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Tim-2 up-regulation and galectin-9-Tim-3 pathway activation in Th2-biased response in *Schistosoma japonicum* infection in mice

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ABSTRACT

T cell immunoglobulin domain and mucin domain (Tim) family, a new gene that expresses on the surface of T cells, plays a critical role in regulation of T cells response. Previous data have shown that Tim-3 expressed on Th1 cells promotes itself apoptosis. Tim-2 is preferentially up-regulated during Th2 differentiation and functions as a potent costimulatory molecule for T-cell immunity. The present study aims to learn whether Tims are responsible for Th2-biased response evoked by *Schistosoma japonicum* infection. The expressions of Tim-2 and Tim-3 in spleen lymphocytes from *S. japonicum*-infected mice were examined, and the possible role of galectin-9-Tim-3 pathway in Th2-biased response triggered by schistosome infection was discussed. Our results showed that Tim-2 mRNAs were up-regulated in the spleen of schistosome-infected mice, which coincided with elevated IL-4 gene expression. Administration of galectin-9 significantly induced apoptosis of naïve spleen lymphocytes with down-regulation IFN-γexpression *in vitro*. Additionally, Tim-3-Fc fusion protein notably enhanced Th1 cells and decreased Th2 cells *in vitro*. Thus, we concluded that pro-apoptotic effects on Th1 population through galectin-9-Tim-3 pathway and the up-regulation of Tim-2 on Th2 cells might be critical to Th2-biased response of host with schistosomiasis japonica.

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1. Introduction

Schistosomiasis is one of the most prevalent parasitic diseases in developing countries and impoverished regions [1]. The most serious outcome of schistosomiasis is the host immune response to schistosome eggs, which comprises the hepatic granuloma followed by hepatic fibrosis [2]. The early stage of infection leads to inflammatory response and initiates granuloma formation. The levels of Th1 cytokines, such as interferon-gamma (IFN-γ) and tumor necrosis factors (TNFs), are higher than Th2 cytokines [3,4]. Five weeks after infection, schistosome worms bloom into sexual maturation; male and female worms are mated and oviposit in the portal venous system. The soluble egg antigen (SEA) secreted by matured schistosome miracidium within eggs is believed to be major stimuli to a fibrotic granulomatous response [4–6]. As SEA is released continuously, the hepatic granuloma is formed around eggs and the immune response switches to Th2-dominated response. Both Th1 and Th2 type cytokines are waned with hepatic fibrosis formation 12 weeks post-infection [4,7]. However, the mechanisms involved are yet to not be fully elucidated.

Tim gene family, which was first cloned in mouse model of asthma [8,9], plays a critical role in autoimmunity, allergy, viral infection, and transplant tolerance [9]. It is located on T cell membrane, consisting of three members (Tim-1, Tim-3, and Tim-4) in humans, and eight members (Tim-1–8) in mouse [10]. Unlike Tim-1which is expressed on all activated $CD4^+$ T cell, Tim-2 is expressed particularly on late differentiated Th2cells at a high level [11], whereas Tim-3 is originally identified as an inhibitory receptor on murine Th1 cell surface [12]. Galectin-9 (Gal-9) is a S-type lectin with two distinct carbohydrate recognition domains expressed on a range of immune cells including T cells, B cells, macrophages,

Abbreviations: Tim, T cell immunoglobulin domain and mucin domain; *S. japonicum*, *Schistosome japonicum*; Gal-9, galectin-9; SEA, soluble egg antigen; Th cells, T helper cells; IFN-γ, interferon gamma; IL-4, interleukin-4.

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endothelial cells, and fibroblasts among the others [9,10,13,14]. Evidence shows that Gal-9 may be negative feedback loop, leading to the death of Th1 cells. Engagement of Tim-3 by Gal-9 leads to Th1 cell apoptosis and a declined IFN-γ production [15,16]. The activation of Gal-9-Tim-3 pathway also resulted in tolerance induction [16]. This pro-apoptotic role in *Schistosoma japonicum* infection, however, has not been demonstrated. In this study, we focus on Tim-2 and Tim-3 as more preferential Th2 makers than Tim-1 in Th2-biased response evoked by *S. japonicum* infection, and possible mechanisms are discussed.

2. Materials and methods

2.1. Animals

Sixty female BALB/c mice (6 weeks of age) were purchased from Anhui Laboratory Animal Center, bred in-house and maintained under specific pathogen-free conditions. All animal experiments were performed in accordance with permission from the Institutional Review Board in Anhui Medical University.

2.2. Mice infection with S. japonicum cercariae

Snail *Oncomelania hupensis* harboring *S. japonicum* cercariae were obtained from the Jiangsu Institute of Parasitic Diseases Control (Wuxi, China). Each mouse in experimental groups was percutaneously infected with 35 cercariae. At week 0, 3, 6, 9, and 12, six mice from each group were sacrificed under anesthesia.

2.3. Separation of spleen lymphocytes

Spleen lymphocytes of normal and schistosome-infected mice were collected and single cell suspension was prepared by density gradient centrifugation using Mouse Lymphocyte Separation Medium (Solarbio, Beijing, China). Then the cells were adjusted to a density of 1×10^8 cells/ml for cell culture or RT-PCR analysis.

2.4. Cell culture

Single cell suspension from schistosome-infected or naïve mice at week 0, 3, 6, 9, and 12 were cultured in RPMI 1640 (GIBCO, Grand Island, NY, USA) containing 10% fetal calf serum (Hyclone, Thermo, Beijing, China), 2×10^{-5} M 2-mercaptoethanol, and 20 mM L-glutamine, 100 U of penicillin/ml, and 0.1 mg/ml streptomycin at a density of 1×10^7 cells/ml in a 12-well plate (Costar, Cambridge, MA, US). After 24 h of culture, the supernatants were collected for IFN-γ and IL-4 detection by ELISA.

To detect the possible role of Gal-9-Tim-3 pathway in Th1 cells apoptosis *in vitro*, spleen lymphocytes from naïve mice were cultured in complete RPMI 1640 medium with 10% FCS at a density of 5×10^6 cells/ml. The cells were pre-activated overnight with anti-CD3 (2 μg/ml, eBioscience, San Diego, CA), anti-CD28 (1 μg/ml, eBioscience, San Diego, CA) for 36 h at 37 °C in 5% CO_2. Subsequently, Gal-9 recombinant protein (0.5 μg/ml, 1.0 μg/ml, 3.0 μg/ml; Abnova, Taipei, Taiwan of China) or the same volume of PBS was added to cultures for 6 or 12 h, respectively. The supernatants were subject to cytokine analysis by ELISA, and the cells to apoptotic analysis by FCM.

Tim-3-Fc fusion protein (eBioscience, San Diego, CA, USA) was used to block Gal-9-Tim-3 pathway. Briefly, spleen lymphocytes from schistosome-infected mice at week 0, 3, 6, 9, and 12 were cultured in RPMI 1640 medium with 10% FCS in presence of 0.5 and 1.0 μg/ml Tim-3-Fc fusion protein or PBS for 48 h. Supernatants were subject to cytokines analysis by ELISA and the cells were intracellularly stained for Th subsets test by FCM.

2.5. Cytokines detection with ELISA

The supernatants from respectively cultured spleen lymphocytes were collected for IFN-γ and IL-4 cytokines detection by ELISA (R&D, Minneapolis, MN, USA), according to manufacturer's guidance.

2.6. FCM (flow cytometry) analysis

For intracellular cytokine staining, either freshly isolated or cultured spleen lymphocytes were stimulated with 25 ng/ml PMA, 1 μg/ml ionomycin, and 1.7 μg/ml monesin for 4 h (all from Alexis, Farmingdale, NY). Cells were harvested, extracellularly stained with FITC-conjugated anti-CD4 (eBioscience, San Diego, CA), fixed and permeabilized with IntraPrep™ Permeabilization Reagent (Beckman Coulter, Marseille, France), and then intracellularly stained with PE-conjugated anti-IFN-γ and anti-IL-4(eBioscience, San Diego, CA) following the manufacturer's instructions. Data were acquired and analyzed on an XL-EPICS MCL with system II (Beckman Coulter, Brea, CA). Corresponding isotype control antibodies were also from eBioscience. Live events were collected based on forward and side scatter patterns.

For apoptosis assay, the pretreated spleen lymphocytes from naïve mice were stained with Annexin V-FITC Apoptosis Detection Kit (Bestbio, Shanghai, China) according to the manufacturer's instructions. The proportion of apoptotic cells was analyzed as previously described.

2.7. RT-PCR analysis

RNA from spleen lymphocytes was isolated with TRIzol (Invitrogen, Carlsbad, CA). RNA was converted to cDNA using the Omniscript reverse-transcription kit (Promega, Madison, WI) according to the manufacturer's protocol. The primers used in RT-PCR were designed using Premier Primer 5.0 software (Premier Biosoft, Palo Alto, CA, USA). Tim-3 sense: ctgagtatctggcgaatg, anti-sense: agagtctggcagtgttgg; Tim-2 sense: gccgccattcttattgta, anti-sense: atcatcccaagggtcatc; IFN-γ sense: ggtggcatagatgtggaag, anti-sense: ggacctgtgggttgttga; IL-4 sense: gttgtcatcctgctcttc, anti-sense: atgctctttaggctttcc; β-actin sense: cagccttccttcttgggtat, anti-sense: ctgtcgccttcaccgttc. RT-PCR reaction was run and PCR-generated products were electrophoresed on a 1.5% agarose gel and quantified by digitized images of DNA gels using Jieda Image Analysis System 8.0 (Nanjing University, Nanjing, China). The target genes expression was normalized against β-actin.

2.8. Histopathologic analysis

Livers from either *S. japonicum* infection mice or age-matched un-infection control mice were fixed for 48 h in 10% buffered formalin. The livers were then embedded in paraffin, and the sections were cut and stained with hematoxylin and eosin (HE). Granuloma diameters were measured using a calibrated measuring eyepiece. The diameters of the 10 largest granulomas in each section were calculated. Only granulomas appearing as circular in section were measured. Granulomas adjacent to areas of hepatocyte necrosis were excluded from diameter measurement.

2.9. Statistics analysis

All data are expressed as mean ± SD. Statistical significance was analyzed by One-way ANOVA and two tailed Student's *t*-test using SPSS software (version 16.0; SPSS Inc., Chicago, IL). $P < 0.05$ was considered statistically significant.

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