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Predominance of IL-10 and TGF-β production from the mouse macrophage cell line, RAW264.7, in response to crude antigens from *Clonorchis sinensis*

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ABSTRACT

Parasitic helminths are well-known to have the ability to modulate host immune responses. In this study, we investigated the fundamental immunoregulatory mechanism of the liver fluke *Clonorchis sinensis* (*C. sinensis*) using a murine macrophage RAW 264.7 (RAW) cell line and mouse bone marrow derived macrophages (BMDMs). We found that *C. sinensis* crude antigen (CA) is able to differentiate macrophage RAW cells into dendritic-like cells that can be detected by morphological observations. In addition, CA induces prominent secretion of anti-inflammatory cytokines such as IL-10 and TGF-β; however, we did not observe changes in cell surface markers that are involved in antigen recognition, antigen presentation, and T cell activation. Additionally, CA treatment induced ERK and JNK phosphorylation, and unexpectedly, elevated levels of IL-10 and TGF-β were inhibited by the addition of an ERK-specific inhibitor. Taken together, these data demonstrate that CA from *C. sinensis* may modulate host immune responses by upregulating anti-inflammatory cytokines via the regulation of ERK.

1. Introduction

Clonorchis sinensis is an Asian human liver fluke that resides in the bile duct. *C. sinensis* is prevalent in Asian countries such as Korea, China, Taiwan, and northern Vietnam [1]. Human clonorchiasis, which is acquired through the consumption of raw or uncooked freshwater fishes, tends to become chronic and can result in cholangitis, bile duct obstruction, and cholangiocarcinoma [2,3]. Due to a high correlation between clonorchiasis and cholangiocarcinoma, *C. sinensis* was classified as a class I carcinogen at the meeting for the International Agency for Research on Cancer (IARC) in 2009.

Helminth infection induces host immune responses. These responses include type 2 responses that are mediated by T_H2 type cytokines such as IL-4, IL-5, and IL-13, and regulatory T (Treg) cell responses that are mediated by IL-10 and TGF-β [4]. Although T_H2-type cytokines and other host protective immune mechanisms attack parasites, the parasites are able to manipulate the host immune system to ensure long-lasting survival, thus resulting in chronic infection [4].

One possible method of evading host immune responses is through stimulating the production of anti-inflammatory cytokines such as IL-10 and TGF-β [5]. Various studies have demonstrated the anti-inflammatory properties of IL-10 and TGF-β in filariasis [6,7], fasciolasis [8], and schistosomiasis [9]. IL-10 is a well-known suppressor of T cell proliferation and cytokine production, and therefore plays an essential role in inducing anergy. IL-10 is produced by various immune cells including T_H1, T_H2, and Treg cells, and other immune cells such as B cells, monocytes, and keratinocytes [5,10]. IL-10-producing macrophages inducing Treg and impaired pathogen control in the case of *Candida albicans* and *Yersinia* spp. Infection [11,12]. Although the source of IL-10 during parasitic infection is unclear, IL-10 is a crucial mediator for the down-regulation of immune responses and immunopathology that facilitate the acute to chronic infection stage switch in a mouse model of schistosomiasis [13].

TGF-β is produced during parasite infections [7–9,14]. Although, it only shows marginal effects in suppressing parasite infection, the combination of IL-10 and TGF-β is reported to suppress hepatic inflammation in schistosomiasis [9]. Moreover, high levels of TGF-β expression were observed in an *Opisthorchis viverrini*-infected hamster model [14], suggesting that TGF-β leads to chronic parasitic infection via anti-inflammatory mechanisms. Immune suppressive properties of TGF-β include inhibition of effector T cell development to the T_H1 cells and the differentiation of naïve $CD4^+$ T cells to Treg and T_H17 cells [15,16].

Only one study has examined the relationship between *C. sinensis* infection and host immune responses [17]. In an FVB and BALB/c mice model of *C. sinensis* infection, IL-4, IL-5 (T_H2 cytokines), and IL-10 levels increased, whereas the levels of a T_H1 type cytokine, interferon (IFN) γ, decreased between 2 and 4 weeks after infection

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with *C. sinensis* metacercariae [17]. Although clonorchiasis may be an important carcinogen, few reports exist regarding clonorchiasis and in vitro and *in vivo* immune responses. Specifically, studying the roles of both the innate and adaptive branches of the immune system will provide us with a greater understanding of chronic helminth infection.

The purpose of this study was to understand the regulation of cytokine production in macrophage RAW cells upon treatment of *C. sinensis* crude antigen. Here we report that antigens from *C. sinensis* are able to upregulate anti-inflammatory cytokines and the regulation of ERK in mouse macrophage cell line, RAW264.7.

2. Materials and methods

2.1. Preparation of crude antigen

Metacercariae of *C. sinensis* were collected from naturally infected freshwater crayfish (*Pseudorasbora parva*) at Shenyang City, Liaoning Province, People's Republic of China, which is an endemic site of clonorchiasis. Pepsin–HCl (0.6%) was used as artificial gastric juice to digest the flesh of the host crayfish in order to obtain the metacercariae. The metacercariae were then introduced into 5-week-old Sprague–Dawley rats. Two months after rats were infected with 50 metacercariae, adult *C. sinensis* worms were collected from the bile ducts and washed several times with phosphate-buffered saline (PBS) containing 100 μg/ml penicillin and 100 U/ml streptomycin. The worms were homogenized within antibiotics containing PBS. PBS-soluble crude antigens were collected after centrifugation at 13,500 rpm and 4 °C for 10 min, and then filtered with a 0.2-μm syringe filter. The protein concentration in the extract was measured using a BCA protein assay kit (Pierce, Rockford, IL, USA).

2.2. Preparation of BMDMs

BMDMs were isolated as previously described [18]. Briefly, bone marrow cells were flushed from the femurs of C57BL/6 male mice with RPMI-1640 (Gibco) containing 2% fetal bovine serum (FBS; Gibco, Grand Island, NY, USA) and washed once with macrophage complete media (RPMI-1640 supplemented with 20% FBS, 55 μM 2-Mercaptoethanol, 100 μg/ml penicillin, 100 U/ml streptomycin and 30% L929 cell cultured supernatant). Cells were cultured for 7 days in macrophage complete media and, on day 3, fresh media was added. On day 7, adherent cells were harvested by removal of media and addition of cold PBS and then used in experiments as a source of BMDMs.

2.3. Cell culture condition and antigen stimulation

Murine macrophage RAW 264.7 cells were cultured in complete DMEM (Gibco) and mouse BMDMs cultured in RPMI-1640 containing 10% FBS, 100 μg/ml penicillin, and 100 U/ml streptomycin at 37 °C under a humidified atmosphere of 5% CO_2. In total, 2×10^5/ml RAW cells and 10^6/ml BMDMs were cultured for the indicated duration with or without CA (20 or 50 μg/ml). The cells and supernatants were then harvested for further analysis. 3 μM of ERK activation inhibitor peptide I (Calbiochem, Laeufelfingen, Switzerland) and 5 nM of JNK inhibitor II (Calbiochem) were used for ERK and JNK pathway blockade, respectively.

2.4. Flow cytometry

After culture with or without CA treatment, cells were harvested with a cell scraper and washed with 0.05% NaN_3 containing PBS. Cells were stained with anti-CD80 (BD-553790) and anti-CD86 (BD-553692), anti-CD40 (BD-553790) and anti-MHC class II (I-A/I-E, BD-557000), anti-CD11c (BD-557400) (BD Biosciences, Franklin Lakes, NJ, USA) and anti-Mac3 (12-5989-83, e-Bioscience, San Diego, CA, USA) monoclonal antibodies. Antibodies were directly coupled to fluorescein isothiocyanate (FITC) or phycoerythrin (PE). Additionally, to confirm the BMDMs phenotype, FITC conjugated anti-CD14 monoclonal antibody (11-0141, e-Bioscience) was used. Cells were analyzed with a FACSCalibur multicolor flow cytometer (Becton–Dickinson, Franklin Lakes, NJ, USA) and electric fluorescence intensity was analyzed using the CellQuest software (Becton–Dickinson).

2.5. Cytokine assay

Cell culture supernatants were collected 2 days after crude antigen treatment for the cytokine assay. To measure the levels of IL-4, IL-5, IL-10, IL-12, IL-13, TGF-β, and IFN-γ, we used commercial sandwich-based enzyme-linked immunosorbent assay (ELISA) kits (e-Bioscience) according to the manufacturer's instructions.

2.6. Western blotting

Cells were harvested with cell scrapers and washed with cold PBS. Cell pellets were lysed under 1% Nonidet P-40 and 0.2% Na deoxycholate in a buffer containing 120 mM NaCl, 0.2% SDS, 1 mM PMSF, and 20 mM Tris at pH 8.0, and were then incubated on ice for 30 min. After the insoluble pellets were removed by centrifugation, the lysates were separated by 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and transferred to a polyvinylidene fluoride (PVDF) membrane (Immobilon; Millipore, Billerica, MA, USA). Polyclonal or monoclonal antibodies were used to detect MAP kinase activity, including phospho-ERK (sc-7383), phospho-JNK (sc-6254), phospho-p38 (sc-7973), ERK (sc-94), JNK (sc-571), p38 (sc-535), and actin (sc-1616) (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 1:1000 dilutions with 5% skim milk and 0.05% Tween 20 containing PBS. Anti-goat, anti-rabbit, and anti-mouse immunoglobulin G antisera conjugated with horseradish peroxidase (HRP) (Dako, Glostrup, Denmark) were used at 1:2000 dilutions and then visualized by ECL™ Western blotting detection reagents (Amersham, Piscataway, NJ, USA).

2.7. Statistical analysis

Statistical analysis was performed by using an independent samples *t*-test.

3. Results

3.1. CA induces morphological changes of RAW cells into dendritic (DC)-like cells

We examined whether CA influences morphological changes of RAW cells after treatment for 48 h. We treated RAW cells with CA 10, 20, 50 and 100 μg/ml and found that some of RAW cells were dying in 100 μg/ml treated group (data not shown) and 50 μg/ml CA concentrate gave a strongest morphological changes. Following dose-dependent experiments, 20 and 50 μg/ml CA concentrate induced the most moderate and strongest responses. RAW cells showed morphological changes from macrophage-like cells into DC-like cells in response to CA in a dose-dependent manner (Fig. 1a).