Published in final edited form as:

Toxicol Appl Pharmacol. 2016 August 01; 304: 110–120. doi:10.1016/j.taap.2016.04.019.

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

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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.

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Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.taap.2016.04.019>.

Conflict of interest

The authors declare no conflicts of interest.

Transparency document

The transparency document associated with this article can be found in online version.

Keywords

Acetaminophen; Hepatotoxicity; Macrophages; Spleen; Myeloid derived suppressor cells

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 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 hematoxylin 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 $720 \times g$ 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 $450 \times g$ 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 F4/80 (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 RNeasy lysis buffer (Qiagen, Valencia, CA) 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- α (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'-GCTTGA GGTGTGGAAAAG-5'; CCR2, 5'-TCCACGGCATACTATCAACATCTC-3' and 5'-GGCCCCCTTCATCAAGCTCTT-3'; and 18S RNA, 5'-CGGCTACCACATCCAAGGAA-3' and 5'-GCTGGAATTACCGCGGCT-3'.

2.7. Measurement of hepatic Cyp activity

To prepare microsomes, liver samples (1 g) were homogenized at 4 °C in 2 volumes (w/v) of 10 mM Tris-base (pH 7.4) containing 1.5% KCl using a Teflon-glass homogenizer. Homogenates were centrifuged at 1000 ×g (10 min, 4 °C), supernatants collected and centrifuged at 12,000 ×g (20 min, 4 °C) to remove cellular debris, and then at 105,000 ×g (1.5 h, 4 °C). Microsomes were resuspended in homogenization buffer containing 0.5 mM phenylmethanesulfonylfluoride and centrifuged at 105,000 ×g (90 min, 4 °C). Pellets were resuspended in 0.25 M sucrose containing 10 mM Tris-base (pH 7.4) and stored at –80 °C until analysis (Cooper et al., 1993). Cyp2e1 was measured by the formation of *p*-nitrocatechol from *p*-nitrophenol (Koop, 1986). Microsomes were incubated with *p*-nitrophenol (200 μM) and NADPH (500 μM) at 37 °C for 30 min, followed by the addition of trichloroacetic acid (20%, w/v) to stop the reaction. Microsomes were then centrifuged (10,000 ×g, 5 min, 4 °C), supernatants collected and mixed with 2 M NaOH. Changes in absorbance were measured spectrophotometrically at 535 nm. Concentrations of *p*-nitrocatechol in the samples were calculated based on a standard curve generated using authentic product. Cyp1a2 and Cyp3a activities were measured as previously described (McLaughlin et al., 2008; Gardner et al., 2012) with some modifications. Briefly, liver microsomes were incubated with 0.1 M potassium phosphate buffer (pH 7.4) containing 1 mM MgCl₂, 0.1 mM EDTA, 0.5 mM NADPH and 5 μM 7-methoxyresorufin for Cyp1a2 or 1 μM dibenzylfluorescein for Cyp3a. Relative fluorescence units were recorded over a 10 min interval at an excitation wavelength of 530 nm and an emission wavelength of 590 nm for methoxyresorufin, and 485 nm and 530 nm for dibenzylfluorescein on a Spectramax M5 fluorescent plate reader (Molecular Devices, Sunnyvale, CA). Rates of product formation were calculated using SoftMaxPro5 software. The concentrations of the reaction products were calculated based on a standard curve generated using authentic resorufin or fluorescein. Each determination was repeated in triplicate for all animals. To assess Cyp isoform specificity, enzyme activities were measured after the addition of 1 μM of the Cyp3a inhibitor, ketoconazole (Sai et al., 2000; McLaughlin et al., 2008).

2.8. Statistical analysis

Data were analyzed using GraphPad Prism 5 (La Jolla, CA). Multiple group comparisons were performed using one-way ANOVA followed by post-hoc Tukey's test or Student's *t*-test. A *p*-value of <0.05 was considered statistically significant.

3. Results

3.1. Infiltration of myeloid cells into the spleen following APAP intoxication

Treatment of mice with APAP resulted in a time related increase in the number of cells in the spleen, which peaked at 72 h, with no change in cell viability (Table 1 and not shown). To characterize these cells, we used techniques in flow cytometry. For these studies, we focused on infiltrating myeloid cells, which were identified based on forward and side scatter, and on expression of the β2 integrin, CD11b. These cells were found to consist of granulocytic (Ly6G⁺) and monocytic (Ly6G[–]) subpopulations; the majority (>90%) were granulocytic (Fig. 2A, E). Following APAP administration, a transient increase in granulocytic cells was observed in the spleen at 24 h (Fig. 2A). By comparison, a persistent

increase in monocytic cells was noted, beginning 24 h after APAP, and remaining elevated for at least 96 h (Fig. 2E). To further characterize these cells, we analyzed their expression of the monocyte/macrophage activation marker, Ly6C and the mature monocyte/macrophage antigen, F4/80 (Mitchell et al., 2014). Fig. 2B shows that 95–99% of the Ly6G⁺ cells expressed high levels of Ly6C, suggestive of a MDSC phenotype (Gabrilovich and Nagaraj, 2009; Hammerich and Tacke, 2015). Most of these cells (>90%) were granulocytic (F4/80⁻) MDSCs (G-MDSCs), with a smaller percentage of monocytic (F4/80⁺) MDSCs (M-MDSCs) (Fig. 2C, D). Whereas G-MDSCs peaked 24 h post APAP, maximum numbers of M-MDSCs were observed at 48 h (Fig. 2C, D). Ly6G⁻ monocytic spleen cells were also analyzed for expression of Ly6C and F4/80. Three subpopulations were identified that expressed high (pro-inflammatory monocytes), low (anti-inflammatory monocytes) and intermediate (transitional monocytes) levels of Ly6C (Supplemental Fig. 1). The majority (>90%) of these cells were F4/80⁻ (not shown), consistent with an immature phenotype (Shi and Pamer, 2011; Lauvau et al., 2014). A significant increase in all three monocytic subpopulations was observed in the spleen after APAP administration (Fig. 2F–H). While pro-inflammatory monocytes increased rapidly after APAP (within 24 h) and remained elevated, anti-inflammatory monocytes increased more gradually for at least 96 h; increases in transitional monocytes were transient peaking 48–72 h post APAP.

3.2. Effects of splenectomy on inflammatory cell accumulation in the liver

In earlier work we showed that both pro- and anti-inflammatory macrophages accumulate in the liver after APAP intoxication (Laskin and Pilaro, 1986; Dambach et al., 2002; Dragomir et al., 2012a; Gardner et al., 2012). To analyze the contribution of the spleen to APAP-induced liver inflammation, we used SPX mice. Treatment of control mice with APAP resulted in increased numbers of leukocytes in the liver, a response observed at 24 h and 48 h (Table 1). Greater numbers of leukocytes were observed in livers of SPX mice at all times after APAP. These cells also persisted in the liver for at least 96 h (Table 1). In both control and SPX mice, approximately 50% of hepatic leukocytes were CD11b⁺, suggesting that they were derived from infiltrating myeloid cells (Fig. 3A). As observed in the spleen, hepatic myeloid cells consisted of monocytic (Ly6G⁻) and granulocytic (Ly6G⁺) subpopulations (Fig. 3B–C). Additionally, three subpopulations of monocytic cells were identified based on expression of Ly6C, pro-inflammatory macrophages (Ly6C^{hi}), anti-inflammatory macrophages (Ly6C^{lo}) and transitional macrophages (Ly6C^{int}) (Fig. 4). Whereas the majority of the pro-inflammatory macrophages were mature, expressing high levels of F4/80, most anti-inflammatory macrophages were immature F4/80⁻ (Fig. 4A–F). Transitional macrophages consisted of both mature and immature subpopulations (Fig. 4C, D). Treatment of control mice with APAP resulted in a rapid (within 24 h) and persistent (up to 96 h) increase in mature pro-inflammatory and mature transitional macrophages in the liver (Fig. 4A, C). In contrast, although mature anti-inflammatory macrophages also increased, this was only observed at 96 h (Fig. 4C). Immature pro-inflammatory and immature transitional macrophages increased in the liver after APAP in a pattern that was generally similar to mature pro-inflammatory and transitional macrophages (Fig. 4B, F). Conversely, APAP had no significant effect on immature anti-inflammatory macrophages (Fig. 4D). Splenectomy resulted in increased numbers of both mature and immature pro-inflammatory and transitional macrophages accumulating in the liver in response to APAP

(Fig. 4A, B, E, F). As observed in control mice, APAP had no effect on numbers of immature anti-inflammatory macrophages in the livers of SPX mice, however, an increase in mature anti-inflammatory macrophages was observed at 72 h (Fig. 4C, D).

We also analyzed the effects of APAP on infiltrating Ly6G⁺ granulocytic cells in the liver. As observed in the spleen, the majority of these cells (>99%) were also positive for Ly6C, suggestive of a MDSC phenotype (Fig. 3D). Additionally, they consisted of F4/80⁺ monocytic (M-MDSC) and F4/80⁻ granulocytic (G-MDSC) subpopulations (Fig. 3E, F). In both control and SPX mice, APAP administration resulted in a significant increase in these subpopulations at 24 h and 48 h. Greater numbers of G-MDSCs were noted in SPX mice, relative to control mice, at 24 h and 72 h post APAP and of M-MDSCs at 72 h (Fig. 3E, F).

3.3. Effects of splenectomy on expression of inflammatory markers in the liver

In our next series of studies, we analyzed the effects of splenectomy on hepatic expression of markers of pro- and anti-inflammatory macrophages and chemokines/chemokine receptors mediating their trafficking. Gal-3 is a β -galactoside binding lectin expressed on mature pro-inflammatory macrophages (Liu et al., 1995). Consistent with our earlier studies (Dragomir et al., 2012a, 2012b), following APAP administration, we observed a time related increase in Gal-3⁺ macrophages in centrilobular regions of the liver which peaked at 72 h–96 h (Fig. 5). Splenectomy resulted in a decrease in APAP-induced Gal-3⁺ macrophage accumulation in the liver, a response most notable at 96 h. APAP administration also resulted in a persistent increase (24–72 h) in mRNA expression of TNF- α , a prototypical marker of pro-inflammatory macrophages (Fig. 6). CCL2 and CCR2, which are known to be important in pro-inflammatory macrophage migration to the liver (Zimmermann et al., 2012), also increased after APAP. Whereas increases in CCL2 were rapid (within 24 h), persisting for 72 h, increases in CCR2 expression were delayed for 48 h, but sustained for at least 96 h. APAP-induced increases in TNF- α expression were reduced in SPX mice at 48 h and 72 h (Fig. 6). In contrast, although there were no major effects of splenectomy on CCL2 expression 24–72 h post-APAP, at 96 h, CCL2 expression increased. In SPX mice, increases in CCR2 were more rapid, when compared to control mice, beginning within 24 h of APAP administration, and were significantly greater at 48 h and 72 h (Fig. 6). FIZZ-1 and YM-1 are markers of anti-inflammatory M2 macrophages (Raes et al., 2002). Consistent with the appearance of CD11b⁺Ly6G⁻Ly6C^{lo} anti-inflammatory macrophages in the liver, both of these markers were up-regulated after APAP (Fig. 6). While FIZZ-1 remained elevated for at least 96 h, at this time YM-1 was at control levels. Splenectomy attenuated the effects of APAP on FIZZ-1 and YM-1 expression. Conversely, APAP-induced increases in the M2 macrophage chemokine receptor, CX3CR1 (Karlmark et al., 2010) were augmented in SPX mice at 24 h and 96 h post treatment (Fig. 6).

3.4. Effects of splenectomy on APAP-induced hepatotoxicity and metabolism

Treatment of control mice with APAP resulted in a time related increase in serum transaminases and histological evidence of hepatotoxicity (Table 2 and Fig. 7). Splenectomy was associated with reduced hepatotoxicity, as measured by decreases in serum transaminases and more limited centrilobular hepatic necrosis. Additionally, APAP-induced mortality was reduced from 8% in control mice to 0% in SPX mice. To exclude the

possibility that differences in the sensitivity of control and SPX mice to APAP were due to alterations in its metabolism to the cytotoxic product NAPQI, we measured the activity of hepatic Cyps important in this response including Cyp2e1, Cyp3a and Cyp1a2. We found that splenectomy had no significant effect on the activity of these enzymes (Table 3).

4. Discussion

Macrophages have been implicated in the pathogenesis of APAP-induced hepatotoxicity, playing roles in both promoting inflammation and tissue injury, and in down regulating inflammation and inducing wound repair (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). Evidence suggests that these divergent activities are mediated by distinct macrophage subpopulations that appear sequentially in the liver following APAP intoxication (reviewed in Laskin, 2009; Laskin et al., 2011). The present studies demonstrate that some populations of pro- and anti-inflammatory macrophages that accumulate in the liver after APAP intoxication originate in the spleen. Findings that hepatotoxicity is reduced in SPX mice suggest that the contribution of the spleen is predominantly pro-inflammatory/cytotoxic cells and/or mediators that regulate the activity of these cells.

The spleen has been shown to function as a reservoir for inflammatory monocytes, which increase in number in response to tissue injury or infection (Swirski et al., 2009; Bao et al., 2010; Robbins et al., 2012; Kim et al., 2014; Liu et al., 2015). Consistent with these findings, we observed increased numbers of inflammatory monocytes in the spleen following APAP administration, which expressed high, low and intermediate levels of Ly6C, a characteristic of pro-inflammatory, anti-inflammatory and transitional monocytes and macrophages, respectively (Robbins and Swirski, 2010; Zimmermann et al., 2012; Mitchell et al., 2014). Our findings that each of these monocyte subpopulations increased after APAP support the idea that the spleen functions as a reservoir for these cells. Transitional monocytes (Ly6C^{int}) that appear in the spleen following APAP administration most likely represent cells at an intermediate stage between pro-inflammatory and anti-inflammatory monocyte subpopulations (Lin et al., 2009). The origin of the infiltrating monocytes in the spleen after APAP is unknown. Cell tracking experiments have demonstrated that bone marrow-derived monocytes circulate through the spleen before accumulating in atherosclerotic lesions (Nahrendorf et al., 2007; Swirski et al., 2009; Robbins et al., 2012). It remains to be determined if this also occurs in the liver in response to APAP-induced injury.

Following APAP administration, increased numbers of pro-inflammatory (Ly6C^{hi}) and anti-inflammatory (Ly6C^{lo}) macrophages were observed in the liver, which is generally similar to previous reports (Dragomir et al., 2012a; Zigmond et al., 2014). Our observation that the majority of pro-inflammatory macrophages were mature F4/80⁺ cells, while anti-inflammatory macrophages were mainly immature F4/80⁻, suggests a potentially greater contribution of macrophages to tissue injury relative to repair. We also noted increased numbers of Ly6C^{int} macrophages in the liver after APAP. The role of these cells in the pathogenesis of APAP hepatotoxicity is unknown. Evidence suggests that they are in transition from a pro-inflammatory to an anti-inflammatory phenotype, expressing intermediate levels of pro- and anti-inflammatory mediators (Gordon and Taylor, 2005;

Lauvau et al., 2014). Further studies are required to determine if these cells play a similar transitional role in the liver after APAP intoxication and contribute to tissue repair. Splenectomy was associated with a heightened response of Ly6C^{hi} pro-inflammatory macrophages to APAP. This may be due to a compensatory increase in the release of these cells from the bone marrow in SPX mice. This is supported by our findings that expression of CCR2, a chemokine receptor important in directing pro-inflammatory monocytes and macrophages to sites of injury from the bone marrow (Zimmermann et al., 2012) increased in livers of SPX mice 24–72 h after APAP.

In contrast to Ly6C^{hi} pro-inflammatory macrophages, Gal-3⁺ pro-inflammatory macrophages were decreased in the livers of SPX mice, relative to control mice, a response most notable 96 h post APAP, suggesting that these cells are derived in part, from the spleen. In earlier studies, we demonstrated that Gal-3 plays a role in APAP-induced hepatotoxicity by promoting pro-inflammatory activation of liver macrophages (Dragomir et al., 2012a, 2012b). The present studies show that decreases in Gal-3⁺ macrophages in livers of SPX mice had little impact on APAP-induced hepatotoxicity. This is most likely a consequence of the increased numbers of Ly6C^{hi} pro-inflammatory macrophages in the livers of APAP treated mice. Billiar et al. (1988) previously showed that splenectomy is associated with reduced sensitivity of liver macrophages to lipopolysaccharide, which was due to the absence of spleen-derived activating cytokines. It is possible that pro-inflammatory macrophages accumulating in the livers of SPX mice after APAP produce lower amounts of pro-inflammatory/cytotoxic mediators, when compared to cells in control mice, which may contribute to reduced hepatotoxicity in these animals. Our findings that TNF- α expression is reduced in SPX mice, when compared to control mice, are consistent with this idea. Recent studies have shown that Ly6C^{hi} macrophages that accumulate in the liver after APAP transition into Ly6C^{lo} macrophages, and that these cells are involved in early resolution of tissue injury (Zigmond et al., 2014). Increased recruitment of Ly6C^{hi} macrophages to the livers of SPX mice may provide a precursor pool of anti-inflammatory/wound repair macrophages, which contribute to decreased hepatotoxicity in these animals. This is supported by our findings of increased numbers of mature anti-inflammatory macrophages and mature and immature transitional macrophages in the livers of SPX mice after APAP administration.

MDSCs consist of a heterogeneous population of myeloid progenitor cells that play a role in suppressing inflammatory reactions in response to infections and injury (Gabrilovich and Nagaraj, 2009; Hammerich and Tacke, 2015). They have also been reported to reside largely in the spleen and to play a role in post-injury wound healing (reviewed in Gabrilovich and Nagaraj, 2009; Cuenca et al., 2011). During homeostasis, immature myeloid cells, originating from hematopoietic stem cells, mature into dendritic cells, macrophages, and neutrophils. Under pathological conditions, however, inflammatory mediators induce the expansion of immature myeloid cells, which develop into MDSCs with a monocytic or granulocytic phenotype (reviewed in Gabrilovich and Nagaraj, 2009; Cuenca et al., 2011; Hammerich and Tacke, 2015). MDSCs suppress inflammatory responses via secretion of immunosuppressive cytokines such as transforming growth factor- β and interleukin (IL)-10 and by inducing apoptosis (Terabe et al., 2003; Nagaraj et al., 2010; Sinha et al., 2011). Moreover, cross-talk between MDSCs and macrophages results in increased production of

IL-10 and decreased production of IL-12 and IL-6 (Ostrand-Rosenberg et al., 2012). Following APAP administration, we identified two MDSC subpopulations in the liver and the spleen displaying a monocytic or a granulocytic phenotype. Greater numbers of these cells were observed in livers of SPX mice 24 h and 72 h after APAP. This may be due to a compensatory increase in release of these cells from the bone marrow to limit tissue injury.

Splenectomy was also associated with alterations in expression of markers of pro-inflammatory M1 (TNF- α) and anti-inflammatory M2 (FIZZ-1 and YM-1) macrophages. Interestingly, each of these was down-regulated in the livers of SPX mice, relative to control mice. Decreases in TNF- α may be explained by reduced numbers of Gal-3⁺ macrophages in the liver, which are known to generate pro-inflammatory cytokines (Dragomir et al., 2012a). The fact that FIZZ-1 and YM-1 were reduced, despite increases in Ly6C^{lo} anti-inflammatory and Ly6C^{int} transitional macrophages in the liver, suggests that multiple subpopulations of anti-inflammatory macrophages respond to APAP, and that their origins are distinct. Consistent with increases in pro-inflammatory and anti-inflammatory cells in the liver after splenectomy is our finding that chemokine receptors, CCR2 and CX3CR1, which play roles in trafficking of pro- and anti-inflammatory macrophages, respectively, to sites of injury (Zimmermann et al., 2012), were also increased. CX3CR1 is highly expressed on anti-inflammatory/wound repair macrophages and it has been shown to play a protective role in models of hepatitis and in carbon-tetrachloride-induced hepatic fibrosis (Aoyama et al., 2010; Karlmark et al., 2010; Inui et al., 2011). We speculate that it plays a similar protective role in APAP-induced hepatotoxicity. The chemokine CCR2 is expressed on pro-inflammatory macrophages (Zimmermann et al., 2012). We found that APAP-induced up-regulation of CCR2 expression in control mice was correlated with increases in both pro-inflammatory Ly6C^{hi} macrophages and Gal-3⁺ macrophages in the liver. However, while splenectomy caused a coordinate increase in APAP-induced CCR2 expression and numbers of Ly6C^{hi} macrophages in the liver, Gal-3⁺ macrophage accumulation was reduced. These data indicate that distinct chemokine receptors mediate the accumulation of pro-inflammatory macrophage subpopulations into the liver. In this regard, earlier studies have demonstrated a key role of CXCL8, CCR8, and CXCL5 in pro-inflammatory macrophage migration into the liver in response to hepatotoxic doses of carbon tetrachloride, thioacetamide and APAP (Heymann et al., 2012; Amanzada et al., 2014; Zigmond et al., 2014). In contrast to the effects of splenectomy on APAP-induced expression of CCR2, expression of the CCR2 ligand, CCL2, was generally unaltered. These data provide additional support for the notion that multiple pathways are involved in regulating inflammatory cell trafficking into the liver in response to APAP-induced liver injury.

In conclusion, the present studies identified multiple myeloid subpopulations in the spleen and the liver following APAP intoxication. We also demonstrate a novel role of the spleen as an extramedullary source of macrophages, monocytes and MDSCs responding to APAP. These findings are consistent with previous reports that the spleen is a reservoir of inflammatory cells in models of myocardial infarction, atherosclerosis, and spinal cord injury (Swirski et al., 2009; Bao et al., 2010; Robbins et al., 2012; Kim et al., 2014; Liu et al., 2015). Inhibition of monocyte release from the spleen may represent a novel approach to altering hepatic inflammation, a key step in mitigating APAP-induced hepatotoxicity.

Supplementary Material

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Acknowledgments

This work was supported by the National Institutes of Health grants GM034310, ES004738, AR055073, ES007148, and ES005022.

Abbreviations

APAP	acetaminophen
NAPQI	<i>N</i> -acetyl- <i>p</i> -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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Fig. 1.

Flow chart of gating strategy for spleen and liver myeloid cells. Viable myeloid cells staining positive for CD11b were analyzed for the expression of Ly6G. Ly6G⁺ and Ly6G⁻ cells were then analyzed for expression of Ly6C, followed by F4/80. MDSC, myeloid derived suppressor cells, M-MDSC, monocytic-MDSC, and G-MDSC, granulocytic-MDSC.

Fig. 2.

Characterization of infiltrating myeloid cells in the spleen following APAP intoxication. Spleen cells, collected 24–96 h after treatment of control (CTL) mice with APAP or PBS, were preincubated with anti-mouse CD16/32 followed by antibodies to CD11b, Ly6C, Ly6G, and F4/80. Cells were then incubated with fixable viability dye and analyzed by flow cytometry as described in the Materials and methods. Bars are the mean $\pm$ SE ($n = 6-8$). ^aSignificantly different ($p < 0.05$) from PBS. Panel A, infiltrating granulocytic cells (CD11b⁺Ly6G⁺); Panel B, myeloid derived suppressor cells (MDSC,

CD11b⁺Ly6G⁺Ly6C⁺); Panel C, granulocytic (G)-MDSC (CD11b⁺Ly6G⁺Ly6C⁺F4/80⁻); Panel D, monocytic (M)-MDSC (CD11b⁺Ly6G⁺Ly6C⁺F4/80⁺); Panel E, infiltrating monocytic cells (CD11b⁺Ly6G⁻); Panel F, infiltrating proinflammatory monocytes (CD11b⁺Ly6G⁻Ly6C^{hi}); Panel G, infiltrating anti-inflammatory monocytes (CD11b⁺Ly6G⁻Ly6C^{lo}); Panel H, infiltrating transitional monocytes (CD11b⁺Ly6G⁻Ly6C^{int}).

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Fig. 3.

Characterization of infiltrating myeloid cells in liver following APAP intoxication. Hepatic leukocytes, collected 24–96 h after treatment of control (CTL) and splenectomized (SPX) mice with APAP or PBS, were preincubated with anti-mouse CD16/32 followed by antibodies to CD11b, Ly6C, Ly6G, and F4/80. Cells were then incubated with fixable viability dye and analyzed by flow cytometry as described in the Materials and methods. Bars are the mean $\pm$ SE ($n = 6-8$). ^aSignificantly different ($p < 0.05$) from PBS. ^bSignificantly different ($p < 0.05$) from control mice. Panel A, infiltrating myeloid cells (CD11b⁺); Panel B, infiltrating monocytic cells (CD11b⁺Ly6G⁻); Panel C, infiltrating granulocytic cells (CD11b⁺Ly6G⁺); Panel D, myeloid derived suppressor cells (MDSC, CD11b⁺Ly6G⁺Ly6C⁺); Panel E, monocytic (M)-MDSC (CD11b⁺Ly6G⁺Ly6C⁺F4/80⁺); Panel F, granulocytic (G)-MDSC (CD11b⁺Ly6G⁺Ly6C⁺F4/80⁻).

Fig. 4.

Effects of APAP on macrophage subpopulations accumulating in the liver. Hepatic leukocytes, collected 24–96 h after treatment of control (CTL) and splenectomized (SPX) mice with APAP or PBS, were preincubated with anti-mouse CD16/32 followed by antibodies to CD11b, Ly6C, Ly6G, and F4/80. Cells were then incubated with fixable viability dye and analyzed by flow cytometry as described in the Materials and methods. Bars are the mean $\pm$ SE ($n = 6-8$). ^aSignificantly different ($p < 0.05$) from PBS. ^bSignificantly different ($p < 0.05$) from control mice. Panel A, mature infiltrating proinflammatory macrophages (CD11b⁺Ly6G⁺Ly6C^{hi}F4/80⁺); Panel B, immature infiltrating proinflammatory macrophages (CD11b⁺Ly6G⁺Ly6C^{hi}F4/80⁺); Panel C, mature infiltrating antiinflammatory macrophages (CD11b⁺Ly6G⁺Ly6C^{lo}F4/80⁺); Panel D, immature infiltrating antiinflammatory macrophages (CD11b⁺Ly6G⁺Ly6C^{lo}F4/80⁺); Panel E, mature

infiltrating transitional macrophages (CD11b⁺Ly6G⁻Ly6C^{int}F4/80⁺); Panel F, immature infiltrating transitional macrophages (CD11b⁺Ly6G⁻Ly6C^{int}F4/80⁻).

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Fig. 5. Effects of APAP on Gal-3 expression in the liver. Livers were collected 24–96 h after treatment of control (CTL) and splenectomized (SPX) mice with APAP or PBS. Gal-3 expression was analyzed by immunohistochemistry as described in the Materials and methods. Representative sections from 6 to 8 mice/treatment group are shown. Original magnification $\times 100$.

Fig. 6.

Effects of APAP on hepatic expression of markers of classical and alternative macrophage activation. mRNA, prepared from liver samples collected 24–96 h after treatment of control (CTL) and splenectomized (SPX) mice with APAP or PBS was analyzed by RT-PCR. Data were normalized to 18S RNA and presented as fold change relative to PBS. Bars are the mean $\pm$ SE ($n = 3-8$). ^aSignificantly different ($p < 0.05$) from PBS. ^bSignificantly different ($p < 0.05$) from control mice.

Fig. 7. Effects of APAP on liver histology. Livers were collected 24–96 h after treatment of control (CTL) and splenectomized (SPX) mice with APAP or PBS. Sections were stained with hematoxylin and eosin. Representative sections from 6 to 8 mice/treatment group are shown. Original magnification $\times 100$.

Table 1

Effects of acetaminophen on numbers of spleen cells and hepatic leukocytes.

Time post APAP	Spleen cells ($\times 10^5$)	Hepatic leukocytes ($\times 10^5$)	
	Control	Control	SPX
PBS	383.2 $\pm$ 39.1	29.5 $\pm$ 6.2	27.4 $\pm$ 10.1
24 h	530.4 $\pm$ 23.2 ^a	75.8 $\pm$ 8.3 ^a	111.1 $\pm$ 19.3 ^a
48 h	630.1 $\pm$ 37.6 ^a	59.2 $\pm$ 5.2 ^a	87.6 $\pm$ 6.4 ^{a,b}
72 h	690.8 $\pm$ 42.7 ^a	33.3 $\pm$ 7.3	73.7 $\pm$ 13.7 ^{a,b}
96 h	535.3 $\pm$ 27.3 ^a	39.9 $\pm$ 12.1	53.2 $\pm$ 9.3 ^a

Cells were collected from the spleen and/or the liver 24–96 h after treatment of control and splenectomized (SPX) mice with APAP or PBS. Viable cells were enumerated using a hemocytometer with trypan blue dye exclusion. Data are the mean $\pm$ SE (n = 5–11 mice).

^aSignificantly different ($p < 0.05$) from PBS.

^bSignificantly different from control hepatic leukocytes.

Table 2

Effects of APAP on serum transaminases.

Time post APAP	ALT (U/L)	
	Control	SPX
PBS	89.7 ± 60.2	78.6 ± 26.4
24 h	12,172.2 ± 1899.4 ^a	9066.4 ± 847.6 ^{a,b}
48 h	1228.8 ± 234.7	848.2 ± 177.9
72 h	225.7 ± 31.2	157.6 ± 15.5 ^b
96 h	91.5 ± 9.0	135.1 ± 37.1
	AST (U/L)	
	Control	SPX
PBS	117.4 ± 42.0	120.1 ± 19.1
24 h	10,718.2 ± 1930.3 ^a	8201.4 ± 994.3 ^a
48 h	654.6 ± 168.6	317.1 ± 60.1 ^b
72 h	257.0 ± 39.2	243.5 ± 41.2
96 h	111.8 ± 17.1	126.6 ± 26.3

Serum was collected 24–96 h after treatment of control and splenectomized (SPX) mice with APAP or PBS and analyzed for ALT and AST. Data are the mean ± SE (n = 5–11 mice).

^aSignificantly different ($p < 0.05$) from PBS.

^bSignificantly different from control.

Table 3

Effects of splenectomy on Cyp450 activity in mouse liver microsomes.

Enzyme	Control	SPX
Cyp2e1	1.2 ± 0.3	1.4 ± 0.1
Cyp1a2	100.0 ± 19.9	114.1 ± 14.9
Cyp3a	15.3 ± 1.1	15.2 ± 0.9
Cyp3a + ketoconazole	2.5 ± 0.1 ^a	1.6 ± 0.2 ^a

Liver microsomes, prepared from control and splenectomized (SPX) mice, were assayed for Cyp2e1, Cyp1a2 and Cyp3a activities as described in the Materials and methods. In some experiments 1 µM ketoconazole was added to the assay mixtures to confirm enzyme specificity. Data are presented as pmol product/min/mg protein. Each value is the mean ± SEM of triplicate determinations from 4 mice.

^aSignificantly different ($p < 0.05$) from enzyme assays in the absence of ketoconazole.