late onset syndrome is mostly associated with the presence of low penetrance mutations, milder phenotypes, and a lower risk of developing amyloidosis (43, 44).

Diagnosis of specific AD relies on patient's clinical history, demonstration of increased acute phase response during inflammatory attacks, and possibly, genetic confirmation (45).

The introduction of biologic agents has strongly changed the course and prognosis od AD. In particular, TNF-α and IL-1 blockers have significantly improved overall patients' quality of life and prognosis with a good control of systemic symptoms (46).

Even though genetic mutations mainly involve proteins implicated in innate immune responses, adaptive immune system might be implicated as well.

In this regard, patients with CAPS have shown higher levels of Th17 cells without a clear autoimmune syndrome (47). According to this, a recent paper by *Arbore et al* has demonstrated that NLRP3 inflammasome activity within $CD4^+$ T cells is necessary to drive Th1 differentiation and for the production of optimal IFN-γ responses during a viral infections. They also showed that patients with CAPS had hyperactive T Th1 responses, and that this was associated with increased IL-1β and IFN-γ production by Th1 cells. (48)

Therefore, it seems that the interplay between the innate and adaptive immune responses may be even stronger than previously thought.

1.4 TRAPS

Tumour necrosis factor (TNF)-α is produced by epithelial and immune cells (49-52) and regulates several biological processes including immune responses, inflammation, apoptosis, cell differentiation and proliferation. This is due to the binding to TNF receptors TNFRSF1A (or TNFR1A p55/p60-TNFR) and TNFR1B (or TNFR2, p75/80-TNFR) (53, 54). TNFRSF1A is constitutively expressed in many cell types including macrophages, neutrophils, mast cells and lymphocytes and mediates TNF-α signalling through the recruitment of various intracellular proteins. This results in the activation of transcription factors such as NF-kB and AP-1, apoptosis pathways and mitogen-activated protein kinase (MAPKs) signalling (55-57). Mutations in the *TNFRSF1A* gene that encode for TNFRSF1A receptor are known to be associated to an autosomal dominant auto-inflammatory syndrome known as tumour necrosis factor-receptor associated periodic syndrome (TRAPS) (58, 59). The auto-inflammatory syndromes are a growing group of conditions characterized by impaired innate immune response associated to generalised systemic inflammation without involvement of auto-antibodies

and auto-reactive T cells (40). TRAPS is clinically characterized by recurrent fever attacks, periorbital oedema, conjunctivitis, a migratory skin rash, myalgia, arthralgia and/or arthritis, polyserositis and amyloidosis as more severe complication (60-65). Several TNFRSF1A mutations have been found associated to TRAPS (http://fmf.igh.cnrs.fr/infevers/). Most of these mutations are heterozygous *missense* and localized in the extracellular portion of the receptor (66). TRAPS-associated mutations can be high-penetrance (HP-TRAPS) also called “structural mutations” mainly affecting cysteine residues. These mutations generally involve the receptor folding and are associated related to a more severe phenotype. On the other side, low-penetrance (LP-TRAPS), defined as "non-structural mutations or variants of unknown significance" mainly associated to milder symptoms, lower risk of amyloidosis and shorter duration of attacks (60, 61, 43, 67, 68). The precise pathogenic mechanism of TRAPS is not well understood. It has been observed that TRAPS-associated mutations can alter TNFRSF1A cell surface expression, shedding, TNF-α binding affinity, TNFRSF1A intracellular trafficking as well as TNFα/TNFRSF1A signalling and autophagy (67, 69-74). Recently it has been demonstrated that peripheral blood leukocytes isolated either from TRAPS patients or mice with TNFRSF1A mutations, showed increased MAPKs activation after LPS stimulation due to intracellular

receptor accumulation (75). These cells also exhibited increased mitochondrial respiration and unconventional unfolded protein response leading to reactive oxygen species (ROS) production, events known to potentiate MAPKs signalling as well as secretion of several pro-inflammatory cytokines (76-78).

Since little is known about the involvement of adaptive immunity in the pathogenic events leading to TRAPS symptoms, we investigated the impact of TNFRSF1A mutations on different T cell subsets. Specifically, we delineated how multiple variants of TNFR1 mutations are able to affect, the T conventional/ T regulatory (Tconv/Treg) balance thus providing novel insights into the detailed molecular mechanisms regulating immune self-tolerance in TRAPS disease.

2. Materials and Methods

2.1 Patients

Serum and blood samples were obtained from 20 HP-TRAPS (C43R 2/35 pts; C43T 1/35 pts; T50M 7/35 pts; C52Y 2/35 pts; C125R 2/35 pts; C55Y 1/35 pts; S59N 1/35 pts; S59P 1/35 pts; C114W 1/35 pts; Del 103-104 1/35 pts; L167-175del 1/35 pts), 15 LP-TRAPS (R92Q 12/35 pts; D12E 1/35 pts; V95M 1/35 pts; R104Q 1/35 pts) and from 32 age-, gender- and BMI-matched healthy controls (HC) without inflammatory diseases (17 males, 15 females) (42 years; 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 workup, in order to rule out any inflammatory, metabolic, and neoplastic disorders. Laboratory assessment included erythrocyte sedimentation rate (ESR), C-reactive protein (CRP), and serum amyloid A (SAA). ESR was measured using the Westergren method (mm/hour), and was considered normal if 15 < mm/hour for males and < 20 mm/hour for females. Serum CRP concentrations were measured using a nephelometric immunoassay, values 0.5 mg/dL were considered normal. Serum amyloid A (SAA) levels were measured by particle-enhanced nephelometry (BNII auto-

analyzer, DadeBehring, Marburg, Germany) and the reference value was 6.4 mg/L.

Written informed consent was obtained both from patients and controls. The study protocol was reviewed and approved by the University of Siena Institutional Ethics Committee in according the Declaration of Helsinki. Clinical and demographic characteristics of HP- and LP-TRAPS patients, at the time of blood collection, are summarized in **Table 2**. All samples have been collected during a remission phase of the disease at routine follow-up.

2.2 Immunephenotype

Immune cell profiling of cells from TRAPS patients and HC subjects was done at the time of the blood draw. Before flow cytometry to determine lymphocyte subsets, whole blood cells were analysed with a clinical grade hematocytometer to determine absolute lymphocyte numbers in each sample. For the HC subjects and TRAPS patients, 100µL blood was incubated 30min at room temperature with the specific antibodies combinations. Erythrocytes were lysed using BD FACS lysing Solution 2 (BD Bioscience) for 10 min and subsequently washed and resuspended in 300µL PBS. Flow cytometry was carried out on cells gated on $CD45^{+}$-SideScatter (SSC). Immunephenotypic analysis was performed with an EPICS XLflow cytometer (Beckman Coulter) using the

Beckman Coulter XLSystem II software program. Triple combinations of different human monoclonal antibodies (i.e., fluorescein isothiocyanate [FITC]– and phycoerythrin [PE]–anti-CD3, PE– and PC-5–anti-CD4, PC5–anti-CD8, PE–anti-CD16, PC5–anti-CD19, PE–anti-CD25, FITC–anti-CD45, and PE–anti-CD56; all from Coulter Immunotech) were used to identify different cell populations.

2.3 Cell cultures and T cell proliferation assays

Human peripheral blood mononuclear cell (PBMCs) were isolated by stratifying 15 mL of heparinized whole blood on 5 mL of Ficoll-Paque PREMIUM (GE Healthcare) and centrifuging the solution at 1.2×g for 20 min. PBMCs (2×10^5 per well) were cultured, in triplicate, in 96-well round-bottomed plates (Becton-Dikinson Falcon), in medium supplemented with 5% (vol/vol) autologous (AS) subject serum or 5% (vol/vol) heterologous (HS) commercial pooled AB human serum (Sigma-Aldrich, St. Louis, MO) and were stimulated or not in parallel with 0.1 µg/mL anti-CD3 monoclonal antibody (OKT3, Orthoclone, Janessen-Cilag SpA). Supernatants were collected from 48h TCR-stimulated PBMCs from TRAPS patients and HC subjects. The human Th1/Th2 11plex kit FlowCytomix (Bender Medsystems GmbH) was used for quantitative detection of human interferon (IFN)-γ, tumor necrosis factor (TNF)-α, TNF-β, interleukin

(IL)-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12p70, by flow cytometry (Becton-Dickinson) according to the manufacturer's instructions.

Human $CD4^+CD25^+$ (Treg) and $CD4^+CD25^-$ (Tconv) T cells were purified from HC and TRAPS patients PBMCs by magnetic cell separation with the Dynal$CD4^+CD25^+$ Treg Kit (Dynal-Biotech) and were rapidly cleaned with the Detach reagent (Dynal-Biotech) from surface-bound CD25 mAb. Magnetic beads-based-based purification technique yielded a highly expressing $CD25^+$ population (98% pure by FACS analysis), 90% of which expressed Foxp3. The Treg:Tconv ratio in the suppression experiments was 1:1. We cultured cells (1×10^4 cells per well) in round-bottom 96-well plates (Becton-Dickinson Falcon) with RPMI 1640 medium supplemented with 100 UI ml^{-1} penicillin and 100 μg ml^{-1} streptomycin (all from Life Technologies) and supplemented with either 5% AS or 5% AB HS. Cells were stimulated for 48h, in the presence of anti-CD3/anti-CD28–coated Dynabeads (0.5 beads per cell) (Invitrogen). On the last day, [^{3}H]thymidine (0.5□Ci/well) (Amersham-Pharmacia Biotech) was added to the cultures and cells harvested after 12h. Radioactivity was measured with a □-cell-plate scintillation counter (Wallac).

2.4 Flow cytometry

On freshly isolated PBMCs from TRAPS patients and HC subjects, we performed FACS analysis (BD FACS-Canto). For staining isolated Treg cells, we used (1:50–100 dilution) APC-H7–labeled anti–CD4 (BD Biosciences). Thereafter, cells were washed, fixed and permeabilized (fixation-permeabilization buffer, eBioscience) and stained with PE-labeled anti-FoxP3 (eBioscience) and FITC-labeled anti-Ki67 (BD Biosciences) monoclonal antibodies. Analyses were performed with Diva software from BD and FlowJo software (Tree Star).

2.5 Molecular signalling and western blot analyses

Tconv cells ($3x10^5$) were lysed in cold radioimmune precipitation assay buffer RIPA (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS,protease and phosphatase inhibitors) for 15 min at 4°C. The lysate was centrifuged (10,000 × g for 10 min); the supernatant was collected, quantized 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 were used according to the protocol provided by the supplier. For all western blot shown 8µg of total protein extracts were loaded; 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); anti-ERK1/2, anti-phospho-ERK1/2, anti-NF-kB p65, anti-actin (Santa Cruz Biotechnology Inc.).

2.6 Statistical analysis

We used non-parametric Mann–Whitney U or Student's T tests. Correlation analyses were performed by using Spearman's correlation coefficients. The statistical software used was GraphPad InStat3 version 4.0. Results are expressed as mean ± S.E.M. p values ≤ 0.05 were considered statistically significant.

3. Results

3.1 $CD4^+$ T cells are increased in HP-TRAPS patients

We analyzed several immune cell subpopulations in peripheral blood from HP-TRAPS (n = 17), LP-TRAPS (n = 13) and HC (n = 32). Total lymphocytes, $CD3^+$ T cells, $CD4^+$ T cells as well as $CD3^+$ T cells with a memory phenotype ($CD45RO^+$) were higher in HP-TRAPS. These patients also showed an increased number of $CD4^+CD45RO^+$ and $CD8^+CD45RA^+$ T cells compared to HC. Statistically significant difference was observed also in the absolute number of circulating $CD4^+CD28^+$ and $CD4^+CD25^+$ cells. They were both increased in HP-TRAPS compared to HC (**Figure 1**, **Table 3**). On the other hand, LP-TRAPS patients had higher number of $CD3^+$, $CD4^+$ and $CD8^+$ T cells with a naïve phenotype ($CD45RA^+$) than HC (**Figure 1**, **Table 3**). Differences between HP- and LP-TRAPS patients were found in the absolute number of total $CD4^+$ T cells and $CD3^+$ and $CD4^+$ T cells with memory phenotype ($CD45RO^+$), as well as circulating $CD4^+CD28^+$ T cells (**Figure 1**, **Table 3**). Finally, the frequency of total $CD4^+CD45RO^+$ T cells and total $CD3^+CD45RO^+$ T cells did not correlate with serum amyloid A (SAA) levels in both HP and LP-TRAPS (**Figure 2**).

3.2 T cell proliferation and cytokine secretion is impaired during T cell receptor stimulation in HP-TRAPS patients

We evaluated T cell proliferative profile in PBMCs, stimulated by physiologic T-cell receptor (TCR) specific stimuli (anti-CD3 – OKT3 mAb), from HP-, LP-TRAPS and HC. In order to understand whether soluble serum factors (either an endogenous factor or related with the drug treatment received by the patients) could affect T cell responses *in vitro*, cells were cultured in medium supplemented with either 5% autologous subject serum (AS) or 5% heterologous pooled healthy human serum (HS) (**Figure 3A**). We found a reduced proliferation in HP-TRAPS compared to HC in both HS- and AS-supplemented culture medium (**Figure 3A**). No significant difference was found between LP-TRAPS and HC, and between HP-TRAPS and LP-TRAPS, respectively (**Figure 3A**).

We next measured the concentration of pro- and anti-inflammatory cytokines in T cell-derived supernatants. HP-TRAPS showed lower levels of IL-5 and IFNγ in both HS and AS-supplemented culture conditions, while lower levels of TNFα, IL-1β, IL-6 and IL-12p70 were observed only in AS-supplemented medium. In addition, significant higher concentration of IL-8 was observed in HP-TRAPS compared to HC in both HS and AS-supplemented medium. Finally, HP-TRAPS showed decreased levels of TNFα, IL-1β, IL-5 and IL-

12p70 in AS-medium, a lower amount of IFNγ in HS-medium, and reduced IL-5 in both HS and AS-media as compared with LP-TRAPS. No significant difference was observed in cytokines production between LP-TRAPS and HC in both HS and AS-supplemented media (**Figure 3B-J**).

3.3 Tconv proliferation is increased in HP-TRAPS patients during TCR stimulation

We then analyzed the impact of TNFRSF1A mutation on Tconv ($CD4^+CD25^-$) cells which are generally increased in both autoimmune and inflammatory disorders (79). Specifically, we evaluated TRAPS Tconv cells proliferation during anti-CD3/anti-CD28 stimulation. HP-TRAPS Tconv cell proliferative rate was higher than that of HC and LP-TRAPS (**Figure 4A**). In addition, HP-TRAPS Tconv cells showed also a higher expression of several intracellular pathways related to proliferation and activation such as ERK1/2, STAT1, STAT3, STAT5 and NF-kB p65 compared to HC. Whereas, LP-TRAPS Tconv cells showed higher expression of STAT1 compared to HC Tconv cells (**Figure 4B**). We further analyzed the mammalian target of rapamycin (mTOR) pathway evaluating the activation of S6, a downstream target of mTOR. We found that S6 phosphorylation was significantly higher in HP-TRAPS than HC, while no significant difference was observed between LP-TRAPS and HC (**Figure 4B**).

3.4 Treg cell frequency and suppressive function is altered in HP-TRAPS patients

To get further insights into the mechanisms involved in a possible break of self-tolerance in TRAPS, we analyzed the Treg cell compartment. Indeed, it is well delineated that Treg cells display a central role in the regulation of immune homeostasis by reducing inflammatory responses and suppressing Tconv cells functions (80, 81). We found that HP-TRAPS had a lower frequency of $CD4^{+}FoxP3^{+}$ Treg cells than HC, while no difference was found between HP- and LP-TRAPS and between LP-TRAPS and HC, respectively (**Figure 5A, B**). In addition, HP-TRAPS Treg cells showed a reduced *ex-vivo* proliferation rate as suggested by a lower expression of the Ki67, an intracellular proliferative marker than HC and LP-TRAPS Treg cells. No difference was found comparing LP-TRAPS and HC Treg cells **(Figure 5C, D**). In contrast, HP-TRAPS Treg cells exhibited a higher proliferative rate *in vitro* during anti-CD3/anti-CD28 stimulation compared to TRAPS-LP and HC Treg cells despite it did not reach a statistical significance ($p = 0.06$) (**Figure 5E**).

Finally, we analyzed the suppressive capacity of Treg cells isolated from HP-, LP-TRAPS and HC. We evaluated the Tconv cell proliferation when co-cultured with Treg cells. HP-TRAPS Treg cells showed reduced suppressive capacity

compared to LP-TRAPS and HC while no significant difference was observed in LP-TRAPS compared to HC (**Figure 4E, F**). A similar trend was also detected in HS medium although it was not significant (**Figure 6A, B**).

3.5 Meta-immunological profiling in TRAPS patients

To characterize the meta-immunological profile of TRAPS, several indicators of inflammatory and metabolic activities were also analyzed in sera from both HP- and LP-TRAPS and HC (leptin, MCP-1, MPO, sCD40L, sICAM-1, resistin and sTNF-R). The serum concentration of leptin was significantly higher in LP-TRAPS than HP-TRAPS ($p=0.0427$) and HC ($p=0.0137$) serum samples. No difference was found between HP-TRAPS and HC. Interestingly sTNF-R levels were lower in HP-TRAPS compared to LP-TRAPS ($p=0.016$) and HC ($p=0.0051$) (**Figure 7**). sICAM-1 resulted higher in both HP- ($p=0.0053$) and LP-TRAPS ($p=0.0046$) than in HC. In addition we observed that MPO levels were higher in HP-TRAPS than HC ($p=0.0117$). Finally, there was no difference in the serum levels of sCD40L, MCP-1, resistin and IL-6 between patients and controls (**Figure 7**).

4. Discussion

In this study we report that TNFRSF1A mutation strongly impacts on adaptive immune compartment thus suggesting a key role of T cells in the pathogenesis of TRAPS. In this regard, HP-TRAPS patients showed a selective increase of $CD4^+$ T cells which displayed a memory ($CD4^+CD45RO^+$) and an effector phenotype with over-expressed markers of T cell activation such as the CD25 (known as the IL-2R alpha chain) and CD28. This is a classic feature of autoimmune disorders where a peripheral conversion of naïve $CD4^+$ T cells into a memory phenotype has often been described. The frequency of peripheral memory $CD4^+CD45RO^+$ did not correlate with disease activity (serum SAA levels) in both HP- and LP-TRAPS confirming that the observed altered immunologic profile was to be ascribed to a defective TNFR signalling pathway more than to the inflammatory milieu (79). In line with this evidence, all patients were in a remission phase of the disease when were enrolled in the study and blood sampling.

We next evaluated TRAPS PBMCs function during a TCR-dependent stimulation, which selectively stimulates the whole T cell compartment. Surprisingly, HP-TRAPS T cells displayed a reduced proliferative rate compared to HC T cells in either HS or AS-supplemented culture medium. This result is may be ascribed to an impairment of T cell antigen

presentation/co-stimulation in the context of PBMCs, secondary to the altered TNFRSF1A signalling. Our hypothesis is related to the finding that anti-CD3 mediated T cell proliferation of PBMCs needs the presence of abundant antigen-presenting cells (APCs) which must carry on their surface MHC-II, and co-stimulatory molecules, whose expression is linked to TNFR-signalling. Clearly, further studies need to be performed to better explain this phenomenon. In line with this result, we also found that anti-CD3 stimulated HP-TRAPS PBMCs secreted lower amounts of several cytokines, indicating a T-cell impaired activation as well. Surprisingly, IL-8 was the only cytokine increased in HP-TRAPS anti-CD3 stimulated PBMCs supernatants. IL-8 is a well-known cytokine involved in neutrophils activation and chemotaxis, thus confirming the involvement of these cells in the pathogenesis of TRAPS as previously reported (82, 67). In line with this evidence, we found in HP-TRAPS serum samples, high levels of myeloperoxidase (MPO). Of note, we ruled out that medications present in TRAPS serum could have an impact on T cell mediated responses. Indeed, we did not find difference in terms of T cell proliferation/cytokine profiles between culture media supplemented either with AS or HS in both HP- and LP-TRAPS.

In order to provide a novel mechanism of inflammation at the basis of TRAPS, we also analyzed functional aspects of

isolated Tconv and Treg cells. Surprisingly, we found an enhanced Tconv proliferative response in HP-TRAPS associated with a high expression of multiple inflammatory signalling pathways such as STAT1, STAT5, STAT3, ERK1/2, NFkb p65, and S6 irrespective of the pro-inflammatory milieu as previously described in TRAPS PBMCs (83). These results could be explained by a "constitutive activation", induced by mutated TNFR1, of the above inflammatory intracellular signalling pathways. This evidence could in part justify the diverse pathophysiology of TRAPS and the limited efficacy of cytokine-blocking biological agents. Furthermore, LP-TRAPS Tconv cells had an intermediate/mild phenotype, since they behaved very similarly to HC Tconv cells, showing only an elevated expression of STAT1. This latter result is of high interest since it has been reported that STAT1 signalling plays a central role in IL-1β activation (84) and helps to understand why symptomatic LP-TRAPS patients benefit from anti-IL-1 treatment. In summary, while the whole T cell population of PBMCs showed a reduced proliferative rate during anti-CD3 stimulation, when $CD4^+$ Tconv cells were isolated from PBMCs they showed a higher proliferative phenotype secondary to an enhanced mTOR-STATs signalling.

On the side of immune tolerance, HP-TRAPS patients showed a reduced frequency of peripheral $CD4^+FoxP3^+$ Treg cells which expressed a defective suppressive capacity

compared to LP-TRAPS and HC Treg cells. One of the immune abnormalities observed in autoimmune disorders, is a reduction in the suppressive function and/or number of natural Treg cells (85-87). Under normal circumstances, Treg cells are typically hyporesponsive to TCR stimulation *in vitro* and highly proliferative *in vivo*. Moreover, it is well delineated that *in vitro* Treg cell anergy (a state of hyporesponsiveness to antigenic stimulation) is crucial for an optimal suppressive function (80). In our study, we observed that HP-TRAPS Treg cells had a higher proliferation after TCR stimulation *in vitro* (reduced anergy) with a lower expression of $Ki67^+$ *ex-vivo*. This may explain the Treg impaired suppressive function and higher Tconv proliferative response in HP-TRAPS. These alterations were not found in patients carried low penetrance mutations. In this context, it has been recently shown that TNFR1 is crucial for Treg cell functions: thus, alterations involving the expression of this receptor on T cell surface may promote inflammatory responses (88) and our data are in agreement with this recent finding.

Taken together, our report unveils a novel mechanism of inflammation in TRAPS involving $CD4^+$ T cells (both Tconv and Treg cells) and reveals a novel *scenario* in which a dysfunction in the regulatory immune-cell compartment is present in the context of auto-inflammatory disorders,

previously considered mainly ascribed to an altered innate immune response.

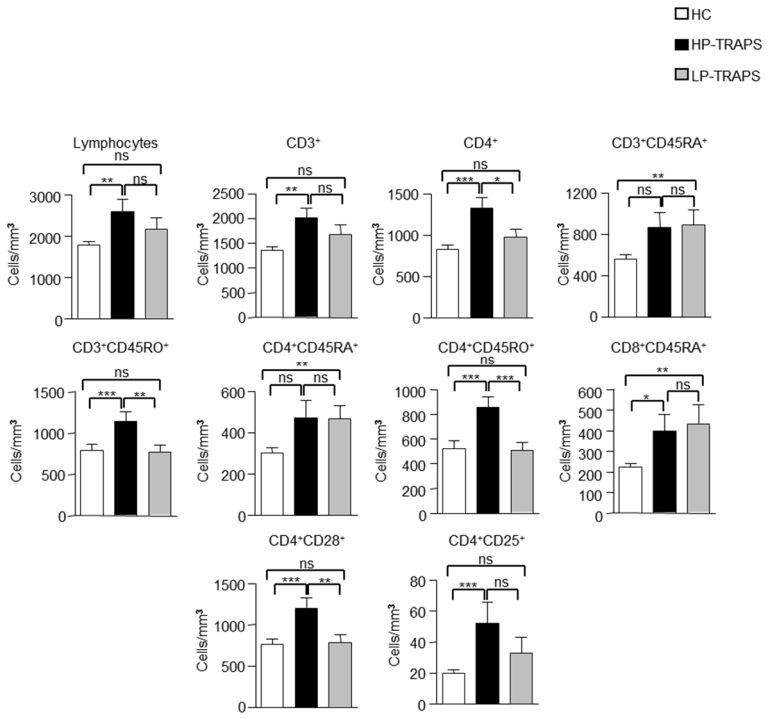

Figure 1. *Immunephenotype of TRAPS patients with low and high penetrance TNFRSF1A variants.* HP-TRAPS (n = 17; 2 C43R, 1 C43T, 7 T50M, 2 C52Y, 2 C125R, 1 C55Y, 1 S59P, 1 Del 103-104); LP-TRAPS (n = 13 ; 10 R92Q, 1 R104Q, 1 D12E, 1 V50M); HC (n = 32).Data are expressed as mean ± s.e.m. Statistical significance was determined using Mann-Whitney non-parametric U-test. *$p \leq 0.05$, **$p \leq 0.03$, ***$p \leq 0.01$, ns = not significant.

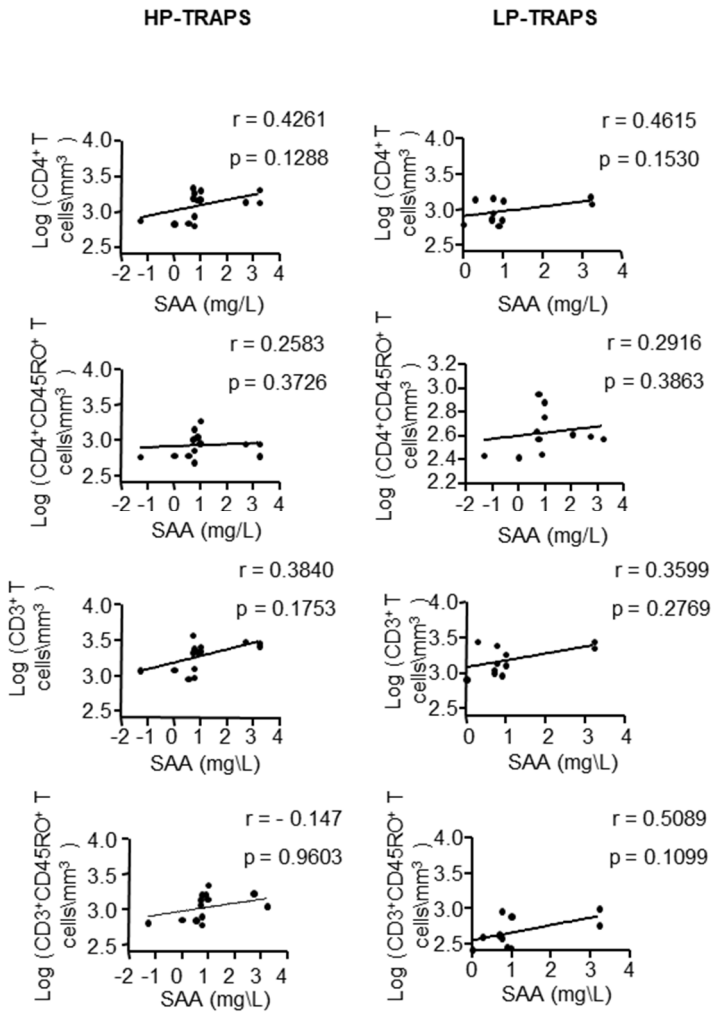

Figure 2. *Correlations of CD3$^+$ and CD4$^+$ phenotypes and serum amyloid A levels from peripheral blood of HP-TRAPS and LP-TRAPS patients*. Total CD3$^+$, CD4$^+$ and CD4$^+$CD45RO$^+$ and CD3$^+$CD45RO$^+$ T cells were correlated with serum amyloid A levels (SAA, mg/L) Values were obtained using Spearman's correlation coefficients. Correlations were considered significant at $p < 0.05$.

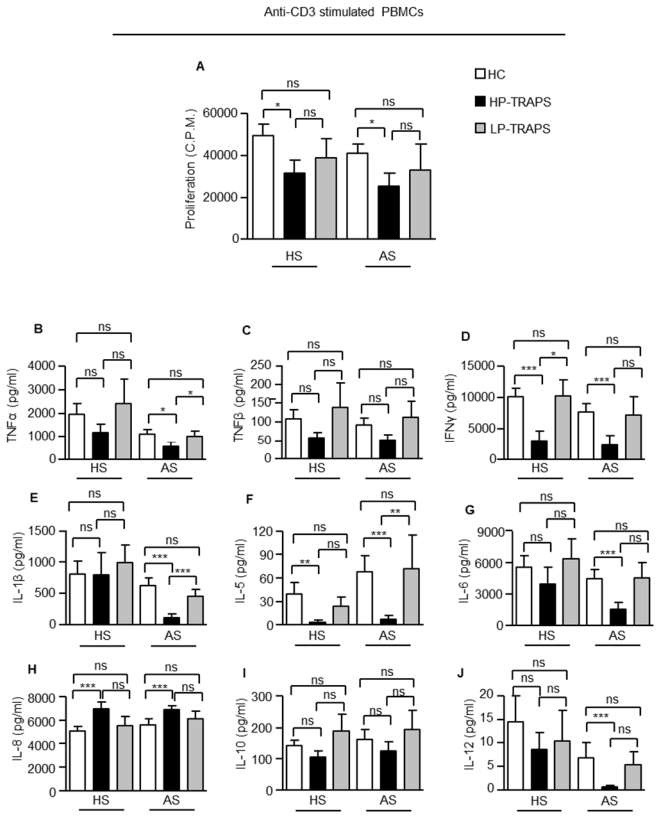

Figure 3. *PBMCs proliferative profile and cytokine release in TRAPS patients and HC during anti-CD3 stimulation.* (A) Proliferation (CPM-3H incorporation) and (B-J) cytokine production (pg/ml) of PBMCs isolated from TRAPS patients with high (n = 10) and low penetrance (n = 7) TNFRSF1A mutation and HC (n = 16) after TCR-mediated stimulation (anti-CD3) in medium supplemented with heterologous (HS) or autologous (AS) serum. Data are expressed as mean ± s.e.m. Statistical significance was determined using Mann-Whitney non-parametric U-test. *$p \leq 0.05$, **$p \leq 0.03$, ***$p \leq 0.01$, ns = not significant.

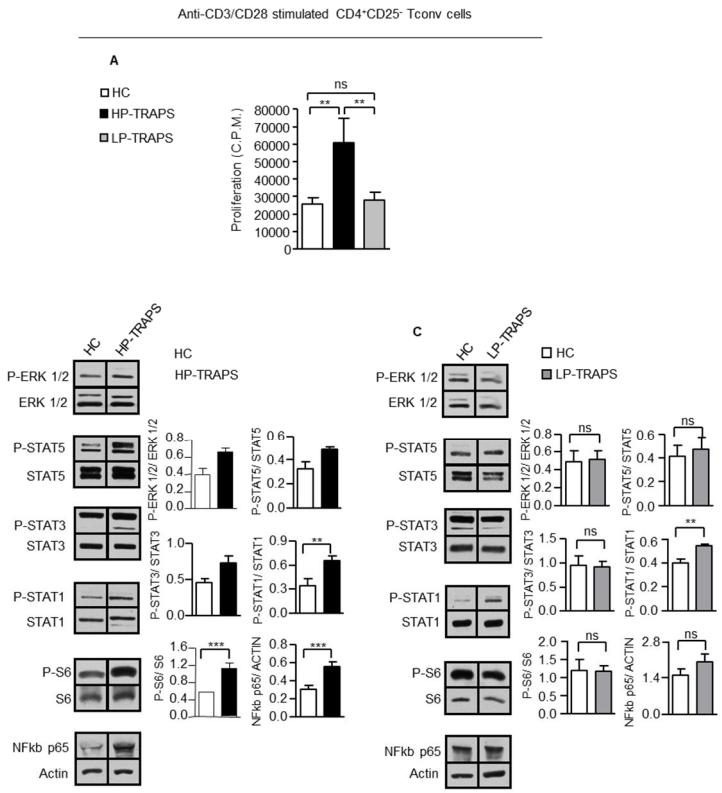

Figure 4. *Enhanced activation and proliferation of Tconv cells in HP-TRAPS associates with an increased STATs-mTOR signalling.* (A) Proliferation (CPM-^{3}H incorporation) of anti-CD3/CD28-stimulated Tconv cells from HP-TRAPS (n = 5; 2 C52Y, 1 T50M, 1 DEL103-104, 1 S59P), LP-TRAPS (n = 5; 3 R92Q, 1 D12E, 1 R104Q) and HC (n = 7). Cells were cultured in 5% AS medium and stimulated with 0.5 beads per cell for 48h. (B, C) Western blot analysis of P-ERK1/2, P-STAT1, P-STAT3, P-STAT5, NF-kB p65 and P-S6 in 24h anti-CD3/CD28-stimulated Tconv cells in HP-, LP-TRAPS and HC. Cells were cultured in 5% AS-supplemented medium and were

stimulated with 0.2 beads per cell. Data are representative of at least three independent experiments and are expressed as mean ± s.e.m. Statistical significance was determined using unpaired Student's T-test. *$p \leq 0.05$, **$p \leq 0.03$, ns = not significant.

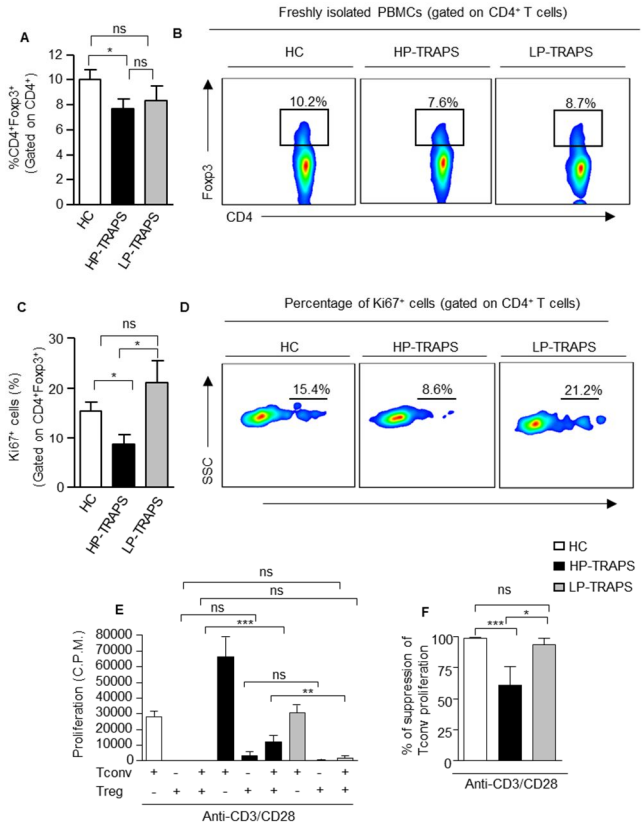

Figure 5. *HP-TRAPS patients show a reduced Treg cell number and suppressive function.* (A) Foxp3 expression in freshly-isolated PBMCs from HP-TRAPS (n = 15; 2 C43R, 1 C43T, 2 C52Y, 2 C125R, 6 T50M, 1 DEL103-104, 1 S59P), LP-TRAPS (n = 12; 9 R92Q, 1 R104Q, 1 D12E, 1 V95M) and HC (n = 15). PBMCs were stained for CD4 and Foxp3 expression. (B) Representative flow cytometry plots of Foxp3 expression in freshly isolated PBMCs from HP-, LP-TRAPS and HC. (C) Ki67 expression in freshly isolated Treg cells defined by gating $CD4^{+}Foxp3^{+}$ cells from HC (n = 11), HP-TRAPS (n = 8; 2 C125R, 4 T50M, 1 DEL103-104, 1 S59P) and

LP-TRAPS (n = 5; 4 R92Q, 1 R104Q). (D) Representative flow cytometry plots of Ki67 expression in freshly isolated PBMCs gated on $CD4^{+}Foxp3^{+}$ from HP-, LP-TRAPS and HC. (E) Proliferation of anti-CD3/CD28 stimulated Tconv and Treg cells from HP- (n = 5; 2 C52Y, 1 T50M, 1 DEL103-104, 1 S59P), LP-TRAPS (n = 5; 4 R92Q, 1 D12E) and HC (n = 7) alone or in co-culture. (F) Percentage of suppression of Tconv cells proliferation when in co-culture with Tconv cells. Treg : Tconv cells ratio in the suppression experiments was 1:1. Cells were cultured in 5% AS-supplemented medium and stimulated with anti-CD3/CD28 (0.5 beads per cell) for 48h. Comparisons were evaluated using the non-parametric Mann Whitney U test. Data are reported as the mean ± s.e.m. *$p \leq 0.05$, **$p \leq 0.03$, ns = not significant

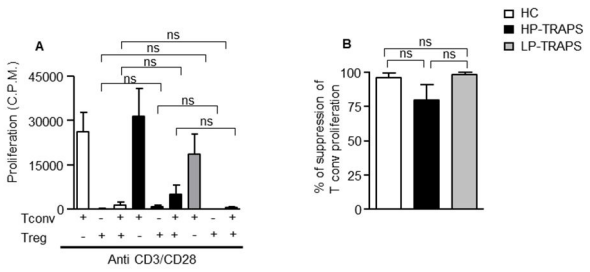

Figure 6. *Tconv, Treg proliferative potential and Treg suppressive capacity in TRAPS patients with low and high penetrance mutation cultured in heterologous serum.* Proliferation (A) and percentage of suppression (B) of anti-CD3/CD28 stimulated Tconv and Treg cells from HP-TRAPS (n=5), LP-TRAPS (n = 3) and HC (n = 5) alone or in co-culture. Treg cells: Tconv cells conventional ratio in the suppression experiments was 1:1. Cells were cultured in 5% HS medium and were stimulated for 48h with anti-CD3/CD28 (0.5 beads per cell). Comparisons were evaluated using the non-parametric Mann Whitney U test. Data are reported as the mean and s.e.m. ns = not significant.

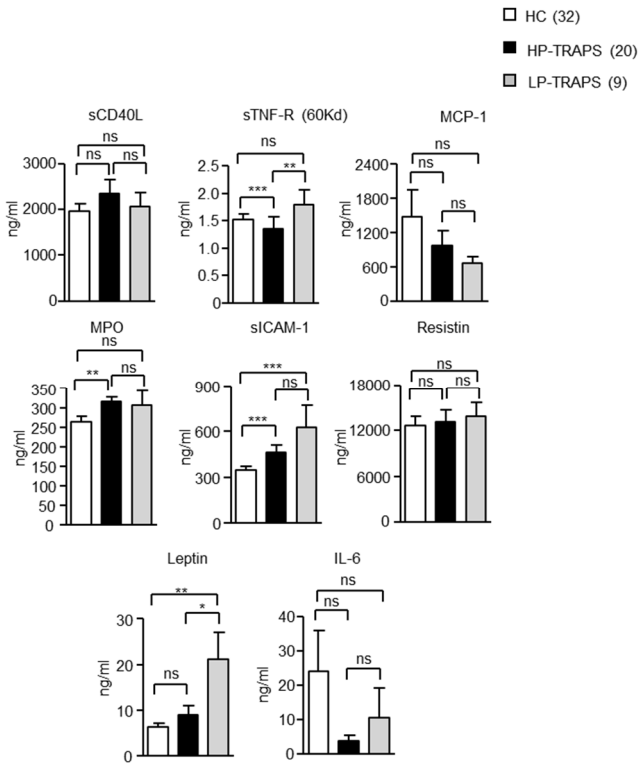

Figure 7. *Meta-immunological profiling in HP-and LP-TRAPS patients and CTR subjects.* Circulating level of sCD40L, TNF-R, MCP-1, MPO, leptin, sICAM-1, resistin, IL-6 in TRAPS patients with HP TNFRSF1A mutation (n = 20; 2 C43R, 1 C43T, 7 T50M, 2 C52Y, 2 C125R, 1 C55Y, 1 S59N, 1 S59P, 1 C114W, 1 Del 103-104, 1 L167-175del), LP mutation (n = 9; 6 R92Q, 1 R104Q, 1 D12E, 1 V50M) and CTR subjects (n = 32). Data are expressed as mean ± s.e.m. statistical significance was determined using Mann-Whitney non parametric U-test. ***p≤0.01, **p≤0.03, *p≤0.05.

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Table 1: Monogenic autoinflammatory disorders

Disease	Gene	Protein	Trasmission	Symptoms	Therapy
FMF	MEFV 16p13.3	Pyrin	AR	Short duration of fever episodes: 24–48 hours. Abdominal and chest pain. Erysipelas-like erythema. High incidence of renal amyloidosis in untreated patients. Good response to colchicine. Possible response to IL-1 blockade.	Colchicine
HIDS	MVK 12q24	Mevalonate kinase	AR	Early onset (usually <12 months). Mean duration of fever episodes: 4–5 days. Poor conditions during fever episodes. Abdominal pain, vomiting and diarrhoea. Splenomegaly. Good response to steroids. High rate of self-resolution during adulthood. Amyloidosis is rare.	FANS, anakinra
TRAPS	TNFRSF1A	p55 TNF-α receptor	AD	Prolonged fever episodes: 1–3 weeks. Periorbital oedema, monocytic fasciitis. Incidence of renal amyloidosis: 15–25%. Response to TNF- and IL-1 blockade.	Etanercept, anakinra
FCAS MWS CINCA	NLRP3	Criopyrin	AD AD AD	FCAS: rash, fever and arthralgia after cold exposure. MWS: recurrent or sub-chronic urticaria-like lesions, sensorineural hearing loss, amyloidosis. CINCA: as above + mental retardation, chronic aseptic meningitis and bone deformities. Good response to IL-1 blockade.	Etanercept, anakinra
PAPAs	PSTPIP1	PSTPIP1	AD	Pyogenic sterile arthritis, pyogenic gangrenosum, cystic acne. Good response to IL-1 blockade.	Corticosteroids, infliximab, anakinra
MS DIRA	LPIN2 18p IL1RN 2q LPIN2	LPIN2 IL-1 receptor antagonist	AR AR	Multifocal osteomyelitis, congenital dyserythropoietic anaemia, inflammatory dermatosis. Neonatal-onset multifocal osteomyelitis, periostitis, and pustulosis. Dramatic response to anakinra.	Corticosteroids, interferon
BS	NOD2/CARD1 516q12.1-13	NOD2/CARD15	AD	Early onset (<5 years). Polyarticular granulomatous arthritis, uveitis, skin rash. Good response to anti-TNF monoclonal antibodies.	Corticosteroids, infliximab

FMF = Familial Mediterranean fever; **MKD** = Mevalonate kinase deficiency; **TRAPS** = TNF receptor associated periodic syndrome; **FCAS** = Familial cold autoinflammatory syndrome; **MWS** = Muckle-Wells syndrome; **CINCA** = Chronic infantile neurological cutaneous and articular syndrome; **PAPA** = Pyogenic sterile arthritis, pyoderma gangrenosum and acne (PAPA) syndrome; **CRMO** = Chronic recurrent multifocal osteomyelitis; **DIRA** = Deficiency of theinterleukin-1-receptor antagonist; AR = autosomal recessive; AD = autosomal dominant.

Table 2. Clinical and demographic characteristics of TRAPS patients with high and low penetrance TNFRSF1A (at the time of sample collection), expressed as median (range).

	HP-TRAPS (n = 20)	**LP-TRAPS (n = 15)**
Age	44 (9-71)	36 (8-56)
Gender	8M:12F	7M:8F
Disease onset (yrs)	6 (1-49)	15 (3-55)
N^{o} of fever attacks per year	5 (0-14)	2 (0-15)
Duration of fever attacks (days)	8 (0-20)	8 (0-25)
ESR (mm/h)	6 (1-45)	12 (1-31)
CRP (mg/dL)	0.65 (0-11.4)	0.31 (0-9)
SAA (mg/L)	5.70 (0.05-1734)	18.5 (17.8-224.8)
BMI (Kg/m^2)	23.6 (19.0-28.0)	23.4 (21.3-28.7)
Amyloidosis	5/20	None
Chronic course	4/20	6/15
Treated with anti-IL1	15/20	5/15
Naïve to anti-IL1 (NSAIDs or corticosteroids on demand)	5/20	10/15

Table 3: Immunophenotype of TRAPS patients with low and high penetrance TNFRSF1A mutation and healthy controls

Cell type	HC (n=32) Cells/mm^3 (%)	HP-TRAPS (n=20) Cells/mm^3	LP-TRAPS (n=13) Cells/mm^3	*p* value (HC *vs* HP-TRAPS)	*p* value (HC *vs* LP-TRAPS)	*p* value (HP-*vs* LP-TRAPS)
Leucocytes	5786.56 ± 1621.32	7142.11 ± 2989.33	5488.75 ±1848.07	0.1362	0.1382	0.2389
Lymphocytes	1775.72 ± 472.41	2574.79 ± 1174.83	1926.38 ± 614.22	**0.0157	0.1225	0.2571
$CD3^+$	1354.44 ± 414.99	1972.11 ± 832.95	1457.86 ± 534.48	**0.0119	0.1172	0.2330
$CD4^+$	821.84 ± 351.83	1288.05 ± 536.32	876.25 ± 327.70	***0.0019	0.0882	*0.0500
$CD8^+$	430.53 ± 155.04	573.89 ± 374.87	408.38 ± 189.51	0.1877	0.2616	0.3794
$CD3^-CD16^+CD56^+$	166.58 ± 113.13	177.42 ± 107.13	240.38 ± 111.24	0.3739	*0.0420	0.1134
$CD19^+$	222.72 ± 108.27	355.42 ± 318.24	199.00 ± 85.31	0.3341	0.4354	0.2697
$CD3^+CD45RA^+$	565.58 ± 218.14	871.45 ± 565.77	646.61 ± 245.54	**0.0288	**0.0162	0.3506
$CD3^+CD45RO^+$	796.62 ± 378.92	1100.53 ± 474.84	811.48 ± 337.70	***0.0062	0.3963	**0.0301
$CD4^+CD45RA^+$	299.81 ± 140.48	802.23 ± 364.10	502.43 ± 224.07	***<0.0001	**0.0117	***0.0013
$CD4^+CD45RO^+$	523.27 ± 353.22	769.37 ± 502.43	502.43 ± 224.07	***0.0015	0.3583	**0.0106
$CD8^+CD45RA^+$	265.76 ± 130.29	379.21 ± 310.00	264.67 ± 141.60	0.1241	***0.0085	0.4919
$CD8^+CD45RO^+$	164.77 ± 129.05	160.50 ± 147.87	149.31 ± 92.98	0.1362	0.2008	0.2854
$CD4^+CD28^+$	766.57 ± 350.48	1172.25 ± 487.60	791.04 ± 282.20	***0.0014	0.4130	**0.0247
$CD4^+CD25^+$	19.67 ± 9.60	37.99 ± 48.04	36.82 ± 49.63	0.1107	**0.0240	0.2195

$CD4^+CD8^+$	19.62 ± 17.98	32.38 ± 67.73	45.77 ± 96.19	0.4737	0.2553	0.2692
$CD4^+DR^+$	17.60 ± 12.70	22.90 ± 22.09	19.62 ± 9.8	0.2663	0.2285	0.4788
$CD8^+DR^+$	14.04 ± 21.36	6.61 ± 11.75	7.03 ± 13.20	*0.0462	0.1839	0.4677
$CD8^+CD11b^+$	94.30 ± 76.30	96.36 ± 96.10	102.40 ± 120.11	0.3710	0.3629	0.2595
$CD3^+CD16^+CD56^+$	44.19 ± 31.49	75.91 ± 101.63	51.66 ± 41.30	0.4402	0.2952	0.3771
$CD3^-CD8^+$	75.62 ± 44.06	99.97 ± 101.11	113.49 ± 101.48	0.2895	0.2336	0.3515

Abbreviations: HC = healthy controls (n =32); HP-TRAPS = high penetrance TRAPS (n = 20); LP-TRAPS = low penetrance TRAPS (n = 13). Data are expressed as mean ± s.e.m. statistical significance was determined using Mann-Whitney non-parametric U-test.*$p \leq 0.05$, **$p \leq 0.03$,***$p \leq 0.01$