

Anti-tumour necrosis factor therapy enhances mucosal healing through down-regulation of interleukin-21 expression and T helper type 17 cell infiltration in Crohn's disease

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Introduction

Crohn's disease (CD) is a chronic intestinal disorder, which is characterized by a patchy, segmental and typically transmural inflammation in any part of the gastrointestinal tract [1]. Although the specific aetiology of CD is still not completely understood, it is well established that chronic inflammation is primarily the consequence of a dysregulation of innate and adaptive immune responses to commensal bacteria in intestinal mucosa leading to an immunological imbalance, characterized by an excessive

Summary

Anti-tumour necrosis factor (TNF) monoclonal antibody (mAb) (infliximab, IFX) has been shown to be highly effective in the management of Crohn's disease (CD). Herein we investigated the potential role of IFX in inducing clinical remission and regulating interleukin (IL)-21 expression and T helper type 17 (Th17) cell infiltration in the intestinal mucosa of CD patients. Twenty-six CD patients were treated with IFX at weeks 0, 2 and 6. Clinical response, mucosal healing, serum C-reactive protein (CRP) and erythrocyte sedimentation rate (ESR) were evaluated at week 10 after IFX administration. Expression of IL-21, IL-17A and retinoic acid-related orphan receptor C (RORC) in intestinal mucosa were analysed by quantitative real-time polymerase chain reaction (PCR) and immunohistochemistry. Peripheral blood and lamina propria CD4⁺ T cells were stimulated with anti-CD3 and anti-CD28 mAbs in the presence of IFX. Cytokine profiles and RORC were determined with enzyme-linked immunosorbent assay (ELISA) and real-time PCR. IL-21 and Th17 cells were found to be expressed highly in inflamed mucosa of active CD patients compared with healthy controls. Ten weeks after IFX infusion, CD activity index, ESR, CRP and intestinal mucosal healing were improved markedly in CD patients, and IL-21 expression and Th17 cell infiltration were decreased significantly compared with those before IFX therapy. *In-vitro* study demonstrated that IFX treatment could suppress IL-21, IL-17A and RORC expression in cultured CD biopsies. Moreover, IFX was also observed to down-regulate markedly IL-17A, IL-21 and RORC expression by CD CD4⁺ T cells. IFX is highly effective in inducing clinical remission and promoting intestinal mucosal healing in CD patients through down-regulation of IL-21 expression and Th17 cell infiltration in intestinal mucosa.

Keywords: Crohn's disease, infliximab, interleukin-21, mucosal healing, Th17 cells

production of proinflammatory cytokines such as tumour necrosis factor (TNF), interleukin (IL)-21 and IL-23 [2–4]. Accumulating evidence has shown that CD bears all the stigmata of an exaggerated T helper type 1 (Th1) immune response, characterized by increased interferon (IFN)- γ and IL-12 [2–4]. However, in past years published data have highlighted the critical role of the Th17 subset in the pathogenesis of CD, as well as the signature cytokine IL-17A, in the development of intestinal mucosal damage [5]. Th17 cells are a recently discovered addition to the Th cell ensemble, characterized by the expression of a cytokine family

(IL-17A, IL-17F, IL-21, IL-22 and IL-26) and transcription factor retinoic acid-related orphan receptor C (RORC) [6]. This cell population subset is plastic, and can be converted into Th1-type cells if they receive appropriate stimuli such as IL-12, although IL-23 was identified originally as a factor necessary for expanding and maintaining the Th17 cell response [7]. Previous work has demonstrated that Th17 cells and their associated cytokines (e.g. IL-17A, IL-21, IL-23) are expressed highly in the inflamed mucosa of patients with inflammatory bowel disease (IBD); genome-wide association studies have indicated that IL-23 receptor (IL-23R) and five additional genes involved in Th17 differentiation [IL-12B, Janus kinase 2 (JAK-2), signal transducer and activator of transcription 3 (STAT-3), chemokine receptor 6 (CCR)-6 and TNF-SF15] are associated with susceptibility to CD and also partly to ulcerative colitis [8]. Taken together, both Th1 and Th17 cells are important mediators of inflammation in the pathogenesis of CD.

IL-21 is a member of the IL-2 family of cytokines, synthesized mainly by a range of differentiated CD4⁺ Th cells, including Th1, Th2 and Th17 cells [9], and mediates its biological effects through interaction with an orphan receptor, IL-21R [10]. IL-21 plays an important role in the control of innate and adaptive immune responses. It exhibits a pleiotropic capacity to regulate T cell differentiation and function, enhances clonal expansion of antigen-activated naive CD4⁺ and CD8⁺ T cells, induces the genes encoding IL-12R, IL-18R, IFN- γ , IL-2R and the Th1-associated transcription factor T-bet in activated memory T cells, drives the differentiation of B cells into memory cells and immunoglobulin (Ig)-secreting plasma cells and promotes the activity of natural killer (NK) cells [11]. Moreover, IL-21 enhances T follicular helper cell and Th17 cell differentiation, down-regulates the induction of T regulatory cells and controls the activity of non-immune cells, including epithelial cells and stromal cells [9–11]. Evidence has shown that IL-21 is associated with the development of several autoimmune diseases such as rheumatoid arthritis, systemic lupus erythematosus and coeliac disease [11,12]. In recent years, IL-21 has been found to be involved in the pathogenesis of human IBD [13,14]. Our recent work has also demonstrated that IL-21R-positive cells were increased significantly in the inflamed mucosa of IBD patients. Importantly, IL-21 could enhance the IBD NK cell cytotoxic response, trigger IBD T cells to produce proinflammatory cytokines and induce IBD CD4⁺ T cells to differentiate into Th17 cells [15]. These data indicate that IL-21 plays an important role in the pathogenesis of IBD, and neutralizing IL-21 may have a therapeutic potential in the management of IBD.

Infliximab (IFX) is an anti-TNF monoclonal antibody (mAb) that binds soluble and cell-surface TNF with high affinity and specificity and which has offered a novel approach for the treatment of active CD, with very encouraging results in terms of efficacy and safety [16]. However, the anti-inflammatory action of IFX is not understood fully,

and it is supposed that several mechanisms contribute to the clinical efficacy of its CD therapy, including triggering apoptosis in monocytes and in lamina propria (LP) T cells [17], restoring epithelial barrier function [18], inducing intestinal fibroblasts motility [19] and down-regulating proinflammatory production in intestinal mucosa [20].

In this study, we evaluated the efficacy of IFX therapy on clinical remission and intestinal mucosal healing in Chinese CD patients, and investigated its role in regulating IL-21 expression and Th17 cell infiltration in intestinal mucosa. We found that IFX administration could induce clinical remission and promote intestinal mucosal healing. Importantly, IFX could down-regulate IL-21 expression and Th17 cell infiltration markedly in inflamed mucosa of CD patients, and suppress proinflammatory cytokine production (e.g. TNF, IFN- γ , IL-17A, IL-21) significantly by CD CD4⁺ T cells and Th17 differentiation.

Materials and methods

Patients and sample collection

Twenty-six patients with active CD (17 male and nine female, age 20–46 years, disease duration 3–46 months) were recruited at the Department of Gastroenterology, the Shanghai Tenth People's Hospital of Tongji University from June 2009 to October 2012. The diagnosis of CD was based on conventional clinical features, radiological and endoscopic features, and finally confirmed by histological examination of ileal and colonic biopsies. None received any immunosuppressants (i.e. azathioprine, 6-mercaptopurine, methotrexate) and targeted biological therapy (e.g. IFX) at initial study. These CD patients were treated with anti-TNF mAb (IFX; Cilag AG, Schaffhausen, Switzerland) intravenously at a dose of 5 mg/kg at weeks 0, 2 and 6, according to the manufacturer's instructions, and infusions were delivered over a 2-h period. The patients were monitored weekly during follow-up; serum samples were collected at week 10 after the first infusion. Clinical response or remission was evaluated at weeks 0 and 10 after the first IFX infusion using the Crohn's disease activity index (CDAI), endoscopic findings and routine biological analysis, e.g. serum C-reactive protein (CRP) and erythrocyte sedimentation rate (ESR) [21]. Response to treatment was defined as a decrease in CDAI from baseline of ≥ 100 points and remission as an absolute value ≤ 150 . Non-response was defined as no improvement and an increase of CDAI score within 10 weeks following administration. Endoscopic biopsies were taken at sites of active inflammation adjacent to ulcerations before and 10 weeks after IFX therapy. The endoscopic assessment of disease activities for mucosal inflammation was performed using the simple endoscopic score for Crohn's disease (SES-CD) [22]. Endoscopic biopsy samples from 18 healthy volunteers paired for sex and age (11 male and seven female, age 21–49 years) were also taken

for comparison. In addition, ethylenediamine tetraacetic acid (EDTA) anti-coagulated blood samples (approximately 20 ml) were obtained from these patients and healthy controls after overnight fasting using a vacuum tube for isolation of peripheral blood mononuclear cells (PBMC). This study was approved by the Institutional Review Board for Clinical Research of the Shanghai Tenth People's Hospital. Written informed consent was also obtained from all subjects before study.

Reagents

RPMI-1640 medium, fetal bovine serum (FBS), penicillin (100 U/ml) and streptomycin (100 µg/ml), L-gentamycin and 2-mercaptoethanol (ME) were all purchased from HyClone (Logan, UT, USA). An untouched human CD4⁺ T cells isolation kit was purchased from Dynal Biotechnology (Oslo, Norway); an RNeasy Kit was purchased from Invitrogen Life Technologies (Grand Island, NY, USA); and a SYBR PrimeScript RT reagent kit was purchased from TaKaRa (Dalian, China). Envision flex peroxidase-blocking reagent was purchased from Dako (Glostrup, Denmark). Rabbit anti-human IL-21 polyclonal antibody was purchased from Novus Biologicals (Littleton, CO, USA), anti-IL-17A mAb (clone eBio64CAP17, mouse IgG1) was purchased from eBioscience (San Diego, CA, USA) and horseradish peroxidase (HRP)-conjugated rabbit anti-mouse IgG and goat anti-mouse IgG were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA, USA). Anti-CD3 (clone UCHT1, mouse IgG1), anti-CD28 (clone CD28.2, mouse IgG1) and enzyme-linked immunosorbent assay (ELISA) kits for TNE, IFN-γ, IL-17A, IL-4, IL-10, IL-13, IL-17A and IL-21 were purchased from eBioscience (San Diego, CA, USA).

Immunohistochemistry

To determine the localization of IL-21- and IL-17A-positive cells in intestinal mucosa, immunohistochemistry was performed on 5-µm-thick sections from fresh-frozen intestinal mucosal tissues. Sections were air-dried overnight, fixed in acetone for 10 min and rinsed in phosphate-buffered saline (PBS) for 5 min. After incubation with Envision flex peroxidase-blocking reagent for 10 min, these sections were incubated with goat anti-human IL-21 polyclonal antibody (dilution 1:100) or anti-human IL-17A mAb (dilution 1:150) at 4°C overnight. After washing, the sections were incubated for 60 min with HRP-conjugated goat anti-rabbit or goat anti-mouse IgG (dilution 1:400) at room temperature. The colour reaction was developed with 3,3'-diaminobenzidine and the sections were counterstained with haematoxylin. As negative controls, sections were treated with PBS instead of primary antibody. To determine the proportion of positive cells, five fields of intestinal mucosa were selected randomly at high power (× 400). The

positive cells and total stromal cells were counted in the LP. The proportion of positive cells was calculated as follows: [(positive cells)/(total cell number)] × 100 [23–25].

Quantitative real-time polymerase chain reaction (PCR)

Total RNA was extracted from the fresh-frozen biopsies using the RNeasy Kit, according to the manufacturer's instructions. Total RNA quantity and quality isolated from each sample was assessed using a NanoVue spectrophotometer (GE Healthcare, Piscataway, NJ, USA), with a 260/280 ratio of > 1.8 and 28S/18S ratio of > 1.4 for the majority of the samples. The cDNA was synthesized with a SYBR PrimeScript reverse transcription (RT) reagent kit, according to the manufacturer's instructions. Quantitative real-time PCR was performed in the ABI prism 7900 HT sequence detector (Applied Biosystems, Foster City, CA, USA) using SYBR green methodology. β-actin was used as the endogenous reference gene. All primers were synthesized and purchased from ShengGong BioTeck (Shanghai, China) and used according to standard methodologies. All PCR reactions were run in triplicate and performed with 40 cycles using the following conditions: 95°C for 1 min, followed by 40 cycles at 95°C for 15 s and 60°C for 30 s. The relative target gene expression levels were calculated as a ratio relative to the β-actin reference mRNA. Quantitative real-time PCR analysis was carried out using the 2-ΔΔCt method [15,23,24].

Culture of intestinal biopsies

Freshly obtained inflamed biopsies from CD patients during endoscopic examination were cultured (two biopsy samples/well) in 1 ml RPMI-1640 medium containing 10% fetal bovine serum, and supplemented with penicillin (100 U/ml) and streptomycin (100 mg/ml) in the presence of IFX or control human IgG (HIG; Sigma Aldrich, St Louis, MO, USA), both at a dose of 50 µg/ml at 37°C in 5% CO₂ humidified air for 24 h. The cultured biopsies were then harvested, followed by extraction of total RNA, and levels of IL-17A, IL-21 and RORC mRNA were analysed by quantitative real-time PCR.

Isolation and culture of CD4⁺ T cells

PBMC and LPMC were isolated as described previously [15,23], and peripheral blood (PB)- or LP-CD4⁺ T cells were then purified by negative selection using the Dynal untouched human CD4⁺ T cells isolation kit (Dynal AS; Invitrogen). Cell purity was evaluated routinely by flow cytometry and ranged from 95 to 99%. Viable cells were determined by 0.1% Trypan blue exclusion (viability ≥ 95%) and counted with a haemocytometer. CD4⁺ T cells were resuspended in RPMI-1640 medium,

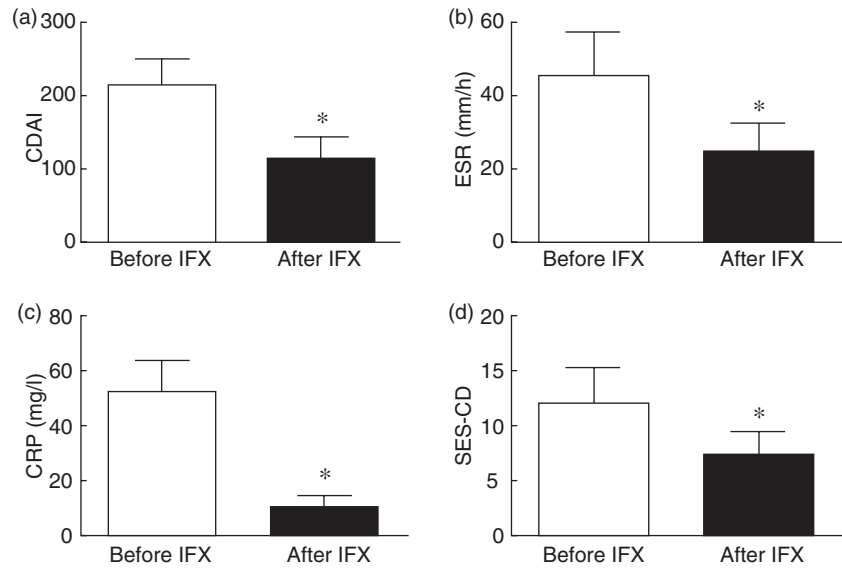


Fig. 1. Infliximab (IFX) therapy induces clinical remission and promotes intestinal mucosal healing in Crohn's disease (CD) patients.

Twenty-six patients with active CD were treated with IFX at weeks 0, 2 and 6; the CD activity index (CDAI), erythrocyte sedimentation rate (ESR) C-reactive protein (CRP) and endoscopic scores were evaluated before and at week 10 after IFX administration. * $P < 0.05$ versus values before IFX therapy.

supplemented with 10% heat-inactivated FBS, penicillin (100 U/ml) and streptomycin (100 µg/ml), 2 mM gentamycin and 50 µM 2-ME.

Purified PB- or LP-CD4⁺ T cells (1×10^6 /ml) from IBD patients and controls were incubated with anti-CD3 (5 µg/ml) and anti-CD28 (2 µg/ml) in the absence or presence of IFX (50 ng/ml) and IL-21R/Fc (20 µg/ml). After 48 h of culture, supernatants were harvested and analysed for TNF, IFN- γ , IL-17A and IL-21 production by sandwich enzyme-linked immunosorbent assay (ELISA) kits according to our previous reports [15,23]. The sensitivity of each assay was 10 pg/ml. Cultured cells were then collected followed by extraction of total RNA; mRNA levels were analysed for evaluation of TNF, IFN- γ , IL-17A, IL-21 and RORC, as described previously [15,23–25].

Statistical analyses

All data are expressed as mean \pm standard error of the mean (s.e.m.). Statistical analysis was performed using SPSS statistics version 13.0 (SPSS, Chicago, IL, USA). Differences between groups were compared using the *t*-test for two independent samples. A value of $P < 0.05$ was considered statistically significant.

Results

IFX induces clinical remission and promotes intestinal mucosal healing

Twenty-six patients with active CD were recruited and treated with IFX as indicated in Materials and methods at weeks 0, 2 and 6; the CDAI and endoscopic scores were evaluated at week 10 after IFX administration. As shown in Fig. 1, the CDAI scores were observed to be decreased

significantly at week 10 after IFX treatment compared with those at the beginning of treatment (112 ± 31 versus 213 ± 34 , $P < 0.005$). The levels of serum ESR and CRP were also found to be decreased markedly from 46.8 ± 6.8 mm/h to 23.6 ± 8.4 mm/h ($P < 0.05$) and from 52.1 ± 12.5 mg/l to 10.3 ± 4.4 mg/l ($P < 0.005$), respectively. Moreover, SES-CD was also performed and demonstrated a marked improvement at week 10 after IFX therapy compared with that the beginning of therapy (7 ± 2 versus 12 ± 3 , $P < 0.05$). Of all 26 CD patients, 12 patients achieved ulcer disappearance (46.2%), seven showed a decreased number of intestinal mucosal ulcer (26.9%), four had a smaller area of ulcer (15.4%) and three showed no response. Taken together, our data indicate that IFX therapy could induce clinical remission and promote intestinal mucosal healing.

IFX administration down-regulates IL-21 and Th17 cell infiltration in inflamed mucosa of CD patients

Because our previous work has demonstrated that potential role of IL-21 is involved in the induction of mucosal immune response and Th17 cell differentiation in the pathogenesis of IBD [15], we further analysed IL-21-positive cells and Th17 cells in the inflamed mucosa of CD patients at transcriptional and translational levels. First, we analysed the mRNA levels of IL-21, TNF, IFN- γ , IL-17A and RORC in intestinal mucosa from 26 active CD patients before and week 10 after IFX therapy, as well as 16 healthy controls by quantitative real-time PCR. Figure 2 shows that the levels of IL-21, TNF, IFN- γ , IL-17A and RORC mRNA were increased significantly in inflamed ileum/colon of CD patients in contrast to those in healthy controls ($P < 0.05$), consistent with our previous data showing that proinflammatory cytokines (e.g. IL-21, TNF, IFN- γ) and Th17 cells

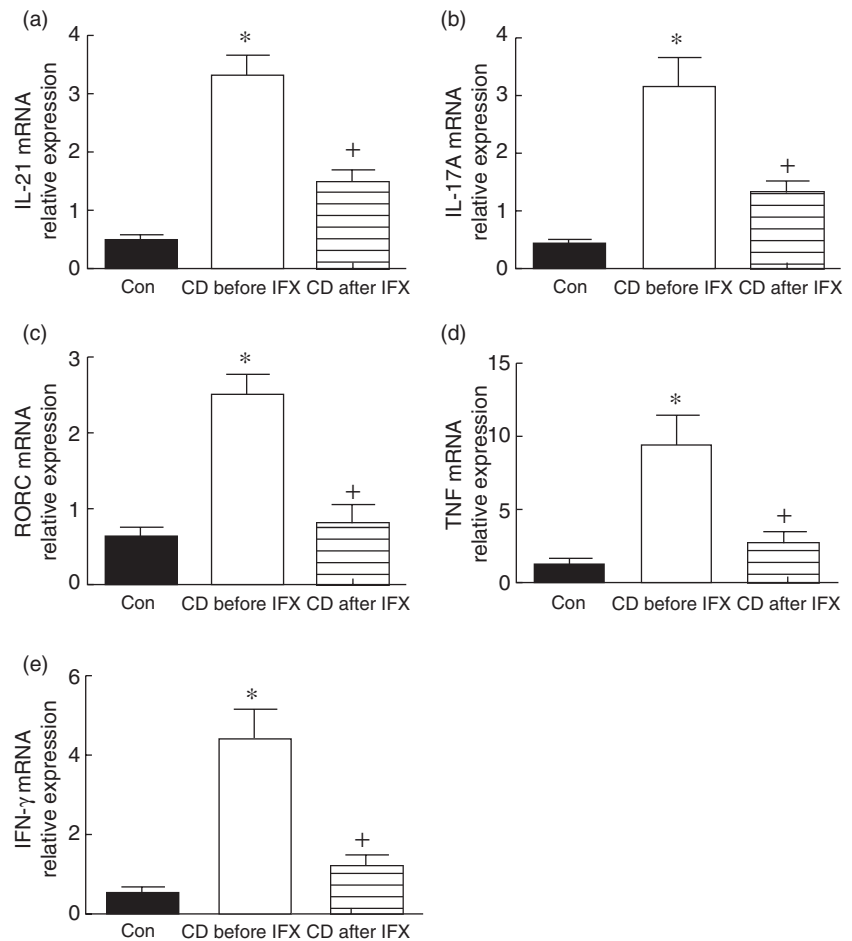


Fig. 2. Infliximab (IFX) down-regulates interleukin (IL)-21 and T helper type 17 (Th17) cell infiltration in the inflamed mucosa of Crohn's disease (CD) patients. Intestinal mucosal biopsies were taken from healthy controls ($n = 16$) and CD patients ($n = 26$) before and 10 weeks after IFX treatment. Total RNA was extracted and analysed for IL-21, tumour necrosis factor (TNF), interferon (IFN)- γ , IL-17A and retinoic orphan receptor C (RORC) mRNA by quantitative real-time polymerase chain reaction (PCR). * $P < 0.005$ versus healthy controls; + $P < 0.05$ versus values before IFX therapy.

play an important role in the pathogenesis of CD [15]. Interestingly, IFX therapy was demonstrated to down-regulate IL-21, TNF, IFN- γ , IL-17A and RORC mRNA expression significantly in the intestinal mucosa of CD patients ($P < 0.05$).

Moreover, immunohistological analysis demonstrated the *in-situ* expression of numerous IL-21⁺ cells with strong cytoplasmic staining in the LP of inflamed mucosa of active CD patients (Fig. 3a). Intestinal epithelial cells showed negative expression of IL-21 in all sections. After treatment with IFX, the proportion of IL-21⁺ cells in the LP of inflamed mucosa from CD patients was found to be decreased markedly compared with that before IFX treatment ($9.4\% \pm 2.8\%$ versus $26.7\% \pm 6.8\%$, $P < 0.005$) (Fig. 3b). These results indicate that IFX could suppress IL-21 expression in the intestinal mucosa of CD patients.

Additionally, we investigated the role of IFX administration in the inhibition of IL-17-expressing cell infiltration in inflamed mucosa of CD patients before and after IFX treatment using immunostaining. As shown in Fig. 3c,d, the levels of IL-17⁺ cells were decreased markedly after IFX treatment compared with those at the beginning of IFX administration ($10.4\% \pm 2.5\%$ versus $31.5\% \pm 5.1\%$, $P < 0.005$). These data indicate that anti-TNF treatment

can also inhibit intestinal mucosal inflammation effectively in CD patients through down-regulation of Th17 cell infiltration.

IFX treatment down-regulates IL-21 and Th17 cell infiltration in intestinal mucosal biopsies of CD patients *in vitro*

To investigate whether anti-TNF mAb treatment could down-regulate expression of IL-17A and Th17 cell differentiation in intestinal mucosa *in vitro*, freshly inflamed biopsies were obtained from 10 CD patients during endoscopic examination, cultured (two biopsy samples/well) in 1 ml RPMI-1640 medium containing 10% FBS and supplemented with penicillin (100 U/ml) and streptomycin (100 mg/ml) in the presence of IFX or control HIg (both at 50 μ g/ml) at 37°C in 5% CO₂ humidified air for 24 h. Total RNA was then extracted and analysed for IL-21, IL-17A and RORC mRNA expression by quantitative real-time PCR. Figure 4 demonstrates that IFX treatment could reduce markedly the expression of IL-21, IL-17A and RORC mRNA in intestinal biopsies. These data confirm further that anti-TNF mAb could suppress IL-21⁺ and Th17 cell infiltration in cultured biopsies.

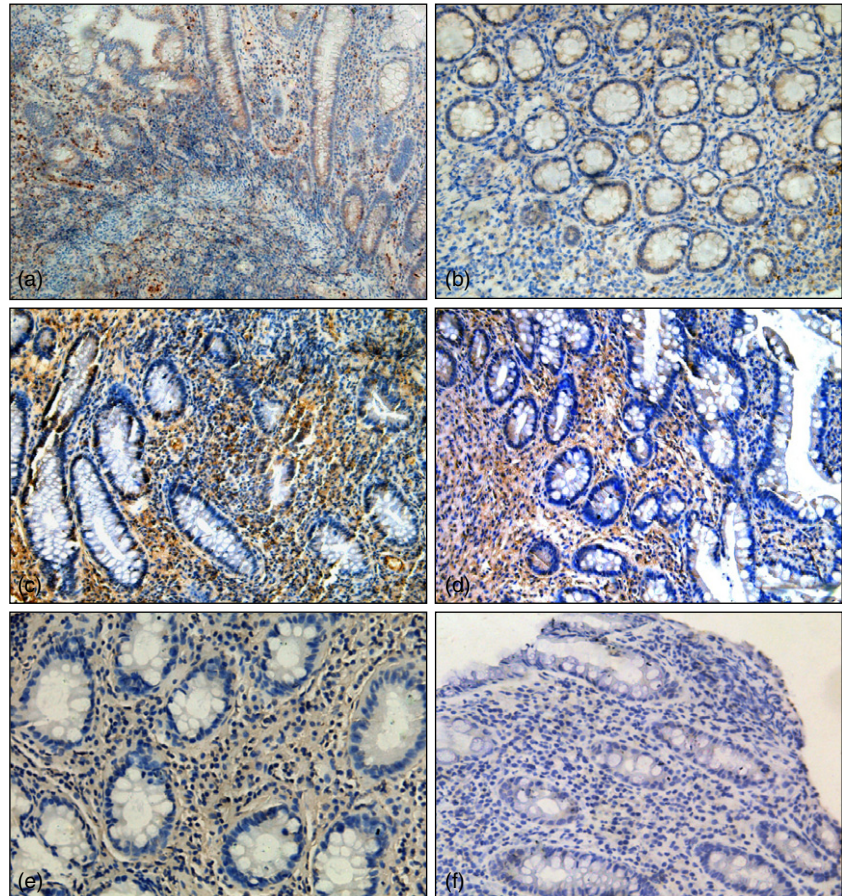


Fig. 3. Infliximab (IFX) decreases interleukin (IL)-21- and IL-17A-positive cell infiltration in the inflamed mucosa of Crohn's disease (CD) patients. Representative frozen sections were obtained from inflamed mucosa of CD patients before (a,c) and at week 10 after IFX treatment (b,d), and stained for IL-21 (a,b) and IL-17A (c,d) by immunohistochemistry. (e,f) The CD sections were stained with a control isotype antibody for IL-21 or IL-17A, respectively. Original magnification $\times 200$.

IFX suppresses CD CD4⁺ T cell differentiation into Th17 cells and IL-21 expression

To investigate further the potential role of IFX in the regulation of CD CD4⁺ T cell differentiation, PB- or LP-CD4⁺ T

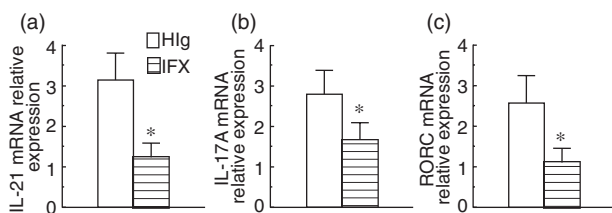


Fig. 4. Infliximab (IFX) treatment decreases interleukin (IL)-21 expression and T helper type 17 (Th17) cell infiltration in intestinal mucosal biopsies from Crohn's disease (CD) patients *in vitro*. Inflamed intestinal biopsies were obtained from 10 patients with active CD during endoscopic examination, and cultured (two biopsy samples/well) in the presence of IFX or control human immunoglobulin (IgG) (Hlg) (both at 50 $\mu\text{g/ml}$) at 37°C in 5% CO₂ humidified air for 24 h. Total RNA was then extracted and analysed for IL-21, IL-17A and retinoic orphan receptor C (RORC) by quantitative real-time PCR. Gene expression was normalized to β -actin mRNA levels in each sample. * $P < 0.05$ versus values before IFX therapy.

cells were isolated from CD patients and healthy controls and stimulated with anti-CD3 (5 $\mu\text{g/ml}$) plus anti-CD28 (2 $\mu\text{g/ml}$) in the absence or presence of IFX (50 $\mu\text{g/ml}$) and IL-21R/Fc (20 $\mu\text{g/ml}$). After 48 h of culture, supernatants were harvested and assayed for IFN- γ , TNF, IL-17A and IL-21. Table 1 demonstrates that the production of IFN- γ , TNF, IL-17A and IL-21 was significantly higher in the patients' cultures than in the cultures from controls when stimulated with anti-CD3 and anti-CD28 ($P < 0.05$). Interestingly, the inclusion of IFX resulted in down-regulating markedly expression of IFN- γ , TNF, IL-17A and IL-21 by CD CD4⁺ T cells ($P < 0.05$). In contrast, low levels of IL-4, IL-5 and IL-13 production were observed in the supernatants by CD4⁺ T cells under stimulation with anti-CD3 and anti-CD28 from all groups (data not shown).

To clarify further whether IFX could regulate CD CD4⁺ T cell differentiation, the cultured CD4⁺ T cells as indicated above were also collected after 48 h of culture, followed by extraction of total RNA, and the mRNA levels were analysed for evaluation of Th17 differentiation and IL-21 expression. As shown in Fig. 5, the mRNA levels of IL-21, IL-17A and RORC were increased markedly in CD CD4⁺ T cells when stimulated with anti-CD3 and anti-CD28. Interestingly, the presence of IFX could prohibit Th17 cell differentiation markedly and reduce the expression of IL-17A and IL-21.

Table 1. Infliximab (IFX) down-regulates Crohn's disease (CD) CD4⁺ T cell proinflammatory cytokine production (pg/ml).

	<i>n</i>		IFN- γ	TNF	IL-17A	IL-21
PB CD4 ⁺						
Con	8	α CD3 + α CD28	1462 \pm 256	167 \pm 42	476 \pm 114	421 \pm 121
		α CD3 + α CD28 + IFX	1024 \pm 149*	15 \pm 2.5	314 \pm 78*	348 \pm 117
		α CD3 + α CD28 + IFX + IL-21R/Fc	892 \pm 210 [†]	14 \pm 3.4	216 \pm 58 [†]	n.d.
CD	9	α CD3 + α CD28	3246 \pm 452 [#]	412 \pm 68 [#]	718 \pm 149 [#]	689 \pm 175 [#]
		α CD3 + α CD28 + IFX	1847 \pm 315*	13 \pm 3.5	458 \pm 108*	386 \pm 102*
		α CD3 + α CD28 + IFX + IL-21R/Fc	1143 \pm 165 [†]	12 \pm 3.1	345 \pm 78 [†]	n.d.
LP CD4 ⁺						
Con	5	α CD3 + α CD28	1843 \pm 387	215 \pm 52	561 \pm 172	568 \pm 147
		α CD3 + α CD28 + IFX	1258 \pm 318*	12 \pm 3.3	378 \pm 106*	383 \pm 96*
		α CD3 + α CD28 + IFX + IL-21R/Fc	788 \pm 215 [†]	11 \pm 2.6	254 \pm 62 [†]	n.d.
CD	5	α CD3 + α CD28	4524 \pm 548 [#]	589 \pm 117 [#]	1325 \pm 319 [#]	1018 \pm 253 [#]
		α CD3 + α CD28 + IFX	2889 \pm 414*	11 \pm 2.8	745 \pm 142*	585 \pm 113*
		α CD3 + α CD28 + IFX + IL-21R/Fc	1752 \pm 325 [†]	13 \pm 3.9	517 \pm 72 [†]	n.d.

* $P < 0.05$ versus data under anti-CD3 + anti-CD28 stimulatory conditions in the same group. [†] $P < 0.05$ versus data under anti-CD3 + anti-CD28 + IFX stimulatory conditions in the same group. [#] $P < 0.05$ versus controls under the same stimulatory conditions. Peripheral blood (PB) or lamina propria (LP) CD4⁺ T cells from CD patients and healthy controls (Con) as indicated were stimulated with anti-CD3 and anti-CD28 monoclonal antibodies (mAbs) in the presence of IFX \pm IL-21R/Fc. After 48 h of culture, supernatants were harvested and assayed for interferon (IFN)- γ , tumour necrosis factor (TNF), interleukin (IL)-17A and IL-21 by enzyme-linked immunosorbent assay (ELISA); n.d.: not done.

Thus, our data suggest that IFX plays an important immunoregulatory role in the treatment of CD patients.

As we and others have reported that IL-21 is overexpressed in inflamed mucosa of IBD and induces IBD CD4⁺ T cell activation to differentiate into Th17 cells [13,15], we asked whether blockage of endogenous IL-21 with recombinant human IL-21R/Fc fusion protein could influence Th17 cell differentiation. To this end, PB- or LP-CD4⁺ T cells were isolated from CD patients and healthy controls and stimulated with coated anti-CD3 and anti-CD28 in the absence or presence of IFX and IL-21R/Fc for 48 h. Levels of mRNA for IL-17A and RORC were analysed by quantitative real-time PCR. As shown Fig. 5, neutralizing endogenous IL-21 production by activated CD4⁺ T cells with IL-21R/Fc fusion protein was demonstrated to decrease further the expression of IL-17A and RORC mRNA. These data suggest that IFX could down-regulate Th17 cell infiltration partially through blockage of the IL-21 signalling pathway.

Discussion

The current study has described the efficacy of IFX in inducing clinical remission and promoting mucosal healing in Chinese CD patients. Importantly, our data have proved that IFX could suppress markedly CD CD4⁺ T cell proinflammatory cytokine production (e.g. IL-17A, IL-21) and Th17 cell infiltration in intestinal mucosa. This work has highlighted the critical role of IFX in the management of human CD patients, and expanded our knowledge of its immunoregulatory role in the control of mucosal inflammation. To date, the precise aetiology of CD remains unknown, but it is well established that there is an infiltration of inflammatory cells focally in the bowel, leading to

the production of proinflammatory cytokines and propagation of the immune response [1–3]. With the recent discovery of the Th17 subset and Th17-associated cytokines, understanding of CD has expanded rapidly because these cells and cytokines have a critical role in the pathogenesis of CD [5–7]. Therefore, Th17-associated cytokines (e.g. IL-17A, IL-21, IL-23) may represent a promising target in the treatment of human CD.

Apart from its direct TNF neutralizing activity, the anti-inflammatory mechanism of IFX is not understood completely, but accumulating evidence has suggested that one of its anti-inflammatory mechanisms may be related to inhibition of the production of proinflammatory cytokines and the infiltration of T cells in intestinal mucosa [26,27]. In the present study, our data have shown that two proinflammatory cytokines, IL-17A and IL-21, which were found to be increased significantly in inflamed mucosa of CD patients compared with healthy controls, were down-regulated markedly in those patients with the improvement of routine clinical parameters and endoscopic scores after IFX infusion. Previous data have demonstrated the association of IL-17A and IL-21 with the pathogenesis of various autoimmune diseases, including CD. Fina *et al.* [28] reported that IL-21-deficient mice are largely protected against intestinal inflammation in both dextran sodium sulphate (DSS)- and 2,4,6-trinitrobenzenesulphonic acid (TNBS)-induced colitis, which was associated with a marked decrease in Th17-related molecules, such as IL-17A and IL-17F. Our previous studies have also shown that IL-21 could promote LP-CD4⁺ T cells to differentiate into Th17 cells in CD patients [15]. Thus, the current results revealed a novel anti-inflammatory activity of IFX by the reduction of IL-21 expression in the inflamed mucosa of CD patients, and the

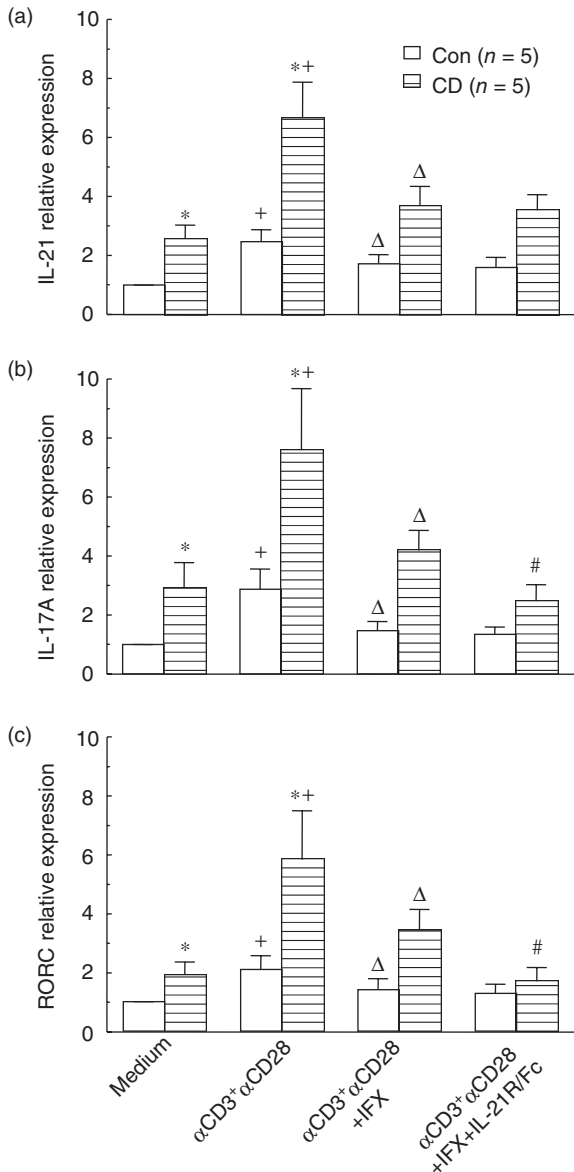


Fig. 5. Infliximab (IFX) suppresses Crohn's disease (CD) lamina propria (LP) CD4⁺ T cell interleukin (IL)-21 and IL-17A mRNA expression and T helper type 17 (Th17) cell differentiation. Purified LP-CD4⁺ T cells (1×10^6 /ml) from CD patients and healthy controls (Con) were stimulated with anti-CD3 (5 μ g/ml) and anti-CD28 (2 μ g/ml) in the absence or presence of IFX (50 μ g/ml) and IL-21R/Fc (20 μ g/ml). After 48 h of culture, the cultured LP CD4⁺ T cells were harvested, followed by extraction of total RNA, and levels of IL-21 (a), IL-17A (b) and Th17 cell transcription factor retinoic orphan receptor C (RORC) (c) were then analysed by quantitative real-time PCR. Gene expression was normalized to β -actin mRNA levels in each sample. Data represent average fold increases or decreases over baseline levels in healthy controls cultured in medium alone (defined arbitrarily as 1.0). * $P < 0.005$ versus healthy controls under the same stimulatory conditions. + $P < 0.05$ versus data from the same group cultured in medium alone. $\Delta P < 0.05$ versus data from the same group stimulated with anti-CD3 and anti-CD28 mAbs. # $P < 0.05$ versus data from the same group stimulated with anti-CD3 and anti-CD28 monoclonal antibodies (mAbs) in the presence of IFX.

possibility that the cascade by which TNF may induce IL-21 production actually occurs *in vivo*. A similar effect of IFX was also reported in the treatment of rheumatoid arthritis patients, showing that the serum level of IL-23 was decreased significantly in those patients after IFX infusion and correlated with ESR, CRP and the disease activity scores of 28 joints (DAS28) [29].

Evidence has shown that Th17 cells are identified as a potential subset of Th cells related to the development of autoimmunity and inflammatory responses such as CD, and that IL-21-driven intestinal pathology is associated with increased Th17 cell differentiation [30,31]. IL-17-producing RORC⁺ CD4⁺ T cells have been found to be constitutively present in the LP in mice [32] and highly infiltrated in the inflamed mucosa of CD patients [33]. A previous report comparing the ability of Th1 and Th17 cells to induce colitis in mice has proved that Th17 cells are significantly more pathogenic than their Th1 counterparts [34]. Recent work [35] has demonstrated that IL-17 knock-out mice with TNBS-induced colitis had significantly lower neutrophil infiltration and inflammatory macroscopic scores in inflamed colon compared to wild-type mice. The treatment of mice with anti-IL-17A neutralizing antibody confirmed a specific role for IL-17A in TNBS-induced acute colitis, suggesting that IL-17 signalling plays a critical role in the local inflammation of TNBS-induced colitis. In the current study, we observed that the mRNA levels of IL-17A and RORC were decreased markedly after IFX treatment in the inflamed mucosa of CD patients, indicating that IFX could inhibit the infiltration of Th17 cells in these patients. Our results are consistent with a previous study [36] showing that IFX could suppress the differentiation of naive CD4⁺ T cells isolated from the peripheral blood into Th17 cells *in vitro*, characterized by the reduction of IL-17A secretion in the culture supernatants. Moreover, another study confirmed further that IFX in combination with methotrexate treatment could decrease the percentage of Th17 cells in peripheral blood mononuclear cells and the concentration of IL-17A in the supernatants from patients with rheumatoid arthritis [37]. Taken together, these data suggest that Th17 cells play a critical role in the pathogenesis of intestinal mucosal inflammation and that IFX plays a profound anti-inflammatory role in modulating Th17 cell differentiation in several autoimmunities, particularly in CD.

Our study has revealed that IFX could improve clinical remission and promote intestinal mucosal healing in active CD patients. One important anti-inflammatory mechanism may contribute to down-regulating the expression of pro-inflammatory cytokines (e.g. IL-17A, IL-21, IL-23) and inhibiting Th17 cell infiltration in the inflamed mucosa of CD patients. This work has shed some light on understanding more clearly the immunoregulatory role of IFX in the control of human IBD. In addition to specific mechanisms, as described previously [17–20], IFX not only suppresses mucosal CD4⁺ T cell activation directly to differentiate into

Th17 cells, but also down-regulates IL-21 expression by mucosal CD4⁺ T cells, which contributes further to the synergistic role in the inhibition of Th17 cell differentiation.

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Disclosure

The authors do not have any conflicts of interest.

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Fig. S1. Infliximab (IFX) suppresses Crohn's disease (CD) peripheral blood (PB) CD4⁺ T cell interleukin (IL)-21 and IL-17A mRNA expression and T helper type 17 (Th17) cell differentiation. Purified PB CD4⁺ T cells (1×10^6 /ml) from

CD patients and healthy controls were stimulated with anti-CD3 (5 μ g/ml) and anti-CD28 (2 μ g/ml) in the absence or presence of IFX (50 μ g/ml) and IL-21R/Fc (20 μ g/ml). After 48 h of culture the cultured PB CD4⁺ T cells were harvested, followed by extraction of total RNA, and levels of IL-21 (A), IL-17 (B) and Th17 cell transcription factor retinoic orphan receptor C (RORC) (C) were then analysed by quantitative real-time polymerase chain reaction (PCR). Gene expression was normalized to β -actin mRNA levels in each sample. Data represent average fold increases or decreases over baseline levels in healthy controls cultured in medium alone (defined arbitrarily as 1.0). * $P < 0.005$ versus healthy controls under the same stimulatory conditions. ⁺ $P < 0.05$ versus data from the same group cultured in medium alone. ^{Δ} $P < 0.05$ versus data from the same group stimulated with anti-CD3 and anti-CD28 monoclonal antibodies (mAbs). [#] $P < 0.05$ versus data from the same group stimulated with anti-CD3 and anti-CD28 mAbs in the presence of IFX.