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TIM-4 is critical for KC homeostatic function in the activation and resolution of liver ischemia reperfusion injury

Ming Ni[*,1,3], **Jing Zhang**[*,1], **Rebecca Sosa**[2], **Hanwen Zhang**[1], **Han Wang**[3], **Dan Jin**[1,4], **Kaitlyn Crowley**[1], **Bita Naini**[1], **Reed, F. Elaine**[2], **Ronald W. Busuttil**[1], **Jerzy W. Kupiec-Weglinski**[1], **Xuehao Wang**[#,3], **Yuan Zhai**[#,1]

[1]Dumont-UCLA Transplant Center, Division of Liver and Pancreas Transplantation, Department of Surgery

[2]Department of Pathology and Laboratory Medicine, David Geffen School of Medicine at University of California-Los Angeles, Los Angeles, CA

[3]Hepatobiliary Center, Key Laboratory of Liver Transplantation of Chinese Academy of Medical Sciences, The First Affiliated Hospital of Nanjing Medical University, Nanjing, Jiangsu Province, China

[4]Department of Obstetrics and Gynecology, Renji Hospital, School of Medicine, Shanghai Jiao Tong University, Shanghai, China

Abstract

Liver ischemia reperfusion injury (IRI) remains an unresolved clinical problem. This study dissected roles of liver resident macrophage Kupffer cells (KCs), with a functional focus on efferocytosis receptor TIM-4, in both the activation and resolution of IRI in a murine liver partial warm ischemia model. FACS results showed that TIM-4 was expressed exclusively by KCs, but not infiltrating macrophages (iMΦs) in IR-livers. Anti-TIM-4 Ab depleted TIM-4^{+} macrophages in vivo, resulting in either alleviation or deterioration of liver IRI, which was determined by the repopulation kinetics of the KC niche with CD11b^{+} macrophages. To determine KC-specific function of TIM-4, we reconstituted clodronate liposome-treated mice with exogenous WT or TIM-4 deficient KCs at either 0h or 24h post reperfusion. TIM-4 deficiency in KCs resulted in not only increases in the severity of liver IRI (at 6h post reperfusion) but also impairment of the inflammation resolution (at 7 days post reperfusion). In vitro analysis revealed that TIM-4 promoted KC efferocytosis to regulate their TLR response by upregulating IL-10 and downregulating TNF-α productions. In conclusion, TIM-4 is critical for KC homeostatic function in both the activation and resolution of liver IRI via efferocytosis.

[#]**Address for correspondence:** Yuan Zhai, MD, PhD. Dumont-UCLA Transplant Center 77-120 CHS, 10833 Le Conte Ave, Los Angeles, CA 90095. Phone: (310) 825-9426; Fax: (310) 267-2367, yzhai@mednet.ucla.edu, Xuehao Wang, MD, Department of Liver Surgery, The First Affiliated Hospital of Nanjing Medical University, 300 Guangzhou Road, Nanjing, P.R.China 210029, Phone: 86-25-68136053; Fax:86-25-84630769, wangxh@njmu.edu.cn.

[*]Ming Ni and Jing Zhang contributed equally to the work.

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Introduction

Liver ischemia reperfusion injury (IRI) is an inevitable pathological consequence of multiple clinical conditions, including hepatic tumor resection, transplantation and trauma. Innate immune-dominated inflammatory response drives the pathogenesis of the disease (1). The danger associated molecular pattern (DAMP), such as HMGB1, DNA and histones, have been shown to engage innate immune cells via pattern recognition receptors, e.g., TLR4 and TLR9, resulting in liver inflammatory activation and hepatocellular damage (2–6). Despite the significant progress in our understanding of the molecular mechanism of liver inflammatory response against IR, the cellular basis of the response remains poorly defined. Additionally, the resolution of liver IRI, which is important for the restoration of liver homeostasis, is largely unknown.

Liver is a unique organ that it has the largest population of tissue resident macrophages, i.e., Kupffer cells (KCs). Liver injuries/infections trigger infiltration of peripheral monocytes. It becomes clear in recent years that both resident and infiltrating MΦs (iMΦs) are involved in liver sterile inflammatory responses by playing potentially distinctive roles. KCs sense hepatocellular damages and initiate the inflammatory cascade. CCR2+ monocytes in the periphery infiltrate into inflamed livers and are activated to perpetuate and amplify local inflammatory cascade (7, 8). Both resident and iMΦs are heterogenous. The original KCs are derived from cells of Yolk sac seeded in the tissue at the embryonic stages and infiltrating MΦs are derived from bone marrow cells (9, 10). In adult animals, however, KCs consist of both embryonic stem cell- and bone-marrow-derived types (11–13).

Tissue resident MΦs express unique markers. Among them, TIM-4 has been identified to mark the resident macrophages of the liver, intestine, heart and kidney (13–16). However, the question of whether TIM-4 plays functional roles in these cells in vivo has not been addressed in the cell-type specific manner. TIM-4 expression is detected in not only macrophages, but also dendritic cells, B cells and NK cells (17). The immune regulatory function of TIM-4 has been documented in at least two aspects: promote T cell proliferation/pro-inflammatory differentiation via TIM-1 and enhance efferocytosis in TIM-4+ macrophage/inhibit macrophage pro-inflammatory activation (17–21). TIM-4 has been shown in vitro as the essential co-receptor of efferocytosis selectively for KCs and peritoneal MΦs (20). Global TIM-4 deficiency and anti-TIM-4 Ab treatment, however, protects livers, kidney and brains from IRI, implicating its pro-inflammatory role (18, 19, 22, 23). In the current study, we focused on KC-specific function of TIM-4 in not only the activation, but also the resolution stage of liver IRI. We first characterized TIM-4 expression in livers in response to IR stress in both experimental and clinical hepatic tissue samples. We then resolved the puzzle why anti-TIM-4 Ab could have both protective or deteriorating effects on the pathogenesis of liver IRI, by analyzing the impact of the Ab on tissue resident TIM-4+ macrophages in vivo. To elucidate the KC-specific function of TIM-4, we established a KC reconstitution mouse model with TIM-4 WT or deficient types. Purified KCs were injected at the distinctive time points of liver IR to determine their impact on the activation and resolution of the disease. KC function was also studied in vitro. Our results show, for the first time, that TIM-4 regulates KC homeostatic function in liver IRI via efferocytosis.

Materials and Methods

Animals.

Male WT C57BL/6J and CD11b-DTR (6–8 weeks old) mice were purchased from the Jackson Laboratory (Bar Harbor, ME); TIM-4 deficient mice were bred in (breeding pairs provided by Dr. Vijay K. Kuchroo, Harvard University, Boston, MA) and housed in the UCLA animal facility under specific pathogen-free conditions, and received humane care, according to the criteria outlined in the "Guide for the Care and Use of Laboratory Animals" prepared by the National Academy of Sciences and published by the National Institute of Health. All animal experiments were approved by the UCLA Office of Animal Research Oversight Committee.

Mouse liver partial warm ischemia model.

After a midline laparotomy, mice were injected with heparin (100 μg/kg) and an atraumatic clip was used to interrupt arterial/portal venous blood supply to the cephalad-liver lobes. After 90 min of ischemia, the clip was removed initiating hepatic reperfusion. Sham controls underwent the same procedure, but without vascular occlusion. Mice were sacrificed after 6h to 7 days of reperfusion, and liver and serum samples were collected. Serum alanine aminotransferase (sALT) levels were measured with an autoanalyzer by IDEXX Laboratories (Westbrook, Maine). Part of liver specimens were fixed at 10% buffered formalin and embedded in paraffin. Liver sections (4μm) were stained with hematoxylin and eosin (HE). The severity of liver IRI was graded blindly using Suzuki's criteria on a scale from 0 to 4 (24). No necrosis, congestion/centrilobular ballooning is given a score of 0, while severe congestion and >60% lobular necrosis is given a score of 4. Anti-TIM-4 Ab (Clone# RMT4–53, BioXCell, West Lebanon, NH) was administered at either 48h or 2h prior to the onset of liver ischemia (0.25mg/mouse i.v.).

Patients and specimens.

Five patients with hepatocellular carcinoma (HCC) undergoing liver partial resection in the Department of Liver Surgery, the First Affiliated Hospital of Nanjing Medical University, were included in the current study. The human liver graft biopsies were collected serially from tumor peripherals at 0, 10, 30 and 50 min after partial hepatic portal occlusion by ligation of branches of the hepatic artery and portal vein into the tumor lobes. The study protocol was approved by the Institutional Review Board of the First Affiliated Hospital of Nanjing Medical University (IRB approval number: 2017-SRFA-138). Informed consent was obtained from each patient.

Macrophage Depletion and Reconstitution.

Macrophage Depletion Kit (Encapsula NanoSciences LLC, Brentwood, TN) was used to deplete KCs according to the manufacturer's protocols. In brief, 200μl/mouse Clodronate encapsulated liposomes injected i.v. at 48h prior to the onset of liver ischemia. Two million anti-F4/80-conjugated magnetic bead-purified KCs were injected via the portal vein at either 0h or 24h post-reperfusion. The purity of KCs and the reconstitution were shown in Sup. Fig.1. To reconstitute BMMs, CD11b-DTR mice were first treated with DT (10μg/g i.v.) 48h

prior to the onset of liver ischemia. Three million mature BMDMs were injected i.v. at 0h or 24h post-reperfusion.

Please refer to the supplements for general methods of liver non-parenchymal cell isolation, bone-marrow-derived macrophage cultures, flow cytometry, quantitative RT-PCR and efferocytosis assay.

Results

1. KCs express TIM-4 in both experimental and clinical ischemia reperfusion settings

TIM-4 has been shown to specifically mark the yolk-sac-derived tissue resident macrophages in liver, heart, intestine and kidney (14–16). We analyzed its expression in liver macrophages (in adult mice) in sham and during IR by FACS (Fig.1a). Based on F4/80 and CD11b expression pattern, we have found previously that liver ischemia triggered the infiltration of $F4/80^{+}CD11b^{high}$ MΦs (iMΦs) and $F4/80^{-}CD11b^{+}$ neutrophils with a simultaneous decrease of KCs ($F4/80^{+}CD11b^{low}$) at 6h post reperfusion (25). KCs in sham livers expressed TIM-4 (Fig.1b). Liver IR downregulated TIM-4 levels in KCs, while iMΦs did not express TIM-4 (vs. isotype controls) at both 6h and day 3 post-reperfusion. The iMΦs and neutrophils were mostly cleared from the IR-livers at day 7 post reperfusion. KCs recovered their numbers and TIM-4 levels at this time point, as shown by the kinetic changes of TIM-4 in liver macrophage subsets during liver IRI (Fig.1b). Quantitative RT-PCR analysis of liver tissues showed similar results that intrahepatic TIM-4 levels were downregulated by IR at 6h and upregulated at day 3–7 post reperfusion (Fig.1c).

To determine the clinical relevance of this observation, we analyzed TIM-4+ cells in human liver biopsies. Immunofluorescence staining showed that CD68+ Kupffer cells, but not hepatocytes, expressed TIM-4 (Fig.1d). The impact of ischemia on liver TIM-4 expression was evaluated in HCC patients during hepatic tumor resection. Both RT-PCR and Western blot analysis of tumor peripheral tissues harvested at different time points post ischemia showed that liver TIM-4 transcript and protein levels reduced progressively with longer ischemia times (Fig.1e, f. 0, 30 and 50 min.). Thus, KCs express TIM-4 and liver IR downregulates TIM-4 in both experimental and clinical settings.

2. The anti-TIM-4 Abs deplete $TIM\text{-}4^{+}$ macrophages in vivo.

The peri-operational administration of anti-TIM-4 Ab has been shown to protect livers, as well as kidneys and brains, from IRI (18, 19, 22, 23), which indicates a pro-inflammatory, rather than homeostatic, role of TIM-4 in these organs. Interestingly, when we tested the Ab in our model, we found that it could either protect or damage livers depending on the time of the Ab administration: liver IRI and inflammatory response were reduced when anti-TIM-4 was injected peri-operationally at 2h, but enhanced when injected pre-emptively at 48h, prior to the onset of liver ischemia (Fig.2a). FACS analysis of liver macrophages revealed that all $F4/80^{+}$ $CD11b^{-}$ cells were gone at 2h, and reappeared at 48h but became $TIM\text{-}4^{-}$ (Fig.2b). To confirm that the anti-TIM-4 Ab did deplete $TIM\text{-}4^{+}$ cells in vivo, we measured the changes in the more accessible TIM-4+ (without collagenase digestion) peritoneal resident macrophages. To identify the potential difference of the Ab

effect at different time points, we sampled peritoneal exudes at 2h and 48h post the Ab-injection. FACS analysis showed that TIM-4+ macrophages were indeed depleted by the anti-TIM-4 Abs. In the control Ig-treated peritoneum, more than 50% of $CD11b^+$ $F4/80^+$ $Gr\text{-}1^-$ macrophages were TIM-4+. All of these TIM-4+ macrophages were gone after the Ab-treatment: majority of CD11b+ cells were $F4/80^-Gr\text{-}1^+$ neutrophils at 2h and $F4/80^{low}$ $Gr\text{-}1^-$ infiltrating macrophages at 48h (Sup Fig.2). These data also indicate that there was a transient period of macrophage deficiency in the peritoneum (at 2h) after the Ab treatment, followed by the repopulation of the emptied niche with infiltrating macrophages (at 48h). To test whether the anti-TIM-4 Ab resulted in a similar kinetic change in liver macrophages, we performed immunofluorescence staining of liver tissues with anti-F4/80, CD11b and Clec4F, harvested after 2h and 48h post anti-TIM-4 injection. Indeed, nearly all F4/80+ or Clec4F + cells, present in control livers, were absent at 2h, and F4/80+ and CD11b+, but not Clec4F+, cells were detected at 48h (Fig.3 Sham), indicating that liver KC niche was indeed emptied by the anti-TIM-4 Abs and repopulated subsequently by $F4/80^+CD11b^+$ $Clec4F^-$ infiltrating macrophages. Thus, the protective effect of the anti-TIM-4 Abs might result from KC depletion, while the deteriorating effect might be due to CD11b+ macrophage repopulation of the KC niche.

3. The effect of anti-TIM-4 on liver IRI is determined by the depletion and repopulation kinetics of KC niche.

To test the hypothesis that the depletion and repopulation of KC niche determined the effect of anti-TIM-4 Ab on liver IRI, we analyzed IR-liver tissues at 6h post reperfusion in these different experiment groups. While IR resulted in increases in both F4/80+ and CD11b+ cells and decrease of Clec4F+ cells in control-Ig-treated livers, significantly less numbers of F4/80 and CD11b cells were found in livers treated with peri-operational anti-TIM-4 Ab regimen (−2h), while significantly higher numbers of CD11b cells were detected in liver treated with the pre-emptive regimen (−48h) (Fig.3 IR6h). No Clec4F+ cells are detected in the Ab-treated IR livers, indicating the depletion of original KCs. These data showed that IR-induced liver infiltration of CD11b+ inflammatory cells was reduced when KC niche was emptied by the Ab. but significantly enhanced when the KC niche was repopulated with CD11b+ macrophages, prior to the onset of liver ischemia. To test whether the CD11b+ macrophage repopulation was responsible for the deteriorating effect in liver IRI, we depleted CD11b+ cells with diphtheria toxin in CD11b-DTR mice simultaneously with the pre-emptive anti-TIM-4 treatment. Indeed, the Ab was no longer able to aggravate hepatocellular injury in the absence of CD11b cells (Fig.2c). These results provide us an answer to the paradoxical effects of anti-TIM-4 in liver IRI. Thus, the anti-TIM-4 Ab depletes KCs in vivo and results in the repopulation of the emptied KC niche with $CD11b^+$ macrophages. The kinetics of KC depletion and repopulation determines the outcome of the Ab effect on liver IRI. These data also indicate that the newly infiltrated $CD11b+F4/80+TIM\text{-}4^-$ $Clec4F^-$ macrophages are more pro-inflammatory than original KCs in the pathogenesis of liver IRI.

The pro-inflammatory property of these new CD11b+ liver macrophages was also demonstrated in two other acute liver injury models induced by either LPS/D-galactosamine or acetaminophen overdose. Significantly higher levels of hepatocellular damages were

induced by these hepatocytotoxic drugs in preemptive anti-TIM-4 vs. control-Ig treated mice (Sup. Fig.3). We also determined whether the repopulated CD11b+ macrophages affected the resolution of liver IRI by extending the reperfusion time to 7–14 days. Clodronate liposomes (CL), which also depleted KCs, was included as a control. Compared with control-Ig and blank liposome treated mice, the resolution of liver IRI was significantly impaired in anti-TIM-4 and CL-treated cohorts, as evidenced by the presence significantly higher amounts of hepatocellular damages (higher Suzuki scores), neutrophils (F4/80-CD11b+) and infiltrating macrophages (F4/80+CD11b+) in IR livers at day 7 post reperfusion, as well as higher intrahepatic levels of pro-inflammatory/pro-fibrosis and lower levels of immune regulatory/reparative gene expressions (Fig.4).

To study the functional differences between TIM-4+ KCs and the TIM-4^{-}CD11b+ liver macrophages, F4/80+ cells were isolated from control and anti-TIM-4 Ab-treated livers (at 48h) by magnetic beads. After incubating with pHrodo-SE-labeled apoptotic thymocytes, macrophage efferocytosis was quantitated under immunofluorescence microscope and by FACS (Fig.5a, Sup.Fig.4). WT KCs had high efferocytosis capacity that more than 50% of them became pHrodo-SE positive. The addition of anti-TIM-4 Ab in the cell cultures inhibited KC efferocytosis. Compared with TIM-4+ KCs, TIM-4^{-} liver macrophages were much inferior in their efferocytosis capacity (80% vs. 30% of pHrodo-SE positivity, Fig.5b). These two types of liver macrophages were also stimulated with LPS in the absence or presence of apoptotic thymocytes. Although they produced similar levels of TNF-a and IL-10 in the absence of apoptotic cells, TIM-4^{+} KCs, but not TIM-4^{-} liver macrophages, reduced TNF-a and increased IL-10 production in the presence of apoptotic cells (Fig.6). The anti-TIM-4 Ab in the cell cultures abrogated the immunoregulatory effect of apoptotic cells in KCs. These data indicate that TIM-4 facilitates KC efferocytosis which regulates their innate immune activation in the context of tissue injury (cell death). This may constitute the key immune regulatory mechanism of TIM-4^{+} KCs in liver inflammatory response against IR. Indeed, when we quantitated efferocytic liver macrophages (F4/80+ cells containing > two DAPI+ nuclei) isolated from IR livers under the fluorescence microscope, significantly lower percentages were found in those from anti-TIM-4-treated vs. control livers day 5 post reperfusion (Sup Fig.5).

4. TIM-4 is critical for the homeostatic function of KCs in liver IRI

To determine the function of TIM-4 in KCs, we compared KCs from WT or TIM-4 deficient mice. Their functional differences were first studied in vitro in their efferocytosis capacity and response to TLR stimulation. As shown in Fig.5c, TIM-4 KO KCs were significantly inferior that approximately 27% of them were pHrodo-SE vs. 61% in WT KCs. These KCs also lost the immune regulatory response to LPS in the presence of apoptotic cells with higher TNF-a and lower IL-10 production, as compared with their WT counterparts (Fig.6).

To determine TIM-4 function in KCs in liver IRI, we reconstituted KC-depleted hosts with purified WT or TIM-4 deficient KCs. Clodronate liposomes were administered at 48h prior to the onset of liver ischemia to deplete endogenous KCs, and exogenous KCs were injected either at 0h or 24 post-reperfusion to evaluate their roles in the activation or resolution stages of liver IRI, respectively. As shown in Fig.7a, mice reconstituted with TIM-4 deficient

KCs developed more severe liver IRI, as evidenced by elevated levels of sALT and worse damaged liver histological architectures with higher Suzuki's scores, compared with those with WT KCs. The liver inflammatory response was enhanced in these TIM-4 deficient KC-reconstituted mice with higher levels of TNF-a, IL-1b, IL-6, iNOS and lowest levels of IL-10 and Arg gene expressions in IR livers at 6h post-reperfusion. Furthermore, the resolution of liver IRI was impaired in TIM-4 deficient KC-reconstituted mice that the repair of hepatocellular damages was significantly delayed with higher Suzuki's scores in IR livers at day 7 post-reperfusion (Fig.7b). This was accompanied with elevated levels of pro-inflammatory and pro-fibrogenic (TNF-a, iNOS, a-SMA and Col1A1/2) and reduced levels of immune regulatory and reparative (IL-10, MerTK and TIM-4) gene expressions in TIM-4 KO vs. WT KC-reconstituted livers.

As a control, we also reconstituted CD11b-depleted hosts (in CD11b-DTR mice, DT injected at 48h prior to the onset of liver ischemia) with either TIM-4 deficient or WT bone marrow-derived macrophages (BMMs) at either 0h or 24h post reperfusion. Both types of BMMs acted equally well in the activation and resolution of liver IRI. Serum ALT levels, histological Suzuki's scores and the gene expression profiles in IR-livers at 6h or day 7 post-reperfusion were similar between the WT and TIM-4 deficient BMM-reconstituted mice (Sup. Fig.6). Thus, BMMs function in liver IRI independent of TIM-4. Overall, our data indicates that TIM-4 regulates homeostatic functions of KCs, but not BMMs, in both the activation and resolution of liver IRI.

Discussion

In the current study, we addressed the question whether TIM-4, as a macrophage efferocytosis co-receptor, plays an immune regulatory role in the pathogenesis of liver IRI. As TIM-4 has been studied in several organ ischemia models (18, 19, 22, 23) and anti-TIM-4 Abs were shown to inhibit tissue inflammatory activation and protect from IR injury (22, 23), the question we clarified first was how the anti-TIM-4 Ab affected liver macrophages in vivo. We revealed the depleting nature of this Ab in mice and find that the repopulation kinetics of the KC niche determines the effect of anti-TIM-4 on liver IRI (Fig.8). Thus, KCs were quickly depleted by the Abs, when the niche was emptied at 2h post the Ab treatment, the liver was protected from IRI; when CD11b+ macrophages repopulated the KC niche at 48h, liver inflammatory response was enhanced. The role of these CD11b cells in mediating anti-TIM-4 induced enhancement of liver IRI was further demonstrated in CD11b-DTR mice.

As TIM-4 regulates functions of different immune cells (17) and macrophage-specific TIM-4 deficient mouse stains are not available, we had to rely on the macrophage reconstitution system with either WT or TIM-4 deficient types to specify roles of TIM-4 in KCs and BMMs in liver IRI. Our results clearly show that TIM-4 is critical for the homeostatic function of KCs, but not BMMs, in both the activation and resolution of liver IRI. Furthermore, we show that TIM-4 regulates KC innate immune activation via efferocytosis which may underly their homeostatic function in the tissue damage-initiated inflammatory response. Fig.8 summarize graphically our finding in the study.

Our finding of TIM-4+ macrophage depletion by the anti-TIM-4 Ab was based on the quantitative FACS and immunobiological analysis in both livers and peritonea. One alternative explanation for the disappearance of these macrophages is their potential reverse migration upon TIM-4 ligation. Tissue macrophages are not motile by definition. However, tissue infiltrating neutrophils, which are traditionally thought to travel one way from the circulation to tissues, have been shown recently to reverse migrate into the vasculature (26). Despite this possibility, our demonstration of the repopulation of KC niche with CD11b+ macrophages is consistent with other findings in the literature (13, 27). These new occupants are clearly more pro-inflammatory than the original KCs, as hepatocellular injuries were significantly elevated in these livers upon not only IR, but also cytotoxic drug stimulations. In adult mice, KCs have been shown to include at least two populations, which are derived from embryonic precursors and circulating monocytes (bone marrow derived). They are phenotypically similar ($F4/80^{+}CD11b^{low}Ly6C^{-}$) with only a few temporally different markers, including Clec4F and TIM-4 (13, 28). It has been shown that bone marrow-derived monocytes are able to become self-renewing KCs and gradually acquire TIM-4 expression (e.g., 23% TIM-4^{+} in 30 days) (13). Functionally, these two types of KCs are similar in their response to LPS, phagocytosis of effete red blood cells, bacteria and parasites, and contain infection and granuloma formation against Leishmania (27). Our study indicates that the newly repopulated CD11b+ liver macrophages are TIM-4^{-}, less capable of efferocytosis and more pro-inflammatory upon TLR activation in the presence of apoptotic cells (context of tissue injury). We have observed that these livers remain hyper-responsive to inflammatory stimuli up to 30 days when TIM-4 levels are recovered in new “KCs” (data not shown).

IR may inhibit TIM-4 function in livers early during IR, due to the necrotic depletion of TIM-4+ KCs (25), as well as TIM-4 downregulation in KCs upon IR and pro-inflammatory stimulation (Fig.1). TIM-4 could also be cleaved by metalloproteinases (29), which are active in inflamed tissues. Thus, it is likely that the immune regulatory function of TIM-4 in KCs is more evident in the late stage of liver IR when its levels are recovered (Fig.1). In our experiments, KCs were isolated from sham livers and reconstituted after liver ischemia. This might help to reveal the TIM-4 function in the activation stage of liver IRI. Therefore, a potential therapeutic target in liver IRI is the mechanism of how IR and inflammatory stimuli downregulate TIM-4 in KCs. One obvious candidate is hypoxia-inducible factor (HIF), which is a master regulator of cellular response to both hypoxia and inflammation (6, 30, 31). Although there are no direct evidence of HIF regulation of TIM-4 in the literature, HIF-1 has been shown to induce TIM-3, another efferocytosis receptor in the same TIM family, in glial cells under hypoxia (32). MicroRNAs are key regulator of gene expression in the liver via translational repression of target mRNAs (33). MiRNA 155 is shown to suppress TIM-3 expression in HCV infected NK cells (34).

Although liver infiltrating CD11b+ macrophages did not express TIM-4 initially (by FACS), they gradually became TIM-4+ in livers, as shown in literatures (13) and our own experiment when we followed their evolution in IR livers in clodronate liposome -treated mice (data not shown). Interestingly, TIM-4 expression was regulated differentially in KCs vs. BMMs. TIM-4 levels were downregulated in KCs, but upregulated in BMMs, upon LPS stimulation in vitro (Sup. Fig.7). Several inflammatory cytokines have been shown to regulate TIM-4 expression in different types of cells. IL-6 is an acute response gene, which

has been shown recently to reprogram liver macrophages toward M2 type (35). In the tumor setting, IL-6 enhances TIM-4 expression via NF-kB signaling pathway (36). TGF-b can also upregulate TIM-4 in tumor cell lines (37). Whether they play roles in the regulation of TIM-4 expression in infiltrating CD11b+ macrophages to facilitate their reprogramming will be of high biological significance. The activation of p38 MAPK has been found recently to suppress TIM-4 expression in macrophages in response to inflammatory stimuli (38). Macrophages from aged individuals are impaired in their pro-resolution function, which can be restored by pharmacological inhibition of p38.

By gene overexpression and knock-down in peritoneal macrophages, TIM-4 has been shown to protect mice against lipopolysaccharide-induced endotoxic shock via suppressing the NF-κB signaling pathway (39). In vitro, TIM-4 overexpression was able to inhibit TNF-a, IL-6, as well as nitro oxide production upon LPS and/or IFN-g stimulations (39, 40). Whether these pro-inflammatory mediators have reciprocal effects on TIM-4 expression in KCs and BMMs remains to be seen.

In global TIM-4 deficient mice, we have found that the protection from liver IRI was independent of KCs, as KC depletion by clodronate liposomes failed to annihilate the resistant phenotype (data not shown). Separate studies have found that anti-TIM-4 Ab could promote CD4+CD25+FoxP3+ expansion in vitro in DC-CD4 T cell co-cultures and adoptive transfer of these iTreg protected livers from IRI (18) and allogeneic skin grafts from rejection (41). TIM-4 was found to regulate T cell proliferation/activation and apoptosis/survival via its interaction with TIM-1 (21) and/or TIM-3 (42). This may constitute a protective mechanism of anti-TIM-4 (19) in IRI, as T cell activation, particularly Th1 type, is critical for the inflammatory response against IR (43, 44). Thus, TIM-4 may exert diverse roles in the pathophysiology of IRI in the cell-type dependent manner.

TIM-4 binds to phosphatidylserine (PtdSer) with high affinity on the surface of apoptotic cells (20). Due to its short cytoplasmic tail, it does not mediate efferocytosis by itself (45), but rather functions as efferocytosis co-receptor for Tyro 3-Axl-Mer (TAM) receptor kinases (20), the primary efferocytosis receptor of macrophages. TIM-4 is required for the efferocytosis by KCs and peritoneal MΦs (20). Efferocytosis is critical for tissue inflammation resolution, responsible for the clearance of used neutrophils and repair of parenchymal damage (46). Additionally, the activation of efferocytosis receptor TAMs has been shown to negatively regulate inflammatory responses of MΦs/dendritic cells against TLRs and type I IFN stimulation (47). In liver disease models, growth arrest-specific gene 6 (GAS6), a ligand for the TAM receptor kinase family, facilitates tissue repair of acute CCl_4–triggered liver injury (48) and protect livers from IRI (49). MerTK+ liver MΦs were found in both human and mouse settings to promote the inflammation resolution in APAP-induced acute liver damage (50). MerTK deficient mice developed more severe and persistent liver damage in response to APAP and liver resident KCs express MerTK, which are critical for the neutrophil clearance (50). Our study demonstrates, both in vitro and in vivo, that TIM-4 regulates KC efferocytosis and their innate activation, which impacts the activation as well as resolution of liver inflammatory response against IR. Importantly, the immune regulatory role of TIM-4 becomes evident only in the context of tissue injury, as neither TIM-4 deficiency nor anti-TIM-4 Ab affects KC response to innate

immune stimulation in the absence of apoptotic cells in vitro. Furthermore, TIM-4 regulates specifically KCs, but not BM-derived macrophages in the mechanism of liver IRI.

In summary, our study demonstrates the homeostatic role of TIM-4 in KCs in liver IRI. TIM-4 regulates KC innate immune activation via efferocytosis. Therapeutic targeting of TIM-4 and efferocytosis may have great clinical potential to alleviate tissue injuries and facilitate the restoration of liver homeostasis after IRI.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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List of Abbreviations:

Ab	antibody
α-SMA	alpha-smooth muscle actin
BMM	bone marrow-derived macrophage
CL	clodronate liposome
DAMP	danger associated molecular pattern
DTR	diphtheria toxin receptor
FACS	fluorescence-activated cell sorting
H/E	hematoxylin and eosin
iMΦ	infiltrating macrophage
iNOS	inducible nitric oxide synthase
IRI	ischemia reperfusion injury
KCs	Kupffer cells
LPS	lipopolysaccharide
MerTK	MER proto-oncogene tyrosine kinase
MΦs	macrophages
NPC	non-parenchymal cell
qRT-PCR	quantitative reverse transcription-polymerase chain reaction
sALT	serum alanine aminotransferase

TIM-4	T cell immunoglobulin and mucin domain containing protein-4
TLR	toll-like-receptor
TNF-α	tumor necrosis factor α

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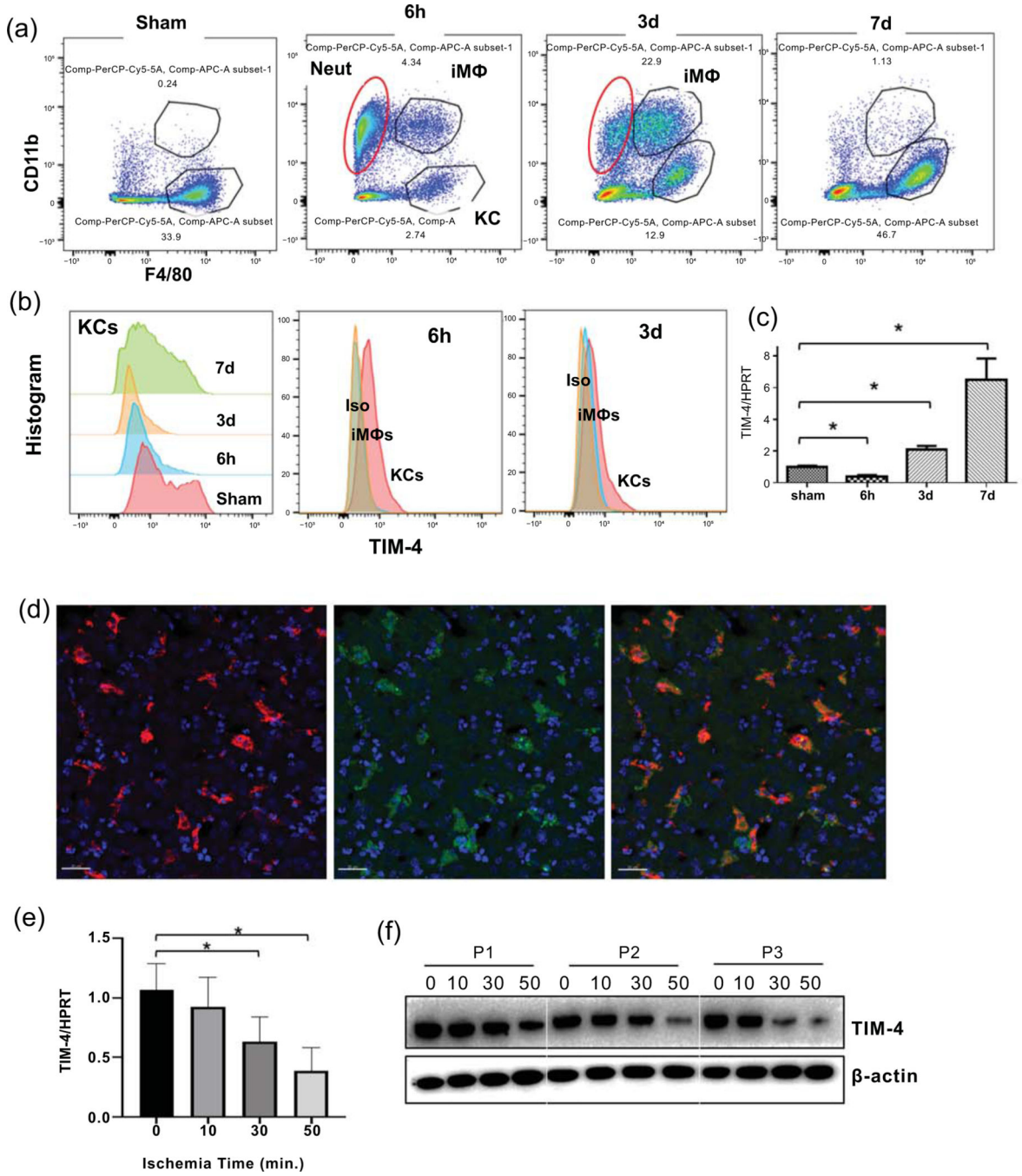

Figure 1.

Liver IR downregulates TIM-4 in KCs. B6 mice were subjected to 90m liver warm ischemia in the cephalad lobes followed by various lengths of reperfusion. Liver NPCs were isolated from the sham or ischemic livers after 6h, 3d and 7d of reperfusion. (a) Liver MΦ subsets based on F4/80 and CD11b expressions (density plots). KCs, infiltrating MΦs (iMΦs) and neutrophils were identified by $F4/80^{+}CD11b^{-}$, $F4/80^{+}CD11b^{+}$ and $F4/80^{-}CD11b^{+}$, respectively. (b) Histograms of KC TIM-4 at different time points of reperfusion, and the TIM-4 level in KCs and iMΦs at 6h and day3 post reperfusion. (c) TIM-4 levels in liver

tissues measured by quantitative RT-PCR, in sham or ischemic livers at different time points post reperfusion. n=4/group, representative results of 2 independent experiments. * $p<0.05$, ** $p<0.005$. (d) Representative immunofluorescence staining of CD68 and TIM-4 in human liver biopsies and their merged images. Frozen sections were stained with FITC-anti-TIM-4, PE-anti-CD68 and DAPI. Scale bar=30μM. (e) Quantitative RT-PCR and (f) Western blotting of TIM-4 in ischemic human liver tissues. Peri-tumor tissues were harvested after various lengths of ischemia (0–50min) during hepatic tumor resection in 3 series of hepatocarcinoma patients.

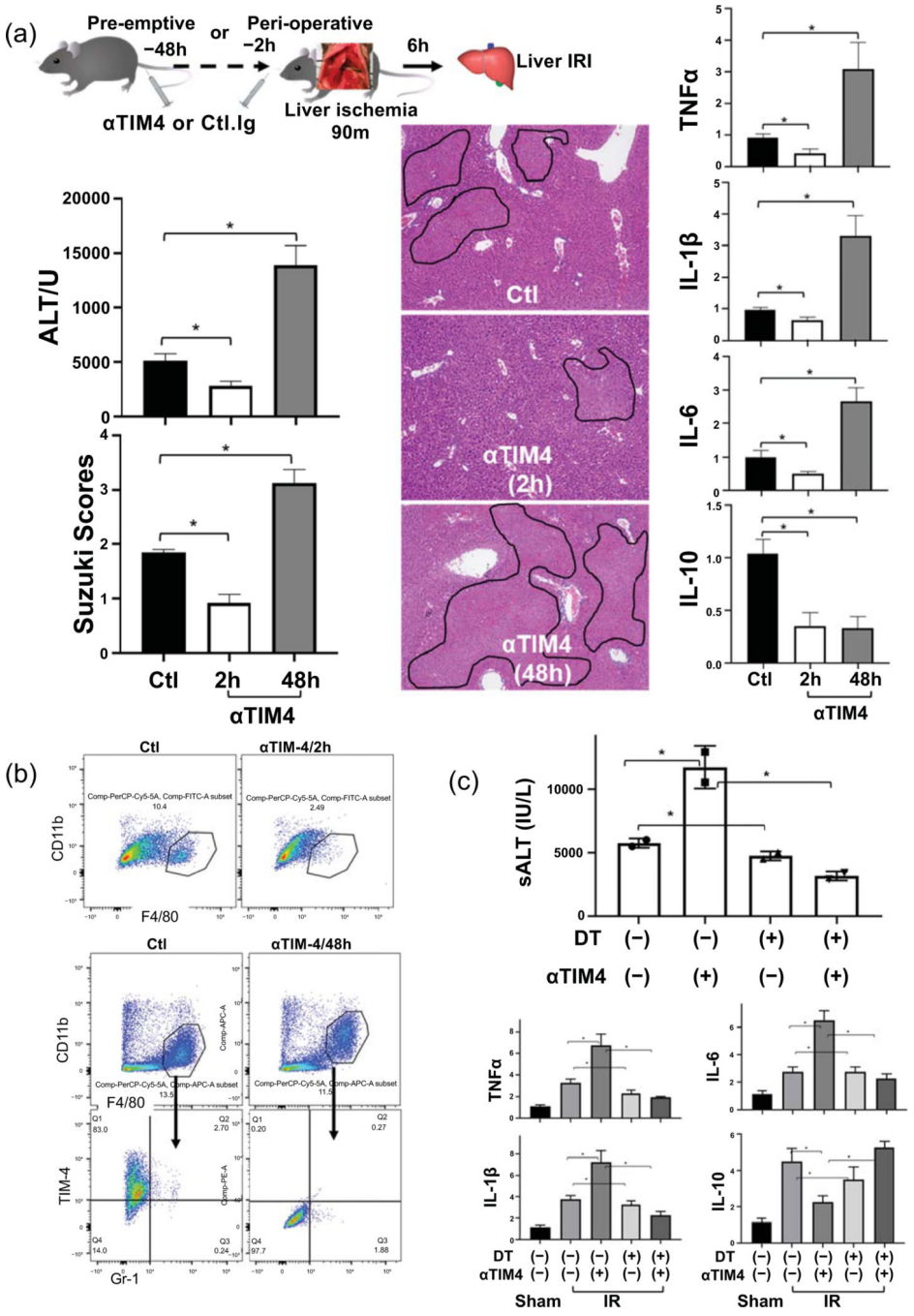

Figure 2.

The opposite effects of peri-operational (−2h) vs. pre-emptive (−48h) anti-TIM-4 Ab treatments on liver IRI. (a) Control or anti-TIM-4 Abs were administered at either 2h or 48h prior to the onset of liver ischemia. Liver IRI was evaluated at 6h post reperfusion. A diagram of the experimental scheme was shown. Average sALT levels and Suzuki scores of different experimental groups were plotted. Representative histological pictures of IR livers (H/E x40) were shown. Average target gene/HPRT ratios in IR livers were plotted. (b) FACS analysis of liver non-parenchymal cells (NPCs). Liver NPCs were isolated from

either control Ig or anti-TIM-4 Ab-treated mice at either 2h (upper panel) or 48h (lower panel) post the treatment, as described in the materials and methods. Cells were stained with fluorochrome-labeled Abs. Representative plots of F4/80 and CD11b expressions in the myeloid cell gate, and TIM-4/Gr-1 expression in the $F4/80^{+}CD11b^{low}$ subset. (c) CD11b-DTR mice were treated with a single dose of DT followed by the control or anti-TIM-4 Abs. Liver ischemia was performed 48h later and IRI was evaluated at 6h post reperfusion. Average sALT levels and target gene/HPRT ratios in IR livers in different experiment groups were plotted. All results are representatives of at least two independent experiments. n=4–6 mice/group. Data were tested for normal distribution and analyzed using a one-way ANOVA with post-test. *$p<0.05$.

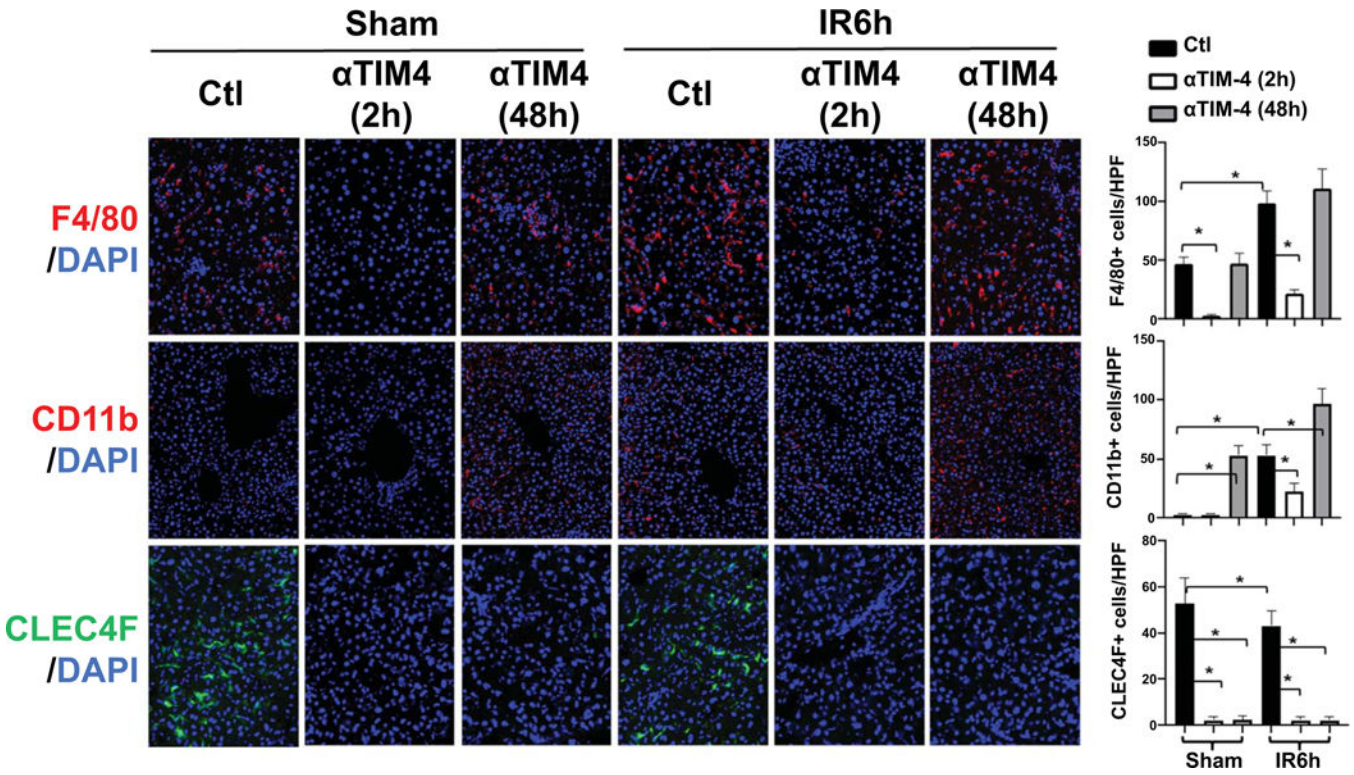

Figure 3.

The anti-TIM-4 Ab depleted KCs in livers and the repopulation kinetics of the emptied KC niche determined the outcome of liver IRI. Control Ig or anti-TIM-4 Ab were administered in separate groups of B6 mice. Liver tissues were harvested (Sham) or liver IR was performed in these treated mice (IR6h), 2h or 48h later after the Ab injection, as described in the Materials and Methods. IR-livers were harvested at 6h post reperfusion. Both sham and IR liver tissues were analyzed by immunofluorescence staining with anti-F4/80, or -CD11b, or -Clec4F Abs and DAPI. Representative images were shown. Average percentages of F4/80, CD11b and Clec4F positive cells in tissue sections of different experimental groups were plotted. All results are representatives of at least two independent experiments. n=4–6 mice/group. Data were tested for normal distribution and analyzed using a one-way ANOVA with post-test. *$p<0.05$.

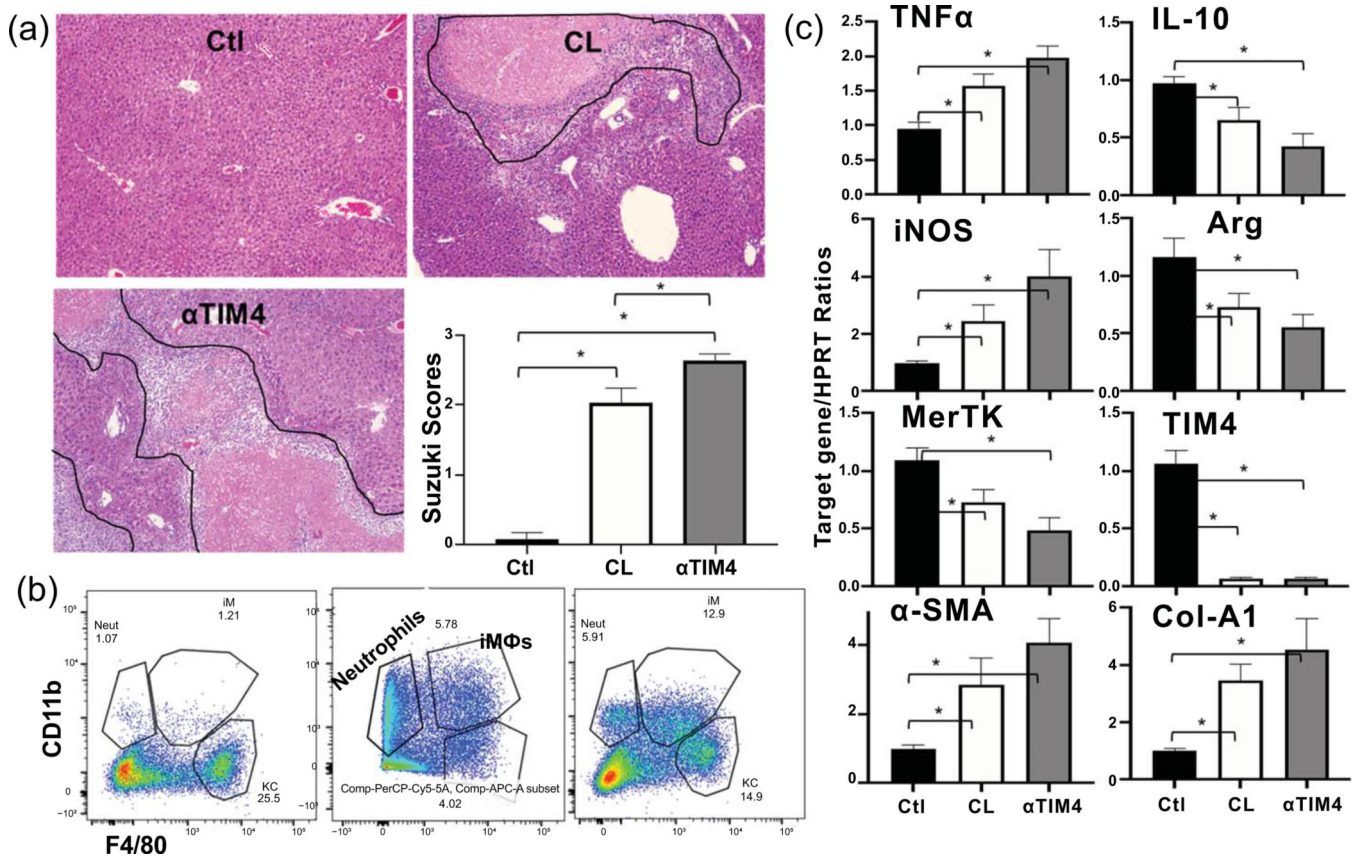

Figure 4.

The depletion of KCs impairs the resolution of liver IRI. B6 mice were treated with either control Ig or blank liposomes (Ctl) or clodronate liposomes (CL) or anti-TIM-4 Ab 48h prior to the onset of liver ischemia. Liver IRI and inflammatory responses were evaluated at 7d post reperfusion. (a) Representative histological pictures of IR livers (H/E x40) were shown. Average Suzuki scores of different experimental groups were plotted. (b) Liver NPCs were isolated from IR livers and analyzed by FACS. Representative cell density plot based on F4/80 and CD11b expressions was shown. (c) Average target gene/HPRT ratios in IR livers were plotted. All results are representatives of at least two independent experiments. n=4–6 mice/group. Data were tested for normal distribution and analyzed using a one-way ANOVA with post-test. *$p<0.05$.

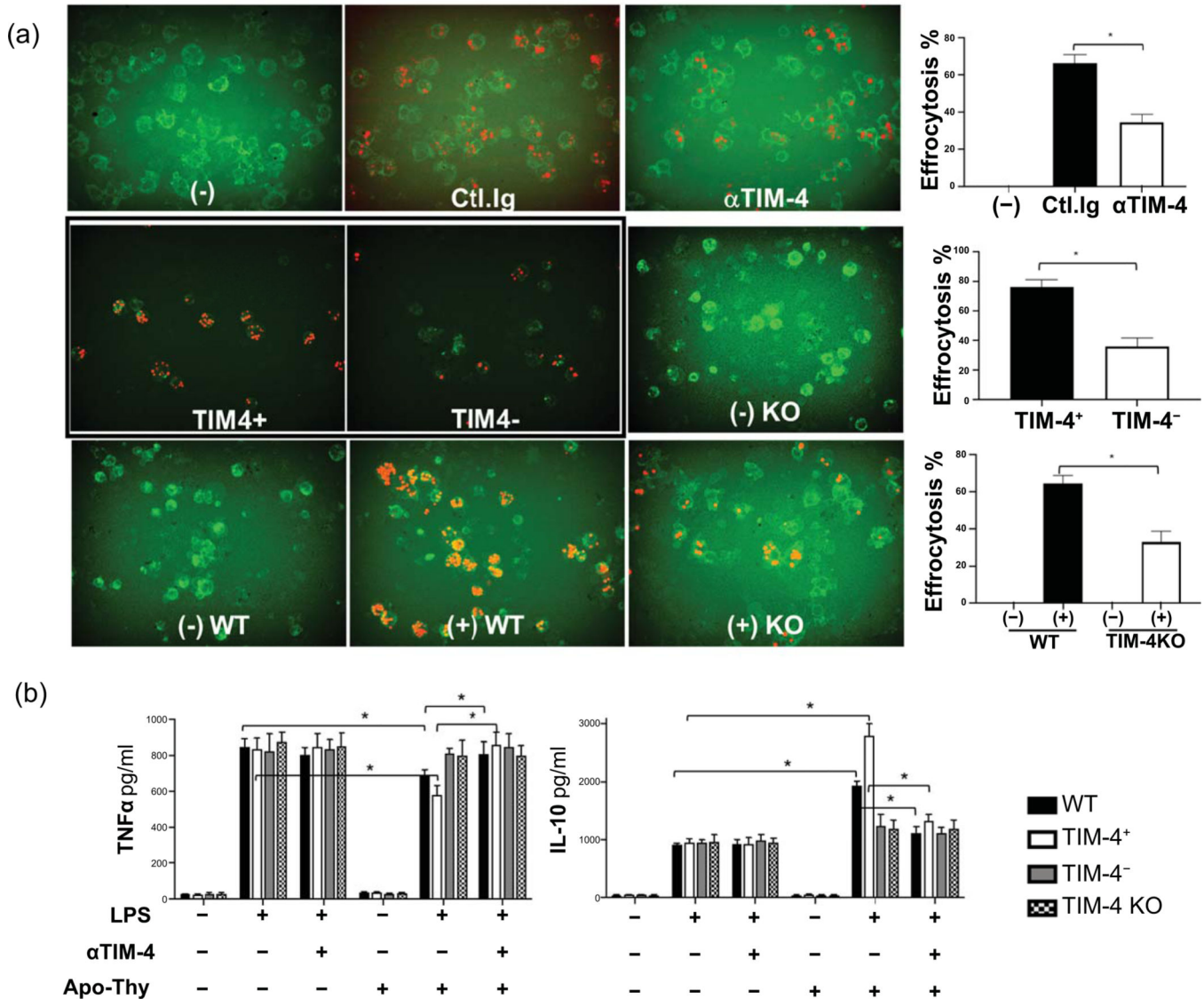

Figure 5.

TIM-4 regulates KC efferocytosis and innate immune activation in vitro. (a) Top panel: KCs were co-incubated with unlabeled (–) or pHrodo-SE-labeled (+) apoptotic thymocytes in the presence of control Ig or anti-TIM-4 (αTIM4) Abs for 2h. Cells were stained with fluorochrome-labeled anti-F4/80, DAPI, and visualized under fluorescence microscope. Middle panel: TIM-4$^+$ KCs and TIM-4$^-$ liver macrophages were isolated from livers treated with control Ig or anti-TIM-4 Ab-treated mice at −48h and measured in the efferocytosis assay. Lower panel: KCs were isolated from WT or TIM-4 KO mice and efferocytosis was measured as above. Representative images were shown and average % of F4/80$^+$/pHrodo-SE$^+$ cells were plotted. (b) WT (TIM-4$^+$), TIM-4 KO KCs and TIM-4$^-$ liver macrophages were isolated as detailed above and stimulated in vitro with LPS in the absence or presence of apoptotic thymocytes (apo-Thy) and/or anti-TIM-4 Abs. Culture supernatants were harvested at 24h post stimulation and TNF-a and IL-10 levels were measured by ELISA. All results are representatives of at least two independent experiments. n=3–5 mice/group. Data

were tested for normal distribution and analyzed using a one-way ANOVA with post-test. *$p<0.05$.

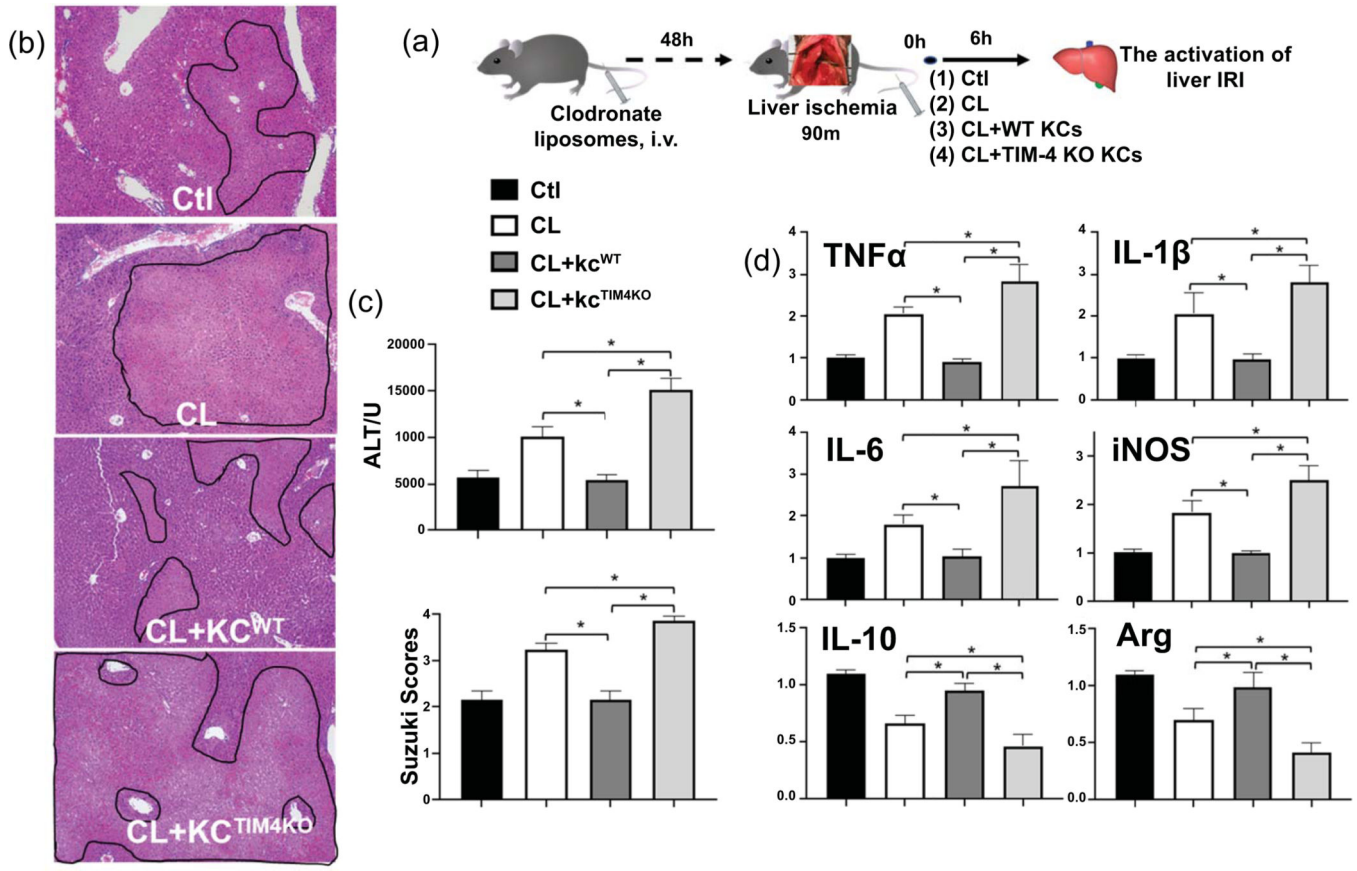

Figure 6.

TIM-4 is critical for the homeostatic function of KCs in the inflammatory activation of liver IRI. KC-depleted mice (by CLs) were reconstituted with either WT or TIM-4 KO KCs at 0h post reperfusion, as described in the Materials and Methods. (a) The diagram of the reconstitution experiment. Liver IRI and inflammatory responses were measured at 6h post reperfusion. (b) Representative histological pictures of IR livers (H/E x40) in KC-intact (Ctl), KC-depleted (CL) and WT or TIM-4 KO KC-reconstituted groups were shown. (c) Average sALT levels and Suzuki scores in different experimental groups were plotted. (d) Average target gene/HPRT ratios in IR livers of different experimental groups were plotted. All results are representatives of at least two independent experiments. n=4–6 mice/group. Data were tested for normal distribution and analyzed using a one-way ANOVA with post-test. *$p<0.05$.

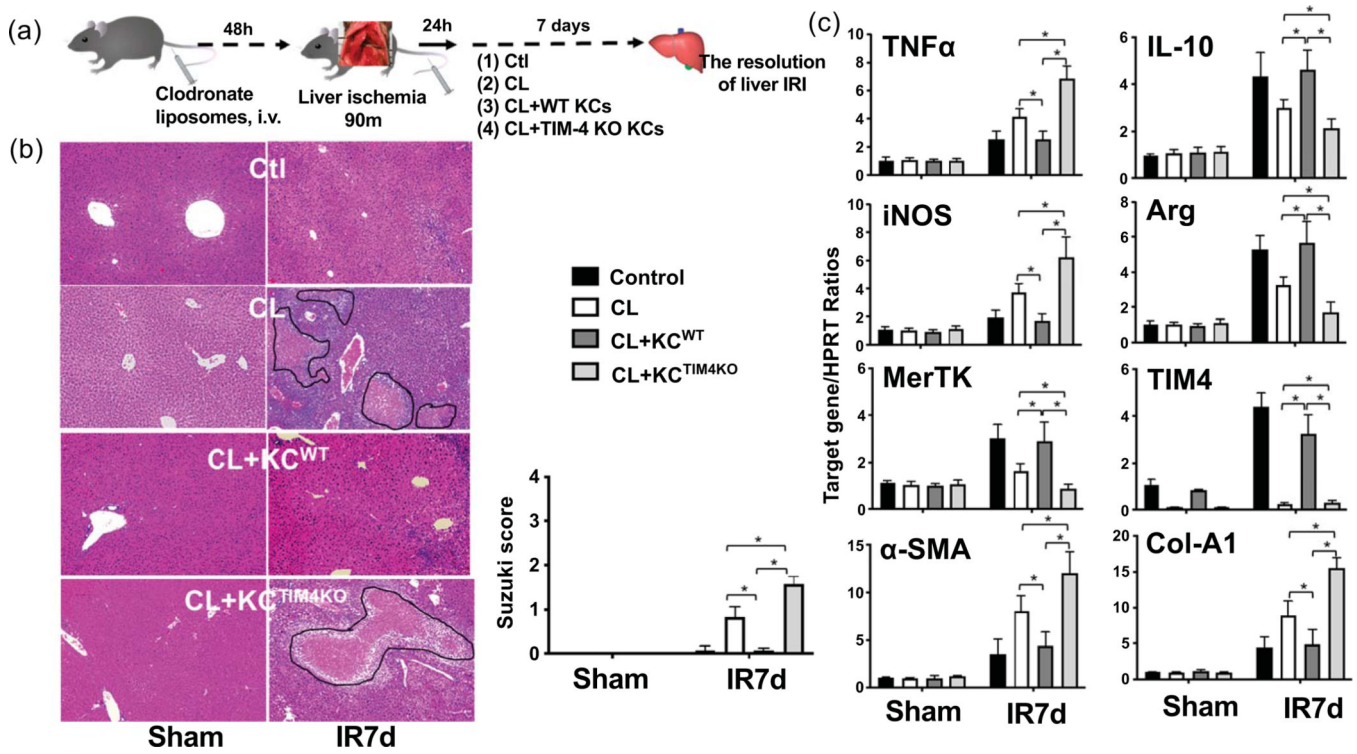

Figure 7.

TIM-4 is critical for the homeostatic function of KCs in the resolution of liver IRI. KC-depleted mice (by CLs) were reconstituted with either WT or TIM-4 KO KCs at 24h post reperfusion, as described in the Materials and Methods. (a) The diagram of the reconstitution experiment. The resolution of liver IRI and inflammatory responses were measured at 7d post reperfusion. (b) Representative histological pictures of IR livers (H/E x40) in KC-intact (Ctl), KC-depleted (CL) and WT or TIM-4 KO KC-reconstituted groups were shown. (c) Average Suzuki scores and (d) average target gene/HPRT ratios in IR livers of different experimental groups were plotted. All results are representatives of at least two independent experiments. n=4–6 mice/group. Data were tested for normal distribution and analyzed using a one-way ANOVA with post-test. *p<0.05.

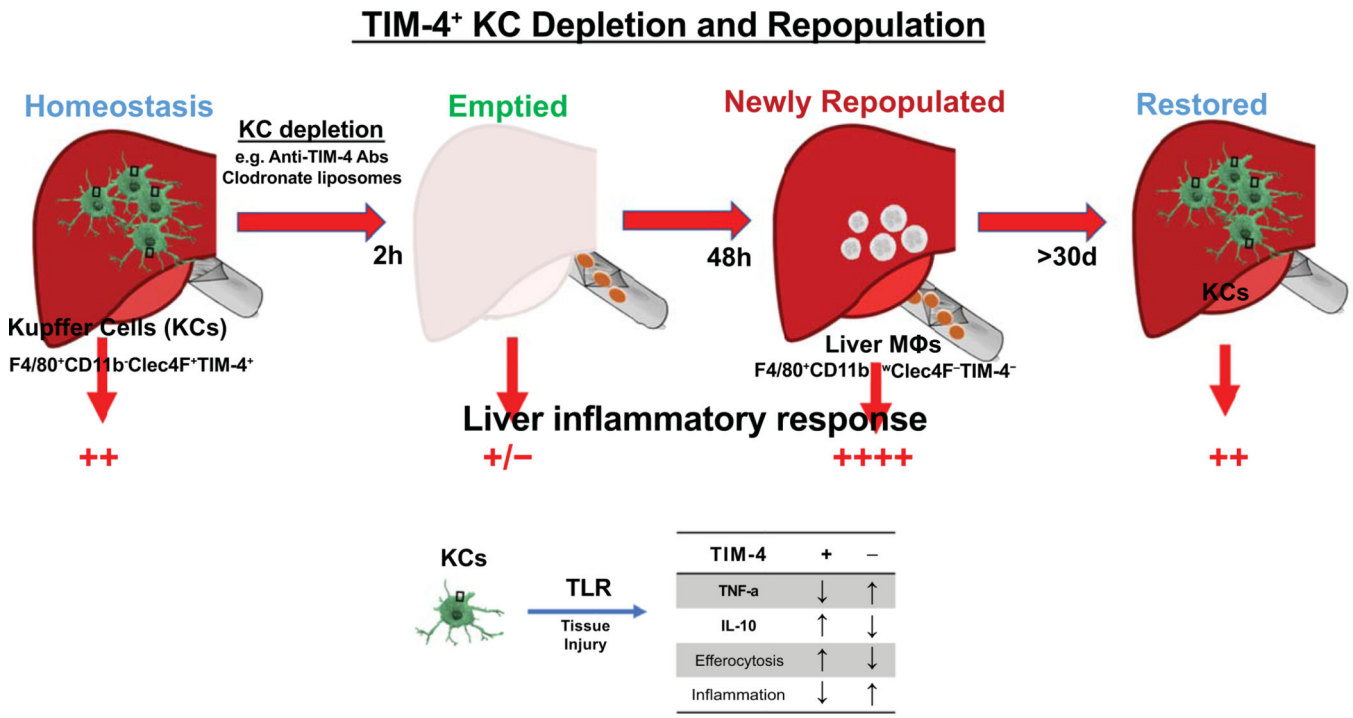

Figure 8.

TIM-4+ KCs and liver IRI. Upper panel: Anti-TIM-4 Abs deplete KCs in vivo and the repopulation kinetics of KC niche determines the Ab-effect on liver IRI. Lower panel: TIM-4 phenotypes determine KC response/function.