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Intestine-derived *Clostridium leptum* induces murine tolerogenic dendritic cells and regulatory T cells *in vitro*

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ABSTRACT

Patients with autoimmune and allergic diseases frequently present with reduced numbers and functionally impaired regulatory T cells (Tregs) and/or tolerogenic dendritic cells (tDCs). tDC-mediated regulation of Treg proliferation (numbers) and activation is crucial to establishing and maintaining an appropriate level of immune tolerance. Colonic colonization of *Clostridium* spp. is associated with accumulation of Tregs, which inhibits development of inflammatory lesions. To investigate whether infection with the *Clostridium leptum* sp. can specifically induce Tregs and/or tDCs bone marrow-derived dendritic cells were cultured in the presence or absence of *C. leptum* then co-cultured with $CD4^+CD25^-$ Tregs or not. Changes in tDC numbers, Treg numbers, percentages of T cell subsets, and expression of cytokines related to Tregs (IL-10 and transforming growth factor-beta (TGF-β1)), DCs (IL-12p40 and IL-6) and effector T cells (IFN-γ, IL-4, IL-5, IL-13, and IL-17A) were measured. In the co-culture system, *C. leptum*-stimulated tDCs were able to increase the percentage and total number of Tregs attenuate activation of T helper cells (Th1, Th2, and Th17), and decrease the amount of secreted IL-4, IL-5, IL-13, IFN-γ and IL-17A. Thus, *C. leptum* exposure can induce the tDC-mediated stimulation of Tregs while disrupting the immune inflammatory response mediated by Th1, Th2 and Th17 cells.

1. Introduction

The intestine is a vital immune organ that plays a central role in gut immune defense against invasion of pathogenic bacteria from the endogenous gut flora. As such, immune cells in the gut have evolved distinct and balanced responses to bacteria in the enteric microbiota and are required for physiological homeostasis [1]. The increase in specific types and genera of bacteria stimulate various types of immune cells to guard against the potential invasion; for example, studies have shown that increased segmented filamentous bacteria lead to generation of T helper (Th)17 cells [2] and that increased bifidobacteria and lactobacilli induce regulatory T cells (Tregs) [3,4].

The dominant bacteria of the human intestinal tract are *Bacteroides* spp. and two members of the *Clostridium* genus, *C. coccoides* and *C. leptum* (*CL*), which account for about one-sixth of the total intestinal bacteria [5,6]. Under normal physiological conditions *CL* maintain the overall balance of the gut microflora and promote appropriate maturation of the immune system [5], possibly through their induction of Tregs [7].

Tregs are specialized T cells that exert their immuno-suppressive function to maintain immune homeostasis of the gut through a variety of mechanisms; these cells are also abundant in the colonic mucosa of mice and readily studied in experimental *in vivo*

Abbreviations: MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; APCs, antigen-presenting cells; BMDCs, bone marrow-derived DCs; *CL*, *Clostridium leptum*; DCs, dendritic cells; ELISA, enzyme-linked immunosorbent assay; FACS, fluorescence-activated cell sorting; FCS, fetal calf serum; Foxp3, forkhead box P3; GAPDH, glyceraldehyde phosphate dehydrogenase; GM-CSF, granulocyte-macrophage colony stimulating factor; iDCs, immature dendritic cells; IBD, inflammatory bowel disease; IL, interleukin; LPS, lipopolysaccharide; MTC, maximal tolerable concentration; mAb, monoclonal antibody; OVA, ovalbumin; PMA, phorbol 2-myristate 13-actate; PBS, phosphate-buffered saline; qRT-PCR, real time reverse transcription-polymerase chain reaction; Tregs, regulatory T cells; SD, standard deviation; sDCs, stimulatory DCs; Th, T helper; tDCs, tolerogenic dendritic cells; TGF-β, transforming growth factor-beta.

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murine models. Recently, dendritic cells (DCs) have also been shown to be involved in the maintenance of gut immune homeostasis. As the most important antigen-presenting cells (APCs) [8], DCs are also widely distributed in various organs. The primary function of DCs is considered to be stimulation of adaptive immunity, and recent evidence has suggested that they also play key roles in establishing and maintaining immunological tolerance [9]. Based on their net effect on T cells, DCs have been grouped into stimulatory DCs (sDCs) and tolerogenic DCs (tDCs) [10]. sDCs are adept at acquiring, processing and presenting antigens to effector T cells in response to antigenic environmental inputs. Conversely, tDCs function to ensure immunological balance in the response.

Accumulated evidence shows that the pool of tDCs is mainly composed of immature DCs (iDCs), and includes other DCs covering a spectrum of different maturation states. The iDCs can promote expansion, function, and differentiation of Tregs [10], and are considered promising manipulable targets of novel molecular therapies. Pathogenic microorganisms, such as influenza virus, Epstein–Barr virus, hepatitis B virus and *Escherichia coli*, are less efficient in inducing the differentiation and maturation of DCs, ultimately promoting an increase in iDCs [11,12]. However, the exact mechanisms underlying the pathogen-induced effects on tDCs remain largely unknown.

Dysfunctional Tregs have been implicated in the development of several common immune-associated disorders including multiple sclerosis, asthma, and inflammatory bowel disease (IBD) [13]. While the exact mechanism of Treg-related pathogeneses remains unknown, it is possible that the functional interaction of tDCs is involved.

Several studies have addressed the potential relationship between Treg numbers, localization and activity, and *CL*. Oral inoculation of *Clostridium* spp. in young mice was shown to induce Treg movement from the colon to immune organs, such as the liver and spleen, causing an inhibition of antigen-induced intestinal inflammation by an unknown mechanism [7,14,15]. Furthermore, the *CL* and *C. coccoides* species were shown to promote Treg amplification and up-regulation of the forkhead box P3 (Foxp3) expression, a key transcription factor involved in programming Treg differentiation [7].

Recent evidence demonstrated that tDCs can manipulate Treg function and differentiation. Since iDCs are the most typical representatives of tDCs, we choose iDCs for the current research objective of investigating the role of *CL* in DC function related to Treg activation and differentiation. In this study, isolated bone marrow-derived dendritic cells (BMDCs) were exposed to *CL* and the effects on DC maturation and ability to convert $CD4^+CD25^-$ T cells to $CD4^+CD25^+Foxp3^+$ Tregs. The results suggest that a counterbalancing dysbiosis caused by deficiency in the commensal bacterium *CL* may underlie immune-related diseases such as asthma and IBD.

2. Materials and methods

2.1. Preparation of inactivated CL

A *CL* isolate from a human feces sample (Jilin Baoxin Biological Technology, Changchun, China) was selected as a single colony and grown in chopped meat broth for 24 h at 37 °C and then inactivated by heating in a water bath at 65 °C for 30 min.

2.2. Mice

BALB/c mice (10 males, 10 females; 6–8 weeks-old; 18–22 g body weight) were obtained from the Animal Research Center of Jilin University and housed in a specific pathogen-free facility under a 12 h/12 h light/dark cycle with *ad libitum* access to water and food. All procedures involving the animals were designed according to the guidelines of the National Institutes of Health Animal Research and Care and were carried out with pre-approval from the Ethics Committee of Jilin University.

2.3. Generation of murine BMDCs

BMDCs were generated according to the method of Inaba et al. [16], with the following minor modifications. BMDCs isolated from the femur and tibia bone marrow of the BALB/c mice were resuspended in RPMI-1640 medium (Gibco, Invitrogen, Carlsbad, CA, USA) supplemented with 20 ng/mL of granulocyte-macrophage colony stimulating factor (GM-CSF) and 10 ng/mL of interleukin (IL)-4 (both from PromoCell, Heidelberg, Germany) and incubated at 37 °C in a 5% CO_2 atmosphere, with medium refreshing (one-half of the medium) on days 3 and 5 of culturing. In addition, on day 3 of culturing non-adherent cells were removed by gentle swirling of the plate and mechanical aspiration, and on day 7 loosely adherent cells, including BMDCs, were harvested by gentle pipetting. The purity of the isolated BMDCs (>80%) was determined by flow cytometric detection of fluorescent-labeled anti-CD11c monoclonal antibody staining.

2.4. Isolation of murine T cells

$CD3^+$ T cells were purified from spleens of the BALB/c mice using a nylon wool column, as previously described [17] and applied to the MACS Treg Isolation Kit (MiltenyiBiotec, USA). After the magnetic bead depletion, the purity of $CD4^+CD25^+$ Tregs and $CD4^+CD25^-$ T cells from BALB/c mice were both >95%; >95% of the $CD4^+CD25^+$ cells were $Foxp3^+$ regulatory T cells, and <5% of the $CD4^+CD25^-$ T cells were $Foxp3^+$ cells.

2.5. Purification of $CD4^+CD25^+$ Tregs of co-cultures

CL-induced $CD4^+CD25^+$ Tregs of co-cultures were purified by the MACS Treg Isolation Kit; the purity of the $CD4^+CD25^+$ Tregs was >95%, and >95% of the cells were $Foxp3^+$ regulatory T cells.

2.6. Cytotoxicity testing

The appropriate dosage of *CL* was first determined by measuring cytotoxicity using the colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Briefly, BMDCs (10^5 cells/well) or T cells were cultured at 37 °C in 5% CO_2 for 72 h in duplicate 96-well plates in the presence or absence of various concentrations of *CL* in quadruplicate. Absorbance was measured at 570 nm using a Synergy plate reader (BioTek, Winooski, VT, USA). The percentage of surviving cells from each tested group was divided by that of the no- *CL* control group and then used to determine the maximal tolerable concentration (MTC) of BMDCs and T cells for *CL*. The *CL* concentration of 20 μg/mL produced no toxicity to both the BMDCs and T cells and was selected for use in all subsequent *in vitro* experiments.

2.7. In vitro analysis of CL-mediated effects on DCs maturation and function

BMDCs were divided into the following four groups: DC group, untreated negative control; DC + lipopolysaccharide (LPS), positive control of BMDCs cultured with 0.5 μg/mL LPS (Serotype 0111:B4; Sigma–Aldrich, St. Louis, MO, USA) [18]; DC + *CL*, BMDCs cultured with 20 μg/mL heat-inactivated *CL*; DC + *CL* + LPS, BMDCs cultured with both the LPS and inactivated *CL*. All groups were incubated at

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