Certolizumab pegol attenuates the pro-inflammatory state in endothelial cells in a manner that is atheroprotective

S.K. Heathfield¹, B. Parker¹,³, L.A.H. Zeef², I.N. Bruce³, M.Y. Alexander¹

¹Cardiovascular Research Group; ²Wellcome Trust Centre for Cell-Matrix Research; ³Arthritis Research UK Epidemiology Unit, University of Manchester, United Kingdom.

Abstract

Objective

Rheumatoid arthritis (RA) is associated with accelerated atherosclerosis and premature cardiovascular death. Anti-TNF therapy is thought to reduce clinical cardiovascular disease risk and improve vascular function in RA patients. However, the specific effects of TNF inhibitors on endothelial cell function are largely unknown. Our aim was to explore the effects of certolizumab pegol (CZP) on TNF-activated human aortic endothelial cells (HAoECs).

Methods

HAoECs were cultured in vitro and exposed to i) TNF alone, ii) TNF plus CZP, or iii) neither agent. Microarray analysis and quantitative polymerase chain reaction were used to analyse gene expression. Activation of NF-κB was investigated using immunocytochemistry, high content analysis and western blotting. Flow cytometry was performed to detect microparticle release from HAoECs.

Results

TNF alone had strong effects on endothelial gene expression, while TNF and CZP together produced a global gene expression pattern similar to untreated controls. In particular, genes for E-selectin, VCAM-1 and ICAM-1 were significantly up-regulated by TNF treatment. Notably, the TNF/CZP cocktail prevented the up-regulation of these genes. TNF-induced nuclear translocation of NF-κB was abolished by treatment with CZP. In addition the increased production of endothelial microparticles in TNF-activated HAoECs was prevented by treatment with CZP.

Conclusions

We have found at cellular level, that a clinically available TNF inhibitor, CZP i) reduces adhesion molecule expression; ii) prevents TNF-induced activation of the NF-κB pathway and iii) prevents the production of microparticles by activated endothelial cells. This could be central to the prevention of inflammatory environments underlying these conditions.

Key words

certolizumab pegol, endothelial cells, tumour necrosis factor, rheumatoid arthritis, microparticles.
**CZP prevents endothelial microparticle release / S.K. Heathfield et al.**

Sarah K. Heathfield, PhD  
Ben Parker, MD  
Leo A.H. Zeef, PhD  
Ian N. Bruce, MD  
M. Yvonne Alexander, PhD  

Please address correspondence to:  
Dr M. Yvonne Alexander,  
The University of Manchester,  
Manchester Academic Health Science Centre, (CMFT),  
School of Biomedicine Cardiovascular Research Group,  
3rd Floor Core Technology Facility,  
46 Grafton Street,  
Manchester M13 9NT, United Kingdom.  
E-mail: yvonne.alexander@manchester.ac.uk  

Received on January 30, 2012; accepted in revised form on July 12, 2012.  
© Copyright CLINICAL AND EXPERIMENTAL RHEUMATOLOGY 2013.

**Introduction**  
Vascular inflammation plays a crucial role in the development of atherosclerosis. Endothelial activation and dysfunction in response to injury are hallmarks of an inflammatory response resulting in lesion formation (1). Rheumatoid arthritis (RA) is a chronic inflammatory arthritis, characterised by chronic erosive synovitis, which can also have systemic features (2). RA is associated with an increased risk of premature atherosclerosis, and cardiovascular disease (CVD) is a leading cause of death in patients with RA (3). A recent meta-analysis of studies of mortality in RA concluded that at least half of the observed excess deaths in RA were due to manifestations of CVD (4). Recent EULAR guidelines suggest that patients should have an annual CV risk assessment and existing risk model scores should be multiplied by 1.5. The guidelines also emphasise the role of aggressive management of the inflammatory component of the disease in reducing long-term CV risk (5).

The cytokine, tumour necrosis factor alpha (TNF), plays a pivotal role in the chronic inflammation and pathogenesis of RA (6) and may contribute to the elevated CV risk by promoting endothelial activation and production of adhesion molecules responsible for leukocyte recruitment (7). Biologic agents, including TNF inhibitors, have been shown to improve endothelial function and decrease cardiovascular events in those patients who respond clinically (8-12), further highlighting the pro-atherogenic role of TNF-alpha in RA.

TNF-binding compounds, including certolizumab pegol, (CZP), are now widely used in clinical practice for patients with active RA who are not responding to conventional disease modifying treatment. CZP has similar efficacy to other TNF antagonist counterparts in terms of neutralisation of soluble and membrane-bound TNF (13). The active portion of CZP is a humanised anti-TNF Fab’ fragment. In contrast to other TNF antagonists, the Fc portion is absent in CZP (13). However, to counteract the consequent loss of mass and to improve solubility and availability, a polyethylene glycol moiety is attached. The structural differences to other anti-TNF treatments remove the ability to activate complement pathways or antibody-dependent cytotoxicity. This reduces immunogenicity (14) and makes CZP an attractive biologic alternative. Clinical studies of CZP treatment in the presence and/or absence of DMARDs have demonstrated significant improvements in disease activity indices and a reduction in radiographic progression of RA (15-18). Although several cellular mechanisms for the beneficial effects of these biologic agents have been described (19), no data are currently available for the specific effects of CZP on vascular function.

Recent studies demonstrate the prognostic value of endothelial microparticles (EMPs) shed from endothelial cells, after activation in patients with cardiovascular disease (20). Concentrations of EMPs (Annexin V pos; CD42bneg/CD31pos) have been shown to be significantly higher in patients with CVD compared to healthy volunteers, and correlate with in vivo measures of endothelial function (20, 21). Therefore, given the anti-inflammatory effects of CZP and the potential of CZP to have significant clinical importance with regard to cardiovascular outcome, our objective was to investigate the role and effects of CZP in an in vitro model of endothelial cells relevant to RA, and to establish whether this anti-inflammatory agent may have beneficial effects on endothelial cell function.

**Materials and methods**

**Cell culture**  
Human Aortic Endothelial Cells (HAoECs) (Promocell) were cultured in Endothelial Basal Growth Medium MV2 (ECBM MV2) supplemented with 5% fetal calf serum (FCS) and penicillin/streptomycin (110 Units/ml and 100 µg/ml respectively) at 37°C in humidified atmosphere with 5% CO₂. At subconfluence HAoECs were trypsinised using DetachKit (Promocell) and re-plated at 10,000 cells/cm². 18 hours later, cells were washed with HBSS (Sigma) and ECBM MV2 containing 1% FCS was added for 3 hours, followed by three treatment conditions, i) untreated con...
trol, ii) 10ng/ml TNF (Promocell), or iii) 10ng/ml TNF in combination with 5μg/ml CZP (UCB). Experiments were conducted with cells at passage 4 to 8.

**Microarray**

--- Sample preparation

Cells in T25 culture flasks were treated for 6 hours and cells were lysed in 1ml TRI Reagent® (Sigma-Aldrich) followed by RNA extraction according to the manufacturer’s instructions. To remove any genomic DNA, RNA was treated with DNase I (Ambion) at 37°C for 30 minutes and the reaction stopped by addition of Phenol:Chloroform:IAA. Following centrifugation at 12,000xg for 5 minutes at 4°C, the aqueous phase was removed and RNA precipitated during a 30 minute incubation on ice with 1/10 ammonium acetate (Ambion) and 3x 100% ethanol. Centrifugation at 12,000xg for 20 minutes at 4°C produced an RNA pellet which was washed in 75% ethanol, centrifuged again for 5 minutes, before air-drying and dissolving in tris-EDTA buffer (Ambion). RNA was quantified using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies). Integrity and purity of the total RNA was checked twice with an RNA 6000 NanoAssay on an Agilent 2100 Bioanalyzer. Only high-quality RNA with an RNA integrity number above 9 was used for microarray analysis. The experiment was performed as a biological duplicate of three 6 hour treatments (untreated control, 10ng/ml TNF alone and in combination with 5μg/ml CZP). 50ng total RNA was used for each Affymetrix Human Exon 1.0 ST Array.

--- Microarray analysis

Microarray data were processed and analysed using Partek Genomics Solution (version 6.5, Copyright 2009, Partek Inc., St. Charles, MO, USA) with the following options: probesets of the core subset were quantile normalised and RNA background correction applied. Exons were summarised to genes by calculating the mean of the exons (log 2). The gene enrichment strategy consisted of the following steps. To establish relationships and compare variability between replicate arrays and experimental conditions, principal components analysis (PCA) and hierarchical clustering was used (22).

Differential expression in response to treatments was calculated by Limma using the functions lmFit and eBayes in Bioconductor (23, 24). This was carried out as three 2-way tests. Correction was made for false discovery rates using the method of QVALUE (25). Differentially expressed genes chosen for subsequent analysis by quantitative real-time PCR met the following criteria: a q value of ≤0.05, a minimum fold change of 5 and a minimum mean fluorescence signal intensity of ≥50 for at least one of the treatment groups.

**Quantitative polymerase chain reaction analysis**

For validation of the microarray, reverse transcription quantitative real-time-PCR (RT-qPCR) was performed using 25ng of complementary DNA (cDNA) to examine gene expression of E-selectin, VCAM-1, ICAM-1, and IkBα in HAOEC in each treatment group. Samples were prepared as for microarray analysis. Quantification and quality control was performed using a NanoDrop ND-1000 spectrophotometer. 1μg RNA was reverse-transcribed using a Precision Reverse transcription kit with oligodT and random nonamer primers according to the manufacturer’s instructions (Primer Design) and then diluted to 10ng/μl. RT-qPCR was performed in duplicate on a 7900 HT Fast Real-Time PCR System (Applied Biosystems) using 2.5μl cDNA, custom designed Gene of Interest as probes and Precision MasterMix with ROX and SYBR green (both Primer Design’s instructions (Primer Design)). The following list of primers were designed and synthesised by Primer Design Ltd; E-selectin (Sense TTCTTGTGCTACTATGCGAGATG; anti-sense AGGAAAGGGCAGCTAGCT), VCAM-1 (sense CAG-GCTAAGTTACATATTGACAT; anti-sense GAGGAAAGGGCAGCTAGCT), ICAM-1 (sense CCTAT-GGCAACGACTCCTTC, anti-sense TCTCTGTCCCTGGTCCT), and IkBα (sense CTATTCTCCCTAC-CAGCCTC; antisense CTCTCCT-CATCTCCTACTCTCTCT). GAPDH was used as a reference gene after validation using a human endogenous control array (Applied Biosystems). The GAPDH reference gene primer sequences from Primer Design are not available for publication. Specificity of the primers and reaction contamination were checked by melt-curve analysis and non-template controls. Data were normalised to the endogenous control gene GAPDH and untreated control at each time point using the 2–ΔΔCT method (26).

**Immunofluorescence**

Immunofluorescence was used to determine the subcellular localisation of NF-κB in HAOECs cultured on chamberslides (BD Falcon). Cells were treated in duplicate with TNF alone and in combination with CZP for 30 minutes. Following treatment, cells were prepared according to previously published protocols (27, 28). Non-specific binding sites were blocked with 5% goat serum (S-1000; Vector Laboratories) in PBS for 1 hour at room temperature (RT). Rabbit anti-human NFκB p65 polyclonal antibody (sc-372; 1:500 dilution; Santa Cruz) and TRITC phalloidin (1μg/ml F-actin stain; Sigma Aldrich) in blocking solution were applied, for 1 hour at RT in the dark. Following further washes in excess PBS, cells were incubated in goat anti-rabbit IgG conjugated to Alexa Fluor® 488 secondary antibody (A11008; Molecular probesTM; Invitrogen) for 1 hour at RT in the dark. Cells were washed, chamberslides dismantled and slides mounted in vectashield with DAPI (H-1200; Vector Laboratories). Images were collected on an Olympus BX51 upright microscope and captured using a Coolsnap ES camera (Photometrics) through [MetaVue Software (Molecular Devices)]. Specific band pass filter sets for DAPI, FITC and Texas red were used to prevent bleed through from one channel to the next. Images were then processed using ImageJ (http://rsb.info.nih.gov/ij). For analysis each chamber was examined using 5 fields of view and representative images taken.
**High content analysis**

HAoECs cultured on a 96 well microplate (DPS-130-020K, Fisher Scientific) were treated in triplicate with TNF alone and in combination with CZP for up to 1 hour. At 10, 20, 30 and 60 minutes after the start of treatment, cells were fixed at RT in 2% paraformaldehyde for 20 mins and washed twice in PBS. Plates were then analysed for NF-κB p65 localisation using high content analysis (HCA) by Imagen Biotech (Manchester, UK); http://www.imagen-biotech.com). Antibodies used were mouse monoclonal anti-NF-κB (sc-8008, Santa Cruz) and fluorophore-conjugated goat anti mouse Alexa488 (A11001, Invitrogen) and were diluted in 500 μg/ml digitonin/PBS solution according to Imagen Biotech proprietary HCA protocols. DNA was stained with Hoechst 33342 at 2.5 μg/mL. Plates were analysed on a Thermosthis Arrayscan (Manchester, UK); http://www.imagen-biotech.com). The Arrayscan Compartmenal Analysis algorithm was used to generate a mask to measure either cytoplasmic or nuclear staining for each fluorescent signal.

**Cell fractionation and western blotting**

HAoECs were cultured in T75 culture flasks. Following a 30 minute treatment, cell fractionation was performed with the FractionPREP™ Cell fractionation kit (Biovision) following the manufacturer’s instructions. Protein concentrations were determined with a detergent-compatible protein assay (Pierce, BCA protein assay reagent, Thermo Fisher Scientific).

Nuclear and cytoplasmic fractions were stored at -80°C until use. Gel electrophoresis and western blotting were performed as described previously (27). Equal amounts of nuclear and cytoplasmic fractions were heated for 10 minutes at 95°C in reducing sample buffer containing β-mercaptoethanol and separated by 10% sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE), using a Mini-Protein system (Bio-Rad). Proteins were transferred onto a nitrocellulose membrane (Protran, Whatman) by tank transfer (Bio-Rad). Non-specific binding was blocked by 5% non-fat milk in Tris-buffered saline with 0.1% tween 20 (TBS-T). Proteins were identified using overnight incubation with NF-κB p65 antibody (sc-372; 1:200 dilution; Santa Cruz) or rabbit anti-human IκBα polyclonal antibody (sc-203; 1:100 dilution; Santa Cruz). Following washes, secondary peroxidase-conjugated antibody was applied (swine anti-rabbit Ig; 1:2000 dilution, DAKO). Chemiluminescent detection was performed using Supersignal West Pico chemiluminescent substrate (Thermo Scientific) and Kodak Biomax XR film. Images were acquired and quantified using a GS-800 calibrated densitometer and Quantity One 1D Analysis Software (BioRad).

**Isolation of microparticles by differential centrifugation**

Using a modification to the technique used by Distler et al. (29), conditioned media from 2x 10^4 HAoECs treated for 24 hours, was centrifuged at 3890g to remove cells. Microparticles were isolated by ultracentrifugation of supernatants for 90 minutes at 100,000xg at 4°C (Beckman L8-M; Beckman Coulter) with a 50.4 Ti rotor (Beckman Coulter). The resulting microparticle pellets were resuspended in 400μl 1x Annexin binding buffer (Annexin V Apoptosis Detection Kit eFluor® 450, EBioscience).

**EMP measurement by flow cytometry analysis**

For quantification of EMPs, isolated microparticles were incubated with PE-labelled anti-human CD31 antibody (BD Pharmingen 55546), eFluor 450 labelled Annexin V (Annexin V Apoptosis Detection Kit, EBiosciene) and APC-labelled anti-human CD42b antibody (BD Pharmingen 551061) as a negative control. 10,000 flow-Count™ Fluospheres (Beckman Coulter) were added to each sample to aid quantification. Flow cytometric analysis was conducted until 2,000 fluorospheres were detected. Gating was employed to eliminate background noise, autofluorescence and fluorospheres. EMPs, (CD31/Annexin V positive events), were identified as particles with a forward angle light scatter smaller than the internal standard of 10 μm sized Flurospheres. Analysis was performed using the Beckman Coulter Cyan ADP flow cytometer with Summit V4.3 software.

**Statistical analysis**

Data were non-parametric and thus Mann-Whitney U-tests were conducted for statistical analysis using SPSS 16.0 software (IBM).

**Results**

Transcriptional profiling of TNF-α and CZP treated HAoECs

In order to develop a model of endothelial dysfunction relevant to rheumatoid arthritis, we treated endothelial cells in monolayer culture with 10ng/ml TNF to mimic the higher levels of TNF found in patient serum (30, 31). Firstly, effects of TNF and CZP on HAoEC viability were examined using MTT and apoptosis assays. No significant effects were identified (data not shown). To validate the model, we performed microarray transcription profiling for HAoECs exposed to three 6 hour treatments; i) untreated, ii) TNF-α and iii) TNF and CZP. Global expression changes were studied by principal components analysis and hierarchical clustering, revealing that TNF has a significant effect on gene expression, while (TNF and CZP) showed a gene expression pattern similar to the untreated control (Fig. 1A). Following further analysis, 211 gene transcripts were found to be significantly altered in response to TNF by two-fold or more (q-value <0.05) and were categorised according to biological function (Fig. 1B). Consistent with results from other human endothelial cell studies (32-34), we found that the levels of several well-characterised TNF-α targets, including cytokine and cytokine receptor genes and adhesion molecules were up-regulated. Gene transcripts for proteins involved in intracellular signalling, transcription and translation were both induced and down-regulated following TNF treatment, compared to untreated controls, as were cell cycle regulatory genes. The most highly up-regulated genes were the adhesion molecules E-selectin and VCAM-1 by 21.6 and 17.8-fold, respectively com-
Fig. 1. Microarray analysis of HAoECs treated with TNF and CZP. Cultured HAoECs were stimulated with 10ng/ml TNF alone (TNF) or in combination with 5μg/ml CZP (T&C) for 6 hours, or remained untreated (UT). Results of gene microarray analysis. (A) Hierarchical clustering of microarray data. Expression data of all genes in log scale was subjected to hierarchical clustering using Euclidean distance. (B) General classification of genes up-regulated or down-regulated in response to TNF treatment, expressed as number of genes in each category. Genes exhibiting differences in expression in excess of two-fold compared to control untreated (q-value <0.05) were included. (C) Selected genes identified by microarray analysis and fold change in gene expression between treatment groups. UT/TNF q<0.00005; TNF/T&C q<0.00005; T&C/UT q=1.0 for all genes in selection.

Compared to untreated controls, ICAM-1 also featured strongly with an 8.8-fold increase in transcript levels (Fig. 1C) (q-values <0.00005) over controls. We then sought to identify the genes that responded to both TNF and CZP. Treatment with a clinically relevant concentration of 5μg/ml CZP prevented the up-regulation of each of these genes (q-values <0.00005 compared to TNF alone).

Time-course changes in gene expression due to TNF and CZP treatment measured by RT-qPCR

RT-qPCR was used to further verify the relative levels of expression of some of the highly differentially expressed genes shown in Figure 1C. Three adhesion molecules E-selectin, VCAM-1 and ICAM-1 were all among the highly ranked genes. Cells were treated with 10ng/ml TNF, this time over a 24 hour time-course, in the presence and absence of CZP and compared to untreated controls. Of the time-points investigated, E-selectin was induced 25.6-fold in HAoECs in response to TNF treatment. Within 30 minutes of treatment with TNF before the CZP challenge still resulted in activation of HAoECs and exhibited a significant increase in levels of E-selectin, VCAM-1 and ICAM-1 gene expression. In addition, cells exposed to the subsequent treatment with TNF abolished the effect of TNF and showed levels of gene expression similar to that seen in untreated control cells (Fig. 2D).

Nuclear translocation of pro-inflammatory transcription factor NF-kB is inhibited by CZP

NF-kB is a well characterised target of the TNF pathway in endothelial cells. We examined nuclear localisation of NF-kB p65 in HAoECs in response to TNF treatment. Within 30 minutes of treatment with TNF, immunofluorescence was used to show an apparent translocation of NF-kB to the nucleus (Fig. 3A-B), which was prevented by CZP (Fig. 3C). This was confirmed by HCA analysis (Fig. 3D) and further val-
lated by western blotting of sub-cellular fractions isolated from the three cell treatment groups, where strong nuclear detection of NF-κB was apparent in the presence of TNF but not in the presence of TNF + CZP (Fig. 4A).

Effects of CZP on TNF-α induced IkBα protein degradation
In unstimulated cells, NF-κB transcription factors typically exist in the cytoplasm in an inactive state by interactions with members of the IkB family of NF-κB inhibitors. This interaction is thought to mask the NF-κB nuclear localisation signal and prevent nuclear translocation of NF-κB. In order for NF-κB translocation to occur, IkB must be phosphorylated, ubiquitinated and subsequently undergo proteosomal degradation (35). Using cell fractionation and western blotting, we showed that in our model at 30 minutes after the start of TNF treatment, NF-κB translocation to the nucleus corresponds with a reduction in IkBα levels, which was not the case for untreated cells or cells treated with TNF in combination with CZP (Fig. 4A). NF-κB is known to induce expression of the NF-κB inhibitor, IkBα as part of a regulatory pathway. We showed that IkBα transcript levels were induced in TNF treated cells, but not in those treated with CZP (Fig. 4B), suggesting that the NF-κB pathway is active in TNF-treated cells, but not during co-treatment with CZP, (Fig. 4B).

Endothelial microparticle production is prevented by CZP
MPs are released from activated and/or apoptotic cells (36), and plasma levels of platelet- and endothelial cell-derived MPs are increased in conditions associated with vascular dysfunction (37). We therefore examined the release of EMPs from HAoECs in response to a 24 hour TNF treatment in the presence and absence of CZP. EMPs were isolated by ultracentrifugation from supernatant obtained from A) untreated cells; B) cells plus TNF and C) cells with TNF and CZP and were then subsequently stained for CD31 and Annexin V and detected using flow cytometry. Positive events expressing phosphotidylserine in the cell membrane following annexin V staining and PE staining for CD31 were defined as EMPs. A mean of 4290 EMPs/mg protein were produced by untreated control HAoECs over 24 hours. In contrast, cells treated with TNF produced 21,890 EMPs/mg protein; 4.79-fold more than control. Co-treatment with CZP reduced this to 8000 EMPs/mg protein, only 1.56-fold more than control. Fig. 5A, B and C show representative scatterplots, which are graphically represented in Figure 5D.
Discussion

This is the first study to assess the effects of an RA biologic on endothelial function at a cellular level. Human umbilical vein endothelial cells (HUVECs) or bovine aortic endothelial cells are often used in cellular studies, due to relatively easy availability and high cell yields, however we chose the more relevant human aortic endothelial cells, since they are arterial and human in origin, the latter being particularly important for a study involving a clinically available treatment.

Transcriptome analysis showed that the endothelial cells used in our study demonstrated a very similar gene expression profile to HUVECs in other microarray studies when stimulated with TNF-α (32-34). While differences in the degree of gene regulation in HAoECs may be apparent when compared to previous studies using endothelial cells from different vascular beds, the actual gene transcripts altered by treatment per se were predominantly constant. Of note, microarray studies have suggested that endothelial cells from different vascular beds are indeed phenotypically different (34, 38, 39), but in addition, variations could be assigned to specifics in experimental conditions.

Transcriptional profiling identified among others, the gene group cytokines and receptors. This group of inflammatory mediators, including members of the TNF receptor superfamily, cytokines such as IL-8, and chemokines responsible for the recruitment of leukocytes (CX3CL1, CXCL10 and 11) was not found to be up-regulated in the presence of CZP. Closer examination of individual genes strengthened the anti-inflammatory profile of this biologic on TNF-induced genes expressed by endothelial cells. Classical genes involved in endothelial activation including the adhesion molecules E-selectin, VCAM-1 and ICAM-1 were highlighted by both microarray analysis and RT-qPCR to be increased in our model and reduced to control levels by CZP treatment. Soluble adhesion molecules E-selectin and VCAM-1 are elevated in RA patients compared to control, and the latter has been associated with carotid intima-media thickness, a marker of pre-clinical atherosclerosis (30). Furthermore, reports from TNF inhibitor studies, using the more established biologics, have shown reductions in soluble fractions of adhesion molecules as early as one week into treatment (30, 40) and higher levels of more novel atheroprotective markers of vascular function and inflammation anti-phosphorylcholine IgM and ApoA1 lipoprotein after 12 months of treatment (41). While these results are from in vitro studies, which cannot mimic all the factors present in the endothelium of a patient with RA, the cell-derived data described here suggest that CZP has the potential to mirror these encouraging clinical data and begin to inform on mechanisms involved.
CZP prevents endothelial microparticle release / S.K. Heathfield et al.

EMPs (Region R6) were identified using flow cytometry. PE (CD31) and Annexin V positive HAoECs were treated for 24 hours and EMPs induced endothelial cell activation. NF-κB in mediating the response of endothelial cells to TNF (42, 43). Here, we demonstrate that cells treated with CZP do not exhibit features of NF-κB activation or an increase in transcripts relating to NF-κB activity, such as IkBα or E-selectin. This supports the anti-inflammatory capacity of CZP and protective effects on the endothelium. Microparticles are released from cells in response to cell activation or apoptosis. We did not observe that TNF had any significant effects on cell viability or that it induced apoptosis. It is generally recognised that TNF alone, in the absence of mRNA or protein synthesis inhibition, does not promote cell death (44). The results presented herein, suggest the reliability of TNF activation of HAoECs, associated with elevated levels of endothelial microparticle release and we demonstrate that CZP acts in a manner that could be considered to be protective, in that CZP prevents the TNF-induced release of EMPs. It is now well documented that the measurement of EMPs may be proposed as a biomarker of CVD and atherosclerotic complication and progression (36). EMP release, and its attenuation by CZP, could reflect the response of endothelial cells to activating stimuli such as TNF, and in addition, act as an indicator of endothelial cell function and damage in patients with RA. Whether EMPs correlate with clinical measures of endothelial function has not yet been investigated in patients with RA but EMPs do correlate with non-invasive clinical measures of endothelial function in other diseases, such as flow-mediated dilatation of the brachial artery in renal failure (45).

Several mechanistic possibilities have been proposed to explain the link between inflammation and endothelial dysfunction in patients with RA, including the release of inflammatory mediators from ECs, as well as increased adhesion molecule expression (46). Based on the data acquired using the in vitro model described in this study, further investigation in patients with RA will clarify whether a correlation exists between EMP release and endothelial dysfunction, which can be prevented by CZP. In conclusion, the data presented herein, suggest that certolizumab pegol has several anti-inflammatory actions on endothelial cells, when applied in the presence of TNF. Our study has shown that endothelial cells treated with TNF have higher concentrations of EMPs compared to untreated cells or those treated with TNF plus CZP. Thus, EMPs may be considered an additional vascular risk factor and show potential as biomarkers for endothelial dysfunction. The evaluation of EMPs may better monitor the degree of the vascular wall damage severity and repair.

Acknowledgements

We thank the Microarray Bioinformatics Group (Faculty of Life Sciences) for their assistance in the analysis of the microarray data and Imagen Biotech Ltd (Manchester: http://www.imagen-biotech.com) for the high content analysis.

References

3. SYMONDS DP, GABRIEL SE: Epidemiology of CVD in rheumatic disease, with a focus on RA and SLE. Nat Rev Rheumatol 2011; 7: 399-408.
8. BILSBOROUGH W, KEEN H, TAYLOR A et al.: Anti-tumour necrosis factor-alpha therapy over conventional therapy improves endothelial function in adults with rheumatoid arthritis.
9. DIXON WG, WATSON KD, LUNT M et al.: Reduc
tion in the incidence of myocardial in
eraction in patients with rheumatoid arthritis
who respond to anti-tumor necrosis factor al-
pha therapy: results from the British Society
10. FOSTER W, CARRUTHERS D, LIP GY, BLANN
AD: Inflammation and microvascular and
macrovacular endothelial dysfunction in
rheumatoid arthritis: effect of treatment. *J
Rheumatol* 2010; 37: 711-6.
11. HURLIMANN D, FORSTER A, NOLL G et al.: An
ti-tumor necrosis factor alpha treatment
improves endothelial function in patients
with rheumatoid arthritis. *Circulation* 2002;
106: 2184-7.
12. SIDIROPoulos PI, SIACKA P, PAGONIDIS K et
al.: Sustained improvement of vascular
endothelial function during anti-TNF alpha
treatment in rheumatoid arthritis patients.
(CDP870): in vitro comparison with other
anti-tumor necrosis factor alpha agents, 
14. TRACZY D, KLARESKOG L, SASSO EH, SAL-
FELD JG, TAK PP: Tumor necrosis factor
antagonist mechanisms of action: a compre-
hensive review. *Pharmacol Ther* 2008; 117:
244-79.
15. FLEISCHMANN R, VENCOVSKY J, VAN
VOLLENHOVEN RF et al.: Efficacy and safety
of certolizumab pegol monotherapy every
4 weeks in patients with rheumatoid arthritis
failing previous disease-modifying antirheu-
matic therapy: the FAST4WARD study. 
16. KEYSTONE E, HEILDE D, MASON D, JI et al.: 
Certolizumab pegol plus methotrexate is sig-
nificantly more effective than placebo plus
methotrexate in active rheumatoid arthritis:
findings of a fifty-two-week, phase III, mul-
ticenter, randomized, double-blind, placebo-
controlled, parallel-group study. *Arthritis
17. PINCUS T, FURER V, KEYSTONE E et al.: 
RAPID3 (Routine Assessment of Patient
Index Data 3) severity categories and response
criteria: Similar results to DAS28 (Disease
Activity Score) and CDAI (Clinical Disease
Activity Index) in the RAPID 1 (Rheumatoid
Arthritis Prevention of Structural Damage)
clinical trial of certolizumab pegol. *Arthritis
18. SMOLEN J, LANDEWIE RB, MEASE P et al.: Ef
cicacy and safety of certolizumab pegol
plus methotrexate in active rheumatoid ar-
thritis: the RAPID 2 study. A randomised
controlled trial. *Ann Rheum Dis* 2009; 68:
797-804.
19. SZKEANECZ Z, KEREKES G, SOLTESZ P: 
Vascular effects of biologic agents in RA and
spondyloarthropathies. *Nat Rev Rheumatol*
2009; 5: 677-84.
20. AMABLE N, BOULANGER CM: Circulating
microparticle levels in patients with coronary
artery disease: a new indicator of vulnerabil-
21. CHRONI GN, SIMON A, BOULANGER CM et al.: 
Circulating microparticles may influence early
carotid artery remodeling. *J Hypertens*
22. QUACKENBUSH J: Computational analysis
of microarray data. *Nat Rev Genet* 2001; 2:
418-27.
23. SMYTH GK: Linear models and empirical
bayes methods for assessing differential
expression in microarray experiments. *Stat
24. WETTENHALL JM, SMYTH GK: limmaGUI:
a graphical user interface for linear modeling
of microarray data. *Bioinformatics* 2004; 20:
3705-6.
25. STOREY JD, TBIRHANI R: Statistical sig-
nificance for genomewide studies. *Proc Nat
Acad Sci USA* 2003; 100: 9440-5.
26. LIVAK KJ, SCHMITTGEN TD: Analysis of
gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T))
27. KIRTON JP, WILKINSON FL, CANFIELD AE,
ALEXANDER MY: Dexamethasone down-
regulates calcification-inhibitor molecules and
accelerates osteogenic differentiation of
vascular pericytes: implications for vascular
28. NDP A, WILLIAMS A, JUDE EB et al.: The
RANKL/RANK/OPG signaling pathway mediates
medial arterial calcification in dia-
betic Charcot neuroarthropathy. *Diabetes*
2011; 60: 2187-96.
29. DISTLER JH, HUBER LC, HUEBER AJ et al.:
The release of microparticles by apoptotic
cells and their effects on macrophages. 
30. FOSTER W, CARRUTHERS D, LIP GY, BLANN
AD: Inflammatory cytokines, endothelial
markers and adhesion molecules in rheuma-
roid arthritis: effect of intensive anti-inflam-
matory treatment. *J Thromb Thrombolysis*
2010; 29: 437-42.
31. KOBAYASHI T, MURASAWA A, KOMATSU
and Rel proteins: evolutionarily conserved
components in vascular biology. *Arterioscler
Thromb Vasc Biol* 2011; 31: 27-
33.
32. CLINNIUK PA, SIERAKOWSKI S, DOMYSLAW-
SKA I, CHWIECKO J: Effect of etanercept on
serum levels of soluble cell adhesion mol-
elcules (sICAM-1, sVCAM-1, and sE-selec-
tin) and vascular endothelial growth factor
in patients with rheumatoid arthritis. 
33. AEGANOVA S, FISKEBLO K, DE FAIRE U,
HAFSTROM I, FROSTEGARD J: Effect of bio-
logical therapy on levels of atheroprotective
antibodies against phospholipidylcholine and
apolipoproteins in rheumatoid arthritis - a
one year study. *Clin Exp Rheumatol* 2011;
29: 942-50.
34. KEMPE S, KESTLER H, LASAR A, WIRTH T:
NF-kappaB controls the global pro-inflammatory
to response in endothelial cells: evidence for
the regulation of a pro-atherogenic program. 
35. VIENNAND M, GOEBELER M, SCHMID S et al.: 
Transcriptional profiling of IKK2/NF-kappa
B- and p38 MAP kinase-dependent gene ex-
pression in TFN-alpha-stimulated primary
human endothelial cells. *Blood* 2004; 103:
3566-73.
36. MADGE LA, POBER JS: TNF signaling in
vascular endothelial cells. *Exp Mol Pathol*
2001; 70: 317-25.
37. AMABLE N, GUERIN AP, LEROYER A et al.: 
Circulating endothelial microparticles are
associated with vascular dysfunction in pa-
tients with end-stage renal failure. *J Am Soc
38. KHAN F, GALARRAGA B, BELCH J: The role
of endothelial function and its assessment
in rheumatoid arthritis. *Nat Rev Rheumatol*
2010; 6: 253-61.