Toxicology and Applied Pharmacology 304 (2016) 110–120

Contents lists available at ScienceDirect

Toxicology and Applied Pharmacology

journal homepage: www.elsevier.com/locate/ytaap

The spleen as an extramedullary source of inflammatory cells responding to acetaminophen-induced liver injury

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ARTICLE INFO

Article history:
Received 26 February 2016
Revised 25 April 2016
Accepted 27 April 2016
Available online 7 May 2016

Keywords:
Acetaminophen
Hepatotoxicity
Macrophages
Spleen
Myeloid derived suppressor cells

ABSTRACT

Macrophages have been shown to play a role in acetaminophen (APAP)-induced hepatotoxicity, contributing to both pro- and anti-inflammatory processes. In these studies, we analyzed the role of the spleen as an extramedullary source of hepatic macrophages. APAP administration (300 mg/kg, i.p.) to control mice resulted in an increase in $CD11b^+$ infiltrating $Ly6G^+$ granulocytic and $Ly6G^-$ monocytic cells in the spleen and the liver. The majority of the $Ly6G^+$ cells were also positive for the monocyte/macrophage activation marker, Ly6C, suggesting a myeloid derived suppressor cell (MDSC) phenotype. By comparison, $Ly6G^-$ cells consisted of 3 subpopulations expressing high, intermediate, and low levels of Ly6C. Splenectomy was associated with increases in mature ($F4/80^+$) and immature ($F4/80^-$) pro-inflammatory $Ly6C^{hi}$ macrophages and mature anti-inflammatory ($Ly6C^{lo}$) macrophages in the liver after APAP; increases in MDSCs were also noted in the livers of splenectomized (SPX) mice after APAP. This was associated with increases in APAP-induced expression of chemokine receptors regulating pro-inflammatory (CCR2) and anti-inflammatory (CX3CR1) macrophage trafficking. In contrast, APAP-induced increases in pro-inflammatory galectin-3^+ macrophages were blunted in livers of SPX mice relative to control mice, along with hepatic expression of TNF-α, as well as the anti-inflammatory macrophage markers, FIZZ-1 and YM-1. These data demonstrate that multiple subpopulations of pro- and anti-inflammatory cells respond to APAP-induced injury, and that these cells originate from distinct hematopoietic reservoirs.

1. Introduction

Acetaminophen (APAP) is a widely used over the counter analgesic, considered safe and effective at therapeutic doses. However, when ingested in excess, APAP causes centrilobular hepatic necrosis, which can lead to acute liver failure. APAP hepatotoxicity is initiated by covalent binding of *N-acetyl-p-benzoquinoneimine* (NAPQI), a highly reactive APAP metabolite generated via cytochrome P450 (Cyp), to liver proteins (Nelson and Bruschi, 2003; Jan et al., 2014). This leads to oxidative stress and the production of pro-inflammatory/cytotoxic mediators, which are thought to be important in the pathogenesis of tissue injury. Evidence suggests that macrophages contribute to APAP hepatotoxicity (reviewed in Laskin, 2009; Laskin et al., 2011). However, the role of these cells in the pathogenic process depends on their phenotype and timing of appearance in the liver. Thus, while initially, classically activated macrophages accumulate in the liver and release pro-inflammatory/cytotoxic mediators that promote hepatotoxicity, subsequently, alternatively activated macrophages appear in the liver and release mediators that suppress inflammation and initiate wound repair. It appears that the outcome of the response to APAP depends, in part, on relative numbers of these different macrophage subpopulations in the liver and their levels of activation (Dambach et al., 2002; Ju et al., 2002; Holt et al., 2008; Dragomir et al., 2012a, 2012b; Gardner et al., 2012; You et al., 2013; Zigmond et al., 2014).

The bone marrow is traditionally considered the major source of inflammatory macrophages. However, studies have shown that the spleen can function as a reservoir of inflammatory monocytes (Swirski et al., 2009; Robbins et al., 2012). Following tissue injury, splenic monocytes enter the circulation migrating to inflammatory sites, where they differentiate into macrophages, participating in both pro- and anti-inflammatory responses (Nahrendorf et al., 2007; Swirski

Abbreviations: APAP, acetaminophen; NAPQI, *N*-acetyl-*p*-benzoquinoneimine; Cyp, cytochrome P450; Gal, galectin; SPX, splenectomized; MDSC, myeloid derived suppressor cell; PBS, phosphate buffered saline; PE, phycoerythrin; AF, AlexaFluor; TNFα, tumor necrosis factor alpha; IL, interleukin; ALT, alanine aminotransferase; AST, aspartate aminotransferase.

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http://dx.doi.org/10.1016/j.taap.2016.04.019
0041-008X/

et al., 2009; Hiroyoshi et al., 2012; Robbins et al., 2012). In this context, a key role of splenic monocytes has been demonstrated in post ischemic brain injury, atherosclerosis, myocardial infarction, and septic peritonitis (Nahrendorf et al., 2007; Swirski et al., 2009; Bao et al., 2010; Kono et al., 2012; Robbins et al., 2012; Kim et al., 2014; Liu et al., 2015).

In the present studies, we analyzed the role of the spleen as an extramedullary source of monocytes responding to APAP-induced liver injury. Following APAP administration to mice, we observed an increase in both pro- ($Ly6C^{hi}$) and anti- ($Ly6C^{lo}$) inflammatory monocytes in the spleen. Splenectomy resulted in an increase in $Ly6C^{hi}$ pro-inflammatory macrophages in the liver, but a decrease in galectin (Gal)-3^{+} pro-inflammatory macrophages, demonstrating the distinct origin of these cells. Increases in cells exhibiting a myeloid derived suppressor cell (MDSC) phenotype were also observed in livers of splenectomized (SPX) mice after APAP. MDSCs consist of a heterogeneous population of immature myeloid cells with immunosuppressive and anti-inflammatory properties (Gabrilovich and Nagaraj, 2009). They have been reported to expand and down regulate hepatic immune responses to infections, acute and chronic inflammation, and fibrosis (Hammerich and Tacke, 2015), and we speculate that they contribute to protecting against APAP-induced hepatotoxicity.

2. Materials and methods

2.1. Animals and treatments

SPX and sham operated (control) C57BL/6 male mice (8–10 wk old) were purchased from The Jackson Laboratories (Bar Harbor, ME). Mice were housed in microisolation cages in a pathogen-free animal facility at Rutgers University. Food and water were provided *ad libitum*. Experimental procedures were approved by the Institutional Animal Care and Use Committee. All mice were fasted overnight prior to intraperitoneal (i.p.) injection of APAP (300 mg/kg) or phosphate buffered saline (PBS); mice were euthanized 24–96 h later with pentobarbital (200 mg/kg, i.p.). Blood was collected from the inferior vena cava for determination of serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) using diagnostic assay kits (Thermo Fisher Scientific, Waltham, MA).

2.2. Histology and immunohistochemistry

Livers were removed and 5 mm sections from the left lateral lobes immediately fixed in 3% paraformaldehyde/2% sucrose solution overnight at 4 °C. Tissues were washed 3 times in PBS/2% sucrose solution for 10 min and stored in 50% ethanol at room temperature. After embedding in paraffin, tissues sections (5 μm) were prepared, stained with hemotoxylin and eosin and examined by light microscopy. Images were acquired using a VS120 Virtual Microscopy System (Olympus, Center Valley, PA). The extent of inflammatory and structural changes in the liver were assessed blindly. Semiquantitative grades (0 to 3) were assigned to the tissues, with Grade 0 = no damage, Grade 1 = mild damage (<2 foci of necrosis), Grade 2 = moderate damage (more widespread necrotic foci, containing infiltrated inflammatory cells), and Grade 3 = severe damage (centrilobular to midzonal necrosis). A minimum of 3 mice per treatment group were analyzed. For immunohistochemistry, sections were rehydrated and incubated with 3% hydrogen peroxide to quench endogenous peroxidase. This was followed by incubation with 20% normal rabbit serum to block non-specific binding and staining with anti-Gal-3 antibody (1:25,000; R&D Systems, Minneapolis, MN) or IgG control (ProSci, Poway, CA). Antibody binding was visualized using a Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA). Slides were washed and incubated with diaminobenzidine (peroxidase substrate), and then counterstained with hematoxylin (Invitrogen, Carlsbad, CA).

2.3. Hepatic leukocyte isolation

Livers were perfused with ice cold PBS through the portal vein, removed and disaggregated through 70 μm nylon mesh cell strainers (Fisher Scientific, Pittsburgh, PA). The resultant cell suspension was layered onto a 2-step (40%/70% in PBS) discontinuous Percoll gradient (GE Healthcare Biosciences Corp, Piscataway, NJ) and centrifuged at 720 x g for 30 min at 24 °C. Hepatic leukocytes were collected at the interface and washed; number and viability were assessed using a hemocytometer with trypan blue dye exclusion.

2.4. Preparation of spleen cells

Spleens were removed, placed in culture plates and teased apart using a 1 ml syringe in ice cold PBS. Cells were then centrifuged at 450 × *g* for 6 min at 4 °C. Spleen cells were washed twice using PBS and then resuspended in buffer consisting of PBS containing 2% FCS and 0.02% sodium azide.

2.5. Flow cytometry

Cells were incubated with anti-mouse CD16/32 (Fc receptor block, clone 93; Biolegend, San Diego, CA) and then with FITC-conjugated anti-mouse CD11b (clone M1/70; Biolegend), PE-conjugated anti-mouse Ly6C (clone HK1.4; Biolegend), PE/Cy7-conjugated anti-mouse *F*4/80 (clone BM8; Biolegend), and AF 647-conjugated anti-mouse Ly6G (clone 1A8; Biolegend) antibodies or appropriate isotypic controls for 30 min at 4 °C. This was followed by incubation with eFluor 780-conjugated fixable viability dye (eBioscience, San Diego, CA). Cells were analyzed on a Beckman Coulter Gallios flow cytometer (Brea, CA); data were analyzed using Kaluza version 1.2 software. Macrophages were separated from contaminating endothelial cells on the basis of forward and side scatter, and on expression of CD11b. Viable $CD11b^{+}$ macrophages were then analyzed for expression of Ly6G. This was followed by analysis of Ly6C and then F4/80 (Fig. 1).

2.6. Real-time PCR

Liver samples (100 mg) were stored at −20 °C in RNAlater (Sigma-Aldrich, St Louis, MO) until RNA isolation. Total cellular RNA was extracted from the samples using RNeasy Mini Kit (Qiagen, Valencia, CA). RNA purity and concentration were measured using a Nanodrop spectrophotometer (Thermo Fisher Scientific, Wilmington, DE). RNA was converted to cDNA using a High Capacity cDNA Reverse Transcription kit according to the manufacturer's instructions (Applied Biosystems, Foster City, CA). cDNA was normalized to GAPDH. Standard curves were generated using serial dilutions from pooled randomly selected cDNA samples. Real-time PCR was performed using SYBR Green PCR Master Mix (Applied Biosystems) on a 7300 thermocycler (Applied Biosystems). All PCR primers were synthesized by Integrated DNA Technologies (Coralville, IA). Gene expression changes were normalized to 18S RNA. Data are expressed as fold change relative to control. Forward and reverse primer sequences were: tumor necrosis factor-alpha (TNF-α), 5′-AGGGATGAGAAGTTCCCAAATG-3′ and 5′-TGTGAGGGTCTGGGCCATA-3′; FIZZ-1, 5′-CAGCTGATGGTCCCAGTGAA-3′ and 5′-TTCCTTGACCTTATTCTCCACGAT-3′; YM-1, 5′-TCTGGTGAAGGAATGCGTAAA-3′ and 5′-GCAGCCTTGGAATGTCTTTCTC-3′; CX3CR1, 5′-TCGGTCTGGTGGGAAATCTG-3′ and 3′-GGCTTCCGGCTGTTGGT-5′; CCL-2, 5′-TTGAATGTGAAGTTGACCCGTAA-3′ and 3′-GCTTGAGGTTGTGGAAAAG-5′; CCR2, 5′-TCCACGGCATACTATCAACATCTC-3′ and 5′-GGCCCCTTCATCAAGCTCTT-3′; and 18S RNA, 5′-CGGCTACCACATCCAAGGAA-3′ and 5′-GCTGGAATTACCGCGGCT-3′.