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# Fatty acid-binding protein 5 limits the anti-inflammatory response in murine macrophages

Sherri M. Moore*, Vivian V. Holt, Lillie R. Malpass, Ian N. Hines, Michael D. Wheeler

*Department of Nutrition Science, College of Human Ecology, East Carolina University, Greenville, NC 27858, United States*

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ABSTRACT

The beginning stages of liver damage induced by various etiologies (i.e. high fat diet, alcohol consumption, toxin exposure) are characterized by abnormal accumulation of lipid in liver. Alterations in intracellular lipid transport, storage, and metabolism accompanied by cellular insult within the liver play an important role in the pathogenesis of liver disease, often involving a sustained inflammatory response. The intracellular lipid transporter, fatty acid binding protein 5 ($FABP_5$), is highly expressed in macrophages and may play an important role in the hepatic inflammatory response after endotoxin exposure in mice. This study tested the hypothesis that $FABP_5$ regulates macrophage response to LPS in male C57bl/6 (wild type) and $FABP_5$ knockout mice, both *in vitro* and *in vivo*. Treatment with LPS revealed that loss of $FABP_5$ enhances the number of hepatic $F4/80^+$ macrophages in the liver despite limited liver injury. Conversely, $FABP_5$ knock out mice display higher mRNA levels of anti-inflammatory cytokines IL-10, arginase, YM-1, and Fizz-1 in liver compared to wild type mice. Bone marrow derived macrophages stimulated with inflammatory (LPS and IFN-γ) or anti-inflammatory (IL-4) mediators also showed significantly higher expression of anti-inflammatory/regulatory factors. These findings reveal a regulatory role of $FABP_5$ in the acute inflammatory response to LPS-induced liver injury, which is consistent with the principle finding that $FABP_5$ is a regulator of macrophage phenotype. Specifically, these findings demonstrate that loss of $FABP_5$ promotes a more anti-inflammatory response.

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## 1. Introduction

Altered lipid composition in the liver may affect the biological activity of macrophages in acute hepatic inflammation. Fatty acid-binding proteins (FABPs), central regulators of both metabolic and inflammatory pathways, may be important targets for understanding the immune response during liver disease (Furuhashi et al., 2007; Maeda et al., 2005). While Adipose FABP ($FABP_4$, FABP-A) and Epidermal FABP ($FABP_5$, FABP-E) are the only known isoforms co-expressed in the macrophage, we report here that macrophage expression of $FABP_5$ limits the anti-inflammatory response in a model of hepatic inflammation and in macrophage polarization studies, *in vitro*.

In the past decade, several studies have uncovered diverse macrophage phenotypes. The once thought of homogeneous population is now known to exhibit an array of functional phenotypes categorized into two broad categories of polarized macrophages: M1, or classically activated, and M2, or alternatively activated (Martinez et al., 2008). The pro-inflammatory (i.e. M1) response is largely activated by interferon gamma (IFN-γ) and/or lipopolysaccharide (LPS) and characterized by an increase in pro-inflammatory cytokines such as tumor necrosis factor alpha (TNF-α), interleukin 12 (IL-12), and interleukin 6 (IL-6) and a decrease in Transforming Growth Factor beta (TGF-β) (Ambarus et al., 2012). Alternatively, a non-classical anti-inflammatory (i.e. M2) response was originally induced by interleukin 4 (IL-4) but other stimuli have been shown as well, such as interleukin 13 (IL-13) and interleukin 10 (IL-10) (Ambarus et al., 2012). Phenotypically, the anti-inflammatory/regulatory response includes increased expression of TGF-β and a decreased IL-12 and IL-6 expression, as well

*Abbreviations:* FABP, fatty acid binding protein; WT, wild type; KO, knockout; IL-10, interleukin 10; YM-1, mouse macrophage secretory glycoprotein; Fizz-1, found in inflammatory zone-1; LPS, lipopolysaccharide; IFN-γ, interferon gamma; IL-4, interleukin 4; M1, pro-inflammatory; M2, anti-inflammatory; TNF-α, tumor necrosis factor alpha; IL-12, interleukin 12; IL-6, interleukin 6; TGF-β, transforming growth factor beta; IL-13, interleukin 13; ALT, alanine transaminase; BMDM, bone marrow derived macrophages; Hp, hepatocytes; HSC, hepatic stellate cells; TC, t-cells; KC, Kupffer cells; iNOS, inducible nitric oxide.

* Corresponding author at: Department of Nutrition Science, College of Human Ecology, East Carolina University, Rivers RW 101, Greenville, NC 27858, United States. Tel.: +1 919 522 8214.

*E-mail addresses:* Mooresh06@students.ecu.edu (S.M. Moore), Holtv14@students.ecu.edu (V.V. Holt), Malpassl14@students.ecu.edu (L.R. Malpass), Hines1@ecu.edu (I.N. Hines), Wheelerm@ecu.edu (M.D. Wheeler).

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as increased chemokines and scavenger and phagocytic activity which are important players of polarized M2 macrophages (Mia et al., 2014). Given the altered metabolic response and the excess lipid accumulation associated with the beginning stages of liver disease, it is important to understand the interaction between lipid and macrophage function.

While some work has been done to elucidate the role of $FABP_5$ in atherosclerosis and obesity, little has been done to investigate the role of FABP in macrophages function. Male wild type (C57Bl/6) and $FABP_5$ knockout mice were used for *in vivo* and *in vitro* experiments to investigate the role of $FABP_5$ in the macrophage response. Livers and bone marrow derived macrophages from $FABP_5^{-/-}$ express an increase in anti-inflammatory markers associated with liver disease yet exhibit resistance to LPS induced liver injury. Therefore, further understanding of how $FABP_5$ regulates macrophage polarization during liver injury is required to better understand liver disease and pathogenesis.

## 2. Materials and methods

### 2.1. Animals

Male wild type C57Bl/6j (Jackson Laboratory, 22–25 g, males) and age- and weight-matched $FAPB_5$-null mice (David Borhlohr, University of Minnesota) on C57Bl/6 background animals were caged under identical housing conditions, given humane care, and maintained in compliance with institutional guidelines (Kremer et al., 2010).

### 2.2. Endotoxin treatment

Mice were given a single exposure to LPS (1 mg/kg, ip) or vehicle (saline). Livers were resected at 0, 3, 9, or 24 h after LPS administration. All treatments with LPS were performed simultaneously in wild type and $FABP_5^{-/-}$ animals. Following sacrifice, serum was collected for ALT evaluation and tissue was collected for subsequent analysis. Liver specimens were fixed in phosphate-buffered formalin and embedded in paraffin, and 7 μm sections were stained with hematoxylin and eosin, F4/80, and MPO for histological/immunohistochemical evaluation.

### 2.3. Clinical chemistry

Serum was collected 0, 3, 9, or 24 h after injection of saline (control) or endotoxin (LPS, 1 mg/kg). Serum alanine transaminase (ALT) levels were measured by standard biochemical assay (Kremer et al., 2006).

### 2.4. Histology/immunohistochemical staining

Double fluorescent staining localizes $FABP_5$ and F4/80 in hepatic tissue. $FABP_5$ staining was performed as described briefly. Formalin fixed hepatic tissue sections (7 μm) were deparaffinized with xylene and rehydrated to water. Antigen retrieval was achieved by proteinase K in 10 mM Tris–HCl. Slides were treated with $H_2O_2$ for 10 min at room temperature, washed, and primary $FABP_5$ (H-45) rabbit polyclonal IgG antibody (Santa Cruz Biotechnologies) was applied (1:100) overnight at 4 °C. Slides were subsequently washed and anti-mouse F4/80 antigen pan macrophage marker, BM8 (eBioscience) was applied (1:100) for 1 h at room temperature. Biotinylated anti-rat IgG secondary was applied to tissue for 45 min followed by simultaneous addition of anti-rabbit IgG Alexa fluor 488 (green) and Alexafluor 546 (red) (1:200) (Cell signaling) for 45 min and coverslipped.

Standard H&E staining was performed as described (Son et al., 2009). Tissue was dehydrated in 100% EtOH and coverslipped. F4/80 protocol was performed as described elsewhere (Kremer et al., 2006). Tissues were incubated with anti-mouse F4/80 antigen (1:100) obtained from eBioscience (San Diego, CA) for 30 min at RT followed by anti-rat biotinylated (Vectastain ABC kit, Burlingame, CA) for 30 min. The hepatic macrophages of each mouse were identified and the F4/80 positive area (×20 objective) was measured using ImageJ. The average for each mouse was determined. Myeloperoxidase staining was performed by using anti-MPO antibody (ThermoFisher Scientific, Waltham, MA). Briefly, tissues were deparaffinized, rehydrated, and incubated in 10 mM Tris–HCl containing 20 μg/ml proteinase K (Thermo Scientific, Waltham, MA) at 37 °C for 30 min. Sections were blocked with 3% $H_2O_2$ for 10 min at room temperature and overnight application of MPO primary antibody (1:200) was applied. Anti-rabbit biotin labeled (1:200) secondary antibody (Cell signaling, Danvers, MA) was applied for 1 h at room temperature. DAB was added to the tissue for 2 min. Sections were dehydrated and cover slipped. Quantitative histological evaluation was performed by counting $MPO^+$ cells.

### 2.5. Bone marrow preparation and culture

Bone marrow cells were harvested from femurs of wild type and $FABP_5^{-/-}$ mice. Cells ($5 \times 10^6$ cells/ml) were cultured in RPMI + 10% FBS + 1% PSG. Cells were treated (day 0) within a 5-day culture with M-CSF (10 ng/ml, Peprotech). M-CSF was used to induce macrophage differentiation of $F480^+$ cells prior to stimulation. On day 4 of culture, cells were moved to a 24-well plate ($0.2 \times 10^6$ cells/ml) and allowed to settle for 24 h. After day-5 of culture, cells were treated for 4 or 24 h with LPS (0.25 mg/ml) and/or IFN-γ (40 ng/ml) to induce an M-1 phenotype or IL-4 (20 ng/ml) to induce an M-2 phenotype.

**Table 1**
RT-PCR primer sequences.

| Primer | Sequence |
|---|---|
| 18s | 5′-CTTAGAGGGACAAGTGGCG-3′ (forward)<br>5′-ACGCTGAGCCAGTCAGTGTA-3′ (reverse) |
| TNF-α | 5′-AGCCCACGTAGCAAACCACCAA-3′<br>5′-ACACCCATTCCCTTCACAGAGCAAT-3′ |
| iNOS | 5′-GAGATTGGAGGCCTTGTG-3′<br>5′-TCAAGCACCTCCAGGAACGT-3′ |
| IL-12 | 5′-CTCACCTGTGACACGCCTGA-3′<br>5′-CAGGACACTGAATACTTCTC-3′ |
| IL-6 | 5′-GAGGATACCACTCCCAACAGACC-3′<br>5′-AAGTGCATCATCGTTGTTCATACA-3′ |
| TGF-β | 5′-TGACGTCACTGGAGTTGTACGG-3′<br>5′-GGTTCATGTCATGGATGGTGC-3′ |
| YM-1 | 5′-TCTGGTGAAGGAAATGCGTAAA-3′<br>5′-GCAGCCTTGGAATGTCTTTCTC-3′ |
| Fizz-1 | 5′-CAGCTGATGGTCCCAGTGAA-3′<br>5′-TTCCTTGACCTTATTCTCCACGAT-3′ |
| Arginase | 5′-TTGGGTGGATGCTCACACTG-3′<br>5′-TTGCCCATGCAGATTCCC-5′ |
| IL-10 | 5′-GGTTGCCAAGCCTTATCGGA-3′<br>5′-ACCTGCTCCACTGCCTTGCT-3′ |
| IRF-4 | 5′-GACCAGTCACACCCAGAAATCCC-3′<br>5′-GTTCCTGTCACCTGGCAAC-3′ |
| CCL-2 | 5′-TTCACAGTTGCCGGCTGG-3′<br>5′-TGAATGAGTAGCAGCAGGTGAGTG-3′ |
| CCL-17 | 5′-TCACTTCAGATGCTGCTCCT-3′<br>5′-TCACCAATCTGATGGCCTTC-3′ |
| Albumin | 5′-GACAAGGAAAGCTGCCTGAC-3′<br>5′-TTCTGCAAAGTCAGCATTGG-3′ |
| F4/80 | 5′-CTGTAACCGGATGGCAAACT-3′<br>5′-CTGTACCCACATGGCTGATG-3′ |
| CD3 | 5′-GGACAGTGGCTACTACGTCTGCTA-3′<br>5′-TGATGATTATGGCTACTGCTGTCA-3′ |
| CD31 | 5′-GGTGGTTGTCATTGGAGTGG-3′<br>5′-GAAGCAGCACTCTTGCAGTC-3′ |
| $FABP_5$ | 5′-AGAGCACAGTGAAGACGAC-3′<br>5′-CATGACACACTCCACGATCA-3′ |

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