Role of Galectin-3 in Classical and Alternative Macrophage Activation in the Liver following Acetaminophen Intoxication

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*J Immunol* published online 21 November 2012
http://www.jimmunol.org/content/early/2012/11/21/jimmunol.1201851

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Inflammatory macrophages have been implicated in hepatotoxicity induced by the analgesic acetaminophen (APAP). In these studies, we characterized the phenotype of macrophages accumulating in the liver following APAP intoxication and evaluated the role of galectin-3 (Gal-3) in macrophage activation. Administration of APAP (300 mg/kg, i.p.) to wild-type mice resulted in the appearance of two distinct subpopulations of CD11b⁺ cells in the liver, which expressed high or low levels of the monocyte/macrophage activation marker Ly6C. Whereas CD11b⁺/Ly6C嗨 macrophages exhibited a classically activated proinflammatory phenotype characterized by increased expression of TNF-α, inducible NO synthase, and CCR2, CD11b⁺/Ly6Cمواد macrophages were alternatively activated, expressing high levels of the anti-inflammatory cytokine IL-10. APAP intoxication was also associated with an accumulation of Gal-3⁺ macrophages in the liver; the majority of these cells were Ly6C嗨. APAP-induced increases in CD11b⁺/Ly6Cمواد macrophages were significantly reduced in Gal-3⁻/⁻ mice. This reduction was evident 72 h post APAP and was correlated with decreased expression of the classical macrophage activation markers, inducible NO synthase, IL-12, and TNF-α, as well as the proinflammatory chemokines CCL2 and CCL3, and chemokine receptors CCR1 and CCR2. Conversely, numbers of CD11b⁺/Ly6Cمواد macrophages increased in livers of APAP-treated Gal-3⁻/⁻ mice; this was associated with increased expression of the alternative macrophage activation markers Ym1 and Fizz1, increased liver repair, and reduced hepatotoxicity. These data demonstrate that both classically and alternatively activated macrophages accumulate in the liver following APAP intoxication; moreover, Gal-3 plays a role in promoting a persistent proinflammatory macrophage phenotype.

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Liver injury caused by overdose of the analgesic acetaminophen (APAP) is the major cause of acute liver failure in the United States (1). APAP intoxication is characterized by centrilobular hepatocellular necrosis, which is initiated by covalent binding of the reactive APAP metabolite, N-acetyl-para-benzoquinoneimine, to critical protein targets in the liver (2). Evidence suggests that activated macrophages contribute to the pathogenic response to APAP. However, the role of these cells in APAP hepatotoxicity depends on their origin, the timing of their appearance in the liver, and the inflammatory mediators they encounter, which control their phenotype and function. On the basis of studies using macrophage inhibitors and transgenic mice, two subpopulations of macrophages that play distinct roles in hepatotoxicity have been identified in the liver after APAP intoxication: classically activated proinflammatory/cytotoxic macrophages and alternatively activated anti-inflammatory/wound repair macrophages (3–7). It appears that the outcome of tissue injury depends on which macrophage subpopulation predominates. Thus, hepatotoxicity results from exaggerated or persistent responses of classically activated macrophages, whereas hepatoprotection is associated with increases in numbers of alternatively activated macrophages (reviewed in Refs. 8, 9). The mechanisms regulating classical and alternative macrophage activation in the liver after APAP intoxication have not been established.

Galactin-3 (Gal-3) is a β-galactoside-binding lectin secreted by macrophages in response to LPS, TNF-α, or IFN-γ (10, 11). Gal-3 acts in an autocrine and paracrine manner to promote macrophage release of proinflammatory mediators, including TNF-α, IL-12, CCL3, and CCL4, as well as reactive nitrogen species generated via inducible NO synthase (iNOS) (10–13). Loss of Gal-3 has been reported to result in reduced susceptibility to Ag-induced arthritis, renal ischemia–reperfusion injury, hypoxic–ischemic brain injury, and Con A–induced hepatotoxicity, pathological conditions associated with exaggerated proinflammatory mediator activity (14–17). These findings led us to hypothesize that Gal-3 plays a role in promoting classical macrophage activation and inflammatory mediator production in the liver following APAP intoxication. This idea is supported by our findings of reduced hepatotoxicity and inflammatory mediator production in response to APAP in mice lacking Gal-3 (18). In the present studies, we extended these observations and characterized the role of Gal-3 in regulating the phenotype of hepatic macrophage subpopulations accumulating in the liver during APAP-induced hepatotoxicity. Results from these studies provide additional support for the contribution of Gal-3 to promoting inflammation in the liver following APAP intoxication.

Materials and Methods

Animals
Male specific pathogen-free C57BL/6J wild-type and Gal-3⁻/⁻ mice (8–12 wk old) were obtained from the Jackson Laboratory (Bar Harbor, ME). Gal-3⁻/⁻ mice were backcrossed to a C57BL/6 background for more than 10 generations. Mice were housed in microisolation cages and allowed free access to food and water. All animals received humane care in compliance with the
institution’s guidelines, as outlined in the Guide for the Care and Use of Laboratory Animals, published by the National Institutes of Health. Mice were fasted overnight prior to i.p. administration of APAP (300 mg/kg) or PBS control. Mice were euthanized 24–72 h later with Nembutal (200 mg/kg). Liver samples (100-mg aliquots) were collected and stored at –20°C in RNA later (InVitrogen, Grand Island, NY) until RNA isolation. The remaining tissue was snap frozen in liquid nitrogen.

Hepatic nonparenchymal cell isolation

Nonparenchymal cells were isolated from the liver as previously described, with some modifications (19). The liver was perfused through the portal vein with warm Ca2+/Mg2+-free HBSS (pH 7.3) containing 25 mM HEPES and 0.5 mM EGTA, followed by Leibowitz L-15 medium containing HEPES, 0.2 U/ml Liberase Blendzyme, and 0.5 mg/ml protease type XIV. The liver was excised, disaggregated, and incubated with 2 mg/ml protease type XIV for 15 min at 37°C. The resulting cell suspension was filtered through a 220-μm nylon mesh. Hepatocytes were separated from nonparenchymal cells by four successive washes (50× g, 3 min). Supernatants containing nonparenchymal cells were centrifuged (300× g, 7 min), and the cells purified by density gradient centrifugation using OptiPrep Medium (Sigma-Aldrich, St Louis, MO). Viability was assessed by trypan blue dye exclusion and was >95%.

Flow cytometry/cell sorting

Cells were analyzed immediately following isolation. Nonspecific binding was blocked by incubation of the cells with anti-mouse–FcRII/III Ab (BD Biosciences, Franklin Lakes, NJ) for 5 min at 4°C. This was followed by 30 min incubation with FITC-conjugated anti-CD11b and PE-conjugated anti-Ly6C Abs or isotype controls (BioLegend, San Diego, CA). Cells were then fixed in 3% paraformaldehyde; permeabilized in buffer containing 0.1% saponin, 0.1% sodium azide, and 1% FBS in PBS; and stained with anti–Gal-3 Ab or goat IgG (R&D Systems, Minneapolis, MN), followed by isotype-specific Alexa Fluor 633–conjugated secondary Ab (Molecular Probes, Carlsbad, CA). Cells were analyzed using an FC500 flow cytometer (Beckman Coulter, Brea, CA). For sorting, cells were incubated with anti-mouse–FcrRII/III Ab, followed by FITC-conjugated anti-CD45, Alexa Fluor 647–conjugated CD11b, and PE-conjugated anti-Ly6C (BioLegend) for 30 min. DAPI was added to the cell suspension immediately before analysis to exclude dead cells. Cells were sorted into DAPI /CD45+/CD11b*/Ly6C– and DAPI /CD45+/CD11b*/Ly6C+ subpopulations, using a Beckman Coulter MoFlo XDP Cell Sorter, and immediately processed for RNA isolation.

Histology and immunohistochemistry

Livers were collected and 5-mm samples of the left lobes immediately fixed overnight at 4°C in 3% paraformaldehyde/2% sucrose. Tissue was washed three times in PBS containing 2% sucrose, and then transferred to 50% ethanol. After embedding in paraffin, 5-μm sections were prepared. For immunohistochemistry, sections were rehydrated and stained with Ab to proliferating cell nuclear Ag (PCNA) (1:800; Abcam, Cambridge, MA), Ym1 (1:450; StemCell, Vancouver, BC, Canada), or IgG control (ProSci, Poway, CA). Binding was visualized using a VECTASTAIN Elite ABC Kit (Vector Laboratories, Burlingame, CA). Three to five random sections of each liver were examined.

Immunofluorescence

Liver samples (5 mm) were snap frozen in liquid nitrogen–cooled isopentane and embedded in OCT medium (Sakura Finetek, Torrance, CA). Then 6-μm sections were prepared and fixed in 90% acetone/10% methanol. For double immunofluorescence, a sequential staining procedure was used (20). Sections were stained with anti-Ly6C Ab (1:50; AbD Serotec, Kidlington, U.K.), followed by isotype-specific Alexa Fluor 488–conjugated secondary Ab (Molecular Probes, Carlsbad, CA). After blocking with 5% rat serum, sections were stained with FITC-conjugated anti-F4/80 Ab (Molecular Probes, Carlsbad, CA). Three to five random sections of each liver were examined.

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Ab (1:50; AbD Serotec, Kidlington, U.K.), followed by anti-FITC Alexa Fluor 488-conjugated secondary Ab. Images were acquired using a Leica TCS SP5 confocal microscope (Leica Microsystems, Wetzlar, Germany). Identical laser power, gain, and offset settings were used for all analyses.

**Western blotting**

Liver samples (30 mg) were lysed in buffer containing 20 mM HEPES, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1.5 mM MgCl2, 1 mM diethylene-triamine pentaacetic acid, 1 mM phenylmethylsulfonyl fluoride, 10 mM sodium pyrophosphate, 50 mM sodium fluoride, 2 mM sodium orthovanadate, and protease inhibitor mixture. Protein concentrations were measured using the Bradford Assay (Bio-Rad, Hercules, CA). Proteins were separated on Tris-glycine polyacrylamide gels (Bio-Rad) and transferred onto nitrocellulose membranes. Nonspecific binding was blocked by incubation of the blots for 1 h at room temperature with buffer containing 5% nonfat milk, 100 mM Tris-base, 200 mM sodium chloride, and 0.1% polysorbate 20. Membranes were then incubated overnight at 4°C with anti-Gal-3 (1:2000) or anti-actin (1:1000) primary Abs, followed by incubation with isotype-specific HRP-conjugated secondary Ab (1:10,000) for 1 h at room temperature. Binding was visualized using an ECL Plus chemiluminescence kit (GE Healthcare, Piscataway, NJ).

**Real-time PCR**

Total RNA was isolated from liver samples using an RNeasy Mini kit, and from sorted monocytes/macrophages using an RNeasy Micro kit (Qiagen, Valencia, CA). RNA purity and concentration were measured using a Nanodrop spectrophotometer (Thermo Fisher Scientific, Wilmington, DE). RNA was converted into cDNA using a High Capacity cDNA Reverse Transcription kit according to the manufacturer’s directions (Applied Biosystems, Foster City, CA). 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 7900HT thermocycler (Applied Biosystems). All PCR primer pairs were generated using Primer Express 2.0 (Applied Biosystems) and synthesized by Integrated DNA Technologies (Corvalle, IA). For each sample, gene expression changes were normalized relative to 18S RNA. Data are expressed as fold change relative to control. Forward and reverse primer sequences were as follows: TNF-α, 5′-AGGAGTGAAGGTTTCCAAGATTCT-3′ and 5′-TGGTGGCCTGGGACCCT-3′; iNOS, 5′-GCCGACCCAGGTTTCTTGGTCT-3′ and 5′-GCCTGGACTCTCTTGT-3′; IL-12, 5′-CCTGAGGACTTCCCCACATCTC-3′ and 5′-TGGCCTGAATTTGCAAAC-3′; CCL2, 5′-GGCTGATGATTGATGACCTTAC-3′ and 5′-GCTGGAATTACCGCGGCT-3′; CX3CR1, 5′-CACGGGAGGTGTAAGAGAAACAG-3′ and 5′-TGTGAGTTCGAGTGGATA-3′; CXCR3, 5′-TGGTGAATTTGAAAG-3′ and 5′-CCGGCCCTTCATCAA-3′; Ym1, 5′-GGAATCAGGCCATTATC-3′ and 5′-GCTGGAATTACCGCGGCT-3′; 18S RNA, 5′-TGAATGCGTGAATATATATGATA-3′ and 5′-TGGATGGGATGGAAGATAGG-3′; Gal-3, 5′-CTGAAGTTGTAGGCACAGGTTGTTG-3′ and 5′-CCGGCGAAGAGCTTTGT-3′; found in inflammatory monocytic cells, 5′-TGTGAGGGTCTGGGCCATA-3′ and 5′-GGCCCCTTCATCAA-3′; CCL3, 5′-TCTTTTCACGCCCAATGGA-3′ and 5′-TCCGGCTGTAGGAGAAGCA-3′; CCR1, 5′-GCACAGGATGCAGGGCTTAC-3′ and 5′-GTCAGAGATGGCCAGGTTGAG-3′; CD11b, 5′-CGGACGCCAAGAGCTTTGT-3′ and 5′-GGGCCCCTTCATCAA-3′; CCR2, 5′-GCCGACCCAGGTTTCTTGGTCT-3′ and 5′-TGGCCTGAATTTGCAAAC-3′; Ly6C, 5′-TGAAGCGTTTCGGGATCTG-3′ and 5′-GGCCTGTGAGACCTTTG-3′; CCR5, 5′-CCGGGAGGTGTAAGAGAAACAG-3′ and 5′-TGTGAGTTCGAGTGGATA-3′; and 5′-TCCGGCTGTAGGAGAAGCA-3′.

**Statistical analysis**

Experiments were repeated two or three times. Data were analyzed using the Student t test or one-way ANOVA followed by Dunn’s post hoc analysis. A p ≤ 0.05 was considered statistically significant.

**Results**

Distinct macrophage subpopulations accumulate in the liver following APAP intoxication

In our initial series of studies, we used techniques in flow cytometry/cell sorting to assess the phenotype of macrophages accumulating in the liver after APAP intoxication. CD11b is the α-chain of the Mac-1 integrin expressed on myeloid cells (21). APAP administration resulted in a time-related increase in CD11b+ cells in the liver (Fig. 1A, 1D). This was evident within 24 h and persisted for at least 72 h after APAP administration. To

![FIGURE 2. Effects of APAP on liver macrophage expression of Ly6C and F4/80. Liver sections, prepared 24–72 h after treatment of wild-type mice with APAP or PBS control (CTL), were sequentially stained with anti-Ly6C and anti-F4/80 Abs, as described in Materials and Methods. Scale bars, 25 μm. One representative section from three or four mice is shown.](http://www.jimmunol.org/doi/abs/10.4049/jimmunol.1700283)
GAL-3 REGULATES MACROPHAGE ACTIVATION AFTER APAP INTOXICATION

As the chemokines CCL2 and CCL3, and the chemokine receptors CCR1 and CCR2, effects most prominent after 72 h (Figs. 5, 6). Delayed CCL4 expression was also noted. Conversely, increases in expression of CX3CL1/CX3CR1 were observed in Gal-3−/− mice relative to wild-type mice at 24 h post APAP administration; however, by 72 h, CX3CR1 expression was reduced in these mice (Fig. 6). APAP-induced expression of CCR5 was not altered by loss of Gal-3 (Fig. 6). The mRNA expression of Ym1 and Fizz-1, markers of alternatively activated anti-inflammatory/wound repair macrophages (23), was also increased in Gal-3−/− mice, when compared with wild-type mice, after APAP administration (Figs. 5, 7). Interestingly, whereas in control mice Ym1 protein was expressed in hepatic sinusoidal endothelial cells, after APAP administration Ym1 was upregulated in macrophages (Fig. 7). This shift was first evident 48 h post APAP and was correlated with decreased Ym1 expression in endothelial cells. Loss of Gal-3 was associated with a more rapid and abundant increase in Ym1 macrophages in the liver, which was apparent within 24 h and remained elevated for at least 72 h. In contrast, expression of the anti-inflammatory lectin Gal-1, which increased in wild-type mice 48 h and 72 h after APAP administration, was not significantly altered by loss of Gal-3 (Fig. 5). Alterations in liver macrophage subpopulations and inflammatory mediator expression in Gal-3−/− mice were also associated with increased repair of APAP-induced injury. Thus, significant increases in hepatocyte proliferation, as measured by the number of PCNA-positive cells and mitotic index, were observed in the livers of Gal-3−/− mice when compared with wild-type mice (Figs. 8, 9).
Discussion
Classically and alternatively activated macrophage subpopulations have been shown to play distinct roles in the pathogenesis of APAP-induced hepatotoxicity. Thus, whereas classically activated macrophages release cytotoxic/proinflammatory mediators that contribute to injury, alternatively activated macrophages downregulate inflammation and promote tissue repair (reviewed in Ref. 8). However, mechanisms regulating phenotypic activation of macrophages in the liver have not been established. The present studies demonstrate that Gal-3 functions in promoting persistent activation of proinflammatory/cytotoxic macrophages in the liver following APAP intoxication. These findings are important, as they suggest a novel mechanism for classical macrophage activation during the pathogenesis of APAP-induced hepatotoxicity.

Evidence suggests that classically and alternatively activated macrophages accumulating in the liver after APAP-induced injury arise from distinct precursors (4–7, 24). The present studies provide additional support for this concept. We confirmed that APAP intoxication results in a time-related increase in CD11b+ macrophages in the liver, which are distinct from resident Kupffer cells (6). In addition, we demonstrated that these CD11b+ cells are heterogeneous with respect to their expression levels of the macrophage activation marker Ly6C. Thus, two subpopulations were identified: cells expressing high levels of Ly6C and cells expressing low levels of Ly6C. Moreover, these CD11b+ subpopulations are functionally distinct. Whereas CD11b+/Ly6C hi macrophages exhibited a classically activated proinflammatory phenotype, characterized by increased mRNA expression of TNF-α, iNOS, and CCR2, CD11b+/Ly6C lo macrophages expressed increased mRNA levels of the anti-inflammatory cytokine IL-10, indicating that they are alternatively activated. Both CD11b+/Ly6C hi and CD11b+/Ly6C lo macrophage subpopulations were found to increase in number in the liver following APAP intoxication; the response of Ly6C hi macrophages was more robust than that of Ly6C lo macrophages. Accumulating Ly6C hi monocytes/macrophages have been reported to contribute to the development of tissue injury and inflammation in the liver induced by high-fat diet and carbon tetrachloride, as well as acute pancreatitis and myocardial infarction (25–28), and we speculate that they play a similar pathogenic role in APAP-induced hepatotoxicity.

APAP-induced increases in CD11b+/Ly6C hi macrophages in the liver were also associated with the appearance of Ym1-positive alternatively activated macrophages in the tissue. Hepatic expression of the anti-inflammatory lectin Gal-1, which is thought to promote alternative macrophage activation (29–31), also increased after APAP administration, suggesting a mechanism mediating their appearance in the liver. In contrast, APAP had no major effect on expression of Fizz-1, another marker of alternative macrophage activation (23). These data support the concept of subpopulation heterogeneity in alternatively activated macrophages (32). Depletion of alternatively activated macrophages with clodronate liposomes is associated with a reduction in APAP-induced IL-10 expression in the liver and exaggerated hepatotoxicity (4). These data, along with reports that IL-10 plays a protective role in the liver following APAP intoxication (33, 34), suggest that IL-10–producing CD11b+/Ly6C lo cells are key to tissue repair in this model. This idea is supported by our findings that in Gal-3+/− mice, increases in CD11b+/Ly6C lo cells were correlated with accelerated tissue repair, as measured by hepatocyte proliferation and reduced hepatotoxicity (18).

Following APAP intoxication we also observed a time-related increase in Gal-3+ macrophages in the liver, which is in agreement with our previous findings (18). The present studies show that these cells express high levels of Ly6C, indicating a proin-

FIGURE 5. Effects of APAP intoxication on expression of markers of classical and alternative macrophage activation. mRNA was prepared from liver samples 24–72 h after treatment of wild-type and Gal-3−/− mice with APAP or PBS control (CTL), and analyzed by RT-PCR. Data were normalized to 18S RNA and presented as fold change relative to control. Each bar represents the mean ± SE (n = 3–8 mice). *Significantly different (p < 0.05) from CTL. **Significantly different (p < 0.05) from wild-type mice.
flammatory phenotype. In accord with this idea is our observation that in the absence of Gal-3 the number of Ly6C\textsuperscript{hi} macrophages appearing in the liver following APAP administration was significantly decreased, and that expression of the classical macrophage activation markers, iNOS, TNF-\(\alpha\), and IL-12, and the proinflammatory chemokines and chemokine receptors, CCL2, CCL3, CCR1, and CCR2, was reduced. The fact that CD11b+/Ly6C\textsuperscript{lo} anti-inflammatory/wound repair macrophages increased in livers of APAP-treated Gal-3\textsuperscript{-/-} mice, and that this was correlated with a more rapid appearance of Ym1-positive macrophages in the tissue, as well as a marked increase in Fizz-1 expression, suggest a shift in the balance of macrophage subpopulations, leading to accelerated tissue repair. Analogous increases in alternatively activated macrophages and hepatoprotection have been described in Gal-3\textsuperscript{-/-} mice treated with Con A (17). Of note is our observation that, in control mice, Gal-3 is expressed at low levels by CD11b+/Ly6C\textsuperscript{lo} cells, supporting previous reports that some liver resident macrophage subpopulations express this protein (18, 35). Expression of Gal-3 on resident Kupffer cells is consistent with findings that these cells are constitutively activated owing to continuous exposure to LPS in the portal circulation (36).

We also noted more rapid increases in peak expression of CX3CR1 and its ligand CX3CL1 in the livers of Gal-3\textsuperscript{-/-} mice treated with APAP. CX3CR1 is highly expressed on anti-inflammatory/wound repair monocytes and macrophages (22), whereas CX3CL1 is produced primarily by hepatocytes and stellate cells following liver injury (37, 38). CX3CL1 and CX3CR1 have been shown to play a protective role in toxin A–induced enteritis and carbon tetrachloride–induced liver inflammation and fibrosis, effects thought to be due to the recruitment of alternatively activated anti-inflammatory macrophages to sites of infection or injury (37, 39). It remains to be determined if the CX3CL1/CX3CR1 signaling pathway is involved in hepatic recruitment of anti-inflammatory/wound repair macrophages following APAP intoxication.

A question arises with regard to the mechanisms underlying the appearance of proinflammatory macrophages in the liver following APAP intoxication. Our findings that peak expression of key proinflammatory mediators, including TNF-\(\alpha\) and IL-12, and chemokines like CCL2 and CCL4, precedes maximal increases in Ly6C\textsuperscript{hi}/Gal-3\textsuperscript{+} macrophages in the liver suggest that the proinflammatory hepatic microenvironment promotes the development of classically activated macrophages. However, at present we cannot exclude the possibility that macrophages are recruited to the liver in response to Gal-3 or other chemokines, or danger-associated molecular patterns, such as high mobility group box-1, released from APAP-injured hepatocytes (40–42). It appears that once localized in the liver, Gal-3\textsuperscript{+} macrophages play a role in

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**FIGURE 6.** Effects of APAP intoxication on expression of chemokines and chemokine receptors. mRNA was prepared from liver samples collected 24–72 h after treatment of wild-type and Gal-3\textsuperscript{-/-} mice with APAP or PBS control (CTL), and analyzed by RT-PCR. Data were normalized to 18S RNA and presented as fold change relative to PBS control. Each bar represents the mean ± SE (\(n = 3–8\) mice). *Significantly different (\(p < 0.05\)) from CTL. **Significantly different (\(p < 0.05\)) from wild-type mice.
maintaining the proinflammatory/cytotoxic microenvironment. In this regard, Gal-3 has been shown to upregulate expression of TNF-α, iNOS, and IL-12 in primary microglia and human monocytes (11, 13). Moreover, reduced susceptibility to injury induced by streptozotocin, Con A, or Ag-induced arthritis in Gal-3−/− mice is associated with decreased expression of these inflammatory proteins (14, 17, 43).

In contrast to our findings, Gal-3 has previously been reported to induce alternative activation in cultured macrophages (44). Differences between these results and our findings may be due to analysis of bone marrow–derived macrophages activated in vitro with defined concentrations of cytokines versus freshly isolated liver macrophages from APAP-injured mice. This difference may be important, as Gal-3 expression and its intracellular distribution have been shown to be altered when primary macrophages are cultured in vitro (45).

In summary, the present studies identify and characterize multiple macrophage subpopulations accumulating in the liver following APAP intoxication, and demonstrate a role for Gal-3 in promoting persistent classical macrophage activation, which contributes to hepatotoxicity. A more detailed understanding of the mechanisms regulating the phenotype of activated macrophages during APAP-induced liver injury may lead to the development of novel approaches to mitigating toxicity caused by this widely used analgesic.

Disclosures
The authors have no financial conflicts of interest.
References


