Research Article

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Macrophage autophagy limits acute toxic liver injury in mice through down regulation of interleukin-1β

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See Editorial, pages 16–18

Background & Aims: Overactivation of the innate immune response underlies many forms of liver injury including that caused by hepatotoxins. Recent studies have demonstrated that macrophage autophagy regulates innate immunity and resultant tissue inflammation. Although hepatocyte autophagy has been shown to modulate hepatic injury, little is known about the role of autophagy in hepatic macrophages during the inflammatory response to acute toxic liver injury. Our aim therefore was to determine whether macrophage autophagy functions to down regulate hepatic inflammation.

Methods: Mice with a LysM-*CRE*-mediated macrophage knockout of the autophagy gene *ATG5* were examined for their response to toxin-induced liver injury from D-galactosamine/lipopolysaccharide (GalN/LPS).

Results: Knockout mice had increased liver injury from GalN/LPS as determined by significant increases in serum alanine aminotransferase, histological evidence of liver injury, positive terminal deoxynucleotide transferase-mediated deoxyuridine triphosphate nick end-labeling, caspase activation and mortality as compared to littermate controls. Levels of proinflammatory tumor necrosis factor and interleukin (IL)-6 hepatic mRNA and serum protein were unchanged, but serum IL-1β was significantly increased in knockout mice. The increase in serum IL-1β was secondary to elevated hepatic caspase 1 activation and inflammasome-mediated cleavage of pro-IL-1β to its active form. Cultured hepatic macrophages from GalN/LPS-treated knockout mice had similarly increased IL-1β production. Dysregulation of IL-1β was the mechanism of increased liver injury as an IL-1 receptor antagonist prevented injury in knockout mice in concert with decreased neutrophil activation.

Conclusions: Macrophage autophagy functions to limit acute toxin-induced liver injury and death by inhibiting the generation of inflammasome-dependent IL-1β.

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Introduction

Liver injury results not only from the direct effects of injurious agents on hepatocytes, but also from cytokines generated by the accompanying innate immune response [1]. Although resident and recruited macrophages mediate the resolution and repair of liver damage, excessive macrophage activation underlies many forms of hepatocyte injury including that from the toxin alcohol and hepatic steatosis [2–4]. The liver presents unique challenges for the proper control of immune responses as this organ contains the vast majority (80–90%) of the resident macrophages in the body. In addition, the intestinal blood supply delivers gut-derived lipopolysaccharide (LPS) and other bacterial products directly to the liver where they can trigger immune cell activation. LPS induces proinflammatory M1 macrophage polarization and production of tumor necrosis factor (TNF) which is cytotoxic to hepatocytes [5]. Additional proinflammatory cytokines may contribute to liver injury including interleukin (IL)-1β and interferon-γ (IFNγ) [6–9]. The large complement of liver macrophages and their exposure to high LPS levels requires that hepatic immune responses be carefully regulated to prevent excessive and injurious macrophage activation.

Recent studies have demonstrated essential protective functions for hepatocyte macroautophagy (hereafter referred to as autophagy) during liver injury [10–12]. In contrast, little is known about the role of autophagy in macrophages [13]. Autophagy was initially linked to inflammatory responses through the ability of this pathway to sequester and eliminate microbial pathogens [14,15]. Autophagy also regulates macrophage responses in sterile inflammation. Recently we

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Abbreviations: LPS, lipopolysaccharide; TNF, tumor necrosis factor; IL, interleukin; IFNγ, interferon-γ; GalN, D-galactosamine; PBS, phosphate-buffered saline; IL-1Ra, IL-1 receptor antagonist; ALT, alanine aminotransferase; TUNEL, terminal deoxynucleotide transferase-mediated deoxyuridine triphosphate nick end-labeling; qRT-PCR, quantitative real-time reverse transcription polymerase chain reaction; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; PARP, poly (ADP-ribose) polymerase; NOS2, nitric oxide synthetase 2; COX2, cyclooxygenase 2.

demonstrated that in obesity-induced hepatic steatosis impaired macrophage autophagy increases liver inflammation and injury from LPS by promoting proinflammatory M1 macrophage polarization [16]. In non-hepatic inflammatory models autophagy inhibits inflammation by down regulating caspase 1-dependent inflammasome cleavage of pro-IL-1β to its active, secreted form [17,18], suggesting another mechanism by which autophagy may limit hepatic inflammation.

This study examined whether macrophage autophagy regulates acute toxin-induced liver injury from D-galactosamine (GalN) and LPS. GalN/LPS liver injury results from GalN-mediated hepatocyte sensitization to cytotoxicity from LPS-induced cytokines [6,9,19]. In mice with a myeloid cell-specific knockout of the autophagy gene *ATG5*, decreased macrophage autophagy amplified GalN/LPS liver injury. With decreased autophagy there was a heightened inflammatory response restricted to increased generation of IL-1β, IL-18 and IFNγ. Higher levels of IL-1β resulted from inflammasome-mediated caspase 1 cleavage of pro-IL-1β and promoted liver injury. These findings establish a new function for macrophage autophagy in protecting against IL-1β-dependent, acute hepatotoxic injury.

Materials and methods

Animal model

Male mice 10–14 weeks old were maintained under 12 h light/dark cycles with unlimited access to food and water. Wild-type C57BL/6 mice were obtained from The Jackson Laboratory (Bar Harbor, ME). *Atg5$^{f/f}$* mice [20] containing floxed alleles for the autophagy gene *Atg5* were crossed with LysM-*CRE* mice [21] to generate *Atg5^{-Amye}* mice with a myeloid cell-specific knockout of *Atg5*-dependent autophagy [16]. Littermate *Atg5$^{f/f}$* mice lacking the *CRE* transgene served as controls. Liver injury was induced by intraperitoneal injections of 100 μg/kg of LPS (*E. coli* 0111:B4) and 700 mg/kg of GalN (Sigma, St. Louis, MO) dissolved in phosphate-buffered saline (PBS), as previously performed [22]. Some mice were pretreated with PBS vehicle or the IL-1 receptor antagonist (IL-1Ra) anakinra (Amgen, Thousand Oaks, CA) 0.5 h before GalN/LPS administration. All animal studies were approved by the Animal Care and Use Committee of the Albert Einstein College of Medicine and followed the National Institutes of Health guidelines for animal care.

Alanine aminotransferase (ALT) assay

Serum ALTs were measured by commercial kit (TECO Diagnostics, Anaheim, CA).

Histology

Mouse livers were fixed in 10% neutral formalin and stained with hematoxylin and eosin. The percentage of apoptosis/necrosis was graded semi-quantitatively in a blinded fashion by a single pathologist on a sliding scale of: 0, absent; 0.5, minimal; 1, mild; 1.5, mild to moderate; 2, moderate; 2.5, moderate to marked; and 3, marked.

Terminal deoxynucleotide transferase-mediated deoxyuridine triphosphate nick end-labeling (TUNEL) assay

The numbers of TUNEL positive cells were detected in liver sections with the DeadEnd Colorimetric System (Promega, Madison, WI) kit. The numbers of TUNEL positive cells were counted under light microscopy in 10 randomly selected high power fields (400× magnification).

Protein isolation and Western blot

Total mouse liver protein was isolated and Western blot preformed, as previously described [23,24]. Membranes were exposed to antibodies that recognized caspase 3 (Cell Signaling, Beverly, MA; #9665), caspase 7 (Cell Signaling, #9492), poly (ADP-ribose) polymerase (Cell Signaling, #9542), tubulin (Cell Signaling, #2148), caspase 1 (Santa Cruz Biotechnology, Dallas, TX; #SC-514) and IL-1β (R&D Systems, Minneapolis, MN; #AF-401-NA).

Caspase 8 activity

Hepatic caspase 8 activity was measured by commercial kit (BioVision, Milpitas, CA). Reactions were carried out with 200 μg of protein at 37 °C for 2 h and fluorescence measured with 400 nm excitation and 505 nm emission filters.

Immunofluorescence microscopy

Livers were frozen in 2-methylbutane, sectioned, blocked for 1 h in 2% donkey serum, 1% bovine serum albumin (Sigma) and 0.05% Tween 20 (Fisher), and incubated overnight with anti-CD68 (Abd Serotec, Raleigh, NC; #MCA1957GA) or anti-Ly6G (Biolegend, San Diego, CA; #127602) antibody at 4 °C. The tissues were then washed with PBS and incubated for 1 h with Cy3-conjugated secondary antibody (Jackson ImmunoResearch, West Grove, PA; #111-165-152). After washing with PBS, tissues were mounted in anti-fading medium containing 4′,6-diamidino-2-phenylindole (Life Technologies, Carlsbad, CA) and visualized by fluorescence microscopy.

Quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR)

Liver and macrophage RNA were isolated with the RNeasy Plus kit (QIAGEN, Valencia, CA). Reverse transcription with 1 μg of RNA was carried out in an Eppendorf Mastercycler (Hamburg, Germany) using a high capacity cDNA reverse transcription kit (ABI, Foster City, CA). qRT-PCR was performed in a 7500 Fast Real-Time PCR System (ABI). The primers in Supplementary Table 1 were purchased from Integrated DNA Technologies (Coralville, IA). Data analysis was performed using the $2^{-\Delta\Delta CT}$ method for relative quantification and normalized to levels of glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

Cytokine assay

Serum and medium cytokines were measured with a multiplex kit (MCYTOMAG-70K) from EMD Millipore (Billerica, MA) and Luminex® xMAP® Technology with the exception of IL-18 which was measured by a standard ELISA (Roche, Basal, Switzerland).

Hepatic macrophage isolation and culture

Mouse liver nonparenchymal cells were isolated by Liberase (Roche) perfusion and centrifuged at 50 g to isolate and remove the hepatocytes. Hepatic macrophages were isolated from the nonparenchymal cell population by differential centrifugation through a 29% Nycodenz (Accurate Chemical & Scientific Corp., Westbury, NY) gradient at 1380 g. Cells were washed with DMEM (Hyclone, Logan, UT) and repelleted at 1380 g. The resuspended cells were plated in DMEM, 10% fetal bovine serum (Atlanta Biological, Atlanta, GA), 2% hepes (Sigma), 1% penicillin/streptomycin (Mediatech, Manassas, VA), 1% non-essential amino acids (Sigma) and 1% sodium pyruvate (Lonza, Walkersville, MD). Non-adherent cells were removed after 1 h by changing the medium to RPMI-1640 (Hyclone) with the same supplements. Some cells were treated with 10 ng/ml of LPS and 5 mM ATP (Sigma) at the time of the medium change, and medium and cells harvested 1 h later. Other cells were left untreated for 18 h prior to harvesting medium and cells. Purity of the macrophage population was insured by mRNA enrichment for macrophage genes and absence of expression for hepatocyte and hepatic stellate cell genes by RT-PCR.

In additional studies hepatic macrophages were separated into Kupffer cells and recruited macrophages by FACS. The nonparenchymal cell fraction was blocked by CD16/32 antibody (eBioscience, #14-0161-82), and then incubated with the following primary antibodies from eBioscience: CD45-FITC (#11-0451-82), CD45-PerCP-Cyanine5.5 (#45-0451-80), Ly6G-PerCP-Cyanine5.5 (#45-5931-80), CD3-PerCP-Cyanine5.5 (#45-0031-80), NK1.1-PerCP-Cyanine5.5 (#45-5941-80), B220-PerCP-Cyanine5.5 (#45-0452-80), F4/80-APC (#17-4801-82), and CD11b-PE (#12-0112-82). Kupffer cells were defined as $CD45^+$ $Ly6G^-$ $NK1.1^-$ $B220^-$ $F4/80^{high}$ $CD11b^{low}$ and infiltrating monocyte-derived macrophages as $CD45^+$ $Ly6G^-$ $NK1.1^-$ $B220^-$ $F4/80^{low}$ $CD11b^{high}$. The FACS sorting was performed using a BD FACS Aria Cell Sorter (BD Biosciences, San Jose, CA). Cells were cultured as above, and the medium assayed for IL-1β 1 h after the medium change.

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