CTLA4-Ig/CD86 interactions in cultured human endothelial cells: effects on VEGFR-2 and ICAM1 expression

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Abstract

Objective

Previous studies have reported the presence of CD86 (B7.2) costimulatory molecule on endothelial cells (ECs) and recent data have shown that CTLA4-Ig (abatacept), used as a biological agent in rheumatoid arthritis, interacts with CD86 expressed on different cells involved in synovitis. Therefore, the effects of CTLA4-Ig/CD86 interaction on VEGFR-2 (vascular endothelial growth factor receptor 2) and ICAM1 expression, were evaluated in cultured ECs.

Methods

Activated ECs (γIFN 500 U/ml or IL-17 100 ng/ml), treated with CTLA4-Ig (10, 100, 500 μg/ml) were analysed for CD86, VEGFR-2 and ICAM1 expression, by flow cytometry (FACS), by western blot (WB) and quantitative real time PCR (qRT-PCR).

Results

Following CTLA4-Ig treatment (10, 100, 500 μg/ml; 24 hrs), activated ECs decreased their CD86-positivity at FACS: 66, 59, 51%, respectively, versus 68% of untreated cells (cnt) (for γIFN-activated cells) and 42, 47, 46% versus 71% (cnt) (for IL-17-activated ECs). Gamma-IFN-activated ECs, treated with CTLA4-Ig, showed a dose-dependent decrease only for ICAM1 fluorescence. Whereas, WB showed a significant decrease ($p<0.05$) for both ICAM1 and VEGFR-2 after CTLA4-Ig 500 μg/ml (3 and 24 hrs) and for VEGFR-2 also after CTLA4-Ig 100 μg/ml (3 hrs). QRT-PCR showed a significant decrease ($p<0.05$) for VEGFR-2 after CTLA4-Ig 500 μg/ml (3 and 24 hrs) and after CTLA4-Ig 100 μg/ml (limited at 3 hrs). QRT-PCR for ICAM1 was negative at 3 and 24 hrs, possibly since it was to late to be detected.

Conclusion

Results support a CTLA4-Ig/CD86 interaction on γIFN and IL-17 activated ECs modulation, in the expression of VEGFR-2 and ICAM1, both relevant for inflammatory and angiogenetic processes, suggesting ECs as a further target for abatacept.

Key words

CD86(B7.2) costimulatory molecule, endothelial cells, VEGFR-2, ICAM1, rheumatoid arthritis, CTLA4-Ig (abatacept)

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Introduction

Angiogenesis and endothelial cell (EC) dysfunction are present in rheumatoid arthtritis (RA) synovitis. ECs undergo activation and proliferation that lead to an increase in endothelial permeability, cytokine and cytokine receptor expression, and protease production (1). These changes lead to migration and homing of leukocytes into the joint with angiogenesis and synovial tissue inflammation, resulting in articular damage.

Actually, it is not clear at what stage of the disease this cascade of events starts to develop. During RA angiogenesis, the propagation of new vessels in the synovial tissue allows its active infiltration into cartilage and results in erosions and destruction of the cartilage and bone (1).

However, the endothelial progenitor cells, which have angiogenic potential, have been shown to possess T-cell costimulatory capability, as demontrated by blocking experiments using CTLA4-Ig (2). Therefore, CD28:CD80(B7.1)/CD86(B7.2) interaction has been identified as a major costimulatory pathway for endothelial progenitor cell-dependent T-cell activation (2).

In addition, abatacept (CTLA4-Ig) is a biologic agent used for the treatment of RA. Originally, CTLA4-Ig was found limited to a selective modulation of T-cell activation (3). Recent studies have reported the presence of CD86 also on synovial macrophages (SM) and CTLA4-Ig exerts anti-inflammatory effects on cultured SM obtained from active RA patients in co-cultures with activated T cell (4). A significant downregulation of cytokine expression from cultured RA SM treated with CTLA4-Ig, compared to untreated macrophages was detected (4-6). On the other hand, CTLA4-Ig/CD86 binding on macrophage surface induced a possible dose-dependent antiinflammatory effect *in vitro*, by downregulating IL-6, TNF-α, IL-1β and TGFβ production, even in the absence of T cell co-cultures (direct effect) (5, 6).

The present study investigated the presence and role of CD86 on human activated ECs, by considering possible costimulatory molecule function on activated microvasculature at the level of the RA synovial tissue. Therefore, we evaluated possible effects of CTLA4-Ig/CD86 interaction, on the expression of specific markers (VEGFR-2: vascular endothelial growth factor receptor 2; ICAM1: intercellular adhesion molecule 1) on activated microvascular ECs.

Materials and methods

Cell culture

Human microvascular endothelial cells (HMVECs, Lonza, Switzerland), were cultured and activated with two different stimuli (γIFN 500 U/ml and IL-17 100 ng/ml for 48 hrs) to reproduce a model for resting and activated ECs.

Then, HMVECs pre-activated with γIFN or IL-17 were treated for 24 hrs with CTLA4-Ig at different concentrations (10, 100, 500 μg/ml). Cells untreated with CTLA4-Ig were used as controls.

A total of nine experiments were performed: three were stopped after 24 hours, for the evaluation of protein by FACS; three stopped after 3 and three after 24 hours for the simultaneous evaluation of RNA by qRTPCR and total proteins by Western blot (WB).

Flow cytometric assay

HMVECs non-activated and activated with γIFN or with IL-17, after CTLA4-Ig treatment (10, 100, 500 μg/ml) or without CTLA4-Ig treatment (controls), were evaluated for CD86 expression at fluorescence-activated cell sorting (FACS, FC500, Coulter, Hialeah, FL) (anti-human CD86-FITC antibody, BD, Biosciences, NY, USA). HMVECs were also stained with anti-human CD31 (Platelet Endothelial cell Adhesion Molecule-1)-PE (Miltenyi Biotec, BO, Italy) antibody and with specific isotype antibody as controls.

In addition, γIFN-activated HMVECs, treated with CTLA4-Ig (10, 100, 500 μg/ml) or untreated (controls), were evaluated for ICAM1 (CD54) (anti-human ICAM1-PE, BD, Biosciences, NY, USA) and for VEGFR-2 (CD309) (anti-human VEGFR-2-PE, Miltenyi Biotec, BO, Italy). GammaIFN-activated HMVECs and untreated, were stained with specific isotype antibodies as controls.

The analysis by FACS, in the absence of permeation cell protocols, detects

the protein ratio expressed on the surface and the fluorescence was measured by considering two main parameters: the percentage of positive cells (%) and the mean fluorescence intensity (MFI, expressed as arbitrary units by FACS software).

Western blot (WB) analysis

Protein lysate from γIFN-activated HMVECs (similar effects expected with IL-17), treated with CTLA4-Ig (10, 100, 500 μg/ml; 3 and 24 hrs) or untreated with CTLA4-Ig (controls) were separated by electrophoresis and transferred into Hybond-C-nitrocellulose membrane (Life Technologies Ltd, Paisley, UK). Membranes were incubated with primary rabbit anti-human ICAM1 or VEGFR-2 antibodies (diluition 1:1000, Cell Signaling Technology, Beverly MA, USA) and subsequently with an anti-rabbit IgG secondary antibody (dilution 1:2000; Cell Signaling Technology, Beverly MA, USA).

Protein detection was performed by the enhanced chemiluminescence system (Immobilon-P, Millipore, CA, USA). Membranes were also incubated with HRP conjugated antibody to human-actin (dilution 1:10000, Santa-Cruz Biotechnology, CA, USA) as controls. The analysis in WB measures the total amount of proteins in the cells.

Quantitative real time polymerase chain reaction (qRT-PCR) analysis

Quantitative real time-polymerase chain reaction (qRT-PCR) with primers (PrimerDesign, UK) for beta-actin (housekeeping gene), CD31, ICAM1 and VEGFR-2 gene expression was performed on mRNA from γIFN-activated HMVECs treated with CTLA4-Ig (10, 100, 500 μg/ml; 3 and 24 hrs) or untreated (controls). The gene expression values were calculated using the comparative ΔΔCT method as previously reported (7).

Statistical analysis

The statistical analysis was performed using the Mann-Whitney non-parametric *t*-test. All the experiments were done at least in triplicate.

Results

CD86 expression and CTLA4-Ig/CD86 interaction on HMVECs

At FACS analysis, activated HMVECs (with γIFN or IL-17), showed an increased percentage of CD86-positivity (68% and 71%, respectively) compared to unstimulated cells (60%) (Fig. 1A-B). After CTLA4-Ig treatment (10, 100, 500 μg/ml; 24 hrs), the γIFN-activated HMVECs decreased their CD86-positivity (66%, 59% and 51%, respectively), compared to untreated cells (68%), but still keeping the same MFI values (Fig. 1A). Results suggest that the CTLA4-Ig/CD86 interaction and related fluorescence decrease are due to molecule masking on cell surface.

Following CTLA4-Ig treatment (10, 100, 500 μg/ml; 24 hrs) of IL-17-activated HMVECs, the CD86-positivity decreased (42%, 47% and 46%, respectively) compared to untreated cells (71%), with no relevant changes in MFI (Fig. 1B). In addition, HMVECs showed a positivity for the phenotypic marker CD31 (95%) and a negative result for specific isotype controls.

ICAM1 and VEGFR-2 expression on activated HMVEC treated with CTLA4-Ig

All the γIFN-activated HMVECs, analysed by FACS, strongly expressed on surface VEGFR-2 (79%) and ICAM1 (99%). After CTLA4-Ig treatment (10, 100, 500 μg/ml; 24 hrs), a dose-dependent decrease in MFI for ICAM1 was observed (196, 187, 178 arbitrary units, AU, respectively) *versus* untreated HMVECs (198 AU), whereas the percentage of ICAM1 positive cells did not change (99%). Otherwise, VEGFR-2 FACS expression were unchanged (Table I).

WB analysis for VEGFR-2 and ICAM1 total protein expression after 3 hrs of CTLA4-Ig treatment of γIFN-activated

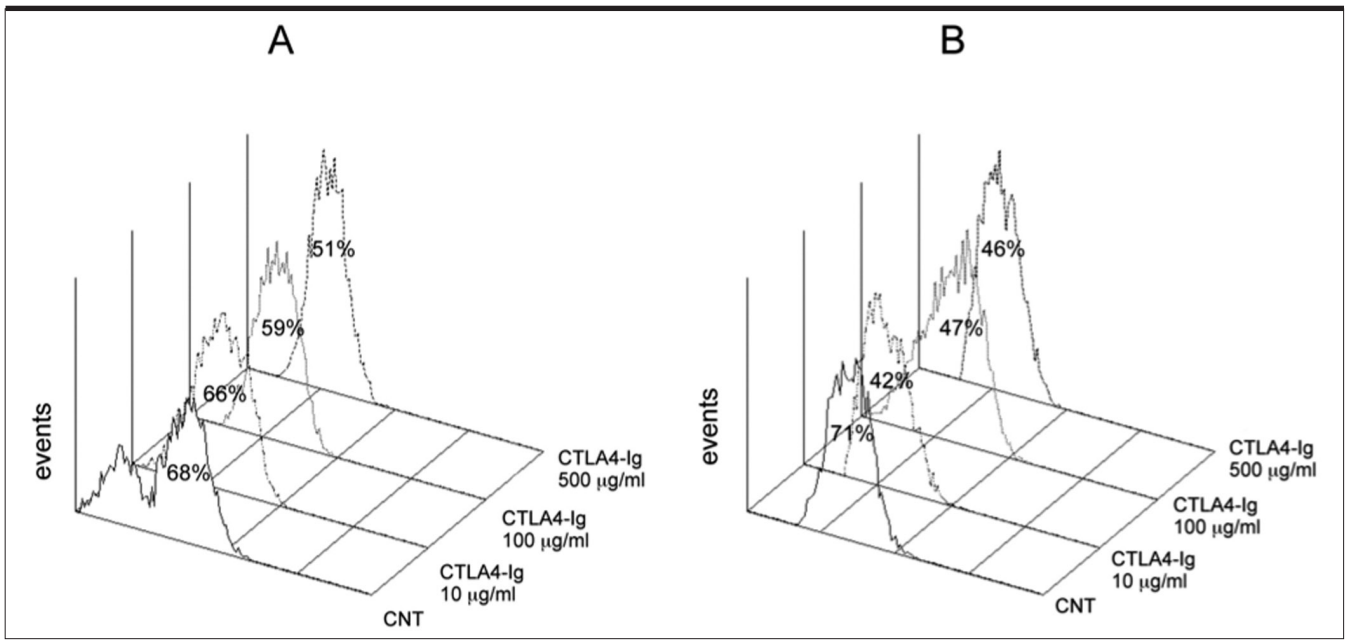

Fig. 1. FACS analysis for CD86 expression on HMVECs, stimulated for 48 hrs with γIFN 500 U/ml (**A**) or with IL-17 100 ng/ml (**B**) and treated for 24 hrs with CTLA4-Ig 10 μg/ml, 100 μg/ml and 500 μg/ml or untreated (CNT).

Table I. FACS analysis for VEGFR-2 and ICAM1 protein expression on HMVECs, stimulated for 48 hrs with γIFN 500 U/ml, and treated for 24 hrs with CTLA4-Ig 10 μg/ml, 100 μg/ml, 500 μg/ml or untreated (CNT). Fluorescence was measured by the percentage of positive cells (%) and the mean fluorescence intensity (MFI).

HMVECs activated with γ-IFN				
Treatments	VEGFR-2		ICAM1	
	% Positive cells	Mean fluorescence intensity (MFI)	% Positive cells	Mean fluorescence intensity (MFI)
CNT	79%	7	99%	198
CTLA4-Ig 10 μg/ml	85%	8	99%	196
CTLA4-Ig 100 μg/ml	86%	8	99%	187
CTLA4-Ig 500 μg/ml	80%	7	99%	178

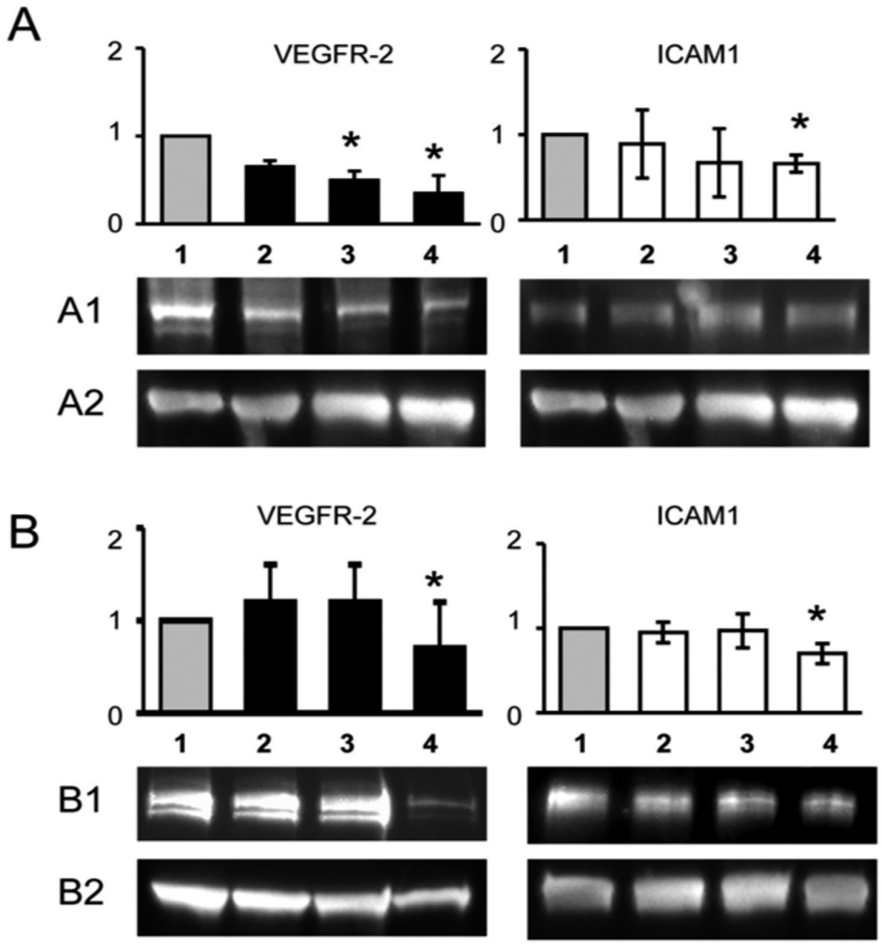

Fig. 2. WB analysis for VEGFR-2 (black bars) and ICAM1 (white bars) protein expression in cultures of HMVECs (stimulated for 48 hrs with γIFN 500 U/ml), untreated (line 1:CNT), and treated for 3 hrs (**A, A1** blotting) and 24 hrs (**B, B1** blotting) with CTLA4-Ig 10 μg/ml (line 2), with CTLA4-Ig 100 μg/ml (line 3) and with CTLA4-Ig 500 μg/ml (line 4). Bar graphs (**A, B**) represent the quantitative densitometric value of the expressed protein vs. actin (**A2, B2**), represented as a percentage of control (assumed equal to 1) and refer to mean values calculated. *: $p<0.05$
The blotting images were acquired using the instrument UVITEC (UVITEC, Cambridge, UK).

HMVECs showed, for the higher doses, a significant decrease ($p<0.05$) compared to the control (CTLA4-Ig-untreated HMVECs). In particular, VEGFR-2 resulted significantly decreased ($p<0.05$) after CTLA4-Ig 100 and 500 μg/ml treatments and ICAM1 showed a significant reduction ($p<0.05$) after CTLA4-Ig 500 μg/ml treatment (Fig. 2 A, A1, A2).

After 24 hrs of CTLA4-Ig treatment, WB for VEGFR-2 and ICAM1 total protein expression still showed a significant decrease ($p<0.05$) in HMVECs treated with CTLA4-Ig at 500 μg/ml (Fig. 2 B, B1, B2).

QRT-PCR for VEGFR-2 gene expression on γIFN-activated HMVECs demonstrated a significant decrease following the 500 μg/ml CTLA4-Ig treatment, both after 3 and 24 hours ($p<0.05$), but only after 3 hrs, following the 100 μg/ml CTLA4-Ig treatment ($p<0.05$). Lower doses of CTLA4-Ig (10 μg/ml) did not induce significant VEGFR-2 gene expression changes.

Otherwise, ICAM1 gene expression was not significantly changed, both after 3 and 24 hrs of treatment, possibly since the time used for the detection was too late. Gene expression of phenotypic marker CD31 was not influenced by CTLA4-Ig treatment at both 3 an 24 hours (Fig. 3A-B).

Discussion

This study reports a CTLA4-Ig/CD86 interaction on cultured activated ECs. In addition, a modulation in the expression of relevant molecules for the inflammatory and angiogenetic processes was observed, at least at protein level. In fact, WB analysis showed a significant decrease for both VEGFR-2 and ICAM1 in the amount of total proteins in ECs, after 3 and 24 hrs of CTLA4-Ig at highest dose treatments.

Results were confirmed at gene level expression for VEGFR-2. Interestingly, ICAM1 gene expression did not change at the tested times (3 and 24 hrs), suggesting that the CTLA4-Ig gene-mediated effects might be exerted earlier.

Otherwise, after 24 hours of treatment with CTLA4Ig, VEGFR-2 protein surface expression were unchanged at the FACS analysis. Probably, we could assume that the proteins expressed on the cell surface (FACS detectable) are not yet influenced by CTLA4Ig gene effects, even after 24 hours, whereas they are already evident after 3 hours of CTLA4Ig treatment, on both gene and intracellular protein expression.

Vascular endothelium is involved in several immune mediated diseases and in chronic inflammation (1). An

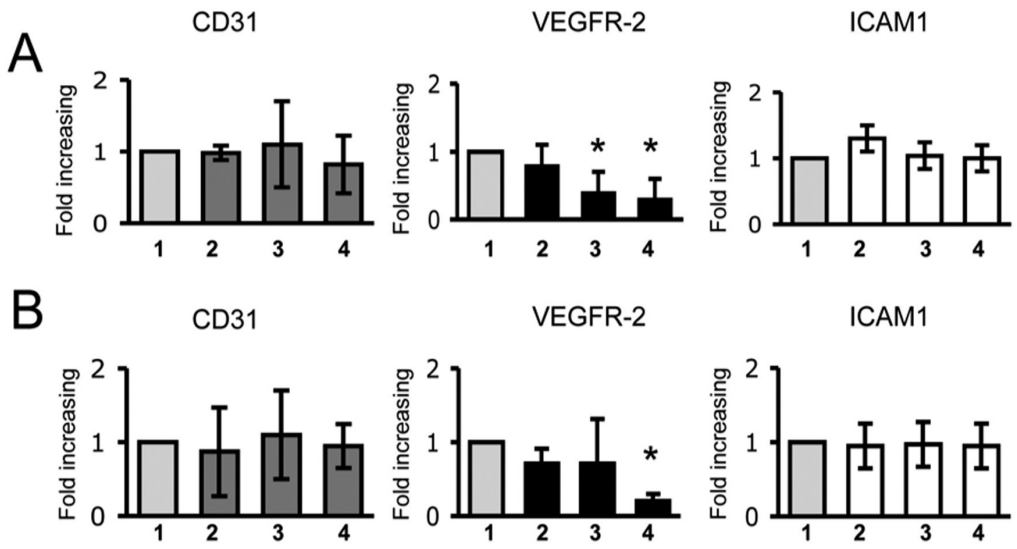

Fig. 3. Quantitative RT-PCR analysis for CD31 (grey bars), VEGFR-2 (black bars) and ICAM1 (white bars) gene expression in cultures of HMVECs (stimulated for 48 hrs with γIFN 500 U/ml), untreated (line 1:CNT), and treated for 3 hrs (**A**) and 24 hrs (**B**) with CTLA4-Ig 10 μg/ml (line 2), with CTLA4-Ig 100 μg/ml (line 3) and with CTLA4-Ig 500 μg/ml (line 4). Bar graphs correspond to the values of fold expression of the target gene compared to the calibrator sample (untreated cells) taken as unit value by definition. * $p<0.05$

important matter of debate is whether ECs, both in resting or activated state, express the repertoire of costimulatory molecules such as CD86, for adequate T cell activation. In fact, through direct interactions with the CD80/CD86 costimulatory molecules, CTLA4-Ig might target, in addition to T cells, macrophages, B lymphocytes and osteoclasts, and also ECs (8-11).

On the other hand, the characteristic expression and distribution of the CD86 costimulatory molecule in cell populations involved in the RA synovitis, indicate that these molecules probably play important role during the progress of RA, and a clear understanding of their functions may further elucidate the pathogenesis of the disease progression (12, 13).

Some recent studies have reported that ligation of CTLA4 on CD4 T cells by CD86 on islet endothelial cells were key to the adhesion of recently activated T cells (14).

The adhesion/transmigration of activated CD4 T cells through the ECs interaction, was completely inhibited by CD86-blocking mAb or CTLA4-Ig, indicating CTLA4 as the T cell ligand for these CD86-mediated effects (14).

In addition, vascular endothelium seems to induce the proliferation of CD8(+) T lymphocytes in a CD86-dependent fashion, suggesting that vascular endothelium can act as an APC (15).

The data reported in our *in vitro* study on cultured ECs, seem in agreement with above discussed evidences and might suggest a further potential modulatory effect for CTLA4-Ig on ECs.

In conclusion, in chronic inflammation situations, such as in RA synovitis, the ECs might be considered a further possible target among other cells, for the modulatory effects exerted by abatacept (CTLA4-Ig)

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