Tocilizumab might prevent cardiovascular disease in rheumatoid arthritis patients through the modulation of endothelial dysfunction, NETosis and monocyte-mediated inflammation

Ruiz-Limón P[1*], Ortega R[1*], Arias de la Rosa I[1], Abalos-Aguilera MC[1], Perez-Sanchez C[1], Jimenez-Gomez Y[1], Peralbo-Santaella E[2], Font P[1], Ruiz-Vilches D[1], Ferrin G[3], Collantes-Estevez E[1], Escudero-Contreras A[1], López-Pedrera Ch[1&] and Barbarroja N[1&]

[1]Maimonides Institute for Biomedical Research in Cordoba (IMIBIC)/Reina Sofia Hospital/University of Cordoba,[2]Microscopy, cytomics and scientific imaging unit, IMIBIC, Cordoba, Spain [3]Biomedical Research Centre Network. Digestive and Liver Diseases (CIBEREHD), Instituto de Salud Carlos III, Córdoba, Spain

*These authors share the first position
&These authors share the senior position

Corresponding author e-mail: nuria.barbarroja.exts@juntadeandalucia.es
Address reprint request to: Nuria Barbarroja PhD, GC05 Group 2º floor, IMIBIC, Avda. Menendez Pidal s/n, E-14006 Córdoba (SPAIN).
Telephone number: (+34) 957 213794

Running title: TCZ prevents CVD through the modulation of endothelial dysfunction, NETosis and monocyte-mediated inflammation.

Number of references: 46
Number of figures: 6
Number of tables: 1

Keywords: Rheumatoid arthritis, cardiovascular disease, tocilizumab, IL6R inhibitor, NETosis, miRNAs

ABSTRACT

Tocilizumab (TCZ) is an effective treatment for rheumatoid arthritis (RA). However, the changes occurred after TCZ therapy on endothelial dysfunction, monocyte activity, NETosis, and oxidative stress, principal effectors of atherosclerosis and cardiovascular disease, have not been analyzed. Whit this objective, twenty RA patients treated with subcutaneous TCZ for 6 months were evaluated. TCZ significantly restored endothelial function and decreased oxidative stress in RA leukocytes. Percentage of low density granulocytes and NETosis generation were reduced. The proinflammatory and prothrombotic status of RA monocytes were also reversed through modulation of specific intracellular pathways. All these results were recapitulated after in vitro treatment with TCZ of RA monocytes and neutrophils, and in co-cultures with endothelial cells. Plasma levels of miRNA-223, miRNA-146 and miRNA-23 were upregulated by TCZ. In conclusion, TCZ prevents CVD through the restoration of the endothelial function, oxidative stress reduction, inhibition of monocytes' prothrombotic and inflammatory profile, and abridged NETosis generation.

INTRODUCTION

Rheumatoid arthritis (RA) is a complex onset autoimmune disease with many associated co-morbidities, including cardiovascular disease (CVD), which significantly contributes to morbidity and mortality in these patients (1). Atherosclerosis at early stage of the disease is considered a potential preclinical manifestation and the risks of carotid plaques and CVD events increase with the progression of the disorder (2). Endothelial dysfunction (ED) is a vascular abnormality frequently present in RA patients, contributing to plaque initiation and progression. It is associated with carotid intima media thickness in long-standing RA (3,4). The phenotypic features of ED comprise upregulated expression of cellular adhesion molecules and compromised barrier function leading to increased leukocyte diapedesis (5). A number of processes have been linked to the development of ED and atherosclerosis in RA. Among them, increased NETosis has been proposed as a relevant mechanism in the occurrence of CVD events. NETosis is a recently described way of cell death, in which occurs the dissolution of internal membranes, followed by the de-condensation of the chromatin and the release of NETs (neutrophil extracellular traps) -networks of chromatin and granular contents of neutrophils, including histones, antimicrobial peptides and oxidant-generating enzymes, such as neutrophil elastase (NE), myeloperoxidase (MPO), NADPH oxidase and nitric oxide synthase, to the extracellular space. A new subset of granulocytes (LDGs) which are more prone to experience NETosis has been identified in the peripheral blood mononuclear cells (PBMCs) fraction of patients with various autoimmune diseases (6).

NETs formation might induce ED and vascular damage in RA patients through stimulation of inflammatory responses, comprising the increased expression of adhesion

molecules, cytokines and chemokines, thus leading to the development of premature atherosclerosis and CVD (7-10).

Oxidative stress is another process frequently altered in RA, which also contributes to atherosclerosis. We have previously described a pro-oxidative status and impairment of antioxidant capacity in RA patients at both, plasma and cellular levels, covering mitochondrial depolarization, increased reactive oxygen species and peroxynitrite levels, and lower levels of intracellular glutathione (GSH) in neutrophils and monocytes from RA patients (11).

Some studies highlighted the role of microRNAs (miRNAs) in regulating processes such as oxidative stress, atherosclerosis, and CVD (12). MicroRNAs are small noncoding RNAs of approximately 19-25 nucleotides in length that negatively regulate gene expression at the post-transcriptional level, by targeting specific mRNAs for degradation or suppressing mRNA translation. Over the past years it has become clear that alterations exist in the expression of miRNAs in patients with RA in plasma and synovial fluid (13). Moreover, we have recently showed that they can be used as biomarkers for therapy effectiveness in this disease (14).

Many different cell components can be considered as key elements in the inflammatory and pro-atherothrombotic status of RA patients. The progressive generation of inflammatory monocytes is an intrinsic element in the immune response mediating RA (15), accelerating the development of advanced atherosclerotic vascular disease. Therefore, treatments targeting monocytes–macrophages might contribute to effectively prevent cardiovascular events.

Interleukin-6 (IL-6) is an upstream inflammatory cytokine that plays a central role in propagating the downstream inflammatory response responsible for atherosclerosis, as it is implicated in ED and arterial stiffening that contribute to accelerated atherosclerosis

process in RA patients (16). High levels of IL-6 may cause a Th17/T-reg cell imbalance during RA, which is corrected upon treatment with tocilizumab (TCZ) (17,18), a recombinant humanized antihuman IL-6 receptor monoclonal antibody that acts by binding both soluble and membrane IL-6 receptor (sIL-6R and mIL-6R), blocking the pro-inflamatory effects of IL-6 (19). It also reduces the expression of atherosclerosis markers, and improves endothelial function by increasing natural killer and regulatory T cells, and decreasing Th1 and Th17 lymphocytes (20).

A number of studies have delineated the effect of TCZ on lymphocyte activation in RA patients. Yet, the changes occurred after anti-IL6R therapy on endothelial dysfunction, monocyte activity, NETosis, and oxidative stress, principal effectors of atherosclerosis and CVD in this autoimmune condition, have not been analyzed. We undertook this study to evaluate the molecular and cellular mechanisms underlying the beneficial effects of TCZ on CVD associated with RA, focusing on the effects of this biological therapy on endothelial dysfunction, neutrophils and monocytes activity.

RESULTS

TCZ improves the disease activity, clinical parameters and endothelial dysfunction in RA patients.

Treatment of RA patients with TCZ (165 mg/ml per week) for 6 months significantly reduced the number of the swollen and tender joints. Moreover, there was a global improvement of the disease showed by a decrease in the VAS and a reduction in DAS28: mean DAS28 changes (± SDs) were 1.35 ± 0.78 (**Table I**). A significant reduction of Rheumatoid factor (RF) levels was observed. However, no effect was detected on the levels of anti-CCP antibodies. RA patients displayed significantly reduced levels of erythrocyte sedimentation rate (ESR) and c-reactive protein (CRP)

after therapy. TCZ also modulated the lipid profile in plasma, increasing the HDL-cholesterol and ApoA1 levels (**Table I**).

Endothelial function improved notably, as shown by the amelioration in the highest perfusion value when occlusion was released (PF) and by the increase of hyperemic area (HA) (**Figure 1A**). In parallel, changes in plasma levels of cellular adhesion molecules such as E-selectin and vascular cell adhesion molecule 1 (VCAM-1)) were found significantly reduced by effect of treatment with TCZ, thus supporting the improvement of the vascular function in these patients (**Figure 1B**).

TCZ reduces oxidative stress in leukocytes from RA patients

Peroxides and peroxynitrites levels were downregulated in leukocytes from RA patients after treatment with TCZ, showing a significant reduction in the mean fluorescence intensity (MFI) of DhRh and DCFHDA in flow cytometry analyses (**Figure 2A and B**).

TCZ therapy downregulates the percentage of low density granulocytes and decreased NETosis

A significant reduction in the percentage of LDGs (phenotypic profile based on $CD14^{-/dim}$ and $CD15^{bright}$ expression) in RA patients treated with TCZ was noticed (**Figure 3C**). In our hands, NE and MPO (primary enzymes stored in azurophilic granules, and found in abundance in NETs) were found reduced in neutrophils from RA patients treated with TCZ (**Figure 3A**). In addition, neutrophils from RA patients treated with TCZ for 6 months displayed a reduced generation of NETs, so that the area of DNA fibers stained with Sytox was significantly reduced by effect of TZC in RA neutrophils treated with PMA (**Figure 3B**).

Moreover, supporting the reduction of NETosis generation by TCZ observed at cellular level, a decreased release of cell-free nucleosomes was detected in serum from RA patients after TCZ therapy (**Figure 3D**).

In vivo treatment with TCZ reduces the procoagulant and inflammatory state, and modulates insulin signalling and lipid storage in monocytes.

Gene expression of molecules involved in inflammation, procoagulant activity and migration was reduced in RA monocytes treated with TCZ (**Figure 4A**). In contrast, *in vivo* inhibition of IL6R signalling greatly increased the mRNA expression of IL6 in monocytes from RA patients. The expression of genes related to insulin signalling, such as IRS1 and IRS2, was also found increased in monocytes from RA patients taking TCZ compared with baseline. Molecules involved in lipid droplets formation (DAGT and PLIN2) were also found significant reduced at mRNA level in monocytes from RA patients after 6 months of TCZ treatment (**Figure 4A**).

TCZ greatly modifies the activation of several intracellular kinases on monocytes from RA patients

Dysfunctional intracellular signaling pathways play a critical role in RA (30). Using a protein array, we analysed the changes promoted by TCZ treatment on the activation of 18 intracellular kinases in monocytes from RA patients. As expected, inhibition of IL6R signalling by TCZ markedly reduced the phosphorylation of STAT3, a kinase directly activated by the binding of IL-6 to its receptor (**Figure 4B and C**). Of note, the phosphorylation of 7 more protein kinases was found significantly downregulated by TCZ on RA monocytes, including AKT, AMPKa, S6 Ribosomal protein, mTOR, HSP27, PRAS40 and GSK3-b.

TCZ increases levels of miRNA223, miRNA23 and miRNA146 in serum of RA patients

Levels of miRNA-23, miRNA-146 and miRNA-223 were found significantly increased in RA sera after 6 months of treatment with TCZ (**Figure 5A**). By using the IPA

software, we further investigated the potential mRNA targets for these three miRNAs in the context of CVD (**Figure 5B**).

In vitro treatment with TCZ improves the inflammatory and thrombotic profile of monocytes, and reduces NETosis generation

In vitro treatment of RA monocytes with TCZ for 18 hours decreased the mRNA expression levels of TF, MCP-1, IL8, TLR4 and TLR2 (**Figure 6E**).

In RA neutrophils, the pre-treatment with IL-6 for 6 hours promoted a significant increase in NE and MPO intracellular levels, which was prevented by addition of TCZ (**Figure 6A and B**). No changes in PAD4 mRNA-expression levels were observed in neutrophils treated with IL6; however there was a significant reduction of PAD4 in neutrophils treated either with IL6 plus TCZ, or with TCZ alone (**Figure 6C**).

We further analysed whether TCZ could diminish NETosis in vitro. The combination of TCZ plus PMA generated significantly less extrusion of DNA fibres in neutrophils comparing to the treatment with PMA alone, suggesting that TCZ might prevent NETosis in RA patients (**Figure 6D**).

TCZ significantly reduced the IL6-induced expression of both adhesion molecules and inflammatory mediators in endothelial cells co-cultured with RA purified monocytes

In order to mimic the in vivo effects of TCZ on the vessel wall, cultured HUVECs were treated with IL6, either alone or in combination with TCZ. A significant increase in the expression of VEGF, ICAM and VCAM mRNAs was noticed in HUVECs after treatment with IL6 for 18 hours. Those high levels were downregulated by addition of TCZ (**Figure 6F**).

On the other hand, by performing co-cultures, we analysed the interaction between endothelial cells and monocytes in the setting of RA. Co-culture of RA monocytes with

HUVEC increased the expression of adhesion molecules (ICAM and VCAM) on endothelial cells. Moreover, these genes, alongside TNFα and IL1ß were significantly upregulated in HUVEC after the addition of IL6 to the co-culture medium. Once more, TCZ significantly reduced the IL6-induced expression of both adhesion molecules and inflammatory mediators in endothelial cells co-cultured with RA purified monocytes (**Figure 6G**), thus underlying the relevant role of this IL6R-inhibitor on both, the improvement of endothelial function, and the decline of the monocytes-mediated proinflammatory profile associated to that autoimmune disorder.

DISCUSSION

The present study describes for the first time how TCZ might ameliorate CVD in RA, exploring the molecular changes related to inflammation, procoagulant properties and intracellular signalling in RA monocytes, the prevention of NETosis, and endothelial dysfunction.

Recently published data show that combination therapy with biologicDMARDs and syntheticDMARDs represents the best therapeutic option for the treatment of RA, since it can slow the progression of the disabling structural damage (21). In our cohort of patients, the combination of a bDMARD, TCZ, with sDMARDs (methotrexate, leflunomide and hydroxichloroquine) globally improved the activity of the disease, with a DAS28 remission, showed by a decrease in the number of both, tender and swollen joints, clinical inflammatory parameters, and the assessment of the pain (VAS) after 6 months of treatment. Regarding autoimmunity, TCZ induced a dramatic change in RF levels with no effect in anti-CCPs levels. A recent study by Iannone and coworkers, which evaluated the effect of several bDMARDs on the levels of RF and anti-CCPs, showed that 12 months of treatment with TCZ significantly reduced both RF and anti-

CCPs serum levels in RA patients (21). Thus, longer treatments with TCZ might be necessary to reduce the levels of anti-CCPs.

Various studies have evaluated the changes occurred in the lipid profile associated with cardiovascular risk in patients with RA after TCZ therapy. In phase II and III trials, moderate alterations of LDLc, HDLc and triglycerides were described. (22-25). In our cohort of patients, there was a significant increase of HDLc levels after 6 months of TCZ treatment. Accordingly, ApoA1 levels were significantly augmented by treatment with TCZ. However this was not related to a significant augmentation of the total cholesterol levels, which might be associated to the unchanged levels found of triglycerides and LDLc. Thus, in our hands, TCZ had no negative effects on the lipid profile of RA patients.

The effect of TCZ on endothelial dysfunction has been minimally explored. Protogerou et al., studied the flow mediated dilatation and aortic stiffness in 16 patients treated with TCZ for 3 and 6 months. They reported an improvement of endothelial dysfunction, showed by a decrease in carotid to femoral pulse wave velocity, and an augmentation of flow mediated dilatation (16). In addition, a comparative study of TCZ monotherapy with other bDMARDs (etanercept and adalimumab), showed that all these bDMARDs decreased the arterial stiffness to a similar extent after 6 months of treatment (26). In accordance, our study showed a significant improvement in the microvascular function, with an increase in the peak flow after post-occlusive reactive hyperaemia. Of notice, we give new evidence about the efficacy of TCZ reducing endothelial dysfunction in combined therapy with sDMARDs. Moreover, levels of CAMs, such as VCAM and e-Selectin were found diminished in plasma of RA patients after treatment with TCZ. Elevated levels of these molecules have been shown to be associated with an increased risk for CVD. Thus, these results evidence the positive effects of TCZ on endothelial

dysfunction, which might be considered in the prevention of cardiovascular events. In agreement, a recent study has shown a decrease in serum VEGF levels after 6 months of TCZ therapy (27). Our results were further supported by *in vitro* studies, on which treatment with TCZ prevented in ECs the IL6-induced expression of VEGF, ICAM and VCAM. Moreover, we could demonstrate that addition of TCZ to the co-culture of RA monocytes with ECs plus IL6 reduced the inflammatory profile (represented by increased TNFα and IL1β expression), and prevented the endothelial dysfunction, (promoting a down regulation of VCAM and ICAM). In agreement with this, Suzuki et al., described the central role of IL6 in increasing the rate of cell migration, and how TCZ was able to reduce the adhesion of monocytic cells (U937) to HUVEC (28). All in all, our study supports the efficacy of TCZ restoring endothelial function, and inhibiting inflammation and cell adhesion in the context of RA.

Alteration in oxidative status has been closely related to CVD. Our group recently reported an altered oxidative status in leukocytes and plasma from RA patients (11). This study shows for the first time the effects of inhibiting IL6R signalling in the reduction of oxidative stress in monocytes and neutrophils of RA patients. *In vivo* treatment with TCZ significantly reduced the levels of peroxinytrites in both cell types. Other bDMARDs, such as etanercept and infliximab have been shown to decrease oxidative stress in serum and urine from patients with RA (29, 30). Yet, only a recent study has evidenced that TCZ is more efficient lowering serum levels of oxidative stress markers in comparison with sDMARDs and anti-TNFα therapy (31). Alongside this article, our study shows the great efficacy of TCZ reducing oxidative status, not only at plasma levels, but also in RA monocytes and neutrophils, thus preventing vascular damage in patients with RA.

A marked reduction of the inflammatory profile, prothrombotic properties, and migratory capacity, was observed on RA monocytes, demonstrated by the downregulation of TF, MCP-1, IL-8, TLR2 and TLR4. However, a significant increase of IL6 mRNA levels was noticed. Our results are in line with Nishimoto et al., who recently described that circulating levels of IL-6 and sIL-6R increased after TCZ administration in RA patients (32). They argued that this effect was probably due to a reduction of their elimination after formation of TCZ/sIL6R immune complexes. In the same way, the increased levels of IL6 mRNA found in our study might derive from that feedback process.

We further provide new evidences about the effect of TCZ on RA monocytes-lipid droplets formation. The accumulation of lipid droplets within leukocytes on inflammatory conditions has been documented (33). Lipid droplets compartmentalize several proteins and lipids involved in the control of biosynthesis and secretion of inflammatory molecules, including leukotriene and PGE2 (34). PLIN2 and DGAT2 are two main genes involved in lipid droplets regulation, of which PLIN2 overexpression results in increased formation of lipid droplets (34). In turn, growth of lipid droplets is linked to functions performed by endoplasmic reticulum mediated by proteins such as DGAT. We found a significant reduction in the expression of these genes on RA monocytes treated with TCZ, which might suggest that TCZ reduced the formation of lipid droplets, inhibiting the inflammation mediated by immune cells. Nevertheless, a deeper research is needed to delineate the effect of TCZ in the lipid metabolism and storage within the immune cells.

The cellular responses observed in monocytes related to cytokine and thrombotic factors production, lipid metabolism and storage, and insulin signaling, depend on the activation of specific signaling pathways. Proteins from the synovial tissue of RA

patients have been reported to be extensively phosphorylated by intracellular tyrosine kinases, supporting the importance of tyrosine kinases in the pathogenesis of RA (35, 36). Yet, this is the first study describing the regulation, promoted in monocytes by TCZ, of the main signaling pathways associated with RA pathogenesis: JAK/ STAT, SAPK/MAPK and PI-3K/AKT/mTOR. Thus, although a number of studies have analyzed the role of specific intracellular pathways on the response to bDMARDs/sDMARDs, the use of an array to identify a wide spectrum of cell signaling molecules, might help to better delineate the regulatory mechanisms modulated by effects of specific drugs.

Neutrophils have recently been recognized as essential actors in the development of atherosclerosis. The effects of IL6 on neutrophils functions remains poorly understood, with conflicting evidence reporting that IL6 can either delay, accelerate, or have no effect on neutrophil apoptosis (37-39). In our hands, the relevance of neutrophils function in CVD and their regulation by TCZ has been exposed by a decrease in the percentage of LDGs and on NETosis generation, both of them determinant in atherosclerosis development. Although further studies are required, our results indicate that NETs might have an important role in the development of inflammation in the context of RA, and that IL6R signaling blockade could be an useful therapy to avoid undesired effects of persistent neutrophil activation.

Finally, analyzing the underlying effects of TCZ in preventing CVD in RA patients, we further describe the upregulation of miRNA-223, miRNA-23 and miRNA-146 in serum of RA patients in response to TCZ therapy. These miRNAs are emerging regulators of inflammation and atherosclerosis. Thus, miR-223 has been implicated in inflammatory diseases such as RA, by modulating the functions of T helper lymphocytes. Its overexpression prevents the production of cytokines and reduces differentation of

monocytes to macrophages (40, 41). It also decreases the expression of TF on endothelial cells (42). On the other hand, miRNA-146 has been extensively associated with the inhibition of NFkB-mediated inflammation, atherosclerosis and endothelial activation (43, 44). We have previously identified these miRNAs as potential biomarkers of therapy effectiveness in anti-TNFα –RA treated patients (14). The present study further underlies their relevance as potential biomarkers of therapy effectiveness in this autoimmune condition.

Our overall data suggest that TCZ improves the pro-atherothrombotic status of RA patients, by simultaneously regulating the dyslipidemia, the endothelial dysfunction and the inflammatory activity of monocytes and neutrophils, through mechanisms involving modulation of oxidative stress, NETosis, intracellular signalling, and altered circulating miRNA expression.

MATERIAL AND METHODS

RA patients

Twenty RA patients were included in this study. RA patients fulfilled at least four 1987 American College of Rheumatology (ACR) and achieved a total score of 6 or greater according to 2010 criteria (45). The patients were taking synthetic DMARDs treatment (**Table 1**). Patients were given TCZ subcutaneous (162 mg/week) for 6 months. The treatment of all patients with synthetic DMARDs had been stable for at least two months before TCZ administration.

Disease activity score 28 (DAS28) index was determined following the guidelines of the American college of Rheumatology indications. Moderate to high activity was defined as DAS28 ≥3.2 (46). All the patients filled the health assessment questionnaire (HAQ) and the visual analog scale (VAS) in order to assess the pain.

For in vitro studies, peripheral blood was withdrawn from 5 RA patients with moderate-high activity (DAS28 4.17±0.6) taking synthetic DMARDs and not having any biologic DMARDs. They all were female, between 36 and 69 years old, CRP range from 5 to 15 mg/ml and ESR range from 7 to 30.

All participants enrolled were Caucasian, were recruited at the department of Rheumatology, and gave their written informed consent approved by the ethical committee of the Reina Sofia Hospital (Cordoba, Spain).

Endothelial function: Laser Doppler linear Periflux 5010

The study of microvascular function was performed by laser Doppler flowmetry.

White blood cells isolation

Neutrophils were isolated from patients by density centrifugation over Dextran-Ficoll Hypaque. Thereafter, the separation of monocytes and lymphocytes from the mononuclear layer was performed by the immunomagnetic depletion of non-monocytes, using a commercially available kit (Monocyte isolation kit II, Miltenyi Biotec, Bergisch Gladbach, Germany).

In vitro studies

Monocytes and neutrophils purified from 5 RA patients were cultured separately in completed RPMI 1640 containing 10% FBS at 37°C in a humidified 5% carbon dioxide (CO_2) atmosphere.

RA monocytes (1×10^6 cells/ml) were pre-treated with FCRII blocking (Miltenyi Biotec) for 15 min. Thereafter, cells were seeded and incubated in the presence or in the absence of TCZ (20 μg/ml) for 18 hours. RA neutrophils (1×10^6 cells/ml) were pre-treated with FCRII blocking as described above. Then, cells were seed and incubated with IL-6 (10 ng/ml) for 3 hours, and thereafter incubated in the presence or in the absence of TCZ (20 μg/ml) for 3 hours.

Human umbilical vein endothelial cells (HUVEC) were cultured in EBM (Endothelial Cell Basal medium, Lonza, Walkersville, MD USA) with 10% FBS, 0.1% human epidermal growth factor (hEGF), 0.1% hydrocortisone, 0.1% gentamicin, amphotericin-B (GA-1000), 0.4% bovine brain extract (BBE), at 37°C in a humidified 5% CO_2 atmosphere.

HUVEC were pre-treated for 15 min with FcR blocking Reagent (Miltenyi Biotec) and incubated with IL-6 (10 ng/ml) for 9 hours in the presence or in the absence of TCZ (20 µg/ml).

Co-cultures of RA monocytes-HUVEC:

Monocytes isolated from RA patients were pre-treated with FcRII blocking reagent for 15 min and seeded into transwell inserts (Sigma Aldrich, Misuri, USA) in EBM Endothelial Cell Basal medium, and added into multiple plate wells preloaded with HUVEC. Thus, HUVEC and monocytes shared the same culture medium but were physically separated. Co-culture was incubated with IL-6 (10 ng/ml) alone or combined with TCZ (20 µg/ml) as described previously.

RNA isolation and quantitative real-time reverse transcriptase PCR

Total RNA was extracted using TRI Reagent (Sigma, St Louis, Missouri, USA) following the manufacturer's recommendations. Gene expression was assessed by real time RT-PCR using a LightCycler Thermal Cycler System (Roche Diagnostics, Indianapolis, Indiana, USA).

Determination of oxidative stress biomarkers in white blood cells

Oxidative stress biomarkers were analysed using a dual-laser FACSCalibur (Becton Dickinson). Cells were incubated with 20.5 µM DCFH-DA (Sigma-Aldrich) and 5 µM DihidroRhodamine123 at 37ºC for 30 min (Sigma-Aldrich).

NETs induction and quantification

Isolated neutrophils from RA patients were seeded in 24 well plates on poly-L-lysine-coated glass coverslips (BD Biosciences, San Jose, CA, USA) in tissue-culture wells. Cells were treated with or without phorphol-12-myristate-13-acetate (PMA, the most potent agent to induce NET formation) (600 nM) (Sigma-Aldrich) for 2 hours in the presence or in the absence of TCZ (20 μg/ml) for 2 hours. Cells were fixed with 4% paraformaldehyde. DNA was stained with 5 μM Sytox orange dye (Life technologies, Netherlands) and NETs were visualized by using a Nikon Eclipse-Ti-S fluorescence microscope, (NIS-Elements imaging software) with a 20x objective. NETs were manually identified on digitalized images as Sytox-positive structures emanating from cells with overall length greater than 2x cell diameter from cells untreated and were counted for at least 3 fields using IMAGE-J software (NIH, Bethesda, MD).

Detection of cell-free nucleosomes

Nucleosomes were measured by using the Human Cell Death Detection ELISAPLUS (Roche Diagnostics, Basel, Switzerland) following the manufacturer's recommendations.

Identification of Low density granulocytes (LDGs)

PMBCs ($5x10^5$) were incubated with PE anti-human CD14 and FITC anti-human CD15 (Biolegend) for 30 minutes at 4ºC in the dark. Cells were acquired on the flow cytometer FACSCalibur.

Neutrophil elastase (NE) and myeloperoxidase (MPO) protein expression

Whole peripheral blood (100 μl) was incubated either with FITC human anti-MPO (BD Biosciences) or with human anti-elastase primary antibody (RbmAb to Neutrophil Elastase (Abcam). Then, for NE analysis, Alexa Fluor conjugated secondary antibody (Abcam) was added for 30 minutes at 4ºC. Cells were acquired on the flow cytometer FACSCalibur.

PathScan intracellular signaling protein array

10 µg of total protein in 75 µl were subjected to PathScan intracellular signaling array following the manufacture's recommendations (Cell signaling technologies, Massachusetts, USA.).

Isolation of microRNAs from serum and quantitative real-time PCR

A total of 200 µl of serum was subjected to RNA isolation and quantitative RT-PCR as described previously (14).

Plasma levels of cellular adhesion molecules

E-selectin and VCAM-1 levels were analyzed in plasma using ProcartaPlex multiplex immunoassay, following the manufacturer's recommendations (Affymetrix eBioscience, Vienna, Austria).

Statistical analysis

All data are expressed as mean ± SEM. Statistical analyses were performed using the statistical software package SPSS (version 17.0 for Windows; SPSS Iberica, Madrid, Spain). Following normality and equality of variance tests, comparisons were made by a parametric test (paired Student's t test) or alternatively by using a non-parametric test (Mann–Whitney rank sum test). Differences were considered significant at $p<0.05$

STUDY HIGHLIGHTS-

- **What is the current knowledge on the topic?** Inhibition of the interleukin IL6 receptor signaling by tocilizumab (TCZ) is an effective treatment for rheumatoid arthritis (RA).

- **What question did this study address?** This study evaluates the molecular and cellular mechanisms underlying the beneficial effects of TCZ on CVD associated with RA.

- What this study adds to our knowledge? TCZ prevents CVD through the restoration of the endothelial function, oxidative stress reduction, inhibition of monocytes' prothrombotic and inflammatory profile, and abridged NETosis generation.

- How this might change clinical pharmacology and therapeutics? TCZ might constitute a relevant therapeutic strategy for patients with chronic inflammatory diseases such as RA, showing increased cardiovascular risk.

ACKNOWLEDGMENTS

We thank all the patients for their kind participation in this study. This work was supported by grants from the 'Junta de Andalucia' (CTS-7940, PI-0191-2013), the Spanish Rheumatology Foundation (FER), the Ministry of Health co-financed with FEDER funds (PI15/01333 and CP15/00158), and Roche Pharma, S.A. CL-P was supported by a contract from the Spanish Junta de Andalucia. NB-P was supported by a Ministry of Health postdoctoral contract.

CONFLICT OF INTEREST/DISCLOSURE

Authors have no conflicts of interest

AUTHOR CONTRIBUTIONS

PR-L and RO drafted the manuscript and discussed the results. PR-L, IAR, MCAA, CP-S performed research. RO, PF and DRV recruitment and followed up patients and performed statistical analysis. CL-P and NB designed the research and wrote the manuscript. EC-E and AE performed clinical analysis, revised the manuscript, and contributed useful suggestions. YJG performed statistical analysis, helped to draft the manuscript, and discussed the results. EP-S and GF contributed technical assistance with microscopy, cytometry and protein studies and discussed the results. All authors read and approved the manuscript.

Authors' information

Patricia Ruiz-Limón and Rafaela Ortega shared first authorship.

Nuria Barbarroja and Chary López-Pedrera shared last authorship.

REFERENCES

1. del Rincón, I.D, Williams K, Stern M.P., Freeman G.L., Escalante A. High incidence of cardiovascular events in a rheumatoid arthritis cohort not explained by traditional cardiac risk factors. *Arthritis Rheum*. 44, 2737–2745 (2001).

2. Gonzalez-Juanatey C., Llorca J., Testa A., Revuelta J., Garcia-Porrua C., Gonzalez-Gay M.A. Increased prevalence of severe subclinical atherosclerotic findings in long-term treated rheumatoid arthritis patients without clinically evident atherosclerotic disease. *Medicine (Baltimore)* 82, 407–413 (2003).

3. Mudau M., Genis A., Lochner A., Strijdom H. Endothelial dysfunction: the early predictor of atherosclerosis. *Cardiovasc J Afr*. 23, 222–231 (2012).

4. Gonzalez-Juanatey C., Llorca J., Gonzalez-Gay M.A. Correlation between endothelial function and carotid atherosclerosis in rheumatoid arthritis patients with long-standing disease. *Arthritis Res Ther*. 13, R101 (2011).

5. Steyers C.M., Miller, F.J. Jr. Endothelial Dysfunction in Chronic Inflammatory Diseases. *Int. J. Mol. Sci.* 15, 11324-11349 (2014).

6. Carmona-Rivera C., Kaplan M.J. Low-density granulocytes: a distinct class of neutrophils in systemic autoimmunity. *Semin Immunopathol*. 35, 455-63 (2013)

7. Khandpur R. *et al*. NETs are a source of citrullinated autoantigens and stimulate inflammatory responses in rheumatoid arthritis. *Sci Transl Med*. 5, 178ra40 (2013).

8. Brinkmann, V. et al., Neutrophil extracellular traps kill bacteria. *Science* 303, 1532–1535 (2004).

9. Steinberg B.E, Grinstein S. Unconventional roles of the NADPH oxidase: signaling, ion homeostasis, and cell death. *Sci STKE*. 379, pe11 (2007).

10. Darrah E., Andrade F. NETs: the missing link between cell death and systemic autoimmune diseases? *Front Immunol*. 3, 428 (2012).

11. Barbarroja N., et al. Anticyclic Citrullinated Protein Antibodies Are Implicated in the Development of Cardiovascular Disease in Rheumatoid Arthritis, *Arterioscler Thromb Vasc Biol*. 34, 2706-2716 (2014)

12. Aranda J.F., Madrigal-Matute J., Rotllan N., Fernández-Hernando C. MicroRNA modulation of lipid metabolism and oxidative stress in cardiometabolic diseases. *Free Radic Biol Med.* 64, 31-39 (2013).

13. Pauley K.M., Satoh M., Chan A.L., Bubb M.R., Reeves W.H., Chan E.K. Upregulated miR-146a expression in peripheral blood mononuclear cells from rheumatoid arthritis patients. *Arthritis Res Ther*. 10, R101 (2008).

14. Castro-Villegas C., *et al*. Circulating miRNAs as potential biomarkers of therapy effectiveness in rheumatoid arthritis patients treated with anti-TNFα *Arthritis Res Ther* 17, 49 (2015).

15. Goronzy J.J., Weyand C.M. Developments in the scientific understanding of rheumatoid arthritis. *Arthritis Res Ther*. *1*1, 249 *(2009).*

16. Protogerou A.D., Zampeli E., Fragiadaki K., Stamatelopoulos K., Papamichael C., Sfikakisa P.P. A pilot study of endothelial dysfunction and aortic stiffness after interleukin-6 receptor inhibition in rheumatoid arthritis. Atherosclerosis 219, 734–736 (2011)

17. Ogura H, *et al*. Interleukin-17 promotes autoimmunity by triggering a positive-feedback loop via interleukin-6 induction. *Immunity*. 29, 628–636 (2008).

18. Samson M., *et al*. Brief report: inhibition of interleukin-6 function corrects Th17/Treg cell imbalance in patients with rheumatoid arthritis. *Arthritis Rheum*. 64, 2499–2503 (2012).

19. Nishimoto N., Kishimoto T. Humanized antihuman IL-6 receptor antibody, tocilizumab. *Handb Exp Pharmacol*. 181, 151-160. (2008)

20. Pasare C., Medzhitov R. Toll pathway dependent blockade of CD4 + CD 25 + T cell mediated suppression by dendritic cells. *Science*. 299, 1033-1036 (2003).

21. Iannone F., Lopalco G., Cantarini L., Galeazzi M., Lapadula G. Efficacy and safety of combination therapy for preventing bone damage in rheumatoid arthritis. *Clin Rheumatol.* 35, 19-23 (2016)

22. Strang A.C. *et al*. Pro-atherogenic lipid changes and decreased hepatic LDL receptor expression by tocilizumab in rheumatoid arthritis. *Atherosclerosis.* 229, 174-81 (2013)

23. Gabay C. *et al*. Comparison of lipid and lipid-associated cardiovascular risk marker changes after treatment with tocilizumab or adalimumab in patients with rheumatoid arthritis. *Ann Rheum Dis*. (2015) [Epub ahead of print]

24. Provan S.A., Berg I.J., Hammer H.B., Mathiessen A., Kvien T.K., Semb A.G. The Impact of Newer Biological Disease Modifying Anti-Rheumatic Drugs on Cardiovascular Risk Factors: A 12-Month Longitudinal Study in Rheumatoid Arthritis Patients Treated with Rituximab, Abatacept and Tociliziumab. *PLoS One.* 10, e0130709 (2015)

25. Souto A., Salgado E., Maneiro J.R., Mera A., Carmona L., Gómez-Reino J.J. Lipid profile changes in patients with chronic inflammatory arthritis treated with biologic agents and tofacitinib in randomized clinical trials: a systematic review and meta-analysis. *Arthritis Rheumatol.* 67, 117-27 (2015)

26. Kume K., Amano K., Yamada S., Hatta K., Ohta H., Kuwaba N. Tocilizumab monotherapy reduces arterial stiffness as effectively as etanercept or adalimumab monotherapy inrheumatoid arthritis: an open-label randomized controlled trial. *J Rheumatol.* 38, 2169-71 (2011)

27. Nishimoto N., *et al.* Study of active controlled tocilizumab monotherapy for rheumatoid arthritis patients with an inadequate response to methotrexate (SATORI): significant reduction in disease activity and serum vascular endothelial growth factor by IL-6 receptor inhibition therapy. *Mod Rheumatol.* 19, 12-9 (2009)

28. Suzuki M., Hashizume M., Yoshida H., Mihara M. Anti-inflammatory mechanism of tocilizumab, a humanized anti-IL-6R antibody: effect on the expression of chemokine and adhesion molecule. *Rheumatol Int.* 30, 309-15 (2010)

29. Kageyama Y., Takahashi M., Ichikawa T., Torikai E., Nagano A. Reduction of oxidative stress marker levels by anti-TNFalpha antibody, infliximab, in patients with rheumatoid arthritis. *Clin Exp Rheumatol*. 26, 73–80 (2008)

30. Kageyama Y., Takahashi M., Nagafusa T., Torikai E., Nagano A. Etanercept reduces the oxidative stress marker levels in patients with rheumatoid arthritis. *Rheumatol Int*. 28, 245–251 (2008)

31. Hirao M. *et al*. Serum level of oxidative stress marker is dramatically low in patients with rheumatoid arthritis treated with tocilizumab. *Rheumatol Int.* 32, 4041-5 (2012)

32. Nishimoto N., Terao K., Mima T., Nakahara H., Takagi N., Kakehi T. Mechanisms and pathologic significances in increase in serum interleukin-6 (IL-6) and soluble IL-6 receptor after administration of an anti-IL-6 receptor antibody, tocilizumab, in patients with rheumatoid arthritis and Castleman disease. *Blood.* 112, 3959-64 (2008)

33. Bozza P.T., Magalhaes K.G., Weller P.F. Leukocyte lipid bodies-biogenesis and functions in inflammation. *Biochim Biophys Acta*. 1791, 540-551 (2009)

34. Herker E., Ott M. Emerging role of lipid droplets in host/pathogen interactions. J Biol Chem. 287, 2280-2287 (2012)

35. Okamoto H., Kobayashi A. Tyrosine kinases in rheumatoid arthritis. *J Inflamm* (Lond). 8,21 (2011)

36. Malemud C.J. Intracellular Signaling Pathways in Rheumatoid Arthritis. *J Clin Cell Immunol*. 4:160 (2013)

37. Biffl W.L., Moore E.E., Moore F.A., Barnett C.C. Jr. Interleukin-6 suppression of neutrophil apoptosis is neutrophil concentration dependent. *J Leukoc Biol.* 58, 5824 (1995)

38. Afford S.C., Pongracz J., Stockley R.A., Crocker J., Burnett D. The induction by human interleukin-6 of apoptosis in the promonocytic cell line u937 and human neutrophils. *J Biol Chem* 267, 216126 (1992)

39. McNamee J.P., Bellier P.V., Kutzner B.C., Wilkins R.C. Effect of pro-inflammatory cytokines on spontaneous apoptosis in leukocyte sub-sets within a whole blood culture. *Cytokine.* 31, 1617 (2005)

40. Taïbi F., Metzinger-Le Meuth V., Massy Z.A., Metzinger L. miR-223: An inflammatory oncomiR enters the cardiovascular field. *Biochim Biophys Acta.* 1842, 1001-9 (2014)

41. Wang J., Bai X., Song Q., Fan F., Hu Z., Cheng G., Zhang Y. miR-223 inhibits lipid deposition and inflammation by suppressing toll-like receptor 4 signaling in macrophages. *Int J Mol Sci.* 16, 24965-82 (2015)

42. Li S. *et al.* MicroRNA-223 inhibits tissue factor expression in vascular endothelial cells. *Atherosclerosis.* 237, 514-20 (2014)

43. Li K., Ching D., Luk F.S., Raffai R.L. Apolipoprotein E enhances microRNA-146a in monocytes and macrophages to suppress nuclear factor-κB-driven inflammation and atherosclerosis. *Circ Res.* 117, e1-e11(2015)

44. Cheng H.S. *et al.* MicroRNA-146 represses endothelial activation by inhibiting pro-inflammatory pathways. *EMBO Mol Med*. 5, 949-66 (2013)

45. Aletaha D. *et al.* 2010 Rheumatoid Arthritis Classification Criteria. An American College of Rheumatology/European League Against Rheumatism Collaborative Initiative. *Arthritis Rheum* 62, 2569-2581 (2010)

46. Prevoo M.L., van't Hof M.A., Kuper H.H., van Leeuwen M.A., van de Putte L.B., van Riel P.L. Modified disease activity scores that include twenty-eight-joint counts. Development and validation in a prospective longitudinal study of patients with rheumatoid arthritis. *Arthritis Rheum*. 38, 44-8 (1995)

FIGURE LEGENDS

Figure 1. A, Microvascular function was measured by Laser Doppler linear Periflux 5010 and was performed before and after TCZ treatment. Normal perfusion (RF), perfusion when occluded (BZ), occlusion area (AO), time to half before hyperaemia (TH1), highest perfusion value after occlusion is released (PF), time to half after hyperaemia (TH2), hyperaemic area (AH), time to max (TM), time to recovery (TR), time to latency (TL). **B,** E-selectin and vascular cell adhesion molecule 1 (VCAM-1) levels were analyzed in plasma from RA patients before and after TCZ therapy by Procarta Plex multiplex immunoassay. Data are presented as mean ± SEM. (*) indicates significant differences vs before TCZ ($p<0.05$).

Figure 2. A, Peroxides production in lymphocytes, monocytes, and neutrophils of RA patients before and after TCZ treatment were determined by addition of the fluorescent probe DCF-DA to the isolated cells and flow cytometry analysis. **B,** Peroxides and peroxynitrites production in lymphocytes, monocytes, and neutrophils of RA patients before and after TCZ treatment were determined by the fluorescent probe dihydrorhodamine-123 and flow cytometry analysis. Bar graphs show the mean ± SEM of median fluorescence intensity (MFI), (*) indicates significant differences vs before TCZ ($p<0.05$).

Figure 3. A, Intracellular MPO and NE protein expression were measured in neutrophils from RA patients before and after TCZ treatment by flow cytometry. Bar graphs show the mean ± SEM of median fluorescence intensity (MFI). **B,** Representative images of neutrophil extracellular traps (NETs) from RA patients before and after TCZ treatment. NETosis was induced by PMA (600 nM) for 2 hours. DNA was stained with Sytox orange dye and NETs were visualized by using a Nikon Eclipse-Ti-S fluorescence microscope 20x objective. NETs were manually identified on

digitalized images as Sytox-positive structures emanating from cells with overall length greater than 2x cell diameter from cells without PMA. **C,** Representative dot plots of low density granulocytes from RA patients before and after TCZ treatment. **D,** Concentration of cell-free nucleosomes in serum by ELISA. Bar graphs show the mean ± SEM, (*) indicates significant differences vs before TCZ ($p<0.05$).

Figure 4. A, Quantitative RT-PCR was performed on a panel of genes related to inflammation (MCP-1, IL-8, IL-6 TLR2 and TLR4,), procoagulant activity (TF), lipid metabolism and storage (DAGT and PLIN2), insulin signal (IRS-1 and IRS-2) in RA monocytes before and after TCZ treatment. TF, tissue factor; MCP-1, monocyte chemotactic protein; IL, interleukin; IRS, insulin signal; TLR, toll like receptor; DGAT-1, diacylglycerolacyltransferase; PLIN-2, adipophilin or ADRP. **B,** Two representative panels of phosphorylation status of kinases using a PathScan intracellular signalling array in RA monocytes. pSTAT3, phospho signal transducer and activator of transcription 3; pAKT, phospho protein kinase B or PKB; pAMPKα, phospho protein kinase AMP-activated catalytic subunit alpha 1 or PRKAA1; pmTOR, phospho mechanistic target of rapamycin; pHSP27, phospho heat shock protein 27; pPRAS40, AKT1 substrate 1 or AKT1S1; p-p38, phospho mitogen-activated protein kinase 14; pGSK-3β, phospho glycogen synthase kinase 3 beta. **C,** Quantification of volume intensity x area (mm^2). Data are presented as mean ± SEM. (*) indicates significant differences vs before TCZ ($p<0.05$).

Figure 5, **A,** Expression levels of miRNAs in serum of RA patients before and after TCZ treatment. Data are presented as mean ± SEM. (*) indicates significant differences vs before TCZ ($p<0.05$). **B,** Interaction Network of miRNAs and mRNA target involved in cardiovascular disease. By using the tool microRNA Target Filter of QIAGEN's Ingenuity Pathway Analysis (IPA®, QIAGEN Redwood

City,www.qiagen.com/ingenuity), the software generated a network including the selected miRNAs and their mRNA targets, filtered by cardiovascular disease. Only targets experimentally observed and predicted with high confidence are shown and related by direct interactions with their specific miRNA regulators.

Figure 6. A and B Intracellular neutrophil elastase (NE) and myeloperoxidase (MPO) protein expression was measured in neutrophils isolated from RA patients untreated and treated *in vitro* with IL-6 (10 ng/ml), TCZ (20 μg/ml) or IL-6 plus TCZ using flow cytometry. Bar graphs show the mean ± SD of median fluorescence intensity (MFI) of five independent experiments. (a) indicates significant differences vs non treated; (b) vs treated with IL-6 ($p<0.05$). **C**, Relative mRNA expression levels of PAD4 (peptide arginine deiminase, type IV). Data are presented as mean ± SD of five independent experiments. (c) indicates significant differences vs non treated and treated with IL-6 ($p<0.05$). **D,** Representative images of neutrophil extracellular traps (NETs) of neutrophils isolated from RA patients untreated and treated *in vitro* with PMA (600 nM), or PMA plus TCZ (20μg/ml). DNA was stained with Sytox orange dye and NETs were visualized by using a Nikon Eclipse-Ti-S fluorescence microscope 20x objective. NETs were manually identified on digitalized images as Sytox-positive structures emanating from cells with overall length greater than 2x cell diameter from cells without PMA. Bar graphs show the mean ± SD of percentage of NETs of five independent experiments. (a) indicates significant differences vs non treated; (b) vs treated with PMA ($p<0.05$). **E,** Quantitative RT-PCR was performed on a panel of genes on monocytes purified from RA and treated *in vitro* in the absence or presence of TCZ (20 μg/ml). Bar graphs show the mean ± SD of five independent experiments. (*) indicates significant differences vs non treated ($p<0.05$). TF, tissue factor; MCP-1, monocyte chemotactic protein; IL, interleukin; TLR, toll like receptor. **F,** Quantitative

RT-PCR was performed on a panel of genes on HUVEC cells treated with IL-6 (10 ng/ml) alone or in combination with TCZ (20 μg/ml). Bar graphs show the mean ± SD of three independent experiments. (a) indicates significant differences vs non treated; (b) vs treated with IL-6 ($p<0.05$). VEGF indicates vascular endothelial growth factor; ICAM, intercellular adhesion molecule-1; VCAM, vascular cell adhesion molecule. **G,** Quantitative RT-PCR chain reaction was performed on a panel of genes on HUVEC cells cultured alone or co-cultured with RA monocytes and treated with or without IL6 (10ng/ml) alone or in combination with TCZ (20 μg/ml). Bar graphs show the mean ± SD of three independent experiments. (a) indicates significant differences vs HUVEC cells cultured alone and non treated; (b) vs co-cultured with RA monocytes; (c) vs co-cultured with RA monocytes treated with IL-6; (d) vs HUVEC cells cultured alone, non treated and co-cultured with RA monocytes ($p<0.05$). TNFα indicates tumor necrosis factor α; IL, interleukin; ICAM, intercellular adhesion molecule-1; VCAM, vascular cell adhesion molecule.

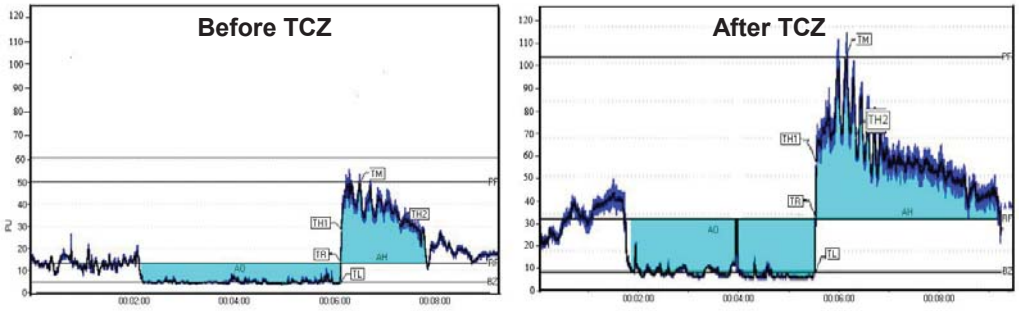
Table I. Clinical details of the Rheumatoid Arthritis patients at baseline and after TCZ treatment

	RA patients	**RA patients**	
Clinical parameters	**Baseline**	**TCZ**	p value
Female/Male (n/n)	16/4		
Age (years)	47.8 ± 2.30		
Duration of the disease (years)	7.6 ± 1.76		
RF levels	**90.84 ± 25.22**	**54.22 ± 13.99**	**p=0.041**
Anti-CCPs levels	761.09 ± 240.19	762.54 ± 251.44	p=0.991
Obesity (n)	6.10 %		
Diabetes (n)	1 %		
Hypertension (n)	2 %		
Menopause (n)	50 %		
Smoker (n)	25.0 %		
Joint damage			
Swollen joints (n)	**6.14 ± 1.01**	**0.71 ± 0.47**	**p=0.001**
Painful joints (n)	**19.00 ± 4.58**	**8.00 ± 2.93**	**p=0.045**
DAS28	**4.25 ± 0.18**	**2.90 ± 0.42**	**p=0.021**
VAS	**71.5 ± 3.94**	**48.33 ± 3.34**	**p=0.001**
HAQ	**1.54 ± 0.31**	**1.04 ± 0.33**	**p=0.046**
Lipid profile			
Total Cholesterol, mg/dl	180.00 ± 9.33	201.20 ± 7.39	p=0.070
HDL-Cholesterol, mg/dl	**45.60 ± 2.51**	**56.10 ± 2.42**	**p=0.001**
LDL-Cholesterol, mg/dl	115.30 ± 8.83	124.30 ± 9.30	p=0.319
Triglycerides, mg/dl	94.00 ± 8.70	102.6 ± 9.57	p=0.273
Apolipoprotein A1	**130.80 ± 5.68**	**151.90 ± 7.31**	**p=0.042**
Apolipoprotein B	73.60 ± 4.44	79.90 ± 6.14	p=0.264
ApoB/ApoA1 ratio	0.57 ±0.051	0.52 ± 0.035	p=0.267
Inflammatory parameters			
ESR	**25.40 ± 6.09**	**4.22 ± 0.70**	**p=0.014**
CRP, mg/dl	**13.29 ± 6.08**	**0.46 ± 0.18**	**p=0.045**
Treatments			
Corticosteroids	62.5 %		
Hydroxychloroquine	12.5 %		
NSAIDS	75.0 %		
Methotrexate	63.5 %		
Leflunomide	30.0 %		
Vitamin D	18.0 %		

[1] Values are means ± SEM. HDL= High density lipoprotein; LDL= Low density lipoprotein; DAS= Disease activity score; anti-CCPs = Anti-cyclic citrullinated proteins; CIMT = Carotide intime media thickness; ESR= Erythrocyte sedimentation rate; CRP= C reactive protein; NSAIDS= Non-steroideal anti-inflammatory drugs; RF= Rheumatoid factor

A

	Before TCZ	After TCZ	p value
PF	71.23 ± 6.06	87.17 ± 10.10*	0.010
AH	2314.03 ± 300.73	3846.54 ± 575.64*	0.041
BZ-PF	1098.23 ± 109.65	1324.37 ± 167.19	0.360
TH1	2.39 ± 0.85	1.48 ± 0.37	0.390
TH2	48.44 ± 9.80	42.11 ± 3.80	0.430



B

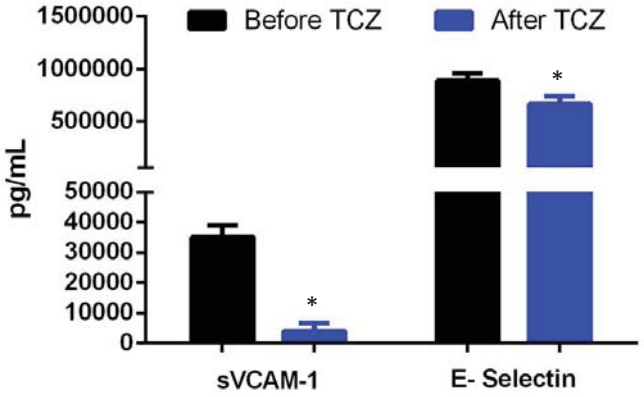


Figure 1

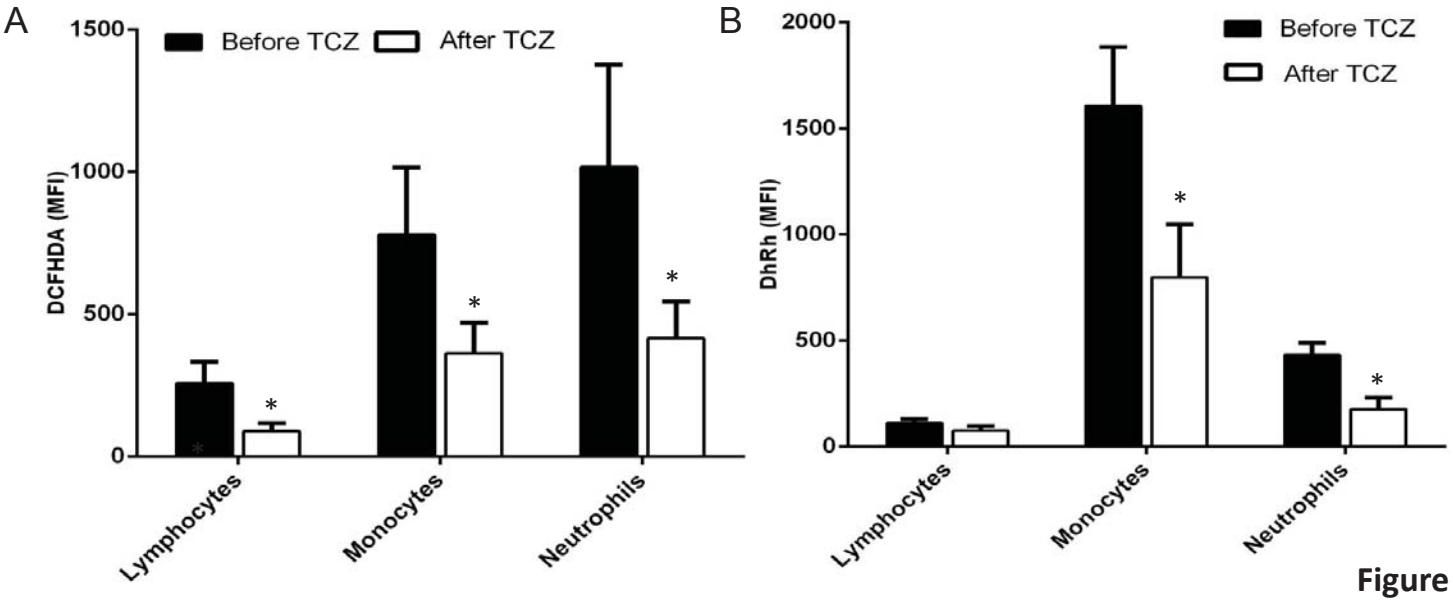

A
1500
1000
500
0
DCFHDA (MFI)
Before TCZ
After TCZ
*
*
*
Lymphocytes
Monocytes
Neutrophils
B
2000
1500
1000
500
0
DhRh (MFI)
Before TCZ
After TCZ
*
*
Lymphocytes
Monocytes
Neutrophils

Figure 2

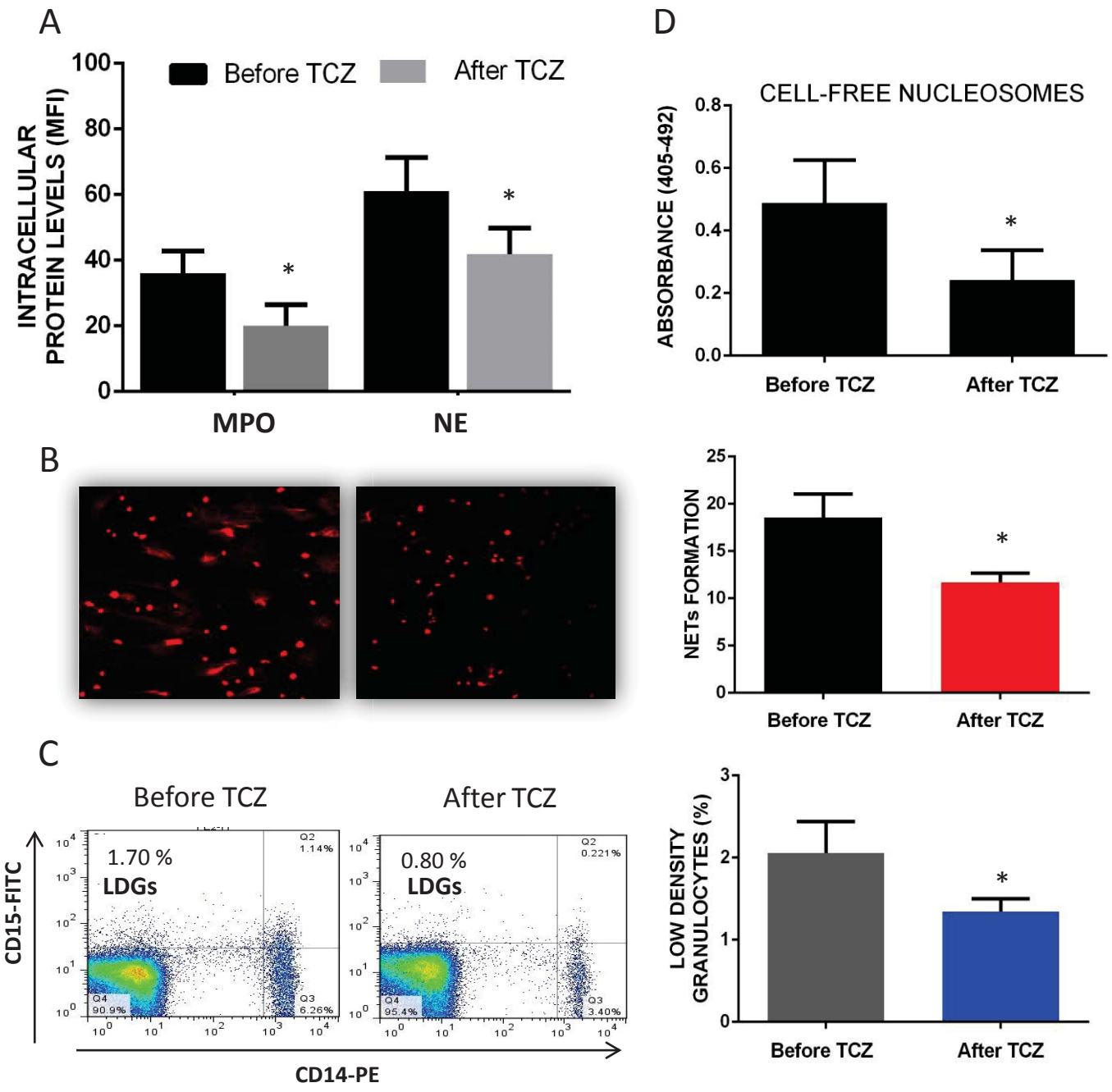
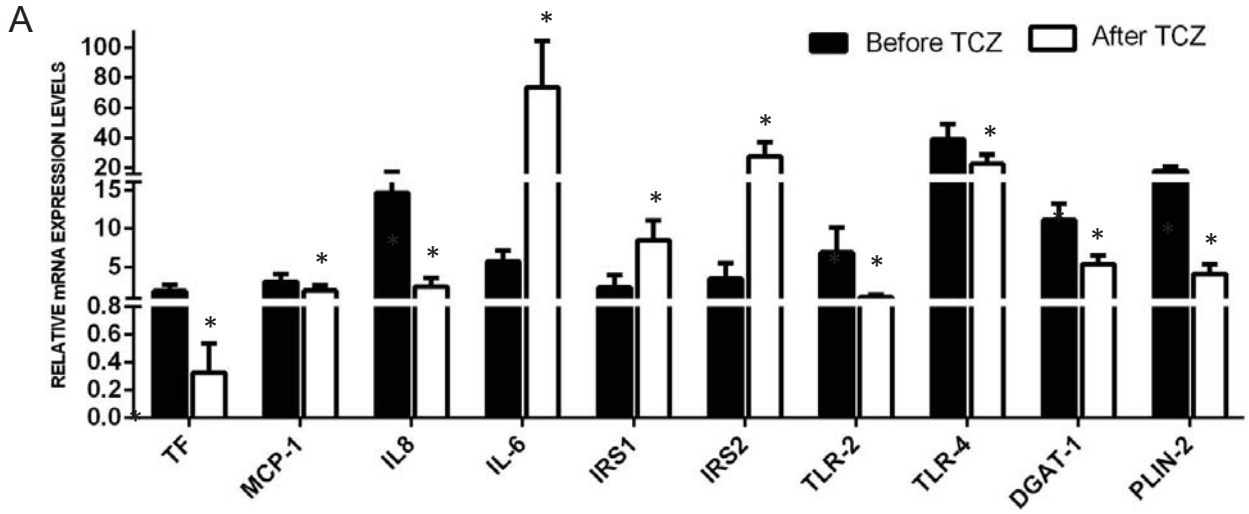
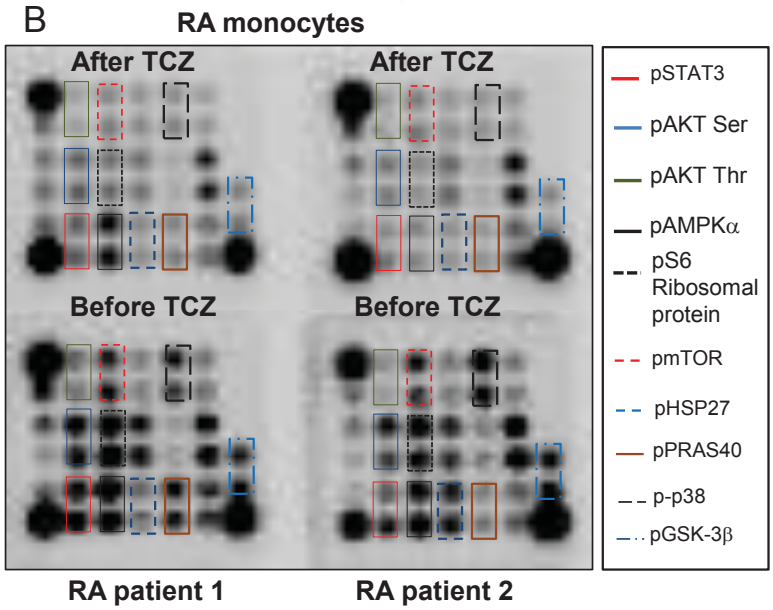

A
Before TCZ
After TCZ
INTRACELLULAR PROTEIN LEVELS (MFI)
100
80
60
40
20
0
*
*
MPO
NE
B
C
Before TCZ
After TCZ
1.70 %
LDGs
0.80 %
LDGs
Q2 1.14%
Q4 90.9%
Q3 6.26%
Q2 0.221%
Q4 95.4%
Q3 3.40%
CD15-FITC
CD14-PE
D
CELL-FREE NUCLEOSOMES
ABSORBANCE (405-492)
0.8
0.6
0.4
0.2
0.0
Before TCZ
After TCZ
NETs FORMATION
25
20
15
10
5
0
Before TCZ
After TCZ
LOW DENSITY GRANULOCYTES (%)
3
2
1
0
Before TCZ
After TCZ

Figure 3


A
Before TCZ
After TCZ
RELATIVE mRNA EXPRESSION LEVELS
100
80
60
40
20
15
10
5
0.8
0.6
0.4
0.2
0.0
TF
MCP-1
IL8
IL-6
IRS1
IRS2
TLR-2
TLR-4
DGAT-1
PLIN-2


B
RA monocytes
After TCZ
After TCZ
Before TCZ
Before TCZ
pSTAT3
pAKT Ser
pAKT Thr
pAMPKα
pS6 Ribosomal protein
pmTOR
pHSP27
pPRAS40
p-p38
pGSK-3β
RA patient 1
RA patient 2

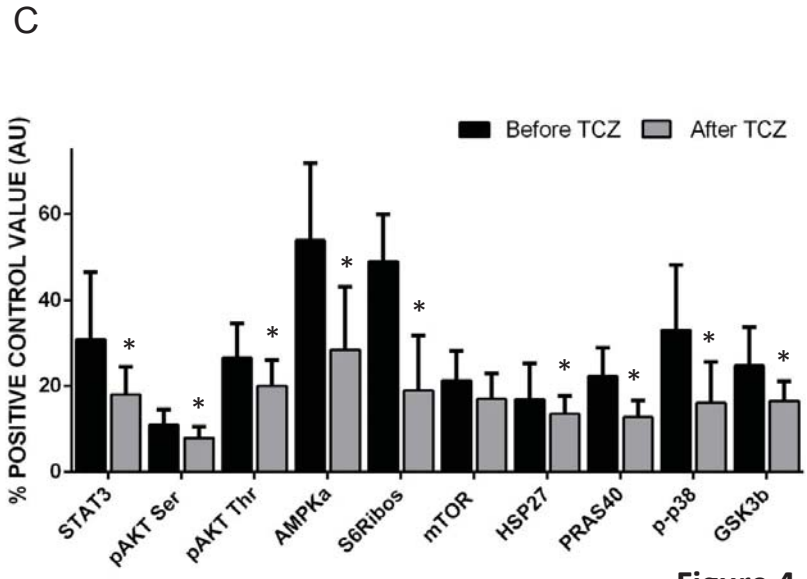
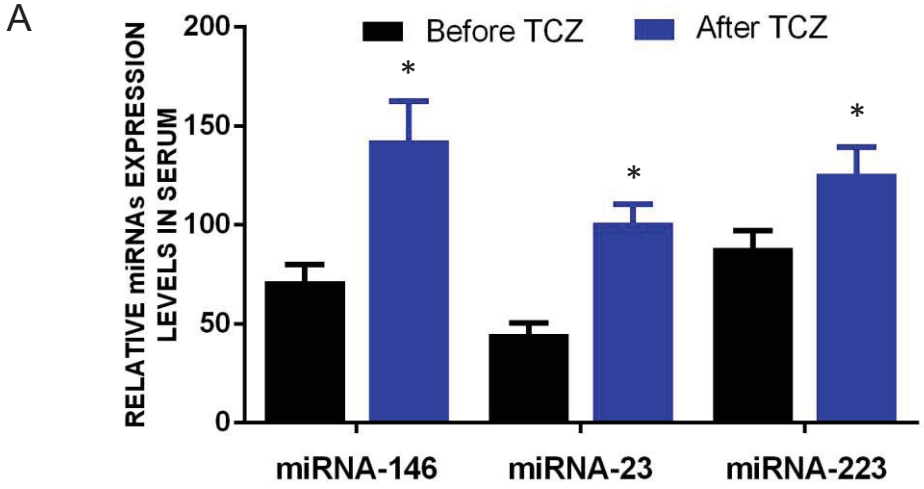

C
Before TCZ
After TCZ
% POSITIVE CONTROL VALUE (AU)
60
40
20
0
STAT3
pAKT Ser
pAKT Thr
AMPKa
S6Ribos
mTOR
HSP27
PRAS40
p-p38
GSK3b

Figure 4

A



B Ingenuity pathways analysis (IPA): mRNAs involved in CVD

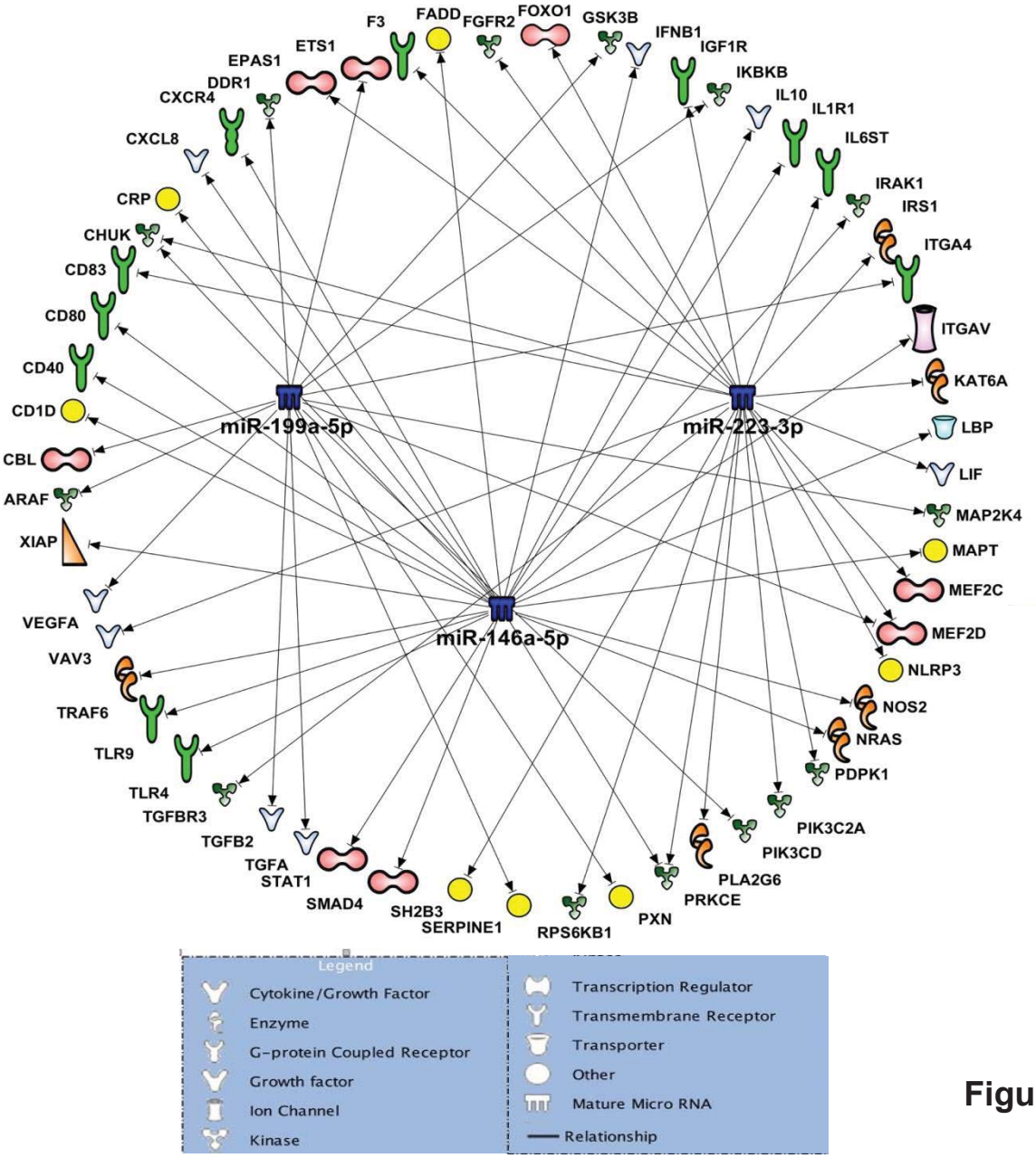
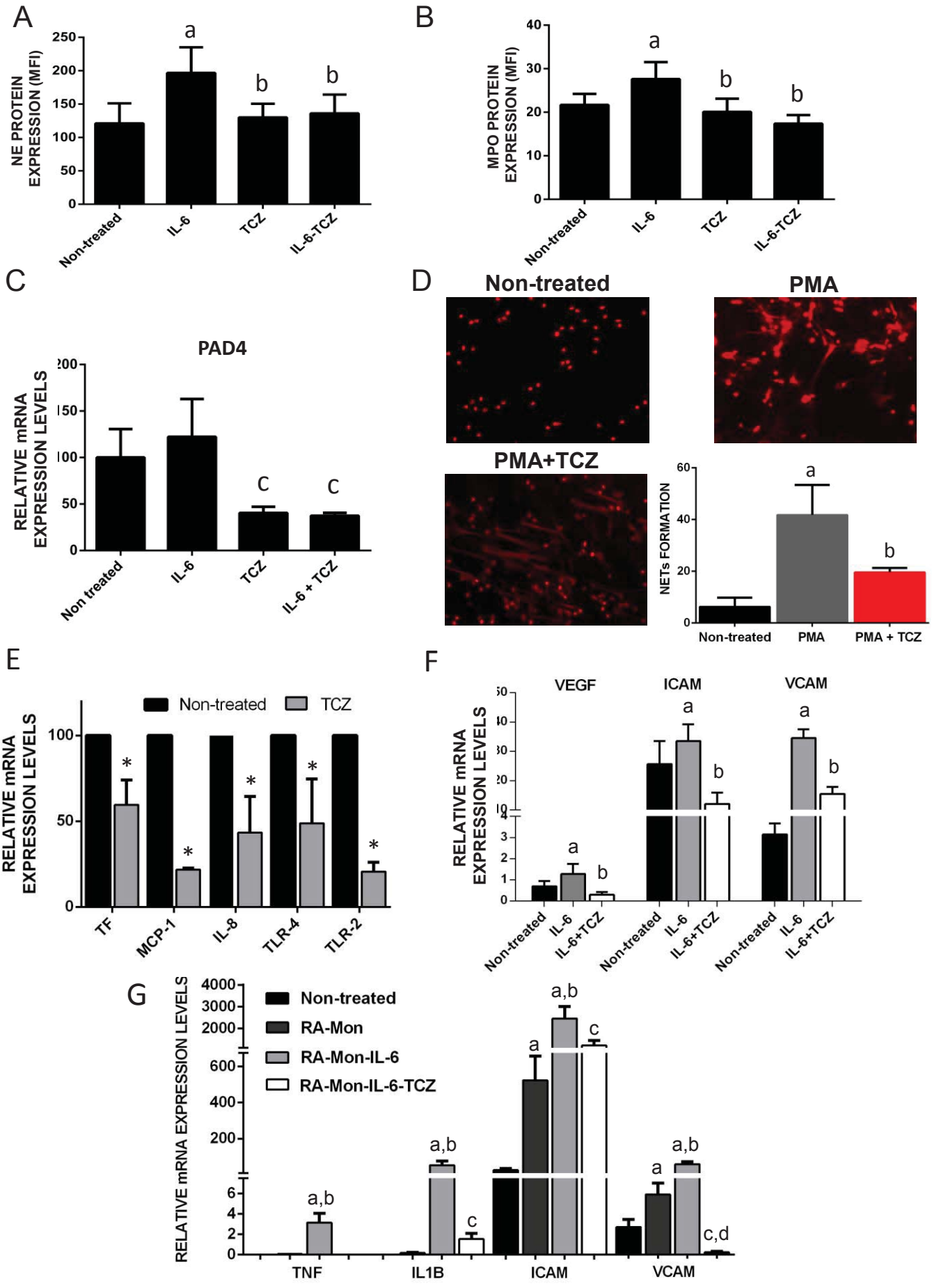


Figure 5


A
NE PROTEIN EXPRESSION (MFI)
250
200
150
100
50
0
a
b
b
Non-treated
IL-6
TCZ
IL-6-TCZ
B
MPO PROTEIN EXPRESSION (MFI)
40
30
20
10
0
a
b
b
Non-treated
IL-6
TCZ
IL-6-TCZ
C
PAD4
RELATIVE mRNA EXPRESSION LEVELS
150
100
50
0
c
c
Non treated
IL-6
TCZ
IL-6 + TCZ
D
Non-treated
PMA
PMA+TCZ
NETs FORMATION
60
40
20
0
a
b
Non-treated
PMA
PMA + TCZ
E
Non-treated
TCZ
RELATIVE mRNA EXPRESSION LEVELS
100
50
0
*
TF
MCP-1
IL-8
TLR-4
TLR-2
F
VEGF
ICAM
VCAM
RELATIVE mRNA EXPRESSION LEVELS
a
b
Non-treated
IL-6
IL-6+TCZ
G
Non-treated
RA-Mon
RA-Mon-IL-6
RA-Mon-IL-6-TCZ
RELATIVE mRNA EXPRESSION LEVELS
4000
3000
2000
600
400
200
6
4
2
0
a,b
c
a
a,b
c
a
a,b
c,d
TNF
IL1B
ICAM
VCAM