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

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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.

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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 (110Units/ml and 100µg/ml respectively) at 37°C in humidified atmosphere with 5% CO_2. At subconfluence HAoECs were trypsinised using DetachKit (Promocell) and re-plated at 10,000 cells/cm^2. 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 RMA 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 IκBα 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 assays and Precision MasterMix with ROX and SYBR green (both Primer Design). The following list of primers were designed and synthesised by Primer Design Ltd; E-selectin (Sense TTCTTGCCTACTATGCCAGATG; anti-sense AGGAAAGGGAACACTGAGTCT), VCAM-1 (sense CAGGCTAAGTTACATATTGATGACAT; anti-sense GAGGAAGGGCTGACCAAGAC), ICAM-1 (sense CCTATGGCAACGACTCCTTC, anti-sense TCTCCTGGCTCTGGTTCC), and IκBα (sense CTATTCTCCCTACCAGCTCAC; antisense CTCTCCTCATCCTCACTCTCT). 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 $2\text{-}\Delta\Delta C_T$ 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 probes™; Invitrogen; 1:500 dilution in blocking buffer) 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 $\mu g.mL^{-1}$. Plates were analysed on a Thermofisher (Cellomics) Arrayscan. The Arrayscan Compartmental 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-Protean 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). Chemiluminscent detection was performed using Supersignal West Pico chemiluminescent substrate (Thermo Scientific) and Kodak Biomax XAR 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 10^6 HAoECs treated for 24 hours, was centrifuged at 3890xg 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, EBioscience) and APC-labelled anti-human CD42b antibody (BD Pharmingen 551061) as a negative control. 10,000 flow-Count™ Fluorospheres (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 Fluorospheres. Analysis was performed using the Beckman Coulter Cyan ADP flow cytometer with Summit V4.3 software.

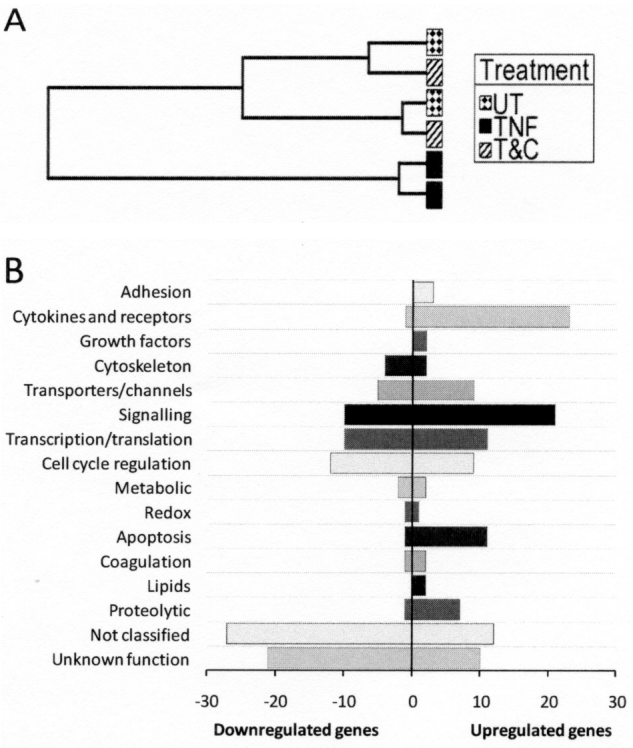
Statistical analysis

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-



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Gene	UT/TNF	TNF/T&C	T&C/UT	Function
E-selectin	**21.6**	*-23.4*	*-1.1*	Adhesion molecule
VCAM-1	**17.8**	*-21.2*	*-1.2*	Adhesion molecule
CX3CL1	**12.7**	*-12.3*	**1.0**	Chemokine
BIRC3	**11.8**	*-12.8*	*-1.1*	Apoptosis inhibitor
CCL20	**11.7**	*-9.0*	**1.3**	Chemokine
CXCL10	**10.9**	*-12.1*	*-1.1*	Chemokine
CXCL11	**10.9**	*-8.3*	**1.3**	Chemokine
TRAF1	**9.3**	*-10.6*	*-1.1*	TNF-receptor associated factor
ICAM-1	**8.8**	*-8.1*	**1.1**	Adhesion molecule
RND1	**7.7**	*-7.2*	**1.1**	Rho Family GTPase
IL-8	**7.2**	*-6.9*	**1.0**	Pro-inflammatory cytokine
UBD	**7.1**	*-6.3*	**1.1**	Ubiquitin D
IκBα	**5.1**	*-4.6*	**1.1**	NF-κB inhibitor
SOD-2	**4.9**	*-4.7*	**1.0**	REDOX enzyme
CXADR	*-5.1*	***5.6***	*-1.0*	Virus receptor
GJA5	*-5.0*	***5.2***	**1.1**	GAP junction protein

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<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.

pared 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 by 1 hour, 93.5-fold by 3 hours, reached 208.5-fold 6 hours after the start of TNF stimulation and had decreased to 12.7-fold by 24 hours, although still significantly higher than control (Fig. 2A). In contrast, cells treated with TNF in the presence of CZP exhibited a very similar gene expression profile to untreated cells; 1.2, 1.4, 1.2 and 1-fold, throughout the time-course respectively. The other adhesion molecules, VCAM-1 and ICAM-1 showed a slightly different pattern of gene expression in response to TNF treatment, with levels highest at 3 hours and remaining high at 6 hours (Fig. 2B-C) (n=3; p≤0.05). Consistent with the microarray analysis, levels were lower than those for E-selectin, furthermore induction of adhesion molecule gene expression was again prevented by co-treatment with CZP. Treatment with CZP in the absence of TNF did not alter gene expression levels (data not shown). To determine dose-dependent effects of CZP on activated endothelial cells, a 6 hour dose-response was conducted with concentrations of CZP ranging from 0.01μg/ml to 100μg/ml. Although 0.01μg/ml had little effect on gene expression, all concentrations from 0.1μg/ml to 100μg/ml significantly eliminated the response to TNF (data not shown).

To further validate the *in vitro* culture system as a model of RA, given that TNF levels are known to be high in patients with rheumatoid arthritis, we pre-conditioned the cells with TNF for six hours to up-regulate adhesion molecule gene expression, followed by a subsequent exposure to CZP for 18 hours. RT-qPCR showed that prior administration of 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 CZP treatment 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-κB is inhibited by CZP

NF-κB is a well characterised target of the TNF pathway in endothelial cells. We examined nuclear localisation of NF-κB 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-κB to the nucleus (Fig. 3A-B), which was prevented by CZP (Fig. 3C). This was confirmed by HCA analysis (Fig. 3D) and further val-

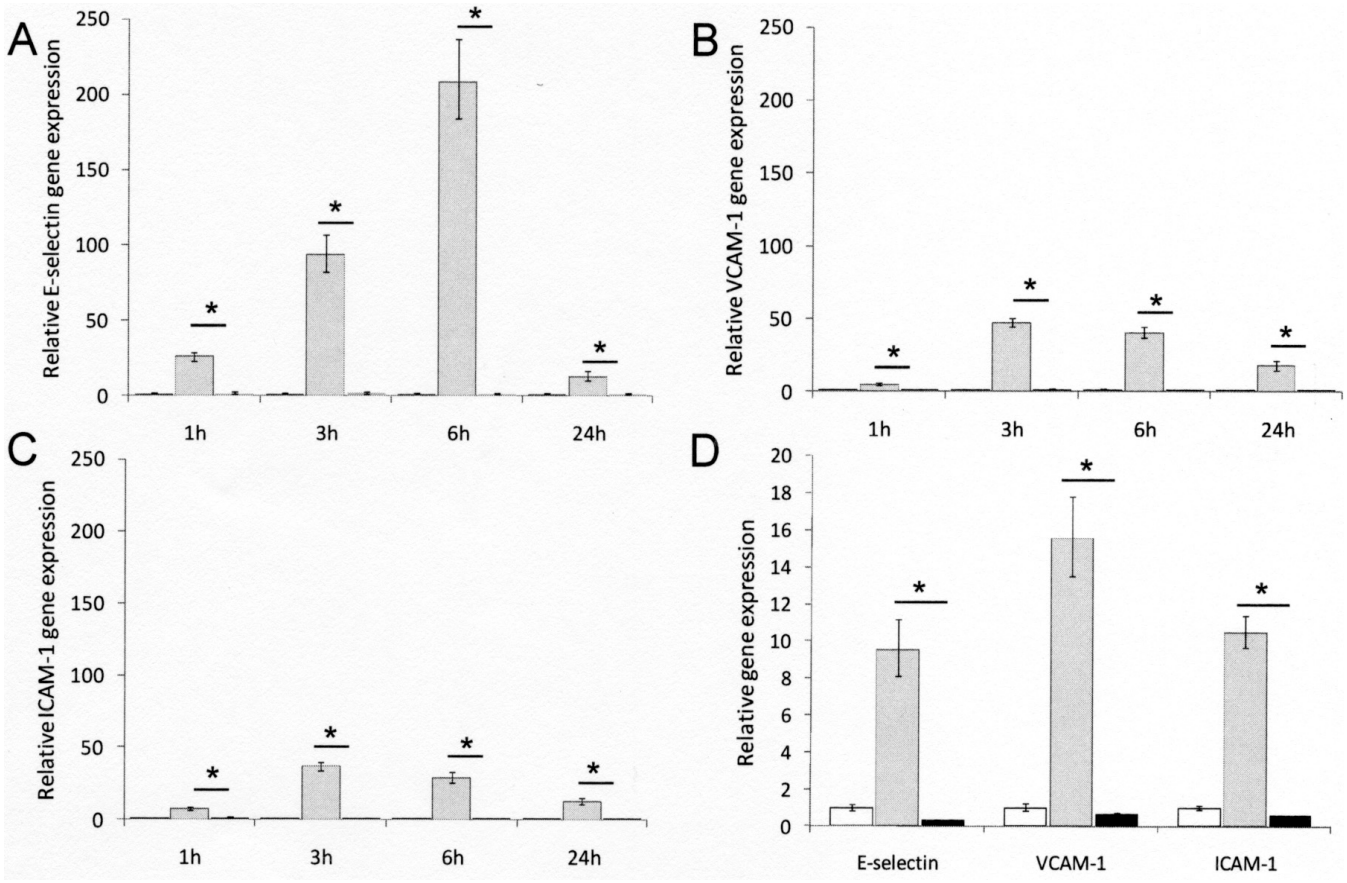

Fig. 2. Prevention of TNF-induced adhesion molecule gene expression by CZP. Expression of E-selectin (**A**), VCAM-1 (**B**) and ICAM-1 (**C**) was examined over 24 hours using RT-qPCR analysis. HAoECs were stimulated with 10ng/ml TNF alone (grey bars) or in combination with 5μg/ml CZP (black bars) for times indicated. Expression was normalised to GAPDH and to untreated control (white bars) at each time-point (n=3). **D** HAoECs were stimulated with 10ng/ml TNF alone for 6 hours to stimulate adhesion molecule gene expression. At this point CZP was added to one sample. All were cultured for a further 18 hours before cell lysis and RNA extraction. Real-time quantitative PCR was used to analyse gene expression in treated samples compared to untreated controls (n=3). Bars show mean ± SEM *: $p \leq 0.05$.

idated 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 IκBα protein degradation

In unstimulated cells, NF-κB transcription factors typically exist in the cytoplasm in an inactive state by interactions with members of the IκB 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, IκB 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 IκBα 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, IκBα as part of a regulatory pathway. We showed that IκBα 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.

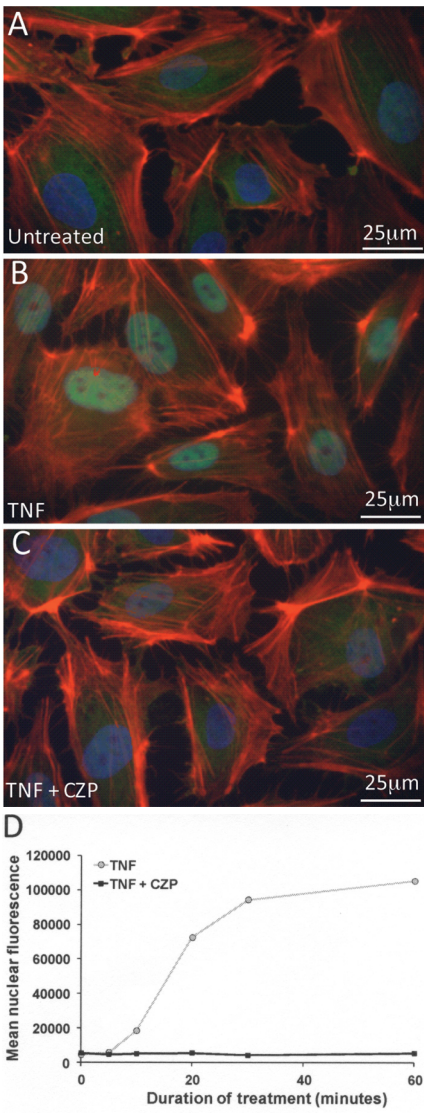

Fig. 3. CZP prevents NF-κB translocation to nucleus. HAoECs were treated with TNF with and without CZP. Nuclear translocation of NF-κB was examined using immunofluorescence for NF-κB (green) 30 minutes after the start of treatment (representative images **A**, **B** and **C**, n=4). Red: phalloidin F-actin; Blue DAPI nuclear stain. **D** High Content Analysis was used to investigate the time-course of NF-κB translocation from baseline to 60 minutes (n=2).

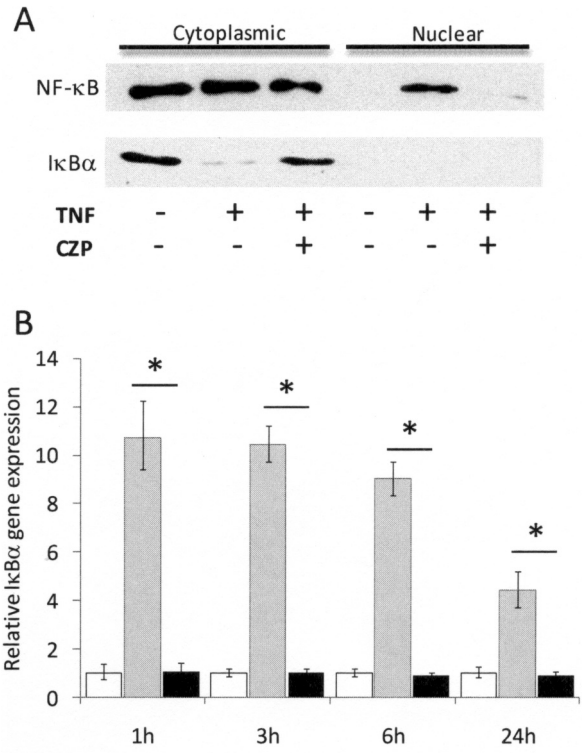

Fig. 4. Effects of CZP on IκB. **A** Lysates of HAoECs treated with TNF for 30 min with and without CZP or left as untreated controls were immunoblotted for NF-κB or IκBα, n=3). **B** Expression of IκBα was examined over 24 hours using RT-qPCR analysis. Cultured HAoECs were stimulated with TNF alone (grey bars) or in combination with CZP (black bars) for times indicated. Expression was normalised to GAPDH and to untreated control (white bars) at each time-point (n=3). Values are mean ± SEM * = $p \leq 0.05$.

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.

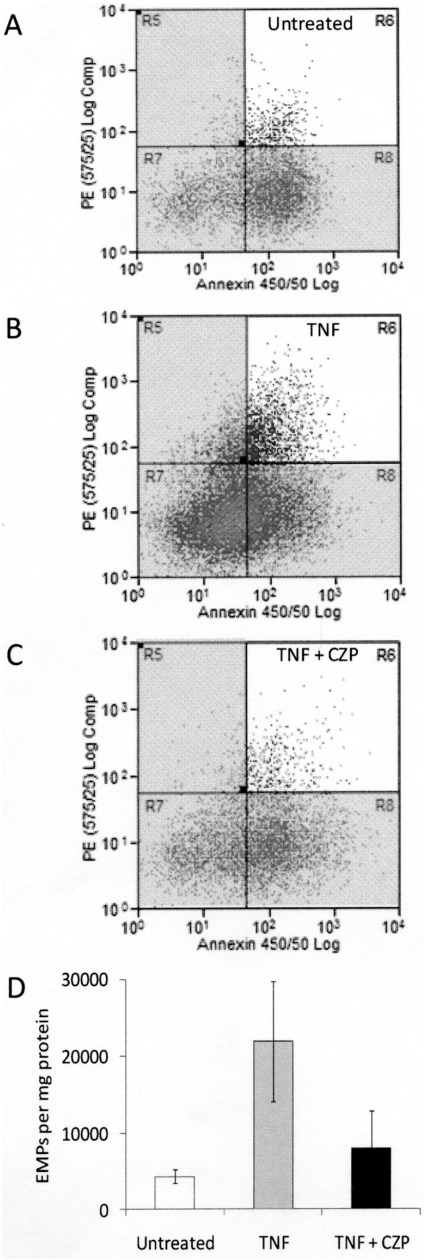

Fig. 5. Generation of EMPs by HAoECs. HAoECs were treated for 24 hours and EMPs collected. PE (CD31) and Annexin V positive EMPs (Region R6) were identified using flow cytometry. Representative scatterplots from treatment groups (**A**,**B**,**C**). **D** The number of EMPs produced during each treatment were normalised to the protein concentration of the cells of origin (n=3). Values are mean ± SEM.

NF-κB is a crucial mediator of TNF-induced endothelial cell activation. Translocation of the transcription factor to the cell nucleus and the expression of NF-κB-dependent genes, facilitates intimal recruitment (IL-8 and CXCL11) and infiltration (E-selectin, VCAM-1 and ICAM-1) of leukocytes, thereby establishing the inflammatory basis underpinning atherogenesis. Two microarray studies have shown that the majority of genes induced by TNF treatment require a functional NF-κB pathway, demonstrating the importance of 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 IκBα 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.

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