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# In vitro production of TNFα, IL-6 and sIL-2R in Chinese patients with ulcerative colitis *

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**Subject headings** tumor necrosis factor alpha; interleukin 6; interleukin 2, receptor; colitis, ulcerative

## Abstract

**AIM** To determine the tumor necrosis factor alpha (TNFα), interleukin 6 (IL-6) and soluble interleukin 2 receptor (sIL-2r) from peripheral blood mononuclear cells (PBMC) in 25 Chinese patients with ulcerative colitis and 20 healthy controls.

**METHODS** PBMC were isolated by density gradient centrifugation of heparinized blood and cultures for 24 or 48 hours by stimulation with LPS or PHA. TNFα and sIL-2r were measured by ELISA method and IL-6 measured by biossay.

**RESULTS** TNFα production stimulated by LPS and sIL-2r production by PHA in ulcerative colitis were significantly lower than in healthy controls (TNFα 509(46-7244)ng/L vs 1995(117-18 950)ng/L, $P<0.05$; sIL-2r 320U/ml±165U/ml *vs* 451U/ml±247U/ml, $P<0.05$). Spontaneous TNFα and sIL-2r production were not significantly different between ulcerative colitis and controls (TNFα 304(46-7044) ng/L *vs* 215(46-4009)ng/L, $P>0.05$; sIL-2r 264U/ml±115U/ml *vs* 236U/ml±139U/ml, $P>0.05$). IL-6 production by spontaneous release from PBMC in ulcerative colitis group was 109U/ml±94U/ml *vs* 44U/ml±39U/ml for those in healthy controls, $P<0.01$. IL-6 stimulated by LPS in ulcerative colitis group was (261U/ml±80U/ml) higher than in healthy controls (102U/ml±54U/ml, $P<0.01$). No correlation of TNFα, IL-6, sIL-2r production was found to disease activity, disease location and medication.

**CONCLUSION** Cytokine production from PBMC was also disturbed in Chinese patients with ulcerative colitis.

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## INTRODUCTION

Ulcerative colitis (UC) is a chronic inflammatory bowel disease (IBD). Its etiology is still unknown so far. Immunological abnormality demonstrated in IBD patients and imbalance between proinflammatory cytokines and anti-inflammatory cytokines play an important role in the initiation and regulation of the immune responses[1,2]. Tumor necrosis factor alpha (TNFα) produced by activated mononuclear cells is a potent proinflammatory and immunoregulatory cytokine[3]. It was named TNF at beginning because of its cytotoxic and anti-tumor activities[4]. Now it is known to have a wide spectrum of importance in IBD, e.g., TNF antibodies have been successfully used for treatment of Crohn's disease[5].

IL-6 is produced by a variety of cells, such as monocytes, macrophages, T lymphocytes, B lymphocytes, fibroblasts, endothelial cells, etc. It may affect the proliferation of epithelial cells and act as an autocrine growth factor for enterocytes[6,7]. It has been shown to stimulate T cell and B cell activation and proliferation[8-10], and to increase immunoglobulin synthesis from epithelium of human intestine[11,12]. It is interesting to know that IL-6 has been proposed as a marker of inflammation in IBD and its concentration was elevated in serum, peripheral blood mononuclear cells (PBMC), mucosa biopsy and lamine propria mononuclear cells (LPMC) in IBD patients[13-16]. Some papers also show that IL-6 is increased in the systemic circulation in Crohn's disease and is not elevated in UC patients[17].

Soluble interleukin-2 receptor (sIL-2r) is one form of IL-2 receptor secreted by activated T cells and other monocytes[18]. It corresponds to the "Tac" antigen (alpha chain) and could bind IL-2, and then participate in the regulation of IL-2 mediated lymphocyte activation[19,20]. Recent studies reported that sIL-2r was increased in vivo and in vitro in active IBD patients and may be an index for IBD activity[21-23]. The present study was to determine in vitro production of TNFα, IL-6 and sIL-2r in PBMC in Chinese UC patients and analyze the relation of these three cytokine production from PBMC to disease activity, location and medication.

## MATERIALS AND METHODS

### *Patients*

Twenty-five patients with UC were studied (13 male,

12 females; mean age 43 and range 23-67 years). Diagnosis of UC was based on the conventional clinical, radiological, endoscopical and pathological criteria designed by Lennard-Jones[24]. Assessment of its activity followed Sutherland's score criteria [25]. Of 25 patients, 7 patients had Sutherland's scores 1-4, 12 had scores 5-8, and 6 patients 9-12; 3 patients had proctitis, 14 left-sided colitis, 8 total colitis; 16 patients were on oral sulphasalazine (SASP) treatment (2 patients were being treated with corticosteroid), 8 patients were on Chinese medicine and one patient was treated with metronidazole.

Twenty healthy volunteers served as controls (9 male, 11 females; mean age 37 and range 21-66 years). There is no statistical difference in age and sex between these two groups.

### *Laboratory methods*

From each patient, five ml of heparinized blood were collected. Vials were coated and PBMC isolated within two hours after blood collection.

**Stimulation of PBMC** PBMC were isolated by density gradient centrifugation of heparinized blood and washed three times with Hank's balanced salt solution (without $Ca^{++}$ and $Mg^{++}$). After washing, cells were suspended in RPMI 1640(GIBCO) with 15% fetal calf serum (GIBCO), 2mM L-glutamine, 100U/ml penicillin and 100 μg/ml streptomycin. The isolated cells were cultured for 24 hours (for TNFα and IL-6 determination) or 48 hours (for sIL-2r determination) at 37℃ under 5% CO2 and 100% humidified air in small sterile culture flasks in 2ml culture medium at a concentration of $1\times10^6$/ml. The cells were cultured in the presence of 100mg/L of LPS (SIGMA) for TNFα and IL-6 stimulation production, and in the presence of 200mg/L PHA (SIGMA) for sIL-2r stimulation production. For spontaneous production of these cytokines, no stimulators were added in cell culture medians. After culturing, cell culture supernatant was harvested, added 1mmol/ml PMSF, aliquoted and stored at -30℃ until assay. Viability was determined using 3% trypan blue. Only more than 95% viable cells were used in this study.

**TNFα measurement** TNFα was determined using specific TNFα ELISA kit (Beijing Biotin Biomedicine Co.). In brief, 100μl/well TNFα standard markers and supernatant samples (in duplicate) were added into immunoplates and incubated for 2 hours at 20℃ and washed three times with 0.01M PBS (pH 7.4) and 0.05% Tween 20. The plates were subsequently incubated with 100 μl/well horseradish labelled polyclonal rabbit-anti-TNFα (1:1 000) for 1 hour and washed three times with 0.01M PBS (pH 7.4) and 0.05% Tween 20. Then 100 μl/well substrate Ophenylenediamine added. Incubation was allowed for 15 minutes in the dark at room temperature. The reaction was stopped by 50 μl/well 2 M H2SO4 solution. Absorption was read at 492nm on ELISA reader. A standard curve was drawn according to OD values of TNFα markers. TNFα concentration of each sample was read to its OD value within standard curve and expressed as ng/L.

**IL-6 bioassay** The IL-6 bioassay was performed using the IL-6 dependent cell line B9 according to Hou[26]. Briefly, each 100 μl of different samples and standard recombinant human IL-6 was added into 96 well microtitration plate (CORNING). Meanwhile, the IL-6 dependent cell line B9 cells were suspended in RPMI 1640 containing 10% fetal calf serum (GIBCO) and regulated at a concentration of $5\times10^4$ cells/ml. Each 100 μl/ml of the cells were added into the plate and cultured at 37℃ in 5% CO2 for 68 hours. Ten μl/well of 5g/L methyl thiazolyl tetrazolium blue (MTT, FLUKA) was added. The culture was continued for another 4 hours. Absorption value was read at 570nm on an automatic ELISA Reader and represented B9 cell proliferation. The concentration of IL-6 in a supernatant sample was calculated as follows: IL-6(U/ml)=the diluted concentration of supernatant of giving rise to half maximal proliferation of B9 cells × standard IL-6 activity (100U/ml) ÷ the diluted concentration of standard IL-6 giving rise to half maximal proliferation of B9 cells.

**sIL-2r measurement** sIL-2r concentration in supernatants from 48-hour culture of PBMC with or without PHA stimulation was determined using specific sIL-2r ELISA kit (Beijing Biotin Biomedicine Co.). In brief, 48-well plate was coated with monoclonal anti-IL-2r alpha antibody and blocked with 1% BSA in PBS. IL-2r markers and samples (in duplicate) were added, and incubated for 2 hours at 37℃. After washing three times with 0.01M PBS (pH 7.4) and Tween 20, 100 μl/well horseradish labelled polyclonal rabbit-anti-IL-2r alpha antibody (1:40) was added and incubated at 37℃ for one hour and a half, then washed three times, and added substrate Ophenylenediamine for 30 minutes at room temperature. The reaction stopped with 50 μl of 2M H2SO4. The absorption was read at 490nm on a ELISA reader. sIL-2r concentration was expressed as U/ml.

### *Statistical analysis*

All values were transformed by log transformation.

Then *t* test was used for comparison of TNFα, IL-6 and sIL-2r between UC and healthy control groups. Relations of TNFα, IL-6 and sIL-2r production to disease activity, disease location and medication were analyzed by linear correlation. *P* value less than 0.05 was considered statistically significant.

## RESULTS

**TNFα,** IL-6 and sIL-2r production The spontaneous and stimulated TNFα, IL-6 and sIL-2r production are shown in Table 1. TNFα production stimulated with LPS and sIL-2r production by PHA in UC group was significantly lower than that in healthy control group. Spontaneous TNFα and spontaneous sIL-2r production were not different significantly between UC and control groups. Large inter individual differences were observed in TNFα production. However, both spontaneous and stimulated IL-6 production in UC group were significantly higher than those in healthy controls.

**Linear correlation of TNFα,** Il-6 and sIL-2r production from PBMC to disease activity, location and medication No significant correlation was found between TNFα, IL-6 and sIL-2r production and disease activity, disease location and medication, but a tendency of correlation was shown between spontaneous IL-6 production and disease activity (r = 0.37) and medication (r = 0.38).

**Table 1 Spontaneous and stimulated production of TNFα, IL-6 and sIL-2r from PBMC**

| | UC (*n* = 25) | HC (*n* = 20) |
|---|---|---|
| TNFα (spontaneous) ng/L | 304(46-7044) | 215(46-4009) |
| TNFα (stimulated) ng/L | 509(46-7244)* | 1995(117-18950) |
| IL-6 (spontaneous) U/ml | 109±94* | 44±39 |
| IL-6 (stimulated) U/ml | 261±80* | 102±54s |
| IL-2r (spontaneous) U/ml | 264±115 | 236±139 |
| sIL-2r (stimulated) U/ml | 320±165* | 451±247* |

*P*<0.05. Median values shown in TNFα, range in brackets; Mean values with standard diviation shown in IL-6 and sIL-2r; HC: heathy controls.

## DISCUSSION

In this study there was a marked decrease in LPS stimulated release of TNFα and a significant decrease in PHA stimulated release of sIL-2r by PBMC from patients with UC as compared with healthy controls. No significant difference was found in spontaneous release of TNFα or sIL-2r by PBMC between these two groups. Our study also showed that both spontaneous and stimulated IL-6 production was increased from PBMC as compared to healthy controls. These implied that the release of TNFα, IL-6 and sIL-2r by activated PBMC may not always be paralleled.

IL-1β, TNFα and IL-6 are three important proinflammatory cytokines which respond to the initial stimulation. Many studies on TNFα production in UC have been reported with a different results[27-31]. Our previous study in Dutch population showed a tendency towards lower TNFα production in PBMC from UC patients[32]. The present study in Chinese UC patients confirmed this result. A large inter-difference was observed in TNFα production in Chinese UC patients.

Several studies have shown that IL-6 concentration is increased in active IBD and may be an index of disease activity[13-16]. Our study confirmed these observations, but only a tendency of correlation between spontaneous IL-6 concentration and disease activity was found in UC patients. The explanation may be that, the patients with mild inflammation often take maintenance dose of SASP or no medicine, only the patients with severe diseases were administered with high dose of SASP and corticosteroid. The latter medicine may have an inhibiting effect on immune reaction of the body. Our data also showed that IL-6 had norelation to the disease location. These results suggested that IL-6 from PBMC may reflect the active stage of the disease.

sIL-2r concentrations were increased in serum, tissue homogenates and PBMC from IBD patients, especially in Crohn's disease[20-22]. However, Schreiber *et al* reported a moderately increased spontaneous release of sIL-2r and significantly less sIL-2r secretion stimulated by pokeweed mitogen in 14 days culture of colonic LPMC from UC patients[33]. Our study had a similar result that wIL-2r concentration was silightly higher in spontaneous release from PBMC in UC patients than in healthy controls. When PBMC were cultured with PHA only for 48 hours, less release of sIL-2r was observed in UC group than in healthy controls.

PBMC is a very heterogeneous cell population. Macrophages and T cells may be mostly responsible for release of these three cytokines. The circulatary changes of proinflammatory cytokines may reflect the original status for cytokine secretion. For this reason, studies on local tissue production of cytokines are more accurate and more exact than studies on circulation. The changes of the proinflammatory cytokines may also reflect different genetic background. Our previous study showed in Dutch population TNF gene polymorphisms are present in five combinations[34] and TNFα production is associated with TNF haplotypes. These data strongly supported the concept that a different immunogenetic background may determine the degree of the immune response in IBD.

Our findings showed that TNFα, sIL-2r in vitro production were reduced and IL-6 was increased

from PBMC activation in Chinese UC patients. Further study is necessary to confirm these in vitro findings to in vivo conditions. Studies at local intestinal level should be undertaken in order to assess the significance of the cytokine dysregulation in the inflammatory response.

## REFERENCES


1 McCabe RP, Dean P, Elson CO. Immunology of inflammatory bowel disease. *Curr Opin Gastroenterol,* 1996;12(4):340-344
2 Sartor RB. pathogenesis and immune mechanism of chronic inflammatory bowel disease. *Am J Gastroenterol,* 1997;92(Suppl 12):5S-11S[a¤]
3 Ruddle NH. Tumor necrosis factor (TNF-A) and lymphotoxin (TNF-A). *Curr Opin Immunol*,1992;4(3):327-332
4 Carswell EA, Old LJ, Kassel RL, Green S, Fiore N, Williamson B. An endotoxin-induced serum factor that causes necrosis of tumors. *Proc Natl Acad Sci USA,* 1975;72(9):3666-3670
5 Derkx B, Taminiau J, Radema S, Stronkhorst A, Wortel C, Tytgat G *et al*. Tumour necrosis-factor antibody treatment in Crohn's disease. *Lancet,* 1993; 342(8864):173-174
6 Krueger J, Ray A, Tamm I, Sehgal PB. Expression and function of interleukin-6 in epithelial cells. *J Cell Biochem,* 1991;45(4):327-334
7 Jones SC, Trejdosiewicz LK, Banks RE, Howdle PD, Axon AT, Dixon MF *et al*. Expression of interleukin-6 by intestinal enterocytes. *J Clin Pathol,* 1993; 46(12):1097-1100
8 Van Seventer GA, Shimizu Y, Horgan KJ, Luce GE, Webb D, Shaw S. Remote T cell co-stimulation via LFA-1 / ICAM-1 and CD2 / LFA-3: demonstration with immobilized ligand / mAb and implication in monocyte mediated co-stimulation. *Eur J Immunol,* 1991;21(7):1711-1718
9 Hilbert DM, Cancro MP, Scherle PA, Nordan RP, Van Snick J, Gerhard W *et al*. Tcell derived IL-6 is differentially required for antigen-specific antibody secretion by primary and secondary B cells. *J Immunol,* 1989;143(12):4019-4024
10 Okada M, Kitahara M, Kishimoto S, Matsuda T, Hirano T, Kishimoto T. IL-6 / BSF-2 function as a killer helper factor in the in vitro induction of cytotoxic T cells. *J Immunol,* 1988;141(5):1543-1549
11 Roldan E, Brieva JA. Terminal differentiation of human bone marrow cells capable of spontaneous and high rate immunoglobulin secretion: role of bone marrow stromal cells and interleukin 6. *Eur J Immunol,* 1991;21(11):2671-2677
12 McGhee JR, Fujihashi K, Beagley KW, Kiyono H. Role of interleukin 6 in human and mouse mucosal IgA plasma cell responses. *Immunol Res,* 1991;10 (3-4):418-422
13 Holtkamp W, Stollberg T, Reis HE. Serum interleukin-6 is related to disease activity but not disease specificity in inflammatory bowel disease. *J Clin Gastroenterol,* 1995;20(2):123-126
14 Susiki Y, Saito H, Kasanuki J, Kishimoto T, Tamura Y, Yoshida S. Significant increase of interleukin 6 production in blood mononuclear leukocytes obtained from patients with active inflammatory bowel disease. *Life Sci,* 1990; 47(24):2193-2197
15 Reinecker HC, Steffen M, Witthoeft T, Pflueger I, Schreiber S, MacDermott RP. Enhanced secretion of tumour necrosis factor-alpha, IL-6, and IL-1β by isolated lamina propria mononuclear cells from patients with ulcerative colitis and Crohn's disease. *Clin Exp Immunol,* 1993;94(1):174-181
16 Hyams JS, Fitzgerald JE, Treem WR, Wyzga N, Kreutzer DL. Relationship of functional and antigenic interleukin 6 to disease activity in inflammatory bowel disease. *Gastroenterology,* 1993;104(5):1285-1292
17 Gross V, Andus T, Caesar I, Roth M, Sch-lmerich J. Evidence for continuous stimulation of interleukin 6 production in Crohn's disease. *Gastroenterology,* 1992;102(2):514-519
18 Rubin LA, Kurman CC, Fritz ME, Biddison WE, Boutin B,Yarchoan R *et al*. Soluble interleukin 2 receptors are released from activated human lymphoid cells in vitro. *J Immunol,* 1985;135(5):3172-3177
19 Rubin LA, Jay G, Nelson DL. The released interleukin 2 receptor binds interluekin 2 efficiently. *J Immunol,* 1986;137(12):3841-3844
20 Jacques Y, Le-Mauff B, Boeffard F, Godard A, Soulillou JP. A soluble interleukin 2 receptor produced by a normal alloreactive human T cell clone binds interleukin 2 with low affinity. *J Immunol,* 1987;139(7):2308-2316
21 Brynskov J, Tvede N. Plasma interleukin-2 and a soluble shed interleukin-2 receptor in serum of patients with Crohn's disease. Effect of cyclosporin. *Gut,* 1990;31(7):795-799
22 Mahida YR, Gallagher A, Kurlak L, Nawkey CJ. Plasma and tissue interleukin 2 receptor levels in inflammatory bowel disease. *Clin Exp Immunol,* 1990;82 (1):75-80
23 Mueller C, Knoflach P, Zielinski CC. T-cell activation in Crohn's disease. Increased levels of soluble interleukin 2 receptor in serum and in supernatants of stimulated peripheral blood mononuclear cells. *Gastroenterology,* 1990;98(3):639-646
24 Lennard-Jones JE. Classification of inflammatory bowel disease. *Scand J Gastroenterol.* 1989;24(Suppl 170):2-6
25 Sutherland LR, Martin F, Greer S. 5-Aminosalicylic acid enema in the treatment of distal ulcerative colitis, proctosigmoiditis, and proctitis. *Gastroenterology,* 1987;92(6):1894-1898
26 Hou J, Kong XT. Interleukin 6 bioassay by MTT colorimetry. *Chin J Med Lab Technol*,1993;16:208-210
27 Maeda M, Watanabe N, Neda H, Yamauchi N, Okamoto T, Sasaki H *et al*. Serum tumor necrosis factor activity in inflammatory lowel disease. *Immunopharmacol Immunotoxical,* 1992;14(3):451-461
28 Murch SH, Lamkin VA, Savage MO, Walker-Smith JA, MacDonald TT. Serum concentrations of tumour necrosis factor alpha in childhood chronic inflammatory bowel disease. *Gut,* 1991;32(8):913-917
29 Mahmud N, O'Connell MA, Stinson J, Goggins MG, Weir DG, Kelleher D. Tumor necrosis factor-alpha and microalbuminuria in patients with inflammatory bowel disease. *Eur J Gastroenterol Hepatol*,1995;7(3):215-219
30 Mazlam MZ, Hodgson H. Peripheral blood monocyte cytokine production and acute phase response in inflammatory bowel disease. *Gut,* 1992;36(6): 773-778
31 Nielsen OH, Brynskov J, Bendtzen K. Circulating and mucosal concentrations of tumour necrosis factor and inhibitor(s) in chronic inflammatory bowel disease. *Dan Med Bull,*1993;40(2):247-249
32 Bouma G, Oudkerk Pool M, Scharenberg JGM, Kolkman JJ, von Blomberg BME, Scheper RJ *et al*. Differences in the intrinsic capacity of T-cells to produce the cytokines tumor necrosis factor alpha and beta in patients with inflammatory bowel disease and healthy controls. *Scand J Gastroenterol,* 1995;30(11):1095-1100
33 Schreiber S, Raedler A, Conn AR, Rombeau JL, MacDermott RP. Increased in vitro release of soluble interleukin 2 receptor by colonic lamina propria mononuclear cells in inflammatory bowel disease. *Gut,* 1992;33(2):236-241
34 Bouma G, Xia B, Crusius JBA, Bioque G, Koutroubakis I, von Blomberg BME *et al*. Distribution of four polymorphisms in the tumour necrosis factor (TNF) genes in patients with inflammatory bowel disease (IBD). *Clin Exp Immunol,* 1996;103(3):391-396