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Research article

Role of antigen presenting cell invariant chain in the development of hepatic steatosis in mouse model

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ABSTRACT

The role of Invariant chain (CD74 or Ii) in antigen presentation via Antigen Presenting Cells (APC), macrophage recruitment as well as survival, T cell activation and B cell differentiation has been well recognized. However, the aspect of CD74 which is involved in the development of hepatic steatosis and the pathways through which it acts remain to be studied. In this study, we investigated the role of CD74 in the inflammatory pathway and its contribution to development of hepatic steatosis. For this, wild type C57BL/6J and CD74 deficient mice ($Ii^{-/-}$ mice) were fed with high fat high fructose (HFHF) diet for 12 weeks. Chronic consumption of this feed did not develop hepatic steatosis, glucose intolerance or change in the level of immune cells in $Ii^{-/-}$ mice. Moreover, there was relatively delayed expression of genes involved in development of non alcoholic fatty liver disease (NAFLD) in HFHF fed $Ii^{-/-}$ mice as compared to that of C57BL/6J phenotype. Taken together, the data suggest that HFHF diet fed $Ii^{-/-}$ mice fail to develop hepatic steatosis, suggesting that Ii mediated pathways play a vital role in the initiation and propagation of liver inflammation.

1. Introduction

NAFLD embraces a wide spectrum of conditions associated with the over-accumulation of lipids in the liver, ranging from steatosis to nonalcoholic steatohepatitis (NASH), which includes hepatitis and fibrosis. In order to realize and develop realistic treatment strategies for different stages of steatosis leading to NASH and cirrhosis, it is indispensible to unravel the underlying mechanism leading to this chronic disease [26]. The summation effects of various hepatic immune and inflammatory pathways have a central role in the pathogenesis of NAFLD [14]. Hence, it is important to thoroughly understand the role of other controlling factors together with oxidative stress, cytokine production and other pro-inflammatory mediators that affect the progression and occurrence of the disease in delivering a second hit during the switch from simple steatosis to NASH [15,22]. Recently, it was suggested that T lymphocytes play an essential role in the development of adipose tissue inflammation [19,20,25]. Although the liver is enriched with cells of the innate immune system, the adaptive immune system is also important, as evidenced by an association between a chronic inflammatory infiltrate and oxidative stress causing NASH and fibrosis.

CD74, also known as the HLA-DR antigens-associated invariant chain or invariant chain (Ii), is a non-polymorphic glycoprotein that has diverse immunological functions and performs diverse activities which affect several cell populations on B cells, T cells and APCs [2]. Various studies have indicated that CD74 is highly expressed in inflammatory disorders and cancers. It also serves as a receptor for macrophage migration inhibitory factor (MIF) that shows pleotropic effects in the immune-pathogenesis of several diseases [4,13]. Recent studies suggested that the absence of CD74 renders T cells inactive and impairs antigen presentation via APCs which in turn attenuate atherogenesis [23]. CD74 also acts through mitogen activated protein kinase (MAPK) and c-Jun amino – terminal kinase (JNK) activation mediated pathways for energy homeostasis and regulation of inflammatory cytokines [13]. Since it has also been reported that CD74 has an indirect role in causing T cell activation, B cell differentiation and macrophage survival [4,17], we hypothesize that the adaptive immune cell function together with a CD74 mediated action on hepatic cells exert an inflammatory effect in HFHF treated mice resulting into hepatic

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steatosis. This has been demonstrated by using $Ii^{-/-}$ mouse model which lacks in CD74 and T cell function and shows a reduced susceptibility towards HFHF diet induced hepatic steatosis supporting our hypothesis.

2. Materials and methods

2.1. Animals

Experimental procedures used in this study were approved by the Institutional Animal Ethics Committee. Six-week old C57BL/6J male mice and CD74 knockout male mice (referred to as $CD74^{-/-}$ mice or $Ii^{-/-}$ Jackson Stock No 002729) (Jackson Laboratories, Bar Harbour, ME, USA) were used in this study. Mice homozygous for the Ii^{tm1Liz} targeted mutation are viable and fertile. Ii-associated invariant chain deficient mice have defects in major histocompatibility complex (MHC) Class II assembly, transport, surface expression, and antigen presentation. Homozygous mutant mice lack mature $CD4^+$ T cells and are deficient in the invariant chain. These mice were housed in a pathogen-free environment under standard light (12 h light, 12 h dark cycle), temperature (23 ± 1 °C), and humidity (50 ± 5%) conditions. Each of C57BL/6J and $Ii^{-/-}$ mice groups (n=10) were divided into two batches i.e. control (n=5) and treated (n=5); (*hereinafter referred to as control C57BL/6J or control $Ii^{-/-}$ group and treated C57BL/6J or treated $Ii^{-/-}$ group*) and the study was performed thrice. The control mice received 4.5% fat and 22% crude protein supplemented diet (total energy 3802 kcal/ kg) while the treated group received 30% fat and 18% crude protein supplemented diet along with an additional 50% (w/v) fructose (HiMedia, Mumbai, India). The total energy content obtained from HFHF diet is 5202 kcal/kg.

2.2. Biochemical analysis

At the end of 12 weeks, the animals in each group were weighed and the blood samples were drawn by cardiac puncture under anesthesia after fasting the animals for 6 h. Serum biochemical parameters such as cholesterol (CHO), triglycerides (TG) and alanine aminotransferase (ALT) levels were analyzed using serum auto-analyzer (Screen Master 3000, Tulip, Alto Santacruz, India). Blood glucose was measured directly by Glucometer (Accu chek, Mumbai, India) via a tail nick. For glucose tolerance test (GTT) mice were fasted for 6 h and injected intraperitoneally with 2 g/kg dextrose, and tail blood glucose was measured at different time points using a glucose monitor (Accu chek) at 0, 30, 60, and 120 min. For ITT, insulin was injected intraperitoneally (1 U/kg), and blood glucose was measured at time points of 0, 30, 60, and 120 min post injection. *These tests were done 48 h before euthanizing mice*.

2.3. Histological analysis

Liver tissues were isolated after euthanasia, fixed overnight in 10% formalin and embedded in paraffin blocks. For Hematoxylin-Eosin (H-E) staining, tissues sections of 4 μm thickness were obtained on poly-L-lysine (Sigma) coated slides using microtome and stained according to standard protocol. Further, NAFLD activity score was calculated essentially as described previously [12]. Collagen was stained using picrosirius red stain as per standard protocol. Oil-Red-O (ORO) staining was performed on liver tissue cryosections (5 μm). Tissue sections were fixed in paraformaldehyde (10%), rinsed with 60% isopropanol and stained with freshly prepared ORO according to standard protocol. Representative photomicrographs of hematoxylin-eosin, picrosirius red and ORO stained slides were captured at 40X magnification.

2.4. Immunodetection of F4/80 and analysis of apoptosis in liver

Liver tissues were fixed in 4% paraformaldehyde or cryoprotected in 30% sucrose solution for 24 h at 4 °C and frozen in optimum cutting temperature (OCT) medium. Five micron sections were obtained using cryostat and treated with 0.15% Triton X-100 for 30 min at room temperature (RT). The sections were then stained with anti-mouse F4/80 (ebioscience) antibody for 1 h at RT. After washing the slides for 3 times with 1% BSA in PBS, sections were treated with secondary antibody (alexa fluor 594 conjugated anti-rabbit IgG) for 1 h at RT. The sections were further washed and the nucleus was counter-stained with 4, 6-diamidino-2-phenylindole (DAPI) diluted to a concentration of 2ug/ml in PBS (1:500) for 5 min. Sections were observed under Olympus fluorescence microscope using LCPlanFl 20X and 60X objective. The Image-Pro software was used for image acquisition. The images were composed and edited in Photoshop 6.0 (Adobe). Apoptosis in liver cells was evaluated through terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay. Paraffin sections of liver were deparaffinized, rehydrated by successive serial washings with ethanol before treating with proteinase K for permeabilization of cells. Fragmented DNA was labeled with terminal deoxynucleotidyl transferase (TdT) and biotin deoxynucleotides (dNTPs) as per manufacturers' recommendation (Dead End™ Colorimetric TUNEL System, Promega, USA). The image acquisition was performed using a bright field microscope (Nikon, Japan) and further analyzed for apoptosis. The total number of intact hepatic cells was established by counting these cells (counterstained in DAPI) in a random collection of the captured 20 X images (10 fields). The apoptotic index was calculated by dividing the total number of TUNEL positive cells by the total number of hepatic cells and multiplying by 100. F4/80 positive cell quantification was performed by assessing at least 10 random fields using Image J program (NIH).

2.5. Measurement of total triglyceride content in liver

Total liver triglyceride (TG) was extracted using Folch method [7]. Briefly, the liver is weighed and homogenized with chloroform/methanol (2/1) to a final volume 20 times the volume of the tissue sample and centrifuged to obtain the liquid phase. The solvent is then washed with normal saline and further centrifuged to obtain two separate phases. The upper phase is removed and lower chloroform phase containing triglycerides is evaporated under vacuum. The total liver TG thus obtained was dissolved in 95% alcohol. Quantification of total TG was done using Coral GPO-PAP kit (CORAL Clinical systems India) using manufacturers' instructions.

2.6. Flow cytometric analysis

Fluorescence-activated cell sorting (FACS) analysis was performed to evaluate the proportion of immune cells in the both peripheral blood and liver of mice. Blood was drawn at the end of 11weeks, from the retro-orbital plexus of anaesthetized mice in tubes containing citrate phosphate dextrose (CPD) buffer. The antibodies used were fluorescein isothiocyanate conjugated anti-mouse CD3, allophycocyanin (APC) conjugated anti-mouse CD8, phycoerethryn (PE) conjugated anti-mouse CD68 and peridinin chlorophyll (PerCp)-Cy5.5 conjugated anti-mouse B220 (all from BD Biosciences). 50 μl of anti-coagulated blood was mixed with 50 μl of a combination of anti-CD3, CD4, CD8 antibodies at a final antibody dilution of 1:200 in phosphate buffered saline (PBS), while anti-B220 and anti CD68 antibodies were singly mixed with 50ul of anti-coagulated blood at a final dilution of 1:200 in PBS and incubated at room temperature (RT) for 30 min. RBC was lysed