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염증성 장질환에서 C/EBPβ 발현을 통한 유도조절T세포의 면역 억제 효과 기능 개선

Improved Immune Suppressive Activity of Induced Regulatory T cells Expressing C/EBPβ in Inflammatory Bowel Disease

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Abstract

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The prevalence of the inflammatory bowel disease(IBD) is increasing worldwide, not to mention in the Western countries but also in Eastern Asia countries considered low risk in the past. The absolute cure for the IBD, however, is not achieved with the current treatments. Notwithstanding the exact cause of IBD is still under investigated, inflammatory nature of IBD is generally accepted idea.

A new therapy using regulatory T(Treg) cell is considered as a promising candidate because Treg cells have capacity for both down-regulating specific pro-inflammatory target cells and repairing the tissue damage. It's previously been reported that the stability of C/EBPβ overexpressed *in vitro* cultured TGFβ induced Treg cells(C/EBPβ_iTreg cells) is enhanced when most of its counterpart control TGFβ induced Treg cells(NGFR_iTreg cells) cultured in the same way turn into pathogenic ex-iTreg cells under acute inflammatory environments.

Therefore, in this study, the stability and anti-inflammatory function of C/EBPβ_iTreg cells were examined in physiological context under severe inflammatory condition employing Dextran Sulphate Sodium(DSS) induced colitis mouse model. A certain degree of the inflammation caused by DSS was being rescued by intraperitoneal injection of C/EBPβ_iTreg cells comparing to control iTreg cells injection. The degree of inflammation was evaluated by the ration of he body weight change, colon length, and histological analysis. Additionally, Type1 T helper related cytokines' mRNA levels(IFNγ, TNFα and IL-12) in the distal colon of the mice with DSS are reduced by injecting C/EBPβ_iTreg cells. In order to find the cause of its improved anti-inflammatory function, the mRNA level of the well-known suppressive cytokines(IL-10, CTLA-4 and TGFβ) were measured. IL-10 mRNA level, among others, was increased *in vitro* cultured C/EBPβ_iTreg cells comparing to the control iTreg cells. All told, IL-10 might be a major player in remitting the colitis. The mechanism how C/EBPβ overexpression in iTreg cells increases IL-10 production is in question and its association with enhanced iTreg cell's stability needs to be investigated further.

Keywords; regulatory T cell, C/EBPβ, inflammatory bowel disease, interleukin-10, Dextran Sulfate Sodium

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Introduction

A. The prevalence of inflammatory bowel disease and its inflammatory nature.

The primary types of Inflammatory Bowel Disease(IBD) are Crohn's Disease(CD) and ulcerative colitis(UC). IBD is multifactorial inflammatory disease with unknown factors in colon and small intestine. The common signs and symptoms of IBD are diarrhea, bloody stool, abdominal cramping and pain(Daniel C Baumgart 2007). The disease put patients with burden of surgery, therapy, hospitality, and social functioning due to its prolonged nature. About 1~1.3 million people are currently estimated to be suffering from IBD in the United States. It's experiencing the steepest increase worldwide, even in the population considered 'low risk', for instance, Japan and India(Ananthakrishnan 2015). The differential diagnosis of the IBD patients is broad, and there isn't any single golden standard maker. So there are various diagnostic markers that are somewhat related to inflammation, such as erythrocyte sedimentation rate, sialic acid, fibrinogen, lactoferrin, β2 microglobulin, serum amyloid A, α2 globulin, and α1 antitrypsin, Especially C reactive protein(CRP) level in the blood, though it shows remarkable heterogeneity between CD and UC, is highly corresponds with IBD progression. It is created by hepatocyte under the influence of tumor necrosis factor α(TNF-α),

interleukin(IL)-6, and IL-1β(S Vermeire 2006). The more reliable markers, including inflammatory cytokines, are the subject of intense investigation based on the inflammatory nature of IBD.

B. Current treatments and a regulatory T cell therapy

There are three major types of treatment currently used for IBD; anti-inflammatory and antibiotic drug, TNF-α blocker, and fecal transplantation. Most commonly used ones are the anti-inflammatory and antibiotic drugs. The commercially available drugs for reducing inflammation are Azathioprine, mercaptopurine, Cyclosporine, and so forth. And conventional antibiotics include Metronidazole and Ciprofloxacin(Gary R. Lichtenstein 2009). However, these agents have not only been unsuccessful in the adequate control of the disease, but it's been also followed by adverse side effects in large portion of the patients(Reinisch W 2012, Lee KM 2013) partly caused by lack of an antigen-specificity. In trial cases, the administration of the broad-spectrum antibiotics to patients causes imbalance in gut flora. The situation goes from bad to worse when certain bacteria become less competitive and gives the patients a high chance to catch a pseudomembranous colitis caused by *Clostridium difficile* infection. Therefore, for those patients unresponsive to the conventional treatments, TNF-α blocker(*Infliximab*) were additionally used. Although it has been shown less severe side effects, enhanced clinical remission and mucosal healing all together, the continuous administration would

result in defenseless against pathogenic infections(Lv R 2014). Lastly, as more evidences are recently reported about the relation between microbiota and autoimmune disease, fecal microbiota transplantation(FMT) was used for some patients with IBD(Colleen R. Kelly 2015). But only a limited number of patients were responsive to the treatment. Moreover, since FMT means transferring not only the advantageous bacteria from one person's bowel to another's but also all the fungi, proteins, and viruses in a person's stool, some of the patients become worsen(Paul Moayyedi 2015). Therefore, the limitation for the commercially available treatments these days is that its inability to cure the disease but mostly to relieve the symptoms, and if the disease is not remedied by the treatments, it should be accompanied with complete removal of the entire intestine. Demands, therefore, for a newer biological therapy for IBD is increasing.

There is a big demand for a new therapeutic approach for the IBD patients. According to the previous reports, an adoptive transfer of regulatory T cells(Treg) prevent miscellaneous autoimmune disease pathogenesis in several mouse models, such as type 1 diabetes, multiple sclerosis, immunodysregulation polyendocrinopathy enteropathy X-linked syndrome(Singh B 2001, A. Mros 2006, Kajsa Wing 2010, Zhou X 2011). These reports suggest that a novel therapeutic approach to treat autoimmune diseases may be achieved by manipulation of Treg cells. However, there are some unsatisfactory factors for the therapeutic use of the Treg cells on

established autoimmune diseases. First, the low frequency and the poor growth of nTreg cells directly obtained from *in vivo* have insufficient numbers for adoptive transfer into the patients. The number could be increased by *in vitro* expansion of naïve T cells and differentiate it into the regulatory T cells under optimized condition. However, its suppressive activity could be influenced by the increased level of inflammatory cytokines in the patient's body so turns it into pathogenic ex-Treg cells(Estelle Bettelli and Kuchroo 2006, Zhou X 2011).

C. A role of CCAAT/enhancer-binding protein beta(C/EBPβ) as a stabilizer of Foxp3 expression in iTreg cells

As mentioned above, the most notable subtypes of Treg cells are natural regulatory T cells(nTreg) and induced regulatory T cells(iTreg). In thymus, thymocytes with intermediate affinity for self-antigens were selected to be differentiated into nTreg cells (Sakaguchi S 1995) and those are directly harvested directly from peripheral blood. Even though the stability of Foxp3 in nTreg is considered to be maintained consistently(Zhou X 2011), the portion of nTreg in the blood is approximately 5~10% of the mature T cells, suggesting that the amount of purified nTreg cell is less than the amount needed to exert immunosuppressive effects when injected to the IBD induced mice model(Wang X 2011). On the other side, naïve $CD4^+$T cells differentiate to regulatory T cells *in vitro* via Treg differentiation condition(TGF-β, anti-IFN-γ and anti-IL-4)

is called TGFβ-induced Treg cells (Huter EN 2008). Greater yield of the TGFβ-induced Treg(called iTreg here) could be achieved by this method. The greatest drawback is under repeated rounds of activation of both nTreg and iTreg cells because they are likely to lose their Foxp3 expression. To make matters worse, a transformation to pathogenic effector TH17/TH1 cells(pathogenic ex-iTreg cells) from iTreg cells under severe inflammatory condition could take place due to its instability. It will result in accelerating the inflammation of IBD patients(Estelle Bettelli and Kuchroo 2006, Xuexian O. Yang 2008, Zhou L 2009, Zhou X 2009).

An initial observation by Dr. Lee showed enhanced Treg stability after a heterologous gene encoding CCAAT/enhancer-binding protein beta(C/EBPβ) was introduced to iTreg cells. C/EBPβ is a bZIP transcription factor encoded by cebpβ gene. It can bind to certain DNA regulatory regions as a homodimer or a heterodimer with C/EBPα, C/EBPδ, and C/EBPϒ(Stein B 1993). The C/EBPβ proteins are previously reported to be a regulator of genes in a various cells with a function of inflammatory response(Sven Heinz 2010, Baldwin 1993).

The transcription factor Foxp3 controls a regulatory T cell differentiation and maintains its suppressive function(Chen W 2003, Fontenot JD 2003, Hori S 2003). However, under *in vivo* pro-inflammatory conditions with inflammatory cytokines, such as IL-4 and INFϒ, it has been reported that the Foxp3 expression level of

the cells is mitigated and an inhibitory cytokine IL-10 level in iTreg cell is also decreased(Estelle Bettelli and Kuchroo 2006, Dardalhon V 2008). According to Dr. Lee, the level of Foxp3 expression of C/EBPβ overexpressed iTreg(called C/EBPβ_iTreg cells in this paper) was sustained *in vitro* under IL-4 and INFγ rich conditions when compared to non-transduced TGF-β induced Treg cells(Lee 2014). Due to its increased stability, the adoptive transfer of C/EBPβ_iTreg cells to the patients could form a promising therapeutic candidate for autoimmune diseases, especially inflammatory bowel disease.

D. Utilization of mouse Dextran Sulphate Sodium(DSS) induced colitis to mimic inflammatory bowel disease in human

Dextran sodium Sulfate(DSS) is polysaccharide with molecular weight ranging from 5 to 1400kDa. It is water-soluble and negatively charged(Okayasu I 1990). The pathology of the DSS colitis is mostly placed in distal colon. Intestinal inflammation is likely to be induced by damaging epithelial monolayer lining of the large intestine resulting in dissemination of pro-inflammatory intestinal contents such as bacteria and their products. According to a recent published article, DSS forms nanometer-sized vesicles which bind to the colonic lumen, by forming complex to medium-chain-length fatty acids(MCFAs). And the DSS/MCFA vesicles go into the cytoplasm and activate inflammatory response(Hamed Laroui 2012). The symptoms and signs of the DSS-induced colitis have many similarities with a human IBD; hunched back, rectal bleeding,

diarrhea, weight reduction, shortness of colon, and endoscopic damage. Additionally, the involvement of the certain cytokines, such as TNFα and IFN$_{\gamma}$, in the disease process is one of the similarities(S E Plevy 1997, Alex P 2009). There are some of the strong points in using DSS. The disease duration is controllable and the onset is immediate. Moreover, artificial genetic manipulation and deletion of the mice is unnecessary to induce the disease like the IBD caused in human(Strober W 2002, Wirtz S 2007). For these reasons, DSS-induced colitis model is one of the most widely used chemically induced colitis models. However, an important limitation of using DSS is that the inflammation could be induced without help of the adoptive immune cells, unlike humans(Chassaing B. 2015).

In this study, the suppressive function of C/EBPβ_iTreg was measured in DSS-induced colitis mouse by checking indicators used to see the disease progression. As a result of it, C/EBPβ_iTreg cells showed an enhanced suppressive response *in vivo*. This paper assumes that the improvement of the cell is associated with the increased IL-10 level of the cell. Moreover, the possible target cells of C/EBPβ_iTreg was supposed by comparing mRNA level of inflammatory cytokines in the mouse colon. Further work on finding an exact mechanism of up-regulating IL-10 by C/EBPβ in the transduced iTreg cells and confirming the target cells of the transduced iTreg cells would lead to a more reliable and safe therapeutic treatment for IBD.

Method

Mice. C57BL/6(H-2b) mice were purchased form The Jackson Laboratory. Foxp3eGFP reporter mice were kindly provided by T.A. Chatila(University of California at Los Angeles). All the mice were bred and maintained in specific pathogen-free barrier facilities at Seoul National University and were used according to protocols approved by the Insititutional Animal Care and Use Committees (IACUC) of Seoul National University.

DNA constructs. *C/ebpβ* was amplified from mouse genomic DNA by PCR. After sequenced, the product was inserted into the restriction site of the MSCV-IRES-hNGFR(MIN) retroviral vector. MIN vector was kindly provided by U.N. Pear(University of Pensylvania).

Purification and *in vitro* iTreg cell transduction and differentiation. The precursor T cells in this experiment were cultured in RPMI-1640 medium supplemented with 10%FBS, 100 U/mL penicillin, 10 μg/mL streptomycin, and 55μM 2-mercaptoethanol. The naïve T cells($CD4^+CD44^{low}GFP^-$) cells from mesenteric lymph node and spleen of 8- to 12- week old Foxp3eGFP reporter mice were sorted via FACS(SH800 SONY Cell Sorter) and placed in the well coated with plate bound α-CD3(2 hours,37C). After activation with soluble α-CD28, α-IL-4, and α-TGFβ, prepared C/ebpb-Min and Min (control) vector was respectively transduced into the activated T cells. The cells were rested in 96-round-bottom plate for 12

hours with α-IL-4, and α-TGFβ. Next, the cells were moved to α-CD3 coated well with soluble α-CD28, α-IL-4, and TGFβ for differentiation. After 48 hours, half of the antibody mixed media was replaced by fresh media. Both transduced and TGFβ induced regulatory T cells(GFP^+Min^+) were sorted using FACS.

Retroviral transduction. Phoenix packing cells(8.5x105/1mL media) were plated for 3hours ahead of the transduction. The retroviral vector was calcium transfected to the cell. The media was replaced by flash media after 24hours. And viral supernatant was collected after 48 hours from the media change. The collected supernatant spin infected the activated T cells with 8㎍/mL polybrene. The viral supernatant was removed, and the appropriate neutralizing antibody(α-TFGβ and α-IL-4) containing media was added for resting condition.

Sorting and adoptive transfer. The differentiated cells were stained with α-CD271-PE and sorted the GFP and PE double-positive cells. $1x10^6$ iTreg cells that successfully transduced and differentiated are obtained using FACS(SH800 SONY Cell Sorter). The cells were placed in 150㎕ of PBS and adoptively transferred to recipient mice by a intrapertoneal injection. The 4 mice were prepared for each set. The mice were subjected to; Control with or without DSS, C/EBPβ or NGFR_iTreg with DSS.

Induction of colitis via administration of DSS water. 2.0% of Dextran

Sulfate Sodium(DSS) salt(MP Biomedicals M.W 36-50kDa, Ref = 160110) was dissolved in autoclaved water. The body weights of C57BL/6J WT mice were equilibrated to range between 23g and 25g before DSS administration. DSS was fed *ad libitum* and it was replaced by the flash one on alternate day. Same drinking water without DSS was given to the control mouse. The body weight of the mouse was measured daily. DSS was given for 7 days and the mice were analyzed on day 8.

Cytokine levels of both the distal colon and the two kinds of iTreg cells were analyzed by qRT-PCR analysis. A piece of colon(50mg) was placed in the Trizol and homogenized(Qiagen TissueLyser / Retsch MM301). RNA was extracted by a standard procedure and reverse transcribed to cDNA for qRT-PCR analysis(Applied Biosystems StepOne™). The mRNA levels of the cytokines increased in DSS induced colitis(TNFα, IFNγ, IL-12, etc.) were measured(Alex P 2009). For the distinct iTreg cells, $1x10^5$ transduced and differentiated iTregs are sorted as it was mentioned above. qRT-PCR analysis was conducted to measure mRNA levels of the well-known suppressive cytokines(IL-10, CTLA-4, and TGFβ)(Dario A. A. Vignali 2008).

Histological analysis of distal colonic section by H&E (=Hematoxylin & Eosin) staining. The each piece of the distal colon was cut and rinsed with PBS. The piece of the organ was fixed in 10% buffered formalin for following 24 hours, and transferred to 70% ethanol to be analyzed. The analysis was based on the degree of epithelial

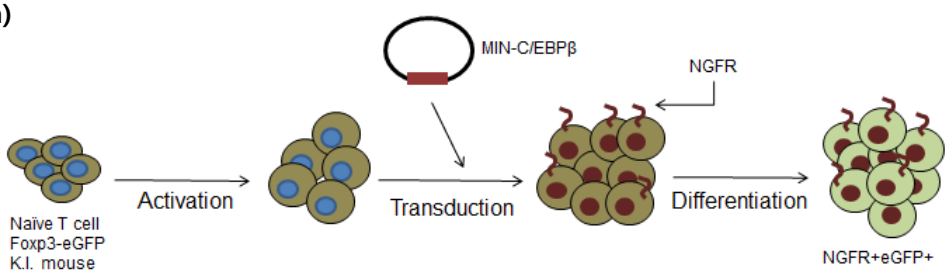
disruption, inflammatory infiltration, lamina muscularis propria, submucosal edema, and muscular thickening in lamina muscularis propria.(original magnification x100)

Result

A. Experimental design for comparing suppressive functions between C/EBPβ_iTreg and NGFR_iTreg in DSS-induced colitis mouse model.

To determine whether the C/EBPβ overexpressed iTreg shows more suppressive function than control iTreg cells in severe *in vivo* inflammation condition, this experiment was designed. The vector transduced iTreg cells were prepared as follows(Fig. 1a). These steps were to get enough number of the transduced and differentiated iTreg cells *in vitro*. The naïve T cells ($CD4^+CD44^{lo}GFP^-$) were sorted from Foxp3eGFP reporter mice. To cause much proliferation of the naïve T cells, those were activated with plate bound α-CD3 and α-CD28 for 24 hours. Then spin transduced by C/EBPβ inserted MIN vector and control MIN vector, respectively. The infected T cells express Nerve Growth Factor Receptor(NGFR) on its surface. After resting for 12 hours, transduced cells were placed in iTreg differentiation condition. After 48 hours, the half of the medium was replaced by a new media, and cultured for additional 48 hours in order to get a sufficient amount of iTreg cells. The $1x10^6$ cells expressing both NGFR and GFP are sorted. To confirm the C/EBPβ transduced iTreg cells have enough purity, mRNA level of C/EBPβ of the iTreg cell were measured and compared to the control iTreg cells (Fig. 1b). The C/EBPβ mRNA level is approximately 12 times

(a)


MIN-C/EBPβ
NGFR
Activation
Transduction
Differentiation
Naïve T cell
Foxp3-eGFP
K.I. mouse
NGFR+eGFP+

(b)

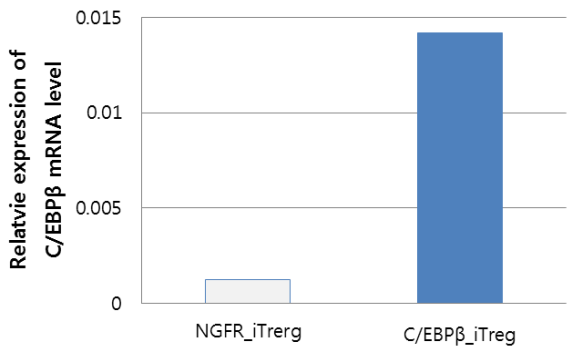
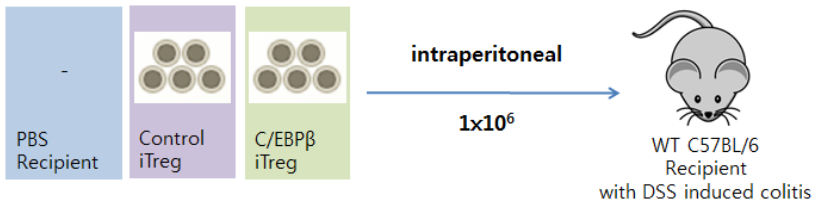

Relatvie expression of
C/EBPβ mRNA level
0.015
0.01
0.005
0
NGFR_iTrerg
C/EBPβ_iTreg

Figure1. Experimental scheme for C/EBPβ & Control (NGFR)_iTreg.

(a) The precursor T cells (CD4+CD44loGFP−) were sorted from mesenteric lymphnode and spleen of Foxp3−eGFP Knock In C57BL/6 mouse. After activation, the prepared virus supernatant was transduced into the cell. The transduced cells were rested and placed under iTreg differentiation condition, with TGFβ. Both transduced and differentiated regulatory T cells (GFP+Min+) were sorted using FACS. (b) To check whether the transduced iTreg cells are expressing C/EBPβ properly, the relative expression of C/EBPβ mRNA levels was measured from the each sorted population.

(a)


-
PBS
Recipient
Control
iTreg
C/EBPβ
iTreg
intraperitoneal
1x10⁶
WT C57BL/6
Recipient
with DSS induced colitis

(b)

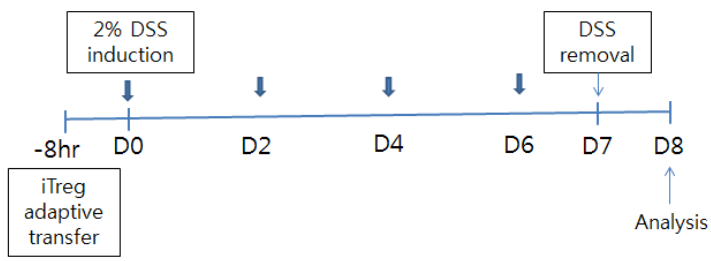

2% DSS
induction
DSS
removal
-8hr
D0
D2
D4
D6
D7
D8
iTreg
adaptive
transfer
Analysis

Figure2. The method for Dextran Sulfate Sodium (DSS)_induced colitis and adaptive transfer of iTreg. (a) Four mice with equilibrated body weights were prepared for each set; control mouse without DSS treatment, control (PBS) mouse with DSS treatment, C/EBPβ_iTreg cells injected mouse with DSS treatment, and control (NGFR)_iTreg injected mouse with DSS treatment. 1x106 iTreg were respectively prepared. The intraperitoneal injection of the cells was conducted to the mouse. (b) 8 hours before DSS induction, iTreg cells were adoptively transferred to the mouse. And 2% DSS was given ad libitum and replaced to the new one every other day. The weight of the mice was measured daily basis. On day 7, DSS was removed and the mice were analyzed on day8.

larger than the control one according to qRT-PCR analysis.

All the mice used for 1 set of the experiment were littermates and weights were equilibrated. Four mice were assigned for 1 experimental set; one without DSS treatment, and the other 3 mice were treated with DSS. One is adoptively transferred with C/EBPβ iTreg cells, another one with control iTreg cells, $1x10^6$ cells were injected to the each mouse(Fig 2a). The mere PBS and the PBS with the prepared cells were injected to the each mouse 8 hours before DSS induction. The weights were measured just before the colitis induction. Then, the DSS was given for 7 days and it was replaced by the fresh DSS every other day. On day8, the mice were sacrificed and analyzed (Fig. 2b).

B. The ratio of the body weight change was reduced in the mouse with adoptively transferred C/EBPβ_iTreg cells during colitis induction

DSS administrated into drinking water for 7 days and the weights were measured daily at the same time point. From day four, the weight began to decrease. The body weight reduction rate of DSS treated mice without transferring iTreg cells was approximately 12% from the original body weight on day 7. Additionally, the mice had hunched backs with signs of diarrhea and blood clotting on the anus. The mice adoptively transferred control iTreg cells showed much more severe symptoms of the inflammation. Its weight declination rate was more than the mice with mere colitis induction. This observation might be explained by the previous report asserting

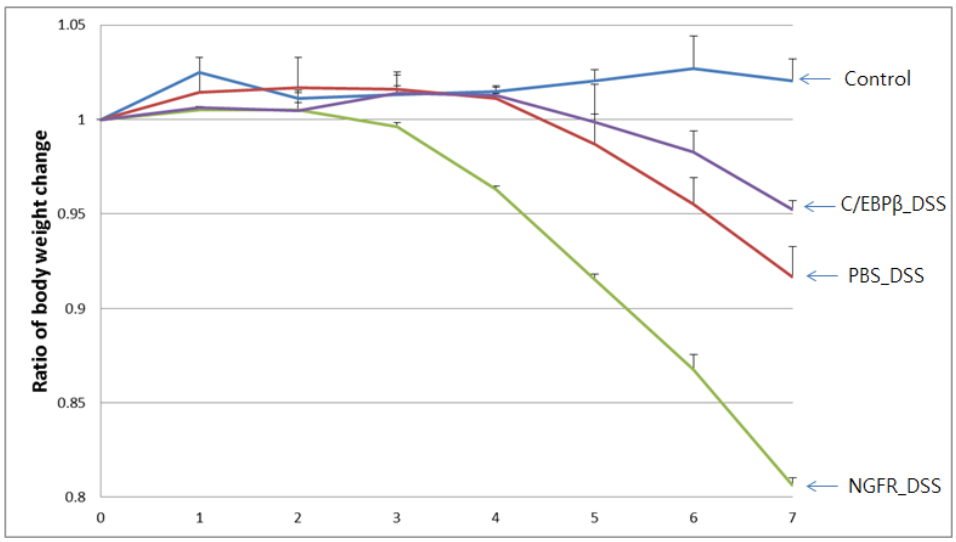


Figure3. The ratio weight loss of DSS colitis induced model. From the start day of DSS induction, the body weights of the mice were measured daily basis. When the weight of the control mice without DSS treatment stayed normal, the weight loss of the mice with DSS induction was observed and compared. Two separate sets (each set contains 4 mice) of the experiments were conducted.

severe inflammatory micronvironment alters iTreg cells to pathogenic ex-Treg cells (Zhou X 2009). Contrarily, the weight reduction rate of the DSS treated mice adoptive transferred with C/EBPβ_iTreg cells was milder than that of the both mere colitis induced mice and control iTreg cell transferred mice with colitis induction (Fig. 3a). The ration of the body weight changes from the largest; NGFR_iTreg with DSS, control with DSS, C/EBPβ_iTreg with DSS control without DSS.

C. **C/EBPβ_iTreg cells transferred mouse shows decreased inflammatory signs in organs and colon tissue among colitis induced mouse.**

On the 8th day from the initial DSS treatment, the mice were sacrificed and analyzed. The lengths of the colons were compared. The control mouse without DSS treatment had the longest colon. The second longest one was the colon treated with DSS with adaptive transferring C/EBPβ_iTreg cells. The colon length of DSS treated mouse without iTreg transfer was shorter than the colon of the C/EBPβ_iTreg cells transferred mouse with DSS. Lastly, the colon length of control (NGFR) iTreg cells transferred mouse with DSS treatment was the shortest among the four, and purplish lump was observed in some of the colon (Fig. 4a).

The superficial inflammation on distal colon was compared via H & E staining. Comparing to the control mouse, the DSS treated mouse characterized by epithelial disruption, lamina muscularis

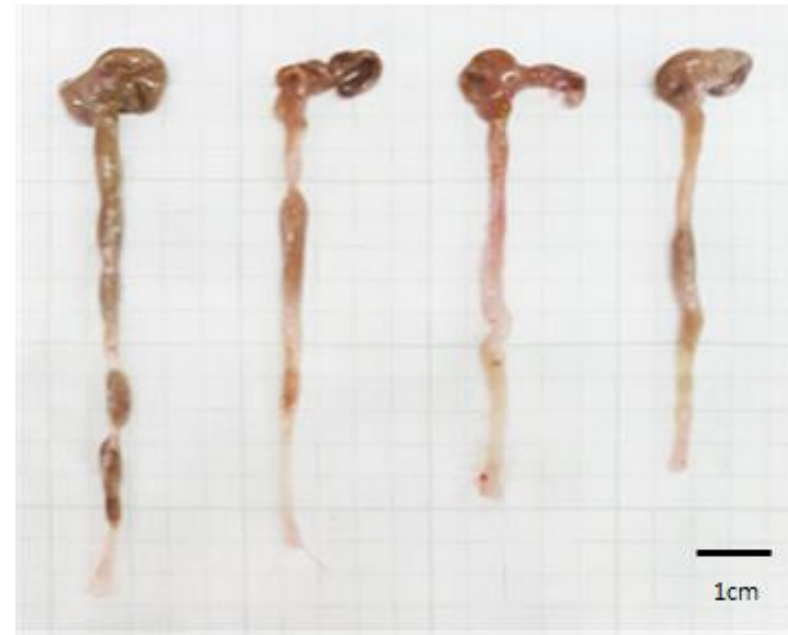

1cm
Control without DSS
C/EBPβ_iTreg with DSS
PBS with DSS
NGFR_iTreg with DSS

Figure 4. Comparison of Shortness of the colons. On day 8 from DSS treatment initiation, the colon length and spleen enlargement were investigated. (a) The length of the colon from each sample was measured and compared since the shortening of the colon is correlated with the degree of an inflammation. The colon length of the mouse from the shortest to the longest; NGFR_iTreg with DSS, PBS with DSS, C/EBPβ_iTreg with DSS, and control.

muscularis mucosae, inflammatory infiltrate and, submucosal edema, muscular thickening in lamina muscularisi propria. The colon of the control iTreg injected mouse with DSS treatment showed even more severe inflammation. It was shown that lamina muscularis mucosa was larger than any other mice. None of the intact goblet cells was shown due to severe epithelial disruption. The colon tissue of C/EBPβ_iTreg cells transferred mouse with DSS showed less degree of inflammation comparing to mere DSS treated mouse. Although mild sub-mucosal edema, epithelial disruption, inflammatory infiltrate, and muscular thickening in lamina muscularis propria were shown, the overall histology of C/EBPβ_iTreg cells transferred distal colon looked more like the intact colon tissue of the control mouse, but none of the colons from other mice did(Fig. 5a).

D. mRNA level of T_H1 associated cytokines are reduced in colitis induced C/EBPβ_iTreg transferred mouse.

The mRNA was isolated from the distal colon of each mouse. Because it has been reported that Treg cells have roles in protecting damaged tissue(Arpaia N 2015), the certain part of the whole distal colon was used in order to figure out which cells are regulated by iTreg cells. The relative expression levels of several inflammatory cytokines(IFNγ, TNFα, IL-12, IL-17, IL-23, Il-1β, IL-6, GM-CSF etc.) were investigated using qRT-PCR. Among other cytokines, the pattern of the three cytokines seemed to be related with the regulatory role of C/EBPβ_iTreg cells; IFNγ, TNF

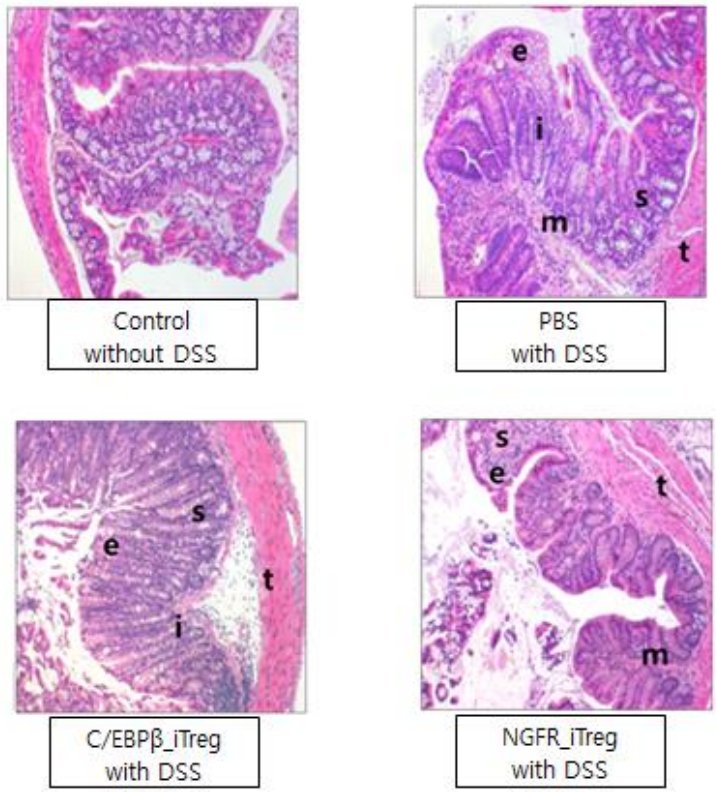


Figure 5. Histological analysis of colonic section. On day 8, distal colon of the each mouse was H&E stained to compare and observe the degree of the inflammation at the tissue in the mice colon; DSS treated mice (mere DSS or with control iTreg transfer and C/EBPβ_iTreg cell transfer) and the control mice. The letters indicate: e, epithelial disruption; i, inflammatory infiltrate; m, lamina muscularis mucosae; s, submucosal edema; t, muscular thickening in lamina muscularis propria (original magnification x100)

α and, IL-12. For all three cytokines, the expression level was the highest in the colon of the mouse adoptively transferred NGFR_iTreg cells with DSS. The next higher level of them was observed in the colon of the mouse treated with mere DSS with PBS. Lastly, it was shown that their levels seemed to be similar between the colons of the mouse injected C/EBPβ_iTreg cells with DSS mice and the control mice (Fig. 6). Thus, the relative amount of these cytokines shows the same trend to the degree of inflammation in the colon as previously mentioned. All three cytokines, IFNγ, TNFα and, IL-12, are somewhere related to the Th1. The IFNγ (type II class of interferon) is secreted by Th1 cells, IFNγ helps its own differentiation by positive feedback and macrophage activation(Bottomly 1989, Ronald B. Smeltz 2002). TNFα, which is secreted by mainly Th1 and macrophage, is likely to induce mucosal inflammation(F. Mackay 1998, D. Kontoyiannis 1999, L.M. Higgins 1999). And IL-12 produced by macrophage works as an initiator of the inflammation, it enhances IFNγ and TNFα production by Th1(S.J. Simpson 1998, I.J. Fuss 1999). Since the mouse adoptively transferred C/EBPβ_iTreg with DSS showed the reduction of those cytokines, it could be assumed that C/EBPβ_iTreg cells alleviate inflammation by suppressing Th1 or Th1 activating macrophage.

E. The C/EBPβ enhances interleukin-10 mRNA level in iTreg cells.

In order to check the mRNA expression level of suppressive cytokines in C/EBPβ_iTreg and NGFR_iTreg cells, naïve T cells

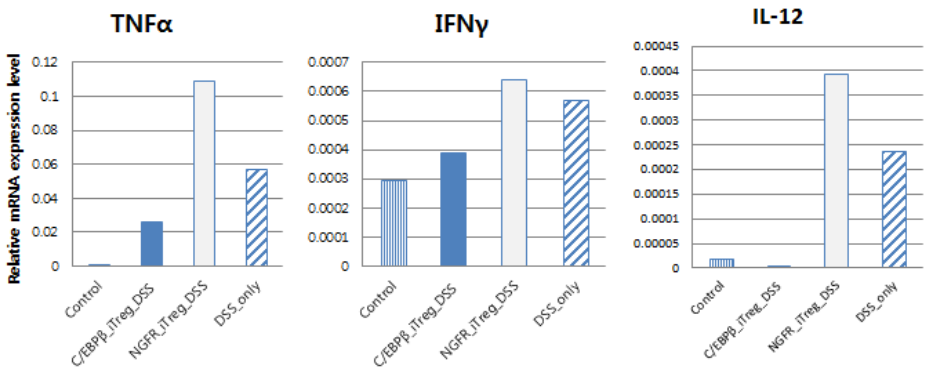


Figure 6. The mRNA level of inflammatory cytokines in distal colon. The mRNA level of pro-inflammatory cytokines of the distal colon was investigated to find the certain kinds of the cells regulated by C/EBPβ_iTreg cells. First, mRNA was isolated from a piece of the distal colon(50mg). Through qRT-PCR, the level of the pro-inflammatory cytokines that are reported to be associated with the DSS induced colitis are assessed(IFNγ, TNFα, IL-12, IL-17, IL-23, Il-1β, IL-6, GM-CSF etc.)(Alex P 2009) (n=1) (data not shown for all the cytokines). Among the examined cytokines, the cytokines(IFNγ, TNFα and IL-12) correspond with the previously stated data displayed in this paper. Normalized to GAPDH

from mesenteric lymph node and spleen of Foxp3eGFP reporter mice were achieved, and transduced with C/EBPβ_Min and Min vectors respectively. These cells were *in vitro* cultured under differentiation condition to iTreg cell. Then, both transduced and differentiated cells(GFP^+Min^+) were sorted. The culturing procedure is the same as stated above. The mRNA level of the well-known suppressive cytokines in Treg(TGFβ, CTLA-4, and IL-10) was measured using qRT-PCR. Among other cytokines, the IL-10 level of C/EBPβ_iTreg cells was more than that of control iTreg cells(Fig. 7). It was reported that IL-10 down-regulates Th1 cytokines, co-stimulatory molecule on macrophage, and NK cells(Joss A 2000, Moore KW 2001, Couper KN 2008). The C/EBPβ overexpression might not only enhances the stability of the iTreg but also increases the IL-10 expression level that remits inflammation.

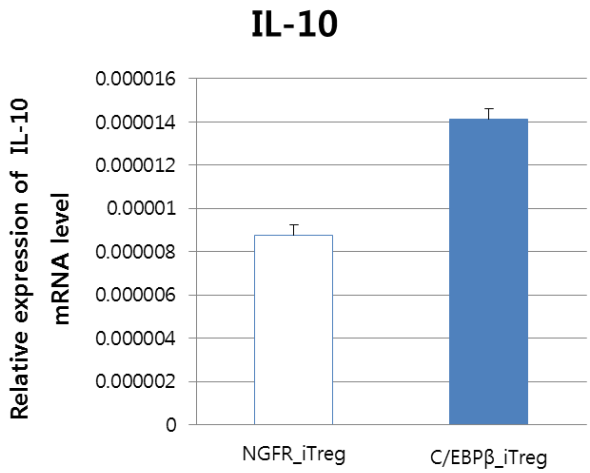


Figure 7. IL-10 mRNA level in C/EBPβ & NGFR-iTreg. Naïve CD4 T cell were isolated from Foxp3eGFP reporter mice and transduced with C/EBPβ_Min Vector and Min(NGFR) vector, and those were differentiated into iTreg cells under TGFβ rich media. The detailed procedure is listed under the figure 1. From each sample, the cells(GFP+Min+) were sorted and the mRNA level of IL-10 was checked from the each sample(n=3).

Discussion

C/EBPβ transduced iTreg cells are reported to have enhanced stability under IL-4 and IFNγabundance(Lee 2014). In order to check its role under severe inflammation, it was intraperitoneally injected to C57BL/6 mice and DSS was treated. The degree of inflammations was assessed with following criteria; percent weight loss, colon length, and histology analysis. The overall inflammatory signs and symptoms were remitted when C/EBPβ_iTreg cells were injected with DSS. The C/EBPβ_iTreg injected moue showed reduced weight loss, normal-like spleen, less shortening of the colon, and less tissue destruction when it was compared to the NGFR_iTreg injected mouse.

In order to investigate what kind of pro-inflammatory cells are affected by C/EBPβ_iTreg cells in colon, a certain part of the whole distal colon was sonicated and mRNA were isolated. Then, the mRNA levels of some pro-inflammatory cytokines(IFNγ, TNFα, IL-12, IL-17, IL-23, Il-1β, IL-6, GM-CSF etc.) were measured. The mRNA levels of IFNγ, TNFα and IL-12 seemed to be correlated with the degree of inflammation. Corresponding to the previous data, those mRNA levels were decreased in the colon of the mouse injected with C/EBPβ_iTreg cells, contrarily, those levels stayed higher in the colon of the other mice with DSS. Thus, there is a strong possibility that C/EBPβ_iTreg down-regulates Th1 cell or Th1 activating macrophage.

However, to confirm the theory, more detailed analyses of

the sample mice are necessary. It would give more credible results if the protein levels of the pro-inflammatory cytokines in the lamina propria or peritoneal cavity were assessed. Although the mRNA level of the cytokines showed the clear correlation, the protein level might produce other possible results. In Addition, the number and proportion of the remaining iTreg cells(GFP^+Min^+), such as iTreg to naïve $CD4^+$ and $CD8^+$T cells, in lamina propria of the mice with DSS could be measured by FACS. The following results would help us to strengthen the theory of the enhanced stability of C/EBPβ_iTreg *in vivo* under DSS treatment.

The inhibitory cytokines produced by Treg cells were one of the well-known arsenals for pro-inflammatory cells (Dorothy K Sojka 2008). So, The mRNA were isolated from C/EBPβ_iTreg and NGFR_iTreg cells, and the level of the inhibitory cytokines (TGFβ and IL-10) and cytotoxic T -lymphocyte antigen 4 (CTLA-4)were measured and compared. IL-10 mRNA was consistently showed higher level in C/EBPβ_iTreg than in NGFR_iTreg. IL-10 was previously reported to control Th1 cytokine and macrophage(Joss A 2000, Couper KN 2008). In addition, IL-10-deficient mice ($Il10^{tm1Cgn}$, $IL10^{-/-}$) are characterized similar to those of human IBD by histological findings (Lydia M. Keubler 2015). Thus, the fact that IL-10 increased in C/EBPβ_iTreg compared to its level of the control iTreg narrows down the list of the possible target cells to Th1 related cells. But the further confirmation is necessary.

The work by the Lee (2014) nicely demonstrated that the C/EBPβ overexpressed iTreg cells maintain its suppressive function when the control iTreg loss suppressive capacity and turn into pathogenic ex-iTreg cells under the severe inflammatory environment. He suggested that this might be due to C/EBPβ prevents the reduction of Foxp3 expression by binding to methyl sequence-CRE in Treg-Specific Demethylated Region(TSDR). In addition to his reports, it was recently suggested by Schmitt et al. (2012) that the frequency of pathogenic ex-iTreg cells in both MLN and colon were reduced when iTreg cells produce IL-10(Schmitt EG 2012). CD4+Foxp3$^-$ IL-10-producing Type 1 regulatory T cells(Tr1) cells and CD4+Foxp3+ regulatory(Treg) restrain Th1+Th17 or Th17cells in IL-10 dependent manner(Huber S 2011), Furthermore, IL-10R depleted Treg cells augment IL-17 production and fail to control pathogenic Th17 response(Chaudhry A 2011). There are several data that supports iTreg cell-produced IL-10 controls the pathogenic potential of ex-iTreg cells. In this paper, IL-10 mRNA level was increased in C/EBPβ_iTreg cells comparing to NGFR_iTreg cells. Beside the assumption that C/EBPβ enhance iTreg stability by directly binding to TSDR, C/EBPβ might enhance its stability by increasing IL-10 production by iTreg cells.

IL-10 is produced from diverse cell types by a number of transcriptional families. For example, there are signal transducers and activators of transcription(STAT), specificity protein(Sp), CCATT enhancer/ binding protein(C/EBP), activator protein(AP),

interferon regulatory factors(IRF), c-musculoaponeurotic fibrosarcoma factor(c-MAF), cAMP response element binding protein(CREB), and nuclear factor κ-B(NF-κB)(Cheng 2012). In monocyte, C/EBPβ is directly involved in IL-10 production (Susanne Brenner 2002, Balázs Csóka 2007). It has been reported that C/EBPβ enhances IL-10 secretion by increasing IL-10 mRNA level by directly binding to IL-10 promoter region(-438 and -376) in macrophage. The key mediator of the transcriptional up-regulation of IL-10 is an A_{2A} receptor in macrophage when it is stimulated by adenosine(Balázs Csóka 2007). Consistent with these observations, many other researchers demonstrated that a number of potent anti-inflammatory activities were mediated by A_{2A} or A_{2B} receptors that signals into lymphocytes(Grace Pinhal-Enfield 2003, Hong Zhang 2004), monocytes/macrophages(Lappas CM 2005), dendritic cells(Panther E 2003), neutrophils(Cronstein BN 1990), and endothelial cells(Yang D 2006). A_{2A} receptor in $CD4^+$T lymphocyte, in particular, down-regulates its IFNγ production (Lappas CM 2005). Moreover, it was recently suggested that $CD4^+$ T cell cultured with adenosine increases Foxp3 expression (Rui Bao 2016). In conclusion, it could be assumed that activating $CD4^+$T lymphocyte up-regulates A_{2A} receptor expression on its surface, and adenosine stimulation can cause C/EBPβ binding to IL-10 promoter that resulting in IL-10 production. The increased IL-10 would enhance iTreg stability and prevent it from turning into ex-iTreg in colon. So, it would be meaningful to test whether the IL-10

level increased following A_{2A} stimulation by using A_{2A} agonist. And if it does, for the next step, the putative binding spot of C/EBPβ and IL-10 promoter could be checked.

In addition, it was reported that extracellular signal regulated kinase (ERK) treatment promote Treg development and prevent Th17 development, so it controls colitis(Liu H 2013). And c-musculoaponeurotic fibrosarcoma factor(c-MAF) affects ERK to produce IL-10 in Tr1 and other cells(Lionel Apetoh 2010, Cheng 2012). According to the previous data, the both ERK and C/EBPβ have the functional roles in producing IL-10 and maintaining stability in iTreg cells. Thus, investigating the association between the redundant roles of the two molecules in Treg might produce fruitful results.

The DSS induced colitis model in this experiment imitates acute inflammation response in the colon so that without other interventions the percent body weight change reaches up to approximately 12% on the day 7. Under the condition of the severe inflammation, all the mice injected with C/EBPβ_iTreg consistently eased the inflammation without an exception during the 4 sets of the separate experiment. This result indicates a certain role of C/EBPβ_iTreg in alleviating excessive inflammation. The IBD patients often have periods of remission followed by periods of active illness (Neeraj Narula 2008). 7 days of DSS treatment and 10 days of normal autoclave water were given for four repetitive cycles to pattern on the chronic IBD. iTreg was transferred by

intravenous injection to the mouse on Day 0, and the alleviation of the disease was observed during the experimental period(data not shown). This indicates iTreg therapy works not only for the acute but also for the chronic IBD.

Even though extended rounds of clinical trial are necessary to be used as a therapeutic application to the patients, the alleviation of the disease makes the study for continued development of iTreg therapy. Since the stable and functional C/EBPβ_iTreg can be harvest extensive amount *in vitro*, the other numerous autoimmune disease such as diabetes mellitus type1, eczema and rheumatoid arthritis, and also an organ transplantations could be benefited from the suppression of inflammation.

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국문 초록

염증성 장질환에서 C/EBPβ 발현을 통한 유도조절T세포의 면역 억제 효과 기능 개선

염증성 장질환 환자는 전세계적으로 증가하는 추세이나, 현재까지 근본적인 치료법은 개발되지 못하고 있는 상황이다. 이 질환이 세포의 염증반응에 의한 것임은 널리 알려진 사실이지만, 복합적인 작용에 의하여 유발되는 염증반응의 원인을 정확히 진단하는 것은 쉽지 않다.

조절T세포는 표적세포에 대한 특이성을 가지고 있고, 항염증 반응을 일으키며 손상된 조직을 복구하는 기능이 있기 때문에 염증성 장질환에 대한 새로운 치료법으로 연구되고 있다. 하지만, 조절T세포는 급성염증반응의 상황에서 Foxp3의 발현을 무화시키고 병원성 ex-iTreg으로 변한다는 단점이 있다. 이 연구는 체외에서 배양된 C/EBPβ를 발현 시켜 TGFβ에 의해 유도된 조절T세포(C/EBPβ_iTreg cells)가 같은 조건에서 배양된 대조군인 유도성 조절T세포(NGFR_ iTreg cells)에 비해 향상된 안정성을 보인 것을 발견한 선행연구를 기반으로 진행된 마우스 체내실험이다.

본 연구는 급성염증반응을 일으키는 Dextran Sulphate Sodium(DSS)로 대장염을 유도하여 생체 내에서 C/EBPβ_iTreg의 안정성과 항염효과의 향상 정도를 관찰하였으며, 대조군 조절T세포에 비해서 C/EBPβ_iTreg을 복강주사로 주입하였을 때 DSS로 유도된 염증반응이 일부 완화됨을 발견하였다. DSS에 의한 염증 정도는 마우스의 몸무게 변화 백분율과

대장의 길이변화, 비장의 확대 정도, 조직학적 분석을 통해서 측정하였다. 더불어 C/EBPβ_iTreg를 주입하고 DSS를 복용시킨 마우스의 대장 말단에서 제1형 도움T세포와 관련한 사이토카인(IFNγ, TNFα and IL-12)의 mRNA양이 대조 군에 비해 감소한 것이 발견되었다. 이에 대한 C/EBPβ_iTreg의 항염작용이 향상된 원인을 조사해보기 위해서 조절T세포의 제어 사이토카인의 mRNA(IL-10, CTLA-4, TGFβ) 양을 측정한 결과, 대조 군 조절T세포에 비해 C/EBPβ_iTreg에서 IL-10의 mRNA 양이 특이적으로 증가함을 확인하였다. 따라서 IL-10이 대장염을 완화시키는 중요한 역할을 한다고 추측해 볼 수 있다. C/EBPβ 발현에 의한 IL-10 증가기작과, 이것의 증가가 어떻게 iTreg의 안정성을 향상시켜주는지는 더 풀어야 할 과제이다.

주요어: 조절 T세포, 염증성 장질환, 인터류킨-10, 덱스트란 황산 나트륨

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