Comparable Immune Function Inhibition by the Infliximab Biosimilar CT-P13: Implications for Treatment of Inflammatory Bowel Disease

Ki Jung Lim, So Jung Lee, Sunghwan Kim, Su Yeon Lee, Min Seob Lee, Yoon A. Park, Eun Jin Choi, Eun Beom Lee, Hwang Keun Jun, Jong Moon Cho, Soo Young Lee, Ki Sung Kwon, Byung Pil Lim, Myung-Shin Jeon, Eui Cheol Shin, Yong Sung Choi, Ella Fudim, Orit Picard, Miri Yavzori, Shomron Ben-Horin, Shin Jae Chang

R&D Division, Celltrion Inc., Incheon, Korea
Translational Research Center and Inha Research Institute for Medical Sciences, Inha University School of Medicine, Incheon, Korea
Laboratory of Immunology and Infectious Disease, Graduate School of Medical Science and Engineering, KAIST, Daejeon, Korea
Department of Gastroenterology, Daehang Hospital, Seoul, Korea
Department of Gastroenterology, Sheba Medical Center & Sackler School of Medicine, Tel-Aviv University, Ramat-Gan, Israel

Corresponding author: Shin Jae Chang, PhD, R&D Division, Celltrion Inc., 13-1 Songdo-dong, Yeonsu-gu, Incheon 406–840, South Korea. Tel.: +82-32-850-4700; fax: +82-32-850-4633; email: ShinJae.Chang@celltrion.com

Abstract

Background and Aims: CT-P13 is the first biosimilar monoclonal antibody to infliximab, and was recently approved in the European Union, Japan, Korea, and USA for all six indications of infliximab. However, studies directly assessing the biologic activity of CT-P13 versus infliximab in the context of inflammatory bowel disease (IBD) are still scanty. In the present study, we aimed to compare the biological activities of CT-P13 and infliximab with specific focus on intestinal cells so as to gain insight into the potential biosimilarity of these two agents for treatment of IBD.

Methods: CT-P13 and infliximab were investigated and compared by in vitro experiments for their neutralisation ability of soluble tumour necrosis factor alpha (sTNFα) and membrane-bound tumour necrosis factor alpha (mTNFα), suppression of cytokine release by reverse signalling, induction of regulatory macrophages and wound healing, and antibody-dependent cell cytotoxicity (ADCC).

Results: CT-P13 showed similar biological activities to infliximab as gauged by neutralisation of soluble TNFα, as well as blockade of apoptosis and suppression of pro-inflammatory cytokines in intestinal Caco-2 cells. Infliximab and CT-P13 equally induced apoptosis and outside-to-inside signals through transmembrane TNFα (tmTNFα). Moreover, regulatory macrophage induction and ensuing wound healing were similarly exerted by CT-P13 and infliximab. However, neither CT-P13 nor infliximab exerted any significant ADCC of ex vivo-stimulated peripheral blood monocytes or lamina propria mononuclear cells from IBD patients.

Conclusions: These findings indicate that CT-P13 and infliximab exert highly similar biological activities in intestinal cells, and further support a mechanistic comparability of these two drugs in the treatment of IBD.

Key Words: CT-P13; infliximab; biosimilar; inflammatory bowel disease; biosimilarity; ADCC
1. Introduction

Tumour necrosis factor alpha [TNFα], an inflammatory cytokine, is known to play a central role in several chronic, immune-mediated inflammatory diseases [IMIDs]. The precursor form of TNFα, transmembrane TNFα [tmTNFα], is expressed as a 26 kDa, type II polypeptide on the cell surface of activated macrophages and lymphocytes as well as other cell types including mast cells, endothelial cells, cardiac myocytes, adipose tissue, fibroblasts, and neurons. tmTNFα functions not only as an activating ligand to trigger forward signalling into target cells but also as a receptor to transmit reverse signals into tmTNFα expressing cells, which can lead to numerous effects including apoptosis and cytokine suppression. tmTNFα is cleaved by the metalloproteinase, TNFα converting enzyme [TACE], which releases the secreted soluble form of TNFα, a 17 kDa polypeptide. Upon release, TNFα induces proliferation and differentiation of target cells by initiating signalling pathways that regulate gene expression including the upregulation of adhesion molecules, and thus promotes the inflammatory response in various diseases.

As a key early mediator of the inflammatory process, blockade of TNFα by anti-TNF agents has emerged as an effective treatment of diverse chronic inflammatory diseases such as rheumatoid arthritis [RA], psoriasis [PsO], ankylosing spondylitis [AS], and Behcet's disease. Similarly, the beneficial effects of anti-TNF therapy have been well established for Crohn's disease [CD] and ulcerative colitis [UC], which are collectively known as inflammatory bowel diseases [IBD].

Infliximab, one of the approved TNF blocking agents, is a chimeric anti-TNFα monoclonal antibody composed of murine variable and human constant regions of IgG1κ. This antibody binds with high affinity and specificity to both soluble TNFα and tmTNFα. After binding to soluble TNFα, infliximab blocks the interaction of this molecule with cellular receptors. In addition, infliximab binding leads to lysis of inflammatory cells expressing tmTNF through both antibody-mediated and complement-mediated mechanisms. Through reverse signalling, infliximab also induces apoptosis in monocytes and lymphocytes and suppresses the cytokine release in patients with CD, further blocking the inflammatory response. These effects, as well as induction of regulatory macrophages, have been proposed as infliximab's mode of action in IBD.

CT-P13 is the first biosimilar therapeutic to infliximab. Following extensive testing of physiochemical comparability and equivalent pharmacokinetic and clinical efficacy of CT-P13 and infliximab in two clinical trials in RA and AS, CT-P13 was recently approved by the European Medicines Agency [EMA], Korean MFDS, and US FDA for use in all indications for which the reference infliximab is approved, including RA, AS, CD, UC, PsA, and PsO. In the present study, we aimed to compare the biological activity of CT-P13 and infliximab in intestinal cells, so as to gain insight into their potential mechanistic biosimilarity for treatment of IBD.

2. Materials and Methods

2.1. Antibodies

Originator infliximab batches were purchased from pharmacies in the EU and USA and stored according to the manufacturer's instructions. The proposed biosimilar antibody, CT-P13, was manufactured in-house at Celltrion Inc., Korea. CT-P13 or infliximab was immediately reconstituted from commercial batches with water for injection [WFI] according to the manufacturing protocol from the vial before experiment and/or if the reconstituted samples were not immediately used, these were frozen with aliquot. In the case of long-term studies, samples were used within 1 month of storing at 2–8°C. There was no issue on stability of experimental materials. No dialysis was employed for the proteins.

2.2. Suppression of cytokine secretion in Caco-2 cells

Caco-2 cells were seeded at a density of 9 x 10⁴ cells/well in 96-well plates for a minimum 14 days, to allow differentiation of the cells. Differentiated cells were treated for 24 h with a mixture of cytokine stimulators [TNFα, 100 ng/ml; IL-1β, 50 ng/ml; LPS, 20 μg/ml; IFN-γ, 100 ng/ml] in order to mimic the intestinal inflammatory process. Cells were simultaneously treated with CT-P13 or infliximab for the same period of time. For IL-6 and IL-8 detection, cells were treated with 10 μg/ml of antibodies; 10 μg/ml concentration was chosen after preliminary experiments showed it to exert maximal inhibition. Following incubation, IL-6 and IL-8 were quantitated in the culture supernatants using a commercially available enzyme-linked immunosorbent assay [ELISA] [R&D Systems, Minneapolis, MN]. Percent inhibitory activity was calculated using the following equation:

\[ *\text{Inhibition} (\%) = 100\% - (\text{mean of IL-6 or IL-8 production from CT-P13 or infliximab} \times \text{treated cells} / \text{IL-6 or IL-8 production of reference material at each concentration} \times 100\% \]

2.3. Suppression of apoptosis in Caco-2 cells

Caco-2 cells differentiated for a minimum of 14 days were treated with a mixture of cytokine stimulators [TNFα, 25 ng/ml; IL-1β, 3.15 ng/ml; LPS, 1.25 μg/ml; IFN-γ, 6.25 ng/ml] for 24 h in order to mimic the intestinal inflammatory process, as described previously, in the presence of CT-P13 or infliximab at concentrations of 10 μg/ml. The concentration was determined in the linear range of the concentration-response curve for each analysis. Following incubation, cells were lysed and the activity of caspase 3/7 was measured using the Caspase-Glo® 3/7 Assay system [Promega, Fitchburg, WI]. Percent inhibitory activity was calculated using the following equation:

\[ *\text{Inhibition} (\%) = 100\% - (\text{mean of caspase activity from CT-P13 or infliximab} \times \text{treated cells} / \text{caspase activation from reference treated cells} \times 100\% \]

2.4. Induction of apoptosis by reverse signalling

The tmTNFα-expressing Jurkat cells were seeded onto six-well plates, and incubated with the antibodies for 24 h. Antibodies were used at 1, 0.6, 0.3, and 0.2 μg/ml.

Monocytes were obtained from peripheral blood mononuclear cells [PBMCs] of healthy volunteers [Cellular Technology Ltd, OH, USA] by positive selection using CD14 MicroBeads [Miltenyi Biotec, Berdisch Gladbach, Germany] and a MACS separator. Isolated CD14⁺ monocytes were incubated with anti-CD64 [FcγRII] mAb [Biolegend, CA, USA] at 4°C for 30 min to block FcR. After cells were washed, seeded to 1 x 10⁶ cells/well into 12 well-plates using RPMI1640 medium [Gibco] supplemented with 10% FBS. Subsequently cells were cultured with LPS 1 μg/ml and respectively with CT-P13 [10 μg/ml], infliximab,
or human IgG [10 μg/ml] for 24 h.24 Percentages of apoptotic cells were analysed using FITC Annexin V Apoptosis Detection Kit II [BD Pharmingen, San Diego, CA] by flow cytometry using a FACSCantoII® flow cytometer and FACSDiva® software. The apoptotic effect of each sample was assessed by calculation of population percentage gated as Annexin V-FITC/PI. Ten batches of CT-P13 and 20 batches of infliximab were used in apoptosis assay.

2.5. Suppression of cytokine [TNFα] secretion through reverse signalling
Antibodies at various concentrations [from 8000 to 468 ng/ml] were mixed with PBMCs from healthy volunteers. Following incubation, the cells were washed and subsequently incubated with complete media containing 1 μg/ml lipopolysaccharide [LPS] for 7 h, and then culture supernatants were harvested and subsequently analysed using the Quantikine™ assay [R&D systems] to quantify the amount of TNFα released from the PBMCs. Seven batches of CT-P13 and 14 batches of infliximab were used in this assay.

2.6. Inhibition of T cell proliferation [mixed lymphocyte reaction]
PBMCs from two Crohn’s disease [CD] patient donors were mixed at a 1:1 ratio and treated with 2.5, 0.625, or 0.156 μg/ml of each antibody. After 6 days, 0.5 μCi/ml (H)-thymidine was added to each well and, after an additional 18 h, cell proliferation based on (H)-thymidine incorporation was assessed. Cell viability was expressed as CPM [counts per minute]. Two batches of CT-P13 and of infliximab were used in this assay.

2.7. Induction of regulatory macrophages
PBMCs from two CD patient donors were mixed at a 1:1 ratio with 2.5 μg/ml of antibodies for 6–8 days. After incubation, the cells were re-suspended with vigorous pipetting and stained with anti-human CD14-PE [eBioscience, San Diego, CA] and anti-human CD206-Alexa Fluor 488 [eBioscience] for 30 min. As CD206 is a common regulatory macrophage marker, the CD206-positive cell population was considered to be the infliximab-induced regulatory macrophages. Expression of this population marker was analysed by flow cytometry using a FACSCantoII® flow cytometer and FACSDiva® software [BD Science] with default acquisition of 10,000 cells. Two batches of CT-P13 and of infliximab were used in this assay.

2.8. Isolation of CT-P13- or infliximab-induced macrophages
CT-P13-induced regulatory macrophages [M regs] and infliximab-induced M regs were isolated from mixed lymphocyte reaction [MLR] cultures from healthy volunteers as previously described.18 In brief, induced M regs were isolated from the MLR based on the expression of CD14 using CD14 microbeads according to the manufacturer’s protocol [Miltenyi Biotec, Bergisch Gladbach, Germany]. Cells were then counted and cultured in RPMI 1640 containing 10% heat-inactivated FBS. Two batches of CT-P13 and of infliximab were used in this assay.

2.9. Wound healing assay
In vitro wound healing assays were performed as previously described.54 One day before the experiment, HCT116 cells were seeded at a density of 8 × 10⁴ cells/well in 12-well plates and cultured up to 80–90% confluency. The HCT116 cell-seeded 12-well plates were marked to create a reference point to identify the location of the wound at time 0 h and 24 h. An artificial wound was made using a plastic tip to scratch the adherently grown cell culture, and images were taken with a phase-contrast microscope [Nikon Eclips TS100, 40×] to define the wound area at time 0 h. CT-P13- or infliximab-induced M regs or CD14 negative non-M regs were then added at a density of 5 × 10⁴ cells/well to the HCT116 cells. After 24 h, images of the wound were taken again to semi-quantitatively assess the degree of wound healing, which is represented by closing of the scratch-induced gap in the monolayer. The area of the wound was calculated as an arbitrary unit and the percentage of wound healing was calculated using Image J software with the following equation:

\[
\text{% Closure} = \frac{\text{area of wound at time 0 h} - \text{area of wound at time 24h}}{\text{area of wound at time 0h}} \times 100
\]

Two batches of CT-P13 and of infliximab were used in wound healing assay.

2.10. Determination of tmTNFα expression levels
To evaluate tmTNFα expression on LPMCs isolated from IBD patients and compared with LPS-stimulated PBMCs from healthy volunteers, FACS analysis using a labelling antibody was performed. Anti-human CD13 APC antibody and anti-human HLA-DR PerCP-Cy5.5 antibody [Bioscience, CA, USA] for detection of monocyte populations within LPMCs from IBD patients and PBMCs from healthy volunteers were used. An FITC-labelled Fab’ fragment of CT-P13 was used for detection of tmTNFα expression levels on the cell surface. The tmTNFα expression levels on the Jurkat cell line and tmTNFα Jurkat cell line were also determined with an FITC-labelled Fab’ fragment of CT-P13. An FITC-labelled human IgG was used as a negative control. The tmTNFα expression level on various target cells was calculated as the mean fluorescence intensity [MFI] of FITC staining.

2.11. ADCC assay
2.11.1. Isolation of lamina propria mononuclear cells and natural killer cells
Lamina propria mononuclear cells [LPMC] were obtained from consecutive IBD patients undergoing colonoscopy in Sheba Medical Center Gastroenterology Department. LPMC were isolated as previously described.54 Briefly, intestinal samples were washed twice with 10 ml PBS with 0.75 mM EDTA and antibiotics [100 μg/ml streptomycin and 100 μg/ml penicillin], and then twice with 10 ml Hanks’ salt solution with 1 mM EDTA and antibiotics. Samples were then passed four times through an 18G needle and incubated twice at 37ºC for 30 min in RPMI with FCS 20% and 300 μg/ml collagenase A [Sigma Aldrich] with 10 μg/ml DNase [Sigma Aldrich] with antibiotics and 25 μg/ml amphotericin with 10 mmol/l HEPES buffer. Cells from the supernatant of the digested samples were then collected, filtered, and washed. Cells were subsequently subjected to centrifugation through a Percoll gradient [Sigma], and the lymphocytes at the 40/80% interface were collected.

In parallel, PBMC were isolated from the same IBD donors by density centrifugation on a Ficoll-Hypaque gradient [Sigma]. Natural killer [NK] cells were purified from PBMC by an NK cell-negative selection magnetic separation kit, according to manufacturer instructions [Miltenyi Biotec, Cologne, Germany].
2.11.2. ADCC in various target cells using NK cells as effector cell populations

For ADCC activity of CT-P13 and infliximab on various target cells, the ADCC effect was measured by fluorescence through calcein-AM release using a Jurkat cell line, a tmtNFαααααJurkat cell line, LPS-stimulated CD14+ monocytes as target cells, and NK cells as the effector cell populations. Antibodies, calcein-AM labelled target cells, and effector cells were plated onto 96-well plates sequentially. After incubation for 4 h, cell lysis could be detected in the supernatant by detection of released fluorescence signals using a SpectraMax M5 plate reader [Molecular devices, CA].

2.11.3. ADCC activity in LPMC

LPMC were re-suspended in RPMI 1640 medium supplemented with 10% inactivated FCS, 2 mM L-glutamine and antibiotics. LPMC were then stimulated by 10 ng/ml LPS [E. coli, L6529-Sigma, USA] and were incubated for 1h in 96 U-shaped culture plate wells at 1 x 10^7 cells/well containing 100 μl of medium. LPMC were then incubated for an additional 30 min after the addition of 50 μg/ml or 10 μg/ml as designated of either US-infliximab batch ECD18012P1, or CT-P13 batch 12B1C005, or control IgG1 [O15IK01, SouthernBiotech, USA].

PB NK cells were loaded with 0.25 μM CFSE [Molecular Probes, USA], and were added to the LPMC at a 2:1 effector:target final ratio and cultured for 4 h in a 37°C 5% CO2 incubator. A negative control well with LPMC alone was also included. Cells were harvested after 4 h of co-culture and stained by 0.3 μg/ml propidium iodide [P4864, Sigma, USA] for 10 min. The percentage of dead [PI-positive] cells in the CFSE-negative gated population of LPMC target cells was detected by flow cytometry [Navius 6 colours/2 lasers, Beckman Coulter] and Kaluza flow cytometry analysis software [Beckman Coulter]. The percentage of NK-mediated lysed cells was computed by the following formula:

\[
\% \text{ Cell lysis} = \left( \frac{(E - S)}{S} \right) \times 100, 
\]

where E designates the percentage of dead [PI-positive] LPMC in the ADCC experiment in the presence of NK cells, and S designates the percentage of spontaneously dead [PI-positive] LPMC in the absence of NK cells.

To exclude the possibility that lack of increased ADCC killing by the NK was due to any inherent incompetence to exert cytotoxicity, an additional experiment was performed. In this experiment, NK cells obtained from three of these IBD donors were loaded with CFSE and used as effector cells against K562 lymphoblastic leukaemic cells [ATCC CCL-243™] as the target cells, employing the same experimental conditions as above. Briefly, K562 cells were similarly cultured alone or with CFSE-stained peripheral blood NK cells from IBD donors, harvested after 4 h as above, and NK-mediated cell lysis was determined by FACS analysis-based determination of the percentage of CFSE-negative PI-positive K562 cells, with or without co-cultured NK cells, as above.

2.12. Statistical analysis

Statistical evaluations were performed using Student’s t-test with GRAPHPAD Prism 4.0 [GraphPad Software, LaJolla, USA]. p-value < 0.05 was considered as statistically significant. Statistical analysis of ADCC assay with LPMC from IBD patients was performed using the Wilcoxon’s rank sum test. All data are expressed as mean ± SEM [standard error of the mean].

3. Results

3.1. CT-P13 and infliximab similarly suppress pro-inflammatory cytokine secretion and induce apoptosis of intestinal epithelial cells by neutralisation of soluble TNFα

In IBD patients, TNFα is highly produced by immune cells and recruits immune cells to inflammation sites. This induces epithelial cells and immune cells to release inflammatory cytokines and mediators. sTNFα neutralisation and sTNFα inhibition of production by infliximab can ameliorate IBD by downregulating the expression of inflammatory cytokines and mediators in intestinal tissues.26

Thus, we evaluated the biosimilarity of CT-P13 and infliximab with respect to the suppression of pro-inflammatory cytokines, such as IL-6 and IL-8, in the human intestinal epithelial Caco-2 cell line activated with several inflammatory cytokines [TNFα, IL-1β, IFN-γ] and LPS.26

As shown in Figure 1, CT-P13 and infliximab similarly inhibited the secretion of representative inflammatory cytokines, IL-8. The IL-8 secretion from Caco-2 cells was decreased 32.1 ± 3.3% and 32.9 ± 1.8% after treatment of CT-P13 and infliximab, respectively [number of tested batches: CT-P13 = 10, infliximab = 20, p-value = 0.8221]. In the same manner, CT-P13 and infliximab also inhibited IL-6 secretion from Caco-2 cells by 43.1 ± 2.1% and 46.4 ± 5.5%, respectively. [Data not shown, number of tested batches: CT-P13 = 2, infliximab = 2, p-value = 0.3011].

TNF is reported to trigger epithelium apoptosis thereby leading to tissue degradation and intestinal wall damage in IBD patients.27,28

Thus, the ability of CT-P13 and infliximab to suppress intestinal epithelium apoptosis through neutralisation of sTNFα was also evaluated using TNFα-stimulated Caco-2 cells and determination of caspase activity with or without the anti-TNFα antibodies. As a result, activation of caspase 3/7 from Caco-2 cells was decreased 59.9 ± 6.1% and 50.8 ± 10.2% by CT-P13 and infliximab, respectively, demonstrating similar capabilities to suppress sTNFα-induced apoptosis of Caco-2 cells [data not shown, number of tested batches: CT-P13 = 2, infliximab = 2, p-value = 0.9468]. Therefore, these results indicate that CT-P13 and infliximab exhibit biosimilarity in their in vitro blocking potency on sequential effects of sTNFα on intestinal epithelium, such as cytokine release and induction of apoptosis.

![Figure 1](https://academic.oup.com/ecco-jcc/article-abstract/11/5/593/2503247)

*Figure 1. Suppression of pro-inflammatory cytokine secretion on human intestinal epithelial Caco-2 cells CT-P13 and Remicade®. The ability of CT-P13 and Remicade® to suppress pro-inflammatory cytokine secretion through neutralization of sTNFα was evaluated using TNFααααα, IL-1β, IFN-γ and LPS-stimulated Caco-2 cells. Cells were treated with 10 μg/mL of each CT-P13 and Remicade®. Secreted IL-8 was quantitated in the culture supernatants.*
3.2. Induction of cell cytotoxicity and cytokine suppression via tmTNFα-mediated reverse signalling by infliximab and CT-P13

As part of their mechanism of action in IBD, TNF antagonists bind to tmTNFα and elicit reverse signalling-induced apoptosis and cytokine suppression in monocytes, T cells, and Jurkat cells.\textsuperscript{14,17,29} Therefore, we first examined apoptosis induced by reverse signalling in the Jurkat cells stably overexpressing tmTNFα. Following exposure to different concentrations of CT-P13 and of infliximab, the percentage of apoptotic cells was found to increase in a concentration-dependent manner [Figure 2A]. No significant difference in relative apoptosis-inducing ability on inflammatory immune cells was observed between CT-P13 and infliximab [number of tested batches: CT-P13 = 10, infliximab = 20, \(p\)-value > 0.05 for all data points for comparisons of CT-P13 and infliximab].

Pre-treatment of monocytes with TNFα blocking agents prevents the LPS-induced release of apoptotic factors and soluble cytokines such as TNFα, IL-1, IL-6, and IL-10 in IBD. Thus, we further examined the effects of CT-P13 and of infliximab on suppression of cytokine [TNFα] release induced from LPS-stimulated PBMCs from healthy volunteers. As shown, the secretion of TNFα from PBMCs similarly decreased with increasing graded concentrations of infliximab and CT-P13 [Figure 2B]. These results indicate that CT-P13 and infliximab exert similar dose-dependent cytokine suppression of inflammatory immune cells, presumably mediated through reverse signalling [number of tested batches: CT-P13 = 7, infliximab = 14, \(p\)-value > 0.05 for all data points for comparisons of CT-P13 and infliximab].

3.3. CT-P13 and infliximab similarly induce differentiation of monocytes into regulatory macrophages and promote wound healing \textit{in vitro}

Recent studies have shown that infliximab decreases the proliferation capacity of activated T cells in the environment of a mixed lymphocyte reaction but not in assays using isolated T cell populations.\textsuperscript{17,29} This observation has been attributed to the role of regulatory macrophages [M regs]. M regs, also called ‘wound healing macrophages’,\textsuperscript{10} are known to mediate immune suppressive effects and are involved in mucosal healing and gut homeostasis.\textsuperscript{14} It has been reported that the percentage of M regs is significantly increased in CD and UC patients who are responsive to infliximab therapy, suggesting that they are linked to a therapeutic response through involvement in the process of mucosal healing.\textsuperscript{19}

Therefore, we assayed T cell proliferation in a mixed lymphocyte reaction [MLR] assay following antibody treatment and observed that CT-P13 and infliximab similarly inhibited T cell proliferation in a concentration-dependent manner [Figure 3A].

Subsequently, the effect of CT-P13 on the induction of M regs and subsequent wound healing was investigated. CT-P13- or infliximab-induced CD14+ macrophages were isolated by a CD14-positive microbeads selection from MLRs cultures of CD patient-derived PBMCs. The M reg populations induced by 2.5 µg/ml CT-P13 or infliximab were assessed by CD206 expression and found to be similar [5.2 ± 1.0% and 5.5 ± 1.2%, respectively, number of tested batches: CT-P13 = 2, infliximab = 2] [Figure 3B].

To assess the wound-healing capacity of these M regs, we performed a wound healing assay using an HCT116 epithelial cell monolayer. CT-P13- or infliximab-induced M regs, CD14-negative PBMCs similarly decreased with increasing graded concentrations of infliximab and CT-P13 [Figure 2B].

3.4. Correlation between expression levels of tmTNFα on various target cells and antibody-dependent cell-mediated cytotoxicity activity of CT-P13 and infliximab

Antigen expression level on the target cell surface is an important factor that affects ADCC, as the potency of cytotoxicity increases according to the density of IgG coating the target cell surface. Thus, we investigated the correlation between ADCC activity and tmTNFα expression on target cells. To this end, the tmTNFα levels were evaluated by fluorescence-activated cell sorting [FACS] analysis using FITC-labelled human IgG and FITC-labelled CT-P13 on
Various target cells: Jurkat cells, tmTNFα-overexpressing Jurkat cells, monocytes within LPS-stimulated PBMCs from healthy volunteers, monocytes within LPS-stimulated PBMC derived from a CD patient, and monocytes within lamina propria mononuclear cells (LPMCs) derived from a CD patient (Figure 4A and B).

The results showed the various target cells to possess similar low expression levels of tmTNFα on the cell surface except for the tmTNFα-overexpressing Jurkat cells (Figure 4A and B). The expression level of tmTNFα Jurkat cells was approximately 80-fold higher than other target cell lines. Even the human monocytes derived from LPS-stimulated PBMC and LPMC from IBD patients both expressed very low levels of tmTNFα. These data indicate that only low levels of tmTNFα are expressed on monocytes in the lamina propria of IBD patients in physiological conditions.

We next compared the ADCC potency of CT-P13 and infliximab by a calcine-AM release assay, using natural killer (NK) cells as the effector cells and different cell populations as the target cells. The results showed that CT-P13 and infliximab similarly induced cell lysis of over-expressing tmTNFα Jurkat cells in a dose-dependent manner [p-value > 0.05 for all data points for comparisons of CT-P13 and infliximab] (Figure 4C). However, ADCC was not induced by either drug in any of the other more physiologically relevant target cell populations, including wild-type Jurkat cells not stably transfected by tmTNFα as well as LPS-stimulated PBMCs obtained from a CD patient.

Furthermore, apoptotic activities of CT-P13 and infliximab was also assessed in LPS-stimulated monocytes obtained from PBMCs of healthy volunteers, to mimic the same target cells used in ADCC assay. Contrary to the ADCC results, 10 µg/ml of CT-P13 and infliximab induced apoptosis in 34.4% and 36.6% of monocytes, respectively, compared with 20.2% apoptosis induction by control IgG [Figure S1, available as Supplementary data at ECCO-JCC online].

These results indicate that CT-P13 and infliximab induce apoptosis of monocytes but do not exert ADCC of these same cells under similar conditions.

Finally, the ADCC efficacy of infliximab and CT-P13 were compared using LPMC obtained from IBD patients as target cells, which are the most representative of the purported target cells for infliximab in vivo resumed. The percentage of ADCC cell lysis of LPMC by NK cells, as mediated by IgG1, infliximab at 50 µg/ml, and CT-P13 at 50 µg/ml, was computed according to the formula described in the Methods section for patients with sufficient numbers of LPMC (n = 6), and is shown in Figure 4D. As shown, there was no statistically significant difference between ADCC of LPMC mediated by CT-P13 and by infliximab [p = 0.3, Wilcoxon rank sum test] nor by CT-P13 or US-infliximab compared with control IgG1. For comparison, NK cells from the same IBD donors were also assessed for their cell-lysis activity towards the K562 erythroleukaemia cell line, which cells are conventional targets for assessing NK-mediated tumour cell killing. As opposed to the results observed with the same NK cells with infliximab towards LPMC, the cell lysis of K562 cells by these CT-P13 or US-infliximab was three orders of magnitude higher, further supporting a negligible infliximab-mediated ADCC activity in vivo [Figure 4D].

Taken together, these findings suggest that significant tmTNFα expression on cells is a prerequisite for ADCC activity. Thus, ADCC is unlikely to be instrumental in infliximab’s mode of action in IBD in vivo due to the very low expression levels of tmTNFα on cells of IBD patients in physiological conditions.

4. Discussion

Although their structure and physiochemical properties have been previously reported to be highly similar,14 there are still scant
data comparing the functionality of CT-P13 and infliximab with respect to inhibiting immune functions, especially in the gut. The present study investigated functional inhibition of immune attributes by CT-P13 and infliximab, and found comparable blockade of both soluble and tmTNFα on intestinal cells, thereby providing in vitro evidence for the potential mechanisms whereby CT-P13 may exert therapeutic effects in patients with IBD, similarly to infliximab.

Figure 4. Expression levels of tmTNFα on various target cells and ADCC activity of CT-P13 and Remicade® against target cell type. Representative Expression levels of tmTNFα on various target cells for ADCC assay were measured by FACS analysis using FITC-labeled CT-P13 and compared with FITC-labeled human IgG treated cells. The results are represented as histograms (A) and MFI values (B). (C) Percentage of ADCC effects of CT-P13 and Remicade® in various target cells with different tmTNFα expression levels. (D) Percentage of NK mediated cell-lysis of LPMC in the presence of the designated antibodies in IBD patients (n=6) compared to IBD-derived NK mediated cell lysis of target K562 cells (n=3).
Although various classes of TNFα-blocking therapies have been developed, they each exhibit unique efficacy profiles. Whereas infliximab\textsuperscript{12,33} and adalimumab\textsuperscript{13,34} were shown to be effective in both inducing and maintaining remission in CD patients, two soluble receptors, etanercept and oncept, were ineffective in inducing remission in CD patients.\textsuperscript{5,24,26} In addition, the humanised anti-TNFα antibody, CDP571, which was designed as an IgG to reduce interaction with Fc receptors in the hope of reducing side effects, also failed to show effectiveness in CD.\textsuperscript{35}

Infliximab is a chimeric human-murine monoclonal antibody directed against TNFα, which has shown particularly high effectiveness in the clinical management of IBD. Since the approval of infliximab in the European Union [EU] in 1999, the biological action of this antibody in IBD has been extensively explored\textsuperscript{28} and can be categorised as affecting two unique pathways. The primary mechanism of action, common to all TNF-blocking agents, involves soluble TNFα neutralisation by direct binding to TNFα. The blockade of soluble TNFα-induced inflammatory activities results in reduced secretion of pro-inflammatory cytokines such as interleukins [IL-1 and IL-6],\textsuperscript{36} inhibition of leukocyte migration by reducing endothelial layer permeability and arrogating expression of adhesion molecules by endothelial cells and leukocytes,\textsuperscript{19,36} inhibiting neutrophil and eosinophil functional activity,\textsuperscript{36,41} diminishing acute phase reactants such as CRP and other liver proteins\textsuperscript{46} as well as tissue-degrading enzymes produced by synoviocytes and/or chondrocytes,\textsuperscript{45} and inhibiting sTNFα-TNFR1-mediated induction of apoptosis of immune tissue cells and intestinal epithelial cells.\textsuperscript{46}

In addition, through a second mechanism consisting of reverse signalling, infliximab downregulates the production of pro-inflammatory cytokines by stimulated monocytes via binding to tmTNFα located on the cell surface of immune cells such as NK cells and monocytes.\textsuperscript{19,47} This binding stimulates the activation of various immune responses including suppression of cytokine release from monocytes, apoptosis of pro-inflammatory cells, and induction of regulatory macrophages which inhibit T cell proliferation and promote wound healing. tmTNFα-mediated reverse signalling is considered an important mechanism of action particularly in IBD. In addition, some evidence in tmTNFα-overexpressing cell lines suggested ADCC or CDC to be important for the efficacy of anti-TNFα agents in IBD via binding to tmTNFα.\textsuperscript{45,46,48}

Here, we first investigated soluble TNFα binding and neutralising activity of CT-P13 and of infliximab, using several orthogonal approaches, first by analysis of the suppression of cytokine release and apoptosis in Caco-2 cells. We found that CT-P13 and infliximab exhibit similar suppressive activity of cytokine release [IL-6 and IL-8] from these intestinal epithelial cell lines. These results provide explanation for the similar clinical efficacy of infliximab and CT-P13 as documented in clinical trials in AS and RA. They also provide a preliminary mechanistic basis for postulating similar such efficacy in IBD. Furthermore, immune effects attributed to reverse signalling were found to be comparable for CT-P13 and for infliximab by the variety of assays employed, including suppression of cytokine release in LPS-stimulated PBMCs, and apoptosis induction in tmTNFα-overexpressing Jurkat cells. In addition, CT-P13 and infliximab showed comparable immunosuppressive properties dependent on tmTNFα binding and their Fc region: suppression of T cell proliferation, induction of Mregs and wound healing.\textsuperscript{18,49} These mechanisms are believed to be important in mediating the effects of anti-TNFα in IBD, bearing in mind that the TNFα-receptor antagonist Enbrel, which is devoid of such reverse-signalling capacity, was found ineffective for the treatment of IBD.\textsuperscript{4,15} Moreover, possibly unique to IBD is the tissue re-modelling that results in mucosal healing mediated by anti-TNFα, which is believed to be important for restoring gut barrier integrity and for preventing continued intrusion of luminal organisms into deeper layers of the gut wall.\textsuperscript{50} Indeed, induction of mucosal healing was shown to be a distinctive feature of anti-TNFα which is associated with improved long-term clinical outcomes\textsuperscript{51} and was recently shown to be orchestrated by regulatory macrophages induced by the anti-TNFα.\textsuperscript{52} In this context, the present findings show that regulatory macrophage induction and sequential inhibition of T cell proliferation in an MLR, and ensuing wound healing were all similarly exerted by CT-P13 and by infliximab. Overall, the results indicate that CT-P13 and infliximab exert similar biological activities in intestinal cells as well as comparable inhibition of the cellular mechanisms implicated in the pathogenesis of IBD. Although these results cannot in themselves attest to the clinical equivalence of infliximab and CT-P13 in IBD patients, they support initial clinical reports showing the comparable efficacies of these antibodies in IBD patients.\textsuperscript{21}

With regard to ADCC, several \textit{in vitro} studies have reported that infliximab can induce ADCC, a process mediated by the Fab region of infliximab binding to tmTNFα on target cells and the Fc region of infliximab binding to the FcγRIIIa on the surface of effector cells such as NK cells. However, all these studies employed as target cells engineered tmTNFα-overexpressing cell lines, primarily a Jurkat cell line which cannot secrete TNFα and shows high levels of tmTNFα by an artificial mutation in TNFα cleavage enzyme [TACE].\textsuperscript{20,52,53} Nevertheless, a trend to lower values of NK ADCC activity was noted for the CT-P13 lots in NK ADCC relative activity in a previous study, and over 92% of CT-P13 lots were within the quality range (mean ± 3 standard deviation [SD]) of infliximab lots at all antibody concentrations\textsuperscript{46}; thus the products were statistically highly similar in NK ADCC activity.

We further investigated if ADCC by infliximab, in particular infliximab versus CT-P13, will be different when the target cells used more closely resemble tmTNFα expression \textit{in vivo}. We first investigated the different tmTNFα expression levels in various target cells. In these staining experiments, tmTNFα-overexpressing Jurkat cells showed significantly higher expression level of tmTNFα than other cells, including stimulated monocytes from healthy volunteers and CD patients or LPMC from CD patients. Moreover, the dose-dependent ADCC effects of CT-P13 and of infliximab were only observed in tmTNFα-overexpressing Jurkat cells. In contrast, no increase of NK-mediated cell lysis by either CT-P13 or infliximab was detected using wild-type Jurkat cells, LPS-stimulated human monocytes from healthy volunteers, or LBMGs from CD patients as target cells. This result corresponds with a previous report which showed that infliximab directly induced apoptosis of a significant percentage of activated lymphocytes from peripheral blood and the lamina propria of patients with CD, but did not exhibit any ADCC activity of these cells.\textsuperscript{25} Moreover, several studies have suggested that ADCC and NK activity are decreased in IBD.\textsuperscript{12,54} Taken together, these results suggest that ADCC is unlikely to be instrumental in mediating the biological effects of infliximab \textit{on IBD in vivo}. Rather, it appears that the mode of action of infliximab is mostly driven by other mechanisms, such as neutralisation of TNFα, reverse signalling effects and, particularly, effects on apoptosis, affected by infliximab in CD and UC patients. In particular these results, arguing against ADCC as a mechanism of action of infliximab in IBD, also do not suggest a difference in ADCC activity conferred by CT-P13 and by infliximab. Collectively, our findings imply that the ADCC activity of infliximab \textit{in vitro} is unlikely to have clinical relevance in
vivo. Indeed, cohort studies in IBD patients to date suggest rates of response to CT-P13 within the range observed with infliximab, further lending support to the above findings.

In conclusion, these findings indicate that infliximab and CT-P13 similarly inhibit cytokine secretion, induce reverse signalling and apoptosis in intestine-derived cells, and promote regulatory macrophages. Moreover, both exert only a negligible and comparable ADCC towards target cells of relevance to IBD pathophysiology, suggesting that ADCC is unlikely as a mechanism of action of infliximab agents. These results provide novel indication for a mechanic similarity of infliximab and CT-P13 for suppressing immune functions believed to be driving the chronic inflammatory process underlying IBD.

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**Conflict of Interest**

The authors declare that they have no competing interests.

**Author Contributions**

SHK, SB-H, and SJC conceived and designed the study protocol; SJL, SYL, MSL, YAP, EJC, EBL, HKJ, EF, OP, and MY performed the experiments; KJL drafted the manuscript; KJL, SJL, and SHK participated in acquisition and interpretation of the data; JMC, SYL, KSK, BPL, MSJ, YSC, and ECS participated in scientific discussion; SB-H and SJC critically revised the manuscript; all the authors approved the final version of the manuscript.

**Supplementary Data**

Supplementary data are available at ECCO-JCC online.

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