Gene expression profile in TNF receptor-associated periodic syndrome reveals constitutively enhanced pathways and new players in the underlying inflammation

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ABSTRACT

Objective. *Tumour necrosis factor (TNF) receptor-associated periodic syndrome (TRAPS) is a multisystemic autoinflammatory condition associated with heterozygous TNFRSF1A mutations, presenting with a variety of clinical symptoms, many of which yet unexplained. In this work, we aimed at deepening into TRAPS pathogenic mechanisms sustained by monocytes.*

Methods. *Microarray experiments were conducted to identify genes whose expression results altered in patients compared to healthy individuals, both under basal condition and following LPS stimulation.*

Results. *An inflammatory state baseline, characterised by constitutive overexpression of IL1β and IL1R1 receptor, has been shown in TRAPS patients compared to controls, including in non-active disease phases. Following LPS stimulation, IL1RN up-regulation is stronger in controls than in patients and inflammatory pathways and microRNAs undergo differential regulation. Genes involved in post-translational modifications, protein folding and ubiquitination result constitutively up-regulated in TRAPS, while response to interferon types I and II is defective, failing to be up-regulated by LPS. TGFβ pathway is down-regulated in untreated TRAPS monocytes, while genes involved in redox regulation result constitutively over-expressed. Finally, additional molecular alterations seem to reflect organ failures sometime complicating the disease.*

Conclusion. *Gene expression profile in resting TRAPS monocytes has confirmed the patients' chronic inflammatory condition. In addition, pathways not yet associated with the disease have been disclosed, such as interferon types I and II response to LPS stimulation and a downregulation of the TGFβ pathway in basal condition. The role of miRNA, suggested by our results, deserves in-depth analyses in light of the possible development of targeted therapies.*

Introduction

Tumour necrosis factor (TNF) receptor-associated periodic syndrome (TRAPS) is an autosomal-dominant multisystemic autoinflammatory condition (OMIM: 191190). Patients present with long-lasting recurrent fevers associated with abdominal pain, arthromyalgias, rashes, fasciitis, periorbital edema, and systemic AA amyloidosis as a long-term complication (1). TRAPS has been associated with more than 100 different mutations of the TNF receptor superfamily 1A gene (*TNFRSF1A*) (http://fmf.igh.cnrs.fr/ISSAID/infevers). TNF-α binding to TNFR1 receptor induces either NF-κB activation or cell death and is able to lead to pleiotropic activities, including increased expression of adhesion molecules, induction of cytokine secretion, activation of leukocytes, and host defense against intracellular pathogens (2, 3).

The extracellular domain of TNFR1 is cleaved upon receptor activation. Such a mechanism, known as shedding, has the role in producing a pool of soluble receptors which can attenuate the inflammatory response by reducing circulating TNF-α. At first, defective shedding was proposed as the cause of the TRAPS phenotype (1, 2, 4). However, the shedding of TNFR1 resulted to differ between cell types bearing the same mutations (4). Other proposed mechanisms are: defective TNF-induced apoptosis (5), TNF-independent NF-κB activation (3) and impaired intracellular TNFR1 traffick-

ing and TNF-binding (6-10). In this latter case, both variant and wt TNFR1 were suggested to work synergistically to generate the full TRAPS phenotype (11). More recently, *TNFRSF1A* mutant cells have been shown to exhibit altered mitochondrial function with enhanced oxidative capacity and ROS generation, whose pharmacological blockade could efficiently reduce inflammatory cytokine production after LPS stimulation in cells from both TRAPS patients and healthy controls (12). Moreover, autophagy has resulted to play a role in the physiological intracellular balance of TNFR related proteins, shown to be impaired in the presence of mutant TNFR1 proteins. It has been proposed that an autophagy defect can cause excessive NF-κB activation and, therefore, enhance IL-1β secretion (13). Despite these advancements, molecular details are still needed to define *TNFRSF1A* mediated TRAPS pathogenesis and to find new pathways which, once impaired, can account for all clinical aspects of this disorder. A remarkable proportion of the above reported observations have been achieved either *in vitro*, in the monocytic THP-1 cell line, or *ex vivo*, in monocytes derived from affected and healthy subjects (13-15), thus reflecting the relevance of this cell type for the disease pathogenesis.

In this work, we aimed at deepening into the pathogenic mechanisms responsible for TRAPS, focusing on the monocyte population. In particular, we sought to verify which molecular pathways are involved in TRAPS by conducting microarray experiments to identify genes whose expression results altered in patients compared to healthy individuals, both under basal condition and following LPS treatment. A similar approach has already been applied successfully to another autoinflammatory disease, the Cryopyrin-Associated Periodic Syndromes (CAPS) (16).

Patients and methods

Sample collections and processing

The present study was conducted on five patients affected with TRAPS and bearing mutations in the *TNFRSF1A* gene and ten control individuals (six individuals for microarray experiments and four independent individuals for quantitative PCR). Our study was approved by the Ethic Committee of the Gaslini Institute. Informed consent (according to the Declaration of Helsinki) was obtained from all individuals before study enrolment.

Monocytes were isolated from frozen vials of peripheral blood mononuclear cells as untouched cell population using Monocyte Isolation Kit II (Miltenyi Biotec) and then diluted at $1x10^6$ cells/ml in serum free medium in the presence of LPS (100 ng/ml) for 4 hours.

Total RNA was isolated using the RNeasy Micro Kit and DNAse1 treatment (Qiagen) and quantified by NanoDrop (Thermo Scientific). RNA integrity was assessed on the 2100 Bioanalyzer using the Nano labchip kit (Agilent Technologies). RNAs were reverse-transcribed into cDNA and cRNA and hybridised to Affymetrix GeneChip™ HuGene1.0 arrays. Raw intensity data (cel-files) were imported into BioConductor and normalised using the RMA algorithm implemented in R/Bioconductor (17) applying quantile normalisation (18). Expression data were filtered to remove non-expressed (log 2 intensity <5) or invariant (lowest 25% of standard deviation) genes. The four conditions analysed were compared pairwise: untreated TRAPS *vs*. untreated controls, LPS-treated TRAPS *vs*. LPS-treated controls, LPS-treated *vs*. untreated controls, LPS-treated *vs*. untreated TRAPS. To compare TRAPS patients and control individuals, average expression ratios were calculated in TRAPS *vs*. controls (untreated TRAPS *vs*. untreated controls, LPS-treated TRAPS *vs*. LPS-treated controls). On the other hand, LPS effects were evaluated for each patient or control sample, calculating the gene expression ratio between the LPS treated *vs*. respective basal condition, and then assessing the mean ratio for each comparison (LPS-treated *vs*. untreated controls, LPS-treated *vs*. untreated TRAPS). In each case, we took into account only probes that displayed at least two-fold differential expression. Gene annotation tracing, through the Affymetrix probe set ID, was performed using IPA software. Statistical testing (student's two tailed, homoscedastic *t*-test) was performed for each dataset.

Validation

Validation of microarray data was performed by quantitative PCR on IQ5 (Biorad) using Assays on-demand (Life technologies) both in control and TRAPS monocytes.

Reference genes (PPIA, RPLPO, GAPDH, RNApol2F) were selected using the GeNOrm software (http://medgen.ugent.be/~jvdesomp/genorm).

After ascertaining that efficiencies of target and reference genes were approximately the same, changes in mRNA amount of target genes were quantified by using the comparative CT Method. Amplification experiments were performed in duplicate and repeated at least two times.

Bioinformatic analysis

Gene lists were analysed by employing the IPA platform (http://www.ingenuity.com) and EnrichR application (19), as well as various gene-set libraries, such as BioCarta (www.biocarta.com/genes/allpathways.org), WikiPathways (http://www.wikipathways.org), KEGG (http://www.genome.jp/kegg), Gene Ontology (http://geneontology.org/), MGI Mammalian Phenotype (http://www.informatics.jax.org/searches/MP_form.shtml). IPA-based analysis takes into account whether

Table I. Features of TRAPS patients analysed.

Patient ID	FP013	FP024	FP103	FP131	FP833
Gender	F	F	F	M	M
TNFRSFA1 mutation	T79M	C81Y	C72R	C84Y	C91Y
Age at disease onset	2y+6m	3m	3m	7y	1y
Age	14.5y	13y+7m	4y	9y	3 y
Therapeutic strategy	Steroid on demand				
Disease activity	Remission				

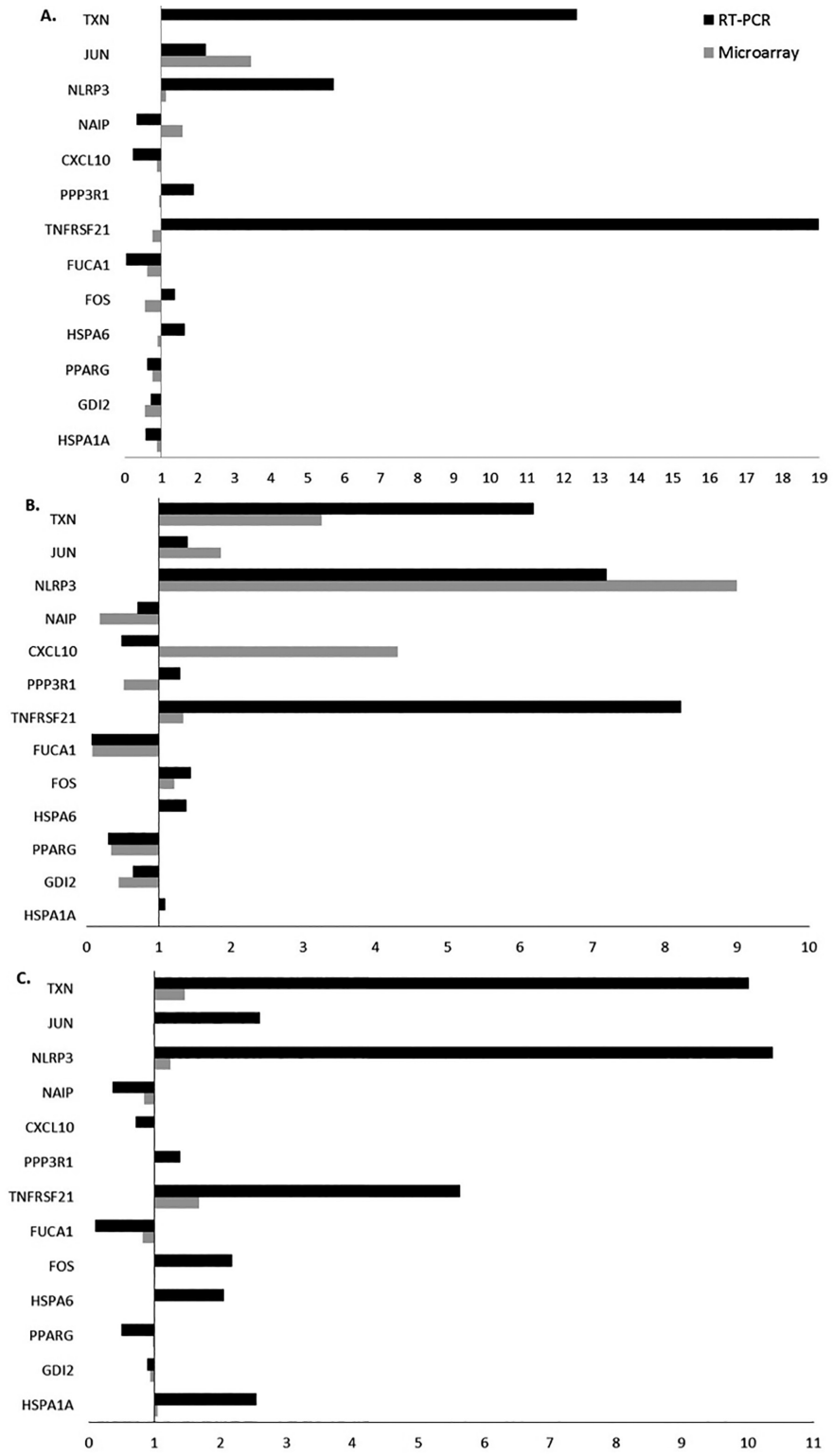

Fig. 1. Analysis of gene expression in TRAPS patients (**A**. FP833; **B**. FP103 father) and in a pool of control individuals (**C**.). Values obtained from microarrays (grey) and quantitative PCR (black) are reported as ratio between samples treated with LPS against samples under basal conditions.

genes are up- or down-regulated in the samples of interest *vs*. related controls, whereas we loaded the plain gene lists in the EnrichR application and evaluated the direction of their modulation.

Results

Identification of modulated genes in TRAPS vs. controls and in LPS-treated vs. -untreated

RNA was extracted, both under basal condition and after LPS stimulation from monocytes of five patients affected with TRAPS and bearing mutations in the *TNFRSF1A* gene (Table I), and six control individuals. Upon RNA hybridisation to Affymetrix chips, the following pairwise comparisons were performed: untreated TRAPS *vs*. untreated controls, LPS-treated TRAPS *vs*. LPS-treated controls, LPS-treated *vs*. untreated controls, LPS-treated *vs*. untreated TRAPS.

We noticed a high variability among samples, both when comparing gene expression levels in TRAPS *vs*. control monocytes, and when analysing the up- or down-regulation induced by LPS among different healthy donors or patients. For these reasons, we took into account only genes that displayed a differential expression of at least a factor of two, both in case of up-regulation (>2-fold) and in case of down-regulation (<0.5-fold) (Supplementary Table SI). First, we compared TRAPS *vs*. controls obtaining 234 genes differentially expressed under basal conditions and 306 genes differentially expressed after LPS stimulation. Moreover, we analysed the effect of LPS stimulation by comparing gene expression under basal condition *vs*. treatment, thus obtaining 604 and 236 genes two fold up- or down-regulated in control individuals and in TRAPS patients, respectively. As expected, in control monocytes, LPS stimulation elicited a gene expression profile consistent with activation of immune system (Supplementary Table SI, see CTR LPS *vs*. CTR UNT).

Validation by quantitative-PCR

To validate results obtained from microarray analysis we performed quantitative PCR. We used new samples obtained from patient FP833 and from patient FP103's father, this latter also affected with TRAPS and carrying the same mutation of the daughter. As, in the meantime, the other patients had started anakinra therapy, they were not eligible to validate pattern of transcription obtained from their monocytes isolated before starting the treatment. We selected thirteen transcripts displaying differential expression upon LPS stimulation for quantitative PCR. Values

Table II. Genes included in IPA categories, related to nefrotoxicity* and modulated in TRAPS patients after LPS treatment.

ID probe¥	Symbol	Fold change
8064779	ADRA1D	-2.012
8155849	ANXA1	2.254
7990818	BCL2A1	2.503
8065403	CST3	-2.540
8156228	CTSL	2.725
8101126	CXCL10	-4.229
8011713	CXCL16	-2.311
7954090	EMP1	2.097
8118310	HSPA1A/HSPA1B	2.522
8179322	HSPA1A/HSPA1B	2.474
8118314	HSPA1A/HSPA1B	2.277
8178086	HSPA1A/HSPA1B	2.255
8179324	HSPA1A/HSPA1B	2.255
8133721	HSPB1	-3.584
8054722	IL1B	2.046
8131803	IL6	2.666
8169949	MST4	2.192
8142120	NAMPT	2.735
7944876	NRGN	-2.833
8077899	PPARG	2.346
8052721	PPP3R1	2.571
7922976	PTGS2	2.614
8037005	TGFB	-2.394

*glomerular injury; kidney failure; nephritis/nephrosis/INFL; renal necrosis/cell death; renal fibrosis; renal proliferation, ¥Affymetrix ID number for the corresponding probe on the microchip.

Table III. Genes related to Post-Translational Modification and Protein Folding differentially expressed in affected *vs*. control individuals under basal conditions.

Gene Symbol	Fold change
CCL4	2.889
CCL5	-2.338
CD244	2.545
DNAJA4	2.121
DNAJB1	2.427
EIF3M	2.033
EIF5	2.570
HSP90AA1	2.467
HSPA1A/HSPA1B	2.255
HSPA6	2.804
HSPA9	2.141
HSPB1	-3.584
HSPH1	3.240
IL1B	2.046
MPDUI	2.332
PSMA6	2.084
RP2	2.774
RPL41	-2.529
RPS23	2.065
RPS26	-2.437
RPS4Y1	-2.177
SEP15	2.363
TGFB1	-2.394

from microarray experiments weighed against values from quantitative PCR have shown concordance for 61.5% of the genes analysed in the first case (FP833, patient) and 84.6% in the second case (FP103, father) (Fig. 1).

The use of blood samples from control individuals in our Institution is subjected to anonymity and little amounts are made available, so we could not obtain further samples from the same individuals included in the microarray analysis to test their gene expression levels through quantitative PCR. In alternative, we analysed RNA from four different healthy donors and compared quantitative PCR data with microarray data using the gene expression means, thus validating 76.9% of the genes.

These results confirmed the reliability of the microarray analysis performed.

TRAPS gene expression in basal conditions vs. CTR subjects

The IPA software allowed to identify TNF as a possible regulator for genes differentially expressed in TRAPS monocytes, among which ATP2B1, BCL2A1, BCL3, GCLC and IL6.

IPA and EnrichR tools pointed out a basal inflammatory profile (Supplementary Table SII). In addition, other pathways relevant for TRAPS pathogenesis did include: oxidative stress (WikiPathways), unfolded protein response (GO_biological processes), apoptosis (KEGG). IPA identified, as pathological correlates of the gene expression profile, inflammatory and immune diseases with possible targets in connective tissues of organs such as the kidney, liver and heart. Indeed, among genes deregulated in TRAPS, many have already been implicated in rheumatic and autoimmune pathologies. Interestingly, renal failure due to AA amyloidosis is the most frequent long-term complication of untreated TRAPS (1). Genes associated with nephrotoxicity include genes involved in the regulation of cell proliferation and apoptosis, pro-inflammatory cytokines (IL1β and IL6, up-regulated in TRAPS), and TGFβ (down-regulated in TRAPS) (Table II).

The IPA platform allowed us to compare the results of different analyses. Therefore, genes differentially expressed in untreated TRAPS *vs*. untreated CTR cells were compared with genes modulated by LPS in control cells. The idea was to identify families, pathways or functional categories of genes associated with TRAPS, besides those underlying the mere inflammatory phenotype. This analysis pointed out alterations within the functional categories of "post-translational modifications" and "protein folding". We took into account all the genes we could find related to these functions in the GO_biological processes and GO_molecular functions databases (Table III). The genes sorted out were mainly molecular chaperones and heat shock proteins (HSPs), and others such as SEP-15 that is a thioredoxin-like, endoplasmic reticulum-resident enzyme exerting a redox function. All these genes are overexpressed in TRAPS, with the notable exception of HSPB1/HSP27 (0.3 x untreated TRAPS/untreated CTR, 0.5 x LPS-treated TRAPS/LPS-treated CTR).

Other genes differentially expressed in TRAPS *vs*. CTR exert their function in the unfolded protein response (UPR), namely genes included in the EIF2 signalling (RPS4Y1, RPS26, EIF5, RPS23, RPL41, EIF3M) and genes involved in the protein ubiquitination (PSMA6, HSPA1A/HSPA1B, HSPH1, HSPA9, HSPA6, HSP90AA1, DNAJB1, HSPB1). Genes involved in the UPR are deregulated in TRAPS patients in basal conditions and they are unable to respond to LPS treatment, while in control individuals most of them are up- or down-regulated by LPS, thus suggesting a specific role during inflammation for the pathway under analysis. On the contrary, with the exception of HSP9 and PSMA6, all genes involved in protein ubiquitination are deregulated in TRAPS patients in basal conditions and do not respond to LPS stimulation in control individuals (Table III).

Response to LPS in TRAPS vs. CTR

To assess the response to LPS, we selected the genes up- or down-regulated by LPS at least two fold in TRAPS and in CTR (Supplementary Table I) and analysed separately the two gene lists, which were then compared taking profit of a specific tool provided by IPA. We also evaluated the results obtained for the two gene sets with the EnrichR software. Response to LPS is grossly similar in the two individual sets, though

Table IV. Modulation of mi-RNA expression as revealed in different pairwise comparisons.

Symbol	TRAPS_UNT/ CTR_UNT	TRAPS_LPS/ CTR_LPS	CTR_LPS/ CTR_UNT	TRAPS_LPS/ TRAPS_UNT
mir-142			-2.264	
mir-146		3.068	3.805	7.026
mir-155	2.832	3.088	26.935	23.027
mir-191		2.486		
mir-21			2.564	
mir-221				2.041
mir-24	2.190	2.693		
mir-27		3.534		
mir-425		2.595		
mir-9				2.016

some subtle differences can be recognised. Genes regulated by LPS in CTR but not in TRAPS include a series of IFN-responsive genes, as well as interferon receptor 1 (IFNAR1). Moreover, TLR4 is exclusively up-regulated in CTR, together with many pro-inflammatory cytokines and chemokines that, though up-regulated in both lists, are induced more in CTR than in TRAPS (Supplementary Table I). Interestingly, some of these genes are already slightly up-regulated in TRAPS in basal conditions. Conversely, there are genes up-regulated more in TRAPS than in CTR monocytes, including CCL4, miR146, PHF19 and TRAF1, while IL1α and IL1β are induced by LPS at a similar level in the two gene sets.

Taking advantage of the present expression data, we decided to investigate the role of microRNAs. In control monocytes, LPS stimulates the up-regulation of miRNAs known for their effects on the immune system, namely mir-146, mir-155 and mir-21, while mir-142 is down-regulated by LPS. Interestingly, mir-146 and mir-155 are 3-fold up-regulated in TRAPS *vs*. CTR after LPS stimulation. Moreover, in TRAPS patients mir-21 and mir-142 failed to be modulated, while a number of other miRNAs are induced by LPS. Noteworthy, mir-155 is up-regulated in TRAPS patients also in basal conditions (Table IV).

As an alternative approach to compare the response to LPS in TRAPS and CTR monocytes, we selected the genes differentially expressed in LPS-treated TRAPS *vs*. LPS-treated CTR. IPA and EnrichR analyses revealed an expression phenotype related to infectious and inflammatory diseases, with manifestations linked to dermatological conditions and connective tissues. Similarly to what we found when analysing gene expression in untreated TRAPS monocytes, also the gene alterations in LPS-treated TRAPS monocytes pointed to toxicity toward kidney and liver. Since the top pathways included "Interferon Signalling", we looked at genes belonging to this pathway and noticed a significant enrichment in IFN-induced genes in LPS-stimulated CTR cells compared to LPS-stimulated TRAPS cells (Table V).

Discussion

In the present study, a microarray analysis has been conducted to find genes and pathways whose deregulation can account for clinical aspects of TRAPS. Unlike previous reports (20-22), our study has been carried out in monocytes purified from patients bearing different mutations in the *TNFRSF1A* gene. Expression data thus obtained are characterised by high variability among samples, both when comparing gene expression levels in TRAPS *vs*. control monocytes and when analysing the up- or down-regulation induced by LPS in the same samples. We have postulated two different sources of expression variability: the physiological diversity of monocytes in their basal condition and LPS-response among patients and control individuals and the different TNFR mutations harbored by TRAPS patients under analysis. Nonetheless, analysis of transcript levels showed that the overall pattern is more homogeneous within each dataset, patients and healthy controls, than between them. For these reasons, we took into account all genes that displayed a differential expression of at least a factor of two. We confirmed the reliability of microarrays analysis by validating the transcript level of a number of genes through quantitative PCR. Unfortunately, we could not assess the corresponding protein levels as further samples from the same patients were not available. Nonetheless, this may be not crucial as correlation between mRNA extent and protein amount has already been demonstrated in TRAPS (21).

The genetic basis of the disease is a mutation in the TNFR1 receptor. Genes regulated by its ligand, TNF-α are indeed over-expressed in unstimulated TRAPS monocytes as compared to controls. Our data indicate a more active inflammatory baseline in the disease, also in non-active phases. Acute episodes can be triggered by an infection or a physical stress, however, response to LPS appears grossly normal in TRAPS monocytes. After looking into details, some interesting differences can be observed in genes that are modulated by LPS in TRAPS *vs*. control monocytes. The differential regulation involves molecules belonging to inflammatory pathways and microRNAs known to be involved in regulation of inflammatory response and NFkB activation, such as miRNA-155, miRNA-146 and miRNA-21 (23, 24). In particular, miRNA-146 exerts an anti-inflammatory effect, through a negative feed-forward loop that disrupts cytokine mRNA translation following TLR4 responses. Indeed, TLR4 and molecules belonging to TLR4 signalling pathways are direct targets of miR146. This observation could explain why TRAPS monocytes fail to upregulate TLR4 upon LPS stimulation (25). Indeed, altered levels of circulating miRNA had already been identified in serum from TRAPS patients, without evaluating LPS response, thus reinforcing the role of these non-coding RNAs in TRAPS pathogenesis (26).

Consistent with the fact that IL1β and IL1R1 are constitutively overexpressed in TRAPS under basal conditions (TRAPS untreated/CTR untreated ratio: 2.04 and 2.3, respectively), TRAPS symptoms can be controlled by IL1 in-

hibitors, such as anakinra. Differently from what expected, LPS stimulation seems to induce transcription of IL1β and IL1β at a similar level in TRAPS and CTR cells. Nevertheless, the pattern of secretion of IL-1β from LPS-stimulated monocytes from TRAPS patients with active disease has been reported up-regulated, thus supporting a role of the NLPR3 inflammasome during the TRAPS active phase (13). LPS induced IL1RN (IL-1 receptor antagonist) up-regulation was stronger in CTR subjects than in TRAPS patients. This is in line with a similar finding reported in CAPS patients, suggesting a possible defective role in the production of the natural inhibitor of IL-1, possibly related to the increased oxidative stress occurring in TRAPS monocytes (12). A hypothesis to explain how TNFR1 mutations cause TRAPS takes into account the possible intracellular accumulation of mutated TNFR, leading to impaired intracellular trafficking, unfolded protein response, and ER stress (6-10). Our data do support this hypothesis, since we found that genes involved in post-translational modifications, protein folding and ubiquitination are constitutively up-regulated in TRAPS. Some of these genes are further up-regulated in response to LPS.

The HSPs family resulted up-regulated in TRAPS patients *vs*. controls in basal condition. Among cellular processes, these proteins are involved in the endoplasmic reticulum workflow, that is related to one of the putative TRAPS pathogenic mechanisms (6-10). HSPs are known to be up-regulated in monocytes by hyperthermia; however, we exclude that the patients were experiencing fever at the moment of blood sampling. Other stimuli for HSPs up-regulation include various types of physical stress, infections, and inflammatory conditions, where HSPs play a protective role regulating magnitude of the innate immune response, survival/apoptosis, and macrophage differentiation. However, many genes of these pathways failed to be regulated by LPS both in TRAPS and in CTR cells, so we are tempted to hypothesise that their deregulation in untreated TRAPS is not merely due to ongoing inflammation. We focused on Heat Shock 27kD Protein 1 (HSPB1) that is down-regulated in untreated TRAPS *vs*. CTR monocytes. HSPB1 is constitutively expressed in monocytes and down-regulated upon heat shock, but overexpressed in case of inflammation. Once phosphorylated, HSPB1 enhances the proteasomal degradation of its targets, for example IkB alpha (27), and cooperates with AUF1 to degrade TNF-α mRNA (28, 29). It is not clear whether HSPB1 exerts a pro- or an anti-inflammatory role, since data in literature are controversial and because of its multiple functions related to cytokine expression, apoptosis regulation, reactive oxygen intermediate (ROI) production, and mitochondria protection (30-32). HSPB1 is necessary for cytokine expression induction downstream of p38, namely pro-inflammatory cytokines and also IL10 (33) that is up-regulated 5.2x in CTR monocytes, and only 2.5x in TRAPS monocytes upon activation with LPS. It has also a role in adhesion-induced cytoskeletal remodeling and cell motility, and thus may provide a sensing mechanism to couple pro-inflammatory cytokine induction with monocyte adhesion and motility (28).

TNFR1 mutant cells exhibit altered

Table V. Modulation of genes belonging to the INTERFERON signalling pathway.

Gene Symbol	TRAPS_UNT/ CTR_UNT	TRAPS_LPS/ CTR_LPS	CTR_LPS/ CTR_UNT	TRAPS_LPS/ TRAPS_UNT
APOL6			2.129	
CXCL10	-4.229			2.104
DDX58			2.953	
DDX60			2.969	
EIF2AK2			6.233	
GBP1		-2.953	5.586	2.385
GBP2			2.746	2.745
HERC5		-4.029	4.559	
HERC6		-2.692	3.345	
HIF1A			2.735	
HMGCS1			2.622	
IFI44		-2.323	8.625	
IFI44L		-2.990	4.704	
IFI6			2.400	
IFIH1			3.596	
IFIT1		-3.302	4.767	
IFIT2			10.675	
IFIT5			2.802	
IFITM1		-3.331		
IFITM3			2.342	
IFNAR1			2.715	
IL10			5.263	2.542
IL6	2.666		62.791	49.594
ISG15			4.140	
MDM2			2.156	
MX1		-2.259	6.127	
OAS1		-2.663	4.571	
OAS2		-4.925	8.594	
OAS3			3.965	
PARP12			2.809	
PLSCR1			3.032	
PMAIP1			2.784	3.285
PML		-2.617	3.271	
SOCS1			2.295	2.667
SOCS3			4.057	4.971
SP100			2.121	
SP110		-2.198	2.976	
STAT1		-2.109	2.292	
TAP1		-2.112	2.920	
TDRD7			3.137	
TNFSF10			2.493	
USP18		-3.422	4.692	
ZC3HAV1			3.710	

mitochondrial function with enhanced oxidative capacity and ROS generation (12). We found genes involved in redox regulation, such as GSR, GCLC, TXN, TXNRD1, SEP15, constitutively over-expressed in TRAPS monocytes. Noteworthy, redox proteins and HSPs are co-regulated, as both gene sets are induced by heat shock. Thioredoxin-interacting protein (TXNIP) is thought to be a negative regulator of the TRX reductase activity. In conditions of oxidative stress TXNIP binds NLRP3 and leads to its activation (34). In particular, the authors suggest that in resting cells, TXNIP interacts with TRX and is therefore unavailable for NLRP3 interaction. After the ROS increase due to NLRP3 activators, TXNIP is released from oxidised TRX and binds in turn NLRP3. Our data displayed that, after LPS treatment, TXNIP transcripts are underrepresented in patients *vs*. control, thus impairing the physiological response to the oxidative stress. Differently from expectation, we could not find autophagy players among deregulated genes, thus suggesting that the expression of autophagic proteins must be under either post-transcriptional or translational control.

Microarray data have been useful to identify pathways that have not yet been linked to TRAPS. We found that interferon type I and type II responses may be defective in TRAPS, since IFN-responsive genes are up-regulated by LPS treatment in CTR, but not in patients cells. Moreover, also interferon receptor 1 (IFNAR1) fails to be up-regulated by LPS treatment in TRAPS cells. LPS treatment down-regulates TRIM8, an ubiquitin ligase which promotes SOCS1 proteasomal degradation thus leading to repression of IFNγ signalling, only in CTR cells. CLEC2D, a protein involved in IFNγ release, is expressed at lower levels in untreated TRAPS. Finally, LPS-treated TRAPS express less USP18, an ubiquitin-specific protease, whose expression is induced by IFNβ. The TGFβ pathway also displayed differences. Both TGFβ1 and RUNX3 (a transcription factor downstream of TGFβ) are down-regulated in untreated TRAPS *vs*. CTR cells. Among other TGFβ targets, ITGB8, an integrin involved in TGFβ1 activation (35), is up-regulated by LPS more strongly in CTR than in TRAPS cells while PMEPA1, a suppressor of the TGFβ pathway (36), is down-regulated in TRAPS monocytes from all the donors in basal conditions.

TRAPS pathology ultimately targets organs through amyloid A deposition. We found gene expression profiles possibly involved in nephrotoxicity, hepatic toxicity and cardiotoxicity in both untreated and LPS-treated TRAPS cells. Genes involved in the regulation of cell proliferation and apoptosis, such as ADRA1D, MST4, BCL2A1, EMP1, NRGN, PPARG, PPP3R1, may indeed be causative agents of renal damage, if we assume that their expression profile is similar in kidney cells and monocytes. On the other hand, IL1β and IL6, elevated in untreated TRAPS, would exert their effect systemically once released into plasma, and would induce aberrant renal cell proliferation, while low TGFβ expression should result in decreased apoptosis. We are therefore tempted to speculate that, besides late amyloid A deposition, also early gene deregulation may predispose these organs to be damaged. However, only a minority of patients with long-standing inflammation actually presents with this complication, pointing to the existence of disease-modifying factors, the best characterised of which is the SAA1 genotype (37). Identifying early markers of tissue stress could allow to assess high-risk patients.

In conclusion, the gene expression profile in resting monocytes has demonstrated that patients affected with TRAPS present at molecular level a chronic inflammation condition and alterations possibly linked to the organs failure sometimes associated with the disease. In this light, the role of miRNA will deserve an in-depth analysis, being generally crucial regulators and possible targets for therapy. Moreover, the present study has confirmed the involvement of pathways already proposed as players in the development of the disease (oxidative stress and UPR) and pointed out to novel deregulated pathways (IFN and TGFβ). These results pave the way for future studies on post-transcriptional and post-translational modifications that, once elucidated, could provide new therapeutic targets for the disease.

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References

1. HULL KM, DREWE E, AKSENTIJEVICH I *et al*.: The TNF receptor-associated periodic syndrome (TRAPS): emerging concepts of an autoinflammatory disorder. *Medicine* (Baltimore) 2002; 81: 349-68.
2. MCDERMOTT MF, AKSENTIJEVICH I, GALON J *et al*.: Germline mutations in the extracellular domains of the 55 kDa TNF receptor, TNFR1, define a family of dominantly inherited autoinflammatory syndromes. *Cell* 1999; 97: 133-44.
3. STOJANOV S, MCDERMOTT MF: The tumour necrosis factor receptor-associated periodic syndrome: current concepts. *Expert Rev Mol Med* 2005; 7: 1-18.
4. HUGGINS ML, RADFORD PM, MCINTOSH RS *et al*.: Shedding of mutant tumor necrosis factor receptor superfamily 1A associated with tumor necrosis factor receptor-associated periodic syndrome: differences between cell types. *Arthritis Rheum* 2004; 50: 2651-9.
5. D'OSUALDO A, FERLITO F, PRIGIONE I *et al*.: Neutrophils from patients with TNFRSF1A mutations display resistance to tumor necrosis factor-induced apoptosis: pathogenetic and clinical implications. *Arthritis Rheum* 2006; 54: 998-1008.
6. TODD I, RADFORD PM, DRAPER-MORGAN KA *et al*.: Mutant forms of tumour necrosis factor receptor I that occur in TNF-receptor-associated periodic syndrome retain signalling functions but show abnormal behaviour. *Immunology* 2004; 113: 65-79.
7. TURNER MD, CHAUDHRY A, NEDJAI B: Tumour necrosis factor receptor trafficking dysfunction opens the TRAPS door to pro-inflammatory cytokine secretion. *Biosci Rep* 2012; 32: 105-12.
8. REBELO SL, BAINBRIDGE SE, AMEL-KASHIPAZ MR *et al*.: 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* 2006; 54: 2674-87.
9. LOBITO AA, KIMBERLEY FC, MUPPIDI JR *et al*.: Abnormal disulfide-linked oligomerization results in ER retention and altered signaling by TNFR1 mutants in TNFR1-associated periodic fever syndrome (TRAPS). *Blood* 2006; 108: 1320-7.
10. DICKIE LJ, AZIZ AM, SAVIC S *et al*.: 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* 2012; 71: 2035-43.

11. SIMON A, PARK H, MADDIPATI R *et al*.: Concerted action of wild-type and mutant TNF receptors enhances inflammation in TNF receptor 1-associated periodic fever syndrome. *Proc Natl Acad Sci USA* 2010; 107: 9801-6.
12. BULUA AC, SIMON A, MADDIPATI R *et al*.: Mitochondrial reactive oxygen species promote production of proinflammatory cytokines and are elevated in TNFR1-associated periodic syndrome (TRAPS). *J Exp Med* 2011; 208: 519-33.
13. BACHETTI T, CHIESA S, CASTAGNOLA P *et al*.: Autophagy contributes to inflammation in patients with TNFR-associated periodic syndrome (TRAPS). *Ann Rheum Dis* 2013; 72: 1044-52.
14. TASSI S, CARTA S, DELFINO L *et al*.: Altered redox state of monocytes from cryopyrin-associated periodic syndromes causes accelerated IL-1β secretion. *Proc Natl Acad Sci USA* 2010; 107: 9789-94.
15. CARTA S, TASSI S, DELFINO L *et al*.: Deficient production of IL-1 receptor antagonist and IL-6 coupled to oxidative stress in cryopyrin-associated periodic syndrome monocytes. *Ann Rheum Dis* 2012; 71: 1577-81.
16. BALOW JE, JR, RYAN JG, CHAE JJ *et al*.: Microarray-based gene expression profiling in patients with cryopyrin-associated periodic syndromes defines a disease-related signature and IL-1-responsive transcripts. *Ann Rheum Dis* 2013; 72: 1064-70.
17. GENTLEMAN RC, CAREY VJ, BATES DM *et al*.: Bioconductor: open software development for computational biology and bioinformatics. *Genome Biol* 2004; 5: R80.
18. IRIZARRY RA, BOLSTAD BM, COLLIN F, COPE LM, HOBBS B, SPEED TP: Summaries of Affymetrix GeneChip probe level data. *Nucleic Acids Res* 2003; 31: e15.
19. CHEN EY, TAN CM, KOU Y *et al*.: Enrichr: interactive and collaborative HTML5 gene list enrichment analysis tool. *BMC Bioinformatics* 2013; 14: 128.
20. FAIRCLOUGH LC, STOOP AA, NEGM OH, RADFORD PM, TIGHE PJ, TODD I: Tumour necrosis factor receptor I blockade shows that TNF-dependent and TNF-independent mechanisms synergise in TNF receptor associated periodic syndrome. *Eur J Immunol* 2015; 45: 2937-44.
21. REBELO SL, AMEL-KASHIPAZ MR, RADFORD PM *et al*.: Novel markers of inflammation identified in tumor necrosis factor receptor-associated periodic syndrome (TRAPS) by transcriptomic analysis of effects of TRAPS-associated tumor necrosis factor receptor type I mutations in an endothelial cell line. *Arthritis Rheum* 2009; 60: 269-80.
22. NEGM OH, MANNSPERGER HA, MCDERMOTT EM *et al*.: A pro-inflammatory signalome is constitutively activated by C33Y mutant TNF receptor 1 in TNF receptor-associated periodic syndrome (TRAPS). *Eur J Immunol* 2014; 44: 2096-110.
23. MA X, BECKER BUSCAGLIA LE, BARKER JR, LI Y: MicroRNAs in NF-kappaB signaling. *J Moll Cell Biol* 2011; 3: 159-66.
24. KANWAL N, JOHN P, BHATTI A: MicroRNA-155 as a therapeutic target for inflammatory diseases. *Rheumatol Int* 2013; 33: 557-60.
25. NAHID MA, SATOH M, CHAN EK: Mechanistic role of microRNA-146a in endotoxin-induced differential cross-regulation of TLR signaling. *J Immunol* 2011; 186: 1723-34.
26. LUCHERINI OM, OBICI L, FERRACIN M *et al*.: First report of circulating microRNAs in tumour necrosis factor receptor-associated periodic syndrome (TRAPS). *PLoS One* 2013; 8: e73443.
27. PARCELLIER A, SCHMITT E, GURBUXANI S *et al*.: HSP27 is a ubiquitin-binding protein involved in I-kappaBalpha proteasomal degradation. *Mol Cell Biol* 2003; 23: 5790-802.
28. SINSIMER KS, GRATACÓS FM, KNAPINSKA AM *et al*.: Chaperone Hsp27, a novel subunit of AUF1 protein complexes, functions in AU-rich element-mediated mRNA decay. *Mol Cell Biol* 2008; 28: 5223-37.
29. KNAPINSKA AM, GRATACÓS FM, KRAUSE CD *et al*.: Chaperone Hsp27 modulates AUF1 proteolysis and AU-rich element-mediated mRNA degradation. *Mol Cell Biol* 2011; 31: 1419-31.
30. LAUDANSKI K, DE A, MILLER-GRAZIANO C: Exogenous heat shock protein 27 uniquely blocks differentiation of monocytes to dendritic cells. *Eur J Immunol* 2007; 37: 2812-24.
31. ZENG L, TAN J, LU W, LU T, HU Z: The potential role of small heat shock proteins in mitochondria. *Cell Signal* 2013; 25: 2312-9.
32. BRUEY JM, DUCASSE C, BONNIAUD P *et al*.: Hsp27 negatively regulates cell death by interacting with cytochrome c. *Nat Cell Biol* 2000; 2: 645-52.
33. DE AK, KODYS KM, YEH BS, MILLER-GRAZIANO C: Exaggerated human monocyte IL-10 concomitant to minimal TNF-alpha induction by heat-shock protein 27 (Hsp27) suggests Hsp27 is primarily an antiinflammatory stimulus. *J Immunol* 2000; 165: 3951-8.
34. ZHOU R, YAZDI AS, MENU P, TSCHOPP J: A role for mitochondria in NLRP3 inflammasome activation. *Nature* 2011; 469: 221-5.
35. EDWARDS JP, THORNTON AM, SHEVACH EM: Release of active TGF-β1 from the latent TGF-β1/GARP complex on T regulatory cells is mediated by integrin β8. *J Immunol* 2014; 193: 2843-9.
36. ITOH S, ITOH F: Inhibitory machinery for the TGF-β family signaling pathway. *Growth Factors* 2011; 29: 163-73.
37. OBICI L, MERLINI G: AA amyloidosis: basic knowledge, unmet needs and future treatments. *Swiss Med Wkly* 2012; 142: w13580.