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Elevated expression of interleukin-21 and its correlation to T-cell subpopulation in patients with ulcerative colitis

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Abstract

Objective: *To investigate the expression of interleukin-21 (IL-21) and its correlation to T-cell subpopulation including Th1, Tc1 and Th17 cells in Ulcerative colitis (UC).*

Material and methods: *We examined the expression of IL-21, IL-17 and IFN-γ in UC patients and controls by enzyme-linked immunosorbent assay (ELISA) and flow cytometry.*

Results: *We found that IL-21 was expressed on $CD3^+CD8^-$T cells by flow cytometry. Plasma IL-21 level and the percentage of $CD3^+CD8^-IL\text{-}21^+$ T cells were significantly elevated in UC patients compared to controls. The percentage of $CD3^+CD8^-IL\text{-}17^+$ T (Th17), $CD3^+CD8^-IFN\text{-}\gamma^+$ T (Th1) and $CD3^+CD8^+IFN\text{-}\gamma^+$ T (Tc1) cells was also significantly increased in UC patients. Moreover, we found a significant positive correlation between $CD3^+CD8^-IL\text{-}21^+$T cells and Th17 cells.*

Conclusions: *Elevated IL-21 and its positive correlation to Th17 cells may play a role in the pathogenesis of UC.*

Key words: *ulcerative colitis, interleukin-21, T helper 1, T helper 17.*

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Introduction

Ulcerative colitis (UC) is a common form of inflammatory bowel disease (IBD) that finally has a high risk of the development of colorectal cancer [1]. The pathophysiology of UC is heterogeneous and complex [2]. Several abnormalities involving the cellular mechanisms of immune modulation, such as the T helper 1 (Th1) bias [3], the decreased number or defective suppressive function of regulatory T cells [4], colonic epithelial cell damage by cytotoxicity T lymphocytes (CTL) [5, 6], have been described. The reason for these abnormalities remains unknown.

T cells are broadly classified as either helper T cells (Th cells, $CD4^+$) or cytotoxic T cells (Tc cells, $CD8^+$). Both $CD8^+$ (Tc) and $CD4^+$ (Th) T lymphocytes can be functionally divided into type 1 (T1) and type 2 (T2) subsets based on the secretion of cytokines. $CD8^+$T cells can differentiate into cells that make IFN-γ but not IL-4 (Tc1 cells) and cells that make IL-4 but not IFN-γ (Tc2 cells) [7, 8] and could kill cells infected with viruses or other intracellular pathogens, suppress immune responses and so on. The cytokine-driven differentiation of distinct lineages of effector and regulatory T cells (Tregs) from naive $CD4^+$ T-cell precursors is a hallmark of the adaptive immune system. Up to date, there are three major effector T cells including T helper 1(Th1), Th2 and recently found Th17 cells. Th1 cells produce interferon-γ (IFN-γ) and tumor necrosis factor-β (TGF-β) and mediate immune responses against intracellular bacteria, viruses and tumor cells, through the activation of macrophages and cytotoxic T cells. Th2 cells make mostly interleukin 4 (IL-4), IL-5 and IL-13, which stimulate humoral responses and are thought to have evolved to enhance resistance against extracellular parasites [9, 10].

In recent years, a distinct T-cell subset, termed Th17 cells, has also been identified and seems to play key roles in the activation of neutrophils and immunity to bacteria, particularly at mucosal surfaces. IL-17, also termed IL-17A, is the signature cytokine of Th17 cells [11]. $CD4^+CD25^+Foxp3^+$Tregs, consist of an anergic lymphocyte population representing 1-10% of total $CD4^+$ T cells in thymus, peripheral blood and lymphoid tissues, and are dominant in control of self-reactive T responses and main-

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taining tolerance in several models of autoimmunity [12]. The decision by the immune system to drive naive T cells in a certain functional direction is influenced not only by the antigen type and concentration but also by signals that act synergistically or antagonistically, through positive or negative feedback loops, to activate or inhibit specific T-cell lineage programs [11]. Skewing of responses toward inflammatory Th1, Th2 or Th17 pathways and away from regulatory T-cell pathways might be responsible for the initiation and progress of immune-mediated diseases [13]. Several articles indicate increased Th1 cells and reduced $CD4^+CD25^+Foxp3^+$Tregs in patients with chronic UC with limited articles on the profile of Tc1 cells in UC patients [14-17]. Recent work from various laboratories indicates a cytokine, IL-21, that plays a critical role in switching between inflammatory and suppressive T-cell types.

Interleukin-21, mainly produced by activated $CD4^+$ T cells and natural killer T cells, is involved in the regulation of central functions of the immune system [15, 16], including promoting T-cell-mediated humoral immune responses and antibody production, increasing the cytolytic potential of NK cells, promoting the differentiation of naive Th cells into Th17 cells. Interleukin-21 and its receptor IL-21R are implicated in the pathogenesis of some autoimmune diseases such as systemic lupus erythematosus (SLE), rheumatoid arthritis (RA) in humans [18, 19]. Although analysis of biopsies from patients with either Crohn's disease (CD) or UC has revealed higher levels of IL-21 protein expression in mucosal samples compared to controls [20], there are limited data about IL-21 in patients with UC and its correlation to T-cell subpopulation. To further investigate the possible role of IL-21 in the pathogenesis of UC, we measured the levels of IL-21 and correlated its levels to Th17 cells, Th1 cells and Tc1 cells.

Material and methods

Patients and controls

The UC patients were fifteen females and eleven males with an age range of 19-58 years and a median age of 38 years. They were enrolled in this study between August 2013 and June 2014 at the Department of Gastroenterology, Provincial Hospital Affiliated to Shandong University (Shandong, China). The diagnosis of UC had been established by clinical, endoscopic, histological, and/or radiological criteria [21]. Infection or the presence of parasites was excluded by stool culture and microscopic examination. The disease activity of active UC was determined using a grading scale including clinical and para-clinical variables. Twenty normal subjects without any appearance of colonic inflammation or tumor under endoscopy were included as a control group. Informed consent was obtained from each participant. Ethical approval for the study was obtained from the Medical Ethical Committee of our hospital.

Flow cytometric analysis

Intracellular cytokines were studied by flow cytometry to reflex the cytokine-producing cells. Briefly, heparinized peripheral whole blood (400 µl) with an equal volume of RPMI 1640 medium was incubated for 4 h at 37°C, 5% CO_2 in the presence of 25 ng/ml of phorbol myristate acetate (PMA), 1 µg/ml of ionomycin, and 1.7 µg/ml GolgiPlug (Monensin) (all from Alexis Biochemicals, San Diego, CA). Phorbol myristate acetate and ionomycin are pharmacological T-cell-activating agents that mimic signals generated by the TCR complex and have the advantage of stimulating T cells of any antigen specificity. Monensin was used to block intracellular transport mechanisms, thereby leading to an accumulation of cytokines in the cells.

After incubation, the cells were stained with PE-Cy5-conjugated anti-CD3 and FITC-conjugated anti-CD8 monoclonal antibodies at room temperature in the dark for 15 min to delimitate $CD4^+$ T cells because CD4 was down-modulated when cells were activated by PMA. After the surface staining, the cells were stained with PE-conjugated anti-IFN-γ monoclonal antibodies, anti-IL17 or anti-IL-21 monoclonal antibodies after fixation and permeabilization according to the manufacturer's instructions. Isotype controls were given to enable correct compensation and confirm antibody specificity. Stained cells were analyzed by flow cytometric analysis using a FACScan cytometer equipped with CellQuest software (BD Bioscience Pharmingen).

Interleukin-21 enzyme-linked immunosorbent assay (ELISA)

All assays were performed on plasma samples. Interleukin-21 levels were determined with a quantitative sandwich enzyme immunoassay technique in accordance with the manufacturer's recommendations (Bender MedSystems, Burlingame, CA). The lower detection limit of this assay was 20 pg/ml.

Statistical analysis

Results were expressed as mean ± standard deviation (SD). The differences between groups were assessed using unpaired *t* test. The Pearson correlation test was also conducted to identify univariate associations between IL-21, Th17 cells, Th1 cells, and Tc1 cells. All tests were performed by SPSS 13.0 system. *P* value less than 0.05 was considered statistically significant.

Results

Elevated interleukin-21 in ulcerative colitis patients

Interleukin-21 was expressed on $CD3^+CD8^-$T cells by flow cytometry. The expression of a typical dot plot

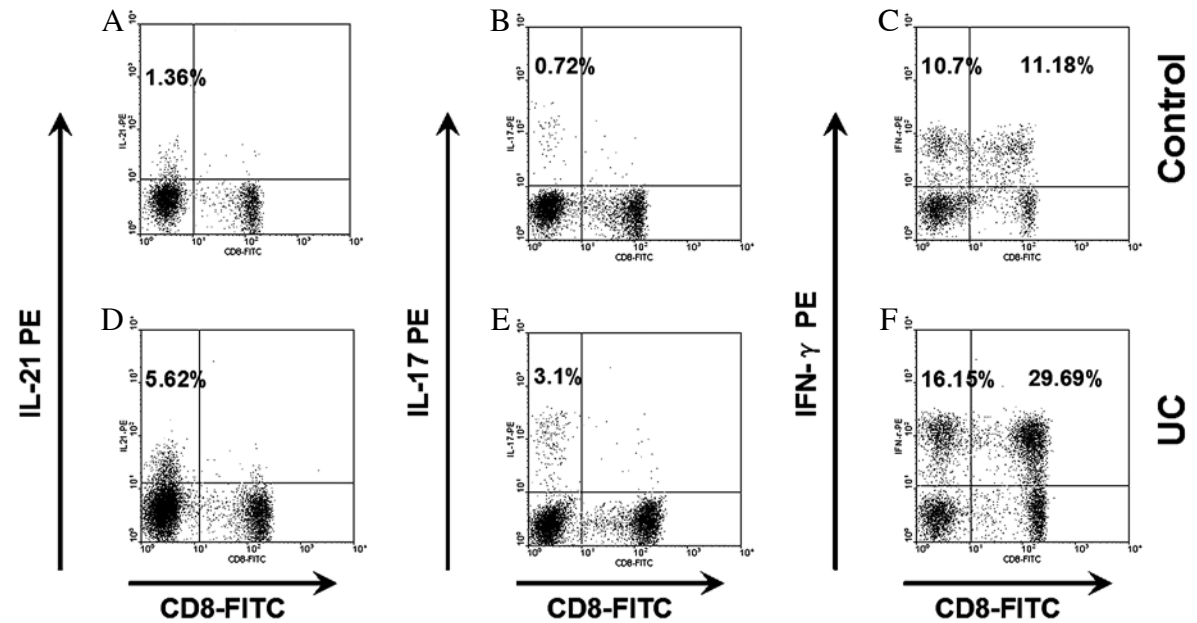

Fig. 1. The percentages of circulating $CD3^+CD8^-IL21^+$T cells, Th17 cells, Th1 cells and Tc1 cells in representative UC patients and controls. Heparinized peripheral whole blood from all subjects was stimulated with PMA, ionomycin and monensin for 4 h, and then stained with labeled antibodies as described in methods. (**A**) and (**B**): Representative IL-21 expression in $CD3^+CD8^-$T subsets from UC patients and controls is shown. (**C**) and (**D**): Representative IL-17 expression in $CD3^+CD8^-$T subsets from UC patients and controls is shown. (**E**) and (**F**): Representative IFN-γ expression in $CD3^+CD8^-$T subsets ($CD4^+$ T subsets) and CD^+CD8^+T subsets from UC patients and controls is shown

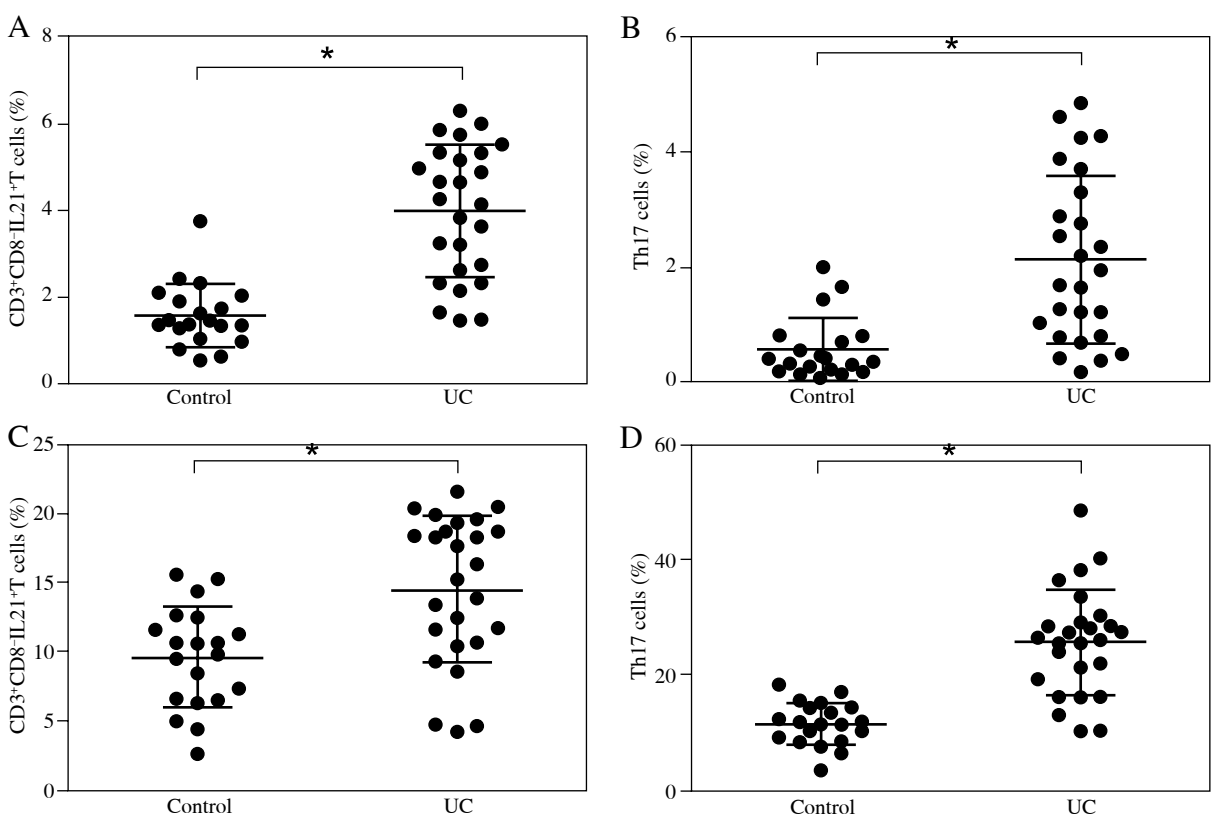

Fig. 2. Increased percentages of $CD3^+CD8^-IL\text{-}21^+$T cells (**A**), Th17 cells (**B**), Th1 cells (**C**) and Tc1 cells (**D**) by flow cytometry in UC patients was found as compared to controls ($p < 0.05$) *$p < 0.05$

of IL-21 gated on $CD3^+$T cells in representative patients and controls is shown in Fig. 1A, B. The percentage of $CD3^+CD8^-IL\text{-}21^+$ T cells was significantly elevated in UC patients (mean ± SD, 3.99 ±1.52%) compared to healthy controls (1.59 ±0.73%, $p < 0.05$). We investigated plasma IL-21 by ELISA. The level of IL-21 was significantly increased in UC patients (129.6 ±97.1 pg/ml) compared to controls (72.0 ±29.0 pg/ml, $p < 0.05$) (Fig. 2A).

Table 1. Clinical characteristics of patients with UC

	***n* = 26**
Gender (Male/female)	15/11
Age (years)	
Median (range)	38 (19-58)
Disease duration (years)	
Median (range)	8 (0-24)
Short-term (< 8 years)	15
Long-standing (≥ 8 years)	11
Extent of disease	
Pan-colitis	14
Proctitis	6
Left-sided colitis	5
Right-sided colitis	1
Daily medication at entry	
Mesalazine (1.5-4.5 g/day)	20
Steroids (5-15 mg/day)	12
Azathioprine (25-50 mg/day)	2
None	3
Past use of steroids	
Yes/no	18/8

Increased expression of Th17 and Th1 cells in ulcerative colitis patients

Interleukin-17 was expressed on $CD3^+CD8^-$T cells and IFN-γ was expressed on both $CD3^+CD8^-$T cells and $CD3^+CD8^+$T cells. The expression of a typical dot plot of IL-17 and IFN-γ gated on $CD3^+$T cells in representative patients and controls is shown in Fig. 1C, D and Fig. 1E, F.

Ulcerative colitis patients had a significantly increased percentage of Th17 cells, Th1 and Tc1 cells. The percentage of Th17 cells was significantly increased in UC patients (2.13 ±1.46%) compared to controls (0.56 ±0.54%, $p<0.05$). Similarly, the percentage of Th1 and Tc1 cells was also increased in UC patients compared to controls (Th1 cells: 14.52 ±5.29% vs. 9.59 ±3.63%, Tc1 cells: 25.75 ±9.17% vs. 11.60 ±3.68%) (Fig. 2B-D).

Correlation between $CD3^+CD8^-IL\text{-}21^+$ T cells, Th17 cells, Th1 cells and Tc1 cells in ulcerative colitis patients

We observed that the percentage of $CD3^+CD8^-IL\text{-}21^+$ T cells positively correlated to Th17 cells ($p < 0.05$). No significant correlation between $CD3^+CD8^-IL\text{-}21^+$ T cells and Th1 or Tc1 cells was found ($p > 0.05$)(Fig. 3).

Correlation of cells with CRP and ESR in ulcerative colitis patients

No significant correlation between cells ($CD3^+CD8^-IL\text{-}21^+$ T cells, Th17 cells, Th1 cells and Tc1 cells) and C-reactive protein (CRP) or erythrocyte sedimentation rate (ESR) was found in UC patients ($p > 0.05$).

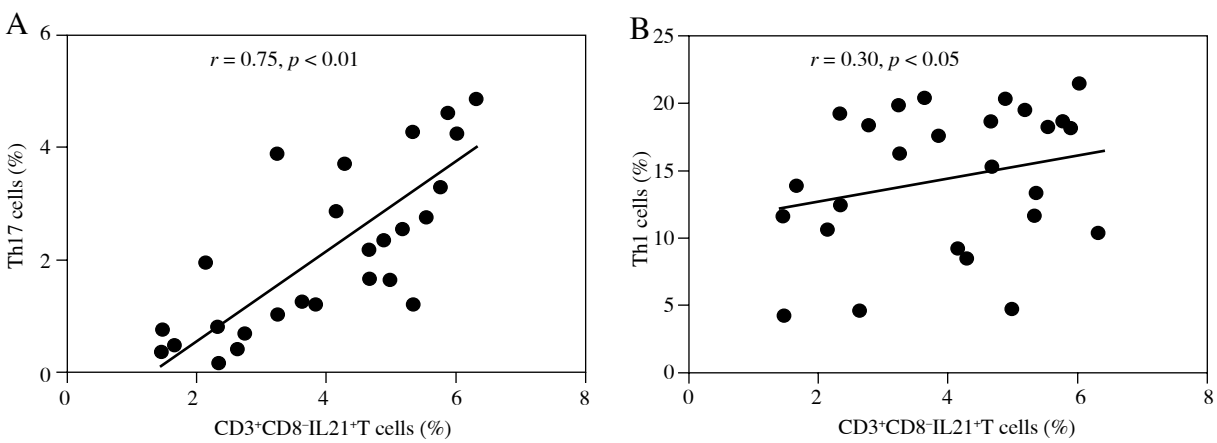

Fig. 3. Correlation between percentages of $CD3^+CD8^-IL\text{-}21^+$T and Th17 or Th1 cells (**A**) Positive correlation between the percentage of $CD3^+CD8^-IL\text{-}21^+$T cells and Th17 cells was found in UC patients ($r = 0.75$, $p < 0.01$). (**B**) No significant correlation between the percentage of $CD3^+CD8^-IL\text{-}21^+$T cells and Th1 cells was found in UC patients ($r = 0.30$, $p > 0.05$)

Discussion

Ulcerative colitis, the major form of IBD, is an immune-mediated heterogeneous disorder. Increasing evidence has shown that T cell-mediated effects play a role in the mechanisms of UC [3, 4]. An increased Th1/Th2 ratio in the peripheral blood has been proposed to correlate with disease activity in UC, and CTL contribute to colonic epithelial cell damage in patients with UC [22]. Recently it has been reported that Th17 is deeply involved in the pathogenesis of multiple animal models of IBD that were thought to be either Th1 or Th2 dominant previously [23].

In addition, accumulating evidence indicates that cytokines produced by immune or non-immune cells play a major role in initiating the pathologic process of UC, one such cytokine seems to be IL-21, which is the most recently discovered member of the family of cytokines which is produced by $CD4^+$ T cells, including Th17 cells, and executes its function through its receptor (IL-21R) [24]. Interleukin-21 is able to modulate both innate and adaptive immune responses [25, 26] including positive effects such as enhanced T cell proliferation, increased cytotoxicity of $CD8^+$ T cells and NK cells, differentiation of B cells into plasma cells and regulation of Th17 development. Conversely, IL-21 also has direct inhibitory effects on the antigen-presenting function of DCs and inhibits the differentiation of naive Th cells into IFN-γ-producing Th1 cells, negatively regulating T helper cell production of IFN-γ in the effector phase [27-29]. Studies have indicated that IL-21 controls the functional activity of epithelial cells and fibroblasts in the gut, thus it is an important mediator in the crosstalk between immune and non-immune cells [30, 31].

Elevated IL-21 was found in many autoimmune diseases and correlated with disease activity [18, 19, 32]. Similarly, we found an increased expression of IL-21 in UC patients in our study, which is consistent with previous studies [20, 33, 34]. Inous *et al.* have shown that in the inflamed intestine of patients with UC, there is enhanced production of IL-21 [33]. These data were confirmed by another study that IL-21 mRNA expression is increased in rectal mucosa from patients with active UC compared to UC patients in remission and healthy controls and that, in UC, IL-21 gene expression correlates with histological activity of the disease [34]. $CD4^+$ T lymphocytes are important mediators of the pathologic response in UC. In both CD and UC patients, IL-21 is made by $CD4^+$ but not $CD8^+$T cells [35]. A recent study also found that IL-21 is mostly produced by $CD4^+$ T intestinal lamina propria lymphocytes co-expressing IFN-γ in IBD [36]. Similarly, we found that IL-21 was expressed on $CD4^+$T cells but not $CD3^+CD8^+$T cells by flow cytometry.

Th17 cells are associated with host defense against extracellular pathogens and the development of autoimmunity and inflammatory response, such as multiple sclerosis, rheumatoid arthritis and IBD [37-40]. The number of Th17 cells significantly increased in the peripheral blood of patients with active UC compared with healthy individuals. Fujino *et al.* found that IL-17^+ cells were markedly increased in the inflamed region of patients with active UC compared with inactive UC patients. Similarly, we also found an increased expression of Th17 cells in UC patients compared to controls.

In addition, a positive correlation between $CD3^+CD8^-$ IL-21^+ T cells and Th17 cells was found in UC patients. Interleukin-21 plays a key role in the differentiation of naive Th cells into Th17 cells, leading to increased IL-17 production and IL-23R expression [41, 42]. In addition, IL-21-deficient and IL-21R-null mice have substantially impaired generation of Th17 cells [42]. Interleukin-21 is highly produced in the gut of wild-type mice with dextran sulfate sodium (DSS)- and trinitrobenzene sulfonic acid-relapsing (TNBS)-induced colitis [29]. Amelioration of both DSS- and TNBS-induced colitis in IL-21-knockout mice is associated with a marked decrease in Th17-related molecules, such as IL-17 and IL-17F. Stimulation of intestinal mucosal T cells with IL-21 results in enhanced activation of transcription factors (i.e. Stat3, Stat4 and T-bet) and marked synthesis of IFN-γ and IL-21 itself [16]. Moreover, treatment of CD mucosal cells with IL-21R/Fc reduces Stat4 and T-bet and inhibits IFN-γ production. Neutralization of IL-21 in CD mucosal cell cultures leads also to a decreased expression of IL-17A [18]. Taken together, these data indicate that IL-21 is able to expand Th1 and Th17 cell responses in the gut [43].

Taken together, our data demonstrate elevated IL-21 in UC patients and its positive correlation to Th17 cells, indicating a possible role of IL-21 in UC and blockade of IL-21 may be a reasonable therapeutic strategy for UC.

The authors declare no conflict of interests.

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