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3,3′-Diindolylmethane alleviates oxazolone-induced colitis through Th2/Th17 suppression and Treg induction

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ABSTRACT

The T cell is pivotal in orchestrating and promoting an immune response during ulcerative colitis (UC). The aryl hydrocarbon receptor (AhR) is involved in the regulation of T cell responses, and 3,3′-diindolylmethane (DIM) is a known ligand of AhR. The aim of this study was to examine the therapeutic effects of DIM in experimental colitis and to investigate the possible mechanisms underlying its effects on mucosal T cell responses. The therapeutic effects of DIM were studied in an oxazolone-induced colitis model. The pathologic markers of colitis were measured, moreover, T-helper cell (Th)- and regulatory T cell (Treg)-related transcription factor expression and associated colonic cytokine production were determined. The impact of DIM on T cell differentiation was further investigated in cultures of naive Th cells that were stimulated with anti-CD3/CD28 monoclonal antibodies (mAbs). The administration of DIM attenuated experimental colitis, as determined by pathological indices. DIM may affect signaling pathways downstream of AhR, leading to decreased Th2/Th17 cells and increased Tregs. Ultimately, this could result in the alleviation of experimental colitis. DIM has shown anti-UC activity in animal models via inhibition of Th2/Th17 cells and promotion of Tregs and may thus offer potential treatments for UC patients.

1. Introduction

Ulcerative colitis (UC), one of the two main subtypes of inflammatory bowel disease (IBD), is a chronic, relapsing and remitting inflammatory disorder within the gastrointestinal tract (Abraham and Cho, 2009). Although the precise molecular basis and pathophysiology of UC remain unclear, it is generally accepted that the interactions between genetic susceptibility and environmental triggers cause hyperactivation of the mucosal immune system, which results in chronic intestinal inflammation. Inflammatory cells during UC show a bias towards T-helper (Th) 2 cell cytokine production, and recent data have shown that an imbalance between regulatory T cells (Tregs) and Th17 effector cells also plays a key role in gut inflammation (Xavier and Podolsky, 2007; Liu et al., 2009).

The aryl hydrocarbon receptor (AhR) is a cytosolic transcription factor that can be activated by certain polycyclic aromatic hydrocarbons, such as 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD). Such activation can lead to a variety of toxic effects, including immunoregulation, tumor promotion and cell differentiation (Puga et al., 2005). Recent findings have indicated that AhR controls T cell differentiation (Marshall and Kerkvliet, 2010). Th17 cells and Tregs both express AhR, although the outcome of AhR signaling on Th17/Treg differentiation differs depending on the particular AhR ligand (Quintana et al., 2008). Additionally, it was reported that another synthetic ligand of AhR, M50354, could suppress Th2 responses by inhibiting GATA3, the master transcription factor for Th2 cell differentiation (Negishi et al., 2005). 3,3′-Diindolylmethane (DIM) is a natural compound formed during the autolytic breakdown of glucobrassicin in cruciferous vegetables. As a promising anti-tumor agent, DIM has been widely studied in laboratory and clinical studies (Weng et al., 2008; Okino et al., 2009). Moreover, recent studies have demonstrated that DIM has the ability to suppress inflammatory responses in some murine models of inflammation (Cho et al., 2008; Kim et al., 2009; Dong et al.,

Abbreviations: AhR, aryl hydrocarbon receptor; CCK-8, Cell Counting Kit-8; DAI, disease activity index; DIM, 3,3′-diindolylmethane; DSS, dextran sodium sulfate; EAE, experimental autoimmune encephalomyelitis; ERα, estrogen receptor α; FICZ, 6-formylindolo[3,2-b]carbazole; H&E, hematoxylin–eosin; IBD, inflammatory bowel disease; IFN-γ, interferon-γ; IL, interleukin; I3C, indole-3-carbinol; mAbs, monoclonal antibodies; MOI, multiplicity of infection; MPO, myeloperoxidase; PAS, Periodic Acid-Schiff; shRNA, short hairpin RNA; TCDD, 2,3,7,8-tetrachlorodibenzo-p-dioxin; TNBS, 2,4,6-trinitrobenzenesulfonic acid; TGF-β1, transforming growth factor-β1; Th, T-helper; Tregs, regulatory T cells; 2-HP-β-CD, 2-hydroxypropyl-b-cyclodextrin.

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2010). Although the mechanisms underlying the immunosuppressive capacities of DIM are far from completely understood, it is established that DIM can modulate the activation of several important immune signals through AhR binding. While recent studies have found that DIM exerts protective effects during inflammation by attenuating NF-κB activation, inhibiting prostaglandin E2 production or reducing the generation of antioxidants (Kim et al., 2009; Huang et al., 2011), none of these studies have examined the potential regulatory role of DIM in T cell differentiation and activation during the inflammation process.

The intracolonic administration of oxazolone induces colitis in mice that is similar to the cytokine and histopathological profile observed in human UC (Boirivant et al., 1998). Recent studies have indicated that, in addition to Th2 cells, Th17/Tregs are also involved in mucosal inflammation during oxazolone-induced colitis (Galitovskiy et al., 2011). Therefore, we used the oxazolone-induced colitis model to simulate human UC in order to investigate whether AhR activation by DIM regulates pathogenic responses in the gut and the Th cell/Treg balance.

2. Materials and methods

2.1. Reagents

DIM was purchased from Ruima Chem Co. (Nanjing, China) (purity > 99%). As previously reported, DIM was formulated in 2-hydroxypropyl-b-cyclodextrin (2-HP-β-CD, Sigma, St. Louis, MO, USA) to improve its water-solubility (Dong et al., 2010). Briefly, 20 mg of DIM was dissolved in 1 ml 2-HP-β-CD solution (molar ratio = 1:10; prepared at 40 °C for 20 min). These stock solutions (20 mg/ml) were serially diluted in sterile PBS to obtain the experimental concentrations (15 μmol/L for cell treatment and 5 mg/ml for animal experiments) on the day of use. RPMI 1640, penicillin/streptomycin, and fetal bovine serum were purchased from Invitrogen (Shanghai, China). Other chemical reagents were purchased from Sangon Biotech (Shanghai, China).

2.2. Naive Th cell preparation and treatment

Naive $CD4^+CD62L^+$ Th cells were positively selected from murine splenocytes using the magnetic-activated cell-sorting system (MiltenyiBiotec, Cologne, Germany) as previously described (Negishi et al., 2005). The purified cells were greater than 95% $CD4^+CD62L^+$ Th cells as assessed by flow cytometry. Naive Th cells were cultured in RPMI 1640 supplemented with 10 mM HEPES (pH 7.4), 10% FBS, 100 U/ml penicillin/streptomycin and anti-CD3/CD28 monoclonal antibodies (mAbs, 1 μg/ml each, BD Pharmingen, San Diego, CA, USA). To evaluate the effect of DIM on Th cells and Treg responses, DIM (15 μmol/L) was added to the culture plates for 48 h in the presence of anti-CD3/CD28 mAbs. Following DIM treatment, the cells and supernatants were collected. ER-α (estrogen receptor α), T-bet, GATA3, RORγt and Foxp3 levels in naive T cells were determined by real-time qPCR or immunofluorescence staining. The levels of related cytokines (interferon-γ (IFN-γ), interleukin-4 (IL-4), IL-5, IL-10, IL-13, IL-17A and TGF-β1 (transforming growth factor-β1)) in the supernatants were determined by ELISA (R&D Systems, MN, USA). Naive T cells that were not stimulated with anti-CD3/CD28 were used as negative controls. To test the role of AhR in the DIM-mediated modulation of Th and Treg responses, naive T cells were pretreated with a lentivirus vector-encoded short hairpin RNA (shRNA) directed at AhR or a control shRNA at a multiplicity of infection (MOI) of 50:1 for 48 h before the incubation with DIM. The transfection efficiency of AhR was examined in the preliminary experiments. The AhR and the control shRNA lentiviral particles were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The Cell Counting Kit-8 (CCK-8, DOJINDO LABORATORY, Kumamoto, Japan) was used to determine the viability of naive T cells following different treatments.

2.3. Establishment and assessment of oxazolone-induced colitis

Male BALB/c mice were obtained from the Laboratory Animal Center of Nanjing University (Nanjing, China, $n = 95$). All of the animals received care according to the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the United States National Institutes of Health. They were housed in standard cages (4–5 mice per cage) and provided with free access to food and tap water. The animals were maintained at a constant temperature (20–21 °C) and a 12 h light/dark cycle. Oxazolone-induced colitis was studied in 6–8-week-old male BALB/c mice as previously described (Wirtz et al., 2007). To presensitize the mice, a 1.5 cm × 1.5 cm field was shaved on the back between the shoulders, and 200 μl of a 3% (w/v) solution of oxazolone (Sigma) in acetone/olive oil (4:1) was applied. Eight days after presensitization, mice were rechallenged intrarectally under ethyl ether general anesthesia with 100 μl of 1% oxazolone in 50% ethanol to induce colitis or 50% ethanol alone (vehicle control).

DIM and the vehicle control were given daily after the induction of colitis at 50 mg/kg via intraperitoneal injection. The mice were checked daily for behavior and body weight. To investigate the therapeutic effect of DIM, mice were sacrificed at day 3 after colitis induction. The colons were excised for macroscopic observation, histopathological study, immunofluorescence analysis, mRNA quantification, cytokine determination and myeloperoxidase (MPO) activity measurement. For weight and survival observation, mice were euthanized 8 days after the establishment of colitis.

2.4. Disease activity index evaluation

The disease activity index (DAI) was used to evaluate intestinal inflammation based on a previously published grading system (Kihara et al., 2003). The score ranged from 0 to 4 based on the following parameters: change in weight (0, ≤1%; 1, 1–5%; 2, 5–10%; 3, 10–15%; 4, >15%), rectal bleeding (0, no blood using hemoccult (Beckman Coulter, Palo Alto, CA, USA); 2, positive hemoccult; 4, gross bleeding) and stool consistency (0, normal; 2, loose stools; 4, diarrhea). The combined scores were then averaged to obtain the final disease activity index score.

2.5. Histopathological analysis

For histopathological examination, a sample of colonic tissue was harvested, fixed in Bouis buffer, embedded in paraffin, sectioned and stained with hematoxylin–eosin (H&E) or Periodic Acid-Schiff (PAS). Inflammation was blindly scored from 0 to 4 as follows: 0, no signs of inflammation; 1, low leukocyte infiltration; 2, moderate leukocyte infiltration; 3, high leukocyte infiltration, moderate fibrosis, high vascular density, thickening of the colon wall, moderate goblet cell loss, and focal loss of crypts; 4, transmural infiltration, massive loss of goblet cells, extensive fibrosis, and diffuse loss of crypts.

2.6. Cytokine analysis

IFN-γ, IL-4, IL-5, IL-10, IL-13, IL-17A and TGF-β1 concentrations in the colonic mucosa were evaluated by ELISA (R&D Systems, MN, USA). MPO activity in the colonic mucosa was also analyzed by commercially available kits (Jiancheng Biotech, Nanjing, China). Colonic ER-α, GATA3, T-bet, RORγt and Foxp3 mRNA levels were determined by real-time qPCR. Immunofluorescence staining was