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Author: Valentina Petito Loris Riccardo Lopetuso Egidio Stigliano Vincenzo Arena Alma Boninsegna Stefano Bibbò Andrea Poscia Sergio Alfieri Fausto Rosa Arianna Amato Giovanni Cammarota Alfredo Papa Alessandro Sgambato Antonio Gasbarrini Franco Scaldaferr

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

Valentina Petito¹, *, Loris Riccardo Lopetuso¹, * MD, PhD, Egidio Stigliano², Vincenzo Arena² MD, Alma Boninsegna² Prof, Stefano Bibbò¹ MD, Andrea Poscia³ MD, Sergio Alfieri⁴ Prof, MD, Fausto Rosa⁴ MD, Arianna Amato¹ MD, Giovanni Cammarota¹ Prof, MD, Alfredo Papa¹ MD, Alessandro Sgambato², # Prof, MD, PhD, Antonio Gasbarrini¹, # Prof, MD, and Franco Scaldaferri¹, # MD, PhD.

¹ Internal Medicine Department, Gastroenterology Division, Catholic University of Sacred Heart, Rome, Italy.
² Institute of Pathology, Catholic University of Sacred Heart, Rome, Italy.
³ Institute of Hygiene, Catholic University of Sacred Heart, Rome, Italy.
⁴ Surgery Department, Catholic University of Sacred Heart, Rome, Italy.
* equal contributors
# equal contributors

Corresponding author:
Franco Scaldaferri
Università Cattolica del Sacro Cuore, “A. Gemelli” Hospital,
Largo A. Gemelli 8, 00168, Rome, Italy
Tel/Fax (+39) 06 3015 5923
francoscaldaferri@gmail.com

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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.

Keywords: Infliximab, TNF-α, mucosal healing, wound repair, cellular proliferation.
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. 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.

The gut wall is characterized by a continuous influx of leukocytes. 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, 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. This TNF inhibition causes apoptosis of Jurkat T cells in vitro, and in vivo of lamina propria mononuclear cells (LPMC) CD3+, through the activation of caspase-8, -9, -3 and the increased transcription of the pro-apoptotic proteins Bax and Bak. 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).

MAPK is one of the major signal transduction pathways. 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)\textsuperscript{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\textsuperscript{10}.

While Caprioli et al. have shown clear changes in immune cells within intestinal biopsies following intravenous IFX treatment \textsuperscript{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.

**MATERIALS AND METHODS**

**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).

**Tissue culture**

Colonic biopsies were washed with phosphate buffer saline (PBS), weighed and put in culture for 18 hours 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 hours, supernatants from colonic specimens were collected and stored at -80 °C for further analysis.

**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), 2mM L-glutamine, 25 U/ml penicillin and 25µg/ml streptomycin.

Human intestinal fibroblasts (HIFs) were isolated as previously shown

**Supernatant generation**

PBMCs were pre-activated with LPS 1µg/ml for 24 hours. PBMCs and HIFs were then cultured in 60mm$^2$ 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 hours supernatants were collected and stored at -80 °C for further analyses as described below.

**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 [(40x) 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.

**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, Massachusetts, 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.

**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.

**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 hours 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 MTT salt was estimated by spectrophotometry at a wavelength of 540 nm, with 630 nm as a reference filter, using the Spectramas plus 384-molecular devices spectrophotometer. Cell viability (%) was shown by comparing the viability of treated cells with control cells treated with human IgG1. The score in the Results section, reported in round brackets, is the difference of Cell viability between two different treatments analyzed by Bonferroni post-hoc analysis.

**Scratch assay**

To evaluate the wound-healing process induced by IFX, and in particular the motility process associated to wound-healing by the intestinal epithelial monolayer, a scratch assay was performed. Four µ-Dish 35 mm, low Culture-Inserts (ibidi GmbH, Germany) were put into 24-wells and after their adhesion on the bottom of the well, CaCo2 cells were cultured into them (7x10^5 cells /mL and applied 70 µL per well of Culture Insert). These inserts allowed a wound of approximately 500 µm width. After 24 hours the inserts were removed and cells were treated with IFX 50 µg/ml and TNF-α 25 ng/ml alone or together or with PBMC or HIF supernatant diluted 1:4. The wound repair was monitored from time 0 until 24 hours using Nikon Eclipse TE2000-S microscopy. The microscopic pictures were analyzed with “WimScratch image analysis” software (ibidi GmbH, Wimasis- Image Analysis Platform, Germany). This software detects the cell-covered area in the picture and calculates the Scratch Area [%], Cell Covered Area [%], Speed [%/hour], and Acceleration [%/14^2]. The score in the Results section, reported in round brackets, is the difference of Cell Covered Area (different measurements were performed to obtain a mean value per condition) between two different treatments. Percentage of covered area was reported per each time point and condition. For statistics, Bonferroni post-hoc analysis correction was applied.

**Western Blot**
Caco2 cells were seeded in 10 cm² cell culture dishes (13x10⁵ cells/mL) After 24 hours
scratches were performed on the bottom of each dish and cells were treated with IFX 50
µg/ml and TNF-α 25 ng/ml alone or together for 6 hours. Phospho p42/p44 ERK (1:1000,
Cell Signalling Technology®) and anti α-Actinin antibody (1:500, Cell Signalling
Technology®) were assessed according to manufacturer. The protein signals were
observed using chemiluminescence kit (ECL Plus Western Blotting. Detection Reagents,
GE Healthcare- Life Sciences, Amersham). Films were scanned for densitometric analysis
with NIH Image J software.

Statistical analysis
ANOVA and adjusted Bonferroni post-hoc analyses were performed in order to assess
scratch and MTT changes during the different type of treatment (Control, TNF-α, IFX,
IFX+TNF-α) in each condition (Normal, with supernatants of PBMC or HIF). A p value of <
0.05 was considered statistically significant. Statistical analysis was performed using IC
STATA 12 for Mac.

RESULTS
Direct effect of IFX on intestinal biopsies from IBD patients
As the aim of this study was to show the effects of IFX administered directly onto colonic
mucosa, an in vitro system was used to culture and treat colonic biopsies from IBD
patients. Hematoxylin/eosin staining showed clearly how IFX can maintain the structure of
glandular epithelium in treated biopsies compared to untreated ones (figure 1, panel A).
This effect is also confirmed by the increased expression of E-cadherin, a component of
cellular junctions¹⁵, and by higher levels of phospho-p42/p44 ERK: (both of which are
markers of mucosal healing¹⁷,¹⁶) seen in areas with better preservation of the epithelium
(figure 1, panel B).
The effect of IFX on immune cells was then assessed. IFX exposure was associated to a reduced count of CD3+ and CD68+ cells (70 CPF in IFX treated biopsies and 870 in controls; figure 1, panel C), confirmed also by TUNEL assay, showing higher apoptosis within immune cells in intestinal mucosa biopsies treated with IFX (figure 1, panel D). These results were also enforced by the lower levels of innate and adaptive immune system cytokines found in supernatant of biopsies, with a clear decrease of TNF-α, INF-γ, Il-1b, Il-6 and Il-8 compared to controls and an higher content of basic FGF (figure 1, panel E).

Direct and indirect effect of IFX on intestinal epithelial cell healing model

Initially, the direct effect of IFX on epithelial cells was assessed. At scratch assay, TNF-α reduced the re-epithelialization time compared to control (-18.0, p<0.01); adding IFX to TNF-α, the re-epithelialization time was similar to control (-4.3, p<0.05). Furthermore, significant differences were found among TNF-α treatment and IFX treatment (-17.0; p<0.01) and IFX+TNF-α treatment (-14.0; p = 0.002; figure 2, Panel A).

Next, the indirect effect of IFX on epithelial cells following exposure to supernatant of PBMC or HIF was assessed. The scratch assay, performed in the presence of supernatant generated from PBMC exposed to TNF-α, was characterized by the absence of re-epithelialization, which was not detectable despite adding IFX. After 12 h, cells exposed to supernatants of PBMC treated with TNF-α alone or in combination with IFX lost vitality (figure 2, panel B). Caco2 treated with supernatants generated from HIFs exposed to TNF-α were characterized by a clear reduction of re-epithelialization rate at scratch assay, that was restored by adding IFX, with a re-epithelialization rate similar to control (figure 2, panel C).

Following completion of experiments dealing with the healing process or cell migration by scratch assay, the effect of IFX on proliferation of Caco2 was then assessed.
The direct effect of IFX on epithelial cells was assessed first. At MTT test, IFX and TNF-α alone or in association did not influence the proliferation rate of Caco2 (figure 3, panel A), while results were significantly different between control and IFX plus TNF-α treatment (-17.0; p = 0.043).

Finally, the indirect effect of IFX on epithelial cells, following exposure to supernatants of PBMC or HIF was assessed. Corresponding to what was observed at scratch assay, an absence of proliferation was found when epithelial cells were exposed to supernatants generated from PBMC treated TNF-α, with or without IFX (figure 3, panel A). In the presence of HIF supernatant, the proliferation of Caco2 cells seemed to be sustained: at MTT assay, adding IFX to TNF-α increased the percentage of cell viability similarly to controls (figure 3, panel A). Using supernatant generated from PBMCs, at MTT and scratch assay differences between baseline and 24 hours were significantly different between control and TNF-α treatment (-55.0; p = 0.036 and -97.6; p < 0.01 respectively), control and IFX+ TNF-α treatment (-76.6 p < 0.01 and -91.3; p <0.01 respectively), TNF-α and IFX treatment (-59.2; p = 0.023 and 81.6; p<0.01, respectively), IFX and IFX+TNF-α treatment (-75.3; p < 0.01 and -80.8; p <0.01 respectively).

At scratch assay results were also significantly different between control and IFX treatment (-16; p < 0.01) and between TNF-α and IFX+ TNF-α treatment (6.3; p < 0.01). When Caco2 cells were treated with supernatant from HIFs the results were significantly different only at scratch assay after 12 hours of treatment between control and TNF-α treatment (-6.6; p <0.01), control and IFX+TNF-α treatment (5.7; p = 0.013), TNF-α and IFX treatment (10.0; p <0.01), TNF-α and IFX+TNF-α treatment (12.3; p <0.01). At MTT and scratch assay, significantly different results were found among the different treatments (control, TNF-α, IFX and IFX+TNF-α) from different origins: from plain medium or PBMC supernatant or HIF supernatant.

**IFX sustains local mucosal healing by activation of MAPK pathway**
As with the human biopsies, p44/p42 ERK activation and expression were evaluated by western blot on CaCo2 cells treated with IFX and TNF-\(\alpha\) (alone or in association; figure 3, panel B and C). The blot showed clearly a lower level of p44/p42 ERK activation by adding TNF-\(\alpha\) in the cell medium; treatment with IFX was associated with a restoration of the p44/p42 ERK expression. Taken together this data suggests a possible role of IFX in enhancing wound repair through ERK pathway activation.

DISCUSSION

Few studies show how IFX acts on intestinal mucosal immune cells and how this influences intestinal epithelial cells behaviour or different cell-cell interactions within the mucosa itself. For the first time we addressed the direct effect of IFX on an entire intestinal biopsy from IBD patients, showing a direct effect of IFX on apoptosis, in particular of CD68\(^+\) and CD3\(^+\) cells, together with a clear action in maintaining the glandular epithelial architecture of the mucosa. IFX has previously been shown to induce apoptosis of monocytes\(^8\) and activated peripheral blood lymphocytes through the binding of membrane-bound TNF-\(\alpha\)\(^17\). These effects lead to the reduction of mucosal T cell proliferation\(^18\) when administered intravenously. As shown previously, cytokines such as TNF-\(\alpha\), IL-6 and IL-1\(\beta\) are considerably reduced in the supernatant of biopsies, with a decrease of their activity on the mucosal components. On the other hand, the increase of FGF, that took place during the regenerative process, matched the overexpression of E-cadherin, a marker of restored cell-cell adhesions\(^16\).

Wound healing is a complex biological process that requires interactions among fibroblasts, myofibroblasts, smooth muscle cells, endothelial cells, keratinocytes and immune cells\(^{19-22}\). These interactions are mediated by numerous factors such as growth factors. FGF is mitogenic and chemotactic for fibroblasts and influences angiogenesis, extracellular matrix deposition, the production of cytokines and the activation of several
pathways such as MAP kinases\textsuperscript{23,24}. Cell-cell contacts among gastrointestinal epithelial cells have a crucial role in tissue architecture to preserve the epithelial barrier from luminal antigens such as food or microorganisms\textsuperscript{25}. E-cadherin is one of the essential transmembrane proteins involved in the cell-cell junctions and its loss is associated with damage of gastrointestinal epithelium\textsuperscript{26}. The small GTPases of the Rho family (\textit{i.e.}, RhoA, Rac1, and Cdc42) are important for cytoskeleton reorganization since these small intracellular proteins are able to interact with downstream effector molecules such as Mek/ERK\textsuperscript{27-30}. Similarly, we demonstrated that local IFX administration increases E-cadherin expression with an up-regulation of ERK signalling. To better understand the action of IFX on intestinal epithelium we used CaCo2 cells incubated for 24 hours with either TNF-\(\alpha\), IFX (alone or in association) or human IgG1 as control. The most interesting data was obtained on CaCo2 treated with supernatants generated from PBMCs or HIFs. The repair process is strongly enhanced by HIF supernatant, which is faster (12 h vs 24 h), in particular when HIFs were previously treated with TNF-\(\alpha\) and IFX together. The wound repair stimulated by HIF supernatant suggests that fibroblasts could have a protective role in gut environment. In terms of whether HIFs sustain the motility of Caco2 at scratch assay, it seems that they reduce epithelial cell proliferation, particularly in the presence of TNF-\(\alpha\). The presence of IFX over TNF-\(\alpha\), partially decreases the inhibitory effect of HIF on Caco2 cell proliferation, obtaining a score at MTT similar to controls. We were surprised to find an apparent opposite effect of HIF on the healing process: the promotion of cell motility at scratch assay and a mild decrease in proliferation rate at MTT test. However these results should not be too much of a surprise as the two different pathways could participate differently to the entire healing process. It could be argued that the reduction of proliferation in the first part of the healing process is something physiologic and favors the healing process, which starts first by “closing the gap” and then inducing a real “regeneration” of the tissue.\textsuperscript{1} In this setting the role of anti-TNF-\(\alpha\) seems to
counterbalance the negative effect of TNF-α, moving things to a more physiologic homeostasis.

Our study also obtained additional important data on the role of PBMCs. PBMCs, when untreated or when exposed to anti-TNF-α, do not really affect intestinal epithelial cell motility at scratch assay and do not really influence the proliferation rate of Caco2 cells. On the contrary, when exposed to TNF-α, mimicking pro-inflammatory milieu, they potently interfere with intestinal epithelial cell function by inducing apoptosis both at scratch assay and MTT assay, suggesting that they are the major driver of mucosal damage. In this setting, IFX is not able to counterbalance their effect, in contrast to what has been shown in other sections of this paper. However, this is not unexpected: functional experiments of scratch and MTT assays are meant to evaluate only contact independent contributions of HIF and PBMC on epithelial cells. It could be argued that PBMCs release such a large number of pro-inflammatory cytokines in response to anti-TNF-α, that even in the presence of anti-TNF-α, the biological effect is still enormous. Data on file (not shown) suggests that reducing the number of PBMCs used to generate supernatant for the assays, also reduces the apoptosis of Caco2 cells in both MTT and scratch assay. The effect of anti-TNF-α on PBMCs, however, has already been studied and is not an aim of the present paper. Furthermore, the clear reduction of inflammatory infiltrate in biopsies from IBD patients exposed to IFX together with a better preservation of epithelial cells, suggest that IFX could act by reducing total number of inflammatory cells and/or perhaps modifying also contact dependent interactions between intestinal epithelial cells and activated leukocytes.

Furthermore our study shows that IFX restores p44/p42 ERK pathway activation on intestinal epithelial cells by western blot, similarly to what has been observed on culture of intestinal biopsies.
The effect of IFX on HIFs as well as PBMCs has been already addressed previously and was not an aim of the present study\textsuperscript{31-32}.

In conclusion our study obtained several important findings. Cell-cell interaction is crucially important in the mucosal healing process and activated PBMCs play a main role in inducing intestinal damage; furthermore they interfere with the healing process driven by intestinal epithelial cells. IFX contributes to the mucosal healing process by acting directly at the intestinal mucosal level where it induces apoptosis of leukocytes and directly sustains epithelial cell migration and proliferation, together with induction of p44/p42 ERK, as a result of the neutralizing effect of TNF-\(\alpha\). Furthermore IFX indirectly affects epithelial cell migration and proliferation by acting on both fibroblasts (HIF) and leukocytes (PBMC).

**CONFLICT OF INTEREST:** None declared.

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**ACKNOWLEDGEMENT**

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REFERENCES


FIGURE LEGENDS

Figure 1. Local effects of Infliximab on intestinal biopsies from inflammatory bowel disease patients.

Panel A) Hematoxilin/eosin staining. Left: biopsy from an active inflammatory bowel disease patient in culture with plain medium (without infliximab) for 18 hours; right: sample from an inactive inflammatory bowel disease patient, as internal control.

Panel B) Expression of E-cadherin (upper panels), and of Phospho-ERK (lower panels) in intestinal biopsies exposed or not to infliximab in vitro.

Panel C) CD68 and CD3 staining in intestinal biopsies cultured for 18 hours, exposed (right side) or not (left side) to infliximab.

Panel D) Lamina propria mononuclear cell apoptosis. Infliximab treatment permits a better preservation of the tissue and a better observation of the nuclei staining of lamina propria immune cells.

Panel E) The supernatants from biopsies cultured in vitro were collected to measure the levels of TNF-α, INF-γ, IL-1b, IL-6, IL-8 and basic FGF after 18 hours with or without infliximab. Results are representative of 5 independent experiments.

CTR, Control; IFX, Infliximab; Hu,: Human; IL, Interleukin; TNF, Tumor Necrosis Factor; IFN, Interferon; bFGF, basic Fibroblast Growth Factor

Figure 2. Infliximab effects on epithelial cells in vitro and wound healing. The pictures, on the left side, and the graphs on the right, indicate the % of cell-covered area for each sample assessed by “Wimasis-Image Analysis Platform” database. The cell covered area refers to where the scratch was performed at time 0 (value at time 0 = 0%)

CaCo2 cells were put in culture in 24-well with TNF-α (25 ng/ml) and infliximab (50 µg/ml) alone or in association for 24 hours in plain medium (Panel A), or from PBMC (Panel B) or...
HIF supernatants (Panel C) previously treated with TNF-α and/or infliximab. Results are representative of 3 independent experiments.

* p < 0.05 indicated in each figure (vs untreated of the same figure, see text for further details)

IFX, Infliximab; TNF, Tumor Necrosis Factor; PBMCs, peripheral blood mononuclear cells; HIFs, Human Intestinal Fibroblasts.

**Figure 3. Infliximab affects specific pathway of proliferation.** The graphs in Panel A indicate the % of cell able to metabolize the MTT salt after the treatment with TNF-α (25 ng/ml) and/or infliximab (50 µg/ml) for 24 hours in plain medium (on the left side), or from PBMC or HIF supernatants (on the right side) previously treated with TNF-α and/or IFX.

Panel B shows the p42/p44 ERK activation after treatment of Caco2 with TNF-α (25 ng/ml) and/or Infliximab (50 µg/ml). B-Actin is used as endogenous control. Panel C

Densitometry was obtained calculating Fold differences for each sample between loading control and target protein.

Results are representative of 3 independent experiments.

*p < 0.05; ****p < .0001

Sup., supernatant; CTR, control; IFX, infliximab; TNF, Tumour Necrosis Factor
Figure 1.

A) Active IBD, without IFX | Active IBD, with IFX | Inactive IBD

B) E-cadherin staining

C) CD68 staining

D) Apoptosis assessment by TUNEL assay

E) 

- Hu TNF-α
- Hu INF-γ
- Hu IL-1b

CTR, IFX
Figure 2.

**A) Unconditioned Media**

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**B) PBMC Supernatants**

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<tr>
<td>24h</td>
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<td><img src="imageB" alt="Image" /></td>
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</table>

**C) HIFs Supernatants**

<table>
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<tr>
<th>Time (h)</th>
<th>CTR</th>
<th>TNF-α</th>
<th>IFX</th>
<th>IFX + TNFα</th>
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<tr>
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<td><img src="imageC" alt="Image" /></td>
<td><img src="imageC" alt="Image" /></td>
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</tr>
</tbody>
</table>
Figure 3.

A) 

Cell viability (%) vs. Time (24h) 

B) 

Beta-Actin and Phospho-ERK protein bands 

C) 

Relative density of protein bands vs. Control 

Contrast images and graphs for cell viability, beta-Actin, and Phospho-ERK expression.