

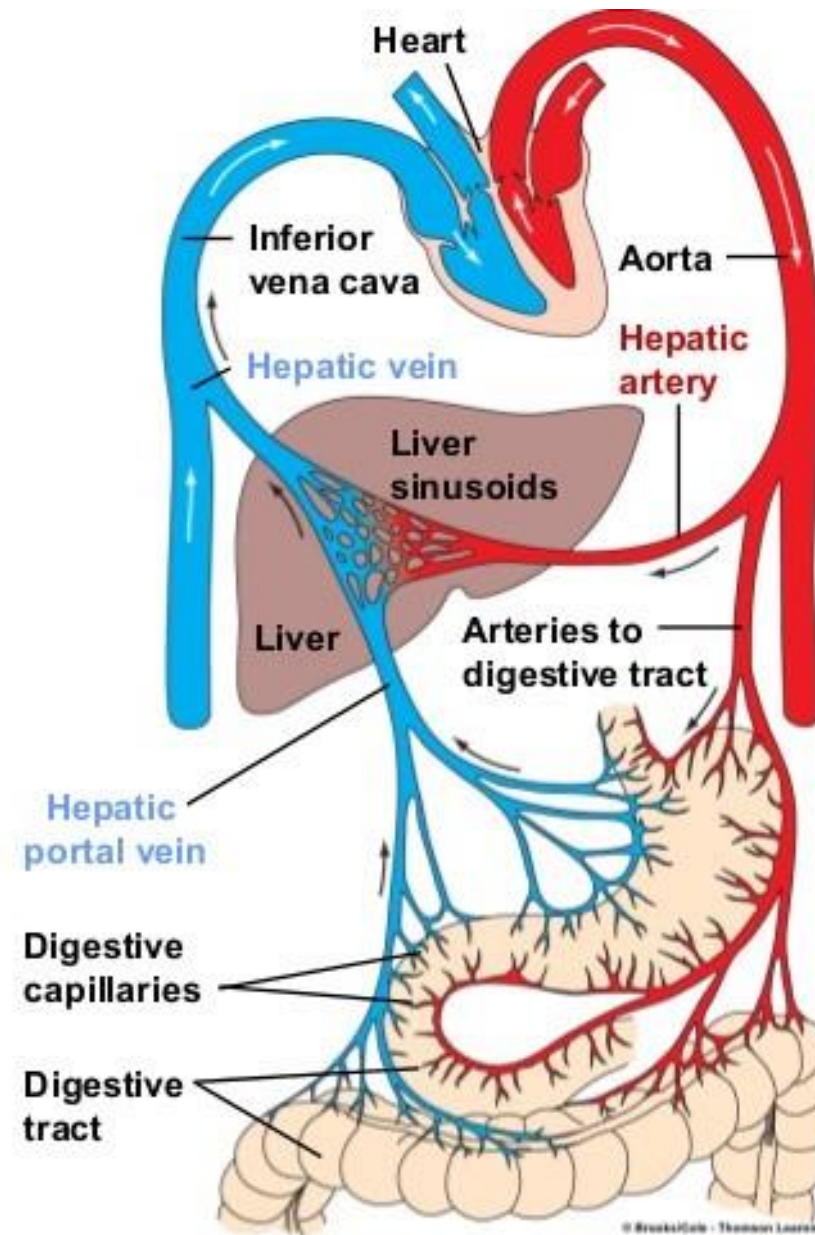
Apoptotic Cells Attenuate Fulminant Hepatitis by Priming Kupffer Cells to Produce Interleukin-10 Through Membrane-Bound TGF- β

Minggang Zhang, Sheng Xu, Yanmei Han and Xuetao Cao

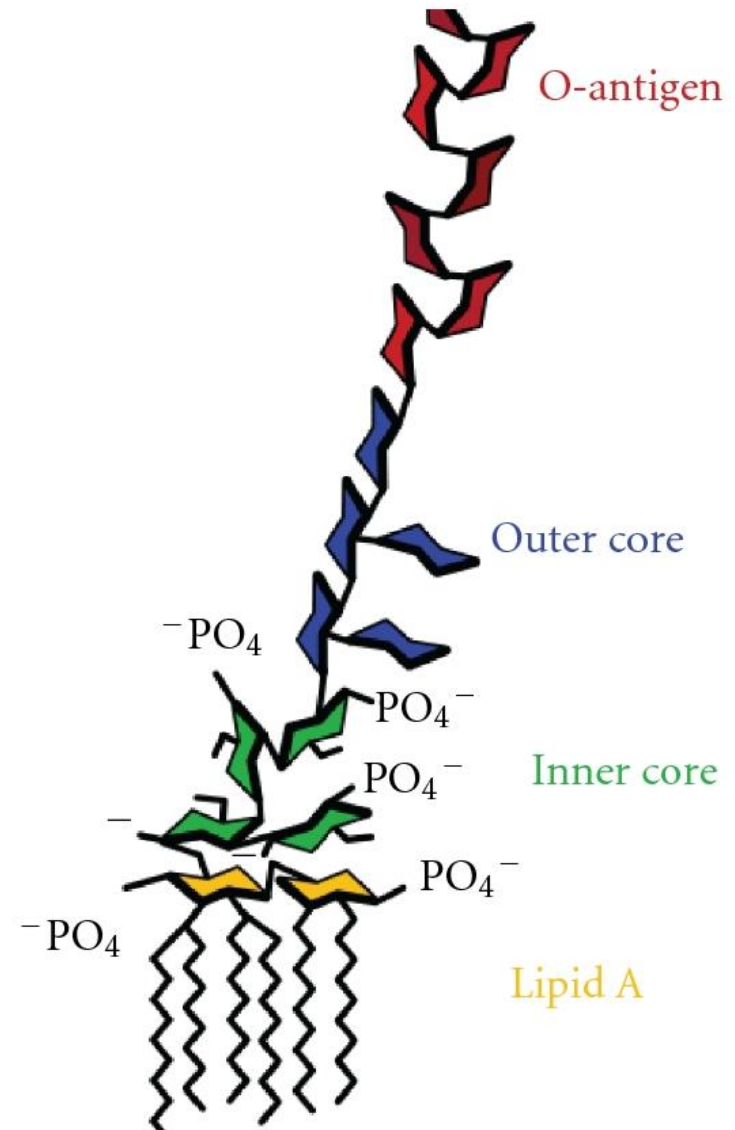
Hepatology (7 December 2010)

Background

The liver is continuously exposed to lipopolysaccharide (LPS) and other pathogenic components from the gastrointestinal (GI) tract via the hepatic portal vein.



- LPS, also known as endotoxin, is found in the outer membrane of Gram-negative bacteria and it is a potent stimulator for immune responses, but normally these products from pathogenic microbes are cleared by the liver, leading to immune tolerance rather than significant immune response and inflammation in the liver.
- Hepatic tolerance is also reflected in immune tolerance toward oral antigens and liver grafts.



- Nonparenchymal liver cells:
 - liver sinusoidal endothelial cells,
 - dendritic cells (DCs)
 - Kupffer cells (KCs),

→ responsible for the creation of a local immunosuppressive microenvironment, permitting immune effector cells, such as T cells, to deliver tolerogenic signals, which contributes to the tolerogenic properties of the liver.
- Exact mechanisms are still unclear.

The Liver

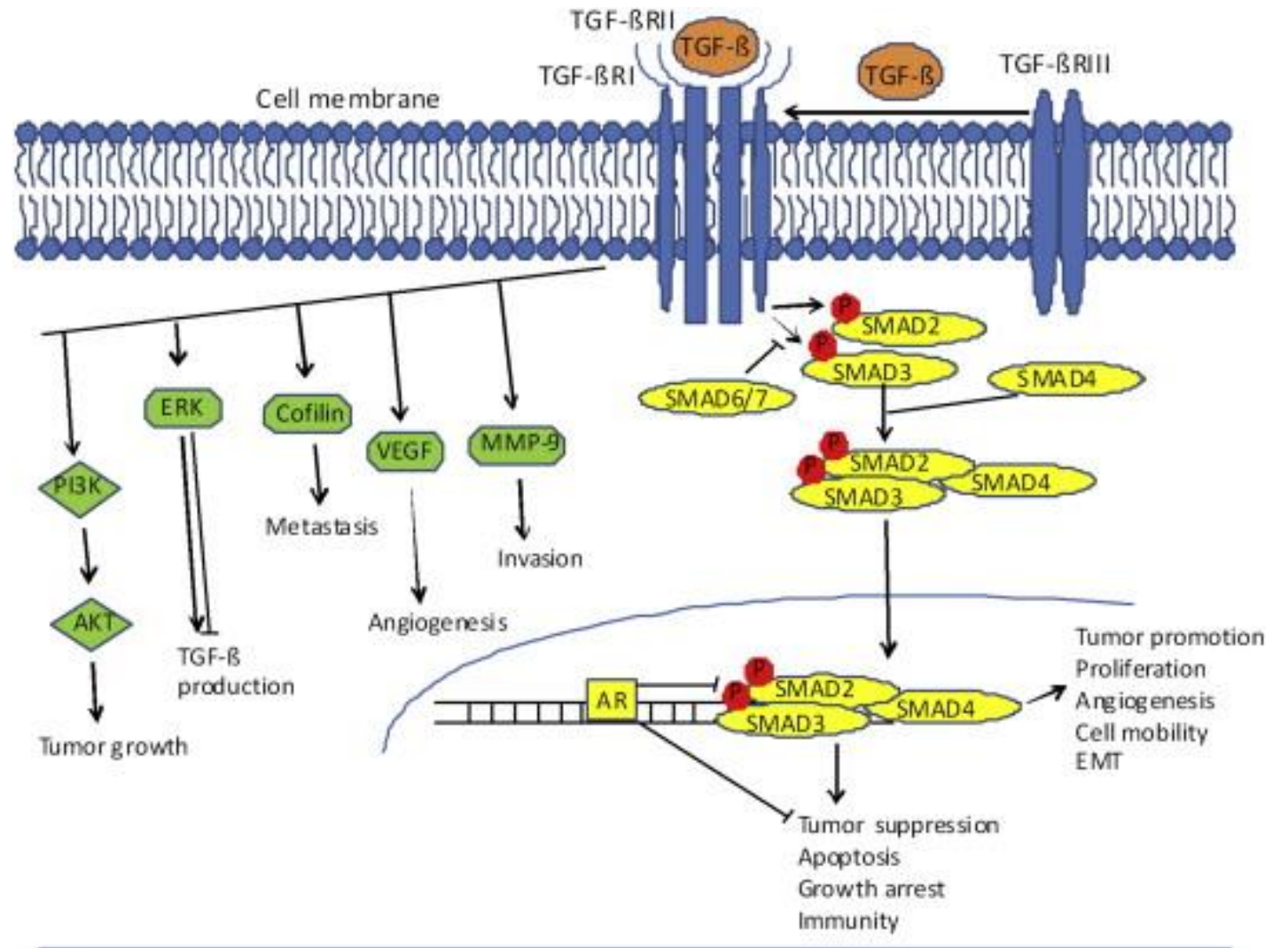
- Major “graveyard” of aging erythrocytes and neutrophils.
- A site for trapping and eliminating activated CD8+ T and CD4+ T cells that have completed their immunological functions and become apoptotic.
 - Phagocytic clearance of apoptotic cells (ACs) generally leads to immunosuppression.
- Contact with ACs during the resolution of inflammation or in remodeling tissue educates macrophages to adopt an immunoregulatory property.
- Ingestion of ACs by DCs and monocytes also stimulates their production of anti-inflammatory cytokines (e.g., transforming growth factor [TGF]- β and interleukin [IL]-10) and inhibits proinflammatory cytokines.
- This interaction is poorly understood, but seems to require recognition of specific molecules on ACs, such as anionic lipid phosphatidylserine, by the phagocytic cells.

- Strong immunological challenges → Liver injury
- Nearly all innate immune cells are associated with diverse liver injury.
- Murine models
 - Natural killer T cells (NKTs) mediate concanavalin A-induced hepatitis.
 - Natural killer (NK) cells mediate polyinosinic:polycytidylic acid-induced hepatitis.
 - KCs are reported to mediate endotoxin induced fulminant hepatitis, which is associated with increased production of tumor necrosis factor (TNF)- alpha.
- However, the exact mechanisms of liver injury and normally how the liver balances the immune response and immunosuppression remain to be further determined.

SMAD3

Mothers against decapentaplegic homolog 3 also known as SMAD family member 3 or SMAD3 is a protein that in humans is encoded by the SMAD3 gene. SMAD3 is a member of the SMAD family of proteins.

- Mediates the signals from the transforming growth factor beta (TGF- β) superfamily ligands that regulate cell proliferation, differentiation and death.
- Related with tumor growth in cancer development.



This study

- Administration of donor ACs can dramatically suppress the immune response and inflammation in a mouse model of endotoxin-induced hepatitis, which is mediated by IL-10 from KCs after priming with membrane-bound TGF- β on ACs.
- The molecular mechanisms for how IL-10 was induced and functioned were determined.
- Priming of KCs by ACs is an important component of the immunosuppressive microenvironment in the liver.

Materials and Methods

Materials

- Adult male C57BL/6 (H-2^b) mice
- Adult male BALB/c (H-2^d) mice
- CD11c-DTR mice (C57BL/6 background)
CD11c.DTR mice, which express simian diphtheria toxin receptor under an Itgax promoter (drives CD11c expression). This makes cells that express CD11c sensitive to diphtheria toxin treatment in transgenic mice, most of which are dendritic cells, but also a few NK cells and a few CD8 memory T cells at low levels.
- IL-10- deficient mice (C57BL/6 background)



- Smad3-deficient mice

Smad3^{-/-} and Smad3^{+/+} (as wild-type control and origin of ACs for Smad3^{-/-} KC priming) homozygous littermates were used (sexuality balanced), which were derived from the filial generation mice of Smad3^{+/-} mice mating with Smad3^{+/-} mice.

All mice were housed in a SPF facility, and used at 6-8 weeks of age.

Ethical approval

Scientific Investigation Board of the Second Military Medical University in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Supporting materials

Antibodies and Reagents

- Recombinant murine TGF- β 1, neutralizing murine anti-IL-10, anti-TNF- α , anti-TGF- β 1 and isotype antibodies
- Fluorescein-conjugated mAbs against Annexin V, F4/80, CD11c, CD80, CD86, CD40, CD14, and B7-H1, biotin-conjugated Abs against TGF- β 1, and the corresponding isotypes
- Antibody 2.42G (rat IgG2b) for blocking CD16/CD32
- Lipopolysaccharide (LPS) (Escherichia coli, O26:B6), 7-AAD, saponin, BSA, CFSE, GdCl₃, D-galactosamine (D-GalN), cytochalasin B, S-methylisothiourea sulfate (SMT) and diphtheria toxin (DT)
- Primers for mouse IL-10 (5'ATCCTGGCTCAGCACTGCTATG3' and 5'GCTTAGCTTTTTCATTTTGATCA3')

Cell Preparations

- Apoptotic splenocytes were obtained using an ultraviolet (UV)-B irradiation method
 - Single-cell suspensions of mouse splenocytes were prepared and dead cells or debris were removed using 35% Ficoll.
 - Five million cells were suspended in 5 mL Hank's balanced salt solution (HBSS) in a 10-cm-diameter Petri plate, irradiated using a 40-W UV-B (320 nm) at a distance of 40 cm for 10 minutes, and then cultured in Roswell Park Memorial Institute 1640 medium (RPMI-1640) (with 10% fetal bovine serum [FBS]) at 37°C in 5% CO₂ for 4 hours.
- For apoptotic cell inoculation via vein or priming KCs in vitro, syngeneic apoptotic splenocytes were used, and 1.5×10^7 ACs were injected per mouse.
- The purity of early ACs (propidium iodide, annexin V+) was 90% to 95%.
- For phagocytosis assay, F4/80⁻ splenocytes were sorted by Dako Moflo-XDP, and irradiated with UV-B after labeling with 5,6-carboxyfluorescein diacetate succinimidyl ester (CFSE).
- All cells were cultured for 1-3 days before being used in the experiments.
- For in vitro priming, KCs were incubated with ACs (1:10) for 4 hours, washed to get rid of the uncaptured ACs, and cultured for 3 days.

Fluorescence-Activated Cell Sorting Analysis

- Fc receptors were blocked using 2.4G2 and stained with fluorescent antibodies.
- Fluorescence Activated Cell Sorting (FACS) analysis was performed using BD LSR II with FACSDiva software (BD Biosciences).
- ERK phosphorylation was examined using a BD phosflow method.

Cell Depletion, Phagocytosis Inhibition, and Cell Adoptive Transfer

- KCs were depleted by intravenous (i.v.) injection of gadolinium chloride (GdCl₃, 20 mg/kg body weight) in mice.
- For depletion of DCs, CD11cDTR mice received intraperitoneal (i.p.) injection of diphtheria toxin (DT, 16 µg/kg body weight) at 24 hours prior to AC infusion.
- An anti-CD25 Ab (PC 61) was used to deplete regulatory T cells from mice at 24 hours before LPS/D-GalN injection.
- Cytochalasin B, a blocker of microfilament formation, was used to inhibit phagocytosis of macrophages and DCs.
- For adoptive cell transfer, mice were treated with GdCl₃, and then received portal vein (p.v.) injection of AC-primed KCs (1x10⁶ /mouse) 24 hours later.

Detection of Cytokines and NO

- Cytokine concentration was measured using commercial enzyme-linked immunosorbent assay (ELISA) kits.
- NO was assayed using a Griess reagent kit.

Endotoxin-Induced Fulminant Hepatitis Model

Fulminant hepatitis in mice was established by injection with LPS (5 µg/kg body weight) and D-galactosamine (400 mg/kg body weight).

Statistical Analysis

- To analyze the statistical significance of differences for paired samples → **Student t test**
- To analyze differences between groups → a post hoc **Bonferroni test**
- To correct for multiple comparisons → One-way analysis of variance (**ANOVA**)
- To analyze the survival data → **Kaplan-Meier analysis**.
- Statistical significance was set at $P < 0.05$.

Results

KCs Are Critical for the Clearance of ACs in the Liver

- Apoptotic naive splenocytes i.v. infused via the tail vein were quickly captured and cleared (within 4 hours) by splenic macrophages (Fig. 1 A).
- Apoptotic activated splenocytes infused i.v. or apoptotic splenocytes infused p.v. (whether naive or activated) were mainly captured by hepatic KCs.
- Pretreatment with cytochalasin B significantly delayed the clearance of ACs in the liver (Fig. 1 B).
- Depletion of KCs with GdCl₃ also significantly delayed the clearance of ACs in the liver.
- In contrast, treatment of CD11cDTR mice with diphtheria toxin did not change AC clearance in the liver.

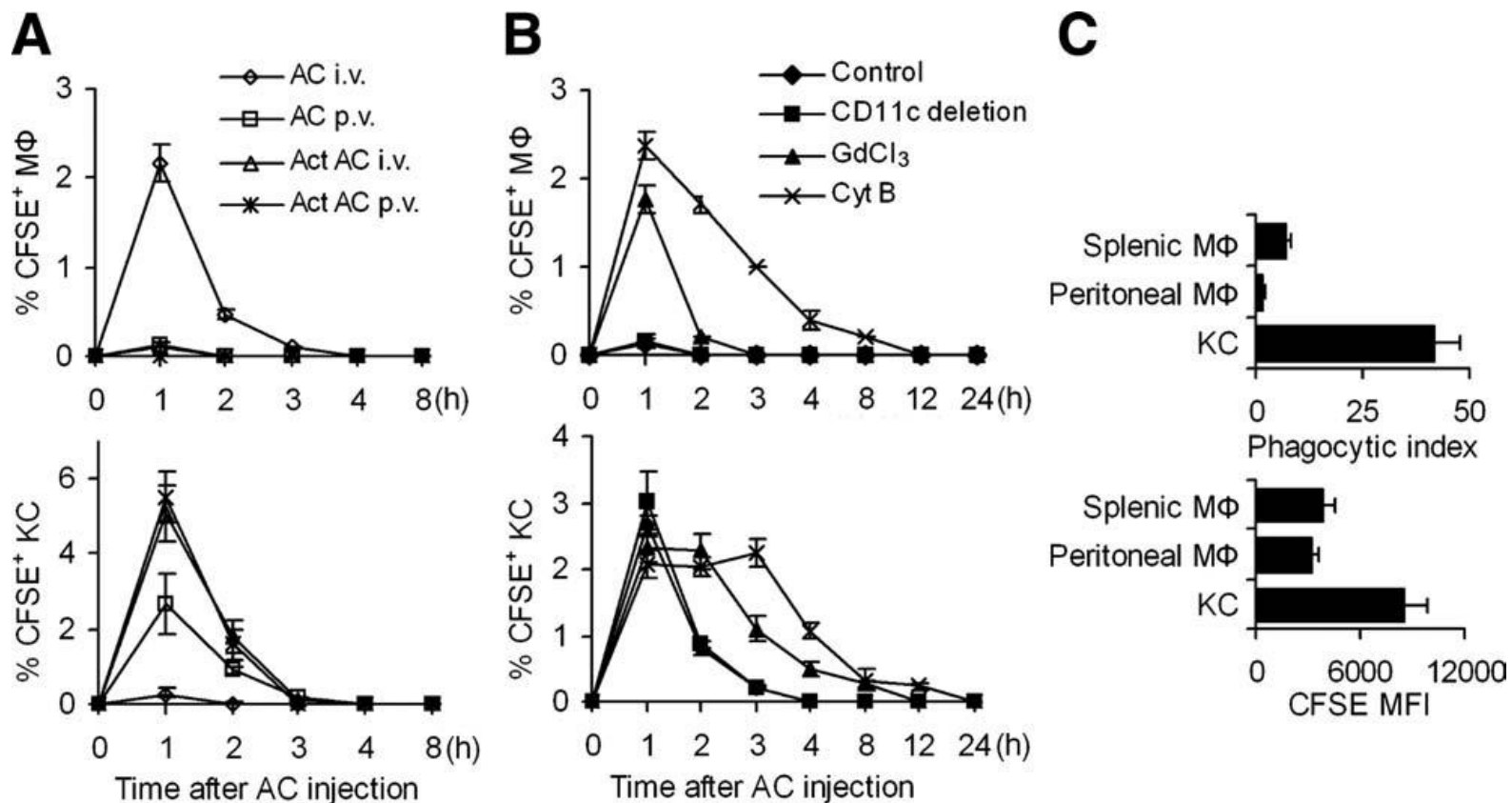
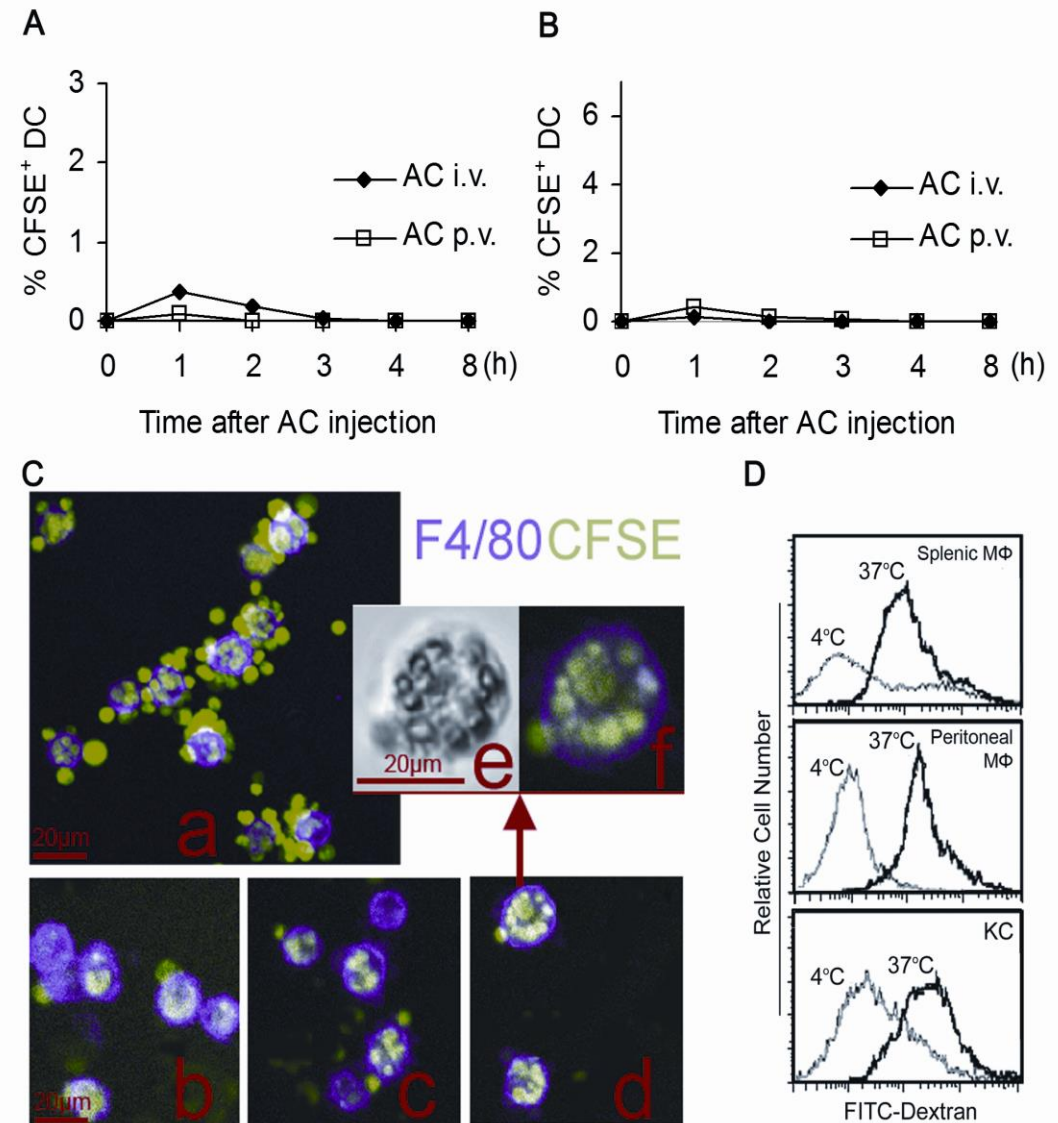


Fig. 1. Kupffer cells are critical for phagocytic clearance of apoptotic cells in the liver. (A) CFSE⁺ F4/80⁺ cells in the spleen (upper panel) and liver (lower panel) of mice (n = 6) were quantified by FACS at indicated time after i.v. (tail vein) or p.v. (portal vein) infusion of CFSE-labeled syngeneic AC (F4/80). (B) CFSE⁺ F4/80⁺ cells in the spleen (upper panel) and liver (lower panel) of mice (n = 6) were quantified by FACS at indicated time after p.v. infusion of CFSE-labeled autologous AC (F4/80). AC was infused into syngeneic mice (control), syngeneic mice pretreated with GdCl₃ (20 mg/kg, at 24 hours), syngeneic mice pretreated with cytochalasin B (Cyt B, 10 mg/kg, at 1 hour), or CD11c-DTR mice pretreated with DT (16 μg/kg, at 24 hours, CD11c deletion); n = 6. (C) CFSE-labeled AC (F4/80) was incubated with F4/80⁺ macrophages from the spleen, peritoneal cavity, or KCs for 4 hours in vitro. Then the phagocytic index and CFSE MFI of macrophages were determined. Experiments in (A) and (B) were performed independently using C57BL/6 or BALB/c mice for 4 times with similar results, and data shown are from C57BL/6 mice; experiments in (C) were performed independently 3 times using C57BL/6 mice with similar results. MU: macrophages.

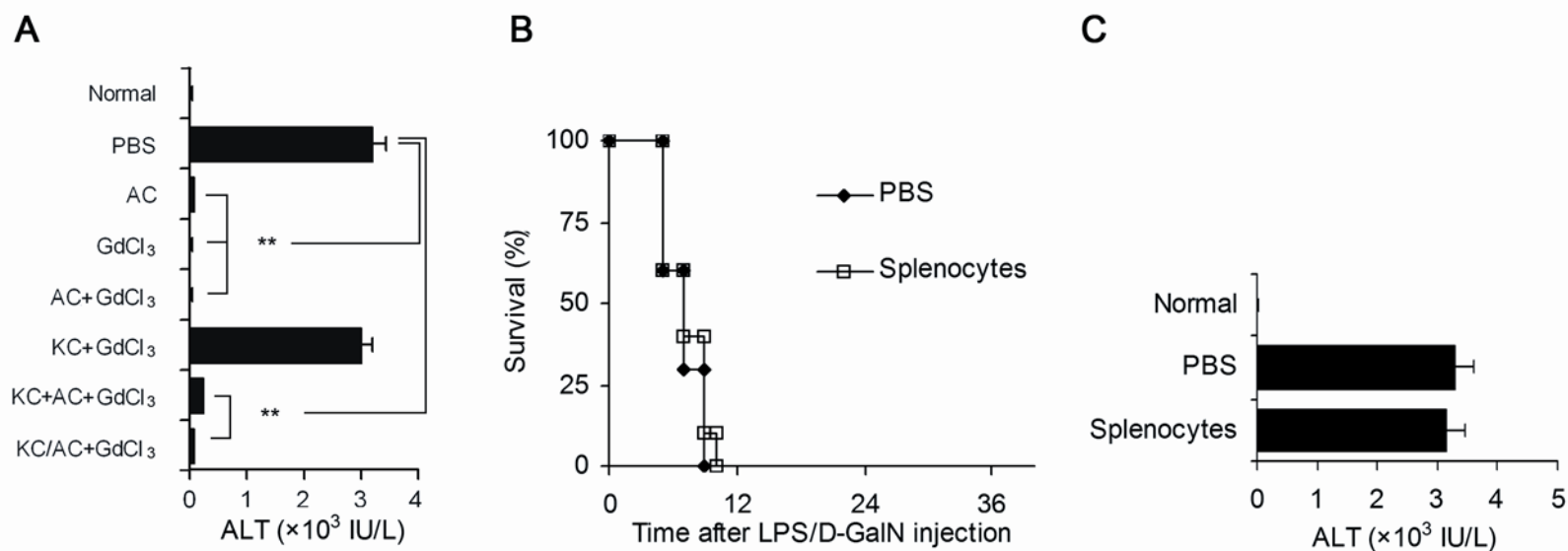
- The AC capture capability of DC in vivo was not so potent compared with that of KCs in the liver or macrophages in the spleen (Supporting Fig. 1A,B).
 - An in vitro experiment demonstrated that liver KCs had higher phagocytic capacity for ACs (Fig. 1C; Supporting Fig. 1C) but had similar pinocytic capacity in comparison to splenic and peritoneal macrophages (Supporting Fig. 1D).
- Liver KCs but not DCs are the major phagocyte for AC capture and clearance in the liver.

Supporting Fig. 1 The AC capture ability of DC in the spleen and liver. **(A,B)** AC were prepared from freshly isolated splenocytes (CD11c-), stained by CFSE and irradiated using UV-B. Four hours after culture in vitro, AC were injected via i.v. (tail vein) or p.v. (portal vein) into syngeneic C57BL/6 mice (n=6). The indicated times later, CFSE+ CD11c+ cells in the spleen (A) and the liver (B) of mice were quantified by FACS. **(C)** CFSE-labeled AC (F4/80-) were incubated with F4/80+ macrophages from the spleen (b), peritoneal cavity (c), or KC (a,d) for 4h in vitro. Adherent AC were detached with (b,c,d) or without (a) trypsin, then the culture was washed with PBS, stained with F4/80-allophycocyanin, and examined under a Leica TCS SP2 confocal laser microscope (Leica Microsystem, Germany). Panels e and f are higher magnification of a single KC in panel d. Bar=20µm. **(D)** FITC-Dextran pinocytosis of splenic macrophages (MΦ), peritoneal macrophages and KC at 37°C vs. 4°C (the control). Experiments were performed for three times with similar results.



Infusion of ACs Suppresses KC-Mediated Endotoxin Induced Fulminant Hepatitis Nonspecifically.

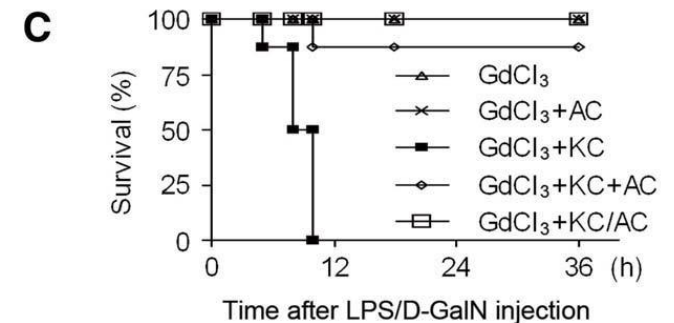
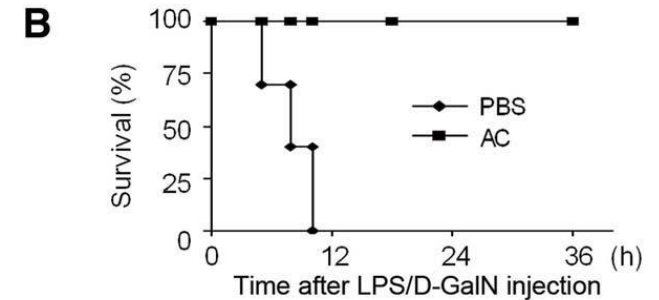
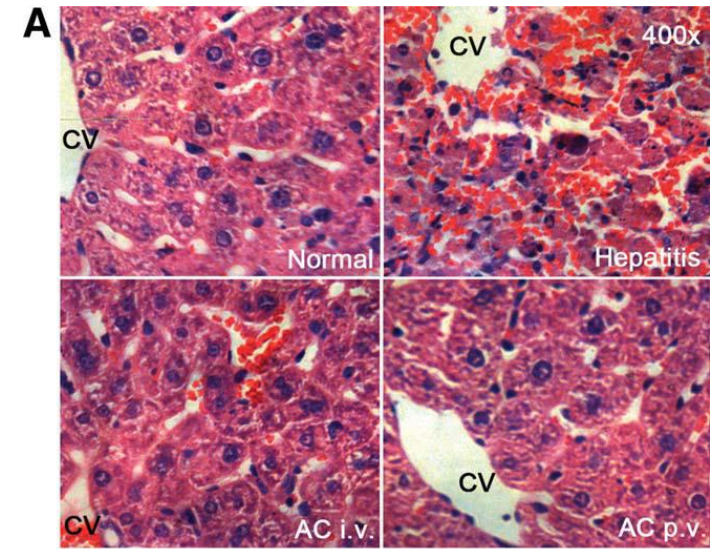
- LPS/D-GalN induced a fulminant hepatitis in mice with a high mortality within 12 hours.
- Infusion of donor ACs but not normal splenocytes (Supporting Fig. 2B,C) prior to LPS/D-GalN challenge, particularly via p.v. infusion, significantly attenuated liver injury (Fig. 2A,B; Supporting Fig. 3A,B).
- The timing of AC infusion was critical, with maximum protective effects at 3-7 days and much less effective within 3 days, prior to the LPS/D-GalN challenge (Supporting Fig. 3C,D).
- Maximum protective effects of the AC infusion were observed at doses of 1×10^7 to 3×10^7 ACs per mouse (p.v.; Supporting Fig. 3E,F). Somewhat surprisingly, a higher dose of the AC infusion at 2×10^8 per mouse had no protective effects. So, p.v. infusion of 1.5×10^7 ACs/mouse was used in all subsequent in vivo experiments.



Supporting Fig. 2 Transfer of normal splenocytes does not affect the pathogenesis of endotoxin-induced hepatitis. **(A)** Serum ALT levels 6h after LPS/D-GalN challenge in mice receiving different treatments. Normal: no challenge; PBS: PBS p.v. before challenge; Gdcl3: C57BL/6 mice (n=8) received GdCl₃. AC was injected at 3 d prior to LPS/D-GalN treatment; KC: p.v. injection of KC at 1 d prior to LPS/D-GalN treatment; KC+AC: KC and AC were injected simultaneously at 3 d prior to LPS/D-GalN treatment; KC/AC: AC-primed KC was injected at 1 d prior to LPS/D-GalN treatment. **(B,C)** C57BL/6 splenocytes or PBS were transferred via p.v. into syngeneic mice (n=10), three days later, LPS/D-GalN were injected. Survival was observed (B) and serum ALT levels were determined after 6h (C). **P<0.01 Experiments were performed for three times with similar results.

Fig. 2. Clearance of apoptotic cells suppresses endotoxin-induced hepatitis via Kupffer cells.

- (A) Histological analysis of liver tissue prior to LPS challenge (normal), at 6 hours after LPS/D-GalN injection alone (hepatitis) or with AC injection through i.v. (AC i.v.) or p.v. (AC p.v.). C57BL/6 mice were used in all above experiments.
- (B) C57BL/ 6 mice (n ¼ 10) were pretreated with PBS or AC (1.5 10⁷ /mouse) via p.v. 3 days prior to the mice were injected with LPS/D-GalN, and their survival was observed.
- (C) Different groups of mice were observed for their survival after LPS/D-GalN treatment. GdCl₃: C57BL/ 6 mice (n ¼ 8) received GdCl₃; AC: ACs were injected at 3 days prior to LPS/D-GalN treatment; KC: p.v. injection of KCs at 1 day prior to LPS/D-GalN treatment; KC+AC: KCs and ACs were injected simultaneously at 3 days prior to LPS/D-GalN treatment; KC/AC: AC-primed KCs were injected at 1 day prior to LPS/D-GalN treatment. All data are representative of 5 independent experiments. CV: central vein.

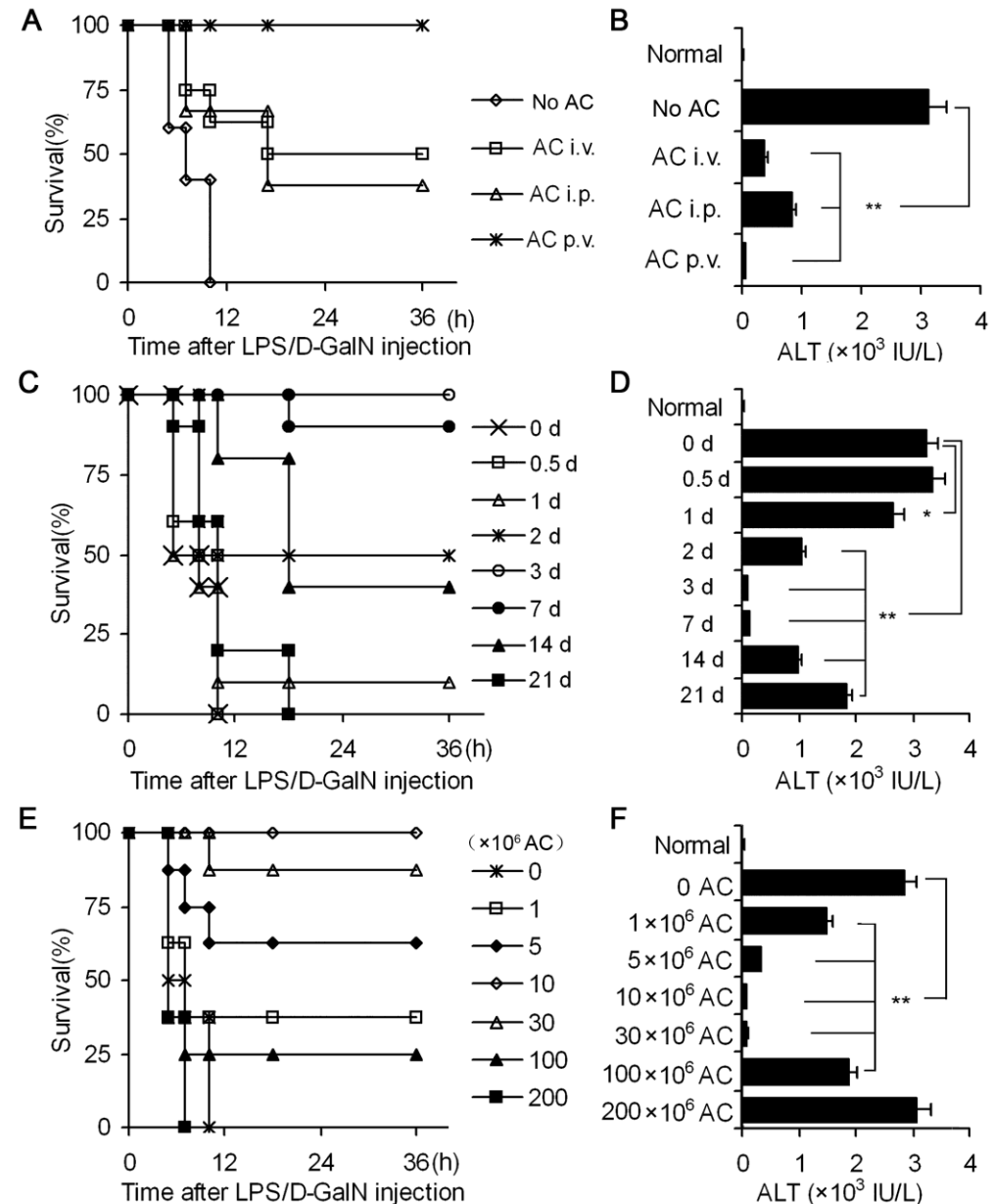


Supporting Fig. 3 Effects of the route, timing, and dosage of AC infusion on pathogenesis of endotoxin-induced hepatitis.

(A, B) Effects of AC infusion via different routes on hepatitis. C57BL/6 ACs (1.5×10^7 /mouse) were i.v., i.p. or p.v injected into syngeneic mice at 3 day prior to LPS/D-GalN. The control received no AC infusion. Survival (A) was observed and serum ALT (B) was detected at 6 h after LPS/D-GalN injection. Normal: naïve mice receiving no treatment. N=8 for i.v., 6 for i.p. and p.v., and 10 for the control (no AC infusion). **P<0.01.

(C, D) Effect of AC infused at different times on hepatitis. C57BL/6 mice (n=10) received syngeneic AC (1.5×10^7 /mouse, p.v.) at indicated time prior to LPS/D-GalN injection. Mice survival (C) and serum ALT (D) were assayed. *P<0.5, **P<0.01.

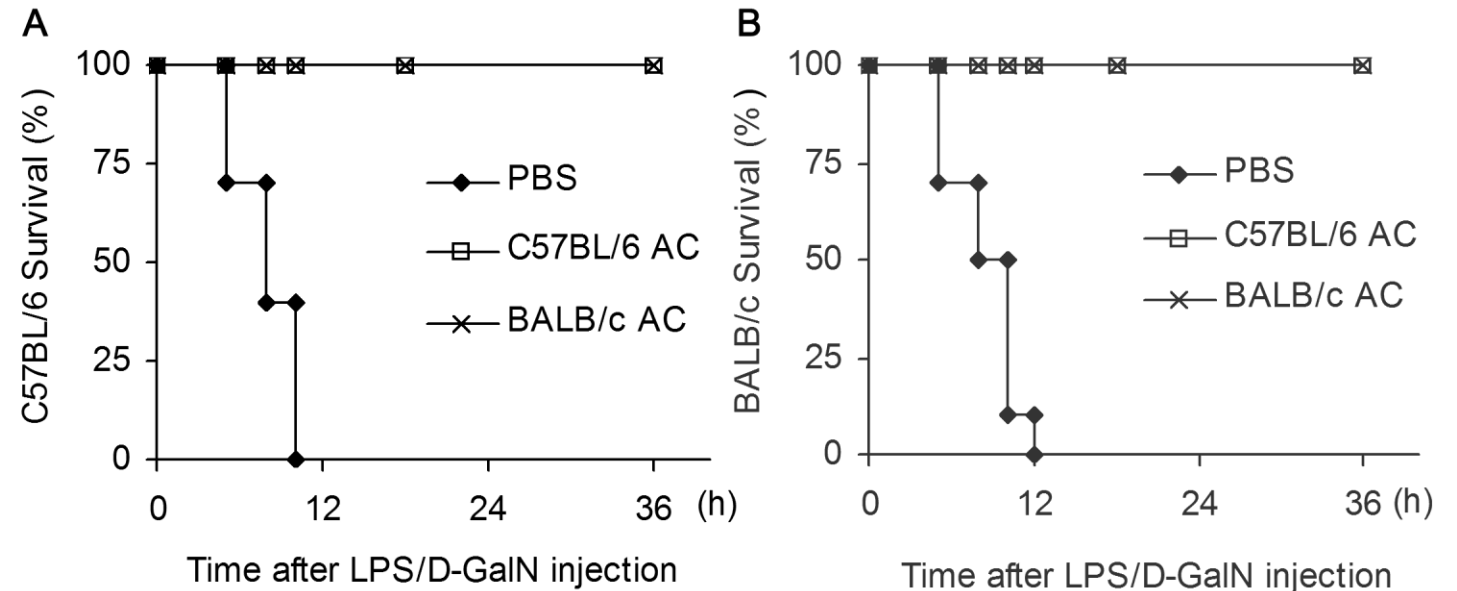
(E, F) Effect of AC infused at different dosages on hepatitis. C57BL/6 mice (n=8) received indicated amount of syngeneic AC (p.v.) at 3 day prior to LPS/D-GalN injection. The survival (E) and serum ALT (F) were assayed. **P<0.01. All data are representative of five independent experiments.



There is cross-inhibition of hepatitis by ACs between C57BL/6 and BALB/c mice (Supporting Fig. 4), indicating that the suppression of hepatitis by ACs is nonspecific.

Supporting Fig. 4 Non-specific suppression of inflammatory response by AC in the liver. **(A)** AC from C57BL/6 or BALB/c mice or PBS was injected into C57BL/6 mice (n=10) via p.v. and LPS/D-GalN were injected as in Fig. 2. The mice survival was determined. **(B)** AC from C57BL/6 or BALB/c mice or PBS was injected into BALB/c mice (n=10) via p.v. and LPS/D-GalN were injected three days later. The mice survival was determined. Experiments were performed for three times with similar results.

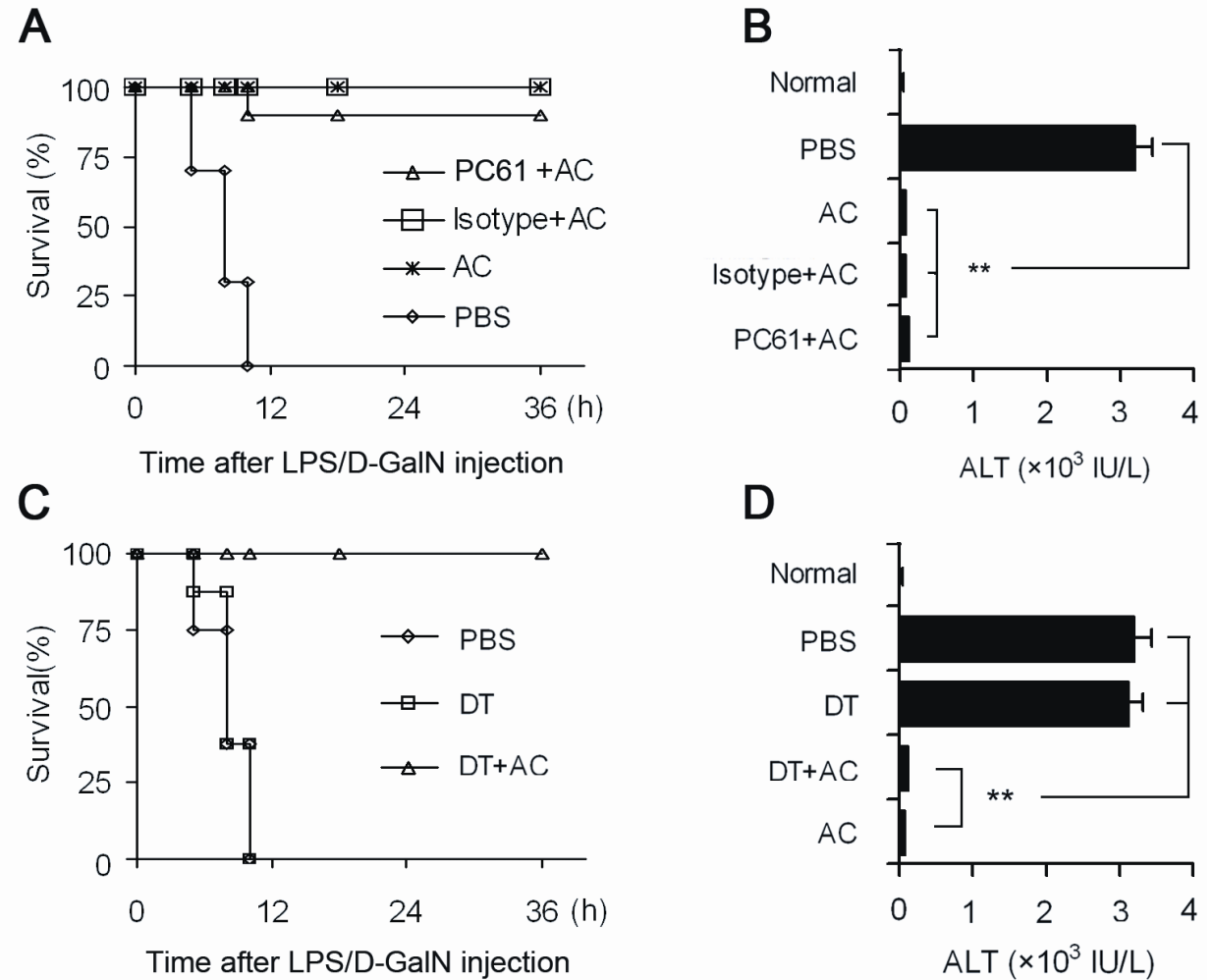
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- Depletion of KCs by GdCl₃ completely prevented LPS/D-GalN-induced hepatitis (Fig. 2C; Supporting Fig. 2A). Adoptive transfer of KCs, but not AC primed KCs, restored the hepatic susceptibility to LPS/D-GalN.
 - Depletion of either regulatory T cells (Supporting Fig. 5A,B) or DCs (Supporting Fig. 5C,D) could not eliminate the protective effects, indicating that regulatory T cells and DCs are not involved in the protective effect of AC infusion.
 - At 3 days after AC inoculation, no change of T helper 1/T helper 2 (Th1/Th2) cells was found (Supporting Fig. 6), suggesting that Th1/Th2 balance may not be involved in the protection by ACs.
- KCs are critical to mediate LPS/DGalN-induced fulminant hepatitis, and AC infusion can suppress KC-mediated LPS/D-GalN-induced fulminant hepatitis.

Supporting Fig. 5 Regulatory T cells and dendritic cells are not involved in the suppression of endotoxin-induced hepatitis by apoptotic cell infusion.

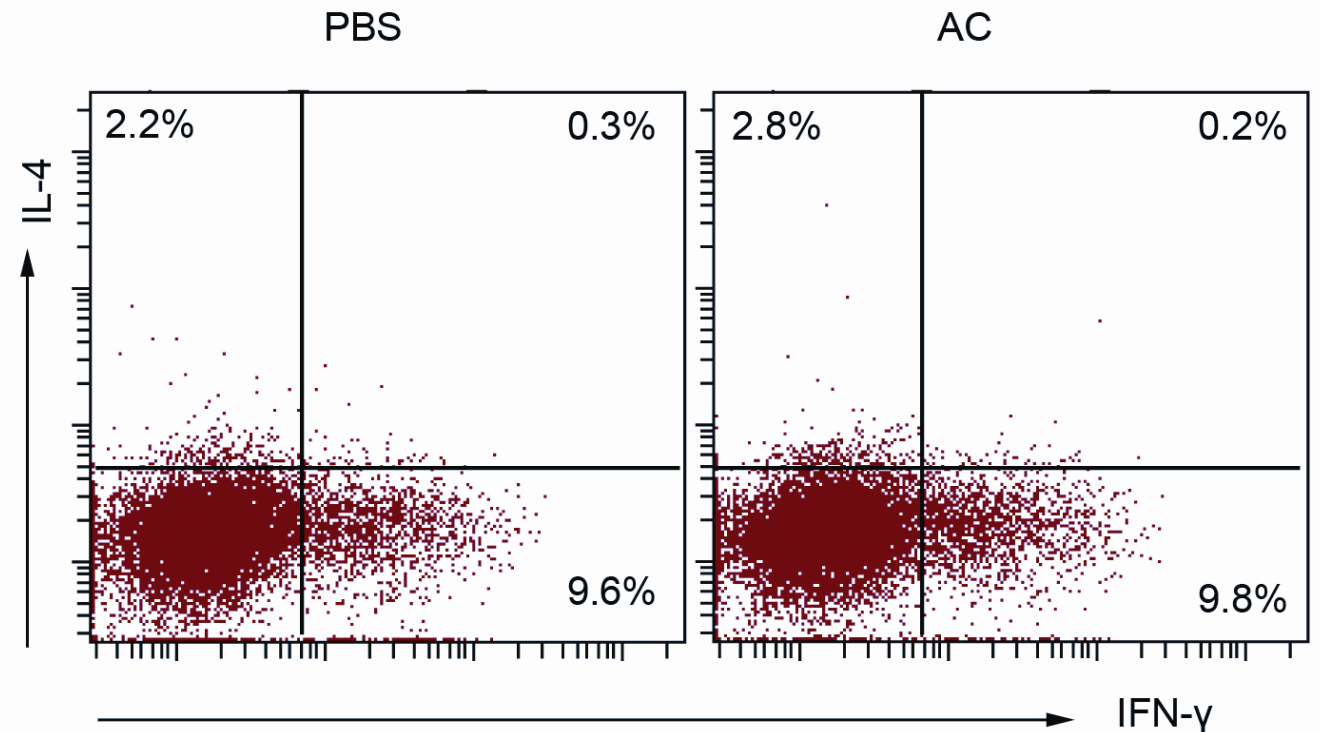
- (A) C57BL/6 mice received anti-CD25 antibody (PC61) or isotype antibody (Isotype) at 24 h prior to LPS/D-GalN treatment (n=10). AC was injected at 3 day prior to LPS/D-GalN treatment. PBS: mice were injected with PBS instead of AC prior to LPS/D-GalN injection.
- (B) Serum ALT level at 6 h after LPS/D-GalN injection in (A). Normal: naïve mice without treatment. **P<0.01.
- (C) CD11c-DTR mice received syngeneic AC at 24 h after DT infusion (n=8). Three days later, LPS/D-GalN were injected. PBS: mice were injected PBS instead of DT prior to LPS/D-GalN injection.
- (D) Serum ALT level in (C). Normal: naïve mice without treatment. **P<0.01. All data are representative of five independent experiments.



Supporting Fig. 6 No change of Th1/Th2 response in the liver 3 days after AC inoculation.

C57BL/6 mice were injected with syngeneic AC (1.5×10^7 /mouse) or PBS, 3 days later mononuclear cells from the liver were stimulated with PMA plus ionomycin for 6 h in vitro. Brefeldin A was added at the last 4 h culture, and the cells were collected and stained with DX5-FITC, IL-4-PE and IFN- γ -APC, assayed using BD LSR II and analyzed using BD FACSDiva software. Data shown are gated on CD4+DX5- cells, and are representative of three independent experiments.

Zhang M, et al. Supporting Fig. 6



Priming by ACs Inhibits Proinflammatory Cytokines but Enhances Anti-Inflammatory Cytokine Production of KCs in Response to LPS Stimulation

- When cultured alone, KCs appeared to be rounded and relatively nonadherent to the culture wall.
- Three days after AC priming, KCs became firmly adherent and ellipsoid in shape (Supporting Fig. 7A).
- Upon overnight culture with 10 ng/mL LPS, KCs became more tightly adherent and the cell skirt stretched out.
- Surface expression of costimulatory molecules (CD40, CD80, and CD86), CD14, and B7-H1 on AC-primed KCs remained comparable to the controls (Supporting Fig. 7B).

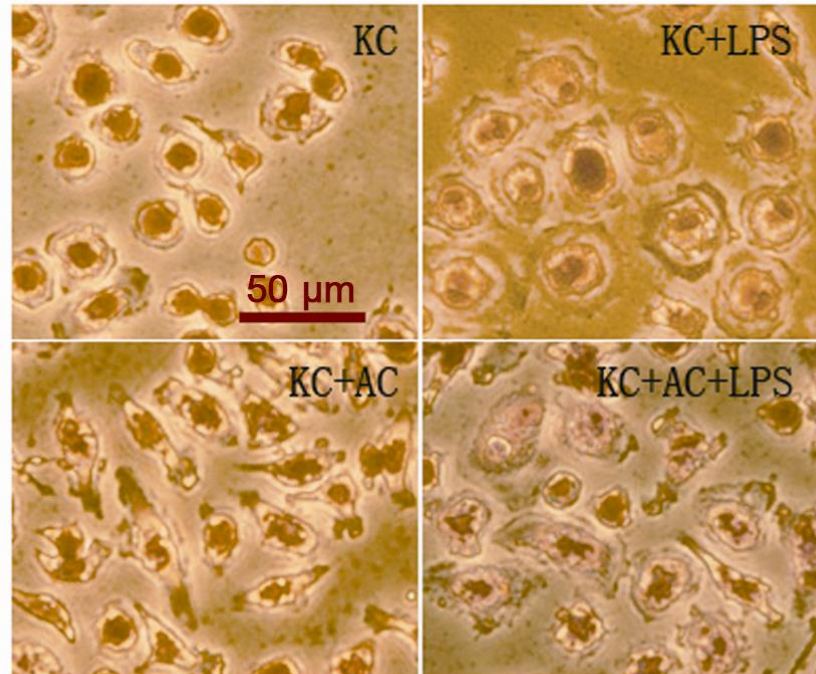
Supporting Fig. 7 Priming with apoptotic cells affects the morphology and phenotype of Kupffer cells.

(A) C57BL/6 KC was cultured with syngeneic AC (1:10) for 4 h, washed and cultured for additional 3 days prior to stimulation with LPS (10 ng/mL for 12 h). Wright-Giemsa staining; bar = 50µm.

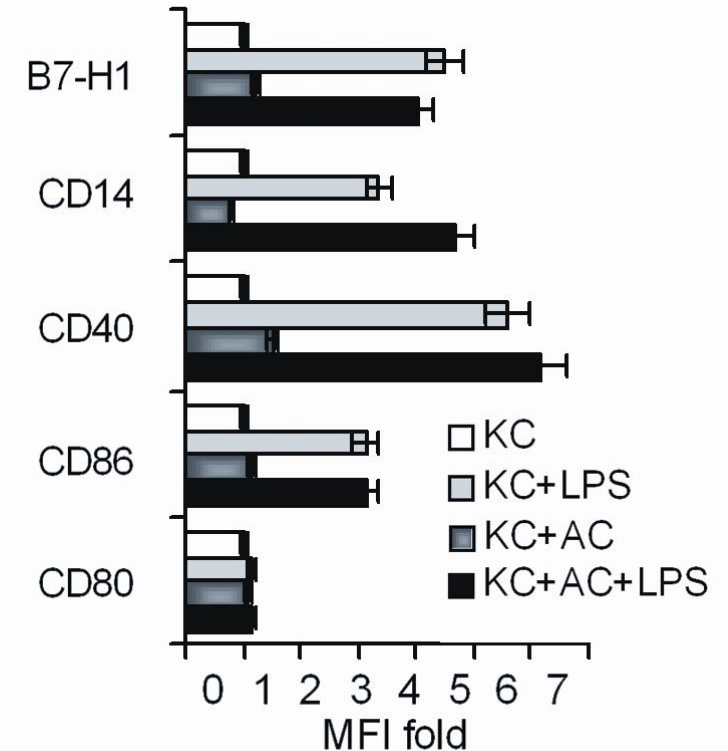
(B) FACS analysis of KC obtained in A for membrane expression of CD80, CD86, CD40, CD14 and B7-H1 on F4/80+ cells was shown. All data are representative of three independent experiments.

Zhang M, et al. Supporting Fig. 7

A

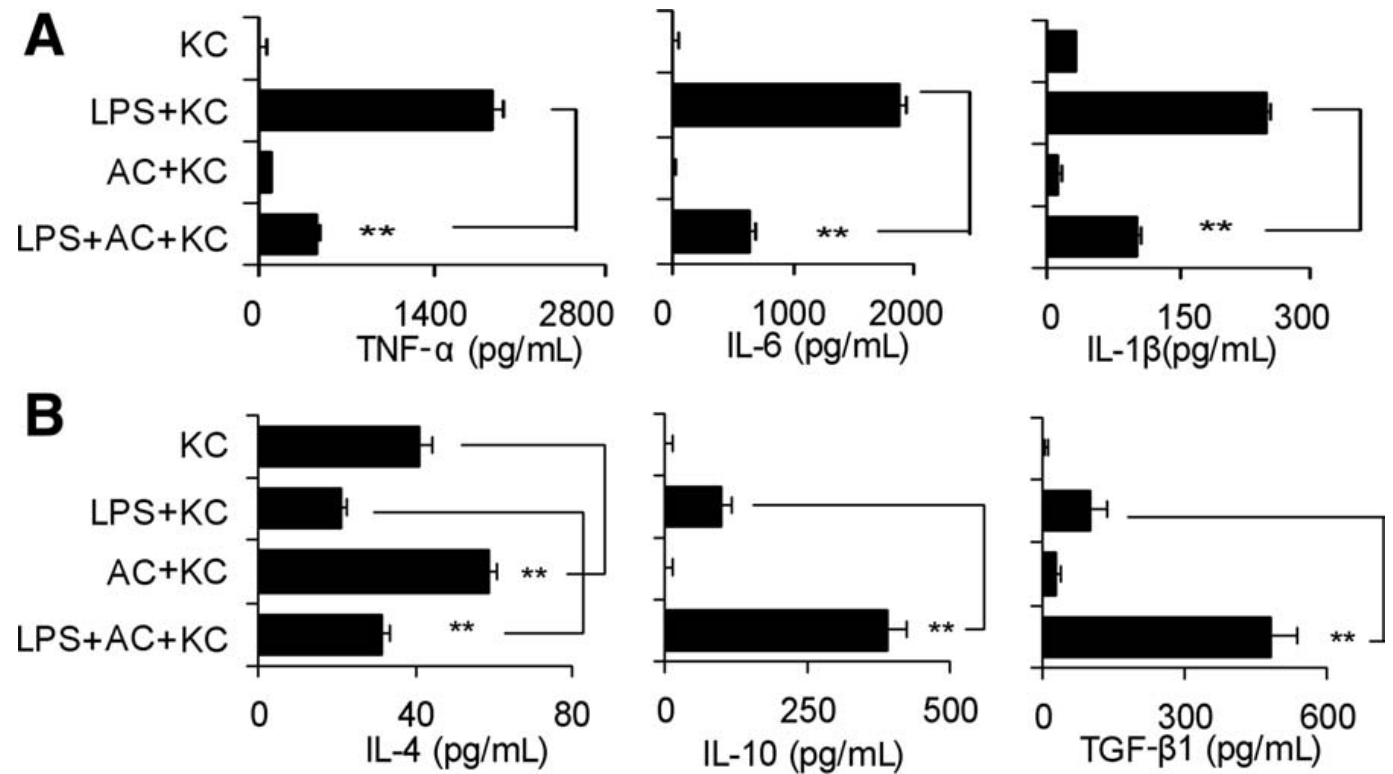


B



AC priming alone did not affect KC production of cytokines. However, LPS induced cytokines were significantly altered by AC primed KCs: proinflammatory cytokines (TNF- α , IL6, IL-1b) were significantly reduced (Fig. 3A); antiinflammatory cytokines (IL-4, IL-10, and TGF- β) were significantly enhanced (Fig. 3B).

Fig. 3. Priming with apoptotic cells affects the cytokine secretion of Kupffer cells. **(A,B)** KCs were cultured with ACs (1:10) for 4 hours, washed, and cultured for an additional 3 days. After stimulation with LPS (10 ng/mL) for 12 hours in fresh culture media, the supernatant was collected, and (A) inflammatory cytokines TNF- α , IL-6, and IL-1b and (B) antiinflammatory cytokines IL-4, IL-10, and TGF- β were assayed. **P < 0.01. All data are representative of 3 independent experiments using C57BL/6 mice, and there are similar results in experiments using BALB/c mice (data not shown).



Priming by ACs Reduces the Cytotoxicity of LPS Triggered KCs to Hepatocytes by IL-10-Mediated Suppression of TNF- α and NO Production

LPS-stimulated KCs exhibited potent cytotoxicity against hepatocytes.

This cytotoxic effect of KCs was significantly attenuated by AC priming or an anti-TNF- α antibody (Fig. 4A,B).

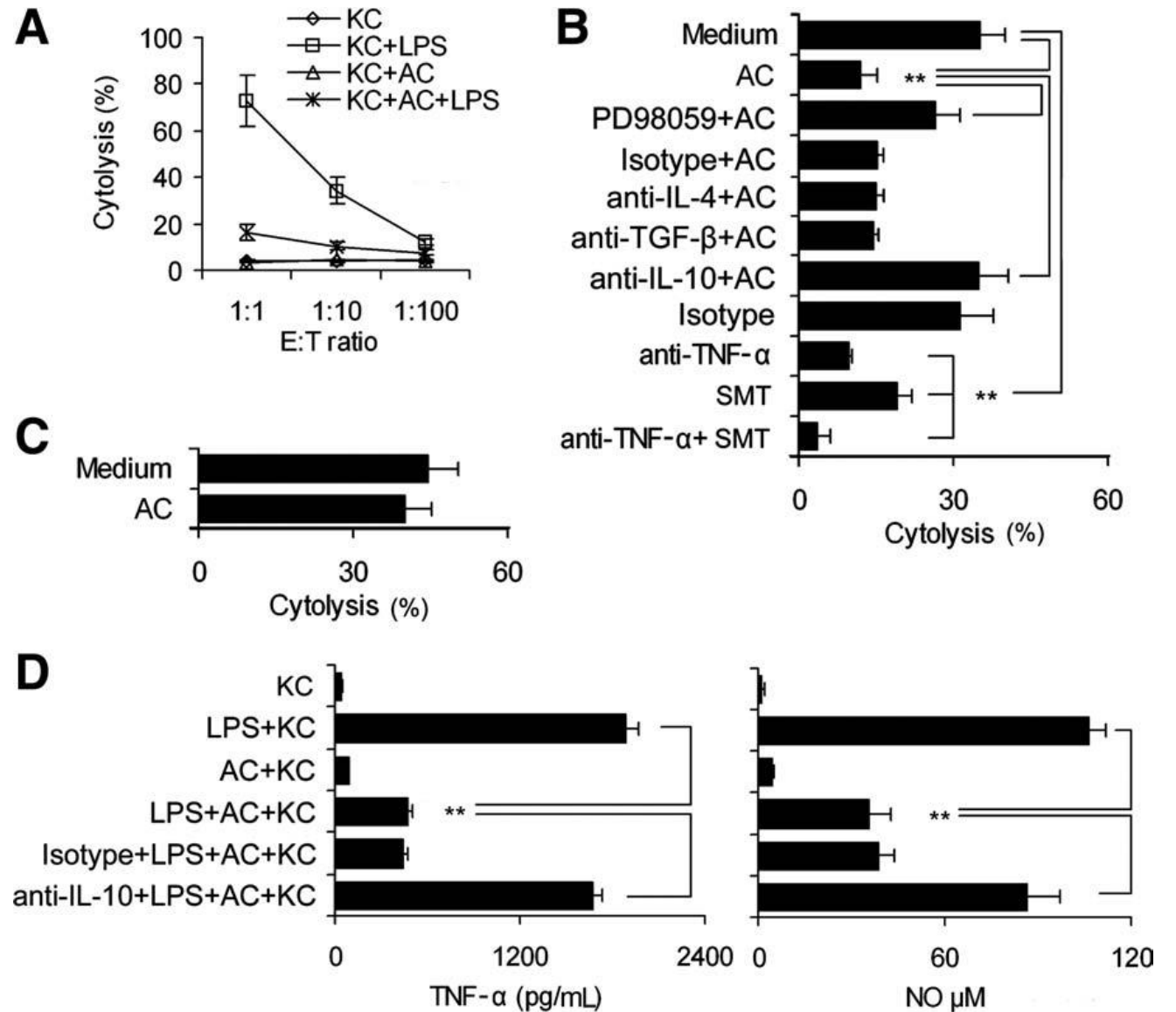
In vitro use of neutralizing anti-IL10 antibodies (Abs), but not anti-IL-4 or anti-TGF- β Abs, could eliminate the inhibition of hepatocytes cytotoxicity by ACs (Fig. 4B).

If IL-10-deficient KCs were used instead of wild-type KCs as above, AC priming could not inhibit the cytotoxicity of KCs and prevent decrease of live hepatocytes number (Fig. 4C).

S-methylisothiourrea sulfate (SMT), a specific inducible nitric oxide synthase (iNOS) inhibitor, also dramatically reduced the death of hepatocytes induced by KCs. An anti-TNF- α antibody significantly enhanced the inhibitory effect of SMT on KC-mediated death of hepatocytes. Furthermore, neutralizing anti-IL-10 Abs reversed the inhibition of TNF- α and NO production of KCs by AC priming (Fig. 4D), consistent with a previous report that IL-10 can target TNF- α messenger RNA (mRNA) to inhibit its translation.

These data suggest that IL-10 derived from AC-primed KCs could inhibit the KC-mediated cytotoxicity of hepatocytes through inhibition of TNF- α and NO production.

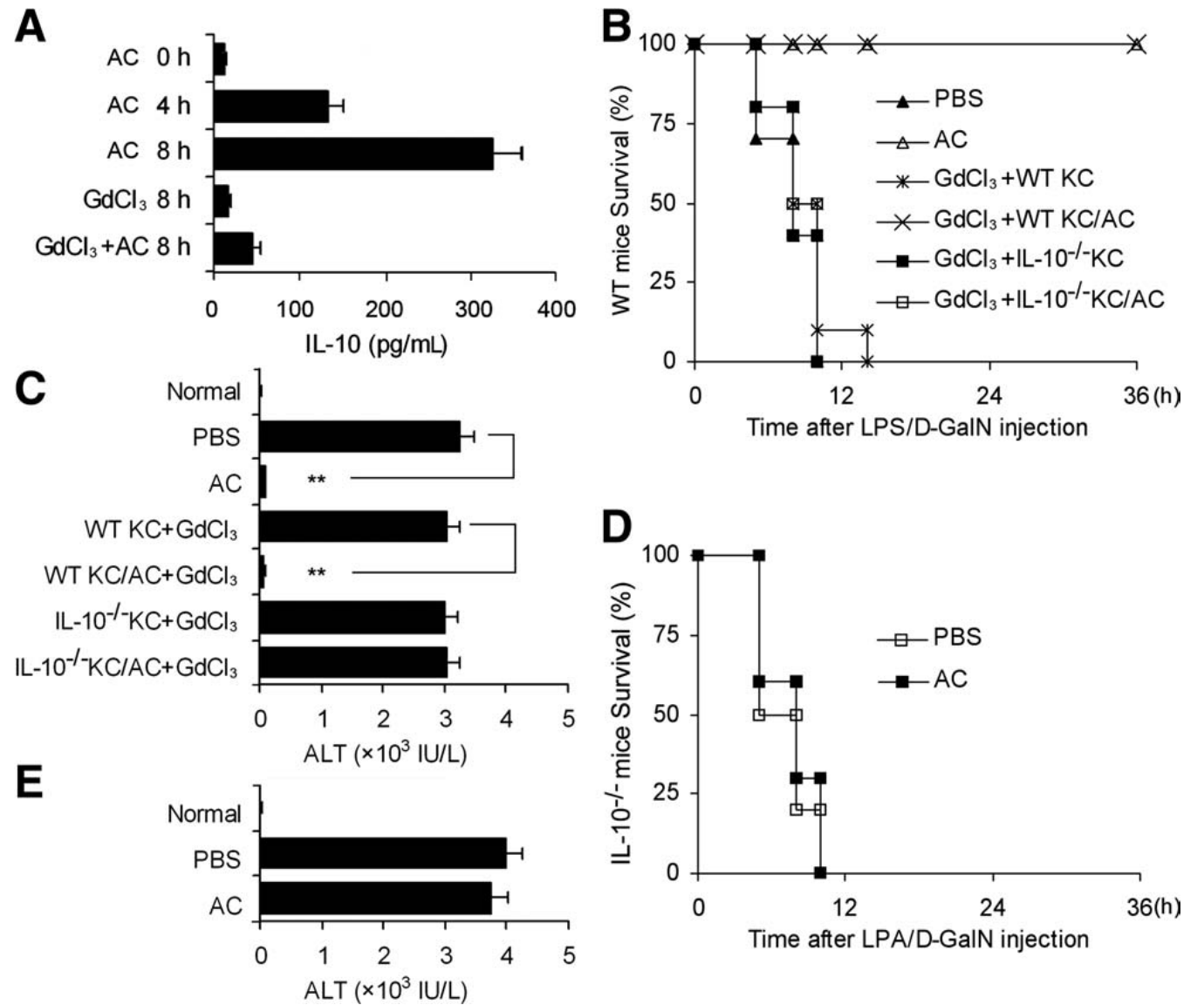
Fig. 4. IL-10 is responsible for reduced cytotoxicity of LPS-stimulated Kupffer cells to hepatocytes through suppression of TNF- α and NO production. **(A)** C57BL/6 KCs were primed with syngeneic ACs for 4 hours, and then cultured alone for 3 days prior to stimulation with LPS for 12 hours. KCs were then cocultured with hepatocytes at the indicated E:T (KC:hepatocytes) ratio. The number of live hepatocytes was assayed using anti-F4/80-APC Ab, Annexin V-FITC, and 7-AAD. The cytotoxicity (%) = $\frac{1}{4}$ (7AADAnnexin V F4/80/F4/80) 100. **(B)** C57BL/6 wild-type (WT) KCs were primed with syngeneic ACs as described in A, and then stimulated with LPS. The syngeneic hepatocytes were mixed with KC cells at an E:T of 1:10. PD98059 (25IM) was added at 1 hour before AC priming. Anti-IL-4 Ab, anti-TGF- β Ab, anti-IL-10 Ab, anti-TNF- α Ab, S-methylisothiourea sulfate (SMT) or anti-TNF- α Ab plus SMT were added immediately prior to LPS stimulation. **P < 0.01. **(C)** IL-10-deficient KCs were primed with ACs as described in A, and then stimulated with LPS. The hepatocytes were mixed with KCs at an E:T of 1:10. The cytotoxicity of hepatocytes is shown. **(D)** C57BL/6 KCs were primed with syngeneic ACs for 4 hours and cultured for 3 days, then stimulated with LPS for 12 hours in the presence or absence of anti-IL-10 Ab. TNF- α and NO production by KCs was assayed. **P < 0.01. Data are representatives of 3 experiments with similar results.



KC-Derived IL-10 Is Responsible for the Suppression of KC-Mediated Endotoxin-Induced Fulminant Hepatitis by ACs

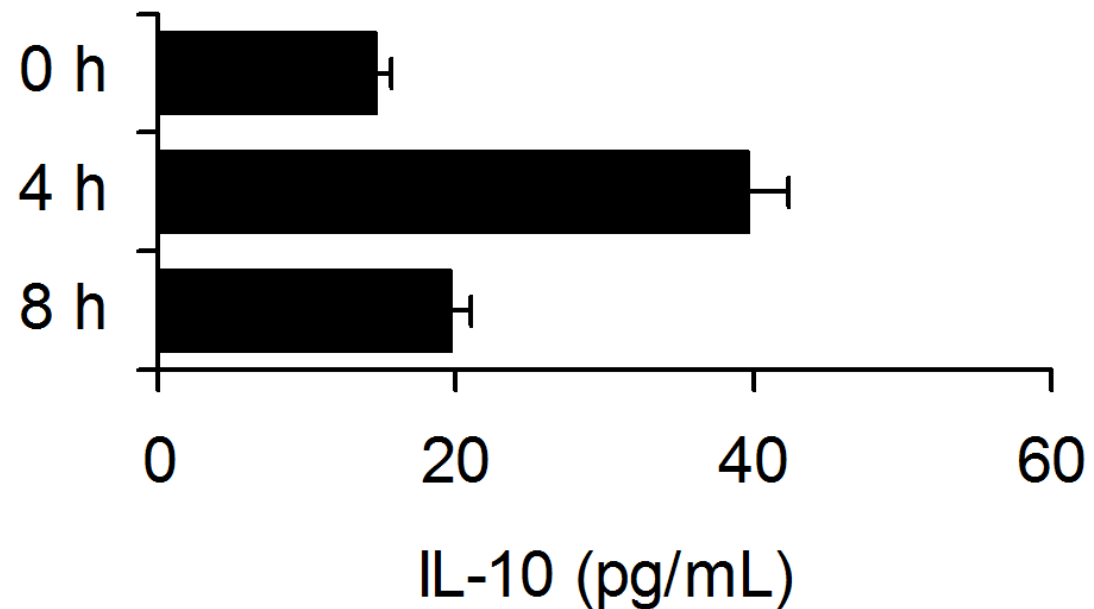
- Considering the importance of IL10 in immune suppression, serum IL-10 level in AC preinjected mice was assayed, showing significant increase after LPS/D-GalN injection (Fig. 5A).
 - In normal mice without AC injection, LPS/D-GalN stimulation could increase the IL-10 production in the serum at a very low level, with increase at 4 hours but decrease at 8 hours after the LPS/D-GalN injection (Supporting Fig. 8).
 - Once KCs were made dysfunctional with GdCl₃ prior to AC infusion, the IL-10 response was significantly inhibited (Fig. 5A), suggesting KCs were the major producer of IL-10 after AC infusion.
 - Infusion of IL-10- deficient KCs, either naive or AC-primed, into mice already depleted of KCs with GdCl₃ did not protect the liver from LPS challenge (Fig. 5B,C).
 - Infusion of ACs to IL-10-deficient mice did not protect the liver from LPS-induced damage (Fig. 5D,E).
- IL-10 derived from KCs is critical for the suppression of endotoxin-induced hepatitis by AC infusion.

Fig. 5. Kupffer cell-derived IL-10 is responsible for the suppression of Kupffer cell-mediated fulminant hepatitis. **(A)** Serum IL-10 level was assayed in C57BL/6 mice (n = 6) at the indicated time after LPS/D-GalN injection. AC: AC (1.5×10^7 /mouse, p.v.) was injected at 3 days prior to LPS/D-GalN injection, GdCl₃: GdCl₃ was injected at 24 hours before LPS/D-GalN injection. GdCl₃+AC: AC was injected p.v. at 24 hours after GdCl₃ injection, and 3 days later, LPS/D-GalN was injected. **(B)** KCs derived from wild-type (WT) mice or IL-10-deficient (IL-10^{-/-}) mice were p.v. injected into WT C57BL/6 mice (n = 10) at 24 hours after GdCl₃ infusion. One day later, these mice were injected with LPS/D-GalN. AC: mice received LPS/D-GalN at 3 days after AC injection; PBS: mice received LPS/D-GalN at 3 days after PBS injection; KC/AC: AC-primed KCs. The survival of the mice receiving the different treatments was observed. **(C)** Serum ALT of the mice in B at 6 hours after LPS/D-GalN injection. Normal: mice receiving no treatment. **P < 0.01. **(D)** IL-10-deficient (IL-10^{-/-}) mice (n = 10) received LPS/D-GalN at 3 days after AC injection. The survival of the mice was observed. **(E)** Serum ALT of mice in D at 6 hours after LPS/D-GalN injection. Normal: mice receiving no treatment. Experiments were performed 3 (A) or 5 (B-E) times with similar results.



Supporting Fig. 8 Serum IL-10 level after LPS/D-GalN injection in mice without AC pretreatment. Normal C57BL/6 mice (without AC pretreatment) were injected LPS/D-GalN, serum IL-10 levels were determined using ELISA kit (R&D systems) at the indicated times later. Data are representative of three independent experiments.

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Membrane-Bound TGF- β on ACs Is Responsible for the Increased IL-10 Production of KCs

Cultured-alone ACs released IL-10 and TGF- β upon UV-B irradiation (Supporting Fig. 9).

However, adding supernatant from culture-alone ACs did not induce IL-10 production of KC culture, suggesting soluble factor(s) from ACs are not responsible for the increased IL-10 production of KCs.

In the KC and AC coculture system, using transwell (0.4 μ m pore size) to separate KCs from ACs dramatically reduced IL-10 production (Fig. 6A,B).

Blockade of phagocytosis by cytochalasin B could not prevent IL-10 production of KC to AC priming, suggesting that cell-cell contact between KCs and ACs, but not phagocytosis, is critical for IL-10 induction.

Neutralizing anti-TGF- β 1 but not anti-IL-10 Ab blocked IL-10 production of KC to AC priming (Fig. 6B).

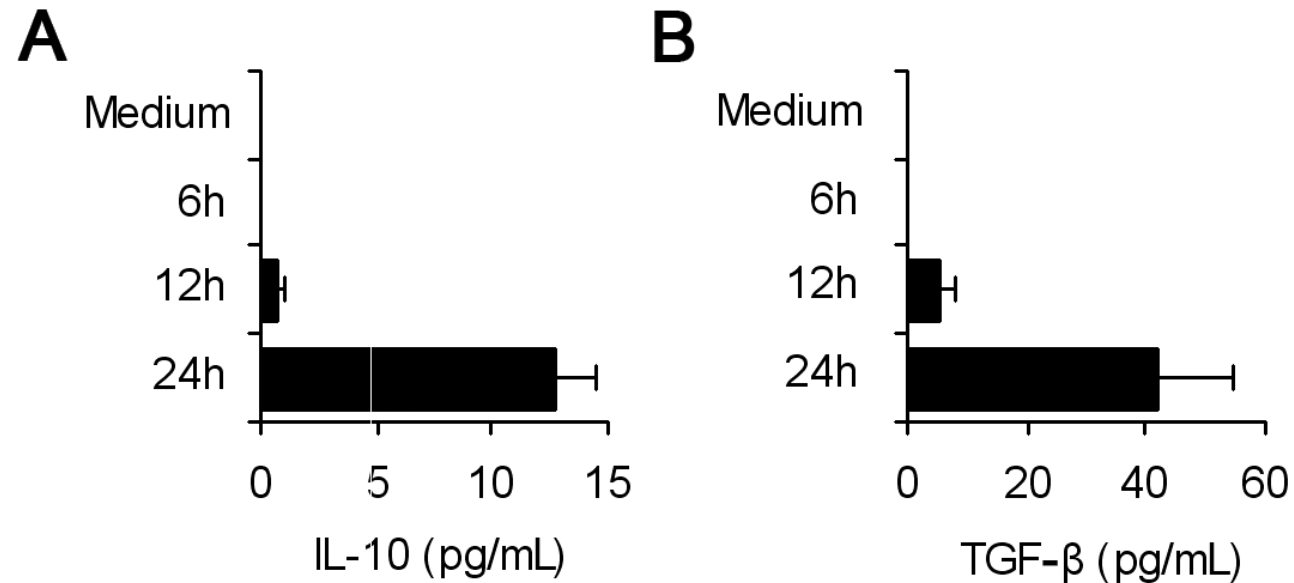
However, the recombinant murine TGF- β 1 did not increase IL-10 production as ACs did.

Smad3-deficient mice are deficient in TGF- β signal transduction and hence were used to further examine the role of TGF- β . AC priming did not increase IL-10 production in KCs from Smad3-deficient mice (Fig. 6C).

Consistently, when Smad3^{-/-} KCs primed with ACs were transferred to GdCl₃-pretreated mice, the protective effect of ACs on hepatitis disappeared (Supporting Fig. 10A,B).

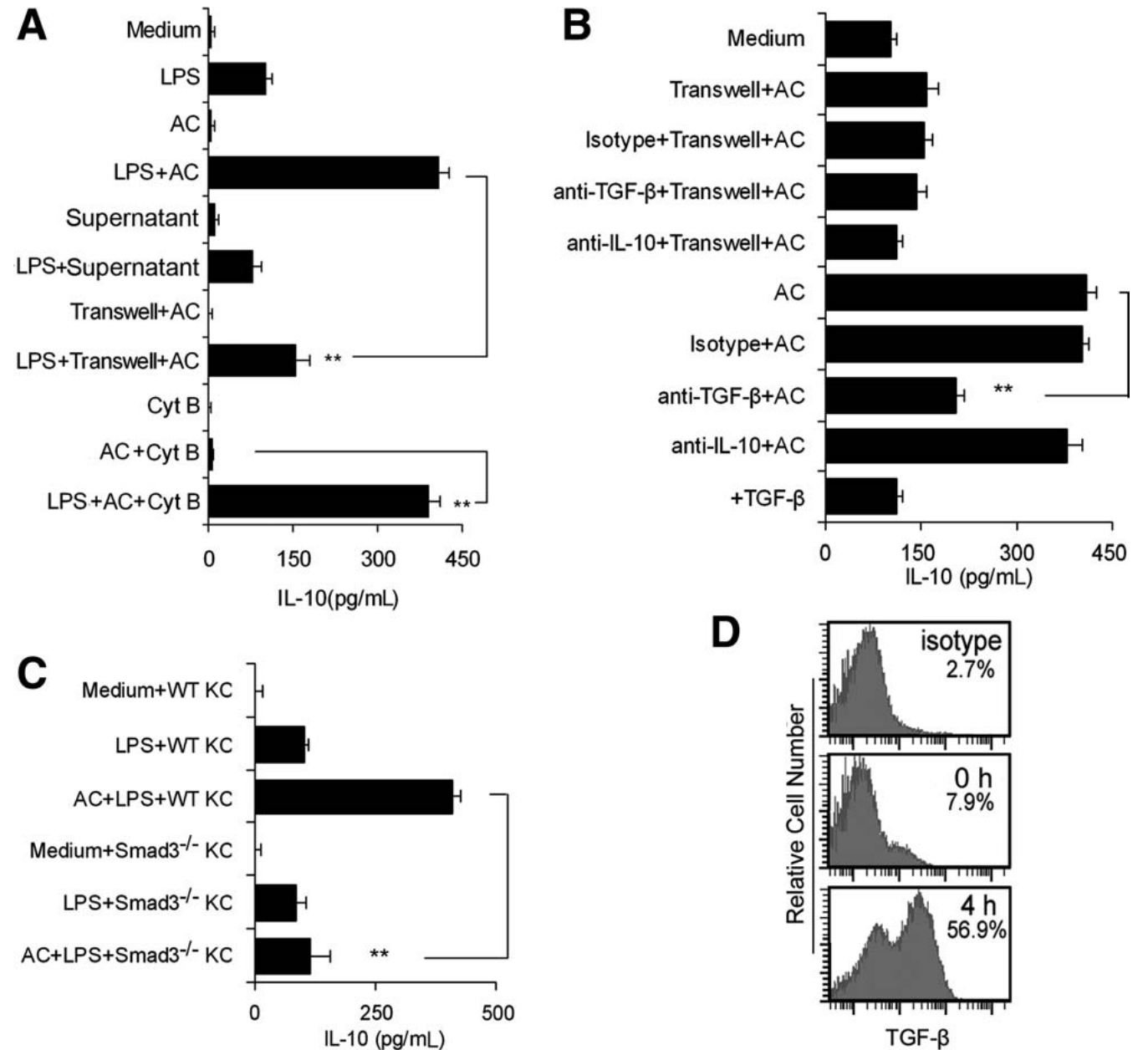
FACS analysis revealed a significant increase of membrane-bound TGF- β 1 on ACs (Fig. 6D), and up to 56.9% cells expressed membrane-bound TGF- β 1 at 4 hours after UV-B irradiation. So, it is membrane-bound TGF- β on ACs that induces IL-10 production from KCs.

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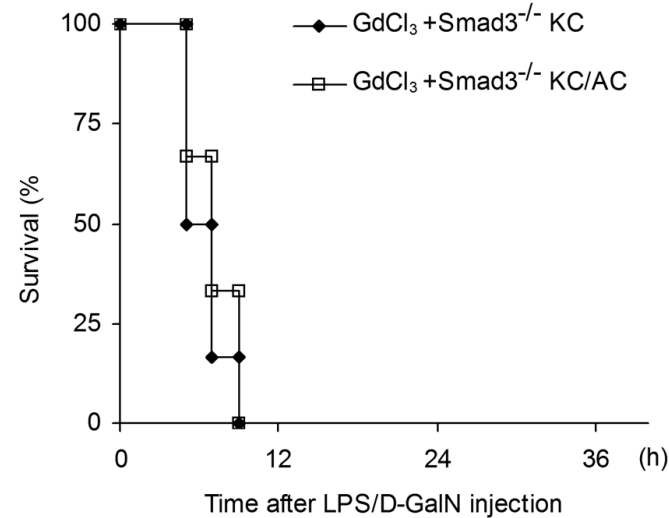
Supporting Fig. 9 IL-10 and TGF-β release from apoptotic cells. After UV-B irradiation, the apoptotic C57BL/6 splenocytes were cultured. IL-10 (A) and TGF-β (B) in the supernatants were measured using ELISA kit at 6 h, 12 h and 24 h.

Fig. 6. Membrane-bound TGF- β on apoptotic cells is responsible for increased IL-10 production by apoptotic cell-primed Kupffer cells. **(A)** Supernatant of ACs cultured for 6 hours (Supernatant) was used to culture KCs. Transwell was used to separate ACs (upper chamber) from KCs (lower chamber). Cytochalasin B (Cyt B) pretreated KCs were cocultured with AC. Four hours later, the KCs were washed and cultured with fresh culture medium for an additional 3 days prior to LPS stimulation (10 ng/mL for 12 hours). IL-10 in the supernatant was measured with ELISA. Both KC and AC were derived from C57BL/6 mice. ****P < 0.01.** **(B)** In the coculture system of KCs and ACs, transwell was used to separate the upper ACs and the lower KCs. Anti-IL-10, anti-TGF- β , or isotype antibody was added to the KC culture just before AC addition. And recombinant murine TGF- β was used to replace ACs (+TGF- β). Four hours later, the KCs were washed and cultured for 3 days with fresh culture medium. IL-10 in supernatant was assayed after LPS stimulation for 12 hours. Both KCs and ACs were derived from C57BL/6 mice. ****P < 0.01.** **(C)** Smad3^{+/+} wild-type (WT) or Smad3-deficient (Smad3^{-/-}) KCs were cocultured with ACs derived from homozygous littermates Smad3^{+/+} WT mice for 4 hours, then washed to get rid of the uncaptured ACs, cultured for 3 days, and stimulated with LPS for 12 hours. IL-10 concentration in the supernatant was measured using ELISA. ****P < 0.01.** **(D)** Membrane-bound TGF- β on ACs was determined by FACS at the indicated time after UV-B irradiation. The membrane-bound TGF- β -positive cell percentage is shown. Isotype: isotype antibody staining of cells 2 hours after UV-B irradiation. Data are representative of 3 independent experiments.

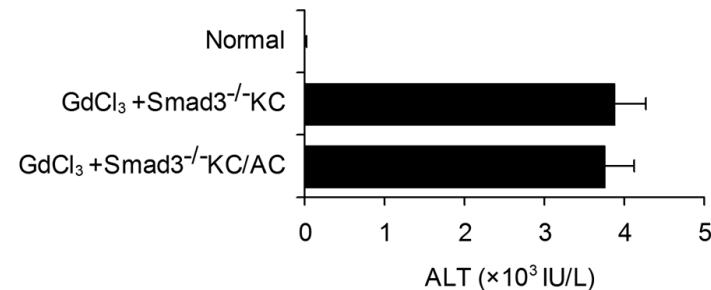


Supporting Fig. 10 TGF- β pathway in Kupffer cells is involved in AC induced protection of hepatitis. (A,B) KC derived from Smad3^{-/-} mice were p.v. injected into Smad3^{+/+} homozygous littermates (wild-type, n=6) at 24 h after GdCl₃ infusion. One day later, these mice were injected with LPS/D-GalN. KC/AC: AC-primed KC. And AC used above is from Smad3^{+/+} homozygous littermates. The survival (A) and the serum ALT levels at 6h after LPS/D-GalN injection (B) of the mice receiving different treatments were observed. (C) Membrane-bound TGF- β on AC was determined by FACS at indicated time after UV-B irradiation. Data are representative of three independent experiments.

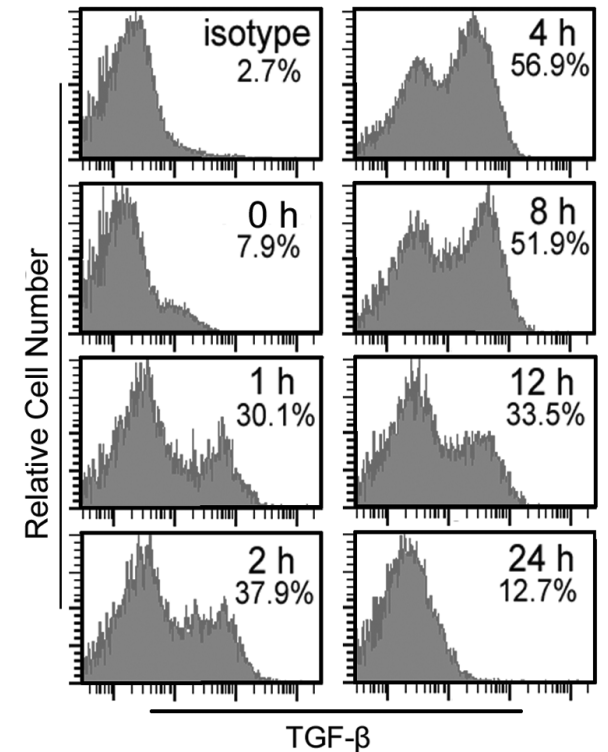
A



B



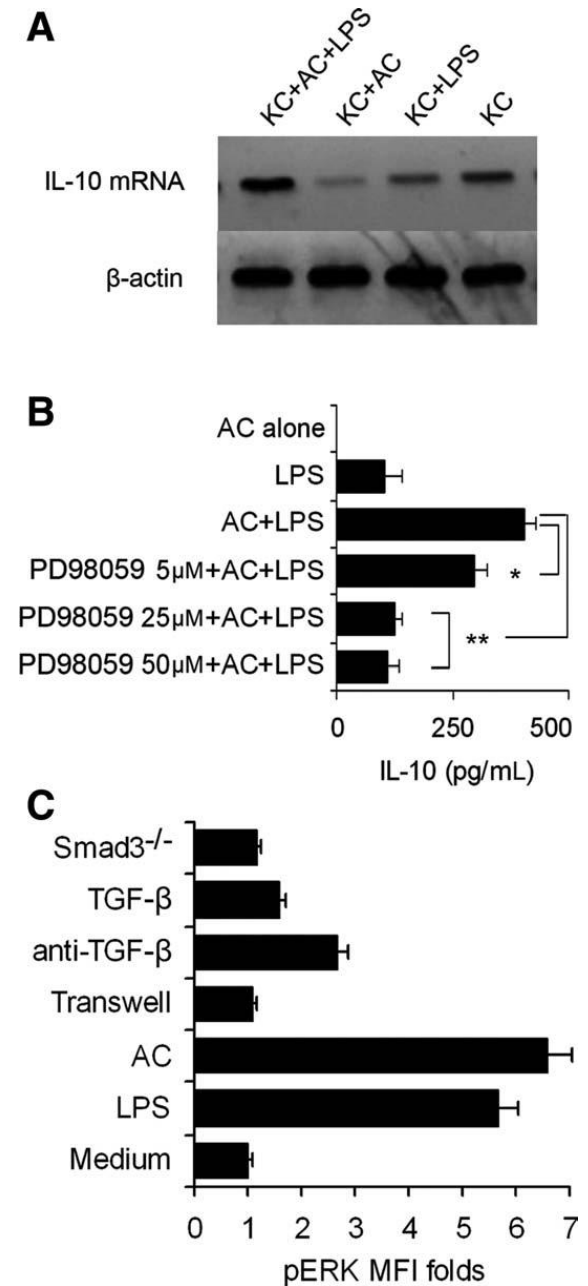
C



ERK Pathway Activated by Membrane-Bound TGF- β on ACs Is Responsible for the Increased IL10 Production in KCs

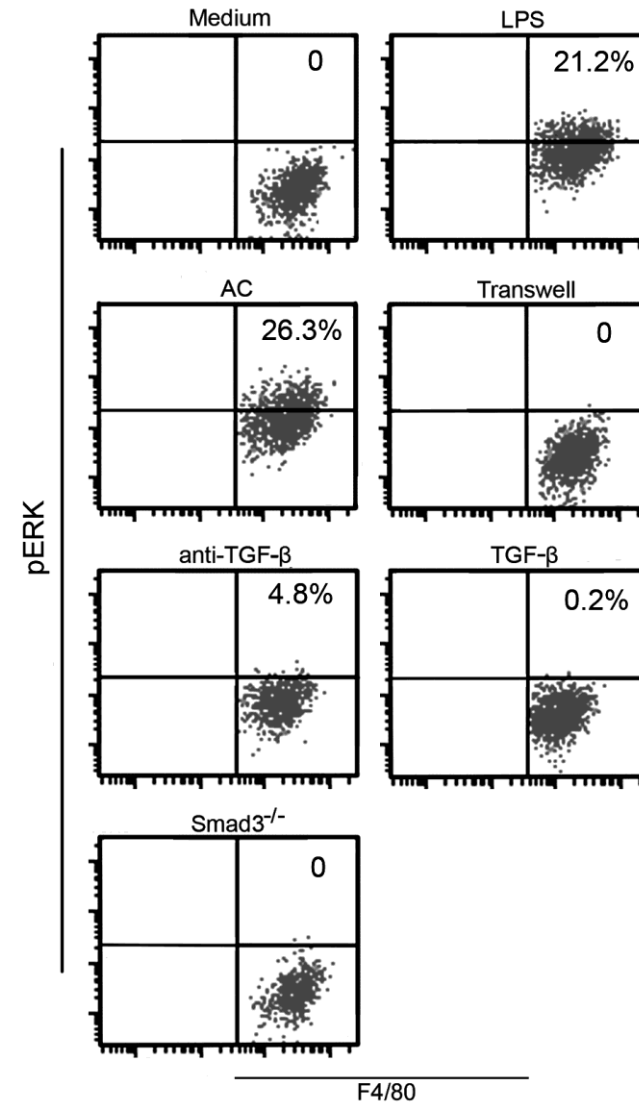
- Reverse transcription polymerase chain reaction (RT-PCR) assays showed that, after AC priming, IL-10 mRNA of KCs increased significantly in response to LPS (Fig. 7A).
 - IL-10 production of KCs in response to AC priming could be significantly reduced when the ERK pathway was suppressed by PD98059, a specific inhibitor of ERK (Fig. 7B), but inhibition of the JNK or p38 pathway could not influence the IL-10 production (data not shown).
 - FACS analysis revealed that contact with ACs increased phosphorylation of ERK in KCs (Fig. 7C; Supporting Fig. 11).
 - A neutralizing antibody against TGF- β 1 eliminated the ERK activation of KCs in response to AC priming. However, ERK in wild-type KCs was not activated by recombinant murine TGF- β , and not in KCs from Smad3-deficient mice in response to AC priming.
- > Membrane-bound TGF- β on ACs activates the ERK pathway in KCs, leading to IL-10 production.

Fig. 7. ERK pathway activated by membrane-bound TGF- β on apoptotic cells is responsible for increased IL-10 production in Kupffer cells. **(A)** C57BL/6 KCs were primed by syngeneic ACs for 4 hours, then uncaptured ACs were removed, and cultured for 3 days, then stimulated by LPS for 12 hours. IL-10 expression was examined with RT-PCR analysis. **(B)** C57BL/6 KC was stimulated with syngeneic ACs and LPS as described above. At 1 hour before adding ACs, PD98059, a specific ERK inhibitor, was added. Three days later, KC was stimulated by LPS for 12 hours. IL-10 in the supernatant was determined using ELISA. * $P < 0.5$, ** $P < 0.01$. **(C)** Thirty minutes after adding ACs or LPS, KCs were collected for analysis of pERK using a Phosflow method; pERK: fluorescence intensity (MFI) of all F4/80-positive cells is shown. Culture medium was used as a negative control; LPS was used as a positive control. Transwell was used to separate the upper ACs and the lower KCs. Anti-TGF- β , the neutralizing anti-TGF- β antibody, was added before AC priming. TGF- β : recombinant murine TGF- β was used instead of ACs. Smad3 $^{-/-}$: Smad3-deficient KCs were primed by ACs. All KCs except for Smad3 $^{-/-}$ mice were derived from C57BL/6 mice stimulated with syngeneic ACs (Smad3 $^{-/-}$ KCs were stimulated with ACs from Smad3 $^{+/+}$ homozygous littermates). All data are representative of 3 independent experiments. pERK: phosphorylated ERK.



Supporting Fig. 11 Membrane-bound TGF- β on apoptotic cells activates ERK pathway in Kupffer cells. Thirty minutes after adding AC or LPS, KC was collected for analysis of phosphorylated ERK (pERK) using a Phosflow method; the percentage of pERK positive cells in F4/80 positive cells was shown. Culture medium was used as a negative control; LPS was used as a positive control. Transwell was used to separate the upper AC and the lower KC. Anti-TGF- β : the neutralizing anti-TGF- β antibody was added before AC priming. TGF- β : recombinant murine TGF- β was used instead of AC. Smad3^{-/-}: Smad3-deficient KC was primed by AC. Data are representative of three independent experiments.

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Discussion

- Dysfunction of AC clearance may result in the pathogenesis of autoimmune diseases, such as systemic lupus erythematosus or diabetes.
- ACs or apoptotic bodies prepared from different systems at different time points may induce different effects, proinflammatory or anti-inflammatory, once infused in vivo.
- Phagocytes may be primed to become suppressive in the early stage after AC injection, releasing antiinflammatory cytokines and forming a nonspecific inhibitory microenvironment. However, 7 days later, donor-specific tolerogenicity may be established following Treg induction.
- ACs themselves could release soluble IL-10 and TGF- β after UV-B irradiation, and it is reported that these cytokines from ACs contribute to an immunosuppressive milieu, but the authors found these soluble factors released from ACs are not involved in the priming of KCs to be immunosuppressive. As for TGF- β , it can work in a membrane-bound manner as well as in a soluble manner.

Conclusion

- Membrane-bound TGF- β on ACs stimulates IL-10 production by KCs via activation of ERK.
- Then, IL-10 inhibits TNF- α and NO generation from KCs, and by doing so inhibits immune/inflammatory responses of the liver to LPS/D-GalN, preventing KC-mediated endotoxin-induced fulminant hepatitis.
- The authors provide a new pathway for suppressing liver injury by AC.

Literature

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