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Alimentary Tract

# Direct effect of infliximab on intestinal mucosa sustains mucosal healing: exploring new mechanisms of action

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ABSTRACT

*Background:* Infliximab is effective in inflammatory bowel disease through several mechanisms, possibly acting at the mucosal level.
*Aim:* To assess the role of infliximab on intestinal mucosa and whether it contributes to mucosal healing.
*Methods:* Human colonic mucosal biopsies were incubated with or without infliximab. Cultured biopsies were evaluated for histological staining, CD68, CD3, E-cadherin and phospho-extracellular signal-regulated kinases (ERK) expression, and apoptosis. A scratch assay and MTT assay were performed with Caco2 cells in the presence of infliximab and/or tumour necrosis factor (TNF)-α or treated with supernatants obtained from human peripheral blood mononuclear cells or human intestinal fibroblasts treated with TNF-α and infliximab alone or in association.
*Results:* Infliximab-treated biopsies displayed a better histological appearance, reduced inflammation with an increase of E-cadherin, phospho-ERK and apoptosis. Supernatants showed lower TNF-α, IL-17, IL-6 and IL-8 concentration, with an increase in fibroblast-growth-factor. Motility at scratch assay and proliferation at MTT assay of Caco2 cells displayed differential modulation by TNF-α and infliximab, directly or through supernatants of human intestinal fibroblasts and human peripheral blood mononuclear cells exposed to them.
*Conclusion:* Infliximab contributes to the mucosal healing process by acting directly at an intestinal mucosal level; infliximab indirectly affects epithelial cell migration and proliferation by acting on both fibroblasts and leukocytes.


## 1. Introduction

Inflammatory bowel disease (IBD) is characterized by chronic colonic mucosal damage associated to an abnormal immune response against food or bacterial antigens in genetically predisposed individuals [1,2]. Tumour necrosis factor-α (TNF-α) is a key player in intestinal inflammation since it is involved in several immune reactions, such as the modulation of intestinal permeability, endothelial expression of adhesion molecules, and matrix metalloproteinase cleavage [3]. The gut wall is characterized by a continuous influx of leukocytes [4,5]. It is likely within the intestinal mucosa that anti-TNF-α agents, such as infliximab (IFX), act to neutralize immune and non-immune cell activations. As previously described by our group [6], measurable levels of IFX have been found within intestinal mucosa and even in faeces of IFX-treated animals.

IFX binds both soluble and membranous TNF (mTNF) and activates in vitro antibody- and complement-dependent cellular cytotoxicity by its Fc portion [7]. This TNF inhibition causes apoptosis of Jurkat T cells in vitro, and in vivo of lamina propria

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mononuclear cells (LPMC) CD3+, through the activation of caspase-8, -9, -3 and the increased transcription of the pro-apoptotic proteins Bax and Bak [8,9]. It is well established that IBD patients after IFX treatment show a different expression of adhesion molecules, such as E-cadherin, and of many leukocyte/endothelial cellular adhesion molecules (CAMs) and chemokines/chemokine receptors. At a sub-cellular level, TNF-α triggers phosphorylation of mitogen-activated protein kinases (MAPK), the up-regulation of vascular cell adhesion molecule 1 (VCAM-1), Intercellular Adhesion Molecule 1 (ICAM-1) and fractalkine (FKN) on human intestinal microvascular endothelial cells (HIMEC), and the production of IL-8 and monocyte chemoattractant protein-1 (MCP-1) by human intestinal fibroblast (HIFs) [10]. MAPK is one of the major signal transduction pathways [11]. Three major groups of MAPK have been identified in mammalian cells: the extracellular signal-regulated protein kinases (p42/44, also known as extracellular signal-regulated kinases or ERK), the p38 MAPK and the c-Jun N-terminal protein kinase (JNK) [12]. The blockade of p38 and p42/44 reduces the production of IL-8 and MCP-1 by HIFs, and the expression of CAM on intestinal endothelial cells are significantly down-regulated [10]. While Caprioli et al. have shown clear changes in immune cells within intestinal biopsies following intravenous IFX treatment [13], the purpose of this study is to show that the majority of these effects is actually promoted by IFX at a local mucosal level.

## 2. Materials and methods

### 2.1. Patient selection

Five patients with a well-established diagnosis of ulcerative colitis since at least 1 year were enrolled. All patients had clinically active disease and for this reason were admitted to the outpatient clinic. Endoscopy was performed as clinically indicated, and all had a Mayo score of 2. Patients had been receiving a stable dose of mesalazine for at least 2 weeks and were not on any other medications. Before the procedure, written informed consent was provided by all patients. For each patient 2 biopsies from an affected area were taken for tissue culture, one of which was snap-frozen, and the other was sent for routine histology. Biopsies from 5 healthy individuals, who underwent colonoscopy for colon cancer screening, were taken as controls. All patients provided written informed consent.

This project was approved by Ethical Committee of the Sacred Heart University of Rome (protocol number P/491/CE/2011).

### 2.2. Tissue culture

Colonic biopsies were washed with phosphate buffer saline (PBS), weighed and put in culture for 18 h on 30 μm PET track-etched membrane in 24 multiwell plates (VWR International PBI srl) with or without IFX (Remicade®, Centocor B.V, 50 μg/ml). In the control group, the same concentration of human IgG1 (Cusabio Biotech Co., LTD) was used. After 18 h, supernatants from colonic specimens were collected and stored at −80 °C for further analysis.

### 2.3. Peripheral blood mononuclear cells and human intestinal fibroblasts isolation

Human peripheral blood mononuclear cells (PBMCs) were isolated using Histopaque 1077 (Sigma–Aldrich Co., LLC). PBMCs were maintained in culture medium composed of RPMI 1640 (Lonza, Switzerland), 10% fetal bovine serum (FBS), 2 mM L-glutamine, 25 U/ml penicillin and 25 μg/ml streptomycin. Human intestinal fibroblasts (HIFs) were isolated as previously shown [10].

### 2.4. Supernatant generation

PBMCs were pre-activated with LPS 1 μg/ml for 24 h. PBMCs and HIFs were then cultured in 60 $mm^2$ cell culture dishes (250,000 cells seeded) in the presence of either: human IgG1 (Cusabio Biotech Co., LTD), IFX 50 μg/ml (Remicade®, Centocor B.V.), TNF-α 25 ng/ml (PrepoTech, London, UK) or IFX and TNF-α together at similar concentrations as previously described. After 24 h supernatants were collected and stored at −80 °C for further analyses as described below.

### 2.5. Histological-staining of cultured biopsies

Cultured biopsies, not treated with IFX, were fixed in 4% formalin and embedded in paraffin. After hydration, all sections were de-paraffinized and haematoxylin/eosin (H&E) staining was performed. For immunohistochemistry (IHC) the staining was carried out using anti-E-cadherin (pre-diluted, Dako Cytomation, Glostrup, Denmark) as the primary antibody. After incubation with secondary antibodies, the expression of the proper antigen in cells was detected with diaminobenzidine (DAB; Sigma, St. Louis, MO, USA). Colonic biopsies (treated with or without IFX) were also stained with anti-phospho p44/p42 ERKs (Cell Signalling technology, Denver, MA), -CD68 and -CD3 (DakoCytomation, Glostrup, Denmark) antibodies and counted using high-power lens [(40×) of the Nikon E400 Eclipse] microscopy. Macrophages and lymphocytes were manually scored in the whole section of the lamina propria and reported as cells/section. Tissue sections stained without primary antibody served as a negative control. The slides were then counterstained with Mayer's haematoxylin.

### 2.6. TUNEL assay

This method was used to assess apoptosis in intestinal biopsies (IFX-exposed and non-exposed) following manufacturer's instructions (ApopTag® Peroxidase Kits, Millipore, MA, USA). Nucleotides, contained in the Reaction Buffer, were enzymatically added to the DNA by terminal deoxynucleotidyl transferase (TdT), and allowed to bind an anti-digoxigenin antibody that is conjugated to a peroxidase reporter molecule.

### 2.7. Measurement of cytokines in supernatants of IFX-treated intestinal specimens from IBD patients

Cytokines from supernatants of intestinal biopsies were measured by Bio-Plex® suspension array. Bio-Plex Manager software was used to analyze data, which are presented as median fluorescence intensity (MFI) or as concentration (pg/ml). The higher and lower limits of detection are 20,000 pg/ml and 0.14 pg/ml (average limits, as for certain cytokines limits may vary, as specified by the manufacturer). Results from supernatants of intestinal specimens were related to the total protein concentration of these specimens, evaluated by spectrophotometry at λ of 650 nm using the Bradford colorimetric method.

### 2.8. Cell viability assay

Human colonic carcinoma cells (Caco2) were seeded at 50,000 into each well of a 24-multi-well plate in 500 μl of culture medium. After 24 h, Caco2 cells were treated with IFX 50 μg/ml and TNF-α 25 ng/ml alone or together, or with PBMC or HIF supernatant (obtained as previously described) diluted 1:4. After 24 h of each incubation period, two washes with medium without FBS were made and 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT, Sigma–Aldrich Co., LLC) was added to each well in 500 ml (0.5 mg/ml in medium without FBS). The amount of