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The NF-kB regulates the SHP-1 expression in monocytes in congestive heart failure

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1. ABSTRACT

It has been shown that functional recovery of patients with acute congestive heart failure (ACHF) after treatment with conventional drugs (CD) is mediated by suppression of inflammation in peripheral blood mononuclear cells. Here, we analyzed gene expression profiles of monocytes from symptomatic ACHF patients (NYHA Class III-IV) before and after pharmacological treatment with CD. The treatment was associated with selective down-regulation of "TNFR signaling" and pro-inflammatory mediators CCL5, MIP-1α receptor, CD14, ITGAM, and significant up-regulation of "TNFR signaling" as evidenced by increase in anti-inflammatory factors including NF-kBIA, TNFAIP3 and SHP-1. In monocyte TNF-alpha-stimulated there is a down-regulation of the phosphatase SHP-1 which induces a significant activation of TAK-1/IKK/NF-kB signaling. These findings suggest that the therapeutic impact of CD treatment in symptomatic ACHF includes negative regulation of the NF-kB signaling in monocytes and the improvement of the SHP-1 activity.

2. INTRODUCTION

Heart Failure (HF) is one of the most common pathological condition in the Western World having the highest impact on survival and on the quality of life of patients. Although there have been significant progress that have reduced the mortality rate of the disease or delayed its progression and made way for quality of life considered acceptable, the prognosis of HF remains poor (1-3). Progression of HF is a result of compensatory mechanisms and modulation of T cells and complement system, and activation of a variety of pro-inflammatory cytokines, such as Tumor Necrosis Factor (TNF)-α and Interleukin (IL)-6 (4-8). This indicates that the control of inflammation is a requisite for prevention of excessive tissue damage.

Thus, there is a need to better understand the processes that contribute to the control and to resolution of inflammation. Inflammation is regulated by natural immunosuppressive mechanisms which are necessary to preserve the defense of the organism and tissue integrity. The regulation of the inflammatory

process can take place not only by using agents that inhibit the activated pro-inflammatory pathway, but also by an approach which aims to activate the natural anti-inflammatory processes. It is, therefore, essential to identify those pathways which contribute to the resolution of inflammation (9,10)

American College of Cardiology and the American Heart Association for Heart Failure recommends Angiotensin-Converting Enzyme Inhibitors (ACEIs), Angiotensin II Receptor Antagonists (ARBs), Beta-adrenoceptor antagonist (beta-blockers, BBs), diuretic, and usually digitalis for pharmacotherapy of Heart Failure. The aim of this treatment is to stabilize patients in a hemo-dynamic state as to improve symptoms, in particular dyspnea. The initial treatment may be important for an early positive outcome. The development of new pharmacological agents for Acute Congestive Heart Failure (ACHF) remains a challenge having brought slight progress in recent years (11).

The drugs used in the ACHF have also shown immune-modulatory activities that could contribute in to the therapeutic efficacy (12-14). It was evidenced that, ACEIs and Beta-blockers exert their anti-inflammatory activity reducing inflammatory cytokines such as TNF-α and IL-6 resulting in the improvement of the cardiac function and of the fraction of ejection in patients with HF (15, 16).

Moreover, angiotensin II levels and sub-clinical inflammation are closely related in Chronic Heart Failure (CHF) patients (12). The ARBs treatment decreases the levels of some inflammation markers in healthy subjects, in hypertensive patients, as well as in CHF patients, suggesting the presence of a complex crosstalk between angiotensin II and inflammation in CHF patients (14,15,17).

Because the monocytes are important for the innate immune system and their activation has a fundamental role in inflammatory pathophysiology of HF, gene expression patterns in peripheral blood have been proven as a basis for detecting diseases such as HF (18,19). This study aims to evaluate the effects of ordinary therapy on the human transcriptome in blood monocytes extracted from ACHF patients in order to identify specific molecular markers. This analysis gave a global view of the genes whose expression can be modulated by drugs usually used.

The objective of the study is to explore the various endogenous pro- and anti-inflammatory pathways of monocytes, so as to understand how conventional pharmacological therapies for ACHF could lead to clinical improvement using positive modulation of a few inflammatory mediators.

3. MATERIALS AND METHODS

3.1. Study population

Between January 2015 and July 2015, all patients diagnosed with ACHF, were admitted to the Intensive Cardiology Unit of San Camillo De Lellis Hospital (Manfredonia, Italy) (ACHF patients), and all the healthy subjects (Control Group) that matched the prerequisites in terms of education, nutrition, socio-demographic status, lifestyle, gender and age with ACHF patients, were invited to participate in this study, approved by the Ethics Committee of the hospital.

Consent was given after having being informed on the purpose of the study. The ACHF was defined according to the European Society of Cardiology guidelines (20). The eligible subjects were between 50–75 years old, with a Left Ventricular Ejection Fractions (LVEF) of ≤35%. Patients were also classified according to the criteria of New York Heart Association (NYHA). A significant hepatic dysfunction was defined as serum aminotransferase levels with twice the upper limit of what is considered normal. Significant renal dysfunction was defined as an Estimated Glomerular Filtration Rate (eGFR) of ≤30 mL/(min/1.73 m^2). The exclusion criteria included acute coronary syndromes, diabetes mellitus, active infection, malignant or inflammatory diseases, chronic renal insufficiency, history of thromboembolism, the use of steroids, immunosuppressive as well as nitrate treatment. Smokers were also excluded. The C-Reactive Protein (CRP) serum levels were measured as a non-specific marker for inflammation (Table 1). For all patients, the HF therapy consisted in ACEIs, ARBs, BBs, diuretics (Table 1). All hospitalized patients were venesected within 24 hours of admission.

The participants of the Control Group were not relative to one another nor to ACHF patients, in which no identifiable data was available. However, general characteristics are based on blood donor criteria. It was necessary for patients to be negative for Hepatitis B and C, HIV, and syphilis, and they were requested to wait up to 6 months after being exposed to risk of blood-borne infection. Intravenous illicit drug users were not allowed to donate blood. The blood and urine tests, such as SGOT, SGPT, hemoglobin, hematocrit, serum electrolytes, blood urea, and creatinine, needed to be within the normal range.

3.2. Echocardiographic-Doppler evaluation

Echocardiography was performed using an ultrasound system (Vivid-e GE Healthcare Fairfield, Connecticut) with a 3.7 MHz transducer (21).

The LVEF was evaluated by apical four-chamber and two-chamber views, using the Simpson's biplane method. Each value was obtained from the average of three measurements in accordance with the American Society of Echocardiography criteria (22).

Table 1. Clinical characteristics ACHF patients pre- and post-treatment

Variable	ACHF group (pre-)	ACHF group (post-)	*p* value
Overall	50	-	
Age (years), mean±SD	65.1±5.8	-	
NYHA functional class, n (%)			
3	26	-	
4	24	-	
SBP (mmHg), mean±SD	125±21	115±17.2	0.131[(1)]
WBC(x103/µl), mean±SD	7.1±1.2	5.6±1	ns
CRP(mg/dl), mean±SD	0.4±0.1	0.4±0.1	ns
Cholesterol (mg/dl), mean±SD	142.8±31.9	165.3±35.6	ns
Triglycerides (mg/dl), mean±SD	85.1±34.1	107.7±27.2	ns
Hb (g/l), mean±SD	11.6±1.4	13.2±1.5	ns
Creatinine (mg/dl), mean±SD	1.5 ± 0.7	1.2±0.9	ns
Glycaemia (mg/dl), mean±SD	103.6±31.0	110.7±45.1	ns
(BMI), mean±SD	29.1±3.5	-	
Treatment, n (%)			
ACEIs	30 (60)	-	
ARBs	10 (20)	-	
BBs	20 (40)	-	
Diuretics	35 (70)	-	
LVEF (%)	37.2±12.1	49.2±12.1	0.001

SBP: Systolic Blood Pressure; HB: Hemoglobin; ACEIs: ACE inhibitors; ARBs: Angiotensin II Receptor Blockers; BBs: Beta-adrenergic receptor blockers. The definition for obesity is having a BMI greater than or equal to 30 kg/m^2 (32). [1]Mann-Whitney U test ACHF (pre-) *vs* ACHF (post-)

The assessment of the valves included the evaluation of the function of the mitral, aortic and tricuspid valves. Color-Doppler echocardiography was performed after optimizing a gain resulting the Nyquist limit, where continuous standard and pulsed-wave Doppler recordings were acquired. Mitral valve stenosis and regurgitation diseases were evaluated according to semi-quantitative and quantitative methods recommended by the American Society of Echocardiography (23, 24).

3.3. Sample collection and isolation of human monocytes, lymphocytes and neutrophils

Venous blood was collected by phlebotomy in the EDTA vacutainers (6mL K2EDTA, Becton Dickinson, USA) and processed within 2 hours of procurement. The serum was isolated from the blood of all patients. Human monocytes were separated from the blood of all patients, both at pre-pharmacological and post-pharmacological treatment, as described previously (25). Briefly, monocytes were isolated by density-gradient centrifugation through Ficoll/Hypaque (Pharmacia), after centrifugation (1500× rpm; 4◦C; ×25 min), while the interphase layer containing Primary Blood Mononuclear Cells (PBMC) was carefully removed, washed in PBS (1×) followed by centrifugation (2500 × rpm; ×15 min) and suspended (4x10^6 cells/mL) in the RPMI-1640 medium with 10% heat-inactivated human serum (Sigma), and then seeded into flasks. After incubation for 1 hour at 37 °C, adherent cells were detached and then suspended (1x10^6 cells/mL) in a medium, supplemented with 10% of fetal bovine serum (Sigma). The cell suspension was constituted by >90% monocytes and was seeded onto six well tissue culture plates. The cell was placed into the Trizol reagent (Invitrogen, Carlsbad, CA) and immediately stored at -80 ◦C until further processing. The cell viability, which was determined by trypan blue exclusion, was > 99%. The same batch of serum and medium were used in all experiments. The content of lipopolysaccharide in media and serum was very low as determined by testing the chromogenic assay using limulus amoebocyte lysate (minimum detection level 0.1. µg/mL; Whittaker Bioproducts, Walkersville, MD, USA). To detect the role of the SHP-1 in regulating the TAK1/IKK/NF-kB signaling, during the study, the cells were treated with the chemical inhibitor of human SHP-1 (Sodium Stibogluconate, SS, 10 µM) (Merck (Darmstadt,

Germany), and/or the TNF-alpha (20 ng/mL) (Sigma-Aldrich, St Louis, MO).

To detect the role of the SHP-1 in regulating nuclear translocation of the NF-kB after the TNF-alpha stimulation, monocytes, lymphocytes and neutrophils from ten (10) healthy volunteers, were transfected with the siRNA against the human SHP-1 or controlled siRNA (Dharmacon, Chicago, IL, USA) at a concentration of 1 microg/10^6 cells. The transfection reagent (Dharmafect 2; Dharmacon) was used as specified by the manufacturer. Cells were incubated in the transfection medium for 24 hours, after which the medium was replaced with a complete growth medium for another 48 hours before the TNF-alpha treatment (20 ng/mL) (Sigma-Aldrich, St Louis, MO). The effectiveness of the SHP-1 siRNA in lowering the SHP-1 expression was evaluated by Western Blotting (WB).

Human monocytes were isolated as described above. Briefly, human lymphocytes were isolated by density-gradient centrifugation through Ficoll/Hypaque (Pharmacia), after centrifugation (1500× rpm; 4°C; ×25 min), the interphase layer containing Primary Blood Mononuclear Cells (PBMC) was carefully removed, washed in PBS (1×) followed by centrifugation (2500× rpm; ×15 min) and suspended (4x10^6 cells/mL) in RPMI-1640 medium with 10% heat-inactivated human serum (Sigma), and seeded into flasks. After incubation for 1 hour at 37 °C, suspension cells were detached, re-suspended (1x10^6 cells/mL) in a medium, supplemented with 10% of fetal bovine serum (Sigma). During the same experiments, human neutrophils were recovered from the ficoll-paque pellet of sedimented Red Blood Cells (RBC) with granulocytes. The RBC pellet was immediately suspended in 5 ml of 1 x HBSS buffer for further processing. The dextran sedimentation was performed with 3% and 6% dextran solution, in order to select the optimal dextran concentration to be used in sedimentation of the neutrophils. Red cell and granulocyte suspension was immediately mixed with 3% and 6% dextran (Fisher Scientific, NJ, and USA) and optimal RBC sedimentation was finally achieved at 30 minutes, RT in the dark room. After sedimentation, the neutrophil rich supernatant at an upper layer was collected and centrifuged for 5 minutes at 600× rpm at RT. The RBC lysis was performed to obtain a pure granulocyte population. After dextran sedimentation, the remnant or trace amount of RBC was lysed using RBC lysis buffer. Ten times (10X) RBC lysis buffer was prepared by adding 8.3 g of NH_4Cl (Sigma, Germany), 1.0 g of $KHCO_3$ (Sigma, Germany), 1.8.ml of 5% EDTA (Sigma, Germany) in 1000 ml of sterile water. The RBC lysis is a sensitive step in the neutrophil isolation, hence optimization of downstream steps were carried out. Based on optimization, the RBC lysis was performed once for 20 seconds (time was strictly monitored using a stopwatch). The lysis process was stopped using 1 x HBSS buffer and centrifuged for 10 minutes at 400× rpm with no brakes. After centrifugation, the supernatant was discarded and the white pellet consisting of granulocytes was obtained and re-suspended immediately in the RPMI complete medium (Gibco, United Kingdom). After the lysis, the morphological examination and trypan blue exclusion tests were performed to determine the cell count and purity of the neutrophils (26).

3.4. Microarray

The total RNA extraction from human monocytes was performed using the Trizol reagent (Invitrogen, Carlsbad, CA). The RNA concentration and purity were determined by measuring absorbencies at 260 and 280 nm, and a 260:280 ratio of 1.7. was considered acceptable for analysis. On a denaturing gel, the extracted RNA was run to highlighted two (2) bright distinct bands, representing the 28S and 18S ribosomal species. This assured that the RNA lacked DNA contamination and that the RNA had not been degraded, both of which could confuse the array results. Analysis was carried out using high density array containing approximately 30968 human genome probes and 1082 experimental control probes (60-mer sense-strand polynucleotide probes) (Eurogentec, Seraing, BE) (27). One µg RNA of each patient (n=11), pre-treatment and post-treatment, was amplified using the "Ammino Allyl MessageAmp^{TM} II aRNA Amplification kit" (Ambion, Austin, TX, USA), able to produce aRNA, containing 5-(3-amminoallyl)-UTP modified nucleotides. The obtained aRNA (5-20 µg) was labelled with Cys3 or Cys5 fluorochromes (Amersham, Pharmacia Biotech, Buckinghamshire, UK) and hybridized on the array. Data showed the results of eleven (11) independent analyses. Fluorescent signals were captured by ScanArray 5000 Packard laser scanning (Packard BioChip Technologies, Billerica, MA) and normalized using the "ScanArray Express" software. Microarray data are deposited in the GEO public database (accession number: GSE51888). All data are MIAME compliant.

3.5. Quantitative real-time PCR

The Quantitative Real-time PCR (qPCR) assay was carried out in an Eppendorf Mastercycler EP Realplex (Eppendorf AG) as described previously (28). Preliminary PCR reactions were run to optimize the concentration and ratio of each primer set. For all the cDNA templates, 2 µL was used in a 20 µL qPCR amplification system of the SYBR Green Real Master Mix Kit according to the manufacturer's directions. Primers for human CCL5, CD14, ITGAM, CCR1, NFKBIA, PTPN6, TNFAIP3 genes and GAPDH as control were designed using GeneWorks software (IntelliGenetix, Inc., Mountain View, CA, U.S.A.). The primer pairs used are shown in Table 2.

Similar amplification procedures and data computation were followed as described above. No PCR products were generated from genomic versus the cDNA template. The fluorescence intensity of the

Table 2. Human primers used for qPCR

Name	Reference	5'-3'	3'-5'
GADPH	NM_002046	GCGCCCAATACGACCAA	GCTCTCTGCTCCTCCTGTT
CCL5	NM_002985	TACCATGAAGGTCTCCGC	GACAAAGACGACTGCTGG
CD14	NM_000591	GCTGTGTAGAAAGAAGCTAAAGCACTT	TGGCGTGGTCGCAGAGA
ITGAM	NM_000632	GCCTTGACCTTATGTCATGGG	CCTGTGCTGTAGTCGCACT
CCR1	NM_001295	TCCTGCTGACGATTGACAGGTA	GTGCCCGCAAGGCAAAC
NFKBIA	NM_020529	TCTCTGGCAGCATCTGAAGGT	CCCAAGCACCCGGATACAG
PTPN6	BC_002523	TGGCGTGGCAGGAGAACAG	GCAGTTGGTCACAGAGTAGGGC
TNFAIP3	NM_006290	TCCTCAGGCTTTGTATTTGAGC	TGTGTATCGGTGCATGGTTTTA

GADPH: Glyceraldehyde 3-phosphate dehydrogenase; CCL5: Chemokine (C-C Motif) Ligand 5; CD14: Cluster of differentiation 14; ITGAM: Integrin alpha M; CCR1: Chemokine (C-C Motif) Receptor 1; NF-kBIA: NFKB inhibitor alpha; PTPN6: Protein Tyrosine Phosphatase Non-Receptor Type 6; TNFAIP3: Alpha-Induced Protein 3

double-strand-specific SYBR Green, reflecting the amount of formed PCR product, was monitored at the end of each elongation step. The melting curve analysis was performed to confirm the purity of the PCR products. The relative expression of CCL5, CD14, ITGAM, CCR1, NFKBIA, PTPN6 and TNFAIP3 was normalized to GAPDH using the ΔCT method (relative expression = $2^{-\Delta CT}$, where ΔCT =CT (CCL5, CD14, ITGAM, CCR1, NFKBIA, PTPN6, TNFAIP3) – CT(GAPDH)). The predicted cycle threshold values were exported directly onto Excel worksheets for analysis. The relative changes in gene expression were determined by the $2^{-\Delta\Delta CT}$ method as described previously and reported as the difference (n-fold) relative to the value for a calibrator cDNA (control) prepared in parallel with the experimental cDNAs. The data represented with three different experiments each run in triplicate and presented as the mean ± SEM of triplicates. The DNA was denatured at 95 °C for 2 minutes followed by 40 cycles of 30 seconds at 95 °C together with 30 seconds at 60 °C. The experiments were repeated twice with consistent results.

3.6. Western blotting

Monocytes were washed one time, in cold Phosphate-Buffered Saline (PBS; 0.5. mol/L sodium phosphate, pH 7.5.), collected by gentle scraping, and used to prepare total protein and nuclear protein extracts. Total protein extracts were prepared by treating cells with the lysis buffer as described previously (29). Nuclear extracts were prepared as previously described (30). The protein concentration of the extracts were determined using the Bradford method (Bio-Rad protein assay, Hercules, CA, U.S.A.).

As regards the Western Blotting (WB) analysis, 50 μg of protein per lane was separated on a 4–12% NuPAGE gradient gel (Gibco Invitrogen), electro-transferred onto a nitrocellulose membrane and blocked with 10% skimmed milk in PBS containing 0.1. % Tween-20. Blots were probed and incubated overnight at 4 °C with polyclonal rabbit IgG anti-SHP-1, TAK-1, pTAK-1, IKK, pIKK, IKB, pIkB, and pNFkB (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.), all at 0.2. microg mL^{-1} in Tris-buffered saline (TBS) ⁄0.1. % Tween-20. A mouse antihuman monoclonal antibody recognizing human beta-actin (A5441; Sigma-Aldrich) was used as the control in all experiments. A rabbit antihuman antibody for beta-laminin was used as the control in experiments with nuclear extract proteins (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.). Blots were then washed and incubated for 1hour with goat anti-rabbit–horseradish peroxidase (Pierce Biotechnology, Rockford, IL, U.S.A.) diluted 1:10 000 in TBS⁄0.1. % Tween-20. Immunoblot signals were developed using the Super Signal Ultra chemiluminescence detection reagents (Pierce Biotechnology). The blot images were analyzed with a gel analysis software package (Gel Doc 1000; Bio-Rad,Milan, Italy). Data was expressed as the mean ± SD intensity of optical density.

3.7. Statistical analysis

The results were reported separately for the ACHF (pre-) group and the ACHF (post-) group. All qualitative variables were summarized as a frequency and as a percentage and all quantitative variables as a mean and Standard Deviation (SD). A non-parametric Mann-Whitney U-test was applied for assessing the comparison of the quantitative variables between the two groups: ACHF (pre-) group and the ACHF (post-) group. In all statistical tests the threshold of statistical significance is assumed to be equal to $p<.05$. Data was analyzed by SPSS ®Advanced Statistical 19.0. software (SPSS Inc, Chicago, Illinois, USA).

In order to identify genes with a certain level of accuracy, the Significance Analysis of Microarrays (SAM) (27) and Fold Change (FC) criteria were employed simultaneously. A gene was considered to be expressed differently if it had a FDR<.5% for the SAM analysis and a FC≥1.5. in the average of the mRNA expression. Ingenuity

Table 3. Comparison of selected genes identified in monocytes of patients as down-regulated or up-regulated after treatment with conventional drugs

Name	Fold change ± SD (Microarray)	Fold change±SD (qPCR)
CCL5	-1.8±0.6[(1)]	−2.1±0.4[(3)]
CD14	-1.8±0.5[(1)]	−1.7±0.3[(4)]
ITGAM	-2±0.6[(1)]	−2.3±0.5[(4)]
CCR1	-2.8±0.7[(2)]	−3.8±0.9[(3)]
NFKBIA	2.5±0.6[(1)]	2.2±0.2[(3)]
PTPN6	1.9±0.6[(2)]	2.4±0.3[(4)]
TNFAIP3	3±0.9[(1)]	3.6±1.1[(3)]

CCL5: Chemokine (C-C Motif) Ligand 5; CD14: Cluster of differentiation 14; ITGAM: Integrin alpha M; CCR1: Chemokine (C-C Motif) Receptor 1; NF-kBIA: NFKB inhibitor alpha; PTPN6: Protein Tyrosine Phosphatase Non-Receptor Type 6; TNFAIP3: Alpha-Induced Protein 3., [(1)]FDR: 0%, [(2)]FDR: 0.1% (n=11); [(3)]p<.01, [(4)]p<.05 (n=50)

Pathway Analysis Application (IPA) (Ingenuity Systems, Mountain View, CA, USA) was used to identify the biological organization of genes in cellular functions. Fisher's exact test was used to calculate a p-value determining the probability of the associations (p<.05). Benjamini-Hochberg multiple testing correction was used to calculate a p-value for significant pathways within the gene list (p<.05) (31).

4. RESULTS

4.1. The demographic, clinical and echocardiographic characteristics

Of the sixty-one (61) patients hospitalized, fifty (50) fulfilled the diagnostic criteria for acute congestive heart failure. The characteristics are shown in Table 1.

4.2. Microarray analysis and qPCR analysis

Global gene expression analysis was performed using a microarray method to investigate the underlying alteration in gene expression from monocytes of patients with ACHF before and after treatment with conventional drugs. The SAM analysis (FDR<.5%) and Fold Change criteria (genes differentially expressed at least 1.5.-fold after treatment) identified a gene list of 481 genes: 165 genes were significantly up-regulated, while 316 genes were significantly down-regulated after treatment (Supplementary File 1). Analysis of the genes list with the IPA indicated that 328 genes are eligible both in functions and pathways. The IPA showed that monocytes were likely to have altered the activity of the immune bio-function: an inflammatory response (P=1.7.6E-06), an immunological disease (P=2.6.E-06) and an inflammatory disease (P= 2.6.E-06). Especially, the analysis reported an expression modulation by treatment of inflammatory key molecules including a down-regulation of the pro-inflammatory mediator CCL5, MIP-1α receptor, CCR1 and two other potential activation markers on these cells, the CD14 and the ITGAM. These molecules have shown to be important in trafficking, in adhesion, in phagocytosis, and in the migration of monocytes at inflammatory sites (33, 34). The analysis also showed an up-regulation of anti-inflammatory molecules NF-kBIA and the gene PTPN6, encoding for the phosphatase SHP-1. Microarray data was confirmed by the qPCR (Table 3). Together, these findings indicated that monocytes by pre-treatment ACHF patients were characterized by a relatively elevated inflammatory state.

Moreover, we employed the IPA in order to investigate which cytokines and relative signaling were associated with the gene list. The analysis revealed that many specific treatment responsive genes linked to the TNF-α (ratio: 74/328), pointing out that this cytokine is the main molecule involved in modulating the monocytes expression profile.

In accordance with these findings, IPA showed that the pathways significantly involved with regulated genes were Tumor Necrosis Factor Receptor 1 (TNFR1) signaling (B-H p-value: 4.9.E-03), TWEAK signaling (B-H p-value: 1.7.9E-02), TNFR2 signaling (B-H p-value: 4.2.6E-02) and CCR5 signaling in macrophages (B-H p-value: 4.7.8E-02). Microarray Analysis revealed that mRNA levels of TNF-α and its receptors (*TNFRSF1A* and *TNFRSF1B* genes) were not significantly modified into monocytes. However, pharmacological treatment significantly up-regulated *NF-kBIA,* and *TNFAIP3* genes, strictly involved in the inhibition of TNF-α to the NF-kB signaling.

4.3. TAK-1/IKK/NF-kB signaling in monocytes of ACHF patients

In the TNF-alpha pathway, the binding of the trimeric TNF-alpha ligand to the TNFR lead to the trimerization of the TNFR and subsequent recruitment of signaling proteins including the protein kinase TAK-1, which is essential for the activation of the NF-kB in response to the TNF-alpha. Following the NF-kB nuclear translocation involved in the activation of the IKKbeta and results in the expression of pro-inflammatory genes (35,36). During the downstreaming of this process, we can speculate the TAK1/IKK/NF-kB activation in monocytes from pre- treatment patients. The TAK-1 and the IKKbeta activity are activated by phosphorylation of the Thr-187 and the serine 181 respectively. Moreover, activating the IKK complex phosphorilates IkBalpha at two serine sites (32 and 36), triggers its ubiquitination and degradation by 26S proteasome. These modifications can be detected by the WB. As shown in Figure 1A-C, the monocytes from pre-treatment patients showed the activation of the TAK-1 and of the IKKbeta by phosphorylation on these sites, whereas un-phosphorylated TAK-1 and IKKbeta levels did not change after treatment. As expected, the level of pIkBalpha (Ser-32) is higher in pre-treated patients

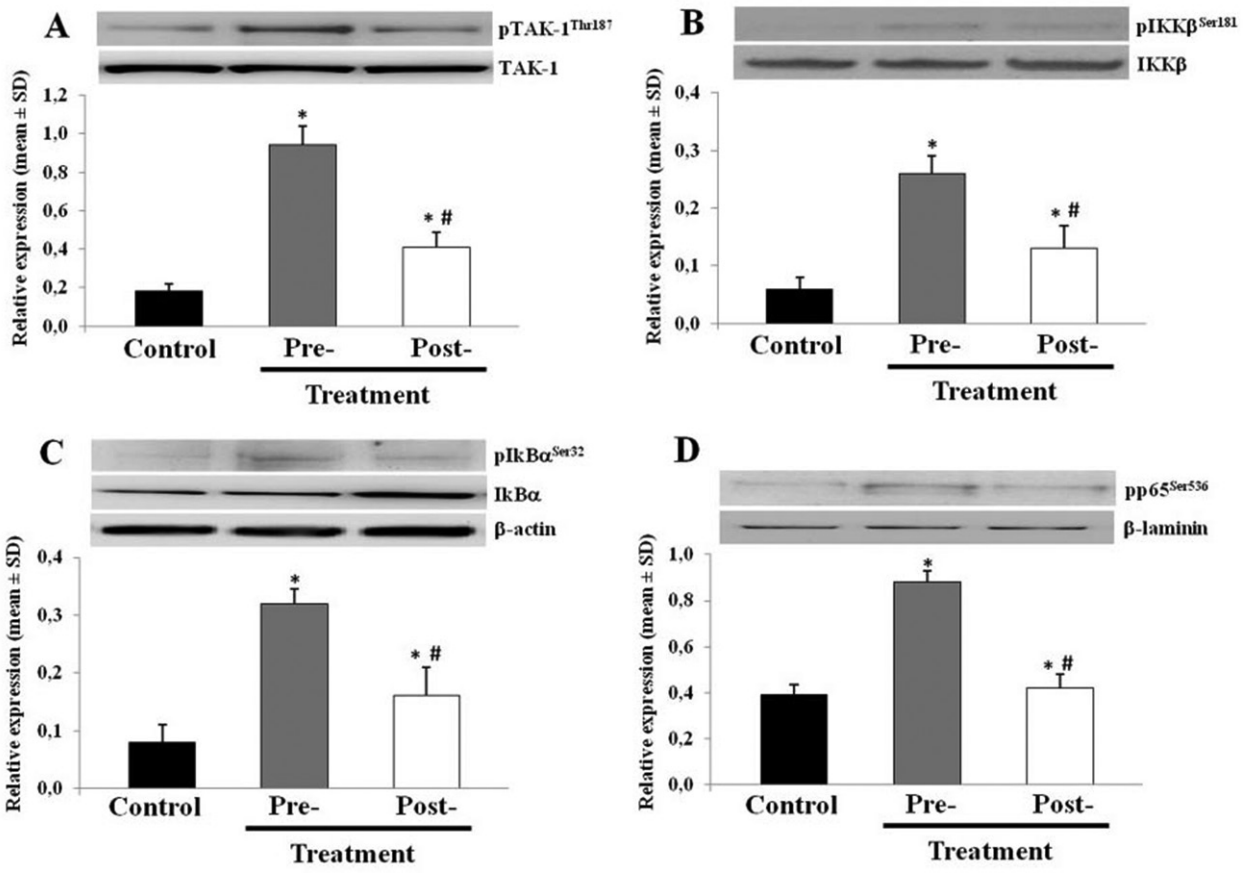

Figure 1. The activation of the TAK-1/IKK/NF-kB signaling in ACHF patients. The representative image of Western blotting experiments on cultured monocytes from healthy donors (Control) and ACHF patients pre- and post- treatment with conventional drugs. Total protein extracts were incubated against antibodies for the pTAK-1^{Thr187} and the TAK-1 (A) or the pIKKbeta^{Ser181} and IKKbeta (B). Densitometric analysis was performed on three different consistent experiments. Data are mean ± SD (n=50, Control; n=50, ACHF patients). C) The representative Western blot and densitometric analysis of IkBalpha and pIkBalpha^{Ser32} by monocytes from Control and ACHF patients pre and post treatment. Relative quantification was performed using ppIkBalpha^{Ser32} in respect to the IkBalpha expression as the standard (bottom). D) At the top end, the representative image of Western blot experiments showing the relative expression of the pp65^{Ser536}on nuclear extract proteins from monocytes of Control and ACHF patients. Beta laminin was used as the internal standard. Data are expressed as mean ± SD of three independent experiments performed in triplicate (bottom). *p<.05 vs. Control; #p<.05 vs. pre-treatment ACHF patients.

with conventional drugs, whereas the WB analysis for un-phosphorylated IkBalpha protein confirmed an up-regulation of the mRNA reported through Microarray analysis as well as the qPCR analysis after therapy. As a post-translational modification of the NF-kB (p65) affecting its transcriptional activities, we performed further studies to determine the phosphorylation of the p65 at Ser-536 by the WB, using specific phospho-p65 antibody on nuclear protein extracts. We observed that treatment with conventional drugs decreased the nuclear translocation of the pp65^{Ser536} (Figure 1D).

4.4. SHP-1 role in regulating TNF-alpha to NF-kB signaling in monocytes of ACHF patients

The SHP-1 is a protein tyrosine phosphatase with two SH2 domains which act as a negative regulator of both innate and acquired immune cytokine signaling. Several reports have documented the negative regulation of the NF-kB by the SHP-1 (37-39). Therefore, it was important to confirm the modulation obtained in the mRNA expression at a protein level. The Western Blotting analysis of the SHP-1 protein of cultured monocytes from post-treatment ACHF patients, significantly showed higher levels (2-fold) than those of pre-treatment patients (Figure 2A).

In order to investigate the role of SHP-1 in regulating TNF-alpha to the NF-kB signaling, monocytes from healthy donors and ACHF patients were treated with medium being TNF-alpha or TNF-alpha and a selective inhibitor of the SHP-1 activity, Sodium Stibogluconate (SS). Nuclear protein extracts were detected with a specific antibody for the pp65^{Ser536}. As showed in Figure 2B, treatment with the TNF-alpha increased the pp65 expression in nuclei. This response was significantly elevated in monocytes of pre-treatment of

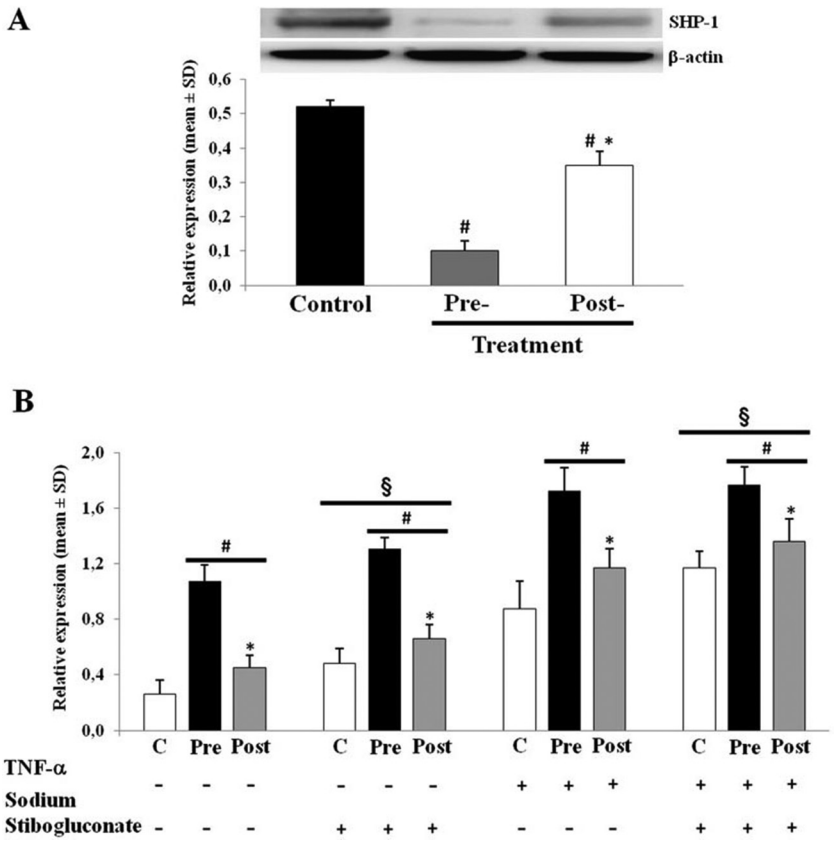

Figure 2. The role of the SHP-1 on the NF-kB signaling in TNF-alpha stimulated monocytes. A) The SHP-1 protein expression levels in freshly isolated monocytes from healthy donors (Control, C), and ACHF patients pre and post treatment with conventional drugs. At the top end, the representative Western blot image of the SHP-1 and beta-actin. At the bottom end, each immuno-reactive band was analyzed by densitometry and normalized to beta-actin levels. Each value is a mean ± SD of three different experiments performed in triplicate (n=50, Control; n=50, ACHF patients. B) the pp65^{Ser536} nuclear expression in cultured monocytes from Control and ACHF patients after treatment with pro-inflammatory cytokine TNF-alpha ng/mL and the chemical inhibitor of the SHP-1 phosphatase activity, Sodium Stibogluconate (SS) (10 microM), as described in the Materials and Methods section. Western immune-blotting was performed on nuclear proteins with antibody anti-pp65^{Ser536} and anti-beta-laminin as internal standard. *p<.05 vs. pre-treatment ACHF patients; $^{\#}$p<.05 vs. C; §p<.05 vs. relative monocytes no treated with SS.

ACHF patients as compared to post-treatment of ACHF patients and healthy donors. Interestingly, incubation with the SHP-1 inhibitor significantly increased the NF-kB nuclear translocation in monocytes from post-treatment patients.

To further confirm whether decreased SHP-1 levels are directly responsible for NF-kB nuclear translocation after TNF-alpha stimulation in monocytes and to verify whether this process affects other blood cell types, monocytes, neutrophils and lymphocytes found in healthy volunteers were treated with siRNA to acutely deplete SHP-1 (Figure 3). Firstly, we observed that SHP-1 protein was substantially depleted by more than a 5-fold in the monocytes treated with theSHP-1 siRNA compared to monocytes treated with controlled siRNA in a scrambled sequence (Figure 3A). The efficiency of SHP-1 depletion showed the same amount in neutrophils and lymphocytes (data not shown). Accordingly, SHP-1 depletion resulted in a significant increase of pp65^{Ser536} nuclear levels both in cells treated with or without the TNF-alpha (Figure 3B).

5. DISCUSSION

The comparison between the monocytes global expression profile of ACHF patients pre- and post-pharmacological treatment with ACEIs, ARBs, BBs or diuretics, was the primary goal of this study. The intention of the study was to investigate the inflammatory pathways modulated by conventional therapy in ACHF patients. We chose monocytes which are one of the main sources of production and secretion of inflammatory mediators (19). From the analysis of the gene expression profile with IPA, it was evident that 328 genes are eligible both in functions and pathways (see Supplementary File 1). As expected, these genes are mainly involved in the inflammatory process (P=1.7.6E-06) and 23% of those are linked to pro-inflammatory cytokines TNF-alpha. TNFR signaling, resulted as mainly modulated within

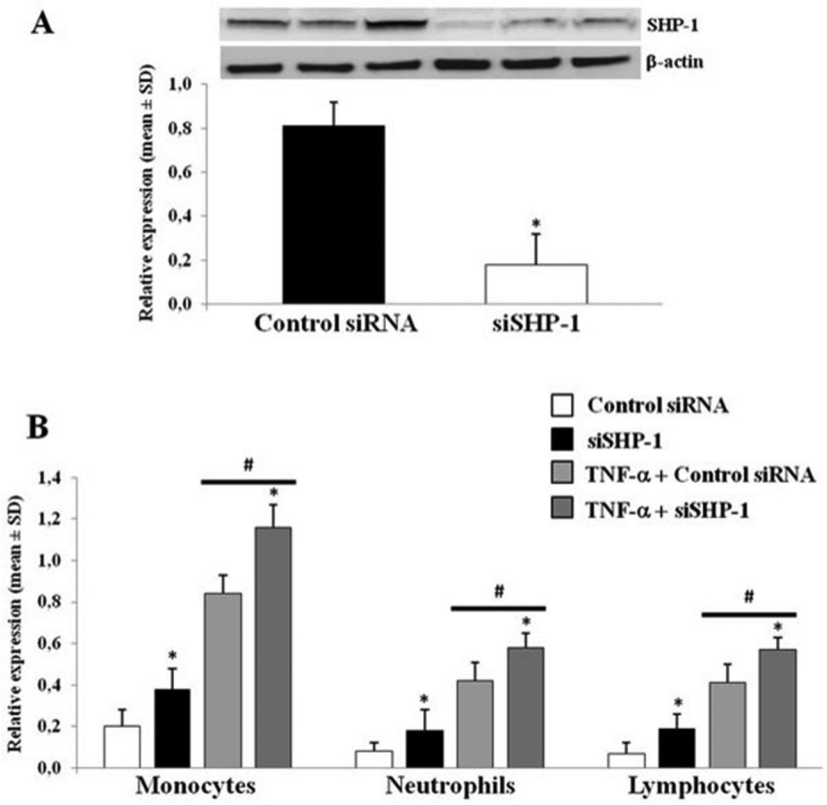

Figure 3. The SHP-1 was depleted in monocytes, neutrophils and lymphocytes from healthy volunteers using the siRNA and the nuclear level of the pp65^{Ser536} with or without TNF-alpha stimulation, was characterized in the context of the SHP-1 deficiency. A) Monocytes from healthy volunteers were treated with the SHP-1 siRNA or controlled siRNA (scramble sequences) and the levels of the SHP-1 and beta-actin protein levels were quantified by the Western immune-blot analysis. In the same panel the amount of SHP-1 measured by the Western immune-blot were quantified by measuring the pixel density relative to beta-actin. B) the pp65^{Ser536} nuclear expression in cultured monocytes, in neutrophils and in lymphocytes from healthy volunteers after treatment with pro-inflammatory cytokine TNF-alpha (20 ng/mL) and the SHP-1 siRNA or controlled siRNA (scramble sequences), as described in the Materials and Methods section. The Western immune-blotting was performed on nuclear proteins with the antibody anti-pp65^{Ser536} and the anti-beta-laminin as internal standard. *p<.05 vs. correspondent Control siRNA treated cells; $^{\#}$p<.05 vs. cells not treated with TNF-alpha.

genes which were significantly considered. Our data confirms that monocytes from pre-treatment patients were characterized by a higher inflammatory state when compared to those from post-treatment.

To validate microarray data and further confirm the dual inflammatory condition pre- and post- conventional treatment, we analyzed using the qPCR, some inflammatory key molecules, namely chemokine CCL5, receptor of MIP-1α, CCR1, and two other potential activation markers of monocytes, CD14 and ITGAM (Table 3). These molecules have been shown to play a pivotal role in the adhesion, phagocytosis, and migration of monocytes at inflammatory sites (40, 41). We reported that, in *ex vivo* monocytes, from ACHF patients treatment with ordinary drugs significantly down-regulated these genes (Table 3). Together, we analyzed the anti-inflammatory genes *NF-kBIA, PTPN6* and *TNFAIP3*, involved in the abrogation of signaling from TNF-alpha to the NF-kB, and reported its up-regulation after pharmacological treatment. *TNFAIP3* was identified as a gene whose expression is rapidly induced by TNF-alpha. The protein encoded by this gene is a zinc finger protein and ubiquitin-editing enzyme, and has been shown to inhibit NF-kB activation as well as TNF-mediated apoptosis. Moreover, the encoded protein, which has both ubiquitin ligase and deubiquitinase activities, is involved in the cytokine-mediated immune and inflammatory responses (42-44).

The role of the *TNFAIP3* in inhibiting nuclear translocation of NF-kB induced by TNFR activation has been thoroughly investigated; mouse embryonic fibroblasts derived from *TNFAIP3*-deficient mice have sustained TNF-induced NF-kB responses as well as an increased sensitivity to TNF-induced cell death (44). Further, deletional analysis of *TNFAIP3* in the NF-kB context has shown that the carboxyl-terminal domain of TNFAIP3 containing seven zinc fingers was sufficient to block the TNF-induced NF-kB activation (43, 45). Therefore, we propose that the up-regulation of *TNFAIP3*, together with NF-kB inhibitor IkBα (*NF-kBIA)*

contributes to the recovery of inflammatory condition that characterizes patients from an acute phase of HF. This is confirmed by the expression data of *NF-kBIA, PTPN6* and *TNFAIP3,* which follow the same trend seen in microarray analysis: in ACHF patients we observed a significant higher level of the mRNA for this gene after pharmacological treatment (Table 3). Our findings reinforce the latter but fragmented studies which show that some beneficial effects of treatment with ordinary drugs were exerted through the reduction of inflammatory condition that identify ACHF patients. Although, the microarray data showed neither the TNF-alpha nor the TNFR expression variation during therapy, more than a fifth of significantly linked genes with some of the TNF-alpha biological functions. This cytokine is probably the most well-characterized inflammatory molecule in HF.

It is important to keep in mind that higher levels of circulating TNF-alpha were initially identified in patients with edematous decompensation (46). Moreover, several studies have reported that this cytokine is already activated at earlier phases of HF and continues to rise in direct relation to the worsening of the NYHA functional class (47-49). Our data has shown that in *ex vivo* monocytes from ACHF patients, treatment with ordinary drugs downsize the intensity of the NF-kB signaling (Figure 1). In most instances, the TNF-alpha binding to TNFR induces an NF-kB response. Indeed, the inactive NF-kB transcription factors in the cytoplasm are bound to inhibitory I-kB proteins, and when stimulated, I-kB is phosphorylated on specific serine residues by the IKK (50). This phosphorylation event targets the I-kB for ubiquitination and degradation by the proteasome, thereby allowing the free NF-kB factors to translocate to the nucleus. Following the NF-kB, the nuclear translocation involved the activation of the IKKbeta and results in the expression of pro-inflammatory genes. During the downstreaming of this process, we can suppose that there is an activation of TAK-1/IKK/NF-kB signaling in monocytes of pre-treatment patients. As shown in Figure 1A and 1B, we observed, in ACHF patients, an increased activation of TAK-1 and of IKKbeta, whereas un-phosphorylated TAK-1 and IKKbeta levels did not change after treatment. As expected, the level of p-IkBalpha was higher prior to the pre-treatment with conventional drugs, whereas WB analysis for un-phosphorylated I-kBalpha protein confirmed an up-regulation of the mRNA reported through the microarray analysis and the qPCR analysis after therapy (Figure 1C). Due to the post-translational modification of NF-kB which affects its transcriptional activities, we further performed studies to determine the phosphorylation of p65 by the WB on nuclear protein extracts. We observed that treatment with conventional drugs decreases the nuclear translocation of $pp65^{Ser536}$ (Figure 1D).

As mentioned above, the treatment with ordinary drugs, up-regulates the expression of PTPN6 gene that encodes for a protein tyrosine phosphatase ubiquitously expressed in hematopoietic and non-hematopoietic cells, named SHP-1 (Table 3). Studies addressing its functions have typically been performed using motheaten (me/me) and motheaten viable (mev/mev) mouse strains, which spontaneously arise the SHP-1-deficient strains (51, 25). These mice display an increased susceptibility to autoimmune and innate inflammatory diseases (52, 53). This phosphatase contains two SH2 domains which act as negative regulators of both innate and acquired immune cytokine signaling and negatively regulating the NF-kB activation and expression as well as its respective pro-inflammatory genes (53-56, 25, 39). Interestingly, our findings were in accordance with the above evidence. As shown in Figure 2A, the monocytes expression levels of SHP-1 were increased by treatment and contribute to the downsize of the nuclear translocation of p65 sub-unit of NF-kB. The role of SHP-1 in regulating TNF-alpha to the NF-kB signaling, is confirmed by its activity inhibition with sodium stibogluconate (Figure 2B), in which we observed significant increase of TNF-alpha induced phosphorylation of p65 subunit at Ser536 and following nuclear translocation. This finding was verified by a further experiment in which $pp65^{Ser536}$ was significantly higher in nuclei of monocytes from healthy donors treated with anti-SHP-1 siRNA and stimulated or not stimulated with TNF-alpha (Figure 3). Remarkably, we observed the same trend of monocytes in neutrophils and lymphocytes which claim to further investigations on these cell types.

Our study underlines the fact that treatment with ordinary drugs down-regulated pro-inflammatory and immune-modulators key molecules (chemokine CCL5 and the receptor of MIP-1alpha, CCR1) which regulate several biological processes such as chemotaxis, the activation and the migration of leukocytes to areas of inflammation, collagen turnover, angiogenesis, and apoptosis (57). Moreover, expression data, combined with functional analysis, demonstrates that treatment with conventional drugs of symptomatic ACHF negatively affects TAK-1/IKK/NF-kB signaling in blood monocytes and that improvement of the SHP-1 activity plays a role in this process. In drawing conclusions, we first proposed the above mentioned genes as potential markers for monitoring the response to therapy in HF. However, despite the significant changes observed in gene expression, data on changes in the circulating levels of protein products, from large-scale trials, are necessary to be correlated over time with the morbidity and mortality in ACHF patients.

These results lay the basis for following analysis on the possible changes of “pro-inflammatory pathway”, highlighted above, which could also be used to identify new therapeutic strategies in non-responsive patients.

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Abbreviations: acute congestive heart failure (ACHF), tumor necrosis factor receptor (TNFR), Chemokine (C-C Motif) Ligand 5 (CCL5), macrophage inflammatory protein 1-alpha (MIP-1α receptor), cluster of differentiation 14 (CD14),

Integrin, Alpha M (Complement Component 3 Receptor 3 Subunit)) (ITGAM), NFKB inhibitor alpha (NF-kBIA), Tumor Necrosis Factor, Alpha-Induced Protein 3 (TNFAIP3), Src homology region 2 domain-containing phosphatase (SHP-1)

Key Words: ACHF, Pharmacological Treatment, Monocytes, Inflammation, NF-kB, SHP-1, TAK-1

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