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The differential organogenesis and functionality of two liver-draining lymph nodes in mice

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ABSTRACT

The liver is an immunological organ. However, fundamental knowledge concerning liver-draining lymph nodes (LNs), which have been newly identified in mice as the portal and celiac LNs, is still lacking. Here, we revealed that the portal LN and celiac LN drain liver lymph through different lymphatic vessels. Although both the portal LN and celiac LN possess typical structures, they have different cell compositions. Interestingly, these two LNs form at different times during fetal development. Moreover, the organogenesis of the celiac LN, but not the portal LN, is controlled by the transcription factor NFIL3. Furthermore, the portal LN and celiac LN also perform different functions. The celiac LN is the predominant site of liver antiviral immune responses, whereas the portal LN functions in the in situ induction of dietary antigen-specific regulatory T cells. In conclusion, the portal LN and celiac LN are two independent liver-draining LNs with different organogenesis histories and separate functions in maintaining immune homeostasis in the liver.

1. Introduction

The liver, the largest solid organ in the human body, also works as an immunological organ [1–3]. The unique anatomical architecture and ample blood supply of the liver not only endow it with its metabolic function but also maximize immune surveillance in this organ [4]. In the liver, blood is extensively filtered to clear harmful exogenous substances, such as microorganism-derived toxins and blood-borne pathogens [5–8]. Simultaneously, substantial evidence has shown that the liver is important for the induction of systemic immune tolerance to nonpathogenic antigens, such as food-derived antigens [9–12]. Thus, a balance between activation and tolerance characterizes the liver as an immunological organ, with key immune functions to clear blood-borne pathogens and induce tolerance to harmless molecules. However, for the liver immune system, the generation of robust immune responses to eliminate pathogens and induction of tolerance to harmless molecules are the best-case scenarios. When the liver immune system is compromised, immune disorders in the liver lead to severe diseases, such as chronic infections, autoimmune hepatitis (AIH) and cancer [13–15]. The mechanisms underlying the maintenance and disruption of immune homeostasis in the liver are still not fully understood.

The liver has well-developed lymphatic networks and produces large amounts of lymphatic fluid that drains into downstream lymph nodes (LNs). Liver lymph accounts for approximately 25–50% of the lymph flowing into the thoracic duct [16]. Antigens in the liver are captured by antigen-presenting cells and are transported to liver-draining LNs [17]. Because draining LNs are specialized secondary lymphoid organs for adaptive immune

Abbreviations: Ad-HBV, adenovirus expressing the hepatitis B virus genome; AIH, autoimmune hepatitis; DAPI, 6-diamidino-2-phenylindole; DC, dendritic cell; HBV, hepatitis B virus; HCV, hepatitis C virus; IFN, interferon; LN, lymph node; NK cell, natural killer cell; NKT cell, natural killer T cell; LCMV, lymphocytic choriomeningitis virus; LTβ, lymphotoxin-β; MAdCAM-1, mucosal addressin cell adhesion molecule-1; OVA, ovalbumin; PNAd, peripheral node addressin; PP, peyer's patch; TNF, tumor necrosis factor; Treg, regulatory T cell.

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responses, the immune outcome for substances entering the liver is the integration of responses in the liver and liver-draining LNs. Therefore, as important parts of the liver immune system, liver-draining LNs play important roles in maintaining immune homeostasis in the liver.

Compared to the immune responses in the liver, the immune responses in the liver-draining LNs are less well studied. The inability to clearly identify liver-draining LNs in mice, the most common experimental animal for immunological research, is the reason for the current knowledge gap. In contrast, the clinical significance of LNs draining the human liver has been extensively reported [18–25]. Generally, human liver-draining LNs in the hepatic hilar region are anatomically separated into two major clusters, including nodes along the common hepatic artery and nodes in the hepatoduodenal ligament [23,25,26]. An enlargement of these LNs has been reported in patients with chronic infections, AIH and cancer [18–25]. Interestingly, several reports studying metastasis in these two clusters of LNs in patients with colorectal liver metastasis showed that the involvement of different LN clusters leads to different survival rates [23,25,27]. Other reports indicate that different LN clusters are involved when neoplastic lesions are observed in different liver lobes [28–30]. These results imply that these two clusters of human liver-draining LNs might have different functions. Although the clinical manifestations of various liver diseases indicate that liver-draining LNs are closely related to liver immune status, how immune responses occur in these specialized places and the separate roles of different LN clusters are still unclear because data are lacking regarding the liver-draining LNs in animal models.

In mice, liver-draining LNs have only recently been strictly identified [31,32]. In the mouse hepatic hilar region, two LNs draining mouse liver, the portal and celiac LNs, were identified as equivalents to the two clusters of liver-draining LNs in humans. Clear identification of the liver-draining LNs in mice makes it possible to investigate the fundamental knowledge of liver-draining LNs and the immune responses that occur there, providing clues for human studies related to liver-draining LNs. Immune responses in specific contexts were observed after their identification [33–36]. However, several studies pooled the portal and celiac LNs together [32,34,36], whereas other studies assessed the portal LN and celiac LN separately and observed substantially different responses induced in these two LNs, although no proper explanation was presented [31,33]. Thus, the relationship between the portal LN and celiac LN remains obscure. In addition, fundamental knowledge regarding their structures, immune cell compositions and organogenesis is still lacking. Moreover, their contributions to the immune homeostasis of the liver and their separate roles in the liver immune system remain unknown.

In this study, we show that the portal LN and celiac LN drain liver lymph through different lymphatic vessels. The portal LN and celiac LN also have different immune cell compositions and different organogenesis histories. Utilizing hepatotropic viral infection models, which mimic blood-borne pathogen invasion, we showed that the celiac LN is the predominant site of liver antiviral immune responses. In contrast, using an established model of oral tolerance, we showed that the portal LN is the site for in situ induction of antigen-specific regulatory T cells (Tregs) after oral antigen administration. Thus, the portal LN and celiac LN are developmentally and functionally different liver-draining LNs that contribute to immune homeostasis in the liver.

2. Materials and methods

2.1. Animals

Male C57BL/6 wild-type mice (4–6 weeks old) were purchased from the Shanghai Experimental Animal Center (Shanghai, China). *Nfil3*$^{+/-}$ mice were provided by Dr. Tak W. Mak (University of Toronto), and *Nfil3*$^{-/-}$ mice were bred in house. *IL2Rγc*$^{-/-}$ mice were purchased from The Jackson Laboratory (Maine, USA). Foxp3-GFP reporter mice were provided by Dr. A.Y. Rudensky (Howard Hughes Medical Institute). OT-II mice were provided by Dr. Xuetao Cao (Second Military Medical University). OT-II×Foxp3-GFP mice were bred in house and maintained on a CD45.1^{+}CD45.2^{+} background. All mice were maintained in specific pathogen-free, temperature- and humidity-controlled environments with 12-h light/dark cycles. Clean Wistar rats were purchased from Vital River Laboratories (Beijing, China). Chinese treeshrews were purchased from the Kunming Institute of Zoology (Kunming, China). All animals received care in compliance with the animal care regulations of the University of Science and Technology of China. All animal studies have been reviewed and approved (USTCACUC1601009).

2.2. Viruses and infections

The replication-deficient adenovirus was purchased from 5 + MMI (5 + MMI, Beijing, China). An adeno-vector expressing the HBV genome was a gift from Dr. Ulrike Protzer (Technical University of Munich). Adenoviruses expressing the HBV genome (Ad-HBV) were packaged and propagated in HEK293A cells. Ad-HBV was purified by discontinuous CsCl density gradient centrifugation and titrated using an Adeno-X™ Rapid Titer Kit (Clontech, California, USA), according to the manufacturer's instructions. Mice were intravenously (i.v.) infected with 2×10^9 infectious units (ifu) of adenovirus and euthanized at various time points. The lymphocytic choriomeningitis virus (LCMV) Armstrong and Clone 13 strains were gifts from R. Ahmed (Emory University). Mice were intraperitoneally (i.p) infected with LCMV Armstrong (2×10^5 plaque-forming units (PFU)) or i.v. infected with LCMV Clone13 (2×10^6 PFU). For infections, mice were randomly divided into infection group and control group. Mice infected with viruses were housed in accordance with institutional biosafety regulations of the University of Science and Technology of China.

2.3. Flow cytometry

Isolation of mononuclear cells from liver, spleen, and LNs are described previously [32]. Single-cell suspensions were incubated with rat serum to block Fc receptors and stained with fluorescent monoclonal antibodies. Monoclonal antibodies (mAbs) against CD3 (145-2C11), CD4 (RM4-5), CD27 (LG.3A10), TNFα (MP6-XT22), IFN-γ(XMG1.2), CD45.1 (A20), CD45.2 (104), CD86 (GL1), CD103 (M290), NK1.1 (PK136), Gr1 (RB6-8C5), I-A/I-E (M5/114.15.2), CXCR3 (CXCR3-173), B220 (RA3-6B2), NKp46 (29A1.4) were purchased from Biolegend. Abs against CD11b (M1/70), CD11c (HL3), CD19 (1D3), CD44 (IM7), CD45 (30-F11), CD80 (16-10A1), ICOS(7E.17G9), TCRγδ(GL3), DX5 (DX5), GL7 (GL7), Fas (Jo2) were purchased from BD. Abs against α4β7 integrin (DATK32), Rorγt (B2D), Foxp3 (FJK-16s) were purchased from eBioscience. CD1d tetramer was obtained from the National Institutes of Health Tetramer Core Facility. For intracellular staining of IFN-γ and TNF-α, cells were stimulated with 30 ng/ml PMA (Sigma) and 1 μg/ml ionomycin (Sigma) for 4 h, and 2 μg/ml monensin (Sigma) was added at the beginning of stimulation to prevent cytokine secretion. Hepatitis B surface antigen (HBsAg)-specific CD4^{+} T cells were detected using a previously described procedure [37]. Customized I-A^{b} tetramer loaded with HBsAg$_{126–138}$ peptide (RGL YFP AGG SSSG) and I-A^{b} tetramer loaded with human class II–associated invariant chain peptide (CLIP) were obtained from the National Institutes of Health Tetramer Core Facility. Data were collected using a flow cytometer (LSR II; BD) and analyzed using FlowJo software.

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