

The anti-TNF- α antibody infliximab indirectly regulates PECAM-1 gene expression in two models of *in vitro* blood cell activation

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Chronic inflammatory bowel diseases can be successfully treated with antibodies against the acute phase mediator TNF- α . The process of activation and of extravasation of inflammatory cells from the blood into the 'stressed' tissue site is controlled by cytokines and chemokines, which attract leukocytes and by adhesion molecules, which mediate their attachment and transmigration toward the affected cell(s). The changes in the gene expression of adhesion molecules taking place in those cells before attachment have been less investigated. Changes of PECAM-1, ICAM-1 and vascular cell adhesion molecule-1 (VCAM-1) gene expression were studied in phytohaemagglutinin (PHA)- and lipopolysaccharide (LPS)-treated human peripheral blood leukocytes (PBLs), granulocytes and the human monocyte cell line U-937. Cells were treated either with PHA or with LPS in the presence or absence of infliximab and incubated with TNF- α , IFN- γ and/or transforming growth factor beta (TGF- β) and treated as above. Activation of PBLs by PHA or LPS treatment triggered a sharp upregulation of ICAM-1, VCAM-1 gene expression and a time-dependent downregulation of PECAM-1 gene expression reaching a minimum 4 h from start of the experiment. The anti-TNF- α antibody infliximab, by neutralizing TNF- α and IFN- γ production, completely reversed PECAM-1 mRNA downregulation and ICAM-1 and VCAM-1 upregulation. Immunostaining of PBLs cytopins with antibodies against PECAM-1 and ICAM-1 confirmed RT-PCR and western blot results. PBLs IFN- γ or TNF- α treatment downregulated PECAM-1 in parallel with the upregulation of ICAM-1 and VCAM-1 gene expression, whereas TGF- β upregulated PECAM-1- and downregulated ICAM-1 and VCAM-1 gene expression counteracting the effect of TNF- α or IFN- γ . Similar results were obtained in human U937 cells and in granulocyte cultures by TNF- α or IFN- γ treatment. Taken together, these results suggest that infliximab, blocking TNF- α and IFN- γ production, exerts its anti-inflammatory effect through inhibiting downregulation of PECAM-1 gene expression and upregulation of ICAM-1 and VCAM-1 expression in leukocytes of the peripheral blood. These results also suggest that TGF- β may thus be of therapeutic importance as an anti-inflammatory agent.

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Impaired intestinal immunoregulation has a central role in the pathogenesis of chronic inflammatory bowel diseases (IBDs) such as ulcerative colitis (UC) and Crohn's disease (CD).¹ Acute flares in IBDs are characterized by an excessive recruitment of leukocytes from the blood circulation into the inflamed gut wall.

Neutrophils are the first line of immune defense^{2,3} as they are recruited from the blood to sites of injury where they contribute to destruction of the initiating noxae and damaged tissue.⁴ During acute flares of UC or CD there is a massive infiltration of neutrophil granulocytes into the affected mucosa, which is manifested clinically by an increase

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in stool granulocytes. Furthermore, lactoferrin, a major component of the secondary granules of neutrophils, is increased in stool and has been shown to correlate with colonoscopic grading systems in patients with IBDs.⁵

In IBDs there is also a marked infiltration of macrophages into the affected mucosa, manifested by an increase in macrophage-derived inflammatory cytokines IL-1 β , IL-6 and TNF- α during acute flares.⁶

In vitro 'activation' of peripheral blood leukocytes (PBLs) is an established model system that mimics acute (and chronic) inflammation.⁷ As such it has been used to define the disease-related profiles occurring in patients affected by various disorders.⁸⁻¹⁰ Importantly the PBLs model faithfully mimics the phenomena observed in inflamed tissues.¹¹

Infliximab is a chimeric monoclonal antibody specific for TNF- α having a murine variable region and a human immunoglobulin constant region. It has been shown to have efficacy against CD, rheumatoid arthritis, psoriasis and ankylosing spondylitis.¹² Treatment with infliximab strongly reduces inflammation in CD¹³ limiting TNF- α and IFN- γ mRNA levels in colorectal mucosa obtained from patients with UC.¹⁴ Several different mechanisms have been proposed to explain the actions of infliximab. It neutralizes TNF- α ,¹⁵ and induces apoptosis of activated T cells and lymphocytes in the lamina propria thereby reducing the levels of IL-1, IL-6, IL-18 and IFN- γ both *in vivo*¹⁶⁻¹⁸ and *in vitro*.¹⁹⁻²¹

In the present investigation we determined the changes in the levels and gene expression of the adhesion molecules PECAM-1, ICAM-1 and vascular cell adhesion molecule-1 (VCAM)-1 in two different *in vitro* models of inflammation, phytohaemagglutinin (PHA)- and LPS-stimulated PBLs, and we defined which adhesion molecules are restored by infliximab treatment in activated leukocytes. In addition, the expression profiles of the genes encoding human PECAM-1 and ICAM-1 in a promonocytic cell line U-937 (a model used to study macrophages biological functions) in response to treatment with PHA and LPS have been defined and using these two models the effect of the anti-TNF- α antibody, infliximab, on PECAM-1, ICAM-1 and VCAM-1 gene expression was determined.

The results of this investigation show that the activation of PBLs and monocytes by PHA or LPS treatment induces a sharp upregulation of ICAM-1 and VCAM-1 gene expression and a time-dependent downregulation of PECAM-1 gene expression.

As TNF- α and IFN- γ downregulate PECAM-1 gene expression in liver sinusoidal endothelial cells and in rat liver macrophages in parallel with ICAM-1 upregulation^{22,23} and both cytokines are inhibited by infliximab,¹⁹ the identity of the cytokines responsible for PECAM-1 downregulation in activated PBLs and in granulocytes was investigated.

The anti-TNF- α antibody infliximab, by neutralizing TNF- α and inhibiting IFN- γ gene synthesis, prevents

PECAM-1 mRNA downregulation as well as ICAM-1 and VCAM-1 upregulation.

Based upon these data it can be concluded that the anti-TNF- α antibody indirectly regulates PECAM-1 gene expression through IFN- γ downregulation whereas TNF- α treatment increases IFN- γ gene expression. Transforming growth factor beta (TGF- β) may exert an anti-inflammatory effect inducing PECAM-1 expression and re-establishing endothelial barrier integrity.

MATERIALS AND METHODS

Isolation and Culture Conditions of Human PBLs and Promonocytic Cell Line U-937

Human PBLs were isolated from five healthy blood donors by Ficoll density gradient centrifugation and dextran sedimentation according to Bøyum.²⁴ Residual red blood cells were hypotonically lysed. Subsequently PBLs were washed three times with phosphate-buffered saline (PBS), pH 7.3. PBLs (1×10^6) were cultured in 1 ml medium (RPMI 1640 with L-glutamine, 10% fetal calf serum, 10 000 U/ml penicillin and 10 000 mg/ml streptomycin (Bio Whittaker, Verviers, Belgium)) and stimulated *in vitro* with PHA (5 μ g/ml) (Roche Molecular Biochemicals, Mannheim, Germany) or LPS (1 μ g/ml) (Sigma-Aldrich Chemie, Taufkirchen, Germany) in the absence or presence of the antibody against human TNF- α infliximab (Remicade, 1000 μ g/ml; MSD, München, Germany) for different lengths of time. Although in preliminary experiments some effects were observed with lower concentrations, the dose of 1000 μ g/ml allowed us to obtain reproducible and significant effects without any sign of cytotoxicity.

In a second set of experiments, PBLs were stimulated with either human IFN- γ (500 U/ml) (Roche, Germany), TGF- β (5 ng/ml) (PeproTech, Hamburg, Germany) or TNF- α (10 ng/ml) (Roche) in the presence or absence of TGF- β .

U937 promonocytic cells were seeded at 2×10^5 cells/ml in RPMI 1640 medium containing 10% (v/v) heat-inactivated fetal calf serum, 2 mM/l L-glutamine, 1 mM/l sodium pyruvate and 1 mM of non-essential amino acids, and cultured at 37°C in an atmosphere of 95% air, 5% CO. They were then stimulated *in vitro* with PHA (5 μ g/ml) or LPS (1 μ g/ml) for different lengths of time.

Isolation of Human Granulocytes and Culture Conditions

Human granulocytes were isolated from the heparinized peripheral blood of healthy donors ($n=3$) using the Histopaque-1077 and Histopaque-1119 gradient density centrifugation (Sigma-Aldrich, USA). Residual erythrocytes were removed by washing with an erythrocyte lysis buffer and monocytes by cell incubation in a culture flask with RPMI medium for 30 min at 37°C. The non adherent cells were washed three times, counted, resuspended in cold PBS at 1×10^6 cells/ml and treated with PHA or LPS as described for PBLs. Granulocytes cultures were stimulated and processed

as described for PBLs in the presence or absence of infliximab. In a separated set of experiments, granulocytes were alternatively treated with TNF- α (10 ng/ml) or with IFN- γ (500 U/ml).

RNA Isolation, Quantitative Real-time RT-PCR

Isolation of total cellular RNA from human PBLs and from granulocytes was performed according to the RNeasy Mini Protocol with the RNeasy total RNA extraction Mini Kit (QIAGEN, Hilden, Germany). The isolated RNA was used for quantitative real-time RT-PCR using the Superscript kit from Invitrogen (Groningen, Netherlands) following the manufacturer’s instructions. Real-time PCR analysis of cDNA was performed at 60°C to 95°C for 45 cycles in the Sequence Detection System of ABI Prism 7000 (Applied Biosystems, Darmstadt, Germany) following the manufacturer’s instructions and by using SYBR Green Reaction Master Mix (ABI Prism; Applied Biosystems) and the primers listed in Table 1. All primers were synthesized by Invitrogen. In every RNA sample, β -actin mRNA was measured as the housekeeping gene. β -actin was chosen as housekeeping gene in according to the experience obtained in previous experiments as the gene expression (Ct values) was constant in different experimental settings.

Values were then compared with those obtained by using the control-RNA obtained from unstimulated cells. The results were normalized to the housekeeping gene and fold change expression was calculated using threshold cycle (Ct) values. During a real-time RT-PCR, the Ct value is known as the number of cycles when the expression of any specific gene under study reaches a predetermined threshold of detection defined as the fractional cycle number at which the fluorescence passes the fixed threshold. The higher the Ct value is for a specific gene at a given time, the lower the amount of specific mRNA is in the preparation. For calculation of the relative changes, gene expression measured in unstimulated cells at 0 h was set at 1.

Table 1 Sequence of human gene-specific primers used for quantitative RT-PCR analysis

Primer	Forward 5' → 3'	Reverse 5' → 3'
PECAM-1	CCAGTGTCCCCAGAAGCAAA	TGATAACCACTGCAATAAGTCCTTTC
ICAM-1	GCAGACAGTGACCATCTACAGCTT	CTTCTGAGACCTCTGGCTTCGT
VCAM-1	GGCAGGCTGTAAAAGAATTGCA	GTCATGGTCACAGAGCCACCTT
IFN- γ	CCAACGCAAAGCAATACATGA	TTTTCGCTCCCTGTTTTAGCT
TNF- α	CCCAGGCAGTCAGATCATCTTC	AGTGCCCTCAGCTTGA
TGF- β	GGGAACACCACCACTCTCATTAG	CAAATTACCACTCGGAAGTTCGT
IL-6	TGGCTGAAAAAGATGGATGCT	AACTCCAAAAGACCAGTGATGATTT
β -actin	CTGGCACCCAGCACAATG	CCGATCCACACGGAGTACTTG

Antibodies

Rabbit polyclonal antibody directed against human ICAM-1 was purchased from Abcam (Cambridge, UK, catalog no. ABIN343417). Mouse monoclonal antibody directed against human PECAM-1 was purchased from Dako (Hamburg, Germany, catalog no. M0823). The CD-31 mouse monoclonal antibody (clone JC70A) recognizes formalin-resistant 130 kDa glycoprotein epitope on extracellular domain 1. The anti-PECAM-1 and anti-ICAM-1 antibodies were used for the double immunofluorescence staining studies.²⁵

Cytospin Preparation, Immunofluorescent Staining and Immunocytochemistry

PBLs and neutrophil granulocytes were placed on poly-L-lysine-coated microscope slides using cytocentrifugation (5 min at 500 rpm). In all, 100 000 cells/cytospin were fixed at room temperature using cold methanol solution, followed by 10 s in cold acetone solution and air dried. After washing in distilled water, endogenous peroxidase activity was blocked by incubating the slides in 3% hydrogen peroxide solution in methanol for 5 min followed by a wash in 10 mM PBS (pH = 7.5). The cytospin preparations were treated with universal blocking serum (Novocastain Super ABC Kit, Novocastra Laboratories, Newcastle upon Tyne, UK) for 20 min at room temperature. The single- and double-immunofluorescence studies were performed according to a protocol described previously²⁶ with the rabbit polyclonal antibody against ICAM-1 and the mouse monoclonal anti-PECAM-1 (1:50), were incubated with the cytospins overnight at 4°C. Following a short washing step in PBS, incubation was carried out with Alexa-Fluor-conjugated goat anti-rabbit and anti-mouse secondary antibody (1:200; Molecular Probes, Germany) at room temperature for 1 h. The sections were then washed three times for 5 min in PBS. Finally, cell nuclei were stained with 4',6-diamidino-2-phenylindole, and the sections were washed and mounted. The number of positive PECAM-1 cells after PBLs incubation with PHA or LPS was counted for 100 cells from 10 different areas of each slide. PECAM-1 APAAP immunostaining of human PBLs was performed as described.²⁷ Negative controls were produced by replacing the primary antibody with murine IgGs.

Protein Extraction and Western Blot Analysis from Human PBLs, Neutrophil Granulocytes and Human Monocyte Cells

Cells (PBLs, neutrophil granulocytes and U-937 cell line) were lysed in 50 mM HEPES, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 10% glycerol, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 1 g/ml aprotinin, 0.5 mM sodium orthovanadate and 20 mM sodium pyrophosphate. Lysates were centrifugated at 14 000 × rpm for 10 min. The protein content of the cellular lysates was calculated by the Coomassie Protein Assay (Pierce, Germany) in which β -actin

was used as the loading control. Proteins were transferred onto Hybond-ECL nitrocellulose hybridization transfer membranes according to Towbin *et al.*²⁸ The immunodetection studies were performed according to the ECL western blotting protocol of GE Healthcare (Germany). The primary rabbit polyclonal antibody to ICAM-1 was used at 1:250 dilution, whereas the mouse monoclonal anti-PECAM-1 was used at a 1:500 dilution and the anti-rabbit and anti-mouse immunoglobulins were each used at a 1:2000 dilution.

Statistical Analysis

The data were analyzed with Prism Graph Pad 4 software (San Diego, USA). All experimental errors are shown as SEM. Statistical significance was calculated by Student's *t*-test, one-way analysis of variance and Dunnett's *post hoc* tests. Significance was accepted at *P* < 0.05.

RESULTS

Changes of ICAM-1, PECAM-1 and VCAM-1 Gene Expression In Vitro PHA- and LPS-Activated Human PBLs

In according to the experience obtained in previous experiments, we used β -actin as a housekeeping gene because its Ct values were constant and did not change in different experimental setting using real-time PCR (data not shown).

PHA treatment induced a time-dependent downregulation of PECAM-1 mRNA, reaching a minimum value within 8 h (20-fold downregulation). LPS treatment produced the same effect but achieved a nadir of 10-fold reduction after 4 h of treatment (Figure 1a).

In contrast to PECAM-1 downregulation, ICAM-1 and VCAM-1 gene expression were sharply increased by PHA

(18 \pm 1.8-fold, 10 \pm 0.03-fold) and LPS (11.7 \pm 1-fold, 10 \pm 0.02-fold) reaching a peak within 4 h (Figure 1b and c). It is noteworthy, that the increase in ICAM-1 and VCAM-1, and the reduction of PECAM-1 occurred as early as 1 h (Figure 1a–c) suggesting that the regulation of the three genes occurs just after cell stimulation and parallels the sharp upregulation of IFN- γ and TNF- α (data not shown). However it is noteworthy, that the amount of VCAM-1 (average of Ct value obtained from real-time PCR = 31) transcripts in unstimulated and in stimulated-PBLs is much lower than that of ICAM-1 (average of Ct value = 22).

TNF- α stimulated IFN- γ gene expression with a maximum effect seen at 8 h (21 \pm 3-fold, Figure 2a). LPS and, at a lower dose, IFN- γ induced a strong upregulation of TNF- α gene expression within 4 h (68 \pm 10- and 30 \pm 4-fold, respectively, Figure 2b). LPS induced IL-6 gene expression within 4 h (16-fold compared with unstimulated cells, Figure 2c). In order of magnitude, TGF- β (four-fold), IFN- γ (two-fold) and TNF- α (two-fold) upregulated IL-6 gene expression (Figure 2c). PBLs treatment with LPS, TNF- α or IFN- γ induced a downregulation of the TGF- β mRNA (Figure 2d).

Changes of ICAM-1, PECAM-1, VCAM-1 Gene Expression in Activated Human PBLs Induced by the anti-TNF- α Antibody Infliximab

As PECAM-1 and ICAM-1 are implicated in the regulation of inflammatory responses, the question arose can an immunosuppressant influence gene regulation of these adhesion molecules. In response to treatment of stimulated PBLs with anti-TNF- α antibody infliximab the prior PHA- and LPS-induced PECAM-1 downregulation was abated (Figure 1a).

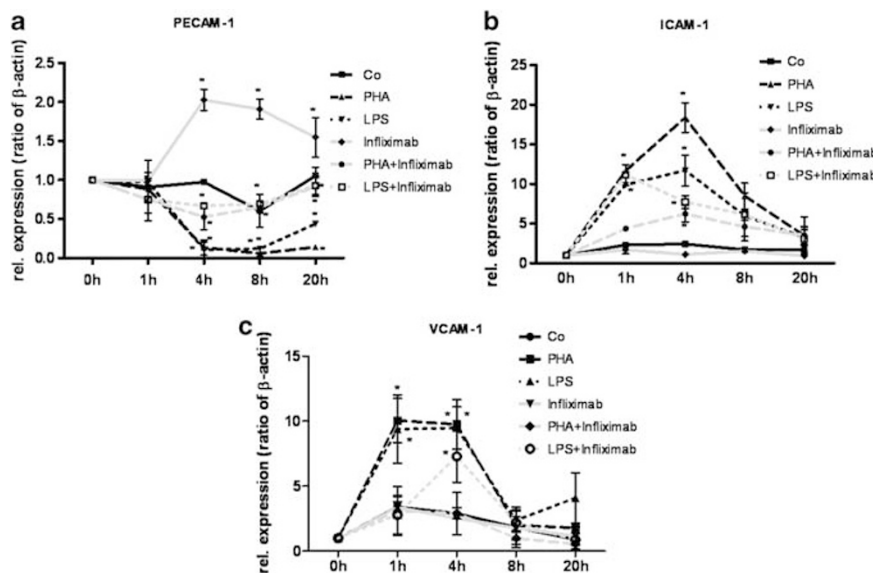


Figure 1 Quantification of mRNA expression of PECAM-1 (a), ICAM-1 (b) and VCAM-1 (c) in PBLs stimulated with infliximab in the presence or absence of PHA and LPS. RT-PCR data were shown as fold changes in mRNA expression normalized with the housekeeping gene β -actin. Results represent the mean value \pm s.e.m. of five separate experiments (**P* < 0.05; *n* = 5).

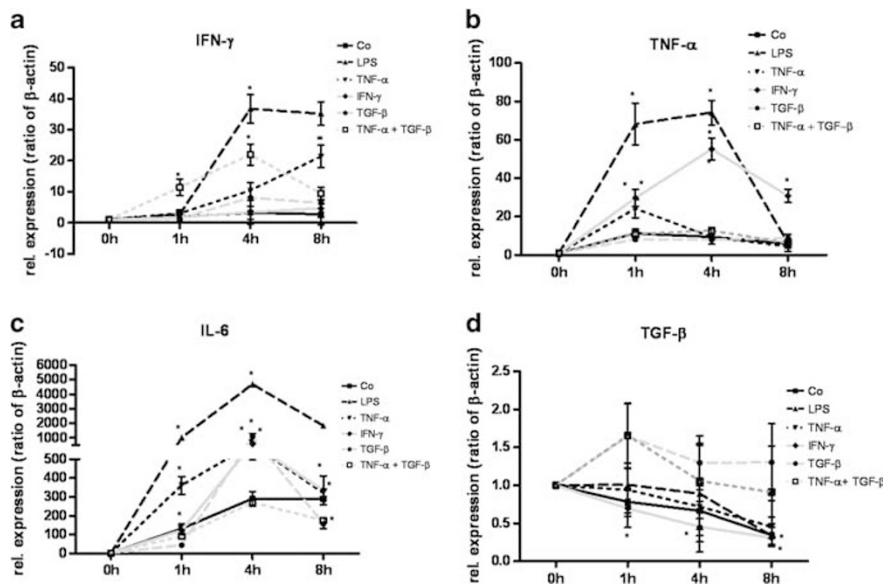


Figure 2 Fold change of mRNA expression of IFN- γ (a), TNF- α (b), IL-6 (c) and TGF- β (d) at different time points in *in vitro* activated human PBLs related to unstimulated cells and after treatment with the cytokines TNF- α , IFN- γ , TGF- β , TNF- α and TGF- β . RT-PCR data, shown as fold changes in mRNA expression normalized with the housekeeping gene β -actin. Results represent the mean value \pm s.e.m. of five separate experiments (* P < 0.05).

Furthermore, the anti-TNF- α antibody suppressed PHA-mediated ICAM-1 induction of gene expression (from 11.6 ± 0.08 - to 4.3 ± 0.1 -fold at 1 h, from 18.3 ± 1.8 - to 6.2 ± 1 -fold at 4 h and from 8.5 ± 0.6 - to 4.5 ± 1.7 -fold at 8 h, Figure 1b) as well as LPS-mediated ICAM-1 upregulation (from 11.7 ± 1.9 - to 7.7 ± 0.9 -fold). Infliximab had the same inhibitory effect on PHA or LPS-induced upregulation of VCAM-1 gene expression. In fact, it totally suppressed PHA and LPS effect at 1 h (from 10- to 3-fold and from 9.5- to 2.5-fold, respectively, Figure 1c).

Changes in Gene Expression of the Adhesion Molecules ICAM-1, PECAM-1, VCAM-1 and of the Pro-inflammatory Cytokines TNF- α , IFN- γ , TGF- β and IL-6 in Cytokine-Stimulated Human PBLs and Neutrophil Granulocytes

To characterize the response of human PBLs to inflammatory conditions, gene expression profiles of isolated PBLs, which were stimulated for 1, 4 and 8 h by TNF- α , IFN- γ , TGF- β , TNF- α and TGF- β in combination, as well as by PHA or LPS were examined. Stimulated samples were compared with unstimulated samples, which were used as control group.

TGF- β was the only stimulus able to induce a time-dependent induction of PECAM-1 gene expression with a peak effect occurred at 8 h (3 ± 0.3 -fold, Figure 3a), whereas TNF- α and IFN- γ inhibited PECAM-1 gene expression. Stimulation of PBLs with IFN- γ induced a sharp upregulation of ICAM-1 and VCAM-1 gene expression at 1 and 4 h after stimulation (37 ± 3 and 21 ± 2 -fold, Figure 3b and c). TNF- α induced ICAM-1 and VCAM-1 gene expression (13 ± 3 - and 21 ± 2 -fold after 1 h, Figure 3b and c).

As PBLs represent a 'co-culture' of leukocytes, pure granulocyte populations (with a very scarce presence of mono-

cytes < 1%) were also studied in the same way as PBLs. Using real-time RT-PCR, no effect on PECAM-1 or ICAM-1 gene expression was detected after treatment with PHA or LPS. The constitutive expression of PECAM-1 *in vitro* PBLs and granulocytes seems to be similar after analyzing the Ct values obtained from real-time PCR. On the contrary, a PECAM-1 downregulation and a ICAM-1 upregulation were observed 2 h after starting treatment with IFN- γ or TNF- α (data not shown). Notably, *in vitro* granulocytes isolation lead to a statistic significant spontaneous upregulation of PECAM-1 gene expression just 2 h after beginning the treatment.

Changes of ICAM-1 and PECAM-1 Gene Expression in PHA- and LPS-Stimulated Human Monocytic Cells, PBLs and Neutrophil Granulocytes

Western blot analysis of PECAM-1 and ICAM-1 in human U-937 and PBLs confirmed the results obtained for mRNA from activated PBLs as PECAM-1 levels decreased 4 h after U-937 stimulation as compared with the control samples (Figure 4a). At the same time, PHA and LPS induced a progressive time-dependent induction of ICAM-1 protein expression with the maximum value achieved at 4 h. Densitometry analysis for PECAM-1 and ICAM-1 western blots from U-937 cell lysates showed a significant downregulation of PECAM-1 after PHA- and LPS-stimulation. A 47 and 60% downregulation for PECAM-1 after PHA- and LPS-treatment was observed, whereas PHA and LPS induced respectively a 112% and 46% upregulation of ICAM-1 expression (Figure 4b).

Furthermore, western blot analysis of cell lysate from unstimulated and stimulated PBLs, using an antibody

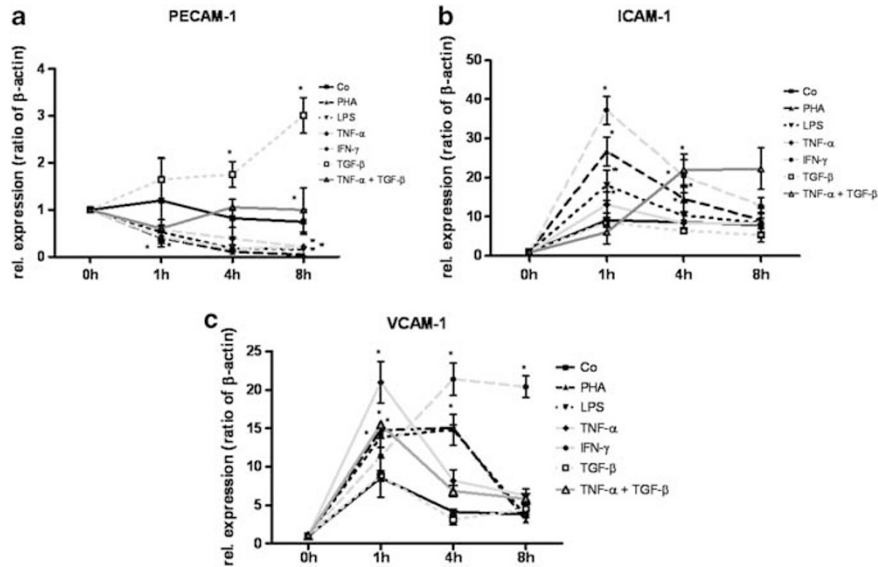


Figure 3 Fold change of mRNA expression of PECAM-1 (a), ICAM-1 (b) and VCAM-1 (c) gene expression at different time points in *in vitro* activated human PBLs related to unstimulated cells and after treatment with the cytokines TNF- α , IFN- γ , TGF- β , TNF- α and TGF- β . RT-PCR data were normalized with the housekeeping gene β -actin. Results represent the mean value \pm s.e.m. of five separate experiments (* $P < 0.05$).

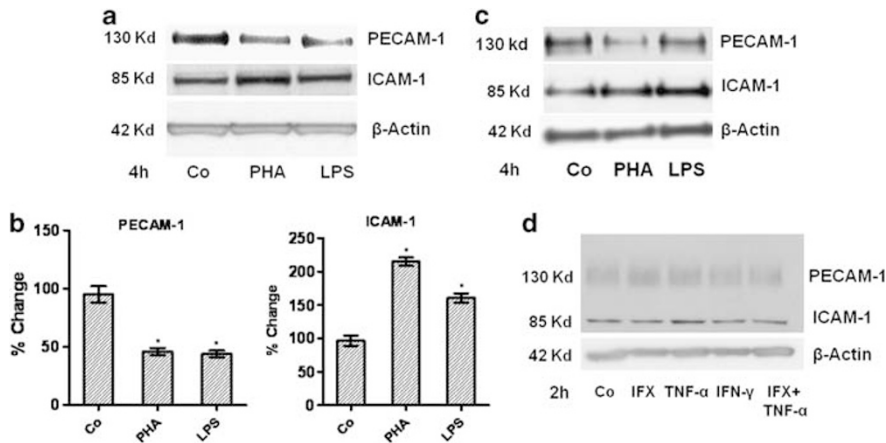


Figure 4 Western blot analysis of cell lysate from unstimulated, PHA- and LPS-stimulated U-937 cell line 4h after treatment with antibodies specific for PECAM-1, ICAM-1 and β -actin, (Co = control) (a). Densitometry analysis of the western blots (b) was performed to show the changes in protein expression of PECAM-1 and ICAM-1. Results represent mean values \pm s.e.m., (* $P < 0.05$, $n = 3$). Western blot analysis of cell lysate from unstimulated, PHA- and LPS-stimulated human PBLs 4h after treatment using antibody against PECAM-1 and ICAM-1 clearly showed a downregulation of PECAM-1 and an increase in ICAM-1 synthesis after treatment with PHA and LPS compared with unstimulated cells ($n = 3$, c). Western blot analysis of cell lysate from human granulocytes after 2h incubation with infliximab (IFX), TNF- α , IFN- γ , infliximab plus TNF- α compared with unstimulated cells ($n = 3$, d).

against PECAM-1 or ICAM-1, clearly showed a downregulation of PECAM-1 and an upregulation of ICAM-1 synthesis after treatment with PHA or LPS compared with unstimulated cells (Figure 4c), confirming real-time PCR results. Western blot analysis of protein lysates from human granulocytes also confirmed real-time PCR results as an induction of ICAM-1 and a suppression of PECAM-1 synthesis after incubation with TNF- α or IFN- γ was observed (Figure 4d).

Identification of PECAM-1⁺ in *In Vitro* Activated Human PBLs by Immunofluorescence Single Staining and by APAAP Immunostaining

By using a monoclonal antibody directed against PECAM-1/CD-31, immunoreactivity of PECAM-1 was detectable in unstimulated and activated PBLs confirming the results, obtained at the RNA and protein level with PECAM-1 expression being seen in stimulated and control PBLs (Figure 5). Compared with unstimulated cells, a significant

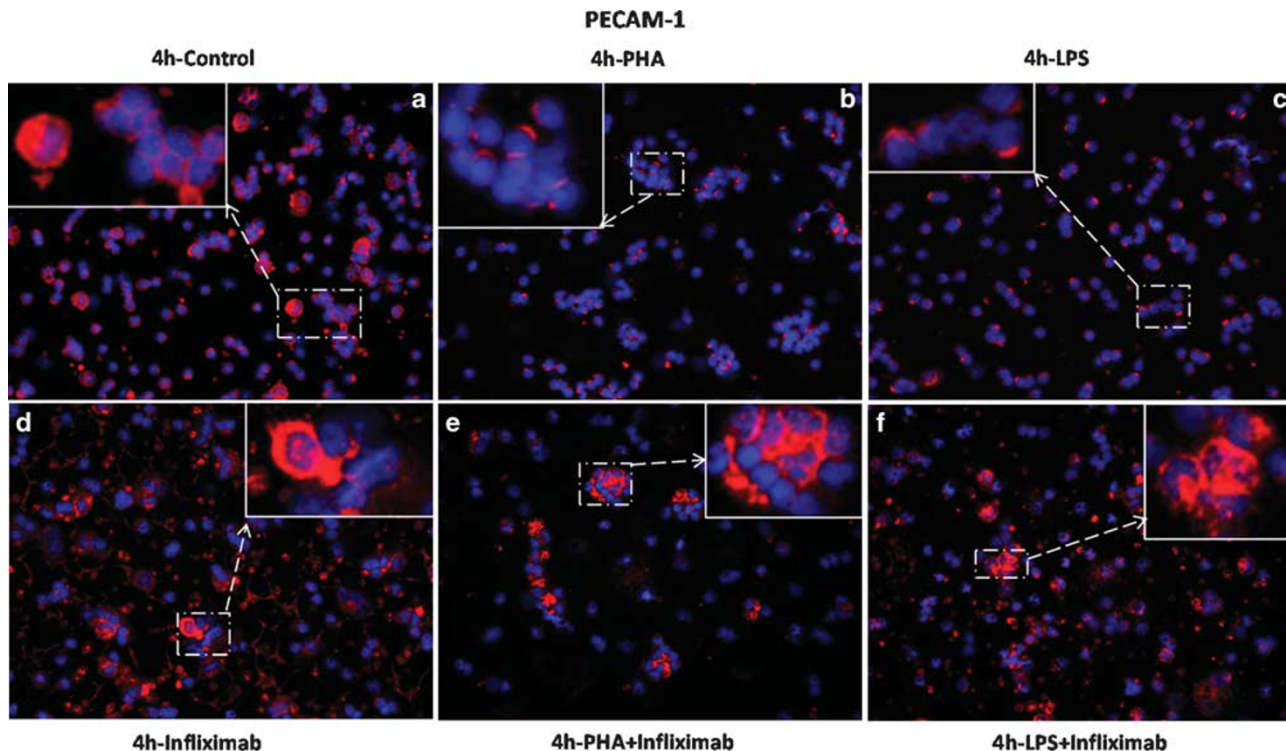


Figure 5 Immunofluorescence staining of human PBLs cytopspins with monoclonal antibody directed against mouse anti-PECAM-1 (red) followed by fluorescence immunodetection in cytopspins of unstimulated PBLs (a) and 4 h after treatment with PHA (b), LPS (c), infliximab (d) alone or in combination with PHA (e) or LPS (f). Original magnification: $\times 200$. Scale bar = $100 \mu\text{m}$.

rapid and progressive reduction of PECAM-1 positivity in PBLs stimulated with PHA or LPS was observed reaching a nadir of inhibition at 4 h, although reduced expression of PECAM-1 was evident already 1 h after stimulation. After PHA treatment a typical agglutination of PBLs occurred but no numerical reduction was observed. ICAM-1 and VCAM-1 gene expression in the same leukocytes were opposite to those obtained for PECAM-1 and were reduced in the presence of the anti-TNF- α antibody (data not shown).

APAAP immunodetection confirmed the results obtained by immunofluorescence staining as PECAM-1 expression in freshly isolated PBLs (Figure 6), consisting of neutrophil granulocytes and mononuclear cells, were PECAM-1 positive.

***In vitro* Identification of PECAM-1⁺/ICAM-1⁺ Cells in Activated Human PBLs and Neutrophil Granulocytes by Immunofluorescence**

Similar to the results obtained for mRNA and protein, ICAM-1 and PECAM-1 expression was observed in unstimulated PBLs (Figure 7a). After 4 h of stimulation with PHA or LPS, a reduction in PECAM-1⁺ cells and an increase in ICAM-1⁺ cells (Figure 7b, e) were observed. After counting the PECAM-1⁺ cells, we could demonstrate a significant reduction of PECAM-1⁺ after PBLs incubation with PHA or LPS. PECAM-1 expression in human PBLs stimulated with the anti-TNF- α antibody infliximab alone or in combination with PHA or LPS was demonstrated (Figure 7c, d, f).

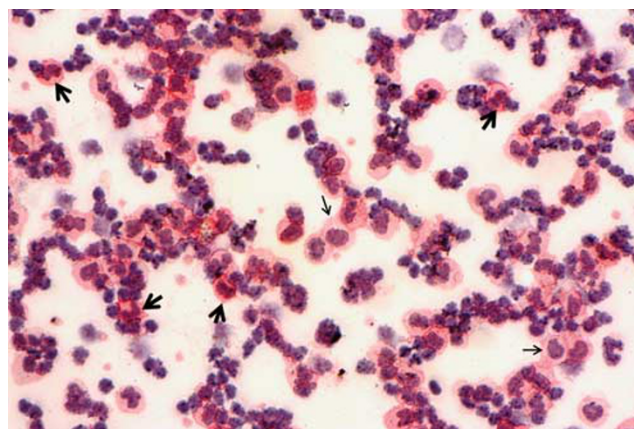


Figure 6 Indirect immunodetection of PECAM-1 in *in vitro* unstimulated human PBLs using the APAAP method. Cytopspins were stained with a mouse monoclonal antibody directed against human PECAM-1 followed by the APAAP immunodetection. The heavy arrows show neutrophil granulocytes but also mononuclear cells (thin arrows), which were PECAM-1 positive. Original magnification: $\times 200$. Scale bar = $100 \mu\text{m}$.

We next asked whether the presence of mononuclear phagocytes is necessary for the induction of the changes of ICAM-1 and PECAM-1 gene expression in granulocytes.

Using antibodies against ICAM-1 and PECAM-1, no significant difference in ICAM-1⁺ and PECAM-1⁺ granulocytes after treatment with PHA or LPS occurred (data not

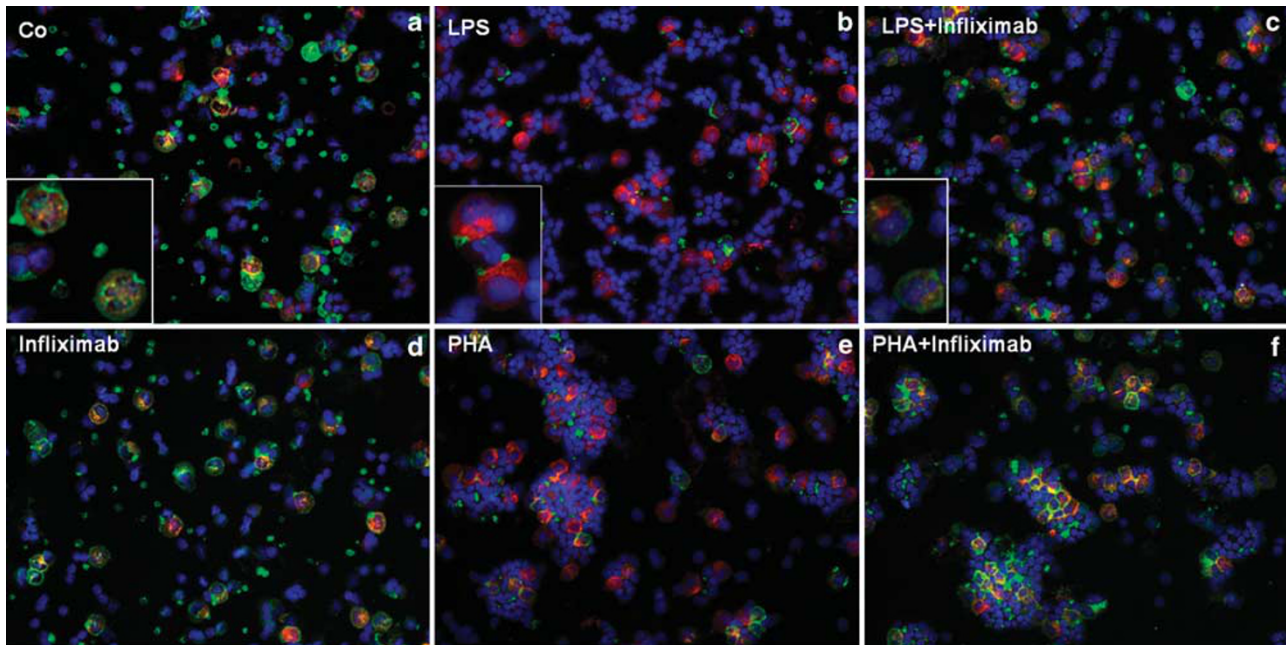


Figure 7 Immunofluorescence double staining of *in vitro* isolated human PBLs cytopspins with monoclonal antibody directed against mouse anti-PECAM-1 (green) and polyclonal antibody against rabbit ICAM-1 (red) followed by fluorescence immunodetection in PBLs unstimulated (a) and stimulated with PHA (e), LPS (b), infliximab alone (d) or in combination with PHA (f) or LPS (c). Original magnification: $\times 200$. Scale bar = $100 \mu\text{m}$.

shown). On the other side, after treating human granulocytes with $\text{TNF-}\alpha$, a progressive decrease of PECAM-1^+ cells and an increase of ICAM-1^+ cells (Figure 8) compared with unstimulated cells was demonstrated by performing separate immunofluorescence single- and double-staining for ICAM-1 and PECAM-1, indicating that the direct interaction of the mononuclear phagocytes with the granulocytes may not be necessary for inducing the observed changes.

It is noteworthy, that PECAM-1 is highly expressed on the cell surface of human PBLs and granulocytes (Figures 5, 7 and 8), on the other side ICAM-1 is only barely detectable in control PBLs and granulocytes (Figures 7 and 8).

DISCUSSION

During the past few years, advances have been made in the understanding of the pathogenesis of IBDs.²⁹ IBDs are characterized by continuous recruitment of leukocytes into the inflamed intestine. Neutrophils, as the first line of immune defense, are thought to combat pathogens in concert with mononuclear cells by phagocytosis, degranulation and the release of neutrophil extracellular enzymes. Intestinal macrophages are thought to orchestrate local inflammatory responses^{30,31} They are recruited, probably by activated macrophages, from the blood to sites of injury where they contribute to destruction of the initiating noxae and damaged tissue.

CD and UC are characterized by continuous recruitment of leukocytes into the inflamed intestine after contact with noxious agents. During acute flares of CD or UC there is a massive infiltration of neutrophil granulocytes into the

affected mucosa, manifested by an increase in stool granulocytes. When stimulated with pro-inflammatory cytokines (eg, $\text{IL-1}\beta$, $\text{TNF-}\alpha$, IL-6) or infected with microbial pathogens, intestinal epithelial cells secrete chemokines³² (eg, IL-8 , MCP-1 , MIP-1), directing migration and activation of neutrophils and mononuclear phagocytes from the capillaries of the intestinal villi into the injured tissue. The macrophage-derived inflammatory cytokines $\text{TNF-}\alpha$, $\text{IL-1}\beta$, $\text{IFN-}\gamma$ and IL-6 play a pivotal role in the induction and amplification of the inflammatory cascade in the gut, producing complex networks that activate neutrophils and mononuclear phagocytes in the capillaries to enter the lamina propria and produce tissue damage (see Supplementary Figure 1).

Several studies showed that macrophage-derived cytokines are more abundant than are T-cell cytokines in inflamed mucosa obtained from UC patients^{33,34} with active disease.

In vitro models of activated PBLs have been established in clinical immunology for several decades allowing the situation in acute and chronic inflammation to be mimicked.^{35–37}

Specifically activation of PBLs *in vitro* reproduces the cytokine expression¹⁹ present in inflamed intestinal mucosa¹¹ of patients with chronic intestinal inflammation.

A quantitative analysis of intestinal mucosal mRNA expression in colonic biopsies and PBL from IBDs patients has been previously performed.¹¹ This study showed that the expression of the acute phase mediators $\text{IL-1}\beta$, IL-6 and $\text{TNF-}\alpha$ is increased in the mucosa obtained from patients with active disease. Moreover the quantitative relationship between the cytokines observed *in vitro* mimics that observed in the inflamed mucosa *in vivo* as IL-6 , $\text{IL-1}\beta$, $\text{TNF-}\alpha$ and $\text{IFN-}\gamma$ are all strongly induced

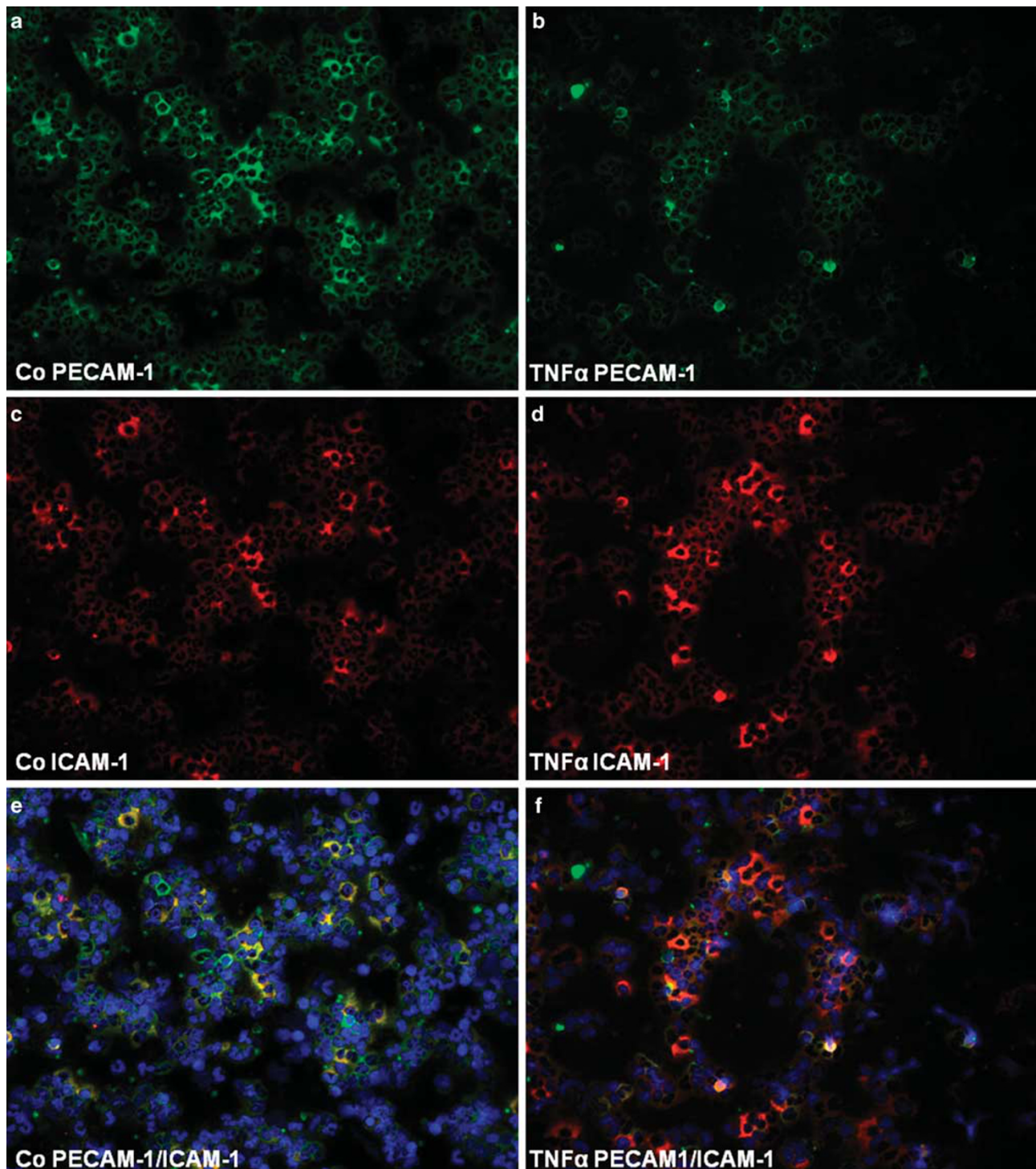


Figure 8 Immunofluorescence staining of *in vitro* isolated human granulocytes cytopspins with monoclonal antibody directed against mouse anti-PECAM-1 (green) and polyclonal antibody against rabbit ICAM-1 (red) followed by fluorescence immunodetection in unstimulated (**a** = control PECAM-1, **c** = control ICAM-1, **e** = double staining control ICAM-1/PECAM-1) and stimulated cells with TNF- α (**b** = TNF- α PECAM-1, **d** = TNF- α ICAM-1, **f** = double staining TNF- α ICAM-1/PECAM-1). Original magnification: $\times 200$. Scale bar = 100 μm .

in *in vitro* by ‘activated’ PBLs, as well as in colonic biopsies obtained from patients with acute flares of UC.¹⁹

The development of the anti-tumor necrosis factor (TNF) antibodies has revolutionized the management of many

chronic-inflammatory diseases and is now an approved treatment for CD, UC, juvenile RA, ankylosing spondylitis, psoriatic arthritis and psoriasis^{38,39} However, the mechanisms of their actions are unclear and $\sim 10\%$ of patients with

'refractory' or newly diagnosed CD fail to respond to anti-TNF treatment.⁴⁰

In normal conditions TNF- α has important roles in the development of the immune system and host protection against infectious pathogens. The altered mucosal immune response, characterized by an increased production of TNF- α by local macrophages or monocytes, is characteristic of intestinal mucosa obtained from individuals with IBD.⁴¹

Several studies have demonstrated that infliximab exerts its anti-inflammatory effect by downregulation of the pro-inflammatory cytokines TNF- α , IL-6, IL-1 β and IL-8.^{42,43}

Several families of molecules, including adhesion molecules and chemokines, regulate the influx of inflammatory cells from the blood into sites of inflammation, these molecules represent an interesting target for the treatment of IBD.

As proinflammatory cytokines induce gene expression of several CAMs, the primary goal of the present study was to investigate changes of gene expression of ICAM-1, PECAM-1 and VCAM-1, in *in vitro* activated human leukocytes and to examine the effect of infliximab on their expression.

The process of leukocyte rolling, adhesion and migration across blood vessels and sinusoids wall (diapedesis) involves various families of chemokines⁴⁴ and cell adhesion molecules, to include selectins,⁴⁵ integrins,⁴⁶ VCAM-1, ICAM-1,⁴⁷ ICAM-2,⁴⁸ junctional adhesion molecules,⁴⁹ VE-cadherin, platelet endothelial cell adhesion molecule-1 (PECAM-1, CD-31) and CD-99.⁵⁰ Although rolling and sticking are supposed to take place by the linking of vascular molecules to ligands expressed on the surface of leukocytes, leukocyte transmigration involves homophilic interactions between inflammatory cell molecules and the adhesion molecules ICAM-1, LFA-1 and ICAM-2.

PECAM-1 belongs to the Ig superfamily of cell adhesion molecules. It has been shown to be vital for the regulation of inflammatory responses, as it activates a variety of pro-inflammatory and anti-inflammatory functions in both leukocytes and endothelial cells.⁵⁰⁻⁵³ Ligands for PECAM-1 include integrin $\alpha v \beta 3$, $\alpha 6 \beta 1$ or CD38. PECAM-1 is also involved in leukocyte migration across the 'second barrier', the subendothelial basal membrane.⁵⁴ Blocking PECAM-1 in mice has been shown to prevent acute inflammation *in vivo*.⁵⁵ In addition antibody-mediated blocking of PECAM-1 reduces transmigration of monocytes, neutrophils and natural killer cells.⁵⁶ It has been suggested that PECAM-1 guides leukocytes through the endothelium via enhanced expression at endothelial cell junctions through recycling of PECAM-1-rich membrane invaginations at the lateral junctions of endothelial cells.⁵²

However the specific role of PECAM-1 during the inflammatory process is controversial. Changes of PECAM-1 gene expression on leukocytes under inflammatory conditions have not been carefully investigated.⁵⁷ It has been suggested that, in response to proinflammatory cytokines that include TNF- α , IL-1 β , IFN- γ and endotoxins, PECAM-1 expression is

not transcriptionally upregulated,⁵⁸ but is redistributed away from the intercellular junction of endothelial cells indicating that the spatial location of PECAM-1 has a regulatory role in the processes mediated by PECAM-1.⁵⁹

PECAM-1 gene expression is decreased *in vivo* in a rat model of liver damage occurring with an upregulation of TNF- α and IFN- γ gene expression, and under *in vitro* 'inflammatory conditions'.^{22,60} In fact, IFN- γ as well as TNF- α downregulates PECAM-1 and upregulates ICAM-1 gene expression in macrophages obtained from liver tissue and in endothelial cell cultures.

These results were confirmed in the present study in human granulocytes as ICAM-1 upregulation and PECAM-1 downregulation occurred after treatment with TNF- α and IFN- γ at RNA level and after performing immunohistochemical studies. The lack of effect by PHA and LPS on ICAM-1 and PECAM-1 gene expression in granulocytes, other than that observed in PBLs and monocytic cells, can be explained on one side by the almost complete lack of monocytes in the granulocyte culture, having as a consequence a lower amount of cytokines mRNA synthesis in these cells, and on the other side by the strong 'activation' of the granulocyte with a consequent downregulation of PECAM-1 gene expression as shown by the high Ct-values in freshly isolated control granulocytes.

By performing real-time PCR, we could clearly demonstrate that the massive production of acute phase cytokines, which is known to have a striking role in leukocytes activation induced by PHA or LPS in PBLs, cannot be shown in PHA or LPS stimulated granulocytes. TNF- α or IFN- γ , at the dosage used in the experiments, however, showed the expected effect on ICAM-1 and PECAM-1 gene expression also in granulocytes.

The late increase in TGF- $\beta 1$ gene expression in rat liver seen during the recovery period after CCl₄-induced damage,^{22,60} suggested that TGF- $\beta 1$ counteracts the effects of both IFN- γ and TNF- α by upregulating PECAM-1 and downregulating ICAM-1 gene expression.

The principal finding of this study is that PECAM-1 gene expression is downregulated under 'inflammatory' conditions in parallel with an increase in ICAM-1 and VCAM-1 expression;^{61,62} PECAM-1 downregulation and ICAM-1 upregulation in activated PBLs were completely suppressed by the anti-TNF- α antibody infliximab. These findings were confirmed by immunofluorescence detection of PECAM-1- and ICAM-1-positive cells, which showed a complete recovery of PECAM-1⁺ and a reduction of ICAM-1⁺ leukocytes and granulocytes after treatment with infliximab, alone or in combination with PHA or LPS. These results suggest that infliximab, by blocking TNF- α and consequently IFN- γ activity, may exert its anti-inflammatory effect also through downregulation of PECAM-1 gene expression and upregulation of ICAM-1 and VCAM-1 gene expression not only on the cells of the intestinal mucosa but also in the blood leukocytes.

The question of the mechanisms by which the anti-TNF- α antibody inhibits IFN- γ synthesis and which cellular mechanism is responsible for PECAM-1 recovery during anti-TNF- α treatment was therefore addressed.

Previously it has been shown that IFN- γ -treatment induces a reduction of PECAM-1 gene expression occurring in parallel with an increase in ICAM-1 expression in isolated sinusoidal endothelial cells and liver tissue macrophages.²² In the present work, we could confirm these results showing that PHA-, LPS-induction of TNF- α and IFN- γ produced a time-dependent PECAM-1 downregulation and TNF- α sharply upregulated IFN- γ gene expression in an *in vitro* leukocyte model. These results match previous findings reporting that the expression of PECAM-1, in *in vitro* isolated endothelial cells, drops due to an increase of TNF- α and IFN- γ .⁶³

Moreover, the results are consistent with the view that infliximab, by blocking soluble TNF- α , indirectly inhibits IFN- γ gene expression, preventing the downregulation of PECAM-1 and upregulation of ICAM-1 and VCAM-1 gene expression, not only on endothelial cells but also on blood leukocytes, a prerequisite for adhesion and transmigration of inflammatory leukocytes into tissue.

This effect of anti-TNFs in this sequence might contribute to a reduction in the inflammation in patients with IBDs.

The second important finding of the present *in vitro* experiments is that incubation of PBLs with TGF- β induced a time-dependent upregulation of PECAM-1 gene expression. TGF- β is a highly pleiotropic cytokine that stimulates most of the processes of wound healing and is considered to be the major profibrotic factor and it promotes angiogenesis both in *in vivo* and *in vitro* models.^{64,65}

The findings of this investigation may lead to reveal novel therapeutic targets in the treatment of both acute and chronic inflammatory disorders.

Supplementary Information accompanies the paper on the Laboratory Investigation website (<http://www.laboratoryinvestigation.org>)

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DISCLOSURE/CONFLICT OF INTEREST

The authors declare no conflict of interest.

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