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ORIGINAL ARTICLE

The proinflammatory effect and molecular mechanism of IL-17 in the intestinal epithelial cell line HT-29

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Summary

Purpose: *To evaluate the proinflammatory effect and molecular mechanism of IL-17 in the intestinal epithelial cell line HT-29.*

Methods: *After culture of HT-29 cells with IL-17 and/or TNF-(a), real-time (RT) PCR and Western blot were used to measure the gene expression level of the neutrophil chemokines CXCL1, CXCL2, CXCL5, CXCL6, IL-8 and the Th-17 chemokine CCL20, the phosphorylation level of P38 and TNF-a, and the expression level of IL-8 after treatment with P38 inhibitor. Act1 stable knockdown HT-29 cell line was established to further test the change of P38 phosphorylation after treatment with IL-17 and TNF-a.*

Results: *When HT-29 cells were cultured with IL-17 and TNF-a, the expression level of neutrophil chemokines (CXCL1, CXCL2, CXCL5, CXCL6, IL-8) and Th17 chemokine (CCL20) was significantly improved (24.96±2.53, 28.47±2.87, 38.08±2.72, 33.47±2.41, 31.7±2.38, 44.37±2.73, respectively) ($p<0.01$). The results of Western blot showed that IL-17 obviously enhanced the phosphorylation of P38 induced by TNF-a. Compared with the control group, the expression level of IL-8 declined significantly (9.47±1.36 vs 3.06±0.67) when HT-29 was cultured together with IL-17 and TNF-a ($p<0.01$). P38 inhibition assay showed that P38 pathway played an essential role in IL-17 induced inflammatory response. The level of P38 phosphorylation could not be changed after treatment with IL-17 and TNF-a in Act1 stable knockdown HT-29 cell line.*

Conclusion: *IL-17 significantly promoted the gene expression level of TNF-a-induced neutrophil chemokines and Th17 cells chemokine. IL-17 and TNF-a have an obvious synergistic effect on P38.*

Key words: *HT-29, IL-17, inflammatory bowel disease, TNF-a*

Introduction

Ulcerative colitis and Crohn's disease are known as inflammatory bowel disease (IBD) [1]. They are chronic inflammatory disorders of the gastrointestinal tract that recur repeatedly. IBD occurs more often in males than females and nowadays the morbidity shows an upward trend. IBD has become one of the most common digestive system diseases [2]. It is widely accepted that the interaction of genetic and environmental factors leads to disease development [3-5], but the pathogenesis is still unclear.

Hundorfean et al. [6] found that the expression level of interleukin-17 (IL-17) increased significantly in the peripheral blood of patients with IBD. This implied that IL-17 may play an important role in the physiological and pathological processes. IL-17 and tumor necrosis factor (TNF) can accelerate the inflammatory response through inducing many kinds of inflammatory cytokines in diseases like IBD [7,8], but the molecular mechanism of the proinflammatory effect is still unknown. Wu et al. [9] reported that IL-17 can induce neutrophil infiltration and the expression of related inflammatory cytokines through P38 pathway in myoblasts and fibroblasts, while the mechanism in epithelial cell is rarely reported.

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Table 1. The primers used in RT-PCR

Gene	*Forward*	*Reverse*
CXCL1	AGATTCTATGTTAATATTTTAGGTGTAAAATAAT	AACTAACTTGGGGTTGACATTTC
CXCL2	CTTCTATTTATTTATTTATTTATTTATTTGTTTGTTTT	GAACTAACTTGGGTTTGACCTAAA
CXCL5	TGGCCCCTTTCACAGAGTAG	CTAAAAACCCGACAGGCATC
CXCL6	AGTTTACAGCTCAGCTAATGAAGTACTAAT	CGGTAAGACTTTAAGGAATGTATGATA
IL-8	GAATTGAATGGGTTTGCTAGA	CACTGTGAGGTAAGATGGTGG
CCL20	CTGGCTGCTTTGATGTCAGT	CGTGTGAAGCCCACAATAAA

To deeply understand the pathogenesis of IBD, the molecular mechanism of the proinflammatory effort of IL-17 and TNF-α on the intestinal epithelial cell line HT-29 was studied. Because the intestinal epithelial cell line HT-29 has normal structure and function of the colonic epithelium, it is the most common cell line used in laboratory to study the immunologic mechanism of intestinal mucosa [10,11]. Although the IL-17 inhibitors were proved to be not so helpful in the treatment of IBD, blocking other molecular targets involved in the IL-17 induced proinflammatory pathway is a potential treatment approach to IBD. Therefore, it is of great importance to explore the mechanism of IL-17 function.

Methods

Materials

Reagents: McCOY's 5A medium (HyClone, Pasadena USA), recombinant human IL-17 and TNF-α (E Biosciences, Shanghai, China), Eastep Universal RNA Extraction Kit (Promega, Beijing, China), Fist Strand cDNA Synthesis kit (Promega), SYBR Premix Ex TaqTM (Takara, Otsu, Japan), P38 inhibitor SB203580 (Sigma, Pasadena, USA), BCA Protein assay kit (Thermo, Massachusetts, USA), Phospho-p38 MAPK antibody (Beyotime, Shanghai, China), HRP-labeled Donkey Anti-Goat IgG (Beyotime), Mouse anti-Human Act1 Antibody (Biolegend, California, USA), Goat Anti-Mouse IgG (Biolegend) were utilized in this study.

Instruments: CO_2 incubator (Thermo), PCR Amplifier (Longgene, Hangzhou, China), Electrophoresis Apparatus (Tanon, Shanghai, China), Gene Genius Bioimaging System (Bio-rad, California, USA), Polyacrylamide Gel Electrophoresis Apparatus (Tanon), centrifuge (Debon, Jiangsu, China, Microplate Reader (Thermo) were utilized in this study.

Cell culture

Intestinal epithelial cell line HT-29 was cultured in McCoy's 5A medium supplemented with 10% fetal bovine serum, 100 mg/ml streptomycin, and 100 U/ml penicillin in humidified atmosphere at 37 ^{O}C and 5% CO_2. Then the cells were cultured to the exponential phase in order to be used.

RT-PCR

HT-29 cells were seeded in 12-well plates (5×10^5 cells/well), and as they reached about 70% confluence, the fetal bovine serum was reduced to 0.5% and the plates were cultured overnight. Then, rhIL-17 (50 ng/ml) and rhTNF-α (0.5 ng/ml), were added into the plates. After being cultured for another 6 hrs, the samples were collected, and TRIzol (1ml TRIzol per 5-10×10^6 cells) was added to lyse the cells. Before the TRIzol was added, it was prohibited to wash the cell with buffer, as RNA can be easily degraded. Total RNA was isolated using Eastep Universal RNA Extraction Kit and reverse transcribed by Fist Strand cDNA Synthesis kit according to the manufacturers' instructions. Finally, RT fluorescence quantitative PCR (SYBR Green I RT PCR method) was performed using 200 ng of cDNA. The reaction system was performed according to the specification of SYBR Premix Ex TaqTM purchased from Takara. The reaction conditions were: a first step initial denaturation at 95 ^{O}C for 10 min, followed by denaturation at 95 ^{O}C for 15 sec, annealing at 60 ^{O}C for 30 sec, cycling 40 times, and then followed by collecting the fluorescence signals. After amplification, the melting curve of 65 ^{O}C to 95 ^{O}C was analyzed to exclude the non-specific product of PCR and the amplification of primer dimers. Then, agarose gel electrophoresis was used to analyze the specificity of the product. GAPDH was amplified as a control for the amount of cDNA in each sample and the expression level of each gene was computed. The gene levels of chemokines of neutrophils and Th-17 (CXCL1, CXCL2, CXCL5, CXCL6, IL-8 and CCL20) were examined, and all the primers used in our study were designed according to the sequence published online (GenBank), and synthesized by Invitrogen (Table 1).

Cells were cultured as we described above, and rhIL-17, rhTNF-α and P38 inhibitor SB203580 were added into the plates of the experimental groups, and then, the same procedure was conducted to detect the gene expression level of IL-8.

Western blot

HT-29 cells were seeded in 6-well plates (8×10^5 cells/well), and after they reached about 70% conflu-

ence, the fetal bovine serum was reduced to 0.5% and cultured overnight. Then, rhIL-17 (50 ng/ml), rhTNF-α (0.5 ng/ml), were added into the plates and the samples were collected after 10, 15 and 30 min. At each time point, the medium was removed and the cells were washed with PBS and then radioimmunoprecipitation assay (RIPA) lysis buffer was added and the plate was placed on ice for 30 min to lyse the cells. Cells were collected into a centrifuge tube with a cell scraper, and then lysed with ultrasonic irradiation for 3 sec, 2 times. Cell debris were removed by centrifugation of the lysates at 12000 g for 2 min at 4 ^{O}C, and protein concentration was determined by using the BCA Protein assay kit (Thermo). The working liquid was prepared according to the manufacturer's instructions, and the standard curve was made, the plate was incubated in 37 ^{O}C for 30 min, and then the optical density (570) was tested by a microplate reader, and finally the protein concentration was computed according to the standard curve. All the specimens were adjusted to the same concentration, after the protein supernatant was mixed with the buffer, the liquid was boiled for 10 min followed by cooling, centrifugation, 12% SDS-PAGE and transferring the protein to nitrocellulose membrane. The Emitter Coupled Logic (ECL) based Western Blot was performed using the anti-P38 antibody, followed by the horseradish peroxidase (HRP)-labeled secondary antibody for chemiluminescence detection. Briefly, 5% Base Station Controller (BSC) blocked membrane was incubated for 12hrs at 4 ^{O}C with primary antibodies (1:1000). The membrane was then washed and incubated with rabbit anti-mouse IgG conjugated to HRP for 60min at room temperature. After washing, the protein was tested with ECL Western Blot analysis detection system.

Establishing Act1 silenced cell line

HT-29 cells were cultured in 96-well plates, and when the cell reached 80% confluence. Metafectene transfection reagents were used to transfer HT-29 targeted small interfering RNA into HT-29. The plasmid with the best knockdown effect (tested through transient transfection) was selected to establish stable cell line, and then the cells cultured for the follow-up experiments. Immunocytochemistry was performed to identify the transfected cells. Briefly, cells were blocked with bovine calf serum (diluted by PBS) for 15min, and then the serum was removed and cells were incubated with mouse anti-human Act1 antibody for 12hrs at 4 ^{O}C. Unbounded antibody was washed off with PBS, and cells were incubated with Goat Anti-Mouse IgG again for 1h at room temperature, followed by mounting and microscopic observation.

The transfected cells were seeded in 6 well plates (8×10^5 cells/well), and after they reached about 80% confluence, fetal bovine serum was reduced to 0.5% and cultured overnight. Then, rhIL-17 (50ng/ml), rhTNF-α (0.5ng/ml), rhIL-17 and rhTNF were added into the plates and the samples were collected after 15min. The same method as described above was used to test the expression level of P-P38 and P-ERK.

Statistics

SPSS 17.0 software (SPSS Inc, Chicago, Ill) was used for statistical analysis in this study. All data were expressed as mean±SD, and differences were assessed using t-test. A p-value was <0.05 considered significant.

Results

The expression of neutrophil chemokines and Th-17 chemokines in HT-29

Figure 1 shows that when IL-17 and TNF-α were used separately, the RNA expression of those chemokines (neutrophil chemokines: a~e and TH-17 chemokines: f) was relatively low. When treated together, the mRNA expression level for all these mediators was strongly upregulated. The difference was statistically significance ($p<0.01$).

The phosphorylation level of P38 and ERK

When IL-17 was used alone, no obvious phosphorylation occurred on P38 and ERK, and when TNF-α was used alone, the phosphorylation of P38 and ERK was very low. In contrast, when IL-17 and TNF-α were used together, obvious phosphorylation could be observed, and the phosphorylation was strengthened overtime (Figure 2) .

The expression level of IL-8 when P38 inhibitor was used

Whether the P38 inhibitor was added or not, the combined treatment of IL-17 and TNF-α could improve the expression level of IL-8 significantly, while compared with the cells cultured without P38 inhibitor, the expression level of IL-8 was reduced significantly in all three groups (Figure 3).

The phosphorylation level of IL-8 in Act1 silenced HT-29 cell line

Immunocytochemistry was performed to identify the Act1 silenced cells. The fluorescent intensity was greatly decreased as compared with the control group (Figure 4), and the positive rate (transfection efficiency or ACT1 expression in the control group) reached 90%. In the control group, the combination of IL-17 and TNF-α increased the phosphorylation level of P38 greatly, while in the

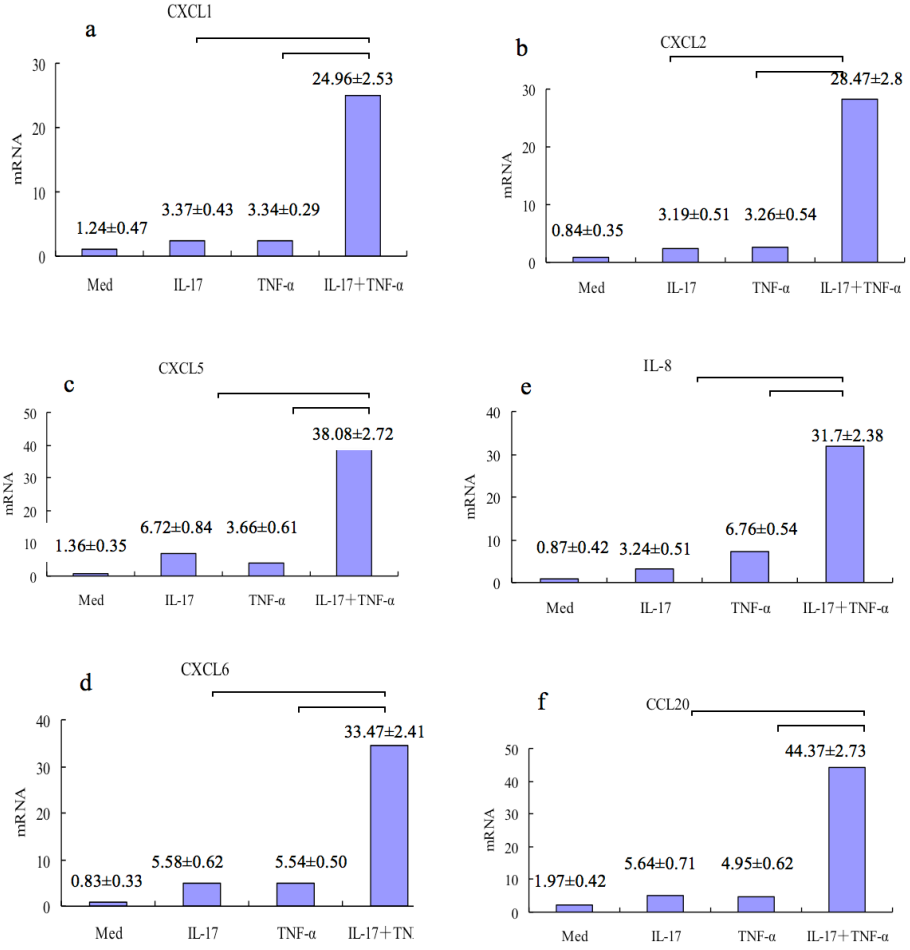

Figure 1. The expression of chemokines when Th-17 cells were cultured with IL-17 and/or TNF-α. When IL-17 and TNF-α were used separately, the expression of CXCL1, CXCL2, CXCL5, CXCL6, IL-8 and CCL20 in Th-29 was comparatively low. When used together, their expression was strongly upregulated. **a:** IL-7+TNF-α vs IL-17, t=30.424, p=0.000; IL-7+TNF-α vs TNF-α t=30.438, p=0.000; **b:** IL-7+TNF-α vs IL-17, t=35.273, p=0.000; IL-7+TNF-α vs TNF-α, t=35.125, p=0.000; **c:** IL-7+TNF-α vs IL-17, t=85.718 p=0.000, IL-7+TNF-α vs TNF-α t=94.199, p=0.000); **d:** IL-7+TNF-α vs IL-17, t=50.165 p=0.000, IL-7+TNF-α vs TNF-α, t=50.224 p=0.000; e: IL-7+TNF-α vs IL-17, t=54.585 p=0.000, IL-7+TNF-α vs TNF-α, t=44.036 p=0.000; f: IL-7+TNF-α vs IL-17, t=56.272 p=0.000, IL-7+TNF-α vs TNF-α, t=57.410 p=0.000.

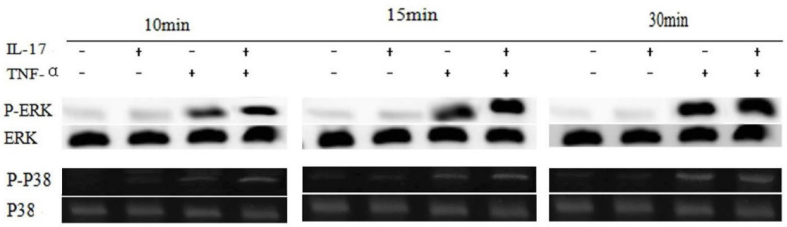

Figure 2. The phosphorylation level of P38 and ERK. When the IL-17 and TNF-α were used separately, the phosphorylation level of P38 and ERK was very low. When IL-17 and TNF-α were combined together, the phosphorylation level of P38 and ERK improved significantly, and with prolongation of time the phosporylation improved greatly.

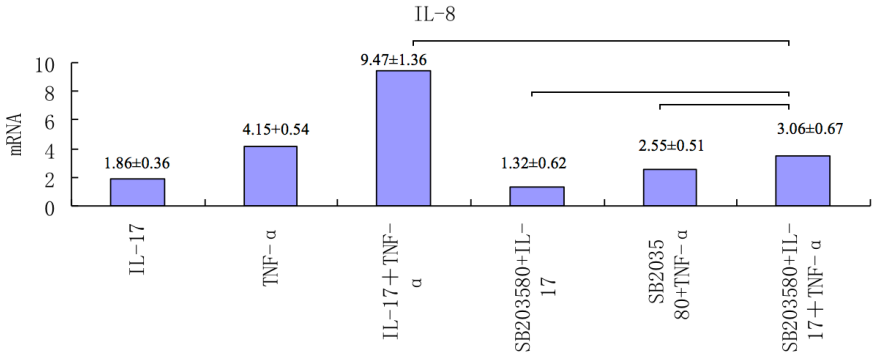

Figure 3. P38 inhibitor decreased the expression of IL-8; when the P38 inhibitor was used, the expression level of IL-8 declined in all three groups: IL-7+TNF-α vs S+IL-17+TNF-α, t=103.701, p=0.000; S+IL-7+TNF-α vs S+TNF-α, t=-4.131, p=0.07; S+IL-7+TNF-α vs S+IL-17, t=-11.509, p=0.000.

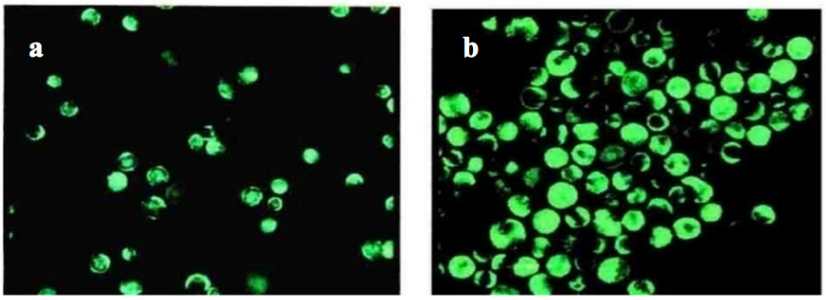

Figure 4. The Act silenced HT-29 cell line. Compared with the control group **(a)**, the expression level of Act 1 was comparatively low in the Act 1 silenced cell **(b)** (x250).

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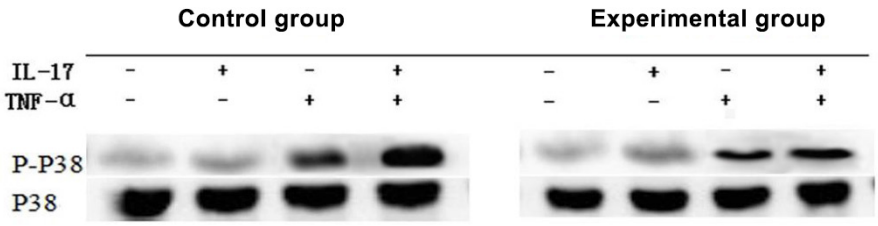

Figure 5. The phosphorylation level of P38 in Act 1 silenced HT-29 cell line. In the control group, IL-17 and TNF-α combined together greatly improved the phosphorylation level of P38. In the Act 1 silenced, cells the phosphorylation level of P38 remained unchanged when IL-17 and TNF-α were used together.

Act1 silenced treatment group no obvious change occurred (Figure 5).

Discussion

To deeply understand the pathogenesis of IBD, the molecular mechanism of the proinflammatory effect of IL-17 and TNF-α on the intestinal epithelial cell line HT-29 was studied. We studied the expression level of neutrophil chemokines and TH-17 chemokines, the phosphorylation level of P38 in HT-29 cells when cultured with IL-17 and/or TNF-α. The results further confirmed that IL-17 can obviously enhance the TNF-α induced P38 phosphorylation and that P38 pathway plays an essential role in IL-17 induced inflammatory response.

Intestinal epithelium is a physical barrier between the intestinal tract and the external environment, playing an important role in intestinal immunity. The cell line HT-29 used in our research is human colonic epithelia, and it is the most common cell line employed in the laboratory to study the immunologic mechanism of intestinal mucosa. As the most representative member of the IL-17 family, IL-17 received much attention these years. Many studies suggested that multiple chemokines can be induced by IL-17 in epithelial cells to promote infiltration of neutrophils in a lesion area [12,13]. It is now known that IL-17 has been involved in the pathological process of autoimmune diseases [14,15]. Anti-IL-17 monoclonal antibodies can be used for the treatment of psoriasis, rheumatoid arthritis and uveitis, and now this treatment is assessed in a clinical trial [16]. On the opposite, usage of monoclonal antibodies in the treatment of IBD exacerbated the illness [17,18]. Results suggested that IL-17 may have a special mode of action in IBD. TNF-α is another cytokine that plays an important role in the immune regulation of IBD [19]. Hence, in our study we combined IL-17 with TNF-α to investigate whether IL-17 acts alone or in concert with TNF-α in HT-29.

The chemokine factors CXCL1, CXCL2, CXCL5, CXCL6 and IL-8 play important roles in the recruitment of neutrophils in inflammation, therefore, we detected their expression level to monitor the proinflammatory effect of IL-17. In our study, we found that when IL-17 or TNF-α were used alone to stimulate the cells, the expression level of these chemokines were relatively low. However, when combined together, their expression level elevated significantly. This result suggested that there may be a synergy between IL-17 and TNF-α. The mechanism of inflammatory response caused by IL-17 is achieved via the MAPK signal pathway by mediating the stability of mRNA [20,21]. TNF-α acts as initiator of genetic transcription, and can promote some transcriptions [22,23]. The mRNA genes regulated by IL-17 possess a similar sequence. There is an AU-rich sequence in the 3 putative untranslated regions of the mRNA, which can be grasped by Zinc finger proteins and thus the mRNA can be recognized and degraded by exosome complex [24]. For relatively stable mRNAs, the lack of the AU-rich sequence makes it unable to be mediated by IL-17. Moreover, IL-17 can elevate the chemokine factor CCL20 through TNF-α, and CCL20 can combine with CCR6 on the cell surface to further recruit TH17 and then more IL-17 will be expressed. This would create a positive feedback and it would further promote the inflammatory reaction [25,26]. Therefore, we can assume that IL-17 may improve the expression level of related cytokines by stabilizing their mRNA through the MAPK pathway together with TNF-α.

In our experiments, P38 inhibitor could decrease the expression level of IL-8 in HT-29 cells. The results suggested that the two cytokines may accelerate the inflammatory response through P38 pathway, and this was similar with the report by Guo's [27]. We also found that when IL-17 and TNF-α were used together to stimulate the Act1 silenced cells, the phosphorylation level of P38 decreased significantly compared with the control group. The result further confirmed that the Act1-dependent P38 pathway is very important for IL-17 mediated proinflammotory response. Act1 is an important receptor protein in IL-17 mediated signal pathway and its expression level will affect the activation of MARK pathway by IL-17 [28,29]. Kang et al. demonstrated that the inflammatory response can be reduced by down-regulating the expression level of Act1 [30]. It is well known that IL-17 alone cannot cause inflammatory response. However, it is widely believed that several factors working together triggering the inflammatory response [31,32].

Inflammation also emerges as a vital etiological factor for colorectal cancer (CRC), especially after chronic inflammatory stimulation [33]. Straus [34] demonstrated that the expression level of HIF-1α and c-myc genes, two factors known to be able to induce the transcription of genes encoding components of the glycolytic pathway, were upregulated in the presence of TNFα plus IL-17 in HT-29 cells. Aerobic glycolysis is a metabolic adaptation that promotes the survival/prolifera-

tion of cancer cells, thus the proinflammatory cytokines TNFα and IL-17 can also contribute to the colorectal tumorigenesis. In another study [35], Th17-type cytokines, IL-6 and TNF-α were found to synergistically activate STAT3 and NF-kB to promote CRC cell growth. IBD, which we aimed to study, can develop colitis associated with cancer over time, which would be more difficult to treat and have a poorer prognosis [36]. These facts raise the necessity to further study the function of TNFα and IL-17 in inflammatory diseases.

In conclusion, we demonstrated that IL-17 could synergistically promote TNF-α -mediated neutrophil chemokines and Th17 cell chemokine gene expression. IL-17 obviously enhanced TNF-α induced P38 phosphorylation and P38 pathway plays an essential role in IL-17 induced inflammatory response. In our study, we only examined the Act1-dependent P38 pathway, but whether other pathways (ERK, PI3K-AKT) or cytokines also participated in the function of IL-17 is still unclear, requiring further studies to be performed.

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