Research Article

EASL EUROPEAN ASSOCIATION FOR THE STUDY OF THE LIVER | JOURNAL OF HEPATOLOGY

Two lymph nodes draining the mouse liver are the preferential site of DC migration and T cell activation

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Background & Aims: Lymph nodes (LNs) play a critical role in host defence against pathogens. In rodents, lymphatic anatomy and drainage have been characterized for many different organs. Surprisingly, the LNs draining the mouse liver have not been clearly identified. This knowledge is of central importance to allow accurate characterization of immune responses to pathogens infecting the liver. It is also important for exploring immune responses in hepatic tumour models, and mechanisms underlying the relative tolerogenic properties of the liver. In this study, we used both anatomical and immunological approaches to identify the LN(s) draining the mouse liver.

Methods: Evans Blue and purified dendritic cells were directly injected into the hepatic parenchyma.

Results: Using Evans Blue, we identified three LNs adjacent to the liver that stained with the dye within the first 5 min, which we termed portal, coeliac, and first mesenteric LNs. We also provide evidence that dendritic cells (DCs) injected under the liver capsule preferentially migrate to the coeliac and portal nodes, leading to local activation of antigen-specific naïve CD8 and CD4 T cells, suggesting this is a route of lymphatic drainage from the liver. Consistent with this result, cell-associated antigen injected under the liver capsule was also cross-presented to CD8 T cells in these nodes.

Conclusions: These results suggest for the first time that the coeliac and portal nodes are the main LNs draining the liver, and that DCs exiting the liver can elicit primary T cell activation within these lymph nodes; first mesenteric nodes play a secondary role. We propose this nomenclature to be used as common designations for the observed structures.

Keywords: Liver; T cells; Draining lymph nodes; Dendritic cells; Transgenic mice.
Received 24 August 2011; received in revised form 8 March 2012; accepted 12 March 2012; available online 25 April 2012

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Abbreviations: B6, C57BL/6; DC, dendritic cell; LN, lymph node; MHC, major histocompatibility complex; TCR, T cell receptor; Gb, gallbladder; IVC, inferior vena cava; PV, portal vein.

Introduction

Lymph nodes (LNs) are specialised compartments located throughout the body that favour interaction of naïve T cells with professional antigen presenting cells (APCs) such as dendritic cells (DCs). Specialised DC subsets capture foreign proteins at the site of infection, and migrate to the draining LN to present antigenic peptides in the context of MHC molecules to antigen-specific T cells. LNs thus play a critical role in host defence against pathogens, tumour antigens, and in the development of immune responses to cross-presented alloantigens following organ transplantation. Lymphatic vessels are also a critical route of tumoral cell migration and metastases formation. In oncological surgery, lymphadenectomy is a vital component of correct tumour staging in the TNM classification [1], with important implications for therapy and prognosis. For instance, in breast cancer the sentinel LN technique, where the first draining LN of the tumour is analyzed to guide the extent of lymphadenectomy, is now widely used [2].

Although LNs draining specific tissues are well characterised in humans, anatomical and functional descriptions in rodents, in particular mice, are less well documented. Exhaustive studies performed in the 1970s [3,4] identified most LNs in rats, and subsequent studies indicated that mice have a similar lymphatic anatomy and drainage [5–7]. However, this is not always the case. Although some authors referred to "hepatic LNs" in their studies [8–12], clear anatomical or functional identification of the LNs draining the mouse liver is surprisingly absent. The most exhaustive study, characterising all murine LNs in BALB/cAnNCrl mice using Indian ink mixed with adjuvant to stain and reveal all LNs, failed to demonstrate the existence of nodes that specifically drained the liver [13]. This basic knowledge is critical for studies of immune responses against pathogens infecting the liver, murine models of hepatic tumour immunity and basic liver immunology, and is required to allow different investigators to have a common designation for the observed structures.

In this study, we used both anatomical and immunological approaches to identify the liver draining LN in mice. We report for the first time that the murine liver is predominantly drained by a chain of three LNs. Two of these are part of the previously described pancreaticoduodenal LNs, and the third LN is actually the first mesenteric LN. However, DCs migrated preferentially

to two of the three LNs, suggesting that these are the main LNs draining the liver, and are previously uncharacterized sites relevant to immune responses to antigens or APCs exiting the liver.

Material and methods

Mice

All mice were maintained in the Centenary Institute under specific pathogen-free conditions. B10.BR (H-2^k, Ly5.2), C57BL/6 and Balb/c mice were purchased from the Animal Resources Centre (Perth, Western Australia, Australia). B10.BR (H-2^k, Ly5.1) homozygous mice were initially derived from B6.SJLPtprc^a (H-2^b, Ly5.1) backcrossed for more than 10 generations with B10.BR (H-2^k, Ly5.2). Des-TCR mice, expressing a T cell receptor (TCR) specific for the mouse MHC class I molecule H-2K^b and self-peptide, have been described elsewhere [14]. Des-TCR *RAG-1*$^{-/-}$ mice (herein referred to as Des-RAG mice) were derived by crossing Des-TCR mice with H-2^k *RAG-1*$^{-/-}$ mice. Des-TCR and Des-RAG mice were maintained on a B10.BR (Ly5.2) background [15]. 178.3 (Ly5.2) transgenic mice express the MHC class I molecule H-2K^b ubiquitously under the control of its own promoter [16], and were originally provided by Drs. W. Heath and M. Hoffmann (WEHI, Melbourne, Australia). H-2^{bm1} mice were a kind gift of W. R. Heath and are bred at the Centenary facility. P25 mice were a kind gift of K. Takatsu (University of Tokyo, Japan) and express a transgenic TCR specific for a peptide from the *Mycobacterium tuberculosis* antigen 85B in the context of I-A^b [17]. The University of Sydney Animal Ethics Committee approved all experimental procedures.

Dye injection and anatomical description

Mice were anaesthetized with 1–2.5% isofluorane. Ten percent Evans Blue dye (Sigma–Aldrich, Pty. Ltd., Castle Hill, NSW, Australia) diluted in a total of 5 μl Hank's Buffered Salt Solution was injected directly into the liver parenchyma/capsule (right, left, and median lobes) using a 27-gauge syringe. Mice were maintained for 5–20 min under continuous anaesthesia to allow the dye to migrate through the lymphatic vessels and reach LNs of interest, and then euthanized by cervical dislocation. Progression of the dye was monitored under a surgical microscope. The different LNs were then harvested separately to compare the blue labelling.

Adoptive transfer of CFSE-labelled Des-RAG T cells

Single-cell suspensions of pooled LN cells from Des-RAG transgenic mice were labelled with CFSE, as previously described [8]. 2×10^6 CFSE-labelled CD8 Des-RAG T cells were injected into the lateral tail vein of recipient mice.

DC culture from bone marrow and immunization protocol

DCs were generated *in vitro* from 178.3 (Ly5.2) and B10.BR (Ly5.2) bone marrow cells as previously described [18]. Briefly, tibiae and femora were flushed with RPMI 1640 supplemented with 10% FCS (Life Technologies, Grand Island, NY), 2 mM L-glutamine and 50 μM 2-mercaptoethanol (ICN Biomedical, Aurora, OH) referred to as T cell medium. Cells were washed twice and 1×10^6 cells cultured in T cell medium with 10 ng/ml GM-CSF and 2.8 ng/ml IL-4 added at day 0 and day 4. At day 7, 0.05 μl/ml of LPS at a concentration of 5 ng/ml was added. DCs were resuspended in RPMI and flow cytometric analysis performed to confirm DC differentiation. They were injected at day 8 into the liver capsule of different lobes as described below. Approximately 20–30% of cultured 178.3 cells and B10.BR cells were CD11c$^+$MHC class II$^+$.

Purification of splenic DC by positive selection using Miltenyi magnetic beads

Spleens were collected into serum-free RPMI, diced finely and digested in RPMI containing 2 mg/ml Collagenase IV (Sigma #C5138) for 40 min with gentle rocking at 37 °C. Digestion was stopped by cold buffer (PBS supplemented with 2.5% FCS and 1 mM EDTA), filtered (40 μm nylon), then centrifuged at 300 *g* for 7 min at 4 °C. The pellet was lysed in red cell lysis buffer, with this reaction stopped by cold buffer, filtered, and again washed before incubation with anti-CD11c magnetic microbeads for 15 min in the fridge (Miltenyi Biotec). Cells were selected on LS columns (Miltenyi Biotec) as per manufacturer's instructions. Cell purity was checked by staining pre- and post-selection with an antibody cocktail of CD11c-PE-Cy7, I-A/I-E-FITC and CD11b-APC. Purified DCs were resuspended in RPMI at 50–100 million cells/ml, and 20 μl was injected into each liver using a Hamilton syringe. In one experiment, DCs were pulsed with 10 μg/ml of the peptide recognized by P25 cells, FQDAYNAAGGHNAVF, for 40 min at 37 °C then washed twice before injection. Approximately 60% of purified cells were CD11c$^+$MHC class II$^+$.

Surgery for dye injection

A transverse laparotomy was performed under general anaesthesia. 1×10^6 cells from the cultured DC suspension were injected into the liver lobes of each mouse. Abdominal closure was performed in two layers. Buprenorphin (0.05 mg/kg 12 hourly for 24 h) was injected intraperitonally for intraoperative (0.1 mg/kg) and postoperative (0.05 mg/kg/12 h) analgesia.

Isolation of liver, spleen, and lymph node lymphocytes for flow cytometry

Mice were euthanized at 24 h or 48 h. LNs were harvested separately as follows: portal, coeliac, first mesenteric, jejunal, colic, and caudal mesenteric (referred to as mesenteric LNs), lumbar aortic and medial iliac (referred to as deep LNs), and axillary and inguinal LNs (referred to as skin LNs). The name "portal", "coeliac", and "first mesenteric" LNs was given to the relevant LN draining the liver; their location is detailed in the Results section. Names in brackets refer to the description published by Van den Broeck *et al.* [13]. Lymphocytes were isolated from LNs, spleens and livers as previously described [8].

DC isolation from spleen and LN for flow cytometry

LNs or spleen were collected in serum-free RPMI containing 2 mg/ml collagenase IV, and diced finely before being digested for 30 min at 37 °C in a shaking incubator. Digestion was stopped with cold buffer (PBS containing 0.1 mM EDTA and 2.5% FCS), washed and filtered before staining with an antibody cocktail of biotinylated-anti-Ly5.2, Ly5.1-V450, anti-H2K^b-FITC, CD11c-PE-Cy7, and CD11b-APC.

Antibodies and flow cytometric analysis

Staining for flow cytometry was carried out as described previously [8]. For cultured DCs, dead cells were excluded by 4′, 6-diamidino-2-phenylindole staining. FITC-conjugated anti-MHC class II monoclonal antibodies and phycoerythrin-Cy7-conjugated anti-CD11c monoclonal antibodies (BD Pharmingen) were used for flow cytometric analysis.

For LN, liver, and splenic lymphocytes, antibodies used for flow cytometric analysis included the clonotypic anti-Des-TCR mAb "Désiré [19] and anti-Ly5.2-biotin, anti-CD8-Pacific Blue, anti-CD44-allophycocyanin-Cy7, and anti-CD69-Peridinin-chlorophyll protein-Cy5.5 (BD Pharmingen). Streptavidin–phycoerythrin–Cy7 and streptavidin–allophycocyanin were also purchased from BD Pharmingen. Flow cytometric acquisition was performed using a FACSCanto II flow cytometer (Becton Dickinson). Analysis was performed using FlowJo software (Tree star Inc) on a Macintosh computer (Apple Computer Inc).

Results

Identification of three LNs adjacent to the liver

To visualize the hepatic lymphatic drainage, the small bowel and the mesenteric axis were placed on the left side of the mouse (B10.BR strain). Three main LNs were observed in close proximity to the porta hepatis (Fig. 1A and B). Two of these LNs were located close to the portal vein: the left one (which will be referred to as the portal LN) was the most superficial/caudal LN, while the right one (which will be referred to as the coeliac LN) was the deeper/cranial LN. The third LN was the jejunal LN most proximal to the portal vein, and belonged to the mesenteric group of LN (this will be referred to as the first mesenteric LN). Similar locations were observed in both C57BL/6 and Balb/c mice (the same background used by Van den Broeck *et al.* in their