Supplemental Material

Myeloid Cell Specific ABCA1 Deletion Protects Mice from Bacterial Infection

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Running title: ABCA1 and macrophage function

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MATERIALS AND METHODS

Animals

Wild type (WT) and myeloid cell specific ABCA1 knockout (MSKO, homozygous) mice were generated as described previously [1, 2]. Mice were backcrossed to C57BL/6 background for six generations before use in the studies. All animal procedures were approved by the Wake Forest School of Medicine Animal Care and Use committee.

Cell culture

Peritoneal macrophages (PMs) were harvested from WT and MSKO littermate mice 4 days after receiving an intraperitoneal injection of 1 ml 10% thioglycolate and plated in RPMI media containing 1% Nutridoma SP (NutSP) media (Roche Applied Science) as previously described [1]. Bone marrow from WT and MSKO littermate mice was isolated and cultured in DMEM media containing 20% FBS and 30% L929 conditioned media for 5–7 days before being used in experiments as bone marrow-derived macrophages (BMDMs) [1].

Bacteria

L. monocytogenes strains used in this study was the wt strain10403S. For Lm infection experiments, bacteria were first grown overnight at 30°C in brain–heart infusion broth (BHI) to reach the stationary phase before washed twice in PBS and resuspended in PBS or medium at indicated concentrations based on experimental designs as described below.

In vivo Lm clearance experiments

Female mice (20-30 wk old) were infected i.p. with Lm 10403S at a dose of 5×10^4 Listeria/mouse. At 36h or 72h post infection, mice were sacrificed using ketamine/xylazine. Blood was taken via cardiac puncture for plasma cytokine and chemokine measurement by ELISA or Bioplex assay. Mouse body weight was monitored before and after 3 days of Lm infection. To determine organ Listeria burden, spleen and liver were homogenized in sterile H2O at day 3 infection. Serial dilutions of homogenate were plated on brain heart infusion agar, and bacterial colony formation was assessed after overnight growth at 37°C. Small portions of liver were fixed in 10% formalin.

Histology and immunohistochemistry (IHC)

Formalin fixed liver tissues were dehydrated in ethanol and embedded in paraffin. Sections (4 μm thick) were cut and stained with hematoxylin and eosin (H&E) for evaluation of pathological changes. For IHC analysis, livers sections were deparafinized and hydrated through ethanol to H2O. Antigen retrieval was achieved using a microwave method with target retrieval solutions (Dako). Endogenous peroxidase activity was blocked in 0.3% H_2O_2 in PBS (20 min). The tissue sections were incubated with an appropriate normal serum (30 min) before incubation for 1 h at room temperature (RT) with the primary antibodies to CD68 (abD Serotec), Ly6B.2 (abD Serotec), cleaved caspase-3 (Cell signaling) and MCP-1 (Novus Biologicals). The sections were then washed and incubated for 30 min at RT with the appropriate biotinylated secondary antibody. ABC reagent was then added to the sections for 30 min (ABC vector kit; Vector) and revealed with AEC (Dako; for CD68, Ly6B.2 and cleaved caspase-3) or DAB substrate chromogen (Dako; for MCP-1). The sections were counterstained with Hemotoxylin and mounted from water using an aqueous mounting medium (Vector). The staining was analyzed by Image Pro software. The percentage of liver sections covered by $CD68^+$ cells (% $CD68^+$ area) was calculated to indicate the intensity of hepatic $CD68^+$ cells.

TUNEL assay

In Situ Cell Death Detection Kit, Fluorescein (Roche) was used to detect hepatic apoptosis in Lm infected mouse liver sections according to the manufacturer's instruction. Lm infected mouse liver sections incubated with Label solution only (without terminal transferase) instead of the TUNEL reaction mixture was used as a negative control. Lm infected mouse liver sections pre-incubated with DNase I prior to labeling procedures was used as a positive control.

Oxysterol measurement

Oxysterol content of thioglycollate-elicited peritoneal macrophages from WT (n=3) and MSKO (n=4) mice was measured by using isotope dilution-mass spectrometry as described by Dzeletovic et al [3] and results were normalized to cellular protein measured by Lowry assay.

Macrophage Migration Assay

In vitro chemotaxis assay was performed as described before[4] with minor modification. Thioglycollate elicited PMs were incubated in RPMI-1640 containing 1% NutSP media overnight. PMs were then gently scraped from dishes and resupended in RPMI-1640 + 1% NutSP media (chemotaxis media) before used in the chemotaxis assay. BMDMs were incubated in chemotaxis media for at least 4 hours before used in the assay. For chemotaxis assay, cells were suspended at a concentration of 2×10^6 cells/mL in chemotaxis media. Fifty microliter of cell suspension was loaded in the upper chamber of a 48-well microtaxis chamber. And 25 μl of MCP-1(1 μM and 10 μM, Pepro Tech Inc) and MIP-1α (10 μM, Pepro Tech Inc) in chemotaxis media was added to the lower chamber. A 5-μm polycarbonate membrane separated the upper and bottom chambers. After a 2-hour incubation at 37°C, macrophages attached to the underside of the membrane were fixed and stained using the Diff-Quick stain set (Dade Behring Inc). The results are expressed as the mean number of cells that migrated in 5 high-power fields (40× objective) in 4 replicate samples. An *in vivo* migration assay was performed as described before with minor modification[5, 6]. Briefly, WT mice were injected intraperitoneally (i.p.) with 1 ml of 10% thioglycollate to elicit sterile peritonitis. Three days later, the mice were i.p. injected with an equal number ($5x10^6$) of BMDMs from WT (labeled with cell tracker green CMFDA) and MSKO (labeled with cell tracker red CMPTX; both from Molecular Probes) mice. Twenty hours later, 400 ng LPS was i.p. injected into the mice. Three hrs later, mice were sacrificed, peritoneal cells were harvested with PBS, and the frequency of fluorescent-labeled macrophages in total peritoneal cells was analyzed by flow cytometry.

Flow cytometry

Blood were collected from uninfected or Lm infected mice via cardio-puncture. Bone marrow cells were harvested from mouse femurs. Erythrocytes were lysed with ACK lysis buffer. After blocking the Fcγ receptor with purified anti-mouse CD16/CD32 antibody (Fcγ receptor III/II; BD Biosciences), blood or bone marrow cells were incubated at 4 °C for 30 min with isotype controls or the following Abs: FITC-anti-CD11b (M1/70, BD phamingen), PE-anti-Ly6C (AL-21, BD phamingen), APC-anti-CD45 (30-F11, BD phamingen) and Percp-Cy5.5-anti-Ly6G (RB6-BC6, eBioscience). Cell fluorescence was determined using a FACSCalibur flow cytometer (BD Biosciences) and analyzed with FlowJo software.

Western Blotting and Real time PCR: In vitro Lm infection was performed as described before [7, 8]. Briefly, 2×10^6 BMDMs in RPMI-1640 containing 10% FBS were seeded on a 60-mm tissue culture dish. Approximately 8×10^6 wt bacteria (10403S) were used to infect BMDMs as described before. One hour post infection, the macrophages were washed with PBS and fresh medium containing 10 μg/ml gentamicin was added to kill extracellular bacteria. After 6, 12, and 24h infection, macrophages were lysed for protein using RIPA buffer containing proteinase inhibitor cocktails (Roche) or RNA using TRIzol reagent (Invitrogen), respectively. Protein expression was examined using western blotting and mRNA expression was examined using real time PCR. In a separate experiment, BMDMs from WT and MSKO mice were incubated with 10 μM TO-901317 or vehicle (DMSO) for 24h before harvesting RNA. Primers for SPα were provided by Dr. Peter Tontonoz (Howard Hughes Medical Institute, University of California, Los Angeles School of Medicine). Other primers can be found in our previous published paper [1] or were listed in **Supplemental Table I**.

Statistics

Differences were compared with two-tailed Student's t -test using GraphPad Prism software. $P < 0.05$ was considered statistically significant. Data are presented as the means ± SD unless indicated otherwise.

Supplemental Table I: Real time PCR primer sequences

ACAT2 F	GACTTGGTGCAATGGACTCG
ACAT2 R	GGTCTTGCTTGTAGAATCTGG
SREBP1c F	GGAGCCATGGATTGCACATT
SREBP1c R	GGCCCGGGAAGTCACTGT

SCD1 F	CCGGAGACCCCTTAGATCGA
SCD1 R	TAGCCTGTAAAAGATTTCTGCAAACC
ACC1 F	TGGACAGACTGATCGCAGAGAAAG
ACC1 R	TGGAGAGCCCCACACACA
PGC1a F	AACCACACCCACAGGATCAGA
PGC1a R	TCTTCGCTTTATTGCTCCATGA
DGAT1 F	GAGGCCTCTCTGCCCCTATG
DGAT1 R	GCCCCTGGACAACACAGACT
CD68 F	CCTCCACCCTCGCCTAGTC
CD68 R	TTGGGTATAGGATTCGGATTTGA
F4/80 F	CTTTGGCTATGGGCTTCCAGTC
F4/80 R	GCAAGGAGGACAGAGTTTATCGTG

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Online Figure I. Lipogenic gene expression in livers in control (uninfected) and Lm infected (3 days) mice. Gene expression was analyzed using real time PCR and normalized to GAPDH. WT: wild type; MSKO: myeloid cell-specific ABCA1 knockout.

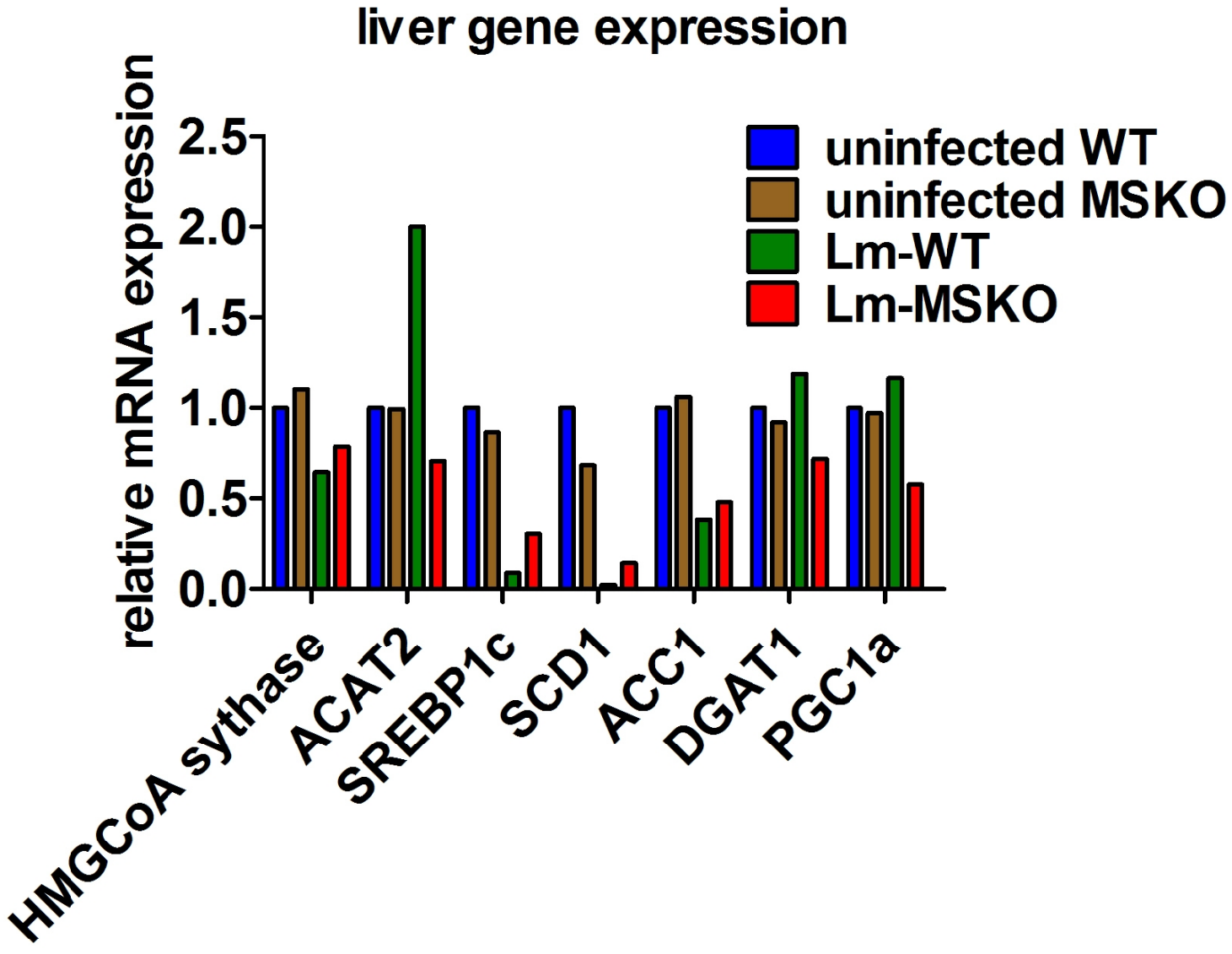
Online Figure II. Neutrophil infiltration in livers 3 days post Lm infection. Immunohistochemistry staining was performed to visualize neutrophils in liver in uninfected and Lm infected mice using antibody against Ly6B.2 (objective magnification 10× and 40×). WT: wild type; MSKO: myeloid cell-specific ABCA1 knockout.

Online Figure III. Inflammatory cytokine and chemokine mRNA expression in livers of control (uninfected) and Lm infected (3 days) mice. Gene expression was analyzed using real time PCR and normalized to GAPDH. Values with different letters are statistically different ($P<0.05$). WT: wild type; MSKO: myeloid cell-specific ABCA1 knockout.

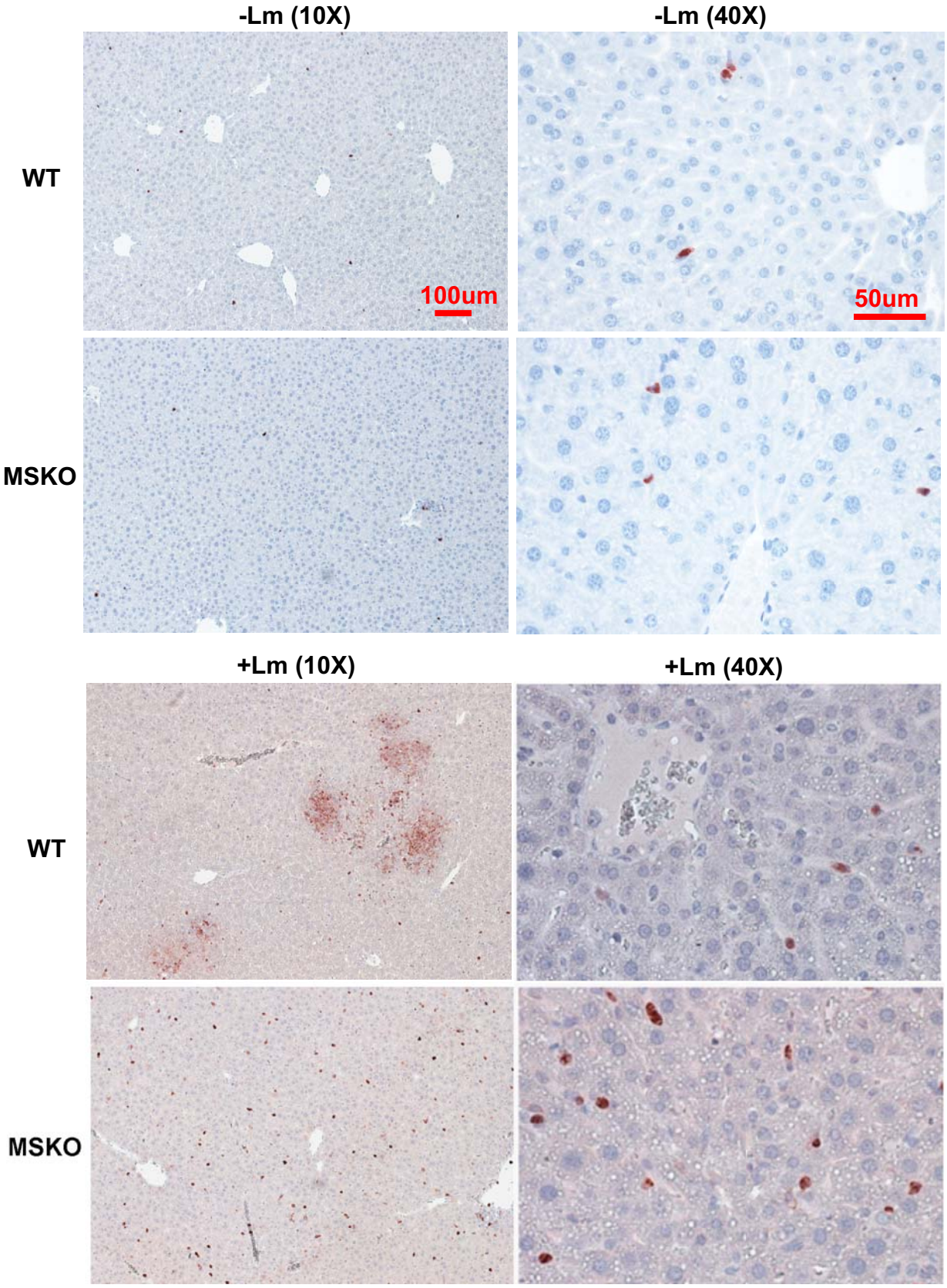
Online Figure IV. Oxysterol and SPα expression in macrophages and apoptosis in livers of Lm infected mice. **(A)** oxysterol species in thioglycollate elicited PMs from WT (n=3) and MSKO (n=4) mice were measured using isotope dilution-mass spectrometry. **(B)** SPα mRNA expression in BMDMs from WT and MSKO mice was measured by real time PCR and normalized to GAPDH. Macrophages were treated with synthetic LXR agonist 10 μM TO-901317 or vehicle DMSO for 24h before RNA isolation. **(C-D)** Mice were i.p. infected with Lm for 3 days. (**C**) Liver sections from Lm infected mice were analyzed by TUNEL staining. Objected magnification: 10×. Lm infected mouse liver section incubated with Label solution only (without terminal transferase) instead of TUNEL reaction mixture was used as negative control. Lm infected mouse liver section pre-incubated with DNase I prior to labeling procedures was used as positive control. (**D**) Immunohistochemical analysis of cleaved caspase-3 revealed very few apoptotic cells (immune cells and hepatocytes) in the livers of mice. WT: wild type; MSKO: myeloid cell-specific ABCA1 knockout.

Online Figure V. ABCA1 deficient peritoneal macrophages display increased chemotaxis towards MIP-1α. The chemotactic response of thioglycollate-elicited peritoneal macrophages from WT and MSKO mice to MIP-1α was tested in a 48-well microchemotaxis chamber as described in the Methods section.

Online Figure I



Online Figure II



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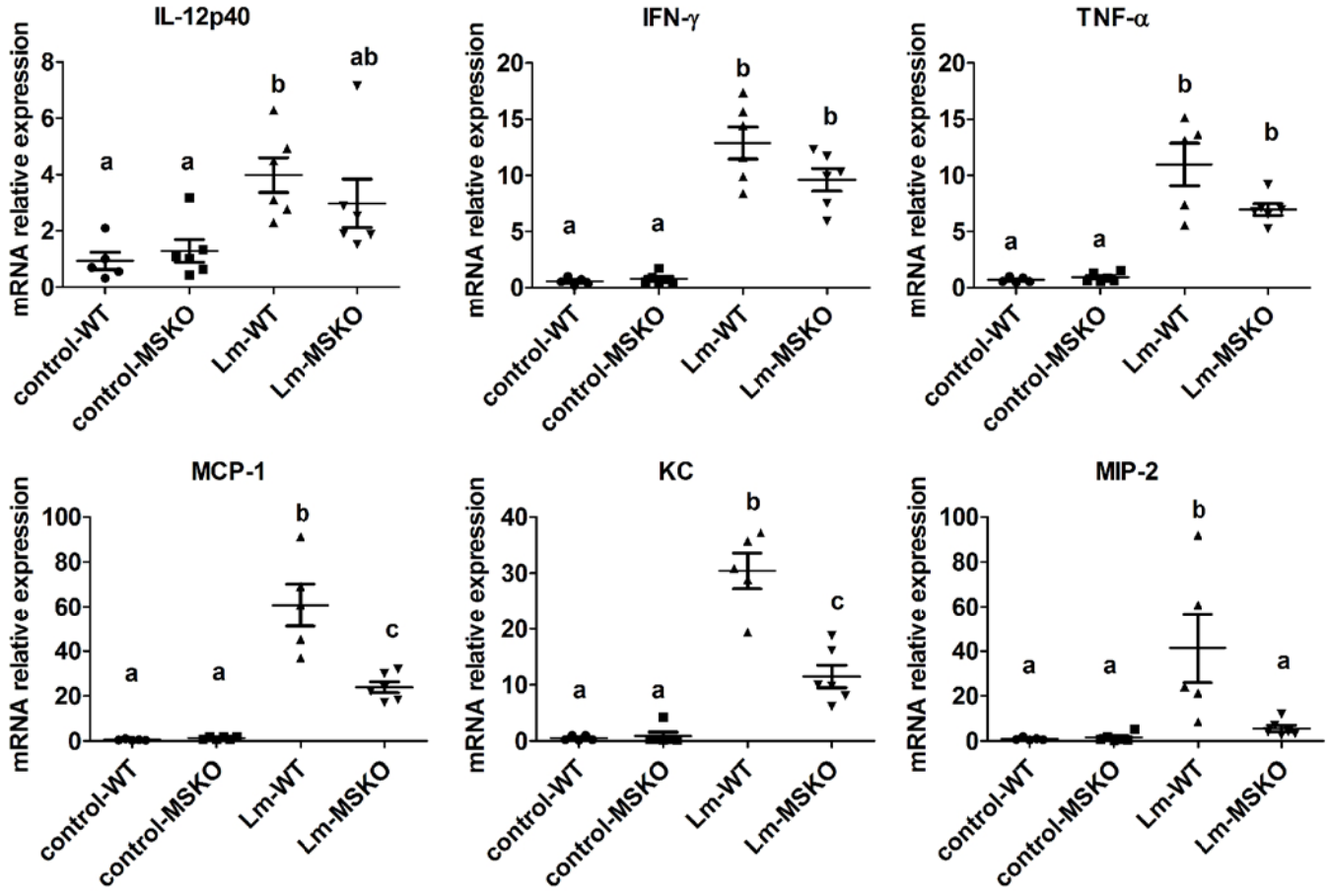

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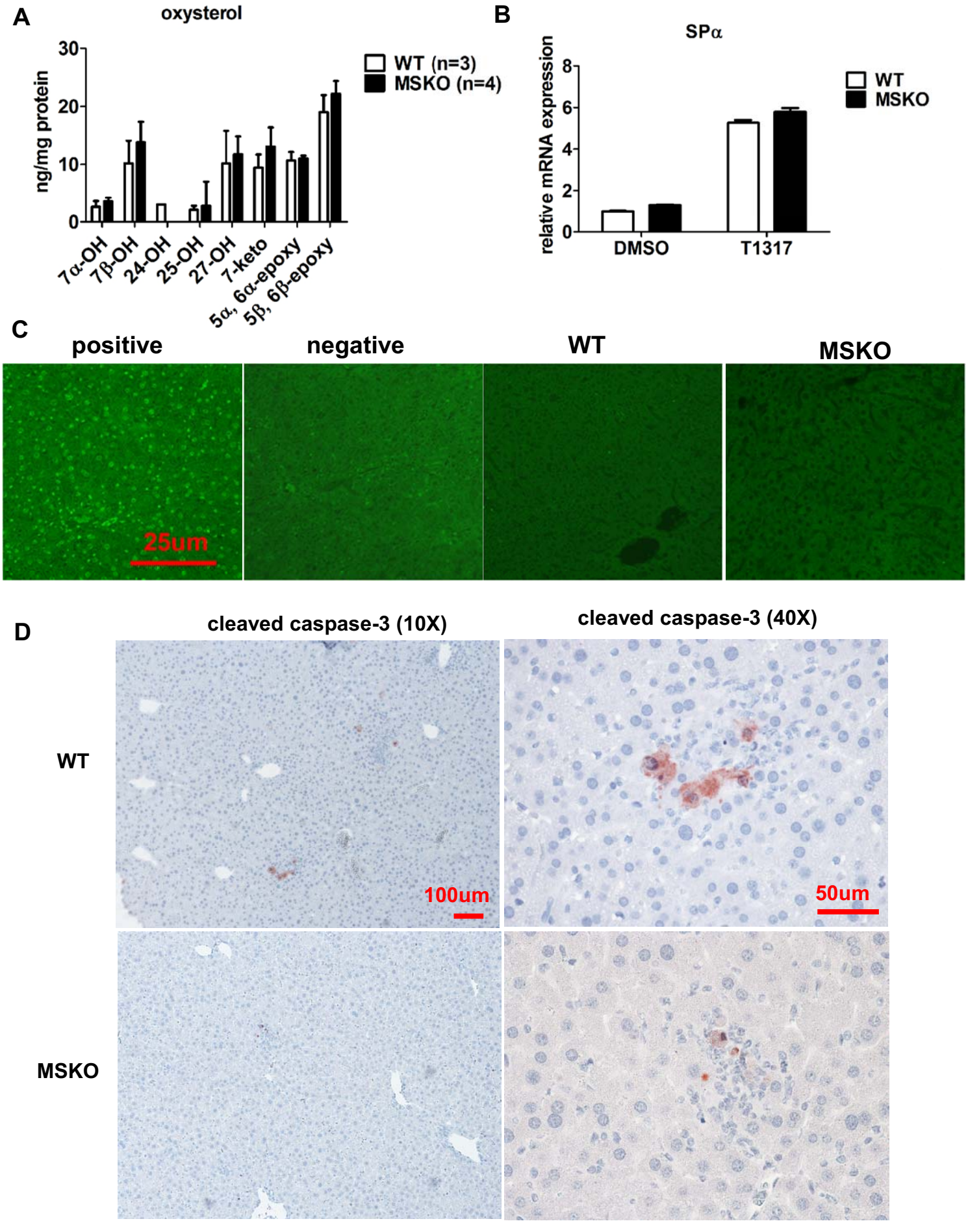

Online Figure V