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CD49a promotes T-cell-mediated hepatitis by driving T helper 1 cytokine and interleukin-17 production

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Summary

It is becoming increasingly clear that the T-cell-mediated immune response is important in many diseases. In this study, we used concanavalin A (Con A) -induced hepatitis to investigate the role of CD49a in the molecular and cellular mechanism of the T-cell-mediated immune response. We found that CD49a$^{-/-}$ mice had significantly reduced levels of serum alanine aminotransferase and were protected from Con A-induced hepatitis. CD49a deficiency led to decreased production of interferon-γ (IFN-γ) and interleukin-17A (IL-17A) after Con A injection. Furthermore, we found that hepatic CD4$^+$ T cells and invariant natural killer T cells up-regulated CD49a expression, along with enhanced activation after Con A injection, leading to production of inflammatory cytokines by these T cells. Blockade of CD49a *in vivo* ameliorated Con A-induced hepatitis with reduced production of IFN-γ and IL-17A. Hence, CD49a promoted Con A-induced hepatitis through enhancing inflammatory cytokine production (IFN-γ and IL-17A) by CD4$^+$ T and invariant natural killer T cells. The protective effect of CD49a blockade antibody suggested a new target therapeutic molecule for intervention of T-cell-mediated liver injury.

Keywords: CD49a; interferon-γ; interleukin-17A; T-cell-mediated hepatitis.

Introduction

T-cell-mediated hepatitis plays a central role as one of the causes of fulminant liver failure. Considerable progress has been made in understanding the underlying mechanism of T-cell-mediated hepatitis using the well-characterized murine model of hepatitis induced by concanavalin A (Con A).[1] CD4$^+$ T cells and invariant natural killer T (iNKT) cells have been demonstrated to be critical in Con A-induced hepatitis through secreting various cytokines;[2–4] among them, interferon-γ (IFN-γ),[2,5–7] tumour necrosis factor-α (TNF-α)[7–9] and interleukin-4 (IL-4)[4,10,11] are key mediators in hepatocyte necrosis. However, the factors responsible for T-cell effector functions in T-cell-mediated hepatitis have not been fully understood.

CD49a, also known as integrin α_1, binds CD29 (integrin β_1) to form the heterodimeric integrin very late antigen 1,[12] which has been found to be widely expressed on various cell types, including NKT cells and T cells.[13] A number of studies have demonstrated a role for CD49a in T-cell-mediated inflammatory diseases.[14–17] Treatment with CD49a blockade antibody *in vivo* has been shown to be therapeutic in CD4$^+$ T-cell-driven diseases, such as experimental rheumatoid arthritis and delayed-type hypersensitivity responses.[18–20] Consistently, CD49a$^{-/-}$ mice exhibited decreased inflammatory responses in models of hypersensitivity and arthritis.[18] CD49a expression parallels high levels of IFN-γ production in effector T cells, and CD49a has been intensively studied for its role in mediating T-cell accumulation, apoptosis and retention within pathological tissues in immune-mediated inflammation.[14–17,21] However, it is unclear whether there are other mechanisms in which CD49a participates in the pathogenesis of T-cell-mediated inflammatory diseases.

CD49a is associated with liver diseases,[22–25] but the role of CD49a in T-cell-mediated hepatitis is still unknown. In this study, we investigated the role of CD49a in the T-cell-mediated immune response using the murine model of Con A-induced hepatitis. We observed that CD49a$^{-/-}$ mice were protected from Con A-induced hepatitis, as evidenced by enhanced survival, lower serum alanine aminotransferase (ALT) levels and less necrosis in the liver. Production of the inflammatory cytokines, such as IFN-γ and IL-17A, was reduced in CD49a$^{-/-}$ mice

after Con A treatment, whereas other cytokines showed no significant differences. Upon Con A stimulation, CD49a expression on $CD4^+$ T cells and iNKT cells from wild-type mice significantly increased and positively correlated with IFN-γ and IL-17A production. $CD4^+$ T cells and iNKT cells in the livers of $CD49a^{-/-}$ mice produced less IFN-γ and IL-17A during Con A-induced hepatitis. CD49a blockade by antibody treatment *in vivo* attenuated the severity of Con A-induced hepatitis and so may be potentially employed as a therapeutic strategy for T-cell-mediated hepatitis.

Materials and methods

Mice

All experiments involving mice were approved by the Animal Care and Use Committee at the University of Science and Technology of China. Male wild-type C57BL/6 (B6) between 6 and 8 weeks old were purchased from the Shanghai Experimental Animal Centre (Shanghai, China). $CD49a^{-/-}$ B6 mice were kindly provided by Paul Rennert (Biogen Idec, Cambridge, MA). All mice involved in experiments were maintained under specific pathogen-free conditions according to the guidelines for experimental animals from the University of Science and Technology of China.

Antibodies and tetramers

The allophycocyanin-conjugated (APC-) CD1d:PBS57 tetramer was kindly provided by the National Institutes of Health tetramer facility. We purchased FITC-anti-CD19 (1D3), FITC-anti-Ly6C (AL-21), FITC-anti-CD25 (7D4), FITC-anti-CD69 (H1.2F3), FITC-rat IgM (R4-22), FITC-hamster IgG1 (G235-2356), phycoerythrin-conjugated (PE-) anti-CD8α (53-6.7), PE-anti-Fas ligand (MFL3), PE-anti-IL-17A (TC11-18H10), PE-anti-CD49a (Ha31/8), PE-rat IgG1 (R3-34), PE-rat IgG2a (R35-95), PE-hamster IgG1 (A19-3), PE-hamster IgG2 (Ha4/8), peridinin chlorophyll protein-conjugated (PerCP-) Cy5.5-anti-CD3 (145-2C11), APC-Cy7-anti-CD4 (GK1.5), APC-Cy7-anti-CD11b (M1/70), PE-Cy7-anti-NK1.1 (PK136), PE-Cy7-anti-IFN-γ (XMG1.2), PE-Cy7-rat IgG1 (R3-34), anti-CD45 (30-F11), Alexa-488-anti-IL-17A (TC11-18H10), Alexa-488-rat IgG1 (R3-34), anti-CD3, anti-CD28, purified-NA/LE hamster anti-rat/mouse CD49a (Ha31/8) and purified-NA/LE hamster IgG2 (Ha4/8) isotype control antibodies from BD Biosciences (San Jose, CA). The PE-anti-TNF-related apoptosis-inducing ligand (TRAIL; N2B2), anti-IL-4 and PerCP-Cy5.5-anti-F4/80 (BM8) antibodies were purchased from eBioscience (San Diego, CA). Goat anti-rat IgG-horseradish peroxidase (HRP) antibody was purchased from Rockland (Gilbertsville, PA). Goat anti-type IV collagen antibody was purchased from SouthernBiotech (Birmingham, AL). Mouse anti-mouse glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibody was purchased from Lk-tag (Shanghai, China). Rabbit anti-goat IgG-HRP and goat anti-mouse IgG-HRP antibodies were purchased from Boster (Wuhan, China). Anti-IFN-γ antibody was purchased from BioLegend (London, UK).

Cytokines

Murine IL-12, IL-6, transforming growth factor-β and IL-1β were purchased from Pepro Tech (Rocky Hill, NJ). Murine IL-23 was purchased from R&D Systems (Minneapolis, MN).

Con A treatment

Wild-type and $CD49a^{-/-}$ mice were injected with Con A (Sigma-Aldrich, St Louis, MO) dissolved in pyrogen-free saline at a dose of 25 or 12 μg/g body weight intravenously. Mouse survival was monitored for 120 hr after injection. CD49a blockade antibody or isotype antibody was intravenously administered 24 hr before Con A injection at a dose of 100 μg/mouse.

ALT assay

Individual mouse serum was collected and diluted with ddH_2O. Serum levels of ALT activity were measured with ALT reagents (Rongsheng Biotech, Shanghai, China) by an automatic biochemical analyser (Rayto Chemray-240, Shenzhen, China) according to the manufacturer's instructions.

Histology

For histological examination, liver tissues were removed from each mouse and fixed in 4% paraformaldehyde for at least 48 hr. Fixed liver tissues were dehydrated using a graded series of alcohol and embedded in paraffin. The specimens were then cut into 7-μm thick sections. The paraffin sections were de-paraffinized and hydrated. Sections were stained with haematoxylin and eosin (H&E). After staining, sections were rinsed in water, dehydrated, cleared, covered with coverslips and examined using light microscopy. The percentage of liver necrosis was analysed using Image-Pro Plus software (Media Cybernetics, Warrendale, PA).

Immunohistochemistry

For immunohistochemistry, paraffin liver sections were de-paraffinized and hydrated. Antigen retrieval was carried out by boiling sections in 10 mM sodium citrate buffer for 20 min. Sections were then cooled in room

temperature, incubated with 3% hydrogen peroxide for 10 min at room temperature to block peroxidase and rinsed three times in PBS. Serum blocking was performed by incubating the sections with goat serum (Santa Cruz Biotechnology, Santa Cruz, CA) for 30 min at room temperature. Sections were then incubated in anti-CD45 in a 1 : 100 dilution overnight at 4° and rinsed three times in PBS. For detection of CD45, the sections were incubated in goat anti-rat IgG-HRP in a 1 : 500 dilution for 30 min at 37° and rinsed three times in PBS. Sections were then incubated in a peroxidase substrate solution using DAB (Vector Laboratories, Burlingame, CA) for stable brown staining and rinsed in water. Finally, sections were stained in haematoxylin for 5 seconds. After haematoxylin staining, sections were rinsed in water, dehydrated, cleared, covered by coverslips and examined by light microscopy.

Preparation of liver mononuclear cells

Livers were collected from killed mice and pressed through a 200-gauge stainless steel mesh. The filtrate containing non-parenchymal cell was washed once. The cells were resuspended in 40% Percoll (GE Healthcare, Uppsala, Sweden) and overlaid onto 70% Percoll. After centrifugation at 1260 ***g*** for 30 min, the interphase was collected and washed once.

Antibody staining and flow cytometry

To block rat Fc receptors, liver mononuclear cells were incubated with rat immunoglobulin for 30 min before staining with antibodies. Cells were incubated with PBS57-loaded CD1d tetramer at 4° for 45 min in the dark and washed in ice-cold PBS buffer. Then cells were stained with a cocktail of other antibodies at 4° for 30 min in the dark and washed in ice-cold PBS buffer. For IFN-γ and IL-17A staining, intracellular staining assays were performed using the fixation/permeabilization buffer kit (eBioscience). Flow cytometry was performed on an LSRII (BD Biosciences) and data were analysed with FlowJo software (Tree Star, Ashland, OR).

Immunoblot analysis

Liver tissues from untreated wild-type and CD49a$^{-/-}$ mice were homogenized with lysis buffer and lysed for 30 min on ice. After centrifugation at 16 000 ***g*** for 10 min at 4°, the protein concentration in the supernatant was quantified with Bradford's reagent (Bio-Rad, Hercules, CA), and protein was denatured by boiling for 10 min. Protein (90 μg) was resolved by SDS–PAGE and then transferred onto nitrocellulose membranes. Blocking was performed using Tris-buffered saline plus Tween-20 buffer containing 5% non-fat dairy milk for 1 hr at room temperature. Primary antibodies (goat anti-type IV collagen or mouse anti-mouse GAPDH) were diluted to 1 : 500 in blocking solution and incubated overnight at 4°. The nitrocellulose membranes were rinsed three times in blocking buffer. HRP-conjugated secondary antibodies (rabbit anti-goat IgG-HRP or goat anti-mouse IgG-HRP) were diluted to 1 : 5000 in blocking solution and incubated for 1 hr at room temperature. Immune complexes were visualized using Super Signal West Pico chemiluminescent substrate (Thermo Scientific, Rockford, IL).

Stimulation of cytokine secretion in vitro

Type IV collagen (100 μl, 25 μg/ml) (Sigma-Aldrich) was coated onto 96-well plates overnight at 4°.[26] Plates were washed twice with PBS. For Con A stimulation, liver mononuclear cells were cultured at a density of 1×10^6 per well in 200 μl of complete RPMI-1640 medium containing 10% calf serum in the presence of Con A (3 μg/ml) for 24 hr in wells pre-coated with type IV collagen. For T helper type 1 (Th1) and Th17 polarization *in vitro*, liver mononuclear cells were suspended at a density of 2×10^5 per well in 200 μl of complete RPMI-1640 medium containing 10% calf serum in the presence of anti-CD3/28 antibodies (2 μg/ml). Cells were stimulated under Th1- or Th17-polarizing conditions for 72 hr in type IV collagen pre-coated wells. Th1 mixture: IL-12 (10 ng/ml), anti-IL-4 antibody (10 μg/ml); Th17 mixture: IL-6 (10 ng/ml), transforming growth factor-β (2 ng/ml), IL-1β (10 ng/ml), IL-23 (10 ng/ml), anti-IL-4 antibody (10 μg/ml) and anti-IFN-γ antibody (10 μg/ml).[27] Supernatants were collected for ELISA.

Cytokine detection in serum and supernatant

Cytokines (IFN-γ, IL-17A, TNF-α, IL-4, IL-10, IL-2 and IL-6) in sera and supernatants were measured using commercially available ELISA kits (Dakewei Biotech, Beijing, China). ELISAs were performed according to the manufacturer's instructions.

Statistical analysis

All data are expressed as the mean ± SEM. Student's unpaired *t*-tests were used to calculate the *P*-values. Values of $P < 0{\cdot}05$ were considered statistically significant.

Results

CD49a deficiency protects mice from Con A-induced hepatitis

To assess the role of CD49a in fulminant liver injury, we used a murine model of acute hepatitis induced by Con A. A high dose of Con A (25 μg/g body weight) was injected into wild-type and CD49a$^{-/-}$ mice, and their

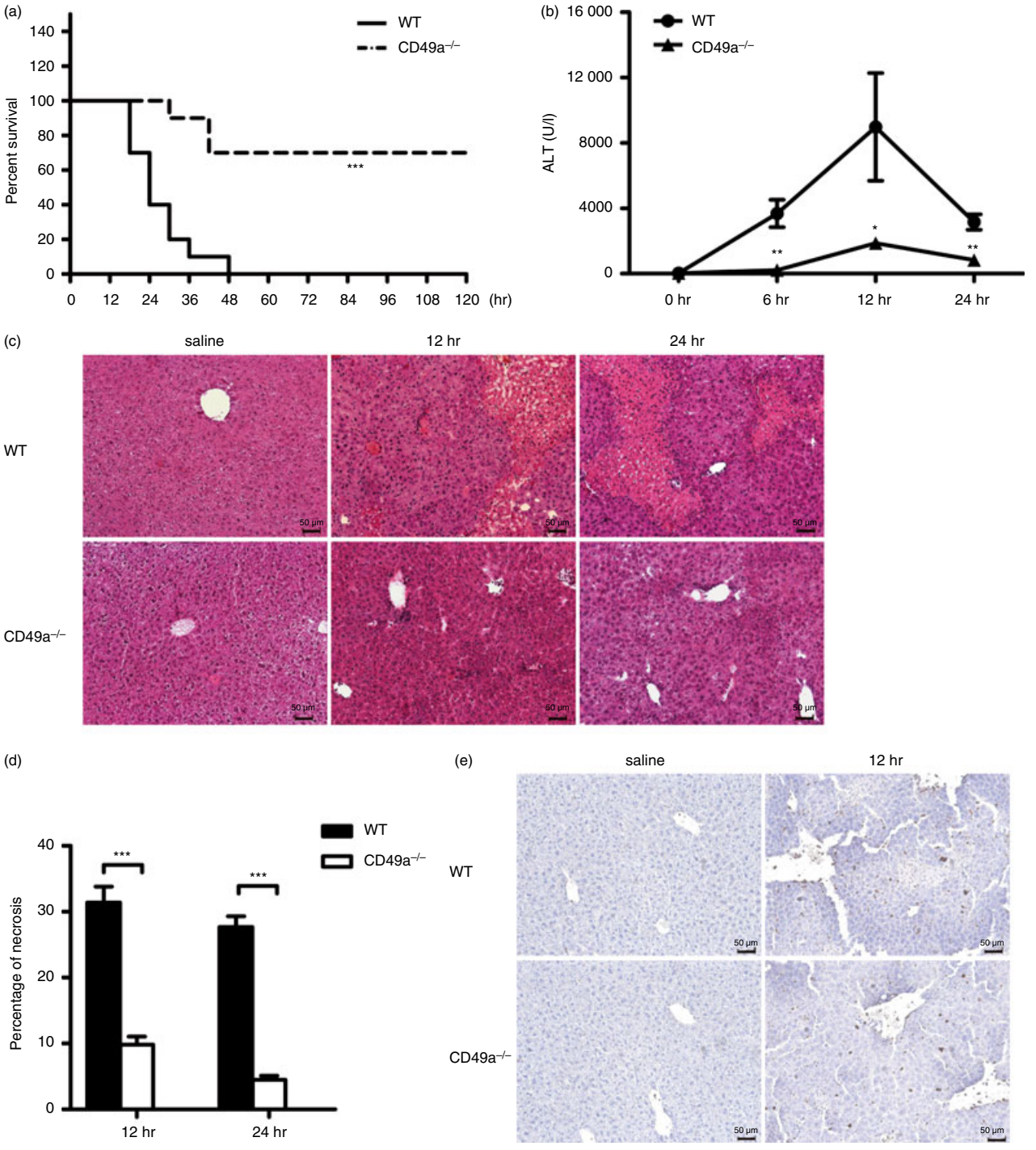

Figure 1. CD49a deficiency ameliorates concanavalin A (Con A) -induced hepatitis. (a) Survival of wild-type and CD49a$^{-/-}$ mice after injection with a lethal dose of Con A (25 μg/g). Survival was monitored every 6 hr. Data show the combined results from two experiments, $n = 10$ per group. (b) Serum alanine aminotransferase (ALT) levels of wild-type ($n = 7$ or $n = 8$) and CD49a$^{-/-}$ ($n = 7$ or $n = 8$) mice 24 hr after intravenous injection of Con A (12 μg/g). Data show the combined results from two experiments (mean ± SEM; $^{*}P < 0{\cdot}05$; $^{**}P < 0{\cdot}01$). (c) Representative photomicrographs of haematoxylin & eosin-stained liver sections from wild-type and CD49a$^{-/-}$ mice 12 and 24 hr after Con A injection. Black scale bars represent 50 μm. (d) The percentage of the necrotic area in the livers of wild-type and CD49a$^{-/-}$ mice 12 and 24 hr after Con A treatment (12 μg/g). The percentage of the necrotic area was measured from three different fields for each mouse ($n = 7$ or $n = 8$ mice/group; mean ± SEM; $^{***}P < 0{\cdot}001$). (e) Representative photomicrographs of liver sections stained with anti-CD45 (brown) from wild-type and CD49a$^{-/-}$ mice 12 hr after Con A injection (12 μg/g). Black scale bars represent 50 μm.

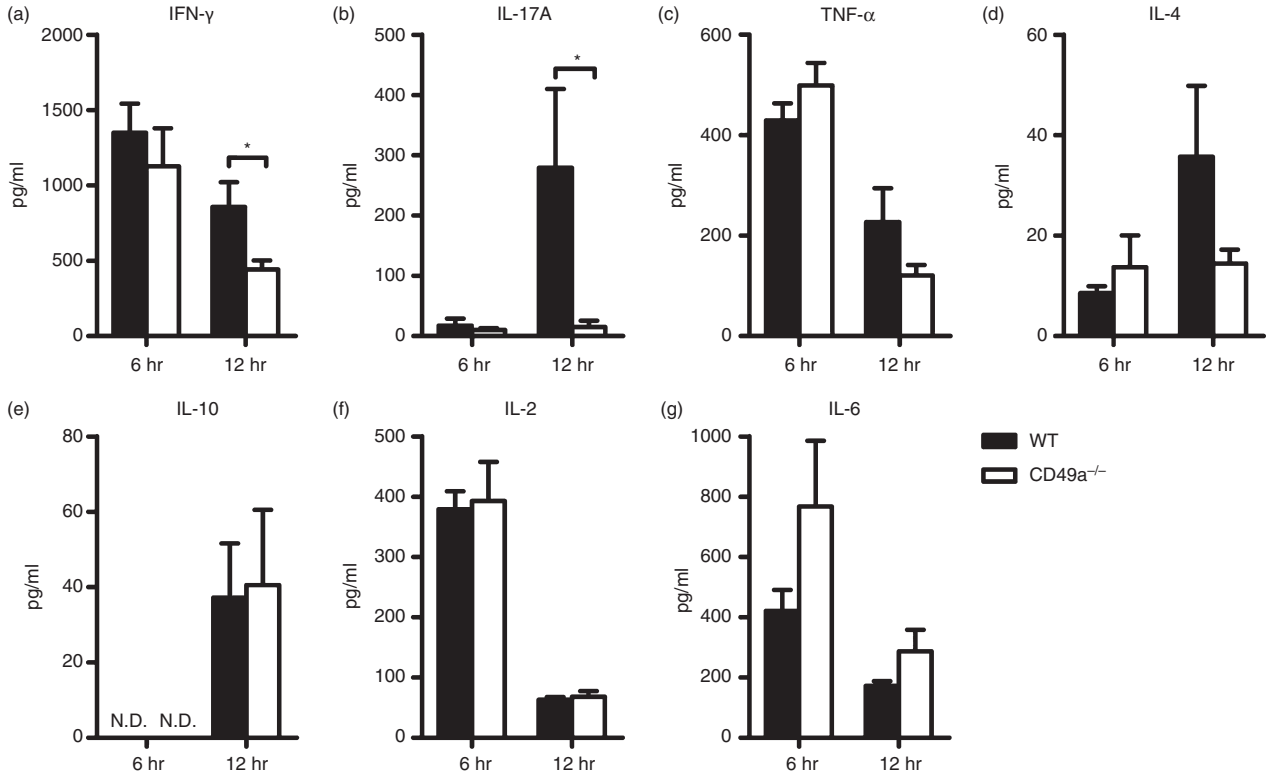

Figure 2. Serum interferon-γ (IFN-γ) and interleukin-17A (IL-17A) levels decrease in CD49a$^{-/-}$ mice after concanavalin A (Con A) injection. Wild-type (n = 6–10) and CD49a$^{-/-}$ mice (n = 6–10) were intravenously injected with Con A (12 μg/g). The serum levels of IFN-γ (a), IL-17A (b), tumour necrosis factor-α (TNF-α) (c), IL-4 (d), IL-10 (e), IL-2 (f) and IL-6 (g) in wild-type (black bars) and CD49a$^{-/-}$ (white bars) mice were detected by ELISA at the indicated time-points (6 and 12 hr) after Con A injection. Data show the combined results from two experiments (mean ± SEM; *$P < 0{\cdot}05$).

survival rates were determined. Consistent with previous results,[28] this dosage of Con A was lethal for wild-type mice, whereas 70% of CD49a$^{-/-}$ mice survived (Fig. 1a), indicating that CD49a deficiency exerts an important protective effect during T-cell-mediated acute hepatitis. We then treated mice with a lower, non-lethal dose (12 μg/g body weight) and compared serum ALT levels between wild-type and CD49a$^{-/-}$ mice. As shown in Fig. 1(b), ALT levels were low and were not significantly different between wild-type and CD49a$^{-/-}$ mice without Con A injection, indicating that CD49a$^{-/-}$ mice did not have liver injury under steady-state. After Con A treatment, CD49a$^{-/-}$ mice had significantly lower levels of serum ALT than wild-type mice at 6, 12 and 24 hr, which was indicative of the ameliorated liver damage (Fig. 1b). Histological analysis of CD49a$^{-/-}$ mice also showed no liver lesions before Con A treatment. In addition, we did not observe any structural abnormality within the livers of CD49a$^{-/-}$ mice. Wild-type mice had more extensive liver necrosis than CD49a$^{-/-}$ mice 12 and 24 hr after Con A treatment (Fig. 1c,d). Histological staining of CD45 in the livers showed that both wild-type and CD49a$^{-/-}$ mice exhibited immune-cell infiltration in the liver 12 hr after Con A injection (Fig. 1e). These results clearly suggested that Con A-induced liver damage was ameliorated in CD49a$^{-/-}$ mice.

CD49a deficiency reduces the production of inflammatory cytokines

Various cytokines are involved in the pathogenesis of Con A-induced hepatitis, so we wondered whether CD49a deficiency affects serum cytokine levels after Con A treatment. As shown in Fig. 2, serum levels of IFN-γ, IL-17A, TNF-α, IL-4, IL-10, IL-2 and IL-6 were all comparable between wild-type and CD49a$^{-/-}$ mice 6 hr after Con A treatment. However, at 12 hr after Con A administration, serum IFN-γ and IL-17A levels were significantly lower in CD49a$^{-/-}$ mice than in wild-type mice, whereas TNF-α, IL-4, IL-10, IL-2 and IL-6 were not significantly different between the two groups.

We also compared the capacity of wild-type and CD49a$^{-/-}$ liver mononuclear cells to produce IFN-γ and IL-17A *in vitro*. Type IV collagen, the ligand for CD49a,[13] existed in the livers of both wild-type and CD49a$^{-/-}$ mice (Fig. 3a). To define the role of CD49a, we precoated 96-well plates with type IV collagen to mimic the intrahepatic microenvironment *in vivo*. Liver mononu-

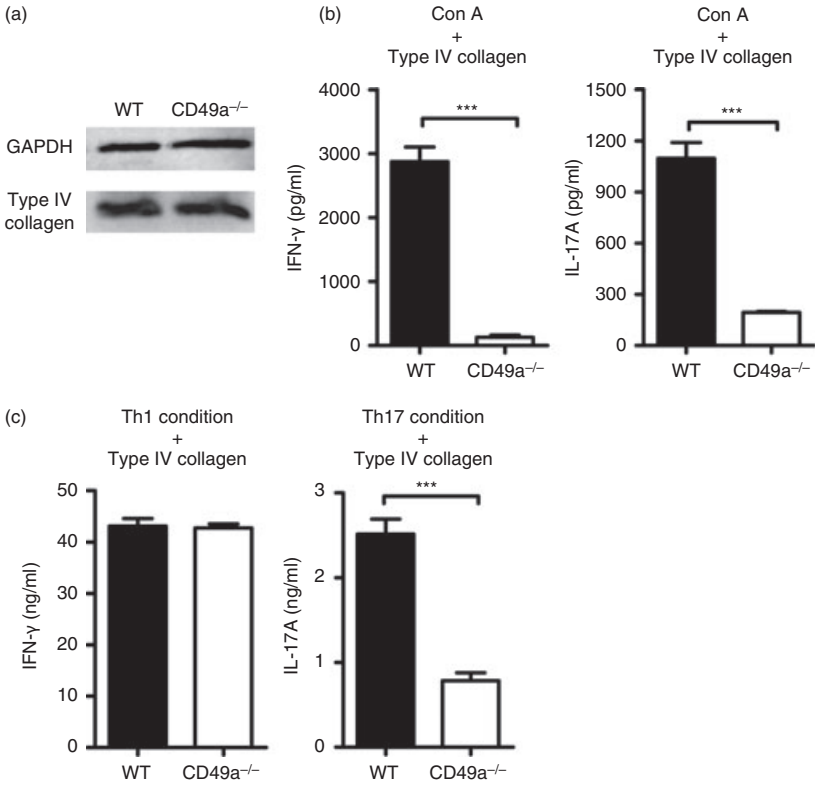

Figure 3. CD49a deficiency decreases production of inflammatory cytokines *in vitro*. (a) Representative Western blotting of type IV collagen in the livers of untreated wild-type and CD49a$^{-/-}$ mice. $n = 3$ per group. (b) Liver mononuclear cells were prepared from untreated wild-type (black bars) and CD49a$^{-/-}$ mice (white bars). Cells were stimulated with concanavalin A (Con A) for 24 hr in 96-well plates pre-coated with type IV collagen. Culture supernatants were collected and assayed for interferon-γ (IFN-γ) and interleukin-17A (IL-17A) production by ELISA. Data are expressed as the mean ± SEM from quintuplicate samples (***$P < 0{\cdot}001$). Data are representative of two independent experiments. (c) Liver mononuclear cells were isolated from untreated wild-type (black bars) and CD49a$^{-/-}$ mice (white bars) and cultured under T helper type 1 (Th1) or Th17 cell-polarizing conditions in the presence of anti-CD3/28 antibodies for 72 hr in 96-well plates pre-coated with type IV collagen. Data are expressed as the mean ± SEM from quadruplicate samples (***$P < 0{\cdot}001$). Data are representative of two independent experiments.

clear cells from wild-type and CD49a$^{-/-}$ mice were isolated and separately incubated with Con A (3 μg/ml) for 24 hr in the wells pre-coated with type IV collagen. We observed markedly decreased levels of IFN-γ and IL-17A (Fig. 3b) in the culture supernatants of liver mononuclear cells from CD49a$^{-/-}$ mice. In addition, to clarify the intrinsic capacity of wild-type and CD49a$^{-/-}$ liver mononuclear cells to produce cytokines, we cultured wild-type and CD49a$^{-/-}$ liver mononuclear cells under Th1 or Th17 cell-polarizing conditions for 3 days in the presence of type IV collagen. Under Th1 cell-polarizing conditions, there was no significant difference in the production of IFN-γ between wild-type and CD49a$^{-/-}$ liver mononuclear cells (Fig. 3c), suggesting differential roles for CD49a in intrinsic and Con A-induced production of IFN-γ. Under Th17 cell-polarizing conditions, CD49a$^{-/-}$ liver mononuclear cells produced less IL-17A than their wild-type counterparts (Fig. 3c). Together, our results indicate that CD49a deficiency impairs the production of inflammatory cytokines by liver mononuclear cells during Con A-induced hepatitis.

CD49a is not essential for the recruitment of mononuclear cells in Con A-induced hepatitis

To further investigate the role of CD49a in the influx of mononuclear cells into the liver after Con A injection, we calculated the numbers of different lymphocyte subsets in the livers of wild-type and CD49a$^{-/-}$ mice 6 hr after Con A injection. As shown in Fig. 4, there was no significant difference in the total number of iNKT cells (CD3^{int} subset positive for PBS57-loaded CD1d tetramer) (Fig. 4a), CD4$^+$ T cells (Fig. 4b), CD8$^+$ T cells (Fig. 4c), NK cells (Fig. 4d), or of B cells (Fig. 4e) between wild-type and CD49a$^{-/-}$ mice. As a previous study reported altered monocyte populations in CD49a$^{-/-}$ mice in an experimental model of colitis,[29] we compared the number of macrophages (Fig. 4f) and inflammatory monocytes (Fig. 4g) between wild-type and CD49a$^{-/-}$ mice and found no significant difference.

CD49a expression is critical for cytokine production by hepatic CD4$^+$ T and iNKT cells

CD4$^+$ T and iNKT cells in the liver can induce rapid liver injury by producing a large amount of cytokines in Con A-induced acute hepatitis.[2,4,5] Previous studies have demonstrated that CD4$^+$ T and iNKT cells are the main sources of IFN-γ and IL-17A in this model.[2,30–32] Since a significant decrease of IFN-γ and IL-17A in CD49a$^{-/-}$ mice has been observed, we speculate that CD49a may be important for the activation of hepatic CD4$^+$ T and iNKT cells. We examined CD49a expression on hepatic CD4$^+$ T cells and found that CD4$^+$ T cells in the liver seldom expressed CD49a under normal conditions. After administration of Con A, CD49a expression on hepatic CD4$^+$ T cells was elevated (Fig. 5a,b) and the absolute number of CD49a$^+$ CD4$^+$ T cells in the liver also increased (Fig. 5c).

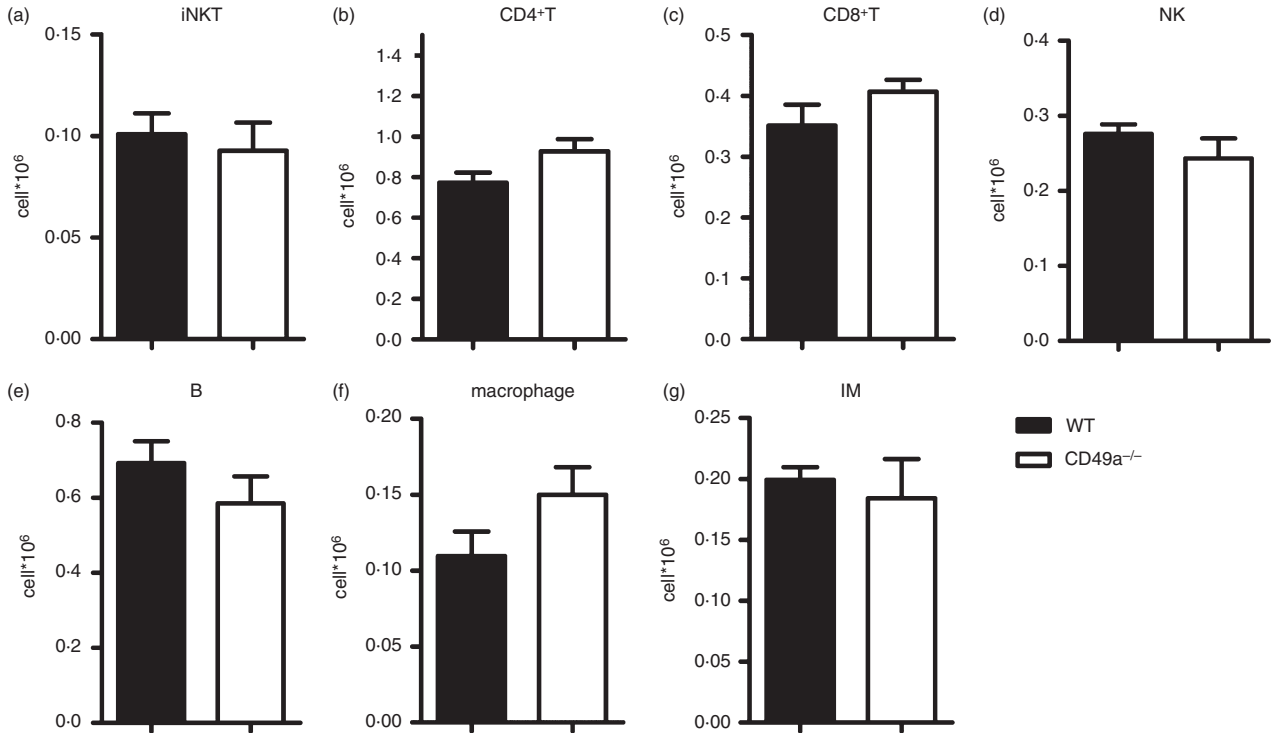

Figure 4. CD49a deficiency does not influence mononuclear cell infiltration. Wild-type and CD49a$^{-/-}$ mice were intravenously injected with concanavalin A (Con A) (12 μg/g). Liver mononuclear cells were isolated 6 hr after injection. Absolute numbers of invariant natural killer T (iNKT) (CD1d tetramer^{+} CD3^{int}), CD4^{+} T (CD1d tetramer^{-} CD3^{hi} CD4^{+}), CD8^{+} T (CD3^{hi} CD8^{+}), NK (NK1.1^{+} CD3^{-}), B (CD19^{+} CD3^{-}), macrophage (CD11b^{+} F4/80^{+}) and inflammatory monocytes (IM) (CD11b^{+} Ly6C^{hi}) cells in the livers of wild-type (n = 8, black bars) versus CD49a$^{-/-}$ (n = 7–8, white bars) mice were calculated. Data show the combined results from two experiments (mean ± SEM).

We observed CD69 up-regulation on hepatic CD4^{+} T cells from both wild-type and CD49a$^{-/-}$ mice after Con A injection (Fig. 5d). CD25, the α-chain of the IL-2 receptor, exhibited reduced expression in the absence of CD49a (Fig. 5d), consistent with a previous study showing that an anti-CD49a antibody reduced surface expression of IL-2 receptor on T cells.[33] To investigate the mechanism underlying the reduced production of IFN-γ and IL-17A in CD49a$^{-/-}$ mice during Con A-induced hepatitis, we first detected expression of CD49a on CD4^{+} T cells producing IFN-γ or IL-17A in the liver of wild-type mice 12 hr after Con A injection and found that CD4^{+} T cells producing IFN-γ or IL-17A were positive for CD49a expression (Fig. 6a). To investigate whether CD49a deficiency impaired the cytokine-secreting capacity of CD4^{+} T cells, we then analysed the intracellular cytokine production by hepatic CD4^{+} T cells from wild-type and CD49a$^{-/-}$ mice. At 12 hr after Con A injection, IFN-γ and IL-17A production by CD4^{+} T cells was much lower in CD49a$^{-/-}$ mice than in wild-type mice. The percentage of IFN-γ^{+} or IL-17A^{+} CD4^{+} T cells was significantly reduced in CD49a$^{-/-}$ mice (Fig. 6b–d). Meanwhile, the mean fluorescence intensity of IFN-γ and IL-17A in CD4^{+} T cells from CD49a$^{-/-}$ mice was significantly lower (Fig. 6c,d).

We then examined CD49a expression on hepatic iNKT cells. Unlike CD4^{+} T cells, hepatic iNKT cells were mostly positive for CD49a expression under normal conditions (Fig. 7a). CD49a expression on liver iNKT cells remained positive after Con A injection (Fig. 7b). The up-regulation of Fas ligand (CD178) and TRAIL (CD253) induced by Con A was similar on iNKT cells from wild-type and CD49a$^{-/-}$ mice (Fig. 7c). CD49a was also expressed on IFN-γ-producing or IL-17A-producing iNKT cells in the livers of wild-type mice 12 hr after Con A injection (Fig. 8a). Consistent with the results from CD4^{+} T cells, intracellular staining analysis showed that IFN-γ and IL-17A production by iNKT cells from CD49a$^{-/-}$ mice was also lower (Fig. 8b–d). Collectively, these results indicated that CD49a deficiency impaired the production of IFN-γ and IL-17A by liver CD4^{+} T and iNKT cells after Con A stimulation *in vivo*.

Blockade of CD49a ameliorates T-cell-mediated hepatitis

To further elucidate the role of CD49a in Con A-induced liver injury, we treated wild-type mice with a Ha31/8 monoclonal antibody,[34] a function-blocking antibody against CD49a. As hepatic iNKT cells expressed CD49a

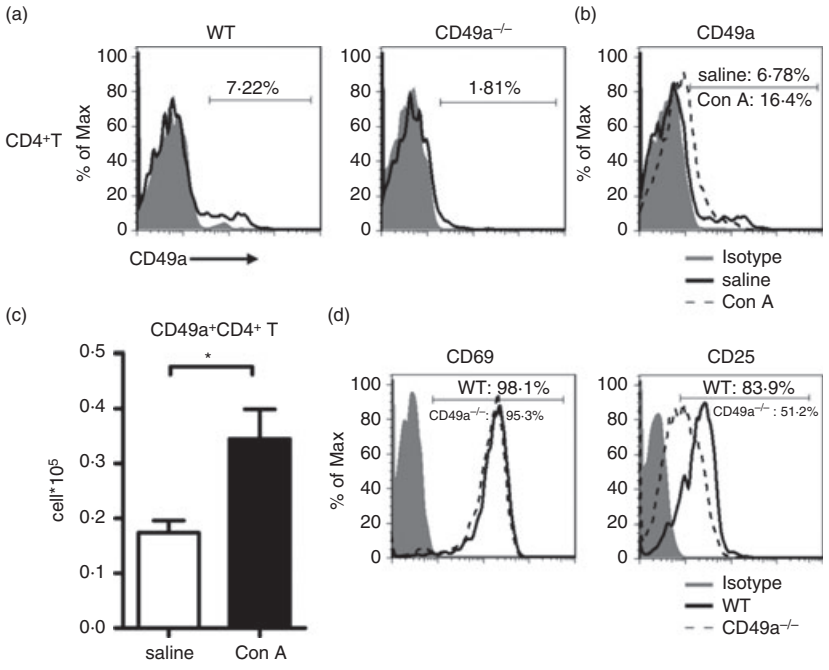

Figure 5. CD49a expression on hepatic $CD4^+$ T cells during concanavalin A (Con A) -induced hepatitis. (a) CD49a expression on liver $CD4^+$ T ($CD1d$ tetramer$^-$ $CD3^{hi}$ $CD4^+$) cells from untreated wild-type and CD49a$^{-/-}$ mice. Data are representative of two independent experiments, $n = 8$ per group. Grey-filled curves, isotype control. (b) CD49a expression on liver $CD4^+$ T ($CD1d$ tetramer$^-$ $CD3^{hi}$ $CD4^+$) cells from saline-treated (black solid line) or Con A-treated (12 μg/g, dashed line) wild-type mice 6 hr post-injection. Data are representative of two independent experiments, $n = 8$ per group. Grey-filled curves, isotype control. (c) Absolute number of CD49a$^+$ $CD4^+$ T ($CD49a^+$ $CD1d$ tetramer$^-$ $CD3^{hi}$ $CD4^+$) cells in the livers of saline-treated (white bar) or Con A-treated (12 μg/g, black bar) wild-type mice 6 hr after injection. Data show the combined results from two experiments, $n = 8$ per group (mean ± SEM; $^*P < 0{\cdot}05$). (d) Expression of CD69 and CD25 on hepatic $CD4^+$ T ($CD1d$ tetramer$^-$ $CD3^{hi}CD4^+$) cells from wild-type (black solid line) and CD49a$^{-/-}$ mice (dashed line) 6 hr after Con A injection (12 μg/g). Data are representative of two independent experiments, $n = 8$–9 per group. Grey-filled curves, isotype control.

under normal conditions, we injected the antibody 24 hr before Con A injection to ensure blockade effectiveness. We found that the blockade of CD49a prevented the increase of serum ALT levels (Fig. 9a). The protective effect was also reflected in fewer necrotic areas being observed in the livers of Ha31/8-treated mice, which was in contrast to the significantly larger necrotic areas observed in the liver parenchyma of mice treated with isotype antibody (Fig. 9b,c). Consistently, blockade of CD49a also significantly decreased the serum levels of IFN-γ (Fig. 9d) and IL-17A (Fig. 9e). Therefore, CD49a blockade is effective in attenuating Con A-induced liver injury.

Discussion

Despite the plethora of knowledge regarding the role of CD49a in several inflammatory diseases,[14,15,18] little is known about the role of CD49a in hepatitis. In this study, we used CD49a$^{-/-}$ mice to assess the role of CD49a in hepatitis *in vivo* for the first time. We used Con A to induce acute hepatitis in mice and observed attenuated liver injury in CD49a$^{-/-}$ mice. Previous studies have demonstrated that either deficiency or blockade of CD49a inhibited T-cell migration[14,15,21] or retention,[17] resulting in decreased inflammatory responses in T-cell-mediated diseases. In this study, however, CD49a deficiency does not impair T-cell accumulation in the liver during Con A-induced hepatitis. We observed a similar infiltration of T cells and other mononuclear cells in the livers of wild-type and CD49a$^{-/-}$ mice. CD49a deficiency attenuated hepatitis by reducing the production of inflammatory cytokines by $CD4^+$ T and iNKT cells in CD49a$^{-/-}$ mice. These results unveil a new immunopathological mechanism by which CD49a participates in T-cell-mediated inflammation.

CD49a expression is often associated with the activated or effector memory status of immune cells.[12,15,17,35] CD49a or Foxp3 expression has been demonstrated to be a mutually exclusive event in $CD4^+$ T cells, and they each respectively define functionally distinct effector and regulatory T-cell subsets.[36] The exclusive expression of CD49a or Foxp3 may explain the adverse effects induced by different anti-adhesive substances. A recent study showed that blockade of integrin α_4 (CD49d) worsened Con A-induced hepatitis by inhibiting $CD49d^+$ suppressor cells.[37] The phenomenon was ascribed to broad expression of CD49d on immunosuppressive populations (regulatory T

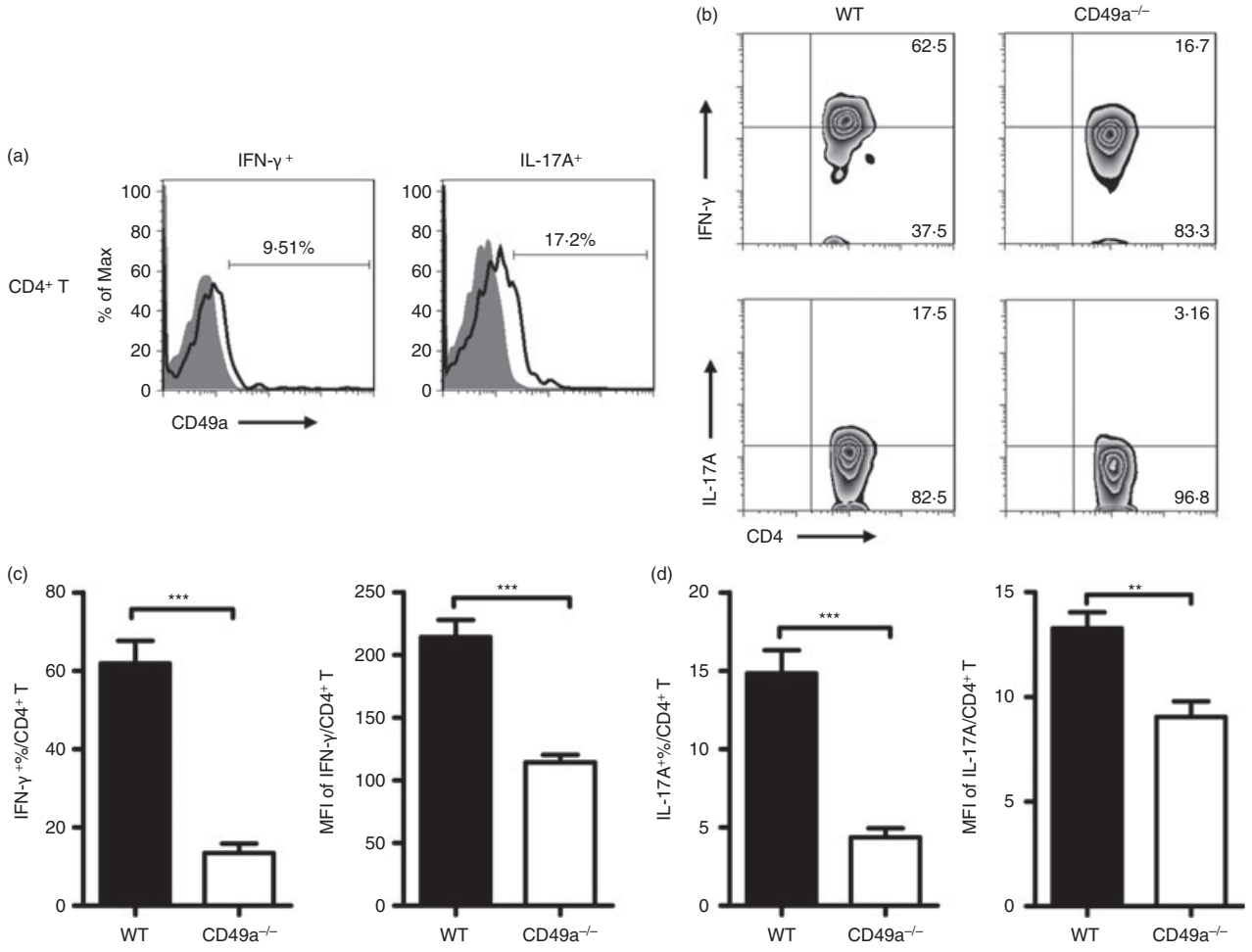

Figure 6. Decreased production of $CD4^+$ T-cell-derived interferon-γ (IFN-γ) and interleukin-17A (IL-17A) in $CD49a^{-/-}$ mice. Wild-type and $CD49a^{-/-}$ mice were intravenously injected with concanavalin A (Con A) (12 μg/g). Liver mononuclear cells were isolated 12 hr after Con A treatment. (a) CD49a expression on liver IFN-γ^+ $CD4^+$ T (IFN-γ^+ CD1d tetramer$^-$ $CD3^{hi}$ $CD4^+$, left) cells and IL-$17A^+$ $CD4^+$ T (IL-$17A^+$ CD1d tetramer$^-$ $CD3^{hi}$ $CD4^+$, right) cells from wild-type mice. Data are representative of two independent experiments, $n = 6$ per group. Grey-filled curves, isotype control. (b) Intracellular staining of IFN-γ and IL-17A in liver $CD4^+$ T (CD1d tetramer$^-$ $CD3^{hi}$ $CD4^+$) cells. Numbers indicate percentages. (c) Statistical analysis of the percentage of IFN-γ^+ cells (left) and mean fluorescence intensity (MFI) of IFN-γ (right) in liver $CD4^+$ T (CD1d tetramer$^-$ $CD3^{hi}$ $CD4^+$) cells. (d) Statistical analysis of the percentage of IL-$17A^+$ cells (left) and MFI of IL-17A (right) in liver $CD4^+$ T (CD1d tetramer$^-$ $CD3^{hi}$ $CD4^+$) cells. Data show the combined results from two experiments, $n = 8$ per group (mean ± SEM; $^{**}P < 0{\cdot}01$; $^{***}P < 0{\cdot}001$).

cells and a monocytic myeloid-derived suppressor cell subset). However, our study detected CD49a expression on immune effector cells (i.e. IFN-γ-producing or IL-17A-producing $CD4^+$ T and iNKT cells), but not on regulatory T cells (data not shown) in the liver after Con A injection. Moreover, CD49a expression on $CD11b^+$ cells has been reported to be restricted to Gr-1^- cells,[29] which suggests that $CD11b^+$ Gr-1^+ monocytic myeloid-derived suppressor cells do not express CD49a. Because of the preferential expression of CD49a on immune effector cells, deficiency or blockade of CD49a inhibited effector T cells but not immunosuppressive cells, leading to attenuated liver injury. Therefore, anti-adhesive substances may exert pro-inflammatory or anti-inflammatory effects according to the repertoire of adhesion molecule expression.

Our study also showed a different pattern of CD49a expression between $CD4^+$ T cells and iNKT cells. In untreated wild-type mice, we detected high expression of CD49a on hepatic iNKT cells, but not on $CD4^+$ T cells. Hepatic iNKT cells express several activation markers such as CD69 and CD44 under homeostatic conditions and appear to be constitutively activated by endogenous ligands *in vivo*.[38] Hence, the constitutive expression of CD49a on hepatic iNKT cells under homeostatic conditions may be due to their constitutively activated characteristic. CD49a has been reported to be expressed on NKT cells,[13] but studies on $CD49a^+$ iNKT cells are rare.

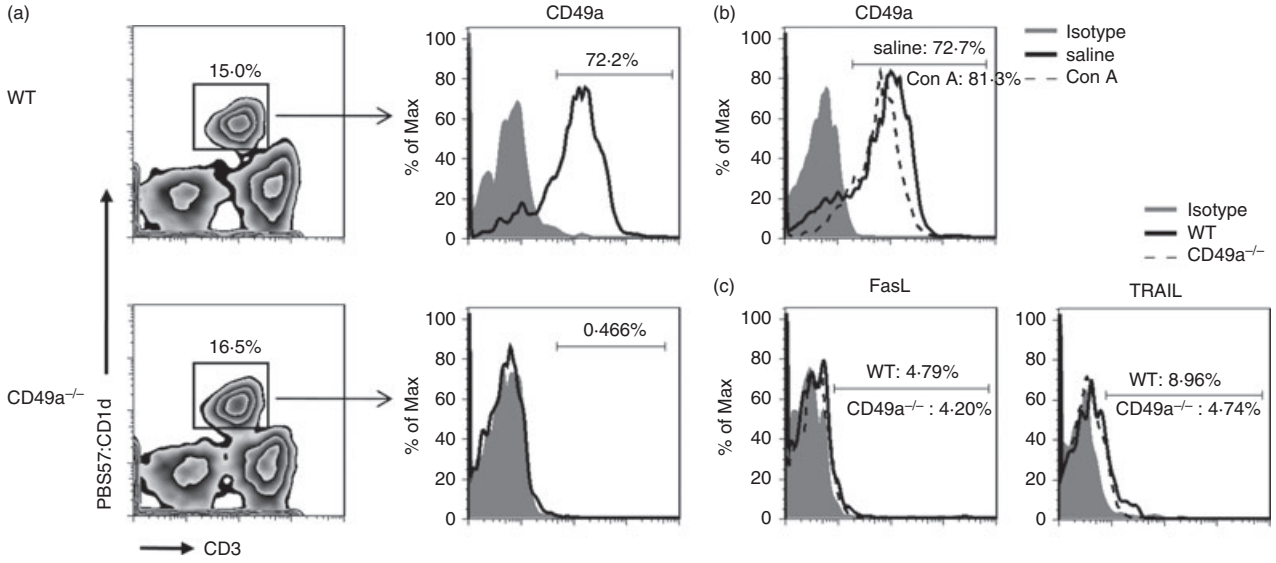

Figure 7. CD49a expression on hepatic invariant natural killer T (iNKT) cells during concanavalin A (Con A) -induced hepatitis. (a) CD49a expression on liver iNKT (CD1d tetramer$^+$ CD3^{int}) cells from untreated wild-type and CD49a$^{-/-}$ mice. Data are representative of two independent experiments, $n = 8$ per group. Grey-filled curves, isotype control. (b) CD49a expression on liver iNKT (CD1d tetramer$^+$ CD3^{int}) cells from saline-treated (black solid line) or Con A-treated (12 μg/g, dashed line) wild-type mice 6 hr post-injection. Data are representative of two independent experiments, $n = 8$ per group. Grey-filled curves, isotype control. (c) Expression of Fas ligand and TRAIL on liver iNKT (CD1d tetramer$^+$ CD3^{int}) cells from wild-type mice (black solid line) and CD49a$^{-/-}$ mice (dashed line) 6 hr after Con A injection (12 μg/g). Data are representative of two independent experiments, $n = 9$ per group. Grey-filled curves, isotype control.

Whether CD49a is involved in the function of iNKT cells is unknown. Here, we found that CD49a was expressed on iNKT cells before and after Con A stimulation, and deficiency of CD49a impaired cytokine production by iNKT cells. In contrast to iNKT cells, CD4$^+$ T cells seldom express CD49a in the resting state, but have elevated CD49a expression after Con A treatment *in vivo*. Similar to iNKT cells, CD49a deficiency also decreases inflammatory cytokine production by CD4$^+$ T cells.

After Con A injection, we found less IFN-γ and IL-17A in the sera of CD49a$^{-/-}$ mice than in wild-type mice. The pro-inflammatory effect of IFN-γ in Con A-induced hepatitis has been well documented. Both neutralization of IFN-γ[6] and targeted disruption of the IFN-γ gene[5] protected mice from Con A-induced liver injury. Though the role of IL-17 remained controversial,[39] blockade of IL-17/IL-17R protected mice during Con A-induced hepatitis,[30] and ameliorated liver injury was also observed in IL-17$^{-/-}$ mice.[5,40,41] Therefore, the reduced liver injury in CD49a$^{-/-}$ mice may be attributed to the decreased levels of IFN-γ and IL-17A. In our study, the intracellular staining assay showed less IFN-γ and IL-17A production by CD4$^+$ T and iNKT cells in the livers of CD49a$^{-/-}$ mice compared with wild-type mice. CD49a has been reported to be expressed on effector CD4$^+$ T cells,[36] and CD49a$^+$ T cells are apt to polarize to Th1 cells and produce IFN-γ.[14,15,35,42] Moreover, signalling via very late antigen 1 can induce IFN-γ production in activated Th1-type cell lines.[35] Consistent with these studies, our study also demonstrated that CD49a was important for IFN-γ production by T cells in Con A-induced acute hepatitis. Moreover, our study suggests a new role for CD49a in the T-cell-mediated immune response. Besides IFN-γ, CD49a was also essential for the production of IL-17A by iNKT and CD4$^+$ T cells. There are hints about the relationship between CD49a and IL-17A in previous reports. A fraction of Th17 cells polarized by IL-23 *in vitro* expressed CD49a.[43] Auto-reactive T-cell lines generated from patients with probable multiple sclerosis homogeneously co-expressing CD45RO and CD49a expressed higher levels of IL-17A mRNA than those from healthy donor cells.[44] Although the exact mechanism by which CD49a promotes IL-17A production remains unclear, this is the first indication to our knowledge that CD49a expression can affect IL-17A production by T cells. Hence, our finding that CD49a is critical for IL-17A secretion by T cells during inflammation sheds new light on the mechanism of CD49a in the T-cell-mediated immune response.

In Con A-induced hepatitis, CD49a blockade by antibody treatment decreased production of IFN-γ and IL-17A, ultimately leading to lower ALT levels and less liver necrosis. These findings suggest that CD49a may be a novel therapeutic target for acute liver failure. It should be noted that reducing the inflammatory response cannot be used as a universal treatment because the role of

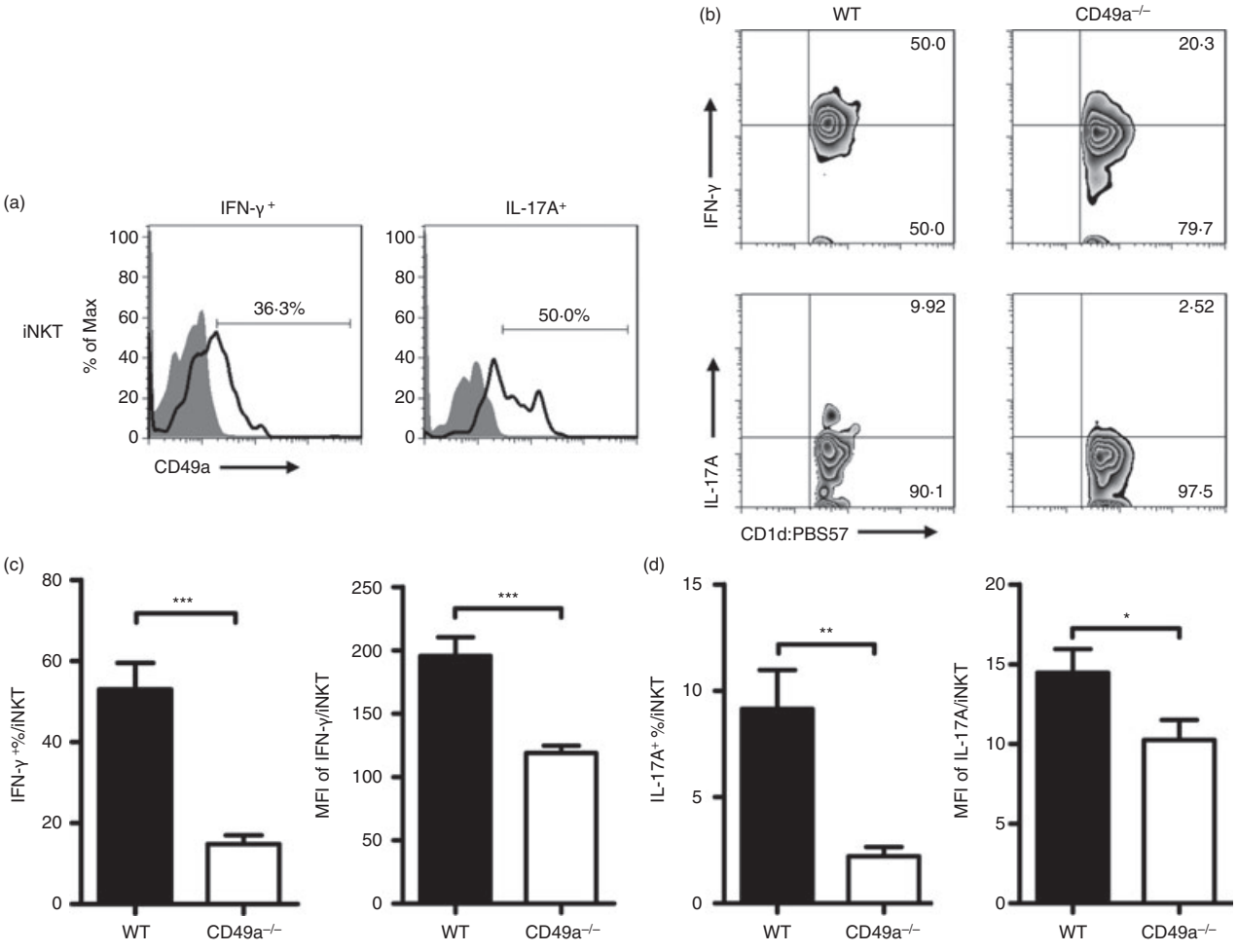

Figure 8. Invariant natural killer T (iNKT) -cell-derived interferon-γ (IFN-γ) and interleukin-17A (IL-17A) are reduced in CD49a$^{-/-}$ mice. Wild-type mice (black bars) and CD49a$^{-/-}$ mice (white bars) were intravenously injected with concanavalin A (Con A) (12 μg/g). Liver mononuclear cells were isolated 12 hr after Con A treatment. (a) CD49a expression on liver IFN-γ^+ iNKT (IFN-γ^+ CD1d tetramer$^+$ CD3^{int}, left) cells and IL-17A$^+$ iNKT (IL-17A$^+$ CD1d tetramer$^+$ CD3^{int}, right) cells from wild-type mice. Data are representative of two independent experiments, $n = 6$ per group. Grey-filled curves, isotype control. (b) Intracellular staining of IFN-γ and IL-17A in liver iNKT (CD1d tetramer$^+$ CD3^{int}) cells. Numbers indicate percentages. (c) Statistical analysis of the percentage of IFN-γ^+ cells (left) and mean fluorescence intensity (MFI) of IFN-γ (right) in liver iNKT (CD1d tetramer$^+$ CD3^{int}) cells. (d) Statistical analysis of the percentage of IL-17A$^+$ cells (left) and MFI of IL-17A (right) in liver iNKT (CD1d tetramer$^+$ CD3^{int}) cells. Data show the combined results from two experiments, $n = 8$ per group (mean ± SEM; $^*P < 0{\cdot}05$; $^{**}P < 0{\cdot}01$; $^{***}P < 0{\cdot}001$).

inflammatory responses differs in hepatitis of various aetiologies. Concanavalin A-induced hepatitis mimics many aspects of autoimmune hepatitis in which a strong inflammatory response aggravates liver injury. As a result, application of anti-inflammatory substances usually ameliorates autoimmune hepatitis and is therapeutic. However, in viral hepatitis, such as hepatitis C virus infection, the situation is quite the opposite, as a strong inflammatory response benefits viral clearance and promotes the outcome towards resolution.[45] Patients who fail to clear the virus and develop chronic persistent hepatitis are characterized by insufficient inflammatory activity.[46] Therefore, application of anti-inflammatory substances impedes resolution of viral hepatitis and is harmful. There is no doubt that advances in therapy for hepatitis need precise and profound understanding of the immune mechanisms governing the pathogenesis of hepatitis.

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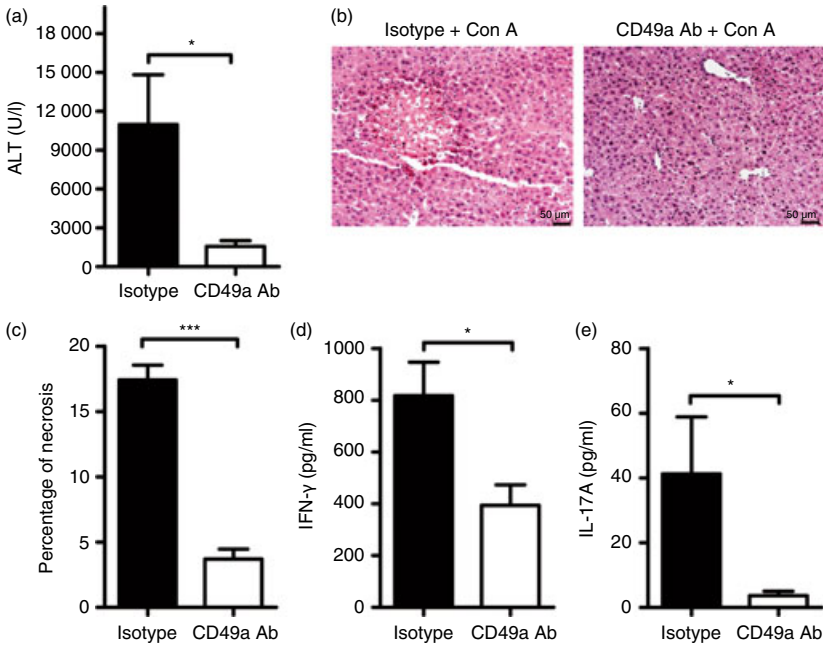

Figure 9. Blockade of CD49a attenuates concanavalin A (Con A) -induced liver injury. Wild-type mice were pretreated with 100 μg of either isotype antibody (black bars) or anti-CD49a monoclonal antibody (Ha31/8, white bars) per mouse 24 hr before Con A (12 μg/g) injection. Blood and liver tissues were collected 12 hr after Con A injection. (a) Serum alanine aminotransferase (ALT) levels were detected. (b) Representative photomicrographs of haematoxylin & eosin (H&E) -stained liver sections. Black scale bars represent 50 μm. (c) The percentage of the necrotic area in the livers 12 hr after Con A treatment was calculated by analysing H&E-stained sections. The percentage of necrotic area was measured from three different fields for each mouse (n = 9 mice/group). (d) Serum interferon-γ (IFN-γ) levels were detected by ELISA. (e) Serum interleukin-17A (IL-17A) levels were detected by ELISA. Data show the combined results from two experiments, n = 9 per group (mean ± SEM; $^*P < 0{\cdot}05$,$^{***}P < 0{\cdot}001$).

Disclosures

The authors have no financial conflicts of interest.

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