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Tim-3/Galectin-9 Pathway Involves in the Homeostasis of CCl4 Induced Hepatitis

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Abstract: Acute liver injury refers to diseases caused by various factors such as explosive liver dysfunction and inflammatory reaction caused by various causes, and the main mechanism is unclear. In this study, we found that T cell Ig and mucin domain protein 3 (Tim-3) play an important role in CCl4-induced acute liver injury. Using antibodies against the Tim-3 antibody (anti-Tim-3Ab), we studied the Tim-3 signal in an animal model of acute liver injury and found that a large number of inflammatory factors were released. Administration of galectin-9 (Gal-9 is a Tim-3 ligand) reduced the production of IL-1β and IL-6 in spleen lymphocytes. This indicates the importance of the Tim-3 / Gal-9 signaling pathway in maintaining liver homeostasis. This finding is potentially important for the treatment of early liver damage.

1. Introduction

Immunological checkpoint receptors play an important role in maintaining immune stability. Over the past few decades, it has been discovered that the activation and proliferation of T cells is recognized by immunological checkpoint molecules that up-regulate or down-regulate T-cell immune responses[1, 2]. Recently, research in the field of tumor immunotherapy is exciting. Tumor immunotherapy aims to block the immune checkpoint molecular signaling pathway by using specific antibodies, restore the effector cells response, and allow effector cells to exert anti-tumor effects.

Tim-3 are one of inhibitory molecules that are currently of considerable interest. Tim-3 belongs to the TIM family and is a novel immunological checkpoint molecule that initially identified Tim-3 on the surface of activated Th1 cells but not on Th2 cells[3]. Subsequently, Tim-3 was found to be expressed on the surface of innate immune cells, such as antigen presenting cells (APCs), dendritic cells (DCs), monocytes, macrophages[4-6], and natural killer cells (NK)[7, 8]. Tim-3 inhibitory receptors are genetically linked to liver disease and their function in regulating T cell responses and maintaining autoimmune tolerance has been extensively studied. Recently, studies have suggested that Tim-3 plays an important role in liver disease, in which Tim-3 receptor is highly expressed on effector cells, affecting the function of effector cells[9-11], therefore, this is a clinical improvement of T cell responses provide new ideas.

Although immunotherapy against the inhibitory receptors CTLA-4 and PD-1 has shown significant efficacy in several cancers and improved survival, many cancer patients still do not respond to these treatments. This has led to intensive research on the targeting of other inhibitory receptors, and Tim-3 is currently the most promising inhibitor. This review will focus on the study of Tim-3 molecules in liver disease, providing power for future treatment of liver disease.

In this study, we designed a role for the Tim-3 signaling pathway in CCl4-induced liver injury, and our results suggest that blocking Tim-3 aggravates local inflammation. This suggests that Tim-3 plays an important role in the maintenance of hepatic homeostasis. A novel mechanism provides a potential basis for the future development of hepatoprotective drugs and early treatment of liver damage.

2. Materials and Methods

2.1 Animals

Male wild-type C57BL/6J was purchased from Experimental Animal Center of Qingdao University. The mice were housed in temperature controlled environment under specific pathogen-free conditions. All mice received humane care according to the guidelines of the Ethical Committee of the Hospital Affiliated to Qingdao University.

2.2 Establish a mouse model of acute liver injury

Carbon tetra chloride（CCl4）was dissolved in olive oil, and mice were intraperitoneally injected with 0.5% CCl4 oil solution (0.1 ml/10g), and the control group was given an equal volume of olive oil. To investigate the role of the Tim-3 signaling pathway in acute liver injury, we used anti-mouse Tim-3 monoclonal antibody (mAb) (RMT3-23; rat IgG2a, Bio-x-cell). Mice were given injection anti-Tim-3 mAb (0.1 mg intraperitoneal) 6 hours before liver injury and then mice were sacrificed at 24 hours after injection of 0.5%CCl4. Controls were injected with IgG2a.

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2.3 Hepatocellular function
Serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST)were measured with an automated biochemical analyzer (Chemray 240).

2.4 Quantitative real-time RT-PCR
Mice were sacrificed 24 hours after treatment, and mouse splenocytes total RNA was extracted using the Trizol method (TaKaRa, Dalian, China) according to the manufacturer's protocol. The PrimeScriptTM RT reagent Kit with gDNA Eraser (TaKaRa, Dalian, China) reagent was used for cDNA synthesis, and the qPCR reaction was carried out according to the TB GreenTM Premix Ex TaqTM (TaKaRa, Dalian, China) specification. The housekeeping gene GAPDH was used as an internal reference, and the reaction conditions were: pre-denaturation at 95℃ for 10 seconds, PCR reaction at 95℃ for 5 seconds, 60℃ for 30 seconds, and 40 cycles. Three replicate wells were set for each sample and the results were analyzed using the mean CT values. The mRNA expression levels of IL-6, IL-1β, IFN-γ, TNF-α were quantitatively analyzed by the $2^{-\Delta\Delta CT}$ method. Primer sequences are listed in (Table 1).

Table 1. PCR Primer sequence

Genes	Forward-primers	Reverse-primers
IL-6	ACTTCCATCCAGTTGCCTTCTTGG	TTAAGCCTCCGACTTGTGAAGTGG
IL-1β	TCGCAGCAGCACATCAACAAGAG	TGCTCATGTCCTCATCCTGGAAGG
TNF-α	GCGACGTGGAACTGGCAGAAG	GCCACAAGCAGGAATGAGAAGAGG
GAPDH	TCTTGGGCTACACTGAGGAC	CATACCAGGAAATGAGCTTGA

2.5 Western blots
Western blotting was used to assess Tim-3 protein levels, and 50 μg of liver tissue was taken from each sample to grind the protein with RIPA lysis buffer. After centrifugation at 12000 rpm for 10 minutes at 4 ° C, the supernatant was collected and the protein concentration was determined using a BCA Protein Assay Kit (Sangon Biotech). Protein samples were electrophoretically separated on a 10% SDS-polyacrylamide gel and subsequently transferred to a PVDF membrane. Block with 5% skim milk powder at 37 ° C for 1 hour. Membranes were incubated overnight with rabbit Abs against Tim-3 (1:2000, Bioss) at 4 °C. β-action (1:2000, Bioss) was used as an internal reference and incubated with HRP-labeled anti-rabbit IgGAbs for 1 hour (1:5000, Bioss).The detection was developed by a gel imaging system (UVP Biospectrum 810, USA) using an ECL luminescence kit.

2.6 Immunohistochemistry
Paraffin-embedded liver tissue was made into 5μm. Sections were incubated with primary antibodies overnight at 4 °C. After PBS washing, biotin-labeled secondary antibody was added, and incubated at 37 ° C for 60 minutes, rinsed with PBS, and finally developed with DAB solution, and the sections were dehydrated and sealed. A negative control was used instead of the primary antibody with PBS. After the above operation was completed, the expression level of Tim-3 protein was observed under a microscope. Randomly take 10 fields of view (×400) for each slice to observe the experimental results, and analyze the optical density using Image J software and calculate the number of positive cells in each field of view

2.7 Enzyme-linked immunosorbent assay (ELISA)
The cell suspension was prepared at a density of 1 x 10^6 / ml, and centrifuged at 3500 rpm for 30 minutes, and the cell supernatant was carefully collected. Measurement of IL-6, IL-1β protein according to manufacturer's ELISA (eBioscience) instructions

2.8 Isolation of splenocytes
Splenocytes were prepared by treating single cell suspensions with Red Cell Lysing Buffer (EpiZyme, China) and resuspended in RPMI-1640 medium (Invitrogen). Splenocytes were adjusted to 1×10^6cells/mL and incubated at 37℃ for 12 hours. T cells were stimulated with ConA (5 μg/mL, EpiZyme) and incubated for 24 hours with or without anti-mouse Tim-3 Ab (10 μg/mL)and recombinant protein GaL-9(0.2μM)[12]

2.9 Statistical analysis
All data are expressed as mean ± SD analysis. Comparisons between the two groups were analyzed using Student's t test. One-way ANOVA was used to assess differences between paired data. At $P < 0.05$, it was considered statistically significant.

3 Results
3.1 Tim-3 upregulation correlates with liver damage
Mice were injected intraperitoneally with 0.5% CCl4 oil solution or olive oil and killed after 24 hours. As shown in Figure 1A, serum ALT/AST was significantly elevated in the 0.5% CCl4 group. Immunohistochemistry results showed that the expression level of Tim-3 protein in the liver tissue of CCl4-treated mice was significantly higher than that of the control group (Fig. 1B). Using the western blot method, we found that the level of Tim-3 in the liver tissue of CCl4 mice was higher than that of the control group (Fig. 1C). Our data show that Tim-3 expression is significantly increased in the mouse model of acute liver injury.

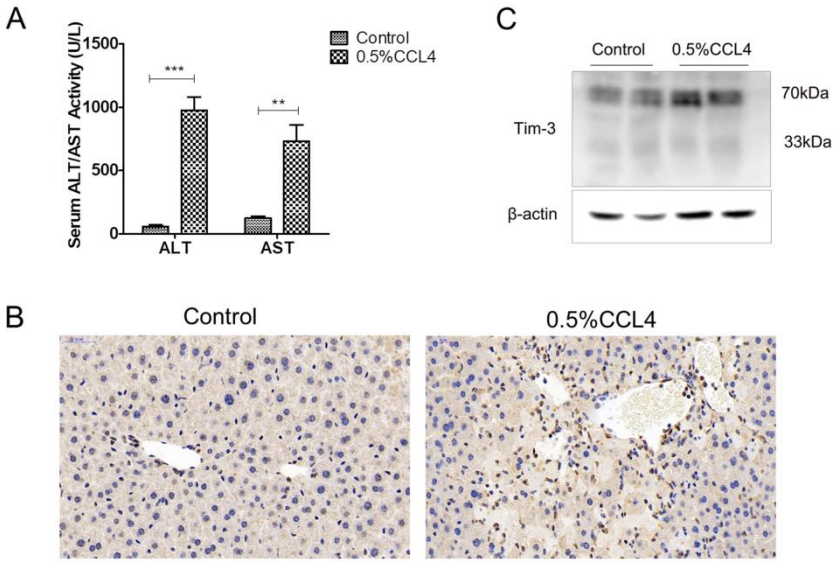

Figure 1. Expression of Tim-3 in the liver of hepatitis mice induced by CCL4. (A) Expression of ALT or AST in serum of CCl4-induced liver injury. (B) Immunohistochemistry results showed the expression level of Tim-3 (×400) in the experimental group and the control group. (C) Western blot to measure Tim-3 protein levels. All data were ± SD, and for three independent experiments, there were 8-10 mice per experimental group. * P <0.05; ** P <0.01.

3.2 Treatment of CCl4-induced liver injury with Anti-TIM-3Ab, and upregulation of inflammatory factors

We studied the role of Tim-3 in acute liver injury, using anti-Tim-3 antibodies to block signaling pathways in mice with acute liver injury, and we found that expression of inflammatory factors was up-regulated 24 hours after liver injury (Fig. 2 P<0.05). The primer sequences of the cDNA are listed in Table 1.

3.3 Tim-3/Gal-9 pathway regulates inflammatory cytokines in T cells

We isolated spleen cell culture from C57BL/6 mice and added anti-Tim-3 Ab (10 μg/ ml) or Gal-9 (0.2 μM) under ConA (5 μg/ml) for 24 hours. We found an increased in IL-6 levels in the anti-Tim-3 Ab group (Fig. 3A). Interestingly, the addition of the recombinant protein Gal-9 reduced the production of IL-6 and IL-1β (Figures 4A and B). These results demonstrate T cell-dependent Tim-3 signaling, and blocking Tim-3 can aggravate the release of inflammatory factors. Tim-3/Gal-9 signaling pathway plays an important regulatory role in liver injury.

4 Discussion

We found that Tim-3 expression is up-regulated in a mouse model of liver injury, and blocking Tim-3 can up-regulate the inflammatory factors IL-1β, IL-6, IFN-γ and TNF-α mRNA levels, aggravating liver damage. This suggests that Tim-3 plays an important negative regulatory role in early liver injury and may be a therapeutic target for intervention in early liver injury.

TIM-3 is a member of the TIM family, and it is identified as selectively expressed on the surface of CD4+T cells and CD8+T cells, as well as in Th17[13], DCs, macrophages, but not on Th2 cells[14-16]. The interaction between Tim-3 and its ligand galectin-9 negatively regulates T cell responses and mediates T cell apoptosis[17]. The dysregulation of Tim-3 expression on immune cells has shown to be associated liver injure . However, the specific mechanism for clarifying

We found that Tim-3 expression is up-regulated in the liver in a mouse model of liver injury. For further testing, Tim-3 was blocked with an antagonist mAb against Tim-3 to detect changes in acute liver injury. We found that interference with the Tim-3 pathway significantly aggravated the production of inflammatory factors (Figure 2). These data suggest that Tim-3 is negatively regulated by inflammatory factors to promote liver homeostasis.

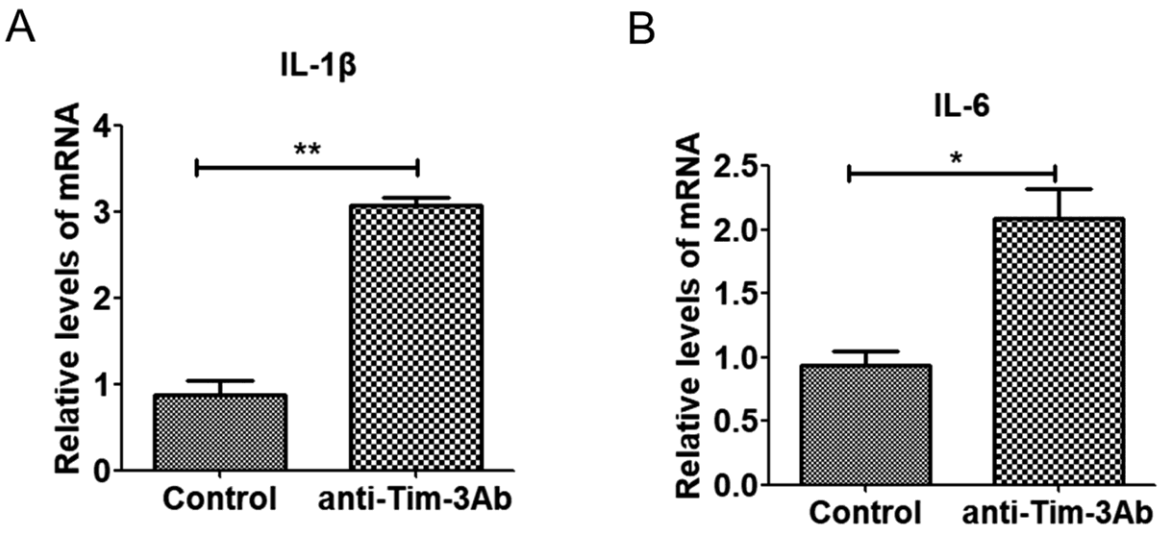

Figure 2 .Blocking Tim-3 aggravates the inflammatory response in the liver. Splenocytes were isolated from anti-Tim-3 Ab or control IgG-treated CCL4 mice and tested for inflammatory factor mRNA levels by real-time PCR. The results we obtained were expressed as mean ± standard deviation, three independent experiments (n = 5-6 / group, * P <0.05, ** P <0.01).

To mimic in vivo scenarios, we used ConA to stimulate the expression of Tim-3 in lymphocytes[18, 19]. We found that IL-6 cytokines were significantly increased in ConA-stimulated spleen T cells (mainly Th1) after addition of anti-Tim-3 antibody (Fig. 3), and the results of in vitro experiments were identical to our in vivo data.

Interestingly, we found that the expression levels of IL-6 and IL-1β were decreased in the recombinant protein Gal-9 treated group (Fig. 4). Inflammatory factors further aggravate the deterioration of liver damage by interacting with Th1 cells by Tim-3 / Gal-9. Our results are consistent with previous reports[20, 21]. Figure 5 summarizes the mechanism of negative regulation of Tim-3 in liver injury. Tim-3 and the ligand Gal-9 pathway regulate the expression of inflammatory factors, which can reduce the production of IL-6, IL-1β, IFN-γ, TNF-β, and reduce liver damage.

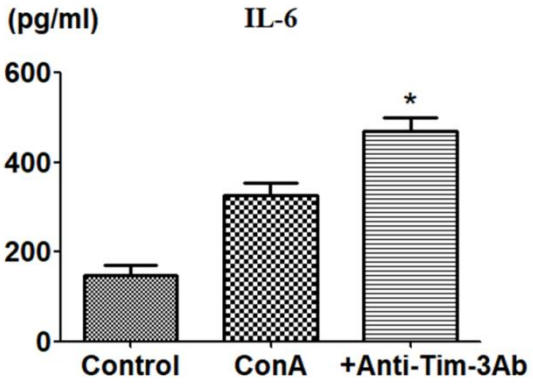

Figure 3 Production of inflammatory cytokines in splenocytes. Spleen cells were cultured alone or in culture medium containing ConA (5 ng / ml) and / or without anti-Tim-3 Ab (10 mg / ml), the supernatant was then tested for IL-6 by ELISA.

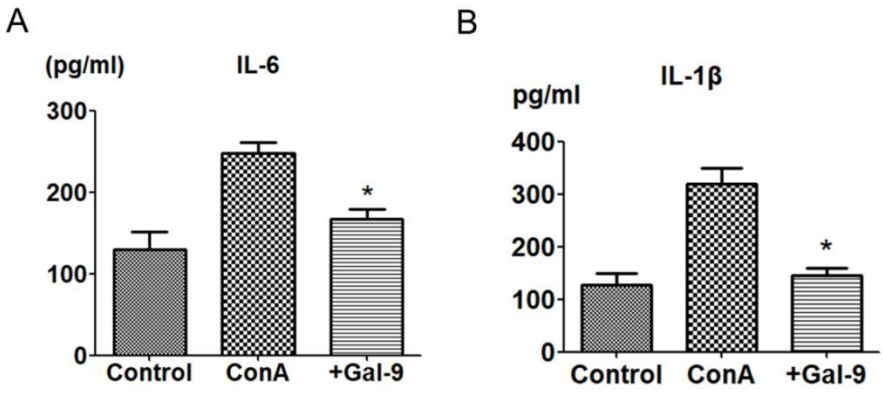

Figure 4 IL-6 and IL-1β levels were measured in ConA-stimulated spleen T cell cultures. The addition of the recombinant protein Gal-9 inhibits IL-6 and IL-1β levels. Data are shown as mean ± SD of three independent experiments with 8 mice per group per experiment. * P <0.05

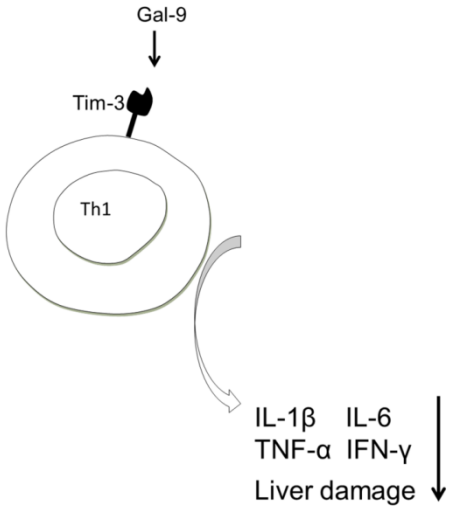

Figure 5. Negative regulation of Tim-3 in liver injury.

Our results suggest that the Tim-3/Gal-9 pathway plays an important role in the liver immune response that maintains liver homeostasis. Tim-3 acts as a negative regulator to regulate immune cell activation and may be an effective targeting molecule for clinical interventions, which will provide new targeted therapies for clinical research.

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