Tocilizumab improves the pro-atherothrombotic profile of rheumatoid arthritis patients modulating endothelial dysfunction, NETosis and inflammation

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ABSTRACT

Tocilizumab (TCZ) is an effective treatment for rheumatoid arthritis. 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 yet. Twenty rheumatoid arthritis patients received 162 mg per week subcutaneous TCZ for 6 months. Endothelial function was measured through post occlusive hyperemia using Laser-Doppler. Oxidative stress markers in monocytes and neutrophils were analyzed by flow cytometry. NETosis was measured through sytox staining of DNA fibers and the expression of myeloperoxidase and neutrophil elastase. Percentage of low density granulocytes was analyzed through flow cytometry. Gene expression and phosphorylation of intracellular pathways was analyzed in monocytes. TCZ improved endothelial function and decreased oxidative stress in rheumatoid arthritis leukocytes. Percentage of low density granulocytes and NETosis generation were reduced. The proinflammatory and prothrombotic status of rheumatoid arthritis monocytes were also reversed through modulation of specific intracellular pathways. All these results were recapitulated after in vitro treatment with TCZ of monocytes and neutrophils purified from rheumatoid arthritis patients, and co-cultured with endothelial cells. TCZ might reduce the pro-atherothrombotic profile in rheumatoid arthritis patients through the restoration of the endothelial function, oxidative stress reduction, inhibition of monocytes prothrombotic and inflammatory profile, and abridged NETosis generation.

Running head: TCZ improves atherothrombosis in RA patients

Abbreviations:

ACR: American College of Rheumatology.

ADRP: Adipophilin.

AH: Hyperaemic area.

AKT1S1: AKT1 substrate 1.

Anti-TNFα: Anti-Tumor Necrosis Factor alpha.

AO:Occlusion area.

BBE: Bovine brain extract.

BDMARDs: Biologic DMARDs.

BZ: Biological zero.

CO2: Carbon dioxide.

CRP: C-reactive protein.

Ct: Threshold cycle value.

CVD: Cardiovascular disease.

DAS28: Disease activity score 28.

DGAT: Diacylglycerolacyltransferase.

EBM: Endothelial Cell Basal medium.

ED: Endothelial dysfunction.

ESR: Erythrocyte sedimentation rate.

FITC: Fluorescein isothiocyanate.

GSH: Intracellular glutathione.

HAQ: Health assessment questionnaire.

HDL: High density lipoprotein.

hEGF: Human epidermal growth factor.

HUVEC: Human umbilical vein endothelial cells.

ICAM-1: Intercellular adhesion molecule-1.

IL: Interleukin.

IRS: Insulin receptor substrate.

LDGs: Low density granulocytes.

LDL: Low-density lipoprotein.

MAb: Monoclonal antibody.

MCP-1: Monocyte chemoattractant protein-1.

MFI: Mean fluorescence intensity.

mIL-6R:Membrane IL-6 receptor.

MPO: Myeloperoxidase.

NE: Neutrophil elastase.

NETs: Neutrophil extracellular traps.

NOS: Nitric oxide synthase.

NOX: NADPH oxidase.

NSAIDS: Non-steroideal anti-inflammatory drugs.

OD: Optical density.

p-p38: Phospho mitogen-activated protein kinase 14.

PAD4: Peptide arginine deiminase, type IV.

PF: Peak flow.

pGSK-3β: Phospho glycogen synthase kinase 3 beta.

pHSP27: Phospho heat shock protein 27.

PKB/AKT: Phospho protein kinase B.

PMBCs: Peripheral mononuclear blood cells.

pmTOR: Phospho mechanistic target of rapamycin.

PORH: Post Occlusive Reactive Hyperaemia.

pPRAS40: Proline-rich Akt substrate.

PRKAA1/pAMPKα: Phospho protein kinase AMP-activated catalytic subunit alpha 1.

pSTAT3: Phospho signal transducer and activator of transcription 3.

RA: Rheumatoid arthritis.

RF: Normal perfusion.

RF: Rheumatoid factor.

sDMARDs: Synthetic DMARDs.

sIL-6R: Soluble IL-6 receptor.

TCZ: Tocilizumab.

TF: Tissue factor.

TH1: Time to half before hyperaemia.

TH2: Time to half after hyperaemia.

TLR: Toll like receptor.

TM: Time to max.

TNFα: Tumor necrosis factor α.

VAS: Visual analogue scale.

VCAM: Vascular cell adhesion molecule.

VEGF: Vascular endothelial growth factor.

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, causing the 39% to 50% of deaths.[1]Atherosclerosis at early stage of the disease is considered a potential preclinical manifestation. In fact, the risk of CVD events, such as myocardial infarction, is increased in the 2 years preceding the formal diagnosis of RA[2] and once the disease is diagnosed, the risk of having carotid plaques and CVD events increase with the progression of the disorder.[3] The mechanisms responsible for the development of premature atherosclerosis in RA are not well understood, but traditional risk factors alone are not fully accountable, and a role for inflammation has been suggested in this process. Thus, it is likely that inflammatory mediators might be causal in the accelerated atherosclerosis observed in this autoimmune disease, which is further secondary to endothelial dysfunction (ED) and CVD.[4]

Endothelial dysfunction (ED) is a vascular abnormality frequently presented in RA patients, contributing to plaque initiation and progression. It is associated with carotid intima media thickness in long-standing RA.[5,6] The phenotypic features of ED comprise upregulated expression of cellular adhesion molecules, compromised barrier function leading to increased leukocyte diapedesis, increased vascular smooth muscle tone - secondary to impaired processing of vasodilator substances such as nitric oxide and prostacyclin-, as well as increased production of vasoconstrictor substances including endothelin.[7]

A number of processes have been linked to the development of ED and atherosclerosis in RA. Among them, increased neutrophil extracellular traps (NETs) have been proposed as a potential mechanism in the occurrence of CVD events. NETosis is a way of cell death, different from necrosis and apoptosis, in which occurs the dissolution of internal membranes, followed by the de-condensation of the chromatin and the release of NETs -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 (NOX) and nitric oxide synthase (NOS)- to the extracellular space. 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[8-11].

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. Moreover, we demonstrated a close relationship between anti-CCP levels and inflammation, so that they act as direct inductors of the pro-oxidative status, the inflammatory, and the atherogenic profile of lymphocytes, monocytes, and neutrophils in patients with RA.[12]

Many different cell components can be considered as key elements in the inflammatory and pro-atherothrombotic status of RA patients. Among them, macrophages have been demonstrated to play an important role in the pathogenesis of atherosclerosis. Various parameters of circulating monocytes, including count, increased adhesive properties, lipid metabolism alterations, phagocytosis and low-density lipoprotein (LDL) cholesterol, are associated with CVD.[13,14]Moreover, monocytes/macrophages are central players in inflammation, and have been found to be activated in RA through the release of cytokines and massive infiltration in the inflammatory sites, such as synovial membranes.[15,16] Thus, the progressive generation of inflammatory monocytes is an intrinsic element in the immune response mediating RA[17], and this response provides lipid deposits with effectors cells that accelerate the development of advanced

atherosclerotic vascular disease. Therefore, treatments targeting monocytes–macrophages might contribute to effectively prevent cardiovascular events.

Anti-tumor necrosis factor alpha (anti-TNFα) therapy has significantly improved the outlook for patients suffering from RA, but a substantial proportion of patients fail to respond to these therapies, which implies that treatment response is likely to be multifactorial.[18] Thus, new therapies are being evaluated. Interleukin-6 (IL-6) is an upstream inflammatory cytokine that plays a central role in propagating the downstream inflammatory response inducing atherosclerosis, as it is implicated in ED and arterial stiffening contributing to accelerated atherosclerosis process in RA patients.[19] Moreover, it has a role in the differentiation of B lymphocyte into auto-antibody producing plasma cells, which participate in the pathogenesis of RA through the formation of immune complexes.[20] High levels of IL-6 may cause a Th1-Th17/T-reg cell imbalance during RA, which is corrected upon treatment with tocilizumab (TCZ),[21,22] a recombinant humanized antihuman IL-6 receptor monoclonal antibody (mAb) that acts by binding both soluble and membrane IL-6 receptor (sIL-6R and mIL-6R),blocking the pro-inflammatory effects of IL-6.[23]

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 monocyte activity, NETosis, and oxidative stress, principal effectors of endothelial dysfunction, atherosclerosis and CVD in this autoimmune condition, have not been analyzed. We undertook this study to evaluate the molecular and cellular mechanisms underlying the effects of TCZ on the pro-atherothrombotic profile associated with RA, focusing on the effects of this biological therapy on endothelial dysfunction, neutrophils and monocytes activity.

MATERIAL AND METHODS

This paper conforms to the relevant ethical guidelines for human research.

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.[24] The patients were taking the following treatments: corticosteroids (62.5%), leflunomide (30.0%), hydroxichloroquine (12.5%), NSAIDs (75.0%), methotrexate (63.5%) and vitamin D (18.0%). Patients having inadequate response or intolerance to conventional DMARDs were given subcutaneous Tocilizumab (162 mg per week) for 6 months. The treatment of those patients with synthetic DMARDs, NSAIDs or/and corticoids had been stable for at least two months before TCZ administration and was not modified during TCZ treatment.

All patients were tested for the presence of anti-CCPs and rheumatoid factor (RF). 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 $\geq$3.2.[25] All the patients filled the health assessment questionnaire (HAQ) and the visual analogue scale (VAS) in order to assess the pain. Changes from baseline in DAS28, VAS, HAQ, erythrocyte sedimentation rate (ESR) and C-reactive protein (CRP) levels, and tender and swollen joints were analyzed.

All participants enrolled were Caucasian, 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, analysing the response to reactive hyperaemia, so as the increase in blood flow occurred after temporary occlusion of blood flow, using a skin probe attached to the inner forearm. Post occlusive reactive hyperaemia (PORH) test consisted of 2 minutes of baseline followed by 4 min occlusion period. The cuff was then released and the PORH response was analysed during 3 minutes.

Several parameters were obtained: normal perfusion (RF), perfusion when occluded (BZ), occlusion area (AO), time to half before hyperaemia (TH1), highest perfusion

value after occlusion was released (PF), time to half after hyperaemia (TH2), and hyperaemic area (AH).

White blood cells isolation. Neutrophils were isolated from patients by density centrifugation over Dextran-Ficoll Hypaque, which allowed cells to keep a non-activated state. 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, MiltenyiBiotec, BergischGladbach, Germany). Purity of the fractions was evaluated via flow cytometry (FACScalibur cytometer), by analysing the size and complexity of each population (forward and size scatters). The purity of monocytes was further evaluated with fluorescein isothiocyanate (FITC)-conjugated anti-CD14 antibody by flow cytometry. By this method, 95.2 ± 4.3 viable monocytes were obtained.

In vitro studies. Monocytes and neutrophils purified from 5 RA patients at baseline (no taking TCZ) were cultured separately in RPMI 1640 containing 10% FBS, 2 mM l-glutamine, 100 U ml^{-1}penicillin, 100 mg ml^{-1} streptomycin and 250 $pgml^{-1}$fungizone (BioWhittaker/MA Bioproducts, Walkersville, Maryland, USA) at 37°C in a humidified 5% carbon dioxide (CO_2) atmosphere.

For mRNA and protein expression analyses, RA monocytes (1×10^6 cells ml^{-1}) were pre-treated with FCRII blocking (Miltenyi Biotec) for 15 min. Then, cells were washed with 1 ml of PBS and centrifuged at 300 g for 10 minutes. Thereafter, cells were seeded and incubated with IL-6 (10 $ngml^{-1}$) for 9 hours and then incubated in the presence or in the absence of TCZ (20 μg ml^{-1}) for 9 hours. RA neutrophils (1×10^6 cells ml^{-1}) were pre-treated with FCRII blocking as described above. Then, cells were seed and incubated with IL-6 (10 $ngml^{-1}$) for 3 hours, and thereafter incubated in the presence or in the absence of TCZ (20 μg ml^{-1}) for 3 hours, at 37°C in a humidified 5% (CO_2) atmosphere. 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), 100 U ml^{-1} penicillin, 100 mg ml^{-1} streptomycin and 250 $pgml^{-1}$fungizone (BioWhittaker/MA Bioproducts, Walkersville, Maryland, USA) at 37°C in a humidified 5% CO_2 atmosphere.

For in vitro studies, HUVECs were seeded into 6-well plates ($4x10^5$ cells per well) in 1.5 ml of complete medium. 24 hours after, cells were pre-treated for 15 min with FcR blocking Reagent (Miltenyi Biotec), and subsequently incubated with IL-6 (10 $ngml^{-1}$) for 9 hours and thereafter incubated in the presence or in the absence of TCZ (20 μg ml^{-1}) for 9 hours. The cultured cells were harvested for total RNA isolation and applied to RT-PCR studies.

Co-cultures of RA monocytes-HUVEC: HUVECs were cultured as described above. Monocytes isolated from 5 RA patients at baseline (no taking TCZ) were pre-treated with FcRII blocking reagent for 15 min. Then, monocytes were washed with 1-2 ml of PBS, centrifuged at 300 g for 10 minutes and seeded into trans well inserts (Corning® Transwell® polycarbonate membrane cell culture inserts, Sigma Aldrich, Misuri, USA) ($1×10^6$ cells per transwell) 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 $ngml^{-1}$) alone or combined with TCZ (20 μg ml^{-1}) as described previously. The co-cultured cells (RA monocytes and HUVEC) were harvested separately for total RNA isolation and applied to subsequent RT-PCR.

RNA isolation and quantitative real-time reverse transcriptase PCR. Total RNA from monocytes, neutrophils and endothelial cells was extracted using TRI Reagent (Sigma, St Louis, Missouri, USA) following the manufacturer's recommendations. The integrity of RNA was verified by optical density (OD) absorption ratio OD260/OD280 between 1.8 and 2.0.

For first strand cDNA synthesis, 1μg of total RNA was reverse transcribed using random hexamers as primers and Transcriptor Reverse Transcriptase (Quiagen, Madrid, Spain). Gene expression was assessed by real time PCR using a LightCycler

Thermal Cycler System (Roche Diagnostics, Indianapolis, Indiana, USA). The reaction was performed, following the manufacturer's protocol, in a final volume of 12µl.

The reactions consisted of an initial denaturing of 10 min at 95 ºC, then 40 cycles of 15 seconds denaturing phase at 95ºC, and 1 min annealing and extension phase at 60ºC. A threshold cycle (Ct value) was obtained for each amplification curve and a ΔCt value was first calculated by subtracting the Ct value for human glyceraldehyde-3-phosphate dehydrogenase cDNA from the Ct value for each sample and transcript. Fold changes compared with the endogenous control were then determined by calculating 2-ΔCt. Samples were analysed in triplicate and negative controls were included in all the reactions. Test reproducibility for all investigated transcripts was less than 0.5% in inter-test experiments and even lower in intra-test experiments.

Genes related to inflammation [tissue factor (TF), monocyte chemoattractant protein-1 (MCP-1), IL-8, TNFα, IL-1β and IL-6], lipid metabolism and storage [toll like receptor (TLR) 2, TLR4, diacylglycerolacyltransferase (DGAT), adipophilin or ADRP (PLIN2), insulin signalling (IRS1 and IRS2)] and cell adhesion (ICAM, VCAM and VEGF) were evaluated.

Determination of oxidative stress biomarkers in white blood cells. Oxidative stress biomarkers were analysed in monocytes and neutrophils using a dual-laser FACSCalibur (Becton Dickinson). Test standardization and data acquisition analysis were performed using the CELL Quest software (Becton Dickinson). For the assessment of ROS generation, including superoxide anion and hydrogen peroxide, cells were incubated with 20.5 µM DCFH-DA (Sigma-Aldrich) at 37°C for 30 min in the dark and 5 μM DihidroRhodamine123 at 37ºC for 30 min (Sigma-Aldrich). The cells were washed, re-suspended in PBS, and then analysed on a dual-laser FACSCalibur. As internal control, the fluorescence of unstained cells was used. To test the positivity of the RA population, monocytes and neutrophils isolated from healthy donors were used.

NETs induction and quantification. Isolated neutrophils from RA patients at baseline and after 6 months of TCZ treatment were seeded in 24 well plates on poly-L-lysine-coated glass coverslips (BD Biosciences, San Jose, CA, USA) in tissue-culture wells and allowed to settle for 1 hour at 37°C under 5% CO2. Then, 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. Neutrophils purified from 5 RA patients with moderate-high DAS28, and not taking any biologic treatment, were treated *in vitro* with FCRII blocking (MiltenyiBiotec) for 15 min. Then, cells were washed with 1 ml of PBS and centrifuged at 300 g for 10 minutes. Thereafter, cells were seeded in the same way as described above and pre-treated with or without TCZ (20 $\mu g\ ml^{-1}$) for 30 minutes and then incubated with IL-6 (100 $ngml^{-1}$) during 15 hours or PMA (600 nM) for 2 hours. After the different treatments, cells were fixed with 4% paraformaldehyde and rinsed three times with PBS. DNA was stained with 5 mM Sytox orange dye (Life technologies, Netherlands) and NETs were visualized using a Nikon Eclipse-Ti-S fluorescence microscope, (NIS-Elements imaging software). Five images selected randomly from different regions of each coverslip per case were taken 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[26,27]and were counted for at least 3 fields using IMAGE-J software (NIH, Bethesda, MD). Results were expressed as percentage of NETs (NETs formation).

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. Monoclonal antibodies against DNA (double and single strand) and histones (H1, H2A, H2B, H3 and H4) were used to detect mono- and oligo nucleosomes in serum from RA patients. Quantification of nucleosomes was performed by photometrical determination of the absorbance at 405 nm, using as reference wavelength 492 nm.

Identification of Low density granulocytes (LDGs). Peripheral mononuclear blood cells (PMBCs) ($5x10^5$) were incubated with PE anti-human CD14 and FITC anti-human CD15 (Biolegend) for 30 minutes at 4ºC in the dark. PE and FITC IgG isotypes from the same company were used as negative controls. Cells were washed, resuspended on 500 μl of PBS and acquired on the flow cytometer FACSCalibur.

Neutrophil elastase (NE) and myeloperoxidase (MPO) protein expression. Whole peripheral blood (100 μl) was incubated with 1 ml of lysis buffer (BD Pharm Lyse Lysing Buffer) for 10 minutes at room temperature in the dark. After centrifugation at 1800 rpm for 5 minutes at 4 ºC, cells were fixed and permeabilized with 250 μl of buffer (BDCytofix/Cytoperm™Fixation/Permeabilization solution Kit with BD GolgiPlug™) for 20 minutes at 4ºC. Then, cells were incubated either with FITC human anti-MPO (BD Biosciences) or with human anti-elastase primary antibody (RbmAb to Neutrophil Elastase (Abcam, Cambridge, UK). Then, for neutrophil elastase analysis, Alexa Fluor conjugated secondary antibody (Abcam) was added for 30 minutes at 4ºC. IgG isotypes were used as negative controls. Cells were washed and 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.). The phosphorylation levels of ERK1/2, STAT1, STAT3, AKT, AMPKa, S6 Ribosomal protein, mTOR, HSP27, Bad, P70 S6 Kinase, PRAS40, p53, p38, SAPK/JNK, PARP, Caspase-3 and GSK-3b were analyzed on monocytes isolated from RA patients at baseline and after 6 months of treatment with TCZ.

Plasma levels of cellular adhesion molecules. E-selectin and vascular cell adhesion molecule 1 (VCAM-1) levels were analyzed in plasma from RA patients before and after TCZ therapy using ProcartaPlex multiplex immunoassay, following the manufacturer's recommendations (AffymetrixeBioscience, Vienna, Austria).

Statistical analysis. All data are expressed as mean ± SD. 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 $p<0.05$

RESULTS

TCZ improved the disease activity, clinical parameters and endothelial dysfunction in RA patients. Treatment of RA patients with TCZ (162mg/ml per week) for 6 months significantly reduced the number of both, 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 from high disease activity to low-moderate disease activity: mean DAS28 changes (± SDs) were 1.35 ± 0.78 in TCZ-treated patients (**Table I**). Autoimmunity was further modulated by treatment *in vivo* with TCZ. Thus, a significant reduction of rheumatoid factor (RF) levels was observed. However, no effect was detected on the levels of anti-CCP antibodies. Regarding inflammatory clinical parameters, RA patients displayed significantly reduced levels of erythrocyte sendimentation 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 (measured by Laser Doppler measurement of post ischemic reactive hyperemia) improved notably, as shown by the augmentation of the highest perfusion value after occlusion was released (peak flow, PF) and by the increase of hyperemic area (HA) (**Fig 1A**). In parallel, changes in plasma levels of parameters closely related to endothelial dysfunction (cellular adhesion molecules such as E-selectin and vascular cell adhesion molecule 1 (VCAM-1)) were found significantly

reduced by treatment with TCZ, thus supporting the improvement of the vascular function in these patients (**Fig 1B**).

TCZ reduced oxidative stress in leukocytes from RA patients. We have recently reported an increased oxidative status in leukocytes from RA patients, directly related to both the titers of anti-CCPs, and the expression of a number of inflammatory parameters, thus contributing to atherosclerosis.[12] Accordingly, peroxides and peroxynitrites levels were downregulated *in vivo* in monocytes and neutrophils from RA patients after 6 months of treatment with TCZ, showing a significant reduction in the mean fluorescence intensity (MFI) of DhRh and DCFHDA in flow cytometry analyses (**Fig 2A and B**).

TCZ therapy in RA patients downregulated the percentage of low density granulocytes and decreased NETosis. A new subset of granulocytes (LDGs) has been identified in the peripheral blood mononuclear cells (PBMCs) fraction of patients with various autoimmune diseases. These cells show a phenotypic profile based on $CD14^{-/dim}$ and $CD15^{bright}$ expression, and are more prone to experience NETosis.[28] In the present study, a significant reduction in the percentage of LDGs in RA patients treated with TCZ was noticed (**Fig3A**).

Previous studies have shown that RA patients exhibit increased spontaneous NETs generation, associated with enhanced NE and MPO expression. In our hands, NE and MPO (main enzymes leading the initiation of NETosis) were found reduced in neutrophils from RA patients treated with TCZ (**Fig3B**).

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 the effect of TCZ in RA neutrophils treated with PMA (**Fig3D**).

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 (**Fig3C**).

***In vivo* treatment with TCZ reduced the procoagulant and inflammatory state, and modulated insulin signalling in monocytes.** Gene expression of molecules involved in inflammation (MCP-1, TLR4 and TLR2), procoagulant activity (TF) and migration (IL-8) was reduced in RA monocytes treated with TCZ (**Fig4A**). In contrast, *in vivo* inhibition of IL-6R signalling by TCZ increased the mRNA expression of IL-6 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 state. Molecules involved in lipid droplets formation (DGAT and PLIN2) were also found significant reduced at mRNA level in monocytes from RA patients after 6 months of TCZ treatment (**Fig4A**).

TCZ modified the activation of several intracellular kinases on monocytes from RA patients. Dysfunctional intracellular signaling pathways plays a critical role in RA, which might account for the immune-mediated chronic inflammation present in those autoimmune patients.[29] 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 IL-6R signalling by TCZ markedly reduced the phosphorylation of STAT3, a kinase directly activated by the binding of IL-6 to its receptor (**Fig4B 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, p38, HSP27, PRAS40 and GSK3-b (**Fig4B and C**).

***In vitro* treatment of purified RA neutrophils and monocytes with TCZ reduced NETosis generation and improved the inflammatory and thrombotic profile of monocytes.** To evaluate the specificity of the effect of TCZ, we conducted *in vitro* studies on monocytes and neutrophils.

In neutrophils isolated from RA patients, the pre-treatment with IL-6 for 6 hours promoted a significant increase in NE and MPO intracellular levels that was prevented by addition of TCZ (**Fig5A and B**).

We further analysed whether TCZ could diminish NETosis induced *in vitro*. The treatment of RA-neutrophils with IL-6 or PMA induced an increase of NETosis formation. The combination of TCZ plus IL-6 or PMA generated significantly less extrusion of DNA fibres in neutrophils comparing to the treatment with IL-6 and PMA alone, suggesting that TCZ might prevent NETosis in RA patients (**Fig5C**).

In vitro treatment of monocytes isolated from RA patients with TCZ reduced their inflammatory and prothrombotic profile demonstrated by the decrease in the high expression of TLR-2, TNF-α, IL-1β, IL-8, MCP-1, VEGF, VCAM and ICAM observed in non-treated monocytes or induced by IL-6 (**Fig 6A**).

TCZ significantly reduced the IL6-induced expression of both adhesion molecules and inflammatory mediators in endothelial cells alone or co-cultured with monocytes purified from RA patients. In order to mimic the *in vivo* effects of TCZ on the vessel wall, cultured HUVECs were treated with IL-6, either alone or in combination with TCZ. A significant increase in the mRNA expression of TLR-2, IL-8, MCP-1, VEGF and VCAM was noticed in HUVECs after treatment with IL-6 for 18 hours. Those high levels were downregulated by addition of TCZ, suggesting that IL-6R blockade might have a beneficial effect on the endothelial function (**Fig6B**).

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 HUVECs increased the mRNA expression of TLR-2, TNF-α, IL-1β, IL-8, TF, MCP-1, ICAM, VCAM and VEGF on endothelial cells. Moreover, these genes were significantly upregulated after the addition of IL-6 to the co-culture medium (HUVECs with RA monocytes). Once more, TCZ significantly reduced the IL6-induced expression of both adhesion molecules and inflammatory and prothrombotic mediators on endothelial cells co-cultured with RA purified monocytes (**Fig6C**), thus underlying the relevant role of this IL-6R-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 the pro-atherotrombotic profile 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 showed that combination therapy with biologic DMARDs (bDMARDs) and synthetic DMARDs (sDMARDs) represents the best therapeutic option for the treatment of RA, since it can slow the progression of the disabling structural damage.[30] In our cohort of patients, the combination of a bDMARD, TCZ, with sDMARDs (methotrexate, leflunomide and hydroxychloroquine) 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, 6 months of treatment with 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.[30] Thus, longer treatment with TCZ might be necessary to reduce the levels of anti-CCPs.

Recently, several studies evaluated the changes 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. Thus, comparing with naïve-biologic therapy and others bDMARDs (including adalimumab, rituximab and tofacitinib), TCZ greatly increased the levels of HDLc, noticed after the first 6 weeks of treatment, which was translated into high levels of total cholesterol.[31-34]Some of these studies further reported an increase in LDLc and triglycerides. In our cohort of patients, there was a significant increase in fasting HDLc levels after 6 months of TCZ

treatment; however this was not related to a significant augmentation of the total cholesterol levels, which might be due to the unchanged levels of triglycerides and LDLc. Accordingly, ApoA1 levels were significantly augmented by treatment with TCZ.

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.[35] In addition, comparison 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.[36] In accordance, our study shows a significant improvement of 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 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 showed a decrease in serum VEGF levels after 6 months of TCZ monotherapy.[37] Our results were further supported by in vitro studies, on which treatment of ECs with TCZ prevented the induced expression of TLR2, IL-8, MCP-1, VEGF, ICAM and VCAM by IL-6. Moreover, we could demonstrate that addition of TCZ to the co-culture of RA monocytes with ECs plus IL-6 reduced the inflammatory profile (represented by a reduction of TNFα, IL1β, IL-8, MCP-1 and TLR2 expression), and improved the endothelial dysfunction, (through a downregulation of VEGF, ICAM and VCAM). In the same way, Suzuki et al., described the important role of IL-6 increasing the rate of cell migration, and how TCZ was able to reduce the adhesion of monocytic cells (U937) to

HUVEC.[38] 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.[12] The present study shows for the first time the effects of the inhibition of IL-6R 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.[39,40] 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-TNFa therapy.[41] 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.

Monocytes from RA patients display a pro-atherothrombotic profile, showing elevated expression of proinflammatory cytokines and procoagulant factors.[12] The effect of TCZ on the atherothrombotic markers associated with RA at plasma level has already been described. Thus, TCZ reduced prothrombotic molecules including D-dimer, prothrombotic fragment 1+1, fibrinogen, lipoprotein A and phospholipase A-2-IIA in plasma from RA patients[32,42,43]. However, there is little evidence about the effect of TCZ on RA monocytes, only few studies described that TCZ induced apoptosis on monocytes from RA patients.[44] We here analysed in depth the molecular changes occurred on monocytes from RA patients treated with TCZ. A marked reduction of the inflammatory prolife, prothrombotic properties and migratory capacity was observed at mRNA levels on RA monocytes, showed by the downregulation of TLR2, TNFα, IL-1β, IL-8, MCP-1, VEGF, VCAM and ICAM.

By contrast, a significant increase of IL-6 mRNA levels was noticed on monocytes from RA patients taking TCZ for 6 months. 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.[45]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 IL-6 mRNA in our study might derive from that feedback process.

In addition, we here provide new evidences about the effect of TCZ on the formation of lipid droplets on RA monocytes. The accumulation of lipid droplets within leukocytes on inflammatory conditions has been documented.[46] In this context, lipid droplets compartmentalize several proteins and lipids involved in the control of biosynthesis and secretion of inflammatory molecules, including leukotriene and PGE2.[47] PLIN2 and DGAT2 are two genes involved in lipid droplets regulation. PLIN2 overexpression results in increased formation of lipid droplets.[47] In turn, growth of lipid droplets is linked to functions performed by endoplasmic reticulum mediated by endoplasmic reticulum proteins such as DGAT. We found a significant reduction in the expression of these genes on RA monocytes treated in vivo 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.[48] Yet, this is the first article describing a downregulation of the main signaling pathways associated with RA pathogenesis in monocytes by TCZ: JAK/ STAT, SAPK/MAPK and PI-3K/AKT/mTOR. 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, constitutes a valuable tool to better

delineate the regulatory mechanisms modulated at cellular level by effects of specific drugs.

Neutrophils have recently been recognized as essential actors in the development of the atherosclerotic plaque. Neutrophils from RA patients are more susceptible to experience spontaneous NETosis.[49] The effects of IL-6 on neutrophils functions remains poorly understood, with conflicting evidence reporting that IL6 can either delay, accelerate, or have no effect on neutrophil apoptosis.[50-52] In our hands, the relevance of neutrophils function in CVD and their regulation by TCZ has been exposed by a decrease in the NETosis generation induced either by IL-6 or PMA, determinant in atherosclerosis development. This data suggests that TCZ improves the overall state of neutrophils so that they can be less prone to experience in vitro induced NETosis.

LDGs in RA are functionally different from RA neutrophils, having enhanced netting capabilities[28] and survival properties and decreased TNF signaling which might contribute to the disease pathology and response to therapy.[53] Moreover, LDGs display an activated phenotype, inducing endothelial cell cytotoxicity and thus playing a relevant role in cardiovascular development.[54] Our results show the beneficial effect of TCZ decreasing the percentage of LDGs in RA patients which might reduce the vascular damage induced by these subtypes of neutrophils.

Although further studies are required, our results indicate that neutrophils might have an important role in the development of inflammation in the context of RA, and that IL-6R signaling blockade could be a useful therapy to avoid undesired effects of persistent neutrophil activation.

This is the first study that evaluates the direct effect of TCZ on monocytes and neutrophils from rheumatoid arthritis patients. By performing in vitro studies, adding TCZ to monocytes and neutrophils isolated from RA patients that were not taking TCZ, the specificity of the effects of this biologic therapy on each cell type and each parameter analyzed was confirmed, something that should be considered a relevant

point since patients were taking other therapies that could also influence the effects attributed to TCZ.

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 and intracellular signalling.

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FIGURES AND FIGURE LEGENDS

Fig 1. (A) Microvascular function was measured by Laser Doppler linear Periflux 5010 and was performed at baseline and after 6 months of 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 ProcartaPlex multiplex immunoassay. Data are presented as mean ± SD (std deviation), n= 20 patients. (*) indicates significant differences vs before TCZ (p<0.05).

Figure 2. (A) Peroxides production in monocytes, and neutrophils of RA patients at baseline and after 6 months of TCZ treatment were determined by addition of the fluorescent probe DCFHDA to the isolated cells and flow cytometry analysis. **(B)** Peroxides and peroxynitrites production in monocytes, and neutrophils of RA patients before and after TCZ treatment were determined by the fluorescent probe dihydrorhodamine-123 and flow cytometry analysis. Upper panels show representative histograms with the mean fluorescence intensity of DCFHDA and DHRH in a healthy donor (full histogram), a RA patient before TCZ treatment (black unfilled histogram) and the same patient after TCZ therapy (grey unfilled histogram). Bar graphs show the mean ± SD of mean fluorescence intensity (MFI),n=20 patients. (*) indicates significant differences vs before TCZ (p<0.05).

Fig 3.(A) Representative dot plots of low density granulocytes from RA patients before and after TCZ treatment.**(B)** Intracellular MPO and NE protein expression were measured in neutrophils from RA patients at baseline and after 6 months of TCZ treatment by flow cytometry. Bar graphs show the mean ± SD of median fluorescence intensity (MFI).**(C)** Concentration of cell-free nucleosomes in serum by ELISA.**(D)** 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. Five images selected randomly from different regions of each coverslip per case were taken 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 without

PMA.NETs formation represents the average percentage of NETs structures from the 5 images taken in each condition. Bar graphs show the mean ± SD, n= 20 patients(*) indicates significant differences vs before TCZ (p<0.05).

Fig 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 (DGAT-1 and PLIN2), insulin signal (IRS-1 and IRS-2) in RA monocytes at baseline and after 6 months of 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 ± SD, n= 20 patients.(*) indicates significant differences vs before TCZ (p<0.05).

Fig5.(A and B) Intracellular neutrophil elastase (NE) and myeloperoxidase (MPO) protein expression was measured in neutrophils isolated from 5 RA patients at baseline (no taking TCZ), non-treated and treated *in vitro* with IL-6 (10 ng/ml), TCZ (20μg/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)** Representative images of neutrophil extracellular traps (NETs) of neutrophils isolated from 5 RA patients at baseline (no taking TCZ), non-treated and treated *in vitro* with IL-6 (100 ng/ml), TCZ or IL-6 plus TCZ (20μg/ml) for 15 hours and also treated with PMA (600 nM) alone or combined with TCZ (20μg/ml) 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. Five images selected randomly from different regions of each coverslip per case were taken 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 untreated cells. NETs formation represents the average percentage of NETs structures from the 5 images taken in each condition. Bar graphs show the mean ± SD of percentage of NETs of 5 independent experiments, n=5 different patients.(a) indicates significant differences vs non treated; (b) vs treated with IL-6; (c) vs treated with PMA (p<0.05).

Fig 6.(A) Quantitative RT-PCR was performed on a panel of genes on monocytes purified from 5 RA patients at baseline (no taking TCZ), non-treated and treated *in vitro* with IL-6 (10 ng/ml), TCZ or IL-6 plus TCZ (20μg/ml) for 18 hours. Bar graphs show the mean ± SD of five independent experiments, n=5 different patients. (a) indicates significant differences vs non-treated monocytes; (b) vs monocytes treated with IL-6; (p<0.05). **(B)** 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 (20μg/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).**(C)** 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 IL-6 (10ng/ml) alone or in combination with TCZ (20μg/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 (p<0.05).

TLR indicates toll like receptor; TNFα, tumor necrosis factor α; IL, interleukin; TF, tissue factor; MCP-1, monocyte chemotactic protein; VEGF, vascular endothelial

growth factor; ICAM, intercellular adhesion molecule-1; VCAM, vascular cell adhesion molecule.

Tocilizumab improves the pro-atherothrombotic profile of rheumatoid arthritis patients modulating endothelial dysfunction, NETosis and inflammation

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ABSTRACT

Tocilizumab (TCZ) is an effective treatment for rheumatoid arthritis. 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 yet. Twenty rheumatoid arthritis patients received 162 mg per week subcutaneous TCZ for 6 months. Endothelial function was measured through post occlusive hyperemia using Laser-Doppler. Oxidative stress markers in monocytes and neutrophils were analyzed by flow cytometry. NETosis was measured through sytox staining of DNA fibers and the expression of myeloperoxidase and neutrophil elastase. Percentage of low density granulocytes was analyzed through flow cytometry. Gene expression and phosphorylation of intracellular pathways was analyzed in monocytes. TCZ improved endothelial function and decreased oxidative stress in rheumatoid arthritis leukocytes. Percentage of low density granulocytes and NETosis generation were reduced. The proinflammatory and prothrombotic status of rheumatoid arthritis monocytes were also reversed through modulation of specific intracellular pathways. All these results were recapitulated after in vitro treatment with TCZ of monocytes and neutrophils purified from rheumatoid arthritis patients, and co-cultured with endothelial cells. TCZ might reduce the pro-atherothrombotic profile in rheumatoid arthritis patients through the restoration of the endothelial function, oxidative stress reduction, inhibition of monocytes prothrombotic and inflammatory profile, and abridged NETosis generation.

Running head: TCZ improves atherothrombosis in RA patients

Abbreviations:

ACR: American College of Rheumatology.

ADRP: Adipophilin.

AH: Hyperaemic area.

AKT1S1: AKT1 substrate 1.

Anti-TNFα: Anti-Tumor Necrosis Factor alpha.

AO:Occlusion area.

BBE: Bovine brain extract.

BDMARDs: Biologic DMARDs.

BZ: Biological zero.

CO2: Carbon dioxide.

CRP: C-reactive protein.

Ct: Threshold cycle value.

CVD: Cardiovascular disease.

DAS28: Disease activity score 28.

DGAT: Diacylglycerolacyltransferase.

EBM: Endothelial Cell Basal medium.

ED: Endothelial dysfunction.

ESR: Erythrocyte sedimentation rate.

FITC: Fluorescein isothiocyanate.

GSH: Intracellular glutathione.

HAQ: Health assessment questionnaire.

HDL: High density lipoprotein.

hEGF: Human epidermal growth factor.

HUVEC: Human umbilical vein endothelial cells.

ICAM-1: Intercellular adhesion molecule-1.

IL: Interleukin.

IRS: Insulin receptor substrate.

LDGs: Low density granulocytes.

LDL: Low-density lipoprotein.

MAb: Monoclonal antibody.

MCP-1: Monocyte chemoattractant protein-1.

MFI: Mean fluorescence intensity.

mIL-6R:Membrane IL-6 receptor.

MPO: Myeloperoxidase.

NE: Neutrophil elastase.

NETs: Neutrophil extracellular traps.

NOS: Nitric oxide synthase.

NOX: NADPH oxidase.

NSAIDS: Non-steroideal anti-inflammatory drugs.

OD: Optical density.

p-p38: Phospho mitogen-activated protein kinase 14.

PAD4: Peptide arginine deiminase, type IV.

PF: Peak flow.

pGSK-3β: Phospho glycogen synthase kinase 3 beta.

pHSP27: Phospho heat shock protein 27.

PKB/AKT: Phospho protein kinase B.

PMBCs: Peripheral mononuclear blood cells.

pmTOR: Phospho mechanistic target of rapamycin.

PORH: Post Occlusive Reactive Hyperaemia.

pPRAS40: Proline-rich Akt substrate.

PRKAA1/pAMPKα: Phospho protein kinase AMP-activated catalytic subunit alpha 1.

pSTAT3: Phospho signal transducer and activator of transcription 3.

RA: Rheumatoid arthritis.

RF: Normal perfusion.

RF: Rheumatoid factor.

sDMARDs: Synthetic DMARDs.

sIL-6R: Soluble IL-6 receptor.

TCZ: Tocilizumab.

TF: Tissue factor.

TH1: Time to half before hyperaemia.

TH2: Time to half after hyperaemia.

TLR: Toll like receptor.

TM: Time to max.

TNFα: Tumor necrosis factor α.

VAS: Visual analogue scale.

VCAM: Vascular cell adhesion molecule.

VEGF: Vascular endothelial growth factor.

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, causing the 39% to 50% of deaths.[1]Atherosclerosis at early stage of the disease is considered a potential preclinical manifestation. In fact, the risk of CVD events, such as myocardial infarction, is increased in the 2 years preceding the formal diagnosis of RA[2] and once the disease is diagnosed, the risk of having carotid plaques and CVD events increase with the progression of the disorder.[3] The mechanisms responsible for the development of premature atherosclerosis in RA are not well understood, but traditional risk factors alone are not fully accountable, and a role for inflammation has been suggested in this process. Thus, it is likely that inflammatory mediators might be causal in the accelerated atherosclerosis observed in this autoimmune disease, which is further secondary to endothelial dysfunction (ED) and CVD.[4]

Endothelial dysfunction (ED) is a vascular abnormality frequently presented in RA patients, contributing to plaque initiation and progression. It is associated with carotid intima media thickness in long-standing RA.[5,6] The phenotypic features of ED comprise upregulated expression of cellular adhesion molecules, compromised barrier function leading to increased leukocyte diapedesis, increased vascular smooth muscle tone - secondary to impaired processing of vasodilator substances such as nitric oxide and prostacyclin-, as well as increased production of vasoconstrictor substances including endothelin.[7]

A number of processes have been linked to the development of ED and atherosclerosis in RA. Among them, increased neutrophil extracellular traps (NETs) have been proposed as a potential mechanism in the occurrence of CVD events. NETosis is a way of cell death, different from necrosis and apoptosis, in which occurs the dissolution of internal membranes, followed by the de-condensation of the chromatin and the release of NETs -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 (NOX) and nitric oxide synthase (NOS)- to the extracellular space. 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[8-11].

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. Moreover, we demonstrated a close relationship between anti-CCP levels and inflammation, so that they act as direct inductors of the pro-oxidative status, the inflammatory, and the atherogenic profile of lymphocytes, monocytes, and neutrophils in patients with RA.[12]

Many different cell components can be considered as key elements in the inflammatory and pro-atherothrombotic status of RA patients. Among them, macrophages have been demonstrated to play an important role in the pathogenesis of atherosclerosis. Various parameters of circulating monocytes, including count, increased adhesive properties, lipid metabolism alterations, phagocytosis and low-density lipoprotein (LDL) cholesterol, are associated with CVD.[13,14]Moreover, monocytes/macrophages are central players in inflammation, and have been found to be activated in RA through the release of cytokines and massive infiltration in the inflammatory sites, such as synovial membranes.[15,16] Thus, the progressive generation of inflammatory monocytes is an intrinsic element in the immune response mediating RA[17], and this response provides lipid deposits with effectors cells that accelerate the development of advanced

atherosclerotic vascular disease. Therefore, treatments targeting monocytes–macrophages might contribute to effectively prevent cardiovascular events.

Anti-tumor necrosis factor alpha (anti-TNFα) therapy has significantly improved the outlook for patients suffering from RA, but a substantial proportion of patients fail to respond to these therapies, which implies that treatment response is likely to be multifactorial.[18] Thus, new therapies are being evaluated. Interleukin-6 (IL-6) is an upstream inflammatory cytokine that plays a central role in propagating the downstream inflammatory response inducing atherosclerosis, as it is implicated in ED and arterial stiffening contributing to accelerated atherosclerosis process in RA patients.[19] Moreover, it has a role in the differentiation of B lymphocyte into auto-antibody producing plasma cells, which participate in the pathogenesis of RA through the formation of immune complexes.[20] High levels of IL-6 may cause a Th1-Th17/T-reg cell imbalance during RA, which is corrected upon treatment with tocilizumab (TCZ),[21,22] a recombinant humanized antihuman IL-6 receptor monoclonal antibody (mAb) that acts by binding both soluble and membrane IL-6 receptor (sIL-6R and mIL-6R),blocking the pro-inflammatory effects of IL-6.[23]

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 monocyte activity, NETosis, and oxidative stress, principal effectors of endothelial dysfunction, atherosclerosis and CVD in this autoimmune condition, have not been analyzed. We undertook this study to evaluate the molecular and cellular mechanisms underlying the effects of TCZ on the pro-atherothrombotic profile associated with RA, focusing on the effects of this biological therapy on endothelial dysfunction, neutrophils and monocytes activity.

MATERIAL AND METHODS

This paper conforms to the relevant ethical guidelines for human research.

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.[24] The patients were taking the following treatments: corticosteroids (62.5%), leflunomide (30.0%), hydroxichloroquine (12.5%), NSAIDs (75.0%), methotrexate (63.5%) and vitamin D (18.0%). Patients having inadequate response or intolerance to conventional DMARDs were given subcutaneous Tocilizumab (162 mg per week) for 6 months. The treatment of those patients with synthetic DMARDs, NSAIDs or/and corticoids had been stable for at least two months before TCZ administration and was not modified during TCZ treatment.

All patients were tested for the presence of anti-CCPs and rheumatoid factor (RF). 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 $\geq$3.2.[25] All the patients filled the health assessment questionnaire (HAQ) and the visual analogue scale (VAS) in order to assess the pain. Changes from baseline in DAS28, VAS, HAQ, erythrocyte sedimentation rate (ESR) and C-reactive protein (CRP) levels, and tender and swollen joints were analyzed.

All participants enrolled were Caucasian, 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, analysing the response to reactive hyperaemia, so as the increase in blood flow occurred after temporary occlusion of blood flow, using a skin probe attached to the inner forearm. Post occlusive reactive hyperaemia (PORH) test consisted of 2 minutes of baseline followed by 4 min occlusion period. The cuff was then released and the PORH response was analysed during 3 minutes.

Several parameters were obtained: normal perfusion (RF), perfusion when occluded (BZ), occlusion area (AO), time to half before hyperaemia (TH1), highest perfusion

value after occlusion was released (PF), time to half after hyperaemia (TH2), and hyperaemic area (AH).

White blood cells isolation. Neutrophils were isolated from patients by density centrifugation over Dextran-Ficoll Hypaque, which allowed cells to keep a non-activated state. 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, MiltenyiBiotec, BergischGladbach, Germany). Purity of the fractions was evaluated via flow cytometry (FACScalibur cytometer), by analysing the size and complexity of each population (forward and size scatters). The purity of monocytes was further evaluated with fluorescein isothiocyanate (FITC)-conjugated anti-CD14 antibody by flow cytometry. By this method, 95.2 ± 4.3 viable monocytes were obtained.

In vitro studies. Monocytes and neutrophils purified from 5 RA patients at baseline (no taking TCZ) were cultured separately in RPMI 1640 containing 10% FBS, 2 mM l-glutamine, 100 U ml^{-1}penicillin, 100 mg ml^{-1} streptomycin and 250 $pgml^{-1}$fungizone (BioWhittaker/MA Bioproducts, Walkersville, Maryland, USA) at 37°C in a humidified 5% carbon dioxide (CO_2) atmosphere.

For mRNA and protein expression analyses, RA monocytes (1×10^6 cells ml^{-1}) were pre-treated with FCRII blocking (Miltenyi Biotec) for 15 min. Then, cells were washed with 1 ml of PBS and centrifuged at 300 g for 10 minutes. Thereafter, cells were seeded and incubated with IL-6 (10 $ngml^{-1}$) for 9 hours and then incubated in the presence or in the absence of TCZ (20 μg ml^{-1}) for 9 hours. RA neutrophils (1×10^6 cells ml^{-1}) were pre-treated with FCRII blocking as described above. Then, cells were seed and incubated with IL-6 (10 $ngml^{-1}$) for 3 hours, and thereafter incubated in the presence or in the absence of TCZ (20 μg ml^{-1}) for 3 hours, at 37°C in a humidified 5% (CO_2) atmosphere. 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), 100 U ml^{-1} penicillin, 100 mg ml^{-1} streptomycin and 250 $pgml^{-1}$fungizone (BioWhittaker/MA Bioproducts, Walkersville, Maryland, USA) at 37°C in a humidified 5% CO_2 atmosphere.

For in vitro studies, HUVECs were seeded into 6-well plates ($4x10^5$ cells per well) in 1.5 ml of complete medium. 24 hours after, cells were pre-treated for 15 min with FcR blocking Reagent (Miltenyi Biotec), and subsequently incubated with IL-6 (10 $ngml^{-1}$) for 9 hours and thereafter incubated in the presence or in the absence of TCZ (20 μg ml^{-1}) for 9 hours. The cultured cells were harvested for total RNA isolation and applied to RT-PCR studies.

Co-cultures of RA monocytes-HUVEC: HUVECs were cultured as described above. Monocytes isolated from 5 RA patients at baseline (no taking TCZ) were pre-treated with FcRII blocking reagent for 15 min. Then, monocytes were washed with 1-2 ml of PBS, centrifuged at 300 g for 10 minutes and seeded into trans well inserts (Corning® Transwell® polycarbonate membrane cell culture inserts, Sigma Aldrich, Misuri, USA) ($1×10^6$ cells per transwell) 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 $ngml^{-1}$) alone or combined with TCZ (20 μg ml^{-1}) as described previously. The co-cultured cells (RA monocytes and HUVEC) were harvested separately for total RNA isolation and applied to subsequent RT-PCR.

RNA isolation and quantitative real-time reverse transcriptase PCR. Total RNA from monocytes, neutrophils and endothelial cells was extracted using TRI Reagent (Sigma, St Louis, Missouri, USA) following the manufacturer's recommendations. The integrity of RNA was verified by optical density (OD) absorption ratio OD260/OD280 between 1.8 and 2.0.

For first strand cDNA synthesis, 1μg of total RNA was reverse transcribed using random hexamers as primers and Transcriptor Reverse Transcriptase (Quiagen, Madrid, Spain). Gene expression was assessed by real time PCR using a LightCycler

Thermal Cycler System (Roche Diagnostics, Indianapolis, Indiana, USA). The reaction was performed, following the manufacturer's protocol, in a final volume of 12µl.

The reactions consisted of an initial denaturing of 10 min at 95 ºC, then 40 cycles of 15 seconds denaturing phase at 95ºC, and 1 min annealing and extension phase at 60ºC. A threshold cycle (Ct value) was obtained for each amplification curve and a ΔCt value was first calculated by subtracting the Ct value for human glyceraldehyde-3-phosphate dehydrogenase cDNA from the Ct value for each sample and transcript. Fold changes compared with the endogenous control were then determined by calculating 2-ΔCt. Samples were analysed in triplicate and negative controls were included in all the reactions. Test reproducibility for all investigated transcripts was less than 0.5% in inter-test experiments and even lower in intra-test experiments.

Genes related to inflammation [tissue factor (TF), monocyte chemoattractant protein-1 (MCP-1), IL-8, TNFα, IL-1β and IL-6], lipid metabolism and storage [toll like receptor (TLR) 2, TLR4, diacylglycerolacyltransferase (DGAT), adipophilin or ADRP (PLIN2), insulin signalling (IRS1 and IRS2)] and cell adhesion (ICAM, VCAM and VEGF) were evaluated.

Determination of oxidative stress biomarkers in white blood cells. Oxidative stress biomarkers were analysed in monocytes and neutrophils using a dual-laser FACSCalibur (Becton Dickinson). Test standardization and data acquisition analysis were performed using the CELL Quest software (Becton Dickinson). For the assessment of ROS generation, including superoxide anion and hydrogen peroxide, cells were incubated with 20.5 µM DCFH-DA (Sigma-Aldrich) at 37°C for 30 min in the dark and 5 μM DihidroRhodamine123 at 37ºC for 30 min (Sigma-Aldrich). The cells were washed, re-suspended in PBS, and then analysed on a dual-laser FACSCalibur. As internal control, the fluorescence of unstained cells was used. To test the positivity of the RA population, monocytes and neutrophils isolated from healthy donors were used.

NETs induction and quantification. Isolated neutrophils from RA patients at baseline and after 6 months of TCZ treatment were seeded in 24 well plates on poly-L-lysine-coated glass coverslips (BD Biosciences, San Jose, CA, USA) in tissue-culture wells and allowed to settle for 1 hour at 37°C under 5% CO2. Then, 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. Neutrophils purified from 5 RA patients with moderate-high DAS28, and not taking any biologic treatment, were treated *in vitro* with FCRII blocking (MiltenyiBiotec) for 15 min. Then, cells were washed with 1 ml of PBS and centrifuged at 300 g for 10 minutes. Thereafter, cells were seeded in the same way as described above and pre-treated with or without TCZ (20 $\mu g\ ml^{-1}$) for 30 minutes and then incubated with IL-6 (100 $ngml^{-1}$) during 15 hours or PMA (600 nM) for 2 hours. After the different treatments, cells were fixed with 4% paraformaldehyde and rinsed three times with PBS. DNA was stained with 5 mM Sytox orange dye (Life technologies, Netherlands) and NETs were visualized using a Nikon Eclipse-Ti-S fluorescence microscope, (NIS-Elements imaging software). Five images selected randomly from different regions of each coverslip per case were taken 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[26,27]and were counted for at least 3 fields using IMAGE-J software (NIH, Bethesda, MD). Results were expressed as percentage of NETs (NETs formation).

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. Monoclonal antibodies against DNA (double and single strand) and histones (H1, H2A, H2B, H3 and H4) were used to detect mono- and oligo nucleosomes in serum from RA patients. Quantification of nucleosomes was performed by photometrical determination of the absorbance at 405 nm, using as reference wavelength 492 nm.

Identification of Low density granulocytes (LDGs). Peripheral mononuclear blood cells (PMBCs) ($5x10^5$) were incubated with PE anti-human CD14 and FITC anti-human CD15 (Biolegend) for 30 minutes at 4ºC in the dark. PE and FITC IgG isotypes from the same company were used as negative controls. Cells were washed, resuspended on 500 μl of PBS and acquired on the flow cytometer FACSCalibur.

Neutrophil elastase (NE) and myeloperoxidase (MPO) protein expression. Whole peripheral blood (100 μl) was incubated with 1 ml of lysis buffer (BD Pharm Lyse Lysing Buffer) for 10 minutes at room temperature in the dark. After centrifugation at 1800 rpm for 5 minutes at 4 ºC, cells were fixed and permeabilized with 250 μl of buffer (BDCytofix/Cytoperm™Fixation/Permeabilization solution Kit with BD GolgiPlug™) for 20 minutes at 4ºC. Then, cells were incubated either with FITC human anti-MPO (BD Biosciences) or with human anti-elastase primary antibody (RbmAb to Neutrophil Elastase (Abcam, Cambridge, UK). Then, for neutrophil elastase analysis, Alexa Fluor conjugated secondary antibody (Abcam) was added for 30 minutes at 4ºC. IgG isotypes were used as negative controls. Cells were washed and 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.). The phosphorylation levels of ERK1/2, STAT1, STAT3, AKT, AMPKa, S6 Ribosomal protein, mTOR, HSP27, Bad, P70 S6 Kinase, PRAS40, p53, p38, SAPK/JNK, PARP, Caspase-3 and GSK-3b were analyzed on monocytes isolated from RA patients at baseline and after 6 months of treatment with TCZ.

Plasma levels of cellular adhesion molecules. E-selectin and vascular cell adhesion molecule 1 (VCAM-1) levels were analyzed in plasma from RA patients before and after TCZ therapy using ProcartaPlex multiplex immunoassay, following the manufacturer's recommendations (AffymetrixeBioscience, Vienna, Austria).

Statistical analysis. All data are expressed as mean ± SD. 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 $p<0.05$

RESULTS

TCZ improved the disease activity, clinical parameters and endothelial dysfunction in RA patients. Treatment of RA patients with TCZ (162mg/ml per week) for 6 months significantly reduced the number of both, 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 from high disease activity to low-moderate disease activity: mean DAS28 changes (± SDs) were 1.35 ± 0.78 in TCZ-treated patients (**Table I**). Autoimmunity was further modulated by treatment *in vivo* with TCZ. Thus, a significant reduction of rheumatoid factor (RF) levels was observed. However, no effect was detected on the levels of anti-CCP antibodies. Regarding inflammatory clinical parameters, RA patients displayed significantly reduced levels of erythrocyte sendimentation 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 (measured by Laser Doppler measurement of post ischemic reactive hyperemia) improved notably, as shown by the augmentation of the highest perfusion value after occlusion was released (peak flow, PF) and by the increase of hyperemic area (HA) (**Fig 1A**). In parallel, changes in plasma levels of parameters closely related to endothelial dysfunction (cellular adhesion molecules such as E-selectin and vascular cell adhesion molecule 1 (VCAM-1)) were found significantly

reduced by treatment with TCZ, thus supporting the improvement of the vascular function in these patients (**Fig 1B**).

TCZ reduced oxidative stress in leukocytes from RA patients. We have recently reported an increased oxidative status in leukocytes from RA patients, directly related to both the titers of anti-CCPs, and the expression of a number of inflammatory parameters, thus contributing to atherosclerosis.[12] Accordingly, peroxides and peroxynitrites levels were downregulated *in vivo* in monocytes and neutrophils from RA patients after 6 months of treatment with TCZ, showing a significant reduction in the mean fluorescence intensity (MFI) of DhRh and DCFHDA in flow cytometry analyses (**Fig 2A and B**).

TCZ therapy in RA patients downregulated the percentage of low density granulocytes and decreased NETosis. A new subset of granulocytes (LDGs) has been identified in the peripheral blood mononuclear cells (PBMCs) fraction of patients with various autoimmune diseases. These cells show a phenotypic profile based on $CD14^{-/dim}$ and $CD15^{bright}$ expression, and are more prone to experience NETosis.[28] In the present study, a significant reduction in the percentage of LDGs in RA patients treated with TCZ was noticed (**Fig3A**).

Previous studies have shown that RA patients exhibit increased spontaneous NETs generation, associated with enhanced NE and MPO expression. In our hands, NE and MPO (main enzymes leading the initiation of NETosis) were found reduced in neutrophils from RA patients treated with TCZ (**Fig3B**).

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 the effect of TCZ in RA neutrophils treated with PMA (**Fig3D**).

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 (**Fig3C**).

***In vivo* treatment with TCZ reduced the procoagulant and inflammatory state, and modulated insulin signalling in monocytes.** Gene expression of molecules involved in inflammation (MCP-1, TLR4 and TLR2), procoagulant activity (TF) and migration (IL-8) was reduced in RA monocytes treated with TCZ (**Fig4A**). In contrast, *in vivo* inhibition of IL-6R signalling by TCZ increased the mRNA expression of IL-6 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 state. Molecules involved in lipid droplets formation (DGAT and PLIN2) were also found significant reduced at mRNA level in monocytes from RA patients after 6 months of TCZ treatment (**Fig4A**).

TCZ modified the activation of several intracellular kinases on monocytes from RA patients. Dysfunctional intracellular signaling pathways plays a critical role in RA, which might account for the immune-mediated chronic inflammation present in those autoimmune patients.[29] 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 IL-6R signalling by TCZ markedly reduced the phosphorylation of STAT3, a kinase directly activated by the binding of IL-6 to its receptor (**Fig4B 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, p38, HSP27, PRAS40 and GSK3-b (**Fig4B and C**).

***In vitro* treatment of purified RA neutrophils and monocytes with TCZ reduced NETosis generation and improved the inflammatory and thrombotic profile of monocytes.** To evaluate the specificity of the effect of TCZ, we conducted *in vitro* studies on monocytes and neutrophils.

In neutrophils isolated from RA patients, the pre-treatment with IL-6 for 6 hours promoted a significant increase in NE and MPO intracellular levels that was prevented by addition of TCZ (**Fig5A and B**).

We further analysed whether TCZ could diminish NETosis induced *in vitro*. The treatment of RA-neutrophils with IL-6 or PMA induced an increase of NETosis formation. The combination of TCZ plus IL-6 or PMA generated significantly less extrusion of DNA fibres in neutrophils comparing to the treatment with IL-6 and PMA alone, suggesting that TCZ might prevent NETosis in RA patients (**Fig5C**).

In vitro treatment of monocytes isolated from RA patients with TCZ reduced their inflammatory and prothrombotic profile demonstrated by the decrease in the high expression of TLR-2, TNF-α, IL-1β, IL-8, MCP-1, VEGF, VCAM and ICAM observed in non-treated monocytes or induced by IL-6 (**Fig 6A**).

TCZ significantly reduced the IL6-induced expression of both adhesion molecules and inflammatory mediators in endothelial cells alone or co-cultured with monocytes purified from RA patients. In order to mimic the *in vivo* effects of TCZ on the vessel wall, cultured HUVECs were treated with IL-6, either alone or in combination with TCZ. A significant increase in the mRNA expression of TLR-2, IL-8, MCP-1, VEGF and VCAM was noticed in HUVECs after treatment with IL-6 for 18 hours. Those high levels were downregulated by addition of TCZ, suggesting that IL-6R blockade might have a beneficial effect on the endothelial function (**Fig6B**).

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 HUVECs increased the mRNA expression of TLR-2, TNF-α, IL-1β, IL-8, TF, MCP-1, ICAM, VCAM and VEGF on endothelial cells. Moreover, these genes were significantly upregulated after the addition of IL-6 to the co-culture medium (HUVECs with RA monocytes). Once more, TCZ significantly reduced the IL6-induced expression of both adhesion molecules and inflammatory and prothrombotic mediators on endothelial cells co-cultured with RA purified monocytes (**Fig6C**), thus underlying the relevant role of this IL-6R-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 the pro-atherotrombotic profile 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 showed that combination therapy with biologic DMARDs (bDMARDs) and synthetic DMARDs (sDMARDs) represents the best therapeutic option for the treatment of RA, since it can slow the progression of the disabling structural damage.[30] In our cohort of patients, the combination of a bDMARD, TCZ, with sDMARDs (methotrexate, leflunomide and hydroxychloroquine) 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, 6 months of treatment with 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.[30] Thus, longer treatment with TCZ might be necessary to reduce the levels of anti-CCPs.

Recently, several studies evaluated the changes 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. Thus, comparing with naïve-biologic therapy and others bDMARDs (including adalimumab, rituximab and tofacitinib), TCZ greatly increased the levels of HDLc, noticed after the first 6 weeks of treatment, which was translated into high levels of total cholesterol.[31-34]Some of these studies further reported an increase in LDLc and triglycerides. In our cohort of patients, there was a significant increase in fasting HDLc levels after 6 months of TCZ

treatment; however this was not related to a significant augmentation of the total cholesterol levels, which might be due to the unchanged levels of triglycerides and LDLc. Accordingly, ApoA1 levels were significantly augmented by treatment with TCZ.

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.[35] In addition, comparison 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.[36] In accordance, our study shows a significant improvement of 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 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 showed a decrease in serum VEGF levels after 6 months of TCZ monotherapy.[37] Our results were further supported by in vitro studies, on which treatment of ECs with TCZ prevented the induced expression of TLR2, IL-8, MCP-1, VEGF, ICAM and VCAM by IL-6. Moreover, we could demonstrate that addition of TCZ to the co-culture of RA monocytes with ECs plus IL-6 reduced the inflammatory profile (represented by a reduction of TNFα, IL1β, IL-8, MCP-1 and TLR2 expression), and improved the endothelial dysfunction, (through a downregulation of VEGF, ICAM and VCAM). In the same way, Suzuki et al., described the important role of IL-6 increasing the rate of cell migration, and how TCZ was able to reduce the adhesion of monocytic cells (U937) to

HUVEC.[38] 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.[12] The present study shows for the first time the effects of the inhibition of IL-6R 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.[39,40] 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-TNFa therapy.[41] 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.

Monocytes from RA patients display a pro-atherothrombotic profile, showing elevated expression of proinflammatory cytokines and procoagulant factors.[12] The effect of TCZ on the atherothrombotic markers associated with RA at plasma level has already been described. Thus, TCZ reduced prothrombotic molecules including D-dimer, prothrombotic fragment 1+1, fibrinogen, lipoprotein A and phospholipase A-2-IIA in plasma from RA patients[32,42,43]. However, there is little evidence about the effect of TCZ on RA monocytes, only few studies described that TCZ induced apoptosis on monocytes from RA patients.[44] We here analysed in depth the molecular changes occurred on monocytes from RA patients treated with TCZ. A marked reduction of the inflammatory prolife, prothrombotic properties and migratory capacity was observed at mRNA levels on RA monocytes, showed by the downregulation of TLR2, TNFα, IL-1β, IL-8, MCP-1, VEGF, VCAM and ICAM.

By contrast, a significant increase of IL-6 mRNA levels was noticed on monocytes from RA patients taking TCZ for 6 months. 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.[45]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 IL-6 mRNA in our study might derive from that feedback process.

In addition, we here provide new evidences about the effect of TCZ on the formation of lipid droplets on RA monocytes. The accumulation of lipid droplets within leukocytes on inflammatory conditions has been documented.[46] In this context, lipid droplets compartmentalize several proteins and lipids involved in the control of biosynthesis and secretion of inflammatory molecules, including leukotriene and PGE2.[47] PLIN2 and DGAT2 are two genes involved in lipid droplets regulation. PLIN2 overexpression results in increased formation of lipid droplets.[47] In turn, growth of lipid droplets is linked to functions performed by endoplasmic reticulum mediated by endoplasmic reticulum proteins such as DGAT. We found a significant reduction in the expression of these genes on RA monocytes treated in vivo 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.[48] Yet, this is the first article describing a downregulation of the main signaling pathways associated with RA pathogenesis in monocytes by TCZ: JAK/ STAT, SAPK/MAPK and PI-3K/AKT/mTOR. 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, constitutes a valuable tool to better

delineate the regulatory mechanisms modulated at cellular level by effects of specific drugs.

Neutrophils have recently been recognized as essential actors in the development of the atherosclerotic plaque. Neutrophils from RA patients are more susceptible to experience spontaneous NETosis.[49] The effects of IL-6 on neutrophils functions remains poorly understood, with conflicting evidence reporting that IL6 can either delay, accelerate, or have no effect on neutrophil apoptosis.[50-52] In our hands, the relevance of neutrophils function in CVD and their regulation by TCZ has been exposed by a decrease in the NETosis generation induced either by IL-6 or PMA, determinant in atherosclerosis development. This data suggests that TCZ improves the overall state of neutrophils so that they can be less prone to experience in vitro induced NETosis.

LDGs in RA are functionally different from RA neutrophils, having enhanced netting capabilities[28] and survival properties and decreased TNF signaling which might contribute to the disease pathology and response to therapy.[53] Moreover, LDGs display an activated phenotype, inducing endothelial cell cytotoxicity and thus playing a relevant role in cardiovascular development.[54] Our results show the beneficial effect of TCZ decreasing the percentage of LDGs in RA patients which might reduce the vascular damage induced by these subtypes of neutrophils.

Although further studies are required, our results indicate that neutrophils might have an important role in the development of inflammation in the context of RA, and that IL-6R signaling blockade could be a useful therapy to avoid undesired effects of persistent neutrophil activation.

This is the first study that evaluates the direct effect of TCZ on monocytes and neutrophils from rheumatoid arthritis patients. By performing in vitro studies, adding TCZ to monocytes and neutrophils isolated from RA patients that were not taking TCZ, the specificity of the effects of this biologic therapy on each cell type and each parameter analyzed was confirmed, something that should be considered a relevant

point since patients were taking other therapies that could also influence the effects attributed to TCZ.

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 and intracellular signalling.

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FIGURES AND FIGURE LEGENDS

Fig 1. (A) Microvascular function was measured by Laser Doppler linear Periflux 5010 and was performed at baseline and after 6 months of 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 ProcartaPlex multiplex immunoassay. Data are presented as mean ± SD (std deviation), n= 20 patients. (*) indicates significant differences vs before TCZ (p<0.05).

Figure 2. (A) Peroxides production in monocytes, and neutrophils of RA patients at baseline and after 6 months of TCZ treatment were determined by addition of the fluorescent probe DCFHDA to the isolated cells and flow cytometry analysis. **(B)** Peroxides and peroxynitrites production in monocytes, and neutrophils of RA patients before and after TCZ treatment were determined by the fluorescent probe dihydrorhodamine-123 and flow cytometry analysis. Upper panels show representative histograms with the mean fluorescence intensity of DCFHDA and DHRH in a healthy donor (full histogram), a RA patient before TCZ treatment (black unfilled histogram) and the same patient after TCZ therapy (grey unfilled histogram). Bar graphs show the mean ± SD of mean fluorescence intensity (MFI),n=20 patients. (*) indicates significant differences vs before TCZ (p<0.05).

Fig 3.(A) Representative dot plots of low density granulocytes from RA patients before and after TCZ treatment.**(B)** Intracellular MPO and NE protein expression were measured in neutrophils from RA patients at baseline and after 6 months of TCZ treatment by flow cytometry. Bar graphs show the mean ± SD of median fluorescence intensity (MFI).**(C)** Concentration of cell-free nucleosomes in serum by ELISA.**(D)** 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. Five images selected randomly from different regions of each coverslip per case were taken 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 without

PMA.NETs formation represents the average percentage of NETs structures from the 5 images taken in each condition. Bar graphs show the mean ± SD, n= 20 patients(*) indicates significant differences vs before TCZ (p<0.05).

Fig 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 (DGAT-1 and PLIN2), insulin signal (IRS-1 and IRS-2) in RA monocytes at baseline and after 6 months of 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 ± SD, n= 20 patients.(*) indicates significant differences vs before TCZ (p<0.05).

Fig5.(A and B) Intracellular neutrophil elastase (NE) and myeloperoxidase (MPO) protein expression was measured in neutrophils isolated from 5 RA patients at baseline (no taking TCZ), non-treated and treated *in vitro* with IL-6 (10 ng/ml), TCZ (20μg/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)** Representative images of neutrophil extracellular traps (NETs) of neutrophils isolated from 5 RA patients at baseline (no taking TCZ), non-treated and treated *in vitro* with IL-6 (100 ng/ml), TCZ or IL-6 plus TCZ (20μg/ml) for 15 hours and also treated with PMA (600 nM) alone or combined with TCZ (20μg/ml) 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. Five images selected randomly from different regions of each coverslip per case were taken 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 untreated cells. NETs formation represents the average percentage of NETs structures from the 5 images taken in each condition. Bar graphs show the mean ± SD of percentage of NETs of 5 independent experiments, n=5 different patients.(a) indicates significant differences vs non treated; (b) vs treated with IL-6; (c) vs treated with PMA (p<0.05).

Fig 6.(A) Quantitative RT-PCR was performed on a panel of genes on monocytes purified from 5 RA patients at baseline (no taking TCZ), non-treated and treated *in vitro* with IL-6 (10 ng/ml), TCZ or IL-6 plus TCZ (20μg/ml) for 18 hours. Bar graphs show the mean ± SD of five independent experiments, n=5 different patients. (a) indicates significant differences vs non-treated monocytes; (b) vs monocytes treated with IL-6; (p<0.05). **(B)** 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 (20μg/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).**(C)** 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 IL-6 (10ng/ml) alone or in combination with TCZ (20μg/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 (p<0.05).

TLR indicates toll like receptor; TNFα, tumor necrosis factor α; IL, interleukin; TF, tissue factor; MCP-1, monocyte chemotactic protein; VEGF, vascular endothelial

growth factor; ICAM, intercellular adhesion molecule-1; VCAM, vascular cell adhesion molecule.

Table(s)

	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 (%)	6.10 %		
Diabetes (%)	1 %		
Hypertension (%)	2 %		
Menopause (%)	50 %		
Smoker (%)	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^{-1}	180.00 ± 9.33	201.20 ± 7.39	p=0.070
HDL-Cholesterol, mg dl^{-1}	**45.60 ± 2.51**	**56.10 ± 2.42**	**p=0.001**
LDL-Cholesterol, mg dl^{-1}	115.30 ± 8.83	124.30 ± 9.30	p=0.319
Triglycerides, mg dl^{-1}	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, mm h^{-1}	**25.40 ± 6.09**	**4.22 ± 0.70**	**p=0.014**
CRP, mg dl^{-1}	**13.29 ± 6.08**	**0.46 ± 0.18**	**p=0.045**
Treatments			
Corticosteroids	62.5 %	62.5 %	
Hydroxychloroquine	12.5 %	12.5 %	
NSAIDS	75.0 %	75.0 %	
Methotrexate	63.5 %	63.5 %	
Leflunomide	30.0 %	30.0 %	
Vitamin D	18.0 %	18.0 %	

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

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

Figure 1

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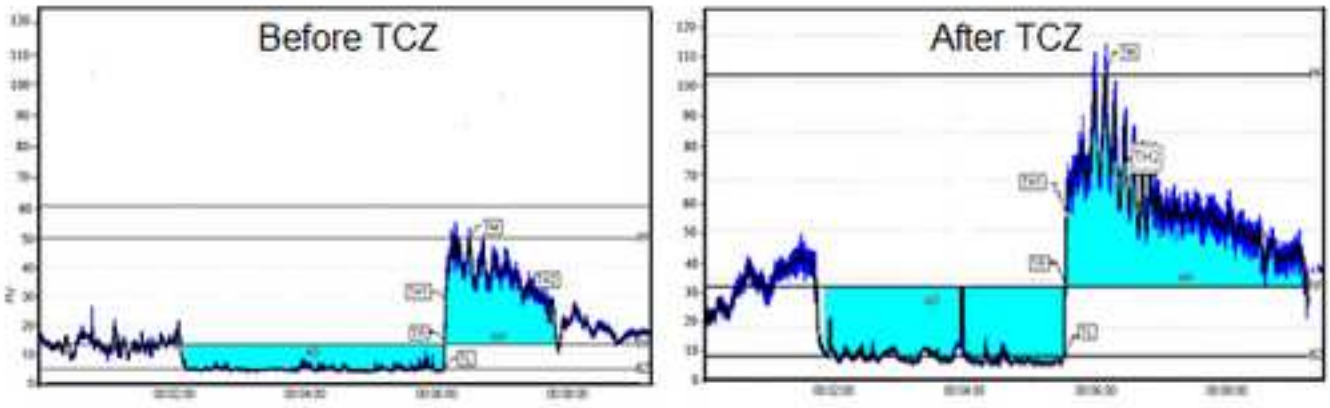
A

	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



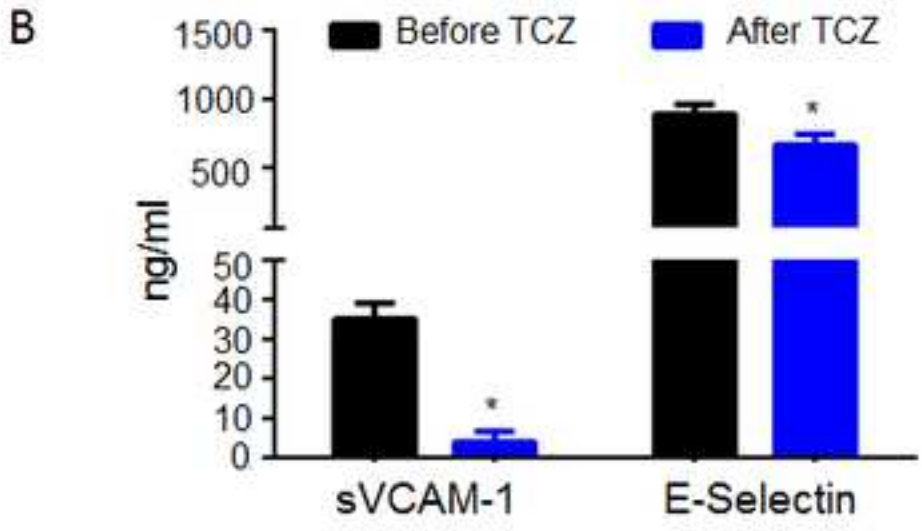


Figure 2

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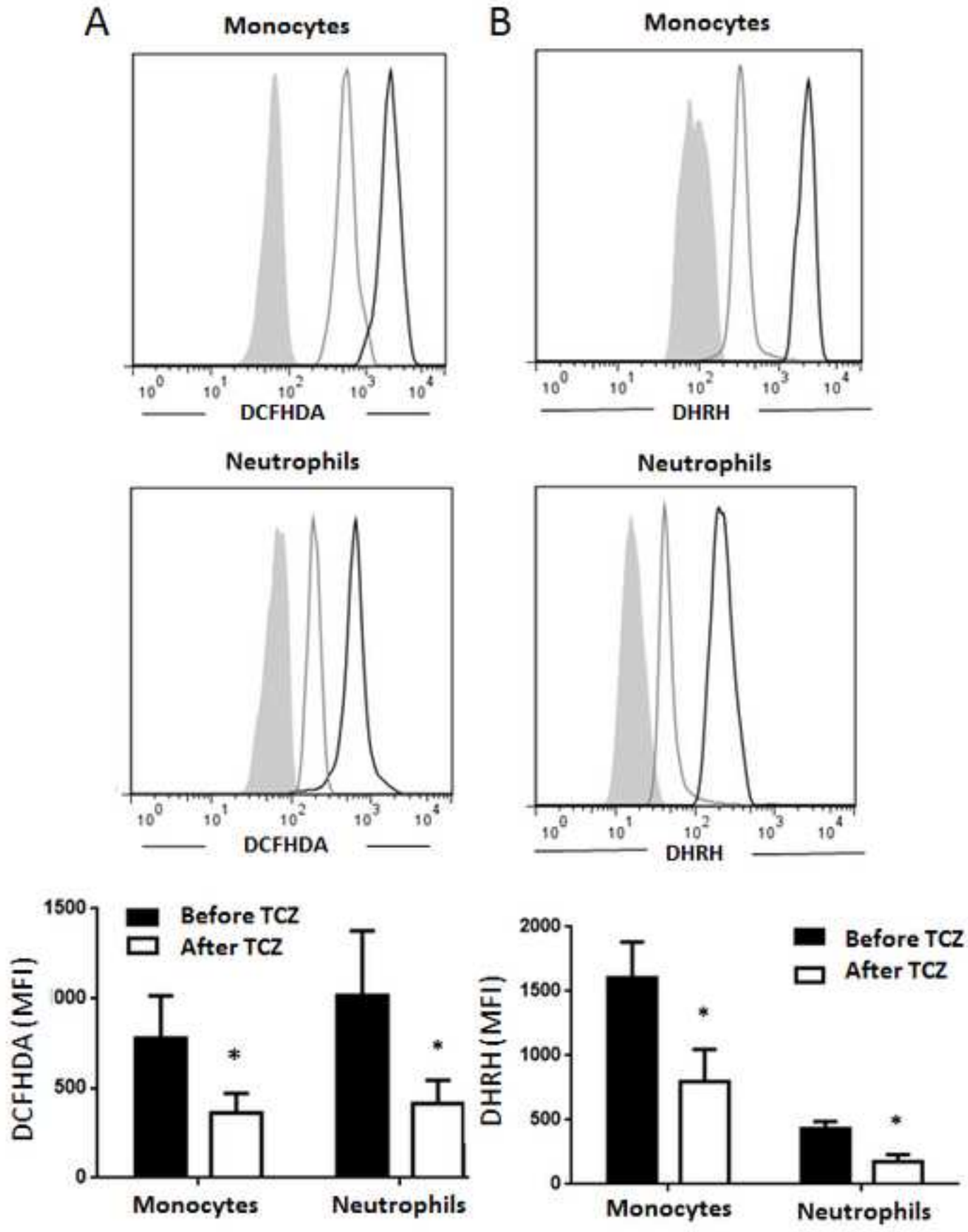

Figure 3R2

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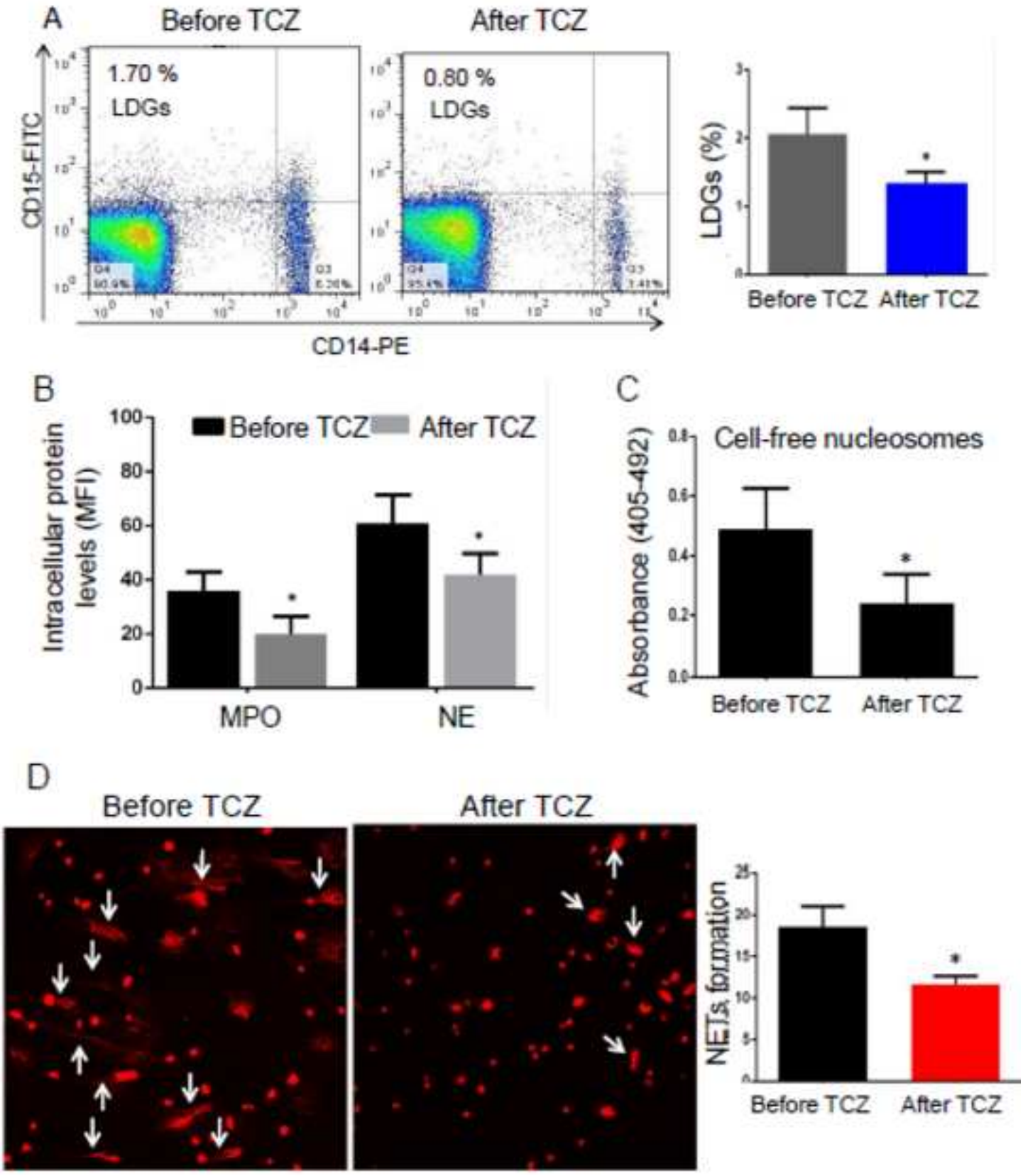

Figure 4

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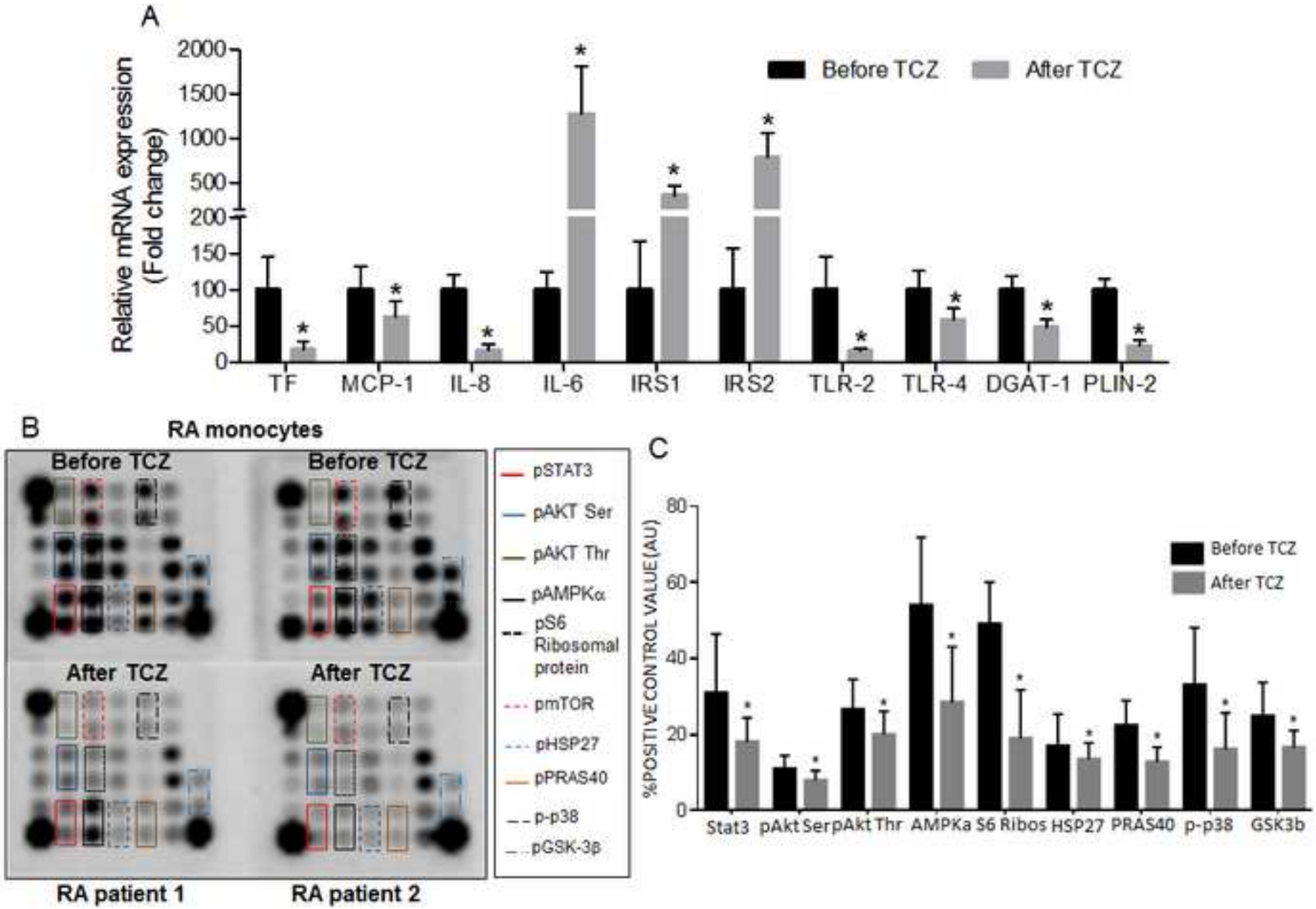

Figure 5R2

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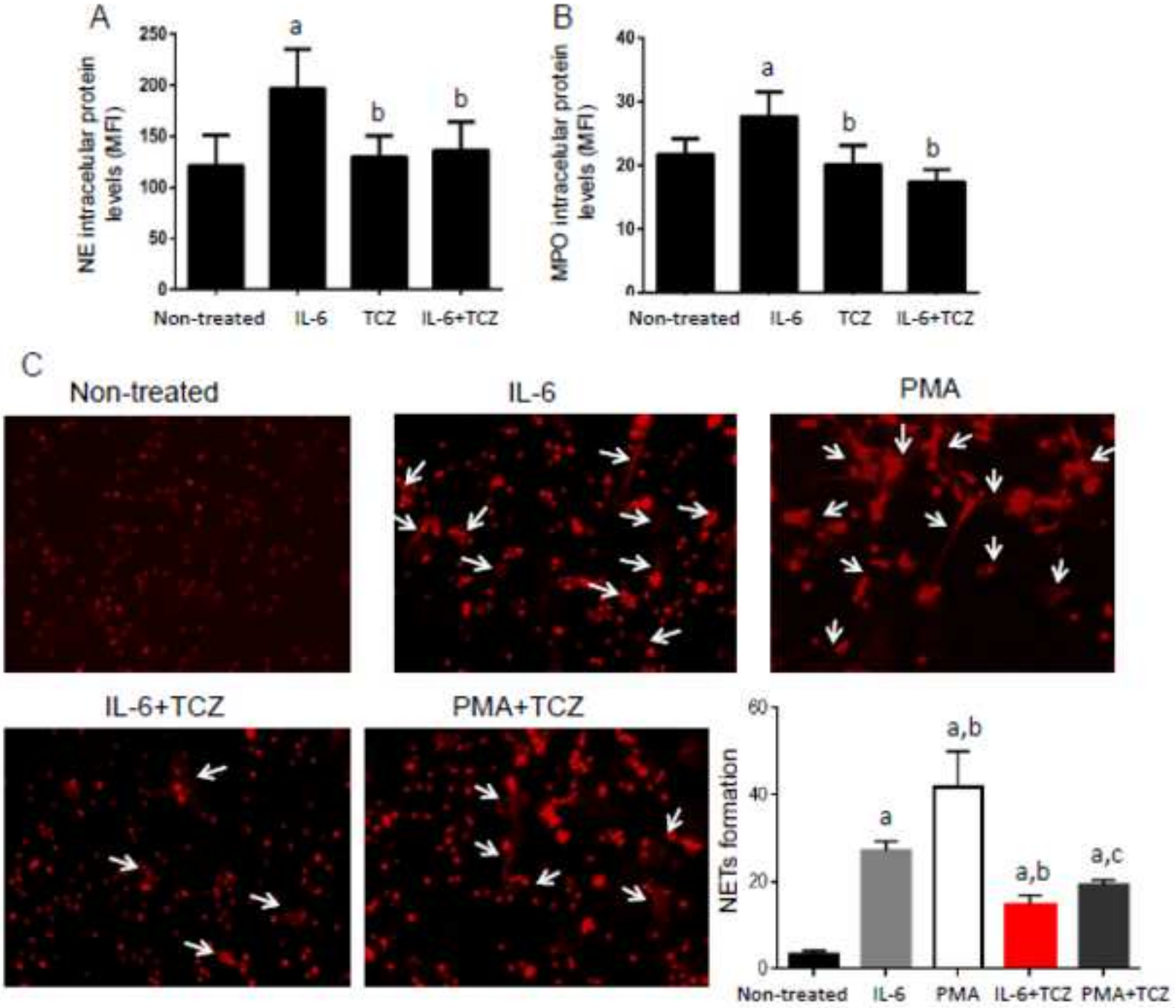

Figure 6

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