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Blockade of IL-6 signal exacerbates acute inflammatory bowel disease via inhibiting IL-17 producing in activated CD4+ Th17 population

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Abstract. – BACKGROUND: Inflammatory bowel disease (IBD) is a common disease in human resulted from a various of factors including genetic background, immune system and environment factors.

OBJECTIVES: Recent studies suggest pro-inflammatory cytokine IL-17 producing cell subset was involved in the disease development and the maintenance of IBD. And the differentiated and activation of IL-17 producing cells were mostly dependent on the cytokines profile secreted by innate cells in intestinal tissues. In this study, we examined the functions of IL-6 signal in regultory of IL-17 production in acute IBD model.

MATERIALS AND METHODS: Wildtype mice were treated with anti-IL-6 neutralizing antibodies to block IL-6 signal And then treated with DSS to induce acute IBD.

RESULTS: Mice treated with anti-IL-6 neutralizing antibodies show severe colitis and high level of pro-inflammatory cytokine IL-17 production in DSS-induced acute IBD model when conpared with control group. Our research suggested blockade of IL-6 signal pathways in acute colitus model resulted in specifical activation of IL-17 producing cell population. Furthermore, CD44+ activated Th17 cell popualtion and CD44- IL-17 producing T cells exhibited different susceptibility to IL-6 signal in our model.

CONCLUSIONS: Blockade of IL-6 signal in DSS-induced acuted IBD model increased IL-17 production level specifically in CD44- T cells and reduced CD44+ Th17 cell population.

Key Words:
Inflammatory bowel disease (IBD), DSS, Th17 cells, IL-6.

Introduction

Inflammatory bowel disease (IBD) including Crohn's disease (CD) and ulcerative colitis (UC) is a increasing public health problem which affects 1/1,000 individuals in human. The symptoms of IBD include diarrhea, abdominal pain, weight loss and result in death in some extreme cases and the development of IBD is considered resulted form a complex interplay among genetic, immunological and environmental factors. In general, CD4+ T cell subsets are extremely important in IBD via regulation of pro/anti-inflammatory cytokines producing according to recent works[1,2]. The balance state of protective immunity and self-tolerance of self-antigens and commensal bacteria in intestine is maintained by Treg/Th17 cells. Moreover, Tfh cells are critical to the mature of antibodies producing B cells and IgA secretion in gut mucosa[3,4].

However, there is growing evidence that Th17 cells are linked form the disease development in IBD. It was reported that cytokines IL-17 and Th17 cells population was increased in murine IBD model[5,6]. Cytokines IL-17 IL-17F, IL-21 and IL-23 which were mostly secreted by Th17 cells or regulated by Th17 cells are now considered crucial factors in murine inflammatory colitis[7-9]. Studies in transgenic mice model suggested IL-17RA deficient mice who lacked of Th17 cells shown higher susceptibility to 2,4,6-trinitrobenzene sulfonic acid (TNBS)-induced acute gut inflammation[10,11], which was consisted with these mice treated with anti-IL-17 neutralizing antibodies. Similar results were also observed in IL-17/IL-17f deficient mice model[12]. By contrast, adoptive transfer of T cells from IL-17a, IL-17f or IL-22 deficient mice into RAG-1 [V(D)J recombination activation gene RAG-1] deficient mice induced severe colitis while comparing these cells form wildtype mice[13]. Similarly, induced intestinal colitis in IL-17 deficient mice shown no difference from that in wildtype mice[14]. Notable, blockade of IL-17 signal pathway with anti-IL-17 neutralizing antibodies reduced colitis in TNBS-induce murine

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acute IBD model but not in the dextran sodium sulphate (DSS)-induced IBD model. The reason why these studies provided us with different results is still unknown. One possible reason is that Th17 cells play multiple roles in the regulation of intestinal colitis and its roles is the disease development dependent on the co-factors such cytokines as IFN-gama, IL-10 and TGF-beta, in the local tissues which was produced by other cells[15]. However, to clarify the importance of Th17 cells in IBD, further data are needed.

Recent data suggest the fundamental role of IL-6 signal pathways in the development and maintenance of IBD and IL-6 also contributes to Th17 differentiation both *in vivo* and *in vitro*[16,17]. Molecular mechanism studies show that IL-6-SATA3 pathway results in T cells accumulation and tolerant against apoptosis and promote Th17 cell development, however, the source of IL-6 and its target cell subset was still unclear. In this study, we treated mice with anti-IL-6 neutralizing antibodies to remove soluble IL-6 and block IL-6 signal in DSS-induced acute IDB model. This model provides a clear background to study T subsets differentiation and their roles in the colitis development.

Materials and Methods

Treatment with Anti-IL-6 Neutralizing Antibodies

For blocking of IL-6 signal *in vivo*, mice were treated with 500 ug anti-IL-6 monoclonal antibodies (clone MP4-20F3, eBioscience, San Diego, CA, USA) or isotype control purified IgG with same amount. Abs were injected i.p. In a volume of 200 ul PBS 1 day after DSS treatment.

DSS Induction of Colitis

For the DSS-induced colitis, 4%(weight/volume) DSS (Sigma Aldrich Co, St Louis, MO, USA) was administered in the drinking water for 5 days as previously described[18] to female B6 wildtype mice aged 6-8 weeks. To assess the extent to which colitis was induced, the mice were examined daily for body weight, stool consistency, and blood in the stool.

RNA Isolation and Quantitative Real-Time RT-PCR (qRT-PCR)

Total RNA was extracted with TRIzol reagent (Invitrogen Co, Carlsbad, CA, USA) according to the manufacturer's protocol. Reverse transcription (RT) was performed with a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Tokyo, Japan) according to the manufacturer's instructions. Quantitative real-time PCR was performed with SYBR Green PCR Master Mix (Applied Biosystems) according to the manufacturer's protocol. The reaction conditions involved 40 cycles of two-stage PCR consisting of denaturation at 95°C for 15 s and annealing at 60°C for 1 min after an initial denaturation step of 95°C for 10 min. The primer sequences were as follows: IFN-gamma, 5′-ACTGGCAAAAGGATGGTGA-3′ and 5′-GCTGTTGCTGAAGAAGGTAG-3'; mouse IL-17A, 5′-ATCAGGACGCGCAAACAT G-3′ and 5′-TGATCGCTGCTGCCTTCAC-3'; and mouse β-actin, 5′-AGAGGGAAATCGTGCGTGAC-3′ and 5′-CAATAGTGATGACCTGGCCGT-3′. To allow comparisons of mRNA expression levels, the real-time PCR data were analyzed with the ΔΔCt method and normalized to the amount of β-actin cDNA as an endogenous control.

Flow Cytometry Analysis

Intracellular expression of IL-17 producing T cells was analyzed using a Cytofix/Cytoperm Kit Plus (with GolgiStop; BD Biosciences, San Diego, CA, USA), according to the manufacturer's instructions. In brief, lymphocytes obtained from mLNs were incubated with 50 ng/ml PMA (Sigma), 5 M calcium ionophore A23187 (Sigma), and GolgiStop at 37°C for 4 h. Surface staining was performed with anti-TCR-beta-FITC, anti-CD44-APC, anti-CD4-PerCP-Cy5.5 (BioLegend, San Diego, CA, USA) for 20 min at 4°C, the cells were permeabilized with Cytofix/Cytoperm solution for 20 min at 4°C, and intracellular cytokine staining was performed with anti-IL-17A-Alexa Fluor 647 (BD Biosciences).

Statistics Analysis

Results were expressed as means ± SD. The statistical significance of the data was evaluated by Student's *t*-test. Data analysis was performed using the SPSS v10.0 (SPSS Inc., Chicago, IL, USA). A value of $p < 0.05$ was regarded as significant.

Results

Treatment with anti-IL-6 Neutralizing Antibodies Exacerbates DSS-Induced IBD in Mice

To studies Th17 cell population and their roles in colitis, we generated DSS-induced acute IBD

model in female B6 wildtype mice. These mice were treated with 4% DSS in daily water for 5 days and began to show classic symptom of IBD such as body weight loss and changes of feces 10 days after DSS treatment. These mice also shown some changes in numbers of lymphocytes, percentages of T cell subsets, cytokines profiles and size of spleen.(data no shown) In this model, mice started to dead from acute IBD 2 weeks after DSS treatment and all the mice dead 4~5 weeks after DSS treatment if they were not treated by drugs to help defense against this disease.

To investigate the role of IL-6 in IBD disease model, these mice were treated with anti-IL-6 neutralizing antibodies 1 day after DSS treatment to block soluble IL-6. In our observation, mice treated with anti-IL-6 neutralizing antibodies had lost more body weight than PBS control group. We next analyzed the intestinal tissues from mice treated with DSS for 2 weeks and found that these mice from anti-IL-6 neutralizing antibodies treatment group had shorter colon than the control group (data not shown), which suggested these mice suffered a more severe IBD than the control group. The survival rate analysis also suggest similar results that anti-IL-6 neutralizing antibodies treated mice began to dead on day 10 and they could not survival for more than 4 weeks. However, the control group still had more than 25% alive 4 weeks after DSS treatment (Figure 1).

Treatment with Anti-IL-6 Neutralizing Antibodies Increases IL-17a Producing Level

We next studied the cytokines producing levels of *mRNA* in intestinal tissues of mice after 10 day treatment of DSS, who show classic IBD symptom. As recent researches reported, IL-17a is the major regulatory factor in IBD development and maintenance. However, Th1 type cytokines such as IFN-gamma is mostly respond to tissue damages in disease development. We focused on these 2 cytokines producing in colon tissues and found that the expression of IFN-gamma was similar in both anti-IL-6 neutralizing antibodies treatment group and control group. However, the Il-17a producing levels in treatment group was significant higher than the control group (Figure 2).

Considering the fact that IL-17a is produced by many cell types including T cells and innate immune cells like gamma-delta T cells and NK cells, and the percentage of T cells in intestinal tissues is very low (less that 0.5% of total cells), it is possible that these IL-17a was mostly produced by non-T cells in intestinal tissues. To investigate the IL-17a producing levels in T cells, we analyzed IL-17 producing *via* FACS (Flow Cytometry) intercellular staining.

Treatment with Anti-IL-6 Neutralizing Antibodies Reduces CD44+ Th17 Cells

We firstly stained IL-17a producing cell *via* FACS intercellular staining in T cells and non-T cells, and found that non-T cells produced more IL-17a than T cells did. However, anti-IL-6 neutralizing antibodies treated seemed to have no effect on these cells population for that the IL-17a positive cells group in non-T cells did not change in antibodies treatment or control group (data not shown).

We next distinguished T cells into CD44+ activated group and CD44- group and examined the IL-17a producing in these two group. The results shown that anti-IL-6 antibodies treatment signifi-

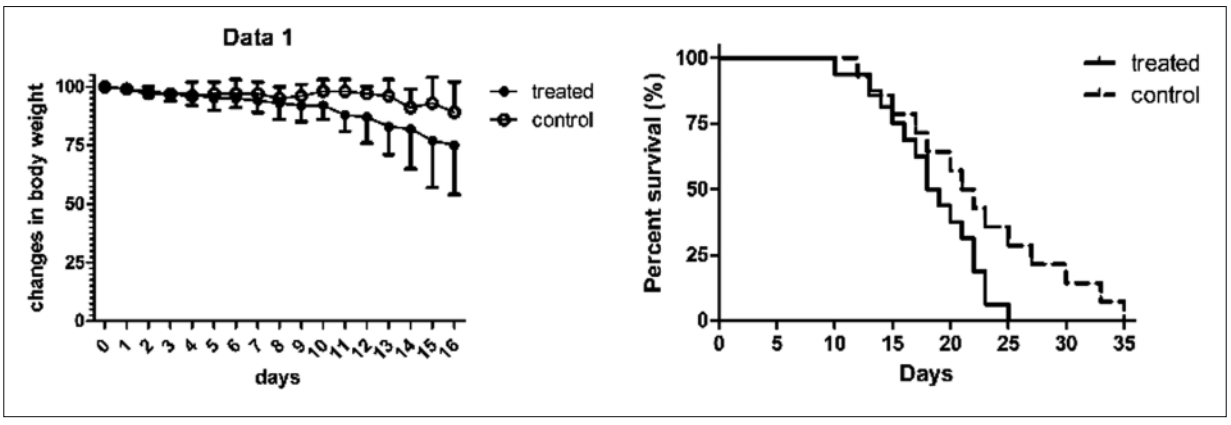

Figure 1. Body weight loss and survival rate in DSS-induced acute IBD mice. Treatment with anti-IL-6 neutralizing antibodies led to more severe symptom of IBD (n=20).

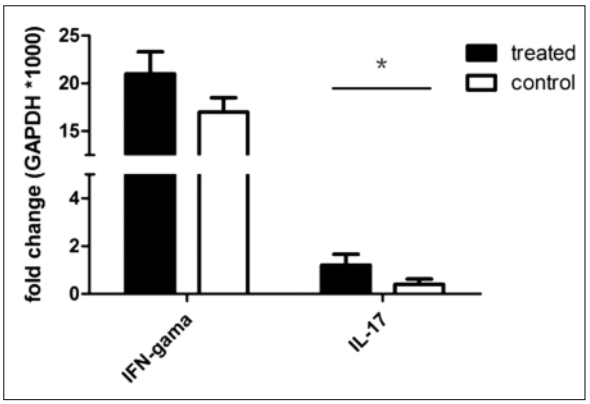

Figure 2. mRNA levels of IFN-gamma and IL-17a in intestinal tissues of IBD mice. Treatment with anti-IL-6 neutralizing antibodies had almost no effect on IFN-gamma expression but significant induced IL-17a expression level in intestinal tissues (n=6).

cantly reduced IL-17a producing in the activated T cells group when compared with the control group. Furthermore, we were interesting to found that the IL-17a producing level in CD44- cells group was increased in antibodies treated group, and these mice had a higher total IL-17a that the control group. The reasons why IL-6 has different effect on activated and non-activated T cells is still unclear. One possible reason is that IL-6 is important in activated T cells survival from apoptosis signals and blockade of IL-6 signal leads to the clearance of IL-17a producing activated T cells in the antibodies treated mice (Figure 3).

Discussion

We generated acute IBD model in female B6 wildtype mice with DSS treatment and treated these mice with anti-IL-6 neutralizing antibodies 1 day after, which efficiently blocked IL-6 signal pathways as reported[19]. These mice treated with anti-IL-6 neutralizing antibodies shown higher susceptibility to DSS treatment and shown severe IBD symptom such as body weight loss, changes in feces. As recent works reported, Th17 cells were crucial to IBD development and IL-6 was the key factor in Th17 cell differentiation, we focused on IL-17a producing in small intestinal tissues in these mice with IBD, and found that mRNA level of IL17a was increased in anti-IL-6 neutralizing antibodies treatment mice. However, whether these increasing IL-17a is directly linked with aggravation of IBD or not is still unclear and more experimental data are needed.

Furthermore, cytokine IL-17a is a multi-functional pro-inflammatory factor and produced by many cells types including T cells and innate cells in intestine tissues, we next investigated Il-17a producing in T cells and non T cells and found that IL-17a was mostly producing by T cells, and anti-IL-6 neutralizing antibodies treatment had no detected effect on IL-17a producing non-T cells. Furthermore, we also found that blockade of IL-6 in IBD mice specifically reduced IL-17a producing levels in activated CD44+ Th17 cells and promoted IL-17a secretion in CD44- Th17 cells population *via* FACS data, which was firstly reported.

Blockade of IL-6 signal did not reduce IL-17a producing in DSS-induced acute IBD model although IL-6 is the key factor in Th17 cells differentiation. One posssible reason is that Th17 cells differentiation rare occurred in acute inflammatory and these cytokines are mostly produced by pro-exist primary Th17 cells.

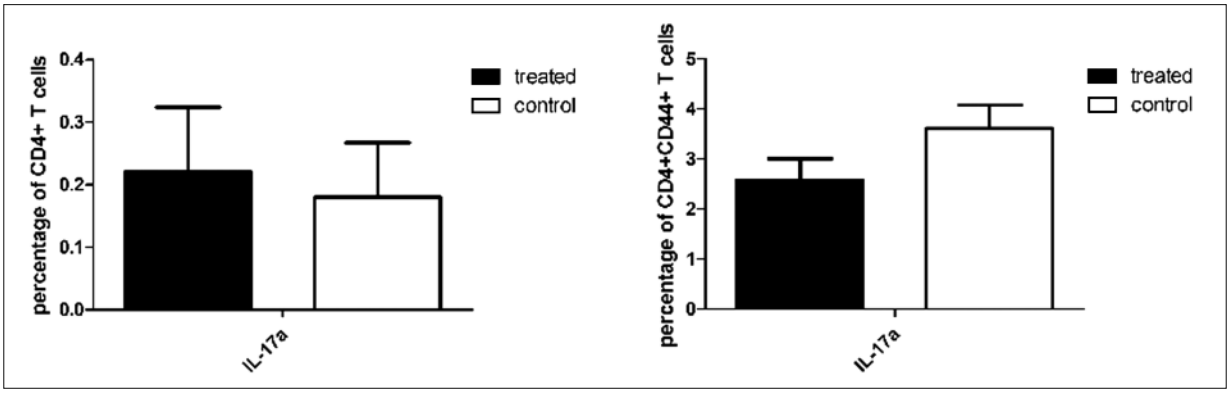

Figure 3. Percentage of IL-17a producing T cells in CD4+ T cells and CD4+ CD44+ double positive cells (cells were gated on TCRbeta+ CD4+ or TCRbeta+ CD4+ CD44+).

Conclusions

In our system, we observed that IL-6 had different effects on CD44+ activated Th17 cells and CD44- Th17 group, which suggested these two cells populations played different roles in colitis development and maintenance. For details, activated Th17 cells were involved in inflammatory control and host defense against colitis. On the other side, IL-17a produced by non-T cells and non-activated T cells was respond to host recognition of infection and local inflammation. However, more experimental data are needed fot us to identify Th17 cell population and their roles in IBD.

Acknowledgements

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Conflict of Interest

The Authors declare that there are no conflicts of interest.

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